Gli3 Controls Corpus Callosum Formation by Positioning Midline Guideposts During Telencephalic Patterning

Dario Magnani[1], Kerstin Hasenpusch-Theil[1], Carine Benadiba[2], Tian Yu[3], M. Albert Basson[3], David J. Price[1], Cécile Lebrand[2] and Thomas Theil[1]

[1]Centre for Integrative Physiology, University of Edinburgh, Edinburgh, UK [2]Department of Cellular Biology and Morphology, University of Lausanne, Lausanne, Switzerland and [3]Department of Craniofacial Development, King's College London, London, UK

Address correspondence to Thomas Theil. Email: thomas.theil@ed.ac.uk

The corpus callosum (CC) represents the major forebrain commissure connecting the 2 cerebral hemispheres. Midline crossing of callosal axons is controlled by several glial and neuronal guideposts specifically located along the callosal path, but it remains unknown how these cells acquire their position. Here, we show that the Gli3 hypomorphic mouse mutant Polydactyly Nagoya (Pdn) displays agenesis of the CC and mislocation of the glial and neuronal guidepost cells. Using transplantation experiments, we demonstrate that agenesis of the CC is primarily caused by midline defects. These defects originate during telencephalic patterning and involve an up-regulation of Slit2 expression and altered Fgf and Wnt/β-catenin signaling. Mutations in sprouty1/2 which mimic the changes in these signaling pathways cause a disorganization of midline guideposts and CC agenesis. Moreover, a partial recovery of midline abnormalities in Pdn/Pdn;Slit2$^{-/-}$ embryos mutants confirms the functional importance of correct Slit2 expression levels for callosal development. Hence, Gli3 controlled restriction of Fgf and Wnt/β-catenin signaling and of Slit2 expression is crucial for positioning midline guideposts and callosal development.

Keywords: corpus callosum, Fgf8, Gli3, Pdn, Slit2

Introduction

The corpus callosum (CC) connects neurons of the 2 cerebral hemispheres and coordinates information between the left and right cortex. CC malformations have been associated with mental retardation involving a wide range of cognitive, behavioral, and neurological consequences (Richards et al. 2004; Paul et al. 2007) and have been identified in over 50 human congenital syndromes (Richards et al. 2004). During CC formation, several guidance events control midline crossing of callosal axons. The midline zipper glia (MZG) have been suggested to initiate the fusion of the dorsal midline producing the substrate on which callosal axons navigate (Silver et al. 1993). Moreover, several guidepost cells are located along the path of callosal axons including the midline glial cell populations composed of the indusium griseum glia (IGG) and the glial wedge (GW) (Richards et al. 2004), and GABAergic and glutamatergic neurons that transiently populate the CC (Niquille et al. 2009). Finally, axons from the cingulate cortex pioneer the CC and function as scaffolds for neocortical axons (Koester and O'Leary 1994; Rash and Richards 2001; Piper, Plachez et al. 2009). Several axon-guidance molecules, including Slit2, that are produced by midline glial cells and by the glutamatergic neurons have essential roles in callosal development (Bagri et al. 2002; Niquille et al. 2009). While these studies reveal complex interactions between callosal axons and their environment, it remains largely unknown how guidepost cells acquire their correct positions and how the expression of essential guidance molecules is regulated.

Gli3 encodes a zinc-finger transcription factor with crucial roles in early patterning of the dorsal telencephalon (Theil et al. 1999; Tole et al. 2000; Kuschel et al. 2003; Fotaki et al. 2006) acting both cell autonomously (Quinn et al. 2009) and cell nonautonomously by controlling the expression of signaling molecules essential for telencephalic development (Grove et al. 1998; Theil et al. 1999; Tole et al. 2000; Aoto et al. 2002). Moreover, Gli3 functions in axon pathfinding in the forebrain. The Gli3 hypomorphic mouse mutant Polydactyly Nagoya (Pdn) shows defects in the corticothalamic and thalamocortical tracts (Magnani et al. 2010) and lacks the CC (Naruse et al. 1990) though for unknown reasons. Using transplantation experiments, we here demonstrate that midline abnormalities are primarily responsible for agenesis of the corpus callosum (ACC). We show that Pdn mutants display mislocated glial and neuronal guidepost cells. The Pdn cingulate cortex contains ectopic glial cells transecting the path of callosal axons. These midline abnormalities are associated with an up-regulation and down-regulation of Fgf and Wnt/β-catenin signaling, respectively. These changes in these signaling pathways are mimicked in Sprouty1/2 double mutants, which display a mislocation of midline guideposts and ACC. Pdn mutants also show an up-regulation of Slit2 expression, and positioning of the neuronal guideposts is largely rescued in Pdn/Pdn;Slit2$^{-/-}$ double mutants suggesting that maintaining correct Slit2 expression levels is crucial for callosal development. Collectively, these analyses reveal a novel role for Gli3 in controlling the positioning of midline guideposts by regulating Fgf and Wnt/β-catenin signaling and Slit2 expression levels and provide new insights into the mechanisms underlying CC pathogenesis.

Materials and Methods

Mice

The mutant mouse lines Pdn, τGFP, Slit2, Sprouty1, and Sprouty2 and mating strategies have been described previously (Naruse et al. 1990; Pratt et al. 2000; Plump et al. 2002; Basson et al. 2005; Shim et al. 2005; Simrick et al. 2011). All experimental procedures involving mice were performed in accordance with local guidelines. In analyses of Pdn mutant phenotypes, heterozygous and wild-type embryos did not show qualitative differences and both were used as control embryos. For quantitative analyses, wildt-ype and Pdn/Pdn embryos were compared to avoid the possible risk of Pdn/+ embryos having subtle defects. For each marker and each stage, 3–5 embryos were analyzed.

In Situ Hybridization and Immunohistochemistry

Antisense RNA probes for *Bmp7* (Furuta et al. 1997), *Msx1* (Hill et al. 1989), *Sema3C* (Bagnard et al. 2000), *Slit2* (Erskine et al. 2000), *Fabp7* (Genepaint. RNA probe 653), *Fgf8* (Crossley and Martin 1995), *Sprouty2* (Minowada et al. 1999), *Axin2* (Lustig et al. 2002), *Wnt7b* (Parr et al. 1993), *Wnt8b* (Richardson et al. 1999), *Nf1b* (IMAGE: 4038233), *Nf1x* (IMAGE: 3491917), *Emx1* (Simeone et al. 1992), and *Six3* (Oliver et al. 1995) were labeled with digoxigenin. In situ hybridization on 12-μm serial paraffin sections of mouse brains were performed as described (Theil 2005).

Immunohistochemical analysis was performed as described previously (Theil 2005) using antibodies against the following molecules: β-III-tubulin (Tuj1 antibody; 1:1000, Sigma); brain lipid-binding protein (Blbp; 1:500, CHEMICON); calbindin (CB; 1:1000, Swant); calretinin (CR; 1:1000, CHEMICON); Glast (1:5000, CHEMICON); glia fibrillary acidic protein (GFAP; 1:1000, DakoCytomation); green fluorescent protein (GFP; 1:1000, Abcam); Nf1a (1:1000, Active Motif); neural cell adhesion molecule L1 (1:1000, CHEMICON); Neurofilament (2H3; 1:5, DSHB); Neuropilin-1 (Npn-1; 1:1000, R&D Systems); Satb2 (1:50, Abcam); Tbr1 (1:2500, CHEMICON). Primary antibodies for immunohistochemistry were detected with Alexa- or Cy2/3-conjugated fluorescent secondary antibodies. For non-fluorescent detection, we used biotinylated goat antimouse antibodies (Dako) followed by avidin-HRP and DAB detection (Vector Labs).

CB^+ neurons in the indusium griseum of E16.5 and E18.5 embryos were quantified by determining total CB^+ cell numbers in this region. For quantifying CR^+ neurons, a box with constant area (170 μm^2 for E16.5 and 297 μm^2 for E18.5 embryos) was placed in the cingulate cortex immediately dorsal to the CC, and the numbers of CR^+ neurons were counted within this box. Numerical values are given as a proportion of CR^+ cells per μm^2. For statistical analyses, an analysis of variance test was used followed by a Bonferroni's multiple comparison test.

Explant Culture

Organotypic slice cultures of rostral levels of the embryonic mouse telencephalon were prepared as previously described (Magnani et al. 2010). Brain slices were cultured on polycarbonate culture membranes (8-μm pore size; Corning Costar) in organ tissue dishes containing 1 mL of medium (Neurobasal/B-27 [Gibco] supplemented with glutamine, glucose, penicillin, and streptomycin). For transplantation experiments, slices were cultured for 72 h, fixed with 4% PFA, and processed for antiGFP immunofluorescence as described above. For Fgf blocking experiments, slices were cultured in the presence of either DMSO or of 25 or 100 μM SU5402 (Calbiochem) for 48 h, fixed with 4% PFA, and processed for in situ hybridization or Blbp immunofluorescence as described above.

