

# Wnt/Rspondin/ $\beta$ -catenin signals control axonal sorting and lineage progression in Schwann cell development

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**During late Schwann cell development, immature Schwann cells segregate large axons from bundles, a process called “axonal radial sorting.” Here we demonstrate that canonical Wnt signals play a critical role in radial sorting and assign a role to Wnt and Rspondin ligands in this process. Mice carrying  $\beta$ -catenin loss-of-function mutations show a delay in axonal sorting; conversely, gain-of-function mutations result in accelerated sorting. Sorting deficits are accompanied by abnormal process extension, differentiation, and aberrant cell cycle exit of the Schwann cells. Using primary cultured Schwann cells, we analyze the upstream effectors, Wnt and Rspondin ligands that initiate signaling, and downstream genetic programs that mediate the Wnt response. Our analysis contributes to a better understanding of the mechanisms of Schwann cell development and fate decisions.**

Axin2-LacZ reporter mice | paracrine mechanism | lamellipodia | myelination | Lgr5 receptor

Development of the neural crest-derived Schwann cells (SCs) proceeds through several embryonic and postnatal stages (1). Around birth, SCs perform radial sorting of axons. During this process, immature SCs that envelop axonal bundles project cytoplasmic processes into the bundles to segregate individual large-diameter axons and to establish a one-to-one relationship with the axons. Subsequently, large sorted axons become myelinated, whereas multiple small axons remain unsorted and surrounded by nonmyelinating SCs (Remak bundles). The precise timing of radial sorting is instrumental for correct nerve development and homeostasis and is achieved through signaling cues provided by axons and by the extracellular environment (1–3). Axonal Neuropilin-1 type III acts through the ErbB/Shp2 signaling system to control SC proliferation, migration along nerves, and terminal differentiation (4–6). Axonal Notch signals promote the formation of immature SCs from precursors (7). Progression of the SC lineage beyond the immature SC stage and radial sorting are controlled by signals from both axons and the extracellular matrix, e.g., laminin/integrin  $\beta$ 1 and the small GTPase Rac1, and by transcription regulators, e.g., Sox10 and Sox2 (8–11).

Canonical Wnt signaling is evolutionarily conserved and takes over critical roles in development, organ maintenance, and regeneration (12, 13). The role of Wnt/ $\beta$ -catenin signals in specification of the early neural crest and in formation of the sensory lineage of the peripheral nervous system has been defined (13–17). Work performed in zebrafish embryos and in SC lines implicated Wnt/ $\beta$ -catenin signals in SC proliferation and apoptosis and as positive regulators of myelination (18–21). In contrast, conditional mouse mutants of  $\beta$ -catenin using *Dhh-cre* suggested that Wnt signaling does not affect SC functions in adult mice (22, 23). To date, it is unclear which role Wnt/ $\beta$ -catenin signals play in late SC development. Recently, Rspondin (Rspo) growth factors were identified as previously undescribed coactivators of Wnt signaling that bind to Lgr4, 5, and 6. Lgr4–6 receptors associate with the Lrp/Frizzled complex to enhance Wnt signaling and control progenitor cell maintenance in different developing

organs (24–27). A role of Rspondins and Lgr4–6 receptors in SC development has not been studied.

Here we define a temporal window of Wnt/ $\beta$ -catenin activity and its role in SC lineage progression using mouse genetics and cell culture techniques. Conditional loss-of-function (LOF) and gain-of-function (GOF) mutations of  $\beta$ -catenin in mouse SCs produce converse phenotypes: a delay and an acceleration of axonal sorting, respectively. Using cultured primary SCs and established SC lines, we delineate the roles of ligands, e.g., axonal Rspondins and Wnts, and of the downstream target genes that mediate the Wnt response.

