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Article

Pluripotent Stem Cell Protein Sox2 Confers Sensitivity to LSD1 Inhibition in Cancer Cells

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SUMMARY

Gene amplification of Sox2 at 3q26.33 is a common event in squamous cell carcinomas (SCCs) of the lung and esophagus, as well as several other cancers. Here, we show that the expression of LSD1/KDM1 histone demethylase is significantly elevated in Sox2-expressing lung SCCs. LSD1-specific inhibitors selectively impair the growth of Sox2-expressing lung SCCs, but not that of Sox2-negative cells. Sox2 expression is associated with sensitivity to LSD1 inhibition in lung, breast, ovarian, and other carcinoma cells. Inactivation of LSD1 reduces Sox2 expression, promotes G1 cell-cycle arrest, and induces genes for differentiation by selectively modulating the methylation states of histone H3 at lysines 4 (H3K4) and 9 (H3K9). Reduction of Sox2 further suppresses Sox2-dependent lineage-survival oncogenic potential, elevates trimethylation of histone H3 at lysine 27 (H3K27) and enhances growth arrest and cellular differentiation. Our studies suggest that LSD1 serves as a selective epigenetic target for therapy in Sox2-expressing cancers.

INTRODUCTION

Lung cancer is the most frequent cause of cancer death worldwide (Jemal et al., 2009; Jones et al., 2006). Squamous cell carcinoma (SCC) of the lung is a major form of frequent and aggressive lung cancer (Jones et al., 2006). Recent studies have shown that the gene amplification of *Sox2*, which encodes a high-mobility group domain-containing transcription factor, is the most frequent and common event in SCCs of the lung, esophagus, and oral cavity at 3q26.33 (Bass et al., 2009; Hussenet et al., 2010; Maier et al., 2011). It is also amplified in a fraction of small cell lung carcinomas (Rudin et al., 2012) and glioblastoma multiforme (GBM) (Alonso et al., 2011). Sox2 is a master regulator of pluripotent embryonic stem cells (ESCs) and adult neural stem cells (Episkopou, 2005; Okita and Yamanaka, 2006). It reprograms somatic cells into induced pluripotent stem cells (iPSCs) with Oct4, Klf4, and c-Myc, or with Oct4, Lin 28, and Nanog (Takahashi et al., 2007; Yu et al., 2007). Sox2 also plays an essential role in the morphogenesis and homeostasis of esophageal, tracheobronchial, and bronchiolar epithelia (Que et al., 2007). Sox2 acts as a lineage-survival oncogene for the expression of pluripotent stem cell (PSC) signatures and the lineage-specific genes in lung SCCs (Bass et al., 2009). Ectopic expression of Sox2 causes the oncogenic transformation of normal tracheobronchial epithelial cells (Bass et al., 2009). Sox2 is frequently expressed in other types of poorly differentiated and aggressive human cancers (Ben-Porath et al., 2008). In lung adenocarcinomas, Sox2 expression is associated with poor prognosis (Sholl et al., 2010). In stem cell-like ovarian cancer cells, Sox2 is also coexpressed with Oct4 and Lin28 (Peng et al., 2010; Zhong et al., 2010). In breast carcinomas, expression of Sox2 is required for mammosphere formation as part of its stem cell-like properties (Leis et al., 2012). In colon cancer, Sox2 expression correlates with lymph-node and distant metastases (Neumann et al., 2011). In these cancers, Sox2 appears to confer certain progenitor/stem cell properties to carcinoma cells to allow them to differentiate.

Histone methylation is a major covalent modification of histones that provides the structural and functional characteristics of chromatin to epigenetically define gene-expression patterns in a cell (Klose and Zhang, 2007; Shi, 2007). LSD1 (also known as KDM1, AOF2, or BHC110) is a highly conserved flavin adenine dinucleotide (FAD)-dependent, lysine-specific demethylase that was initially found to specifically remove mono- and dimethyl groups from methylated histone H3 at lysine 4 (H3K4) to suppress gene expression. In prostate cancer cells, it also demethylates the repressive mono- and dimethylated lysine 9 (H3K9) in an androgen-receptor-dependent manner (Metzger et al., 2005; Shi et al., 2004). Recent studies indicated that LSD1 is an essential epigenetic regulator of pluripotency in ESCs (Adamo

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et al., 2011; Whyte et al., 2012). We previously showed that LSD1 levels are significantly elevated in cells derived from pluripotent germ cell tumors such as teratocarcinoma, embryonic carcinoma, and seminoma cells (Wang et al., 2011). Our LSD1 inhibitors selectively inhibited the growth of ESCs and pluripotent germ tumor cells (Wang et al., 2011). LSD1 is also essential for maintaining the oncogenic potential of MLL-AF9 leukemia stem cells and acute myeloid leukemia (Harris et al., 2012; Schenk et al., 2012). However, the critical downstream targets of LSD1 in ESCs and cancer cells remain unclear. Here, we report that Sox2 serves as the critical target of LSD1 in a wide variety of human carcinoma cells.

RESULTS

LSD1 Expression Is Elevated in Human Lung SCCs that Overexpress Sox2

Our previous studies indicated that germ tumor cells such as teratocarcinoma and embryonic carcinoma cells expressed elevated levels of LSD1. They were also highly sensitive to our LSD1-specific inhibitors that noncovalently interact with LSD1 (Wang et al., 2011). Given that these cells usually express the PSC proteins Oct4, Sox2, and Lin28, which are also expressed in other human cancers (Leis et al., 2012; Peng et al., 2010; Zhong et al., 2010), we examined LSD1 in various human cancer tissues that also coexpress some of the PSC markers (only deidentified samples were used, which were acquired with the approval of the Ethics Committee of the First Affiliated Hospital, Shihezi University School of Medicine). In 13 independent cases of human lung SCC, we observed highly elevated levels of Sox2 and LSD1 in five poorly differentiated cases, moderate Sox2 and LSD1 increases in seven moderately differentiated cancer cases, and low LSD1 expression in a single Sox2-negative and moderately differentiated cancer (Figure 1A; Figure S1A available online; Table S1). Statistical analysis revealed a significant correlation between Sox2 and LSD1 expression (Pearson's correlations: $R^2 = 0.4372$ and $p = 0.014$). In contrast, Sox2 expression was low or nondetectable in all 17 cases of lung adenocarcinoma carcinomas. In the lung adenocarcinoma samples, only two cases of poorly differentiated cancers had moderately increased levels of LSD1, and LSD1 was low in the remaining 15 moderately differentiated cancers. As a control, the surrounding normal lung tissues expressed undetectable levels of LSD1 and Sox2 proteins (Figure 1A).

Our finding that the levels of LSD1 are significantly elevated in Sox2-expressing SCCs is consistent with previously reported studies. For example, in a search of publicly reported tumor microarray data from Oncomine (http://www.oncome.org), we found that in 21 SCC cases reported by Bhattacharjee et al. (2001), Sox2 was overexpressed in 18 cases, whereas LSD1 was co-overexpressed in 16 cases. In another six small cell lung carcinoma cases with elevated LSD1, Sox2 was also overexpressed in four of them. In 17 cases of esophagus carcinomas (Hu et al., 2010) with high LSD1 levels, Sox2 was overexpressed in 14 cases. The overexpression of Sox2 and LSD1 was also found in other cancers. For example, in 19 cases of large cell lung carcinomas (Hou et al., 2010), 16 cases overexpressed LSD1 and 15 overexpressed Sox2. In a study of cervical SCCs (Scotto et al., 2008), Sox2 was overexpressed in 50% of 84 cases and LSD1 was coelevated in 48% of all cases. In 122 cases of ductal breast carcinomas (Sorlie et al., 2003), Sox2 was overexpressed in ~52% of them and LSD1 was co-overexpressed with Sox2 in ~49% of all cases. In a single undifferentiated breast carcinoma case, both Sox2 and LSD1 were overexpressed. Our studies suggest that there is a significant correlation between Sox2 and LSD1 expression in a wide array of human cancers.

Lung SCC Cells that Contain Amplified *Sox2* Gene or Other Lung Carcinoma Cells that Express Sox2 Are Particularly Sensitive to LSD1 Inactivation

Our observation that LSD1 levels are elevated in Sox2-expressing lung SCCs prompted us to investigate the functional relationship between Sox2 and LSD1 in lung carcinomas. For this purpose, we used human SCC NCI-H520 cells that contain the *Sox2* gene amplification at 3q26.33, and human lung adenocarcinoma NCI-H1437 cells that do not express Sox2 (Bass et al., 2009; Figure 1B). H520 and H1437 cells were treated with LSD1 inhibitors for 24–30 hr, and cell growth was analyzed. We found that the LSD1 inhibitor CBB1007 selectively and specifically inhibited the growth of H520 cells, but had no detectable effects on H1437 cells (Figures 1C and 1D).