Quantitative Reverse Transcription PCR

Total RNA was prepared from the E14.5 rostromedial telencephalon of wild-type or *Pdn/Pdn* embryos. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a TaqMan® Gene Expression Assay (Applied Biosystems) for *Slit2* (Mm00662153.m1, probe dye FAM-MGB) with ACTB (#4352933, probe dye FAM-MGB) and GAPDH (#4352932, probe dye FAM-MGB) as endogenous controls and a 7000 Sequence Detection System. The abundance of each transcript in the original RNA sample was extrapolated from PCR reaction kinetics using sequence detection software (SDS) version 1.2.3 running an absolute quantification protocol including background calibrations.

Results

CC Midline GuidePost Cells are Severely Disorganized in Pdn/Pdn Brains

Neurofilament, Tuj1 and L1 immunohistochemical stainings, and cortical DiI labeling confirmed a previous description of CC malformation in *Pdn* mutants (Naruse et al. 1990),

showing that the path of callosal axons is disrupted at several positions in the cingulate cortex and that those axons which approach the midline fail to cross it, forming Probst bundles instead (Fig. 1 and Supplementary Fig. 1). To gain insights into the origins of these defects, we analyzed the navigation of the cingulate pioneer axons and the formation of glial and neuronal guideposts that are essential for callosal development (Paul et al. 2007). In P0 control animals, the cingulate pioneer axons are immunopositive for Npn-1 occupying the dorsal-most part of the CC (Fig. 1A,B). In *Pdn* mutants, Npn-1$^+$ axons fail to project to the contralateral hemisphere, but form dense bundles ipsilaterally (Fig. 1C,D). Glutamatergic guidepost neurons express Tbr1, CR, or CB (Niquille et al. 2009). In control embryos, CR^+ and CB^+ neurons are both located in the IG region, and CR^+ neurons are also found within the CC where they delineate its ventral and dorsal parts (Niquille et al. 2009; Fig. 1A,B,E,F). In *Pdn* mutants, CR^+ neurons are dramatically disorganized, but maintain their spatial association with callosal axons, with clusters of CR^+ neurons surrounding the Probst bundles (Fig. 1C,D). CB^+ neurons remain concentrated in the medial cortex although they are more diffusely distributed and clusters of CB^+ neurons intermingle abnormally with callosal axons (Fig. 1G, H). Finally, GFAP immunostaining labels the GW, the IGG, and the MZG in control embryos (Fig. 1I,J). In *Pdn* brains, several GFAP$^+$ fascicles are formed ectopically in the cingulate cortex (Fig. 1K,L). Some fascicles span the whole cortical width and transect the path of callosal axons. The IGG could not be identified and the MZG expands into more ventral regions of the septum. Taken together, these data show a dramatic disorganization of glial and neuronal guidepost cells.

Given the severity of this disorganization, we started to study the origins of these midline defects by investigating the formation of the midline guideposts and of the cingulate pioneer neurons at earlier stages. In E16.5 control embryos, cingulate pioneer axons approach the midline and start to cross it (Supplementary Fig. 2A,B). In *Pdn* mutants, few axons have reached the corticoseptal boundary (CSB) and many have abnormally formed clusters in the cingulate cortex (Supplementary Fig. 2C,D). Tbr1$^+$, CR$^+$, and CB$^+$ glutamatergic guidepost neurons form a well organized band of neurons at the CSB of control embryos, but their organization is severely disturbed in *Pdn* embryos with less CB$^+$ neurons in the IG region (Supplementary Figs 2A–L and 7P). In the cingulate cortex, the cortical plate is disrupted in several positions where callosal axons stop their navigation (Supplementary Fig. 2G,H). Finally, radial glial cells (RGCs) at the CSB, which co-express GFAP and the RGC marker Glast, have started to differentiate into GW cells, to translocate to the pial surface, and to form the IGG in control embryos (Supplementary Fig. 2M,N). In *Pdn* mutants, GFAP$^+$;Glast$^+$ cells are present ectopically in the cingulate cortex and extend projections from the ventricular to the pial surface (Supplementary Fig. 2O,P). Taken together, these findings suggest that midline guidance cues are already disorganized in *Pdn* mutants when callosal axons approach the CSB.

Agenesis of the CC in Pdn Mutants is Caused by CC Midline Defects

Since *Gli3* is widely expressed in progenitor cells that give rise to callosal neurons and to guidepost cells, agenesis of the
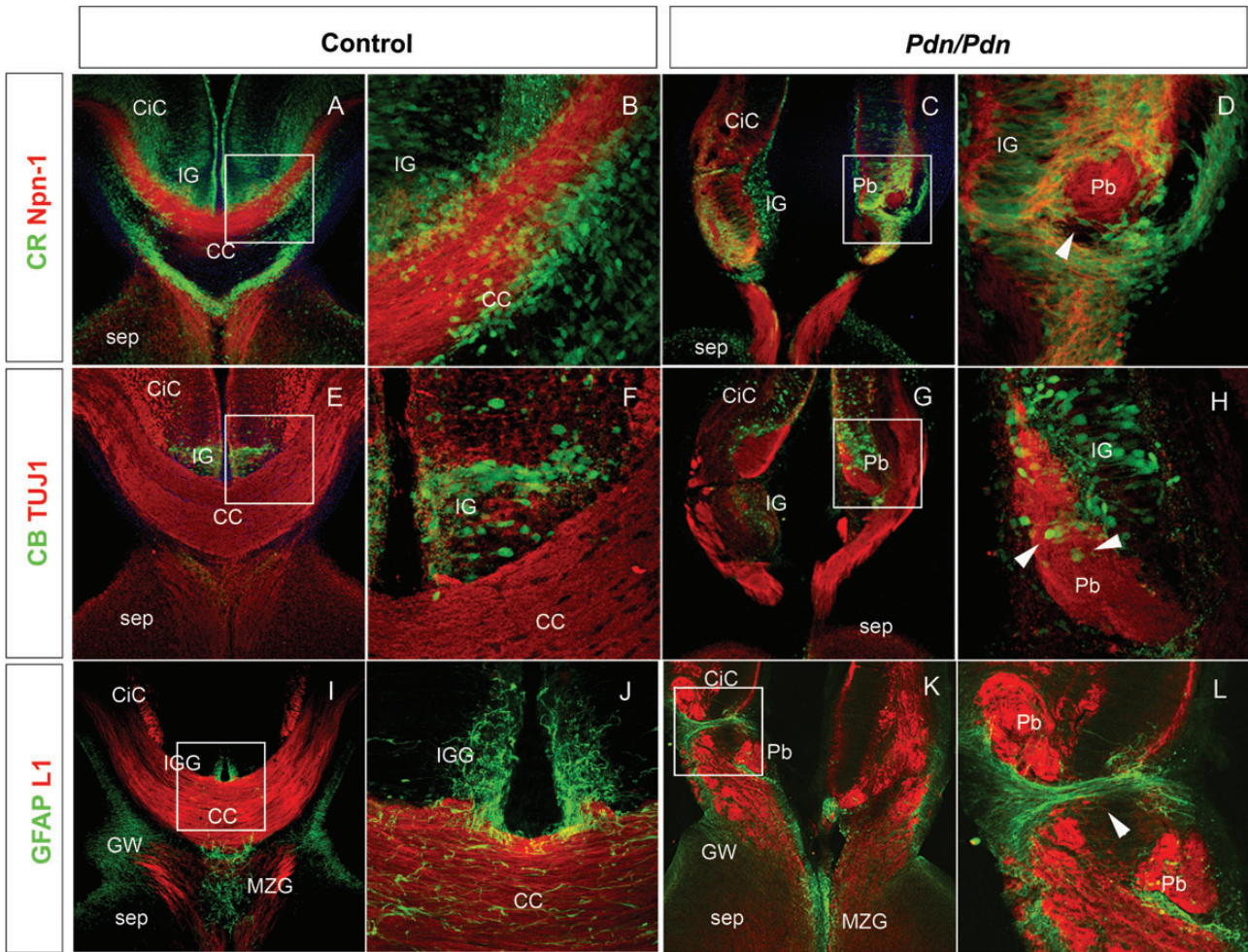
Figure 1. Disorganization of midline structures in P0 *Pdn/Pdn* brains. (*A–D*) Npn-1 stains the pioneer axons of the cingulate cortex (CiC) in control and *Pdn/Pdn* mutants. *Pdn/Pdn* Npn-1^{+} axons fail to reach the contralateral hemisphere, instead forming Probst bundles (*C* and *D*). (*A* and *B*) CR labels glutamatergic guidepost neurons. (*C* and *D*) In *Pdn/Pdn* brains, CR^{+} neurons are disorganized and clusters of CR^{+} neurons are associated with Probst bundles (arrowhead in *D*). (*E* and *F*) CB labels guidepost neurons located in the IG. (*G* and *H*) CB^{+} neurons abnormally cluster with Tuj1^{+} callosal axons in the *Pdn/Pdn* cortex (arrowheads in *H*). (*I–J*) GFAP immunofluorescence labels the GW, the IGG, and the MZG in control brains. (*K* and *L*) In *Pdn/Pdn* brains, GFAP[+] fascicles are ectopically formed within the CiC (arrowhead in *L*) and transect the path of callosal axons. The MZG expands into more ventral regions of the septum.

