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Ldb1 Is Essential for the Development of Isthmic Organizer and Midbrain Dopaminergic Neurons

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LIM domain-binding protein 1 (Ldb1) is a nuclear cofactor that interacts with LIM homeodomain proteins to form multiprotein complexes that are important for transcription regulation. Ldb1 has been shown to play essential roles in various processes during mouse embryogenesis. To determine the role of Ldb1 in mid- and hindbrain development, we have generated a conditional mutant with a specific deletion of the *Ldb1* in the *Engrailed-1*-expressing region of the developing mid- and hindbrain. Our study showed that the deletion impaired the expression of signaling molecules, such as fibroblast growth factor 8 (FGF8) and Wnt1, in the isthmic organizer and the expression of Shh in the ventral midbrain. The midbrain and the cerebellum were severely reduced in size, and the midbrain dopaminergic (mDA) neurons were missing in the mutant. These defects are identical to the phenotype that has been observed previously in mice with a deletion of the LIM homeodomain gene *Lmx1b*. Our results thus provide genetic evidence supporting that Ldb1 and Lmx1b function cooperatively to regulate mid- and hindbrain development. In addition, we found that mouse embryonic stem cells lacking Ldb1 failed to generate several types of differentiated neurons, including the mDA neurons, serotonergic neurons, cholinergic neurons, and olfactory bulb neurons, indicating an essential cell-autonomous role for Ldb1 in the development of these neurons.

Introduction

The patterning of the mid- and hindbrain and generation of midbrain dopaminergic (mDA) neurons are complex processes regulated by a large number of signaling molecules and transcription factors [1–4]. Two closely related LIM homeodomain proteins, Lmx1a and Lmx1b, play important roles in these processes. The *Lmx1b* gene, expressed in the isthmic organizer, is essential for patterning of the mid- and hindbrain by controlling the expression of secreted signaling molecules, such as fibroblast growth factor 8 (FGF8) and Wnt1, in embryos of various vertebrate species, including chick, zebrafish, and mouse [5–7]. *Lmx1b*, together with *Lmx1a*, is also expressed in mDA neuron progenitors and required for the proliferation, specification, and differentiation of the mDA progenitors [8,9]. In addition, gain-of-function studies have shown that ectopic expression of *Lmx1a* or *Lmx1b* induces the generation of mDA neurons *both in* embryos and in differentiating embryonic stem (ES) cells [10–12].

The function of LIM homeodomain proteins is largely dependent on the formation of multiprotein complexes through interactions between these proteins and other nuclear factors [13,14]. A key component of these complexes is a transcription coregulator called ''LIM domain-binding protein 1'' [(Ldb1), also called ''NLI'' or ''CLIM2''] [15–17]. A number of previous studies have revealed that Ldb1 is essential for the regulation of a variety of processes in mouse embryogenesis, including the head and heart formation, limb patterning, and forebrain and cerebellum development [18–21].

In this study, to determine the role of *Ldb1* in mid- and hindbrain development, we generated a conditional mouse mutant to delete *Ldb1* more specifically in the mid- and hindbrain regions during embryonic development by crossing the mutant $Ldb1^{fl-}$ [20] with mice expressing the Cre recombinase under the control of the regulatory element of the *Engrailed-1* gene ([$En1^{Cre}$], [22]). Our analysis of the *Ldb1* mutant revealed a phenotype similar to that observed previously in *Lmx1b* null mutant [7]. Our results provide genetic evidence suggesting that *Ldb1* cooperates with *Lmx1b* in regulation of mid- and hindbrain development. In addition, we provide evidence supporting that *Ldb1* is also

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required for the generation of several types of terminally differentiated neurons, including the mDA neurons, from differentiation of the mouse ES cells.

Materials and Methods

Mouse lines and genotyping

For mouse care and experiment, we followed the guideline of the Korea University Animal Care and Use Committee, and our IRB number is KUIACUC-20111024-2.

To generate conditional mutants with a specific deletion of *Ldb1* in the mid- and hindbrain regions, the *Engrailed-1*$^{+/Cre}$ (*En1*$^{+/Cre}$) mouse line, which contains an insertion of the Cre recombinase gene into the *En1* locus [22], was first crossed to *Ldb1*$^{+-}$ mice [18]. Offspring containing one *Ldb1* null allele and one En1^{Cre} allele (*Ldb1*$^{+/-}$ and *En1*$^{+/Cre}$) were selected and mated to either heterozygous or homozygous *Ldb1* floxed (*Ldb1*$^{+/f}$ or *Ldb1*$^{f/f}$) mice [20] to produce *Ldb1* conditional mutants (*Ldb1*$^{f/-}$ and *En1*$^{+/cre}$) and controls (wild type, *Ldb1*$^{+/f}$, and *Ldb1*$^{+/f}$ and *En1*$^{+/Cre}$) for analysis. Mouse genotypes were determined by polymerase chain reaction (PCR) as described in previous studies [23] using the following primers: *Ldb1* wild-type and floxed alleles: 5′-CAGCAAACG GAGGAAACGGAAGATGTCAG and 5′-CTTATGTGACC ACAGCCATGCATGCATGTG; *Ldb1* null allele: 5′-ACGA GTTCTTCTGAGGGGATC and 5′-TGCCACACAGAATC TGCTCTGAACGTCT; and *En1*^{Cre} allele: 5′-CACCCTGT TACGTATAGCCG and 5′-GAGTCATCCTTAGCGCCG.

