LSD1 demethylase and the methyl-binding protein PHF20L1 prevent SET7 methyltransferase–dependent proteolysis of the stem-cell protein SOX2

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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) 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 demethylase 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 (equivalent to Lys-119 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.

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The lysine-specific demethylase 1 (LSD1,[2] also called KDM1A) was originally identified as a histone demethylase that removes the methyl group from the mono- and dimethylated 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 down-regulation 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 (9). SOX2 is also a key factor for various adult stem/progenitor cells in the brain, retina, and trachea and in the epithelium of the cervix, tongue, and testes, allowing for tissue regeneration and repair. Recently, *Sox2* has been identified as a major oncogene that is commonly and recurrently amplified at 3q26.33 in squamous cell carcinomas of the lung, esophagus, and oral cavity (10–14). Gene amplification of *Sox2* also occurs in small-cell lung carcinomas and glioblastoma multiforme (15, 16). SOX2 is overexpressed 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, are monomethylated on specific lysine residues by SET7 (SETD7, KMT7, SET7/9, or SET9) (3, 28–33), a methyltransferase that was originally identified for its activity to monomethylate H3K4. A novel function of these methylation events in a group of proteins, such as DNMT1, E2F1, NFκB/RelA, FOX3A, 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

[2] The abbreviations used are: LSD1, lysine-specific demethylase 1; H3K4, histone H3 Lys-4; K42me1, monomethylated Lys-42; K117me1, monomethylated Lys-117; mES, mouse embryonic stem cells.

monomethylated on lysine 119 (equivalent to Lys-117 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 down-regulation 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 down-regulating 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

Knockdown 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 (long terminal repeat in pMSCV) in human ovarian teratocarcinoma cell line PA-1 (35), which abundantly expresses endogenous SOX2 (35, 36, 39). Reduction of LSD1 by two independent siRNAs led to the marked down-regulation of endogenous SOX2 protein (Fig. 1*A*). However, we found that knockdown of LSD1 also led to the significantly reduced level of FLAG-tagged SOX2 protein (Fig. 1*A*). The reduced levels of both FLAG-tagged and 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 (Fig. 1*B*). We also found that endogenous SOX2 and LSD1 proteins physically interact with each other (Fig. 1*C*), suggesting that LSD1 may directly regulate SOX2 protein stability through protein-protein interactions. Because the FLAG-tagged SOX2 is ectopically expressed under the retroviral long terminal repeat 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 (Fig. 1*D*), whereas the endogenous SOX2 protein was also significantly restored (Fig. 1*D*). These studies raised the possibility that LSD1 may act as a demethylase for SOX2, as the reported methylated Lys-117 motif in SOX2 is similar to that of methylated H3K4, a substrate of LSD1 (Fig. 1*E*) (31, 32, 34).

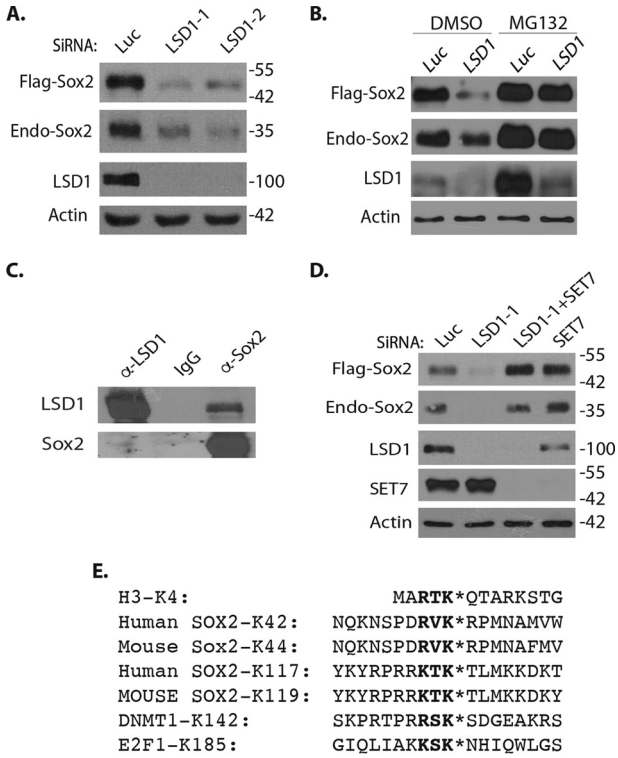

Figure 1. Loss of LSD1 destabilizes both endogenous and ectopically expressed SOX2 in the SET7-dependent manner. *A*, LSD1 knockdown reduces SOX2 proteins. PA-1 cells that stably express a FLAG-SOX2 protein were transfected with 50 nM luciferase (*Luc*, control) siRNA or two independent LSD1 siRNAs for 48 h. The cells were directly lysed in an SDS-containing lysis buffer, equalized, and 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 result, and one example is shown. Protein molecular mass markers in kDa were indicated on the *right* of the *panels*. *B*, the 26S proteasome inhibitor MG132 prevents SOX2 degradation in LSD1-knockdown cells; same as in *A*, except at 43 h post-siRNA transfection, one set of cells was treated with 5 μg/ml MG132 for an additional 5 h, whereas the other set was treated with DMSO (control). *C*, LSD1 and SOX2 physically interact *in vivo*. Actively growing PA-1 cells were lysed in the Nonidet P-40–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 h. The lysates were equalized, and proteins were analyzed by antibodies against each indicated protein on the *left* of the *panels*, as in *A*. Experiments were repeated three independent times with the same result, 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. Lys-42 in SOX2 is located in a highly conserved motif containing the RVK* motif, as indicated.

