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Human LSD2/KDM1b/AOF1 Regulates Gene Transcription by Modulating Intragenic H3K4me2 Methylation

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SUMMARY

Dynamic histone H3K4 methylation is an important epigenetic component of transcriptional regulation. However, most of our current understanding of this histone mark is confined to the regulation of transcriptional initiation. We now show that human LSD2/KDM1b/AOF1, the human homolog of LSD1, is an H3K4me1/2 demethylase that specifically regulates histone H3K4 methylation within intragenic regions of its target genes. Genome-wide mapping reveals that LSD2 associates predominantly with the gene bodies of actively transcribed genes, but is markedly absent from promoters. Depletion of endogenous LSD2 results in an increase of H3K4me2 as well as a decrease of H3K9me2 at LSD2-binding sites and a consequent dysregulation of target gene transcription. Furthermore, characterization of the LSD2 complex reveals that LSD2 forms active complexes with euchromatic histone methyltransferases G9a and NSD3 as well as cellular factors involved in transcription elongation. These data provide a possible molecular mechanism linking LSD2 to transcriptional regulation after initiation.

INTRODUCTION

The interplay and dynamics of histone modifications govern the structural diversity of chromatin and the accessibility of DNA, thus representing an important epigenetic mechanism underlying regulation of eukaryotic gene transcription (Goldberg et al., 2007; Jenuwein and Allis, 2001; Strahl and Allis, 2000). Many studies have addressed how histone modifications are established and maintained at gene promoters to regulate transcription initiation. However, once transcription initiation events commence, RNA polymerase II (Pol II) can either continue forward with productive elongation, as is the case for constitutively active genes, or demonstrate promoter-proximal pausing, depending on gene context, cellular demands, or environmental stimuli (Core and Lis, 2008; Sims et al., 2004). Several regulatory mechanisms converge at this stage, including the activity of negative and positive elongation factors. While the negative elongation factor complex (NELF) functions by effectively stalling Pol II, positive transcription elongation factor b (P-TEFb), comprised of CDK9 and cyclin T1, phosphorylates the C-terminal domain of Pol II, NELF, DSIF, and other targets, thereby promoting productive elongation (Brès et al., 2008; Peterlin and Price, 2006).

Although the functional role and dynamic regulation of histone modification during active elongation remain largely unknown, increasing evidence indicates a distinct epigenetic contribution to postinitiation transcriptional regulation. Both NELF and P-TEFb have been reported to play important roles in coordinating cotranscriptional histone modifications, such as histone acetylation, H3K4 and H3K36 methylation, and H2B ubiquitination during Pol II elongation (Pirngruber et al., 2009). Further, studies ranging from yeast to humans have suggested that several histone-modifying enzymes are associated with elongating Pol II, functioning to control proper histone methylation within coding regions for productive transcription or mRNA processing. For example, in yeast, SET2 associates with elongating Pol II and mediates cotranscriptional H3K36 trimethylation within the coding regions of actively transcribed genes (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). The Eaf3/Rpd3C deacetylase complex can recognize this H3K36 methylation mark and remove histone acetylation immediately subsequent to Pol II transcription, thus maintaining a repressive chromatin structure and preventing unwanted intragenic transcription initiation or cryptic transcription. In mammalian cells, H3K9 methylation and its associated HP1γ have been shown to be dynamically regulated within actively transcribed regions; it has been speculated that this mark, in coordination

with modifications at H3K4, H3K36, and H3K79, comprises a unique histone modification pattern characteristic of actively transcribed chromatin (Brinkman et al., 2006; Vakoc et al., 2005). Collectively, these findings not only illustrated the interplay and cooperative nature of various histone modifications during elongation but also led to the unexpected finding that maintenance of a "repressive" environment may be critical for optimal transcription (Berger, 2007).

The functions and mechanisms of several LSD1/KDM1a family histone demethylases have been intensively studied in multiple model organisms, including human, mouse, *Drosophila*, *C. elegans*, and *S. pombe*. Yet our knowledge of the precise roles of LSD1 family members remains mostly limited to promoter association and regulation. Specifically, one key function of LSD1 is balancing promoter H3K4/H3K9 methylation for activation or repression of LSD1 target genes (reviewed in Chosed and Dent, 2007).

LSD2/KDM1b/AOF1 is the only mammalian homolog of LSD1. Here, we present evidence for an important function of LSD2 in active gene transcription. LSD2 specifically associates with the coding region of its target genes. Removal of endogenous LSD2 promotes an increase in H3K4me2 levels and concurrent decrease in H3K9me2 levels, with a consequent downregulation of targeted gene transcription. We propose a model where LSD2, possibly through association with elongation factors and phosphorylated Pol II, is required for coordinating the dynamics of H3K4 and H3K9 methylation within elongating regions of genes, maintaining repressive chromatin structure, and thus contributes to the fine-tuning of gene expression.

RESULTS

LSD2 Is an H3K4-Specific Demethylase with Molecular and Functional Properties Distinct from LSD1

LSD2/KDM1b/AOF1, the only homolog of LSD1/KDM1a/AOF2 in the human genome, shares a similar domain homology with LSD1, but exhibits an overall sequence identity of less than 31%. Both LSD1 and LSD2 contain a SWIRM domain, a FAD coenzyme-binding motif, and a C-terminal amine oxidase domain, all of which are integral to the enzymatic activity of LSD1 family members (Figure 1A). In contrast to LSD1, LSD2 contains a CW-type zinc finger domain in its N-terminal region; the corresponding region in LSD1 is unstructured. Additionally, LSD2 lacks the "tower domain" present in LSD1. Thus, we predicted that the unique structure of LSD2 may confer biochemical and biological properties distinct from those of LSD1.

Using a series of in vitro and in vivo assays, we confirmed that LSD2, like its human homolog LSD1, possesses H3K4me1/2-specific histone demethylase activity. As shown in Figure 1B, LSD2 can remove mono- and dimethyl H3K4 modifications from histone substrates without affecting other marks, including dimethyl H3K36, H3K79, H3K9, H3K27, and H4K20. Robust H3K4me2 demethylase activities were observed on synthetic H3 peptides (Figure 1C) and nucleosomes (Figure 1D), but only weak or negligible activity toward H3K4me1 under these conditions, suggesting that H3K4me2 is the preferred substrate. While it is possible that optimal demethylase activity of LSD2 may still require additional cofactor(s), LSD2 alone could demonstrate significant H3K4 demethylase activity on nucleosomal substrates—a clear distinction from LSD1.

We next examined a series of mutations to further dissect the critical residues and domains involved in demethylation activity. The panel of proteins includes two point mutations that ablate the predicted FAD-binding site (LSD2.M1) and catalytic residue (LSD2.M2), as well as deletions that remove part of the amine oxidase and SWIRM domain (LSD2.1, a splicing isoform, NM_153042.3) or the upstream zinc finger (LSD2.2) (Figure 1E). In vitro demethylase activity assays of these LSD2 mutants demonstrate the requirement of the SWIRM, amine oxidase, and zinc finger regions, as well as critical residues, for robust enzymatic activity of LSD2 protein (Figures 1F and S1A). We also examined LSD2 demethylase activity in vivo by staining for global dimethylated H3K4 levels after GFP-LSD2 overexpression. We observed a significant reduction in total cellular H3K4me2 levels in cells expressing GFP-LSD2, whereas GFP-LSD1 was unable to do so, as previously observed (Shi et al., 2005). H3K4me1 demethylase activity was undetectable under identical experimental conditions (compare Figures S1B and S1C). As expected, LSD2.M1 and LSD2.M2 did not demonstrate demethylase activity in vivo (Figure 1G).

