S. pombe LSD1 Homologs Regulate Heterochromatin Propagation and Euchromatic Gene Transcription

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SUMMARY

LSD1 represses and activates transcription by demethylating histone H3K4me and H3K9me, respectively. Genetic ablation of the S. pombe homologs, splsdl and splsd2, resulted in slow growth and lethality, respectively, underscoring their physiological importance. spLsd1 and spLsd2 form a stable protein complex, which exhibits demethylase activity toward methylated H3K9 in vitro. Both proteins were associated with the heterochromatin boundary regions and euchromatic gene promoters. Loss of spLsd1 resulted in increased H3K9 methylation accompanied by reduced euchromatic gene transcription and heterochromatin propagation. Removal of the H3K9 methylase Clr4 partially suppressed the slow growth phenotype of splsd1Δ. Conversely, catalytically inactivating point mutations in the splsd1 and splsd2 genes partially mimicked the growth and heterochromatin propagation phenotypes. Taken together, these findings suggest the importance of both enzymatic and nonenzymatic roles of spLsd1 in regulating heterochromatin propagation and euchromatic transcription and also suggest that misregulation of spLsd1/2 is likely to impact the epigenetic state of the cell.

INTRODUCTION

Histone N-terminal tails are subject to multiple posttranslational modifications including methylation, which occurs on both lysine (K) and arginine (R) residues. A number of histone lysine residues are methylated, including histones H3K4, 9, 27, 36, and 79 as well as histone H4K20. Methylation at these sites is associated with distinct chromatin structure and promoter activities (Kouzarides, 2002; Fischle et al., 2003; Margueron et al., 2005; Lachner et al., 2004; Martin and Zhang, 2005). For instance, methylation of H3K4 is associated with active transcription, while methylation of H3K9 is linked to transcriptional repression and heterochromatin. Lysine residues can be mono-, di-, and trimethylated, and these different methylation states may also play important roles in the regulation of chromatin structure and gene transcription (Bannister and Kouzarides, 2004). Furthermore, depending on the location, the same methylation event may have different functional outcomes. Specifically, the repressive H3K9 methylation, when occurring within the body of the gene, has been shown to positively regulate transcriptional elongation (Vakoc et al., 2005). Thus, the site and the extent of methylation, as well as the location where methylation occurs at the gene locus, can all impact the functional outcome, attesting to the complexity of histone methylation in chromatin regulation.

Recent studies have shown that histone methylation is reversible by demethylases. LSD1, which is evolutionarily conserved from S. pombe to human, represses transcription via demethylation of H3K4me (Shi et al., 2004). The numbers of LSD1-related genes vary in different species. The Arabidopsis has four while both S. pombe and human have two LSD1-related genes (Shi et al., 2004). LSD1-mediated repression in vivo requires the associated protein Co-REST, which is a SANT domain-containing molecule (Shi et al., 2005; Lee et al., 2005). Co-REST stabilizes LSD1 (Shi et al., 2005) and provides LSD1 access to nucleosomal substrates via interactions with LSD1 (Lee et al., 2005; Shi et al., 2005), as well as contacts with DNA (Yang et al., 2006). In contrast, in the presence of the androgen receptor (AR), LSD1 is associated with active transcription and demethylation of H3K9me at the target promoters (Metzger et al., 2005). These findings demonstrate that protein-protein interactions play an important role in the regulation of the activity and substrate specificity of LSD1.
Histone methylation is associated with important biological processes, including DNA damage response and heterochromatin formation (Lehnertz et al., 2003; Peters et al., 2001; Sanders et al., 2004). In the fission yeast \textit{S. pombe}, heterochromatin is present at a number of different genomic loci, including centromere, telomere, and the mating type loci (Grewal, 2000; Pidoux and Allshire, 2004). In the mating type and centromere loci, heterochromatin assembly has been shown to be initiated by siRNAs and requires the RNAi effector complex RITS and the RNAi machinery (Hall et al., 2002; Verdel et al., 2004; Volpe et al., 2002). This is followed by recruitment of the H3K9 histone methylase Clr4, the \textit{S. pombe} homolog of Suv39H (Ivanova et al., 1998; Rea et al., 2000). Heterochromatin propagation is believed to involve the \textit{S. pombe} HP1 homolog Swi6, which binds methylated H3K9 as well as the methylase Clr4 (Nakayama et al., 2001). In general, \textit{S. pombe} heterochromatin is characterized by H3K9 hypermethylation and H3K4 hypomethylation, but the heterochromatin boundaries are associated with hypermethylated H3K4 and hypomethylated H3K9, respectively (Noma et al., 2001). Because human LSD1 mediates demethylation of both H3K4 and H3K9 (Metzger et al., 2005; Shi et al., 2004), these findings collectively raise the question of whether \textit{S. pombe} LSD1 homologs may play a role in regulating H3K4 and/or H3K9 methylation at heterochromatin, therefore impacting heterochromatin assembly and/or propagation.

