Development/Plasticity/Repair

Stage-Specific Regulation of Oligodendrocyte Development by Wnt/β-Catenin Signaling

Zhong-Min Dai,[1]* Shuhui Sun,[1]* Chunyang Wang,[1] Hao Huang,[1] Xuemei Hu,[2] Zunyi Zhang,[1] Qing Richard Lu,[3] and Mengsheng Qiu[1,2]

[1]Institute of Developmental and Regenerative Biology, Key Laboratory of Organ Development and Regeneration of Zhejiang Province, College of Life Sciences, Hangzhou Normal University, Hangzhou, 310029, People's Republic of China, [2]Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, Kentucky 40292, and [3]Department of Pediatrics, Division of Experimental Hematology and Cancer Biology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229

Oligodendrocytes are myelin-forming glia that ensheath the axons of neurons in the CNS. Recent studies have revealed that *Wnt*/β-catenin signaling plays important roles in oligodendrocyte development and myelin formation. However, there are conflicting reports on the specific function of *Wnt* signaling components in oligodendrocyte specification and differentiation. In the present study, we demonstrate that activation of β-catenin in neural progenitor cells before gliogenesis inhibits the generation of oligodendrocyte progenitors (OLPs) in mice. Once OLPs are formed, β-catenin becomes necessary for oligodendrocyte differentiation. Disruption of β-catenin signaling instead leads to a significant delay of oligodendrocyte maturation. These findings suggest that *Wnt*/β-catenin pathway regulates oligodendrocyte development in a stage-dependent manner.

Key words: **β-catenin; oligodendrocyte differentiation; OLPs; spinal cord; Wnt**

Introduction

Myelin ensheathment of neuronal axons enables rapid and accurate transmission (salutatory conduction) of electrical currents along axons. In the CNS, myelin sheaths are elaborated by specialized glial cells termed oligodendrocytes (OLs) (Baumann and Pham-Dinh, 2001). Impairment of OL function is found in many neurological disorders including multiple sclerosis (Van der Walt et al., 2010; Prineas and Parratt, 2012), schizophrenia, and bipolar disorder (Tkachev et al., 2003). Elucidation of signaling pathways that control OL differentiation and myelin formation is a crucial prerequisite for developing novel strategies for myelin repair in these neurological diseases.

Recent studies have revealed that canonical *Wnt* signaling plays vital roles in OL development. Despite the extensive work outlining the function of *Wnt* signaling in OL development, there are some conflicting reports on the role of the *Wnt* pathway in oligodendrogenesis. For instance, inactivation of *Wnt*/β-catenin signaling with dominant-negative forms of *Tcf/Lef* (Ye et al., 2009; Langseth et al., 2010) or *Wnt* antagonism (Shimizu et al., 2005; Langseth et al., 2010) increases the production of OL progenitors (OLPs). Activation of *Wnt*/β-catenin signaling by ways such as ΔExon3 mutation of β-catenin (Fancy et al., 2009; Feigenson et al., 2009; Ye et al., 2009), *Wnt3a* treatment (Shimizu et al., 2005; Feigenson et al., 2009; Azim and Butt, 2011), loss of function of the *Wnt* pathway inhibitor Apc (Apc^{Min}; Fancy et al., 2009), or Apc knock-out (Lang et al., 2013) significantly inhibit the maturation of OLs. Moreover, stabilization of *Axin2* via inhibiting tankyrase with small molecule XAV939, or reduction of β-catenin concentration, accelerates OLP differentiation and myelination after hypoxic and demyelinating injury (Fancy et al., 2011). Together, these studies demonstrate that increased *Wnt* signaling inhibits the production and differentiation of OLP. However, the following studies illustrate that *Wnt* signaling is vital for the production and differentiation of OLP. For instance, activation of *Wnt* signaling in postnatal brain by inhibiting *Gsk3β* or Wnt3a treatment increases OLPs and promotes myelination (Azim and Butt, 2011), whereas overexpression of the dominant-negative form of *Tcf7l2/Tcf4* decreases the number of *Olig2* and *Pdgfrα*-positive cells (Ortega et al., 2013). *Wnt*/β-catenin signaling has also been shown to be an essential direct driver of myelin gene expression in both Schwann cells and OLs (Tawk et al., 2011; Makoukji et al., 2012). In addition, *Tcf7l2/Tcf4* is expressed specifically in premyelinating OLs in spinal cord (Fu et al., 2009), and knock-out of *Tcf7l2* causes a myelin deficit phenotype (Fu et al., 2009; Ye et al., 2009). These studies suggest that the *Wnt*/β-catenin pathways functions to promote OL differentiation.

To address the perplexing roles of *Wnt*/β-catenin signaling in OL development, we investigated OL specification and differentiation in various genetically modified mutant mice with both

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*Z.-M.D. and S.S. contributed equally to this work.

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Correspondence should be addressed to Mengsheng Qiu, Hangzhou Normal University, Hangzhou, 310029, PR China. E-mail: m0qiu001@yahoo.com.

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gain of function (β-catenin ΔExon3, *Axin2*$^{-/-}$) and loss of function (β-catenin ΔExon2–6) of β-catenin. Activation of β-catenin signaling resulted in an inhibition of the specification of OLP from neural stem cells. Conversely, loss of β-catenin function had little effect on OLP generation, but caused a significant delay and reduction of OL differentiation. Together, these results demonstrate the stage-specific effects of *Wnt*/β-catenin signaling on OL development.

