Mechanisms of Development 160 (2019) 103577

Contents lists available at ScienceDirect

Mechanisms of Development

journal homepage: www.elsevier.com/locate/mod

# The Hedgehog receptor Patched1 regulates proliferation, neurogenesis, and axon guidance in the embryonic spinal cord

Angelo Iulianella*, Danielle Stanton-Turcotte

*Department of Medical Neuroscience, Faculty of Medicine and Brain Repair Centre, Dalhousie University, Life Science Research Institute, 1348 Summer Street, Halifax, Nova Scotia B3H-4R2, Canada*

A B S T R A C T

The formation of the vertebrate nervous system depends on the complex interplay of morphogen signaling pathways and cell cycle progression to establish distinct cell fates. The Sonic hedgehog (Shh) signaling pathway is well understood to promote ventral cell fates in the developing spinal cord. A key regulator of Shh signaling is its receptor Patched1 (Ptch1). However, because the *Ptch1* null mutation is lethal early in mouse embryogenesis, its role in controlling cell cycle progression, neurogenesis, and axon guidance in the developing spinal cord is not fully understood. An allele of Ptch1 called *Wiggable* (*Ptch1*$^{Wig}$), which was previously shown to enhance Shh signaling, was used to test its ability to regulate neurogenesis and proliferation in the developing spinal cord. *Ptch1*$^{Wig/Wig}$ mutants displayed enhanced ventral proneural gene activation, and aberrant proliferation of the neural tube and floor plate cells, the latter normally being a quiescent population. The expression of the cell cycle regulators p27$^{Kip1}$ and p57$^{Kip2}$ were expanded in *Ptch1*$^{Wig/Wig}$ mutant spinal cords, as was the number of mitotic and S-phase nuclei, suggesting enhanced cell cycle progression. However, *Ptch1*$^{Wig/Wig}$ mutants also showed enhanced apoptosis in the ventral embryonic spinal cord, which resulted in thinner spinal cords at later embryonic stages. Commissural axons largely failed to cross the floor plate of *Ptch1*$^{Wig/Wig}$ mutant embryos, suggesting enhanced Shh signaling in these mutants led to a dorsal expansion of the chemoattraction front. These findings are consistent with a role of Ptch1 in regulating neurogenesis and proliferation of neural progenitors, and in restricting the influence of Shh signaling in commissural axon guidance to the floor plate.

## 1. Introduction

The coordination between growth, specification, and neurogenesis is crucial for the proper formation and integration of neuronal circuits during development. Signaling by morphogens play a powerful role in regulating neuronal growth and specification, but how they influence neuronal birth and cell cycle progression is less clear. The Sonic hedgehog pathway is essential for the specification of ventral cell identities in the vertebrate neural tube. While it has a potent mitogenic influence leading to oncogenesis when it is over-expressed, *Shh* loss-of-function of does not necessarily lead to inhibiting growth of the developing nervous system (Chiang et al., 1996; Matise, 2013). It remains unclear how it can coordinately influence both neural progenitor proliferation and differentiation. A possibility may involve bifurcating pathways acting downstream of Shh signaling to separately influence neurogenesis and growth control. For instance, Shh can activate atypical guanine-nucleic exchange factors and kinase signaling to influence axonal growth and targeting independently of its patterning and mitogenic roles (Izzi et al., 2011; Makihara et al., 2018; Yam et al., 2012; Yam et al., 2009). Activation of the principal transcriptional effectors for Shh signaling, the Gli proteins, leads to the specification of the ventral-most cell types in the developing nervous stems: namely, the floor plate, V0–V3 interneurons, and motor neurons (Briscoe and Small, 2015; Iulianella et al., 2018).

Shh signaling principally acts by binding to a 12-pass transmembrane protein called Patched1 (Ptch1). It functions by inhibiting a co-repressor, Smoothened (Smo), which in turn is responsible for promoting the processing of full-length Gli factors into potent transcriptional activators (Bai et al., 2004; Balaskas et al., 2012; Chen and Struhl, 1996; Cohen et al., 2015; Gorojankina, 2016; Lei et al., 2004). In the absence of ligand binding, Ptch1 remains at the cell surface prevent Smo activation of downstream events necessary for the activation of Shh target genes. The binding of lipid-modified Shh results in the endocytosis of the Shh-Ptch1 complex, allowing for the activation of Smo-regulated downstream events (Denef et al., 2000; Incardona et al., 2002; Myers et al., 2017; Torroja et al., 2004; Weiss et al., 2019). As such Ptch1 acts a key regulator of the Shh pathway, dynamically regulating the response to the Shh signaling gradient during neural development (Iulianella et al., 2018). Despite this, our knowledge of Ptch1 receptor function is incomplete. For instance, despite homologies to Niemann-Pick C1 disease protein (Carstea et al., 1997; Loftus et al., 1997), we have yet to small lipophilic molecules that may pass through it, allowing for an activation of Smo. However, recent structural evidence suggests the possibility that Ptch1 may function as a hetero-tetrameric channel complex that allows the transport of cholesterol-like small molecules intracellularly, that could presumably activate Smo

* Corresponding author.
*E-mail address:* angelo.iulianella@dal.ca (A. Iulianella).

https://doi.org/10.1016/j.mod.2019.103577
Received 23 July 2019; Received in revised form 3 September 2019; Accepted 2 October 2019
Available online 18 October 2019
0925-4773/ 

(Qian et al., 2019). It is a complex multi-functional protein, harboring a sterol-sensing domain, extracellular Hh-binding loops, and a C-terminal domain that interacts with several proteins, include pro-apoptotic XIAP proteins (Aoto and Trainor, 2015; Marigo et al., 1996; Qi et al., 2018; Rohatgi et al., 2007; Stone et al., 1996).

To more fully explore the role of Shh signaling in ventral neurogenesis and cell cycle progression, a previously reported Ptch1 allele called *Wiggable* was used to create excess Shh signaling in the embryonic spinal cord. This allele was produced by ethylnitrosourea (ENU)-mediated mutagenesis in a mouse embryonic forward genetic screen, and results in a Carboxyl (C)-terminal truncation of Ptch1 (Iulianella et al., 2018; Kurosaka et al., 2014; Kurosaka et al., 2015; Sandell et al., 2011). The C-terminus is crucial for the regulation of Smo activity, thus the Ptch1$^{Wig}$ protein behaves genetically as a Shh gain-of-function mutation (Iulianella et al., 2018). Indeed, the *Ptch1*$^{Wig}$ allele was recently used to explore the role of Shh activity gradient in the ventral neural specification (Iulianella et al., 2018). It was reported to act as a Shh gain-of-function mutation, showed reduced Gli-repressor formation, and as a consequence an expansion of ventral neural progenitor patterning in the developing spinal cord. However, the effect of the *Ptch1*$^{Wig}$ mutation on proliferation, neurogenesis, and commissural axon guidance in the developing spinal cord has yet to be investigated.

The formation of neurons requires the activation of a proneural gene expression program and cell cycle exit of specified progenitors. The proneural factors, which include basic-helix-loop-helix domain-containing transcription factors such as Neurod1, Ngn1, Ngn2, and Ascl1, act downstream of Notch signaling to drive the switch from progenitor maintenance to differentiation in the developing spinal cord (Iulianella et al., 2008; Iulianella et al., 2009; Sugimori et al., 2007; Yamamoto et al., 2001). Interestingly, previous findings have suggested a link between Notch and Shh signaling crosstalk in regulating embryonic neurogenesis (Dave et al., 2011; Kong et al., 2015; Stasiulewicz et al., 2015). In this study, the *Ptch1*$^{Wig}$ allele was used to reveal a role for the Ptch1 receptor, and Shh signaling in general, in the regulation of the proliferation and neurogenesis in the developing nervous system, including the floor plate cells. It is reported here that *Ptch1*$^{Wig/Wig}$ mutants displayed aberrantly enhanced proliferation, and expanded expression of a proneural gene expression program and cell cycle exit regulators in the embryonic spinal cord. As a consequence, commissural axon guidance towards the midline was greatly perturbed. Based on these findings, it is concluded that Ptch1 influences proneural gene activation and cell cycle progression, facilitating the rapid specification of ventral neural cell types during spinal cord development.

## 2. Results

Shh signaling plays crucial roles in the patterning and growth of the embryonic nervous system. The *Ptch1*$^{Wig}$ allele is a carboxyl (C)-terminal truncation of Ptch1 protein that results in a loss negative feedback control of Shh signaling during development. *Ptch1*$^{wig/wig}$ mutants are viable until E11.5, allowing for an analysis of some of the key events in neural and craniofacial patterning (Iulianella et al., 2018; Kurosaka et al., 2015). It was recently reported that *Ptch1*$^{wig/wig}$ mutants displayed expanded ventral progenitor domains in eth embryonic spinal cord, while maintaining dorsal specification, suggesting it functioned as a hypomorphic allele relative to the much more severe *Ptch1* complete loss-of-function phenotype (Iulianella et al., 2018; Rohatgi et al., 2007). It is thus a useful allele to explore the consequences of excess Shh signaling during development. While the role of Shh signaling in the specification ventral cell identities is well established, its regulation of proneural gene activity during neural growth it is less understood. The *Ptch1*$^{Wig}$ allele was used to explore the consequences of a ventral expansion of Shh signaling during early neurogenesis in the mouse embryo.

