LSD1 demethylase and the methyl-binding protein PHF20L1 prevent SET7 methyltransferase-dependent proteolysis of the stem-cell protein Sox2

Chunxiao Zhang¹², Nam Hoang¹, Feng Leng¹², Lovely Saxena¹, Logan Lee¹, Salvador Alejo¹, Dandan Qi¹², Anthony Khal¹, Hong Sun¹, Fei Lu², Hui Zhang¹*

From ¹Department of Chemistry and Biochemistry, University of Nevada, Las Vegas, NV89154, USA. ²Laboratory of Chemical Genomics, School of Chemical Biology & Biotechnology, Peking University Shenzhen Graduate School, Shenzhen 518055, China.

Running title: Regulation of Methylated Sox2 by LSD1 and PHF20L1

*To whom correspondence should be addressed: Dr. Hui Zhang, Department of Chemistry and Biochemistry, University of Nevada, 4505 S. Maryland Parkway, SEB 4138, Las Vegas, NV89154, Telephone: 702-774-1489, Fax: 702-895-4072, E-mail: hui.zhang@unlv.edu.

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ABSTRACT

The pluripotency-controlling stem-cell protein SRY-box 2 (SOX2) plays a pivotal role in maintaining the self-renewal and pluripotency of embryonic stem cells and also of teratocarcinoma or embryonic carcinoma cells. SOX2 is monomethylated at lysine 119 (Lys-119 or K119) in mouse embryonic stem cells by the SET7 methyltransferase, and this methylation triggers ubiquitin-dependent SOX2 proteolysis. However, the molecular regulators and mechanisms controlling SET7-induced SOX2 proteolysis are unknown. Here, we report that in human ovarian teratocarcinoma PA-1 cells, methylation-dependent SOX2 proteolysis is dynamically regulated by the LSD1 lysine demethylation and a methyl-binding protein, PHD finger protein 20–like 1 (PHF20L1). We found that LSD1 not only removes the methyl group from monomethylated Lys-117 (K117, equivalent to K119 in mouse Sox2), but it also demethylates monomethylated Lys-42 in SOX2, a reaction that SET7 also regulated and that also triggered SOX2 proteolysis. Our studies further revealed that PHF20L1 binds both monomethylated Lys-42 and Lys-117 in SOX2 and thereby prevents SOX2 proteolysis. Down-regulation of either LSD1 or PHF20L1 promoted SOX2 proteolysis, which was prevented by SET7 inactivation in both PA-1 and mouse embryonic stem cells. Our studies also disclosed that LSD1 and PHF20L1 normally regulate the growth of pluripotent mouse embryonic stem cells and PA-1 cells by preventing methylation-dependent SOX2 proteolysis. In conclusion, our findings reveal an important mechanism by which the stability of the pluripotency-controlling stem-cell protein Sox2 is dynamically regulated by the activities of SET7, LSD1, and PHF20L1 in pluripotent stem cells.

The Lysine-Specific Demethylase 1 (LSD1, also called KDM1A) was originally identified as a histone demethylase that removes the methyl group from the mono- and di-methylated lysine 4 in histone H3 (H3K4)(1), which is associated...
with active chromatin structure for gene activation (2). Mouse deletion of both LSD1 gene alleles causes embryonic lethality, indicating that LSD1 is essential for embryonic development (3). LSD1 is also required for the self-renewal and pluripotency of embryonic stem cells and loss or reduced levels of LSD1 cause transcriptional downregulation of pluripotent stem cell protein Sox2, Oct4, and other essential pluripotent stem cell proteins, promoting cellular differentiation (4-6).

Sox2 belongs to a family of SRY-related HMG box (Sox) transcription factors that play key roles in development and differentiation (7,8). Sox2 is a master stem cell protein that is essential for the maintenance of pluripotency and self-renewal of embryonic stem cells and induced pluripotent stem cells (iPSCs) (9). Sox2 is also a key factor for various adult stem/progenitor cells in brain, retina, trachea, and the epithelium of the cervix, tongue, and testes, allowing for tissue regeneration and repair. Recently, Sox2 is identified as a major oncogene that is commonly and recurrently amplified at 3q26.33 in squamous cell carcinomas (SCCs) of the lung, esophagus, and oral cavity (10-14). Gene amplification of Sox2 also occurs in small cell lung carcinomas and glioblastoma multiforme (GBM) (15,16). Sox2 is over-expressed in other poorly differentiated and aggressive human cancers (17), including breast, ovarian, gastric, and colon carcinomas (14,18-27).

Emerging evidence indicates that many non-histone proteins, such as p53, DNMT1, E2F1, ERα, NFκB/RelA, FOX3A, RB, GLI3, Lin28A, and STAT3, by SET7 is to trigger the ubiquitin-dependent proteolysis of the methylated proteins (28,31,32). A recent report indicated that mouse Sox2 is also monomethylated on lysine 119 (K119, equivalent to K117 in human Sox2) by SET7 in mouse embryonic stem cells and this methylation also triggers the ubiquitin-dependent proteolysis of modified Sox2 protein (34). However, how the methylation-dependent degradation of Sox2 is regulated remains unclear.

We have previously developed a novel class of LSD1 inhibitors and our studies showed that these inhibitors potently inhibited the self-renewal of pluripotent mouse embryonic stem cells and teratocarcinoma and embryonic carcinoma cells through transcriptional downregulation of Sox2 and other pluripotent stem cell proteins such as Oct4 (6,35). We also found that inactivation or inhibition of LSD1 also impeded the growth of many Sox2-expressing lung, breast, and ovarian cancer cells by downregulating Sox2 expression (36). In this report, we found that LSD1 acts as a demethylase that removes the multiple methyl groups on the methylated Sox2 to prevent the methylation-dependent proteolysis of Sox2 protein. Our studies further indicate that the protein stability of methylated Sox2 is also regulated by PHF20L1, a protein that contains a methyl-binding domain (37,38). These LSD1- and PHF20L1-dependent regulatory mechanisms are also conserved in mouse embryonic stem cells. Our studies indicate that the methylation-dependent proteolysis of Sox2 is highly regulated in embryonic stem cells and pluripotent cancer cells.

**RESULTS**

Knocking down of LSD1 reduced the protein level of Sox2. To investigate the effects of LSD1 deficiency on Sox2, we stably expressed a Flag-tagged Sox2 under a retroviral promoter control
(LTR in pMSCV) in human ovarian teratocarcinoma cell line PA-1 (35), which abundantly express endogenous Sox2 (35,36,39). Reduction of LSD1 by two independent siRNAs led to the marked downregulation of endogenous Sox2 protein (Figure 1A). However, we found that knockdown of LSD1 also led to the significantly reduced level of Flag-tagged Sox2 protein (Figure 1A). The reduced levels of both Flag-tagged or endogenous Sox2 proteins in LSD1 knockdown cells can be restored by treating cells with MG132, an inhibitor of the 26S proteasome, indicating that loss of LSD1 might induce the ubiquitin-dependent proteolysis of Sox2 protein through the 26S proteasome (Figure 1B). We also found that endogenous Sox2 and LSD1 proteins physically interact with each other (Figure 1C), suggesting that LSD1 may directly regulate Sox2 protein stability through protein-protein interactions. Since the Flag-tagged Sox2 is ectopically expressed under the retroviral LTR promoter control (35,36), we examined the possibility that loss of LSD1 may trigger the SET7-dependent proteolysis of Sox2. Indeed, co-knockdown of LSD1 and SET7 by specific siRNAs fully restored the Flag-tagged Sox2 protein levels (Figure 1D), whereas the endogenous Sox2 protein was also significantly restored (Figure 1D). These studies raised the possibility that LSD1 may act as a demethylase for Sox2, as the reported methylated K117 motif in Sox2 is similar to that of methylated H3K4, a substrate of LSD1 (Figure 1E) (31,32,34).

