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The Shh coreceptor Cdo is required for differentiation of midbrain dopaminergic neurons

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Abstract Sonic hedgehog (Shh) signaling is required for numerous developmental processes including specification of ventral cell types in the central nervous system such as midbrain dopaminergic (DA) neurons. The multifunctional coreceptor Cdo increases the signaling activity of Shh which is crucial for development of forebrain and neural tube. In this study, we investigated the role of Cdo in midbrain DA neurogenesis. Cdo and Shh signaling components are induced during neurogenesis of embryonic stem (ES) cells. *Cdo*$^{-/-}$ ES cells show reduced neuronal differentiation accompanied by increased cell death upon neuronal induction. In addition, *Cdo*$^{-/-}$ ES cells form fewer tyrosine hydroxylase (TH) and microtubule associated protein 2 (MAP2)-positive DA neurons correlating with the decreased expression of key regulators of DA neurogenesis, such as Shh, Neurogenin2, Mash1, Foxa2, Lmx1a, Nurr1 and Pitx3, relative to the *Cdo*$^{+/+}$ ES cells. Consistently, the *Cdo*$^{-/-}$ embryonic midbrain displays a reduction in expression of TH and Nurr1. Furthermore, activation of Shh signaling by treatment with Purmorphamine (Pur) restores the DA neurogenesis of *Cdo*$^{-/-}$ ES cells, suggesting that Cdo is required for the full Shh signaling activation to induce efficient DA neurogenesis.
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Introduction

The promise of stem cell therapies in neurodegenerative diseases such as Parkinson's disease has revived the pursuit to gain insights into molecular mechanisms that regulate the differentiation of stem/progenitor cells into specific neuronal populations, such as midbrain dopaminergic (DA) neurons (Barzilai and Melamed, 2003; Lang and Lozano, 1998; Tzschentke and Schmidt, 2000). These DA neurons regulate

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diverse physiological functions, including movement, attention, and reward behaviors and their loss is associated with Parkinson's disease. The DA neurons are first detected in the ventral medial midbrain at embryonic day (E) 10.5 and their generation is continued until E13.5 (Ono et al., 2007). The development of DA neurons is a multi-step process from the patterning of the mesencephalon, followed by specification and differentiation of DA neuronal precursors to mature and functional DA neurons. Each step is regulated in a complex but coordinated fashion by cell-intrinsic and extrinsic soluble factors [for review, see (Smits et al., 2006)]. Various soluble factors such as, Sonic hedgehog (Shh), Fibroblast growth factor 8 (FGF8), and Wnt1 have been shown to regulate the initial stages of DA neuronal development to specify DA progenitors in the medial ventral area of the midbrain (Cooper et al., 2010; Yang et al., 2013). These extrinsic signals control a number of key transcription factors such as Neurogenin2 (Ngn2), Mash1, Foxa2, Lmx1a, Lmx1b, Nurr1, and Pitx3 that are critical for various developmental stages of DA neurogenesis (Andersson et al., 2006a,b; Ferri et al., 2007; Kele et al., 2006; Martinat et al., 2006; Park et al., 2006; Smidt et al., 2000). Among these extrinsic factors, the spatio-temporal regulation of Shh signaling activities defines multiple progenitor pools in the developing midbrain (Joksimovic et al., 2009). Shh, expressed originally in the notochord and successively in the floor plate, functions as a ventral morphogenetic signal and regulates the generation of DA neurons from the floor plate of the midbrain. Shh signaling seems to initiate a cascade of transcription factors involved in promoting early specification and differentiation of DA neurons (Wu et al., 2012). Shh signaling has been reported to regulate directly the expression of Foxa2 and Mash1, and indirectly Ngn2 and Nurr1 expression through Foxa2 thereby promoting DA neurogenesis as well as regulating survival and maintenance of DA neuronal progenitors in the ventral midbrain (Bae et al., 2011; Lee et al., 2010; Lo et al., 2002; Sasaki et al., 1997; Voronova et al., 2011). In addition, Ngn2 appears to be critical for differentiation of DA progenitors into Nurr1-positive postmitotic DA precursors (Thompson et al., 2006) and in turn Nurr1 and Pitx3 promote the differentiation of DA precursors into tyrosine hydroxylase-positive (TH^+) DA neurons (Kim et al., 2007). Thus the tight control of Shh signaling activation will be critical to initiate this transcription factor cascade for efficient DA neurogenesis.

The binding of Shh to its primary receptor Patched1 (Ptch1) is regulated through signaling by a second membrane component, Smoothened and the activation of Gli family transcription factors which will turn on the downstream target gene expression, such as Ptch1 and Gli1 (Bayly et al., 2007; Fogel et al., 2008). The expression levels of Shh and its activities are tightly regulated during embryonic development via multiple mechanisms (Allen et al., 2011). Cdo, a member of the immunoglobulin (Ig) superfamily, functions as a coreceptor for Shh to induce full activation of Shh signaling together with two other coreceptors, Boc and Gas1 (Bae et al., 2011; Kang et al., 2007; Zhang et al., 2006a). During early development of the CNS, Cdo is expressed in two regions important for patterning; transiently in the prechordal plate and the notochord of the developing CNS, and more persistently in the dorsal region of the brain and the roof plate of the neural tube (Allen et al., 2011; Zhang et al., 2011). Consistent with this expression pattern, Cdo-deficient mice exhibit multiple defects, including holoprosencephaly, hydrocephalus and reduced cortical thickness (Zhang et al., 2006b, 2011). Based on our previous studies, Cdo appears to regulate neurogenesis via two independent pathways, p38MAPK and Shh signaling (Oh et al., 2009; Tenzen et al., 2006; Zhang et al., 2006b). Cdo promotes neurogenesis by activation of p38MAPK that can positively regulate the activity of the neurogenic bHLH (basic helix–loop–helix) factor, Ngn1 (Oh et al., 2009). In addition, Cdo-deficient mice exhibit defects in ventral neural cell fate specification in the neural tube and ventral forebrain in correlation with decreased Shh signaling (Allen et al., 2011; Tenzen et al., 2006; Zhang et al., 2006b). For full activation of Shh signaling, the transient expression of Cdo in the prechordal mesoderm or notochord appears to be critical to specify the ventral cell fates in the ventral forebrain and neural tube (Tenzen et al., 2006; Zhang et al., 2006b). Since Shh signaling is required for DA neurogenesis and Cdo can promote neuronal differentiation via activation of neural bHLH factors, we examined a potential role of Cdo in DA neurogenesis. Several studies have shown the usefulness of ES cell differentiation to investigate the molecular regulatory mechanism of DA neurogenesis. In this study, we utilize in vitro differentiation system of ES cells isolated from $Cdo^{+/+}$ and $Cdo^{-/-}$mice to examine the role and molecular mechanisms of Cdo in DA neurogenesis.

Materials and methods

Isolation of mouse embryonic fibroblasts (MEFs) and ES cells, and cell cultures

MEFs were derived from days 12.5–13.5 post coitus fetuses of strain ICR mice as previously described (Bae et al., 2011). The second passage of MEFs was mitotically inactivated by gamma irradiation and used as feeders for ES cells. To generate ES cells, blastocysts from intercrosses of $Cdo^{+/-}$mutant mice on a mixed 129SV/C57BL6 background were used. Briefly, blastocysts were allowed to attach to MEFs on 0.1% gelatin coated plates in ES cells growth medium [DMEM supplemented with 15% FBS, 2 mM glutamate, 0.1 mM nonessential amino acids, 20 mM HEPES (pH 7.3), 0.1 mM β-mercaptoethanol, 1% gentamicine and LIF (1000 U/ml)] for 3 days. The expanded inner cell mass cells were trypsinized and seeded onto fresh feeder cells on 0.1% gelatin coated plate. The culture media was replaced every second day for several days after the first trypsinization. By appearance of typical ES cell colonies, cells were trypsinized and expanded. ES cells were routinely propagated on feeder layers. We have established at least two independent ES cell lines for each genotype and these ES cells exhibited normal morphology and proliferation properties, compared to R1 ES cells.

