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Histone Demethylation Mediated by the Nuclear Amine Oxidase Homolog LSD1

Yujiang Shi,[1] Fei Lan,[1] Caitlin Matson,[1] Peter Mulligan,[1] Johnathan R. Whetstine,[1] Philip A. Cole,[2] Robert A. Casero,[3] and Yang Shi[1,*]

[1]Department of Pathology
Harvard Medical School
77 Avenue Louis Pasteur
Boston, Massachusetts 02115

[2]Department of Pharmacology and Molecular Sciences
Johns Hopkins School of Medicine
Baltimore, Maryland 21205

[3]The Sidney Kimmel Comprehensive Cancer Center
Johns Hopkins School of Medicine
Baltimore, Maryland 21231

Summary

Posttranslational modifications of histone N-terminal tails impact chromatin structure and gene transcription. While the extent of histone acetylation is determined by both acetyltransferases and deacetylases, it has been unclear whether histone methylation is also regulated by enzymes with opposing activities. Here, we provide evidence that LSD1 (KIAA0601), a nuclear homolog of amine oxidases, functions as a histone demethylase and transcriptional corepressor. LSD1 specifically demethylates histone H3 lysine 4, which is linked to active transcription. Lysine demethylation occurs via an oxidation reaction that generates formaldehyde. Importantly, RNAi inhibition of LSD1 causes an increase in H3 lysine 4 methylation and concomitant derepression of target genes, suggesting that LSD1 represses transcription via histone demethylation. The results thus identify a histone demethylase conserved from *S. pombe* to human and reveal dynamic regulation of histone methylation by both histone methylases and demethylases.

Introduction

The histone N-terminal tails are subjected to multiple covalent modifications that affect chromatin structure and consequently transcription. One of the best-characterized modifications is acetylation, which is controlled by both histone acetyltransferases (HATs) and deacetylases (HDACs), suggesting that acetylation regulation is a dynamic process (Kouzarides, 2000). More recently, histone methylation has also emerged as a form of posttranslational modification that significantly impacts chromatin structure (Rice and Allis, 2001; Zhang and Reinberg, 2001). Unlike histone acetylation, which takes places only on lysine (K), methylation occurs on both lysine and arginine (R). While acetylation is generally correlated with active transcription (Roth et al., 2001), histone methylation is linked to both transcriptional activation and repression (Zhang and Reinberg, 2001). For instance, histone H3 K9 (H3-K9) methylation is associated with heterochromatin formation (Nakayama et al., 2001; Peters et al., 2002; Rea et al., 2000) and also euchromatic gene repression (Nielsen et al., 2001; Shi et al., 2003). In the case of heterochromatin assembly, H3-K9 is first methylated by Suv39H, and the methylated K9 is then recognized and bound by the chromodomain protein HP1 (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). The Suv39H-HP1 methylation system is proposed to be responsible for heterochromatin propagation. In contrast, methylation of histone H3 K4 (H3-K4) is linked to active transcription (Liang et al., 2004; Litt et al., 2001; Noma et al., 2001; Santos-Rosa et al., 2002; Schneider et al., 2004), as is methylation of arginine residues of histones H3 and H4 (Zhang and Reinberg, 2001). Mechanisms that underlie methylation-dependent transcriptional activation are not completely understood, although H3-K4-specific methylases have recently been shown to associate with RNA polymerase II (Hamamoto et al., 2004; Ng et al., 2003b).

While histone acetylation is dynamically regulated by HATs and HDACs, histone methylation has been considered a "permanent" modification. At least two models are currently being considered to explain the turnover of methyl groups on histones. The first one suggests that a cell may remove histone methylation by clipping the histone tail (Allis et al., 1980) or by replacing the methylated histone with a variant histone in the case of methyl group turnover at H3-K9 (Ahmad and Henikoff, 2002; Briggs et al., 2001; Johnson et al., 2004). However, this mechanism would not allow for dynamic regulation of histone methylation and the plasticity that may be essential for gene transcription regulation in some biological processes. The second model proposes the existence of histone demethylases that function to remove the methyl groups from lysine and arginine, which would make dynamic regulation possible. Recently, a human peptidyl arginine deiminase, PADI4/PAD4, has been shown to antagonize methylation on the arginine residues by converting arginine to citrulline (Cuthbert et al., 2004; Wang et al., 2004). PADI4/PAD4 catalyzes the deimination reaction irrespective of whether the arginine residue is methylated or not. These findings suggest that histone methylation can be dynamically regulated through the opposing actions of histone methylases and enzymes such as PADI4/PAD4. However, since PADI4/PAD4 catalyzes deimination but not demethylation, it remains unclear whether bona fide histone demethylases exist. The search for histone demethylases began in the 1960s when Paik and colleagues first reported an enzyme that can demethylate free mono- and di-N-methyllysine (Kim et al., 1964). Subsequently, the same investigators partially purified an activity that can demethylate histones (Paik and Kim, 1973, 1974). These early studies suggested the possibility that histone demethylases may exist, but the molecular identities of these putative histone demethylases have remained elusive for the past four decades.

Classical amine oxidases play important roles in metabolism and their substrates range from small mole-

*Correspondence: yang_shi@hms.harvard.edu

cules (e.g., spermine and spermidine) to proteins. More recently, amine oxidases have also been proposed to function as histone demethylases via an oxidation reaction that removes methyl groups from lysine or arginine residues of histones (Bannister et al., 2002). KIAA0601 encodes a protein that shares significant sequence homology with FAD-dependent amine oxidases (Humphrey et al., 2001; Shi et al., 2003). We identified KIAA0601/NPAO as a component of the CtBP corepressor complex (Shi et al., 2003), and it has also been found in a number of other corepressor complexes, including NRD (Tong et al., 1998), Co-REST (You et al., 2001), and subsets of the HDAC complexes (Hakimi et al., 2002, 2003; Humphrey et al., 2001). Recent studies of the *C. elegans* homolog, SPR-5, provided genetic evidence for a role in transcriptional repression (Eimer et al., 2003; Jarriault and Greenwald, 2002). However, its exact role in transcriptional regulation has been unclear.

To understand the function and mechanism of action of KIAA0601, we undertook molecular, biochemical, and enzymological analyses of the protein. Using multiple experimental approaches, we provide evidence that KIAA0601 is a lysine-specific demethylase with substrate specificity for K4-methylated histone H3. We now refer to this protein as LSD1 (Lysine Specific Demethylase 1) to reflect this newly identified role. We show that LSD1 functions as a transcriptional corepressor that participates in the silencing of endogenous neuron-specific genes. Significantly, RNAi knockdown of LSD1 resulted in an increase in H3-K4 methylation and a concomitant derepression of the target genes. These findings suggest a model where LSD1 represses transcription by demethylating histone H3 at K4, whose methylation is linked to active transcription (Liang et al., 2004; Litt et al., 2001; Noma et al., 2001; Santos-Rosa et al., 2002; Schneider et al., 2004). Since LSD1 and its related proteins are present from *S. pombe* to mammals, demethylation is likely an evolutionarily conserved function for this family of proteins. The identification of LSD1 as a histone demethylase indicates that, similar to acetylation, histone methylation is also a dynamic process and is subject to regulation by both methylases and demethylases.

Results

LSD1 Is a Transcriptional Corepressor that Is Evolutionarily Conserved

Figure 1A shows a schematic diagram of the predicted domains of LSD1 and its related proteins. The C-terminal 2/3 of LSD1 displays significant sequence homology with FAD-dependent amine oxidases. The N terminus of LSD1 has a SWIRM domain, which is found in a number of proteins involved in chromatin regulation (Aravind and Iyer, 2002). Although the function of the SWIRM domain is currently unclear, the domain sets LSD1 and its family members apart from the conventional amine oxidases involved in metabolism. By searching for proteins that have both the amine oxidase and the SWIRM domains, we identified a LSD1-like protein AOF1 in human (Figure 1A). In addition, we found three LSD-like proteins in *C. elegans*, one in *Drosophila*, four in *Arabidopsis*, and two in *S. pombe* (Figure 1A). Some members such as SPAC23E2.02 of *S. pombe* contain an additional HMG box, suggesting possible DNA binding activity. The amine oxidase homology region was used for the construction of a phylogenetic tree shown in Figure 1B. Interestingly, LSD1 homologs appear to be absent in *S. cerevisiae*.