Materials and Methods

Animals. Use of the animals was approved by the Committee of Laboratory Animals, Hangzhou Normal University. *Olig1*^{Cre} without *Neo* cassette has been previously described (Xin et al., 2005). Mouse lines for *Axin2*^{LacZ} (Lustig et al., 2002), β-catenin$^{loxP(Exon3)}$ (Harada et al., 1999), β-catenin$^{loxP(Exon2–6)}$ (Brault et al., 2001), Rosa26R-LacZ (Soriano, 1999), and Rosa26Sor-GNZ (Schüller et al., 2008) were obtained from The Jackson Laboratory. Mice of either sex were used for sampling.

In situ *hybridization.* Samples were fixed in 4% paraformaldehyde/PBS at 4°C overnight, followed by 20% sucrose infusion for cryoprotection, and finally embedded in Tissue-Tek O.C.T Compound (Sakura Finetek). Samples were cryosectioned at 18 μm for *In situ* hybridization (ISH). DIG-labeled RNA probes were transcribed by T7, T3, or SP6 RNA polymerase using DIG RNA Labeling Mix or Fluorescein RNA Labeling Mix (Roche Diagnostics). Standard ISH was performed according to manufacturer's instruction.

Quantitative reverse-transcription PCR. RNA from the spinal cord of four wild-type (WT) mice and four β-catenin gain-of-function (Cat$^{G/+}$) mice was purified individually using RNAiso Plus [TaKaRa Biotechnology (Dalian)]. RNA was reverse transcribed into cDNA using PrimeScript II first Strand cDNA Synthesis Kit [TaKaRa Biotechnology (Dalian)]. Real-time PCR was performed using SsoFast EvaGreen Supermix with CFX96 Real-Time PCR Detection System (Bio-Rad). Primer sequences used for gene expression analysis were obtained from PrimerBank (Wang et al., 2012). Student's *t* test was used for statistical analysis.

Results

Activation of *Wnt*/β-catenin signaling impaired the generation of OLPs

To systematically investigate the role of *Wnt*/β-catenin signaling in OLP specification, we first examined the expression of several OLP markers in the spinal cords of β-catenin$^{loxP(Exon3)}$/*Olig1*^{Cre} transgenic mice in which β-catenin is selectively activated in the ventral pMN domain and its OLP progenies. At embryonic day 13.5 (E13.5), while many *Olig1*+ and *Olig2*+ cells were produced from the ventral pMN domain in the control tissues (Fig. 1*a*,*e*), only a few *Olig1*+/*Olig2*+ cells migrated away from the ventricular zone in the spinal cord of Cat$^{G/+}$ mice (Fig. 1*a*′,*e*′). At E14.5, *Olig1/2*+ cells in the control animals proliferated rapidly and dispersed into the entire tissue, but only a small number of *Olig1/2*+ oligodendrocyte precursor cells (OPCs) were observed adjacent to the ventral and dorsal ventricular zone in Cat$^{G/+}$ mice (Fig. 1*b*′,*f*′). Even at later stages, the number of *Olig1/2*+ cells did not appear to increase with time in Cat$^{G/+}$ mice despite their wider distribution (Fig. 1*c*–*d*′,*g*–*h*′). No significant differ-