Whole-mount in situ hybridization

Embryos were fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) and processed for whole-mount in situ hybridization with digoxigenin-labeled RNA probes according to a well-established protocol [24]. The hybridization signal was detected with alkaline phosphatase-conjugated antidigoxigenin antibodies and BM purple substrate (Roche Diagnostics). *Fgf8* probe (nucleotides 591–943, GenBank D38752) was previously described [25]. Shh and Wnt1 probes were a generous gift from Dr. Andrew McMahon.

Whole-mount immunohistochemistry

Embryos were fixed in 4% PFA, 0.15% picric acid, and 0.1% Tween 20 in PBS for 5 h at 4°C. Fixed embryos were washed with PBS containing 0.1% Tween 20 (PBT) two times at 4°C, dehydrated in methanol, and bleached with 3% hydrogen peroxide in 80% methanol and 20% DMSO for 3 h at room temperature (RT). The embryos were washed at RT in 1% Tween 20/PBS for 3 h, 1% Triton X-100/PBT for 20 min, and 0.5% Triton X-100/PBT three times each for 15 min. Embryos were blocked in 5% skim milk, 0.5% Triton X-100, and 5% DMSO/PBT for 1 h at RT, followed by incubation with primary antibodies diluted in the same blocking solution for 2 days at RT. The following primary antibodies from Santa Cruz Biotech were used at a dilution of 1:100: goat anti-FGF8 and goat anti-sonic hedgehog (SHH). Afterward, embryos were washed in 0.1% Triton X-100/PBT and incubated with horseradish peroxidase-conjugated secondary antibodies (Dako) overnight at 4°C, washed in 1% Tween 20/0.5% Triton X-100/PBS, and developed for 10–30 min in DAB (Sigma).

Mouse ES cell culture

Ldb1$^{-/-}$ ES cells were generated as described previously [23]. ES cell culture medium is composed of Dulbecco's modified Eagle's medium mixed with 15% fetal bovine serum, 100 mM nonessential amino acids, 0.5% penicillin–streptomycin, 0.55 mM 2-mercaptoethanol, and 1,000 U/mL leukemia inhibitory factor (Chemicon). Both wild-type and *Ldb1*$^{-/-}$ ES cells were cultured in 0.1% gelatin-coated dishes at 37°C in an incubator containing 5% CO_2. For differentiation of the ES cells to neurons, the adherent monolayer culture method was followed as previously described without any modifications [26].

Immunocytochemistry

Cells were fixed with 4% PFA in PBS (pH 7.4) for 20 min at RT, followed by washes in PBS. The cells were treated with 1% sodium dodecyl sulfate for 5 min for antigen retrieval. After

Table 1. List of Primer Sets for Reverse Transcription Polymerase Chain Reaction

	Primer sequences		
Gene	*Upper primer sequence*	*Lower primer sequence*	*Length (bp)*
GAPDH	5′-GTGTTCCTACCCCCAATGTG-3′	5′-TGTGAGGGAGATGCTCAGTG-3′	400
Nurr1	5′-CGGTTTCAGAAGTGCCTAGC-3′	5′–CTGGGTTGGACCTGTATGCT-3′	420
Pitx3	5′-ACAAAGTGGAACCCCTATGAG-3′	5′-TTCTTGGCCAATCTGTAGGA-3′	255
TH	5′-TTGGCTGACCGCACATTTG-3′	5′-ACGAGAGGCATAGTTCCTGAGC-3′	336
DAT	5′-CACTCTGGGTATCGAC-3′	5′-ATGGCATAGGCCAGTTTCTC-3′	537
Ldb1	5′-TGCTGACCATCACTTTCTGC-3′	5′-GGCTGAGGCTGTAGGTCTTG-3′	480
Lhx2	5′-TCAACTGCTTCACATGCACA-3′	5′-TTTCCTGCCCTAAAAGGTTG-3′	590
Lhx5	5′-GACCTCATCGGACAAGGAAA-3′	5′-ACCCCAACATCTCAGACTCG-3′	352
Lhx6	5′-CTGTGCGGCAGACAAATCTA-3′	5′-CTCTCAATGTAGCCGTGCAA-3′	572
Lhx8	5′-CAGTTCGCTCAGGACAACAA-3′	5′-CTTATTGGCAGCTGGGTCAT-3′	331
Lmx1b	5′-CTGCTGTGCAAGGGTGACTA-3′	5′-GGCTTGACAGAACCTCTTGG-3′	420
OMP	5′-GAAGCAGGATGGTGAGAAGC-3′	5′-CACAGAGGCCTTTAGGTTGG-3′	361
Calretinin	5′-CTCCTGAAGAAGGCCAACAG-3′	5′-GGTCTGGGAAGGAGTGTCAA-3′	488
GAD2	5′-GGGTTTGAGGCACACATTGATAAG-3′	5′-GCGGAAGAAGTTGACCTTGTCC-3′	279
ChAT	5′-GCCAATCCATTCCCACTGAC-3′	5′-CATCCAAGACAAAGAACTGG-3′	198
Sert	5′-GTGACAGCCACCTTCCCTTA-3′	5′-CTAGCAAACGCCAGGAGAAC-3′	200