## Results

**Expression of Wnt Pathway Components and Activity of Wnt/ $\beta$ -Catenin Signaling in Mouse Schwann Cell Development.** We first determined the expression of Wnt signaling components in the developing SCs and neurons by *in situ* hybridization of dorsal root ganglia (DRG) and spinal and sciatic nerves and by quantitative real-time PCR (qRT-PCR) of primary embryonic SCs. Genes encoding the ligands Wnt2, 6, and 9b and Rspondin 1–4 were expressed in DRG neurons starting at E12.5; Wnt2 was also expressed in SCs of the spinal and sciatic nerves starting at E14.5 (Table S1, Fig. 1 A–F, and Fig. S14). Genes encoding the receptors Fzd1, 3, 7, and 8 and Lgr4–6 as well as the transcription factors Tcf1, 3, and 4 were expressed in SCs from E12.5 on (Table S1 and Fig. 1 A–G; see also Fig. 1 A–F, Lower). Thus, in the peripheral nervous system, neurons express several Wnt and Rspondin ligands that can signal to SCs in a paracrine manner; in addition, SCs might receive autocrine signals by Wnt2.

## Significance

**Schwann cells (SCs) are the glial cells of the peripheral nervous system essential for nerve ensheathment and myelination. Deregulation in SC development is often associated with diseases in humans. Around birth, immature SCs segregate axons, a process called “axonal radial sorting.” Here we unravel the mechanism of Wnt/ $\beta$ -catenin signaling in axonal sorting of SCs in mice. Loss- and gain-of-function mutations of  $\beta$ -catenin in SCs impede and accelerate radial sorting of axons, respectively. Moreover, down- or up-regulated Wnt/ $\beta$ -catenin signaling inhibits or promotes cell spreading and lamellipodia formation of cultured SCs. Our research contributes to a better understanding of the mechanisms of SC development and disease.**

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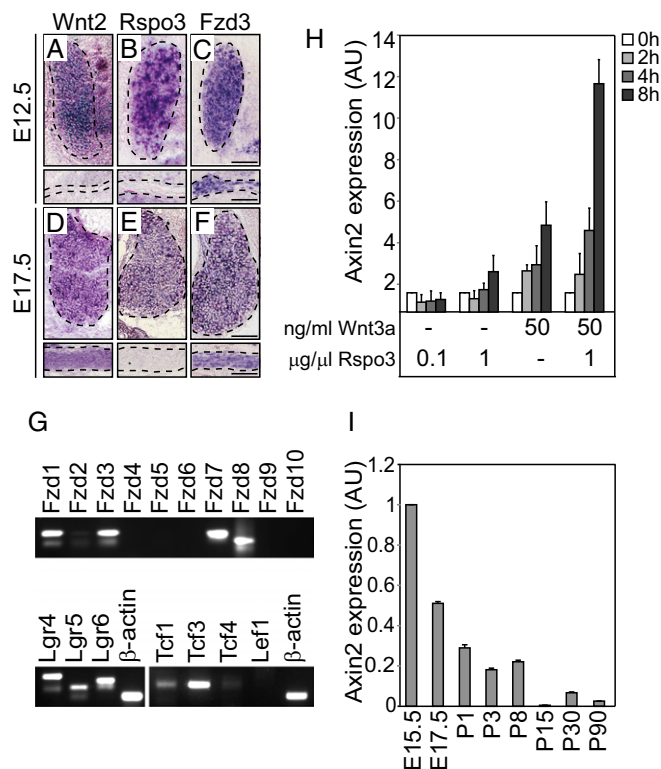
The authors declare no conflict of interest.

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Data deposition: Microarray data have been deposited in the ArrayExpress database, [www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress) (accession no. E-MTAB-1943).

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**Fig. 1.** Expression of the components and activity of Wnt signaling during SC development. Analysis of the expression of canonical Wnt signaling components (A–F) by in situ hybridization at two different stages of SC development (in each panel, *Upper* shows dorsal root ganglia, and *Lower* shows nerves) and (G) by qRT-PCR in primary SC cultures. Canonical Wnt signaling is synergistically activated by Wnt and Rspodin ligands in cultured SCs. (H) Dose-dependent coactivation of canonical Wnt signals by Wnt3a and Rspo3, determined by increase in the expression of the classical Wnt target gene *Axin2*. (I) Endogenous expression of *Axin2* in mouse sciatic nerves measured by qRT-PCR is highest at E15.5 and decreases postnatally. Error bars represent SD ( $n = 5$ ); the value at E15.5 is from embryonic sciatic nerves from 10 embryos pooled as a single probe. AU, arbitrary units. (Scale bars: 100 μm.)

