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Identification of Myelin Transcription Factor 1 (MyT1) as a Subunit of the Neural Cell Type-specific Lysine-specific Demethylase 1 (LSD1) Complex*

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Background: The composition of tissue-specific complexes that modify histone is largely unknown.
Results: We identified myelin transcription factor 1 (MyT1) as a lysine-specific demethylase 1 (LSD1) complex component by biochemical approaches using Neuro2a cells. This complex directly regulated the phosphatase and tensin homolog (*Pten*) gene expression.
Conclusion: MyT1 is a neuro-specific LSD1 complex component.
Significance: A tissue-specific complex component of LSD1 complex was identified.

Regulation of spatiotemporal gene expression in higher eukaryotic cells is critical for the precise and orderly development of undifferentiated progenitors into committed cell types of the adult. It is well known that dynamic epigenomic regulation (including chromatin remodeling and histone modifications by transcriptional coregulator complexes) is involved in transcriptional regulation. Precisely how these coregulator complexes exert their cell type and developing stage-specific activity is largely unknown. In this study we aimed to isolate the histone demethylase lysine-specific demethylase 1 (LSD1) complex from neural cells by biochemical purification. In so doing, we identified myelin transcription factor 1 (MyT1) as a novel LSD1 complex component. MyT1 is a neural cell-specific zinc finger factor, and it forms a stable multiprotein complex with LSD1 through direct interaction. Target gene analysis using microarray and ChIP assays revealed that the *Pten* gene was directly regulated by the LSD1-MyT1 complex. Knockdown of either LSD1 or MyT1 derepressed the expression of endogenous target genes and inhibited cell proliferation of a neuroblastoma cell line, Neuro2a. We propose that formation of tissue-specific combinations of coregulator complexes is a critical mechanism for tissue-specific transcriptional regulation.

In multicellular organisms tissue structures are highly organized and show cell type-specific gene expression. This differential gene profile requires highly precise control of gene expression at the transcriptional level (1, 2). However, the underlying molecular mechanism responsible for such specificity in each cell type, such as neural cells, remains elusive.

One explanation for transcriptional regulation is chromatin reorganization, a process that is controlled by transcriptional coregulators (3–5). These transcriptional coregulators can be classified into two groups. The first group comprises ATP-dependent chromatin remodeling factors that reorganize nucleosomal arrays and potentiate promoter accessibility of transcription factors (6–8). The other group consists of histone modifiers that covalently modify histone tails by acetylation, methylation, ubiquitylation, and phosphorylation (9–11). Using biochemical approaches, these transcriptional coregulators have been found in many cases to act as multisubunit protein complexes that possess distinct enzymatic activities (3, 5, 12).

Of these chromatin modifications, histone lysine methylation is generally regarded as one of the most significant histone modifications to trigger alterations in chromatin structure (13). Methylation of histone H3K9 leads to chromatin silencing, whereas histone H3K4 methylation induces chromatin activation (9, 11, 14). A number of histone methyltransferases like G9a (15) and SUV39H1 (16) as well as demethylases like LSD1 (17) and KIAA1718 (18) have been reported to coregulate transcriptional controls through regulation of histone methylation states. In contrast, little is known about how these histone mod-

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ifiers, despite their global expression pattern, contribute to cell type-specific transcriptional regulation.

Until recently, the composition of the general transcriptional machinery, including core promoter recognition complexes and transcriptional coregulator complexes, were held to be invariant. However, new evidence suggests that significant changes in complex constituents could facilitate global changes in cell type-specific transcription (19, 20). For example, the general transcription factor complex TFIID appears to be exchanged into TRF3-containing complexes in myogenesis (21). Furthermore, components of SWI/SNF chromatin remodeling complexes have been shown to vary depending on the specific cell type (22, 23). Nevertheless, the possibility that histone modifier complex components vary depending on the cell type in question has not been well explored.

Lysine-specific demethylase 1 (LSD1[2]/KDM1a) is the first identified histone demethylase that can demethylate both H3K4me1/2 and H3K9me1/2 as substrates (24, 25). From previous studies, it is clear that LSD1 has significant roles in transcriptional regulation such as transcriptional repression and gene activation by nuclear receptor ligand (13, 26–29). With regard to tissue-specific regulation, in brain tissue, LSD1 is reportedly enriched in the neurons of the hippocampus, cerebral cortex, striatum, and amygdala (30, 31). LSD1 has also been reported to regulate adult neural stem cell proliferation in cooperation with TLX, an orphan nuclear receptor (17, 32). Precisely how LSD1 exerts its function in neural cells, especially in protein complex formation, remains to be investigated.

In the present study we isolated LSD1-containing protein complexes from neural cells as a model of a specific cell type and performed proteomic analysis of the purified complexes. From this analysis, we identified myelin transcription factor 1 (MyT1)/neural zinc finger factor 2 (NZF2) as a novel neural cell-specific LSD1 complex component. MyT1 directly interacted with and formed stable protein complexes with LSD1 (designated as the nLSD1 complex). Microarray and chromatin immunoprecipitation (ChIP) experiments revealed that both MyT1 and LSD1 directly regulated the same target genes, including *Pten*. Thus, we propose that the neural cell-specific nLSD1 complex has a crucial role in neural cell-specific gene expression.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and siRNAs—Full-length mouse *Lsd1* cDNA was amplified by PCR from a Neuro2a cell cDNA library and was cloned in-frame into a pQCXIN-FLAG vector and a pGEX-4T1 vector. Full-length and deletion mutants of mouse *Myt1* with an N-terminal HA tag were amplified from a Neuro2a cell cDNA library and were cloned in-frame into a pcDNA vector. Anti-FLAG and anti-FLAG M2 agarose were purchased from Sigma. Antibodies for immunoblot and ChIP analyses included anti-LSD1 (Abcam, Cambridge, UK, ab17721), anti-MyT1 (Abcam, ab30997), anti-CoREST (Millipore, Billerica, MA, #07-455), anti-HDAC1 (ABR, Golden, CO, #PAI-860), anti-CtBP1 (Santa Cruz, Santa Cruz, CA, sc-17805), anti-SRC1 (Santa Cruz, sc-8995), anti-H3K4me2 (Millipore, #07-212), anti-H3K9me2 (Abcam, ab1220), and anti-H3 (Abcam, ab10799). Antibodies for immunohistochemistry included anti-LSD1 (Abcam, ab31954) and anti-MyT1 (Abcam, ab154516). Pre-designed SMART pool small interfering RNAs (siRNAs) for *Myt1* were purchased from Dharmacon (Lafayette, CO), and those for *Lsd1* and control siRNA were from Qiagen (Hilden, Germany).

Cell Culture and Transfection—Neuro2a cells were cultured in DMEM plus 10% FBS and antibiotics. To establish *Neuro2a FLAG-LSD1* stable transformants, Neuro2a cells were infected with retrovirus carrying the *FLAG-LSD1* gene. For transfection, we used Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. FLAG-tagged mouse LSD1-expressing retroviruses were generated using PLAT-E cells (33). The PLAT-E cells were kindly provided by Dr. Toshio Kitamura (University of Tokyo).