In this report, we investigated the biological functions of LSD1 in the model organism \textit{S. pombe}, which contains two LSD1 homologs, spLsd1 and spLsd2. Genetic ablation of splsd1 or splsd2 resulted in significant growth retardation or lethality, respectively, underscoring their physiological significance. Global gene expression profiling as well as genomically localized spLsd1/2 by chromatin immunoprecipitation coupled microarray (ChiP-chip) implicated spLsd1/2 in both heterochromatin and euchromatin functions. Specifically, loss of spLsd1 resulted in a modest global increase of methylation at H3K9, but not at other lysine residues. In \textit{splsd1} \\textit{J} cells, the increase of H3K9 methylation at the heterochromatin boundary appeared to be associated with heterochromatin expansion. ChiP-chip analysis identified spLsd1/2 at the target sites, suggesting that spLsd1/2 directly regulate heterochromatin propagation and euchromatin gene transcription by antagonizing H3K9 methylation. Several lines of evidence supported this model. First, the growth phenotype was rescued specifically, albeit incompletely, by suppression of the H3K9 methylase Clr4, but not other methylases. Second, the growth and heterochromatin propagation phenotypes were mimicked partially by introduction of catalytically inactive point mutations in the \textit{splsd1} and \textit{splsd2} genes. Third, recombinant spLsd1 and the spLsd1/2 complex exhibited H3K9 demethylase activity in vitro. Taken together, these findings identified critical functions of spLsd1 and spLsd2 in the regulation of heterochromatin propagation as well as transcription of a large number of euchromatic genes, in part by antagonizing H3K9 methylation through the H3K9 demethylase activity.

### RESULTS

**spLsd1 and spLsd2 Are Important for \textit{S. pombe} Growth and Viability**

\textit{S. pombe} has two LSD1-like open reading frames (ORFs), spbc146.09c and spac23e2.02. For simplicity, we refer to these two genes as \textit{splsd1} and \textit{splsd2}, respectively (Figure 1A). We succeeded in knocking out \textit{splsd1} (\textit{splsd1} \\textit{J}) but were unable to obtain transformants for \textit{splsd2} \\textit{J} using PCR mediated strategy, suggesting that spLsd2 was likely to be required for cell viability. We also failed to isolate \textit{splsd2} \\textit{J} strain through sporulation of a heterozygote diploid strain. Interestingly, the \textit{splsd1} \\textit{J}-- diploid cells were also defective in sporulation with only a very small fraction of the diploid cells producing viable spores, suggesting that both spLsd1 and spLsd2 may be involved in regulating \textit{S. pombe} meiosis. Consistent with this hypothesis, the expression of both \textit{splsd1} and \textit{splsd2} is increased significantly during the S phase of meiosis (Mata et al., 2002). \textit{splsd1} \\textit{J} exhibited an array of phenotypes including severely retarded growth (doubling time of 8 hr as opposed to 2.1 hr for the wild-type [WT] cells), flocculation, heterogeneity, and significant loss of viability during growth (Figures 1B and 1C). Reintroduction of WT \textit{splsd1} restored normal growth, indicating that the slow growth phenotype was likely to be the result of genetic inactivation of the endogenous \textit{splsd1} \\textit{J} gene. Taken together, these findings identified an essential function for spLsd1 and spLsd2 in \textit{S. pombe} growth and viability and suggested that these two proteins may have both redundant and nonredundant functions.

**spLsd1 Demethylates and Antagonizes H3K9 Methylation**

Human LSD1 functions as a histone demethylase that reverses H3K4 and H3K9 methylation, respectively (Metzger et al., 2005; Shi et al., 2004). We investigated whether spLsd1 and/or spLsd2 regulated \textit{S. pombe} growth as histone demethylases, using recombinant protein purified from insect cells as well as spLsd complex purified from \textit{S. pombe}. To purify spLsd1/2 complex, we first generated yeast strains carrying C-terminally TAP-tagged \textit{lsd1} \\textit{J} and \textit{lsd2} \\textit{J} genes, respectively, in place of the endogenous genes. These yeast cells were indistinguishable from the WT \textit{S. pombe}, suggesting that the C-terminal TAP did not alter the functions of spLsd1 or spLsd2. As shown in Figure 2A, spLsd1 and spLsd2 are present in the same, stable protein complex. The core spLsd1/2 complex components included approximately stoichiometric quantity of spLsd1, spLsd2, and two PHD domain proteins (SPCC4G3.07c and SPAC3D11.08c, see Table S4 in the Supplemental Data available with this article online), which we refer to as Phf1 (PHD finger 1) and Phf2, respectively. Consistent with the fact that they are components of the same protein complex, the expression patterns of
Phf1 and Phf2 during meiosis were found to be similar to that of spLsd1 and spLsd2 (Mata et al., 2002). Furthermore, we were unable to obtain the knockout strains for them either by the PCR-mediated strategy or through heterozygote sporulation, suggesting that, similar to spLsd1 and spLsd2, Phf1 and Phf2 were also likely to be required for cell viability and meiosis. The precise roles of Phf1 and Phf2 remain to be investigated.