Since LSD1 has been found in a number of corepressor complexes (Hakimi et al., 2002, 2003; Humphrey et al., 2001; Shi et al., 2003; Tong et al., 1998; You et al., 2001), we wished to determine whether it plays a direct role in transcriptional repression. We first asked whether LSD1 functions as a repressor when directed to a target promoter. As shown in Figure 1D, when fused to the GAL4 DNA binding domain (G4LSD1), LSD1 repressed G4-TK-Luc reporter gene in a dose-dependent manner. As a control, G4 DNA binding domain alone (G4DBD) had no repressive effect on the same promoter and instead activated the promoter slightly (Figure 1D). Furthermore, G4LSD1 had no effect on TK-Luc reporter lacking the G4 binding sites, suggesting that repression was not due to squelching (data not shown). Importantly, a C-terminal deletion mutant (G4LSD1ΔC) that lacks a large portion of the amine oxidase homologous region (diagrammed in Figure 1C) and is therefore enzymatically inactive (see below) was significantly compromised in its ability to repress transcription, although some residual repression activity was observed for this mutant (Figure 1E). Since repression mediated by LSD1 requires the C-terminal amine oxidase homology domain, the transcriptional function of LSD1 may therefore be linked to its enzymatic activity.

LSD1 Is a Lysine-Specific Histone Demethylase

LSD1 is a flavin-containing protein based on its ability to bind FAD (Humphrey et al., 2001 and data not shown). Its sequence homology with amine oxidases predicts that LSD1 may catalyze oxidation reactions of biogenic amines including monoamine, polyamines, or N-methylated protein substrates (such as histones) (Bannister et al., 2002). Amine oxidation catalyzed by flavin-containing amine oxidases is characterized by oxidative cleavage of the α-carbon bond of the substrate to form an imine intermediate, which, in turn, is hydrolyzed to form an aldehyde and amine via a nonenzymatic process. In a complete catalytic cycle, the cofactor FAD is reduced to $FADH_2$ and then is likely to be reoxidized by oxygen to produce hydrogen peroxide (Binda et al., 2002). We hypothesized that, as a flavin-containing amine oxidase homolog, LSD1 may catalyze the conversion of mono- or dimethylated K (or R) to nonmethylated K (or R) and formaldehyde (Figure 2A). Since LSD1 is a transcriptional corepressor, we further speculated that it might specifically remove methyl groups from lysine (or arginine) whose methylation is linked to active transcription. We chose to focus on H3-K4 methylation since this is one of the best-characterized sites where both di- and trimethylation have been linked to active transcription (Liang et al., 2004; Litt et al., 2001; Noma et al., 2001; Santos-Rosa et al., 2002; Schneider et al., 2004). To investigate this possibility, a histidine epitope-tagged LSD1 (HIS-LSD1) was expressed in bacteria and purified to near homogeneity (Figure 2B). FAD was found to copurify with LSD1 rendering the purified protein yel-

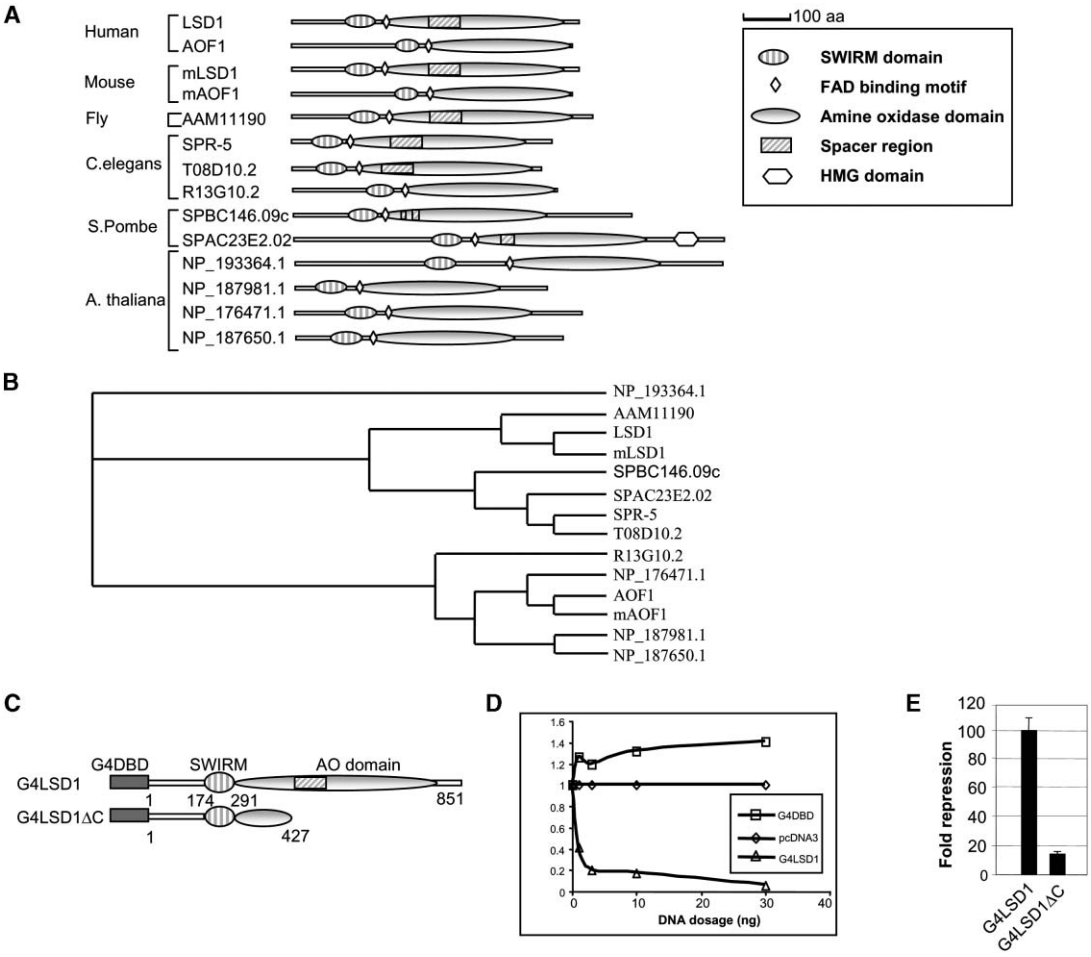

Figure 1. LSD1 Is a Transcriptional Corepressor and Is Evolutionarily Conserved

(A) Diagram of the LSD1-like amine oxidase family members in different species. The deduced amino acid sequences are retrieved from NCBI GenBank and analyzed by the NCBI Conserved Domain Search Program. The SWIRM (oval with vertical stripes), amine oxidase domain (filled oval), and FAD binding motif (diamond) are drawn proportionally. Some family members contain a spacer region in their amine oxidase domain, which is shown by rectangles with diagonal stripes. The *S. pombe* protein SPAC23E2.02 contains an HMG domain (hexagon). NP_187650.1 has been described previously as FLD that is involved in regulating flowering time (He et al., 2003).

(B) Two subfamilies of LSD1-like proteins. The amine oxidase domains of these proteins are classified into two subfamilies based on ClustalW-aligned phylogenetic tree. A noted difference is that the LSD1 subfamily (seven members) contains the spacer region but not the AOF1 subfamily (six members) (except NP_193364.1).

(C) Diagrams of G4LSD1 and the C-terminal deletion mutant G4LSD1ΔC. AO: amine oxidase.

(D) G4LSD1 represses transcription. Various amounts of G4LSD1 and G4DBD plasmids were transfected into HeLa cells together with the G4TK-Luc reporter gene, and their repression activities were analyzed by measuring the luciferase activity. The reporter activity in presence of the pcDNA3 vector (pcDNA3) was designated as 1. The data are the means of two independent experiments.