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 in DNA methyltransferase I (DNMT1) to prevent DNMT1 proteolysis triggered by SET7-mediated monomethylation of Lys-142 (37, 38). Using two independent specific siRNAs against PHF20L1, we found that knockdown of PHF20L1 also led to the

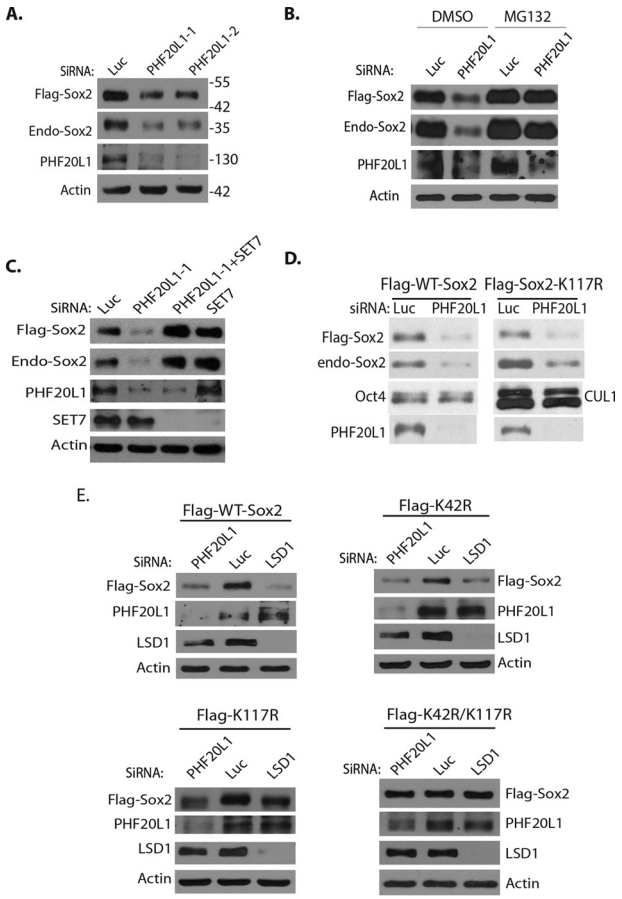

Figure 2. Both Lys-42 and Lys-117 in SOX2 regulate SOX2 protein stability in LSD1- and PHF20L1-knockdown cells. *A*, PHF20L1 knockdown reduces the protein level of SOX2. PA-1 cells stably expressing a FLAG-SOX2 protein were transfected with 50 nM luciferase siRNA or two independent PHF20L1 siRNAs for 48 h. 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 result, and one example is shown. *B*, MG132 prevents SOX2 degradation in PHF20L1-knockdown cells; same as in *A*, except at 43 h post-siRNA transfection, one set of cells was treated with 5 μg/ml MG132 for an additional 5 h, whereas the other set was treated 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 h. The protein levels of FLAG-tagged and endogenous SOX2, SET7, PHF20L1, and actin in cell lysates were detected by the indicated antibodies. Experiments were repeated three independent times with the same result, 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 WT FLAG-SOX2 or the K117R SOX2 mutant protein were transfected with 50 nM siRNAs of luciferase or PHF20L1 for 48 h. 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 result, and one example is shown. *E*, both Lys-42 and Lys-117 are required for SOX2 degradation in LSD1- or PHF20L1-knockdown cells. PA-1 cells stably expressing a WT FLAG-SOX2 or K42R, K117R, or K42R/K117R SOX2 mutant protein were each transfected with 50 nM siRNAs of luciferase, LSD1, or PHF20L1 for 48 h. 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 result, and one example is shown.

markedly reduced levels of FLAG-SOX2 and endogenous SOX2 protein in PA-1 cells (Fig. 2*A*). This destabilization of SOX2 protein was prevented when PHF20L1-knockdown cells were treated with MG132 (Fig. 2*B*). We also tested the involvement of SET7 in PHF20L1 deficiency–induced proteolysis of SOX2. We again found that the destabilization of SOX2 protein was prevented when SET7 was co-knocked down with PHF20L1 siRNA-treated cells (Fig. 2*C*). The effect of PHF20L1 knockdown is specific for the SOX2 protein, since the levels of other proteins, such as OCT4 and CUL1, did not significantly change after the down-regulation of PHF20L1 in PA-1 cells (Fig. 2*D*). 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 Lys-119 in mouse SOX2, equivalent to Lys-117 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 Lys-117 in human SOX2, we converted the highly conserved Lys-117 to arginine (K117R; Fig. 1*E*) and stably expressed the FLAG-tagged K117R mutant of SOX2 in PA-1 cells. Surprisingly, we found that the K117R mutant was still down-regulated after the loss of PHF20L1 or LSD1 (Fig. 2, *D* and *E*), suggesting that there may be additional methylation sites in the human SOX2 protein for its methylation-dependent proteolysis.

SOX2 protein stability is regulated by methylation at lysine 42

Because 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 Fig. 1*E*) (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 Lys-42 in human SOX2 within an RVK motif that shares substantial homology to the consensus (R/K)(S/T)K motif in SET7 methylation substrates (Fig. 1*E*). We therefore tested the possibility that Lys-42 may potentially serve as a novel methylation site for SET7, LSD1, and PHF20L1 by converting Lys-42 to arginine to produce the K42R mutant of SOX2. We stably expressed the FLAG-tagged wildtype 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 toward the loss of LSD1, PHF20L1, and SET7, as compared with the wildtype SOX2. Our studies showed that whereas the K42R or K117R single mutation did not significantly cause resistance to SOX2 degradation in LSD1 or PHF20L1-knockdown cells, the double SOX2 mutant of Lys-42 and Lys-117 (K42R/K117R) was significantly resistant toward the loss of LSD1 or PHF20L1 (Fig. 2*E*). Thus, our results suggest that the methylated Lys-42 and Lys-117 residues in SOX2 may together serve as the methylated degradation signals for SOX2 proteolysis.