We wondered whether the elevated expression of Sox2 is responsible for such sensitivity to LSD1 inhibitors in lung cancer cells. To identify additional Sox2 or other PSC-specific gene-expression signatures in carcinoma cells, we searched the microarray mRNA data of the Cancer Genome Anatomy Project database (http://cgap.nci.nih.gov/Microarray/GeneList) in 60 cancer cell lines collected by the National Cancer Institute. Using this approach, we found that approximately one-third of these cancer cells express Sox2 or other PSC proteins. Treatment of A549, a Sox2-expressing human lung adenocarcinoma cell derived from adenocarcinomic alveolar basal epithelial cells, and H1299, a Sox2-negative human non-small cell lung carcinoma cell (Figure 1B), with LSD1 inhibitors revealed that A549 cells are highly sensitive to LSD1 inhibitors, whereas H1299 cells are not (Figures 1C and 1D). We also ablated the expression of LSD1 using specific siRNAs in H520, H1437, A549, and H1299 cells, and these studies showed that ablation of LSD1 phenocopied the selective growth-inhibitory effects of LSD1 inhibitors on H520 and A549 cells, but not on H1437 and H1299 cells (Figures 1C and 1D). Our results indicate that lung carcinoma cells that express Sox2 are particularly sensitive to LSD1 inactivation, whereas Sox2-negative cells are not. In addition, both Sox2-expressing H520 and A549 cells appeared to express higher levels of LSD1 (Figure 1B).

Breast and Ovarian Carcinoma Cells Can Be Distinguished by their Sensitivity to LSD1 Inhibition

Although LSD1 inhibitors specifically target lung carcinoma cells that overexpress Sox2, it remains unclear whether lung carcinoma cells are uniquely sensitive to LSD1 inhibition. A search of the Cancer Genome Anatomy Project database and published reports revealed that several breast and ovarian carcinoma cells may also express the key PSC proteins Oct4,

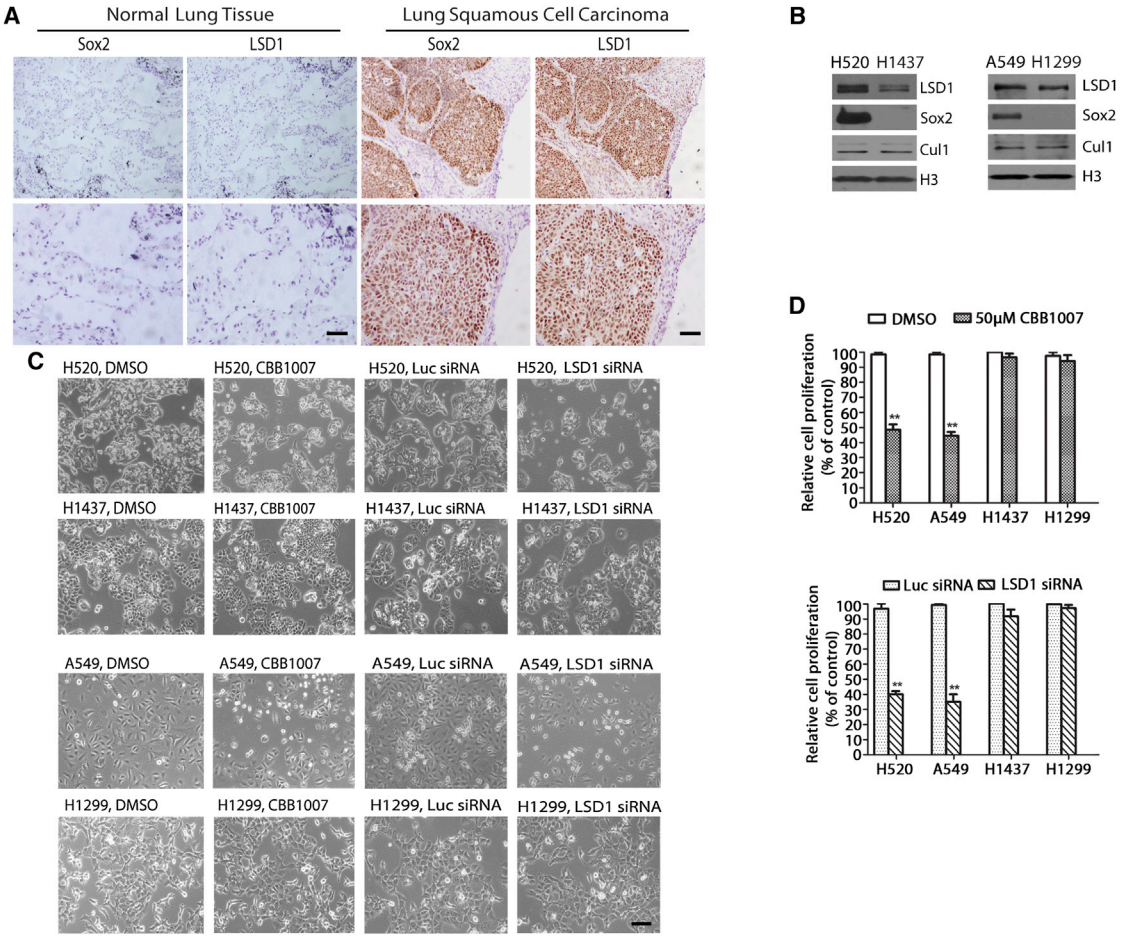

Figure 1. LSD1 and Sox2 Expression in Lung SCCs and Lung Carcinoma Cells that Express Sox2 Are Selectively Sensitive to LSD1 Inactivation

(A) LSD1 and Sox2 expression in lung SCCs. One example of serial tissue sections from clinical lung SCC patients (n = 13) immunostained with Sox2 or LSD1 antibodies. LSD1 and Sox2 were strongly stained in pathological tissues (right), but weakly stained or nondetectable in normal lung tissue (left) surrounding the pathological areas. Scale bar: 100 μm. Lower panels represent magnified images (4× of upper panels; total magnification: 400×). See also Figures S1A and S1B.

(B) Sox2 and LSD1 expression in human lung SCC NCI-H520, lung adenocarcinoma A549, and lung carcinoma NCI-H1437 and H1299 cells. CUL1 and histone H3: loading control.

(C) LSD1 inactivation specifically inhibited the growth of H520 and A549 cells, but not that of H1437 or H1299 cells. Actively growing lung cancer cells were treated with 50 μM LSD1 inhibitor CBB1007 for 30 hr or were transfected with 50 nM control luciferase (Luc) or LSD1 siRNAs for 60 hr. Cell growth was examined by microscopy.

(D) Percent viability of cells treated with LSD1 inhibitors or siRNAs compared with control cells, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data are presented as mean ± SD. The statistical differences between inhibitor-treated and control groups were analyzed by one-way ANOVA. Scale bar: 100 μm. **$p < 0.01$.

See also Figures S2, S7, and Table S1.

Sox2, Lin28, Nanog, and Sall4 either alone or in combination (Leis et al., 2012; Peng et al., 2010; Zhong et al., 2010). We therefore examined a panel of ovarian, breast, and other carcinoma cells for their response to LSD1 inhibition (Table S2). Although the growth of some cells was not inhibited by LSD1 inhibitors such as human ovarian carcinoma cells OVCAR8, Hs38.T, and ES-2, and breast carcinoma cells MDA-MB-231, BT549, and SK-BR-3, several of them were highly sensitive to LSD1 inhibitors such as ovarian carcinoma cells OVCAR3, A2780, SKOV-3, and IGROV-1; ovarian teratocarcinoma PA-1 cells; and breast carcinoma cells MDA-MB-468, T47D, and MCF-7 (Figures 2A, 2B, S1C, S1D, and S2A–S2C). We confirmed the selective effects of LSD1 inhibitors by ablation of LSD1 using LSD1 siRNAs (Figures 2A and 2C). Analysis of the cell-cycle effects by fluorescence-activated cell sorting (FACS) revealed that loss of LSD1 induced significant G1 cell-cycle arrest in cancer cells that are sensitive to LSD1 inhibition, such as A549, T47D, and IGROV1, which was associated with decreased expression of the cell-cycle regulatory proteins c-Myc and various cyclins, whereas such an arrest was not observed in cancer cells that are not sensitive to LSD1 inhibitors, such as H1437 (Figures 2E, 3G, and S3A).