We next assayed the recombinant spLsd1 and the purified spLsd1 complex for histone demethylase activities. Bulk histones were methylated at H3K4 and H3K9 by the recombinant methylases Set7/9 and Clr4, respectively, in the presence of radioactively labeled [3H]methyl-methionine. The demethylation reactions were monitored by the production of the radioactively labeled formaldehyde, which has shown to be a demethylation reaction product (Shi et al., 2004). As shown in Figure 2B, WT, but not the predicted, catalytically inactive spLsd1 point mutant (illustrated in the top panel of Figure 2B), demethylated H3K9, but not H3K4 on the bulk histones. Likewise, the spLsd1 protein complex also demethylated H3K9, but not H3K4. Importantly, the spLsd1 complex purified from the strain carrying the mutations predicted to inactivate both spLsd1 and spLsd2 (spLsd1/2mm) also failed to demethylate H3K9 (Figure 2B). Although the Clr4-labeled histone substrates may include both radioactive H3K9me2 and H3K9me3, the released formaldehyde was likely to have resulted from demethylation of H3K9me2 due to the inherent chemistry of LSD1 family of demethylases (Shi et al., 2004). Consistently, analysis of bulk histones isolated from the WT and the splsd1Δ strains also showed loss of spLsd1 associated with a mild but significant increase in the total H3K9me2 level without overt effects on the levels of H3K4me2, H3K36me2, or H4K20me2 (Figure S1).

We next carried out genetic suppression experiments to determine whether ablation of an H3K9 methylase may suppress the growth phenotype associated with the splsd1Δ cells. Consistent with the biochemical data, deletion of clr4, which encodes the main, if not the only, S. pombe H3K9 methylase (Cam et al., 2005), partially suppressed the growth retardation phenotype (Figure 2C, doubling time of ~5 hr). In contrast, deletion of the other methylase genes including set1 (H3K4) and set2 (H3K36) enhanced the growth retardation defect, suggesting possible collaborations between spLsd1 and these methylases (Figure 2C). Deletion of the H4K20 methylase gene set9 suppressed the growth phenotype slightly, and the significance of this remained unclear. As controls, we found that growth of the individual methylase knockout strains used in the suppression experiments was comparable to that of the WT S. pombe (data not shown). Taken together, these findings suggested that spLsd1 functioned in part by antagonizing Clr4, which mediates H3K9 methylation in S. pombe. However, the weak suppression also suggested splsd1 functions that were independent of antagonizing H3K9 methylation.
To further explore the possibility that spLsd1 functioned as a histone demethylase, we analyzed the catalytic mutant strains carrying either mutated spLsd1 or spLsd2 singly (spLsd1m and spLsd2m), or together (spLsd1/2mm). We found that while single gene mutants behaved like WT, the double mutant spLsd1/2mm was mildly growth retarded but with a significantly reduced severity compared to spLsd1m (Figure 2D; spLsd1/2mm doubling time, 2.5 hr;...
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WT, 2.1 hr; and splsd1Δ, 8 hr). This double mutant also displayed slight flocculation similar to that of splsd1Δ cells (Figure 2E). Thus, mutations that inactivate the demethylase activity partially phenocopied splsd1Δ, suggesting that the biological function of spLsd1/2 was associated with both the demethylase activity and the nonenzymatic roles of spLsd1/2.

**Genomic Locations of spLsd1 and spLsd2 and Alteration of H3K9 Methylation Associated with the Loss of spLsd1**

To further investigate the role of spLsd1/2 in regulating H3K9 methylation, we turned to the genome-wide ChiP-chip approach in order to determine whether (1) H3K9me3 level and reduced Pol II occupancy at the target promoters are associated with spLsd1 deletion. H3K9me3 and Pol II ChiP assays were carried out using the multiplex PCR approach (see Experimental Procedures), and the results are presented as ratios of the experimental to control PCR products (mean + SEM). (B) Significant overlap of spLsd1 and spLsd2 target genes. A total of 560 genes exhibited spLsd1 or spLsd2 enrichment at their promoters of >4-fold of the array mean value. A total of 93 promoters were enriched for both spLsd1 and spLsd2. (C) Microarray analysis comparing expression profiles with spLsd1/2 distribution for the 68 highest-confidence target genes. In this visualization of microarray data, the leftmost column compares spLsd1Δ expression to that of WT cells, while the other columns show spLsd1 and spLsd2 enrichment at promoter, 5′ end, middle, and 3′ end of the genes. Green indicates decreased transcription or depletion, and red indicates enhanced transcription or enrichment. Both spLsd1 and spLsd2 are highly enriched at promoters and 5′ of the coding regions of their targets, and a majority of spLsd1/2 targets display reduced transcription upon deletion of spLsd1.

DNA tiling array constructed based on the genome sequence of *S. pombe*. We found that spLsd1 and spLsd2 shared similar, but not identical genomic distribution patterns (Figures 3A and 4B). We found spLsd1 and spLsd2 to be present at a large number of euchromatic promoters and at the boundary regions of pericentromeric heterochromatin, suggesting that spLsd1 might antagonize H3K9 methylation at both heterochromatic boundaries and euchromatic regions. This hypothesis was further explored.