more washes in PBS, the cells were incubated in a blocking solution containing 0.3% Triton X-100 and 3% bovine serum albumin (BSA) for 45 min and then in primary antibody diluted with the same blocking solution overnight at 4°C. After washes in PBS, the cells were incubated in secondary antibodies diluted in the blocking solution for 1 h at RT. After washes in PBS, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with Fluoromount-G (Southern Biotech). The primary antibodies used were rabbit anti-MAP2 (Chemicon), rabbit anti-GFAP (Chemicon), mouse anti-TH (Chemicon), and rabbit anti-LDB1 (a gift from Dr. Paul Love, NIH, Bethesda, MD) [27]. Images were captured using a Zeiss LSM 510 confocal laser scanning microscope.

Histology and immunohistochemistry

Brains of E14.5 and E18.5 mouse embryos were dissected and fixed in 4% PFA/PBS overnight at 4°C. After washes in PBS, the tissue was either dehydrated in ethanol and embedded in paraffin or soaked with 30% sucrose/PBS and frozen in OCT compound (Sakura Finetek). For histological analysis, 5-μm-thick paraffin sections were cut and stained with hematoxylin and eosin (Sigma). For immunohistochemistry, frozen sections (14 μm) were cut and mounted on silane-coated slides (Muto Pure Chemicals Co., Ltd.). Sections were washed in PBS and incubated in a blocking solution containing 0.3% Triton X-100 and 3% BSA for 45 min. The sections were incubated with primary antibodies diluted with the same blocking solution overnight at 4°C. After washes in PBS, the sections were incubated in secondary antibodies diluted with the same blocking solution for 1 h at RT. After washes in PBS, the sections were stained with DAPI and mounted with Fluoromount-G. Images were taken by a Zeiss LSM 510 confocal laser scanning microscope. The primary antibodies used were rabbit antityrosine hydroxylase (Chemicon), rabbit anti-Pitx3 (Chemicon), rabbit anti-Nurr1 (Santa Cruz Biotech), rabbit anti-GABA (Sigma), rabbit anti-LDB1 (a gift from Dr. Paul Love, NIH, Bethesda, MD), and rabbit anti-caspase 3 (Merck Millipore).

Real-time reverse transcription PCR analysis

Total RNA was isolated from cells using a ToTALLY RNA™ Kit (Ambion) by following the manufacturer's guidance. One microgram of the RNA template was reverse transcribed by a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the instruction from the manufacturer. Real-time PCR was performed using 2 μL aliquot of the reverse-transcribed product for each 20 μL sample reaction mixture containing 4 mM $MgCl_2$, 10 pmole of upstream and downstream primers, and 2 μL of 10X Light Cycler Fast Start DNA Master SYBR Green 1 (Roche Diagnostics). Light Cycler software (version 3.5) was used to analyze the data. The list of primer sets used for reverse transcription PCR (RT-PCR) is shown in Table 1.

Results

Defects in development of the midbrain and cerebellum in Ldb1 *conditional mutant*

Homozygous *Ldb1* null mutant embryos die at E9.5 [18]. To study the function of *Ldb1* in the development of the mid- and hindbrain in later stages of embryos, we generated *Ldb1* conditional mutant embryos that carried one floxed (f) allele [20] and one null (–) allele of the *Ldb1* gene in addition to a knock-in allele containing a targeted insertion of the *Cre* recombinase gene into the *En1* locus ($En1^{Cre}$) [22] ($Ldb1^{f/-}$ and $En1^{+/Cre}$). As revealed by X-gal staining of embryos from crossing between the $En1^{+/Cre}$ and the *Rosa26R* ($Rosa26^{LoxP\text{-}stop\text{-}LoxP\text{-}LacZ}$) reporter line [28], the Cre recombinase was active in the mid- and hindbrain junction of the embryo as early as E8.5 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). As shown by in situ hybridization, the *En1* expression domain at the mid- and hindbrain junction was reduced in size in *Ldb1* conditional mutant embryos in comparison with wild-type embryos at E9.5 (Fig. 1A, A′). The conditional deletion of *Ldb1* was also confirmed by the absence of