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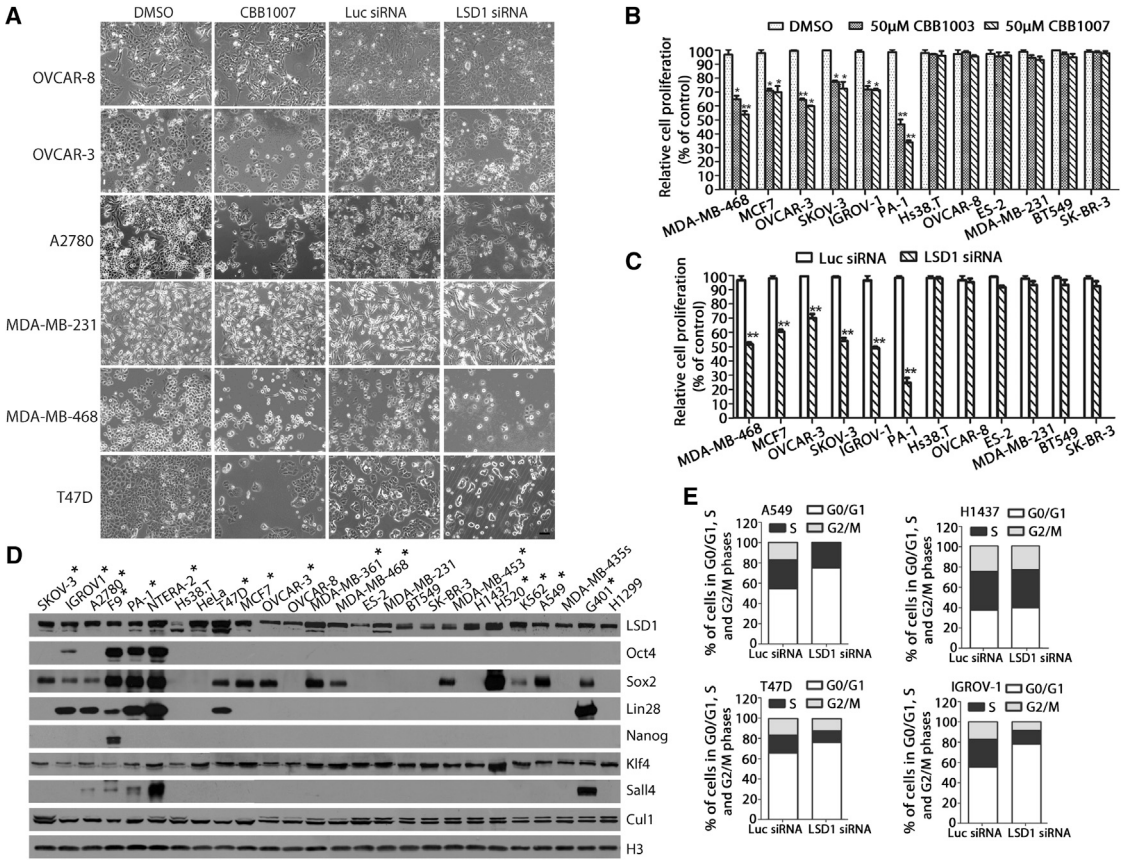

Figure 2. LSD1-Inactivation-Induced Growth Inhibition Is Associated Only with Sox2 Expression in Lung, Breast, Ovarian, and Other Types of Carcinoma Cells

(A) Effects of LSD1 inhibitor CBB1007 and siRNAs on the growth of ovarian carcinoma OVCAR-8, OVCAR-3, and A2780 cells, and breast carcinoma MDA-MB-231, MDA-MB-468, and T47D cells. See also Figures S1C, S1D, S2A–S2C, S2E, S2F, and S7B–S7D.

(B and C) Quantitative analyses of the sensitivity of a panel of breast, ovarian, and other human carcinoma cells to LSD1 inhibitors (B) and LSD1 siRNA-mediated ablation (C). The statistical differences between inhibitor-treated and control groups were analyzed by one-way ANOVA. *$p < 0.05$, **$p < 0.01$.

(D) Analysis of the expression of Oct4, Sox2, Nanog, Lin28, Sall4, and other proteins in lung, breast, ovarian, teratocarcinoma, embryonic carcinoma, and other carcinoma cells as indicated. Cells that are sensitive to LSD1 inactivation are indicated by *. Among PSC proteins, only the expression of Sox2 correlates with the growth-inhibitory effects of LSD1 inactivation. See also Table S2 for summary.

(E) Ablation of LSD1 by siRNAs induces G1 cell-cycle arrest in Sox2-expressing A549, T47D, and IGROV1 cells, but not in Sox2-negative H1437 carcinoma cells. The distributions of the cell-cycle population were as follows:

A549 cells, Luc siRNA: G0/G1: 54.64%, S: 28.42%, G2/M: 16.94%; and A549, LSD1 siRNA: G0/G1: 74.81%, S: 24.55%, G2/M: 0.65%.

IGROV1 cells, Luc siRNA: G0/G1: 55.48%, S: 27.48%, G2/M: 17.04%; and IGROV1, LSD1 siRNA: G0/G1: 78.07%, S: 13.37%, G2/M: 8.56%.

T47D cells, Luc: G0/G1: 65.87%, S: 17.91%, G2/M: 16.22%; and T47D, LSD1 siRNA: G0/G1: 76.34%, S: 11.49%, G2/M: 12.17%.

H1437 cells, Luc siRNA: G0/G1: 39.53%, S: 23.30%, G2/M: 37.17%; and H1437 cells, LSD1 siRNA: G0/G1: 37.28%, S: 24.97%, G2/M: 37.75%.

See also Figure S3A.

Sox2 Is the Only PSC Protein whose Expression Correlates with Sensitivity to LSD1 Inactivation in Carcinoma Cells

Our previous studies indicated that sensitivity to LSD1 inactivation is usually associated with cells derived from germ cell tumors that express PSC proteins (Wang et al., 2011). Because some breast and ovarian cancer cells in our collection were reported to express PSC proteins such as Oct4, Sox2, Lin28, Nanog, and/or Sall4 (Leis et al., 2012; Peng et al., 2010; Zhong et al., 2010), a direct correlation between the expression of these proteins and the sensitivity to LSD1 inactivation has not been established. To determine the mechanism by which various carcinoma cells are sensitive to LSD1 inactivation, we analyzed the expression of known PSC proteins in our collected cell lines and correlated their expression with the sensitivity to LSD1 inactivation, using teratocarcinoma/embryonic carcinoma F9 and NTERA-2 cells and cervical carcinoma HeLa cells as controls (Figure 2D; Table S2) (Wang et al., 2011).

We made several notable findings using this approach. First, we found that Oct4, Lin28, Sall4, and Nanog were each expressed in breast and ovarian carcinoma IGROV1, A2780, and T47D cells, and all teratocarcinoma/embryonic carcinoma F9, PA-1, and NTERA-2 cells (Figure 2D). We did not find a single cell line that expressed these PSC proteins independently of

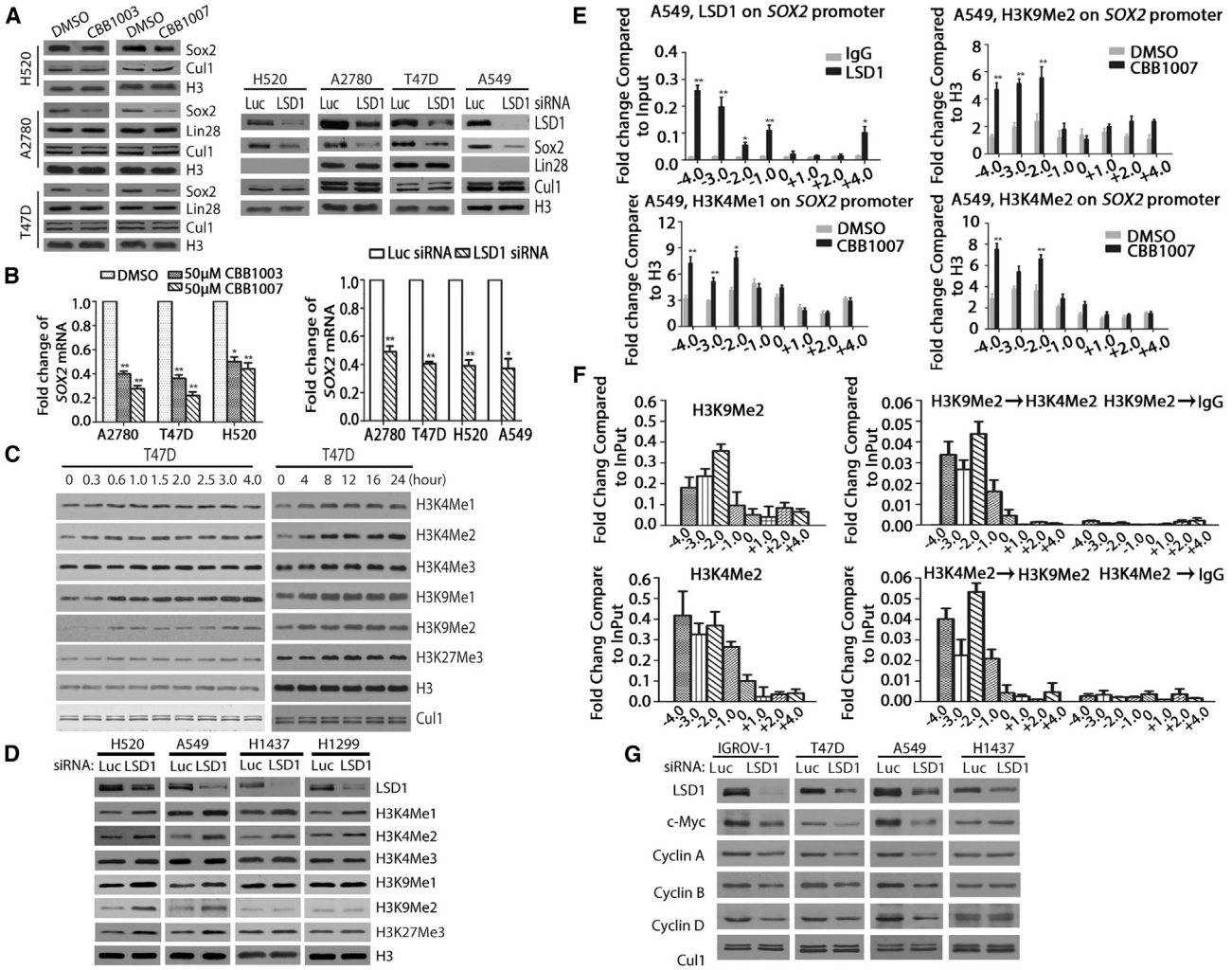

Figure 3. LSD1 Regulates Sox2 Expression by Modulating Bivalent H3K9 and H3K4 Methylations

(A) Inactivation of LSD1 by LSD1 inhibitors or siRNAs caused the downregulation of Sox2. The indicated carcinoma cells were treated with 50 μM of the LSD1 inhibitors CBB1007 and CBB1003 for 30 hr or with 50 nM of Luc or LSD1 siRNAs for 60 hr. The protein levels of LSD1, Sox2, Lin28, CUL1, and histone H3 were analyzed by western blotting. See also Figure S4B.