(E) G4LSD1ΔC mutant is defective in repression. The repression activity was expressed as fold of repression (means ± SD from three independent experiments) relative to the G4DBD control. The repression activity of wt G4LSD1 was arbitrarily designated as 100.

low, which is characteristic of FAD bound proteins (data not shown). The HIS-LSD1 proteins were incubated with histone H3 peptides carrying dimethylated K4 (diMeK4H3) or K9 (diMeK9H3), and the methylation status was determined using a diMeK4H3- or diMeK9H3-specific antibody, respectively. As shown in Figure 2C, even the lowest amount of LSD1 used (lane 2, 1 μg = 10 pmole) effectively reduced dimethylation level at K4 (1 nmole of diMeK4H3) but had no effect on nonmethylated H3 (not shown). This represented approximately 1:100 molar ratio of LSD1 to diMeK4H3, consistent with this being an enzyme-driven reaction. In contrast, LSD1 failed to reduce the dimethylation level at K9 (Figure 2C, panel C2, compare lanes 7 and 8), indicating substrate specificity of this enzyme. The significant reduction of the methylation signal on K4 in the presence of LSD1 was not due to degradation of the diMeK4H3 peptides since LSD1 had no effect on the stability of the H3 peptides (Figure 2C, panel C3, compare lane 1 with lanes 2–4). This putative enzymatic activity is abolished upon heat treatment, which caused protein denaturation, consistent with the possibility that LSD1 was the enzyme responsible for the observed demethylation (Figure 2D, top panel, compare lanes 2 and 3 with lane 1). As a control, FMS1, which is an amine oxidase related to LSD1 in sequence, failed to catalyze the same enzymatic

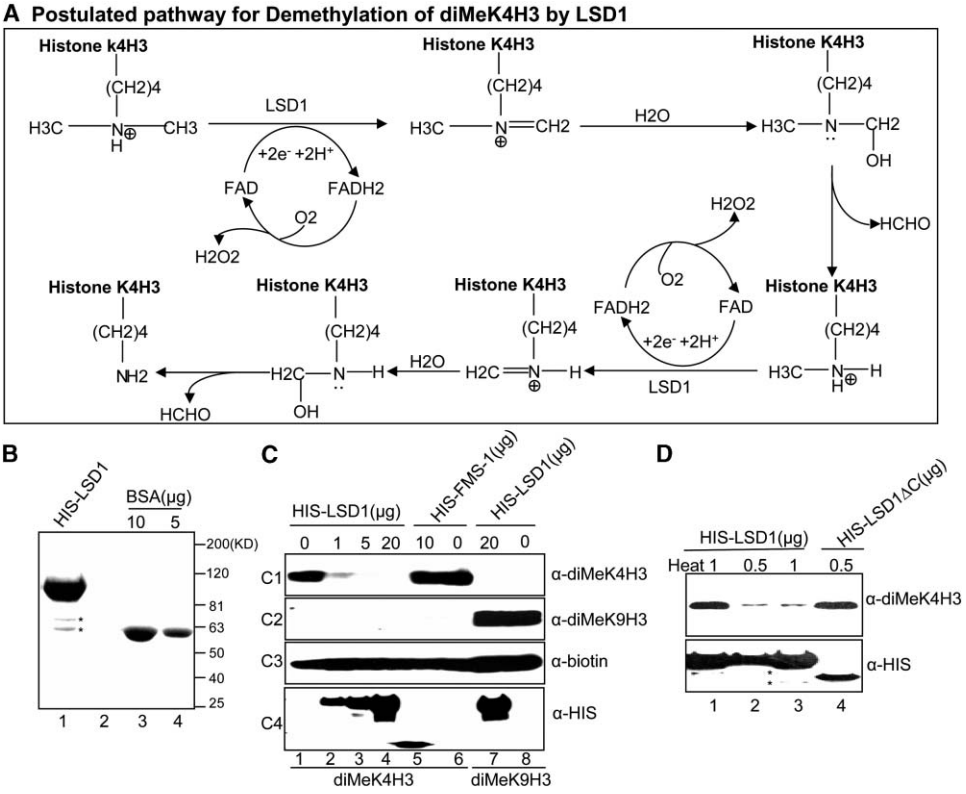

Figure 2. Demethylation of diMeK4H3 Peptides by LSD1

(A) Possible chemical reactions for LSD1-catalyzed demethylation. Only diMeK4H3 is shown, but the proposed reactions are also compatible with monomethylated lysines or methylated arginines.

(B) Purification of HIS-LSD1 from bacteria. Coomassie blue staining of affinity-purified HIS-tagged human LSD1 (HIS-LSD1). LSD1 protein concentration was estimated by comparing with the BSA standard (lanes 3 and 4). Lane1: 10 μl HIS-LSD1, lane 2: empty vector control, lanes 3 and 4: 10 and 5 μg of BSA. Estimated HIS-LSD1 concentration is 3 μg/μl. The asterisk (*) indicates possible minor breakdown products of HIS-LSD1.

(C) Demethylation assay using diMeK4H3 and diMeK9H3 peptides as substrates. Different amounts of purified HIS-LSD1 and HIS-FMS1, a yeast polyamine oxidase, were incubated with diMeK4H3 or diMeK9H3, respectively, and analyzed by Western blot analysis with either a diMeK4H3- or diMeK9H3-specific antibody (panel C1 and C2). The biotin and HIS antibodies were used to detect the histone peptides, which were conjugated to biotin (panel C3), and HIS-LSD1 or HIS-FMS1, respectively (panel C4).

(D) Heat treatment and analysis of the LSD1 C-terminal deletion mutant HIS-LSD1ΔC. Heat treatment at 75°C as well as deletion of aa 428–851 inactivated the enzymatic activity of LSD1 (top panel, compare lanes 1 and 4 with lanes 2 and 3, respectively). The amount of HIS-LSD1 used in the assays was visualized by Western blotting using an anti-HIS epitope tag antibody (lower panel). Asterisks (*) denote possible breakdown products of HIS-LSD1.

reaction (Figure 2C, panel C1, compare lane 5 with lanes 2–4). In contrast, FMS1 has previously been shown to catalyze oxidation of polyamine (Landry and Sternglanz, 2003). Importantly, HIS-LSD1 had barely detectable polyamine oxidation activity, yielding only a 2-fold above background signal, which was about a thousand-fold less active than FMS1 (data not shown). Therefore, LSD1 is likely a histone demethylase but not a polyamine oxidase. Significantly, the same C-terminal deletion mutant LSD1ΔC, which was compromised transcriptionally (Figure 1E), also failed to demethylate diMeK4H3 peptides (Figure 2D, top panel, lane 4), suggesting that LSD1-mediated transcriptional repression may be linked to this potential histone demethylase activity.

We next asked whether LSD1 can mediate demethylation reactions when native histones isolated from HeLa cells were used as substrates. As shown in Figure 3A, wild-type LSD1, but not LSD1ΔC, significantly reduced the signals detected by the diMeK4H3 antibody (panel A1 compare lanes 2 and 3 with lane 1). The same blot was re-probed by a pan H3 acetylation antibody, which detected similar levels of acetylation with or without LSD1 (Figure 3A, panel A2, compare lane 2 with lane 1), suggesting that the loss of the methylation signal was not due to fortuitous degradation of histone H3. We next determined whether LSD1 could catalyze demethylation of histone H3 with either mono- or trimethylated K4, the latter modification being also linked to active transcription. As shown in Figure 3B, while LSD1 reduced the signal representing monomethylated K4 of histone H3, it had no effect on trimethylated K4 (compare panel B2 with panel B3). The inability of LSD1 to convert trimethylated K4 to an unmodified product is likely to be due to the inherent chemistry of the flavin-containing amine oxdases, which requires a protonated nitrogen in the substrates, thus restricting the substrates to mono- or dimethylated peptides (Figure 2A). The modification-specific antibodies used in the above assays were either