Human LSD1 was first characterized as a transcriptional repressor and has well-studied functions in promoter regulation. To explore any potential corepressor activity of LSD2, we utilized a Gal4-TK-luciferase reporter assay where LSD2 was tethered to an active promoter via a Gal4-DNA binding domain (DBD). In both transiently transfected and stably integrated reporter systems, LSD2 displays little impact on luciferase reporter expression. Experiments confirmed the proper expression, nuclear localization and enzymatic activity of Gal4-LSD2 in vivo (Figures S1D and S1E), hence excluding the possibility that the lack of repressive activity of the Gal4 fusion LSD2 is caused by instability or inactivation of LSD2 protein. These results suggest that LSD2 may act differently from LSD1 in epigenetic gene regulation.

LSD2 Is Absent from Gene Promoters but Preferentially Associates with Gene Bodies Downstream of the Promoters

LSD2/KDM1b has been reported to regulate the establishment of maternal imprinting in mouse oocytes (Ciccone et al., 2009). However, little is known about how LSD2 functions in non-oocyte cells, although LSD2 is expressed in multiple tissues and cell lines (Figures S2A and S2B). To address this question, we employed a chromatin immunoprecipitation (ChIP)-chip tiling array strategy to identify LSD2-binding loci on a genome-wide scale in HeLa cells. To ensure ChIP efficacy and specificity, we incorporated native ChIP with tandem affinity purification (TAP-ChIP) from HeLa cells stably expressing FLAG-HA-tagged LSD2. TAP-ChIP DNA was processed for tiling array analysis using an Affymetrix Human Tiling 2.0R Array chip that covers chromosomes 3, 21, 22, X, and Y (representing roughly one-seventh of the human genome). Utilizing the model-based analysis of tiling arrays (MAT) algorithm, we identified >800 LSD2-binding sites ($p < 0.0001$) scattered along the arms of these chromosomes (Figure 2A). Remarkably, as exemplified by *DNAJB11* and *CCNL1* (Figure 2B), the vast majority of

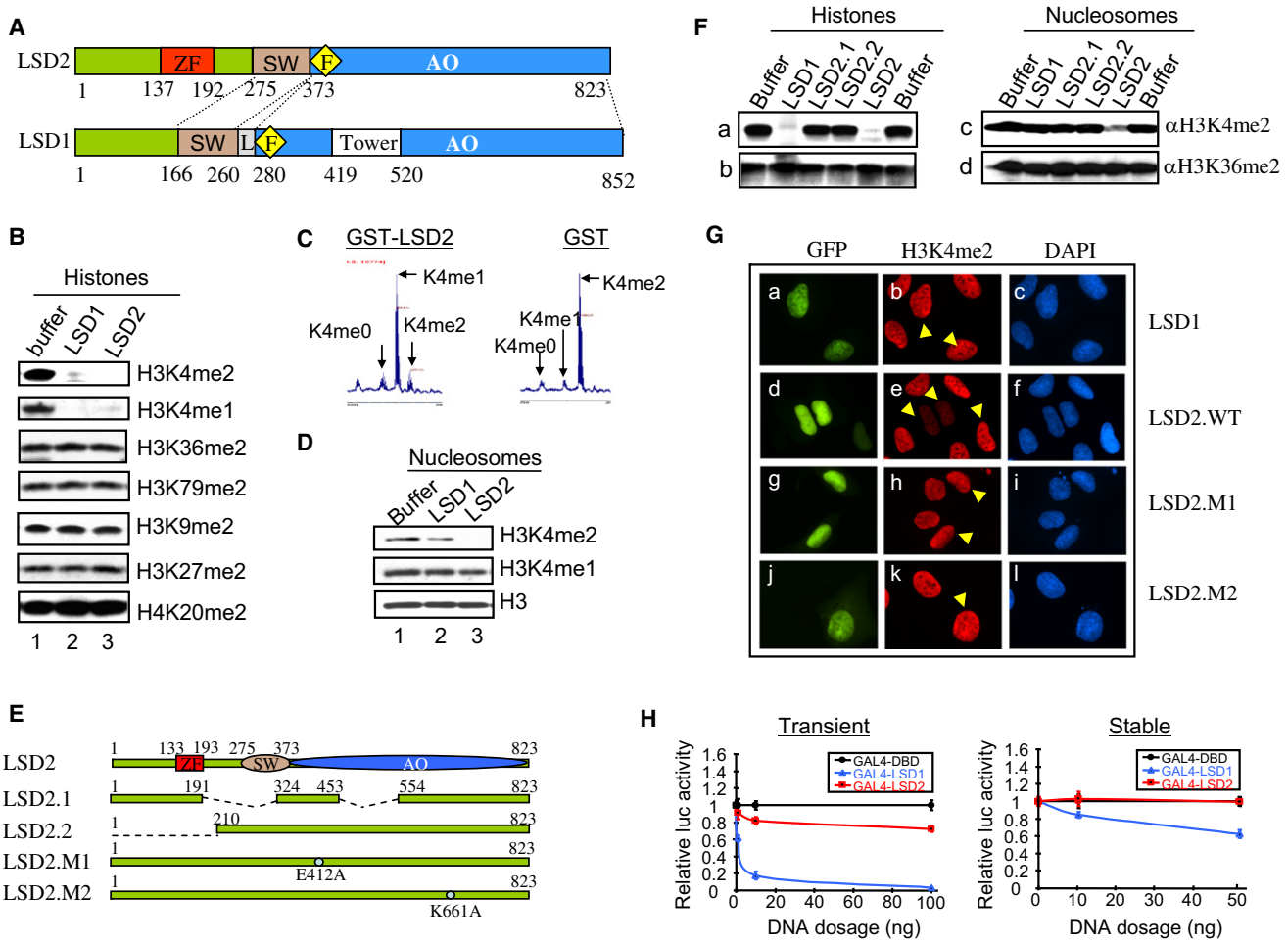

Figure 1. LSD2 Is an H3K4me2-Specific Demethylase with Distinct Properties from LSD1

(A) Schematic representation of LSD2 and LSD1. ZF, zinc finger domain; SW, SWIRM domain; AO, amine oxidase domain; F, FAD-binding motif; L, linker.

(B) LSD2 specifically demethylates mono- and dimethylated H3K4. Bulk histones were incubated with purified recombinant LSD2 and LSD1 and subjected to immunoblot analysis with methylation-specific anti-histone antibodies as indicated.

(C) MALDI-TOF mass spectrometry analysis of LSD2 demethylation of H3K4me2 histone peptides. Peptide masses corresponding to unmethylated (K4me0), monomethylated (K4me1), and dimethylated (K4me2) peptides are denoted.

(D) In vitro demethylation of nucleosomes.

(E) Schematic representation of LSD2 variants and mutants.

(F) Demethylation analysis of LSD2 variants using bulk histone (left) and nucleosome (right) substrates.

(G) Immunofluorescence analysis of in vivo demethylation activities. Arrows denote cells transfected with GFP fusion proteins. Green, GFP fusion protein; red, H3K4me2; blue, DAPI counterstain of DNA.

(H) LSD2 does not repress reporter gene expression when artificially tethered to promoters. Luciferase activity relative to GAL4-DBD control is presented. Transient, transient transfected; stable, stably integrated reporters.

LSD2-binding signal was found to be enriched at introns or exons (herein defined as intragenic regions or gene bodies) but absent from promoters. Statistical analysis showed that more than 80% of LSD2 targets were located either at the exons (33.9%) or introns (50.5%) of ~450 genes, while 0.45% of LSD2 was found at promoters and 15.1% at intergenic regions (Figure 2C). It is noted that 1.1%–1.4% of the human genome is spanned by exons, 24.4%–36.4% by introns, and 0.9%–1.4% by gene promoters (Venter et al., 2001). Clearly, LSD2 does not show any enrichment at promoters, which is strikingly different from the predominant promoter association of all other LSD1 family histone demethylases characterized so far (Lan et al., 2007; Wang et al., 2007). To rule out any potential artifacts due to ChIP-chip technology or native ChIP conditions, we performed conventional ChIP and validated the specific association of LSD2 with coding regions versus gene promoters on all the targets examined (Figure 2D).