CC in *Pdn* mutants could either be caused by the disorganization of midline guideposts or by a primary failure of callosal axons to navigate in the midline region leading to the formation of Probst bundles and to a secondary redistribution of guideposts. Previous marker and BrdU birthdating analyses in *Pdn/Pdn* mutants failed to find major defects in cortical lamination (Magnani et al. 2010). Moreover, Satb2 upper layer callosal projection neurons are borne at E15.5 (Supplementary Fig. 3), suggesting that these neurons are specified correctly. To test directly whether *Pdn* mutant callosal axons are capable of following midline guidance cues we performed in vitro transplantation experiments using mice ubiquitously expressing a τGFP fusion protein (Pratt et al. 2000). Homotopical transplantation of frontal cortex of E17.5 GFP[+] embryos into cortical sections of age-matched GFP[−] embryos resulted in growth of axons into the host tissue and in midline crossing of callosal axons (*n* = 8 of 9; Fig. 2*A*). After transplantation of *Pdn/Pdn*;GFP[+] cortex into control cortex, *Pdn/Pdn* axons also migrated across the midline (*n* = 7 of 8; Fig. 2*B*). However, control;GFP[+] callosal axons did not grow into *Pdn/Pdn*;GFP[−] dorsomedial cortex (*n* = 0 of 7; Fig. 2*C*) and only a

few axons projected along the surface of the mutant host tissue (*n* = 4 of 7; Fig. 2*C*). In contrast, corticofugal axons project into the lateral cortex and striatum under these conditions (Magnani et al. 2010). These results show that normal levels of *Gli3* are not required to generate callosal neurons with the ability to project their axons across the midline, but indicate a requirement for *Gli3* in the generation of the midline guideposts.

The Pdn Mutation Affects the Patterning in the Rostromedial Telencephalon

Next, we became interested in identifying causes underlying these midline defects. Our previous analyses showed that *Pdn* mutants display patterning defects during early telencephalic development (Kuschel et al. 2003). We therefore hypothesized that these defects might cause the defective positioning of the midline guidance cues. To test this idea, we started to analyze the development of the E12.5 corticoseptal region where callosal axons later cross the midline. We showed previously that expression of the *Emx1* homeobox gene is lost in
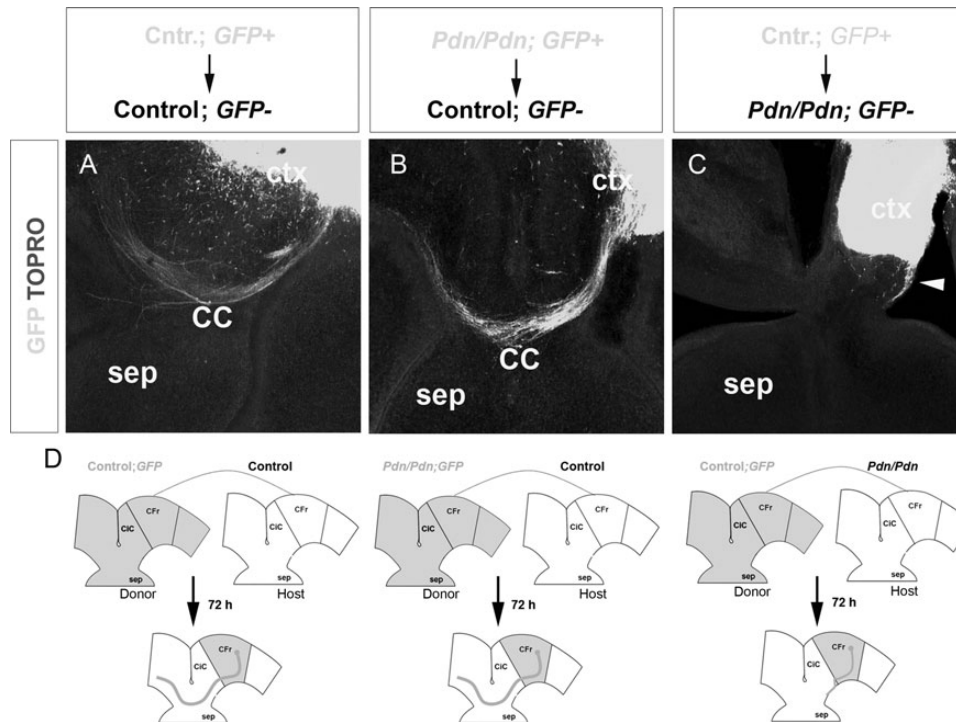
Figure 2. *Pdn/Pdn* callosal axons are able to cross the cortical midline in a control environment. (*A*) Transplantation of E17.5 control GFP$^+$ frontal cortex leads to the migration of GFP$^+$ callosal axons across the midline (*n* = 8 of 9). (*B*) *Pdn/Pdn*;GFP$^+$ cortex transplantation into control cortex also shows crossing callosal axons (*n* = 7 of 8). (*C*) After transplantation of control; GFP$^+$ cortical tissue into *Pdn/Pdn*; GFP-frontal cortex callosal axons do not project into the intermediate zone (*n* = 0 of 7), only few axons project along the surface of the mutant tissue (arrowhead; *n* = 4 of 7). (*D*) Experimental procedure illustrating transplant experiments: Tissue from the frontal cortex of E17.5 GFP$^+$ embryos was homotopically transplanted into cortical sections of age-matched GFP$^-$ embryos and the migration pattern of callosal axons was monitored using GFP immunofluorescence after 72 h of culture.

Pdn mutants (Kuschel et al. 2003). Moreover, *Emx1* mutants display ACC (Qiu et al. 1996; Yoshida et al. 1997) and Emx1 has recently been shown to belong to a group of transcription factors including Six3 and Nfia whose expression domains delineate the regions where the CC, the hippocampal, and anterior commissures cross the midline at E16.5 (Moldrich et al. 2010). As these genes have important roles in forebrain and/or callosal development (Qiu et al. 1996; das Neves et al. 1999; Lagutin et al. 2003; Shu et al. 2003; Campbell et al. 2008; Plachez et al. 2008; Piper, Moldrich et al. 2009), we investigated their expression at the E12.5 CSB where callosal axons later cross the midline. In control embryos, *Six3* is expressed in the septum, but *Six3* expression expands dorsally in *Pdn* mutants (Supplementary Fig. 4*A*,*E*). Nfia, Nfib, and Nfix, are expressed at high levels in the cortex and at lower levels in the dorsalmost septum (Shu et al. 2003; Plachez et al. 2008; Supplementary Fig. 4*B*–*D*). In *Pdn* mutants, their cortical expression domains are lost, while low-level septal expression remains except for Nfia which is strongly expressed in the septum (Supplementary Fig. 4*F*–*H*). Taken together, these data indicate that the expression of several transcription factors with important roles in callosal development is altered in *Pdn* mutants, suggesting that the CSB is poorly defined.

Previous analyses had also shown a requirement of *Gli3* for the correct expression of several signaling molecules in the telencephalon, including *Shh*, *Bmp/Wnt* genes, and *Fgf8* (Grove et al. 1998; Theil et al. 1999; Tole et al. 2000; Aoto et al. 2002; Kuschel et al. 2003; Magnani et al. 2010). We

therefore analyzed the expression of these signaling molecules specifically at the E12.5 CSB. This analysis revealed a slight extension of *Shh* expression into the ventral-most part of the septum in *Pdn/Pdn* embryos, but Shh signaling as judged by *Ptc1* expression remains confined to the septum and does not reach the CSB (Supplementary Fig. 5). Moreover, *Bmp7*, which is essential for callosal development (Sanchez-Camacho et al. 2011), and its target gene *Msx1* are expressed on the cortical side of the CSB though only at caudal levels with no obvious difference between control and *Pdn/Pdn* embryos (Fig. 3*A*,*B*,*F*,*G*). In contrast, we observed severe changes in the *Wnt7b/8b* expression patterns. In control embryos, *Wnt7b* and *Wnt8b* expression are confined to the dorsomedial telencephalon with a sharp expression boundary at the CSB (Fig. 3*C*,*D*), while *Wnt7b* and *Wnt8b* expression is nearly absent from the *Pdn* dorsomedial telencephalon, and *Wnt7b* transcription is increased in the septum (Fig. 3*H*,*I*). Consistent with reduced Wnt/β-catenin signaling, expression of the *Wnt* target gene *Axin2* is severely reduced in *Pdn* mutants (Fig. 3*E*,*J*).