Monolayer differentiation of ES cells into neurons was carried out as described previously (Oh et al., 2009; Ying et al., 2003). To remove MEFs from the ES cell cultures, ES cells were trypsinized for 5 min and plated on uncoated plates. After 2 h, when most of the MEFs were attached to plates, unattached ES cells were replated onto 0.1% gelatin-coated plates at a density of $0.5–1.5 \times 10^4/cm^2$ in the culture medium with LIF for 24 h and cells were transferred into the differentiation medium, DMEM/F12 plus N2/B27 supplement containing vitamin A (Invitrogen) without LIF. The

differentiation medium was replaced every second day throughout 24 days of the differentiation time course. To modulate Shh signaling activities, ES cells were treated with Shh agonists, recombinant Shh with a concentration of 200 ng/ml (R&D systems), 2 mM Pur, or a Shh antagonist 10 μM SANT1 (Calbiochem) either for the first two days of induction or for two days starting at D14 as indicated in the figure legend.

RNA extraction, RT-PCR and real-time PCR analysis

Total RNA was extracted from cells using easyBLUE total RNA extraction kit according to manufacturer's instructions (Intron). The first strand of cDNA was synthesized from 0.5 μg of total RNA using Superscript II Reverse Transcriptase (Invitrogen). RT-PCR reaction was done as previously described (Bae et al., 2008), and quantification was performed with Image-Gauge software (Fuji Film). The real-time PCR reaction was carried out using a real-time PCR system with the SYBR Green PCR master mix according to the manufacturer's instructions (Invitrogen). The relative amount of each transcript was normalized to *Gapdh* level. All experiments were carried out as duplicates and repeated at least twice. The forward (F) and reverse (R) primers used in this study are listed below.

Genes in Shh signaling:

Patched1 F; 5′-TTTTGGTTGTGGGTCTCCTC, R; 5′-TCCACCGTAAAGGAGGCTTA.

Gli1 F; 5′-GAAGGAATTCGTGTGCCATT, R; 5′-GGATCTGTGTAGCGCTTGGT.

Cdo F; 5′-GTGTGCTTGGGGTTATGGTC, R; 5′-CTCCATTTATGTTTCCACTC.

Boc F; 5′-TGCTCTGGGTGTCTTCATCA, R; 5′-ATGGCATGATCAGGTAGTTG.

Gas1 F; 5′-GGAACACTGACCCACACTC, R; 5′-AAAGACCCCCACCGTTCAG.

Stemness genes:

Oct4 F; 5′-AAGCCCTCCCTACAGCAGAT, R; 5′-GGTGCCTCAGTTTGAATGC.

Nanog F; 5′-GCTGCTCCGCTCCATAACTT, R; 5′-CTGGCTTTGCCCTGACTTTA.

Sox2 F; 5′-CTGGAGTGGGAGGAAGAGGT, R; 5′-AACGGCAGCTACAGCATGA.

Markers and regulators for neuronal differentiation:

β-tubulin III F; 5′-CAACGTCAAGGTAGCCGTGT, R; 5′-TCCGATTCCTCGTCATCA TC.

MAP2 F; 5′-AGCCGCAACGCCAATGGATT, R; 5′-TTTGGTCCGAGGCTGGCGAT.

NFM F; 5′-AGACCTTTGAGGAGAAGCTGG, R; 5′-TTCCCTCATATTGCACAAAGG.

NeuroD F;5′-TGACCTTTCCCATGCTGAAT, R; 5′-AAGTGCTAAGGCAACGCAAT.

Ngn2 F; 5′-GATGCCAAGCTCACGAAGAT, R; 5′-ACGTGGAGTTGGAGGATGAC.

Mash1 F;5′-TAACTCCAAACCACTAACAGGC, R; 5′-TGAGGAAAGACATCAACG CAGT.

TH F;5′-GCCGTCTCAGAGCAGGATAC, R; 5′-AGCATTTCCATCCCTCTCCT.

Lmx1a F;5′-ACGAACAGAACACCCAGAGG, R; 5′-ACCATAGGGGTGCATGTGAT.

Nurr1 F;5′-CGGTTTCAGAAGTGCCTAGC, R; 5′-TTGCCTGGAACCTGGAATAG.

Pitx3 F;5′-GAGGACGGCTCTCTGAAGAA, R; 5′-CTCAGTGAGGTTGGTCCACA.

FoxA2 F;5′-GGCACCTTGAGAAAGCAGTC, R; 5′-GACATACCGACGCAGCTACA.

Olig2 F;5′-CTGGTGTCTAGTCGCCCATC, R; 5′-AGGAGGTGCTGGAGGAAGAT.

Nestin F;5′-CCAGAGCTGGACTGGAACTC, R; 5′-ACCTGCCTCTTTTGGTTCCT.

GFAP F; 5′-CACGAACGAGTCCCTAGAGC, R; 5′-ATGGTGATGCGGTTTTCTTC.

Immunocytochemistry and microscopy

Immunostaining was carried out as described previously (Oh et al., 2009; Lee et al., 2012; Takaesu et al., 2006). The primary antibodies used in this study are as follows: β-tubulin III (1:200; Sigma-Aldrich), MAP2 (1:200; Cell signaling), Nestin (1:200; Invitrogen), TH (1:200; Invitrogen), Alexa Fluor 568-Phalloidin (1:400; Invitrogen), and BrdU (1:200; Chemicon). Immunofluorescent images were obtained either with a Nikon ECLIPS TE-2000U microscope and NIS-Elements F software (Nikon) or with Zeiss LSM-510 Meta confocal and Axiophot2 fluorescence microscopes were used for imaging and measurement of neurite at SKKU School of Medicine-Microscopy Shared Resource Facility. Quantification of the percentage of differentiated $Cdo^{+/+}$ and $Cdo^{-/-}$ ES cells/colonies that possess neurites, and positive for β-tubulin III, Nestin or MAP2 were counted and plotted. More than 6 fields and at least 200 cells/field or 10 colonies/field were counted. Quantification of TH and MAP2-double positive colonies relative to total colonies is plotted as percentile. More than 5 fields and at least 10 colonies/field were counted.

TUNEL assay and BrdU incorporation

To monitor cell death, $Cdo^{+/+}$ and $Cdo^{-/-}$ ES cells from differentiation day 2 were fixed with 4% PFA followed by the terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) according to the manufacturer's instructions (Invitrogen). To monitor cell proliferation, cells were grown with bromodeoxyuridine (BrdU; Sigma-Aldrich) for 5 min and then fixed with 4% paraformaldehyde followed by treatment with 2 N HCl for 30 min at 37 °C and neutralization with 0.1 M borate buffer (pH 8.5). Cells were then immunostained as described above.

Mice, immunohistochemistry, whole mount in situ hybridization, and β-galactosidase staining

All mouse work was carried out as previously described (Zhang et al., 2006a, 2011). The heterozygous Cdo mutant mice were maintained on a C57BL/6 background and this study was reviewed and approved by the IACUC of SKKU School of Medicine (SUSM). SUSM is an AAALAC International accredited facility and abide by the ILAR guide. For Nurr1 (1:200; Abcam) and TH (1:200; Cell Signal) immunohistochemistry, embryos at E13.5 were fixed with 4% paraformaldehyde overnight at 4 °C and cryoprotected through a successive sucrose incubation followed by embedding in OCT

and cryosectioning at 8 μm thickness. Immunohistochemistry was carried out as previously described (Zhang et al., 2006b) with an exception of the antigen retrieval. For the antigen retrieval, the tissues were treated with 10 μg/ml proteinase K at 37 °C for 10 min. Whole mount in situ hybridization of E9.5 embryos for Shh was carried out as previously described (Zhang et al., 2006a,b). The stained embryos were then embedded in paraffin, sectioned at 10 μm and stained with hematoxylin and eosin as previously described (Zhang et al., 2011). Cdo mutant mice were crossed with Ptch1$^{+/tm1Mps}$ mice to monitor the Ptch1-lacZ reporter activity and the whole mount β-galactosidase staining is carried out as previously described (Zhang et al., 2006a).