LSD1 demethylates the monomethylated Lys-42 and Lys-117

To facilitate the detection of the methylated Lys-42 and Lys-117 in SOX2, we developed specific anti-monomethylated Lys-42 (K42me1) and monomethylated Lys-117 (K117me1) peptide antibodies using procedures described previously (31, 33, 34). These antibodies were affinity-purified and tested for

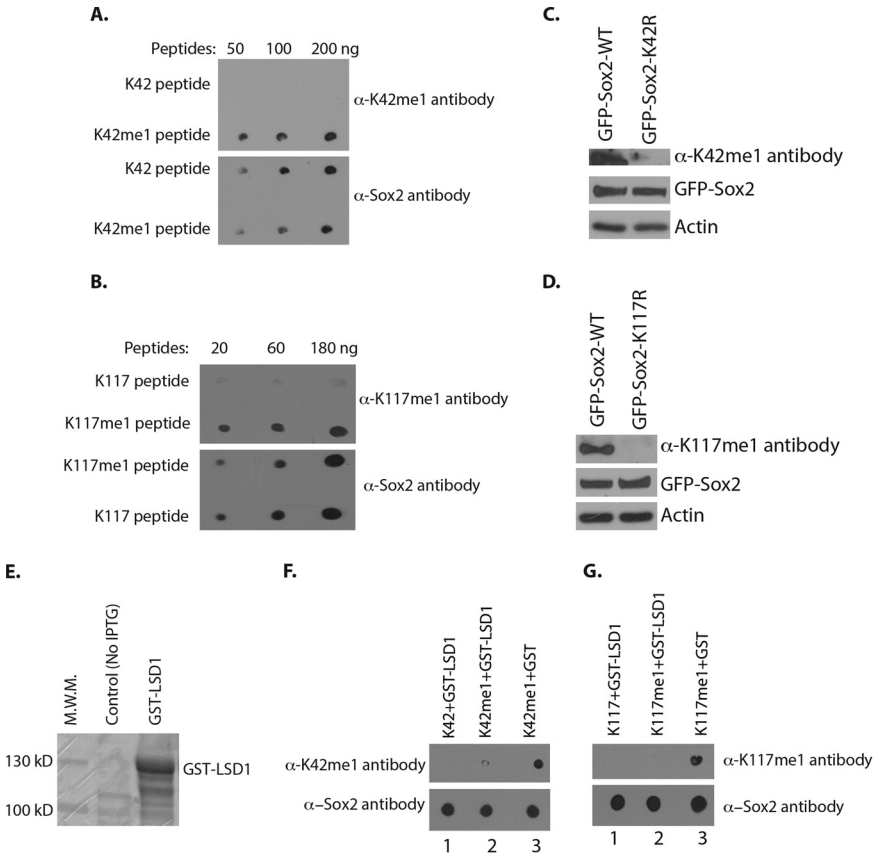

Figure 3. LSD1 demethylates the methyl group from monomethylated Lys-42 and Lys-117 in SOX2. *A*, development of specific anti-K42me1 peptide antibodies for SOX2. The rabbit polyclonal sera against the monomethylated Lys-42 peptide were depleted of the antibodies against the unmethylated peptide using the unmethylated Lys-42 peptide chromatographic resins. The antibodies were then affinity-purified by binding to the monomethylated Lys-42 peptide chromatographic resins. To test the specificity of the methylation antibodies, the unmethylated and monomethylated Lys-42 peptides at the indicated concentrations were blotted onto nitrocellulose membrane and immunoblotted with the affinity-purified anti-monomethylated Lys-42 antibodies or anti-SOX2 antibodies that recognize both unmethylated and methylated peptides. *B*, development of anti-monomethylated Lys-117 (K117me1) peptide antibodies for SOX2. The affinity purification of anti-monomethylated Lys-117 peptide antibodies was similar to that shown in *A*, except unmethylated or monomethylated Lys-117 peptide chromatographic resins were used. The unmethylated and monomethylated Lys-117 peptides at the indicated concentrations were blotted onto nitrocellulose membrane and immunoblotted with the affinity-purified anti-monomethylated Lys-117 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 h. The GFP-SOX2 and K42R proteins were examined in equalized cell lysates by anti-monomethylated Lys-42 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 h post-transfection by anti-monomethylated Lys-117 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 β-D-1-thiogalactoppyranoside induction is shown, and the protein was purified by glutathione-Sepharose. *F*, 1 μg of purified GST (*lane 3*) or GST-LSD1 proteins (*lanes 1* and *2*) were incubated with 50 ng of unmethylated (*lane 1*) or monomethylated Lys-42 (*lanes 2* and *3*) peptides for 30 min at room temperature, and the resulting peptides were blotted onto nitrocellulose membrane. The demethylated products were detected by immunoblotting with anti-monomethylated Lys-42 or SOX2 antibodies, as indicated on the *left*. *G*, same as in *F*, except purified GST (*lane 3*) or GST-LSD1 proteins (*lanes 1* and *2*) were incubated with 50 ng of unmethylated (*lane 1*) or monomethylated Lys-117 (*lanes 2* and *3*) peptides, and the resulting peptides were blotted with anti-monomethylated Lys-117 or SOX2 antibodies, as indicated on the *right*.

their specific antigenic recognition of the methylated Lys-42 or Lys-117 peptides (Fig. 3, *A* and *B*). We found that anti-K42me1 antibodies recognized only the methylated Lys-42 peptide and not the unmethylated peptide. It can also detect wildtype SOX2 but not the K42R mutant isolated from transfected 293 cells (Fig. 3, *A* and *C*). Similarly, the anti-K117me1 antibodies only detected the methylated Lys-117 peptide and not the unmethylated peptide (Fig. 3*B*). The K117me1 antibodies also detected the wildtype SOX2 but not the K117R mutant protein from transfected 293 cells (Fig. 3*D*). Our characterization indicated that the anti-K42me1 or anti-K117me1 antibodies are specific for the methylated Lys-42 or Lys-117 residues of SOX2 both *in vitro* and *in vivo*, as mutation of Lys-42 or Lys-117 in SOX2 abolished their respective reactivity *in vivo*.