### 2.1. *Ptch1*$^{Wig/Wig}$ mutants displayed enhanced neuroepithelial proliferation and floor plate expansion

In order to evaluate the effect of *Ptch1*$^{Wig/Wig}$ mutation on the formation of ventral midline tissues, *Shh mRNA* expression was examined by whole mount ISH (Fig. 1A–C). Shh expression was increased in ventral midline tissues in the *Ptch1*$^{Wig/Wig}$ mutant, with a greater increase observed in the hindbrain to forebrain regions (Fig. 1A–C). Shh immunohistochemistry confirmed expanded Shh expression from the *Ptch1*$^{Wig/Wig}$ floor plate, especially in more anterior regions of the neural tube, which displayed exencephaly (Fig. 1D, E, H, I). This was accompanied by an expansion of the floor plate marker FoxA2 in the mutants, especially in more anterior regions of the developing neural tube (*, Fig. 1G, K). These findings suggested that the expanded ventral identity of *Ptch1*$^{Wig/Wig}$ mutants may be due to enhanced proliferation of ventral neural progenitors. To explore this possibility, BrdU pulse labeling of DNA synthetic nuclei was conducted following a short (1 h) pulse of dams at E9.5 and E11.5. In conjunction, mitotic nuclei were labeled with pH 3 antibodies. The neuroepithelium of the mouse embryo at E9.5 consists mostly of rapidly diving progenitor cells lining the ventricles in a pseudostratified cellular organization. At this stage very few cells have exited the cell cycle, and as such the marginal zone has yet to develop (Fig. 2A). Proliferation of neuroepithelium at E9.5 was largely unaffected in *Ptch1*$^{Wig/Wig}$ mutants, although there was a non-significant trend for enhanced BrdU+ and pH 3-positive mitotic nuclei (Fig. 2B, H). The spacing between the BrdU- and pH 3-positive domains in the mutant embryonic neuroepithelium was decreased, and DNA synthetic and mitotic nuclei intermingled at their respective boundaries instead of being separated by a layer of unlabeled nuclei, suggesting aberrant cell cycle progression in the mutants (Fig. 2A, B). By E11.5 significant increases were observed in both BrdU+ and pH 3+ nuclei of *Ptch1*$^{Wig/Wig}$ mutants relative to wild type littermates (Fig. 2C–F, ***$P < .0001$, Fig. 2G, H). Interestingly, in the forebrain, the *Ptch1*$^{Wig/Wig}$ neuroepithelium was aberrantly kinked, superficially resembling the organization of the cerebellum (Fig. 2D). This abnormal forebrain morphogenesis could be a result of the hyperproliferation of the neuroepithelium or aberrant floor plate development in the *Ptch1*$^{Wig/Wig}$ embryo. These findings illustrated that the loss of *Ptch1* led to unrestricted proliferation of the early embryonic neuroepithelium.

In contrast to the developing neuroepithelium, floor plate cells are normally quiescent, showing very little (if any) proliferation during development (E9.5–11.5; Fig. 2A, E, I, K). It was therefore surprising to see the accumulation of mitotic floor plate cells in the *Wiggable* mutant (Fig. 2I, J, arrows). Antibodies against acetylated-α-tubulin, which is found in stabilized microtubules, can be used to detect monocilia and kinetochore of mitotic chromosomes, and the latter was clearly observed in the *Ptch1*$^{Wig/Wig}$ floor plate cells (arrow, Fig. 2L). This led to a deviation from the normal pseudostratified organization of the floor plate, revealed by high magnification confocal images of DAPI and acetylated-α-tubulin staining (Fig. 2K, L). Thus the mutant floor plate was proliferative, accounting for the dorsal expansion of the Shh gradient and FoxA2 cells (Fig. 1).

Despite the increased proliferation of *Ptch1*$^{Wig/Wig}$ mutant floor plate cells, they appeared to maintain the presence of monocilia in ependymal region (turquoise arrowhead, Fig. 2K, L), suggesting that mutant floor plate cells retained their appropriate cytoarchitecture. While FoxA2 expression is used to infer floor plate identity, its levels can also reflect a direct transcriptional response to Shh signaling (Iulianella et al., 2018; Sasaki et al., 1997). Thus, to unambiguously determine if the increased FoxA2 expression in *Ptch1*$^{Wig/Wig}$ mutants reflected floor plate expansion, we utilized another marker, Hes1, to outline the floor plate boundary region (Baek et al., 2006). While *Ptch1*$^{Wig/Wig}$ mutant floor plate cells displayed a sharp ventrally restricted Hes1-positive floor plate boundary region, low Hes1 expression was observed throughout the basal plate, likely reflecting an expanded competency to form floor plate cells (Fig. 2N, cyan bar). This is consistent with the

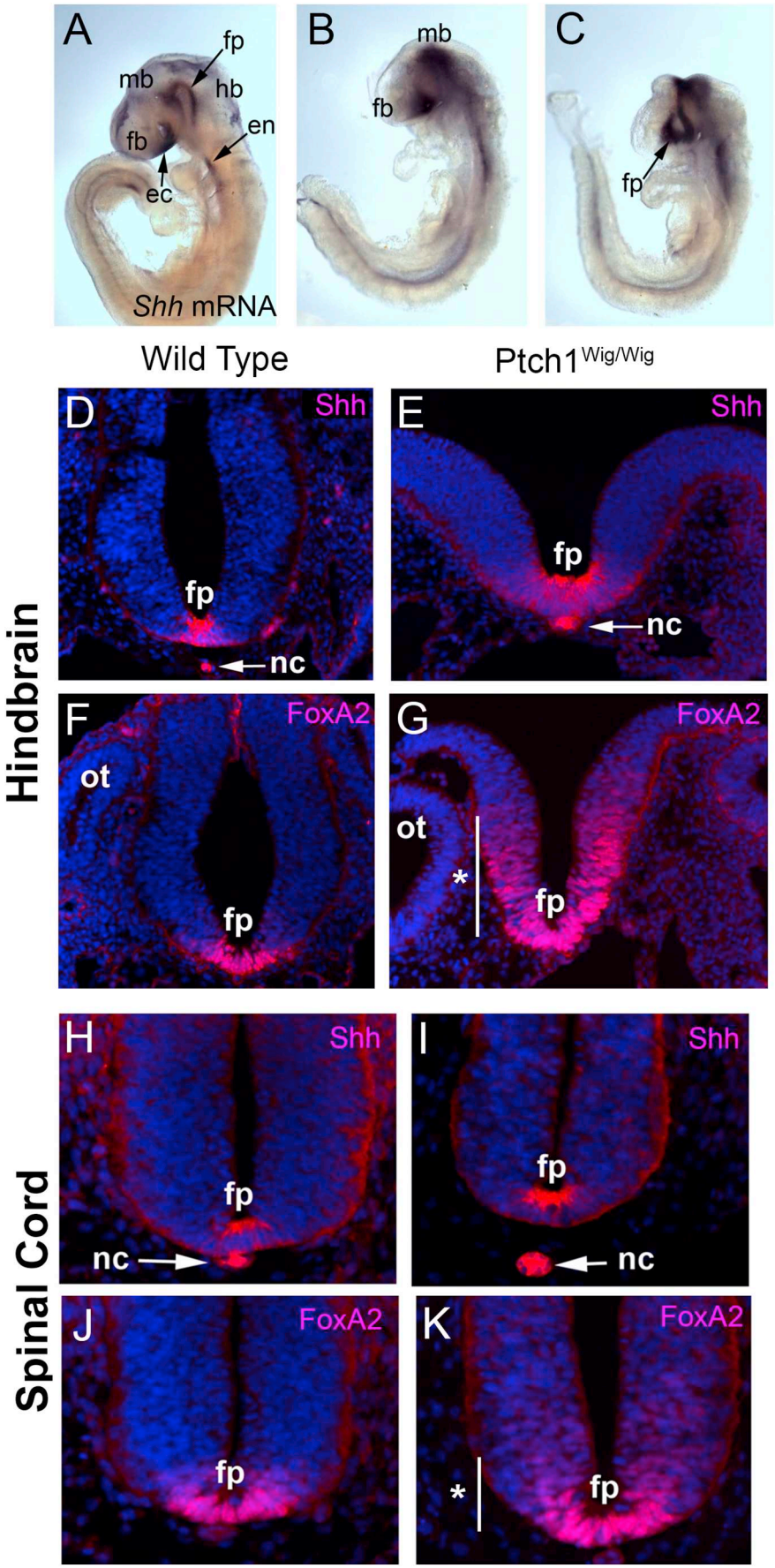
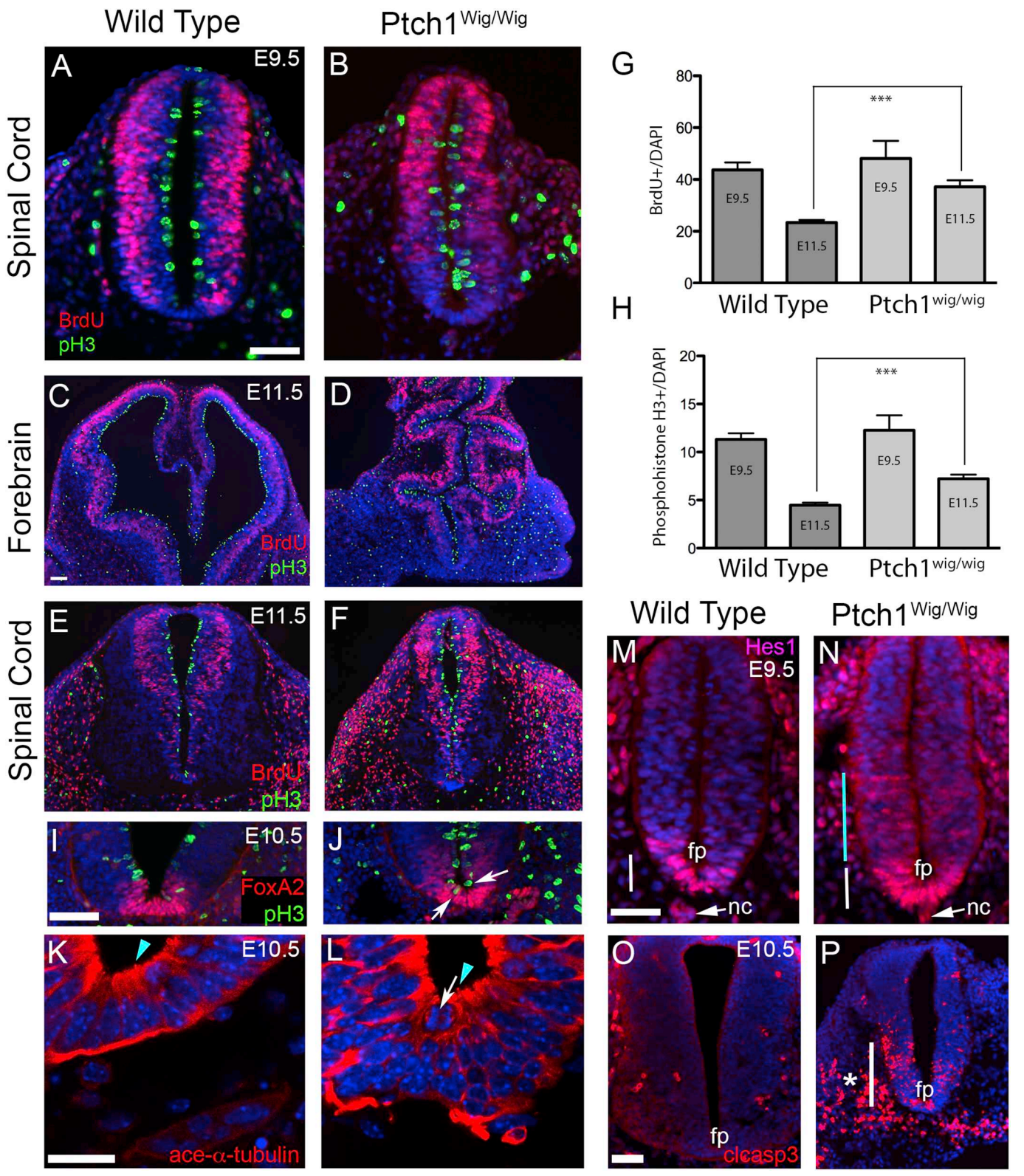