We found that Wnt3a and Rspodin ligands synergistically activated transcription of the classical Wnt target gene *Axin2* in mouse primary SCs and, similarly, the Wnt-responsive luciferase gene (*Topflash* assay) in rat RT4 cells, an established SC line (Fig. 1H and Fig. S1B) (28, 29). Endogenous Wnt signaling in developing SCs was assessed by analyzing two independent Wnt-reporter mouse strains, *Axin2-LacZ* (Fig. S1C–E) (30) and *Topgal* (31), and by measuring the expression of the classical Wnt target gene *Axin2* in developing sciatic nerves by qRT-PCR (Fig. 1I). These analyses indicate that endogenous Wnt signaling activity in SCs peaks in the late fetal period (E15.5–E17.5) and declines subsequently.

**Schwann Cell-Specific  $\beta$ -Catenin Loss- and Gain-of-Function Mutations in Mice Lead to Disrupted Axonal Sorting.** To genetically analyze canonical Wnt signaling in the SC lineage, we generated  $\beta$ -catenin LOF and GOF mutations using *Cnp-cre* in mice (32). Two different alleles of  $\beta$ -catenin were used: an allele with “floxed” exons 3–6 ( $\beta$ -cat<sup>fl/fl</sup>) whose recombination leads to a frameshift mutation that prevents production of functional protein and an allele with “floxed” exon 3 ( $\beta$ -cat<sup>Ex3fl/fl</sup>) whose recombination leads to the expression of an N-terminally truncated, nondegradable, and constitutively active  $\beta$ -catenin (33, 34). Cre-mediated recombination was assessed using a YFP-reporter transgene (Fig. S2A).  $\beta$ -Catenin LOF mutations led to a strong reduction of full-length  $\beta$ -catenin protein (86% reduction), whereas GOF mutations

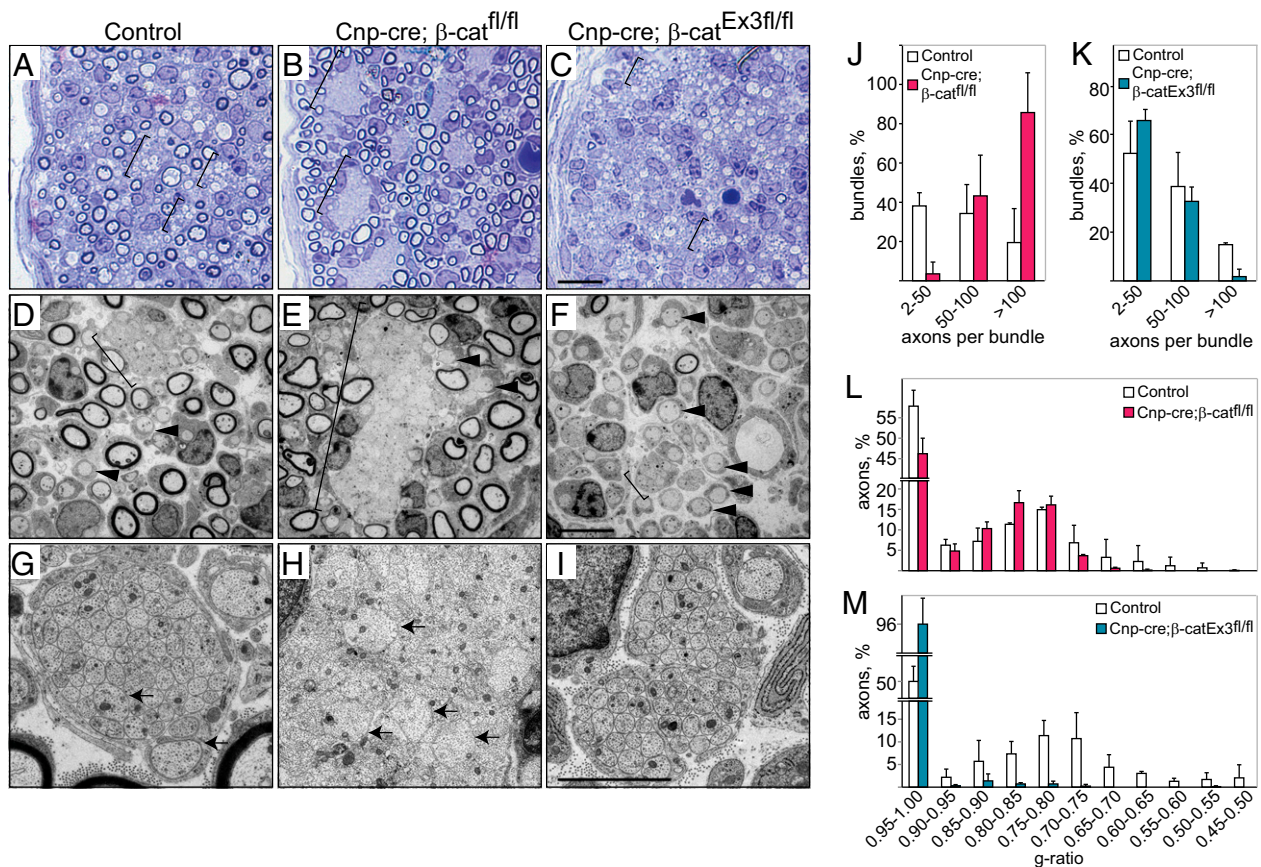
resulted in formation of a stable, activated protein (140% of wild-type level) at postnatal day (P) 1 (Fig. S2B–D). Residual full-length protein in mutants was likely due to the presence of perineurial fibroblasts and other cell types that do not express *Cnp-cre* and/or of SCs that escaped recombination (35).