Since telencephalic patterning is controlled by a balance between Bmp/ Wnt/β-catenin and Fgf signaling (Theil et al. 1999; Kuschel et al. 2003; Shimogori et al. 2004) and since *Fgf8* is required for callosal development (Huffman et al. 2004; Moldrich et al. 2010), we also investigated *Fgf8* expression in *Pdn* mutants. In control embryos, *Fgf8* transcripts are confined to the commissural plate, but expand further dorsally in the E12.5 *Pdn* corticoseptal region (Fig. 3*K*,*O*) consistent with our previous whole-mount
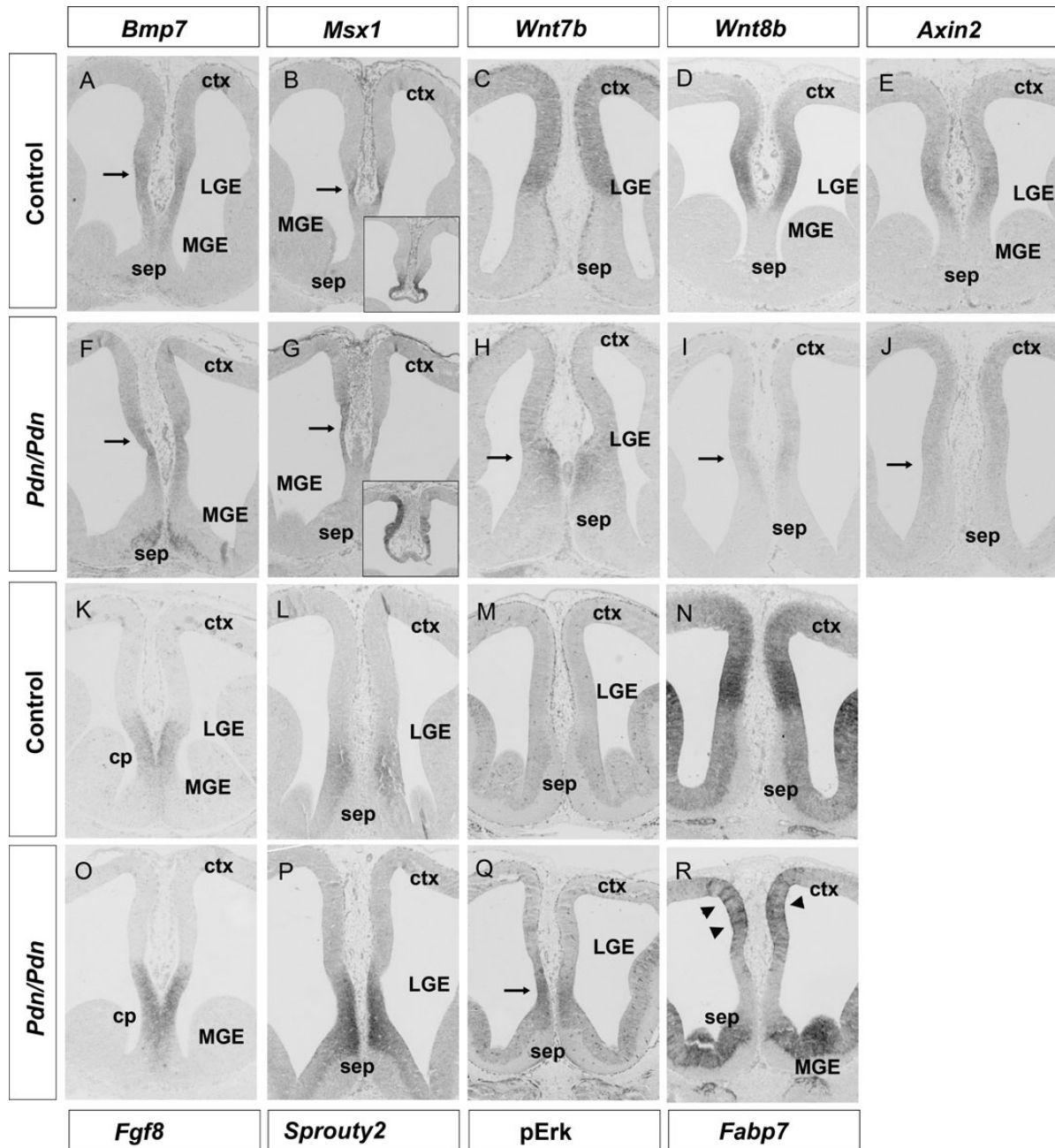
Figure 3. Altered Wnt/β-catenin and Fgf signaling in the E12.5 *Pdn* rostromedial telencephalon. (*A, B, F,* and *G*) *Bmp7* expression and that of its target gene *Msx1* are detected on the cortical side of the CSB at caudal levels in both control and *Pdn/Pdn* mutants. Arrows in (*A*) and (*F*) demarcate the *Bmp7* expression domain. The arrows in (*B*) and (*G*) point at the Msx1 expression domain and the insets show *Msx1* expression in the telencephalic roofplate. (*C, D, H,* and *I*) *Wnt7b* and *Wnt8b* are expressed in the dorsomedial telencephalon with a sharp expression boundary at the CSB. In *Pdn* mutants, cortical *Wnt7b* and *Wnt8b* expression are strongly reduced (arrows in *H* and *I*), and *Wnt7b* expression is shifted ventrally into the septum. (*E* and *J*) *Axin2* expression is severely reduced in the *Pdn* dorsomedial cortex (arrow in *J*). (*K–M* and *O–Q*) *Fgf8, Sprouty2,* and pErk expression are normally confined to the commissural plate (cp) and septum, respectively, but are shifted dorsally and expressed at higher levels at the *Pdn/Pdn* corticoseptal boundary. (*N* and *R*) *Fabp7* is expressed at high levels in the dorsomedial cortex of control embryos with a sharp expression boundary at the CSB. The *Pdn* dorsomedial cortex lacks this *Fabp7* high-level expression domain, but shows clusters of cells expressing high levels of *Fabp7* (arrowheads in *R*).

expression analysis (Kuschel et al. 2003). Expression of and phospho-Erk (pErk), targets of Fgf signaling, also extends dorsally into the cortex (Fig. 3L,M,P,Q). A similar expansion of *Fgf8* and *sprouty2* expression were already observed in E11.5 *Pdn* embryos (data not shown), indicating that Fgf signaling is ectopically activated during patterning. We also analyzed *Fabp7* expression which in control embryos marks neurogenic RGC on the cortical side of the CSB (Fig. 3N) and which is increased upon up-regulation of Fgf signaling in the rostromedial telencephalon (Faedo et al. 2010). Interestingly, the *Pdn* dorsomedial cortex lacks this high-level *Fabp7* expression domain, but shows clusters of RGCs with high levels of *Fabp7* expression next to cells having little *Fabp7* transcripts (Fig. 3R) reminiscent of the ectopic Glast⁺ fibers which we observed at E16.5. Taken together, these analyses indicate severe changes in Fgf and Wnt/β-catenin signaling in the rostromedial telencephalon of *Pdn* mutants.

Sprouty1/2 Double Mutants Show Agenesis of the CC

To investigate the importance of these changes in Fgf and Wnt/β-catenin signaling for callosal development, we made use of *Sprouty1/2* double mutants. *Sprouty1* and *Sprouty2* encode negative feedback regulators of Fgf signaling (Kim and Bar-Sagi 2004). In the E12.5 rostromedial telencephalon of *Sprouty1/2* double mutants, Fgf signaling is up-regulated which in turn leads to a down-regulation of Wnt/β-catenin signaling (Faedo et al. 2010) similar to the situation in *Pdn* mutants. We first determined the effects of these alterations to the signaling pathways on the development of guidepost neurons. At E14.5, prior to the arrival of callosal axons, the CR$^+$ guidepost neurons accumulate at the CSB forming a well organized band of neurons which, however, is largely missing in *Sprouty1/2* double mutants (Fig. 4A,B). The mutants also lack CB$^+$ neurons that can already be detected in the midline region of control embryos (Fig. 4C,D), suggesting that the development of guidepost neurons is disturbed in these mutants before callosal axons approach the CSB. Next, we analyzed CC formation in E18.5 embryos. While the formation of Satb2$^+$ callosal projection neurons and their positioning in the upper cortical layers is not affected (Fig. 4E,F). Neurofilament and Tuj1 staining revealed agenesis of the CC in *Sprouty1/2* mutants (Fig. 4G–L,O,P). Callosal fibers project toward the midline, but fail to cross and form ectopic axon bundles. The analysis of the midline guideposts showed no dramatic differences in the distribution of CB$^+$ neurons, but CR$^+$ neurons formed abnormal fibers in the ectopic axon bundles (Fig. 4I–N). Several GFAP$^+$ glia fibers abnormally cluster at the CSB, transecting the path of callosal axons, while the IGG could not be identified (Fig. 4O,P). Taken together, these data show that up-regulation of Fgf signaling is sufficient to induce callosal malformation.

Fgf Signaling is Reduced in the E16.5 Pdn Cingulate Cortex

A recent analysis had shown that Fgf signaling is required between E15.5 and E17.5 for the translocation of glial cells toward the indusium griseum (Smith et al. 2006). Since interfering with Fgf signaling at this stage leads to glial translocation defects very similar to those in E18.5 *Pdn* mutants, we investigated *Fgf8* expression and that of its target gene *sprouty2* in E16.5 *Pdn* embryos. In the rostral cortex of control embryos, both genes are expressed in the IGG and in the GW and *sprouty2* expression expands into the cingulate cortex (Supplementary Fig. 6A,B). At more caudal levels, *Fgf8* and *sprouty2* transcripts were detected in the septum and in the stria medullaris thalami (Supplementary Fig. 6C,D). In contrast, *Fgf8* expression is absent from the IG region and from the GW of *Pdn* embryos and is confined to the caudal septum (Supplementary Fig. 6E,G). At this caudal level, septum and cingulate cortex are only connected by a thin bridge of tissue. This abnormal morphology and the absence of *Fgf8* expression in the GW and IG region suggests that Fgf8 might not signal to the cingulate cortex. Consistent with this idea, *sprouty2* is only expressed in the septum but not in the cingulate cortex of *Pdn* mutants (Supplementary Fig. 6F, H). Taken together with the results of our E12.5 analysis, these data strongly suggest an early phase when Fgf signaling is up-regulated in the E12.5 rostromedial *Pdn* telencephalon causing patterning defects and a clustering of RGCs followed by a later phase with a down-regulation of Fgf signaling in the E16.5 cingulate cortex due to an abnormal morphology of the *Pdn* rostral midline tissue. This down-regulation coincides with the glial translocation defect in *Pdn* mutants.

