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Destabilizing LSD1 by Jade-2 Promotes Neurogenesis: An Antibraking System in Neural Development

Xiao Han,[1] Bin Gui,[1] Cong Xiong,[3] Linnan Zhao,[4] Jing Liang,[1] Luyang Sun,[1] Xiaohan Yang,[1] Wenhua Yu,[1] Wenzhe Si,[1] Ruorong Yan,[1] Xia Yi,[1] Di Zhang,[1] Wanjin Li,[1] Lifang Li,[1] Jianguo Yang,[1] Yan Wang,[2] Yi Eve Sun,[5,6] Dai Zhang,[4] Anming Meng,[3] and Yongfeng Shang[1,2,*]

[1]Key Laboratory of Carcinogenesis and Translational Research, Ministry of Education, Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, China

[2]2011 Collaborative Innovation Center of Tianjin for Medical Epigenetics, Tianjin Key Laboratory of Medical Epigenetics, Department of Biochemistry and Molecular Biology, Tianjin Medical University, Tianjin 300070, China

[3]State Key Laboratory of Biomembrane and Membrane Engineering, Tsinghua-Peking Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China

[4]Key Laboratory of Mental Health, Ministry of Health, Peking University Health Science Center, Beijing 100191, China

[5]Translational Center for Stem Cell Research, Tongji Hospital, Stem Cell Research Center, Tongji University School of Medicine, Shanghai 200065, China

[6]Departments of Psychiatry and Behavioral Sciences and Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA 90095, USA

*Correspondence: yshang@hsc.pku.edu.cn

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SUMMARY

Histone H3K4 demethylase LSD1 plays an important role in stem cell biology, especially in the maintenance of the silencing of differentiation genes. However, how the function of LSD1 is regulated and the differentiation genes are derepressed are not understood. Here, we report that elimination of LSD1 promotes embryonic stem cell (ESC) differentiation toward neural lineage. We showed that the destabilization of LSD1 occurs posttranscriptionally via the ubiquitin-proteasome pathway by an E3 ubiquitin ligase, Jade-2. We demonstrated that Jade-2 is a major LSD1 negative regulator during neurogenesis in vitro and in vivo in both mouse developing cerebral cortices and zebra fish embryos. Apparently, Jade-2-mediated degradation of LSD1 acts as an antibraking system and serves as a quick adaptive mechanism for re-establishing epigenetic landscape without more laborious transcriptional regulations. As a potential anticancer strategy, Jade-2-mediated LSD1 degradation could potentially be used in neuroblastoma cells to induce differentiation toward postmitotic neurons.

INTRODUCTION

LSD1 was the first histone demethylase identified to catalyze the removal of the mono- and dimethyl moieties from H3K4 (Shi et al., 2004). It is subsequently identified in a number of corepressor complexes, including REST/CoREST (Shi et al., 2005), Mi-2/NuRD (Wang et al., 2009), and SIRT1/HDAC (Mulligan et al., 2011) functioning in transcription repression. The LSD1-REST/NRSF complex has been described as a master regulator of neuronal gene expression (Ballas et al., 2005; Lunyak et al., 2002). Consistently, LSD1 is reported to maintain the silencing of several developmental genes in embryonic stem cells (ESCs) (Adamo et al., 2011; Sun et al., 2010). Interestingly, it has been reported that neurospecific LSD1 (nLSD1) isoforms LSD1-8a and LSD1-2a/LSD1-8a exist and are highly expressed in the nervous system in order to promote neurite morphogenesis (Zibetti et al., 2010). Moreover, recent studies found that the expression of LSD1 is elevated in pluripotent cancer cells (Wang et al., 2011) and that LSD1 is also highly expressed in poorly differentiated neuroblastoma (Schulte et al., 2009). Collectively, current literatures point a critical role for LSD1 in stem cell biology particularly in the maintenance of the silencing of differentiation genes. However, how LSD1 is regulated and the brake is relieved in neural development is largely unknown.

Gene for apoptosis and differentiation in epithelia (Jade) family proteins contain one canonical C_4HC_3 plant homology domain (PHD) followed by a noncanonical extended PHD domain. On the basis of expressed sequence tags, Jade-1, Jade-2, and Jade-3 proteins are homologous at N-terminal and PHD domains but variable at C-terminal portions (Tzouanacou et al., 2003). Jade-1, a short-lived and kidney-enriched protein, was the first and only characterized protein in the Jade family and was physically associated with the von Hippel-Lindau tumor suppressor pVHL and the ING4/ING5/HBO1 histone acetyltransferase complex and functionally linked to apoptosis and DNA replication (Doyon et al., 2006; Panchenko et al., 2004; Tzouanacou et al., 2003; Zhou et al., 2002, 2005). More recently, Jade-1 was reported to inhibit Wnt signaling through its E3 ubiquitin ligase activity toward β-catenin (Chitalia et al., 2008) and was therefore defined as a PHD-finger-type E3 ubiquitin ligase.

However, the molecular function of other members of the Jade family is uncharacterized, and the gene encoding for Jade-2 is not cloned.

Here, we report that elimination of H3K4 demethylase LSD1 promotes ESC differentiation toward neural lineage. We characterized Jade-2 as an E3 ubiquitin ligase specifically targeting LSD1 for degradation. We demonstrated that Jade-2-mediated LSD1 degradation promotes pluri- or multipotent stem cell differentiation toward the neural lineage in vitro as well as in vivo during mouse embryonic cerebral cortical development and neural induction in zebra fish embryos. We showed that the Jade-2-LSD1 pathway is implicated in neuroblastoma in order to induce differentiation of tumor cells into postmitotic neurons.

RESULTS

Elimination of LSD1 Promotes Neural Differentiation

To further explore the role of LSD1 in neural differentiation, R1 mouse ESCs were effectively induced for neural commitment in N2B27 medium (Ying and Smith, 2003; Ying et al., 2003) (Figure 1A). Western blotting analysis and real-time quantitative RT-PCR (qRT-PCR) measurements indicate that the protein level of Lsd1 declined during this process, which was not a result of downregulation of Lsd1 mRNA (Figure 1B). In addition, analysis of mouse ESCs and cortical progenitors (NPCs) by western blotting indicated that the expression of Lsd1 is downregulated in NPCs (Figure 1C). These observations suggest that LSD1 is eliminated during neural differentiation.

To further support this notion, ESCs were infected with lentiviruses carrying control (shCTR) or Lsd1 small hairpin RNA (shLsd1) and selected for stable clones (Figure S1A available online). These clones were subjected to alkaline phosphatase (AP) or immunofluorescent (IF) staining for neural progenitor marker Nestin in undifferentiated cultural environment. It was found that Lsd1 knockdown led to a loss of pluripotency of the ESCs, evidenced by the inability of the cells to grow in clones and by weaker staining for AP and to the induction of Nestin (Figure 1D). When the ESCs were induced for neural differentiation, knockdown of Lsd1 accelerated the emergence of neural progenitors and mature neurons, evidenced by the enhanced expression of Nestin and mature neuron markers βIII-tubulin, Gap43, and Map2 (Figure 1D). On the other hand, overexpression of LSD1 via doxycycline (DOX)-inducible lentiviral system (Brambrink et al., 2008) (Figure S1B) in differentiating ESCs decelerated the derivation of neural progenitors and mature neurons (Figure 1D).

To investigate the role of Lsd1 in cortical progenitor differentiation, shCTR or shLsd1 vectors were electroporated along with GFP expression plasmids in utero into developing embryonic day (E) 13.5 mouse cortices (Figure S1C). The cortices were subsequently analyzed by IF staining for βIII-tubulin or Nestin at E15.5. In cortices electroporated with shCTRs, the expression of βIII-tubulin was absent in the ventricular zone (VZ) but was readily detected in the intermediate zone (IZ), whereas the expression of Nestin was mostly detected in the VZ (Figure 1E). However, a dramatic increase of βIII-tubulin expression and decrease of Nestin expression was detected in shLsd1-electroporated cells (and their progeny) in the VZ (Figure 1E). Image analysis of the whole cortex showed that the proportion of $GFP^+/\beta III\text{-}tubulin^+$ cells was higher and the proportion of $GFP^+/Nestin^+$ cells was lower in shLsd1-electroporated cells in comparison to control cells (Figure 1E). Altogether, these data support a notion that Lsd1 inhibits neuronal differentiation of pluri- or multipotent stem cells, and thus the elimination of Lsd1 promotes neural differentiation.

To answer the question of whether the ubiquitin-proteasome pathway is implemented in the downregulation of LSD1, we transfected U2OS cells with FLAG-LSD1 and treated with DMSO or proteasome inhibitor MG132. Western blotting analysis revealed a time-dependent increase in LSD1 level in the presence of MG132, which was not a result of increased LSD1 mRNA expression (Figure 1F), suggesting that LSD1 is a liable protein and subjected to regulation by the ubiquitin-proteasome-mediated process. To support this, we investigated whether LSD1 could be polyubiquitinated in vivo. U2OS cells were cotransfected with FLAG-LSD1 and HA-tagged ubiquitin. Immunoprecipitation (IP) of the cell lysates with anti-FLAG and immunoblotting (IB) with anti-HA revealed a robust increase of LSD1 polyubiquitination in the presence of MG132 (Figure 1F), supporting the argument that the steady-state level of LSD1 is controlled by the ubiquitin proteasome pathway.

To further strengthen this argument, we treated ESCs or cortical progenitor cells with DMSO or MG132. Western blotting analysis showed that the decreased protein level of Lsd1 in cortical progenitor cells could be restored almost to the same level as ESCs when the activity of proteasome is inhibited by MG132 (Figure 1G). Consistently, in vivo ubiquitination assays with nickel bead precipitation for His-tagged ubiquitin showed an increase in Lsd1 polyubiquitination during neural differentiation (Figure 1H). Altogether, these results support the notion that LSD1 is downregulated via the ubiquitin-proteasome system during neural differentiation.