*Cnp-cre*;  $\beta$ -cat<sup>fl/fl</sup> and *Cnp-cre*;  $\beta$ -cat<sup>Ex3fl/fl</sup> mutant mice were born at the expected Mendelian ratios; however, ~90% of LOF and ~30% of GOF mutants died within 1 wk after birth, likely due to defects in the central nervous system where *Cnp-cre* is also expressed (23, 32). Light microscopy of toluidine blue-stained semithin sections, and electron micrographs of sciatic nerves were used to define axonal sorting. In control mice, the majority of large-diameter axons were sorted from bundles and engaged in 1:1 relationship with SCs, and many SCs had begun to myelinate at P3 (Fig. 2A, D, and G). Remarkably, nerves of the *Cnp-cre*;  $\beta$ -cat LOF mutants contained very large axonal bundles with more than 100 axons per bundle, including large-caliber axons that were unsorted (Fig. 2B, E, and H, marked by brackets, and quantification in Fig. 2J). In addition, SCs often failed to fully envelope the bundles and to extend processes between axons (Fig. 2E and H). Conversely, in nerves of *Cnp-cre*;  $\beta$ -cat GOF mutants, Remak bundles were smaller and contained fewer axons compared with controls (Fig. 2C, F, and I and quantification in Fig. 2K), and the majority of large-diameter axons were sorted (Fig. 2F). Thus, LOF mutations of  $\beta$ -catenin in SCs impede and GOF mutations promote axonal radial sorting. Comparison of the g ratios (ratio between axonal diameter and outer diameter of the myelinated fiber) indicated a minor myelination deficit in  $\beta$ -cat LOF mutants compared with control littermates (Fig. 2L). However, *Cnp-cre*;  $\beta$ -catenin GOF mutants showed an unexpected paucity of myelination at P3, with 96% of axons displaying g ratios between 0.95 and 1 (Fig. 2C, F, and M). Even at P25, axons exhibited markedly thinner myelin, indicating that sustained  $\beta$ -catenin activity interferes with (but does not fully block) myelination, possibly by delaying myelination onset (Fig. S2E–J). Thus, the sustained  $\beta$ -catenin activity in GOF mutants enhances sorting and additionally impedes late SC differentiation and myelination.