If LSD1 acts as a demethylase to remove the methyl group from the methylated Lys-42 or Lys-117 in SOX2, loss of LSD1 should lead to increased levels of methylated SOX2 protein and consequently promote SOX2 degradation. To directly test whether LSD1 can demethylate monomethylated Lys-42 or Lys-117 in SOX2, we purified recombinant GST-LSD1 protein from bacteria (Fig. 3*E*) and examined its ability to demethylate the K42me1 or K117me1 peptides, as we have previously described for analyzing the demethylase activity of LSD1 toward the methylated H3K4 (6). Our *in vitro* biochemical analysis revealed that LSD1 indeed demethylated both the methylated Lys-42 and Lys-117 peptides *in vitro* (Fig. 3, *F* and *G*). In PA-1 cells, our studies also revealed that loss of LSD1 led to the accumulation of the endogenous methylated Lys-42– and Lys-117–containing SOX2 protein (Fig. 4*D*), indicating that LSD1

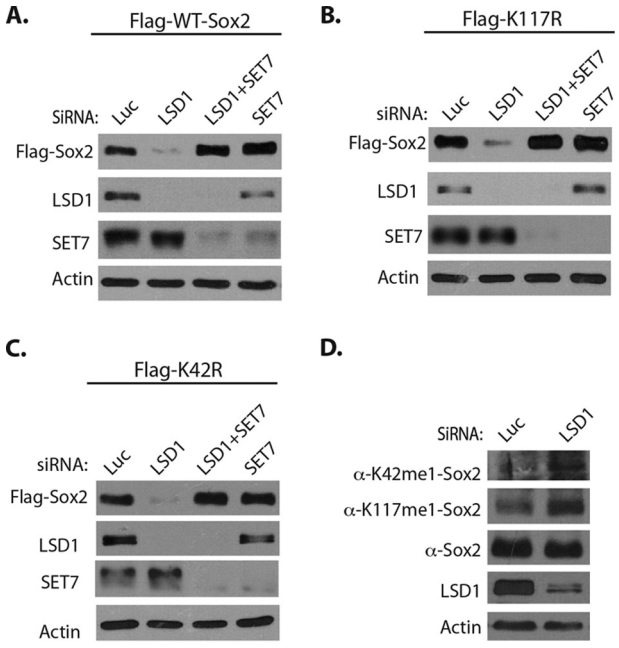

Figure 4. The monomethylated Lys-42 and Lys-117 in SOX2 are regulated by SET7. *A–C*, PA-1 cells that stably express WT FLAG-SOX2 (*A*), K117R (*B*), or K42R (*C*) SOX2 mutant proteins were each transfected with 50 nM siRNAs of luciferase, LSD1, LSD1 + SET7, or SET7 for 48 h. The levels of FLAG-SOX2 (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 result, and one example is shown. *D*, the SOX2 protein containing the monomethylated Lys-42 and monomethylated Lys-117 accumulated in LSD1-knockdown cells. PA-1 cells were transfected with 50 nM siRNAs of luciferase or LSD1 for 43 h and then treated with 5 μg/ml MG132 for last 5 h. The cells were lysed, and the levels of the monomethylated Lys-42 and monomethylated Lys-117 in endogenous SOX2 were analyzed by Western blotting with anti-monomethylated Lys-42 and anti-monomethylated Lys-117 bodies. Total protein levels of SOX2, LSD1, and actin were also monitored by specific antibodies, as indicated on the *left* of the *panels*. Experiments were repeated three independent times with the same result, and one example is shown.

normally removes the methyl group from the methylated Lys-42 and Lys-117 residues in SOX2 *in vivo*.

SET7 regulates the methylation of Lys-42 and Lys-117

Because 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 down-regulation prevented the degradation of both SOX2 K42R and K117R single mutants in LSD1-deficient cells, indicating that Lys-42 and Lys-117 in SOX2 are substrates of SET7 methyltransferase and LSD1 *in vivo* (Fig. 4, *A–C*).

PHF20L1 binds to either monomethylated Lys-42 or Lys-117

It has been shown that the MBT domain of PHF20L1 can bind to the monomethylated Lys-142 in DNMT1 (37). We tested whether the MBT domain of PHF20L1 can also recognize the monomethylated Lys-42 or Lys-117 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 Lys-42 and Lys-117 peptide resins, but not to the non-methylated cognate peptides (Fig. 5, *B* and *C*), indicating that PHF20L1 specif-