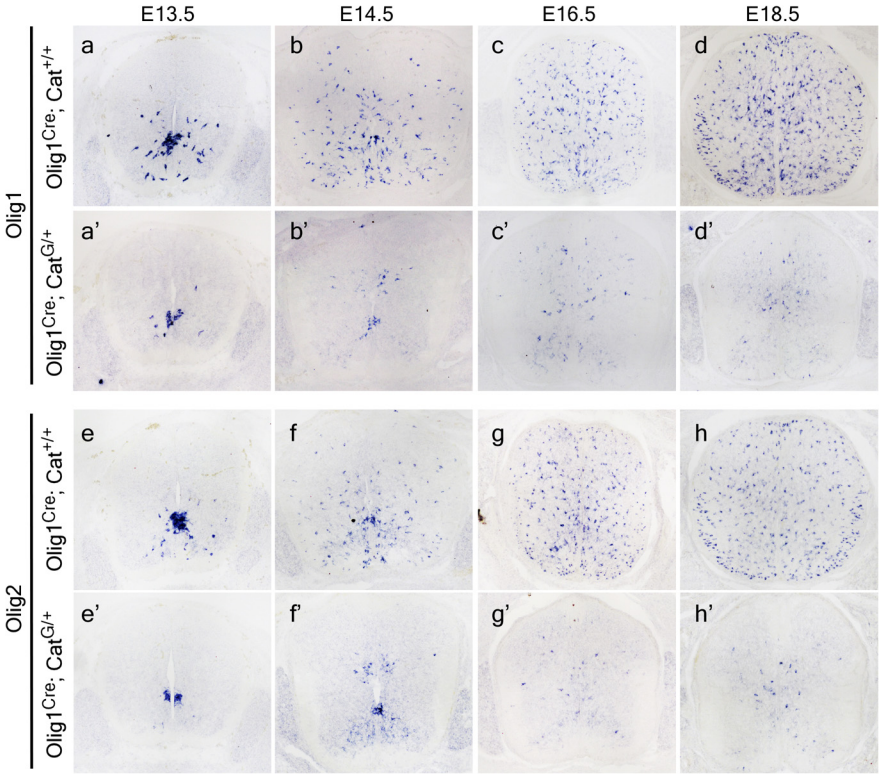

Figure 1. Activation of β-catenin impairs the generation and migration of *Olig1* and *Olig2*-positive cells. Transverse sections of spinal cord from E13.5 (***a–a′***, ***e–e′***), E14.5 (***b–b′***, ***f–f′***), E16.5 (***c–c′***, ***g–g′***), and E18.5 (***d–d′***, ***h–h′***), WT (***a–h***), and Cat$^{G/+}$ (***a′–h′***) mice are subjected to ISH with *Olig1* and *Olig2* riboprobes as OL lineage cell markers. The total number of *Olig1* and *Olig2*-positive cells in Cat$^{G/+}$ mice is severely reduced.

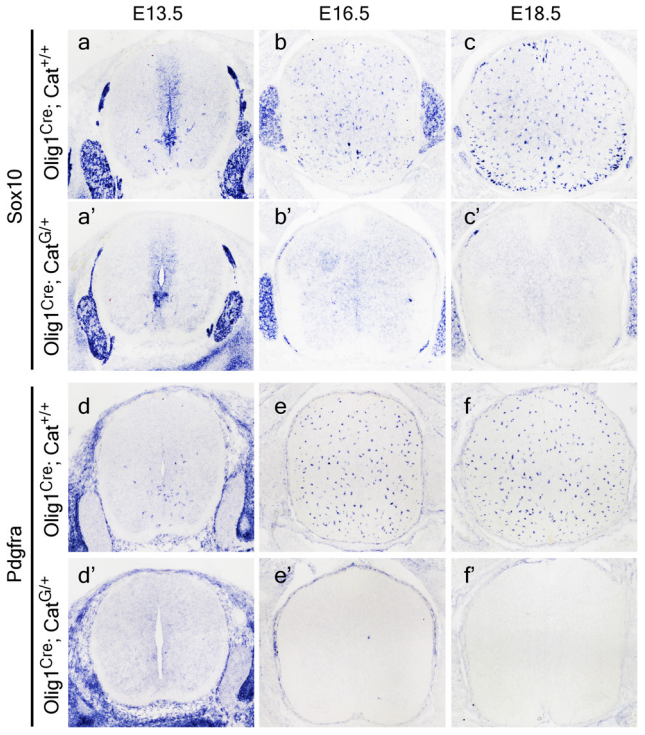

Figure 2. Activation of *Wnt*/β-catenin signaling inhibits the generation of OLPs. Transverse sections of spinal cord from E13.5 (***a–a′***, ***d–d′***), E16.5 (***b–b′***, ***e–e′***), and E18.5 (***c–c′***, ***f–f′***) of WT (***a–f***) and Cat$^{G/+}$ (***a′–f′***) mice are subjected to ISH with *Sox10* and *Pdgfrα* riboprobes. Lack of expression of *Sox10* and *Pdgfrα* in the spinal cord of Cat$^{G/+}$ mice (***b′–f′***) indicated that specification of OLPs from neural stem cells was impaired.

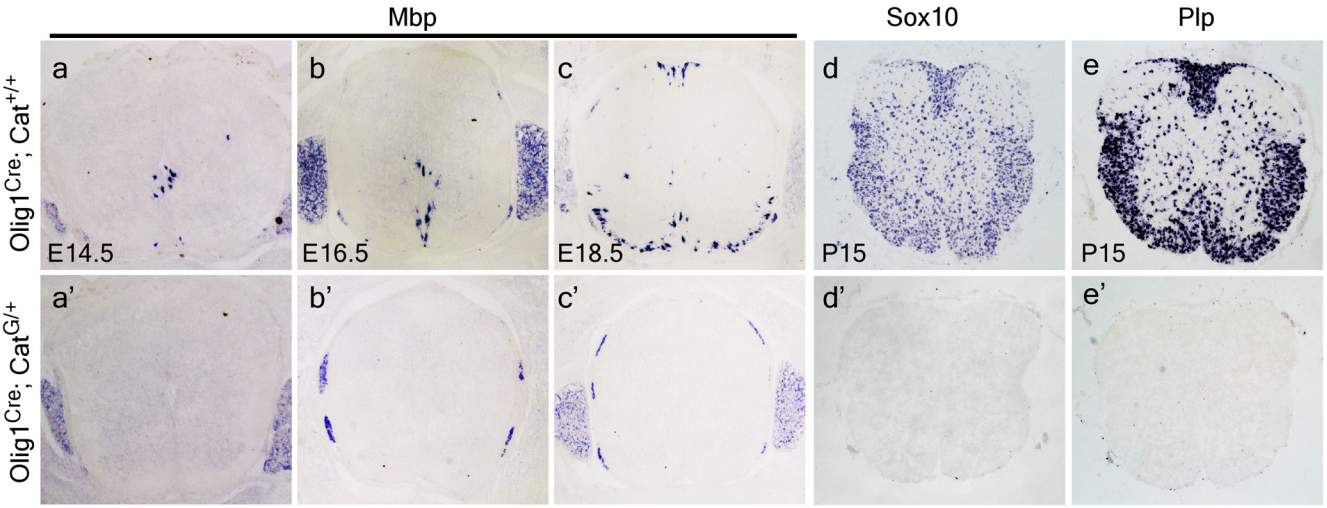

Figure 3. Lack of OLs in Cat$^{G/+}$ mice. Transverse sections of spinal cord from E14.5 (***a–a′***), E16.5 (***b–b′***), E18.5 (***c–c′***), and P15 (***d–e′***) of WT (***a–e***) and Cat$^{G/+}$ (***a′–e′***) mice are subjected to ISH with *Sox10*, *Mbp*, or *Plp* riboprobes as OLP or mature OL marker. Expression of *Sox10*, *Mbp*, and *Plp* is absent in Cat$^{G/+}$ tissue.