### Mechanisms of Axonal Sorting Deficits in $\beta$ -Catenin Mutant Schwann Cells.

The radial sorting deficit in  $\beta$ -cat LOF and GOF mutant mice might be caused by aberrant SC proliferation, migration, process extension, or differentiation (9, 10, 36, 37). At early developmental stages (E14.5–17.5), no significant changes in proliferation or migration of SCs were observed in  $\beta$ -cat LOF or GOF mice compared with controls (Fig. S3A–H). In contrast, at P3, proliferation of  $\beta$ -catenin LOF SCs was reduced, and conversely, in the GOF sciatic nerves, proliferation was increased (Fig. 3A–D). To study the mechanisms further, we inhibited Wnt signaling by introducing LOF mutations in isolated primary  $\beta$ -cat<sup>fl/fl</sup> SCs using the membrane-penetrable cre protein, His-Tat-NLS-cre (HTN-cre; refs. 4 and 38); this resulted in a 76% reduction of  $\beta$ -catenin protein after 4 d culture (Fig. S3J). To activate canonical Wnt signaling in primary SC cultures, we used a small-molecule chemical inhibitor of GSK3 $\beta$ , Chir98014 (39), or recombinant Wnt3a together with Rspo3. No significant changes were observed in proliferation or migration of cultured SC (Fig. S3J–V). However, both HTN-cre and Chir 98014 treatments markedly affected cell shape and process extension, as visualized by immunolabeling of focal adhesions by anti-vinculin antibody and staining of polymerized actin by phalloidin. HTN-cre-treated SCs ( $\beta$ -cat LOF) showed reduced cell spreading, whereas treatment with Chir98014 (mimicking  $\beta$ -cat GOF) increased spreading (Fig. 3E–J and quantification in Fig. 3K). Moreover, the number of peripheral lamellipodia was significantly reduced in  $\beta$ -cat LOF and increased in Chir98014-treated cells (Fig. 3E–J, quantification in Fig. 3L, and enlargements in Fig. S4A–C). Combined treatment with Wnt3a and Rspo3 reproduced the effect of Chir98014: the number of lamellipodia in





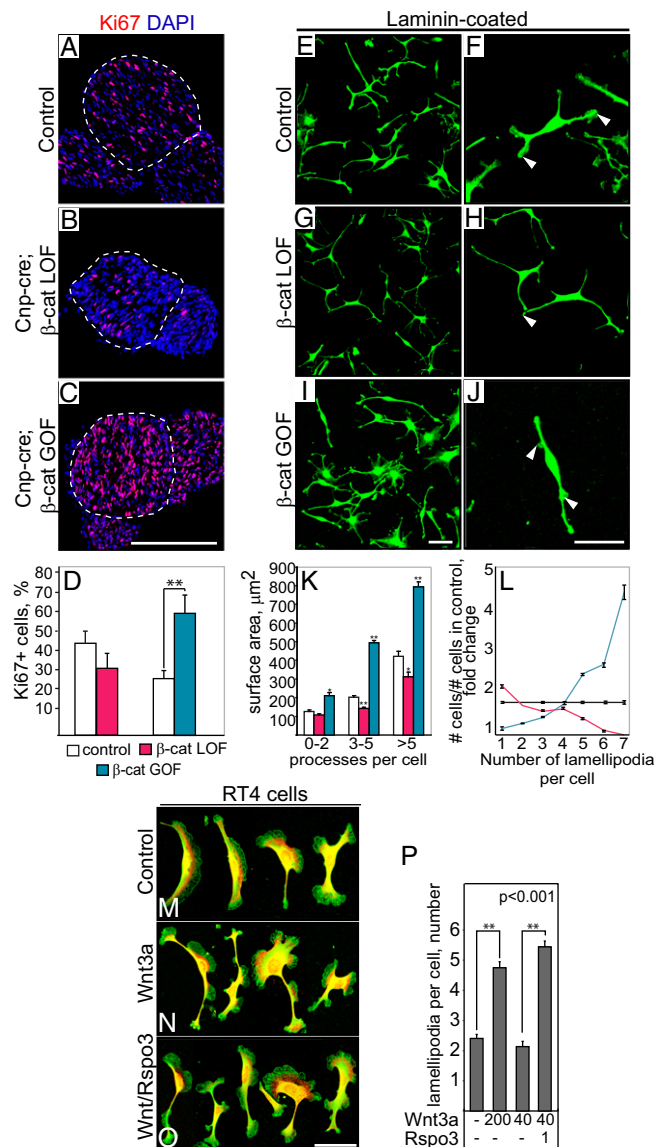
**Fig. 2.** Impaired Wnt signaling in SCs causes defects in the sorting of axons and delays myelination. (A–C) Transverse semithin sections and (D–I) electron micrographs of peripheral nerves of control, *Cnp-cre; β-cat<sup>fl/fl</sup>*, and *Cnp-cre; β-cat<sup>Ex3fl/fl</sup>* mice at P3. Note the large size of the axonal bundles in the *Cnp-cre; β-cat<sup>fl/fl</sup>* nerves and the relatively small size of bundles in the *Cnp-cre; β-cat<sup>Ex3fl/fl</sup>* nerves. Sorted axons are marked with arrowheads, axonal bundles are marked with brackets, and unsorted large axons within the bundles are marked with arrows. (Scale bars: 10 μm in A–C and 5 μm in D–I.) Quantification of the axonal bundle size in control mice along with (J) *Cnp-cre; β-cat<sup>fl/fl</sup>* (red) and (K) *Cnp-cre; β-cat<sup>Ex3fl/fl</sup>* (blue) mice. Shown is the percentage of bundles that contain a particular range of numbers of axons. Quantification of the g ratio in control mice along with (L) *Cnp-cre; β-cat<sup>fl/fl</sup>* (red) and (M) *Cnp-cre; β-cat<sup>Ex3fl/fl</sup>* (blue) mice. Shown is the percentage of axons that display a particular g ratio. Errors bars indicate SD calculated from at least three mice per group.