When the threshold was set at log2Enrichment ≥ 2 (4-fold), we found 93 spLsd1 and spLsd2 co-occupying promoters, spLsd1 and spLsd2 also occupied 277 and 190 unique promoters, respectively (Figure 3B), consistent with the different phenotypes associated with the deletion of *spLsd1* and *spLsd2*. The ChiP-chip results were confirmed by individual ChiP experiments (Figure 3A and data not shown). Some of these direct targets are actively transcribing housekeeping genes, including actin and ubiquitin, as determined by genome-wide expression
profiling experiments (Table S1). Using conventional ChIP, we found that lack of spLsd1 is associated with an increased H3K9 tri-, but not dimethylation, at 4 out of the 5 target promoters examined, but not the coding regions (Figure 3A). Cluster analysis found ~60% of the spLsd1/2 common targets to be downregulated upon lsd1 deletion (Figure 3C), indicating spLsd1 may play a direct role in active transcription. Consistent with this view, we observed reduction of RNA polymerase II (Pol II) occupancy at the target gene loci in the absence of spLsd1 (Figure 3A). Twenty-five of the spLsd1/2 target genes were represented on the spotted microarrays used by Hansen et al. (2005), and six of them were upregulated more than 1.5 x in clr4-681 cells (Table S2). These data suggested that spLsd1 activates transcription of target genes by preventing trimethylation of histone H3K9 and/or by facilitating recruitment of an H3K9 tridemethylase.

We also investigated spLsd1 and spLsd2 function at the subtelomeric region of TEL2L including a cluster of malate permease genes (Figure 4A). Both proteins were highly enriched at the promoters of the malate permease genes and mildly enriched at the surrounding regions. A significantly reduced transcription was associated with the leftmost 13 ORFs of TEL2L including the malate permease genes in splsd1D (Figure 4B). Using conventional ChIP, a significant increase of H3K9me1, 2, and 3 at the promoters of the malate permease genes was also detected in splsd1D (Figure 4C). This increase was accompanied by ~2-fold
increase of Swi6 binding at the promoter regions, and a significant reduction of Pol II occupancy at both the promoter and coding regions (Figure 4C). Interestingly, we did not detect a similar regulation of H3K9me or any enrichment of spLsd1 or spLsd2 at the other subtelomeric regions including TEL1R and TEL1L (data not shown). Finally, we also found increased H3K9me3, but not H3K9me1/2, and increased Swi6 binding in the region surrounding the malate permease genes in splsd1Δ (Figure 4D, lanes 1–4). Consistently, reduced Pol II occupancies were also observed (Figure 4D, lanes 1–4). Collectively, these findings suggested that reduced transcription in the subtelomeric region of TEL2L might be due to a direct action of spLsd1/2 at this euchromatic region.

In addition to direct targets of spLsd1 and spLsd2, expression analysis revealed a number of indirect targets, whose expression was either elevated or reduced in splsd1Δ but which did not bind either spLsd1 or spLsd2. Interestingly, of the 67 genes upregulated by more than 1.5× in splsd1Δ, 46 were also upregulated in Δclr3Δcitr6-1 double mutant strains (Hansen et al., 2005) (Table S3). These genes included noncoding RNA from the dg and dh centromeric repeats, which were investigated further.

spLsd1 and spLsd2 Regulate Pericentromeric Heterochromatin Propagation

Our ChIP-chip analysis detected spLsd1 and spLsd2 at the heterochromatic boundary regions flanking each of the three centromeres (Figure 5A and Figure S2). Specifically, spLsd1/2 was localized to the imr repeats flanking both ends of the centromeric dg-dh repeats but was absent within the silent heterochromatin. These regions contain the tRNA genes, which have been previously reported to act as boundary elements for heterochromatin (Noma et al., 2006; Scott et al., 2006).

The spLsd1 and spLsd2 localization patterns at the heterochromatic regions prompted us to investigate the possibility that spLsd1 and spLsd2 may function together with the boundary elements to regulate heterochromatin propagation. Consistent with this model, the histone methylation profiling showed that the heterochromatin epigenetic marker H3K9me2 was shifted toward the imr region at all three centromeres in splsd1Δ (Figure 5A and Figure S2).

That is, H3K9me2 was reduced over the dg and dh heterochromatic repeats but spread over the imr repeats. Similar H3K9me2 spreading has been shown in an imr tRNAAsu-Δ mutant, in which the barrier is impaired (Scott et al., 2006). Interestingly, this imr tRNAAsu-Δ also displays meiotic chromosome segregation defects, and in combination with the deletion of another imr tRNAAsu, results in cell death (Scott et al., 2006). Thus, it is possible that growth retardation and lethality associated with the loss of spLsd1 and spLsd2, respectively, may be due in part to inappropriate regulation of heterochromatin at the centromeric regions.