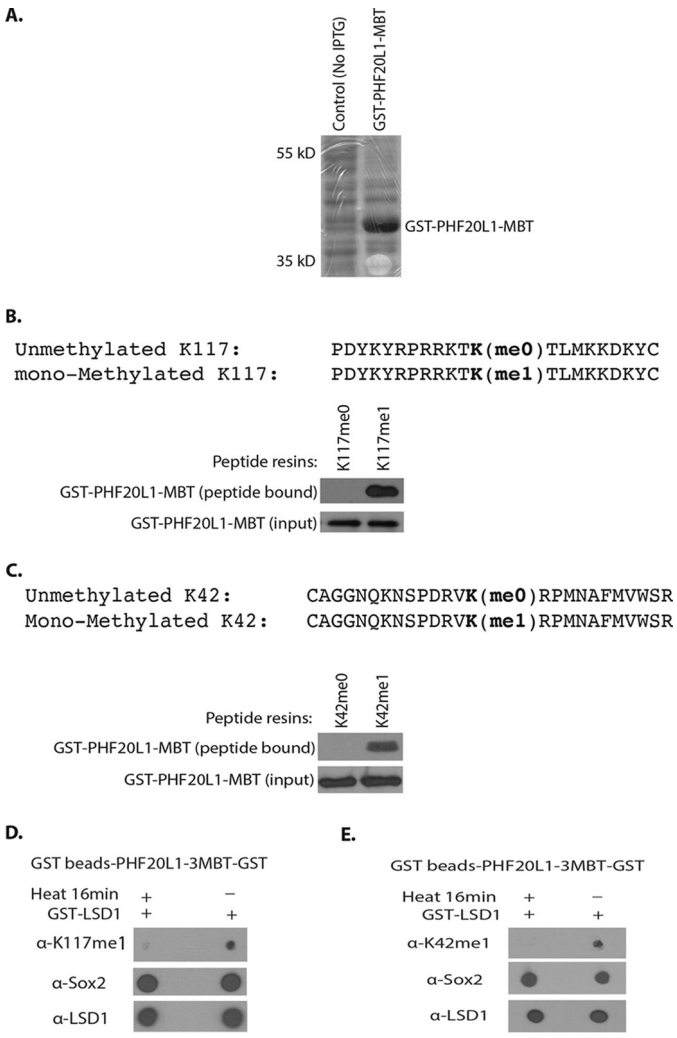

Figure 5. The MBT domain of PHF20L1 binds to the monomethylated Lys-42 and Lys-117 in SOX2. *A*, expressed GST-PHF20L1-MBT domain (residues 1–138) protein in bacteria. The GST-PHF20L1-MBT protein was purified by glutathione-Sepharose. *B*, the MBT domain of GST-PHF20L1 directly and specifically interacts with the monomethylated Lys-117 peptide resin of SOX2 but not the unmethylated cognate peptide resin. The GST-PHF20L1-MBT protein was purified. The unmethylated Lys-117 (*K117me0*) and monomethylated K117me1 peptide resins (30 μl) were incubated with 1 μg of GST-PHF20L1-MBT protein (input) for 1 h 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 result, and one example is shown. *C*, the MBT domain of GST-PHF20L1 directly and specifically interacts with the monomethylated Lys-42 peptide resin of SOX2 but not the unmethylated cognate peptide resin; same as in *B*, except the GST-PHF20L1-MBT protein was incubated with the unmethylated Lys-42 (*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 result, and one example is shown. *D*, the GST-PHF20L1-MBT protein protects monomethylated Lys-117 from LSD1 demethylase. 10 μg of 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 of GST or GST-LSD1 for 5 h at room temperature. For the peptide-release control, the washed K117me1 peptide-GST-PHF20L1-MBT beads were heated at 95 °C for 16 min to release the methylated Lys-117 peptide before the addition of GST-LSD1. *E*, same as in *D*, except the monomethylated Lys-42 peptide was used.

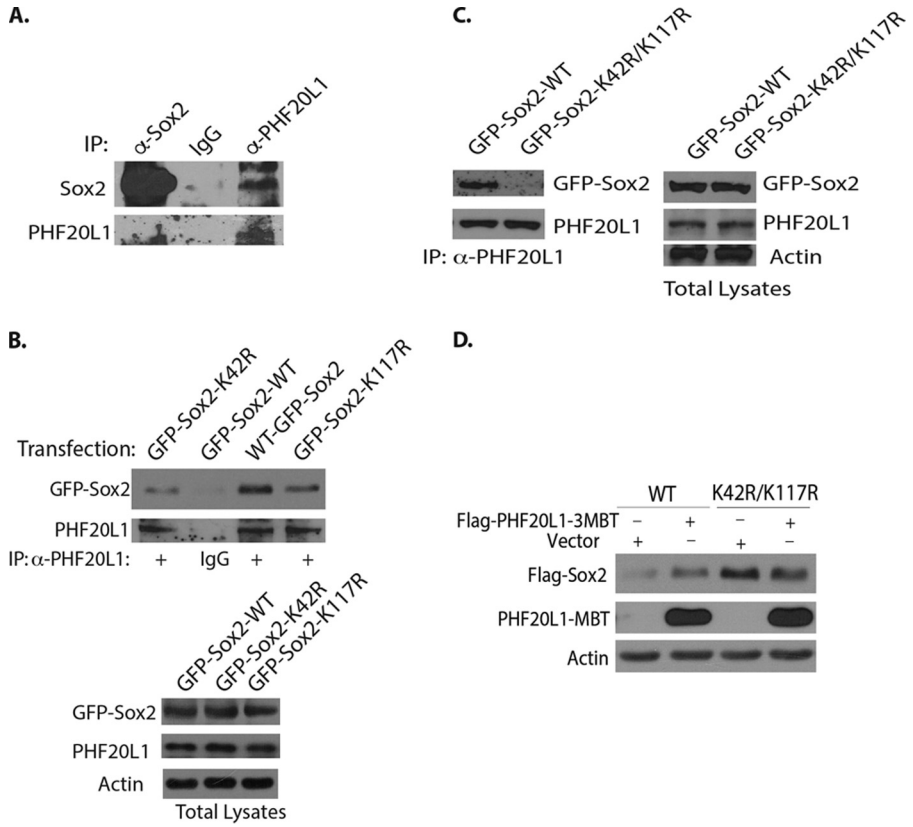

Figure 6. The interaction between PHF20L1 and SOX2 requires the presence of Lys-42 and Lys-117 in SOX2 *in vivo*. *A*, endogenous PHF20L1 and SOX2 proteins interact. The endogenous PHF20L1 and SOX2 protein complexes were immunoprecipitated (*IP*) from PA-1 cells with anti-PHF20L1 and SOX2 antibodies. The protein complexes were blotted with anti-PHF20L1 and SOX2 antibodies. IgG serves as an antibody control. *B*, 293 cells were transfected with the WT GFP-SOX2, GFP-K42R, or GFP-K117R mutant expression constructs for 48 h. 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 WT GFP-SOX2 and GFP-K42R/K117R mutant constructs and expressed for 48 h. 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 PHF20L1 antibodies, as in Fig. 6*B*. The expressed wildtype SOX2 and K42R/K117R mutant proteins in total lysates were also examined. *D*, the PHF20L1-MBT domain stabilizes the wildtype 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 h. The protein levels of FLAG-WT SOX2, K42R/K117R SOX2 mutant, PHF20L1-MBT, and actin were analyzed by Western blotting as indicated.

ically binds to both the methylated Lys-42 and Lys-117 in SOX2 *in vitro*.