Preparation of Nuclear Extracts and LSD1 Complex Purification—LSD1-containing complexes were purified from Neuro2a cells stably expressing FLAG-LSD1. The cells were cultured in fifty 500-cm^2 TC-treated culture dishes (Corning, Corning, NY). Empty virus-infected Neuro2a cells were used as a negative control. Nuclear extracts were prepared by a modification of the previously described method (17). Briefly, collected cells were swollen in hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT), and 10% Nonidet P-40 was added to a final concentration of 0.6%. Then cells were vortexed for 10 s to remove the cell membrane. Isolated nuclei were collected and dissolved in a 0.5 nuclear pellet volume of low salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM KCl). Finally, nuclear proteins were extracted by dropwise addition of 0.5 nuclear pellet volume of high salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.9 M KCl). Prepared nuclear extracts were further fractionated by cation exchange column (SP XL, GE Healthcare) chromatography using a stepwise KCl elution technique. Flow-through fractions were mixed with a 100-μl slurry of FLAG M2 resin and incubated for 6 h in a cold room. After washing with BC300 buffer (20 mM Hepes, pH 7.6, 300 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol), bound proteins were eluted with 200 μl of 400 μg/ml FLAG peptide (Sigma) in BC100 buffer (20 mM Hepes, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Size fractionation of purified complexes by glycerol density gradients was performed as described previously (17).

Mass Spectrometric Analysis—Interactants were excised from the gel and analyzed by LC-MS/MS. Eluted proteins were also precipitated by the methanol-chloroform method, trypsinized, and then directly subjected to LC-MS/MS analysis as previously described (18). Briefly, a total of 10 μl of extracted peptides was analyzed by ESI-MS/MS using a Finnigan LTQ instrument (Thermo Fisher Scientific, Pittsburgh, PA). HPLC was performed with a Zaplous system (AMR, Tokyo, Japan) equipped with C-18 ESI capillary column. The gradient con-

[2] The abbreviations used are: LSD1, lysine-specific demethylase 1; MyT1, Myelin transcription factor; qPCR, quantitative reverse transcription-PCR; DAB, 3,3-diaminobenzidine; nLSD1 complex, neural-specific LSD1 complex; HDAC, histone deacetylase.

sisted of 0.1% formic acid in 2% acetonitrile (A) and 0.1% formic acid in 90% acetonitrile (B): 5–45% B from 0 to 33 min, 45–95% B from 34 to 43 min, and 5% B from 44 to 60 min. The flow rate was 1.3 μl/min from 0 to 60 min. MS spectra were recorded over a range of 450–2000 m/z followed by data-dependent collision-induced dissociation MS/MS spectra generated from the five highest intensity precursor ions.

For protein identification, spectra were processed using BioWorks Version 3.3 (Thermo Fisher Scientific) against the SEQUEST algorithm. For database searches, mouse.fasta built from the nr.fasta (January 25, 2008) was used. The following parameters were used for the searches: tryptic cleavage up to 2 missed cleavage sites and tolerances of $\pm$2.0 atomic mass units for precursor ions and $\pm$1.0 atomic mass units for MS/MS fragment ions. SEQUEST searches were performed allowing optional methionine oxidation and fixed cysteine carbamidomethylation. Proteins that contained peptides with peptide probability >1E-3, Sf (final score) > 0.85, and number of top matches = 1 were selected.

Glutathione S-Transferase (GST) Pulldown Assays—For GST pulldown assays, recombinant GST-fused full-length LSD1 proteins were expressed in *Escherichia coli*, strain BL21. We purified GST fusion proteins on glutathione-Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare). MyT1 and its mutants were translated *in vitro* using the TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, WI) according to the manufacturer's instructions and incubated with GST-fused LSD1 immobilized on GST beads for 1 h at room temperature. The beads were washed and boiled with Laemmli sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and assessed by autoradiography.

RNA Isolation, cDNA Synthesis, and Quantitative Reverse Transcription-PCR (qPCR)—Total RNA was extracted by TRIzol (Invitrogen), and cDNA was synthesized using a One Step PrimeScript RT-PCR kit (Takara Bio, Tokyo, Japan). Total RNA (400 ng) was used for reverse polymerase chain reaction (PCR) using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). qPCR was performed with the Thermal Cycler Dice Real Time System II (Takara Bio) according to the manufacturer's instructions. The primer sequences for each gene were as follows: *Pten* (forward, 5′-ggcactgctgtttcacaaga-3′) and reverse (5′-gggtcctgaattggaggaat-3′); *Bst2* (forward, 5′-tcaggagtccctggagaaga-3′) and reverse (5′-attctccagctcctg-gttca-3′); *Ptgr1* (forward (5′-cactggaggaagctttgagg-3′) and reverse (5′-accacagatggcaatccttc-3′). For mouse *Rplp0*, *Lsd1*, and *Myt1*, pre-designed primer sets were purchased from Takara Bio. RNA levels were normalized using *Rplp0* gene as an internal standard.

ChIP Assays—Neuro2a cells were cross-linked for 10 min at room temperature with 0.75% formaldehyde-containing PBS; cross-linking was stopped with PBS-glycine (0.3 M final). Cells were washed twice with ice-cold PBS, scraped, centrifuged for 10 min at 3000 rpm, resuspended in cell lysis buffer (50 mM Tris, pH 8.1, 1% SDS, 10 mM EDTA), and incubated for 10 min on ice. Lysates were then sonicated to obtain DNA fragments averaging 200–500 bp in length. Sonicated lysates were cleared by centrifugation and diluted in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and immunoprecipitated overnight with 2 μg of indicated antibodies. Immunoprecipitated DNA was analyzed by qPCR (KAPA SYBR FAST Universal 2× qPCR Master Mix, KAPA Biosystems, Woburn, MA) together with 2.7% of the input chromatin. Specific primer pairs were designed to amplify the promoter region of the mouse *Pten* gene: [a] (5′-tgatccagtgtttcagcttagg-3′ and 5′-gttcttttcccaggggactt-3′), [b] (5′-caggtttgttctgggctgac-3′ and 5′-gctggttaggcttaccgatg-3′), [c] (5′-gcacacagataggggaatgg-3′ and 5′-gcggtcggaactactttcag-3′) and [d] (5′-agttggccagtcattgctct-3′ and 5′-ggggtccttattgccattct-3′).