We further analyzed H3K9 methylation at the heterochromatin boundaries in splsd1Δ by conventional ChIP. We first confirmed H3K9me2 expansion at imr1 and imr2 regions (Figures 5C and 5E). Interestingly, in addition to H3K9me2, we also detected a significant increase and expansion of H3K9me3, but not H3K9me1, at these regions (Figures 5C and 5E). Consistently, we also detected an elevated level of Swi6 (Figures 5C and 5E), suggesting that heterochromatin components were also spreading over the boundaries as well as H3K9me2/3. Notably, these increases correlated well with spLsd1 and spLsd2 localization at these regions, indicating that they were probably the direct consequence of the loss of spLsd1. Because trimethyl group is not a substrate of the LSD1-like enzymes due to the inherent chemistry (Shi et al., 2004), the increase of H3K9me3 at these regions may be a result of increased H3K9me2. We speculate that upon spLsd1 loss, H3K9me2 demethylation cannot be effectively executed at the heterochromatin boundaries, thus resulting in the accumulation of H3K9me2 and further conversion into H3K9me3 and subsequent Swi6 recruitment. The abnormal expansion of the heterochromatin domain in splsd1Δ suggested that spLsd1 may function as a boundary factor by regulating H3K9 methylation levels.

In contrast to the increased H3K9me at the boundaries, the H3K9me2 level in dg and dh repeats was reduced (Figure 5A and Figure S2). This reduction was also confirmed by conventional ChIP (Figure S3A). Transcripts from this region were elevated (Figures S3C and S3D), but the mature 23 nt small interfering RNA levels remained unchanged, suggesting that these transcripts were elevated at the transcriptional rather than the posttranscriptional level. Similar upregulation of these transcripts was previously observed in clr3Δcitr6-1 double mutant strains (Hansen et al., 2005).

Functional Analysis of Heterochromatin Expansion at the MAT Locus in the splsd1Δ Strain

We further investigated a potential role of spLsd1 in heterochromatin, taking advantage of a strain that carries an Ade6 reporter inserted into the boundary region between REIL and IR-L at the MAT locus (Figure 6A; Shankaranarayana et al., 2003). This region is subject to weak silencing in WT cells, which results in a low level of expression of the ade6 gene, and when cultured in low adenine medium, the parental strain produces a heterogeneous population of white and red colonies. Red color indicates silencing of the ade6 gene, while white color indicates expression of the ade6 gene and thus a loss of heterochromatin-mediated gene silencing. The splsd1Δ strain has a very slow growth rate, making it virtually impossible to carry out the Ade6 reporter assay in this knockout strain. To circumvent this problem, we asked whether a reduction of spLsd1 or spLsd2 protein levels might affect heterochromatin propagation. In the ade6Δ indicator strain, we replaced the endogenous splsd1 or splsd2 promoters with the nmt81 promoter, which allowed for a significantly reduced level of spLsd1 or spLsd2 expression (Figure 6B). Interestingly, the strains with low levels of spLsd1 or spLsd2 showed a decrease in ade6 gene expression as reflected by the appearance of more red colonies (Figure 6B), suggesting an
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A

Centromere II

Relative expr. profile (splsd1 Δ/NWT)

spLsd1-TAP

spLsd2-TAP

H3K9me2, WT

H3K9me2, splsd1 Δ

H3K4me2, WT

H3K4me2, splsd1 Δ

B

C

D

E
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increase in silencing of the ade6+ gene. Consistent with this, conventional ChIP detected elevated H3K9me3 and Swi6 binding at the same IR-L region in splsd1m and ChIP-chip also found spLsd1 localization at both ends of the MAT locus (data not shown).

We next asked whether this gain of silencing was dependent on the histone demethylase activity of spLsd1 and spLsd2. To address this issue, the ade6+ indicator strains carrying the catalytically inactive alleles of splsd1m and splsd2m either singly or together were tested in the silencing assay. As shown in Figure 6C, mutations of the catalytically important lysine residues in spLsd1 (splsd1m) resulted in an enhanced silencing of the ade6+ reporter. Although strains carrying splsd2m did not display significant alterations of the reporter level, strains carrying mutations in both splsd1 and splsd2 (splsd1/2mm) further enhanced silencing, suggesting that spLsd1 and spLsd2 collaboratively regulate heterochromatin boundaries through their demethylase activities. However, the gain of silencing in splsd1/2mm was less than that in the strains with reduced levels of spLsd1 or spLsd2, indicating that the enzymatic independent functions of spLsd1 and spLsd2 are involved in their boundary activities as well. Taken together, our analysis at the centromere and the MAT locus suggested that spLsd1 and spLsd2 function to prevent heterochromatin propagation into adjacent regions, and this function is at least in part if not all through H3K9 demethylation.

DISCUSSION

We have provided a number of insights into functions of LSD1-like demethylases. Importantly, our findings showed that spLsd1 is an H3K9 demethylase, which regulates heterochromatin in fission yeast by preventing spreading of histone H3K9 methylation at heterochromatin boundaries. We demonstrated that spLsd1 also functioned to activate target euchromatic gene transcription, by antagonizing euchromatic H3K9 methylation and by facilitating Pol II recruitment. Interestingly, the H3K9me3 level increase is more significant than that of H3K9me2 at its target sites/promoters in the lsd1+ strain, suggesting that spLsd1 functions to balance H3K9me2 and H3K9me3 levels. Our studies thus uncovered an important function for spLsd1/2 in heterochromatin barrier regulation as well as a direct transcriptional activation role for euchromatic genes.