Statistical analysis

Statistical analyses of the results are expressed as means ± SE from at least three independent experiments. Error bars represent means ± SE. For comparison between multiple groups, statistical significance was tested by a Mann–Whitney U test for 2 groups or Kruskal–Wallis test for over 3 groups using SPSS (18.0 version; SPSS, Chicago, IL, USA).

Results

Cdo and Shh signaling are involved in neuronal differentiation of ES cells

To examine the role of Cdo and Shh signaling in DA neurogenesis, we utilized in vitro differentiation protocols for mouse ES cell lines which allow differentiation into neurons. To establish an effective in vitro differentiation protocol we employed a well characterized R1 ES cell line. Fig. 1A describes the in vitro differentiation protocol that we used in this study for R1 ES cells as well as ES cells derived from *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ blastocysts. R1 ES cells of the indicated differentiation time points were analyzed by immunostaining for neuronal differentiation and the RT-PCR analysis for expression of Shh signaling components (Cdo, Boc, Gas1, Gli1 and Patched1) and a stemness gene, Oct4. At early differentiation day 6 (D6), neuronal differentiation of these cells was defined by β-tubulin III-positive cells containing long neurites (Fig. 1B), and colonies were formed at middle and late differentiation days (D14–D24) (Fig. 1G). RT-PCR analysis revealed that the Shh signaling coreceptors, Cdo, Boc and Gas1, and the primary receptor Patched 1 were induced at D2, but the induction of Patched1 and Boc expression was more robust at D4 and the expression of these genes was sustained until D8 when β-tubulin III-positive neuronal precursors were observed (Figs. 1B and C). Gli1 expression was detected in proliferating ES cells and decreased slightly starting at D6, and was not detected at D16 (Figs. 1C, E and H). Currently the role of Gli1 in the proliferation of ES cells is unknown. As predicted, Oct4 was expressed at the highest level in pluripotent, proliferating cells and decreased slowly during neuronal differentiation. To examine the role of Cdo in neuronal differentiation of ES cells, we have established two independent ES cell lines from *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ blastocysts, and *Cdo*$^{+/+}$ ES cells were used for positive control in place of R1 ES cells. An initial analysis did not show any difference in their morphological and proliferative characteristics between *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells (Fig. 1L). To verify the differentiation potential of newly established ES cells, *Cdo*$^{+/+}$ ES cells were induced to differentiate using the same differentiation protocol shown in Fig. 1A and analyzed by RT-PCR. As shown in Figs. 1E and H, the expression profile of the Shh signaling components, Cdo, Boc, Gas1, Patched1, Gli1, and a stemness gene, Oct4 resembled that of R1 ES cells. Other stemness genes (Sox2 and Nanog) were shown in similar expression pattern of Oct4. Moreover, neuronal differentiation markers, microtubule-associated protein 2 (MAP2), Neurofilament M (NFM), NeuroD, TH, Olig2 and β-tubulin III were induced and shown similar patterns (Figs. 1E and H). Neuronal differentiation and morphological characteristics also did not show any difference between R1, and *Cdo*$^{+/+}$ ES cells (Figs. 1J and K).

Cdo is required for the neuronal differentiation of ES cells

Next we analyzed the neuronal differentiation capacities of *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells. *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ cells were induced to differentiate for 6 days or 16 days followed by immunostaining with antibodies recognizing β-tubulin III, Nestin and MAP2, and Phalloidin staining for investigating the distribution of F-actin in cells (Figs. 2A and B). At D6, *Cdo*$^{+/+}$ ES cells exhibited clusters of cells with long neurites and a strong immunoreaction to β-tubulin III, MAP2 or Nestin, suggesting efficient differentiation into neuronal progenitor cells. In contrast, *Cdo*$^{-/-}$ ES cells showed fewer cell clusters positive for β-tubulin III, MAP2 or Nestin and signals were generally weaker. At D16, most of the *Cdo*$^{+/+}$ ES cell clusters contained β-tubulin III and MAP2-positive neurons with very long neurites, while *Cdo*$^{-/-}$ ES cells showed only few clusters with β-tubulin III and MAP2-positive neuronal precursors, suggesting impaired neuronal differentiation. We determined the proliferation rate by BrdU incorporation and cell death by TUNEL staining at D2. *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells exhibited no significant difference in BrdU incorporation (Fig. 2E). On the other hand, *Cdo*$^{+/+}$ ES cells displayed 12 ± 3.0% of TUNEL-positive cells while *Cdo*$^{-/-}$ ES cells had 21 ± 1.4% in TUNEL-positive cells (Figs. 2C and D). These data suggest that the deregulation of stemness genes during differentiation and increased cell death upon differentiation may contribute to impaired neuronal differentiation of Cdo-deficient ES cells.

Cdo is required for the efficient DA neuronal lineages of ES cells

We then examined the differentiation capability of *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells into DA neurons. *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells were induced to differentiate for 24 days and immunostained for the expression of TH, a DA neuronal marker, and MAP2 followed by Dapi staining. As shown in Figs. 3A and B, *Cdo*$^{+/+}$ ES cells formed 34 ± 1.1% of clusters containing double positive cells for TH and MAP2. In contrast, clusters of *Cdo*$^{-/-}$ ES cells were generally smaller and only 18 ± 2.2% of clusters contained double positive cells and the number of TH-positive cells per cluster were fewer. To quantify this decrease in DA neuronal differentiation, RNAs harvested from ES cells at D24 were subjected to real-time PCR. The