(B) The mRNA levels of Sox2 in (A) were analyzed by real-time quantitative RT-PCR. Data are presented as mean ± SD. $^{**}p < 0.01$.

(C) T47D cells were treated with 50 μM CBB1007. They were subsequently harvested at various time points as indicated. The effects of the LSD1 inhibitor on H3K4, H3K9, and H3K27 methylations were examined.

(D) The indicated cells were transfected with 50 nM Luc or LSD1 siRNAs, and histone methylations were analyzed as in (C). See also Figures S2C, S2D, and S7F.

(E) ChIP assays for the presence of LSD1, H3K4me1/2, and H3K9me2 on the *Sox2* gene in A549 cells. Chromatin-associated proteins were crosslinked to chromatin, sonicated (average 500–1,000 bp DNA), and immunoprecipitated with control rabbit immunoglobulin G (IgG), LSD1, or H3K9me2 antibodies as indicated. The DNA fragments were purified and used for real-time quantitative PCR with various primers along the *Sox2* promoter. Data are presented as mean ± SD. The statistical differences for increased H3K4 and H3K9 methylations along the *Sox2* gene between inhibitor-treated and control groups were analyzed by one-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$. See also Figure S6C.

(F) Re-ChIP assays for the copresence of H3K9Me2 and H3K4Me2 on the *Sox2* gene in A549 cells. Top panels: chromatin fragments were first immunoprecipitated with H3K9me2 antibodies or control IgG. The fragments were eluted with H3K9me2 peptide and reimmunoprecipitated with H3K4me2 antibodies. Bottom panels: the chromatin fragments were first immunoprecipitated with H3K4me2 and then H3K9me2 antibodies. Real-time quantitative PCR ratios reflect the relative enrichment to the input of the indicated histone methylations on *Sox2* after sequential immunoprecipitations. Data are presented as the mean ± SD of at least two independent assays.

(G) Effects of LSD1 inactivation as in (D) on the protein levels of c-Myc and cyclins.

See also Table S3.

Sox2, suggesting the importance of Sox2. Second, Sox2 was the only PSC protein that was expressed alone and independently of other PSC proteins in SKOV-3, OVCAR-3, MCF-7, MDA-MB-361, MDA-MB-468, MDA-MB-453, NCI-H520, and A549 cells, again indicating that Sox2 is unique for these carcinoma cells. Third, and most importantly, our analyses revealed that all

Sox2-expressing carcinoma cells were sensitive to LSD1 inactivation, whereas all Sox2-negative cancer cells were insensitive (Figure 2D; Table S2). Consistently, we also found that K562, a human myelogenous leukemia cell, and G401, a human rhabdoid-tumor-derived cell, also expressed Sox2, and both were sensitive to LSD1 inhibition, whereas MDA-MB-435S, a melanoma cell that does not express Sox2, was insensitive (Figures 2D, S2E, and S2F; Table S2). Whereas K652 cells only express Sox2, G401 cells also express Lin28 and Sall4. Our studies thus indicate that there is a strong correlation between Sox2 expression and sensitivity to LSD1 inactivation.

LSD1 Inactivation of LSD1 Suppresses Sox2 Expression and Increases the Mono- and Dimethylation of H3K9 and Trimethylation of H3K27 Only in Sox2-Expressing Carcinoma Cells

Analysis of the effects of LSD1 inactivation revealed that LSD1 inactivation consistently reduced the expression of Sox2 in H520, A2780, T47D (Figures 3A and 3B), and other Sox2-expressing cancer cells (Figure S4B). In contrast, LSD1 inactivation did not change the levels of Lin28, another PSC protein, which is often coexpressed with Sox2 in a fraction of carcinoma cells (Figures 2D, 3A, and S4B). Our results suggest that LSD1 is required for the expression of Sox2 in these carcinoma cells.

As LSD1 is a histone demethylase that removes the mono- and dimethyl groups from methylated H3K4 (H3K4me1/me2)(Shi et al., 2004), we found that LSD1 inactivation caused a dose-dependent increase of H3K4me1/me2 in both Sox2-postive and -negative cells (Figures 3C, 3D, S2C, and S2D), indicating that the inhibitors specifically blocked LSD1 demethylase activity in all cancer cells.

Because LSD1 interacts with the androgen receptor to act as an H3K9-specific demethylase to remove the mono- and dimethyl groups from methylated H3K9 (H3K9me1/me2) in certain prostate cancer cells (Metzger et al., 2005), we also examined whether LSD1 inactivation affected H3K9me1/me2 as well as the trimethylation of histone H3 at lysine 27 (H3K27me3), which is not a target of LSD1. Strikingly, we found that LSD1 inactivation induced global increases of both H3K9me1/me2 and H3K27me3 only in Sox2-expressing carcinoma cells, but not in Sox2-negative cancer cells such as H1299 or H1437 (Figures 3C and 3D). A kinetic analysis of induction revealed that the increases of both H3K4me1/2 and H3K9me1/2 occurred early (<1 hr) and simultaneously, whereas H3K27me3 was induced much later (4–8 hr; Figure 3C) after LSD1 inhibition, suggesting that the increases of H3K4me1/2- and H3K9me1/2 are likely a direct consequence of LSD1 inhibition, and H3K27me3 elevation may occur as a secondary event.

LSD1 Binds Directly to the Transcriptional Regulatory Region of *Sox2* to Regulate Bivalent H3K4 and H3K9 Methylation

Because LSD1 inactivation reduced Sox2 expression, we tried to determine whether *Sox2* is a direct target of LSD1 by using a chromatin immunoprecipitation (ChIP) assay (Whyte et al., 2012) to determine whether LSD1 binds to the *Sox2* gene. Our ChIP analysis revealed that LSD1 is enriched in the transcriptional regulatory region (−3.0 to −4.0 kb) of the *Sox2* gene (Figure 3E), a region that was reported to act as a distal enhancer for Sox2 expression in breast cancer cells (Leis et al., 2012). This enrichment of LSD1 appears to be specific for *Sox2*, as no enrichment was observed on *Lin28, Klf4*, or the pericentromeric heterochromatin region (Dovey et al., 2010; Figures 7G and S6D), which are not regulated by LSD1.

To determine whether LSD1 binding is associated with the demethylase activity on *Sox2*, we examined the presence and changes of the characteristic H3K4me1/me2 and H3K9me1/me2 on *Sox2* after LSD1 inactivation. Repeated ChIP analyses in Sox2-expressng cells, such as A2780 and A549 cells, consistently revealed that H3K9me2 (we did not have good ChIP-grade H3K9me1 antibodies) and H3K4me1/me2 were present in the *Sox2* regulatory region, and inhibition of LSD1 caused significantly increased levels of both H3K9me2 and H3K4m1/me2 on *Sox2* (Figure 3E). Reciprocal re-ChIP of H3K9me2 or H3K4me2-enriched chromatin fragments revealed that H3K9me2 and H3K4me2 coexisted on the same *Sox2* regulatory fragment (Figure 3F). Although H3K27me3 was also induced on *Sox2*, the major site was located farther down the gene within the coding region (+2.0; Figure S6C). Thus, our results indicate that the *Sox2* regulatory region is regulated directly by the bivalent H3K4 and H3K9 methylations by LSD1 demethylase. Sox2 downregulation after LSD1 inactivation is likely to be directly caused by increased repressive H3K9 methylations on the *Sox2 gene*, even though H3K4me1/2 also increased on *Sox2* (see below).

Our ChIP analysis also indicated that LSD1 also binds to the *cyclin A*, *cyclin B*, and *cyclin D1* genes, and inactivation of LSD1 caused the increased levels of H3K4me1/me2, H3K9me2, and H3K27me3 on the cyclin promoters (Figures 7F, S3B, and S6C). Thus, increased levels of H3K9me2 on the cyclin genes may also repress the expression of cyclins, which may contribute to the G1 cell-cycle arrest after LSD1 inactivation (Figures 2E, 3G, S3A, and S3B).