Figure 6. spLsd1 and spLsd2 Regulate Heterochromatin Silencing at IR-L of the MAT Locus
(A) Schematic diagram of the mating-type region and the position of integrated ade6+ reporter.
(B) Endogenous promoters of spLsd1 and spLsd2 were replaced by the inducible nmt81+ promoter. Cells were streaked on the YE plates and incubated at 30°C for 3 days. The levels of spLsd1 and spLsd2 were monitored by western analysis using an antibody that recognizes the C-terminal TAP tag. Note that reduced levels of spLsd1 and spLsd2 resulted in increased silencing at the IR-L boundary element, as reflected by the red color of the cells.
(C) Same analysis as (B) of the point mutants carrying catalytically inactivating mutations in spLsd1 and spLsd2. The mutants displayed increased silencing at the IR-L boundary element, and the silencing effect was additive in cells carrying mutations in both splsd1 and splsd2 (splsd1/2mm).

spLsd1/2 and Heterochromatin Boundary
Heterochromatic H3K9 methylation is guided by the RNAi machinery, which recruits the histone H3K9 methylase Clr4 and the H3K9 methyl-binding protein Swi6 (Martienssen et al., 2005). Spreading of these modifications from the repeats into the reporter genes depends on readthrough transcription and “slicing” by Argonaute (Irvine et al., 2006), but mechanisms that control propagation outside the heterochromatin boundary are largely unknown. Nine
of the 12 pericentromeric boundary regions have tRNA genes, which are required for boundary function (Scott et al., 2006). TFIIC recognizes the B box regions in the tRNA genes (Noma et al., 2006; Scott et al., 2006). B boxes are also found outside tRNA genes within the inverted repeats that flank heterochromatin surrounding the silent mating type cassettes. Deletion of these B boxes abolishes TFIIC recruitment, resulting in abnormal H3K9me spreading beyond the boundaries (Noma et al., 2006; Scott et al., 2006). These observations suggest that histone modifications at these boundaries are dynamically regulated and important for heterochromatin propagation. This dynamic histone methylation at the boundaries is likely to involve spLsd1/2, based on our findings that spLsd1/2 are specifically enriched at some of tRNA genes located at imr regions of centromeres, and that spLsd1 deletion is associated with the expansion of H3K9me2, H3K9me3, and Swi6 into the imr regions (Figure 5).

The observation that loss of spLsd1 causes H3K9 methylation and Swi6 spreading suggests that a physiological role of spLsd1 may be to block heterochromatin spreading at the boundary. How do spLsd1/2 carry out this function? The simplest model is that spLsd1/2 located at the heterochromatin boundaries mediate demethylation of H3K9me. This is supported by the finding that spLsd1 is an H3K9 demethylase. The inverse relationship that we have identified between spLsd1/2 binding at the boundaries and the expansion of H3K9 methylation is also consistent with this hypothesis. An enzymatic role for spLsd1/2 in this regulation is further supported by our finding that silencing of the Ade6 reporter at the IR-L boundary is enhanced in the spLsd1 and spLsd2 point mutant strains (Figure 6C). An alternative, but not mutually exclusive, possibility is that spLsd1/2 may restrict access of Cir4, either as a consequence of its ability to antagonize H3K9 methylation or via a yet unknown mechanism. However, it should be noted that most likely the biological roles of spLsd1/2 involve both the demethylase activity as well as enzymatic independent functions because (1) cir4Δ only partially suppressed the splsd1Δ growth phenotype, (2) the splsd1Δ/2 catalytically inactive double mutant strain showed only mild growth retardation, and (3) the splsd1Δ/2 catalytically inactive double mutant affected target gene transcription to a lesser extent than that of the deletion mutant (data not shown).

spLsd1 is required for optimal cell growth, but spLsd2 is essential for viability, suggesting important physiological functions for both proteins. Because of the lethality, it was not possible to directly investigate the role of spLsd2 in heterochromatin expansion. However, three lines of evidence are consistent with the possibility that it too is involved in this regulation. First, purification of spLsd1 and spLsd2 demonstrated that most of spLsd1 and spLsd2 in the cell are in the same biochemical complex (Figure 2A). Second, ChIP analysis showed significant colocalization of both proteins (Figure 3B). Third, reduced level of either spLsd1 or spLsd2 had a similar effect on IR-L ade6 reporter transcription, and catalytically critical lysine mutations of spLsd1 and spLsd2 had an additive effect on the reporter expression (Figure 6).

A Role for spLsd1/2 in Activating Gene Transcription
Loss of spLsd1 is associated with a significant decrease of transcription of most euchromatic target genes identified by ChIP (Figure 3C and Table S1), suggesting an activating function for spLsd1/2. The correlation between spLsd1/2 promoter occupancy and transcript levels suggests a direct role of spLsd1/2 in mediating transcriptional activation. Significantly, spLsd1 promoter occupancy is inversely correlated with H3K9me increase, suggesting that spLsd1 antagonizes H3K9 methylation at the target promoters as well. We speculate that loss of spLsd1 resulted in an initial increase in H3K9me2 level, which is converted to H3K9me3 by a yet-to-be-identified histone methylase. The fact that the increase in H3K9me3 is accompanied by a reduced Pol II occupancy at the target promoters suggests regulation of Pol II function by spLsd1/2 in mediating transcriptional activation. In recent reports, profiling of H3K9me2 and spLsd1/2 in the genome of S. pombe has detected enrichment in heterochromatin and euchromatin, respectively, in patterns that are broadly comparable to the ones reported here (Cam et al., 2005; Nicolas et al., 2006). However, the presence of methylated H3K9 in euchromatic regions, and of spLsd1 at heterochromatin boundaries, as well as a role for spLsd1 in regulating heterochromatin propagation and euchromatic gene transcription by antagonizing H3K9 methylation as an H3K9 demethylase, was not detected (Nicolas et al., 2006).