**Fig. 1.** *Ptch1*$^{Wig/Wig}$ mutants display an expanded FoxA2+ territory in the ventral embryonic nervous system. (A–C) *Shh* mRNA *in situ* hybridization of E9.5 wild type (A) and *Ptch1*$^{Wig/Wig}$ mutant (B, C) mouse embryos. (D, E) Shh immunohistochemistry in the hindbrain region in E9.5 wild type (D) and a *Ptch1*$^{Wig/Wig}$ mutant (E). (F, G) FoxA2 immunohistochemistry in E9.5 wild type (F) and *Ptch1*$^{Wig/Wig}$ mutant (G) embryos, showing expanded FoxA2+ cells in the mutant (bar, *). (H, I) Shh immunohistochemistry in the floor plate and notochord within the spinal cord region of E9.5 wild type (H) and *Ptch1*$^{Wig/Wig}$ mutant (I) embryos. (J, K) FoxA2 immunohistochemistry in E9.5 wild type (J) and *Ptch1*$^{Wig/Wig}$ mutant (K), showing expanded FoxA2+ cells in mutant spinal cords (bar, *). DAPI counterstain. Abbreviations: ec, ectoderm; en, endoderm; fb, forebrain; fp, floor plate; hb, hindbrain; mb, midbrain; nc, notochord.




*(caption on next page)*

**Fig. 2.** *Ptch1*$^{Wig/Wig}$ mutant neural tubes displayed abnormal proliferation of floor plate cells and enhanced apoptosis of ventral tissues. (A-F) Phosphohistone H3 (pH 3, green) and 5-bromo-2′-deoxyuridine (BrdU, red) immunohistochemistry following a 1-hour pulse in wild type (A, C, E) and *Ptch1*$^{Wig/Wig}$ mutant (B, D, F) embryos at E9.5 (A-B) and E11.5 (C–F). (G-H) Quantification of BrdU+ (G) and phosphohistone H3+ (pH 3+; H) cells relative to total DAPI+ cells in the developing spinal cord at E9.5 and E11.5 in wild type and *Ptch1*$^{Wig/Wig}$ mutants. *Ptch1*$^{Wig/Wig}$ mutants displayed enhanced proliferation of neuroepithelial cells revealed by pH 3+ ($N = 7$, $^{***}P = .0002$) and BrdU+ cells at E11.5 (N = 7, $^{***}P = .0003$). (H) *Ptch1*$^{Wig/Wig}$ mutants (N = 7) showed non-significant increases in pH 3+ cells (N = 7, $P = .5834$) and BrdU+ cells (N = 7, $P = .5596$) at E9.5. (I, J) pH 3 (green) and FoxA2 (red) immunohistochemistry if E10.5 wild type (I) and *Ptch1*$^{Wig/Wig}$ mutant (J) embryos displaying aberrant mitotic nuclei (arrows) in the floor plate of mutant embryonic spinal cords. (K, L) High magnification image of acetylated-α-tubulin immunostaining of the floor plate of E10.5 wild type (K) and *Ptch1*$^{Wig/Wig}$ mutant (L) embryos revealed a non-pseudostratified appearance and mitotic nuclei (arrow). Monocilia were present in floor plate cells of wild type (K) and *Ptch1*$^{Wig/Wig}$ mutant embryos (turquoise arrowhead). (M, N) Hes1 immunostaining of E9.5 wild type (M) and *Ptch1*$^{Wig/Wig}$ mutant (N) spinal cords revealed the floor plate boundary region (bar). (N) *Ptch1*$^{Wig/Wig}$ embryo displayed an expanded domain of low Hes1 levels in the basal neural tube (cyan bar). (O, P) Cleaved Caspase-3 (clcasp3) immunostaining of E10.5 wild type (O) and *Ptch1*$^{Wig/Wig}$ mutant (P) embryos, displaying enhanced apoptosis in the ventral neural tube and mesenchyme (bar, *). DAPI counterstain. Scale bars: (A, I, M, O), 50 μm; (K), 12.5 μm. Abbreviations: fp, floor plate; nc, notochord.

expansion of FoxA2 as a marker for floor plate identity observed in *Ptch1*$^{Wig/Wig}$ mutants (Fig.1). Altogether, these findings confirmed that despite the enhanced proliferation of floor plate cells in *Ptch1*$^{Wig/Wig}$ mutants, they nonetheless maintained their proper identity as floor plate tissue.

The increased proliferation of the *Ptch1*$^{Wig/Wig}$ mutant neuroepithelium during contrasted with its thinning later in the development, suggesting elimination of excess cells by death (Fig. 2A–F). To investigate this possibility, Cleaved Capsase-3 immunostaining was used to identify apoptotic nuclei. *Ptch1*$^{Wig/Wig}$ mutants showed greatly enhanced cell death in the basal neuroepithelium and surrounding ventral mesenchymal tissues, which are tissues sensitive to Shh levels (Fig.2O, P, *). Taken together these findings demonstrate that enhanced Shh signaling in *Ptch1*$^{Wig/Wig}$ mutants resulted in a limited expansion of neuroepithelial proliferation, concomitant with a ventralized basal plate.

### 2.2. Enhanced neurogenesis in *Ptch1*$^{Wig/Wig}$ mutant spinal cords

Given the increased proliferation of neural progenitors in the *Ptch1*$^{Wig/Wig}$ mutant neural tube, cell cycle exit was explored by immunohistochemistry for the two main cell cycle inhibitory kinases active in the developing spinal cords: P27$^{Kip1}$ and P57$^{Kip2}$ (Gui et al., 2007; Iulianella et al., 2008; Iulianella et al., 2009). P27$^{Kip1}$ was expressed throughout the marginal zone and adjacent intermediate region surrounding the ventricular zone at E10.5 (Fig. 3A). The levels of p27$^{Kip1}$ appeared to be more intense in the basal plate of *Ptch1*$^{Wig/Wig}$ mutants (Fig. 3B) (Iulianella et al., 2008), likely reflect a contracted marginal zone region. P57$^{Kip2}$ labels neuroblasts exiting the cell cycle in two separate clusters in the dorsal and ventral embryonic neural tube (Fig. 3C). Interestingly the spacing between these two P57$^{Kip2}$ expression clusters was expanded in the *Ptch1*$^{Wig/Wig}$ mutants, as was and its ventral expression domain (*, Fig. 3D). This suggested that the basal half of the mutant embryonic spinal cord displayed enhanced formation of neuroblasts relative to the alar plate. To confirm this phenotype, Ascl1/Mash1 immunostaining was used to explore the formation of neurogenic progenitors one day earlier at E9.5. As with p57$^{Kip1}$, Ascl1 was normally expressed in two domains, one identifying p3 progenitors in the ventral neural tube, the other a cluster of dorsal commissural neurons (Fig. 3E) (Iulianella et al., 2009). In contrast, E9.5 *Ptch1*$^{Wig/Wig}$ mutants showed an expanded ventral p3 Ascl1 domain, but a severe reduction of the dorsal Ascl1-positive cluster, signifying enhanced formation of ventral progenitors and delayed development of dorsal progenitors. This is consistent with a recent report demonstrating expanded ventral neural progenitor regions in the *Ptch1*$^{Wig/Wig}$ mutant (Fig. 3F) (Iulianella et al., 2018). Because of the dramatic expansion of neural progenitors in the E9.5 *Ptch1*$^{Wig/Wig}$ mutant spinal cord, it was expected that the mutants should show expanded neuroblast formation at neurogenic phases of spinal cord development (E10.5–11.5). To evaluate this, antibodies against the proneural protein Neurod1 was used in conjunction with the neural progenitor marker Pax6 in immunostaining experiments. In control wild type embryos, Neurod1 was highly expressed in neuroblasts, while Pax6 was restricted to neural progenitors in the ventricular zone (Fig. 3G). *Ptch1*$^{Wig/Wig}$ mutant embryos displayed expanded Neurod1 levels in the basal plate and dorsalized Pax6 expression (Fig. 3H), confirming enhanced neurogenesis. Altogether theses findings demonstrate that the loss of Ptch1 function led to unrestricted proliferation of ventral neural progenitors, which led to expanded neurogenesis within the basal neural plate. This led to the expectation that targeting of axons to the ventral midline should be affected during the wiring of the developing spinal cord.