**PHF20L1 also regulates the protein stability of Flag-tagged Sox2.** To further test whether Sox2 is regulated by the methylation-dependent proteolysis in PA-1 cells, we also examined the potential involvement of Sox2 regulation by PHF20L1, a protein that contains a methyl binding motif, the MBT domain, that has been shown to bind to the monomethylated lysine 142 (K142) in DNA methyltransferase 1 (DNMT1) to prevent DNMT1 proteolysis triggered by SET7-mediated monomethylation of K142 (37,38). Using two independent specific siRNAs against PHF20L1, we found that knockdown of PHF20L1 also led to the markedly reduced levels of Flag-Sox2 and endogenous Sox2 protein in PA-1 cells (Figure 2A). This destabilization of Sox2 protein is prevented if PHF20L1-knockdown cells were treated with MG132 (Figure 2B). We also tested the involvement of SET7 in PHF20L1 deficiency-induced proteolysis of Sox2. We again found that the destabilization of Sox2 protein is prevented if SET7 is co-knocked down with PHF20L1 siRNA-treated cells (Figure 2C). The effect of PHF20L1 knockdown is specific for Sox2 protein, as the protein levels such as Oct4 or CUL1 did not significantly change after the downregulation of PHF20L1 in PA-1 cells (Figure 2D). These results suggest that PHF20L1 may bind to the methylated lysine residues in Sox2 to prevent the proteolysis of Sox2 protein.

**The K117R mutant Sox2 protein is still sensitive to LSD1 or PHF20L1 deficiency.** It was reported that in mouse embryonic stem cells, SET7 monomethylates K119 in mouse Sox2, equivalent to K117 in human Sox2, to trigger the proteolysis of Sox2 (34). To test whether LSD1, a demethylase, and PHF20L1, a methyl binding protein, act through the methylated K117 in human Sox2, we converted the highly conserved K117 to arginine (K117R, Figure 1E) and stably expressed the Flag-tagged K117R mutant of Sox2 in PA-1 cells. Surprisingly, we found that the K117R mutant is still downregulated after loss of PHF20L1 or LSD1 (Figure 2D and 2E), suggesting that there may be additional methylation sites in human Sox2 protein for its methylation-dependent proteolysis.

**Sox2 protein stability is regulated by methylation at lysine 42 (K42).** Since SET7 usually monomethylates H3K4 and a critical lysine residue in other non-histone proteins such
as DNMT1 and E2F1 on an R/K-S/T-K consensus motif (K* in Figure 1E) (28,31,32), we tried to determine whether Sox2 contains additional methylation sites that are regulated by SET7, LSD1 and PHF20L1. Our examination revealed the highly conserved lysine 42 (K42) in human Sox2 within an R-V-K motif that shares substantial homology to the consensus R/K-S/T-K motif in SET7 methylation substrates (Figure 1E). We therefore tested the possibility that K42 may potentially serve as a novel methylation site for SET7, LSD1 and PHF20L1 by converting K42 to arginine to produce the K42R mutant of Sox2. We stably expressed the Flag-tagged wild-type Sox2, K42R, K117R or double K42R/K117R mutants in PA-1 cells by the retroviral delivery system. We then examined the sensitivity of these mutant Sox2 proteins towards the loss of LSD1, PHF20L1, and SET7, as compared to the wild-type Sox2. Our studies showed that while K42R or K117R single mutation did not significantly cause resistance to Sox2 degradation in LSD1 or PHF20L1 knockdown cells, the double Sox2 mutant of K42 and K117 (K42R/K117R) was significantly resistant towards the loss of LSD1 or PHF20L1 (Figure 2E). Thus, our results suggest that the methylated K42 and K117 residues in Sox2 may together serve as the methylated degradation signals for Sox2 proteolysis.

**LSD1 demethylates the monomethylated K42 and K117.** To facilitate the detection of the methylated K42 and K117 in Sox2, we developed specific anti-monomethylated K42 (K42me1) and monomethylated K117 (K117me1) peptide antibodies using procedures described previously (31,33,34). These antibodies were affinity purified and tested for their specific antigenic recognition of the methylated K42 or K117 peptides (Figure 3A and 3B). We found that anti-K42me1 antibodies recognized only the methylated K42 peptide but not the unmethylated peptide. It can also detect wild-type Sox2 but not the K42R mutant isolated from transfected 293 cells (Figure 3A and 3C). Similarly, the anti-K117me1 antibodies only detected the methylated K117 peptide but not the unmethylated peptide (Figure 3B). The K117me1 antibodies also detected the wild-type Sox2 but not the K117R mutant protein from transfected 293 cells (Figure 3D). Our characterization indicated that the anti-K42me1 or anti-K117me1 antibodies are specific for the methylated K42 or K117 residues of Sox2 both in vitro and in vivo, as mutation of K42 or K117 in Sox2 abolished their respective reactivity in vivo.

If LSD1 acts as a demethylase to remove methyl group from the methylated K42 or K117 in Sox2, loss of LSD1 should lead to the increased levels of methylated Sox2 protein and consequently promote Sox2 degradation. To directly test whether LSD1 can demethylate monomethylated K42 or K117 in Sox2, we purified recombinant GST-LSD1 protein from bacteria (Figure 3E) and examined its ability to demethylate the K42me1 or K117me1 peptides, as we have previously described for analyzing the demethylase activity of LSD1 towards the methylated H3K4 (6). Our in vitro biochemical analysis revealed that LSD1 indeed demethylated both the methylated K42 and K117 peptides in vitro (Figure 3F and 3G). In PA-1 cells, our studies also revealed that loss of LSD1 led to the accumulation of the endogenous methylated K42 and K117-containing Sox2 protein (Figure 4D), indicate that LSD1 normally removes the methyl group from the methylated K42 and K117 residues in Sox2 in vivo.

**SET7 regulates the methylation of K42 and K117.** Since the single K42R or K117R mutant protein was still sensitive to LSD1 knockdown, we analyzed the effect of SET7 knockdown on K42R or K117R mutants in LSD1 siRNA-treated cells. Our results indicated that SET7
downregulation prevented the degradation of both Sox2 K42R and K117R single mutants in LSD1 deficient cells, indicating that K42 and K117 in Sox2 are substrates of SET7 methyltransferase and LSD1 in vivo (Figure 4A-4C).

**PHF20L1 binds to either monomethylated K42 or K117.** It has been shown that the MBT domain of PHF20L1 can bind to the monomethylated K142 in DNMT1 (37). We tested whether the MBT domain of PHF20L1 can also recognize the monomethylated K42 or K117 residue in Sox2 in vitro using a GST-PHF20L1-MBT domain fusion protein (37). Our studies revealed that the MBT domain of PHF20L1 preferentially binds to the monomethylated K42 and K117 peptide resins, but not to the non-methylated cognate peptides (Figure 5B and 5C), indicating that PHF20L1 specifically binds to both the methylated K42 and K117 in Sox2 in vitro.

**PHF20L1 binding to the methylated K42 and K117 prevents LSD1 demethylation.** Since LSD1 demethylates the methylated K42 or K117, we tested whether the binding of PHF20L1 to these methylated lysine residues affects the ability of LSD1 to remove these methyl groups from the methylated peptides. The methylated K42 or K117 peptides were first allowed to bind to the GST-PHF20L-MBT protein immobilized on the glutathione resins. The unbound methylated peptides were washed off and the methylated K42 or K117 peptide on the GST-PHF20L-MBT protein beads was incubated with purified LSD1 protein. As a control, the methylated K42 or K117 peptide on the GST-PHF20L-MBT protein beads was released from the GST-PHF20L-MBT protein beads by heating the peptide-bound beads at 95 °C for 16 minutes. We found that while the heat-released methylated K42 and K117 peptides were fully demethylated by LSD1, a significant fraction of methylated K42 or K117 was protected by their binding to the GST-PHF20L-MBT protein beads (Figure 5D and 5E), indicating that the binding of these methylated peptides to PHF20L1 prevents LSD1 from demethylating these peptides.

**PHF20L1 binds to the endogenous Sox2 protein through K42 and K117 to prevent Sox2 degradation.** We found that the endogenous PHF20L1 interacts with Sox2 protein in PA-1 cells by co-immunoprecipitation analysis (Figure 6A). Our analysis further revealed that while the K42R or K117R mutant each displayed reduced binding to PHF20L1 (Figure 6B), the K42R/K117R double mutation abolished the binding of PHF20L1 to Sox2 (Figure 6C), indicating that PHF20L1 binding to both K42 and K117 in Sox2 in vivo. We also examined the effects of ectopically expressed PHF20L1-MBT domain on stably expressed wild-type Sox2 and the K42R/K117R mutant. We found that expression of the MBT domain of PHF20L1 caused an increased level of the wild-type Sox2 protein, but not the K42R/K117R mutant protein, indicating that the binding of the MBT domain of PHF20L1 to the methylated K42 and K117 in wild-type Sox2 prevents its proteolysis (Figure 6D).