PHF20L1 binding to the methylated Lys-42 and Lys-117 prevents LSD1 demethylation

Because LSD1 demethylates the methylated Lys-42 or Lys-117, 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 Lys-42 or Lys-117 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 Lys-42 or Lys-117 peptide on the GST-PHF20L-MBT protein beads was incubated with purified LSD1 protein. As a control, the methylated Lys-42 or Lys-117 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 min. We found that whereas the heat-released methylated Lys-42 and Lys-117 peptides were fully demethylated by LSD1, a significant fraction of methylated Lys-42 or Lys-117 was protected by their binding to the GST-PHF20L-MBT protein beads (Fig. 5, *D* and *E*), indicating that the binding of these methylated peptides to PHF20L1 prevents LSD1 from demethylating these peptides.

PHF20L1 binds to the endogenous SOX2 protein through Lys-42 and Lys-117 to prevent SOX2 degradation

We found that the endogenous PHF20L1 interacts with SOX2 protein in PA-1 cells by co-immunoprecipitation analysis (Fig. 6*A*). Our analysis further revealed that whereas the K42R or K117R mutant each displayed reduced binding to PHF20L1 (Fig. 6*B*), the K42R/K117R double mutation abolished the binding of PHF20L1 to SOX2 (Fig. 6*C*), indicating that PHF20L1 binds to both Lys-42 and Lys-117 in SOX2 *in vivo*. We also examined the effects of ectopically expressed PHF20L1-MBT domain on stably expressed wildtype SOX2 and the K42R/K117R mutant. We found that expression of the MBT domain of PHF20L1 caused an increased level of the wildtype SOX2 protein, but not the K42R/K117R mutant protein, indicating that the binding of the MBT domain of PHF20L1 to the methylated Lys-42 and Lys-117 in wildtype SOX2 prevents its proteolysis (Fig. 6*D*).

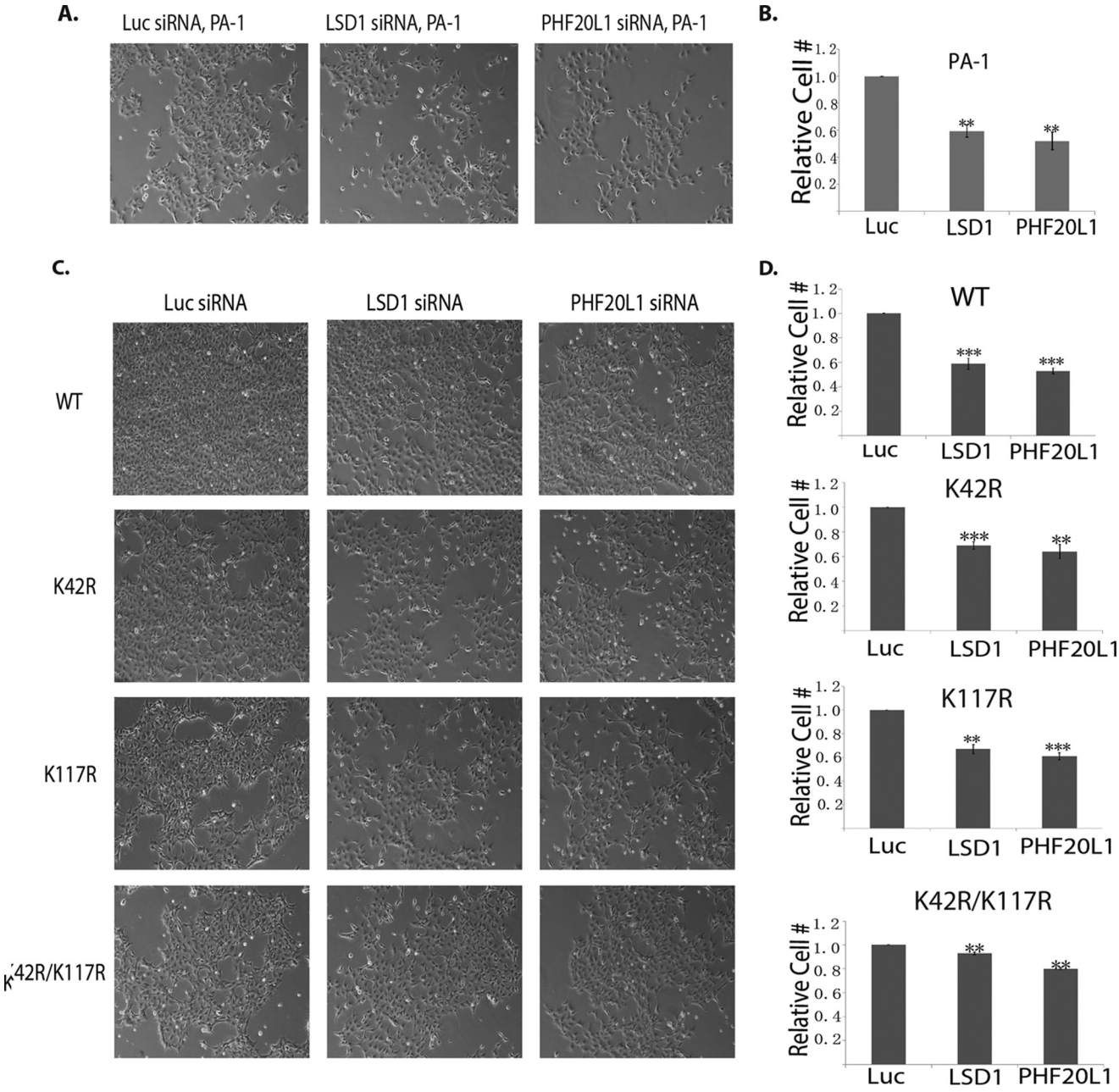

Figure 7. Loss of either LSD1 or PHF20L inhibits the growth of PA-1 cells. *A*, PA-1 cells were transfected with 50 nM siRNAs of luciferase, LSD1, or PHF20L1 for 48 h. Cells were examined, and cell images were acquired with the 10×/0.30 lens of a Nikon ECLIPSE Ti-S microscope equipped with NIS-Elements BR 3.1 software. Triplicated cells (technical repeats) were used for examination, and one set of representative treated cells is shown. *B*, transfected cells from *A* 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 h, as in Fig. 2*E*. Cells were examined, and cell images were acquired as described in *A*. *D*, the treated cells in triplicates from *C* were quantified and plotted as in *B*. *Error bars*, S.D. ***, $p < 0.001$.