Loss of Sox2 Phenocopies the Growth-Inhibitory Effects of LSD1 Inactivation on Carcinoma Cells

Although *Sox2* acts as an amplified lineage-survival oncogene in lung SCCs (Bass et al., 2009), the role of Sox2 in other carcinoma cells remains largely uncharacterized. Because LSD1 inactivation reduced Sox2 expression, we further investigated the role of Sox2 in regulating cancer cell growth. Ablation of Sox2 expression using specific siRNAs consistently showed that it caused G1 cell-cycle arrest and growth inhibition in Sox2-expressing carcinoma cells that are sensitive to LSD1 inactivation, but not in Sox2-negative cancer cells (Figures 4A, 4B, 4E, S4A, and S4C). Loss of Sox2 also downregulated c-Myc and cyclin A, cyclin B, and cyclin D1, and induced the expression of genes for differentiation, including FOXA2, HNF4A, BMP2, EOMES, and Sox17 (Figures 4C and 4D). However, loss of Sox2 increased only the levels of trimethylated H3K27, and not those of H3K4 and H3K9 methylations, suggesting that induction of H3K27 trimethylation after LSD1 inactivation might be an indirect consequence of Sox2 downregulation (Figures 3C, 3D, and 4C). ChIP analysis revealed that Sox2 inactivation induced elevated H3K27me3 on *Sox2* and *cyclin* promoters after Sox2 ablation (Figure 4F), suggesting that increased H3K27me3 on these

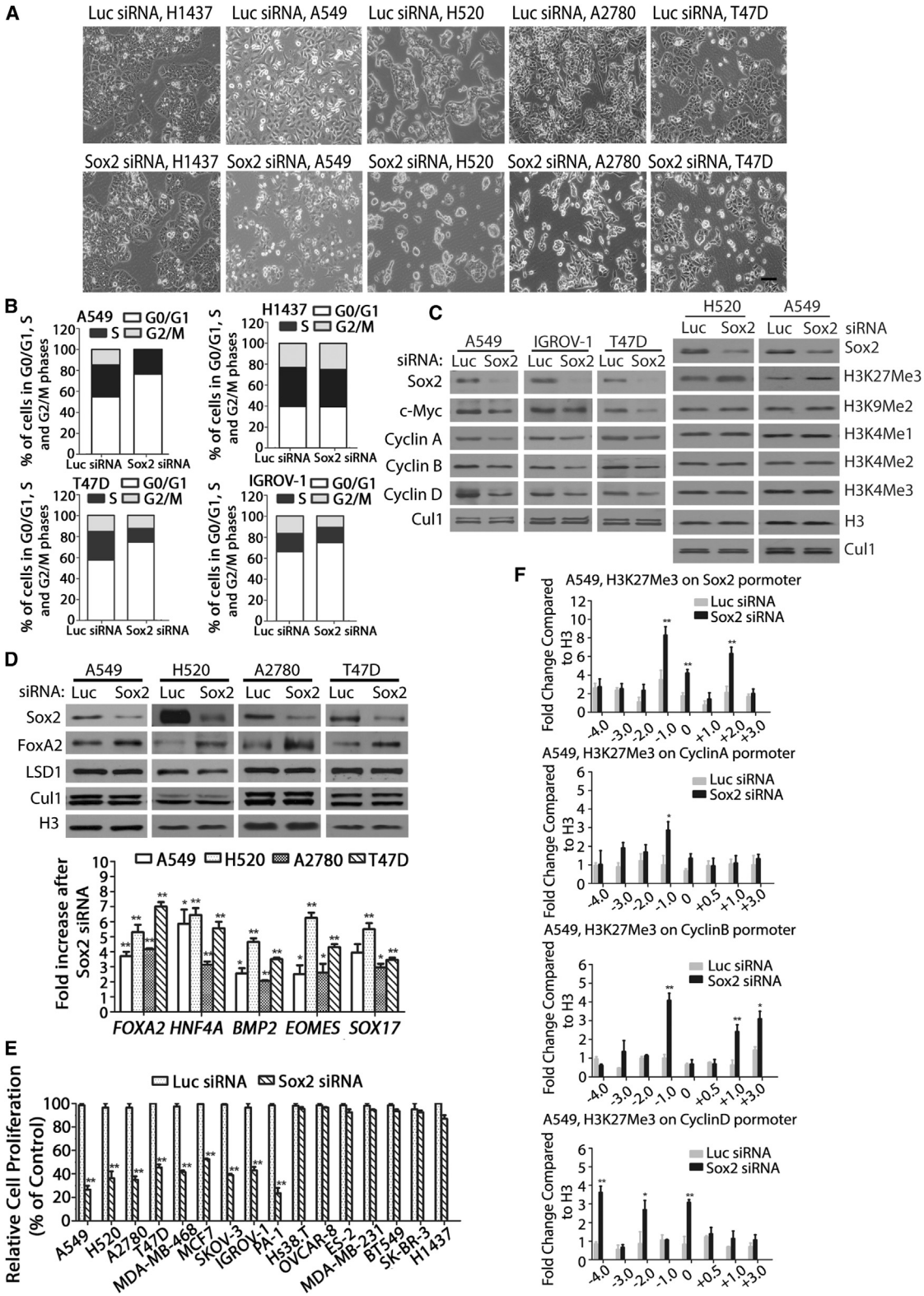

Figure 4. Inactivation of Sox2 Causes G1 Cell-Cycle Arrest, Inhibits the Cell Growth of Sox2-Expressing Carcinoma Cells, and Induces Genes for Differentiation

(A) A549, NCI-H1437, T47D, and IGROV1 cells were transfected with 50 nM luciferase or Sox2 siRNAs for 60 hr and the cell growth of control and Sox2-ablated cells was examined by microscopy. See also Figure S4C.

(legend continued on next page)

genes suppressed their expression. Thus, our data indicate that Sox2 serves as a primary and key direct target of LSD1 inactivation for growth inhibition and differentiation, because downregulation of Sox2 further amplifies and enhances the effects of LSD1 inactivation through the increased levels of H3K27me3. Our observation is consistent with previous reports of critical thresholds and phenotypes associated with haploid insufficiency and hypomorphic mutations of *Sox2* in animals and human diseases (Episkopou, 2005). Mutations of human Sox2 that compromise one allele of the *Sox2* genes were shown to cause anophthalmia-esophageal-genital (AEG) syndrome and exhibited neurological phenotypes, including seizures (Fantes et al., 2003; Williamson et al., 2006), whereas hypomorphic deletion of the enhancer of the mouse *Sox2* genes, which reduced Sox2 mRNA and protein levels by 20%–30% compared with wild-type levels, exhibited lower birth frequency and neurological phenotypes in the mouse (Ferri et al., 2004).

Sox2 Is Involved in Mediating the Growth-Inhibitory Effects of LSD1 Inactivation

To further determine whether reduced expression of Sox2 is responsible for the growth inhibition caused by LSD1 inactivation, we tried to ectopically express Sox2 in Sox2-expressing carcinoma cells. In both Sox2-expressing ovarian A2780 and lung A549 carcinoma cells, stable and ectopic expression of Sox2 led to a significant resistance of these cells to LSD1 inhibition as compared with control cells (Figure 5A). Coinactivation of Sox2 and LSD1 in Sox2-expressing cancer cells by siRNAs or LSD1 inhibitors also did not reveal any additive or synergetic effects on growth inhibition induced by the loss of LSD1 or Sox2 alone (Figures 5D and 5E), suggesting that LSD1 and Sox2 act in the same pathway to control cell growth.

LSD1 was shown to interact with several proteins, such as CoREST, in various cells (Shi et al., 2005). To confirm that the effects of LSD1 inactivation are mediated through its interaction with cellular proteins, we also ablated the expression of CoREST. We found that loss of CoREST caused the same selective growth-inhibitory effects and induction of H3K4, H3K9, and H3K27 methylations on Sox2-expressing cancer cells, but not Sox2-negative cancer cells, as LSD1 inactivation (Figure S7). It is likely that LSD1 acts through the CoREST complex to selectively regulate the growth of Sox2-expressing cancer cells, although loss of CoREST slightly reduced the level of LSD1 protein, possibly because of their in vivo association.

Because Sox2-expressing carcinoma cells may be heterogeneous in Sox2 expression and different cells may vary in their responses to LSD1 inactivation, we examined the clonal response of Sox2-expressing carcinoma cells. To isolate single cells, we used serial dilution to separate A549 cells. Each isolated single cell was clonally expanded and characterized. We found that the isolated single-cell clones exhibited different growth rates represented by their differences in colony size (Figure 5B). Examination of Sox2 expression in various clones revealed that smaller clones usually contained much higher levels of Sox2 expression than the larger clones (Figure 5B). Notably, the smaller clones also expressed more LSD1 and were more sensitive to LSD1 inhibitors than the larger clones that expressed less Sox2 and LSD1 (Figures 5B and 5C). These studies again underscore the correlation between Sox2 and LSD1 expression, and indicate that cancer cells that express higher levels of Sox2 are more sensitive to LSD1 inhibition.