Microarray Analysis—Percellome microarray analysis was performed to calculate mRNA copy numbers per cell as previously described (34, 35). Briefly, cells treated with each siRNAs for 48 h (n = 3) were lysed in RLT buffer (Qiagen). The DNA concentration was measured, and the appropriate amount of spike RNA mixture was added to the samples in proportion to their concentration. Then total RNAs were purified using an RNeasy Mini kit (Qiagen). First-strand cDNAs were synthesized by incubating five μg of total RNA with SuperScript II reverse transcriptase (Invitrogen). After second-strand synthesis, the double-stranded cDNAs were purified using a MinElute Reaction Cleanup kit (Qiagen) and labeled by *in vitro* transcription using a BioArray High Yield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY). The labeled cRNA was then purified using an RNeasy Mini kit (Qiagen) and treated with fragmentation buffer at 94 °C for 35 min. For hybridization to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA), 7.5 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD, and 100 pM Cre), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, and 1× manufacturer-recommended hybridization buffer in a 45 °C rotator for 16 h. Washing and staining were performed in a GeneChip Fluidics Station (Affymetrix). The phycoerythrin-stained arrays were scanned as digital image files that were then analyzed with GeneChip Operating Software (Affymetrix). The expression data were converted to copy numbers of mRNA per cell by the Percellome method, quality-controlled, and analyzed using Percellome software (34).

Cell Proliferation Assay—Cell proliferation was determined using a PreMix WST-1 Cell Proliferation Assay System (Takara Bio) according to the manufacturer's instructions. Briefly, cells were treated with siRNA for 24 h in 6-cm dishes, then 2×10^4 cells were seeded in a 96-well plate. For WST-1 assays, the cells were incubated for 0.5 h in medium containing the 10% Premix WST-1 reagent at 37 °C. Absorbance was then measured using a microplate reader at a wavelength of 450 nm with a reference wavelength of 600 nm.

Immunohistochemistry—A whole brain from an adult mouse (ICR, 8-week-old, male) was fixed overnight with 10% neutral-buffered formalin at room temperature. Antigen retrieval was performed by heating the slides in an autoclave at 121 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). Histofine mouse staining kit (Nichirei Biosciences, Tokyo, Japan) and Histofine SAB-PO Kit

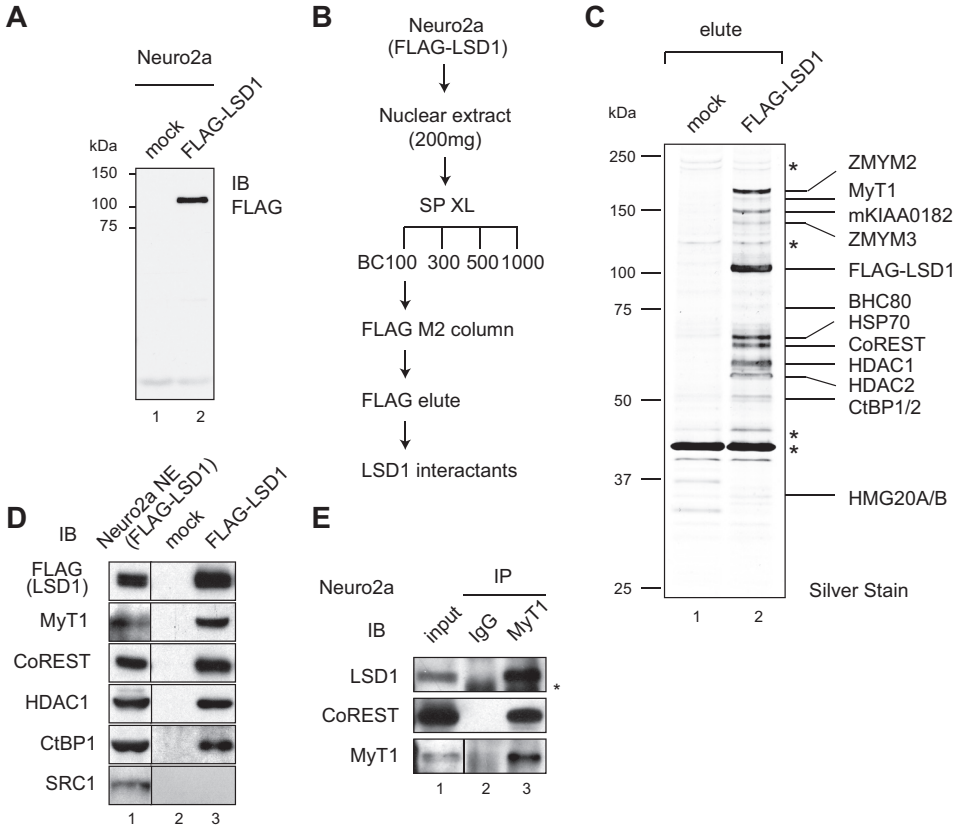


FIGURE 1. **Proteomic analysis of LSD1 interactants from Neuro2a nuclear extracts.** *A*, establishment of Neuro2a cells stably expressing FLAG-LSD1. LSD1 expression was validated by Western blotting with anti-FLAG antibody. Empty virus-infected Neuro2a cells were used as a negative control. *IB*, immunoblot. *B*, schematic diagram of the purification of FLAG-LSD1-associated proteins. Nuclear extracts from Neuro2a stably expressing FLAG-LSD1 were loaded on a column described under "Experimental Procedures." Bound proteins were eluted with 400 μg/ml FLAG peptide in BC100. Empty virus-infected Neuro2a cells were used as a negative control. *C*, identification of FLAG-LSD1-interacting proteins in Neuro2a cells. Eluted proteins were subjected to SDS-PAGE followed by silver staining. The results of ion trap mass spectrometric analysis of the indicated proteins are shown on the *right side*. Molecular mass of each protein marker is indicated on the *left side*. The *asterisks* indicate background peptides. *D*, proteins identified by mass spectrometry were confirmed by Western blotting with indicated antibodies. FLAG eluates of Fig. 1*C* were used, and eluates from empty virus-infected cells were used as a negative control. SRC1 antibody was used as a negative control for Western blotting. *NE*, nuclear extracts. *E*, reverse co-immunoprecipitation (*IP*) from Neuro2a cells was performed using anti-MyT1 antibody. IgG was used as a negative control. The *asterisk* indicates background proteins.

(Nichirei Biosciences) were used for LSD1 and MyT1 immunohistochemistry, respectively. Dilutions of primary antibodies used in this study were as follows: LSD1 (ab31954), 1/100; MyT1 (ab154516), 1/200. The antigen-antibody complex was subsequently visualized with 3,3-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer, pH 7.6, and 0.006% H_2O_2) and counterstained with hematoxylin. For double immunostaining, after antigen retrieval, immunoreactivity of LSD1 was first visualized as brown by DAB as described above. The section was subsequently incubated with anti-Myt1 antibody and visualized as blue by NBT/BCIP (Roche Applied Science) using biotin-labeled secondary antibody (Nichirei) and alkaline phosphatase-labeled streptavidin (Roche Applied Science).