**Loss of LSD1 or PHF20L1 leads to the growth inhibition of PA-1 cells.** Our studies revealed that loss of LSD1 or PHF20L1 led to the proteolysis of Sox2 protein. Since Sox2 is essential for the self-renewal of teratocarcinoma cells such as PA-1 (6,35,36), we monitored the effects of loss of LSD1 and PHF20L1 on PA-1 cell growth (Figure 7A and 7B). We found that knockdown of LSD1 or PHF20L1 by specific siRNAs led to the significant growth inhibition of PA-1 cells (Figure 7A). We also examined whether the expression of mutant Sox2 proteins has any effects on the LSD1- and PHF20L1-knockdown induced growth inhibition. We found...
that while expression of the wild-type Sox2, K42R or K117R mutant protein did not prevent the growth inhibition, expression of the double K42R/K117R Sox2 mutant significantly prevented growth inhibition in LSD1 or PHF20L1 knockdown cells (Figure 7C and 7D), indicating that the expression of the double K42R/K117R mutant causes resistance to the loss of LSD1 or PHF20L1 for PA-1 cell growth (Figure 2E, 7C and 7D).

**LSD1 and PHF20L1 also regulate the protein level of Sox2 in mouse embryonic stem cells.** Since PA-1 is a teratocarcinoma cell line, we tried to determine whether Sox2 protein in pluripotent mouse embryonic stem (mES) cells is also regulated by LSD1 and PHF20L1. We found that the mouse Sox2 protein from mES cells was also recognized by the anti-monomethylated K42 and K117 antibodies, as these methylated lysine residues are highly conserved in human and mouse Sox2 proteins (Figure 1E and 8D), suggesting that mouse Sox2 is also methylated at these conserved lysine residues (Figure 1E). Like PA-1 cells, knockdown of LSD1 or PHF20L1 in mES cells also triggered the downregulation of Sox2 protein (Figure 8B and 8C), resulted in significant growth inhibition of mES cells (Figure 8A). Co-knockdown of SET7 fully restored the protein levels of Sox2 and significantly prevented the growth inhibition in either LSD1- or PHF20L1-knockdown mES cells (Figure 8A-8C). Loss of SET7 alone also induced an increased level of Sox2 protein (Figure 8B and 8C). We also found that endogenous PHF20L1 and Sox2 interact with each other in mES cells (Figure 8E). These studies indicate that SET7, LSD1 and PHF20L1 also regulate the protein levels of Sox2 protein, likely through the methylation-dependent proteolysis of Sox2 in mouse embryonic stem cells. However, while we did not observe significant reduction of Oct4 protein in PHF20L1 knockdown PA-1 cells (Figure 2D), we found that knockdown of LSD1 or PHF20L1 reduced the protein level of Oct4 in mouse embryonic stem cells (Figure 8F). It is known that in pluripotent embryonic stem cells, Oct4, Sox2, and Nanog form a pluripotent stem cell transcriptional circuitry to regulate their own expression, as well as other stem cell proteins, to maintain pluripotency and self-renewal (40). However, we previously found that Nanog is not expressed in PA-1 and several other teratocarcinoma or embryonic carcinoma cells (36). It is possible that lack of Nanog in PA-1 cells may mis-regulate such a critical stem regulatory mechanism so that downregulation of Sox2 may not greatly affect the level of Oct4. Thus, our studies in PA-1 cells suggest that Sox2 is the primary stem cell protein that is regulated by LSD1 and PHF20L1. Our studies are consistent with our working model that LSD1 and PHF20L1 maintain the protein stability of methylated Sox2 to control the self-renewal and pluripotency of embryonic stem cells and teratocarcinoma cells including PA-1.

**DISCUSSION:**
We and others have previously shown that LSD1 acts as a histone demethylase to regulate the transcriptional expression of Sox2 in mouse embryonic stem cells, teratocarcinoma or embryonic carcinoma cells, and many Sox2-expressing lung, breast, and ovarian cancer cells (4-6,35,36). In this report, we found that LSD1 regulates the proteolysis of Sox2 through the post-translational methylation of K42 and K117 residues in human Sox2 (Figure 1-4). Our studies strongly support a model in which LSD1 acts as a demethylase to remove the methyl group from both methylated K42 and K117 to stabilize Sox2 protein, whereas SET7 methylates these lysine residues to destabilize Sox2 protein. Importantly, previous studies showed that mouse Sox2 is methylated at K119 (equivalent to K117 in human Sox2) by SET7 (34). However, we found...
that K117 is only one of multiple methylation sites in Sox2. We found that a novel methylated lysine residue, K42, in Sox2 that is recognized by LSD1 for demethylation and this lysine is also regulated by SET7 (Figure 2). Both methylated K42 and K117 in Sox2 play a key role in the methylation-dependent proteolysis of Sox2 protein. In addition, our studies also revealed that the protein stability of the methylated Sox2 is further regulated by PHF20L1 (Figure 2 and 5), encoded by a gene that is amplified or over-expressed in aggressive basal-like breast or luminal B breast cancers (38). PHF20L1 contains a MBT methyl binding domain, which was shown to interact with the methylated K142 in DNMT1 (37). Our studies revealed that PHF20L1 recognizes both the monomethylated K42 and K117 residues in Sox2 and its binding to these methylated lysine residues prevents the degradation of Sox2 protein. Our studies thus indicate that Sox2 is regulated by multiple methylation events and its protein stability is dynamically controlled by SET7 and LSD1, and PHF20L1. Furthermore, our studies revealed that the protein stability of mouse Sox2 is similarly regulated by LSD1 and PHF20L1 in mouse embryonic stem cells (Figure 8). Since Sox2 is a master regulator of pluripotency and self-renewal of embryonic stem cells and many adult stem/progenitor cells in various tissues (8), the levels of Sox2 expression are highly regulated to maintain the stem cell properties of these cells. Elevated expression or gene amplification of Sox2 is associated with various cancers (14). Our studies suggest that the regulation of Sox2 by its multiple methylation events may play a critical role in Sox2 function in various stem cells and altered regulation of Sox2 by the methylation-dependent processes may contribute to tumorigenesis in various tissues.

EXPERIMENTAL PROCEDURES

Cell culture: Human ovarian carcinoma PA-1 cells and embryonic kidney 293 cells were purchased from American Type Cell Collection (ATCC). Mouse embryonic stem cells (CMTI-2, strain C57/Bl6J, passage 11) were obtained from Millipore-Sigma. PA-1 cells were cultured in Minimum Essential Medium (MEM), 293 cells were in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and penicillin/streptomycin (36). Mouse embryonic stem cells were cultured on the mitomycin C-treated mouse fibroblast feeder layer in Knockout DMEM and Knockout Serum Replacement, supplemented with leukemia inhibitory factor (LIF), GlutaMax, β-mercaptoethanol, MEM non-essential amino acid solution, and penicillin/streptomycin (all from Life Technologies) as described before (6,35). The cells have been recently authenticated and tested based on protein markers such as Sox2 or Oct4 in mouse embryonic stem cells and PA-1 cells, and p53 and p21 in 293 cells (35,36).

Antibodies, affinity purification, and immunological procedures: The specific anti-monomethylated K42 and anti-monomethylated K117 antibodies were raised using synthetic peptides. The monomethylated K42 (CAGGNQKNSPDRVK(me1)RPMNTAFMVWSR) and cognate unmethylated peptides, and the monomethylated K117 (PDYKYRPRRTK(me1)TLMKKDNYC) and cognate unmethylated peptides of Sox2 were synthesized at ABI Scientific. We also synthesized two human PHF20L1 peptides (PHF20L1M1: KEKDKERREKRDKDHYRPKC and PHF20L1C1: LKRHIKQLLDKGMVQKDIATLCSVC) for raising anti-PHF20L1 antibodies. The methylated K42 peptide and K117 peptides, as well as the peptides for PHF20L1, were chemically coupled to keyhole limpet hemocyanin (KLH) to raise specific polyclonal antibodies in rabbits (41,42). For affinity
antibody purification, the unmethylated and methylated peptides were immobilized to Sulfolink-coupled-resins through a cysteine residue on the end of each peptide as described previously (41-43). The antibodies were affinity purified using previously described procedures (31,33,34), by first passing the rabbit anti-sera through the unmethylated peptide chromatographic resins to deplete the antibodies against the unmethylated peptides. The flow-through fraction of anti-sera was then affinity purified by the monomethylated peptide chromatographic resins. The bound anti-methylated antibodies were eluted by 0.1 M glycine, pH2.5, and immediately neutralized by a buffer containing 1 M Tris, pH8.0. Anti-LSD1 (A300-215A), Sox2 (A301-741), Oct4 (A304-591A), Actin (A300-491A), and SET7 (A301-747A) antibodies were purchased from Bethyl Laboratories whereas anti-PHF20L1 (HPA028417) and anti-Flag (F1804) antibodies were from Sigma. We also generated polyclonal anti-Sox2 and anti-PHF20L1 antibodies using purified GST-human Sox2 or PHF20L1 peptide-KLH conjugates as antigens in rabbits using procedures as previously described (44). Other antibodies, as well as immunoprecipitation and western blotting analysis procedures, were described previously (41).