### 2.3. Mis-targeting of commissural axons towards the ventral midline of *Ptch1*$^{Wig/Wig}$ mutant spinal cords

The floor plate is enriched in guidance molecules such as Netrin and Shh, which act coordinately to attract growing axons from dorsal commissural neurons during spinal cord development (Sloan et al., 2015; Wu et al., 2019). The absence of either Netrin or Shh leads to a mis-targeting commissural axons at a distance away from the floor plate (Charron et al., 2003; Kennedy et al., 1994). However, in the case of the Shh mutant, the aberrant targeting of commissural axons could be a secondary consequence of the lack of floor plate tissues (Charron et al., 2003; Chiang et al., 1996). Recently, the endocytic adapter molecule Numb was shown to regulate the internationalization of a receptor complex involving Ptch1 to allow for growth cone turning during commissural axon guidance (Ferent et al., 2019). This suggests that Ptch1 is critically required for axon guidance decisions during spinal cord development, but a definite conclusion required a genetic analysis using *Ptch1* loss-of-function mutations.

*Ptch1*$^{Wig/Wig}$ mutant embryos maintain floor plate identity, but also showed an expansion of Shh and FoxA2 in this tissue, suggesting expanded floor plate identity (Fig. 1). A previous report demonstrated that *Ptch1*$^{Wig/Wig}$ mutants displayed enhanced Shh signaling in the ventral neural tube, acting as a Shh gain-of-function mutation (ref). Thus, the *Ptch1*$^{Wig/Wig}$ mutant it is an appropriate model to examine the effect of an enhanced Shh signaling gradient on axon guidance. The anatomy of the embryonic spinal cord was visualized using H&E staining at E10.5 (Fig. 4). White matter tracts containing axons preferentially stained with Eosin (pink) revealed a dramatic loss of fibers coursing through the ventral commissure of *Ptch1*$^{Wig/Wig}$ mutants (Fig. 4B, D). Tag-1 used to reveal commissural axonal tracts, which generally failed to target the mutant ventral midline, which stained for Shh protein, and instead ended aberrantly in the basal plate (arrowheads, Fig. 5D, F). Although some Tag-1-positve axons made it to the mutant floor plate and crossed the ventral commissure (Fig. 5F), midline targeting was largely abnormal. Furthermore, ventral roots of motor axons exited the spinal cord in more dorso-lateral positions in *Ptch1*$^{Wig/Wig}$ mutants relative to wild type littermates (arrows, Figs. 4 and 5B, D). Given that Shh levels were expanded in the *Ptch1*$^{Wig/Wig}$ mutants (Fig.1, Fig. 5A–D), it is likely that the chemoattraction front expanded dorsally in the mutant, allowing for the premature ending of commissural axonal growth throughout the basal plate instead of reach and crossing the midline. Taken together, these results demonstrate the

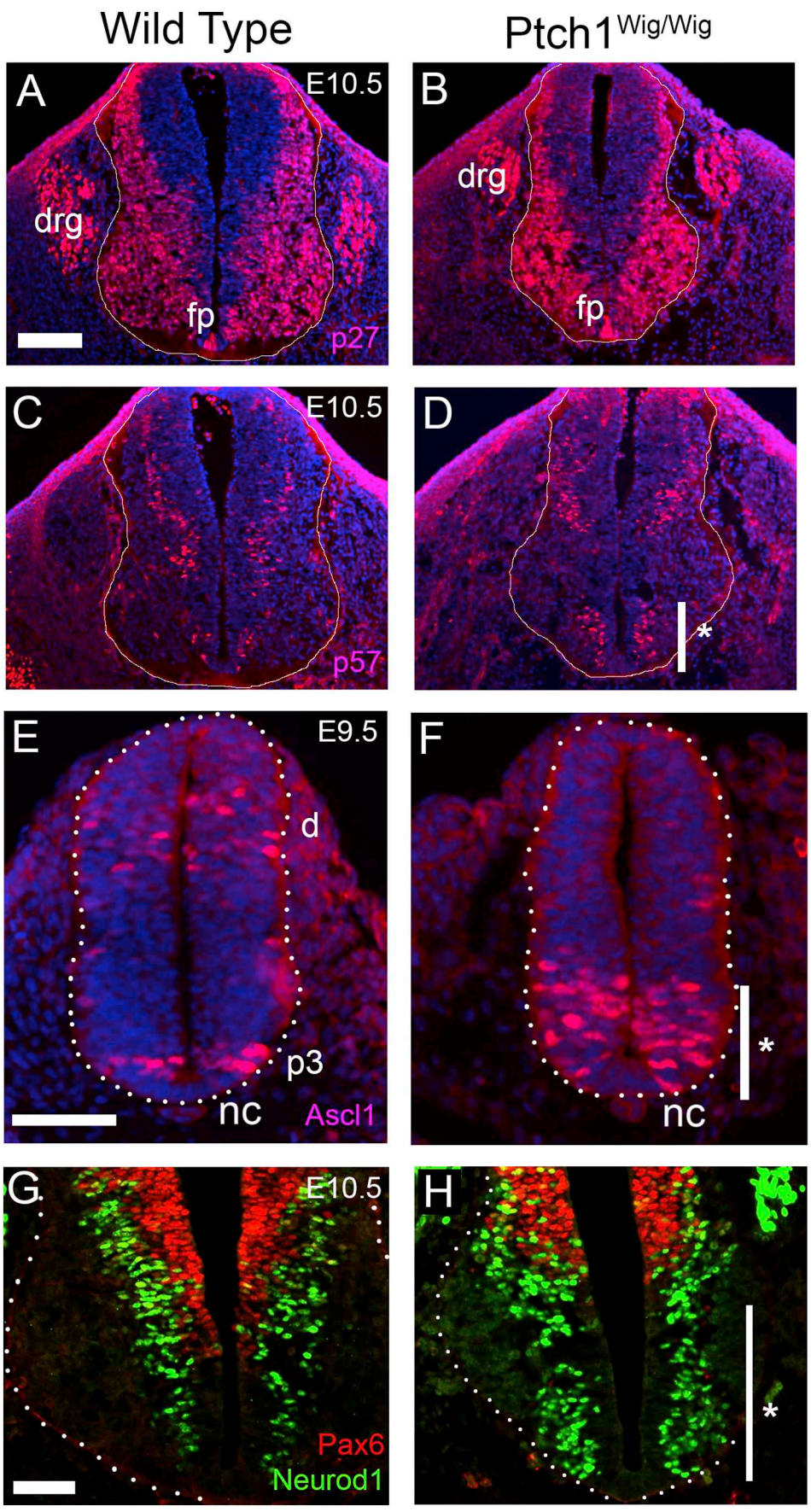



**Fig. 3.** *Ptch1*$^{Wig/Wig}$ mutants displayed enhanced neurogenesis and cell cycle exit in embryonic spinal cords. (A, B) p27$^{Kip1}$ immunohistochemistry in E10.5 wild type (A) and *Ptch1*$^{Wig/Wig}$ mutant (B) spinal cords. (C, D) p57$^{Kip2}$ immunohistochemistry in E10.5 wild type (C) and *Ptch1*$^{Wig/Wig}$ mutant (D) spinal cords, revealing enhanced cell cycle exit in ventral neural progenitors (bar, *). (E, F) Ascl1 expression in E9.5 wild type (E) and *Ptch1*$^{Wig/Wig}$ mutant (F) spinal cords, revealed enhanced neurogenesis in the ventral neuroepithelium (bar, *). (G, H) Pax6 (red) and Neurod1 (green) immunohistochemistry in E10.5 wild type (G) and *Ptch1*$^{Wig/Wig}$ mutant (H) spinal cords, revealed expanded Neurod1 in the ventral neuroepithelium (bar, *). DAPI counterstain. Scale bars: (A), 100 μm; (E, G), 50 μm. Abbreviations: d, dorsal commissural progenitors; drg, dorsal root ganglion; fp, floor plate; nc, notochord; p3, V3 interneuron progenitor domain.