RESULTS

Identification of Myelin Transcription Factor 1 as a Novel LSD1 Interactant—To investigate the role of the LSD1 complex in neural cells, we utilized a neuroblastoma cell line, Neuro2a. This neural cell line has been used as a model for verification of gene regulation via epigenetic changes such as histone acetylation (36, 37). To explore the composition of the LSD1 complex in this cell, we biochemically isolated LSD1-containing protein complexes from Neuro2a nuclear extracts. To purify LSD1-containing protein complexes, we first established a Neuro2a-derived cell line stably expressing FLAG-tagged LSD1 by retroviral infection (Fig. 1*A*). FLAG-LSD1-containing protein complexes were affinity-purified from ~200 mg of Neuro2a-FLAG LSD1 nuclear extracts using anti-FLAG antibodies following the chromatographic scheme indicated in Fig. 1*B*. Neuro2a-FLAG LSD1 nuclear extracts were affinity-purified with an anti-FLAG antibody column using the flow-through fractions from a cation exchange SP XL column. The anti-FLAG affinity eluate was subjected to SDS-PAGE and silver-staining (Fig. 1*C*). Neuro2a FLAG-LSD1 cell-specific bands were cut from the gel and then subjected to ion trap mass spectrometric analysis for protein identification. As anticipated, we identified known interactants of LSD1 such as CoREST, HDAC1/2, and CtBP1/2 (26) that have transcriptionally repressive features. In addition, our analysis revealed the association of LSD1 with MyT1/neural zinc finger 2 (NZF2), a neural cell-specific zinc finger factor, the role of which was largely unknown (38, 39). The association between these proteins and LSD1 was specific, as the affinity eluate from a control cell lacked their presence (Fig. 1*D*). The reverse co-immunoprecipitation using Neuro2a cells showed that immunoprecipitation

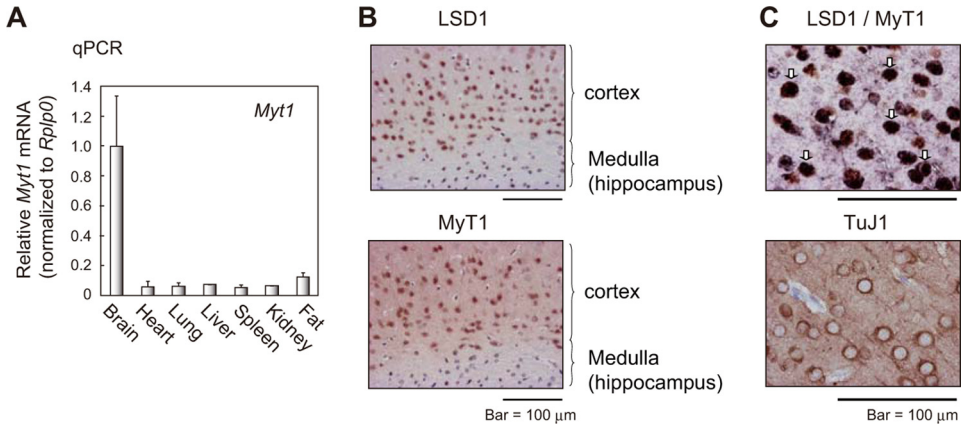


FIGURE 2. **MyT1 and LSD1 co-localized in the nuclei of TuJ1-positive neuron.** *A*, tissue distribution of *Myt1* mRNA was assessed by qPCR using a cDNA library from adult murine ICR animals (8 weeks old; $n = 2$). The expression levels of the *Myt1* gene were normalized to the endogenous expression of the *Rplp0* gene. The *error bars* indicate standard deviations. *B*, immunohistochemistry of adult mouse brain sections (ICR, 8-week- old male). A brain section was stained with the indicated antibody. These panels were from the same area. Each *scale bar* corresponds to 100 μm. *C*, co-immunohistochemistry of adult mouse brain sections (ICR, eight-week-old male, cerebral cortex). A brain section was stained with anti-LSD1 (DAB, brown) and anti-MyT1 (NBT/BCIP, *blue*) antibodies (*top panel*). *White arrows* indicate the double-positive cells. The *lower panel* shows the TuJ1 staining of the same area. Each *scale bar* corresponds to 100 μm.

with anti-MyT1 antibody also brought down endogenous LSD1 and CoREST (Fig. 1*E*).

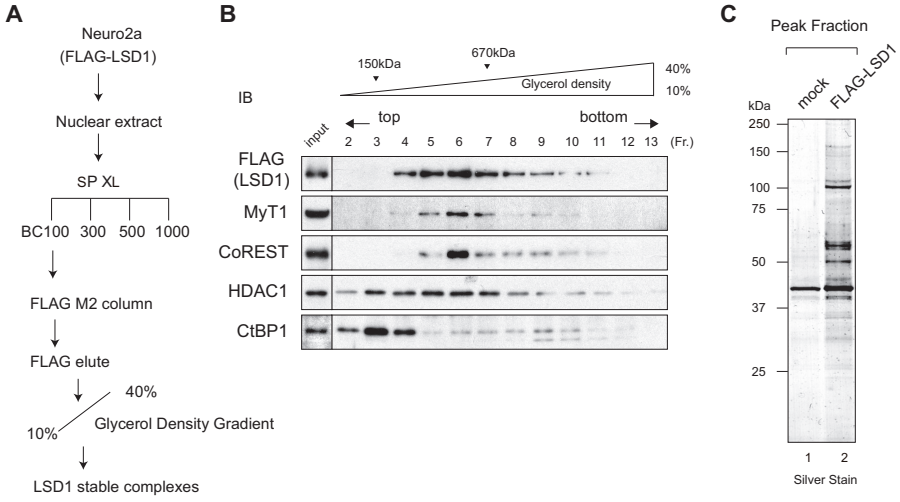
To examine the tissue distribution of *Myt1* mRNA, we carried out real-time qPCR analysis using an adult mouse cDNA library. Consistent with previous reports, *Myt1* mRNA was specifically expressed in brain tissue (Fig. 2*A*). Next, we performed immunohistochemical staining for LSD1 and MyT1 using adult mouse brain sections and found that both factors were positive in the nuclei of neural cells in the same region (cerebral cortex) as shown in Fig. 2*B*. Furthermore, double immunostaining using MyT1 and LSD1 antibodies showed that the expression of both factors significantly overlapped in the nuclei of TuJ1-positive neurons (Fig. 2*C*) but not in glial fibrillary acidic protein-positive glial cells (data not shown). Together, these data show that LSD1 and MyT1 form a protein complex in TuJ1-positive neurons in the adult mouse brain.