**Peptide binding and demethylation assays:** For peptide binding assays, the monomethylated K42 and K117 and their cognate unmethylated peptides of Sox2 were covalently coupled to the Sulfolink-coupled-resins (Thermo Fisher) (41,43,45) through the disulfide bond between the cysteine residue at the end of the peptides and the resins. For peptide binding assays, 20-30 µl of peptide-coupled resin were prewashed with the binding buffer (0.1% NP40, 50 mM Tris-Cl, pH7.5, and 150 mM NaCl) and the resins were pre-blocked with 2 µg GST protein at room temperature for 2 hours. The peptide-coupled resins were then incubated with 1 µg each of GST or indicative GST-PHF20L1-MBT fusion proteins in the binding buffer at room temperature or overnight at 4 °C. The resin beads were extensively washed (4-5 times) and the proteins associated with the resins were analyzed by Western blotting with anti-GST or PHF20L1 antibodies (45). The GST-human LSD1, GST-PHF20L1-MBT domain (amino acid residues 1-138), and GST in expressing plasmid construct, pGEXKG, were transformed into E. coli BL21 strain and their expression is induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (6,42). The GST-LSD1, PHF20L1-MBT, and GST proteins were purified by glutathione agarose (GE Healthcare), as previously described (6).

For PHF20L1 protection assays, 500 µg GST-PHF20L1-MBT protein was pre-bound to 500 µl Glutathione Sepharose and washed extensively with the wash buffer (150mM NaCl and 50mM Tris, pH7.4). Each of K42me1 or K117me1 peptide (10 µg) was mixed with the GST-PHF20L1-MBT- Glutathione Sepharose and rotated at 4°C overnight and unbound peptides were washed away extensively. For each demethylation reaction, 50 µl of beads were mixed with 2.7 µg GST or GST-LSD1 for 5 hours at room temperature.

**Transfection and siRNA-mediated gene silencing:** Cells were usually transfected with 50 nM siRNAs for 48 hours using DharmaFECT Transfection Reagent (#T-2001-03, GE Healthcare) as previously described (6,36). To prevent potential off-target effects, at least two siRNAs against each gene target were designed and analyzed. For mouse embryonic cell transfection with siRNAs, Cells were examined and cell images were acquired with 10X10 lens of Nikon ECLIPSE Ti-S microscope equipped with NIS-Elements BR 3.1 software. Triplicated cells (technical repeats) were harvested by trypsin digestion, diluted, and blindly spotted onto a hemacytometer. Cells in four corners of the hemacytometer were counted to obtain
average cells per dish (36). The differences between control and LSD1 or PHF20L1 siRNA-treated cells were compared and plotted. Experiments were normally repeated for three independent times with the same conclusion. The sequences of siRNAs are human/mouse LSD1-1: GGAAGAAGAUAGUAAACUU; human LSD1-2: AGUGAAAACUCAGGAAAUU human PHF20L1-1: UGGGGuUGAUGgUGCUGAuuu; human PHF20L1-2: GAUGAGAGAAAGGUUUAUU; mouse PHF20L1: UCCAGCUUCAGGGAAUAA; human SET7: GGGCAGUAUAAAGAUAACAUU; Mouse SET7: GGUAGCAGUUGGACCUAAU. Other siRNAs were previously reported (36). All siRNAs were synthesized from GE Healthcare.

ExpressioN of Sox2 and site-directed mutagenesis: Human Sox2 cDNA was cloned into pMSCV-puro-Flag (Addgene) in which the Flag epitope was fused in frame with the amino terminus of Sox2 protein. The Flag-Sox2 fusion protein is stably expressed after transfection of pMSCV-Puro-Flag-Sox2 construct by pMSCV expression protocol with Lipofectamine 2000 (Thermo Fisher) in PA-1 cells using puromycin as the selection (36). The Sox2 mutants were generated using the site-directed mutation procedure, as described previously (45). For the expression of GFP-Sox2 fusion protein, Sox2 cDNA was cloned into pcDNA-EGFP (Addgene) with in frame fusion between the carboxyl terminus of GFP protein and the amino-terminus of Sox2.

Statistical Information: Experiments were usually performed with at least three independent repeats (biological replicates) to ensure the results. For cell number assays, triplicated cell samples were used to determine and compare the statistically significant differences between means of control (luciferase siRNA) and LSD1 or PHF20L1 siRNA-treated cells using two-tailed paired t-test (35,46). Quantitative data are expressed by bar graph and standard deviations (S.D.) are expressed as mean and error bars. Different data sets were considered to be statistically significant when the P-value was <0.05 (*), 0.01 (**) or 0.001 (***) (47).

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Author Contributions: HZ conceived the concept, organized, designed, and coordinated experiments. CZ did many significant experiments, with help from NH, FL, LS, SA, DQ, H.S., LL, AK, and LF on various parts of experiments at University of Nevada, Las Vegas. HZ analyzed and wrote the manuscript. All authors approved the final version of the manuscript.

References:
1. Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., and Casero, R. A. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119, 941-953
2. Mosammaparast, N., and Shi, Y. (2010) Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem 79, 155-179
3. Wang, J., Hevi, S., Kurash, J. K., Lei, H., Gay, F., Bajko, J., Su, H., Sun, W., Chang, H., Xu, G.,
Gaudet, F., Li, E., and Chen, T. (2009) The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. *Nat Genet* **41**, 125-129

4. Adamo, A., Sese, B., Boue, S., Castano, J., Paramonov, I., Barrero, M. J., and Izpisua Belmonte, J. C. (2011) LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells. *Nat Cell Biol* **13**, 652-659

5. Whyte, W. A., Bilodeau, S., Orlando, D. A., Hoke, H. A., Frampton, G. M., Foster, C. T., Cowley, S. M., and Young, R. A. (2012) Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. *Nature* **482**, 221-225

6. Wang, J., Lu, F., Ren, Q., Sun, H., Xu, Z., Lan, R., Liu, Y., Ward, D., Quan, J., Ye, T., and Zhang, H. (2011) Novel Histone Demethylase LSD1 Inhibitors Selectively Target Cancer Cells with Pluripotent Stem Cell Properties. *Cancer Research* **71**, 7238-7249

7. Kamachi, Y., and Kondoh, H. (2013) Sox proteins: regulators of cell fate specification and differentiation. *Development* **140**, 4129-4144

8. Sarkar, A., and Hagedoorn, K. (2013) The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* **12**, 15-30

9. Driessens, G., and Blanpain, C. (2011) Long live sox2: sox2 lasta lasts a lifetime. *Cell Stem Cell* **9**, 283-284

10. Bass, A. J., Watanabe, H., Merrel, C. H., Yu, S., Perner, S., Verhaak, R. G., Kim, S. Y., Wardwell, L., Tamayo, P., Gat-Viks, I., Ramos, A. H., Woo, M. S., Weir, B. A., Getz, G., Beroukhim, R., O’Kelly, M., Dutt, A., Rozenblatt-Rosen, O., Dzinjycz, P., Komisarof, J., Chirieac, L. R., Lafargue, C. J., Scheble, V., Wilbertz, T., Ma, C., Rao, S., Nakagawa, H., Stairs, D. B., Lin, L., Giordano, T. J., Wagner, P., Minna, J. D., Gazdar, A. F., Zhu, C. Q., Brose, M. S., Cecconello, I., Jr, U. R., Marie, S. K., Dahl, O., Shivdasani, R. A., Tsao, M. S., Rubin, M. A., Wong, K. K., Regev, A., Hahn, W. C., Beer, D. G., Rustgi, A. K., and Meyerson, M. (2009) SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nat Genet* **41**, 1238-1242