Loss of LSD1 Suppresses Sox2-Dependent, Lineage-Specific Gene Expression and Reduces Sox2-Mediated Repression of Genes for Differentiation

In lung SCCs, Sox2 acts as a lineage-survival oncogene that is required for the expression of lineage-specific genes such as TP63 and KRT6A (Bass et al., 2009). Our studies revealed that loss of either LSD1 or Sox2 led to the downregulation of TP63 and KRT6A expression in Sox2-expressing H520 and A549, but not in Sox2-negative H1299 lung carcinoma cells (Figures 6A and 6B). We further used ChIP assays to determine whether LSD1 inactivation affects the ability of Sox2 to bind the promoters of *TP63* and *KRT6A*. In H520 cells, we found that Sox2 bound directly to the promoters of *TP63* and *KRT6A* genes (Figure 6C), and inhibition of LSD1 significantly reduced the binding of Sox2 to these lineage-specific genes (Figure 6D).

Our studies also revealed that ablation of Sox2 induced the expression of differentiation genes such as *FOXA2* and *Sox17* (Figure 4D). ChIP assays indicated that Sox2 bound directly to these promoters and inactivation of LSD1 significantly decreased Sox2 binding to these promoters (Figures 6C and 6D), suggesting that Sox2 normally acts as a repressor of these differentiation genes. Thus, our data confirm that LSD1 inactivation acts directly on a Sox2-dependent transcriptional program to reduce the lineage-survival oncogene function of Sox2 and to impair Sox2-mediated repression of differentiation genes.

(B) Cell cycles after Sox2 inactivation were analyzed by FACS. The cell-cycle distribution of cells was as follows:
A549 cells, Luc siRNA: G0/G1: 54.68%, S: 30.52%, G2/M: 14.80%; and A549, Sox2 siRNA: G0/G1: 76.40%, S: 23.41%, G2/M: 0.19%.
H1437 cells, Luc siRNA: G0/G1: 39.69%, S: 23.11%, G2/M: 37.20%; and H1437 cells, Sox2 siRNA: G0/G1: 39.39%, S: 25.10%, G2/M: 35.51%.
IGROV1 cells, Luc siRNA: G0/G1: 57.29%, S: 27.32%, G2/M: 15.39%; IGROV1, Sox2 siRNA: G0/G1: 74.62%, S: 12.87%, G2/M: 12.52%.
T47D cells, Luc: G0/G1: 66.02%, S: 17.27%, G2/M: 16.71%, T47D, Sox2 siRNA: G0/G1: 74.65%, S: 14.48%, G2/M: 10.86%. See also Figure S4A.
(C) The effects of Sox2 inactivation as in (B) on c-Myc, cyclins, and methylated H3K4, H3K9, and H3K27 proteins were analyzed by western blotting.
(D) Induction of differentiation genes FOXA2, HNF4A, BMP2, EOMES, and Sox17 by Sox2 deficiency in A549, H520, A2780, and T47D cells, analyzed by western blotting and real-time quantitative RT-PCR.
(E) The indicated lung, breast, ovarian, and other carcinoma cells were transfected with 50 nM luciferase or Sox2 siRNAs for 48 hr and cell growth was monitored by MTT assay. *$p < 0.05$, **$p < 0.01$.
(F) ChIP arrays on A549 cells with or without Sox2 siRNA-mediated ablation as in (B) for increases of H3K27me3 on *Sox2* and cyclin A, cyclin B, and cyclin D1 genes.
See also Table S3.

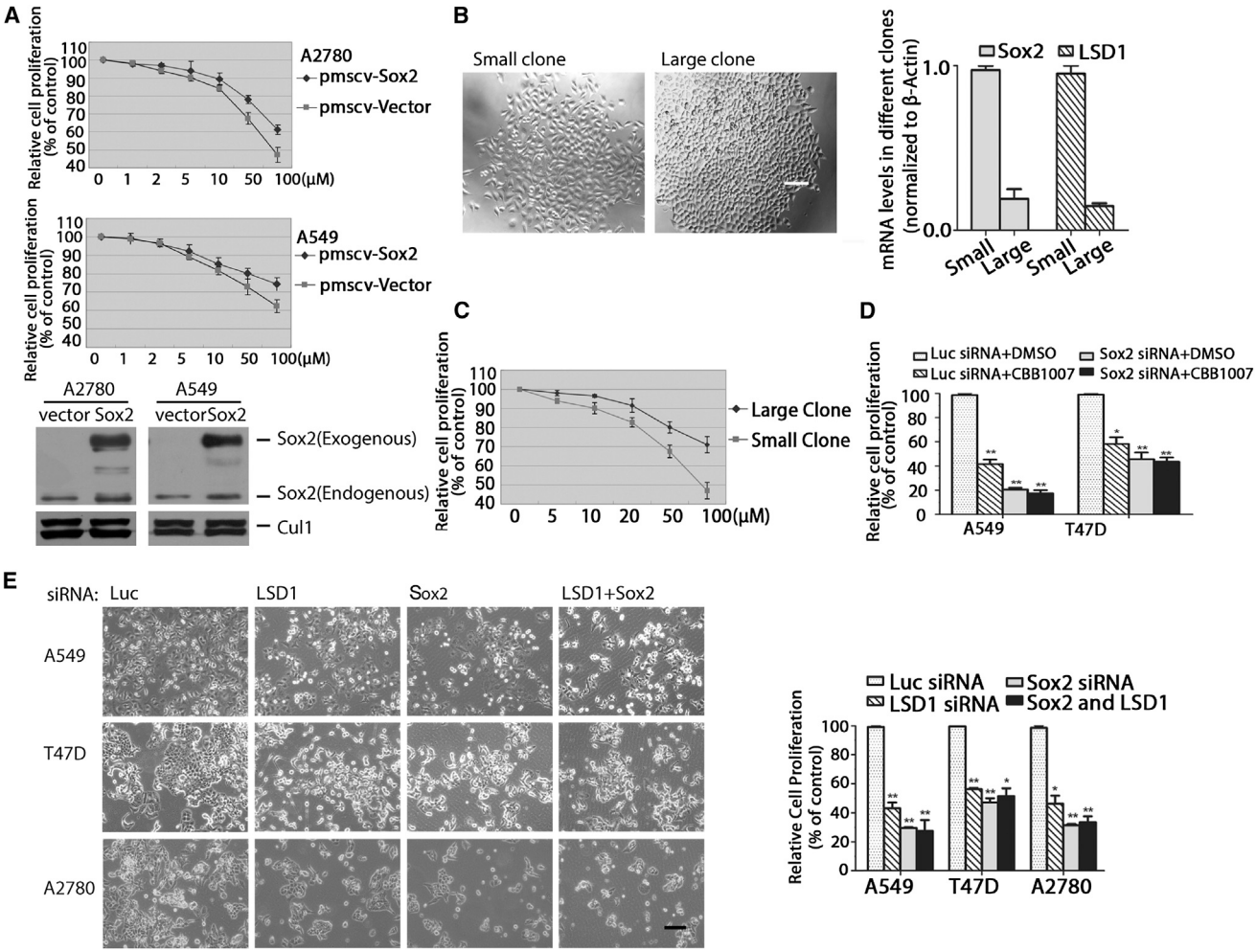

Figure 5. Sox2 Is a Target of LSD1 Inactivation in Sox2-Expressing Carcinoma Cells

(A) Ectopic expression of Sox2 conferred resistance to LSD1 inhibitors. Human Sox2 cDNA was tagged with Flag epitope at the amino terminus and stably expressed in A2780 or A549 cells using the retroviral pMSCV vector. Control and Flag-Sox2-expressing cells were treated with various concentrations of CBB1007 for 30 hr, and cell viability was assayed and compared.

(B) Sox2-expressing A549 carcinoma cells were separated by serial dilution into single cells. The single-cell clones were expanded and two representative clones are shown. The mRNA levels of Sox2 and LSD1 in the small and large clones were analyzed by real-time quantitative RT-PCR.

(C) The responses of the single small and large clones of A549 cells in (B) to various concentrations of CBB1007 were examined.

(D and E) Sox2-expressing A549, T47D, or A2780 cells were treated with 50 μM CBB1007 for 30 hr (D) or transfected with 50 nM luciferase, LSD1, Sox2, or LSD1+Sox2 siRNAs for 48 hr (E), and the effects on their growth were monitored and quantified.

See also Table S3.

Loss of LSD1 Induces the Expression of Genes for Differentiation by Selectively Increasing the Levels of Methylated H3K4, but Not Methylated H3K9 or H3K27, on the Promoters

LSD1 inactivation in germ tumor cells or ESCs induced the expression of genes for differentiation (Wang et al., 2011). To determine whether the function of LSD1 is preserved in Sox2-expressing carcinoma cells, we analyzed the effects of LSD1 inactivation on the expression of differentiation genes. We found that LSD1 inactivation led to the induction of differentiation genes such as *FOXA2*, *HNF4A*, *BMP2*, *EOMES*, and *Sox17* only in Sox2-expressing cancer cells, and not in Sox2-negative cancer cells (Figures 7A–7D and S5). Although loss of LSD1 caused G1 cell-cycle arrest (Figures 2E and S3A), the induction of differentiation genes did not appear to be the consequence of LSD1 inactivation-induced G1 cell-cycle arrest, as arresting Sox2-expressing A549 cells in the G1/S border alone did not promote the induction of differentiation genes or the suppression of Sox2 expression (Figure S3C).