Jade-2 Is an E3 Ubiquitin Ligase that Specifically Targets LSD1 for Degradation

To search for the E3 ubiquitin ligase for LSD1, we employed affinity purification and mass spectrometry in order to identify proteins that potentially interact with LSD1 in vivo. To this end, FLAG-tagged LSD1 was stably expressed in U2OS cells. Then, whole-cell extracts were prepared and subjected to affinity purification with anti-FLAG affinity gel. Mass spectrometry did not detect any known E3 ubiquitin ligase in the purified complex (Figure 2A). However, interestingly, Jade-2, an uncharacterized member of Jade family protein whose gene has not been cloned yet, was identified in the LSD1-containing complex (Figure 2A). The presence of Jade-2 in the LSD1-containing complex was confirmed by western blotting with antibodies generated on the basis of the expressed sequence tag of Jade-2 (Figure 2B).

To further support the observation that LSD1 is physically associated with Jade-2 in vivo, we extracted total proteins from HeLa cells, mouse ESCs, or mouse cortical neurons and performed co-IP experiments. IP with antibodies against LSD1 followed by IB with antibodies against Jade-2 showed that LSD1 was efficiently coimmunoprecipitated with Jade-2

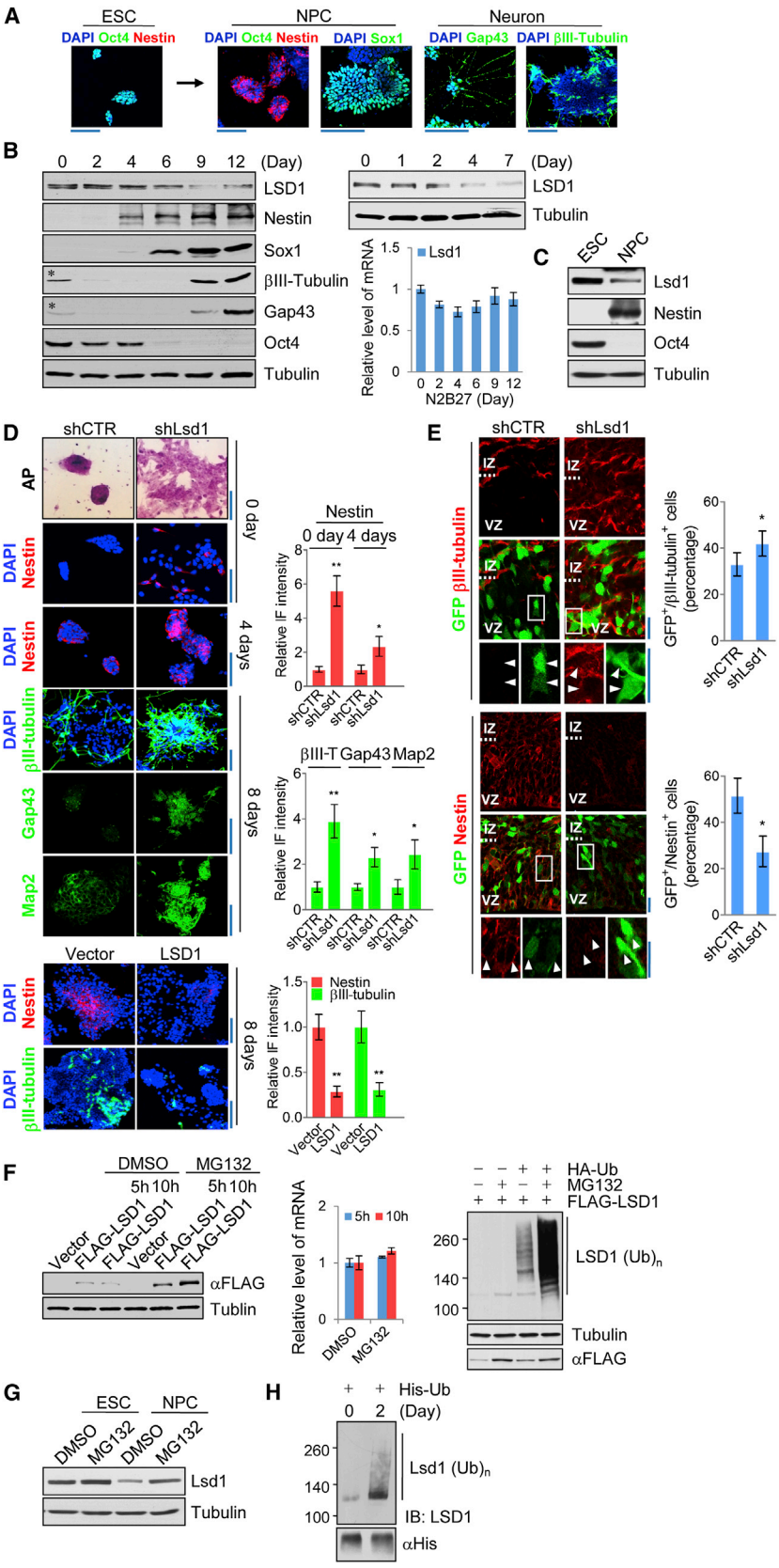

Figure 1. LSD1 Is Eliminated via Proteasome-Dependent Process during Neural Differentiation

(A) R1 mouse ESCs were induced for neural differentiation for 6 days and were replated on PDL and laminin for terminal neuronal differentiation for another 6 days. The expression of neural progenitor markers (NPC) and mature neuron markers was detected by IF microscopy. Oct4, ESC marker; Nestin and Sox1, NPC markers; Gap43 and βIII-tubulin, mature neuron markers. Scale bar represents 100 μm.

(B) ESCs were induced for neural differentiation (left, replated at day 7; right, replated at day 5). The protein level of Lsd1 was detected at the indicated days by western blotting. The pluripotent or neural markers were examined in order to indicate the course of differentiation. Asterisks indicate the expression of the proteins by feeder cells. Alternatively, the mRNA level of Lsd1 was analyzed by qRT-PCR. Error bars represent mean ± SD for triplicate experiments.

(C) Mouse ESCs (ESC) or E12.5 mouse cortical progenitor cells (NPC) were subjected to western blotting for Lsd1 expression.

(D) shCTR or shLsd1 stable expressing ESC clones were induced for neural differentiation for 0, 4, or 8 days (replated at day 5) before AP staining or analysis by IF microscopy for neural markers. Alternatively, ESCs were infected with DOX-inducible lentiviruses carrying vector or LSD1. Then, cells were induced for neural differentiation and treated with DOX for 8 days before they were analyzed by IF microscopy for Nestin or βIII-tubulin. Scale bar represents 100 μm. The relative IF intensity of each staining was quantified by ImageJ software. The results represent mean ± SD of ten sections from each slice. *$p < 0.05$ and **$p < 0.01$ (two-tailed unpaired Student's t test).

(E) shCTR- or shLsd1-electroporated cortical slices were analyzed by IF microscopy for βIII-tubulin or Nestin expression. The images of VZs and IZs are shown. Scale bar represents 20 μm. The percentage of GFP$^+$/βIII-tubulin$^+$ or GFP$^+$/Nestin$^+$ cells to GFP$^+$ cells was quantified by ImageJ. Slices from two independent experiments were processed for each experimental condition. The results represent mean ± SEM of three sections from each experiment. *$p < 0.05$ (two-tailed unpaired Student's t test).

(F) U2OS cells were transfected with vector or FLAG-LSD1 and then treated with DMSO or MG132 (10 μM). Cells were collected for western blotting or qRT-PCR analysis for LSD1 expression. Error bars represent mean ± SD for triplicate experiments. Alternatively, U2OS cells were cotransfected with FLAG-LSD1 and HA-tagged ubiquitin (HA-Ub). Twenty-four hours after transfection, cells were treated with DMSO or MG132 for 10 hr before in vivo ubiquitination assays.

(G) Mouse ESCs or NPCs were treated with DMSO or MG132 (3 μM) for 20 hr before the protein level of Lsd1 was analyzed by western blotting.

(H) ESCs were electroporated with His-tagged ubiquitin (His-Ub) and were induced for neural differentiation for 0 or 2 days before cellular extracts were prepared for Ni-NTA bead precipitation. Whole ubiquitin was loaded as a control. See also Figure S1.