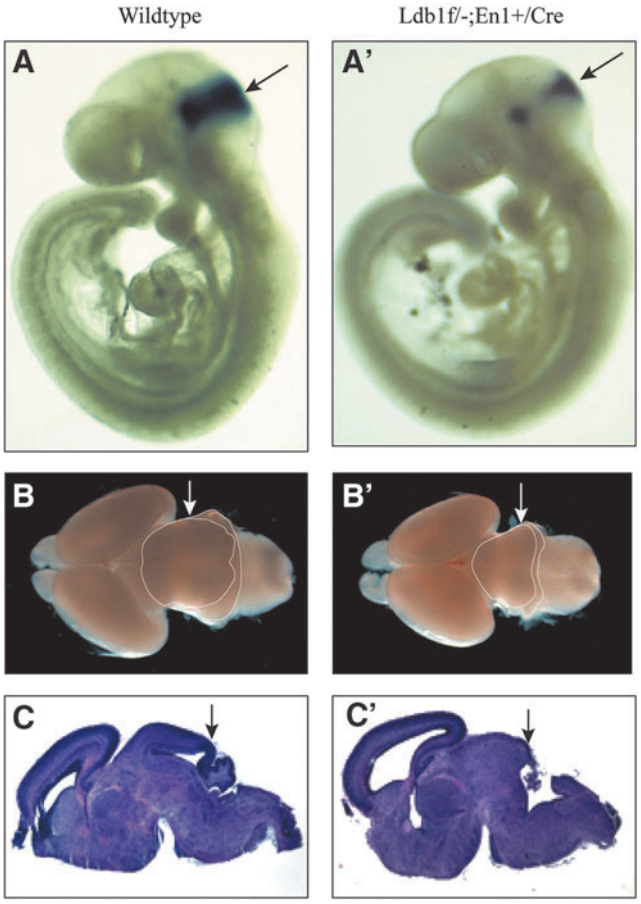

FIG. 1. Defects in the development of the mid- and hindbrain structures in *Ldb1* conditional mutant. **(A, A′)** Whole-mount in situ hybridization showing a reduction in the expression of *En1* in the mid- and hindbrain junction in an E9.5 *Ldb1* mutant embryo **(A′)** compared with a control **(A)**. **(B, B′)** Dorsal view of brain dissected from E18.5 control **(B)** and *Ldb1* mutant **(B′)** embryos showing a truncation of the midbrain and the cerebellum in the mutant. **(C, C′)** Hematoxylin and eosin-stained sagittal sections of the brain showing a severe reduction in size of the dorsal midbrain and missing of the cerebellum in the *Ldb1* mutant **(C′)** compared with the control **(C)** at E18.5.

immunostaining of Ldb1 in the midbrain of the mutant embryo (Supplementary Fig. S2). At E18.5, the *Ldb1* conditional mutant embryo showed a clear truncation of the midbrain and missing of almost the entire cerebellum (Fig. 1B, B′, C, C′). Thus, the conditional *Ldb1* deletion severely impaired the development of the midbrain and the cerebellum.

Impaired Fgf8, Wnt1, *and* Shh *gene expression in the isthmus organizer and ventral midbrain in Ldb1 conditional mutant*

Signaling molecules, such as Fgf8, Wnt1, and Shh, from the isthmus organizer or the ventral midbrain play important roles in the development of the mid- and hindbrain (see [3,4] for a detailed review). By performing whole-mount in situ hybridization analysis, we examined the expression of these molecules in *Ldb1* conditional mutant embryos. At E10.5, while *Fgf8* RNA was detected in the forebrain and the pharyngeal regions, it was absent at the mid- and hindbrain junction in *Ldb1* mutant embryos compared with wild-type controls (Fig. 2A, A′). Similarly, the expression of *Wnt1* was unaffected in the forebrain and dorsal midbrain, but the expression in a sharp semicircular domain at the mid- and hindbrain junction was missing in the mutant embryos compared with the controls (Fig. 2B, B′). *Shh* was expressed in the ventral diencephalon and ventral midbrain. The *Shh* mRNA expression in the ventral midbrain was reduced in *Ldb1* mutant embryos compared with the controls (Fig. 2C, C′). We also performed whole-mount immunostaining of embryos with antibodies directed against Fgf8 and Shh. The staining revealed that Fgf8 and Shh proteins were either missing or reduced in the midbrain in *Ldb1* mutant embryos compared with the controls (Fig. 2D, D′, E, E′). The absence of Fgf8 expression in *Ldb1* mutant embryos was further confirmed on sagittal sections (Fig. 2d, d′). These results indicate that the conditional deletion of *Ldb1* impairs the expression of multiple signaling molecules required for mid- and hindbrain development.

Defects in generation of mDA neurons in Ldb1 *conditional mutant*

The isthmus organizer and signaling molecules, such as Fgf8, Wnt1, and Shh, are important for the patterning of the mid- and hindbrain and the specification of mDA neurons. To investigate whether the defect in mid- and hindbrain patterning and the impaired expression of the signaling molecules affect the generation of mDA neurons in *Ldb1* mutant embryos, we examined the expression of a number of the factors that are involved in mDA neuron fate determination, differentiation, or dopamine biosynthesis. At E14.5, neurons positive for tyrosine hydroxylase (TH), Pitx3, or Nurr1 were detected abundantly in the ventral region of the midbrain in wild-type control embryos (Fig. 3A, B, C), but these cells were either missing or greatly reduced in number in *Ldb1* mutant embryos (Fig. 3A′, B′, C′). However, there was not a significant difference in the number of GABA-positive neurons between control and *Ldb1* mutant embryos (Fig. 3D, D′). In line with the results from staining of sections, sagittal view of the brains stained by whole-mount immunohistochemistry also showed that neurons positive for TH, Pitx3, and Nurr1 were absent in the ventral midbrain in *Ldb1* mutant embryos (Fig. 3E′, F′, G′) compared with the controls (Fig. 3E, F, G). No difference in the number of $GABA^+$ cells was detected between the control and *Ldb1* mutant embryos (Fig 3H, H′). The absence of mDA neurons in *Ldb1* mutant embryos was further confirmed by anti-TH staining of a series of coronal sections through the ventral midbrain of E18.5 embryos (Fig. 4A, B). We also examined the expression of cleaved caspase 3 to determine whether there was an increase of cell apoptosis in association with the defect in the generation of mDA neurons. At E14.5, we did not detect a clear increase in the number of cells labeled positive by cleaved caspase 3 (Supplementary Fig. S3).