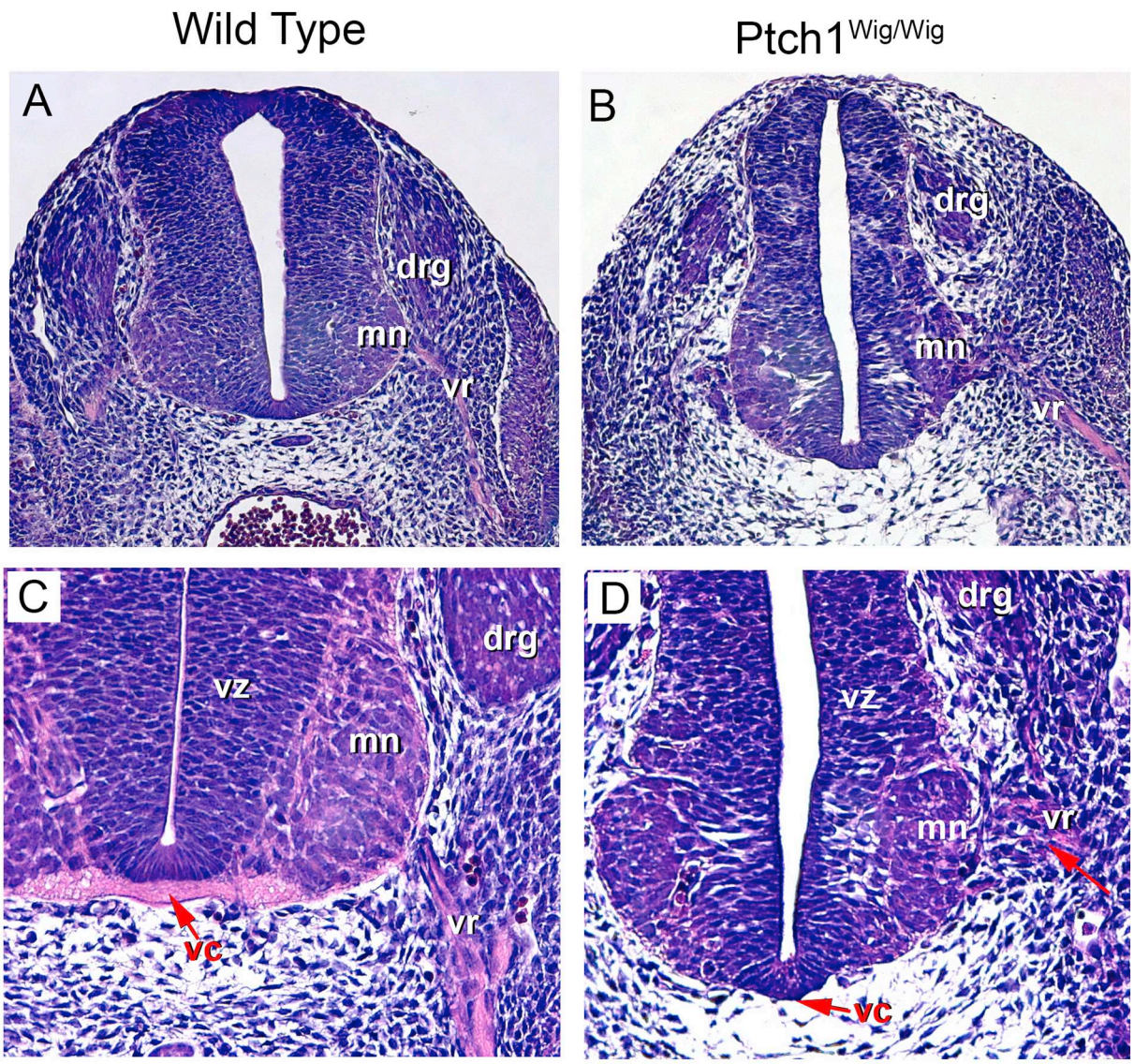



**Fig. 4.** Reduced ventral commissure in *Ptch1*$^{Wig/Wig}$ mutant embryonic spinal cords. (A–D) Hematoxylin-Eosin (H&E) staining of E10.5 wild type (A–C) and *Ptch1*$^{Wig/Wig}$ mutant spinal cords. (B, D) *Ptch1*$^{Wig/Wig}$ mutants displayed abnormal dorsally positioned exit point for the ventral root and reduced Eosin-positive white matter tracts in the ventral commissure (red arrows). Abbreviations: drg, dorsal root ganglion; mn, motor neuron domain; vr, ventral root; vz, ventricular zone.

importance of Ptch1 in controlling the response to Shh signaling in ventral neurogenesis and targeting of commissural axons to the midline.

## 3. Discussion

The contribution of the Hedgehog receptor Ptch1 in embryonic neurogenesis is unknown as the Ptch1 null allele is lethal early in embryogenesis (Goodrich et al., 1997). This study takes advantage of a previously reported ENU allele of Ptch1 called *Wiggable* (*Ptch1*$^{Wig}$) to explore its role neurogenesis and floor plate development and function (Kurosaka et al., 2015; Iulianella et al., 2018). This is possible as *Ptch1*$^{Wig/Wig}$ mutants are viable until E11.5–12.5, allowing for an assessment of spinal cord neurogenesis and commissural axon guidance. Previous work has revealed the utility of the *Ptch1*$^{Wig}$ allele to explore the role of the Shh activity gradient during neural patterning (Iulianella et al., 2018), and craniofacial development (Kurosaka et al., 2014), and in regulating neural crest and placodal cell interactions during the formation of cranial ganglia (Kurosaka et al., 2015). The findings reported here reveal a role for Ptch1 in restricting the proliferation of ventral spinal cord progenitors and floor plate cells and regulating neurogenesis in the ventral spinal cord. As a consequence, defects in commissural axon guidance were observed. These results refine our view of the dynamic regulation of Shh signaling during the formation and integration of neuronal circuits during development.

### 3.1. *Ptch1*$^{Wig}$ embryos displayed enhanced Shh signaling, proliferation and apoptosis in the ventral embryonic spinal cord

Shh signaling regulates both proliferation and patterning during neural development. Ptch1 can also regulate cell cycle progression at least *in vitro* through an association with the cell cycle regulator cyclin B1 (Barnes et al., 2001), but this has not been explored *in vivo*. To ensure the proper formation and integration of neuronal circuits in the spinal cord, progenitor cell neurogenesis necessitates a withdrawal the cell cycle exit and migration of neuroblasts to the forming marginal zone (Iulianella et al., 2008). Any perturbation of this process will lead to aberrant neuronal maturation and cell survival. It is therefore not surprising that *Ptch1*$^{Wig/Wig}$ mutants exhibited unrestricted Shh signaling in the ventral nervous system (Iulianella et al., 2018), and showed enhanced proliferation and neurogenesis in the ventral spinal cord (this study). Interesting, these mutants also showed greatly enhanced apoptosis, accounting for the thinning of the neuroepithelium

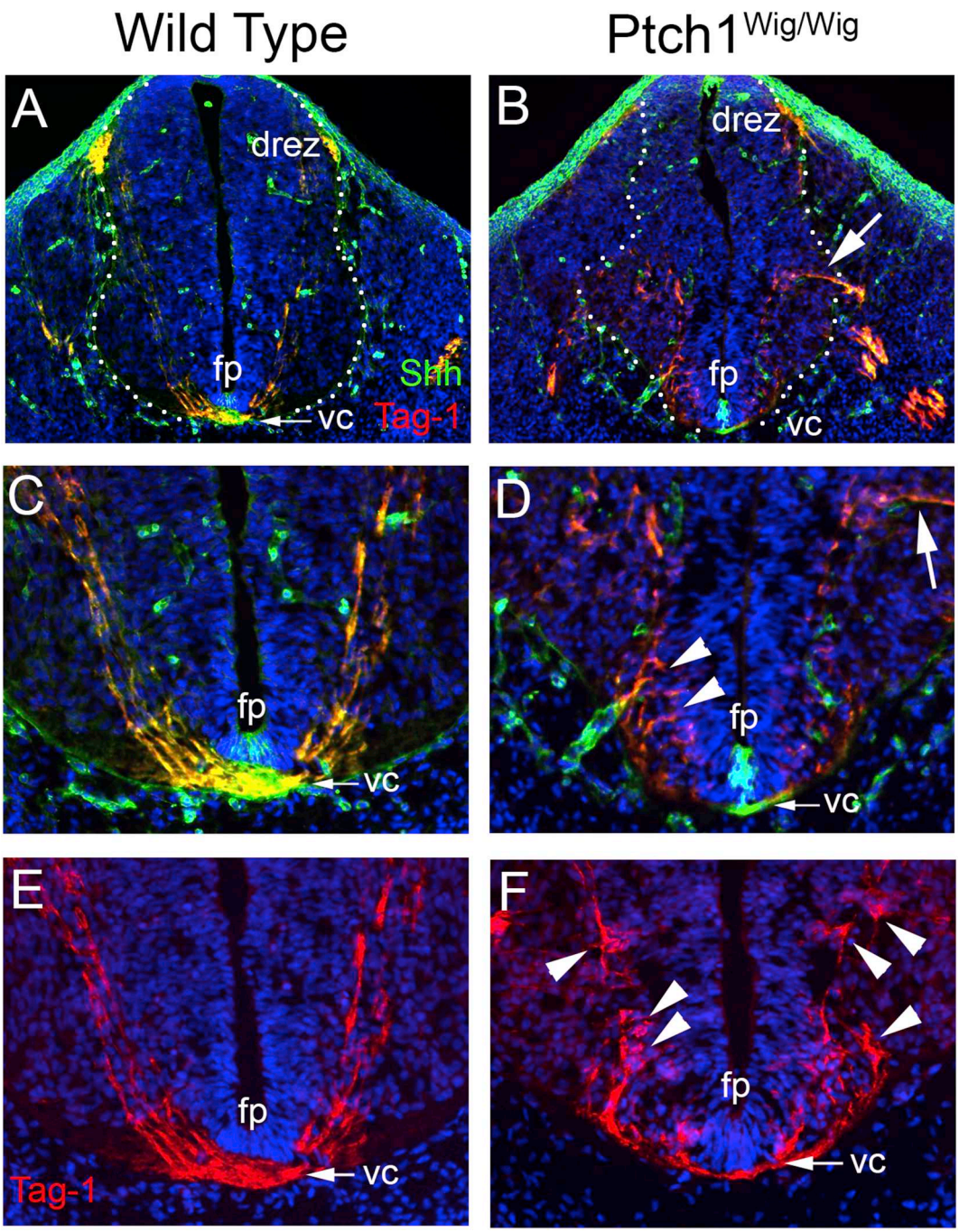



**Fig. 5.** Mis-targeting of commissural axons in ventral embryonic spinal cord of *Ptch1*$^{Wig/Wig}$ mutants. (A–D) Shh (green) and Tag-1 (red) immunostaining in E10.5 wild type (A, C) and *Ptch1*$^{Wig/Wig}$ mutant (B, D) spinal cords revealed aberrant dorsal exit of ventral motor roots (arrow, B, D) and reduced crossing of commissural fibers in the vc. (D) *Ptch1*$^{Wig/Wig}$ mutant displayed aberrant targeting of commissural axons towards the fp (arrowheads) and dorsal exit of the ventral root (arrow). (E, F) Tag-1 immunhistochemistry revealed a failure of *Ptch1*$^{Wig/Wig}$ mutant commissural axons to target the ventral midline (arrowheads, F). DAPI counterstain. Abbreviations: drez, dorsal root entry zone; fp, floor plate, vc, ventral commissure.