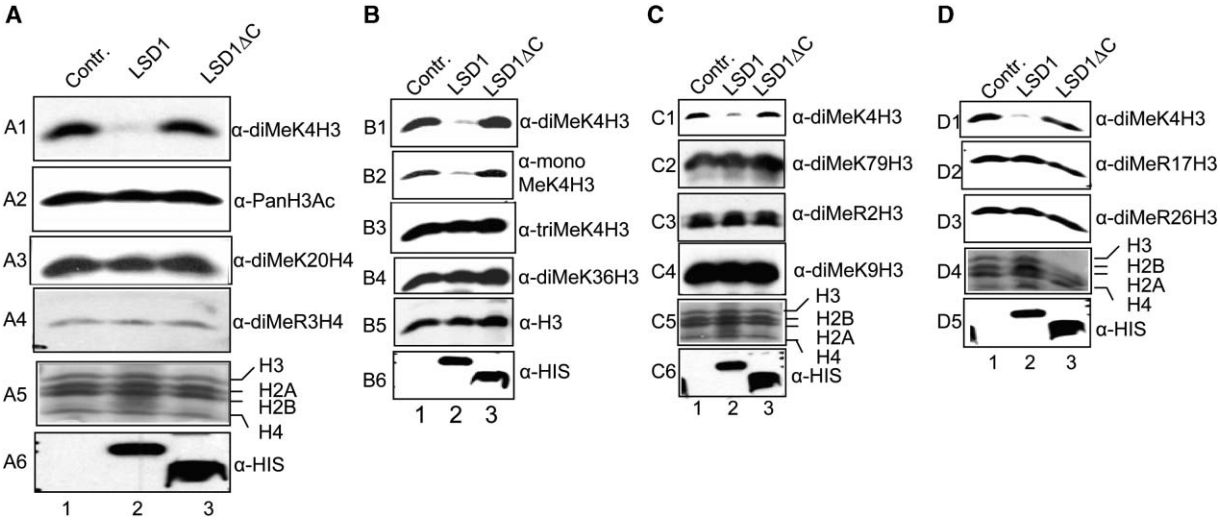

Figure 3. Specific Demethylation at K4 of Histone H3 by LSD1 but Not LSD1ΔC

Native histones were isolated from HeLa cells, incubated with purified HIS-LSD1 or HIS-LSD1ΔC, respectively, and analyzed by Western blotting using antibodies that recognize methylation at specific lysines and arginines. The same blots in (A), (B), (C), and (D) were first probed with the anti-diMeK4H3 antibody and then stripped and reprobed with the antibodies indicated on the right. Equal amounts of histones were used within each set of reactions (Control, LSD1-, or LSD1ΔC-treated) as shown by Ponceau S staining of the blots (panels A5, C5, and D4) or by blotting with a histone H3-specific antibody (panel B5). The amounts of LSD1 or LSD1ΔC used in the demethylation reactions were visualized by an anti-HIS epitope tag antibody (panels A6, B6, C7, and D5). Note the dramatic reduction of the methylation signal detected only by the α-diMeK4H3 antibody (A1, B1, C1, and D1).

commercial antibodies (see Experimental Procedures) or antibodies that have been reported in the literatures (e.g., anti-diMeK79H3 and anti-diMeK20H4 [Feng et al., 2002; Fang et al., 2002]). Additional specificity tests are documented in Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/7/941/DC1/.

To further determine the substrate specificity of LSD1, we examined a number of other amino acid residues on histones whose methylation is likely to be linked to active transcription; these residues included K36 and K79 of histone H3 (Feng et al., 2002; Krogan et al., 2003; Ng et al., 2003a; Schaft et al., 2003), R2, R17 and R26 of histone H3 (Bauer et al., 2002; Chen et al., 1999; Schurter et al., 2001), and R3 of histone H4 (Strahl et al., 2001). We found no difference in the signal intensity detected by Western blotting, in the presence or absence of LSD1, using the modification-specific antibodies designed to visualize methylation at these sites (Figure 3A, panel A4; Figure 3B, panel B4; Figure 3C, panels C2 and C3; and Figure 3D, panels D2 and D3, compare lane 2 with lane 1), suggesting a high level of substrate specificity of this putative enzymatic activity. LSD1 also failed to remove the methyl groups from H3-K9, H3-K27, and H4-K20 (Figure 3C, panel C4; Figure 3A, panel A3; and data not shown), modifications that are linked to transcriptional silencing (Cao et al., 2002, Czermin et al., 2002 #2921; Fang et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002; Nishioka et al., 2002; Rea et al., 2000). Similar to the bacterially purified LSD1, endogenous LSD1 isolated from HeLa cells also displayed the same substrate specificity as the recombinant HIS-LSD1 protein (data not shown). Taken together, these findings support our model that LSD1 functions as a transcriptional corepressor by demethylating sites associated with active transcription but not repression.

To confirm the above results, we turned to mass spectrometry. As predicted by the chemical reaction outlined in Figure 2A, demethylation of a dimethyl-K4 histone H3 by LSD1 is expected to regenerate an unmodified histone H3 with the net loss of 28 Da equal to the molecular weight of 2 CH2. K4- and K9-dimethylated histone H3 peptides were incubated with purified HIS-LSD1, and the reaction mixtures were analyzed by mass spectrometry. As shown in Figure 4, the diMeK4H3 peptide peaked at a molecular mass of 2863 Da as expected. Significantly, upon incubation with HIS-LSD1, but not HIS-LSD1ΔC, a new peak appeared at a molecular mass of 2835 Da, which corresponded to the molecular weight of the unmodified histone H3 peptide (Figures 4B and 4C). As a control, the K9-dimethylated H3 peptides were found to be unaffected by HIS-LSD1 (Figure 4E), consistent with the Western blotting results described earlier. Taken together these findings strongly suggest that LSD1 is a histone demethylase with a substrate preference for methylated K4 over K9 of histone H3.

LSD1-Mediated Histone Demethylation Generates Formaldehyde

We used a third independent method to investigate the possibility that LSD1 is a histone demethylase. As shown in Figure 2A, the demethylation reaction mediated by LSD1 is predicted to generate formaldehyde. To determine whether formaldehyde was produced in LSD1-mediated enzymatic reactions, we first used the formaldehyde dehydrogenase (FDH) assay to detect the presence of formaldehyde (Lizcano et al., 2000). This assay employs formaldehyde dehydrogenase to convert formaldehyde to formic acid using NAD^+ as the electron acceptor (Figure 5A), whose reduction to NADH can be spectrophotometrically measured at OD 340 nm. Thus,

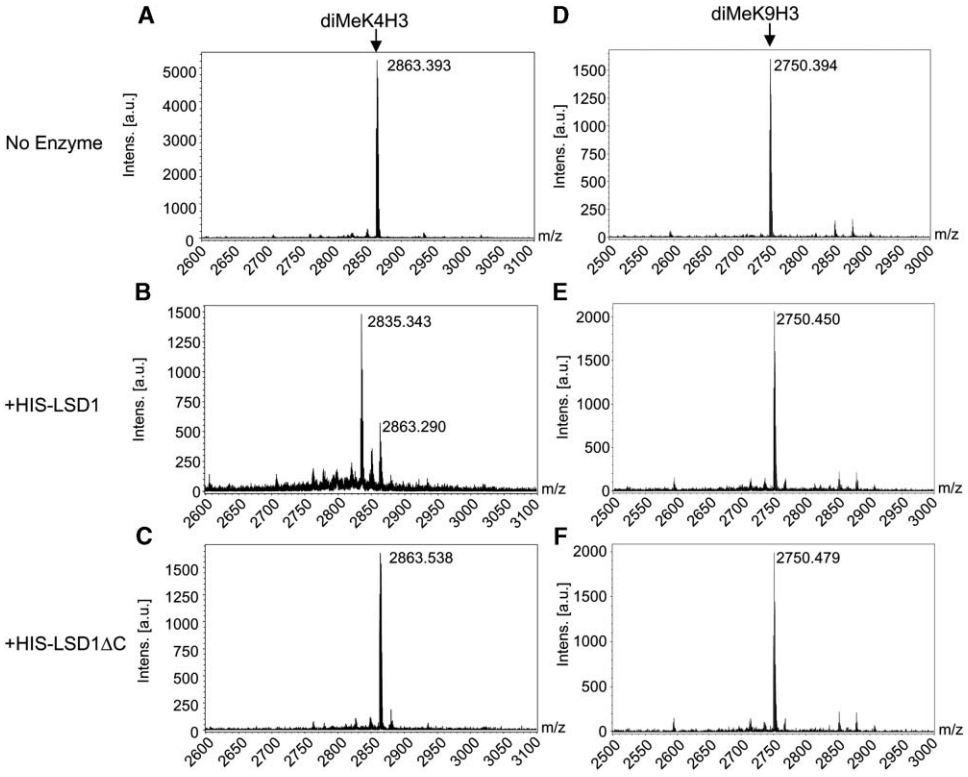

Figure 4. LSD1 Converts diMeK4H3 Peptides to Peptides with Molecular Weight Corresponding to Unmodified H3

Shown are MALDI mass spectrometry results of diMeK4H3 (A, B, and C) or diMeK9H3 peptides (D, E, and F) after incubation with buffer (No Enzyme, A and D), HIS-LSD1 (B and E), or HIS-LSD1ΔC (C and F). Note the appearance of a 2835.343 Da peak in (B) only, which corresponds to H3 peptide removed of two methyl groups from the input diMeK4H3 peptides.

when the demethylation reaction is coupled with the FDH assay, the enzymatic activity of LSD1 and reaction kinetics can be determined by measuring the production of NADH. A standard curve was first generated using purified FDH (EC 1.2.1.46), NAD^+, and different amounts of formaldehyde ranging from 1 pmole to 10 nmole, within which a linear relationship was found between the production of NADH and the range of formaldehyde used in the assay (Supplemental Figure S2 on the *Cell* website). Subsequently, the coupled demethylation-FDH assays were carried out within this linear range and were initiated with the addition of the diMeK4H3 substrates. The continuous production of the formaldehyde as the demethylation proceeded was monitored by OD measurement at 340 nm at different time points. As shown in Figure 5B, a robust increase of absorbance at 340 nm was observed within the first 5 min of the reaction, indicating that substantial amounts of formaldehyde were produced in the LSD1-catalyzed demethylation reaction. The fact that formaldehyde was generated in the demethylation reaction strongly suggests that the reaction had occurred as proposed in Figure 2A. Increasing the amount of either the enzyme (LSD1) or the substrates (diMeK4H3) in the demethylation reaction resulted in a dose-responsive increase in the conversion of NAD to NADH, respectively (Figures 5C and 5D). We next used the demethylation-FDH-coupled spectrophotometric assay as another independent means to investigate the substrate specificity of LSD1. As shown in Figure 5E, only when HIS-LSD1 was incubated with diMeK4H3, but not diMeR2H3 or diMeK9H3, did we detect a robust increase in the absorbance at OD 340 nm, indicating the production of formaldehyde and thus successful demethylation. Furthermore, we failed to detect formaldehyde when triMeK4H3 was used as substrate, suggesting that LSD1 is also unable to catalyze demethylation of the triMeK4H3 peptide (Figure 5F). This result is consistent with the Western blotting assays using modification-specific antibodies shown in Figure 3B.