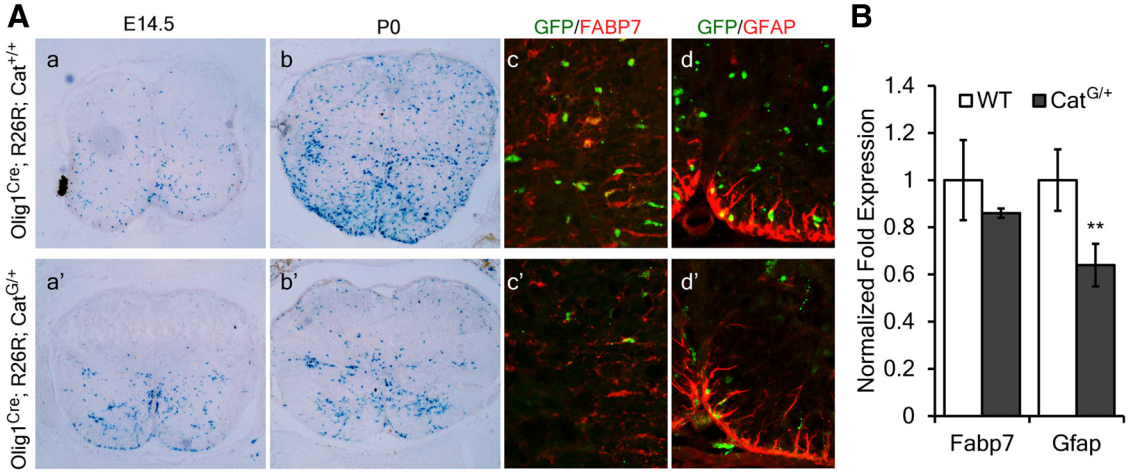

Figure 4. Altered migration pattern of LacZ+ cells in Cat$^{G/+}$ mice. ***A***, Spinal cord tissues from *Olig1*^{Cre}; Rosa26 (***a–d***) or *Olig1*^{Cre}; Rosa26; Cat$^{G/+}$ (***a′–d′***) are stained for β-galactosidase activity (***a***, ***b***, ***a′***, ***b′***) or subjected to immunofluorescent labeling with FABP7 or GFAP (***c***, ***d***, ***c′***, ***d′***). LacZ+ cells in Cat$^{G/+}$ background are less abundant and largely confined to the ventral spinal cord. ***B***, Quantitative reverse-transcription PCR analysis of the expression level of *Fabp7* and *Gfap* in E18.5 spinal cord from WT and Cat$^{G/+}$ mice. All values are presented as means ± SD. $^{**}p < 0.01$.

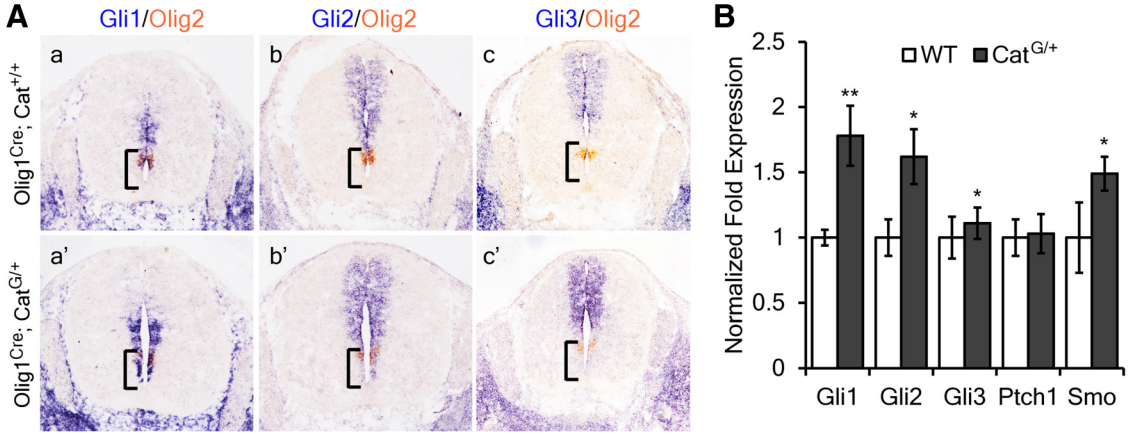

Figure 5. Expression of *Gli* genes in the Cat$^{G/+}$ embryonic spinal cords. ***A***, Transverse sections of spinal cord from E13.5 of WT (***a–c***) and Cat$^{G/+}$ (***a′–c′***) mice are subjected to ISH with *Gli1*, *Gli2*, and *Gli3* riboprobes, followed by ISH with *Olig2* riboprobe. ***B***, Quantitative reverse-transcription PCR analysis of the expression level of *Gli1*, *Gli2*, *Gli3*, *Smo*, and *Ptch1* in E18.5 spinal cord from WT and Cat$^{G/+}$ mice. All values are presented as means ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

