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Lsd1 and Lsd2 Control Programmed Replication Fork Pauses and Imprinting in Fission Yeast

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SUMMARY

In the fission yeast *Schizosaccharomyces pombe*, a chromosomal imprinting event controls the asymmetric pattern of mating-type switching. The orientation of DNA replication at the mating-type locus is instrumental in this process. However, the factors leading to imprinting are not fully identified and the mechanism is poorly understood. Here, we show that the replication fork pause at the *mat1* locus (*MPS1*), essential for imprint formation, depends on the lysine-specific demethylase Lsd1. We demonstrate that either Lsd1 or Lsd2 amine oxidase activity is required for these processes, working upstream of the imprinting factors Swi1 and Swi3 (homologs of mammalian Timeless and Tipin, respectively). We also show that the Lsd1/2 complex controls the replication fork terminators, within the rDNA repeats. These findings reveal a role for the Lsd1/2 demethylases in controlling polar replication fork progression, imprint formation, and subsequent asymmetric cell divisions.

INTRODUCTION

Haploid *S. pombe* cells exist as two mating types (MTs), P (for plus) and M (for minus), that switch during cell divisions. A chromosomal imprinting event at the MT locus, *mat1*, controls the asymmetric pattern of MT switching in a cell lineage (reviewed in Klar, 2007). It was shown that the polarity of DNA replication of *mat1* is instrumental in the establishment of the imprint (Dalgaard and Klar, 1999) on one of the two sister chromatids and for MT switching during the following DNA replication (Arcangioli, 1998; Figure 1A). One replication termination site (*RTS1*) located on the proximal side of the *mat1* locus, together with the nearby origin of DNA replication *ARS756*, located a few Kbps distal to *mat1*, restrict the DNA replication of *mat1* from its distal side (Dalgaard and Klar, 2001). In this configuration, the second replication pause site (*MPS1*) located on the distal side of *mat1* is optimized and competent for the establishment of imprinting (Klar, 1987; Dalgaard and Klar, 1999).

The imprint is made on the newly synthesized lagging strand during the resumption of DNA synthesis at *MPS1* (Dalgaard and Klar, 1999; Holmes et al., 2005) and has been mapped at the nucleotide level (Nielsen and Egel, 1989). Several *cis*-acting elements and *trans*-acting factors are required for imprint formation (Egel et al., 1984; Arcangioli and Klar, 1991; Kaykov et al., 2004; Sayrac et al., 2011). Four gene products, Swi1, Swi3, Swi7, and Sap1, are necessary for imprinting (Egel et al., 1984; Arcangioli et al., 1994). Swi1 and Swi3 promote imprinting by pausing the replication fork at *MPS1* (Dalgaard and Klar, 2000) and accumulate during S-phase at *MPS1* (Kaykov et al., 2004; Holmes et al., 2005). The Swi1/3 complex stabilizes replication fork blocks at three locations, *MPS1* and *RTS1* at the *mat1* locus and at *RFB*s at the rDNA loci. The stabilized fork at *mat1* promotes gene conversion and subsequent MT switching (Klar, 2007), whereas the stabilized fork at the rDNA maintains the number of copies (Sommariva et al., 2005). It was also shown that *RTS1* is not recombinogenic at its endogenous locus, but, when placed ectopically, it generates replication instability (Lambert et al., 2005; Ahn et al., 2005). Thus, Swi1/3 functions differentially to control the recombinogenic potential of site-specific replication fork barriers (Pryce et al., 2009). The *swi7* gene encodes for the essential large catalytic polymerase α subunit (Singh and Klar, 1993) and Sap1 is an essential gene product important for replication termination (Arcangioli et al., 1994; Mejía-Ramírez et al., 2005; Krings and Bastia, 2005; Zaratiegui et al., 2011).

LSD1/KDM1B is a flavine adenine dinucleotide-dependent lysine-specific demethylase enzyme that represses transcription by demethylating histone H3 (H3K4me1 and H3K4me2) (Shi et al., 2004) and activates transcription by demethylating histone H3 (H3K9me1 and H3K9me2) (Metzger et al., 2005). LSD1 specificity, and therefore downstream function, is dictated by the associated DNA-binding transcription factors. LSD1 also demethylates nonhistone proteins, such as the tumor suppressor