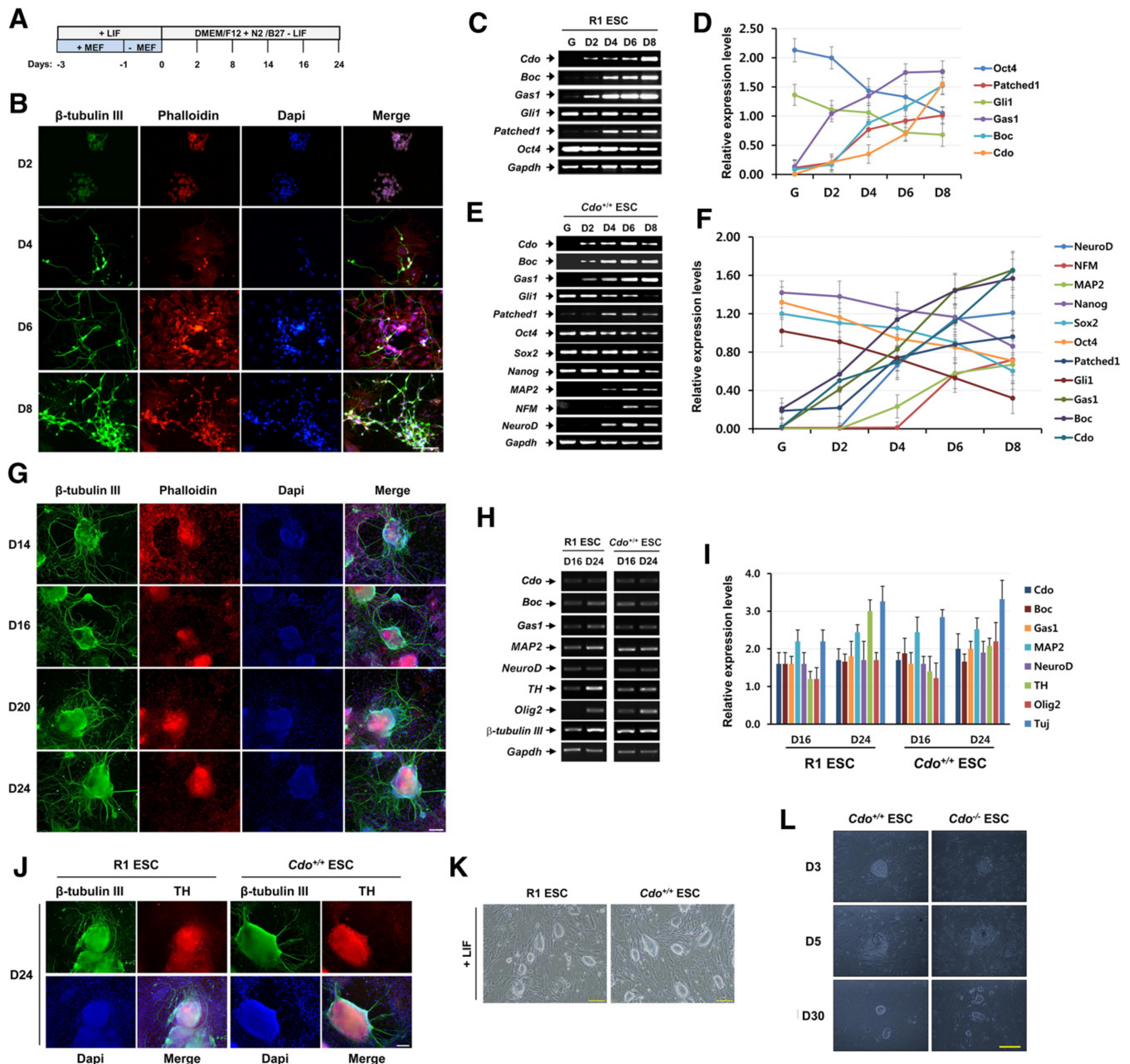

Figure 1 Cdo and Shh signaling are induced during neuronal differentiation of ES cells. (A) The schematic representation of the protocol used in this study. (B) R1 mouse ES cells are induced to differentiate by differentiation medium (N2/B27) containing Vitamin A, and immunostained with a β-tubulin III antibody (a neuronal marker, green). Actin filaments (red) and nuclei were stained by each Phalloidin and Dapi at the indicated early differentiation time points (D2–D8). Size bar = 100 μm. (C) RNAs isolated from R1 cells cultured in growth medium (G) or differentiation medium (N2/B27) for the indicated days (G2–D8) were subjected to RT-PCR for the expression of Cdo, Boc, Gas1 (Shh coreceptors), Gli1, Patched 1 (Shh targets), Oct4 (a stemness gene) and Gapdh as an internal control. (D) Quantificational analysis of the experiment in panel C. The intensity of each band was quantified relative to Gapdh. The experiment was repeated three times with similar results. (E) RNAs isolated from $Cdo^{+/+}$ ES cells at various differentiation time points were analyzed by RT-PCR for the expression of Shh signaling components (Cdo, Boc, Gas1, Gli1 and Patched1), stemness genes (Oct4, Sox2 and Nanog), neuronal markers (MAP2, NFM and NeuroD) and Gapdh as an internal control. (F) Quantification of representative experiment shown in panel E. The intensity of each band was quantified relative to Gapdh. The experiment was repeated three times with similar results. (G) Immunostaining of R1 ESCs at the time points between 14 and 24 days with a β-tubulin III antibody, and actin filaments and nuclei were stained by each Phalloidin and Dapi. Size bar = 100 μm. (H) Semi-quantitative RT-PCR analysis for expression of Shh coreceptors and neuronal marker genes with RNAs isolated from R1 and $Cdo^{+/+}$ ESCs at D16 and D24. (I) Quantification of the experiment in panel H. The intensity of each band was quantified relative to Gapdh. The experiment was repeated three times with similar results. (J) R1 and $Cdo^{+/+}$ ESCs were immunostained with β-tubulin III and TH followed by differentiation for 24 days. Size bar = 100 μm. (K) Morphologies of R1 ESCs and established $Cdo^{+/+}$ ESCs on MEF cells with LIF containing growth medium. Size bar = 50 μm. (L) Photomicrographs of $Cdo^{+/+}$ and $Cdo^{-/-}$ ESCs at various days during establishment. Blastocysts grown on MEF cells (upper panel), hatched blastocysts (middle panel) and colonies at passage 3. Size bar = 25 μm.

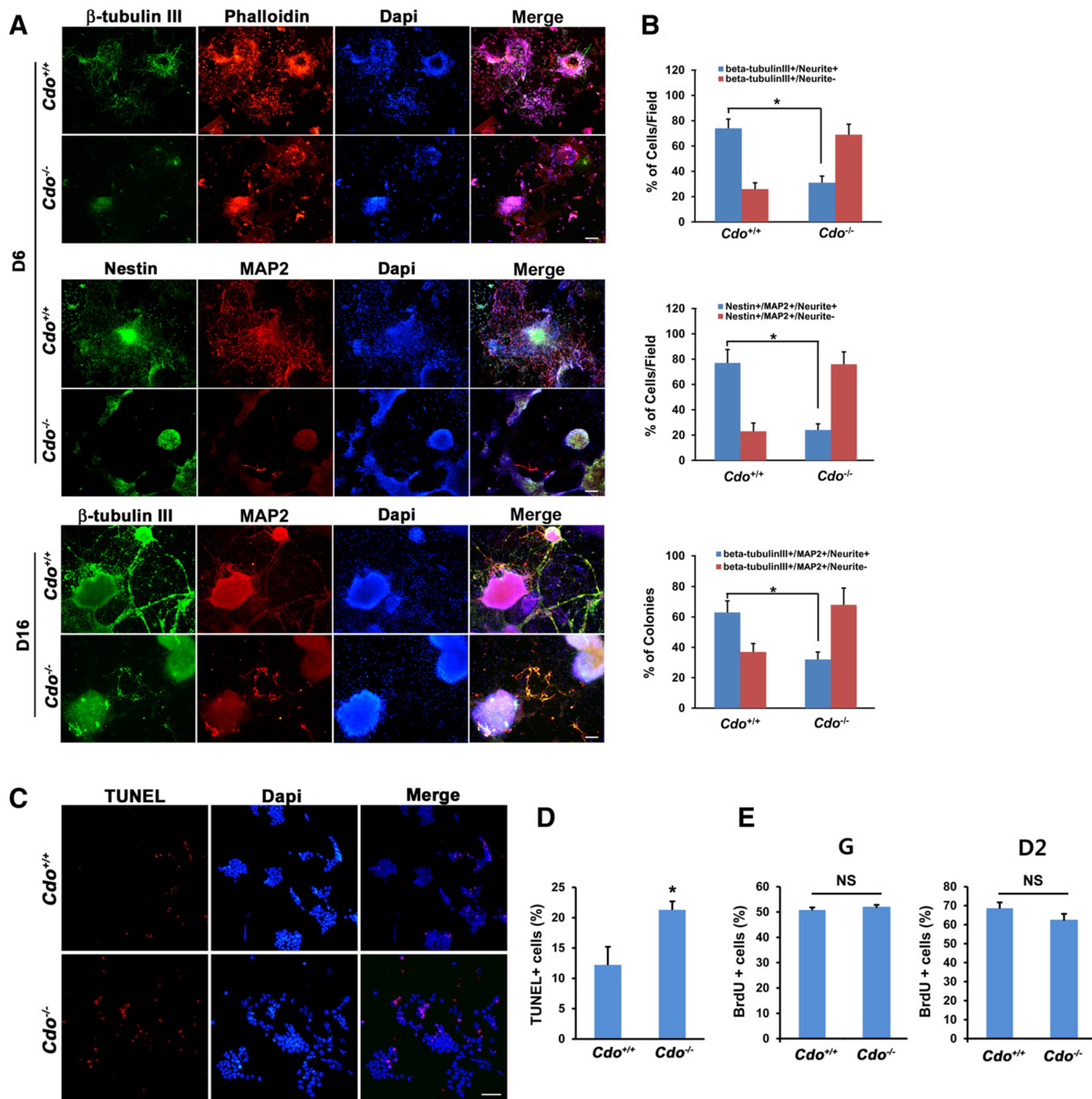

Figure 2 *Cdo*$^{-/-}$ ES cells display impaired neuronal differentiation. (A) *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells were induced to differentiate into neurons for 6 (D6) or 16 days (D16) and immunostained with antibodies recognizing β-tubulin III, Nestin, or MAP2, and actin filament was stained with Phalloidin followed by Dapi staining. These experiments were repeated 3 times with similar results. Size bar = 100 μm. Cdo deficiency resulted in decreased DA neurogenesis. (B) Quantification of the percentage of differentiated *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells/colonies that possess neurites, and positive for β-tubulin III, Nestin or MAP2 were counted and plotted as described for (A). More than 6 fields and at least 200 cells/field or 10 colonies/field were counted. Data are means ± SE (n = 4). *$P < 0.01$. (C) *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells were induced to differentiate for 2 days and cell death was determined by TUNEL assays. Size bar = 100 μm. (D) TUNEL-positive cells were quantified relative to total cells and plotted as percentile. Data are means ± SE of 4 independent experiments. *$P < 0.05$. (E) *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells at G and D2 were analyzed for BrdU incorporation. This experiment was repeated three times with similar results. Data are means ± SE. NS; non-significant.