MyT1 Forms a Stable Multiprotein Complex with LSD1, CoREST, and HDAC—To determine whether MyT1 formed a stable complex with LSD1, the purified FLAG LSD1-interacting proteins were separated on 10–40% glycerol density gradients to fractionate the protein complexes by molecular size (Fig. 3*A*). Each separated fraction was enriched by TCA precipitation and then subjected to SDS-PAGE. As shown in Fig. 3*B*, Western blotting demonstrated that some of the proteins (MyT1, CoREST, and HDAC1) co-fractionated with FLAG LSD1, peaking in fractions five and six. On the other hand, CtBP1 peaked in fraction three, suggesting that CtBP1 was present in other protein complexes or could not form a stable complex with MyT1 and FLAG LSD1 under these conditions (40). Next, the peak glycerol gradient fraction (six) of MyT1 protein was subjected to further ion trap mass spectrometric analysis (Fig. 3*C*), and the identified proteins are listed in Fig. 3*D*. These data suggest that MyT1 and LSD1 form a transcriptionally repressive stable multiprotein complex containing several proteins such as CoREST and HDAC1/2 but not CtBP1.

In addition, our *in vitro* binding experiments using recombinant GST-fused LSD1 proteins (Fig. 4*A*) indicated direct interaction between LSD1 and MyT1 through the 361–794-amino acid region of MyT1 protein (Fig. 4*B*). Taken together, these studies suggested that LSD1 and MyT1 interacted directly with each other to form a novel complex that is present specifically in neural cells. Thus, this complex is a neural lineage-specific LSD1 complex that we designated "nLSD1 complex" (neural cell-specific LSD1 complex).

MyT1 and LSD1 Regulate Neural-specific Genes as a Protein Complex—To explore the targeted genes of the nLSD1 complex in Neuro2a cells, we performed global expression analysis using RNA from Neuro2a cells. For this expression analysis, we utilized the Percellome microarray method to quantify the mRNA molecules per cell based on the measurement of cell number (34). Total RNA from Neuro2a cells treated with siRNA against either *Lsd1* or *Myt1* genes were used in gene expression profiling analysis (Fig. 5, *A* and *B*). A total of 598 and 443 known genes showed at least 2-fold up-regulation ($p < 0.05$) in si*Myt1*- and si*Lsd1*-treated cells, respectively (Fig. 5*C*, *upper figure*). And 266 and 601 genes showed >2-fold down-regulation in siMyt1 and siLsd1, respectively (Fig. 5*C*, *lower figure*). 69 and 48 genes in each group matched, respectively, which are candidate target genes of nLSD1 complex. Fig. 5*D* lists the genes in which the transcript copy number was above 0.1 per cell. In this list several genes such as *Acp1* (41), *Pten* (42), *Nek3* (43), *St18* (44), and *Prkg2* (45) were reported to have a role in neural cells. Notably, *Acp1*, *Pten*, and *St18* genes were reported to negatively regulate cell proliferation (46–48). As the nLSD1 complex had transcriptionally repressive components such as CoREST and HDACs, we focused on up-regulated genes in this experiment. To validate the results of the expression analysis, we selected three genes (*Pten*, *Bst2*, and *Ptgr1*) for qPCR analysis. Consistent with the microarray analysis, all three genes were up-regulated by both *Myt1* and *Lsd1* knockdown (Fig. 5*E*). Furthermore, double knockdown failed to further enhance gene expression compared with single knockdown (Fig. 5*E*), implying that both factors were in the same pathway and that MyT1 and LSD1 proteins co-regulated these genes as a complex.

The nLSD1 Complex Directly Targeted Pten Gene Expression through Epigenetic Regulation—From the expression analysis, we selected the *Pten* gene as a candidate target of the nLSD1



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Protein name	Coverage	MW	Accession	Peptides identified	P (pep)
CoREST	11.40	36416.8	26006101	3	7.28E-12
KIAA0182	5.90	112976.4	28972089	3	1.37E-11
LSD1	23.40	95605.2	37360004	9	5.96E-11
ZMYM2	10.00	113138.9	28175571	5	8.29E-11
HDAC2	13.50	55266.8	3023934	3	1.26E-10
MyT1	6.00	123499.0	23346429	3	3.12E-09
PHF21B	4.30	52968.7	81899423	1	1.36E-08
Hsp60	20.10	60917.5	51702230	6	3.20E-07
HDAC1	3.50	55039.8	6680193	1	8.32E-07
ZFP217	1.70	113528.1	75677492	1	8.33E-07
tubulin, alpha8	4.50	50019.6	8394493	1	4.68E-06
desmoplakin	0.50	332704.8	82950147	1	1.42E-05
elongation factor 1-alpha 1	5.20	50024.2	26328693	1	1.61E-05
tubulin, beta 3	10.20	50386.3	12963615	2	2.15E-05

FIGURE 3. **MyT1 as a neural cell-specific LSD1 complex component.** *A*, schematic diagram of the purification of LSD1-containing stable complexes. LSD1 interactants were purified from Neuro2a nuclear extracts as described in Fig. 1*B*. Eluted proteins were further separated by 10–40% glycerol density gradients to fractionate complexes by their molecular sizes. *B*, LSD1 formed a multiprotein complex with MyT1, CoREST, and HDAC1. Fractionated proteins were subjected to Western blotting (*IB*) using the indicated antibodies. *C*, the peak fraction of MyT1 protein (fraction 6) was visualized by SDS-PAGE and silver staining. Molecular mass of protein markers are indicated on the *left side*. *D*, total identified peptides are listed. Identified proteins were sorted by peptide probability scores (*P (pep)*). Coverage means the percent sequence coverage identified from MS/MS results. *MW*, molecular weight; *Accession*, accession number in NCBI; *Peptides identified*, number of the identified peptides by LC-MS/MS.