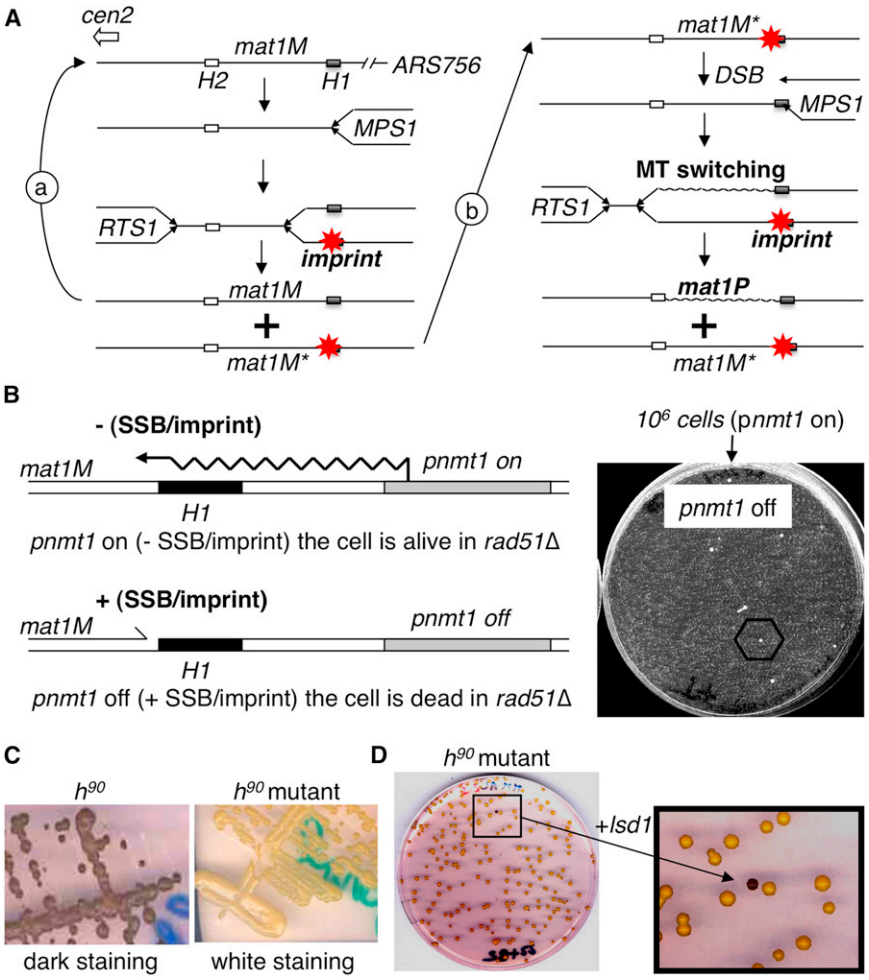


Figure 1. Selection and Complementation of an Imprinting Mutant

(A) Schematic representation of two consecutive rounds of replication at *mat1* starting from a virgin *mat1M* locus (left). The close proximity of *ARS756* with *RTS1*, constrains the polarity of *mat1* replication to ensure optimal imprinting (*) at *MPS1*. Following DNA replication only one of the two *mat1M* loci is imprinted. During the following DNA replication: (a) replication of the virgin *mat1M*, (b) replication of the imprinted *mat1M*, the fork transforms the imprint or single-strand break into a polar one ended double-strand break (DSB) that promotes MT switching (*mat1P*) and the imprint is formed on the unswitched *mat1M* sister locus. The transformation of the imprint into a DSB requires homologous recombination (i.e., Rhp51) for cell survival.

(B) Selection of spontaneous survivors in the selection system. Left: By introducing the thiamine repressible promoter (*pnmt1*) upstream of *mat1*, we can force transcription through the imprinted region, erasing the imprint (-SSB), in a reversible manner (+SSB). Right: 10^6 cells grown under permissive conditions (*pnmt1* on) are plated in the presence of thiamine (*pnmt1* off) and only a few spontaneous mutants can form colonies.

(C) Iodine staining of mutants. By crossing the initial *nbt-18* mutants with the wild-type h^{90} strain, we introduced the mutation in the wild-type background and assayed by iodine staining. The left panel shows the dark staining of the wild-type h^{90} strain, and the right panel shows the white staining of the h^{90} strain carrying the mutant allele.

(D) Functional complementation. The h^{90} strain carrying the mutation (white) is transformed with a genomic library and colonies are assayed for coloration by iodine vapors in order to reveal the complemented cells (black colony in the enlarged view).

See also Table S1.

protein p53 (Huang et al., 2007) and DNA methyltransferase 1 (Dnmt1) (Wang et al., 2009). A second protein related to LSD1 named KDM1B is thought to contribute to the resetting of epigenetic marks in germ cells (Katz et al., 2009; Ciccone et al., 2009). In fission yeast, two histone demethylases, Lsd1 and Lsd2, have been identified within a complex (Nicolas et al., 2006). Lsd1 is required for optimal cell growth, whereas Lsd2 is essential for viability. Lsd1 has been copurified along with Lsd2 and two plant homeo domain finger proteins, Phf1 and Phf2, also essential for viability (Nicolas et al., 2006; Gordon et al., 2007; Opel et al., 2007; Lan et al., 2007). Lsd1 exhibits a weak in vitro H3K9 demethylation activity consistent with a slight in vivo global increase in H3K9 methylation of the *lsd1* catalytically dead mutant. Furthermore, the double catalytically inactive lsd1-ao and lsd2-ao strain is viable, and hence does not fully phenocopy the deletion of lsd1 (or lsd2), strongly indicating a nonenzymatic role for these two proteins (Opel et al., 2007; Gordon et al., 2007; Lan et al., 2007). Finally, Lsd1 acts at boundary elements between euchromatin and heterochromatin in fission yeast and *Drosophila* (Lan et al., 2007; Gordon et al., 2007; Rudolph et al., 2007; Li et al., 2008).

Almost 30 years ago, a genetic screen in *S. pombe* identified switching (swi) mutants (Egel et al., 1984). In this study, we used an inducible MT switching system (Holmes et al., 2005; Roseaulin et al., 2008) to identify new imprinting mutants. Among the imprinting candidates discovered, we found Lsd1. Combining genetic and molecular approaches, we examined which step of the MT switching process Lsd1 intervenes. Our findings are predicted to have important implications for cellular differentiation and development in eukaryotes.