expression level of TH in *Cdo*$^{-/-}$ ES cells was reduced to 42% of the control level (Fig. 3C). These data suggest that *Cdo*$^{-/-}$ ES cells displayed reduced capabilities to differentiate into DA neurons. To examine this defect in detail, the expression of several key regulators of DA neurogenesis (Lmx1a, Nurr1, Pitx3, Ngn2, Mash1 and Foxa2) and Shh signaling components (Shh, Gli1 and Patched1 (Ptch1)) was analyzed by real-time PCR. As shown in Fig. 3D, *Cdo*$^{-/-}$ ES cells displayed a substantial decrease in all genes analyzed, relative to the *Cdo*$^{+/+}$ ES cells. Next we harvested RNAs at the indicated time points

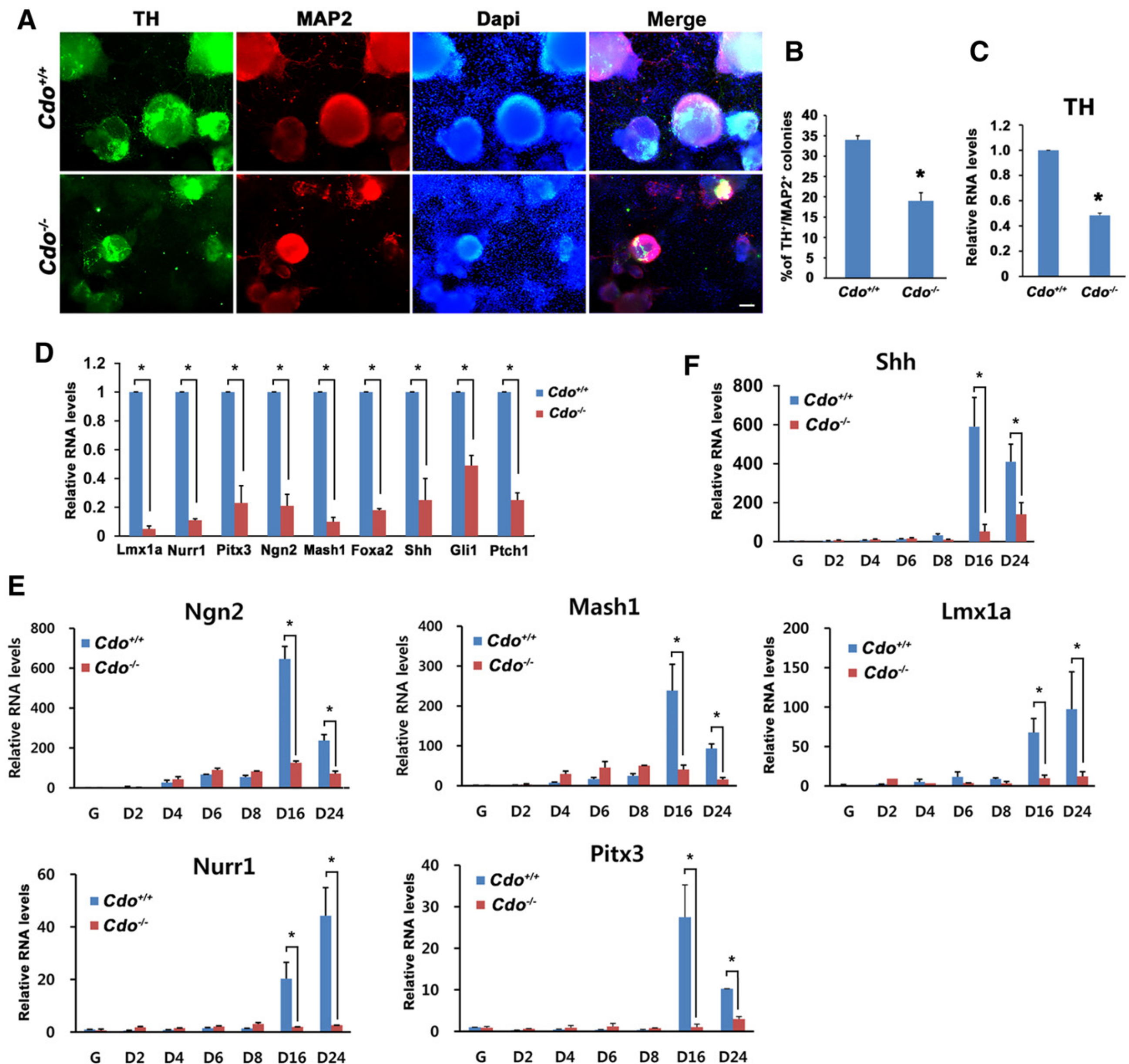

Figure 3 Cdo is required for DA neuronal lineages. (A) Immunostaining of *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells at differentiation day 24 for TH and MAP2. Size bar = 100 μm. (B) Quantification of TH and MAP2-double positive colonies relative to total colonies are plotted as percentile. This experiment was repeated at least 3 times with similar results. Data are means ± SE. $*P < 0.05$. (C) The real-time PCR analysis of ES cells at D24 for the TH expression. The RNA levels of the TH from *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells were quantified and normalized to levels in *Cdo*$^{+/+}$ ES cells. This experiment was carried out as triplicate and repeated three times with similar results. Data are means ± SE. $*P < 0.01$. (D) The real-time PCR analysis of ES cells at D24 for key regulators of DA neurogenesis (Lmx1a, Nurr1, Pitx3, Ngn2, Mash1) and Shh signaling components (Shh, Gli1, Ptch1, Foxa2). The RNA level of each was normalized to a level in *Cdo*$^{+/+}$ ES cells. This experiment was performed in triplicate and repeated independently. Data are means ± SE (n = 4). $*P < 0.01$. (E, F) *Cdo*$^{+/+}$ and *Cd*$^{-/-}$ ES cells were induced to differentiate into neurons for a total of 24 days and total RNAs are harvested at the indicated time points followed by real-time PCR for the expression of Ngn2, Mash1, Lmx1a, Nurr1, Pitx3 and Shh. The RNA level of each was normalized to a level in the G (growth) stage of *Cdo*$^{+/+}$ ES cells. This experiment was performed in triplicate and repeated independently. Data are means ± SE (n = 3). $*P < 0.01$.

from day 0 to day 24 of the differentiation time course employed real-time PCR to determine the expression of Ngn2, Mash1, Lmx1a, Nurr1, Pitx3 and Shh. As shown in Fig. 3E, both Ngn2 and Mash1 were strongly induced at D16 in *Cdo*$^{+/+}$ ES cells and decreased at D24. In contrast, *Cdo*$^{-/-}$ ES cells exhibited a different dynamic of the expression of these genes. Ngn2 and Mash1 were induced only slightly at D4 or D6 and stayed relatively constant until D16 without the robust increase that was seen in the *Cdo*$^{+/+}$ ES cells. In addition, *Cdo*$^{+/+}$ ES cells exhibited a dramatic increase in the expression of Lmx1a, Nurr1, Pitx3 and Shh at D16. At D24, the level of Lmx1a and Nurr1 was further increased, while Pitx3

and Shh were decreased (Figs. 3E and F). In contrast, we failed to observe this dramatic increase for all 6 genes in $Cdo^{-/-}$ ES cells at D16 and with the exception of Shh, the other 5 genes were expressed with low levels at D24 (Figs. 3E and F). These data suggest that Cdo-deficient ES cells fail to differentiate efficiently into neural lineages.