LSD2 Binds to Highly Transcribed Coding Regions Enriched in H3K36me3

To explore the nature of LSD2 association with genes, we further analyzed the intragenic distribution of LSD2-binding sites. Each

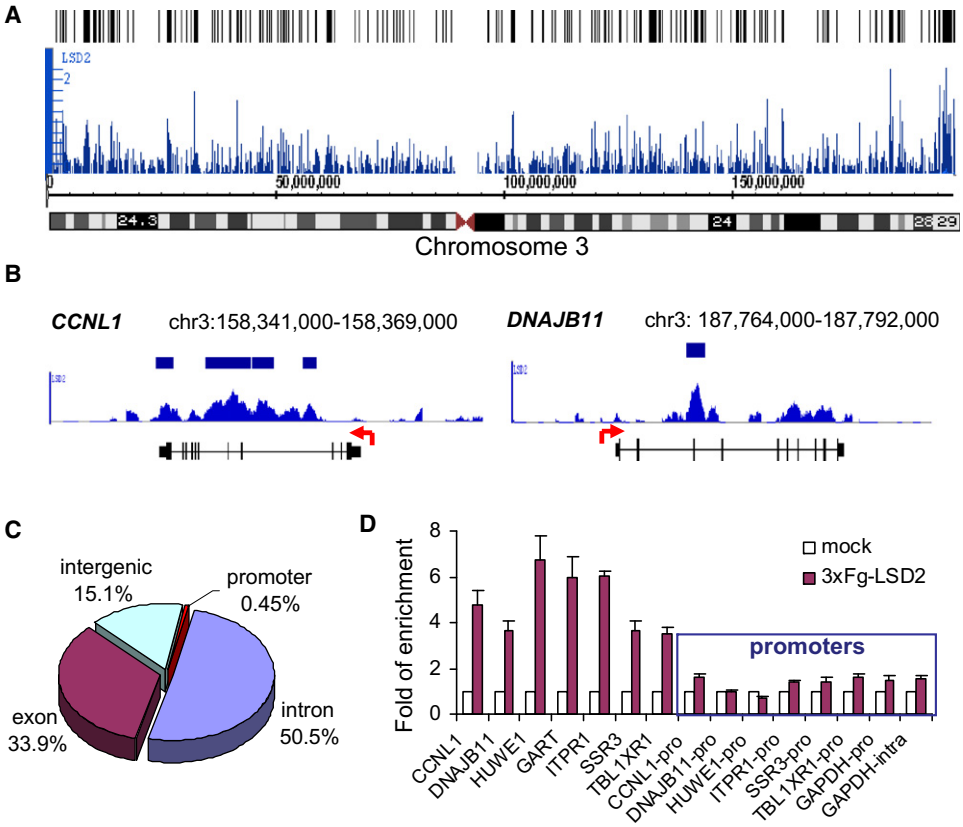

Figure 2. ChIP-Chip Analysis Indicates Enrichment of LSD2-Binding Sites within the Coding Regions but Not at Promoters of Genes

(A) Whole-genome tiling array analysis of LSD2 binding across human chromosome 3. Vertical bars mark positions of LSD2 target sites ($p < 0.0001$). ChIP-chip signals (MAT score) are shown in log 2 scale.

(B) ChIP-chip signals of LSD2 binding across *CCNL1* and *DNAJB11*. Arrow denotes promoter orientation.

(C) Distribution of LSD2-binding sites.

(D) Confirmation of the preferential LSD2 binding to gene bodies. Crosslinked ChIP using anti-FLAG antibody was performed from 3xFLAG-LSD2 expressing HeLa (purple bars, 3xFg-LSD2) and empty vector transduced cells (open bars, mock). Fold of enrichment relative to mock is shown. PCR primers specific to coding region or the promoter (-pro, boxed) of each gene are denoted on the x axis. Error bars, SEM of triplicates from representative experiment. LSD2 did not show significant binding to the promoter (GAPDH-pro) or an intragenic region (GAPDH-intra) of *GAPDH*.

target gene (defined in Ensembl 56 gene database GRCh37; http://www.ensembl.org) was divided into ten equal units, and the number of LSD2-binding sites within each segment was counted and plotted versus the distance from the transcription start site (TSS) (Figure 3A). We found that the majority of LSD2 binding occurs downstream of promoter regions, peaking toward the 3′ end of genes. Negligible binding was observed beyond the up- or downstream gene boundaries.

The distinct distribution of LSD2, showing intragenic binding preferentially at the 3′ end, is reminiscent of the pattern of H3K36me3 deposition (Barski et al., 2007; Hampsey and Reinberg, 2003). We therefore examined the correlation of LSD2 binding with H3K36me3 modifications. As shown in Figure 3B, >60% of LSD2-binding sites are located within 0.5 kb of an H3K36me3 site, demonstrating a strong correlation between LSD2 localization and H3K36me3 enrichment ($p < 10^{-72}$). In contrast, no apparent correlation of LSD2 loci with the repressive mark H3K27me3 was found (p is nearly equal to 1) (Figure 3B). To illustrate this finding on individual genes, we examined LSD2 binding, as well as H3K36me3 and H3K4me2 levels, across LSD2 target genes *CCNL1* and *DNAJB11* by ChIP (Figure 3C). Typical for actively transcribed genes, H3K4me2 peaks at the TSS and rapidly decreases further downstream; H3K36me3 is low at promoters and peaks in the 3′ region. The LSD2-binding profile at these loci mirrors that of H3K36me3 but inversely correlates with H3K4me2 along the gene body, consistent with the H3K4me2 histone demethylase activity of LSD2.

The correlation with H3K36me3 strongly suggests an association of LSD2 with active gene transcription. To test this hypothesis, we divided the annotated genes present on the tiling array into high and low expression groups and examined LSD2 ChIP-chip signals relative to the TSS within each group. As shown in Figure 3D, a significant enrichment of LSD2-binding signals was observed downstream from the TSS of highly expressed genes (blue line within ~0–30 kb), whereas no obvious enrichment was observed either up- or downstream of the TSS in the low-expressing group (green line). These observations together indicate that LSD2 preferentially associates with the coding

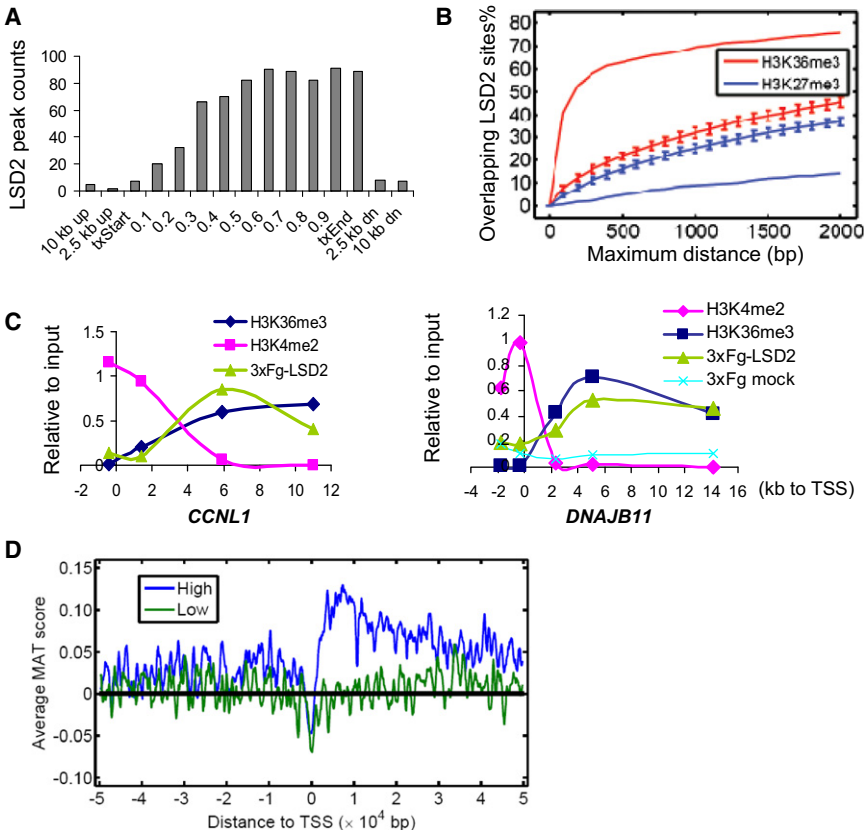

Figure 3. LSD2 Binding Correlates with High Levels of Trimethylated H3K36 within the Coding Regions of Actively Transcribed Genes