RESULTS

Lsd1: An MT Switching Mutant

We recently showed that the homologous recombination machinery is essential for viability when the replication fork collides with the imprint at *mat1* (Roseaulin et al., 2008). This allowed us to convert the inducible imprinting strain (Holmes et al., 2005) into a conditional strain, by deleting the *rhp51* gene, a critical protein involved in the process of homologous recombination. We reasoned that imprinting mutants, arising spontaneously during permissive growth conditions, would

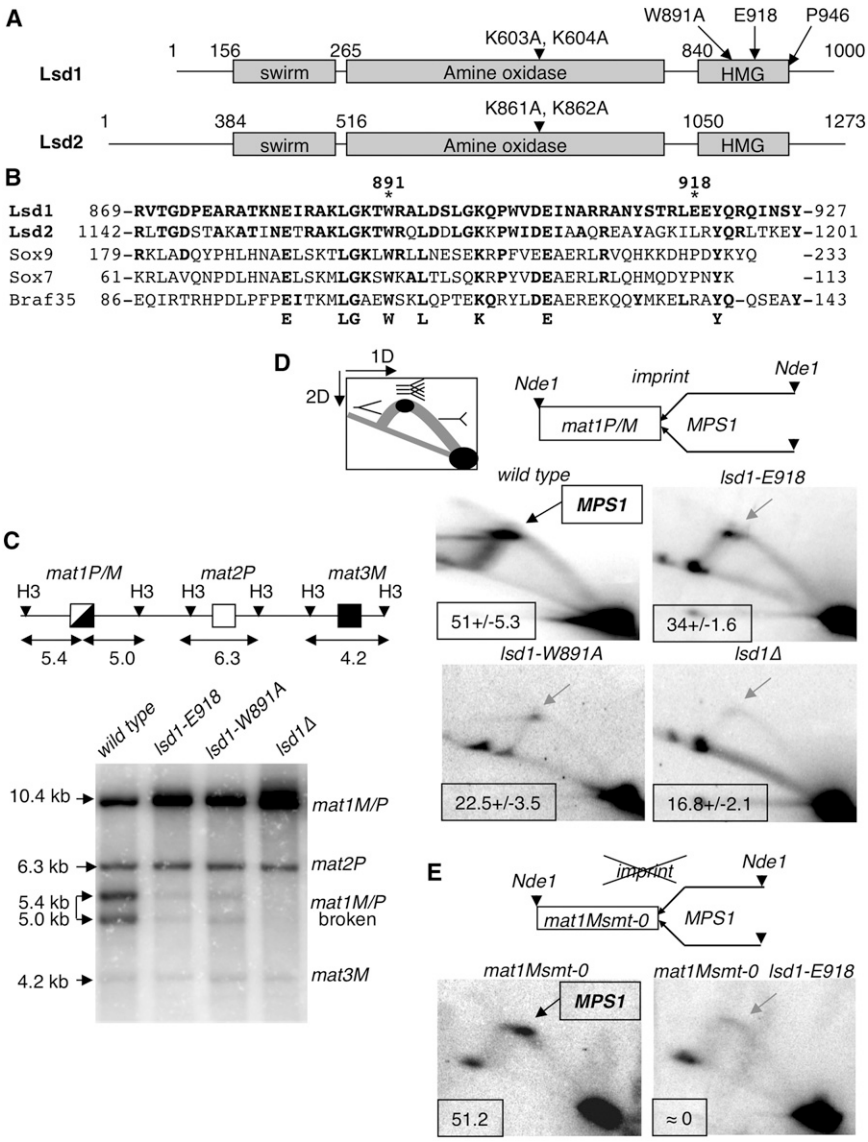


Figure 2. The Lsd1 HMG Domain Is Required for DSB and *MPS1* Activity at *mat1*

(A) Schematic representation of Lsd1 and Lsd2. The protein domains and the mutations in *lsd1* and *lsd2* are indicated.

(B) Alignment of the Lsd1 HMG domain with HMG domains from Lsd2, Sox9, Sox7, and Braf35 from mouse. The conserved residues are indicated.

(C) *lsd1* mutants affect the level of the break at *mat1*. Top panel: Schematic representation of the *mat* region, showing *mat1P/M*, *mat2P* and *mat3M* cassettes. The sizes of the HindIII fragments (H3) are indicated (Kbp). Lower panel, Southern blot of HindIII-digested DNA from the *lsd1*, *lsd1-E918*, *lsd1-W891A*, and *lsd1Δ* strains, hybridized with the labeled *mat1P* HindIII-containing fragment. The *mat1P* probe also hybridizes with the *mat2P* and *mat3M* cassettes. The sizes and identities of the DNA fragments are indicated.

(D) Lsd1 is required for *MPS1* activity at *mat1P/M*. Top panel: diagram of the migration pattern of the replication intermediates detected in 2D gel electrophoresis. The position of the imprint, *MPS1* pause site and the polarity of DNA replication are indicated. DNA replication intermediates at *mat1* from the *wild-type*, *lsd1-E918*, *lsd1-W891A*, and *lsd1Δ* strains are analyzed (lower panel). In the *wild-type* panel, the DNA replication intermediates accumulating at *MPS1* are indicated by an arrow. The percentage of pause is shown for each strain from two to three independent 2D gels.