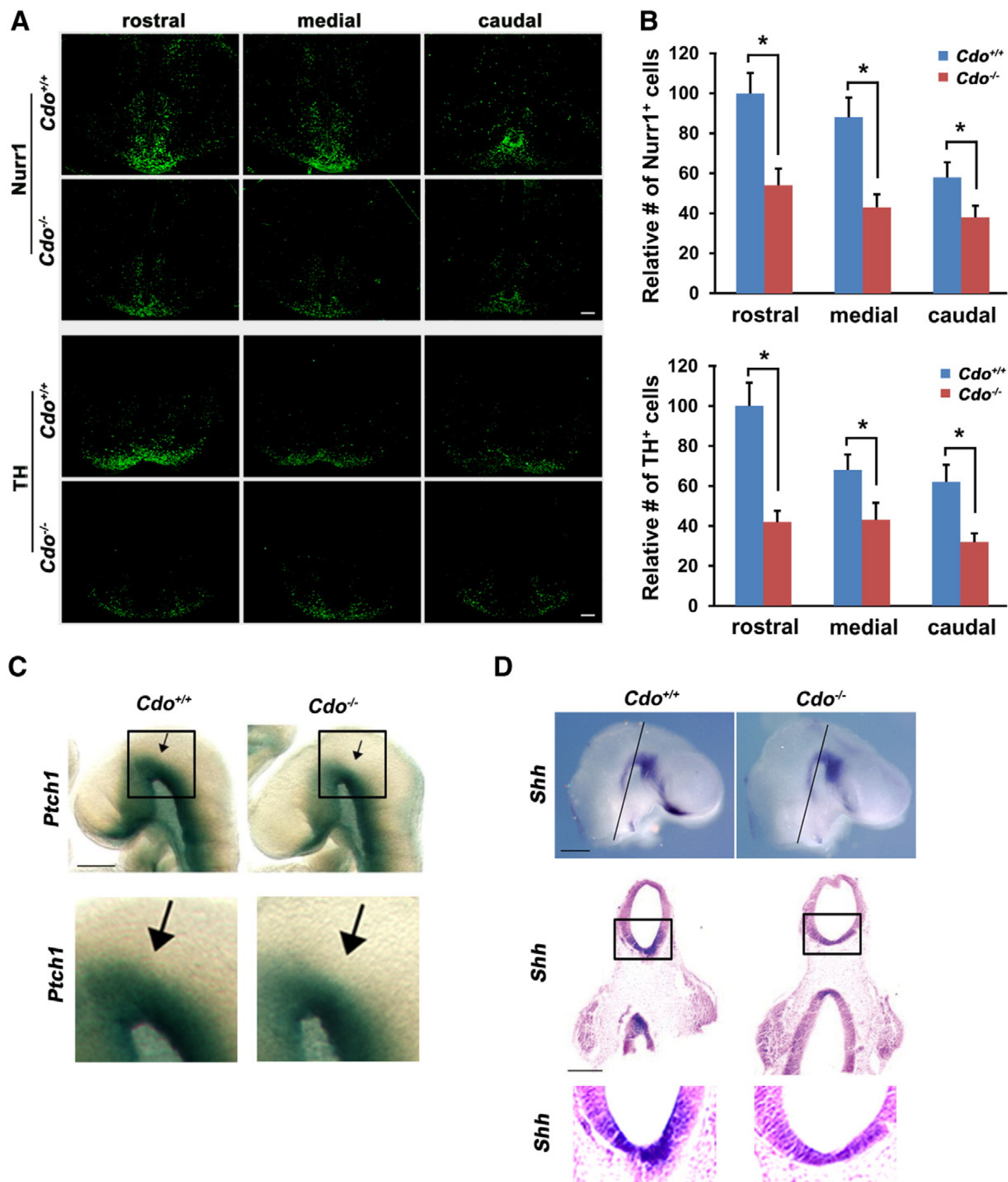

Figure 4 Cdo-deficient midbrain displays decreased Shh signaling and DA neurogenesis. (A) Immunostaining of $Cdo^{+/+}$ and $Cdo^{-/-}$ midbrains at E13.5 for the expression of Nurr1 and TH. Size bar = 100 μm. (B) Quantitation of the relative numbers of Nurr1^{+} and TH^{+} cells (n = 4). Nurr1^{+} or TH^{+} cells from $Cdo^{+/+}$ rostral region set to 100%. Bars represent standard error (SE). * indicates a significant difference in the number of Nurr1^{+} and TH^{+} cells between $Cdo^{+/+}$ and $Cdo^{-/-}$ embryos. *$P < 0.01$. (C) Wholemount staining of the Ptch1-lacZ transgene in $Cdo^{+/+}$ and $Cdo^{-/-}$ embryos at embryonic day 9.5. Size bar = 500 μm. The black boxed midbrain areas (arrowed) in upper panels are shown in lower panels. (D) Wholemount in situ hybridization of $Cdo^{+/+}$ and $Cdo^{-/-}$ embryos at E9.5 for Shh expression and sections of embryos (middle panels) shown in the upper panels. The line in the upper panels indicates the sectional plane. Size bar = 500 μm. The black boxed areas in the middle panels are shown in the lower panels.

Cdo is required for the full activation of Shh signaling in DA neurogenesis

As a next step we analyzed the role of Cdo in DA neurogenesis in vivo. Cryosections of $Cdo^{+/+}$ and $Cdo^{-/-}$ midbrains of embryos at E13.5 were prepared and subjected to immunostaining with antibodies to Nurr1 and TH. As shown in Figs. 4A and B, the $Cdo^{-/-}$ midbrain exhibited substantially reduced expression of Nurr1 and TH proteins compared to the $Cdo^{+/+}$ midbrain, suggesting that Cdo is required for midbrain DA neuronal development. We have previously shown that Cdo is required for full activation of Shh signaling in the early developing ventral forebrain and neural tube (Allen et al., 2011; Tenzen et al., 2006) however it is unknown whether Cdo is required for Shh signaling in the ventral midbrain. Therefore we examined the Shh signaling activities in the developing midbrain of $Cdo^{+/+}$ and $Cdo^{-/-}$ embryos at E9.5, prior to the first appearance of TH-positive neurons. To analyze the Shh signaling activity, we have utilized the compound mutant carrying a Patched1-LacZ reporter transgene and the Cdo mutation. The Patched1-LacZ activity was detected throughout the ventral brain areas of $Cdo^{+/+}$ and $Cdo^{-/-}$ embryos at E9.5 (Fig. 4C). However the staining intensity in the $Cdo^{-/-}$ midbrain was significantly reduced, relative to that in the $Cdo^{+/+}$ midbrain. In addition, $Cdo^{+/+}$ and $Cdo^{-/-}$ embryos at E9.5 were subjected to wholemount in situ hybridization for Shh expression. Consistently, the Shh mRNA expression was also detected throughout the $Cdo^{-/-}$ ventral brain area and in coronal sections of the midbrain but showed a significant reduction in Shh mRNA levels, compared to that of the $Cdo^{+/+}$ embryos (Fig. 4D). Taken together, these data suggest that Cdo is required for the full activation of Shh signaling in the ventral midbrain.