11. Hussenet, T., Dali, S., Exinger, J., Monga, B., Jost, B., Dembele, D., Martinet, N., Thibault, C., Huelsken, J., Brambilla, E., and du Manoir, S. (2010) SOX2 is an oncogene activated by recurrent 3q26.3 amplifications in human lung squamous cell carcinomas. *PLoS One* **5**, e8960

12. Hussenet, T., and du Manoir, S. (2010) SOX2 in squamous cell carcinoma: amplifying a pleiotropic oncogene along carcinogenesis. *Cell Cycle* **9**, 1480-1486

13. Maier, S., Wilbertz, T., Braun, M., Scheble, V., Reischl, M., Mikut, R., Menon, R., Nikolov, P., Petersen, K., Beschorner, C., Moeh, H., Kakies, C., Protzel, C., Bauer, J., Soltermann, A., Fend, F., Staehler, A., Lengerke, C., and Perner, S. (2011) SOX2 amplification is a common event in squamous cell carcinomas of different organ sites. *Hum Pathol* **42**, 1078-1088

14. Weinai, K., and Utikal, J. (2014) SOX2 and cancer: current research and its implications in the clinic. *Clinical and translational medicine* **3**, 19

15. Rudin, C. M., Durinck, S., Stawiski, E. W., Poirier, J. T., Modrusan, Z., Shames, D. S., Bergbower, E. A., Guan, Y., Shing, J., Guillory, J., Rivers, C. S., Foo, C. K., Bhatt, D., Stinson, J., Gnad, F., Haverty, P. M., Gentleman, R., Chaudhuri, S., Janakiraman, V., Jaiswal, B. S., Parikh, C., Yuan, W., Zhang, Z., Koeppepin, H., Wu, T. D., Stern, H. M., Yauch, R. L., Haffeman, K. E., Paskulin, D. D., Illie, P. B., Varella-Garcia, M., Gazdar, A. F., de Sauvage, F. J., Bourgon, R., Minna, J. D., Brock, M. V., and Seshagiri, S. (2012) Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. *Nat Genet* **44**, 1111-1116

16. Alonso, M. M., Diez-Valle, R., Manterola, L., Rubio, A., Liu, D., Cortes-Santiago, N., Urquiza, L., Jauregi, P., Lopez de Munain, A., Sampron, N., Aramburu, A., Tejada-Solis, S., Vicente, C., Odero, M.
11. Bandres, E., Garcia-Foncillas, J., Idoate, M. A., Lang, F. F., Fueyo, J., and Gomez-Manzano, C. (2011) Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. *PLoS One* **6**, e26740

17. Ben-Porath, I., Thomson, M. W., Carey, V. J., Ge, R., Bell, G. W., Regev, A., and Weinberg, R. A. (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* **40**, 499-507

18. Sholl, L. M., Barletta, J. A., Yeap, B. Y., Chirieac, L. R., and Hornick, J. L. (2010) Sox2 protein expression is an independent poor prognostic indicator in stage I lung adenocarcinoma. *Am J Surg Pathol* **34**, 1193-1198

19. Sholl, L. M., Long, K. B., and Hornick, J. L. Sox2 expression in pulmonary non-small cell and neuroendocrine carcinomas. *Appl Immunohistochem Mol Morphol* **18**, 55-61

20. Peng, S., Maihle, N. J., and Huang, Y. (2010) Pluripotency factors Lin28 and Oct4 identify a subpopulation of stem cell-like cells in ovarian cancer. *Oncogene* **29**, 2153-2159

21. Zhong, X., Li, N., Liang, S., Huang, Q., Coukos, G., and Zhang, L. (2010) Identification of microRNAs regulating reprogramming factor LIN28 in embryonic stem cells and cancer cells. *J Biol Chem* **285**, 41961-41971

22. Neumann, J., Bahr, F., Horst, D., Kriegl, L., Engel, J., Luque, R. M., Gerhard, M., Kirchner, T., and Jung, A. (2011) SOX2 expression correlates with lymph-node metastases and distant spread in right-sided colon cancer. *BMC Cancer* **11**, 518

23. Kim, J. B., Sebastiano, V., Wu, G., Arauzo-Bravo, M. J., Sasse, P., Gentile, L., Ko, K., Ruau, D., Ehrich, M., van den Boom, D., Meyer, J., Hubner, K., Bernemann, C., Ortmeier, C., Zenke, M., Fleischmann, B. K., Zehres, H., and Scholer, H. R. (2009) Oct4-induced pluripotency in adult neural stem cells. *Cell* **136**, 411-419

24. Leis, O., Eguiara, A., Lopez-Aribillaga, E., Alberdi, M. J., Hernandez-Garcia, S., Elorriaga, K., Pandiella, A., Rezola, R., and Martin, A. G. (2012) Sox2 expression in breast tumours and activation in breast cancer stem cells. *Oncogene* **31**, 1354-1365

25. Matsuoka, J., Yashiro, M., Sakurai, K., Kubo, N., Tanaka, H., Muguruma, K., Sawada, T., Ohira, M., and Hirakawa, K. (2012) Role of the stemness factors sox2, oct3/4, and nanog in gastric carcinoma. *The Journal of surgical research* **174**, 130-135

26. Sotlo, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C. M., Lomning, P. E., Brown, P. O., Borresen-Dale, A. L., and Botstein, D. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* **100**, 8418-8423

27. Tsukamoto, T., Mizoshita, T., Mihara, M., Tanaka, H., Takenaka, Y., Yamamura, Y., Nakamura, S., Ushijima, T., and Tatatematsu, M. (2005) Sox2 expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes. *Histopathology* **46**, 649-658

28. Zhang, X., Wen, H., and Shi, X. (2012) Lysine methylation: beyond histones. *Acta Biochim Biophys Sin (Shanghai)* **44**, 14-27

29. Kim, S. K., Lee, H., Han, K., Kim, S. C., Choi, Y., Park, S. W., Bak, G., Lee, Y., Choi, J. K., Kim, T. K., Han, Y. M., and Lee, D. (2014) SET7/9 methylation of the pluripotency factor LIN28A is a nucleolar localization mechanism that blocks let-7 biogenesis in human ESCs. *Cell Stem Cell* **15**, 735-749

30. Fu, L., Wu, H., Cheng, S. Y., Gao, D., Zhang, L., and Zhao, Y. (2016) Set7 mediated Gli3 methylation plays a positive role in the activation of Sonic Hedgehog pathway in mammals. *eLife* **5**
31. Esteve, P. O., Chang, Y., Samaranayake, M., Upadhyay, A. K., Horton, J. R., Feehery, G. R., Cheng, X., and Pradhan, S. (2011) A methylation and phosphorylation switch between an adjacent lysine and serine determines human DNMT1 stability. *Nat Struct Mol Biol* 18, 42-48

32. Kontaki, H., and Talianidis, I. (2010) Lysine methylation regulates E2F1-induced cell death. *Mol Cell* 39, 152-160

33. Esteve, P. O., Chin, H. G., Benner, J., Feehery, G. R., Samaranayake, M., Horwitz, G. A., Jacobsen, S. E., and Pradhan, S. (2009) Regulation of DNMT1 stability through SET7-mediated lysine methylation in mammalian cells. *Proc Natl Acad Sci U S A* 106, 5076-5081

34. Fang, L., Zhang, L., Wei, W., Jin, X., Wang, P., Tong, Y., Li, J., Du, J. X., and Wong, J. (2014) A methylation-phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation. *Mol Cell* 55, 537-551

35. Yin, F., Lan, R., Zhang, X., Zhu, L., Chen, F., Xu, Z., Liu, Y., Ye, T., Sun, H., Lu, F., and Zhang, H. (2014) LSD1 Regulates Pluripotency of Embryonic Stem/Carcinoma Cells through Histone Deacetylase 1-Methylated Deacetylation of Histone H4 at Lysine 16. *Mol Cell Biol* 34, 158-179

36. Zhang, X., Lu, F., Wang, J., Yin, F., Xu, Z., Qi, D., Wu, X., Cao, Y., Liang, W., Liu, Y., Sun, H., Ye, T., and Zhang, H. (2013) Pluripotent Stem Cell Protein Sox2 Confers Sensitivity to LSD1 Inhibition in Cancer Cells. *Cell reports* 5, 445-457