by E10.5–11.5. Ptch1 is a large multifunctional transmembrane protein that can positively regulate programmed cell death (Aoto and Trainor, 2015; Guerrero and Ruiz i Altaba, 2003; Katic et al., 2017; Mille et al., 2009; Thibert et al., 2003). It is therefore surprising that the *Ptch1*$^{Wig/Wig}$ mutant displayed enhanced cell death in the basal plate and ventral mesenchymal cells; populations that are normally exposed to high levels of Shh signaling. The *Ptch1*$^{Wig}$ allele encodes a stable C-terminal deletion protein of approximately 70 kDa (Kurosaka et al., 2015). Since the apoptotic function of Ptch1 has been mapped to the C-terminal tail of the protein (Aoto and Trainor, 2015; Thibert et al., 2003), it is possible that *Ptch1*$^{Wig/Wig}$ ventral neural and mesenchymal cells exhibit high levels of cell death because they are extremely sensitive to the enhanced Shh levels. A previous study has shown this enhanced sensitivity is responsible for a failure of integration of neural crest with placodal cells during cranial ganglia formation in *Ptch1*$^{Wig/Wig}$ mutants (Kurosaka et al., 2015). The enhanced sensitivity of *Ptch1*$^{Wig/Wig}$ mutants to Shh levels likely is a contributing factor to the dys-regualted proliferation and apoptosis of ventral tissues during development. Another possibility is that the Ptch1 may directly regulate apoptosis-promoting factors in the remaining regions of the Ptch1$^{Wig}$ protein, but at this time there is no evidence of such interactions.

### 3.2. Enhanced neurogenesis and cell cycle exit in *Ptch1*$^{Wig/Wig}$ mutants

Recent work revealed expanded ventral patterning in *Ptch1*$^{Wig/Wig}$ mutants (Iulianella et al., 2018). Specifically, the mutants showed slightly expanded FoxA2+ floor plate cells and more significant expansion of progenitor domains for V3 interneurons and motor interneurons, while keeping dorsal patterning largely intact. In the *Ptch1*$^{Wig/Wig}$ mutants the entire basal neural plate was shifted dorsally, constraining dorsal progenitors to a reduced region that nonetheless maintained all the diversity of progenitor cell types typically contained in the region, including commissural neurons. This contrasted with the conclusion drawn from the *Ptch1* null mutation, which exhibited earlier lethality relative to the *Ptch1*$^{Wig}$ allele and resulted in the formation of mostly floor plate cells in the embryonic neural tube (Goodrich et al., 1997). The latter finding was interpreted as a near full ventralization of the character of the developing neural tube. Thus, the *Ptch1*$^{Wig}$ mutation is much less severe and allowed the full range of neural progenitor types to develop in the embryonic spinal cord. It is thus a useful mouse allele to explore basic mechanisms of Shh gradient responsiveness during development (Iulianella et al., 2018).

The purpose of this study was to explore the contribution of Ptch1 to neurogenesis and cell cycle exit in the neural tube. As expected from the ventralized spinal cords, *Ptch1*$^{Wgi/Wig}$ mutants exhibited expanded proneural protein expression in the basal plate, along with enhanced expression of cell cycle exhibit regulators in neuroblasts, identified by p57$^{Kip2}$ staining. Ptch1 is the Shh receptor protein but also acts to restrict Shh signaling in the absence of ligand *via* its inhibition of Smo (Briscoe and Small, 2015; Chen and Struhl, 1996; Cohen et al., 2015; Incardona et al., 2000; Rohatgi et al., 2007; Taipale et al., 2002). The truncated Ptch1$^{Wig}$ receptor, while stably expressed, losses the ability to repress Shh signaling downstream and respond to Smo activation within the monocilia, reducing Gli3 repressor formation (Iulianella et al., 2018). As such the resulting mutant embryos exhibited enhanced sensitivity to Shh signaling. Confocal images of floor plate cells revealed that *Ptch1*$^{Wig/Wig}$ mutants still possess what appear to be monocilia *in vivo* (Fig. 2K, L). Thus, despite the enhanced proliferation of floor plate tissues, *Ptch1*$^{Wig/Wig}$ mutants still retained the cytoarchitectural and transcriptional features of floor plate cells. What is changed, however, is in inability to restrict Shh signaling in the ventral neural tube, as was argued in a previous study (Iulianella et al., 2018). A consequence of this unrestricted proliferation of neural progenitors was the expanded production of neuroblasts emanating from the ventral progenitor domains. Altogether, these findings along with those of a previous study (Iulianella et al., 2018) indicates that proliferation and cell cycle exit cannot be uncoupled downstream of Shh signaling *in vivo*. It also suggests that the relationship between Shh signaling, monocilia morphogenesis, and neuroepithelial proliferation is complex, and the exact mechanisms coordinately regulating these processes have yet to be ascertained. With respect to proliferation control and neurogenesis, an interesting possibility is that Shh signaling regulates components of the Notch signaling pathway, allowing of the integration of cell growth and segregation of distinct neuronal subtype identities. Indeed, previous work has revealed the ability of Shh signaling to regulate Notch downstream components in the developing spinal cord (Dave et al., 2011; Kong et al., 2015; Stasiulewicz et al., 2015). As such the upregulation of Ascl1 and Neurod1 in the *Ptch1*$^{Wig/Wig}$ mutants reported here can be taken as evidence of crosstalk between the Shh pathways and the proneural program during the formation of spinal cord circuits.

### 3.3. Floor plate proliferation of commissural axon guidance defects in *Ptch1*$^{Wig/Wig}$ mutants

The floor plate is a quiescent tissue that displays extremely limited growth during development. This is presumably to maintain architectural stability of the basal plate, but also to provide a source of chemoattractive signals to guide commissural neurons during the wiring of the developing nervous system (Charron et al., 2003; Dominici et al., 2017; Kennedy et al., 1994; Morales, 2018; Moreno-Bravo et al., 2019; Sloan et al., 2015; Varadarajan and Butler, 2017; Varadarajan et al., 2017; Wu et al., 2019). Interestingly, *Ptch1*$^{Wig/Wig}$ displayed aberrant proliferation of the floor plate, breaking the normal pseudostratified anatomy of the ventral midline of the nervous system. Instead mutant floor plate cells were mitotic and disorganized. There are not many known regulators of floor plate quiescence, therefore study adds significant to our understanding of this process. The findings report herein support the view that Shh signaling in the floor plate may have numerous roles, including axon guidance and pattern formation. But its effect on proliferation needs to be normally counteracted by Ptch1 to prevent uncontrolled proliferation and cytoarchitectural disarray. Unlike full length Ptch1, Ptch1$^{Wig}$ protein cannot translocate to monocilia upon the activation Smo (Iulianella et al., 2018; Rohatgi et al., 2007). Proteins implicated in ciliary biogenesis and function have important roles in regulating developmental signaling, and particularly Shh signaling (Eggenschwiler and Anderson, 2007; Huangfu and Anderson, 2005; Huangfu et al., 2003). They also show disorganized neuroepithelial architecture and aberrant proliferation, suggesting these two processes are linked (Caspary et al., 2007; Higginbotham et al., 2013; Su et al., 2012). It is possible that cilia are abnormal *Ptch1*$^{Wig/Wig}$ mutants. However, close examination of acetylated-α-tubulin staining in the *Ptch1*$^{Wig/Wig}$ floor late revealed the existence of small extensions from floor plate cells, as was the case for wild type controls (turquoise arrowheads, Fig. 2K, L), suggesting monocilia are intact in the mutants. Another possibility is that monocilia function is somehow perturbed in *Ptch1*$^{Wig/Wig}$ mutants leading to aberrant floor plate proliferation; a possibility that cannot at present be dismissed.

Since *Ptch1*$^{Wig/Wig}$ mutants displayed expanded Shh signaling in the basal plate (Fig. 1) (Iulianella et al., 2018), it was expected that this would lead to aberrant axonal targeting towards the midline. While there is some debate as to the importance of floor plate tissues (*vs.* ventral neural progenitors) in Netrin1-mediated axon guidance, it clearly plays a role in commissural axon targeting to the midline by virtue of the combinatorial expression of Netrin1 and Shh, both key guidance cues (Charron et al., 2003; Dominici et al., 2017; Kennedy et al., 1994; Morales, 2018; Moreno-Bravo et al., 2019; Sloan et al., 2015; Varadarajan and Butler, 2017; Varadarajan et al., 2017; Wu et al., 2019). Consistent with this commissural axons (revealed by Tag-1 staining) failed to target the midline and projected aberrantly throughout the basal plate (Fig. 5). As such white matter tracts in the ventral commissure were severely attenuated. These results add to our view of the importance of floor plate signaling in chemoattraction, but also revealed a direct role for the Shh receptor Ptch1 in this procedure, which was hitherto unknown.

### 3.4. Conclusions

This study provides evidence for the role of Shh signaling in regulating the balance between proliferation and cell death in neural progenitors of the developing spinal cord. Using a mouse ENU mutant for the Shh receptor Ptch1 (*Ptch1*$^{Wig}$), it was shown that this balance could be disturbed resulting in both enhanced neurogenesis and cell death in the spinal cord. It was also discovered that Shh signaling through this receptor normally acts to limit proliferation in the floor plate and keep it largely quiescent. In the absence of a normal Ptch1 protein, floor plate proliferation ensued and led to expanded Shh signaling. Consequently commissural axon guidance towards the midline was perturbed, with axons ending throughout the basal plate instead of crossing the ventral commissure. *Ptch1*$^{Wig/Wig}$ mutants also displayed an aberrant dorsal positioning of the ventral root exit point, reflecting the expanded basal plate. Lastly, this study confirms the utility of using forward genetic screens to create new alleles to refine our knowledge of key developmental processes such as ventral patterning and neurogenesis.