To further confirm the production of formaldehyde in the LSD1-mediated demethylation reaction, we next used electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS) to detect formaldehyde. The formaldehyde produced in the demethylation reaction was captured by dimedone to irreversibly form the dimedone adduct, formaldemethone, which can be detected by the absorbance at OD 254 nm (Rozylo et al., 2000). The formaldemethone was eluted from an HPLC column and the mass of the formaldehyde derivative was analyzed by LC-MS. Using this assay, we identified formaldehyde in the LSD1-, but not LSD1ΔC-mediated demethylation reaction (Figure 6, compare panel G with panel F). Taken together, mass spectrometry and the FDH assay identified formaldehyde and unmodified histone H3 peptides as the products of the demethylation reaction catalyzed by LSD1.

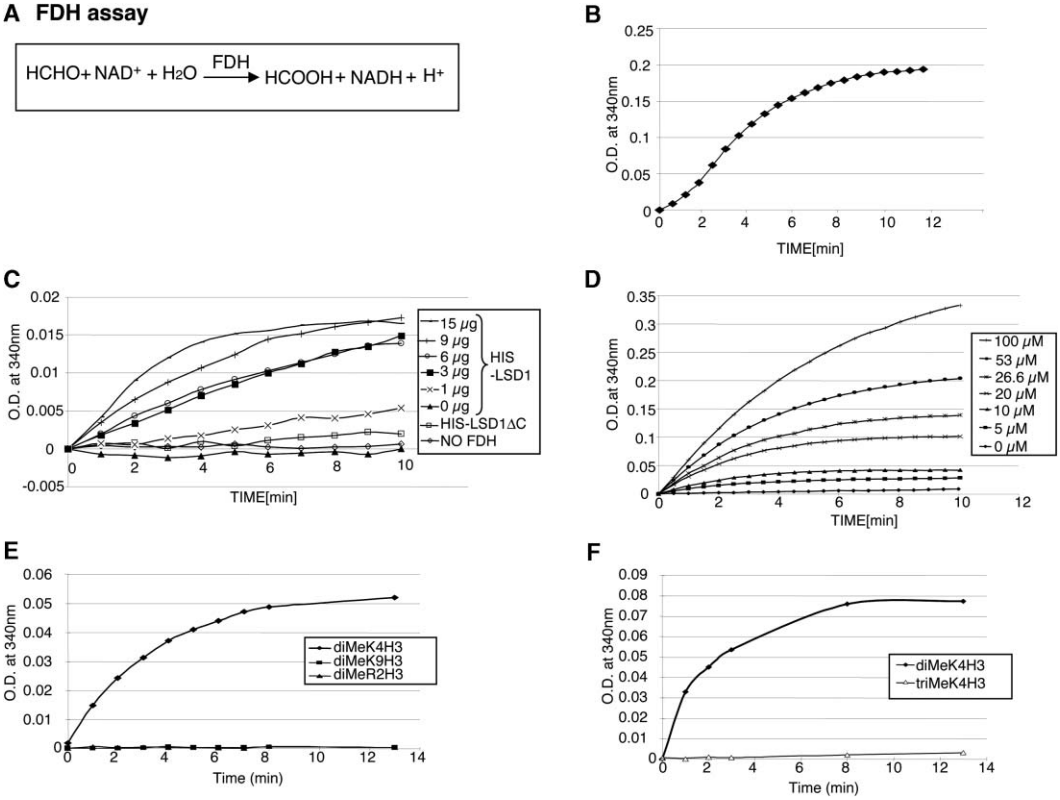

Figure 5. LSD1-Mediated Histone Demethylation Generates Formaldehyde

(A) Chemical reaction for formaldehyde dehydrogenase (FDH)-based formaldehyde detection assay.

(B) Detection of formaldehyde in the demethylation-FDH-coupled assays. The FDH assay was coupled with the demethylation reaction using 15 μg of LSD1 and the substrate diMeK4H3 peptides at an initial concentration of 50 μM. The NADH production was measured at OD 340 nm.

(C) LSD1 dose-dependent formaldehyde production. The demethylation-FDH-coupled assays were carried out using fixed amount of the substrate (diMeK4H3 peptides, 5 μM) but varying amounts of the enzyme LSD1. No LSD1 (0 μg), no FDH, or HIS-LSD1ΔC (15 μg) was used as negative controls.

(D) Demethylation-FDH-coupled assays with fixed amount of the enzyme LSD1 but varying amounts of the substrate diMeK4H3 peptides. The production of formaldehyde was measured with the substrates varying from 0–100 μM. Twenty micrograms of HIS-LSD1 were used in all reactions.

(E and F) Demethylation-FDH-coupled assays to determine LSD1 substrate specificity. (E) Formaldehyde production was monitored by measuring NADH production at OD 340 nm in reactions containing 10 μg of HIS-LSD1 together with 10 μM of diMeK4H3, diMeR2H3, or diMeK9H3 peptides as substrates, respectively. (F) Same assays as in (B) but the substrates were 10 μM of diMeK4H3 or triMeK4H3.

LSD1 Regulation of Endogenous Target Gene Transcription and H3-K4 Methylation In Vivo

We next asked whether native LSD1 regulates endogenous target gene transcription and histone demethylation in vivo. Previous studies identified LSD1 in the Co-REST complex whose primary function is to silence neuronal specific genes in non-neuronal cells (Ballas et al., 2001). A number of Co-REST target genes have been reported including genes that encode the sodium channels (SCNs) and acetylcholine receptors (AchR) (Lunyak et al., 2002). We asked whether these promoters can be derepressed when LSD1 was knocked down by DNA vector-based RNAi (Sui et al., 2002). The *lsd1* RNAi plasmid reduced LSD1 expression efficiently, as judged by immunostaining (Figure 7A) and Western blotting (Figure 7B). Concomitant with the decrease in LSD1 expression, we observed an increase in M4 AchR, SCN1A, SCN2A, and SCN3A expression as determined by RT-PCR (Figure 7C). Derepression of these target genes in the LSD1 knockdown cells indicates that LSD1 is an essential component of the Co-REST complex and is likely to be required for silencing specific neuronal genes in non-neuronal cells. However, LSD1 targets are probably not limited to neuron-specific genes. As shown in Figure 7, we also identified $p57^{KIP2}$, a cyclin-dependent kinase inhibitor (Lee et al., 1995), as a potential LSD1 target gene whose transcription also appeared to be negatively regulated by LSD1. Interestingly, $p57^{KIP2}$ has recently been shown to play a role in developing dopamine cells (Joseph et al., 2003).