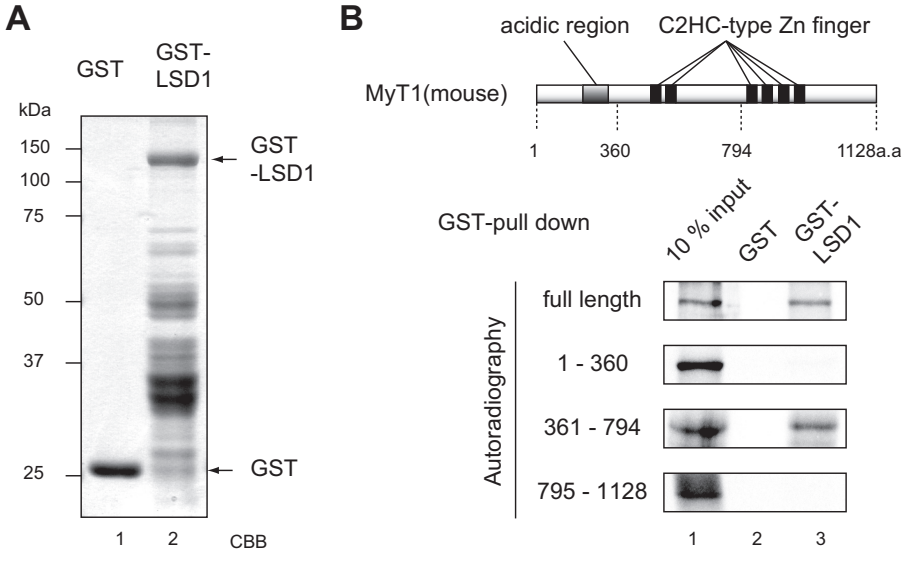
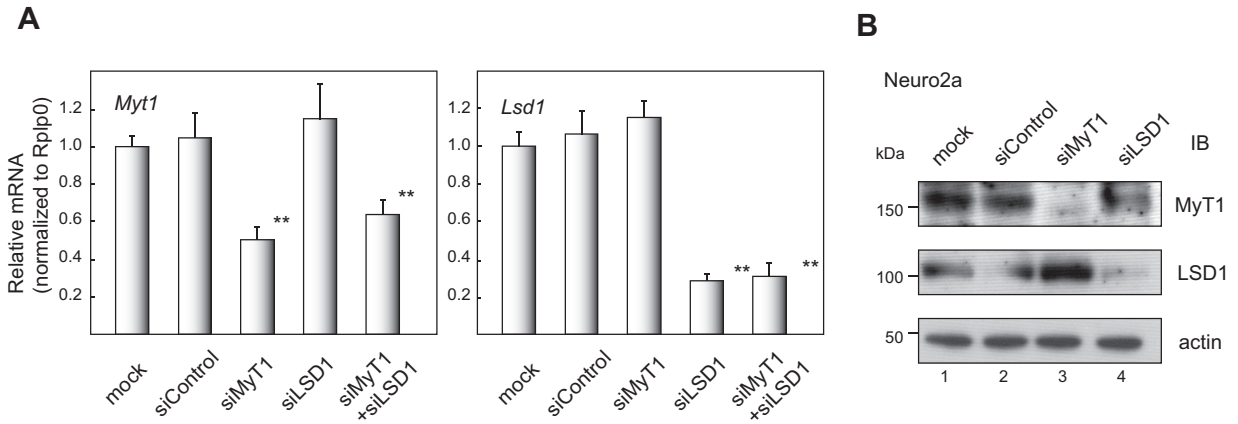
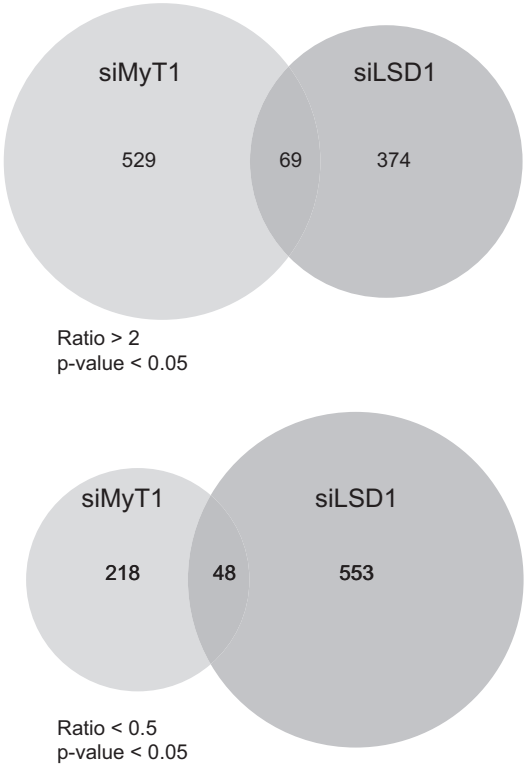


FIGURE 4. **Direct interaction between MyT1 and LSD1.** *A*, Preparation of GST full-length LSD1 protein from *E. coli*. Purified GST-LSD1 and GST proteins were subjected to SDS-PAGE and then visualized by Coomassie Brilliant Blue (*CBB*) staining. Each protein is indicated with a *black arrow*. *B*, *in vitro* interaction between LSD1 and MyT1. GST pulldown assays were performed with ^{35}S-labeled MyT1 or its deletion mutant and bacterially expressed GST-LSD1 fusion proteins as described. GST proteins were used as negative controls. Schematic representation of full-length mouse MyT1 protein containing 1128 amino acids is shown in the *upper panel*. The acidic region and six C2HC-type zinc finger domains are marked in *gray* or *black*, respectively. *a.a.*, amino acids.





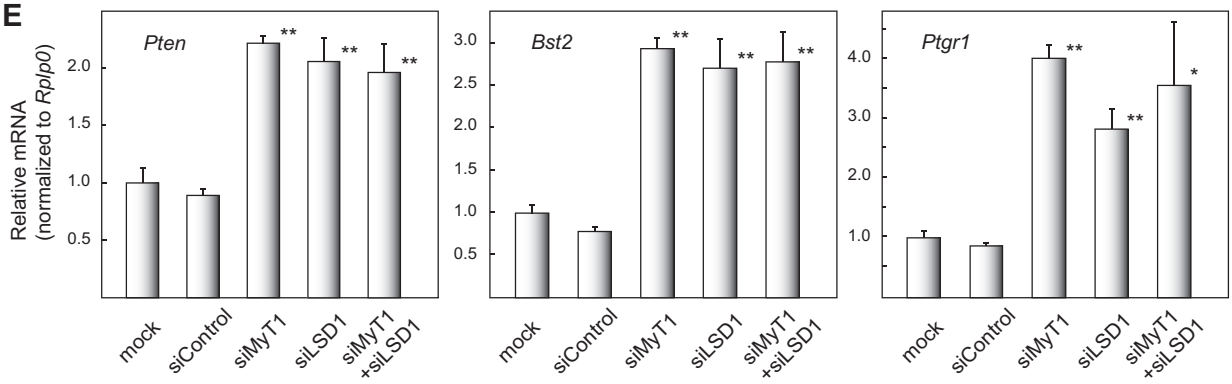
D

Ratio > 2, p-value < 0.05, transcript > 0.1

Probe Set ID	Gene Symbol	GenBank ID	siControl	siMyT1	siLSD1
1450721_at	Acp1	AW554436	18	46	39
1450655_at	Pten	AA214868	9.2	21	19
1426454_at	Arhgdib	AK002516	3.5	8.7	6.9
1424921_at	Bst2	BC008532	2.4	5.5	5.1
1418947_at	Nek3	NM_011848	1.7	4.0	4.0
1417777_at	Ptgr1	BC014865	1.5	4.7	3.0
1455123_at	St18	BB178719	0.99	2.2	2.1
1435460_at	Prkg2	BB363188	0.79	2.5	1.8
1445687_at	Gm885	BE629676	0.30	1.1	0.90
1450958_at	Tm4sf1	BQ177170	0.25	0.68	0.74
1421937_at	Dapp1	NM_011932	0.34	0.75	0.70
1453498_x_at	Steap3	AK003108	0.19	0.49	0.53
1437460_x_at	Rin1	BB264363	0.25	0.58	0.52
1429403_x_at	Glt8d2	AK003894	0.19	0.52	0.51
1429153_at	6530406A20Rik	AK018324	0.19	0.48	0.44
1449508_at	Il27ra	NM_016671	0.13	0.41	0.39
1427486_at	Ptprb	AF157628	0.13	0.38	0.33