## 4. Methods

### 4.1. Animal husbandry

*Ptch1*$^{Wig/+}$ mice were generated as described previous (Kurosaka et al., 2015) and maintained as heterozygotes through breeding with FVB strain of mice. The mice were genotyped by sequencing for the presence of the *Wiggable* allele in amplicons surrounding the mutation site as previously described in Kurosaka et al. (2015). *Ptch1*$^{Wig/Wig}$ embryos are lethal at E11.5–12.5, slowing for an analysis of neurogenesis during spinal cord development. *Ptch1*$^{Wig/+}$ heterozygous mice were intercrossed and examined for the presence of a vaginal plug the following morning. Litters were harvested at E9.5-E11.5 and processed for immunohistochemistry. For 5-Bromo-2′-deoxyuridine (BrdU) analysis, 3 mg/Kg BrdU solution in PBS was injected intraperitoneally into pregnant E9.5 and E11.5 *Ptch1*$^{Wig/+}$ intercross dams, sacrificed 1 h following injection to capture S-phase nuclei, and litters were processed for immunohistochemistry as described below.

### 4.2. Immunohistochemistry, in situ hybridization, and histology

Embryos were collected from E9.5–11.5 and fixed in 4% paraformaldehyde (PFA; Sigma) in PBS for 4–8 h depending on embryonic stages. Wild type *vs.* *Ptch1*$^{Wig/Wig}$ embryos were cryoprotected in sucrose and embedded in Optimal Cutting Temperature compound (O.C.T., VWR) for sectioning at 12 µm using a cryostat, and serial sections were collected in Superfrost plus slides (VWR). Sections were subjections to fluorescent immunohistochemistry using the following primary antibodies: Acetylated α-Tubulin (Sigma, 1:1000), Ascl1/Mash1 (BD Transduction labs, 1:400), BrdU (Abcam 1:200), Cleaved Caspase-3 (Cell Signaling Technology, 1:300), Cyclind1 (Upstate, 1:500), FoxA2 (Developmental Studies Hybridoma Bank or DSHB, 4D1 concentrate, 1:200), Hes1 (Neuromics, 1:400), Neurod1 (1:500, kind gift of Dr. Jacques Drouin, IRCM), p27$^{Kip1}$ (BD transduction labs, 1:300), p57$^{Kip2}$ (LabVision Neomarkers, 1:50), Phosphohistone H3 (pHH3, Millipore, 1:300), Sonic hedgehog (Shh, DSHB, 5E1 concentrate, 1:200), Tag-1 (DSHB, 4D1 concentrate, 1:300). Signal was revealed using the appropriate species-specific fluorescent secondary antibodies conjugated to either AlexaFluor 594 or 488 (Invitrogen/ThermoFisher), or DyLight 594 or 488 (Jackson ImmunoResearch) at 1:1000–1:2000. All slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and images were taken with a Zeiss Axiovert 200 M upright microscope or a Zeiss LSM510 confocal microscope and processed for brightness levels using Adobe Photoshop CS6.

Whole mount Shh mRNA *in situ* hybridization (ISH) was performed as previously described (Kurosaka et al., 2014) and embryos were photographed using a Zeiss V8 dissecting microscope equipped with an ERc5 color camera (Zeiss). To reveal overall grey (soma/nuclei) and white matter (axons) anatomy, E10.5 wild type and *Ptch1*$^{Wig/Wig}$ mutant embryos were sectioned at 12 µm using a cryostat and stained using a Hematoxylin-Eosin (H&E) solution (Sigma, St. Louis) according to manufacturer's recommendation. Slides were mounted with Permount (Fisher Scientific) and photographed in bright field using a Zeiss Axiovert 200 M upright microscope.

### 4.3. Statistical analysis

For quantification of BrdU and pH 3 immunopositive (pH 3) cells, cell counts were conducted for the entire neural tube and normalized for total cell numbers using DAPI staining. At least 2 adjacent sections from 7 different wild type and *Ptch1*$^{Wig}$ mutants were used for each stage of analysis (E9.5 and 11.5), and represented as mean BrdU +/DAPI+ and pH 3+/DAPI+ cell numbers. Significance was evaluated using a Student's *t*-test, 2 sample, unequal variance in Prism (Graphpad). The level for statistical significance was set at $P < .05$, and *** reflected a $P < .001$. Values were plotted as mean with error bars reflecting variance (Fig. 2G, H).

## Funding

Natural Sciences and Engineering Council of Canada grant RGPIN-2015-04475 and the Plum Foundation.

## Acknowledgements

I acknowledge funding support from NSERC (grant #RGPIN-2015-04475) and the Plum Foundation. I am grateful for the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD that supplied the FoxA2, Pax6, and Tag-1 antibodies, and Dr. Jacques Drouin (Clinical Research Institute of Montreal) who provided the Neurod1 antibody.

## References

Aoto, K., Trainor, P.A., 2015. Co-ordinated brain and craniofacial development depend upon Patched1/XIAP regulation of cell survival. Hum. Mol. Genet. 24, 698–713.
Baek, J.H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T., Kageyama, R., 2006. Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. Development. 133, 2467–2476.
Bai, C.B., Stephen, D., Joyner, A.L., 2004. All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Dev. Cell 6, 103–115.
Balaskas, N., Ribeiro, A., Panovska, J., Dessaud, E., Sasai, N., Page, K.M., Briscoe, J., Ribes, V., 2012. Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. Cell. 148, 273–284.
Barnes, E.A., Kong, M., Ollendorff, V., Donoghue, D.J., 2001. Patched1 interacts with cyclin B1 to regulate cell cycle progression. EMBO J. 20, 2214–2223.
Briscoe, J., Small, S., 2015. Morphogen rules: design principles of gradient-mediated embryo patterning. Development. 142, 3996–4009.
Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M.A., Pavan, W.J., Krizman, D.B., Nagle, J., Polymeropoulos, M.H., Sturley, S.L., Ioannou, Y.A., Higgins, M.E., Comly, M., Cooney, A., Brown, A., Kaneski, C.R., Blanchette-Mackie, E.J., Dwyer, N.K., Neufeld, E.B., Chang, T.Y., Liscum, L., Strauss 3rd, J.F., Ohno, K., Zeigler, M., Carmi, R., Sokol, J., Markie, D., O'Neill, R.R., van Diggelen, O.P., Elleder, M., Patterson, M.C., Brady, R.O., Vanier, M.T., Pentchev, P.G., Tagle, D.A., 1997. Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. Science. 277, 228–231.
Caspary, T., Larkins, C.E., Anderson, K.V., 2007. The graded response to Sonic Hedgehog depends on cilia architecture. Dev. Cell 12, 767–778.
Charron, F., Stein, E., Jeong, J., McMahon, A.P., Tessier-Lavigne, M., 2003. The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. Cell. 113, 11–23.
Chen, Y., Struhl, G., 1996. Dual roles for patched in sequestering and transducing Hedgehog. Cell. 87, 553–563.
Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 383, 407–413.
Cohen, M., Kicheva, A., Ribeiro, A., Blassberg, R., Page, K.M., Barnes, C.P., Briscoe, J., 2015. Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms. Nat. Commun. 6, 6709.
Dave, R.K., Ellis, T., Toumpas, M.C., Robson, J.P., Julian, E., Adolphe, C., Bartlett, P.F., Cooper, H.M., Reynolds, B.A., Wainwright, B.J., 2011. Sonic hedgehog and notch signaling can cooperate to regulate neurogenic divisions of neocortical progenitors. PLoS One 6, e14680.
Denef, N., Neubuser, D., Perez, L., Cohen, S.M., 2000. Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell. 102, 521–531.
Dominici, C., Moreno-Bravo, J.A., Puiggros, S.R., Rappeneau, Q., Rama, N., Vieugue, P., Bernet, A., Mehlen, P., Chedotal, A., 2017. Floor-plate-derived netrin-1 is dispensable for commissural axon guidance. Nature. 545, 350–354.
Eggenschwiler, J.T., Anderson, K.V., 2007. Cilia and developmental signaling. Annu. Rev. Cell Dev. Biol. 23, 345–373.
Ferent, J., Giguere, F., Jolicoeur, C., Morin, S., Michaud, J.F., Makihara, S., Yam, P.T., Cayouette, M., Charron, F., 2019. Boc acts via Numb as a Shh-dependent endocytic platform for Ptch1 internalization and Shh-mediated axon guidance. Neuron. 102 (1157–1171), e5.
Goodrich, L.V., Milenkovic, L., Higgins, K.M., Scott, M.P., 1997. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science. 277, 1109–1113.
Gorojankina, T., 2016. Hedgehog signaling pathway: a novel model and molecular mechanisms of signal transduction. Cell. Mol. Life Sci. 73, 1317–1332.
Guerrero, I., Ruiz i Altaba, A., 2003. Development. Longing for ligand: hedgehog, patched, and cell death. Science. 301, 774–776.
Gui, H., Li, S., Matise, M.P., 2007. A cell-autonomous requirement for Cip/Kip cyclin-kinase inhibitors in regulating neuronal cell cycle exit but not differentiation in the developing spinal cord. Dev. Biol. 301, 14–26.