Activation of Shh signaling restores DA neurogenesis of Cdo-deficient ES cells

To verify whether Cdo is required for DA neurogenesis by regulating Shh signaling, we treated cells with a Shh agonist or an antagonist for the initial two days of differentiation or between D14 and D16 during DA neurogenesis, as shown in Fig. 5A. Our initial experiment suggested that early Shh signaling seems to be important. Therefore, $Cdo^{+/+}$ and $Cdo^{-/-}$ ES cells were treated with an Shh antagonist SANT1 for 2 days upon induction of differentiation and RNAs were harvested at D24, followed by real-time PCR for key regulators of DA neurogenesis (Fig. 5B). The levels of Shh, Foxa2, Lmx1a, Nurr1 and Pitx3 were affected by SANT1 treatment to variable degrees. However among these genes Lmx1a expression was most severely affected in $Cdo^{+/+}$ ES cells. In agreement with previous data, the control-treated $Cdo^{-/-}$ ES cells displayed significantly reduced expression of these genes that were further decreased by SANT1-treatment, except Lmx1a. These data suggest that Shh signaling is required for the initiation stage of neurogenesis. Thus we asked whether the treatment with Pur, an agonist of Shh signaling independent of the ligand-receptor action, can restore DA neurogenesis in Cdo-deficient ES cells. To do so, ES cells were treated with the vehicle DMSO or Pur for 2 days upon induction of differentiation and cells were then subjected to immunostaining for TH and MAP2 at D19 (Figs. 5C and D). $Cdo^{+/+}$ ES cells treated with DMSO displayed differentiation capabilities with 49 ± 1.4% of MAP2 positive clusters per field. Similar to the previous results, the control DMSO treated $Cdo^{-/-}$ ES cells showed a stark reduction in MAP2 and/or TH-positive clusters per field, while the Pur-treatment of $Cdo^{-/-}$ ES cells resulted in 52 ± 6.2% of MAP2 and 8 ± 1.4% of MAP2/TH-positive clusters per field which is similar to the control ES cells. However the signal intensity for TH appears to be weaker than in control ES cells. These data suggest that reactivation of Shh signaling in $Cdo^{-/-}$ ES cells at the initial stage of neurogenesis can restore the differentiation potential of $Cdo^{-/-}$ ES cells into DA neurons. To analyze in detail, RNAs from $Cdo^{+/+}$ and $Cdo^{-/-}$ ES cells treated with DMSO or Pur were harvested at the various time points indicated and analyzed by real-time PCR for the expression of Shh and Foxa2 (Fig. 5E). Consistently, Shh was strongly enhanced at D16 and this was decreased at D24 in the control-treated $Cdo^{+/+}$ ES cells. $Cdo^{-/-}$ ES cells treated with Pur showed an increased expression of Shh at D16 and D24, compared to control-treated $Cdo^{-/-}$ ES cells however the increased levels were only roughly 60% of that in control-treated $Cdo^{+/+}$ ES cells at D24. The expression of Foxa2 was induced strongly in control-treated $Cdo^{+/+}$ ES cells however the Pur-treated $Cdo^{-/-}$ ES cells showed a three-fold increase of Foxa2 expression relative to the control-treated cells thereby reaching 50% of that in the control-treated $Cdo^{+/+}$ ES cells at D8 (Fig. 5E). In addition, we have assessed the expression level of Lmx1a, Nurr1 and Pitx3 in these cells at D24. The Pur-treated $Cdo^{-/-}$ ES cells exhibited an increase to the level that is equivalent to the control ES cells (Fig. 5F). Furthermore, $Cdo^{-/-}$ ES cells were treated with soluble Shh proteins for the initial 2 days of differentiation or for two days starting at D14. RNAs harvested at D24 were subjected to real-time PCR. As shown in Fig. 5G, the expression of Shh, Lmx1a, Nurr1 and Pitx3 was significantly increased in the Shh-treated cells for the initial two days except Foxa2, relative to the control cells, while Shh treatment at D14 was much less effective. Taken together, these data suggest that Shh signaling activation at the initiation stage of neuronal differentiation is critical for DA neurogenesis and Cdo is required for full activation of Shh signaling at the critical initiation step of DA neurogenesis.

Discussion

The potential of ES cells to differentiate into DA neurons in vitro offers a simplified model system to dissect the mechanisms of cell intrinsic and extrinsic factors in DA neurogenesis, compared to the complex mammalian CNS (Baizabal and Covarrubias, 2009; Murry and Keller, 2008). Furthermore, ES cell-derived DA neurons are an attractive therapeutic tool to treat neurodegenerative diseases like Parkinson's disease due to their great potential to proliferate and differentiate (Kriks et al., 2011). Therefore, understanding the regulatory mechanisms of ES cell differentiation is beneficial to establish more efficient protocols to generate DA neurons from ES cells. Several soluble factors and overexpression of key regulators by genetic manipulation have been used to induce DA neuronal specification and differentiation of ES cells (Bayly et al., 2007; Prakash and Wurst, 2006; Vernay et al., 2005). Among these factors, Shh signaling has been proposed to be required for DA neurogenesis and exogenous Shh is