RT4 cells and in primary mouse SCs increased upon addition of recombinant Wnt3a in concentration-dependent manners, and Rspo3 acted synergistically (Fig. 3 *M–O*, quantification in Fig. 3*P*, and Fig. S4 *D–P*). Wnt-induced changes in spreading and lamellipodia formation in cultured primary cells and SC lines were independent of β1 integrin or of signaling through Rac1, Rho, Fak, and p38, as assessed by culturing control and β-catenin mutant SCs on the β1 integrin-independent substrate vitronectin, or in the presence of pharmacological inhibitors of Rac1, Rho-kinase, Fak, or p38 (Figs. S4 *Q–Y* and S5). Changes in axonal sorting in β-catenin LOF mutant mice were not accompanied by alterations in adherens junctions. Main components of the adherens junctions, E-cadherin and N-cadherin, were detectable in myelinating fibers starting at P3 and persisted into adulthood in regions of noncompact myelin such as Schmidt–Lantermann incisures (Fig. S6 *A–D'*). Conditional ablation of E-cadherin in SCs did not lead to abnormal bundle size or abnormal myelination (Fig. S6 *F–M*). Thus, β-catenin exerts its role in SC sorting by mediating Wnt signals, rather than stabilizing cadherin-dependent adhesion.

We next examined the differentiation of cultured SCs in vitro by measuring with qRT-PCR the mRNA levels of genes known to be differentially expressed at various stages of SC differentiation. Compared with control cells, β-catenin LOF mutant SCs up-regulated *Ncam*, *Gfap*, *p75*, and *Gap43* after 6 d culture; these genes are known to be expressed at high levels in the immature SCs (Fig. 4*A*) (1). This indicates that the β-catenin LOF mutation

interferes with the maturation of cultured SC. β-Catenin GOF cells showed up-regulated expression of *p75* and *Gap43* but also of *Sox2*, a marker of late immature SCs, and of *GalC*, a marker of differentiated SCs in culture (Fig. 4*B*) (1, 40). Similarly, addition of recombinant Wnt3a to primary SCs enhanced the expression of those late immature SC markers in a dose-dependent manner (Fig. S7*A*). Thus, activation of Wnt signaling drives the differentiation from an early to a late immature SC stage. The expression of the myelin genes *Pmp22*, *P0*, and *Prx* was reduced in both LOF and GOF cells in culture (Fig. 4*A* and *B*).