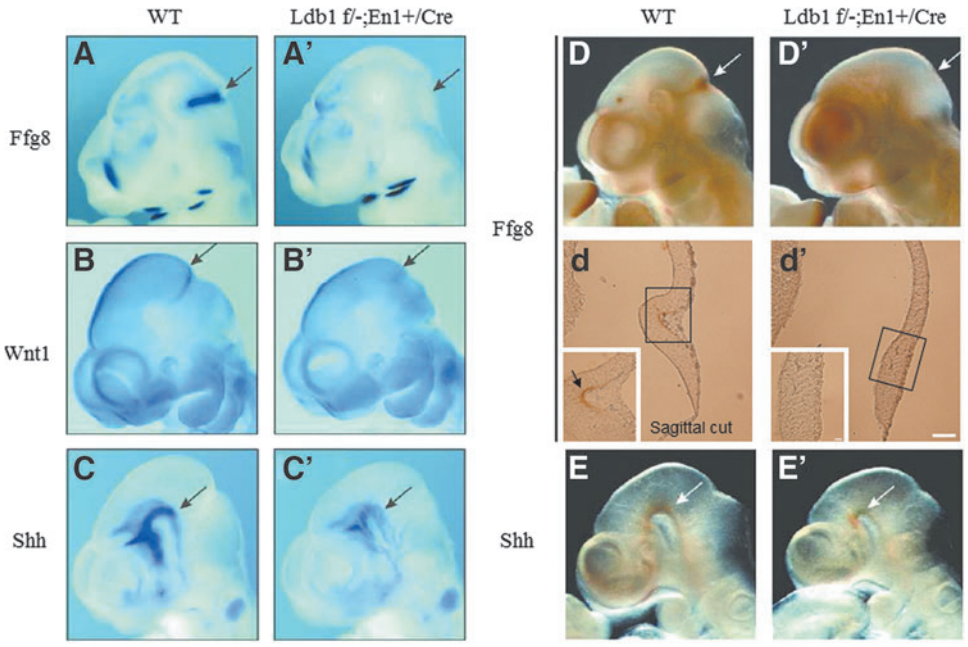

FIG. 2. Reduced expression of *Fgf8*, *Shh*, and *Wnt1* in *Ldb1* mutant embryos. Whole-mount in situ hybridization (**A–C**, **A′–C′**), whole-mount immunostaining (**D**, **E**, **D′**, **E′**), and sagittally sectioned brain (**d**, **d′**) of E10.5 embryos show missing of *Fgf8* and *Wnt1* expression in the isthmus in *Ldb1* mutant embryos (**A′**, **B′**, **D′**) compared with wild-type controls (**A**, **B**, **D**). The expression of *Shh* in the ventral region of the midbrain was reduced in *Ldb1* mutant embryos (**C′**, **E′**) compared with controls (**C**, **E**). Fgf8, fibroblast growth factor 8.

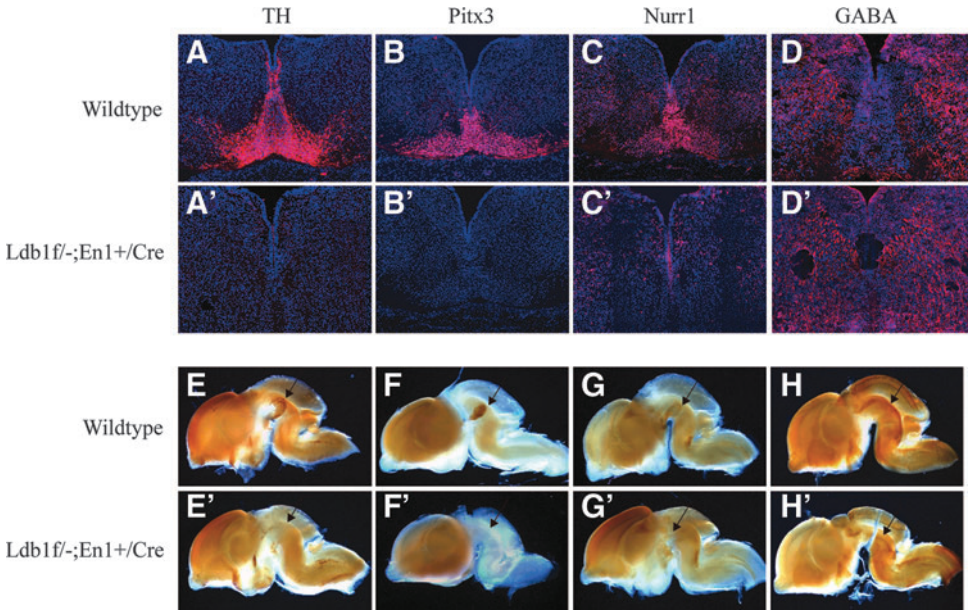

FIG. 3. Defects in the expression of mature mDA neuron markers in *Ldb1* mutant embryos at E14.5. (**A–D**, **A′–D′**) Coronal brain sections were stained with anti-TH, -Pitx3, -Nurr1, and -GABA antibodies. Staining of TH, Pitx3, and Nurr1 was missing or greatly reduced in *Ldb1* mutant embryos (**A′**, **B′**, **C′**) compared with the controls (**A**, **B**, **C**). No change in GABA staining was detected in *Ldb1* mutant (**D′**) compared with the control (**D**). (**E–H**, **E′–H′**) Similar results were shown by whole-mount immunostaining of brains from wild-type (**E–H**) and *Ldb1* mutant (**E′–H′**) embryos. mDA, midbrain dopaminergic.