Ratio < 0.5, p-value < 0.05, transcript > 0.1

Probe Set ID	Gene Symbol	GenBank ID	siControl	siMyT1	siLSD1
1437056_x_at	Crispld2	BB558800	1.5	0.67	0.71
1437233_x_at	LOC100047869	AV339269	0.52	0.20	0.16
1441034_at	Itsn1	BB184062	0.41	0.17	0.15
1433178_at	4933427C19Rik	AK016944	0.33	0.12	0.10
1419532_at	Il1r2	NM_010555	0.31	0.13	0.069
1425733_a_at	LOC632638	BC016890	0.31	0.15	0.13
1432380_s_at	Gsdmcl2	AK019629	0.30	0.13	0.13
1450305_at	4930591A17Rik	NM_026596	0.27	0.13	0.12



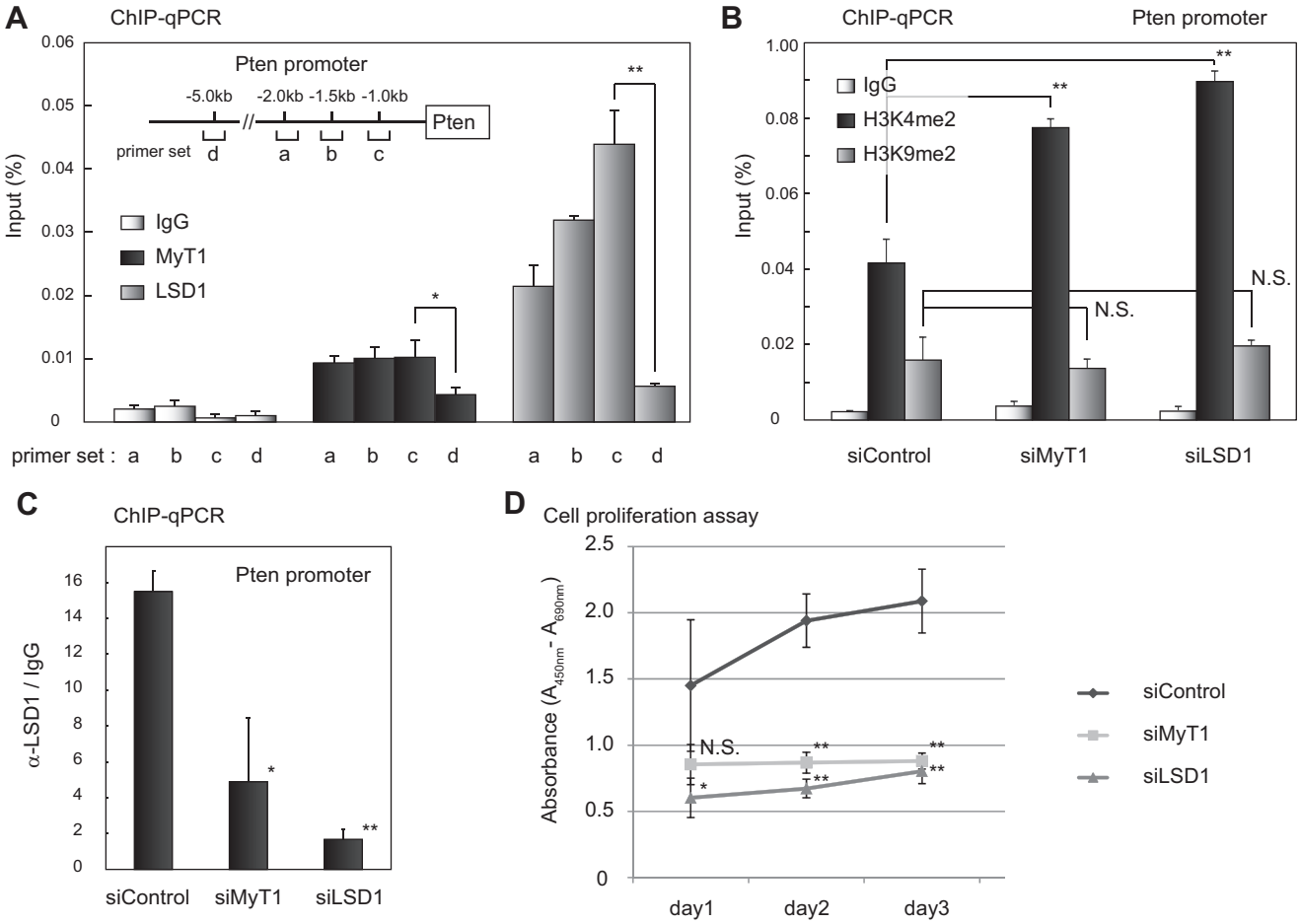


FIGURE 6. **The nLSD1 complex was recruited to the endogenous *Pten* gene promoter via MyT1.** *A*, chromatin immunoprecipitation analysis of MyT1 and LSD1 at the indicated promoter regions. DNA fragments in Neuro2a cells were precipitated with anti-MyT1 and anti-LSD1; rabbit IgG was used as a negative control for the immunoprecipitation. Precipitated DNA fragments were assessed by qPCR using indicated primer sets. The *error bars* indicate standard deviations ($n = 3$). *. $p < 0.05$; **, $p < 0.01$. *B*, the effect of *Myt1* and *Lsd1* RNAi on histone tail modification. ChIP-qPCR analysis was performed using the indicated antibodies and primers for *Pten* promoter c region. siControl and IgG constituted the negative controls. The *error bars* indicate standard deviations ($n = 3$). **, $p < 0.01$. *N.S.*, not significant. *C*, the effect of MyT1 knockdown for recruitment of the LSD1 protein to the *Pten* gene promoter (primer: c). The *error bars* indicate standard deviations ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *D*, cell proliferation assays were performed using the WST-1 Cell Proliferation Assay System. The absorbance of each cell at indicated time points is shown. The *error bars* indicate standard deviations ($n = 4$). *, $p < 0.05$; **, $p < 0.01$. *N.S.*, not significant.

complex. To determine whether the nLSD1 complex directly controlled this gene, we conducted ChIP assays using Neuro2a cells. Using antibodies shown in Fig. 6*A*, the immunoprecipitated chromatin samples were subjected to qPCR using primers corresponding to the indicated promoter regions of the *Pten* gene. The distal region of the *Pten* gene promoter (5.0 kb upstream from the transcription start site) was used as a negative control. As shown in Fig. 6*A*, MyT1 and LSD1 were both detected in the promoter region of the *Pten* gene but not in the distal region, suggesting that this gene was directly targeted by the nLSD1 complex.

Next, we used ChIP analysis to determine if the recruited nLSD1 complex modified histones at the *Pten* promoter. Knockdown of either *Myt1* or *Lsd1* attenuated demethylation of H3K4me2 without affecting the H3K9 methylation state (Fig. 6*B*). Furthermore, knockdown of *Myt1* by siRNA decreased the amount of LSD1 recruitment at the *Pten* gene promoter (Fig. 6*C*). Therefore, it appears that the nLSD1 complex directly bound to the *Pten* gene promoter through MyT1 protein and demethylated H3K4me2 at its promoter. It is well established that Pten protein negatively regulates cell proliferation and its overexpression alone can induce cell cycle arrest (47, 49, 50).