37. Esteve, P. O., Terragni, J., Deepti, K., Chin, H. G., Dai, N., Espejo, A., Correa, I. R., Jr., Bedford, M. T., and Pradhan, S. (2014) Methyllysine reader plant homeodomain (PHD) finger protein 20-like 1 (PHF20L1) antagonizes DNA (cytosine-5) methyltransferase 1 (DNMT1) proteasomal degradation. *J Biol Chem* 289, 8277-8287

38. Jiang, Y., Liu, L., Shan, W., and Yang, Z. Q. (2016) An integrated genomic analysis of Tudor domain-containing proteins identifies PHD finger protein 20-like 1 (PHF20L1) as a candidate oncogene in breast cancer. *Molecular oncology* 10, 292-302

39. Gao, C., Miyazaki, M., Ohashi, R., Tsuji, T., Inoue, Y., and Namba, M. (1999) Maintenance of near-diploid karyotype of PA-1 human ovarian teratocarcinoma cells due to death of polyploid cells by chromosome fragmentation/pulverization. *Int J Mol Med* 4, 291-294

40. Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., Wong, K. Y., Sung, K. W., Lee, C. W., Zhao, X. D., Chiu, K. P., Lipovich, L., Kuznetsov, V. A., Robson, P., Stanton, L. W., Wei, C. L., Ruan, Y., Lim, B., and Ng, H. H. (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* 38, 431-440

41. Higa, L. A., Wu, M., Ye, T., Kobayashi, R., Sun, H., and Zhang, H. (2006) CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. *Nat Cell Biol* 8, 1277-1283

42. Higa, L. A., Mihaylov, I. S., Banks, D. P., Zheng, J., and Zhang, H. (2003) Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. *Nat Cell Biol* 5, 1008-1015

43. Higa, L. A., Banks, D., Wu, M., Kobayashi, R., Sun, H., and Zhang, H. (2006) L2DTL/CDT2 interacts with the CUL4/DDB1 complex and PCNA and regulates CDT1 proteolysis in response to DNA damage. *Cell Cycle* 5, 1675-1680

44. Yu, Z. K., Gervais, J. L., and Zhang, H. (1998) Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. *Proc Natl Acad Sci U S A* 95, 11324-11329
45. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999) p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* 9, 661-664

46. Lu, F., Wu, X., Yin, F., Chia-Fang Lee, C., Yu, M., Mihaylov, I. S., Yu, J., Sun, H., and Zhang, H. (2016) Regulation of DNA replication and chromosomal polyploidy by the MLL-WDR5-RBBP5 methyltransferases. *Biology open* 5, 1449-1460

47. Fay, D. S., and Gerow, K. (2013) A biologist's guide to statistical thinking and analysis. *WormBook: the online review of C. elegans biology*, 1-54

**FIGURE LEGENDS**

**Figure 1. Loss of LSD1 destabilizes both endogenous and ectopically expressed Sox2 in the SET7-dependent manner.**

A. LSD1 knockdown reduces Sox2 proteins. PA-1 cells that stably express a Flag-Sox2 protein were transfected with 50 nM of luciferase (luc, control) siRNA or two independent LSD1 siRNAs for 48 hours. The cells were directly lysed in an SDS-containing lysis buffer, equalized, separated in protein SDS gel. The Flag-Sox2, endogenous Sox2, LSD1 and Actin (loading control) proteins were detected by anti-Flag, Sox2, LSD1, and Actin antibodies as labeled on the left of the panels. Experiments were repeated three independent times with the same conclusion and one example is shown. Protein molecular weight markers in kDa were indicated on the right of the panels.

B. The 26S proteasome inhibitor MG132 prevents Sox2 degradation in LSD1 knockdown cells. The same as 1A except at 43 hours post-siRNA transfection, one set of cells was treated with 5 µg/ml MG132 for additional 5 hours while the other set with dimethyl sulfoxide (DMSO, control).

C. LSD1 and Sox2 physically interact in vivo. Actively growing PA-1 cells were lysed in the NP40-containing lysis buffer and the lysates were used for immunoprecipitation by anti-LSD1 and Sox2 antibodies, using IgG as a control. The presence of LSD1 and Sox2 in the immunoprecipitated protein complexes was detected by Western blotting using anti-LSD1 or Sox2 antibodies.

D. Loss of SET7 prevents Sox2 degradation. The PA-1 cells expressing Flag-Sox2 protein were transfected with 50 nM siRNAs of luciferase, LSD1, LSD1+SET7, or SET7 for 48 hours. The lysates were equalized and proteins were analyzed by antibodies against each indicated proteins on the left of the panels, as in Figure 1A. Experiments were repeated three independent times with the same conclusion and one example is shown.

E. The conserved lysine residues (K*) methylated by SET7 in a methylation motif with the R/K-S/T-K* consensus sequences in histone H3, human and mouse Sox2, DNMT1, and E2F1. The K42 in Sox2 are located in a highly conserved motif containing the R-V-K* motif, as indicated.

**Figure 2. Both K42 and K117 in Sox2 regulate Sox2 protein stability in LSD1 and PHF20L1 knockdown cells.**

A. PHF20L1 knockdown reduces the protein level of Sox2. PA-1 cells stably expressing a Flag-Sox2 protein were transfected with 50 nM of luciferase siRNA or two independent PHF20L1 siRNAs for 48 hours. The levels of Flag-tagged and endogenous Sox2, PHF20L1 and Actin were examined by Western blotting using specific antibodies as indicated. Experiments were repeated three independent times with the same conclusion and one example is shown.
B. MG132 prevents Sox2 degradation in PHF20L1 knockdown cells. The same as Figure 2A except at 43 hours post-siRNA transfection, one set of cells was treated with 5 µg/ml MG132 for 5 additional hours while the other set with DMSO as indicated.

C. Loss of SET7 prevents Sox2 degradation. The PA-1 cells expressing Flag-Sox2 protein were transfected with 50 nM siRNAs of luciferase, PHF20L1, PHF20L1+SET7, or SET7 for 48 hours. The protein levels of Flag-tagged and endogenous Sox2, SET7, PHF20L1 and Actin in cell lysates were detected by indicated antibodies. Experiments were repeated three independent times with the same conclusion and one example is shown.

D. Conversion of lysine 117 to arginine (K117R) in Sox2 does not prevent Sox2 degradation. PA-1 cells that stably express a wild-type (WT) Flag-Sox2 or the K117R Sox2 mutant protein were transfected with 50 nM siRNAs of luciferase or PHF20L1 for 48 hours. The levels of Flag-Sox2 or the mutant Sox2, endogenous Sox2, Oct4, PHF20L1 and CUL1 (loading control) were examined by Western blotting using specific antibodies as indicated. Experiments were repeated three independent times with the same conclusion and one example is shown.

E. Both K42 and K117 are required for Sox2 degradation in LSD1 or PHF20L1 knockdown cells. PA-1 cells stably expressing a wild-type Flag-Sox2 (WT), K42R, K117R, or K42R/K117R Sox2 mutant proteins were each transfected with 50 nM siRNAs of luciferase, LSD1, or PHF20L1 for 48 hours. The protein levels of Flag-Sox2 (WT) or mutant Sox2 proteins, LSD1, PHF20L1 and Actin were examined by Western blotting using specific antibodies as indicated. Experiments were repeated three independent times with the same conclusion and one example is shown.

Figure 3. LSD1 demethylates the methyl group from monomethylated K42 and K117 in Sox2.

A. Development of specific anti-monomethylated K42 (K42me1) peptide antibodies for Sox2. The rabbit polyclonal sera against the monomethylated K42 peptide were depleted the antibodies against the unmethylated peptide using the unmethylated K42 peptide chromatographic resins. The antibodies were then affinity purified by binding to the monomethylated K42 peptide chromatographic resins. To test the specificity of the methylation antibodies, the unmethylated and monomethylated K42 peptides at indicated concentrations were blotted onto nitrocellulose membrane and immunoblotted with the affinity purified anti-monomethylated K42 antibodies or anti-Sox2 antibodies that recognize both unmethylated and methylated peptides.

B. Development of anti-monomethylated K117 (K117me1) peptide antibodies for Sox2. The affinity purification of anti-monomethylated K117 peptide antibodies was similar to Figure 3A except unmethylated or monomethylated K117 peptide chromatographic resins were used. The unmethylated and monomethylated K117 peptides at indicated concentrations were blotted onto nitrocellulose membrane and immuno-blotted with the affinity purified anti-monomethylated K117 antibodies or anti-Sox2 antibodies.