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. Because 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 (Fig. 7, *A* and *B*). We found that knockdown of LSD1 or PHF20L1 by specific siRNAs led to the significant growth inhibition of PA-1 cells (Fig. 7*A*). 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 whereas expression of the wildtype 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 (Fig. 7, *C* and *D*), indicating that the expres-

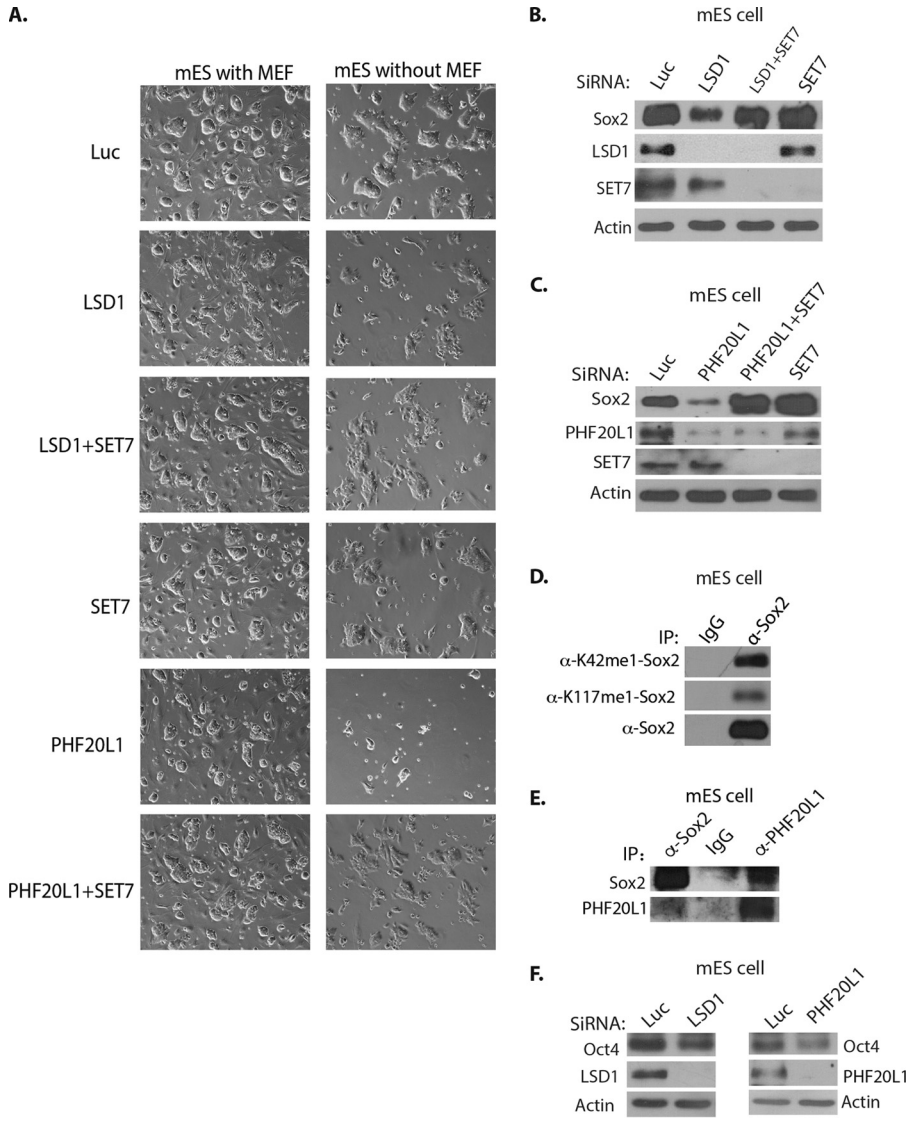

Figure 8. The SOX2 protein is regulated by LSD1 or PHF20L in mouse embryonic stem cells. *A*, mouse embryonic stem cells grown on mitotically inactivated mouse embryonic fibroblasts (*MEF*; *left panels*) or on gelatin-coated culture dishes without MEF (*right panels*) were transfected with 50 nM siRNAs of luciferase, LSD1, LSD1 + SET7, SET7, PHF20L1, or PHF20L1 + SET7 for 44 h. Cells were examined, and cell images were acquired with the 10×/0.30 lens of a 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 *A* 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 *A* 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 (*IP*) by anti-SOX2 antibodies. The immunoprecipitated SOX2 proteins were Western blotted with anti-monomethylated Lys-42, anti-monomethylated Lys-117, 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 protein complexes were blotted with anti-PHF20L1 and SOX2 antibodies, as indicated. IgG serves as an antibody control. *F*, loss of LSD1 or PHF20L1 causes down-regulation 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 h. The protein levels of OCT4, LSD1, PHF20L1, and actin were analyzed by the respective antibodies, as indicated.

sion of the double K42R/K117R mutant causes resistance to the loss of LSD1 or PHF20L1 for PA-1 cell growth (Figs. 2*E* and 7 (*C* and *D*)).