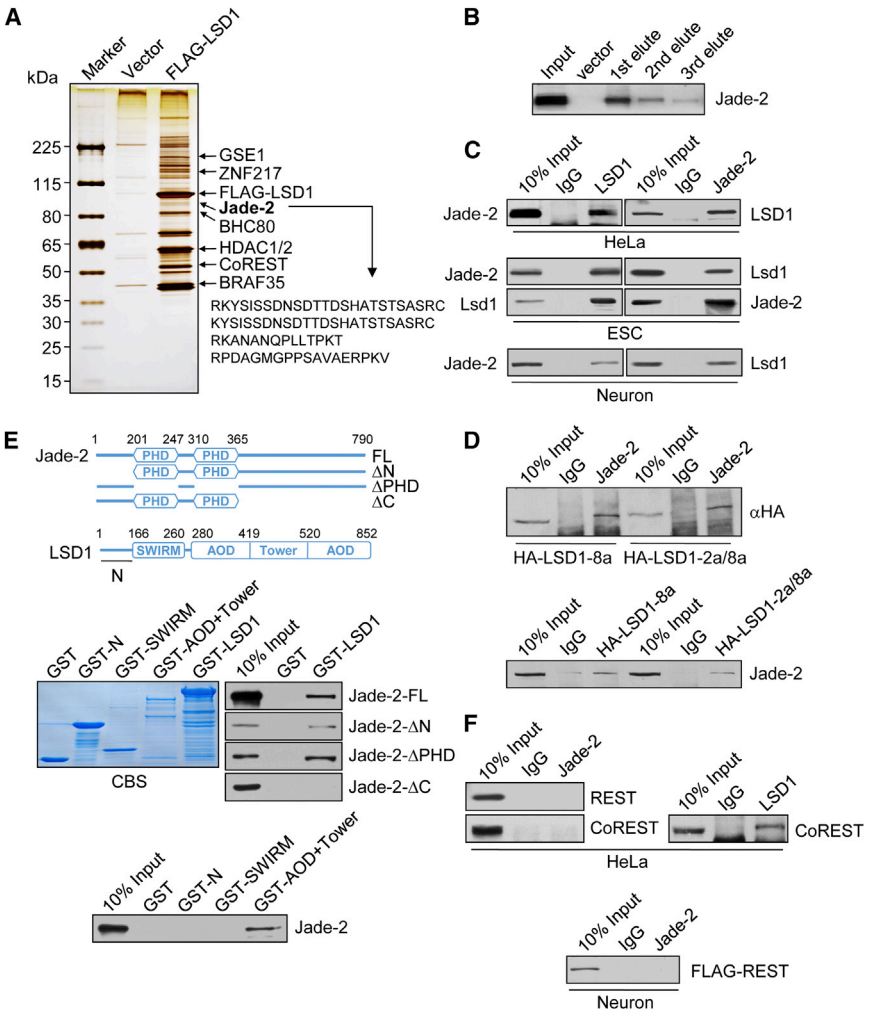

Figure 2. LSD1 Is Physically Associated with Jade-2

(A) Whole-cell extracts from U2OS cells stably expressing FLAG-LSD1 were subjected to affinity purification with anti-FLAG immobilized on the agarose beads. The purified protein complex was resolved on SDS-PAGE and silver stained. The bands were retrieved and analyzed by mass spectrometry.

(B) Western blotting of Jade-2 in FLAG-LSD1-purified protein complex.

(C) Whole-cell lysates from HeLa cells, ESCs, or mouse E17.5 cortical neurons were prepared, and IP was performed with anti-LSD1 or anti-Jade-2 followed by IB with anti-Jade-2 or anti-LSD1, respectively. IB with anti-Lsd1 or anti-Jade-2 in ESCs is showed as a positive control.

(D) Cortical neurons were electroporated with HA-LSD1-8a or HA-LSD1-2a/8a, and IP was performed with anti-Jade-2 or anti-HA followed by IB with anti-HA or anti-Jade-2, respectively.

(E) Schematic representations of WT and deletion mutants of Jade-2 and LSD1. GST pull-down assays were performed with GST-LSD1 and in vitro transcribed and translated full-length Jade-2 or its deletion mutants or with GST-fused deletion constructs of LSD1 and in vitro transcribed and translated Jade-2. Coomassie brilliant blue (CBB) staining of the GST-fused LSD1 WT, and its deletion constructs is shown.

(F) HeLa cell extracts were subjected to IP with anti-Jade-2 or anti-LSD1 followed by IB with anti-REST or anti-CoREST as indicated. The association of LSD1 and CoREST is shown as positive control. Alternatively, cortical neurons were electroporated with FLAG-REST and subjected to co-IP assays with the indicated antibodies. See also Figure S2.

(Figure 2C). Reciprocally, IP with anti-Jade-2 followed by IB with anti-LSD1 revealed that Jade-2 was also coimmunoprecipitated with LSD1 (Figure 2C). Co-IP experiments showed that Jade-2 is also associated with nLSD1 isoforms LSD1-8a and LSD1-2a/LSD1-8a in cortical neurons (Figure 2D). In addition, glutathione S-transferase (GST) pull-down assays indicated that LSD1 is capable of interacting with Jade-2 in vitro (Figure 2E) and the C terminus of Jade-2, which is variable between Jade proteins (Figure S2A) and that the AOD/Tower domains of LSD1 are responsible for the molecular interaction between Jade-2 and LSD1 (Figure 2E). Importantly, Jade-2 is not associated with REST or CoREST (Figure 2F).

To test the hypothesis that Jade-2 is an E3 ubiquitin ligase specific for LSD1, we performed experiments with gain- and loss-of-function of Jade-2 in U2OS cells. Western blotting analysis revealed that increased expression of Jade-2 was associated with a decreased level of LSD1 protein (Figure 3A), an effect that requires the PHD domains and C terminus of Jade-2 (Figure 3B, left). In addition, the decreased LSD1 protein level under Jade-2 overexpression was not a result of downregulation of LSD1 mRNA (Figure 3B, right). Moreover, Jade-2-associated destabilization of LSD1 protein occurred only in the absence of MG132 (Figure 3A). Consistently, knockdown of Jade-2 expression (Figure S2B) resulted in an increased half-life time of LSD1 in U2OS, evidenced by cycloheximide (CHX) chase assays (Figure 3C, top), and the half-life of Lsd1 was extended in mouse ESCs (Figure 3C, bottom) when Jade-2 was depleted (Figure S1A). Furthermore, Jade-2 had no or only marginal effect on the expression of other histone modification enzymes or their cofactors, and LSD1 is not targeted by β-TRCP and Jade-1, the E3 ubiquitin ligases for REST and β-catenin, respectively (Chitalia et al., 2008; Westbrook et al., 2008) (Figures 3D–3F). Altogether, these results support the notion that Jade-2 specifically targets LSD1 for destruction through the proteasome-mediated process.

To further substantiate the argument, in vitro ubiquitination assays with baculovirally purified Jade-2, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme UbcH6 (E2), ubiquitin, and other reaction constituents showed that Jade-2 is able to polyubiquitinate itself (autopolyubiquitination) (Figure 4A), a signature of proteins with E3 ubiquitin ligase activity (Pickart, 2001). Consistently, incubation of bacterially expressed

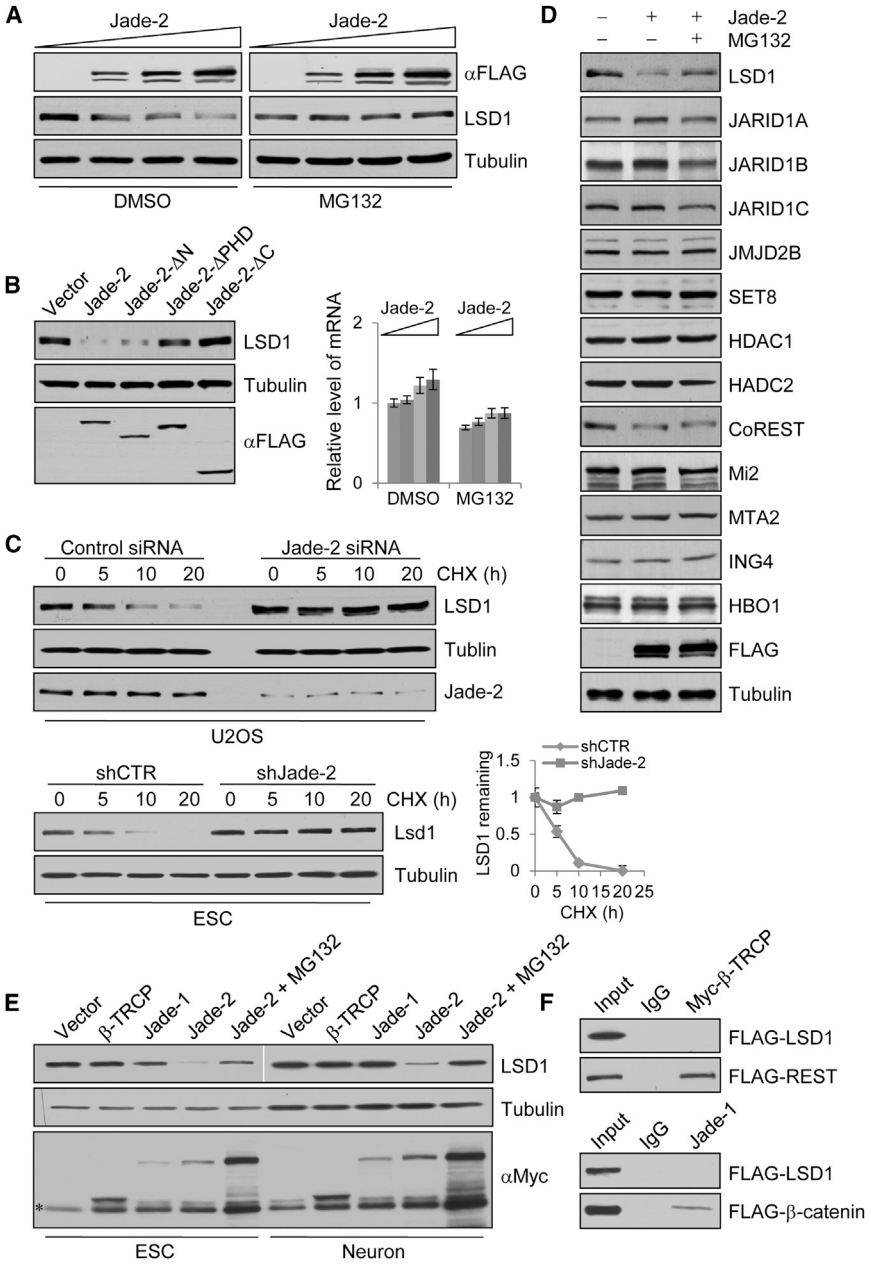

Figure 3. Jade-2 Is a E3 Ubiquitin Ligase that Specifically Targets LSD1 for Degradation

(A) U2OS cells were transfected with increasing amounts of FLAG-Jade-2 for 40 hr and then treated with DMSO or MG132 (10 μM) for 10 hr before cells were collected for western blotting analysis for LSD1.

(B) U2OS cells were transfected with the indicated plasmids before being analyzed for LSD1 expression by western blotting (left). Cells in the experiments in (A) were analyzed for LSD1 mRNA by qRT-PCR (right). Error bars represent mean ± SD for triplicate experiments.