(E) Lsd1 is required for *MPS1* activity in *mat1Msmt-0* (deletion of 263 bp distal to *mat1*). DNA replication intermediates from the *Msmt-0* and *Msmt-0 lsd1-E918* strains were analyzed and show the decrease of *MPS1* activity in the *lsd1-E918* background.

See also Table S1.

allow the *rhp51Δ* strain to survive, when plated in non-permissive growth conditions (Figure 1B). Using this approach, 100 spontaneous mutants were isolated from 100 independent cultures.

Each mutant was backcrossed with the wild-type (h^{90}, MT switching proficient) strain in order to introduce the mutated allele into the wild-type background for assaying MT switching phenotypes. Our first assay relied on the staining of starch, produced during sporulation with iodine staining (Figure 1C). Using this assay, we observed that all of the mutants exhibited white or streaky iodine staining patterns, indicating a drastic reduction of MT switching and sporulation. As Swi1 and Swi3 were previously characterized as imprinting genes, all of the mutants were individually transformed with plasmids expressing Swi1 or Swi3 (more than 90% of the mutants). The mutant *nbt-18* was complemented neither by *swi1* nor *swi3* and was therefore transformed with a bank of plasmid-containing genomic DNA. Among more than 40,000 white colonies, a single black colony was isolated (Figure 1D) and the partial sequence of the corresponding plasmid-inserted genomic DNA identified the lysine-specific demethylase gene, *lsd1/swirm1* (SPBC146.09c) (Nicolas et al., 2006).

The HMG Domain of Lsd1 Is Essential for Replication Fork Pausing at *MPS1*

Sequence analysis of the *lsd1* gene, from the *nbt-18* strain, revealed a nonsense mutation (GAA into TAA) at position E918 (called *lsd1-E918*) within the high mobility group (HMG/B) domain (Figure 2A). To determine whether the HMG domain or the C-terminal end of Lsd1 is required for function at *mat1*, two mutants were constructed. The *lsd1-W891A* mutation converts tryptophan at position 891 into an alanine and the *lsd1-P946* mutation converts proline at position 946 into a stop codon (Figures 2A and 2B). Subsequently, we replaced the endogenous wild-type *lsd1* gene with these mutated alleles. We found that the *lsd1Δ*-null mutant strain confers white iodine staining along with severe growth defects, whereas *lsd1-E918* and *lsd1-W891A*

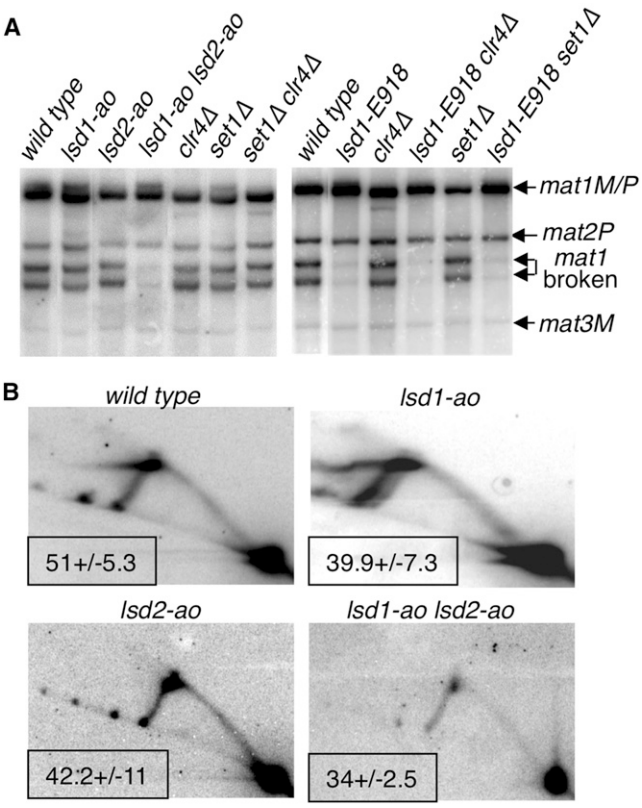


Figure 3. Lsd1/2 Amine-Oxidase Activities Are Required for the Replication Fork Pausing at *MPS1* and Imprinting

(A) Analysis of the DSB at *mat1*. The genomic DNA is analyzed by Southern blot, as in Figure 2. The relevant genotypes of the strains and the sizes and identities of the DNA fragments are indicated.

(B) *MPS1* activity in demethylase mutants. 2D gel analysis of the replication fork intermediates at *mat1*, showing that the accumulating replication material at the apex of the Y-arc is not significantly reduced in single *lsd1-ao* and *lsd2-ao* mutants but is diminished in the double-mutant strain. The gels are treated as in Figure 2 and the relevant genotypes are indicated. The percentage of pause is indicated for each strain from two independent 2D gels. See also Table S1.

confer white staining and mildly reduced growth and *lsd1-P946* cells behave like the wild-type strain. This indicates that the C-ter region is dispensable, whereas the HMG domain is necessary for wild-type functions.