Positioning of Midline Guidance Cues is Rescued in Pdn/Pdn;Slit2$^{-/-}$ Embryos

The findings described above indicate that altered Fgf signaling plays an important part in the development of the *Pdn* callosal phenotype. However, the callosal phenotype of *sprouty1/2* embryos appears relatively mild compared with that of *Pdn* mutants, suggesting additional abnormalities in *Gli3* mutants. We therefore started to analyze the expression of axon guidance molecules in *Pdn* mutants. In E16.5 control embryos, *Sema3c* is expressed in glutamatergic guidepost and cingulate neurons thereby attracting callosal axons toward the midline (Niquille et al. 2009; Piper, Plachez et al. 2009), but its expression is only slightly reduced in *Pdn* mutants (Supplementary Fig. 7). *Slit2* normally prevents callosal axons from projecting into the septum (Bagri et al. 2002) and is already expressed in the commissural plate of E9.5 embryos (Yuan et al. 1999) and in the septum of E12.5 control embryos (Fig. 5A). Interestingly, our in situ hybridization showed a slight expansion of *Slit2* expression into the cortical region of E12.5 *Pdn* embryos (Fig. 5E). This expansion became more prominent by E14.5 when strong *Slit2* expression is confined to the septum of control embryos with a graded but weaker expression in cortical midline progenitors. In contrast, *Slit2* expression is up-regulated in the rostromedial *Pdn* cortex and *Slit2* transcripts were ectopically detected in the septal midline (Fig. 5B,C,F,G). To confirm this potential increase in *Slit2* expression, we used qRT-PCR on rostromedial telenchepic tissue to show a significant increase in *Slit2* mRNA expression levels (Fig. 5I). Moreover, expanded *Slit2* expression is maintained in the E16.5 cingulate cortex (Fig. 5D,H). Thus, *Pdn* mutants show an expansion of *Slit2* expression in the rostromedial cortex from patterning stages until time points when callosal axons approach the CSB.

To test for a role of this expanded *Slit2* expression, we analyzed CC development in *Pdn/Slit2* double mutants. Initially, we determined the positioning of guidepost cells in E16.5 embryos. This analysis showed that the organization of the cortical midline is much improved in *Pdn/Pdn;Slit2$^{+/-}$* and in *Pdn/Pdn;Slit2$^{-/-}$* embryos. The positioning of the CB$^+$ and CR$^+$ guidepost neurons is largely rescued (Supplementary Fig. 8B–D,G–I). The numbers of CB$^+$ neurons are increased in double mutants, though not to wild-type levels, while CR$^+$ neurons are present in normal numbers in the double mutants (Supplementary Fig. 8P,Q). The formation of GFAP$^+$ GW cells is restricted to the CSB, although the GFAP staining appears more irregular with a few isolated GFAP$^+$ fascicles (Supplementary Fig. 8L–N). Moreover, in contrast to *Pdn/Pdn* embryos, L1$^+$ callosal axons progress through the cingulate cortex without disruption in double-mutant embryos (Supplementary Fig. 8B–D;G–I;L–N). We also analyzed the positioning of guidepost cells in *Slit2$^{-/-}$* embryos (Supplementary Fig. 8E,J,O). While the CB$^+$ and many CR$^+$ guidepost neurons acquire their correct position in the prospective IG region of *Slit2$^{-/-}$* mutants, some CB$^+$ and CR$^+$ neurons intermingle ectopically with callosal axons in the septum, where callosal axons are misdirected. In addition, there is a dramatic
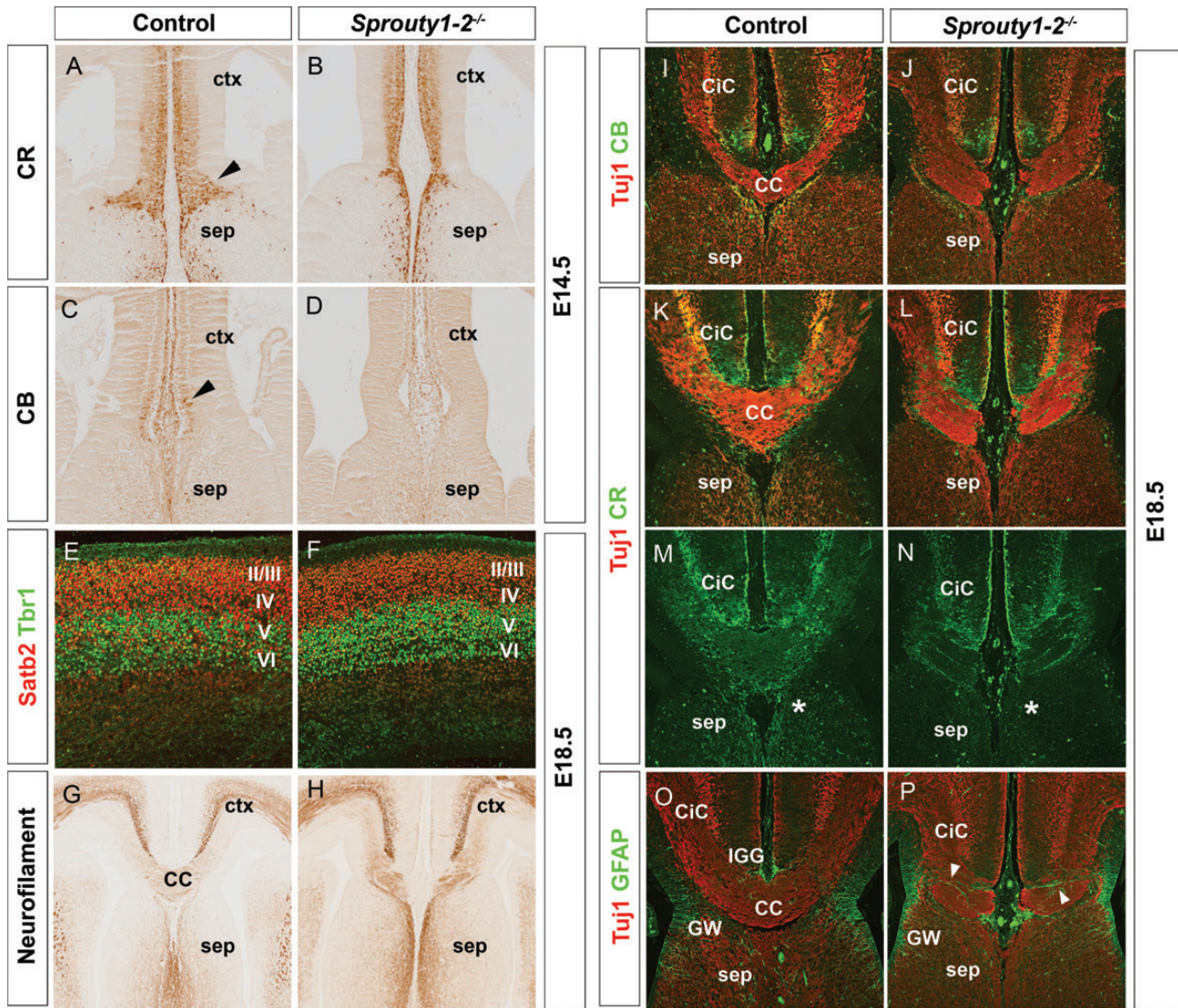
Figure 4. *Sprouty1–2* double mutants lack the CC. (*A* and *B*) In E14.5 control embryos, CR$^+$ guidepost neurons form a well organized band of neurons at the CSB, which is largely missing in *Sprouty1/2* double mutants. (*C* and *D*) Unlike control embryos, CB$^+$ guidepost neurons were not detected at the CSB of *Sprouty1/2* double mutants at E14.5. (*E* and *F*) At E18.5, Satb2$^+$ callosal neurons are normally positioned in the upper cortical layers II/III and IV above the Tbr1$^+$ neurons in layer V and VI. (*G–L, O,* and *P*) Neurofilament and Tuj1 staining reveal agenesis of the CC in E18.5 *sprouty1-2* mutants. Callosal fibers fail to cross the midline and form ectopic axon bundles. (*I–J*) No obvious differences in the distribution of CB$^+$ guidepost neurons are detected in the dorsomedial cortex of *Sprouty1-2* double mutants. (*K–N*) CR$^+$ guidepost neurons form abnormal fiber bundles within the Probst bundles (*O* and *P*) GFAP immunofluorescence reveals abnormally formed midline glia populations. Several GFAP$^+$ glia fibers abnormally cluster at the CSB (arrowheads in *J*), while the IGG cannot be identified.

increase in the number of callosal axons reaching the midline region in *Slit2*$^{-/-}$ embryos as reported previously (Bagri et al. 2002).