C. 293 cells were transfected with DNA constructs expressing the GFP-Sox2 and the GFP-Sox2-K42R mutant for 48 hours. The GFP-Sox2 and K42R proteins were examined in equalized cell lysates by anti-monomethylated K42 antibodies for methylated Sox2, anti-GFP for total GFP-Sox2 and Actin antibodies.

D. 293 cells were transfected with expression constructs expressing the GFP-Sox2 and the GFP-Sox2-K117R mutant. The GFP-Sox2 and K117R proteins were examined 48 hours post-transfection by anti-monomethylated K117 antibodies for methylated Sox2, anti-GFP for GFP-Sox2 total protein and Actin antibodies.
E. Purification of GST-human LSD1 protein from bacteria. The specific expression of GST-LSD1 under IPTG induction is shown and the protein was purified by glutathione sepharose.

F. Purified 1 µg of GST (lane 3) or GST-LSD1 proteins (lanes 1 and 2) were incubated with 50 ng of unmethylated (lane 1) or monomethylated K42 (lanes 2 and 3) peptides for 30 minutes at room temperature and the resulting peptides were blotted onto nitrocellulose membrane. The demethylated products were detected by immuno-blotting with anti-monomethylated K42 or Sox2 antibodies, as indicated on the left.

G. Same as Figure 3F, except purified GST (lane 3) or GST-LSD1 proteins (lanes 1 and 2) were incubated with 50 ng of unmethylated (lane 1) or monomethylated K117 (lanes 2 and 3) peptides and the resulting peptides were blotted with anti-monomethylated K117 or Sox2 antibodies, as indicated on the right.

Figure 4. The monomethylated K42 and K117 in Sox2 are regulated by SET7.

A-C. PA-1 cells that stably express a wild-type (WT) Flag-Sox2 (A), K117R (B), or K42R (C) Sox2 mutant proteins were each transfected with 50 nM siRNAs of luciferase, LSD1, LSD1+ SET7, or SET7 for 48 hours. The levels of Flag-Sox2 (WT) or mutant Sox2 proteins, LSD1, SET7 and Actin were examined by Western blotting using specific antibodies as indicated on the left. Experiments were repeated three independent times with the same conclusion and one example is shown.

D. The Sox2 protein containing the monomethylated K42 and monomethylated K117 accumulated in LSD1 knockdown cells. PA-1 cells were transfected with 50 nM siRNAs of luciferase or LSD1 for 43 hours and then treated with 5 µg/ml of MG132 for last 5 hours. The cells were lysed and the levels of the monomethylated K42 and monomethylated K117 in endogenous Sox2 were analyzed by Western blotting with anti-monomethylated K42 and anti-monomethylated K117 bodies. Total protein levels of Sox2, LSD1 and Actin were also monitored by specific antibodies, as indicated on the left of panels. Experiments were repeated three independent times with the same conclusion and one example is shown.

Figure 5. The MBT domain of PHF20L1 binds to the monomethylated K42 and K117 in Sox2.

A. Expressed GST-PHF20L1-MBT domain (1-138) protein in bacteria. The GST-PHF20L1-MBT protein was purified by glutathione Sepharose.

B. The MBT domain of GST-PHF20L1 directly and specifically interacts with the monomethylated K117 peptide resin of Sox2 but not the unmethylated cognate peptide resin. The GST-PHF20L1-MBT protein were purified. The unmethylated K117me0 and monomethylated K117me1 peptide resins (30 µl) were incubated with 1 µg of GST-PHF20L1-MBT protein (input) for 1 hour at room temperature as indicated. The resins were subsequently washed extensively and blotted with anti-PHF20L1 antibodies. Experiments were repeated three independent times with the same conclusion and one example is shown.

C. The MBT domain of GST-PHF20L1 directly and specifically interacts with the monomethylated K42 peptide resin of Sox2 but not the unmethylated cognate peptide resin. The same as Figure 5B except the GST-PHF20L1-MBT protein was incubated with the unmethylated K42me0 and monomethylated K42me1 peptide resins. The resins were subsequently washed extensively and blotted with anti-PHF20L1 antibodies. Experiments were repeated three independent times with the same conclusion and one example is shown.

D. The GST-PHF20L1-MBT protein protects monomethylated K117 from LSD1 demethylase. 10 µg...
K117me1 peptide was mixed with the GST-PHF20L1-MBT protein bound to the glutathione Sepharose resin and the unbound peptide was washed away extensively. For each LSD1 demethylation reaction, 50 µl of methylated peptide-protein beads were mixed with 2.7 µg GST or GST-LSD1 for 5 hours at room temperature. For the peptide-release control, the washed K117me1 peptide-GST-PHF20L1-MBT beads were heated at 95 °C for 16 minutes to release the methylated K117 peptide before the addition of GST-LSD1.

E. The same as Figure 5D except the monomethylated K42 peptide was used.

**Figure 6. The interaction between PHF20L1 and Sox2 requires the presence of K42 and K117 in Sox2 in vivo.**

A. Endogenous PHF20L1 and Sox2 proteins interact. The endogenous PHF20L1 and Sox2 protein complexes were immunoprecipitated from PA-1 cells with anti-PHF20L1 and Sox2 antibodies. The proteins complexes were blotted with anti-PHF20L1 and Sox2 antibodies. IgG serves as an antibody control.

B. 293 cells were transfected with the wild-type GFP-Sox2 (WT), GFP-K42R or GFP-K117R mutant expression constructs for 48 hours. The interactions between PHF20L1 and WT and mutant Sox2 proteins were analyzed by immunoprecipitation with anti-PHF20L1 antibodies and Western blotting with anti-Sox2 and anti-PHF20L1 antibodies. The expressed wild-type Sox2 and Sox2 mutant proteins in total lysates were also examined.

C. The K42R/K117R mutant Sox2 abolished its binding to PHF20L1 in vivo. 293 cells were transfected with the wild-type (WT) GFP-Sox2 and GFP-K42R/K117R mutant constructs and expressed for 48 hours. The interactions between PHF20L1 and WT and the mutant Sox2 proteins were analyzed by immunoprecipitation with anti-PHF20L1 antibodies and Western blotting with anti-Sox2 and anti-PHF20L1 antibodies, as in Figure 6B. The expressed wild-type Sox2 and K42R/K117R mutant proteins in total lysates were also examined.

D. The PHF20L1-MBT domain stabilizes the wild-type but not the K42R/K117R mutant Sox2 proteins. The Flag-tagged PHF20L1-MBT domain expressing construct or an empty vector was transfected into 293 cells that stably express Flag-WT Sox2 or the K42R/K117R double mutant for 48 hours. The protein levels of Flag-WT Sox2, K42R/K117R Sox2 mutant, PHF20L1-MBT, and Actin were analyzed by Western blotting as indicated.

**Figure 7. Loss of either LSD1 or PHF20L inhibits the growth of PA-1 cells.**

A. PA-1 cells were transfected with 50 nM siRNAs of luciferase, LSD1 or PHF20L1 for 48 hours. Cells were examined and cell images were acquired with 10X10 lens of Nikon ECLIPSE Ti-S microscope equipped with NIS-Elements BR 3.1 software. Triplicated cells (technical repeats) were used for examination and one set of representative treated cells was shown.

B. Transfected cells from Figure 7A were harvested by trypsin digestion and counted on a hemacytometer. Cells in four corners of the hemacytometer were counted to obtain average cells per dish. The differences between control siRNA and LSD1 siRNA- or PHF20L1 siRNA-treated cells in triplicated samples were plotted. Statistically significant differences were determined using a two-tailed equal-variance independent t-test. Different data sets were considered to be statistically significant when the P-value was <0.01 (**).

C. The expression of K42R/K117R mutant partially rescues the growth inhibition in LSD1 or PHF20L1 knockdown cells. The PA-1 cells stably expressing the Flag-WT, K42R, K117R, or
K42R/K117R mutant Sox2 proteins were transfected with 50 nM siRNAs of luciferase, LSD1 or PHF20L1 for 48 hours, as in Figure 2E. Cells were examined and cell images were acquired as described in Figure 7A.

D. The treated cells in triplicates from Figure 7C were quantified and plotted as in Figure 7B.

Figure 8. The Sox2 protein is regulated by LSD1 or PHF20L in mouse embryonic stem cells.