To determine when Lsd1 is required for the MT switching process, we examined the imprint at *mat1*. The imprint (SSB or ribonucleotides) behaves as a fragile site and is transformed into a DSB (breaking the HindIII restriction fragment of 10.4 kb containing *mat1* into two fragments of 5.4 and 5.0 kb, Figure 2C), when the genomic DNA is extracted by the conventional DNA purification procedure (Beach, 1983; Arcangioli, 1998; Dalgaard and Klar, 1999). Southern blot analysis shows that the level of DSB formation at *mat1* is drastically reduced in the *lsd1-E918*, *lsd1-W891A*, and *lsd1*Δ strains compared to the wild-type (Figure 2C). We then examined the same mutants for their role in promoting replication fork pausing at *MPS1* by native 2D gel electrophoresis. In contrast to the prominent pause site induced by *MPS1* in wild-type cells, *MPS1* activity is significantly reduced in *lsd1-E918*, *lsd1-W891A*, and in *lsd1*Δ strains (Figure 2D). We also analyzed the activity of *MPS1* in the *mat1-Msmt-0* strain, which contains a 263 bp deletion of the *cis*-acting elements necessary for imprint formation/maintenance but not for *MPS1* activity. We found that *MPS1* activity in this context was also Lsd1 dependent (Figure 2E). Taken together, these results demonstrate that the HMG domain of Lsd1 is required for replication pausing at *MPS1*, a prerequisite and early step of imprinting.

MPS1 Activity Requires Either Lsd1 or Lsd2 Amine Oxidase Activities

Because a nonenzymatic role for Lsd1 has been proposed (Lan et al., 2007, Gordon et al., 2007), we examined mutant strains, harboring the catalytically dead, *lsd1-ao* or *lsd2-ao*, in which K603 and K604 or K861 and K862 are substituted with alanine residues, respectively (Shi et al., 2004; Lan et al., 2007, Gordon et al., 2007; Figure 2A). We found that the two catalytically dead mutants exhibit DSB and *MPS1* activities similar to the wild-type strain (Figures 3A and 3B), whereas the double mutant exhibits a strong reduction in DSB levels and a more subtle reduction of the *MPS1* levels (Figures 3A and 3B). Taken together, these findings indicate that either Lsd1 or Lsd2 amine oxidase activities are required to control replication fork progression at *MPS1* and imprint formation/maintenance.

Contrary to higher eukaryotes, the fission yeast Set1 and Clr4 methyltransferases are the dominant (if not the only) methyltransferases for H3K4 and H3K9, respectively (Noma and Grewal, 2002; Nakayama et al., 2001; Cam et al., 2005). Therefore, we analyzed the methyltransferase and demethylase single and double-mutant strains for imprinting by Southern blot analysis (Figure 3A). We found that single *set1Δ*, *clr4Δ* and double *set1Δ clr4Δ* mutants exhibit DSB levels similar to the wild-type (Figure 3A), indicating that H3K4 and H3K9 demethylation by Lsd1 and Lsd2 is not involved in imprint formation. Moreover, we found that *lsd1-E918 set1Δ* and *lsd1-E918 clr4Δ* double mutants exhibit similarly low DSB (Figure 3A) and *MPS1* pause levels (data not shown) at *mat1* as compared to the single *lsd1-E918* mutants, indicating that the lysine methylation status at H3K4 and H3K9 is not involved in DSB or imprinting formation.

Lsd1 Is Directly Controlling *MPS1* within the *mat1* Locus

We confirmed that Lsd1-HA- and Lsd2-MYC-tagged proteins are greatly enriched at the imprinted site at *mat1* (Nicolas et al., 2006; Lan et al., 2007; Gordon et al., 2007) but are lost in the *lsd1-HMG* mutant background (data not shown). We reasoned that the dramatic decrease of Lsd1 and Lsd2 enrichment in the context of the *lsd1-HMG* mutations might result from protein instability rather than problems of direct recruitment at *mat1*. Western blot experiments showed that the lsd1-W891A-HA protein is unstable. Similarly, the Lsd2-myc protein in the presence of the *lsd1-E918* mutated allele is strongly destabilized (Figure 4A). Knowing that Lsd2 is essential for cell viability, we infer that the remaining level of Lsd2 in the *lsd1-E918* mutant (Figure 4A) is sufficient to ensure cell viability, but not imprinting at *mat1*. It was previously shown that *lsd1-ao* and *lsd2-ao* single- and double-mutant strains exhibit wild-type