spLsd1/2 and Histone Demethylation
We were able to show that spLsd1 demethylates H3K9me2 using radioactively labeled bulk histones (Figure 2B). However, we were unable to detect demethylation by the highly sensitive mass spectrometry method (Whetsell et al., 2006) using methylated histone peptides, suggesting that spLsd1 may not be active toward peptide substrates. We were also unable to detect demethylation of nonradioactive bulk histones by measuring demethylation using modification-specific antibodies. It is possible that western blot may not be sensitive enough to detect low levels of demethylation as measured by a reduction of the methylation signal. In both cases, we used recombinant spLsd1 purified from bacteria and insect cells or spLsd1 protein complex from S. pombe. This suggests that spLsd1-mediated demethylation reaction was relatively inefficient in the in vitro assays, raising the question of whether the enzymatic activity is regulated. It is also interesting to note that spLsd1 displays H3K9, but not H3K4 demethylase activity, consistent with the observation that it acts primarily as a transcriptional activator in S. pombe. In contrast, the promoters of those genes that appeared to be repressed by spLsd1 are not bound by spLsd1, suggesting that they are indirect targets of spLsd1.

Human LSD1, through its “tower” domain (Yang et al., 2006), interacts with the SANT domain-containing
Co-REST for H3K4 demethylation and transcriptional repression. Unlike LSD1, spLsd1/2 do not have the tower domain and are not associated with any SANT domain-containing proteins, consistent with the fact that spLsd1 is an H3K9 rather than H3K4 demethylase, and that its primary function is activation and not repression. Human LSD1 can also function as H3K9 but only in the presence of the AR (Metzger et al., 2005). Moreover, a recent genome-wide localization study in human MCF7 cells also identified a general activator role of LSD1 at ERα-target promoters in a ligand-dependent manner, which is in part through its H3K9 demethylase activity (Garcia-Bassets et al., 2007). Thus, further investigation of the spLsd1 H3K9 substrate specificity will provide valuable insights into the dual specificity of human LSD1.

In sum, our studies demonstrated that spLsd1/2 regulate both heterochromatin expansion and euchromatic gene transcription in S. pombe in part by antagonizing H3K9 methylation through the H3K9 demethylase activity. In addition, a number of genes and noncoding RNA were upregulated rather than downregulated in the isl1 mutant. These genes are almost certainly indirect targets, as spLsd1 was not associated with them by ChIP-chip, but these genes are almost certainly indirect targets, as spLsd1 does not interact with histone deacetylases for repression in other species.

**EXPERIMENTAL PROCEDURES**

**Strain Construction**

Strains with deletion of splsd1, point mutations in splsd1 and splsd2, C-terminal TAP-tagged splsd1, splsd2, Pht1, and Pht2, and pmnt81-splsd1 and -splsd2 were generated by using a PCR-based module method (Bahl et al., 1998).

**ChIP Analysis**

S. pombe cells were fixed by 1% formaldehyde for 30 min at 30 °C and quenched by 125 mM glycine for 5 min at room temperature. ChIP experiments were carried out as previously described (Verdel et al., 2004) by using antibodies against Swi6 (Abcam, 14988-100), monο-, di-, and trimethylated H3K9 (Upstate 07-450, 09-441, 09-442), and IgG-Sepharose beads (GE Healthcare). Genome-wide mapping of spLsd1, spLsd2, H3K4me2 (Upstate, 07-330), and H3K9me2 was performed according to manufacturer instruction (Nimblegen). Sequences of the primers used in conventional ChIP assays were listed in Table S5. Conventional ChIP results were presented as the means with standard deviations from three independent experiments.

For ChIP analysis in Figures 2A, 3C, and 3D, we used multiplex PCR approach. Briefly, oligo pairs of the test regions were mixed with reference oligo pairs, which were chosen from euchromatic regions that show no transcriptional change in the splsd1 mutant. The absolute signal levels of the experimental as well as the control may vary from experiments to experiments and are dependent on the amount of input material. However, the ratio of the control and the experimental signal should remain relatively constant, since these two PCR reactions occur in the same test tube. Two microliters out of 100 μl recovered ChIP DNA was used for each PCR reaction. Semi-quantitative analyses were achieved by using 32P-dCTP incorporation during amplification, and intensities of amplicons were acquired by phosphor image screen and ImageQuant TL program (Amersham Biosciences). Relative folds of enrichment were calculated as (test-IP/reference-IP)/(test-whole-cell extract [WCE]/reference-WCE). For ChIP analysis in Figures 5C and SE, we used single pairs of oligos in PCR reactions with 32P-dCTP incorporation, and the results were presented as percentage of WCE. The multiplex approach was not used because of the high H3K9me enrichment at heterochromatin boundaries, which resulted in a relative low level of amplification of the reference amplicon, making the internal reference less reliable.