We also assessed the differentiation of mutant SCs in vivo. Immunohistological analysis of *Cnp-cre; β-cat* GOF mice showed that protein levels of myelin basic protein (MBP) and the transcription factor cMaf were reduced compared with the controls, in line with the observed myelin disruption (Fig. 4*C–F*). Another marker of myelinating SCs, Krox20, was unaffected (Fig. 4*G* and *H*). In contrast, markers of immature SCs, L1 and Sox2, were up-regulated in the β-catenin GOF cells (Fig. 4*I–L*). This confirmed that SCs reached a late immature stage, but their entry into the myelination program was impaired when Wnt signaling was activated in a sustained manner. *Cnp-cre; β-cat* LOF mice showed a mild reduction in MBP and cMaf, in line with the slightly reduced myelination, as well as a decrease in Sox2 and an increase in L1 (Fig. S7 *B–I*). We also used gene expression profiling to identify sets of genes that are controlled by Wnt/β-catenin signaling in SCs. First, we focused on genes that were regulated in opposing manners by reduction or stimulation of canonical Wnt



**Fig. 3.** Defects in cell cycle exit and lamellipodia formation in  $\beta$ -catenin mutant SCs in vivo and in vitro. (A–C) Proliferating SCs at P3, visualized by immunostainings using antibodies against proliferation marker Ki67. Dotted outlines surround axons. (Scale bar: 100  $\mu$ m.) (D) Quantification of the proliferating cells. Shown is the percentage of the Ki67+ cells per nerve cross-section. (E–J) SC shape and lamellipodia formation were analyzed by immunostaining using antibodies against vinculin. SCs carrying  $\beta$ -catenin LOF mutations fail to produce terminal lamellipodia, whereas SCs mimicking  $\beta$ -catenin GOF mutations produce more lamellipodia. (Scale bars: 40  $\mu$ m.) (K) Measurement of surface area of control,  $\beta$ -catenin LOF, and GOF SCs on laminin. Shown is the surface area quantified separately for cells containing zero to two, three to five, and more than five processes per cell. (L) Quantification of the number of terminal lamellipodia per cell in the  $\beta$ -catenin LOF and GOF cells, shown as fold changes, normalized to controls. (M–O) Lamellipodia formation in RT4 cells in the absence and presence of 40–200 ng/mL Wnt3a and 1  $\mu$ g/ $\mu$ L Rspo3 ligands as assessed by immunostaining using Phalloidin (red) and antibodies against vinculin (green). (Scale bar: 40  $\mu$ m.) (P) Quantification of lamellipodia in M–O. Error bars in D indicate SD ( $n = 3$ ), and error bars in K, L, and P indicate SEM.  $t$  test: \*\*\* $P < 0.01$ .

signaling, i.e., that were significantly down-regulated in  $\beta$ -cat LOF and up-regulated in Chir98014- or Wnt3a-treated SCs. Among these were the known Wnt target and regulator genes, e.g., *Axin2*, *Nkd1*, and *Ccnd1* (Fig. 4M and refs. 30 and 41); genes involved in cytoskeletal rearrangement and actin-binding, like

*Arpc1a*, *Syn1*, *Fyn*, *Afap1*, and *Fez1*; and potential glial differentiation genes, like *Olig1*, *Shh*, *Jun*, and *Fgf5* (Fig. 4N and Table S2). The mRNA levels of differentiation-associated genes were confirmed by qRT-PCR (Fig. 4N). Second, we analyzed a group of genes that were significantly deregulated upon Chir98014 or Wnt3a treatment only. Among these, *Gap43* and *p75* were up-regulated, whereas myelin genes such as *Pmp22* and *Pmp2* and genes encoding enzymes that control lipid and fatty acid metabolism, e.g., *Pex5*, *Cpt2*, and *Cpt1c*, were down-regulated (Fig. 4M and Table S3).

### Discussion

Here we show that conditional LOF and GOF mutations of  $\beta$ -catenin in mouse SCs impede and accelerate radial sorting of axons, respectively. We further show that down- or up-regulated Wnt/ $\beta$ -catenin signaling inhibits or promotes cell spreading and lamellipodia formation in cultured SCs. A comprehensive expression analysis of the components of Wnt/ $\beta$ -catenin signaling suggests that during axonal sorting, paracrine signal exchange between axons and SCs may occur. Analysis of in vivo Wnt-reporter activity and target gene expression indicated that perinatal SCs transiently receive Wnt signal during radial sorting. Wnts and Rspodins are produced by neurons and potentially released along axons, where they could interact with Lgr4–6 and Lrp/Frizzled receptors on SCs. We suggest a model where Wnt and Rspodin signals are essential to promote axonal sorting and SC lineage progression beyond the immature stage.