(C) U2OS cells were transfected with control or Jade-2 siRNA for 40 hr and then treated with CHX (50 μg/ml) for the designated times before they were analyzed for LSD1 expression by western blotting (top). Alternatively, stable shCTR- or shJade-2-expressing cell clones were treated with CHX for the indicated hours before they were harvested for western blotting analysis for Lsd1. Quantitation of the western blotting results was performed by densitometry and expressed as signals of Lsd1 to tubulin. Each point represents the mean ± SD for triplicate experiments (bottom).

(D) U2OS cells were transfected with vector or FLAG-Jade-2 and then treated with DMSO or MG132. Cells were analyzed by western blotting with antibodies against the indicated proteins.

(E) ESCs were electroporated with Myc-tagged β-TRCP, Jade-1, or Jade-2, or cortical neurons were electroporated with the indicated Myc-tagged plasmids along with FLAG-LSD1 for 30 hr. Cells were treated with DMSO or MG132 (2 μM) for 15 hr before cellular extracts were prepared for analysis for LSD1 by western blotting. Asterisks indicate nonspecific bands.

(F) Cortical neurons were electroporated with the indicated plasmids, and IP with anti-Myc or anti-Jade-1 followed by IB with anti-FLAG was performed. The association between β-TRCP and REST or Jade-1 and β-catenin is shown as a positive control.

See also Figure S2.

GST-LSD1 with bacterially expressed GST-Jade-2 resulted in the detection of LSD1 polyubiquitination but only when both E2 and ubiquitin were present, whereas incubation of GST-LSD1 with GST-Jade-2-ΔPHD resulted in no detectable LSD1 polyubiquitination, even when E2 and ubiquitin were included (Figure 4B). In vivo ubiquitination assays in ESCs showed that depletion of Jade-2 resulted in a dramatic decrease in the level of LSD1 polyubiquitination (Figure 4C) and that overexpression of Jade-2, but not Jade-1, led to a pronounced increase in the level of LSD1 polyubiquitination in SH-SY5Y cells (Figure 4D). Moreover, when wild-type (WT) ubiquitin used in in vivo ubiquitination assays was replaced with an ubiquitin mutant UbK48R, which is defective in the assembly of polyubiquitin chain that is recognized by proteasome for degradation (Chau et al., 1989; Thrower et al., 2000), high-molecular-weight LSD1 conjugates were no longer detected (Figure 4D). Detailed experiments with Jade-2 and LSD1 mutants indicated that the first PHD of Jade-2 is essential for its E3 ubiquitin ligase activity toward LSD1 (Figures 4E and 4F), although in vitro ubiquitination assays, for some reason, detected a slight decrease in Jade-2's ubiquitination activity when the second PHD was deleted (Figures 4G and S2C) and that Jade-2-promoted polyubiquitination of LSD1 occurs on lysine 503 of LSD1 (Figures S2D and 4H). Altogether, these data support the arguments that Jade-2 targets LSD1 for degradation and that Jade-2 does so via its E3 ligase activity and through promoting LSD1 polyubiquitination mainly via its first PHD domain. In vivo ubiquitination assays showed that Jade-2 also promotes the polyubiquitination of nLSD1 doublets (Figure 4I).

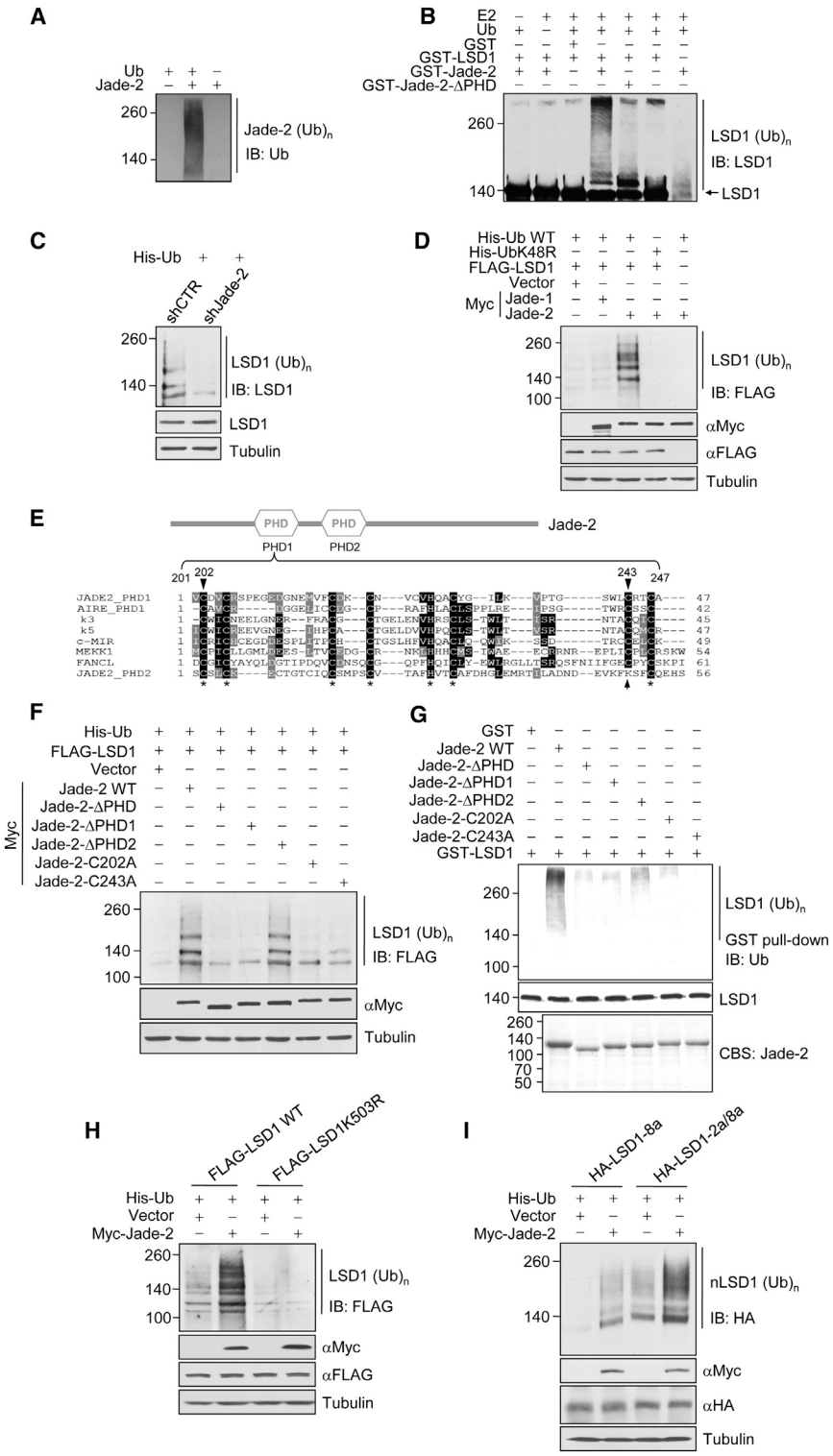

Figure 4. Jade-2 Promotes LSD1 Polyubiquitination

(A) In vitro autoubiquitination assays of Jade-2 were carried out, and the reaction mixture was then subjected to western blotting with anti-ubiquitin.

(B) In vitro ubiquitination assays with GST-LSD1, GST-Jade-2, or GST-Jade-2-ΔPHD in the presence or absence of ubiquitin or E2 were performed. Then, the reaction mixture was subjected to western blotting with anti-LSD1.

(C) Stable shCTR- or shJade-2 -expressing ESC clones were electroporated with His-Ub. Then, cells were treated with MG132 (2 μM) for 15 hr before Ni-NTA bead precipitation.

(D) SH-SY5Y cells were cotransfected with the indicated plasmids and treated with MG132 (10 μM) for 10 hr. Cellular extracts were harvested for Ni-NTA bead precipitation.

(E) ClustalW sequence alignment of the PHD domains of human Jade-2, AIRE, MEKK1, FANCL, c-MIR, viral k3, and k5. Asterisks indicate conserved cysteines or histidines between these domains. Arrows indicate the absence of cysteine in the second PHD domain of Jade-2. Triangles indicate the mutation sites designed in our experiments.

(F) SH-SY5Y cells were cotransfected with the indicated plasmids and treated with MG132. Then, cellular extracts were prepared for Ni-NTA bead precipitation.

(G) In vitro ubiquitination assays with GST-LSD1, baculovirally purified Jade-2 WT, or its mutants E1, E2, and ubiquitin. Then, GST pull-down was performed before the beads were subjected to western blotting with antiubiquitin. CBB staining of baculovirally purified Jade-2 WT and mutants is shown.

(H) SH-SY5Y cells were cotransfected with FLAG-LSD1 WT or FLAG-LSD1K503R along with the indicated plasmids. Cells were treated with MG132 before cellular extracts were prepared for Ni-NTA bead precipitation.

(I) SH-SY5Y cells were cotransfected with HA-LSD1-8a or HA-LSD1-2a/8a along with the indicated plasmids. Cells were treated with MG132 before cellular extracts were prepared for Ni-NTA bead precipitation.

See also Figure S2.