A. Mouse embryonic stem cells grown on mitotically inactivated mouse embryonic fibroblasts (MEF, left panels) or on gelatin-coated culture dishes without MEF (right panels) were transfected with 50 nM siRNAs of luciferase, LSD1, LSD1+ SET7, SET7, PHF20L1, or PHF20L1+ SET7 for 44 hours. Cells were examined and cell images were acquired with 10X10 lens of Nikon ECLIPSE Ti-S microscope equipped with NIS-Elements BR 3.1 software. Triplicated cells were used for examination and one set of representative treated cells was shown.

B. The proteins in mouse embryonic stem cells transfected with siRNAs of luciferase, LSD1, LSD1+ SET7, and SET7 in Figure 8A were analyzed by Western blotting with anti-Sox2, LSD1, SET7 and Actin antibodies.

C. The proteins in mouse embryonic stem cells transfected with siRNAs of luciferase, PHF20L1, PHF20L1+ SET7, and SET7 in Figure 8A were analyzed by Western blotting with anti-Sox2, PHF20L1, SET7 and Actin antibodies.

D. The methylated mouse Sox2 protein is recognized by anti-K42me1 and K117me1 methylation antibodies. Mouse embryonic cells were lysed and Sox2 protein was immunoprecipitated by anti-Sox2 antibodies. The immunoprecipitated Sox2 proteins were Western blotted with anti-monomethylated K42, anti-monomethylated K117, or anti-Sox2 antibodies as indicated.

E. Endogenous PHF20L1 and Sox2 interact in mouse embryonic stem cells. The endogenous PHF20L1 and Sox2 protein complexes were immunoprecipitated from mouse embryonic stem cells with anti-PHF20L1 and Sox2 antibodies. The proteins complexes were blotted with anti-PHF20L1 and Sox2 antibodies as indicated. IgG serves as an antibody control.

F. Loss of LSD1 or PHF20L1 causes downregulation of Oct4 protein in mouse embryonic stem cells. Mouse embryonic stem cells were transfected with 50 nM siRNAs of luciferase, LSD1, or PHF20L1 for 44 hours. The protein levels of Oct4, LSD1, PHF20L1, and Actin were analyzed by respective antibodies as indicated.
**Figure 1**

| H3-K4: | MARTK*QTARKSTG |
|-------|----------------|
| Human SOX2-K42: | NQKNSPD *RVK*RPMNAMVW |
| Mouse Sox2-K44: | NQKNSPD *RVK*RPMNAFMV |
| Human SOX2-K117: | YKYPR *KTK*TLMKDDK |
| MOUSE SOX2-K119: | YKYPR *KTK*TLMKDDKY |
| DNMT1-K142: | SKPRTPR *RSK*SDGEAKRS |
| E2F1-K185: | GIQLIAK *KSK*NHIQWLGS |

**A.**

| SIRNA: | Luc | LSD1-1 | LSD1-2 |
|--------|-----|--------|--------|
| Flag-Sox2 |        |        |        |
| Endo-Sox2 |        |        |        |
| LSD1 |        |        |        |
| Actin |        |        |        |

**B.**

| **DMSO** | **MG132** |
|-----------|-----------|
| Flag-Sox2 |        |        |
| Endo-Sox2 |        |        |
| LSD1 |        |        |
| Actin |        |        |

**C.**

| **α-LSD1** | **IgG** | **α-Sox2** |
|------------|---------|------------|
| LSD1 |        |            |
| Sox2 |        |            |

**D.**

| **SIRNA:** | **Luc** | **LSD1-1** | **LSD1-1+SET7** | **SET7** |
|------------|---------|------------|----------------|---------|
| Flag-Sox2 |        |            |                |         |
| Endo-Sox2 |        |            |                |         |
| LSD1 |        |            |                |         |
| SET7 |        |            |                |         |
| Actin |        |            |                |         |

**E.**
Figure 2

A. 

SIRNA: LucPHF20L1
Flag-Sox2
Endo-Sox2
PHF20L1
Actin

B. 

DMSO   MG132
SIRNA: LucPHF20L1
Flag-Sox2
Endo-Sox2
PHF20L1
Actin

C. 

SIRNA: LucPHF20L1
Flag-Sox2
Endo-Sox2
PHF20L1
SET7
Actin

D. 

Flag-WT-Sox2 Flag-Sox2-K117R
siRNA: Luc PHF20L1 Luc PHF20L1
Flag-Sox2 endo-Sox2 Oct4 CUL1
PHF20L1

E. 

Flag-WT-Sox2
SIRNA: PHF20L1 Luc LSD1
Flag-Sox2 PHF20L1 LSD1 Actin

Flag-K42R
SIRNA: PHF20L1 Luc LSD1
Flag-Sox2 PHF20L1 LSD1 Actin

Flag-K117R
SIRNA: PHF20L1 Luc LSD1
Flag-Sox2 PHF20L1 LSD1 Actin

Flag-K42R/K117R
SIRNA: PHF20L1 Luc LSD1
Flag-Sox2 PHF20L1 LSD1 Actin
Figure 3

A. Peptides: K42 peptide, K42me1 peptide, K42 peptide, K42me1 peptide. Alpha-K42me1 antibody and alpha-Sox2 antibody.

B. Peptides: K117 peptide, K117me1 peptide, K117 peptide, K117me1 peptide. Alpha-K117me1 antibody and alpha-Sox2 antibody.

C. GFP-Sox2-WT, GFP-Sox2-K117R, GFP-Sox2. Alpha-K117me1 antibody, GFP-Sox2, Actin.

D. Peptides: 50, 100, 200 ng. Alpha-K42me1 antibody and alpha-Sox2 antibody.

E. Control (No IPTG) and GST-LSD1. M.W.M. and GST-LSD1. 130 kD and 100 kD.

F. Peptides: K42+GST-LSD1, K42me1+GST-LSD1, K42me1+GST, K42+GST-LSD1, K42me1+GST-LSD1, K42me1+GST. Alpha-K42me1 antibody and alpha-Sox2 antibody.

G. Peptides: K117+GST-LSD1, K117me1+GST-LSD1, K117me1+GST. Alpha-K117me1 antibody and alpha-Sox2 antibody.
Figure 4

A. Flag-WT-Sox2

B. Flag-K117R

C. Flag-K42R

D. SIRNA: α-K42me1-Sox2, α-K117me1-Sox2, α-Sox2

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A. 

Control (No IPTG) GST-PHF20L1-MBT

55 kD

GST-PHF20L1-MBT

35 kD

B. 

Unmethylated K117:       PDYKYRPRRKT(me0)TLMKKDKYC
mono-Methylated K117:    PDYKYRPRRKT(me1)TLMKKDKYC

Peptide resins:
GST-PHF20L1-MBT (peptide bound) 
GST-PHF20L1-MBT (input) 

C. 

Unmethylated K42:      CAGGNQKNSPDRV(me0)RPMNAFMVWSR
Mono-Methylated K42:   CAGGNQKNSPDRV(me1)RPMNAFMVWSR

Peptide resins:
GST-PHF20L1-MBT (peptide bound) 
GST-PHF20L1-MBT (input) 

D. E. 

GST beads-PHF20L1-3MBT-GST

Heat 16min
GST-LSD1  +  +  -  +

α-K117me1
α-Sox2
α-LSD1

Heat 16min
GST-LSD1  +  +  -  +

α-K42me1
α-Sox2
α-LSD1

Figure 5
Figure 6
Figure 7

Luc siRNA, PA-1 LSD1 siRNA, PA-1 PHF20L1 siRNA, PA-1

A. B. C. D.

WT
K42R
K117R
K42R/K117R

Relative Cell #

Luc LSD1 PHF20L1

K42R/K117R

Relative Cell #

Luc LSD1 PHF20L1

K42R

Relative Cell #

Luc LSD1 PHF20L1

K117R

Relative Cell #

Luc LSD1 PHF20L1
Figure 8
LSD1 demethylase and the methyl-binding protein PHF20L1 prevent SET7 methyltransferase-dependent proteolysis of the stem-cell protein Sox2
Chunxiao Zhang, Nam Hoang, Feng Leng, Lovely Saxena, Logan Lee, Salvador Alejo, Dandan Qi, Anthony Khal, Hong Sun, Fei Lu and Hui Zhang

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