Higginbotham, H., Guo, J., Yokota, Y., Umberger, N.L., Su, C.Y., Li, J., Verma, N., Hirt, J., Ghukasyan, V., Caspary, T., Anton, E.S., 2013. Arl13b-regulated cilia activities are essential for polarized radial glial scaffold formation. Nat. Neurosci. 16, 1000–1007.
Huangfu, D., Anderson, K.V., 2005. Cilia and Hedgehog responsiveness in the mouse. Proc. Natl. Acad. Sci. U. S. A. 102, 11325–11330.
Huangfu, D., Liu, A., Rakeman, A.S., Murcia, N.S., Niswander, L., Anderson, K.V., 2003. Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature. 426, 83–87.
Incardona, J.P., Lee, J.H., Robertson, C.P., Enga, K., Kapur, R.P., Roelink, H., 2000. Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. Proc. Natl. Acad. Sci. U. S. A. 97, 12044–12049.
Incardona, J.P., Gruenberg, J., Roelink, H., 2002. Sonic hedgehog induces the segregation of patched and smoothened in endosomes. Curr. Biol. 12, 983–995.
Iulianella, A., Sharma, M., Durnin, M., Vanden Heuvel, G.B., Trainor, P.A., 2008. Cux2 (Cutl2) integrates neural progenitor development with cell-cycle progression during spinal cord neurogenesis. Development. 135, 729–741.
Iulianella, A., Sharma, M., Vanden Heuvel, G.B., Trainor, P.A., 2009. Cux2 functions downstream of Notch signaling to regulate dorsal interneuron formation in the spinal cord. Development. 136, 2329–2334.
Iulianella, A., Sakai, D., Kurosaka, H., Trainor, P.A., 2018. Ventral neural patterning in the absence of a Shh activity gradient from the floorplate. Dev. Dyn. 247, 170–184.
Izzi, L., Levesque, M., Morin, S., Laniel, D., Wilkes, B.C., Mille, F., Krauss, R.S., McMahon, A.P., Allen, B.L., Charron, F., 2011. Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation. Dev. Cell 20, 788–801.
Katic, J., Loers, G., Tosic, J., Schachner, M., Kleene, R., 2017. The cell adhesion molecule CHL1 interacts with patched-1 to regulate apoptosis during postnatal cerebellar development. J. Cell Sci. 130, 2606–2619.
Kennedy, T.E., Serafini, T., de la Torre, J.R., Tessier-Lavigne, M., 1994. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell. 78, 425–435.
Kong, J.H., Yang, L., Dessaud, E., Chuang, K., Moore, D.M., Rohatgi, R., Briscoe, J., Novitch, B.G., 2015. Notch activity modulates the responsiveness of neural progenitors to sonic hedgehog signaling. Dev. Cell 33, 373–387.
Kurosaka, H., Iulianella, A., Williams, T., Trainor, P.A., 2014. Disrupting hedgehog and WNT signaling interactions promotes cleft lip pathogenesis. J. Clin. Invest. 124, 1660–1671.
Kurosaka, H., Trainor, P.A., Leroux-Berger, M., Iulianella, A., 2015. Cranial nerve development requires co-ordinated Shh and canonical Wnt signaling. PLoS One 10, e0120821.
Lei, Q., Zelman, A.K., Kuang, E., Li, S., Matise, M.P., 2004. Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord. Development. 131, 3593–3604.
Loftus, S.K., Morris, J.A., Carstea, E.D., Gu, J.Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M.A., Tagle, D.A., Pentchev, P.G., Pavan, W.J., 1997. Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. Science. 277, 232–235.
Makihara, S., Morin, S., Ferent, J., Cote, J.F., Yam, P.T., Charron, F., 2018. Polarized dock activity drives Shh-mediated axon guidance. Dev. Cell 46 (410–425), e7.
Marigo, V., Davey, R.A., Zuo, Y., Cunningham, J.M., Tabin, C.J., 1996. Biochemical evidence that patched is the Hedgehog receptor. Nature. 384, 176–179.
Matise, M.P., 2013. Molecular genetic control of cell patterning and fate determination in the developing ventral spinal cord. Wiley Interdiscip. Rev. Dev. Biol. 2, 419–425.
Mille, F., Thibert, C., Fombonne, J., Rama, N., Guix, C., Hayashi, H., Corset, V., Reed, J.C., Mehlen, P., 2009. The Patched dependence receptor triggers apoptosis through a DRAL-caspase-9 complex. Nat. Cell Biol. 11, 739–746.
Morales, D., 2018. A new model for netrin1 in commissural axon guidance. J. Neurosci. Res. 96, 247–252.
Moreno-Bravo, J.A., Roig Puiggros, S., Mehlen, P., Chedotal, A., 2019. Synergistic activity of floor-plate- and ventricular-zone-derived Netrin-1 in spinal cord commissural axon guidance. Neuron. 101 (625–634), e3.
Myers, B.R., Neahring, L., Zhang, Y., Roberts, K.J., Beachy, P.A., 2017. Rapid, direct activity assays for Smoothened reveal Hedgehog pathway regulation by membrane cholesterol and extracellular sodium. Proc. Natl. Acad. Sci. U. S. A. 114, E11141–E11150.
Qi, X., Schmiege, P., Coutavas, E., Wang, J., Li, X., 2018. Structures of human Patched and its complex with native palmitoylated sonic hedgehog. Nature. 560, 128–132.
Qian, H., Cao, P., Hu, M., Gao, S., Yan, N., Gong, X., 2019. Inhibition of tetrameric Patched1 by Sonic Hedgehog through an asymmetric paradigm. Nat. Commun. 10, 2320.
Rohatgi, R., Milenkovic, L., Scott, M.P., 2007. Patched1 regulates hedgehog signaling at the primary cilium. Science. 317, 372–376.
Sandell, L.L., Iulianella, A., Melton, K.R., Lynn, M., Walker, M., Inman, K.E., Bhatt, S., Leroux-Berger, M., Crawford, M., Jones, N.C., Dennis, J.F., Trainor, P.A., 2011. A phenotype-driven ENU mutagenesis screen identifies novel alleles with functional roles in early mouse craniofacial development. genesis. 49, 342–359.
Sasaki, H., Hui, C., Nakafuku, M., Kondoh, H., 1997. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development. 124, 1313–1322.
Sloan, T.F., Qasaimeh, M.A., Juncker, D., Yam, P.T., Charron, F., 2015. Integration of shallow gradients of Shh and Netrin-1 guides commissural axons. PLoS Biol. 13, e1002119.
Stasiulewicz, M., Gray, S.D., Mastromina, I., Silva, J.C., Bjorklund, M., Seymour, P.A., Booth, D., Thompson, C., Green, R.J., Hall, E.A., Serup, P., Dale, J.K., 2015. A conserved role for Notch signaling in priming the cellular response to Shh through ciliary localisation of the key Shh transducer Smo. Development. 142, 2291–2303.
Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J.E., de Sauvage, F., Rosenthal, A., 1996. The tumour-suppressor gene patched encodes a candidate receptor for sonic hedgehog. Nature. 384, 129–134.
Su, C.Y., Bay, S.N., Mariani, L.E., Hillman, M.J., Caspary, T., 2012. Temporal deletion of Arl13b reveals that a mispatterned neural tube corrects cell fate over time. Development. 139, 4062–4071.
Sugimori, M., Nagao, M., Bertrand, N., Parras, C.M., Guillemot, F., Nakafuku, M., 2007. Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. Development. 134, 1617–1629.
Taipale, J., Cooper, M.K., Maiti, T., Beachy, P.A., 2002. Patched acts catalytically to suppress the activity of Smoothened. Nature. 418, 892–897.
Thibert, C., Teillet, M.A., Lapointe, F., Mazelin, L., Le Douarin, N.M., Mehlen, P., 2003. Inhibition of neuroepithelial patched-induced apoptosis by sonic hedgehog. Science. 301, 843–846.
Torroja, C., Gorfinkiel, N., Guerrero, I., 2004. Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. Development. 131, 2395–2408.
Varadarajan, S.G., Butler, S.J., 2017. Netrin1 establishes multiple boundaries for axon growth in the developing spinal cord. Dev. Biol. 430, 177–187.
Varadarajan, S.G., Kong, J.H., Phan, K.D., Kao, T.J., Panaitof, S.C., Cardin, J., Eltzschig, H., Kania, A., Novitch, B.G., Butler, S.J., 2017. Netrin1 produced by neural progenitors, not floor plate cells. Is Required for Axon Guidance in the Spinal Cord. Neuron. 94 (790–799), e3.
Weiss, L.E., Milenkovic, L., Yoon, J., Stearns, T., Moerner, W.E., 2019. Motional dynamics of single Patched1 molecules in cilia are controlled by Hedgehog and cholesterol. Proc. Natl. Acad. Sci. U. S. A. 116, 5550–5557.
Wu, Z., Makihara, S., Yam, P.T., Teo, S., Renier, N., Balekoglu, N., Moreno-Bravo, J.A., Olsen, O., Chedotal, A., Charron, F., Tessier-Lavigne, M., 2019. Long-range guidance of spinal commissural axons by Netrin1 and Sonic Hedgehog from midline floor plate cells. Neuron. 101 (635–647), e4.
Yam, P.T., Langlois, S.D., Morin, S., Charron, F., 2009. Sonic hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. Neuron. 62, 349–362.
Yam, P.T., Kent, C.B., Morin, S., Farmer, W.T., Alchini, R., Lepelletier, L., Colman, D.R., Tessier-Lavigne, M., Fournier, A.E., Charron, F., 2012. 14-3-3 proteins regulate a cell-intrinsic switch from sonic hedgehog-mediated commissural axon attraction to repulsion after midline crossing. Neuron 76, 735–749.
Yamamoto, S., Nagao, M., Sugimori, M., Kosako, H., Nakatomi, H., Yamamoto, N., Takebayashi, H., Nabeshima, Y., Kitamura, T., Weinmaster, G., Nakamura, K., Nakafuku, M., 2001. Transcription factor expression and Notch-dependent regulation of neural progenitors in the adult rat spinal cord. J. Neurosci. 21, 9814–9823.