We next investigated whether LSD1 regulates histone demethylation in vivo. Using chromatin immunoprecipitation (ChIP), we found LSD1 located at the target gene promoters (within 2 kb of the transcription initiation site) in HeLa or control RNAi-treated cells (Figure 7D, panel D3, lanes 1, 3, 4, 6, 7, 9, 10, 12, 13 and 15), but LSD1 promoter occupancy was significantly reduced in the *lsd1* RNAi cells (Figure 7D, panel D3, lanes 2, 5, 8, 11,

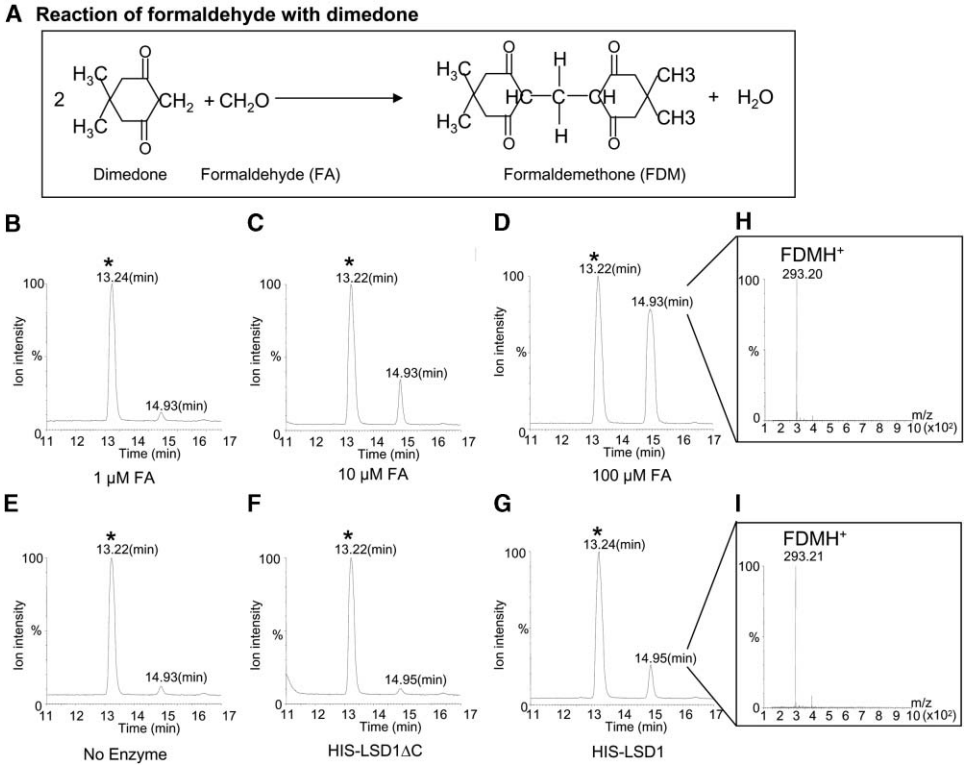

Figure 6. Identification of Formaldehyde in the LSD1-Mediated Demethylation Reaction by ESI-LC-MS Mass Spectrometry

(A) Chemical reaction of formaldehyde (FA) with dimedone to form the dimedone adduct formaldemethone.

(B, C, and D) Selected ion monitoring (SIM) Chromatogram (11–17 min) of formaldemethone (FDM) derived from pure FA at concentration of 1 μM (B), 10 μM (C), and 100 μM (D). The FDM is detected at the 14.93 min peak fraction, which shows dose-responsive ion intensity (compare [B], [C], and [D]). FDM (plus one proton, therefore, $FDMH^+$) was also identified by MS analysis based on its molecular weight (H).

(E, F, and G) SIM Chromatogram (11–17 min) of FDM derived from the demethylation reactions catalyzed by No Enzyme (E), HIS-LSD1ΔC (F), or HIS-LSD1 (G). Based on the peak position for the standard FDM shown above, the 14.95 min peak detected in the LSD1-catalyzed demethylation reaction represents FDM, and its molecular mass (panel I, 293.2 g/mole) further confirmed its identity as $FDMH^+$. The asterisk (*) denotes the nonspecific signal that peaks at 13.24 min in all chromatographic profiles. Note that a significant amount of FDM was detected only in the reaction catalyzed by HIS-LSD1.

and 14). Importantly, concomitant with the decrease of LSD1 occupancy at the target promoters, we observed an increase in H3-K4 dimethylation (Figure 7D, panel D4, lanes 2, 5, 8, 11, and 14) that coincided with the increase in the promoter activity (Figure 7C). Thus, LSD1 promoter occupancy appears to be inversely correlated with promoter activity and H3-K4 dimethylation. Taken together, these findings support the hypothesis that LSD1 regulates histone K4 demethylation at specific loci in vivo, which is correlated with LSD1-mediated repression of target gene transcription.

Discussion

We have provided multiple lines of evidence that support the conclusion that LSD1 is a histone lysine demethylase. These include the direct demethylation assays (Figures 2 and 3); mass spectrometry (Figures 4 and 6); and the demethylation-FDH-coupled spectrophotometric assays (Figure 5) that revealed the demethylation products, i.e., demethylated histone peptides (mass spectrometry) and formaldehyde (FDH and mass spectrometry). We have also shown that LSD1 functions as a transcriptional corepressor and plays an important role in restricting neuron-specific gene transcription in non-neuronal HeLa cells (Figure 7). Importantly, RNAi inhibition of LSD1 resulted in an increase in H3-K4 methylation (Figure 7), which is linked to active transcription, and a concomitant derepression of the target genes, suggesting that LSD1 mediates transcriptional repression via histone demethylation in vivo.

Strikingly, as a histone demethylase, LSD1 displays stringent substrate specificity, which is manifested at two different levels. First, LSD1 is able to distinguish histone H3 peptides with the same type of methylation (dimethylation on lysine) that occurred on different lysine residues (K4 versus K9, K36, and K79). It is possible that the sequences surrounding these lysine residues may contribute to this selectivity. Second, the substrate specificity of LSD1 is further highlighted by its ability to discriminate between di- and trimethylation on the same lysine H3-K4. The inability to demethylate triMeK4H3 is consistent with the chemical nature of the amine oxidation reaction catalyzed by flavin-containing amine oxidases; this reaction requires a protonated nitrogen and thus precludes triMeK4H3 as a substrate (Figure 2A and Bannister et al., 2002). This suggests that triMeK4H3 turnover is accomplished either by histone replacement

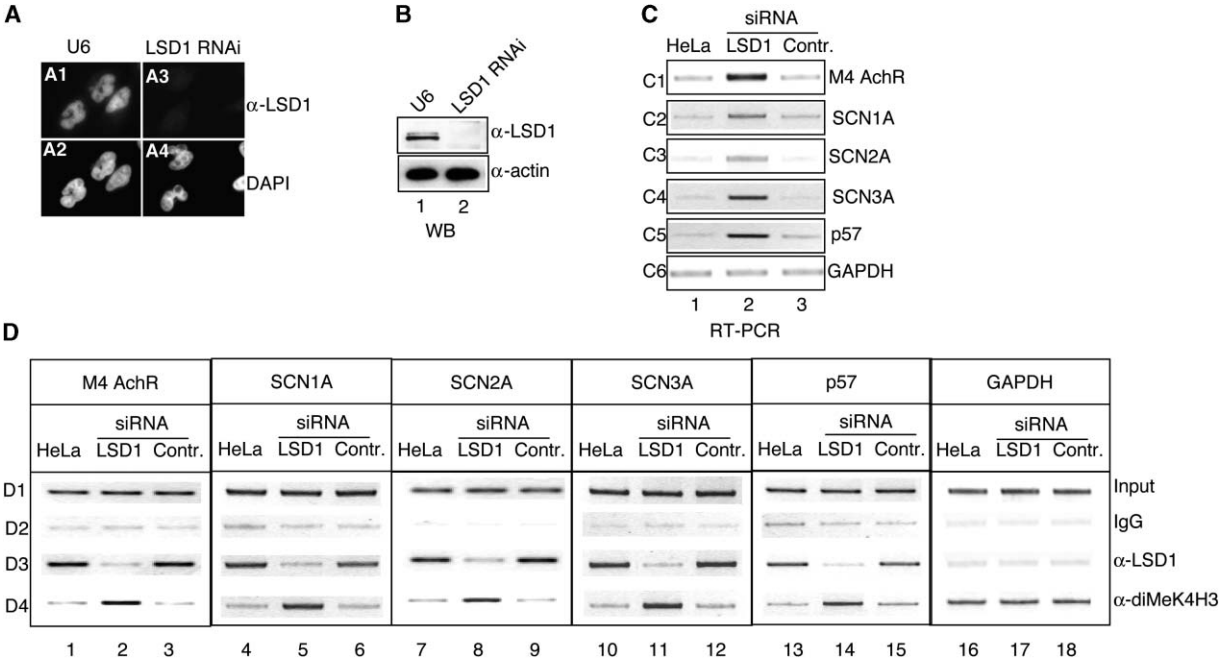

Figure 7. LSD1 Regulation of Endogenous Target Gene Transcription and H3-K4 Methylation In Vivo

(A and B) An *lsd1* RNAi plasmid efficiently knocked down LSD1 expression. (A) Immunostaining of LSD1 protein in HeLa cells transfected with either the U6 control (A1 and A2) or the *lsd1* RNAi plasmids (A3 and A4).