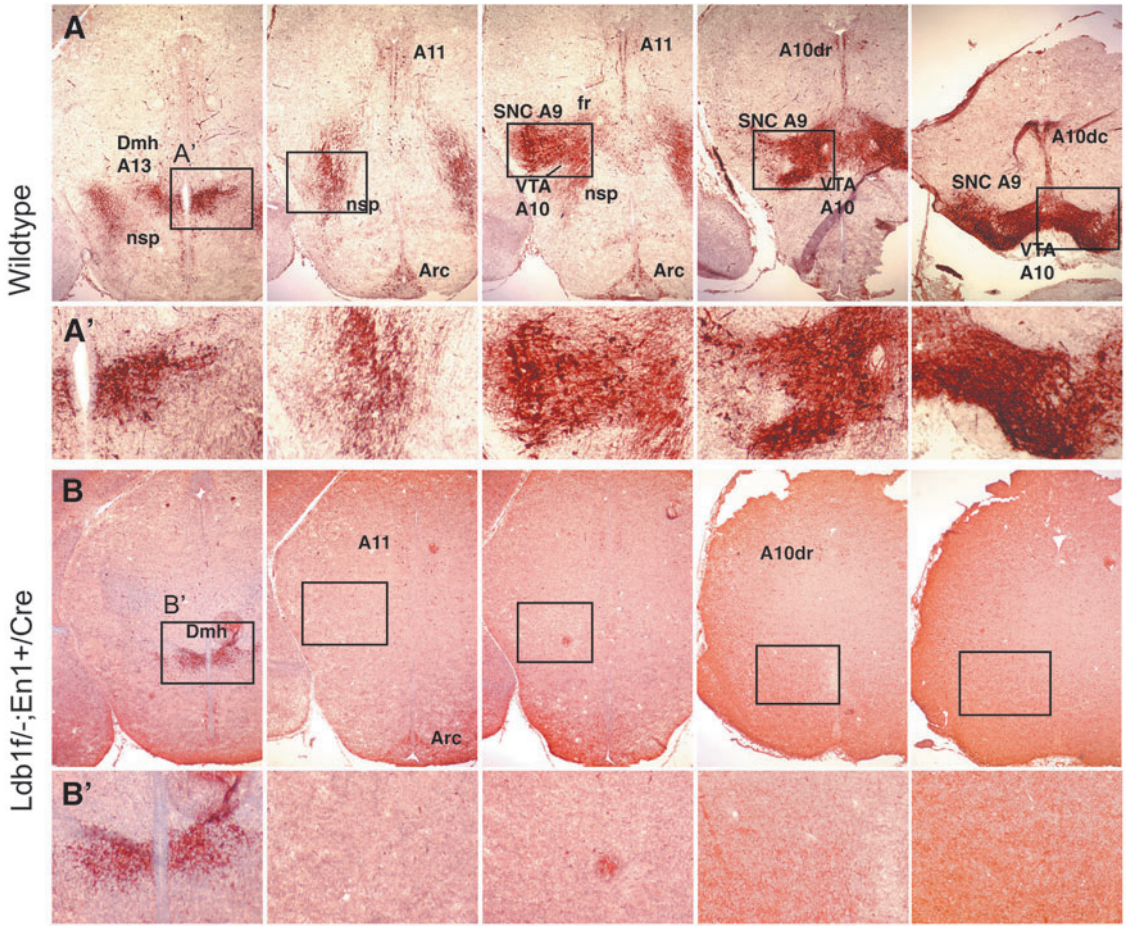

FIG. 4. Missing of mDA neurons in *Ldb1* mutant embryos. Anti-TH staining of a series of sections through the midbrain of E18.5 embryos shows missing of major groups of mDA neurons in *Ldb1* mutant (**B**) compared with the control (**A**). Sections from the mutant were overdeveloped to ensure no staining was detected. Higher magnified images of the *boxed area* in (**A**) and (**B**) are shown in (**A′**) and (**B′**). Arc, arcuate nucleus; Dmh, dorsomedial hypothalamus; nsp, nigra striatal projection; SNC, substantial nigra compacta; VTA, ventral tegmental area. A9, A10, A10dr (dorsorostral), A10dc (dorsocaudal), and A11 indicate the various TH^+-cell groups assigned by the alphanumeric system of Dahlstrom and Fuxe.