(A) Distribution of LSD2-binding sites across genes. The open reading frame of each LSD2 target gene was divided into ten equal segments; regions corresponding to 0–2.5 kb and 2.5–10 kb up- or downstream fo each gene were also studied. The number of LSD2-binding sites within each defined segment is shown. txStart, transcription start site; txEnd, transcription end.

(B) Correlation of LSD2 binding to histone modifications. Enrichment of LSD2-binding sites, presented as the fraction of total peaks, was plotted against the relative distance to known H3K36me3 (red line) and H3K27me3 (blue line) sites. Permuted distribution models are shown as hatched lines.

(C) LSD2 binding at *CCNL1* and *DNAJB11* loci overlaps with H3K36me3 and inversely correlates with H3K4me2. Enrichment relative to input is presented in arbitrary units. Distance of quantitative PCR primers relative to the transcription start site (TSS) is shown.

(D) LSD2 preferentially associates with the gene bodies of actively transcribed genes. Genes present on the tiling array were divided into high-expressing (top 50%, blue line) and low-expressing (low 50%, green line) groups. Average enrichment ratios within ±50 kb regions relative to TSS were calculated based on MAT scores.

regions but not the promoters of actively transcribed genes, further suggesting a positive role of LSD2 in active gene transcription.

LSD2 Is Required for Maintenance of H3K4 and H3K9 Methylation Status within Transcribing Regions of Active Genes

To explore the mechanism(s) of action of LSD2 in active gene transcription, we next examined the role of LSD2 in regulating histone marks within coding regions of its target genes. We depleted endogenous LSD2 using shRNA and first examined the changes in H3K4 methylation levels on the identified LSD2 targets. LSD2 shRNA significantly depleted LSD2 expression at both mRNA and protein levels, but had no effect on the expression of LSD1 (Figure 4A). Changes to the H3K4me2 profile across *CCNL1* and *DNAJB11* were examined by quantitative ChIP subsequent to LSD2 depletion. In agreement with the lack of LSD2 association with gene promoters, no significant increases of H3K4me2 levels were noted at these active promoters; coinciding with LSD2 binding within gene bodies, we consistently observed an increase in H3K4me2 levels across the coding regions of these genes (Figure 4B). Examination of additional targets revealed a similar pattern of H3K4me2 changes (Figure 4C). Significantly, no obvious changes in H3K4me2 levels were found within the coding region of *SCG10*, a repressed gene that does not associate with LSD2 in HeLa cells. The specific H3K4me2 increases at coding regions were confirmed by a second LSD2 shRNA (Figure S3A). Thus, these data suggest that reduction of LSD2 within coding regions elevates the otherwise depressed H3K4me2 levels of these regions.

We next examined changes of other histone modifications at the same regions to further explore the consequences of LSD2 removal on chromatin structure. Of particular interest, it has been reported that H3K9 methylation, typically a mark of repressive chromatin, is associated with actively transcribed regions and required for active transcription (Brinkman et al., 2006; Vakoc et al., 2005). In contrast to the increase of H3K4me2, a consistent decrease in H3K9me2 (Figure 4D) was detected at LSD2-binding sites. Despite a strong coalignment with LSD2 binding, no significant change of the H3K36me3 profile was observed upon LSD2 depletion (Figure S3B). In addition, we did not observe significant changes in the levels of acetylated H3 (Figure S3C) or trimethylated H3K4 (data not shown).

Depletion of LSD2 or Inhibiting Its Demethylase Activity Results in Downregulation of a Subset of LSD2 Target Genes

To interrogate the role of LSD2 in gene transcription, we examined genome-wide mRNA expression profile changes upon LSD2 depletion using microarray analysis. We identified 461 genes that are differentially expressed (DE) after LSD2 depletion in HeLa cells, using criteria of >1.5-fold changes in expression values. Interestingly, the majority (77%) of differentially expressed genes were downregulated after LSD2 depletion. Similar results were observed using more stringent criteria (>2-fold change), where 89 of the 107 target genes were

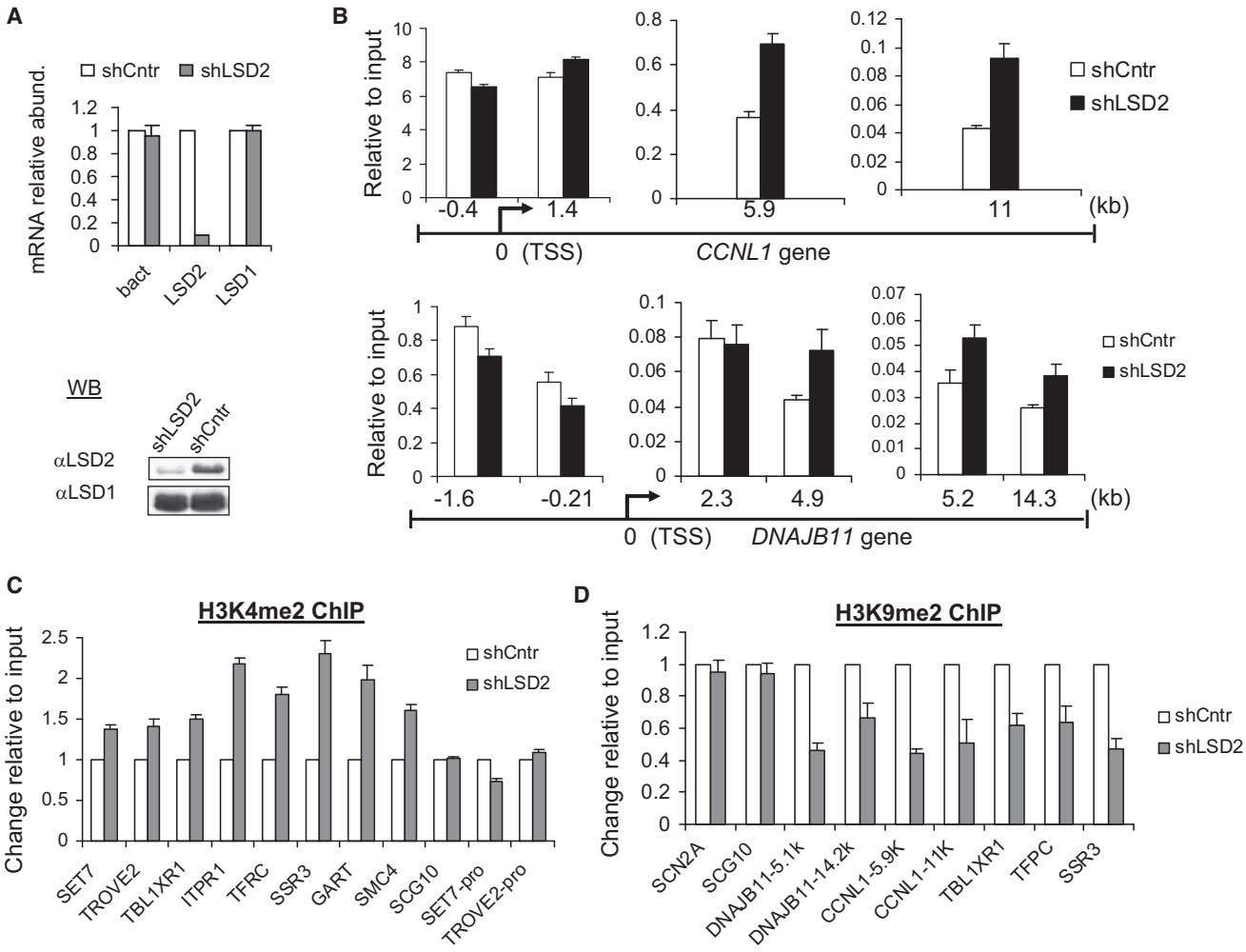

Figure 4. LSD2 Maintains the Balance of H3K4 and H3K9 Methylation within Intragenic Regions

(A) Specific LSD2 depletion by shRNA. Quantitative RT-PCR result normalized with β-actin (upper) and immunoblot of whole-cell lysates (lower) are presented. shCntr, control shRNA; shLSD2, LSD2-specific shRNA. Error bars, SEM of triplicates from representative experiment.