(B) Western blots of LSD1 expression in the control and the *lsd1* RNAi-treated HeLa cells. DAPI staining was used to mark the nuclei of the cells.

(C) Upregulation of neuron-specific and the CDK inhibitor $p57^{KIP2}$ RNA levels in *lsd1* RNAi cells. RT-PCR shows derepression of the neuronal-specific M4 AchR (panel C1) and SCN1A-3A (panels C2–C4) genes as well as the CDK inhibitor $p57^{KIP2}$ (panel C5) in cells where LSD1 expression was inhibited by RNAi. GAPDH was used as a negative control (panel C6).

(D) LSD1 promoter occupancy is inversely correlated with H3-K4 methylation. LSD1 occupancy at the above promoters (+1 to −2 kb) (Figure 7C) was shown by chromatin immunoprecipitation (ChIP) in the *lsd1* and control RNAi-treated HeLa cells (panel D3). The H3-K4 methylation status was analyzed by ChIP using an anti-diMeK4H3 antibody (panel D4). The input and IgG controls were indicated on the right (panels D1 and D2). The GAPDH promoter was used as an additional ChIP control.

or by an unidentified triMeK4H3-specific demethylase. Alternatively, additional mechanisms, such as direct hydroxylation of the methyl groups, may be involved in converting triMeK4H3 to an unmodified product. Our findings further suggest that additional histone demethylases are yet to be identified that would catalyze demethylation reactions at other lysine and/or arginine residues that are associated with either activation or repression of transcription.

Kinetic analysis of LSD1 provided further support that LSD1 is a histone demethylase. The apparent Km for the diMeK4H3 substrates is approximately 30 μM, which is comparable to other histone-modifying enzymes, such as the NAD-dependent histone deacetylase Sir2 (Borra et al., 2004). The actual Km for the demethylation reaction in mammalian cells is likely to be lower since not all purified HIS-LSD1 proteins are expected to be fully active. Possible posttranslational modifications of LSD1 as well as interacting proteins of LSD1 may further enhance its activity in mammalian cells. The fact that the physiological substrates of LSD1 in vivo are nucleosomes may also influence the activity of LSD1, as could other posttranslational modifications on histones. Regardless, these findings provide important kinetic information that substantiates the idea that LSD1 is a histone demethylase.

Another crucial piece of information that supports the conclusion that LSD1 is a histone demethylase is our ability to identify the demethylation reaction products, i.e., formaldehyde and the unmodified histone H3 peptides. Thus we have accounted for the major reaction products during an amine oxidase-mediated demethylation reaction. In this oxidation reaction, the cofactor FAD is likely to be reduced to $FADH_2$ and then reoxidized to FAD by oxygen with the generation of H_2O_2. It will be important in the future to determine the fate of formaldehyde and H_2O_2, which could have potentially deleterious effects when present near promoters. Recently, a significant number of metabolic enzymes and coenzymes have been found to play central roles in regulating gene transcription (Shi and Shi, 2004). Further investigation of proteins such as LSD1 will provide insight into a possible direct link between metabolism and transcription.

Our finding that LSD1 regulates H3-K4 methylation at its target promoters (Figure 7) but not global K4 demethylation (unpublished data) suggests that LSD1 is a locus-specific histone demethylase. However, since LSD1 has been identified in numerous repressor complexes (Hakimi et al., 2002, 2003; Humphrey et al., 2001; Shi et al., 2003; Tong et al., 1998; You et al., 2001), we expect LSD1, much like the HDACs, to play a widespread and central role in establishing repressive chromatin environment as a histone demethylase. We have previously shown that the CtBP repressor complex contains a num-

ber of potential enzymatic activities, including HDACs and HMTases that function coordinately to induce H3-K9 methylation, which is linked to transcriptional repression (Shi et al., 2003). We now show that another component of the CtBP complex, i.e., LSD1/nPAO, demethylates diMek4H3 that is linked to active transcription. Taken together, these findings suggest that the establishment of a repressive environment mediated by the CtBP complex is likely to involve not only the process that confers the repressive modifications (HDACs and HMTases) but also events that erase histone modifications (LSD1) associated with active transcription. This level of complexity is consistent with the histone code hypothesis (Jenuwein and Allis, 2001) and is likely to represent a general principle underlying transcriptional regulation in eukaryotes. Lastly, in addition to H3-K9 methylation, H3-K4 hypomethylation has also been correlated with heterochromatin formation in *S. pombe* (Noma et al., 2001). It would be interesting to determine whether LSD1 homologs play a role in heterochromatin silencing as well as in euchromatic gene repression.

As with any fundamental biological processes, histone demethylation is expected to be conserved through evolution. In support of this hypothesis, we have identified LSD1 orthologs and homologs throughout the eukaryotic kingdom, ranging from *S. pombe* to human (Figure 1). Curiously, LSD1-like proteins appear to be absent in *S. cerevisiae* where histone methylation also plays an important role in chromatin structure and transcriptional regulation. Thus, it is possible that *S. cerevisiae* may have evolved a different strategy to remove methyl groups from histones. Alternatively, different types of enzymes yet to be identified may be involved in demethylating histones in *S. cerevisiae*. In this regard, it is interesting to note that the *S. cerevisiae* genome, as do all the other eukaryotic genomes, has a large number of genes predicted to encode amine oxidases. It is possible that in addition to LSD1 family members, amine oxidases with a different architecture may also function as histone demethylases in *S. cerevisiae* and other organisms. Importantly, our findings documenting an amine oxidase functioning as a histone demethylase lay the foundation for investigation of other amine oxidases as candidates for histone demethylases. It will be exciting to determine if LSD1-related proteins and other types of oxidases function as histone demethylases with different substrate specificities to impact chromatin structure and gene transcription. Given our finding that histone demethylases exist, it will also be exciting to explore other types of enzymes that are also predicted to convert methylated peptides (such as histones) to unmethylated products (Chinenov, 2002).

Finally, recent studies provided a potential important connection between methylation at H3-K4 and cancer. The trithorax group protein MLL, which methylates H3-K4, is found to be frequently involved in chromosomal translocation in both acute lymphoid and myeloid leukemia (Ayton and Cleary, 2001). Another H3-K4 histone methylase, SMYD3, has been shown to be upregulated in colorectal and hepatocarcinoma cells (Hamamoto et al., 2004). Overproduction of SMYD3 increases cell proliferation dependent on the histone methylase activity, consistent with the possibility that SMYD3 is a candidate oncogene (Hamamoto et al., 2004). These findings support the hypothesis that H3-K4 methylation regulation may play a crucial role in tumorigenesis. With the identification of LSD1 as a H3-K4 demethylase, we are now poised to investigate if LSD1 or related histone demethylases play a role in cancer, and if so, whether the demethylase activity is essential for this regulation.

Experimental Procedures

Peptides, Histones, Antibodies, and Chemical Reagents

Synthetic histone peptides with specific modifications as well as antibodies (Ab) that recognize different histone modifications were purchased from either Upstate Group, INC (Lake Placid, New York) (UP) or Abcam Ltd (Cambridge, United Kingdom) (Ab). They are diMeK4H3(1–21 aa) (UP12-460), diMeK9H3 (1–21 aa) (UP12-430), H3 (1–21 aa) (UP12-403), PanH3Ac (1-21 aa) (UP12-402), anti-diMeK4H3 Ab (UP07-030), anti-diMeK9H3 Ab (UP05-768), anti-panH3Ac (UP06-599), anti-monoMeK4H3 Ab (UP07-436), anti-H3 Ab (UP06-755), anti-diMeR2H3 Ab (Ab8046), anti-diMeR3H4 (UP07-213), anti-diMeK79H3 Ab (UP07-366), anti-diMeR17H3 (UP07-214), anti-diMeR26H3 (UP07-215), and triMeK4H3 (Ab1342). Anti-diMeK36H3 and Anti-diMeK20H4 antibodies were gifts from Y. Zhang. Bulk histones were either purchased from Sigma (catalog # H9250) or isolated from HeLa cells according to the protocol provided by Upstate. Formaldehyde dehydrogenase (EC1.2.1.46) purified from *Pseudomonas putida* was purchased from Sigma (F1879). Purified recombinant yeast polyamine oxidase FMS1 was a kind gift from Dr. Rolf Sternglanz.