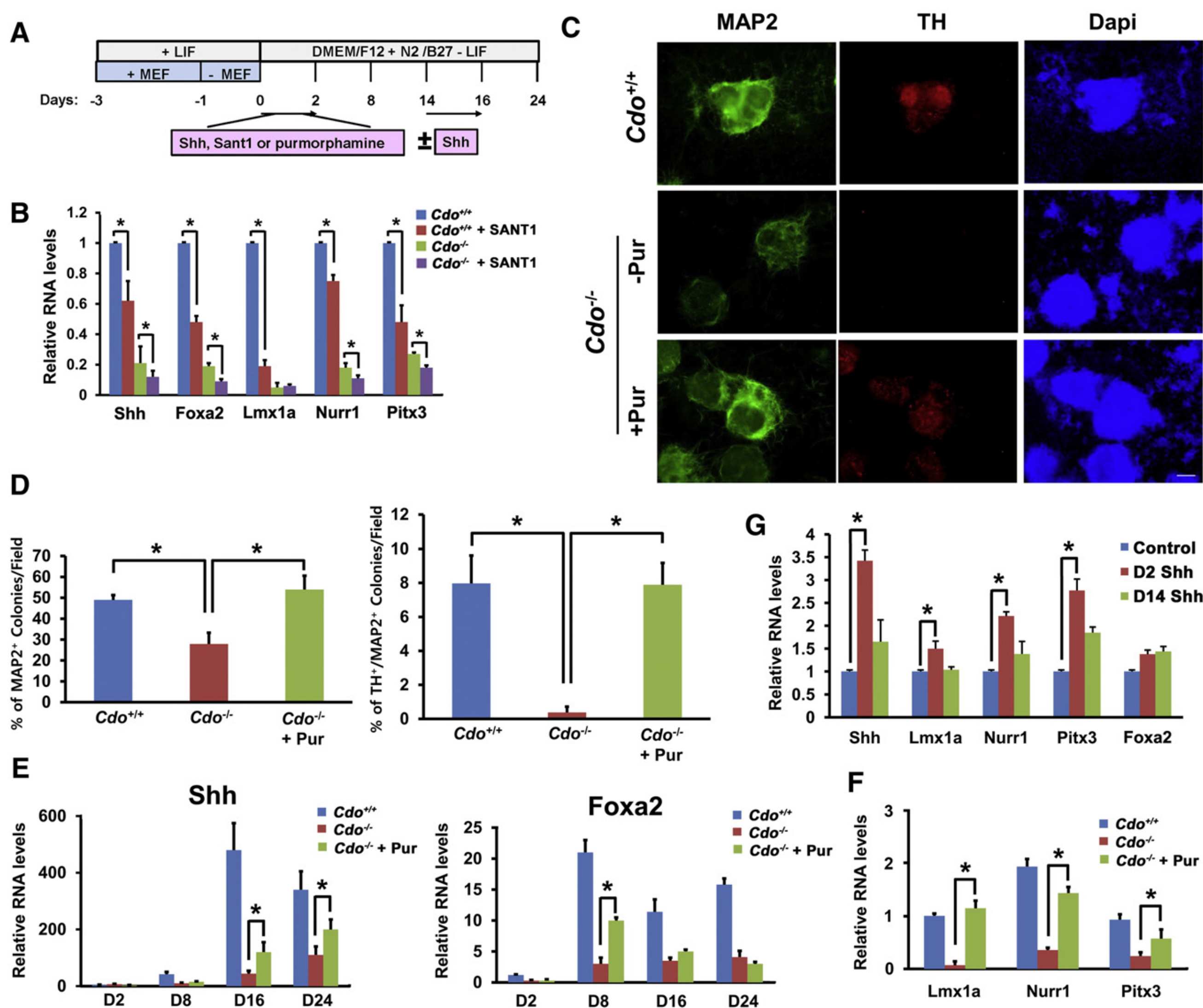

Figure 5 Activation of Shh signaling by the treatment with agonists restores DA neurogenesis of Cdo-deficient ES cells. (A) The schematic representation of protocols used in this study. The time period for the treatment with Shh, SANT1 and Pur is indicated. (B) RNAs isolated from *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells at D24 treated with either DMSO or 10 μM SANT1 for the initial 2 days were analyzed by real-time PCR. The RNA level of each was normalized to a level in *Cdo*$^{+/+}$ ES cells. Data are means ± SE, *$P < 0.05$ (C) *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells were treated with either DMSO (−Pur) or 2 mM Purmorphamine (+Pur) for the initial 2 days in a differentiation medium for 19 days and immunostained for MAP2 and TH expression. Size bar = 100 μm. (D) Quantification of MAP2 or TH positive colonies is plotted as percentile in the experiment shown in panel C. More than at least 5 fields were quantified. Data are means ± SE, ($n = 3$) *$P < 0.005$ (E) RNAs obtained from *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells at indicated differentiation time points, treated with DMSO or Pur for the initial 2 days were analyzed by real-time PCR for the expression of Shh and Foxa2. The RNA level of each was normalized to a level in the D2 (differentiation day 2) stage of *Cdo*$^{+/+}$ ES cells. Data are means ± SE ($n = 3$). *$P < 0.01$. (F) Real-time PCR analysis of *Cdo*$^{+/+}$ and *Cdo*$^{-/-}$ ES cells at D24 by Pur for Lmx1a, Nurr1 and Pitx3. The RNA level of the Lmx1a was normalized to a level in *Cdo*$^{+/+}$ ES cells. Data are means ± SE ($n = 3$). *$P < 0.01$. (G) *Cdo*$^{-/-}$ ES cells were treated with 200 ng/ml soluble Shh protein either for the initial 2 days (D0) or for two days starting at D14, RNAs were obtained at D24 and subjected to real-time PCR. The RNA level of each was normalized to a level in the D2 (differentiation day 2) stage of *Cdo*$^{-/-}$ ES cells. Data are means ± SE ($n = 3$). *$P < 0.01$.

generally added to induce differentiation of ES cells into DA neurons (Lindvall, 2012; Wu et al., 2012). In addition, a number of studies have shown that DA neurogenesis of ES cells in vitro appears to recapitulate the temporal process of DA neuron development in vivo (Chung et al., 2009; Fasano et al., 2010; Martinat et al., 2006). The molecular mechanism of Cdo in specification of ventral cell types of the forebrain and neural tube has been well characterized while its role in midbrain DA neurogenesis was entirely unknown. We thus utilized ES cells to assess the role of Cdo in DA neurogenesis and Shh signaling in the embryonic midbrain. The present study is the first to show that Cdo is required for the specification of DA neurons in the ventral midbrain through Shh signaling. Recent studies have suggested that the responsiveness to Shh and the Shh expression domain in the ventral midbrain alters dynamically during DA neurogenesis and the

Shh expression domain expands laterally during DA neuronal development, suggesting that the tight regulation of Shh signaling activities is important for DA neurogenesis (Hayes et al., 2013; Tang et al., 2013). In particular, a transient but strong activation of Shh signaling from notochord is essential for the floor plate induction at the early patterning stages of embryos which will be crucial for DA neuron specification (Ribes et al., 2010). In general, Cdo is excluded from the ventral regions of the developing CNS, most likely through inhibition by Shh signaling (Tenzen et al., 2006; Zhang et al., 2006a). However one exception of this inhibition by Shh on Cdo expression is the notochord at early embryonic development and Shh originating from the notochord activates Shh signaling in the floor plate (Jeong and Epstein, 2003). Previously we have shown that Cdo is transiently expressed in the prechordal plate and notochord throughout developing CNS and this transient expression appears to be required for the specification of the ventral cell types in the forebrain and neural tube (Tenzen et al., 2006; Zhang et al., 2006a). Consistent with this notion, Cdo is induced in ES cells at the initial specification stages of DA neurogenesis coinciding with Shh signaling activation. However a robust Shh expression is observed later at day 16 of ES cell differentiation coinciding with the expression of key regulators of DA neurogenesis, such as Ngn2, Mash1, Lmx1a, Nurr1 and Pitx3. This robust increase may reflect the expansion of the Shh-expressing domain into the lateral region of the ventral midbrain where TH-positive neurons will be generated. Since the defective DA neurogenesis of Cdo-deficient ES cells is corrected by activation of Shh signaling by Pur as well as Shh treatment at the initial specification stage, the reduction of Shh signaling activity at the specification stage may be a major cause for reduced DA neurogenesis in Cdo-deficient ES cells. This is further supported by the decreased Shh activity and Shh expression in the Cdo-deficient ventral midbrain correlating with the decreased DA neurogenesis.

At early developmental stages, Foxa2 has been shown to be a direct downstream target of Shh signaling. In addition, Foxa2 is required for turning on Shh in the midbrain (Sasaki et al., 1997). The current study shows that the expression of Foxa2 is decreased in Cdo-deficient ES cells and the Pur treatment leads to a significant increase of Foxa2 transcript at D8 which precedes the increased expression of Shh at D16, suggesting that Foxa2 may contribute to the expansion of the Shh expression domain during DA neurogenesis. In accordance with the current study, it has previously been reported that Foxa2 expression in the medial region of the neural tube is reduced in $Cdo^{-/-}$ embryos (Allen et al., 2011; Tenzen et al., 2006) correlated with reduced Shh activation in the floor plate of the neural tube. Since the function of these coreceptors is to activate Shh signaling by increasing the affinity of Shh to the primary receptor we added a high concentration of Shh ligand in Cdo-deficient ES cell cultures for 2 days at the initiation stage or at D14 to mimic the hike of Shh expression seen in $Cdo^{+/+}$ cells at D16. Consistently, the exogenous Shh ligand at the early time point restored the expression of Shh and key regulators of DA neurogenesis in Cdo-deficient ES cells. Unexpected profiles of expression of Lmx1a and Foxa2 in $Cdo^{-/-}$ treated with Shh agonist/antagonist might be caused by the presence of other Shh coreceptors (Figs. 5B and E). Previous studies have suggested that Cdo, Boc and Gas1, three coreceptors of Shh signaling have both specific and overlapping functions and they are necessary for Shh activation in early embryos (Allen et al., 2011). Boc and Gas1 are also expressed at the early stage of DA neurogenesis in ES cell cultures suggesting that these proteins may contribute to the rescue effect of added Shh ligands on DA neurogenesis in Cdo-deficiency.

Conclusions

Cdo expression is induced in the initiation stage of DA neurogenesis of ES cells and Cdo deficiency leads to the reduction of key regulators of DA neurogenesis correlating with decreased Shh signaling activation. Furthermore reactivation of Shh signaling at the initial stage restores DA neurogenesis of Cdo-deficient ES cells, suggesting that Cdo is required for DA neurogenesis via full activation of Shh signaling. These findings further support the notion that Cdo is required for the ventral cell type specification throughout the CNS via activation of Shh signaling.

Author contributions

YRK and MHJ made the concept and design, collection and/or assembly of data, data analyses and interpretation. YEL, SJL and HJK handled the data analyses and interpretation. GUB and JSK also contributed to the concept and design, financial support, collection and/or assembly of data, data analyses and interpretation, manuscript writing, and final approval of manuscript.

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