LSD1 and PHF20L1 also regulate the protein level of SOX2 in mouse embryonic stem cells

Because 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 Lys-42 and Lys-117 antibodies, as these methylated lysine residues are highly conserved in human and mouse SOX2 proteins (Figs. 1*E* and 8*D*), suggesting that mouse SOX2 is also methylated at these conserved lysine residues (Fig. 1*E*). Like PA-1 cells, knockdown of LSD1 or PHF20L1 in mES cells also triggered the down-regulation of SOX2 protein (Fig. 8, *B* and *C*), resulting in significant growth inhibition of mES cells (Fig. 8*A*). 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 (Fig. 8, *A*–*C*). Loss of SET7 alone also

induced an increased level of SOX2 protein (Fig. 8, *B* and *C*). We also found that endogenous PHF20L1 and SOX2 interact with each other in mES cells (Fig. 8*E*). These studies indicate that SET7, LSD1, and PHF20L also regulate the protein levels of SOX2 protein, probably through the methylation-dependent proteolysis of SOX2 in mouse embryonic stem cells. However, whereas we did not observe significant reduction of OCT4 protein in PHF20L1-knockdown PA-1 cells (Fig. 2*D*), we found that knockdown of LSD1 or PHF20L1 reduced the protein level of OCT4 in mouse embryonic stem cells (Fig. 8*F*). 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 the lack of Nanog in PA-1 cells may misregulate such a critical stem regulatory mechanism so that down-regulation 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 or teratocarcinoma or embryonic carcinoma cells and in 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 Lys-42 and Lys-117 residues in human SOX2 (Fig. 1–4). Our studies strongly support a model in which LSD1 acts as a demethylase to remove the methyl group from both methylated Lys-42 and Lys-117 to stabilize SOX2 protein, whereas SET7 methylates these lysine residues to destabilize SOX2 protein. Importantly, previous studies showed that mouse SOX2 is methylated at Lys-119 (equivalent to Lys-117 in human SOX2) by SET7 (34). However, we found that Lys-117 is only one of multiple methylation sites in SOX2. We found that a novel methylated lysine residue, Lys-42, in SOX2 is recognized by LSD1 for demethylation, and this lysine is also regulated by SET7 (Fig. 2). Both methylated Lys-42 and Lys-117 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 (Figs. 2 and 5), encoded by a gene that is amplified or overexpressed 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 Lys-142 in DNMT1 (37). Our studies revealed that PHF20L1 recognizes both the monomethylated Lys-42 and Lys-117 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 SOX2 protein stability is regulated by multiple processes mediated by SET7, 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 (Fig. 8). Because 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, and 293 cells were in Dulbecco's modified Eagle's medium, 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 Dulbecco's modified Eagle's medium and knockout serum replacement, supplemented with leukemia inhibitory factor, GlutaMax, β-mercaptoethanol, minimum essential medium 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 Lys-42 and anti-monomethylated Lys-117 antibodies were raised using synthetic peptides. The monomethylated Lys-42 (CAGGNQKNSPDRVK(me1)RPMNAFMVWSR) and cognate unmethylated peptides and the monomethylated Lys-117 (PDYKYRPRRKTK(me1)TLMKKDKYC) and cognate unmethylated peptides of SOX2 were synthesized at ABI Scientific. We also synthesized two human PHF20L1 peptides (PHF20L1M1 (KEKDKERREKRDKDHYRPKC) and PHF20L1C1 (LKRHIKQLLIDMGKVQQIATLCSVC)) for raising anti-PHF20L1 antibodies. The methylated Lys-42 peptide and Lys-117 peptides, as well as the peptides for PHF20L1, were chemically coupled to keyhole limpet hemocyanin 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 procedures described previously (31, 33, 34), by first passing the rabbit antisera through the unmethylated peptide chromatographic resins to deplete the antibodies against the

unmethylated peptides. The flow-through fraction of antisera was then affinity-purified by the monomethylated peptide chromatographic resins. The bound anti-methylated antibodies were eluted by 0.1 M glycine, pH 2.5, and immediately neutralized by a buffer containing 1 M Tris, pH 8.0. 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 described previously (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 Lys-42 and Lys-117 and their cognate unmethylated peptides of SOX2 were covalently coupled to the Sulfolink-coupled resins (Thermo Fisher Scientific) (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% Nonidet P-40, 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl), and the resins were preblocked with 2 μg of GST protein at room temperature for 2 h. The peptide-coupled resins were then incubated with 1 μg each of GST or the indicated 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 *Escherichia coli* BL21 strain, and their expression was induced by 0.4 mM isopropyl β-D-1-thiogalactoppyranoside (6, 42). The GST-LSD1, PHF20L1-MBT, and GST proteins were purified by glutathione-agarose (GE Healthcare), as described previously (6).

For PHF20L1 protection assays, 500 μg of GST-PHF20L1-MBT protein was prebound to 500 μl of glutathione-Sepharose and washed extensively with the wash buffer (150 mM NaCl and 50 mM Tris, pH 7.4). Each 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 of GST or GST-LSD1 for 5 h at room temperature.

Transfection and siRNA-mediated gene silencing

Cells were usually transfected with 50 nM siRNAs for 48 h using DharmaFECT transfection reagent (catalog no. T-2001-03, GE Healthcare) as described previously (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 the 10 × 10 lens of a 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 three independent times with the same result. The sequences of siRNAs were as follows: human/mouse LSD1-1, GGAAGAAGAUAGUGAAAACUU; human LSD1-2, AGUGAAAACUCAGGAAGAAUU; human PHF20L1-1, UGGGGUUGAUGGUGCUGAAUU; human PHF20L1-2, GAUGAGAGAAAGUGGUUUAUU; mouse PHF20L1, UCCAGCUUCAGGGAAUAAA; 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 the SOX2 protein. The FLAG-SOX2 fusion protein is stably expressed after transfection of the pMSCV-Puro-FLAG-SOX2 construct by the pMSCV expression protocol with Lipofectamine 2000 (Thermo Fisher Scientific) 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 the 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 a 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).

Author contributions—H. Z. conceived the concept, organized, designed, and coordinated experiments. C. Z. did many significant experiments, with help from N. H., F. Leng, L. S., S. A., D. Q., H. S., L. L., A. K., and F. Lu. on various parts of experiments. H. Z. analyzed and wrote the manuscript. All authors approved the final version of the manuscript.

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