The Jade-2-LSD1 Pathway Is an Antibraking System in Neural Differentiation

In order to explore the biological significance of Jade-2-promoted LSD1 degradation in neural differentiation, R1 mouse ESC clones stably expressing shCTR or shJade-2 were induced for neural differentiation in N2B27 medium. Western blotting analysis indicated that the decrease of Lsd1 protein level was significantly delayed upon Jade-2 depletion (Figure 5A). Correspondingly, the emergence of neural progenitors and mature neurons from these cells was decelerated, as evidenced by decreased expression of Sox1, Nestin, and βIII-tubulin and the postponed elimination of pluripotency marker Oct4 (Figure 5A). Morphologically, Jade-2-depleted ESCs grew in aggregates with smoother-edged,

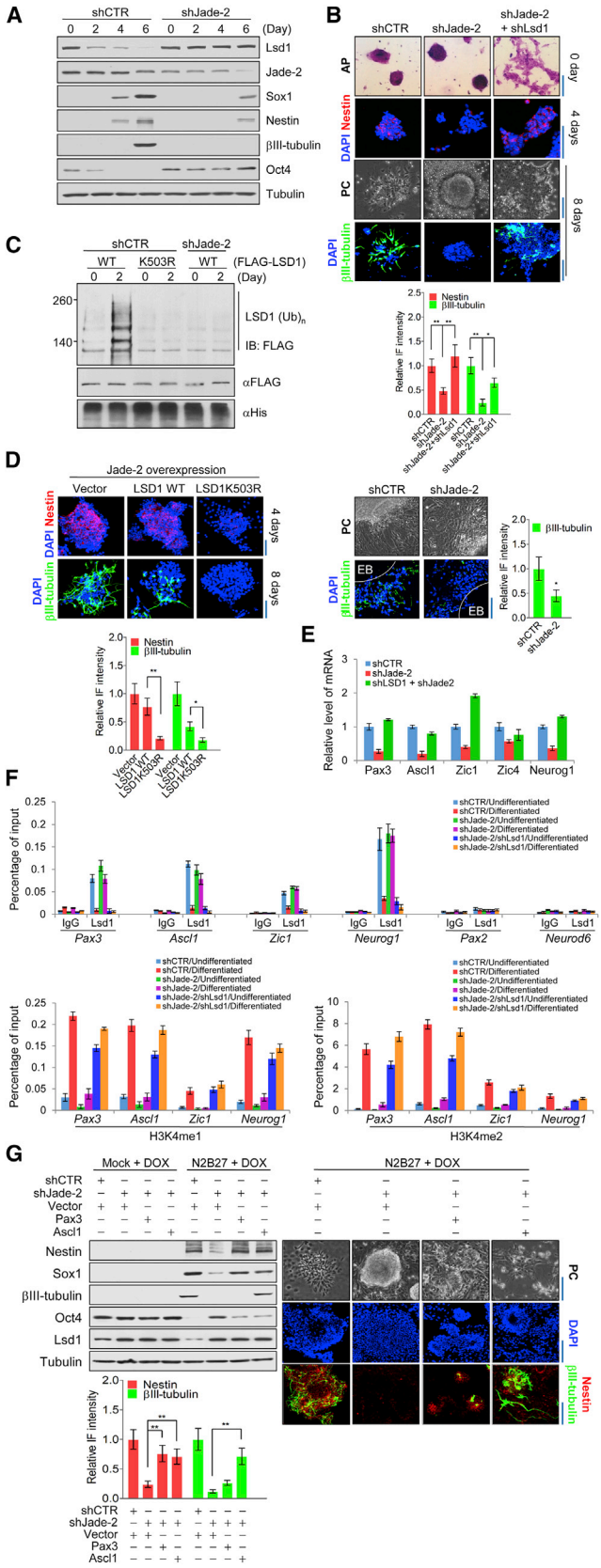

Figure 5. The Jade-2-LSD1 Pathway Is an Antibraking System in Neural Differentiation

(A) ESC clones stably expressing shCTR or shJade-2 were induced for neural differentiation for the indicated days (replated at day 5) and analyzed by western blotting for the expression of the indicated proteins.

(B) shCTR or shJade-2 stably expressing ESC clones or shJade-2+shLsd1-transfected cells were subjected to AP staining (the image of "shCTR" refers to that in Figure 1D, given that the experiments with shLsd1, shJade-2, or shJade-2+shLsd1 were compared to the same control), or cells were induced for neural differentiation (replated at day 6) before being examined by phase-contrast microscopy or IF microscopy. Alternatively, stable shCTR- or shJade-2-transfected ESC clones were detached for embryoid body (EB) formation and treated with RA. Then, EBs were reattached for 3 days and examined by phase-contrast microscopy or IF microscopy. Scale bar represents 100 μm. The relative IF intensity of each staining was quantified by ImageJ. The results are mean ± SD of ten sections from each slice. *$p < 0.05$ and **$p < 0.01$ (two-tailed unpaired Student's t test).

(C) Stable shCTR- or shJade-2-expressing ESC clones were electroporated with His-Ub along with FLAG-LSD1 or FLAG-LSD1K503R before being induced for neural differentiation. Cellular extracts were then harvested for Ni-NTA bead precipitation.

(D) ESCs were infected with DOX-inducible lentiviruses carrying Jade-2 along with lentiviruses carrying vector, LSD1 WT, or LSD1K503R before being induced for neural differentiation in the presence of DOX. Then, cells were examined by IF microscopy. Scale bar represents 100 μm. The relative IF intensity of each staining was quantified by ImageJ. The results are mean ± SD of ten sections from each slice. *$p < 0.05$ and **$p < 0.01$ (two-tailed unpaired Student's t test).

(E) Stable shCTR- or shJade-2-transfected ESC clones or shJade-2^{+}shLsd1-transfected cells were induced for neural differentiation for 4 days before being analyzed by qRT-PCR with the indicated primers. Error bars represent mean ± SD for triplicate experiments.

(F) Stable shCTR- or shJade-2-expressing ESC clones, or shJade-2^{+}shLsd1-transfected cells were induced for neural differentiation for 0 or 3 days. Soluble chromatin was prepared and qChIP was performed with anti-Lsd1, anti-H3K4me1, or anti-H3K4me2 on the promoters of *Pax3*, *Ascl1*, *Zic1*, *Neurog1*, *Pax2*, or *Neurod6*. Error bars represent mean ± SD for triplicate experiments.

(G) Stable shCTR- or shJade-2-transfected ESC clones were infected with DOX-inducible lentiviruses carrying vector, Pax3, or Ascl1. Cells were then cultured in ESC media (mock) or induced for neural differentiation and treated with DOX for 8 days. Cells were harvested for western blotting analysis, observed by phase-contrast microscopy, or analyzed by IF microscopy. Scale bar represents 100 μm. The relative IF intensity of each staining was quantified by ImageJ. The results are mean ± SD of ten sections from each slice. **$p < 0.01$ (two-tailed unpaired Student's t test).

See also Figure S3.

rounder-shaped, and deeper-AP-stained cell clones (Figure 5B). Concurrent infection of lentiviruses carrying shLsd1 (Figure S1A) was able to rescue the phenotype of Jade-2-deficient cells (Figure 5B).

Immunofluorescent staining showed that, during neural differentiation, although most (~70%) of the control cells expressed neural markers and organized in rosettes with surrounding cells exhibiting neuronal morphology with extensive arborization, Jade-2-depleted cells grew in aggregates, failed to attach plates, and display decreased expression of neural markers (Figure 5B). However, simultaneous depletion of Lsd1 could restore the expression of the neural markers and rescue the morphological manifestations (Figure 5B). In addition, all-*trans* retinoic acid (RA)-induced neuronal differentiation of embryoid bodies (EBs) was suppressed upon Jade-2 depletion, evidenced by decreased numbers of βIII-tubulin-expressing neurites around

EBs (Figure 5B). Accordingly, in vivo ubiquitination assays detected no increase in polyubiquitination of either LSD1K503R in control cells or LSD1 in Jade-2-depleted cells during neural differentiation (Figure 5C). In addition, IF staining showed that, although LSD1K503R counteracted Jade-2 overexpression-promoted neuronal differentiation, manifested by the repressed neural markers, LSD1 had limited effect (Figures S3A and 5D). Altogether, these data support a notion that the Jade-2-LSD1 pathway plays an important role in neural differentiation in which Jade-2 targets LSD1 for degradation, relieving the braking function of LSD1 on neural commitment.

Real-time quantitative RT-PCR analysis showed that several LSD1 target genes known to be associated with neurogenesis, including *Pax3*, *Ascl1*, *Zic1*, *Zic4*, and *Neurog1* (Adamo et al., 2011; Whyte et al., 2012), are regulated by Jade-2 (Figure 5E). Consistently, quantitative chromatin immunoprecipitation (qChIP) showed that neural differentiation of ESCs was associated with dramatic decreases in the recruitment of Lsd1 and increases in levels of H3K4me1 and H3K4me2 on the promoters of *Pax3*, *Ascl1*, *Zic1*, and *Neurog1* genes, changes that were abrogated when Jade-2 was depleted (Figure 5F). Significantly, ectopic expression of Pax3 or Ascl1 (Figure S3B), which are both identified targets of the Jade-2-LSD1 pathway, in differentiating ESCs could, at least partially, rescue Jade-2 depletion-restrained derivation of neural progenitors and rosettes formation (Figure 5G), although Jade-2 depletion-associated repression of neuron maturation and neurite extension could be relieved by ectopic expression of Ascl1 but not Pax3 (Figure 5G). These results are consistent with the notion that Pax3 promotes early neural induction (Goulding et al., 1991; Li et al., 1998; Liem et al., 1995), although Ascl1 is involved in neuron maturation (Casarosa et al., 1999; Guillemot et al., 1993). Altogether, these data support the importance of the Jade-2-LSD1 pathway in neural differentiation.

The Jade-2-LSD1 Pathway Is Implicated in Nervous System Development and Neuroblastoma Differentiation

To explore a broad functionality for the Jade-2-LSD1 pathway in nervous system development during embryogenesis, Jade-2 or shJade-2, along with GFP, were electroporated in utero into E13.5 mouse cortices. Subsequent IF staining at E15.5 showed that Jade-2-overexpressing cells in the VZ exhibited a precocious expression of βIII-tubulin resembling the pattern with that of Lsd1-depleted cells, whereas Jade-2 knockdown led to a reduced βIII-tubulin expression of the electroporated cells in the IZ, which could be rescued by simultaneous electroporation of shLsd1 plasmid (Figure 6A). Quantification analysis of the whole cortex showed that Jade-2 overexpression was associated with a higher proportion of $GFP^+/\beta III\text{-}tubulin^+$ cells, whereas Jade-2 knockdown was accompanied by a lower proportion of $GFP^+/\beta III\text{-}tubulin^+$ cells, which could be elevated by simultaneous depletion of Lsd1 (Figure 6A). In addition, Jade-2 overexpression led to a decrease, whereas Jade-2 knockdown resulted in an increase in the expression of Nestin in the electroporated cells, which could be offset when Lsd1 was coknocked down (Figure 6A).