(B) LSD2 depletion causes an increase of H3K4me2 within gene bodies but not at promoters of *CCNL1* and *DNAJB11*. Data are presented as relative enrichment compared to input. Distance of quantitative PCR primers from the TSS is denoted. Error bars, SEM of triplicates from representative experiment.

(C) Effect of LSD2 depletion on H3K4me2 levels at additional LSD2-binding sites. Data are presented as fold of change relative to input, comparing shLSD2 HeLa to shCntr HeLa. Primers targeting the 3′ regions or promoters (-pro) of the genes are denoted on the x axis. Error bars, SEM of triplicates from representative experiment.

(D) LSD2 depletion results in an increase of intragenic H3K9me2 levels of LSD2-binding targets. Data are presented as in (C). Negative controls, *SCN2A* and *SCG10*, are two repressed genes not associated with LSD2.

downregulated (Figure 5A). Out of the 461 DE genes identified by microarray, 37 are located on the four chromosomes examined by LSD2 ChIP-chip (3, 21, 22, and X). Of these 37 DE genes, 11 (~30%) are direct LSD2 targets as determined by ChIP-chip analysis, and 9 of those are downregulated after LSD2 depletion, compared to 2 genes that are upregulated. Microarray results were confirmed by quantitative RT-PCR (Figure 5B, blue and purple bars show down- and upregulated genes, respectively). The LSD2-specific knockdown effects on downregulation of these genes were further validated by a second LSD2 shRNA (Figure S4A). Together, these data indicate that LSD2 is important for active gene transcription.

To ascertain whether the enzymatic activity of LSD2 is important for its role in gene transcription, we studied the effect of a monoamine oxidase inhibitor, tranylcypromine, on the expression of LSD2-regulated genes. Since tranylcypromine inhibits the demethylase activity of both LSD1 and LSD2 (Karytinos et al., 2009; Lee et al., 2006b), we decided to isolate the effect of tranylcypromine inhibition on LSD2 by focusing on genes that are specifically regulated by LSD2, but not LSD1 (Figure S4B, blue bars). Similar to the effect of LSD2 depletion, the majority of LSD2 targets examined were downregulated after tranylcypromine treatment (Figure 5C). This result links LSD2 demethylase activity to active gene transcription.

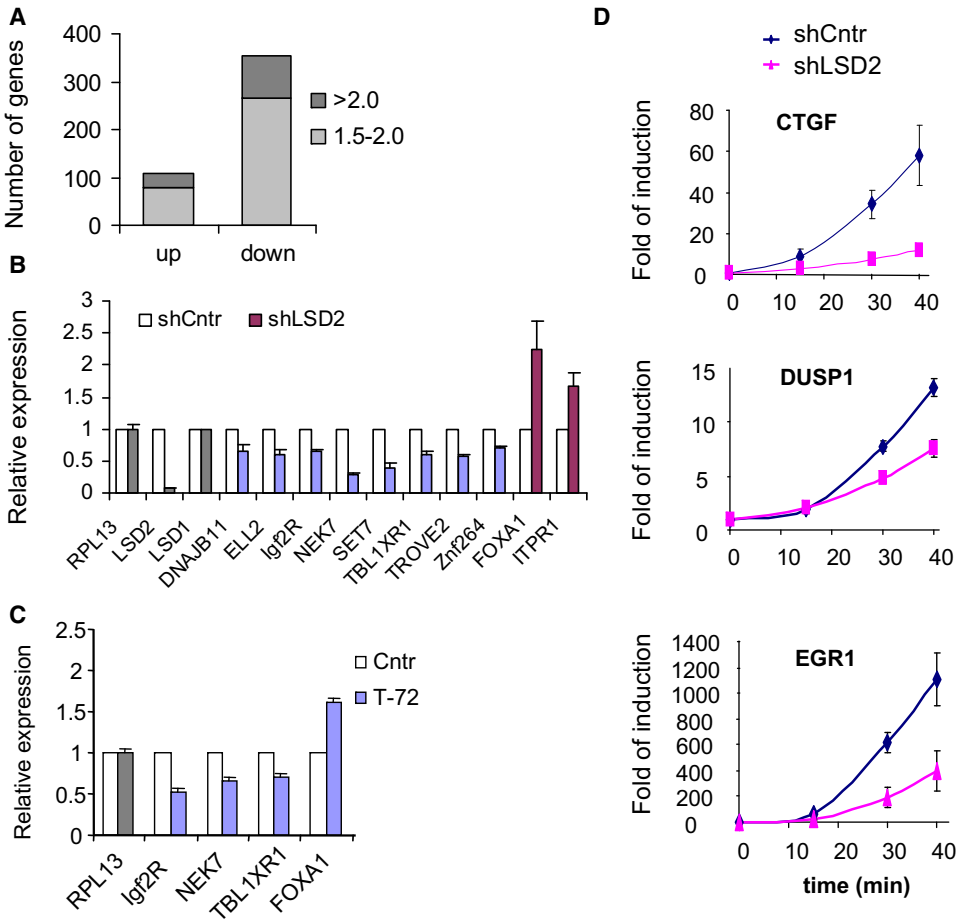

Figure 5. Depletion of LSD2 or Inhibition of Its Demethylase Activity Results in Downregulation of a Subset of Actively Transcribed Genes

(A) The majority of differentially expressed (DE) genes after LSD2 depletion are downregulated. The number of DE genes filling the criteria of 1.5- to 2.0-fold (light gray) or ≥2-fold (dark gray) changes is shown. Up, upregulated; down, downregulated.

(B) Quantitative RT-PCR confirms the downregulation of a large subset of LSD2 targets after LSD2 depletion. Blue bars, downregulated genes after LSD2 depletion; purple bars, upregulated genes. Error bars, SEM of triplicate independent experiments.

(C) Inhibition of demethylase activity by tranylcypromine causes downregulation of LSD2 target genes. T-72, HeLa treated with tranylcypromine for 72 hr; cntr, 72 hr treatment with vehicle only. Error bars, SEM of biological triplicates.

(D) LSD2 is required for optimal induction of immediate-early genes. Control and LSD2 shRNA-transduced HeLa were treated with 50 ng/ml EGF for up to 40 min. Data are presented as mRNA abundance relative to time 0. Error bars, SEM of triplicates.