Defects in generation of dopaminergic and other differentiated neuronal cell types from the Ldb1$^{-/-}$ *ES cells*

Our previous study revealed that *Ldb1*$^{-/-}$ ES cells maintain neuronal differentiation potential [23]. As shown in Figure 5A and A′, at 2 weeks after differentiation, cells derived from both the *Ldb1*$^{-/-}$ and wild-type ES cells grown as adherent monolayer culture expressed the general neuronal cell marker MAP2 or the glial cell marker GFAP. To determine whether the deletion of *Ldb1* affects the potential of the ES cells to generate dopaminergic neurons, we

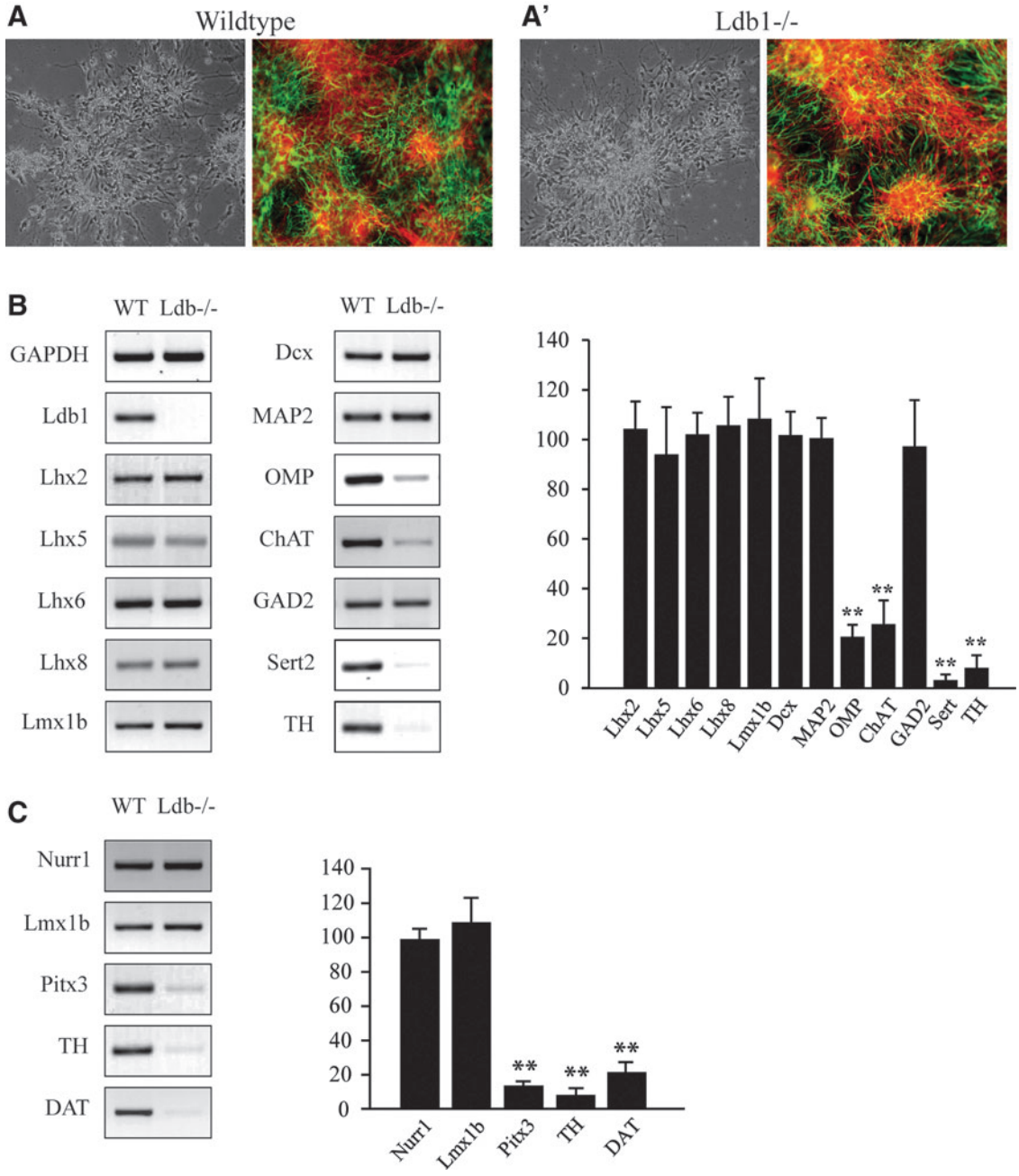

FIG. 5. Difference in gene expression between neurons derived from wild-type (WT) and *Ldb1*$^{-/-}$ ES cells. **(A, A′)** Both wild-type and *Ldb1*$^{-/-}$ ES cells were successfully differentiated into neurons (MAP2^{+}) and glial cells (GFAP^{+}) by the adherent monolayer culture method. Cells were immunostained with anti-MAP2 (*green*) and anti-GFAP (*red*) antibodies. (**B**) *Left*, quantitative RT-PCR products of molecular markers expressed in neurons derived from wild-type and *Ldb1*$^{-/-}$ ES cells. *Right*, graph shows the relative expression levels of the various markers compared with wild type, normalized with GAPDH, and presented as mean ± SEM. (**C**) *Left*, quantitative RT-PCR products of mDA neuron-specific markers expressed in neurons derived from wild-type and *Ldb1*$^{-/-}$ ES cells. *Right*, graph shows the relative expression levels of the markers compared with wild type, normalized with GAPDH, and presented as mean ± SEM. The mark ''**'' indicates a difference with statistical significance examined by Student's *t*-test ($P < 0.01$) between neurons derived from wild-type and *Ldb1*$^{-/-}$ ES cells. RT-PCR, reverse transcription polymerase chain reaction.