Finally, we analyzed CC formation in E18.5 *Pdn/Slit2* double mutants. This analysis confirmed our findings on the much improved organization of midline guidepost neurons, but callosal axons do not cross the midline (Fig. 6C,D,H,I,M, N). In the *Pdn* cingulate cortex, the intermediate zone is disrupted by several, large Probst bundles (Fig. 6B,G,L). In *Pdn/ Pdn;Slit2*$^{+/-}$ and in *Pdn/Pdn;Slit2*$^{-/-}$ embryos, callosal axons migrate uninterrupted through the cingulate cortex without forming Probst bundles (Fig. 6C,D,H,I,M,N). CB$^+$ neurons are located in the IG region similar to control embryos, but are scattered in the *Pdn* cortex (Fig. 6A–D). CR$^+$ neurons occupy positions in the dorsomedial cortex of the double mutants, while these cells are mostly associated with the Probst

bundles in *Pdn* mutants (Fig. 6F–I). In addition to their correct position, normal numbers of CB$^+$ and CR$^+$ neurons are present in the midline region of double mutants (Fig. 6P,Q). In contrast, the midline glia develops abnormally in *Pdn/Slit2* double mutants (Fig. 6K–N). The IGG is missing and ectopic glial fascicles are still formed at the CSB but only in the ventralmost part of the cortex (Fig. 6M,N). Interestingly, the guidepost neurons are also severely affected in *Slit2*$^{-/-}$ mutants. Few CR$^+$ neurons occupy their normal position in the IG, while large clusters of CR$^+$ neurons were detected ventrally to the callosal axons crossing the midline (Fig. 6E,J,O). In addition, 2 large ectopic bundles of fibers were also found at either side of the CC as described previously (Bagri et al. 2002). Taken together, these analyses show a remarkable recovery of midline morphology in *Pdn/Slit2* double mutants.
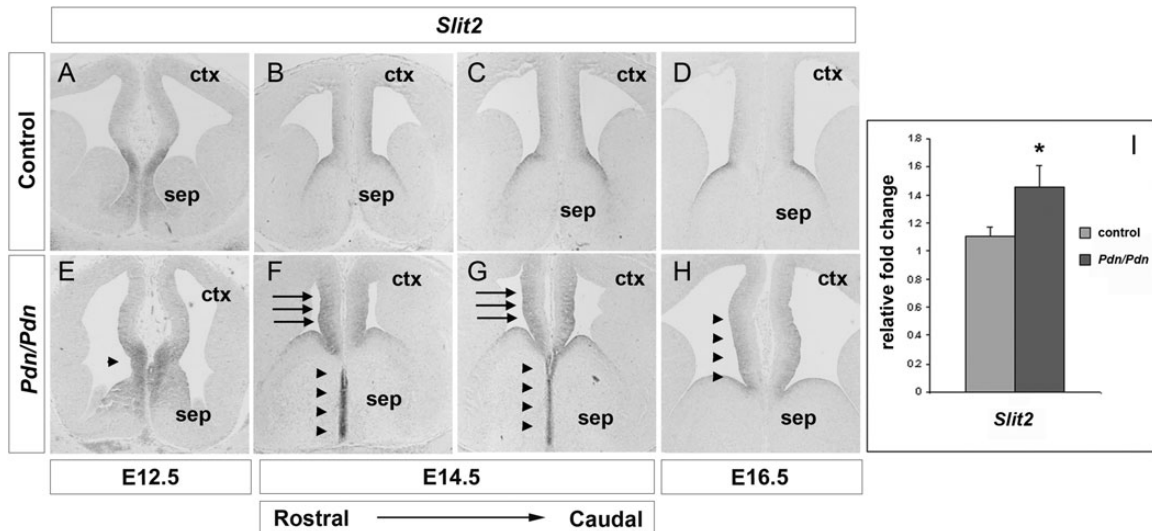
Figure 5. *Slit2* expression expands into the rostromedial cortex of *Pdn/Pdn* embryos. (*A* and *E*) In E12.5 control embryos, *Slit2* expression is confined to the septum, but expands into cortical regions of Pdn mutants (arrowhead in *E*). Note the decreased size of the LGE in *Pdn* mutants as described previously (Magnani et al. 2010). (*B–D*) In E14.5 and E16.5 control embryos, *Slit2* transcripts are detected in the septum and in the prospective cingulate cortex in a graded fashion. (*F–H*) In *Pdn/Pdn* embryos, *Slit2* expression is increased in the future cingulate cortex (arrows). There is also ectopic *Slit2* expression in the midline of the septum, which is enlarged in E14.5 *Pdn/Pdn* embryos (arrowheads in *F* and *G*). (*I*) Quantification of *Slit2* expression levels in the rostromedial telencephalon of E14.5 wild-type and control embryos. Asterisk (*) denotes statistically significant changes with $P \leq 0.05$ (Mann–Whitney test).

Up-Regulation of Fgf Signaling Controls Slit2 Expression and is Required for RGC Clustering in Pdn Mutants

Taken together, our analyses demonstrate roles for Fgf signaling and *Slit2* in positioning callosal guidance cues raising the possibility that both pathways are interconnected. To test for this, we employed an ex vivo explant assay in which we prepared coronal sections of E13.5 control and *Pdn/Pdn* rostral telencephalon, including the commissural plate as the Fgf8 signaling centre, and maintained these sections in culture for 48 h in the presence of DMSO or various concentrations of SU5402, which selectively inhibits Fgf signaling. We first determined the effects of these treatments on the expression of *sprouty2*. Under control conditions, *sprouty2* expression is detected in the septum on sections of control and *Pdn/Pdn* embryos (Fig. 7*A,B*). While the addition of 100 µM SU5402 severely disrupted tissue morphology (data not shown), *sprouty2* expression was abolished in the presence of 25 µM SU5402 (Fig. 7*C*), indicating that this concentration is sufficient to block Fgf signaling in this ex vivo explant culture assay. Next, we analyzed the expression of *Slit2* after SU5402 treatment. In the presence of DMSO, *Slit2* transcripts are confined to the septum of control embryos (Fig. 7*D*), but *Slit2* expression expands into the cortex and into the ventral-most septum on *Pdn/Pdn* sections (Fig. 7*E*). SU5402 treatment of *Pdn* mutant sections resulted in a loss of *Slit2* expression in this latter tissue and in reduced expression in the cortex (Fig. 7*F*), suggesting that up-regulated Fgf signaling in *Pdn* mutants plays at least a partial role in controlling *Slit2* expression. Finally, we used the same assay to determine a role for Fgf signaling in the formation of the ectopic RGC clusters. Immunofluorescence for the Blbp antigen which is encoded by *Fabp7* revealed RGCs in the cortex dorsally to the CSB on control sections and widespread RGC clusters on *Pdn* mutant sections (Fig. 7*G,H*) similar to our in vivo findings (compare with Fig. 3*N,R*). In contrast, addition of 25 µM SU5402 nearly completely abolished the formation of RGC clusters on *Pdn/Pdn* sections (Fig. 7*I*) strongly suggesting that their formation depends on up-regulated Fgf signaling.

Discussion

Several glial and neuronal guidepost cells are organized in strategic positions at the CSB and play crucial roles in the midline crossing of callosal axons, but it remains largely unknown how the guideposts acquire their correct position. The *Gli3* hypomorphic mutant *Pdn* provides an interesting model to address this as the normal distribution of callosal guideposts is severely affected in this mutant. The cortical midline region contains ectopic glial fibers that transect the path of callosal axons and shows an up-regulation of the *Slit2* guidance molecule. Several lines of evidence strongly suggest that the ACC in *Pdn* mutants is caused by these midline defects rather than by defects in callosal axons. Cortical layering, the expression of the callosal determinant Satb2 (Alcamo et al. 2008; Britanova et al. 2008) and the birthdate of upper layer callosal neurons are not affected in *Pdn* embryos (Magnani et al. 2010). Moreover, *Pdn* mutant callosal axons are capable of midline crossing in a wild-type environment. Finally, molecular changes in the cortical midline relevant to the callosal malformation occur as early as E12.5. As these alterations occur well before callosal axons arrive at the midline, our findings strongly suggest that *Gli3*-controlled early patterning events are crucial for setting up the spatial organization of midline guideposts and hence for callosal development.