In addition to the constitutively active genes studied, we examined the effect of LSD2 depletion on inducible gene expression. We chose to investigate a set of immediate-early (IE) genes, since without stimulation these genes are "preinitiated" but blocked at an early stage of elongation, with RNA Pol II paused proximal to TSSs (Wang et al., 2005). Upon EGF treatment, the transcription of a number of IE genes is greatly induced within minutes, independent of promoter initiation and de novo protein synthesis. HeLa cells were treated with control and two independent shLSD2 constructs, followed by EGF treatment. Induction efficiencies of *CTGF*, *DUSP1*, and *EGR1* were significantly reduced after LSD2 depletion, with the most obvious effect occurring at 30 and 40 min after treatment (Figure 5D). Endogenous LSD2 binding within the gene bodies of these genes was detected by ChIP (Figure S4C). These data together strongly support the finding that LSD2 likely functions to modulate the chromatin structure of coding regions to facilitate active elongation.

Factors in LSD2-Associated Complex Physically and Functionally Link LSD2 to Active Transcription Elongation

To investigate how LSD2 is physically and functionally linked to the transcription machinery, we isolated and characterized the LSD2-containing cellular complex using TAP (Shi et al., 2005; Tahiliani et al., 2007). The LSD2 complex eluted from the second affinity purification step (anti-HA affinity column) is highly specific (Figure 6A, compare lanes 3 and 4). LSD2-associated proteins were identified by MS/MS proteomic analysis.

The composition of the LSD2 complex is notably distinct from the LSD1 complex (Shi et al., 2005). LSD2-associated proteins fall into several functional groups, including DNA replication

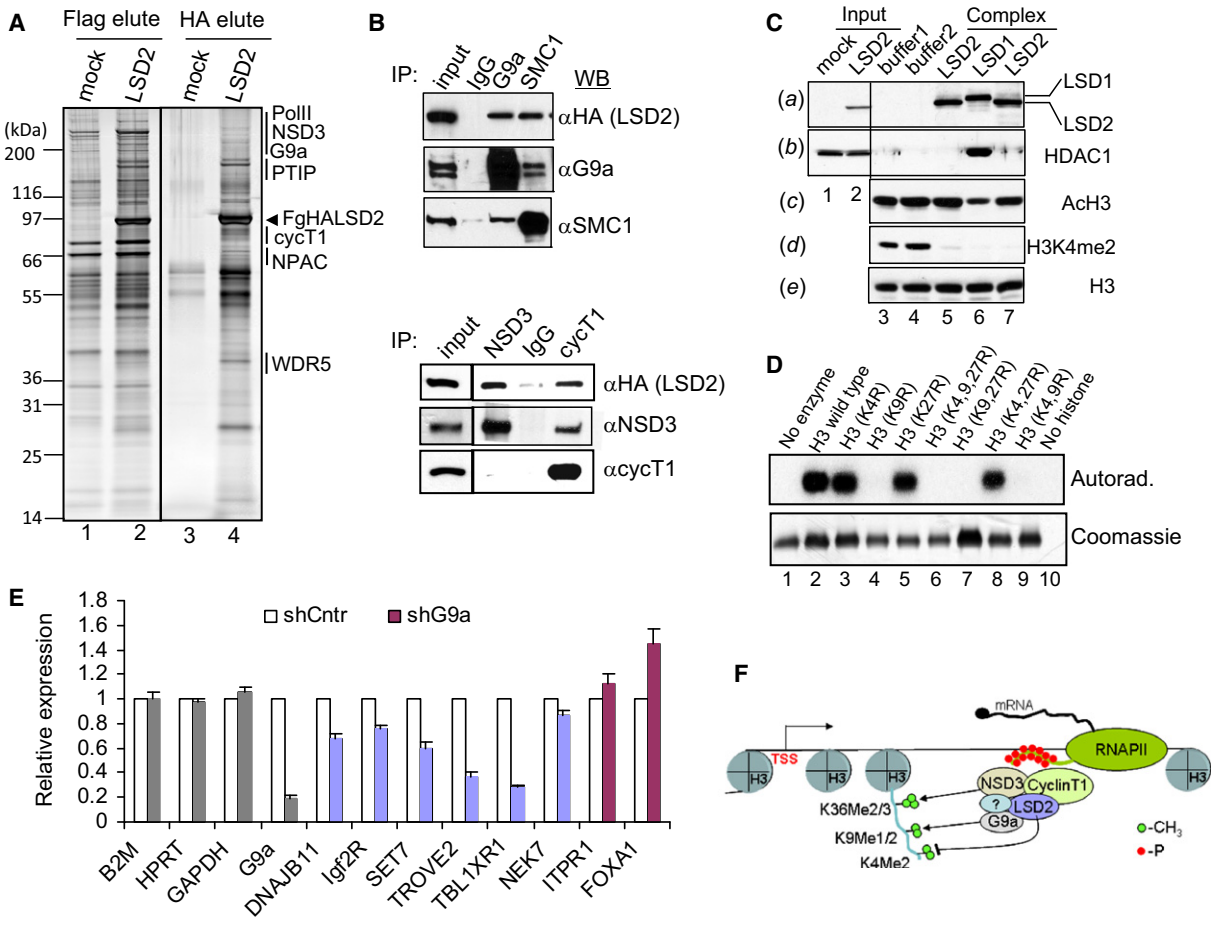

Figure 6. The LSD2 Complex Contains Proteins Involved in Active Gene Transcription and Functionally Links LSD2 to Intragenic Regions

(A) Tandem affinity purification (TAP) and mass spectrometric analysis of LSD2 complex. Silver staining of FLAG elutes (lanes 1 and 2) and HA elutes (lanes 3 and 4) of FgHA-LSD2 and mock purification is shown. A comprehensive list of associated polypeptides identified by MS/MS is shown in Figure S5.

(B) Reciprocal immunoprecipitation. Nuclear extracts from FLAG-HA(FgHA)-LSD2 HeLa were immunoprecipitated with either IgG control or indicated antibodies. Input and precipitated proteins were analyzed by immunoblot with the indicated antibodies.

(C) LSD2 complex demethylates H3K4me2 but lacks deacetylase activity. Bulk histones were incubated with buffer alone (lane 3 and 4), LSD1 complex (lane 6), or two independent preparations of LSD2 complex (lanes 5 and 7) and immunoblotted with the indicated antibodies. Inputs, nuclear extract of mock (lane 1) and FgHA-LSD2 HeLa (lane 2).

(D) LSD2 complex possesses H3K9 methyltransferase activity. Wild-type GST-histone H3 and a panel of arginine substitutions were used. Autoradiography of methylation signal is shown (upper); histone inputs are stained by Coomassie blue (lower).

(E) G9a regulates LSD2 target genes. Quantitative RT-PCR data were normalized with β2-microglobulin (*β2M*) and are expressed as relative abundance comparing G9a shRNA (shG9a)-treated HeLa with control (shCntr). Effects of shG9a on the expression of housekeeping genes *HPRT* and *GAPHD* are shown. Blue bars, genes downregulated after LSD2 depletion shown in Figure 5B; purple bars, upregulated genes. Error bars, SEM of triplicates.

(F) Model of gene transcription regulation by LSD2 complex. LSD2 localizes to the 3′ end of actively transcribed genes, where it can interact with phosphorylated RNA Pol II and elongation factors. The LSD2 complex coordinates intragenic H3K4me2 and H3K9 methylation and potentially H3K36me2 levels via its own intrinsic demethylase activity and through interaction with histone methyltransferases G9a and NSD3.

and damage repair, nucleosome remodeling and histone modification, and active gene transcription (summarized in Figure S5), suggesting that LSD2 may be involved in multiple cellular functions. The presence of a number of proteins from each functional category was confirmed by immunoblot (Figure S6A).