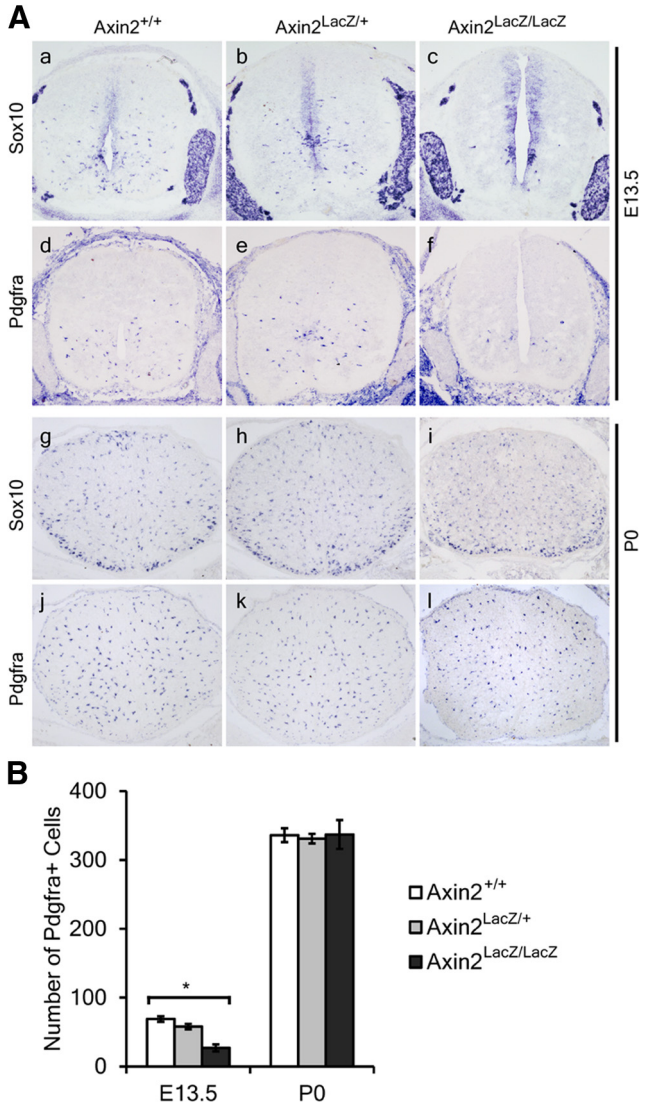

Figure 6. Reduced generation of OLPs in *Axin2* mutant mice. ***A***, Transverse sections of spinal cord from E13.5 (***a–f***) and P0 (***g–l***) WT (***a***, ***d***, ***g***, ***j***), heterozygous (***b***, ***e***, ***h***, ***k***) and mutant (***c***, ***f***, ***i***, ***l***) *Axin2* mice are subjected to ISH with *Sox10* (***a–c***, ***g–i***) and *Pdgfrα* (***d–f***, ***j–l***) riboprobes as OLP markers. ***B***, Quantification of the *Pdgfrα*+ cells in E13.5 and P0 spinal cord from WT heterozygous and mutant *Axin2* mice. All values are presented as means ± SD. *$p < 0.05$.

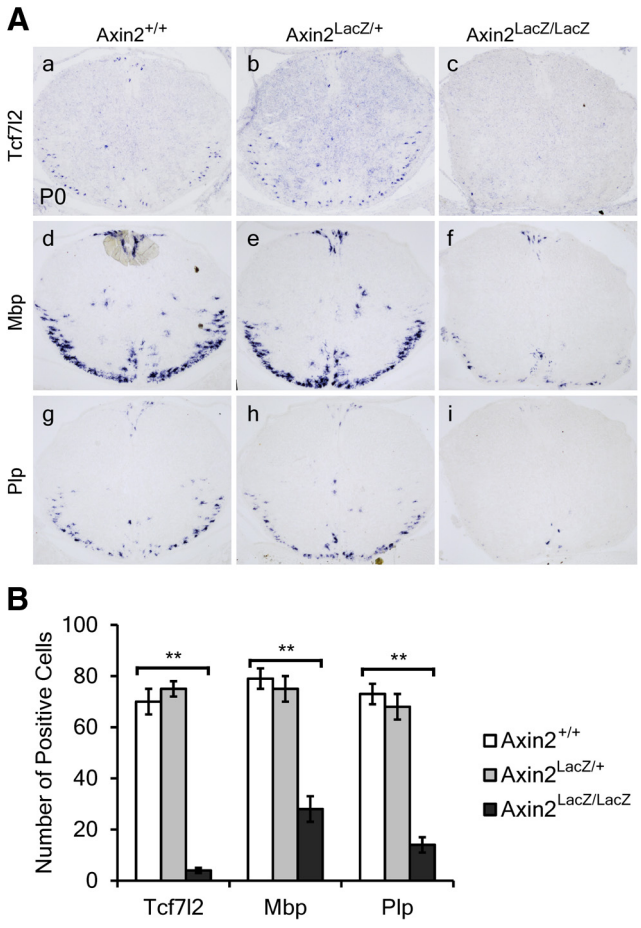

Figure 7. Delay of OL differentiation in *Axin2* mutant mice. ***A***, Transverse sections of spinal cord from P0 WT (***a***, ***d***, ***g***), heterozygous (***b***, ***e***, ***h***), and mutant (***c***, ***f***, ***i***) *Axin2* mice are subjected to ISH with *Tcf7l2* (***a–c***), *Mbp* (***d–f***), and *Plp* (***g–i***) riboprobes. ***B***, Quantification of the *Tcf7l2*+, *Mbp*+, and *Plp*+ cells in P0 spinal cord from WT, heterozygous, and mutant *Axin2* mice. All values are presented as means ± SD. **$p < 0.01$.