FIGURE 5. **Global expression analysis of nLSD1-regulated genes by the Percellome method.** *A*, confirmation of RNAi specificity for *Myt1* and *Lsd1*. Neuro2a cells were transfected with each siRNA and examined for expression of *Myt1* and *Lsd1* by qPCR. The *error bars* indicate standard deviations ($n = 3$). **, $p < 0.01$ *B*, confirmation of RNAi specificity for MyT1 and LSD1 by Western blotting (*IB*). The actin band was used as a loading control for Western blotting. *C*, Venn diagram of the number of genes altered in each knockdown experiment. The criteria for increased gene expression were the following: (i) for each siRNA-treated cell, the ratio of the expression level of each gene to the expression level in the control sample was >2; (ii) the *p* value was <0.05, $n = 3$ (*upper figure*). The Venn diagram of the number of genes down-regulated in each knockdown experiment (ratio <0.5, *p* value < 0.05, $n = 3$) is shown in the *lower figure*. *D*, the altered gene list by Percellome microarray analysis. The numbers shown in the tables are estimated transcript numbers per cell. Of the 69 genes that were increased in both si*Lsd1*- and si*Myt1*-treated samples (ratio >2, *p* value < 0.05, $n = 3$), we show those genes with a transcript copy number >0.1 per cell in the *upper table*. The genes decreased in both si*Lsd*1- and si*Myt*1-treated cells are shown in the *lower table*. *E*, qPCR analysis of candidate target genes expression in si*Lsd1*-, si *Myt1*-, and si*Lsd1* + si*Myt1*-treated cells. Gene expression was normalized to that of *Rplp0* and presented as the fold-change compared with the expression levels in non-treated cells (mock). The *error bars* indicate standard deviations ($n = 3$). *, $p < 0.05$; **, $p < 0.01$.

Finally, we explored the effects of *Myt1* and *Lsd1* RNAi on cell proliferation of Neuro2a cells. As shown in Fig. 6*D*, Neuro2a cell proliferation decreased due to down-regulation of either *Myt1* or *Lsd1* expression. Taken together, it appears that the nLSD1 complex controls cell proliferation, presumably via *Pten* gene regulation in Neuro2a cells.

DISCUSSION

Gene regulation requires basic transcriptional machinery as well as transcriptional coregulators (2, 51, 52). Transcriptional coregulators include ATP-dependent chromatin remodeling factors and histone modifiers (5). Biochemical approaches have shown that these transcriptional coregulators act in many cases as multisubunit complexes that possess distinct enzymatic activities (3). However, there had been little information about the compositional exchanges in these coregulators in a developmental stage- and cell type-specific manner. In this context, studies of the TRF3 complex (myogenesis-specific TFIID-like complex) and nBAF complex (neurogenesis-specific SWI/SNF complex) suggested that significant changes in these transcriptional regulators might facilitate tissue-specific transcriptional regulation (19–22, 53, 54). However, it has remained elusive whether switching of these complex components occurs in histone modifier complexes.

In the present study we demonstrated that histone demethylase LSD1 forms a neural cell-specific complex (nLSD1) that is critical for transcriptional regulation in Neuro2a cells. The nLSD1 complex is composed of LSD1, CoREST, HDAC1/2, and MyT1, a neural cell-specific zinc finger factor that directly interacts with LSD1. Previously, neurospecific LSD1 isoforms were reported to be essential for neurite maturation (31), although binding of MyT1 is not limited to a specific LSD1 isoform (data not shown). The identification of the nLSD1 complex shows that histone modifying factor complexes can also form a tissue-specific multiprotein complex like other transcriptional apparatus such as the TFIID complex and the SWI/SNF complex (19). This finding supports and extends the recent evidence that significant switching in general transcription machinery facilitates global changes in cell type-specific transcription.

The *Myt1* gene was initially cloned from a fetal brain-derived cDNA library as a *cis*-regulatory element of the *Plp1* gene binding factor (38). It is one of the three C2HC-type zinc finger factors that include MyT1, MyT1L, and MyT3 (39, 44, 55). All these paralogues are highly expressed in developing neuronal cells (39, 55, 56). MyT1 can behave either as a transcriptional activator or a repressor in a cell context-dependent manner, although the precise mechanisms by which MyT1 regulates specific target genes remain obscure (39, 57). Using a biochemical approach in this study, we found that LSD1 forms a multiprotein complex containing MyT1 as a neural cell-specific complex component. This result can explain the transcriptional repression mechanism of MyT1 for its targeted genes. From the present data (Fig. 6*C*), it is possible that MyT1 recognizes its targeted DNA sequences and recruits the complex to the sequences, a function that is analogous to DNA binding transcription factors. This hypothesis stems from the fact that MyT1 contains C2HC-type zinc finger motifs and that MyT1L, a paralogue of MyT1, reportedly binds to specific DNA sequences based on *in vitro* DNA binding experiments (39, 58).

Recently, *Myt1l* was reported to be one of the three genes necessary for the efficient *in vitro* conversion of mouse fibroblasts into functional neurons (59). We have confirmed that the interaction between MyT1 and LSD1 is conserved among the MyT family of proteins (data not shown). Thus, LSD1 might be involved in this direct conversion process as a partner of MyT1L. It is possible that the MyT family of proteins coordinately modulates neuro-specific genes by regulating each specific target gene forming complexes with LSD1.

In the microarray analysis, a large fraction of the genes were specific targets of either MyT1 or LSD1 (Fig. 5*C*). We attribute this result to the likelihood that there are multiple protein complexes that contain MyT1 or LSD1. In fact, MyT1 is reported to form a complex with Sin3B (57), and LSD1 is reported to form a complex with many factors such as Mi-2 and MLL1 (60, 61). We believe that this is why a large fraction of the genes were not observed in common. ChIP-seq experiments for both MyT1 and LSD1 would add important information in this regard.[3]

Importantly, most previous studies isolating histone modifier complexes used widely utilized cell types such as HeLa cells and S2 cells (26, 28). In contrast, the present study is the first case in which histone modifier complexes were purified from lineage-specified cells (Neuro2a). Therefore, it is conceivable that as yet unidentified tissue-specific factors could form complexes with LSD1 in different cell types to facilitate tissue-specific transcriptional regulation. Future identification of another tissue-specific LSD1 complex would provide additional details of specific transcriptional regulation. Furthermore, because LSD1 is reported to be a potential drug target in many diseases such as cancer and mental disorders (24, 62–65), our findings provide an attractive rationale for tissue-specific drug actions. Thus, further cell type-specific components of transcriptional coregulator complexes are expected to be identified in the near future.

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