Because LSD1 inactivation promotes the primary global methylations of H3K4me1/2, H3K9me1/2, and H3K27me3 in Sox2-expressing cancer cells (Figures 3C and 3D), and increased levels of these methylations on the promoters of *Sox2* and *cyclin* genes (Figures 3E, 7F, S3B, and S6C), we examined these methylations in the regulatory regions of differentiation genes. In sharp contrast to the increased H3K4,

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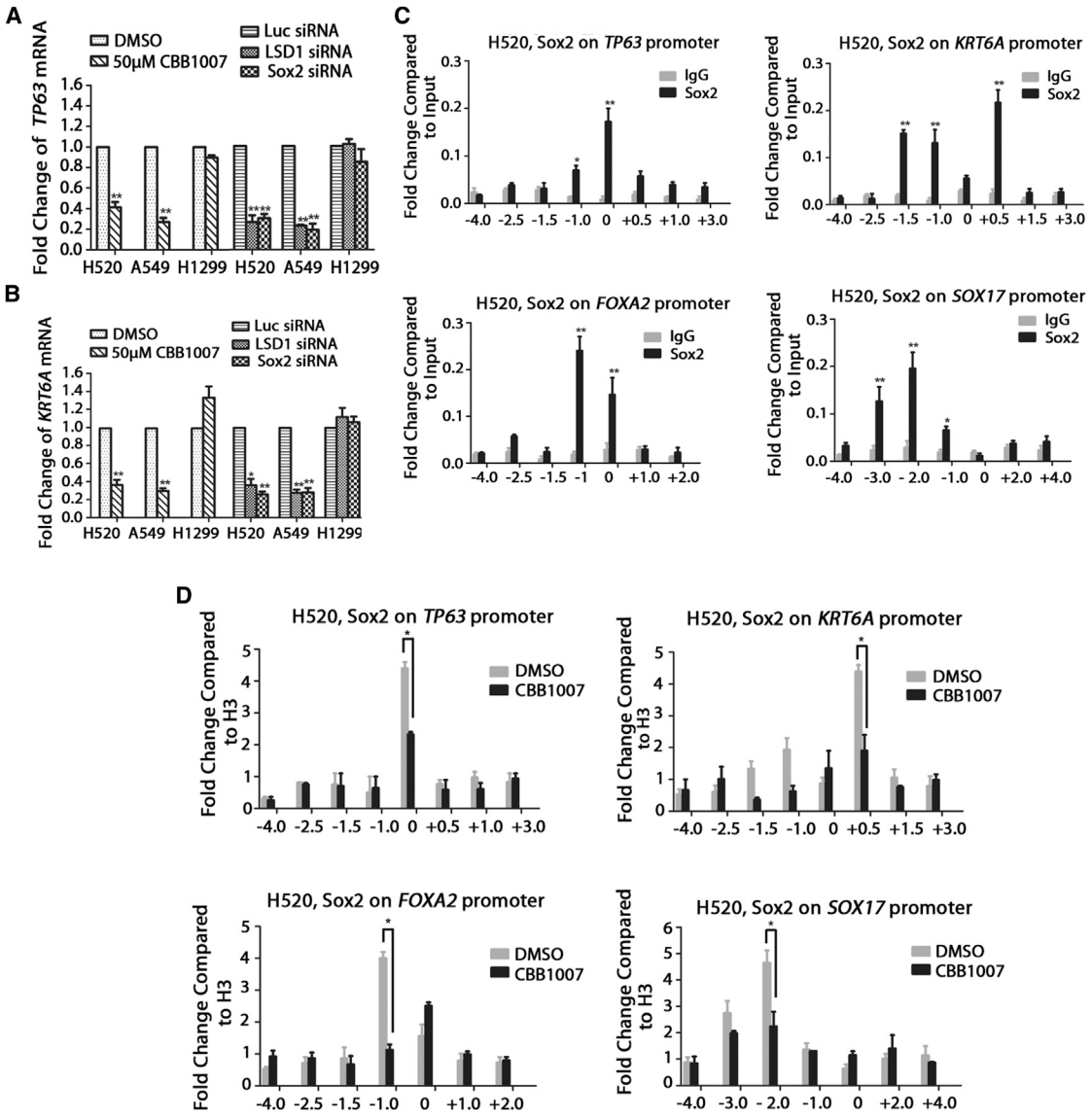

Figure 6. LSD1 Inactivation Impairs Sox2 Regulation of the Expression of Lineage-Specific Genes and Genes for Differentiation

(A and B) The lineage-specific genes TP63 (A) and KRT6A (B) were downregulated after inactivation of LSD1 or Sox2. Lung carcinoma NCI-H520, A549, and H1299 cells were either treated with 50 μM CBB1003 for 30 hr or transfected with Luc-, LSD1-, or Sox2-specific siRNAs for 48 hr as indicated. The mRNA levels of TP63 and KRT6A were measured by real-time quantitative RT-PCR. Downregulation of TP63 and KRT6A occurred in Sox2-ablated H520 and A549 cells, but not in H1299 cells. Statistical differences between experimental and control groups were analyzed by one-way ANOVA. *$p < 0.05$, **$p < 0.01$.

(C) ChIP analysis of Sox2 binding to the promoters of TP63, KRT6A, FOXA2, and Sox17 genes using control IgG and specific Sox2 antibodies in NCI-H520 cells. *$p < 0.05$, **$p < 0.01$.

(D) The LSD1 inhibitor CBB1007 reduced Sox2 binding to TP63, KRT6A, FOXA2, and Sox17 genes in H520 cells.

See also Figures S5, S6, and Table S3.

H3K9, and H3K27 methylations on the *Sox2* or *cyclin* genes after LSD1 inactivation, LSD1 inhibition consistently increased the mono- and dimethylations of H3K4 in the regulatory regions of *FOXA2*, *BMP2*, and *Sox17* genes, but not the H3K9 and H3K27 methylations in Sox2-expressing carcinoma cells (Figures 7E, S6A, and S6B). Thus, our studies reveal a mechanism by which LSD1 inactivation suppresses the expression of *Sox2* and cyclins by increased H3K9 and H3K27 methylations on their regulatory regions/promoters, whereas it causes the induction of differentiation genes by selectively elevating the levels of H3K4me1/me2 on differentiation genes, but not methylated H3K9 and H3K27. The effects of LSD1 inhibitors on differentiation may be enhanced by Sox2 down-regulation, which derepresses Sox2-mediated suppression of differentiation genes and causes further cellular differentiation (Figure 6).