A number of protein factors in the LSD2 complex are involved in the regulation of active gene transcription and Pol II elongation and may therefore provide physical links for LSD2 to the regulation of the transcription machinery. Of particular interest, the Ser2-phosphorylated RNA Pol II, the active form of elongating polymerase, as well as components of P-TEFb, cyclin T1, and CDK9, are detected in the LSD2 complex. We also identified chromatin-modifying factors that have been linked to gene coding regions, including the NSD3 H3K36 methyltransferase (Li et al., 2009), euchromatin H3K9 methyltransferases EHMT1/2, and SWI/SNF-like nucleosome remodeling factor SNF2H. Association of G9a/EHMT2 and SMC1, as well as NSD3 and cyclin T1 with LSD2, was validated by reciprocal coimmunoprecipitation (Figure 6B). Glycerol gradient ultracentrifugation analysis showed that TAP-purified LSD2 complex separates into multiple

peaks (marked A–E in Figure S6B), suggesting the existence of more than one subcomplex of LSD2. Importantly, G9a and NSD3 comigrate with LSD2 in high-density fractions (peak E in Figure S6B), indicating that these proteins are components of the same complex.

To further characterize the LSD2-associated complex, we examined the enzymatic activities of the purified complex. As expected, LSD2 complex readily demethylates H3K4me2 in vitro using bulk histone as substrates (Figure 6Cd). Contrary to the LSD1 complex, no histone deacetylase activity was observed for LSD2 complex (Figure 6Cc). The absence of HDAC activity is consistent with MS/MS proteomic analysis and confirmed by immunoblot (Figure 6Cb).

The histone methyltransferase (HMTase) activity of the complex was assayed in vitro using recombinant GST-histone H3 proteins. LSD2 complex demonstrated robust HMTase activity on recombinant wild-type GST-H3 (Figure 6D, lane 2). This signal is completely abolished when residue Lys9 is replaced with arginine (H3K9R, Figure 6D, lanes 4, 6, 7, and 9), while other mutations (K4R, K27R, lanes 3 and 5, respectively) do not adversely affect HMTase activity, demonstrating that LSD2 complex indeed possesses H3K9 HMTase activity. Recent studies on the nuclear receptor SET domain-containing (NSD) family of methyltransferases suggested that a nucleosomal substrate (or octamers in conjunction with dsDNA) is required for their H3K36-specific HMTase activities (Li et al., 2009). Taking this into account, we modified the histone methylation assays by using reconstituted nucleosomes. Under these conditions, we again detected methylation activity specific to the LSD2 complex (Figure S6C, lanes 1 and 2). Importantly, this signal, although reduced, persisted when MLA nucleosomes carrying H3Kc9me3, an analog of H3K9me3 nucleosomes (Simon et al., 2007), were used (Figure S6C, lane 3). We propose that this activity could be attributed to NSD3 acting on H3K36.

The physical association of G9a with LSD2 and the H3K9 HMTase activity of LSD2 complex are particularly interesting, given the concomitant H3K9 methylation changes at LSD2-binding loci observed upon LSD2 depletion. We next asked if G9a might coordinate with LSD2 to regulate active gene transcription. As expected, we detected G9a binding at the coding region of a subset of LSD2 target genes by ChIP (Figure S6D). Importantly, upon G9a depletion, a large majority of LSD2 targets tested showed transcription changes similar to LSD2 knockdown, with most of the LSD2 target genes downregulated (Figure 6E).

Taken together, we conclude that LSD2, in association with elongating Pol II and elongation factors such as cyclin T1 (P-TEFb complex), and additional LSD2 complex-associated histone-modifying activities may be coordinated to set the proper histone modifications at coding regions, thus facilitating Pol II-mediated transcription elongation (Figure 6F).

DISCUSSION

Histone methylation and the recently identified histone demethylases play important roles in gene transcription regulation. The first identified histone demethylase, LSD1/KDM1a/AOF2, was originally characterized as a transcriptional repressor, functioning in part by removing active H3K4me2 marks from promoter regions. Here, we describe an important function of human LSD2/AOF1/KDM1b in active gene transcription. LSD2 shares similar substrate specificity with LSD1 and demethylates mono- and dimethylated H3K4. Instead of functioning as a corepressor, LSD2 is important for optimal gene transcription. LSD2 is unique in associating specifically with the gene bodies of actively transcribed genes, but not at promoters. A specific function of LSD2 is to maintain low levels of H3K4 methylation within elongation regions. Furthermore, LSD2 forms complexes with H3K9 and H3K36 methyltransferases. These enzymes together orchestrate appropriate histone modifications in order to maintain a repressive chromatin structure at elongation regions, which may be important for optimal transcription elongation.

During the preparation of this manuscript, the enzymatic activity and functional studies of LSD2 were reported by others (Ciccone et al., 2009; Karytinos et al., 2009; Yang et al., 2010). Work by Ciccone et al. has shown that LSD2 knockout mice fail to establish a subset of maternal imprints. These results likely reflect the functional relationship between DNA methylation and H3K4 methylation, although the mechanism underlining how LSD2 directly regulates DNA methylation has yet to be discovered. It is noted that this finding is confined to a particular stage of oocyte maturation and a small subset of imprinted genes, while global DNA methylation is unchanged. To explore the function of LSD2 outside of oocytes, we examined more than 90 genes in HeLa cells after LSD2 depletion (including LSD2 targets and a collection of cancer-related genes) and found no significant alterations in DNA methylation on the CpG islands of corresponding promoters (data not shown). These findings indicate multiple functions of LSD2 at different stages of development.

What is the function of LSD2 in somatic cells, given that LSD2 expression persists postdevelopment? A study by Yang et al. suggests a functional role of LSD2 in direct regulation of gene transcription, mainly toward transcriptional repression, albeit independent of its own H3K4 demethylase or HDAC activity (Yang et al., 2010). However, it is worth noting that this study failed to demonstrate dosage-dependent effects on reporter gene activity or identify any endogenous targets of LSD2. On the other hand, we observed a predominant downregulation of transcription upon depletion of endogenous LSD2, consistent with a positive role for LSD2 in transcription.

The positive role of LSD2 in transcription regulation may at first seem to be at odds with its innate enzymatic activity, which removes H3K4me2/1 marks of active genes. Nonetheless, the incorporation of canonical ''repressive'' marks into regions of active transcription does represent an intriguing development. In yeast, Eaf3 of the Rdp3C complex binds to trimethylated H3K36 and recruits HDAC activity to the loci (Carrozza et al., 2005; Joshi and Struhl, 2005). It is proposed that this HDAC activity counteracts the spreading of acetylation from promoters and is required to maintain a repressive state of chromatin after elongating Pol II. It is tempting to speculate that the LSD2-H3K4me2 relationship within gene bodies at least in part resonates with the aforementioned role of HDAC acetylation on active transcription. Interestingly, we did not detect any HDAC activity from the LSD2 complex, nor did we detect any changes in intragenic acetyl-H3 levels upon LSD2 depletion. It is therefore

probable that LSD2 function in active gene transcription in mammals is independent of HDAC activity. Instead, it may provide a complementary epigenetic mechanism for maintaining a cotranscriptional "repressive" chromatin state.

In addition to H3K4 demethylase activity, LSD2 also contributes to maintaining an optimal "repressive" environment of actively transcribed regions through its interaction with the H3K9 methyltransferase G9a. We show that G9a forms stable complexes with LSD2, regulates H3K9 methylation at LSD2-binding sites within coding regions and is required for active gene transcription. Consistent with our findings, it has also been reported that G9a binds within actively transcribed genes and can function as a coactivator of nuclear receptors (Lee et al., 2006a), in addition to its well-known role as a repressor (Roopra et al., 2004). It is plausible that in mammals, besides HDACs, LSD2 and G9a provide additional layers of control to maintain the repressive chromatin structure of elongating chromatin that could be important for efficient and faithful transcription.

Additional factors present in the LSD2 complex may also shed light on the mechanism by which LSD2 positively regulates transcription. We observed and validated the presence of P-TEFb in purified LSD2 complex as well as Ser2-phosphorylated RNA polymerase. The functional link between elongating Pol II and LSD2 is further supported by our observation that LSD2 depletion impedes the full induction of IE responsive genes, such as *EGR1*, which are known to be regulated postinitiation, at the level of elongation (Wang et al., 2005).