Pdn mutants showing a very severe callosal phenotype present an interesting tool to identify pathways controlling patterning of the CSB. In fact, our analyses led to the identification of altered activities in key signaling pathways and of changed expression patterns of several transcription factors emphasizing this link between patterning and callosal development. First, several transcription factors with important
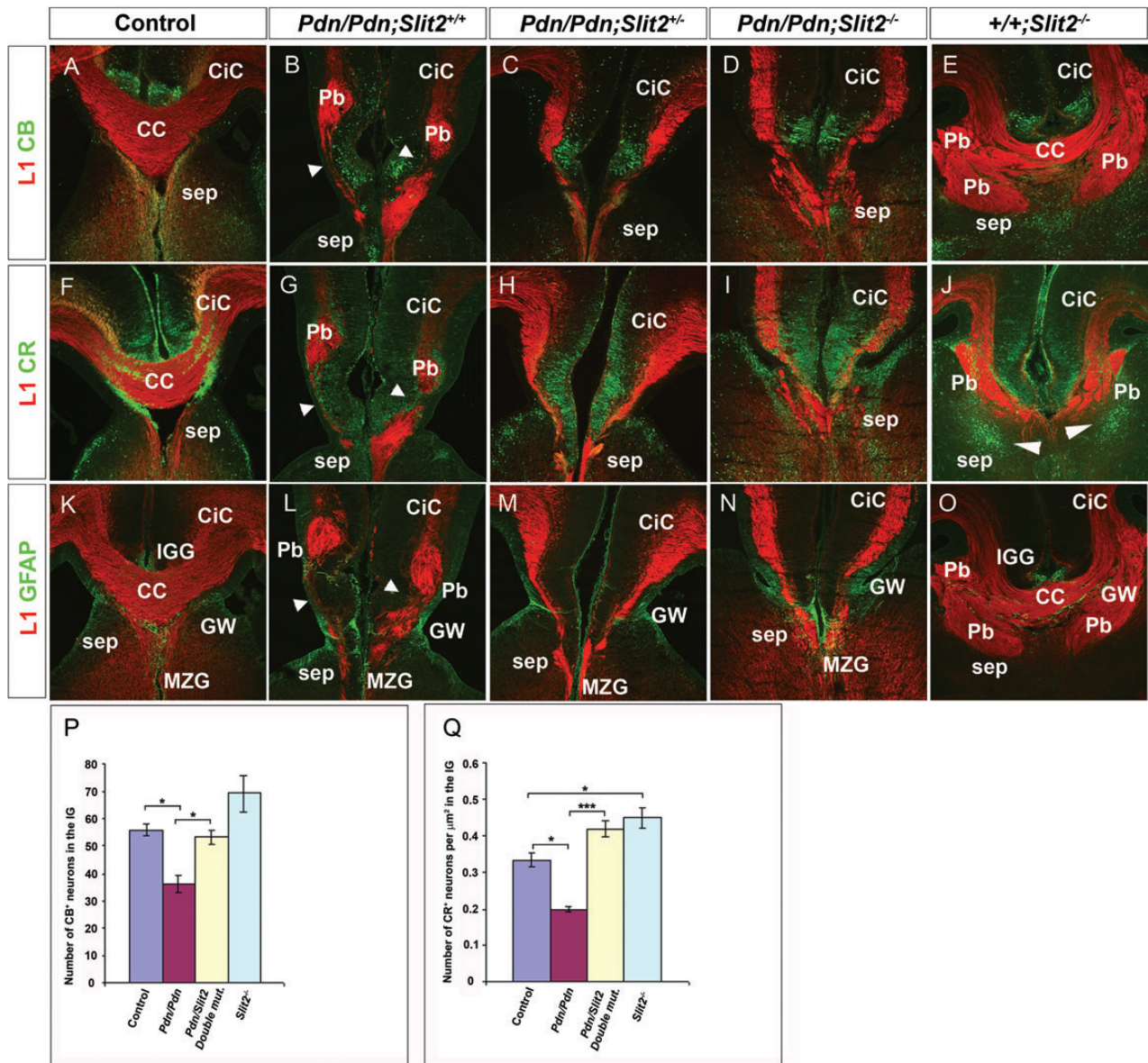
Figure 6. CC development in E18.5 *Pdn/Slit2* double mutants. (*A*, *F*, and *K*) Immunostaining on control brain sections revealing L1⁺ callosal axons, CR⁺ and CB⁺ guidepost neurons and GFAP⁺ midline glia cells. (*B*, *G*, and *L*) In the *Pdn* cingulate cortex, the path of L1+ axons is interrupted at several positions (arrowheads), large L1⁺ Probst bundles are formed and midline glia and neuronal populations are disorganized. (*C*, *D*, *H*, *I*, *M*, and *N*) In *Pdn/Pdn;Slit2⁺/⁻* and in *Pdn/Pdn;Slit2⁻/⁻* embryos, callosal axons reach the CSB without forming Probst bundles, but do not cross the midline. Also, organization and positioning of midline guideposts is partially rescued in the *Pdn/Slit2* double mutants. (*C* and *D*) In *Pdn/Pdn;Slit2⁺/⁻* and in *Pdn/Pdn;Slit2⁻/⁻* embryos, CB⁺ neurons are normally located in the IG region similar to control embryos. (*H* and *I*) *Pdn/Pdn;Slit2⁺/⁻* and in *Pdn/Pdn;Slit2⁻/⁻* CR⁺, sling neurons are normally localized in the cingulate cortex. (*M* and *N*) In *Pdn/Slit2* double mutants, the IGG is absent and ectopic glial fascicles are formed at the CSB. (*E*, *J*, and *O*) Formation of guidepost neurons in *Slit2⁻/⁻* mutants. CR⁺ neurons form large ectopic clusters adjacent to large Probst bundles (arrowhead), but are largely missing from their normal position in the IG (*J*). Note the presence of glial fibers intermingling with the callosal axons crossing the midline (*O*). (*P* and *Q*) Quantification of CB⁺ (*P*) and CR⁺ (*Q*) neurons in the IG region. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (Bonferroni's multiple comparison test).

functions in early forebrain and callosal development have altered expression patterns in the corticoseptal region of E12.5 *Pdn* embryos. Mutations of the human and mouse *SIX3* genes lead to holoprosencephaly (Wallis et al. 1999) and to severe truncations of the prosencephalon (Lagutin et al. 2003), respectively, but the severity of these phenotypes might obscure potential role(s) in callosal formation. In contrast, *Emx1* mutants show ACC due to a lack of the indusium griseum (Qiu et al. 1996; Yoshida et al. 1997). Furthermore, Nfia, *Nfib*, and *Nfix* have high expression level domains dorsally to the CSB (Shu et al. 2003; Campbell et al. 2008;

Plachez et al. 2008) overlapping with the domains of *Wnt7b/8b* expression, suggesting regulatory relationships between these genes. Mutations in *Nfia* and *Nfib* lead to callosal defects due to malformations in the midline glial cell populations and to defective development of the cingulate pioneer neurons (Shu et al. 2003; Steele-Perkins et al. 2005; Piper, Moldrich et al. 2009). Our data suggest that these factors have an earlier patterning role that might be obscured by redundancy between these factors.

Secondly, we identified altered Fgf signaling and Wnt/β-catenin signaling at the CSB in E12.5 *Pdn* mutants as
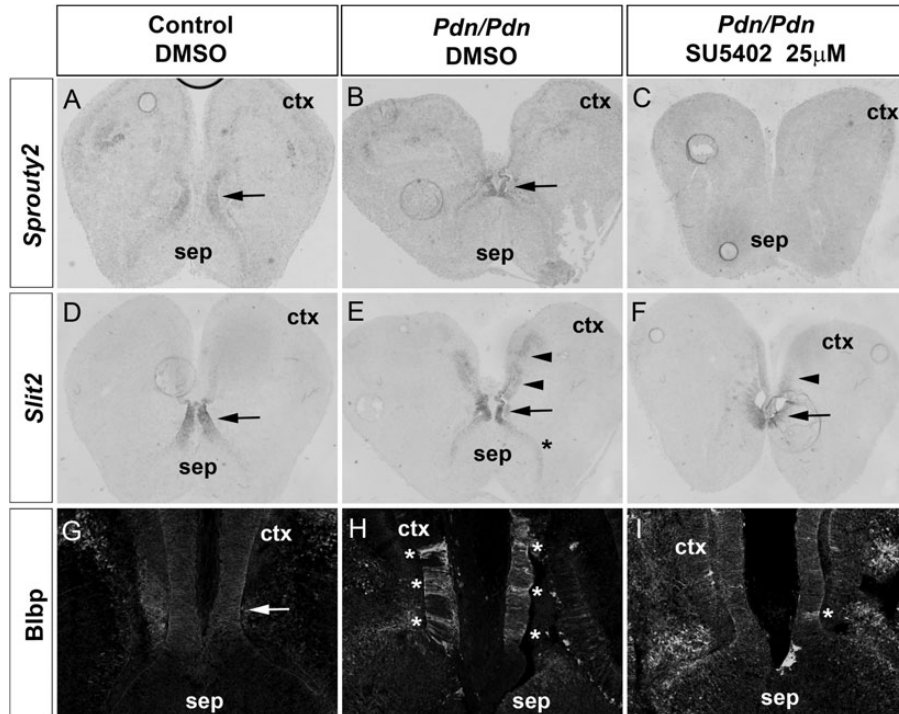
Figure 7. Effects of blocking Fgf signaling on midline development. (*A–C*) *Sprouty2* expression is detected in the septum of DMSO treated control and *Pdn/Pdn* sections (arrows in *A* and *B*) but completely abolished after treatment with 25 μM SU5402. (*D–E*) Under control conditions, *Slit2* expression is confined to the septum (arrows in *D*), but expands into the cortex (arrowheads in *E*) and into the ventralmost septum (asterisks in *E*) of *Pdn/Pdn* mutant sections. (*F*) Treatment of *Pdn/Pdn* sections with 25 μM SU5402 resulted in reduced *Slit2* expression in the cortex (arrowhead) and to a loss of expression in the ventralmost septum. (*G–I*) Up-regulation of Fgf signaling is required for RGC cluster formation. (*G*) Blbp marks RGCs in the cortex dorsal to the CSB (arrow). (*H* and *I*) In *Pdn/Pdn*, sections treated with DMSO, Blbp+ cells form widespread cluster (asterisks in *H*), while their formation is nearly completely abolished after treatment with 25 μM SU5402 (*I*).