ence in cell death was observed between Cat$^{G/+}$ mice and WT mice at these stages (data not shown).

In agreement with the reduced generation of *Olig1/2*+ cells, expression of two OLP markers, *Sox10* and *Pdgfrα*, was also impaired by catenin activation. *Sox10*+ and *Pdgfrα*+ cells were only detected in the ventral ventricular zone of E13.5 Cat$^{G/+}$ spinal cord, contrary to their wide distribution in control tissues (Fig. 2*a–a′*,*d–d′*). At E16.5 and later stages, *Sox10*+ and *Pdgfrα*+ cells were not found in the Cat$^{G/+}$ tissues (Fig. 2*b–c′*,*e–f′*), indicating that *Sox10*+ and *Pdgfrα*+ cells failed to migrate out of the ventricular zone and progress along the OLP lineage. Together, these results suggest that activation of the β-catenin pathway dramatically inhibits the specification of neural stem cell-derived OLPs. Concomitant with the lack of OLPs, expression of the mature OL marker, *Mbp* or *Plp*, was completely absent at all stages (from E14.5 to P15 when animals fail to survive) in the spinal cord tissue of Cat$^{G/+}$ mice (Fig. 3).

Intriguingly, in the Cat$^{G/+}$ spinal cord, the *Olig1*+/*Olig2*+ cells derived from the pMN domain followed a radial migratory trajectory similar to astrocyte precursor cells (Fig. 1*b′*,*f′*), raising the possibility that these cells may have changed identity to astrocyte precursor cells. To examine this possibility, we traced the fate of these cells in *Olig1*^{Cre}; R26R; Cat$^{G/+}$ transgenic mice. Consistent with the ISH study, LacZ+ cells in E14.5 Cat$^{G/+}$ embryos were restricted to the ventral spinal cord, in contrast to the widespread distribution in the WT spinal cord (Fig. 4*a–a′*). Even at P0, the LacZ+ cells remained largely confined to the ventral spinal cord in Cat$^{G/+}$ tissue as would be expected for astrocytes, whereas LacZ+ cells in the control tissue were widely dispersed (Fig. 4*b–b′*). However, double immunofluorescent labeling in *Olig1*^{Cre}; R26R; Cat$^{G/+}$ mice revealed that expression of two astrocyte markers *Fabp7* and *Gfap* was not increased (Fig. 4*c*,*d*,*c′*,*d′*); instead, *Gfap* expression was significantly reduced (Fig. 4*B*). These results suggest the Olig1-Cre+ cells may become an unknown glial cell type or unknown stage of glial development, as suggested by a different study (Ye et al., 2009).

It has been previously shown that OLP generation in the ventral spinal cord is promoted by *Shh* signaling from the ventral midline structures (Pringle et al., 1996). To test the possibility that *Wnt* signaling represses OLP generation by inhibiting *Shh* signaling, we examined the expression of three *Gli* genes (*Gli1–3*), the downstream components of *Shh* pathway, in the transgenic spinal cord. It was found that expression of all *Gli* genes, especially *Gli1*, which functions as a transcriptional activator for *Shh*

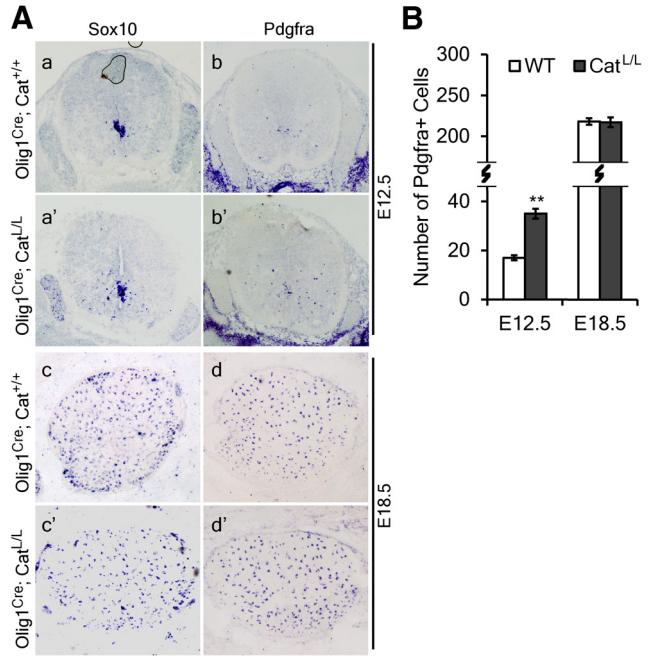

Figure 8. Precocious generation of OLPs in β-catenin mutant mice. ***A***, ISH showed that at E12.5 more *Sox10*+ and *Pdgfrα*+ cells migrated away from the ventricular zone in Cat$^{L/L}$ mice (***a′***, ***b′***) than in WT mice (***a***, ***b***). At E18.5, a similar number of *Sox10*+ and *Pdgfrα*+ cells was observed in Cat$^{L/L}$ mice (***c′***, ***d′***) and WT mice (***c***, ***d***). ***B***, Quantification of the *Pdgfrα*+ cells in E12.5 and E18.5 spinal cord from WT and Cat$^{G/+}$ mice. All values are presented as means ± SD. $^{**}p < 0.01$.