To investigate the functional importance of the Jade-2-LSD1 axis in neural development in vivo, we injected morpholino (MO) antisense oligonucleotides against phf15 (Jade-2 ortholog) and/or kdm1a (LSD1 ortholog) into one-cell-stage embryos of zebrafish (Figure S4), and the mRNA expression of a series of neuroectoderm markers was detected by in situ hybridization followed by statistical analysis in midgastrulation period (75% epiboly). Notably, the expressions of early neuroectoderm markers sox2, sox3, otx2, and hoxb1b decreased in phf15 morphants but increased in kdm1a morphants (Figure 6B). Coinjection of kdm1a MO offset the effect of phf15 MO (Figure 6B). These data indicate that the Jade-2-LSD1 pathway plays an important role in cortical progenitor differentiation and neuroectoderm induction.

Next, we generated a series of SH-SY5Y cell lines stably expressing Jade-2 or its mutants and/or LSD1 by retroviral infection (Figure S5A) and verified Jade-2-mediated LSD1 degradation in these cells (Figure 7A). qRT-PCR analysis showed that ZIC4, PAX3, and TPM1 (Schulte et al., 2009), but not ASCL1, are regulated by the Jade-2-LSD1 pathway in SH-SY5Y cells (Figure 7B). Then, SH-SY5Y cells were induced for differentiation by RA (Sidell, 1982). The average length of neurites was quantified, and the induction of neuronal markers MAP2, TAU, and GAP43 were analyzed by western blotting. These experiments showed that overexpression of LSD1 inhibited neurite extension and the expression of neuronal markers in differentiating SH-SY5Y cells, effects that could be abolished by co-overexpression of Jade-2 but not Jade-2-C202A or Jade-2-C243A (Figures 7C and 7D). Full differentiation of SH-SY5Y cells by sequential exposure to RA and brain-derived neurotrophic factor (BDNF0) (Encinas et al., 2000; Le et al., 2009) (Figure S5B) showed that both mRNA and protein levels of LSD1 decreased, whereas the expression of Jade-2 did not change during neuronal differentiation (Figure S5C). Subsequently, we showed that LSD1 inhibits neuronal differentiation of SH-SY5Y cells, whereas Jade-2 promotes this process, which could be offset by simultaneous overexpression of LSD1 (Figures S5D and S5E). Furthermore, knockdown of LSD1 target gene ZIC4 or PAX3, but not ASCL1, abrogated Jade-2-promoted neurite outgrowth and the expression of neuronal markers (Figures S5F, 7E, and 7F). Therefore, it is possible that different proneural genes are implicated in neural differentiation of ESCs versus neuroblastoma cells. Nevertheless, these experiments support an argument that Jade-2 promotes neuroblastoma cell differentiation by targeting LSD1 for degradation.

DISCUSSION

It has been reported that LSD1 in ESCs occupies promoters of a portion of proneural genes that contain bivalent domains, a chromatin environment containing both H3K4me2/H3K4me3 and H3K27me3 marks, where it controls the levels of H3K4 methylation and maintains the silence of their expressions (Adamo et al., 2011). Consistently, we showed that the protein level of LSD1 is decreased via posttranscriptional regulation during neural differentiation of ESCs. By gain- and loss-of-function studies, we demonstrated that LSD1 inhibits ESC differentiation toward the neural lineage in vitro as well as cortical progenitor differentiation and neuroectoderm induction in vivo. Clearly, LSD1 is an important regulator in the maintenance of pluripotency and in

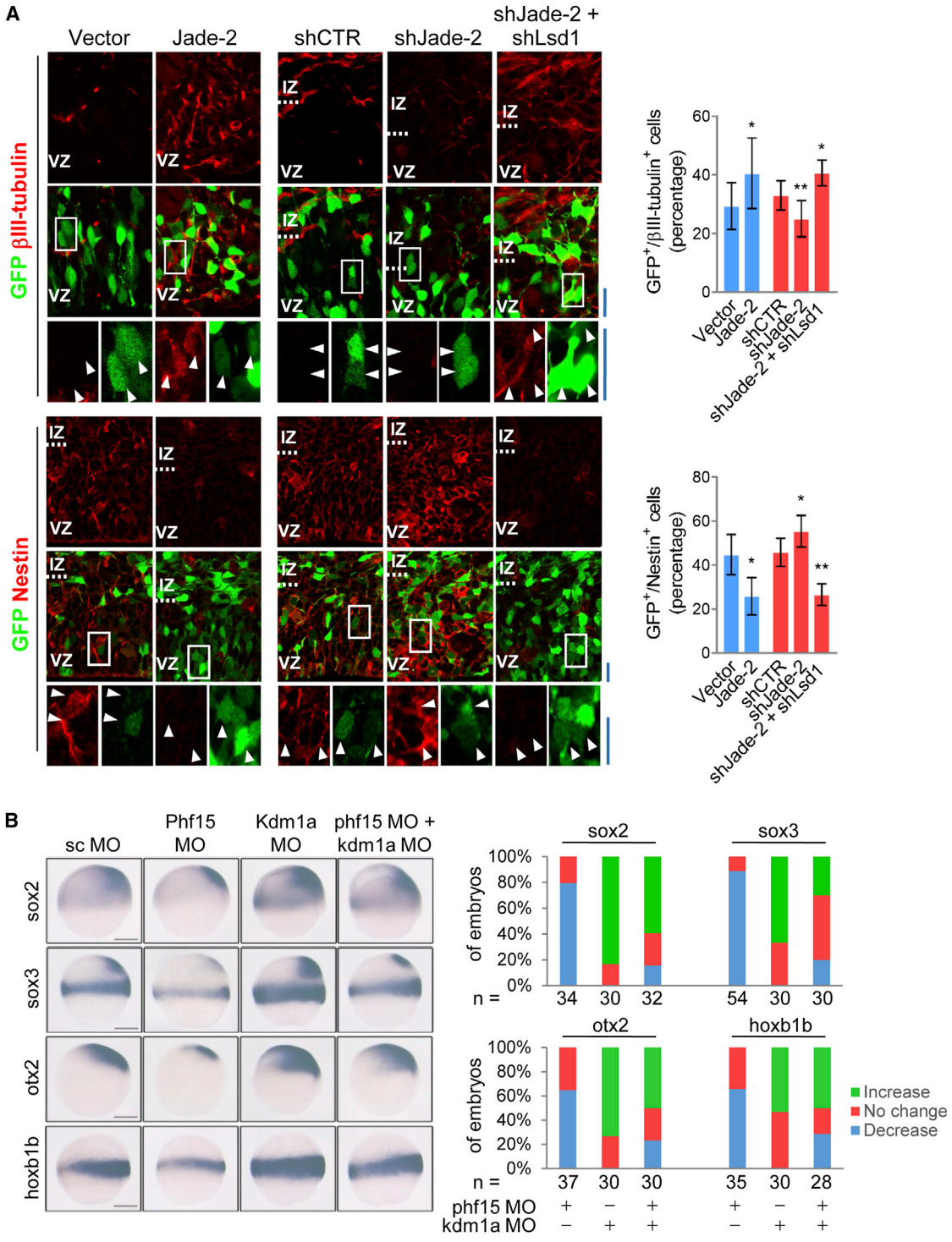

Figure 6. The Jade-2-LSD1 Pathway Is Implicated in Nervous System Development

(A) The indicated electroporated cortical slices were analyzed by IF microscopy for βIII-tubulin or Nestin. The images of VZs and/or IZs are shown. Scale bar represents 20 μm. The percentage of $GFP^+/\beta III\text{-}tubulin^+$, or $GFP^+/Nestin^+$ cells to GFP^+ cells was quantified by ImageJ. Slices from two independent experiments were processed for each experimental condition. The results are mean ± SEM of three sections from each experiment. *$p < 0.05$ and **$p < 0.01$ (two-tailed unpaired Student's t test). The images of "shCTR" refer to that in Figure 1E, given that the experiments with shLsd1, shJade-2, or shJade-2+shLsd1 were compared to the same control.