important regulators of callosal development. In fact, *Sprouty1/2* double mutants, in which increased Fgf signaling down-regulates Wnt/β-catenin signaling in the rostromedial telencephalon (Faedo et al. 2010), display agenesis of the CC. Interestingly, these mutants already show defective development of CR+ and CB+ guidepost neurons at the E14.5 CSB. Although we cannot exclude the possibility that elevated Fgf signaling after E14.5 might further disrupt callosal formation, this altered development of guidepost neurons prior to the arrival of callosal axons strongly suggest that increased levels of Fgf signaling at patterning stages already interfere with guidepost and hence callosal development. This idea is supported by recent findings on callosal development in *Rfx3* mutant mice in which a mild up-regulation of Fgf signaling underlies a mislocalization of glutaminergic guidepost neurons (Benadiba et al. 2012). Consistent with recent findings on a regulatory role of Fgf signaling in RGC development (Kang et al. 2009; Sahara and O'Leary 2009), we also show here that up-regulating Fgf signaling is required for the formation of RGC clusters in the rostromedial telencephalon of *Pdn* mutants. Our Blbp/GFAP double staining further indicates that these RGC clusters give rise to the ectopic glial cells in the E16.5 *Pdn* cingulate cortex, which due to morphological alterations lacks Fgf signaling at this state. This lack is likely to result in a failure of ectopic glial cells to translocate (Smith et al. 2006). Taken together, these findings indicate 2 phases for Fgf signaling in callosal development. During a newly identified, early patterning phase Fgf signaling sets the CSB and positions glial and neuronal guidepost cells. In a second phase, Fgf signaling is required for glial cell transloca-tion (Smith et al. 2006). These data also demonstrate that a reduction and an increase in Fgf signaling can cause ACC, strongly suggesting that regulating *Fgf8* expression levels is crucial for callosal development. This regulation might involve a positive feedback loop with *Shh* (Ohkubo et al. 2002) and/or an interaction with *Wnt7b* and *Wnt8b* which have complementary expression patterns to *Fgf8* at the CSB. Previous analyses have implicated *Wnt5a* and Ryk-mediated Wnt/Ca²⁺ signaling in promoting the escape of callosal axons from the midline into the contralateral hemisphere (Keeble et al. 2006; Hutchins et al. 2011). Moreover, the meninges and neurons of the cingulate cortex use a cascade of signals in-cluding *Wnt3* to regulate midline crossing of cingulate pioneer axons (Choe et al. 2012). In contrast, *Wnt8b* mutant mice show normal callosal development probably due to re-dundancy with other Wnt molecules (Fotaki et al. 2010). However, *Wnt7b/8b* expression is already down-regulated before the onset of ectopic *Fgf8* expression in the E9.0 *Pdn* telencephalon (Ueta et al. 2008). This and the reduced Wnt/β-catenin signaling in the *sprouty1/2* double mutants (Faedo et al. 2010) suggest an antagonistic interaction between Fgf and Wnt/β-catenin signaling to control *Fgf8* expression levels in the commissural plate, thereby regulating patterning of the CSB and positioning of midline guideposts (Fig. 8).

Finally, the up-regulation of *Slit2* expression represents a major cause of the *Pdn* callosal phenotype. *Pdn/Slit2* double mutants show a dramatic improvement in the growth of corti-cal axons toward the midline and in midline organization
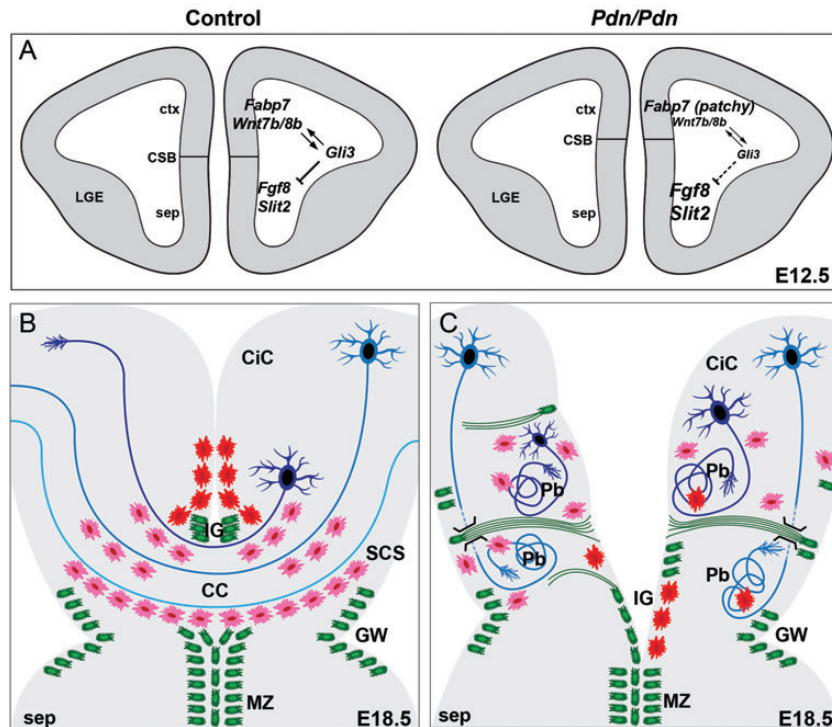
Figure 8. Schematic representation of the major changes during CSB formation and midline crossing of callosal axons in *Pdn/Pdn* mutants. (*A*) During patterning stages, *Gli3* restricts the expression of *Fgf8* and *Slit2* to the commissural plate and is required for *Wnt7b/8b* expression in the rostromedial cortex of control embryos thereby establishing the CSB and controlling a balance between Fgf and Wnt/β-catenin signaling. Arrows and lines indicate genetic interactions except for the direct regulation of *Gli3* expression by Wnt/β-catenin signaling (Hasenpusch-Theil et al. forthcoming 2012). Reducing *Gli3* expression levels in *Pdn/Pdn* mutants leads to a down-regulation of *Wnt7b/8b* expression and a concomitant up-regulation of *Slit2* expression and Fgf signaling, which in turns results in RGC clustering. (*B*) Schematic drawing representing the organization of glial cell populations (green) CR$^+$ (pink) and CB$^+$ (red) guidepost neurons in control embryos to channel callosal axons (blue) across the midline. (*C*) Disorganization of guideposts results in the formation of Probst bundles (Pb) in *Pdn/Pdn* embryos. Ectopic glial fascicles span the width of the cortex and provide a barrier to the migration of callosal axons. Callosal axons may migrate in and out of the image plane to circumvent the ectopic glial fascicles as indicated by the dotted lines. Guidepost neurons associate with the Probst bundles.

suggesting 2, mutually non-exclusive roles for *Slit2* in callosal development. First, *Slit2* could control the permissiveness of the cingulate cortex for the growth of callosal axons. Indeed, callosal axons approach the CSB without forming Probst bundles in *Pdn/Slit2* double mutants, and many callosal axons approach the midline but miss-project into the septum in *Slit2$^{-/-}$* mutants (Bagri et al. 2002). This idea is also consistent with the temporal expression profile of *Slit2*, which becomes down-regulated in the control cingulate cortex after E14.5 (Fig. 5). Alternatively, *Slit2* could regulate the migration of guidepost neurons into the cortical midline (Niquille et al., 2009) similar to its effect on the migration of LGE guidepost cells (Bielle et al. 2011). The positioning and the numbers of guidepost neurons are largely rescued in the *Pdn/Slit2* double mutants, while CR$^+$ neurons form ectopic clusters in *Slit2$^{-/-}$* embryos. Taken together, these findings raise the interesting possibility that a major role of *Slit2* in callosal development is to coordinate the migration of callosal axons with that of the guidepost neurons.

Interestingly, *Slit2* expression is already expanded in E12.5 *Pdn* embryos suggesting that early patterning regulates its expression. *Slit2* could be a downstream target of Fgf signaling given its coexpression with *sprouty2* (Yuan et al. 1999) and its down-regulation in the septum of *Fgfr1* mutant mice (Tole et al. 2006) and after blocking Fgf signaling on rostromedial tissue sections (Fig. 7). Alternatively, Gli3 or transcription factors downstream of Gli3, such as Emx1 or the Nfi transcriptional regulators, could repress *Slit2* expression in the

rostrodorsal telencephalon. Irrespective of the exact mechanism, the up-regulation of *Slit2* provides a link between early patterning and the coordination of midline development.

In summary, our analyses provide insights into how early patterning of the cortical midline controls the organization of midline guideposts and the formation of a permissive environment allowing callosal axons to approach the CSB. In this process, *Gli3* takes centre stage by controlling Fgf and Wnt/β-catenin signaling at the rostral midline and the expression of several transcription factors and of the *Slit2* axon guidance molecule. Interestingly, the human *GLI3* gene is mutated in Acrocallosal syndrome patients who lack the CC (Elson et al. 2002). CC malformations are also a frequent hallmark of ciliopathies in which the function of the primary cilium and hence Gli3 processing is affected (Tobin and Beales 2009). Therefore, our findings provide a framework for understanding the defective processes underlying the ACC in Acrocallosal syndrome and in ciliopathies.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Funding

Notes

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