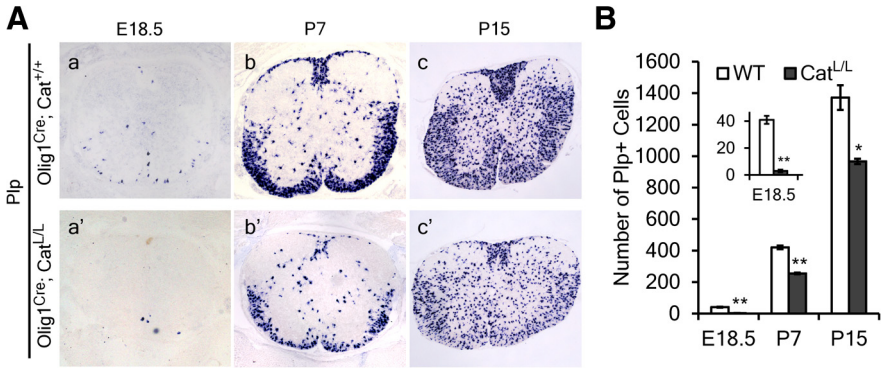

Figure 9. Delay of OL differentiation in β-catenin mutant mice. ***A***, Transverse sections of spinal tissues from E18.5 (***a–a′***), P7 (***b–b′***), and P15 (***c–c′***) control (***a–c***) and Cat$^{L/L}$ (***a′–c′***) mice are subjected to ISH with *Plp* riboprobe. Expression of *Plp* is delayed and reduced. ***B***, Quantification of the *Plp*+ cells in WT and Cat$^{G/+}$ spinal cord tissues. All values are presented as means ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

signaling, was upregulated in the spinal cord of Cat$^{G/+}$ mice (Fig. 5). Moreover, the expression of *Smo* and *Ptch1*, the coreceptors for *Shh*, was not reduced in the spinal cord of Cat$^{G/+}$ mice (Fig. 5). Together, these results indicated that *Shh* signaling was not compromised by increased *Wnt*/β-catenin activity.

Axin2$^{LacZ/LacZ}$ mice showed reduced OLP cell number and delayed maturation of OL

Considering that β-catenin with ΔExon3 mutation may result in dysregulation of the cadherin/catenin-mediated cell—cell adhesion signaling pathway (Nelson and Nusse, 2004) or partial loss of its function, we used the *Axin2*$^{LacZ/LacZ}$ mice to further verify the effects of increased *Wnt*/β-catenin signaling on OL development. Consistent with the finding in Cat$^{G/+}$ embryos, a significantly smaller number of *Sox10* and *Pdgfrα*-positive cells were produced at E13.5 from the ventricular zone in the spinal cord of *Axin2*-null mice than in their WT or heterozygous littermates (Fig. 6*a–f*,*B*). However, expression of both *Sox10* and *Pdgfrα* returned to normal in *Axin2*-null mice at P0, probably due to the increased proliferation of *Pdgfrα*+ OLPs in the mutants (Fig. 6*g–l*). Differentiation of OLs was also inhibited in the *Axin2* mutants, as evidenced by the dramatically decreased number of *Tcf7l2/Tcf4*+, *Mbp*+, and *Plp*+ mature OL cells at P0 (Fig. 7).

Together, data from both the Cat$^{G/+}$ and *Axin2* mutant mice demonstrate that activation of β-catenin signaling at the early stage of glial development represses the specification and generation of OLPs from neural progenitor cells and their subsequent differentiation.

β-catenin is required for the timely differentiation of OLPs

We next examined if β-catenin is required for the development of OLs by selectively disrupting the function of β-catenin in OLPs in the *Olig1*^{Cre}/β-catenin$^{loxP(Exon2-6)/loxP(Exon2-6)}$ conditional mutant mice (Cat$^{L/L}$). Compared with WT littermates, a mild but significant increase of *Pdgfrα*-positive cells was observed in the spinal cord of Cat$^{L/L}$ mutant mice at E12.5 (Fig. 8*b–b′*,*B*), suggesting that OLPs were specified precociously. At E18.5, Cat$^{L/L}$ mice displayed a normal number of *Sox10*+ and *Pdgfrα*+ OPCs in the spinal cord (Fig. 8*c–d′*,*B*), but the expression of mature OL marker *Plp* was dramatically reduced (Fig. 9*a–a′*,*B*). However, at later stages (P7 and P15), the number of differentiated OLs in Cat$^{L/L}$ mice increased markedly, but remained significantly smaller than that in WT mice (Fig. 9*b–c′*,*B*). Thus, maturation of OLs was delayed rather than absent in the Cat$^{L/L}$ mice, indicating that *Wnt*/β-catenin signaling is required for the timely differentiation of OLPs, but not absolutely essential for their maturation.