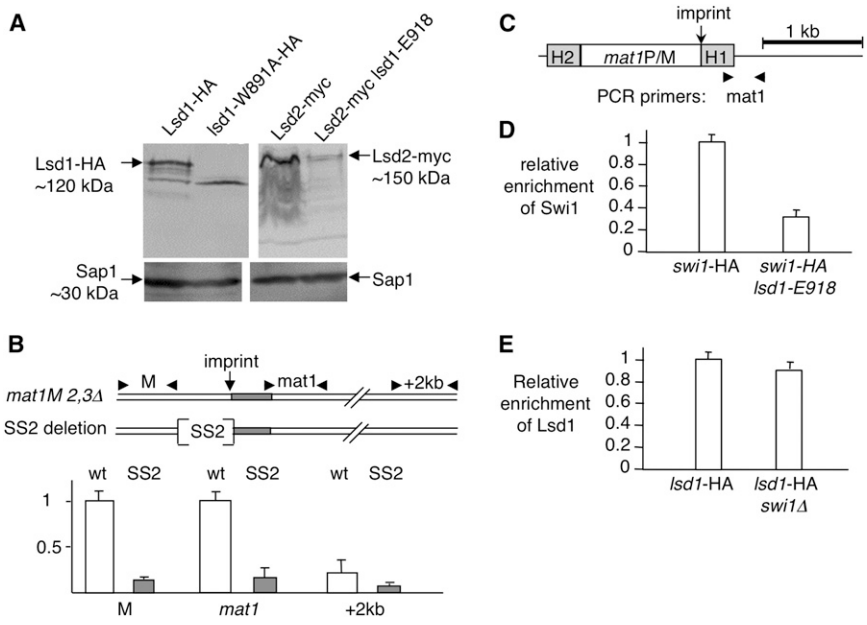


Figure 4. Lsd1 Functions at *mat1* to Promote Replication Fork Pausing at *MPS1*

(A) The HMG domain of Lsd1 stabilizes the Lsd1/2 complex. Western blot of Lsd1-HA- and Lsd2-myc-tagged proteins in WT and HMG mutant strains. Sap1 protein is used as a loading control.

(B) Lsd1 binds to the SS2 *cis*-acting element proximal to the imprint site at *mat1*. Schematic representation of the WT and deletion (SS2: 110 bp) region and primers used for the ChIP experiment are shown. The two sets of primers are localized ~200 bp to the left (M) and ~100 bp to the right of the imprint (mat1). Quantitative PCR analysis of the ChIP for both *wt lsd1-myc* and *SS2 lsd1-myc* strains are shown.

(C) Schematic representation of the *mat1* region and primers used for the ChIP experiment. The PCR primer pair (*mat1*) overlaps the imprinting regulatory region from *mat1*.

(D) Swi1 recruitment is Lsd1 dependent. Quantitative PCR analysis of the ChIP of Swi-HA in the *lsd1*$^+$ and *lsd1-E918* backgrounds. Swi1-HA *lsd1*$^+$ was set to 1.

(E) Lsd1 recruitment is Swi1 independent. Quantitative PCR analysis of the ChIP of Lsd1-HA in the *swi1*$^+$ and *swi1Δ* backgrounds. Lsd1-HA *swi1*$^+$ was set to 1.

See also Table S1.

levels of both proteins (Gordon et al., 2007 and this work, data not shown), indicating that the catalytic activity of both proteins are not necessary for protein stability and that the HMG domain of Lsd1 contributes to the nonenzymatic role of the Lsd1/2 complex.

Another way to directly implicate Lsd1 function at *mat1* is to assay the enrichment of Lsd1 by ChIP on strains containing deletions of *cis*-acting elements required for imprinting. Recently, a small deletion (called SS2) within the *mat1*-M locus has been reported to be important for both the replication fork pause (*MPS1*) and the imprint at *mat1* (Sayrac et al., 2011). Thus, we compared the enrichment of Lsd1 in the wild-type and SS2 mutant backgrounds by ChIP (Figure 4B). Both strains are deleted for the silent donors, *mat2-P* and *mat3-M* cassettes, to avoid removal of the deletions at *mat1* during MT switching and interference during PCR amplification. The *mat2-mat3* deletion strain retains wild-type pausing and imprinting functions (Klar and Miglio, 1986; Dalgaard and Klar, 1999; Roseaulin et al., 2008). The data (Figure 4B) shows that Lsd1 enrichment depends on SS2, indicating that the Lsd1/2 complex is directly controlling replication fork pausing at *MPS1*, hence imprinting through this *cis*-acting element.

Lsd1 Functions Upstream of Swi1 to Control *MPS1* Activity

Consistent with the association of Swi1 with the replication fork, Swi1 accumulates at *MPS1* during S-phase and is released during early G2 of the cell cycle (Holmes et al., 2005). Using the inducible MT switching strain, we found that Lsd1-HA is present at *mat1* before S phase, i.e., before Swi1 accumulation, and remains stably associated with *mat1* during the entire length of the cell cycle (Figure S1). To further investigate the relative order of action of Lsd1 and Swi1 in this process, we analyzed by ChIP-qPCR (Figure 4C) the presence of Swi1-HA at MPS1 in *lsd1-E918* mutant background (Figure 4D) and conversely the presence of Lsd1-HA at MPS1 in the *swi1Δ* mutant background (Figure 4E). We found that Swi1 is not required for the enrichment of Lsd1-HA at *MPS1*, whereas Lsd1 is required for the accumulation of Swi1-HA at *MPS1*, consistent with Lsd1 controlling *MPS1* upstream of Swi1/3 activity.

Swi1 and Swi3 not only stabilize replication forks at *MPS1*, but also are required to preserve genomic integrity when the cells are treated with drugs affecting DNA replication or destabilizing microtubules (Noguchi et al., 2004). We observed (Figure S2) little or no effect for the cells mutated for Lsd1, indicating that Lsd1/2 activity does not simply overlap with all Swi1/3 functions.