We note that not all of the LSD2-associated genes identified by ChIP-chip were dramatically affected by LSD2 depletion, despite robust increases of H3K4me2 levels at LSD2-binding regions. We believe that this finding is not unexpected. In fact, inactivation of other elongation-associated histone-modifying enzymes, such as the deacetylase Rpd3 or H3K36 methyltransferase SETD2, also cause only moderate transcriptional changes in a subset of their associated genes in both yeast and *C. elegans* (Edmunds et al., 2008; Sharma et al., 2007; Wang et al., 2002). This could be explained in part by the understanding that transcription elongation is regulated at multiple levels. The activity of elongating Pol II can be regulated directly by its associated factors, such as P-TEFb and NELF. In addition, Pol II activity is also influenced by chromatin-remodeling and -modifying enzymes, which govern local chromatin structure (Core and Lis, 2008; Sims et al., 2004). Each layer of regulatory factors likely differentially or cooperatively contributes to the transcriptional outcomes of specific genes, providing flexibility and fidelity to ensure proper programs of gene expression essential for cellular and biological processes.

It is of great interest to find several proline-tryptophan-tryptophan-proline (PWWP) domain-containing proteins in association with the LSD2 complex, including the H3K36 methyltransferase NSD3; MSH6, a component of DNA mismatch repair machinery; and an uncharacterized protein, NPAC. The PWWP domain is a loosely conserved protein module found in eukaryotic nuclear proteins, which are often associated with chromatin (Maurer-Stroh et al., 2003). Recently, specific recognition of histone methylation marks by PWWP domains has been reported (Vezzoli et al., 2010; Wang et al., 2009). NSD3 is a major component of the LSD2 complex and demonstrates a strong biochemical interaction (Figures 6B and S6B). We detected NSD3 binding to a small subset of LSD2 targets by ChIP (Figure S6E) and observed a moderate effect of NSD3 depletion on the expression of LSD2 target genes (Figure S6F). It is possible that NSD3 and LSD2 form complexes in vivo and coordinate the dynamics of H3K4 and H3K36 methylation for transcription elongation of certain genes; however, further investigation is required to fully define the nature of this interaction. We are tempted to speculate that PWWP domain-containing factors in LSD2 complex may function in LSD2 targeting through binding to specific histone methylation marks. Future investigation into these possibilities is warranted to further our understanding of the role of PWWP-containing proteins in the recruitment of LSD2 and their potential in regulating LSD2 functions.

Human LSD2 is a candidate cancer-related gene located at chromosome 6p22, a genomic region with a high incidence of chromosomal translocations, deletions, or amplifications in multiple cancer types (Heidenblad et al., 2008; Orlic et al., 2006). Furthermore, data from the ONCOMINE cancer database (Compendia Bioscience; Ann Arbor, MI) show that human LSD2 levels are significantly lower in certain types of leukemia, seminoma, and a few classes of ER-negative breast cancers. These correlations suggest that the biological function of LSD2 and its exact role in human cancer are likely tissue or cell-type dependent. The function of LSD2 in transcription regulation may provide insight to the epigenetic mechanism involving gene regulation in tumorigenesis, therefore offering a molecular basis for future development of demethylase inhibitor-based anticancer drugs.

EXPERIMENTAL PROCEDURES

Plasmids

Full-length *LSD2/KDM1b/AOF1* was amplified from HeLa cDNA, cloned into XhoI and NotI sites of pOZ-N, and expressed as N-terminal FLAG-HA double-tagged fusion proteins. *LSD2* was subcloned into pBlueBac (Invitrogen; Carlsbad, CA) for protein expression and purification from Sf9 insect cells as described (Tahiliani et al., 2007).

Histone Demethylase Assays

In vitro and in vivo histone demethylase assays were performed as described previously (Tahiliani et al., 2007).

HMTase Assays

HMTase activity assays were performed as described (Shi et al., 2003). In brief, samples were incubated for 60 min at 30°C in a final volume of 40 μl methylation assay buffer (50 mM Tris-HCl [pH 8.5], 20 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 10 mM 2-mercaptoethanol, 1 mM PMSF, 8 pmol S-adenosyl-[methyl-^{3}H]methionine). Samples were resolved on SDS-PAGE and analyzed by autoradiography or scintillation counting.

ChIP and Tiling Array Analysis

Native chromatin of HeLa-S stably expressing FLAG-HA(FgHA)-LSD2 was prepared by micrococcal nuclease digestion as previously described (Umlauf et al., 2004) and was primarily composed of mono-, di-, and trinucleosomes. Native chromatin fragments were sequential immunoprecipitated using anti-FLAG and anti-HA agarose beads (Sigma; St. Louis). After extensive washing, FLAG or HA peptides (Sigma) were used for elution after each immunoprecipitation. DNA from the final step was purified and processed for tiling array hybridization to Human Genome Tiling 2.0 Array (Affymetrix; Santa Clara, CA) per the manufacturer's instructions. Input DNA was used as control.

Regions enriched by TAP N-ChIP were identified using MAT algorithm (Johnson et al., 2006) and displayed by Integrated Genome Browser (IGB). CEAS (*cis*-element annotation system) was used for statistic analysis of the distribution pattern of LSD2-binding sites on promoter and nonpromoter regions (Ji et al., 2006). To estimate the statistical significance of colocalization between LSD2 and histone modification marks previously identified (Barski et al., 2007), we estimated the null distribution of overlap by generating 100 randomly permutated samples of the histone modification loci, and the overlap was calculated for each permutated sample.

Conventional ChIP was performed as previously described (Shi et al., 2003) using formaldehyde-crosslinked chromatin. Anti-H3K4me2 (Millipore, 07-030; Billerica, MA), anti-H3K9me2 (Upstate, 05-768), anti-H3K36me3 (Abcam, ab9050; Cambridge, MA), anti-G9a (Abcam, ab40542), and anti-NSD3 (GeneTex; Irvine, CA) antibodies were used for ChIP. Enrichment of each target was determined by quantitative PCR. Sequences of primers are available upon request.

RNAi and Microarray Gene Expression Profile Analysis

Retroviral shRNA targeting human LSD2 (5′-GTGGGACCACAATGAATTCTT-3′) and control shRNA was used to infect HeLa. RNA was purified 70 hr after infection and processed for Human Genome U133 Plus 2.0 Array (Affymetrix) hybridization per the manufacturer's instructions. Biological duplicates were analyzed. A common set of 461 DE genes with at least 1.5-fold changes was obtained by normalization with either Quantile or Invariant Set method using dChip. Microarray results were confirmed by quantitative RT-PCR. Sequences of quantitative RT-PCR primers are available upon request. An independent LSD2 shRNA construct (5′-GCCGTGTTCTATGACATGGATCTC GAGATCCATGTCATAGAACACGGC-3′) was used to confirm the specific effects of LSD2 depletion. G9a and NSD3 shRNAs were purchased from ThermoScientific (Waltham, MA).

Luciferase Reporter Assays

Luciferase reporter assays were performed 36 hr after transfection and normalized with the activity of cotransfected β-galactosidase as described (Shi et al., 2004).

Complex Purification and Proteomics Analysis by Mass Spectrometry

LSD2-associated protein complexes were purified from HeLa-S stably expressing FLAG-HA-LSD2 by sequential immunoprecipitation using anti-FLAG and anti-HA Affinity Gel (Sigma A2220) as previously described (Shi et al., 2003). HeLa-S transformed with pOZ-N vector was used to purify mock complex using identical procedures. LSD2 complex was sequenced by MS/MS at the Harvard Medical School Taplin Biological Mass Spectrometry Facility.

ACCESSION NUMBERS

Data derived from microarray analysis and ChIP-chip experiments have been deposited in the Gene Expression Omnibus (GEO) database, as reference series GSE22872.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.molcel.2010.07.008.

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