Discussion

In this study, we systematically investigated the seemingly discrepant roles of *Wnt*/β-catenin signaling in OL development by examining OL specification and differentiation under various *Wnt*/β-catenin signaling conditions. Our results suggest that *Wnt*/β-catenin signaling exhibits a stage-specific function in the control of OL lineage development.

Activation of *Wnt*/β-catenin signaling in neural progenitor cells inhibits the specification of OLPs

Previous studies have demonstrated that declined expression of *Wnt* in the dorsal spinal cord shows a strong correlation with the emergence of OLPs, suggesting an inhibitory role for *Wnt*/β-catenin signaling in OLP specification (Shimizu et al., 2005; Ye et al., 2009; Langseth et al., 2010). In agreement with these earlier studies, our work shows that loss of β-catenin function also leads to precocious expression of OLP markers in the ventral spinal cord. Moreover, increased β-catenin function or expression in the Cat$^{G/+}$ transgenic mice (Figs. 1, 2) or *Axin2* mutant mice (Fig. 6) suppresses the fate specification of OLPs from neural stem cells, as shown by the lack or reduction of expression of *Sox10* and *Pdgfrα* in spinal cord tissues. Since expression of the *Shh* pathway components, *Smo*, *Ptch1*, and *Gli* genes, is not compromised in Cat$^{G/+}$ mice (Fig. 7), it is possible that *Wnt*/β-catenin signaling inhibits the specifica-

tion of OLPs from neural stem cells through some unknown factors that override the positive effects of *Shh* signaling on specification of OLPs.

Interestingly, Ortega et al. (2013) demonstrated that activation of canonical Wnt signaling in culture and in adult brain by *Wnt3a* treatment significantly increased the number of *Olig2*+ and *Pdgfrα*+ cells, and inhibition of canonical Wnt signaling by overexpression of dnTcf4 decreased the number of these cells. Their findings differ from this and other studies, which showed that inhibition of Wnt signaling by disruption of β-catenin, and overexpression of dnTcf4, dnLef1, or Dkk1 increases OLP generation during development (Ye et al., 2009; Langseth et al., 2010). At this stage, we do not understand what causes this discrepancy; one possibility is that neural stem cells in developing embryos may behave differently from those in adult tissues or in culture.

It was previously observed that reduced or delayed generation of OLPs is invariably associated with delayed differentiation of OLs in many unrelated mutant mice, such as *Nkx6.1* and *Gli2* mutants (Liu et al., 2003; Qi et al., 2003), suggesting that an intrinsic timing mechanism may also operate during the *in vivo* development of OLs. Thus, the absent expression of OL differentiative markers in the $Cat^{G/+}$ and $Axin2^{-/-}$ tissues is likely to be secondary to the defective generation of OLPs, and therefore should not be interpreted as inhibition of OL differentiation by β-catenin. In further support of this notion, activation of β-catenin after OLP specification in Cnp^{Cre}; $Cat^{G/+}$ mice only led to a mild delay of OLP differentiation and axonal myelination (Feigenson et al., 2009).

Wnt/β-catenin signaling is required for the timely differentiation of OLPs

It was previously shown that selective inhibition of *Wnt* components blocks the expression of myelin protein zero (*Mpz*) and peripheral myelin protein 22 (*Pmp22*) in Schwann cells as well as *Plp* in OLs, while activation of *Wnt* signaling by *Wnt1* treatment increased the expression of *Mpz*, *Pmp22*, and *Plp* (Tawk et al., 2011). Moreover, treatment with *Wnt1* enhanced the recruitment of β-catenin to the *Tcf/Lef* transcription factor binding sites present in the promoters of *Mpz* and *Pmp22* (Tawk et al., 2011). These results suggest that *Wnt*/β-catenin signaling may directly drive the expression of myelin gene expression and is essential for OLP differentiation (Tawk et al., 2011). Consistently, *Tcf7l2* and β-catenin colocalize in the nuclei of premyelinating OLs (Fu et al., 2009, 2012). In addition, BAT-gal reporter mice show that *Wnt*/β-catenin signaling is active during developmental myelination and remyelination (Fancy et al., 2009). More importantly, mutation of *Tcf7l2/Tcf4* gene results in an inhibition of OL maturation (Fu et al., 2009; Ye et al., 2009). In the present study, we have demonstrated that the $Olig1^{Cre}$-mediated disruption of β-catenin in OLPs does not alter the expression of OLP markers such as *Sox10* and *Pdgfrα*, but causes a significant delay in the expression of mature OL markers such as *Mbp* and *Plp* (Fig. 9). Thus, *Wnt*/β-catenin signaling is required for the timely differentiation of OLPs.

These observations, together with others, suggest that *Wnt*/β-catenin signaling fulfills multiple functions during OL development. At early CNS development, *Wnt*/β-catenin signaling in the dorsal neural tube functions to inhibit OLP specification from neural progenitor cells, and that the generation of OLPs is accompanied by declined expression of *Wnts*. After OPCs are produced and migrate into the white matter regions, *Tcf7l2/Tcf4* expression is upregulated and coordinates with β-catenin to promote differentiation of OLPs. Finally, once OLs are fully differentiated, *Apc* is upregulated in mature OLs to suppress *Wnt*/β-catenin signaling (Lang et al., 2013).

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