Lsd1 and Lsd2 Control the Replication Fork Barriers at *RFB* within the rDNA Repeats

The Swi1/3 complex controls two other known site-specific replication fork barriers in fission yeast *RTS1* located 700 bp proximal to *mat1* and *RFB,* within the rDNA locus (Dalgaard and Klar, 2000; Sanchez et al., 1998; Noguchi et al., 2003). The *lsd1-W891A* and *lsd1Δ* mutants exhibit a significant reduction of the replication fork arrest at *RTS1* and *RFB* as observed by 2D gel electrophoresis analysis (Figure 5B). These results show that Lsd1 controls several site-specific replication pauses, revealing an epigenetic program controlling DNA replication fork progression. We found that Lsd1-HA is significantly enriched at *RFB* but only weakly at *RTS1* by ChIP-qPCR analysis (Figure 5C). As observed at *MPS1*, Swi1 enrichment at *RFB* is severely reduced (≈7-fold) in the *lsd1-E918* background. The result for *RTS1* is intriguing and suggests an indirect role of Lsd1 at this site. To further assess the role of Lsd1 at *RTS1*,

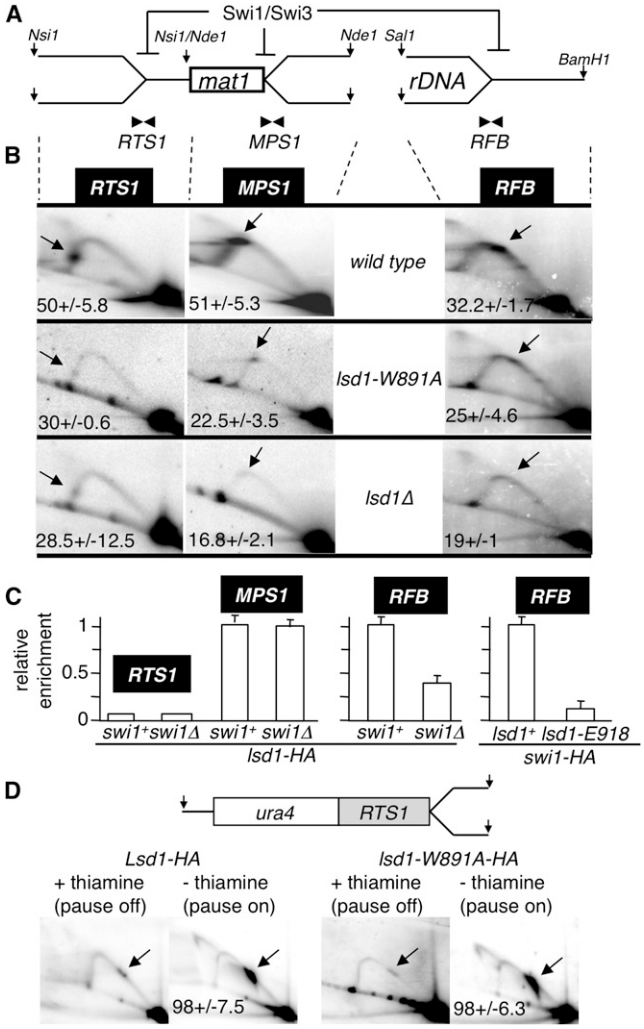


Figure 5. Lsd1 Controls Directly *RFB* and Indirectly *RTS1* Replication Fork Barriers

(A) Schematic representation of the Swi1/Swi3-dependent DNA replication fork pauses and arrests on the proximal (*RTS1*) and distal (*MPS1*) sides of *mat1* and at the rDNA (*RFB*) loci. The restriction enzymes used for genomic digestions are indicated.

(B) 2D gel autoradiograms of *RTS1* (left), *MPS1* (middle), and *RFB* (right) in the wild-type (top), *lsd1-W891A* (middle), and *lsd1Δ* (bottom) strains. The restriction enzymes used are indicated. Arrows show the accumulating DNA replication material at pause/arrest sites.

(C) ChIP analysis of Lsd1-HA at *RTS1*, *MPS1*, and *RFB*. Quantitative PCR analysis of the ChIP of lsd1-HA in *swi1*$^+$ and *swi1Δ* backgrounds using primer pairs close to the pause/arrest sites (*RTS1*, *MPS1*, and *RFB*). On the left panel, Lsd1-HA *swi1*$^+$ was set to 1 at *MPS1* (as a single copy locus), and, on the right panel, Lsd1-HA *swi1*$^+$ was set to 1 at RFB, independently due to the high rDNA copy number.

(D) *RTS1* activity is *lsd1* independent. Schematic representation of the *Ase1* restriction fragment containing the *RTS1* pause at the *ura4* locus on chromosome 3 and 2D gel autoradiograms. *RTS1* activity is "off" by repressing (+ thiamine) and "on" by inducing (− thiamine) expression of the barrier protein Rtf1, as indicated. 2D gels were probed using the *ura4* fragment.

See also Table S1.

we used the ectopic *RTS1* system on chromosome 3, known to induce fork pausing and homologous recombination (Lambert et al., 2005; Ahn et al., 2005). *RTS1* activity is controlled by the expression of the *rtf1* ORF under control of the thiamine repressible promoter *nmt41* (Lambert et al., 2005; Eydmann et al., 2008). We found that in cells harboring the *lsd1-W891A* mutant, ectopic *RTS1* activity is similar to wild-type cells (Figure 5D), ruling out a direct action of Lsd1 at *RTS1*. A simple model is that the lack of *MPS1* activity in the absence of Lsd1 allows the replication fork to replicate through *RTS1,* in a nonactive orientation, prior to the arrival of the converging fork. In this context, the polar terminating *RTS1* activity will appear Lsd1 dependent, but indirectly through *MPS1* inactivation.