(legend continued on next page)

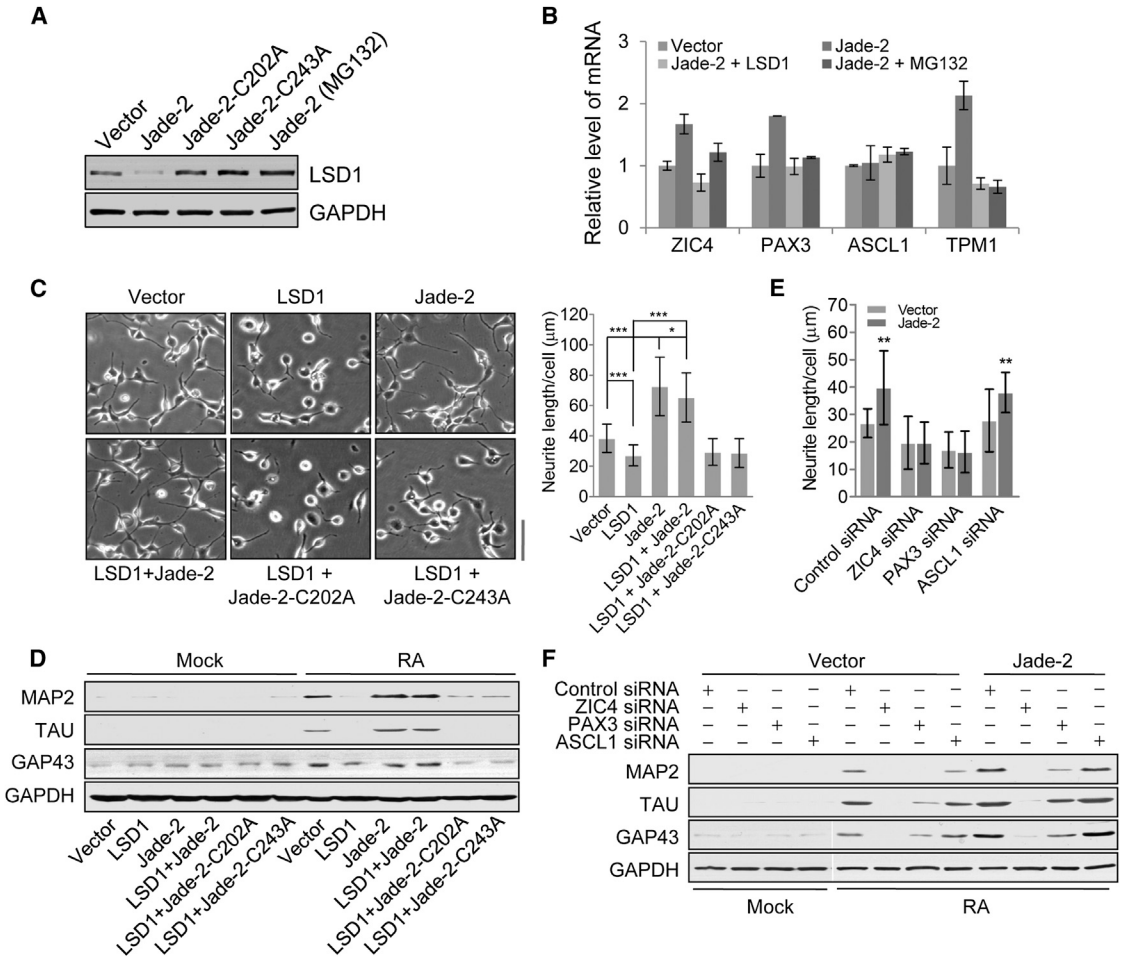

Figure 7. The Jade-2-LSD1 Pathway Is Implicated in Neuroblastoma Differentiation

(A) The indicated stable SH-SY5Y cell clones were treated with DMSO or MG132 (10 μM) for 10 hr before they were harvested for western blotting analysis of LSD1 expression.

(B) The indicated stable SH-SY5Y cell clones were treated with DMSO or MG132 before qRT-PCR analysis. Error bars represent mean ± SD for triplicate experiments.

(C) The indicated SH-SY5Y stable clones were induced for differentiation with 10 μM RA for 9 days before phase-contrast microscopic examination. Scale bar represents 100 μm. The average neurite length per cell was quantified with ImageJ (n = 50, error bars ± SD). *$p < 0.05$ and ***$p < 0.001$ (two-tailed unpaired Student's t test).

(D) The undifferentiated cells or differentiated cells shown in (C) were harvested for western blotting analysis for the expression of MAP2, TAU, or GAP43.

(E) Vector or Jade-2 stably transfected SH-SY5Y cell clones were treated with specific siRNA against ZIC4, PAX3 or ASCL1 before they were induced for differentiation. The average neurite length per cell was examined by phase-contrast microscopy and was quantified by ImageJ (n = 50, error bars ± SD). **$p < 0.01$ (two-tailed unpaired Student's t test).

(F) The undifferentiated or differentiated cells shown in (E) were subsequently harvested for western blotting analysis for the expression of MAP2, TAU, or GAP43.

See also Figure S5.

specification of neural or neuronal commitment of pluri- or multipotent cells.

Cell differentiation is dictated by spatial and temporal accumulation and/or elimination of transcription regulators including histone modification enzymes. Indeed, accumulating evidence indicates that the proper progression of neurogenesis depends on the strict control of the expression of several transcription factors, and, recently, targeted proteolysis of transcriptional regulators via the ubiquitin-proteasome system has emerged as an important regulatory principle in neural lineage

(B) Ten nanograms of each of the indicated morpholinos were injected into one-cell stage embryos before the mRNA level of sox2, sox3, otx2, or hoxb1b was examined at 75% epiboly stage by whole-mount in situ hybridization. Lateral view of dorsal to the right is shown. Scale bar represents 200 μm. The number of observed live embryos (n) was quantified and the ratio of the affected embryos is shown.

See also Figure S4.

specification. However, these studies largely focused on neurogenic transcription factors such as N-Myc, REST, and PAX6 or their downstream gene products such as CCND2, DLL3, and Neurog2, and on several E3 ubiquitin ligases of HECT-domain-type, single- or multiple-subunit RING finger type (Tuoc and Stoykova, 2008; Westbrook et al., 2008; Zhao et al., 2008, 2009). Given that distinct transcriptional programs governing cell fate determination are defined by, in addition to, transcription factors, chromatin architectures, the accumulation and/or elimination of transcriptional coregulators or chromatin modifiers such as LSD1 is also important, if not more important, in the expression of lineage-specific genes. In current study, we reported that a PHD-finger-type E3 ubiquitin ligase, Jade-2, targets LSD1 for degradation through the ubiquitin-proteasome pathway. We demonstrated that Jade-2-mediated LSD1 elimination plays an important role in ESC differentiation toward the neural lineage, during which process Jade-2 is required to relieve the braking function of LSD1 on neural commitment. Significantly, our discovery of Jade-2 as an E3 ubiquitin ligase for LSD1, which is different from the E3 ubiquitin ligase $SCF^{\beta\text{-}TRCP}$ for REST (Westbrook et al., 2008), adds a clue to the understanding of the suggestion that LSD1 might regulate a distinct neural transcriptome from REST in ESCs (Adamo et al., 2011; Whyte et al., 2012). Moreover, we demonstrated that the Jade-2-LSD1 pathway plays a critical role in cortical development and neuroectoderm induction, highlighting the importance of the Jade-2-LSD1 pathway in a broad scope of nervous system development. Interestingly, it has been observed that the inhibition of LSD1 results in excessive apoptosis of nerve cells in 24 hr postfertilization zebrafish embryos because of the activation of p53 pathway (Jie et al., 2009). It is possible that the Jade-2-LSD1 pathway is implemented in controlling cell fate determination during early embryogenesis; in late neural development and after neuron maturation, the level of LSD1 protein is maintained to ensure the survival of neurons. In addition, although it is not the focus of our current study, it is interesting to note that nLSD1 isoforms were reported to be upregulated via alternative splicing during cortical development in perinatal stage and are required for neurite morphogenesis (Zibetti et al., 2010). Consistently, our experiments showed that the overexpression of LSD1-8a in differentiating ESCs was associated with promoted neurite outgrowth (Figure S6). It is possible that Jade-2 mainly promotes LSD1 destabilizing in early neuronal differentiation during embryogenesis, in which time the expression of nLSD1 isoforms is low (Zibetti et al., 2010). It is also likely that the expression of nLSD1 isoforms is mainly controlled via transcriptional regulation rather than posttranscriptional mechanisms.

Although neural induction during embryogenesis was described as the "neural default" model (Muñoz-Sanjuán and Brivanlou, 2002), more recent findings indicate that the initiation of neural differentiation is likely to be triggered by positive signals such as fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), or Wnts (Bally-Cuif and Hammerschmidt, 2003; Stern, 2005). It remains to be investigated whether other post-translational modification enzymes such as acetyltransferases HBO1 and MORF (Doyon et al., 2006), which were identified to be associated with Jade-2, act as upstream stimulators in order to regulate the functionality of Jade-2. It is also reasonable to postulate that the Jade-2-LSD1 pathway might be triggered by yet to be discovered signals such as FGFs, IGFs, or Wnts at the beginning of neural induction.

Our findings show that Jade-2 is a critical regulator of both physiological and pathological LSD1 activities, adding to the understanding of biological functions of the Jade proteins as important regulators in nervous system development and as potential tumor suppressors (Tzouanacou et al., 2003; Zhou et al., 2005). In addition, as stated before, LSD1 is associated with several protein complexes and implicated in various cellular activities such as cell cycle progression and apoptosis (Dietrich et al., 1997; Huang et al., 2007; Scoumanne and Chen, 2007). Therefore, it will be important to investigate the scope and the variety of the functionality of the Jade-2-LSD1 pathway in future studies. Nevertheless, our studies identify an E3 ubiquitin ligase for LSD1, discover a mechanism that links the ubiquitin-proteasome system to epigenetic regulation in nervous system development, and provide a potential target for the therapeutic intervention of neuroblastoma.

EXPERIMENTAL PROCEDURES

In Vivo Ubiquitination Assay

In vivo ubiquitination assays were performed under a denaturing condition as described previously (Laney and Hochstrasser, 2011; Xirodimas et al., 2001). In brief, cells were lysed in denatured buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl [pH 8.0], 5 mM imidazole, and 10 mM β-mercaptoethanol) and were incubated with Ni-NTA agarose beads for 4 hr at room temperature. The beads were washed, eluted with 100 μl elution buffer (50 mM NaH_2PO_4 [pH 8.0], 100 mM KCl, 20% [v/v] glycerol, 0.2% (v/v) NP-40, and 200 mM imidazole), and analyzed by western blotting.