Several ligands of canonical Wnt signaling are expressed in developing peripheral ganglia and in SCs: Wnt2, 6, and 9b and Rspodins 1–4 in neurons and Wnt2 also in SCs. Addition of exogenous Wnt ligands to primary cultured SCs activated the canonical Wnt pathway in a dose-dependent manner, demonstrating that the downstream Wnt cascade was functional. SCs express the three Lgr4–6 receptors, and Rspodins synergistically promote Wnt signaling and lamellipodia formation in SC culture. This suggests that Rspodins that are expressed neuronally during the sorting process could contribute to the sorting also in vivo. The expression of Wnts and Rspodins in nerves and their receptors in SCs was observed starting at E12.5, but Wnt reporter activity was detected in the peripheral nerves starting at E15.5. The absence of Wnt responses at earlier stages may indicate the existence of specific regulatory mechanisms that suppress precocious Wnt activity downstream of Wnt/Rspodin receptors in vivo. Alternatively, a more complex interaction may exist; that is, Wnt2 that is expressed late in SCs may initiate, whereas axonal Rspodins may modulate Wnt signaling in SCs. Postnatally, no Wnt reporter activity could be detected in SCs. Thus, canonical Wnt signaling is active during a small window in the late fetal period of SC development.

Conditional LOF and GOF mutations of  $\beta$ -catenin in mouse SCs were introduced in vivo and in vitro, respectively, using *Cnp-cre* and HTN-cre. In both systems,  $\beta$ -catenin was not required for SC early proliferation or migration; instead, mutant cells showed abnormal extension of cytoplasmic processes. Electron microscopic analyses showed that conditional  $\beta$ -catenin LOF mutant SCs failed to fully envelope axonal bundles and to extend processes between axons, leading to abnormal radial sorting and accumulation of large unsorted bundles containing more than 100 axons. Conversely, in  $\beta$ -catenin GOF nerves, bundles were smaller than in controls and contained fewer axons, pointing to an accelerated sorting. We could exclude Wnt-independent roles of  $\beta$ -catenin, for instance, in cadherin-mediated cell adhesion, because axonal sorting was normal in *Cnp-cre*; *E-cadherin* LOF mutant mice. Previous studies demonstrated that axonal sorting largely depends on signals from the extracellular matrix, e.g., of *Fak*, *Rac1*,  $\beta$ 1 *integrin*, and *Integrin-linked kinase* (9, 10, 42). However, Wnt-dependent changes of the cultured SCs persisted on  $\beta$ 1-integrin-independent substrates. Moreover, pharmacological inhibitors of Rac1, Fak, and p38 signaling could not rescue Wnt-induced phenotypes. Together, this suggests that the SC phenotypes induced by Wnt are independent of the matrix.





light microscopy, 250-nm sections were stained with toluidine blue. For electron microscopy, 60-nm sections were contrasted with lead citrate and examined using a LEO 906E transmission electron microscope. Axon diameters and g ratios were determined from electron micrographs. Immunofluorescence was performed as described (4).

Primary SCs were isolated from E13.5 DRGs as described (52). HTN-cre protein was produced as described (38). To induce homologous recombination, homozygous floxed  $\beta$ -catenin SCs were incubated with 2  $\mu$ M HTN-cre for 20 h and subsequently cultured for 3 d to deplete the  $\beta$ -catenin protein. RT4 cells (28) were cultured in high-Glucose DMEM (Gibco) supplemented with 10% (vol/vol) FBS and antibiotics.

For the microarray, total RNA was isolated from cultured primary SCs using TRIzol (Invitrogen), purified by RNeasy kit (Qiagen), biotin-labeled using Illumina TotalPrep DNA amplification kits (Ambion) according to the manufacturer's

instructions, and hybridized to Illumina MouseRef-8 whole genome expression bead chips. Microarray data were analyzed using Partek Genomics Suite. Normalization was performed using the gcRMA method; subsequently, probe sets with expression levels  $<64$  were eliminated as not expressed. Genes showing differential expression using Benjamin Hochberg false discovery rate (FDR)  $< 0.05$  in ANOVA were selected. Functional analysis and gene ontology classification were performed using the Database for Annotation, Visualization, and Integrated Discovery v6.7 (53).

For more details, see also *SI Methods*.

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