DISCUSSION

In this work, we have identified the lysine-specific demethylase 1 (*lsd1*) gene as an MT switching player. We found that Lsd1 and Lsd2 work in a redundant manner, upstream of the Swi1/3 complex to promote replication fork pausing at *MPS1* and imprinting at *mat1*. We further showed that Lsd1 interacts in vivo with the *cis*-acting element, SS2, within *mat1* (Figure 4B), demonstrating its role in the initial pausing step, prior to imprinting. Furthermore, the well-conserved replication fork block (RFB) at the rDNA loci was also found to be under the control of Lsd1/2 in *S. pombe*. Altogether, these results lead to the idea that the histone H3K4 or H3K9 methylation status might control replication fork progression. Intriguingly, the dedicated methyltransferases (Set1 and Clr4) were not involved in this regulation, indicating a novel mechanism for regulating replication fork progression in eukaryotes. Thus, Lsd1 and Lsd2 control *MPS1* pausing activity, the epigenetic initiator event marking the newly replicated lagging strand at the *mat1* locus to ensure asymmetric cell division (Klar, 1987).

In mammals, the LSD1 protein is not fused to an HMG domain, as in *S. pombe*, but instead the LSD1 complex contains an HMG-containing protein called BRAF35 (Hakimi et al., 2002 and Figure 2B). We found that mutation of the HMG domain of Lsd1 significantly decreases the level of Lsd1 and Lsd2 proteins. Similarly, in mammals, it was shown that CoREST recruits LSD1 to chromatin and protects LSD1 from proteasomal degradation in vivo, suggesting that in fission yeast and mammals chromatin-free Lsd1 is unstable (Shi et al., 2005, Perillo et al., 2008).

In addition to Set1 and Clr4 methyltransferases, we further showed that Set6 (unknown target) and Set9 (H4K20) (Sanders et al., 2004) play no significant role in imprinting formation (Figures 3 and S3). Furthermore, we found that a mutation in the JmjC domain of Lid2 (*lid2-j*) does not affect MT switching and Lsd1 recruitment at *MPS1*, indicating that Lid2, a trimethyl H3K4 demethylase (Li et al., 2008), is not involved in the imprinting process at *mat1* (Figure S4). Therefore, the well-characterized methylated lysine targets within histone H3 and H4 do not appear to be required for imprinting at *mat1*.

In *S. pombe*, the imprint requires *MPS1* pausing activity and marks the lagging strand after replication restart. This implies that Lsd1 functions within the chromatin prior to or upon the arrival of the replication fork machinery. Interestingly, it was recently proposed in *Caenorhabditis elegans* that short

truncations within the C-terminal domain of histone H3 prevent efficient nucleosomal assembly on newly replicated DNA in a chromatin assembly factor-1 (CAF-1)-dependent manner (Nakano et al., 2011). This supports the notion that Lsd1 in *S. pombe* could target the C terminus of histone H3 to generate different sister genomes, although any nonhistone proteins involved in replication fork pause, progression, or stabilization at *MPS1* are also potential targets. Primary candidates could be barrier proteins, replicative DNA helicases, the Swi1/3 complex or CAF-1 assembly factor.

EXPERIMENTAL PROCEDURES

Fission Yeast Strains and Genetic Procedures

The *S. pombe* strains used in this study are listed in Table S1. Standard molecular genetic protocols for fission yeast were previously described (Moreno et al., 1991). A C-terminal HA tag of *lsd1* was constructed using the PCR-based gene tagging system (Bähler et al., 1998).

Drop Assays

Cells are grown at mid-exponential phase, diluted, and lawn on YES plate. The incubation was performed 3 days at 32°C.

ChIP Assays

Exponential cultures were fixed with 1% formaldehyde for 15 min. Spheroplastes were sonicated and the chromatin (0.2–0.5 Kbp) was immunoprecipitated by antibodies (anti-HA affinity matrix from Roche and anti-MYC coupled to protein G-Sepharose 4B from Sigma). Recovered DNA was analyzed by PCR, and the amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. Relative enrichments were obtained by first normalizing the ChIP DNA value by the total input DNA value (WCE). The normalized ChIP DNA value of the wild-type background was set to 1 and compared to the normalized ChIP DNA value of the mutants. The ChIP experiments were repeated at least twice, with independent cultures and provided similar results. The error bars were calculated by the software MxPro v4.10 from Stratagene. The sequence of the oligonucleotides is available upon request.

Southern Blot Analysis

Extracted genomic DNA (Moreno et al., 1991) was digested with the restriction enzyme HindIII, separated by 0.8% agarose gel electrophoresis and blotted onto Hybond-N+ nylon membranes. The probes were labeled with alpha-^{32}P and the blot visualized with a phosphoImager.

2D Gel Analysis

2D gel analysis of replication intermediates was carried out as described (Brewer and Fangman, 1988). DNA was prepared and digested, with the indicated restriction enzymes, in agarose plugs (Kaykov et al., 2004). Enriched fractions for replication intermediates were obtained using BND cellulose columns. Gels were blotted on Hybond-N+ nylon membrane. The probes were labeled with alpha-^{32}P and the blot visualized with phosphoImager and quantified with ImageQuant software (pause/Y arc + pause in percentage).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.10.011.

LICENSING INFORMATION

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