Neural Differentiation

R1 mouse ESCs were cultured on mitomycin C-inactivated mouse embryo fibroblasts for at least two passages and plated on gelatin-coated plates for another two passages. Cells were then dissociated and plated at a density of 1×10^4 cells/cm^2 in N2B27 medium supplemented with 1 mM L-glutamine and 0.1 mM β-mercaptoethanol for 4–6 days. N2B27 is a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 supplemented with N2 and 50 μg/ml BSA and Neurobasal medium supplemented with B27. For neuron derivation, cells were subsequently dissociated and plated on poly-d-lysine (PDL)/laminin-coated plates in the same cultural conditions for another 3–6 days. Embryoid bodies were obtained by plating ESCs on ultra-low-attachment 6-well plates at 5×10^5 cells/well in ESC cultural medium without leukemia inhibitory factor. All-*trans* RA (Sigma-Aldrich; 1 μM) was added from days 4–8. For SH-SY5Y cell differentiation, cells were treated with 10 μM RA and were replated every other day. For full neuronal differentiation of SH-SY5Y cells, cells were seeded on collagen-coated plates and treated with 10 μM RA for 5 days before exposing to 50 ng/ml BDNF for another 7 days.

In Utero Electroporation and Microinjection

The detailed experimental procedures are described in Supplemental Information. Animal handling and procedures were approved by Institutional Animal Care and Use Committees of Peking University Health Science Center and Tsinghua University.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.06.006.

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REFERENCES

Adamo, A., Sesé, B., Boue, S., Castaño, 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.

Ballas, N., Grunseich, C., Lu, D.D., Speh, J.C., and Mandel, G. (2005). REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. Cell *121*, 645–657.

Bally-Cuif, L., and Hammerschmidt, M. (2003). Induction and patterning of neuronal development, and its connection to cell cycle control. Curr. Opin. Neurobiol. *13*, 16–25.

Brambrink, T., Foreman, R., Welstead, G.G., Lengner, C.J., Wernig, M., Suh, H., and Jaenisch, R. (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell *2*, 151–159.

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development *126*, 525–534.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576–1583.

Chitalia, V.C., Foy, R.L., Bachschmid, M.M., Zeng, L., Panchenko, M.V., Zhou, M.I., Bharti, A., Seldin, D.C., Lecker, S.H., Dominguez, I., and Cohen, H.T. (2008). Jade-1 inhibits Wnt signalling by ubiquitylating beta-catenin and mediates Wnt pathway inhibition by pVHL. Nat. Cell Biol. *10*, 1208–1216.

Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C., and Dangl, J.L. (1997). A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death. Cell *88*, 685–694.

Doyon, Y., Cayrou, C., Ullah, M., Landry, A.J., Côté, V., Selleck, W., Lane, W.S., Tan, S., Yang, X.J., and Côté, J. (2006). ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Mol. Cell *21*, 51–64.

Encinas, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Ceña, V., Gallego, C., and Comella, J.X. (2000). Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. J. Neurochem. *75*, 991–1003.

Goulding, M.D., Chalepakis, G., Deutsch, U., Erselius, J.R., and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J. *10*, 1135–1147.

Guillemot, F., Lo, L.C., Johnson, J.E., Auerbach, A., Anderson, D.J., and Joyner, A.L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell *75*, 463–476.

Huang, J., Sengupta, R., Espejo, A.B., Lee, M.G., Dorsey, J.A., Richter, M., Opravil, S., Shiekhattar, R., Bedford, M.T., Jenuwein, T., and Berger, S.L. (2007). p53 is regulated by the lysine demethylase LSD1. Nature *449*, 105–108.

Jie, Z., Li, T., Jia-Yun, H., Qiu, J., Ping-Yao, Z., and Houyan, S. (2009). Trans-2-phenylcyclopropylamine induces nerve cells apoptosis in zebrafish mediated by depression of LSD1 activity. Brain Res. Bull. *80*, 79–84.

Laney, J.D., and Hochstrasser, M. (2011). Analysis of protein ubiquitination. Curr. Protoc. Protein Sci. *Chapter 14*, 5.

Le, M.T., Xie, H., Zhou, B., Chia, P.H., Rizk, P., Um, M., Udolph, G., Yang, H., Lim, B., and Lodish, H.F. (2009). MicroRNA-125b promotes neuronal differentiation in human cells by repressing multiple targets. Mol. Cell. Biol. *29*, 5290–5305.

Li, M., Pevny, L., Lovell-Badge, R., and Smith, A. (1998). Generation of purified neural precursors from embryonic stem cells by lineage selection. Curr. Biol. *8*, 971–974.

Liem, K.F., Jr., Tremml, G., Roelink, H., and Jessell, T.M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell *82*, 969–979.

Lunyak, V.V., Burgess, R., Prefontaine, G.G., Nelson, C., Sze, S.H., Chenoweth, J., Schwartz, P., Pevzner, P.A., Glass, C., Mandel, G., and Rosenfeld, M.G. (2002). Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. Science *298*, 1747–1752.

Mulligan, P., Yang, F., Di Stefano, L., Ji, J.Y., Ouyang, J., Nishikawa, J.L., Toiber, D., Kulkarni, M., Wang, Q., Najafi-Shoushtari, S.H., et al. (2011). A SIRT1-LSD1 corepressor complex regulates Notch target gene expression and development. Mol. Cell *42*, 689–699.

Muñoz-Sanjuán, I., and Brivanlou, A.H. (2002). Neural induction, the default model and embryonic stem cells. Nat. Rev. Neurosci. *3*, 271–280.

Panchenko, M.V., Zhou, M.I., and Cohen, H.T. (2004). von Hippel-Lindau partner Jade-1 is a transcriptional co-activator associated with histone acetyltransferase activity. J. Biol. Chem. *279*, 56032–56041.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503–533.

Schulte, J.H., Lim, S., Schramm, A., Friedrichs, N., Koster, J., Versteeg, R., Ora, I., Pajtler, K., Klein-Hitpass, L., Kuhfittig-Kulle, S., et al. (2009). Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res. *69*, 2065–2071.

Scoumanne, A., and Chen, X. (2007). The lysine-specific demethylase 1 is required for cell proliferation in both p53-dependent and -independent manners. J. Biol. Chem. *282*, 15471–15475.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941–953.

Shi, Y.J., Matson, C., Lan, F., Iwase, S., Baba, T., and Shi, Y. (2005). Regulation of LSD1 histone demethylase activity by its associated factors. Mol. Cell *19*, 857–864.

Sidell, N. (1982). Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells in vitro. J. Natl. Cancer Inst. *68*, 589–596.

Stern, C.D. (2005). Neural induction: old problem, new findings, yet more questions. Development *132*, 2007–2021.

Sun, G., Alzayady, K., Stewart, R., Ye, P., Yang, S., Li, W., and Shi, Y. (2010). Histone demethylase LSD1 regulates neural stem cell proliferation. Mol. Cell. Biol. *30*, 1997–2005.

Thrower, J.S., Hoffman, L., Rechsteiner, M., and Pickart, C.M. (2000). Recognition of the polyubiquitin proteolytic signal. EMBO J. *19*, 94–102.

Tuoc, T.C., and Stoykova, A. (2008). Trim11 modulates the function of neurogenic transcription factor Pax6 through ubiquitin-proteosome system. Genes Dev. *22*, 1972–1986.

Tzouanacou, E., Tweedie, S., and Wilson, V. (2003). Identification of Jade1, a gene encoding a PHD zinc finger protein, in a gene trap mutagenesis screen for genes involved in anteroposterior axis development. Mol. Cell. Biol. *23*, 8553–2.

Wang, Y., Zhang, H., Chen, Y., Sun, Y., Yang, F., Yu, W., Liang, J., Sun, L., Yang, X., Shi, L., et al. (2009). LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer. Cell *138*, 660–672.

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 Res. *71*, 7238–7249.

Westbrook, T.F., Hu, G., Ang, X.L., Mulligan, P., Pavlova, N.N., Liang, A., Leng, Y., Maehr, R., Shi, Y., Harper, J.W., and Elledge, S.J. (2008). SCFbeta-TRCP controls oncogenic transformation and neural differentiation through REST degradation. Nature *452*, 370–374.

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.

Xirodimas, D., Saville, M.K., Edling, C., Lane, D.P., and Laín, S. (2001). Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 in vivo. Oncogene *20*, 4972–4983.

Ying, Q.L., and Smith, A.G. (2003). Defined conditions for neural commitment and differentiation. Methods Enzymol. *365*, 327–341.

Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat. Biotechnol. *21*, 183–186.

Zhao, X., Heng, J.I., Guardavaccaro, D., Jiang, R., Pagano, M., Guillemot, F., Iavarone, A., and Lasorella, A. (2008). The HECT-domain ubiquitin ligase Huwe1 controls neural differentiation and proliferation by destabilizing the N-Myc oncoprotein. Nat. Cell Biol. *10*, 643–653.

Zhao, X., D' Arca, D., Lim, W.K., Brahmachary, M., Carro, M.S., Ludwig, T., Cardo, C.C., Guillemot, F., Aldape, K., Califano, A., et al. (2009). The N-Myc-DLL3 cascade is suppressed by the ubiquitin ligase Huwe1 to inhibit proliferation and promote neurogenesis in the developing brain. Dev. Cell *17*, 210–221.

Zhou, M.I., Wang, H., Ross, J.J., Kuzmin, I., Xu, C., and Cohen, H.T. (2002). The von Hippel-Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. J. Biol. Chem. *277*, 39887–39898.

Zhou, M.I., Foy, R.L., Chitalia, V.C., Zhao, J., Panchenko, M.V., Wang, H., and Cohen, H.T. (2005). Jade-1, a candidate renal tumor suppressor that promotes apoptosis. Proc. Natl. Acad. Sci. USA *102*, 11035–11040.

Zibetti, C., Adamo, A., Binda, C., Forneris, F., Toffolo, E., Verpelli, C., Ginelli, E., Mattevi, A., Sala, C., and Battaglioli, E. (2010). Alternative splicing of the histone demethylase LSD1/KDM1 contributes to the modulation of neurite morphogenesis in the mammalian nervous system. J. Neurosci. *30*, 2521–2532.