examined the expression of multiple markers of the dopaminergic neurons in cells derived from wild-type or *Ldb1*$^{-/-}$ ES cells after differentiation by quantitative RT-PCR. Our analysis revealed that the expression of all these markers, including *Nurr1*, *Pitx3*, *DAT* (encoding a dopamine transporter), and *TH*, was significantly reduced in cells derived from the *Ldb1*$^{-/-}$ ES cells compared with those derived from the control ES cells (Fig. 5B, C). In addition, the generation of other differentiated neuronal cell types, such as the cholinergic neurons, serotonergic neurons, and olfactory bulb neurons from the *Ldb1*$^{-/-}$ ES cells, was also impaired, as shown by the reduction in expression of their respective markers, choline acetyltransferase (*ChAT*), serotonin transporter (*Sert*), and olfactory marker protein (*OMP*) (Fig. 5B). In contrast, the expression of members of the LIM homeodomain gene family, including *Lhx1*, *Lhx2*, *Lhx5*, *Lhx6*, *Lhx8*, and *Lmx1b*, the general neuronal cell marker *MAP2*, and the neural stem cell marker *Dcx* was not significantly changed in cells derived from the *Ldb1*$^{-/-}$ ES cells compared with those derived from the control ES cells (Fig. 5B).

Discussion

Our study revealed that a conditional deletion of *Ldb1* in the *En1*-expressing region of the developing mid- and hindbrain severely impaired the function of the isthmic organizer as demonstrated by the absence in the expression of Fgf8 and Wnt1 (Supplementary Fig. S4). Consistent with the crucial roles of FGF8 and Wnt1 in patterning of the mid- and hindbrain in mouse embryonic development [29–33], the midbrain was drastically reduced in size, and the cerebellum was almost entirely missing in *Ldb1* conditional mutant. In a previous study, identical phenotype was observed in *Lmx1b* mutant embryos [7]. The identical phenotypes observed in both *Lmx1b* and *Ldb1* mutants support the idea that Lmx1b and Ldb1 work cooperatively to maintain or regulate the function of the isthmic organizer for mid- and hindbrain patterning.

In addition to the defect in patterning of the mid- and hindbrain regions, the mDA neurons were largely missing in the *Ldb1* conditional mutant, as shown by the absence of the staining of TH, Pitx3, and Nurr1. Lmx1b and the closely related Lmx1a are required for the specification, proliferation, and differentiation of the mDA progenitors in the ventral midbrain [8,9,34]. The function of Lmx1a and Lmx1b in the mDA progenitors may also be dependent on Ldb1. However, the missing of mDA neurons analyzed in this study could also be caused by the disruption of the signaling of Fgf8, Wnt1, and Shh and the early patterning defect of the midbrain. A clear demonstration of the cell-autonomous role for Ldb1 in development of the mDA progenitors in vivo will require the generation and analysis of mutant with a more specific deletion of the *Ldb1* in the mDA progenitors using other mouse line, such as the *Shh*^{Cre} line, to drive the expression of the Cre recombinase.

To address the issue whether Ldb1 plays a cell-autonomous role in the specification and differentiation of mDA neurons, we instead took an alternative approach by assessing the potential of the *Ldb1*$^{-/-}$ mouse ES cells for the generation of mDA neurons. Our result showed that while the *Ldb1*$^{-/-}$ ES cells still retained the ability to generate cells expressing the general neuronal or glial cell markers, they failed to produce cells expressing specific markers for mDA neurons. A previous gain-of-function study has revealed that overexpression of *Lmx1a* induces robust generation of mDA from the mouse ES cells [10]. Based on our result, this induction of ES cells for generation of mDA neurons by the Lmx1a may be dependent on the presence of Ldb1. Despite the impaired expression of specific markers of mDA neurons, RT-PCR analysis showed that *Lmx1b* and other LIM homeodomain genes, including *Lhx2*, *Lhx5*, *Lhx6*, and *Lhx8*, were expressed in neurons derived from the *Ldb1*$^{-/-}$ ES cells. This indicates that while these genes are expressed, the proper function of their products is critically dependent on Ldb1. Previous studies have shown that Ldb1 plays a central role in the formation of protein complexes that are essential for the function of the various LIM homeodomain proteins [13,20,21,35]. Lmx1b has been shown to interact with Ldb1 [36]. Therefore, although the expression of Lmx1b was not significantly affected, its function in development of mDA neurons was impaired by the deletion of the Ldb1.

Interestingly, in addition to mDA neurons, the cells derived from the *Ldb1*$^{-/-}$ ES cells were also impaired in expression of specific markers for other terminally differentiated neuronal cell types, including the cholinergic (ChAT^{+}), serotonergic (Sert^{+}), and olfactory bulb (OMP^{+}) neurons. It has been shown that various LIM homeodomain factors, such as Lhx8, Lmx1b, and Lhx2, are required, respectively, for the generation of these neurons [37–40]. Our results present here suggest that the generation of these neurons may also require Ldb1. Recently, analysis of the *Ldb1/Nkx2.1-Cre* conditional mutant showed that Ldb1 is indeed required in vivo for the development of the cholinergic neurons in the telencephalon [21]. The role of Ldb1 in development of the serotonergic and olfactory bulb sensory neurons in vivo remains to be determined.

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Author Disclosure Statement

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