**Microarray Design and Hybridization**

A whole-genome tiling array was implemented using a total of distinct 191,581 55-mer tiles with 65 bp spacing, designed using the forward strand of 2003 S. pombe genome assembly. No Tm matching was performed. Each spot was replicated twice on the array, and random GC content probes were included for quality control purposes. The array platform design can be found at the Gene Expression Omnibus (GEO) at NCBI as array platform GPL4749.

The ChIP samples and input DNA were prepared for microarray analysis by random amplification as described (Lippman et al., 2005). Reference designs including dye reversal were used to examine spLsd1, spLsd2, WT versus splsdJ H3K4me2, and WT versus splsdJ H3K9me2. Briefly, 7 μl of the immunoprecipitated DNA or a 1/100 dilution of input DNA was subjected to two rounds of sequencing primer extension with primer A, and later PCR amplified for 40 cycles with primer B. The PCR product was purified and concentrated to 0.5 μg/μl. Labeling, hybridization, and primary quantification were performed using the previously described microarray platform by Nimblegen.

**RT-PCR and Expression Microarray**

A reference design including dye reversal was used to examine genome-wide expression in splsd1J compared to WT. Total RNA from cells cultured to OD600 at 1.2 was isolated by hot acid phenol and further purified by Absolutely RNA Miniprep Kit (Stratagene). For RT-PCR, cDNA was synthesized by using random N9 primer. For microarray hybridization, cDNA was produced from 10 μg of total RNA using the Superscript II Double-Stranded cDNA Synthesis Kit (Invitrogen) and oligo dT primer. Labeling, hybridization, and primary quantification were performed by Nimblegen.

**Analysis of Microarray Data**

Nimblegen-supplied data files were imported into the Bioconductor computing environment (http://bioconductor.org/). After performing quantile normalization, genotype-dependent transcriptional effects and ChIP enrichments were estimated for each probe by fitting a fixed linear model accounting for array, dye, and genotype effects to the data using the limma package (Smyth, 2004). Moderated t statistics and the log-odds score for differential expression or ChIP enrichment were computed by empirical Bayes shrinkage of the standard errors with the false discovery rate controlled to 0.05. Results of these computations were loaded into a relational MySQL database for subsequent positional analyses and also into a customized version of the
Genetic Genome Browser, which is available for public inspection at [http://chromatin.cshl.edu/demethylases/](http://chromatin.cshl.edu/demethylases/). Files containing per-probe signal estimates are available for public access at [http://chromatin.cshl.edu/supplemental_data/demethylases/](http://chromatin.cshl.edu/supplemental_data/demethylases/). In addition, raw microarray data have been deposited at NCBI GEO as series GSE6753.

To examine the physical distribution of splSld1 and splSld2 relative to transcriptional units, genes were divided into thirds. Promoters were considered to span the transcription start site to 500 bp upstream or the end of the closest gene, whichever was smallest. Average ChiP enrichment values were calculated over the promoter region and the segments of each gene. Per-gene transcriptional response in splSld1 was computed as an average of all probes within the transcriptional unit. Genes with average log2(fold) promoter enrichment for splSld1 and/or splSld2 $\geq$ 2 were considered to be major splSld targets. Exploratory hierarchical and k-means clustering was performed on expression and splSld1/2 distribution profiles using TIGR Multiple Experiment Viewer software (http://www.tm4.org/).

**Complex Purification and Mass Spectrometry**

A total of 5 x 10^{10} cells were used in each TAP purification as described previously (Verdel et al., 2004). Whole protein mixtures were digested in solution and analyzed by LC-MS/MS. Peptides were separated and on-line analyzed on a LTO-F hybrid mass spectrometer (ThermoElectron, San Jose, CA). Peptide matches were filtered to <0.5% false positives using a target-decoy database strategy. Final lists of proteins involved in the various complexes were obtained by subtracting protein matches found also in an untagged control sample.

**Demethylation Assay and Mass Spectrometry**

All histone substrates were radioactively labeled by GST-SET7/9 and GST-Chd4 purified from bacteria. Equal counts of the labeled substrates were used in histone demethylation reactions in the presence of recombinant Flag-splSld1 purified from insect cells or the endogenous splSld1 complex (Tsukada et al., 2006). Mass spectrometry analysis of the reaction products was described previously (Whetstine et al., 2006).

**Reporter Assay**

To monitor Ade6 expression, fresh colonies after growing on rich medium for 3 days (YEA) were streaked on EMM medium with low concentration of adenine (10 mg/l). Plates were incubated at 32°C for 3 days.

**Supplemental Data**

Supplemental Data include three figures, five tables, and Supplemental References and can be found with this article online at [http://www.molecule.org/cgi/content/full/26/1/89/DC1/](http://www.molecule.org/cgi/content/full/26/1/89/DC1/).

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