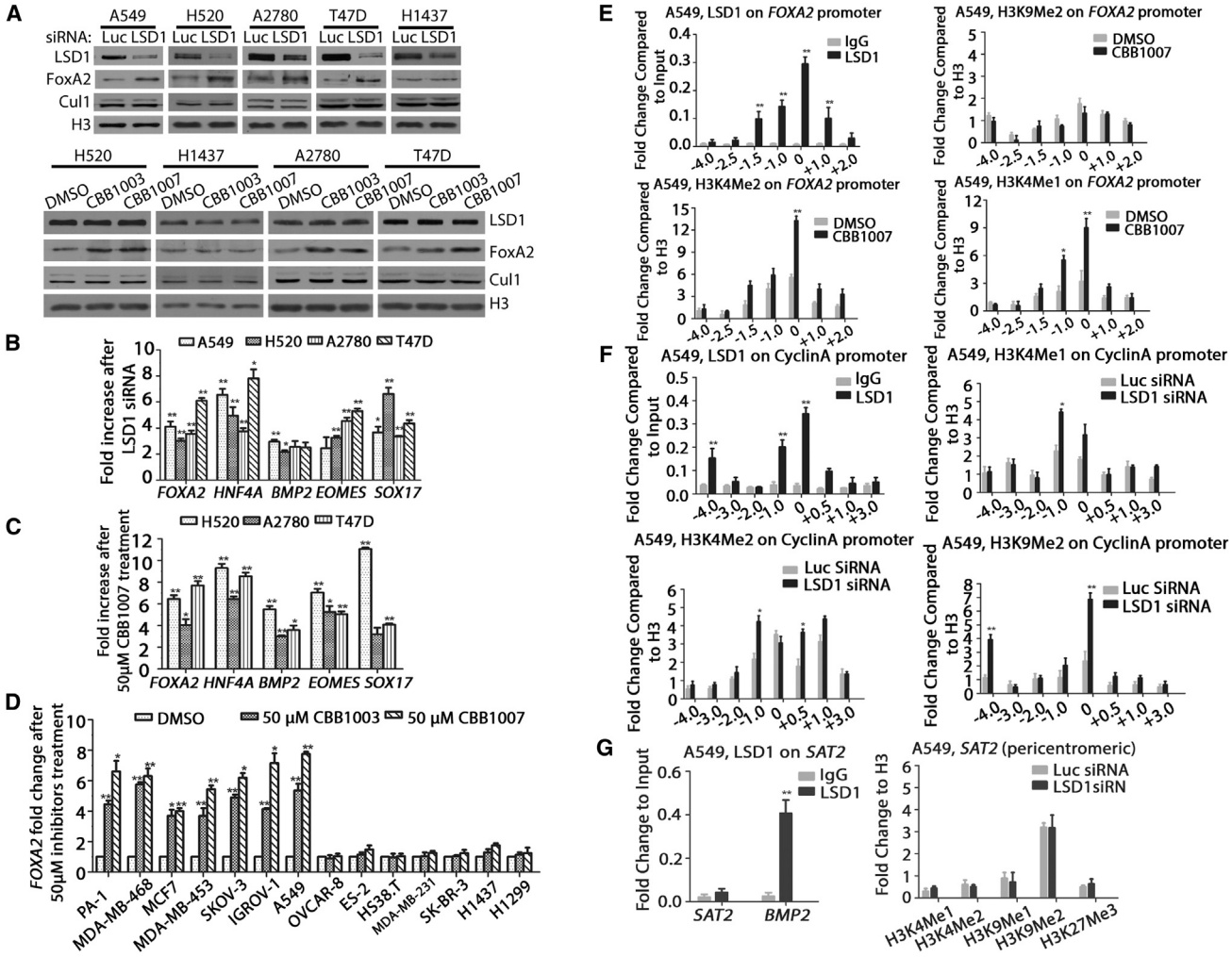

Figure 7. Inactivation of LSD1 Induces the Expression of Differentiation Genes by Increased Methylation of H3K4, but not Methylated H3K9, and Suppression of the Cyclin A Gene by Increased H3K4me1/2 and H3K9me2

(A–C) A549, NCI-H520, H1437, A2780, and T47D cells were transfected with 50 nM luciferase or LSD1 siRNAs for 48 hr or treated with 50 μM LSD1 inhibitors CBB1003 and 1007 for 30 hr. The effects of LSD1 inactivation on the induction of FOXA2 (A) or on various differentiation genes (*FOXA2*, *HNF4A*, *BMP2*, *EOMES*, and *Sox17* [B and C]) were analyzed by western blotting (A) or real-time quantitative RT-PCR (B and C). See also Figure S5A.

(D) The indicated lung, breast, ovarian, and other carcinoma cells were treated with 50 μM CBB1003 or CBB1007 for 30 hr, and induced expression of FOXA2 mRNAs was monitored, quantified, and compared between control and inhibitor-treated cells using real-time quantitative RT-PCR. *$p < 0.05$, **$p < 0.01$. See also Figure S5B.

(E–G) ChIP assays for the presence of LSD1, H3K9me2, H3K4me1, and H3K4me2 in the regulatory regions of the differentiation gene *FOXA2* with or without LSD1 inhibitors for 30 hr (E; see also Figures S6A–S6C) or on the *cyclin A* gene (F; see also Figures S3B and S6C) and the pericentromeric heterochromatin region *SAT2* and *BMP2* genes (G; see also Figure S6D) after LSD1 inactivation in A549 cells. Chromatin fragments were immunoprecipitated with control rabbit IgG-, LSD1-, H3K9me2-, H3K4me1-, and H3K4me2-specific antibodies as indicated. The DNA fragments were purified and used for real-time quantitative PCR with various primers along the *FOXA2* and *cyclin A* genes or the *SAT2* and *BMP2* regions. Data are presented as mean ± SD.

See also Table S3.

DISCUSSION

Although we previously found that LSD1 inactivation selectively inhibited the growth of pluripotent ESCs and germ tumor cells (Wang et al., 2011), the mechanism by which these cells are selectively sensitive to LSD1 inhibitors remains unclear. In this study, we found that tissue-specific carcinoma cells can be divided into two groups based on their sensitivity to LSD1 inactivation. Although some sensitive carcinoma cells express Oct4, Lin28, and/or Sall4, we found that all carcinoma cells that are sensitive to LSD1 inactivation consistently express Sox2, often alone and independently of other known PSC proteins (Figures 1, 2, and S1–S3; Table S2). This correlation strongly suggests that the growth-inhibitory effects of LSD1 inhibitors are dependent on Sox2, but not other PSC proteins. Our finding that Sox2 is the sole PSC protein expressed in a wide array of lung,

breast, ovarian, and other cancer cells suggests that Sox2 is a master regulator of the growth, differentiation, and survival of these cancers.

LSD1 acts primarily as a histone demethylase that removes the methyl groups from H3K4me1/me2 to suppress gene expression (Shi et al., 2004). Only in some prostate cancer cells can LSD1 bind to androgen receptor in a ligand-dependent way to remove the methyl groups from H3K9me1/me2 to activate gene expression (Metzger et al., 2005). Strikingly, we found that LSD1 inactivation induced a global increase of both H3K4me1/me2 and H3K9me1/me2 in Sox2-expressing cancer cells (Figure 3), which form bivalent chromatin domains in the regulatory regions of *Sox2* and cell-cycle regulatory genes such as cyclins to suppress their expression (Wang et al., 2009). Although trimethylated H3K27 was also induced, it appears that this increase is caused by Sox2 downregulation after LSD1 inactivation, as loss of Sox2 induced an increase of trimethylated H3K27, but not of methylated H3K4 and H3K9 (Figure 4C). However, this increase in H3K27me3 appears to be critical for the response to LSD1 inactivation, since inactivation of *Sox2* produced the same growth-inhibitory effects that resulted from LSD1 inhibition in cancer cells (Figures 4 and S4). In contrast, our studies revealed that LSD1 inactivation caused increased levels of H3K4me1/me2, but not H3K9me1/2 and H3K27me3, in the regulatory regions of differentiation genes to induce their expression (Figures 7 and S6). Thus, our work suggests a mechanism whereby LSD1 inactivation causes selective increased methylations of H3K4 and H3K9 on the *Sox2* and cell-cycle genes, but only induces increased H3K4 methylation on the promoters of differentiation genes, resulting in the downregulation of Sox2 and cyclins and the induction of genes for differentiation. The downregulation of *Sox2* consequently reduces lineage-specific oncogenic potential and further promotes cellular differentiation. A key event of Sox2 downregulation by LSD1 inactivation is the induction of H3K27 trimethylation, which further reduces the expression of Sox2 and cell-cycle genes to amplify the growth-inhibitory and differentiation-promoting effects of LSD1 inactivation. Our studies collectively indicate that LSD1 is a key regulator of Sox2 expression, cell-cycle progression, and cellular differentiation in ESCs and Sox2-expressing cancer cells, and suggest that LSD1 can serve as a specific and selective target for treatment of Sox2-expressing cancers.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture

Lung, breast, ovarian, and other carcinoma cells were obtained from the American Type Culture Collection (ATCC) or from the DTP and DCTD Tumor Repository of the National Cancer Institute, as listed in Table S2. They were cultured according to instructions and treated with various concentrations of LSD1 inhibitors for 24–30 hr for cell viability or expression assays (Wang et al., 2011). Additional procedures are described in the Supplemental Experimental Procedures.

Western Blot, Immunohistochemistry, and Antibodies

Log-phase growing cancer cells were directly lysed in a buffer containing 0.5% SDS. Proteins in the lysates were equalized and analyzed by western blotting using specific antibodies as described previously (Wang et al., 2011). For immunohistochemical staining, rabbit monoclonal LSD1 and Sox2 antibodies were obtained from Cell Signaling and immunostaining was conducted as previously described (Wang et al., 2011). Additional experimental methods are described in Supplemental Experimental Procedures.

Small RNAi

Cells were transfected with 50 nM siRNAs using DharmaFECT reagent 1 (Dharmacon) for 48–60 hr as described previously (Wang et al., 2011). The siRNA sequences were GGAAGAAGAUAGUGAAAAC (for human LSD1) and CGCUCAUGAAGAAGGAUAA (for human Sox2).

ChIP Assays

ChIP assays were carried out according to published protocols (Whyte et al., 2012). After the chromatin proteins were crosslinked with formaldehyde, chromatin DNA was sonicated to an average 500–1,000 bp and used for immunoprecipitation by specific antibodies. DNA was isolated for quantitative real-time PCR after the crosslinking on immunoprecipitated chromatin fragments was reversed. The real-time PCR primers used are listed in Table S3. ChIP-grade H3K4me2 (Ab32356), H3K4me1 (Ab8895), H3K9me2 (Ab1220), and LSD1 (Ab17721) antibodies were obtained from Abcam. Re-ChIP for co-occupancy of methylated H3K4 and H3K9 was conducted as previously described (Geisberg and Struhl, 2004).

Statistical Analyses

Statistical analysis was performed using GraphPad Prism v4.0 software as previously described (Wang et al., 2011). Data are presented as mean ± SD. One-way ANOVA was used for comparisons.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.09.018.

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