Protein Expression and Purification

Full-length (1–851 aa) and C-terminal deleted (1–427 aa) human LSD1 cDNAs were cloned into N-terminal 6× HIS-tag bacterial expression vector pET15b. The plasmids were transformed into bacteria and expression of the recombinant proteins was induced by 0.2 mM IPTG at 37°C for 6 hr. The HIS-tagged proteins were purified by Ni-NTA affinity column (Qiagen, Valencia, California). After washing the column, the bound proteins were eluted from the column by 200 mM imidazole. The eluate was then extensively dialyzed in PBS with changes three times at 4°C. The homogeneity and concentration of the protein were estimated on SDS-PAGE by Coomassie blue staining using BSA as standard.

Demethylase Assay

Bulk histones or histone peptides were incubated with purified HIS-LSD1 or HIS-LSD1ΔC in the histone demethylase activity (HDM) assay buffer 1 (50 mM Tris pH 8.5, 50 mM KCl, 5 mM MgCl, 0.5% BSA, and 5% glycerol) from 30 min up to 4 hr at 37°C. For a typical reaction, the volume of the reaction is 100 μl, in which either 20 μg of purified bulk histones or 3 μg of modified histone peptides were used as substrates. Different amounts of HIS-LSD1 ranging from 1–20 μg were used in the reaction. The reaction mixture was analyzed by SDS-PAGE/Western blotting using methyl-specific antibodies, or by formaldehyde formation assay to examine the removal and conversion of the methyl group to formaldehyde, or by mass spectrometry to identify the demethylated peptide.

MALDI Mass Spectrometry (Matrix-Assisted Laser Desorption/Ionization Mass Spectroscopy)

Two microliters of the 100 μl demethylation reaction mixture was desalted by passing through a C_{18} ZipTip (Millipore). Prior to desalting, the ZipTips were activated and equilibrated using 10 μl of 50% acetonitrile/0.1% TFA (2×), followed by 10 μl of 0.1% trifluoroacetic acid (TFA) (3×). The reaction mixture was then loaded onto the activated ZipTips. The ZipTips were washed with 10 μl of 0.1% TFA (5×), and the bound material was eluted from the ZipTip using 2 μl of 70% acetonitrile containing 1 mg/ml α-cyano-4-hydroxycinnamic acid MALDI matrix and 0.1% TFA. The eluates were spotted onto a circle of open MALDI target areas to allow solvent evaporation and peptide/matrix cocrystallization. The samples were analyzed by a MALDI-TOF/TOF mass spectrometer (Ultraflex, Bruker Daltonics, Billerica, Massachusetts) at the PFPC core facility of Department of Pathology, Harvard Medical School.

Formaldehyde Dehydrogenase Assay

Formaldehyde formation was continuously monitored by a coupled spectrophotometric assay (Lizcano et al., 2000) using FDH. HIS-LSD1 was first incubated in buffer containing 50 mM potassium phosphate, pH 7.2, 2 mM NAD^+, and 0.1 U FDH (100 μl reaction volume) at 37°C for 5 min without substrates. The demethylation-FDH-coupled reaction was initiated by the addition of the substrates. The absorbance at 340 nm ($\epsilon_{340} = 6.22\ mM^{-1}cm^{-1}$ for NADH) was measured at each time point in a 0.5 min interval using Beckman DU640 spectrophotometer. The OD 340 nm absorbance at the moment of the substrate addition was considered as 0 and this was used as the 0 min time point. Over a 10 min period, a kinetic software program automatically recorded the absorbance at each time point. The data were analyzed using the Excel program. Standard curves were obtained using various concentrations of formaldehyde diluted from 37% formaldehyde solution (Fisher). K_m and V_{max} values for the purified LSD1 catalyzing demethylation of the diMeK4H3 substrates were estimated using Lineweaver–Burk transformation of the Michaelis-Menten kinetic equation.

ESI-LC-MS standard formaldehyde or formaldehyde (FA) produced in the demethylation reaction was converted to formaldemethone (FDM) by the addition of dimedone, which has a strong absorbance at OD 254 nm and an increased mass suitable for MS detection. In a demethylation assay, 10 μg of enzyme and 15 μg of diMeK4H3 peptide were used in a 100 μl demethylation reaction. To convert FA to FDM, dimedone was added to the demethylation reaction (500 μl final volume with final concentration 0.0125%). For detection of FDM, samples were subjected to a reverse-phase high-pressure liquid chromatography (HPLC) system (Agilent 1100) equipped with an analytical column (Waters Symmetry C18, 2.1 × 50 mm) at a flow rate of 0.4 ml/min. The HPLC system was directly coupled to a LCT mass spectrometer (MS) (Waters/Micromass). Analysis was performed in positive-ion electrospray (ESI) mode with acquisition across a mass range of 100 to 1000 Da. The FDM were identified by the presence of a unique ion having a mass to charge ratio of 293.2, corresponding to the calculated molecular mass, with the addition of a single proton $(M+H)^+$.

RT-PCR

Total RNA samples were isolated from 2×10^6 cells by Trizol reagent (Sigma). After DNase treatment, the RNA samples were purified by phenol-chloroform extraction and ethanol precipitation. Thirty-eight PCR cycles were used for SCN1A, SCN2A, SCN3A, and M4 AchR and 28 PCR cycles for GAPDH. Primers used in RT-PCR were as follows: SCN1A up (5′-gcgaaatagcagaacaagcc-3′), down (5′-ctcat tgctcgttgcctttg-3′); SCN2A up (5′-gatgaggatgatgaaaatggc-3′), down (5′-ctaattttctaatagggttgaaggg-3′); SCN3A up (5′-caccacttcctacttt aatggca-3′), down (5′-aaatagagacaggaaagcccag-3′); $p57^{KIP2}$ up (5′-ggcgatcaagaagctgtcc-3′), down (5′-caccttgggaccagtgtacc-3′); GAPDH up (5′-gaaggtgaaggtcggagtc-3′), down (5′-gaagatggtgatgggatttc-3′).

Chromatin Immunoprecipitation Analysis

ChIP assays were carried out in IP buffer without SDS due to the sensitivity of the LSD1 antibody to SDS. Briefly, 3×10^7 cells were used per LSD1 ChIP and 3×10^6 cells per H3K4diMe ChIP. After 10 min of 0.75% formaldehyde treatment, cells were harvested and sonicated in the ChIP lysis buffer (1% Triton X-100, 10 mM EDTA, 50 mM Tris-HCl and protease inhibitors) to produce soluble chromatin with average sizes between 300 and 1000 bp. The chromatin samples were then diluted 10-fold in the dilution buffer (5 mM EDTA, 25 mM Tris-HCl, 167 mM NaCl, and cocktails of protease inhibitors) and pre-cleaned for 1 hr using salmon sperm DNA/protein-A agarose beads. Ten micrograms of rabbit anti-LSD1, 3 μl of anti-H3K4diMe, or control antibodies were then added to each sample and incubated overnight at 4°C. To collect the immunocomplex, 40 μl of salmon sperm DNA/protein-A agarose beads were added to the samples for 1 hr at 4°C. The beads were washed three times in the wash buffer 1 (0.1% Triton X-100, 5 mM EDTA, 30 mM Tris-HCl, 150 mM NaCl) and one time in wash buffer 2 (1% Triton X-100, 5 mM EDTA, 30 mM Tris-HCl, 300 mM NaCl). The bound protein-DNA immunocomplexes were eluted with 100 μl elution buffer (1% SDS, 0.1 M NaHCO3, 250 mM NaCl, and 0.2 μg/μl protease K) and de-crosslinked at 65°C for 4 hr. The de-crosslinked chromatin DNA was further purified by QIAquick PCR Purification Kit (Qiagen) and eluted in 100 μl TE buffer. Four microliters of eluted DNA sample was used for each PCR reaction. Thirty-six PCR cycles were used for LSD1 ChIP and 32 PCR cycles for H3K4diMe ChIP. Primers used for amplifications were as follows: M4 AchR forward (5′-gaacagaacacctccct cca-3′), reverse (5′-gagtcagaaggcaggacagg-3′); SCN1A forward (5′-taaagcccagtcaagacagc-3′), reverse (5′-gacacacccagaagatggag-3′); SCN2A forward (5′-cgtgtttcaaggctacagca-3′), reverse (5′-ctctagcct cccaaccttcc-3′); SCN3A forward (5′-ctctgtcacagggaggaaag-3′), reverse (5′-agactagagcaggccacaag-3′); $p57^{KIP2}$ forward (5′-ccgtggtg ttgttgaaactg-3′), reverse (5′-tgtccggtggtggactcttc-3′); GAPDH forward (5′-tcctcctgtttcatccaagc-3′), reverse (5′-tagtagccgggcccta cttt-3′).

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