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

Gli3 controls corpus callosum formation by positioning midline guideposts during telencephalic patterning

Abbreviated title: Corpus callosum development requires *Gli3*

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ABSTRACT

The corpus callosum (CC) represents the major forebrain commissure connecting the two 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 corpus callosum and mislocation of glial and neuronal guidepost cells. Using transplantation experiments we demonstrate that agenesis of the corpus callosum 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 signalling. Mutations in *sprouty1/2* which mimic the changes in these signalling 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 signalling and of *Slit2* expression is crucial for positioning midline guideposts and callosal development.

INTRODUCTION

The corpus callosum (CC) connects neurons of the two 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, behavioural and neurological consequences (Paul LK et al., 2007; Richards LJ et al., 2004) and have been identified in over 50 human congenital syndromes (Richards LJ *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 J et al., 1993). Moreover, several guide-post 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 LJ *et al.*, 2004), and GABAergic and glutamatergic neurons that transiently populate the CC (Niquille M et al., 2009). Finally, axons from the cingulate cortex pioneer the CC and function as scaffolds for neocortical axons (Koester SE and DD O'Leary, 1994; Piper M, C Plachez et al., 2009; Rash BG and LJ Richards, 2001). Several axon guidance molecules including Slit2 which are produced by midline glial cells and by the glutamatergic neurons have essential roles in callosal development (Bagri A et al., 2002; Niquille M *et al.*, 2009). While these studies reveal complex interactions between callosal axons and their environment, it remains largely unknown how guide-post 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 (Fotaki V et al., 2006; Kuschel S et al., 2003; Theil T et al., 1999; Tole S et al., 2000) acting both cell autonomously (Quinn JC et al., 2009) and cell non-autonomously by controlling the expression of signaling molecules essential for telencephalic development (Aoto K et al., 2002; Grove EA et al., 1998; Theil T *et al.*, 1999; Tole S *et al.*, 2000). 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 D et al., 2010) and lacks the corpus callosum (Naruse I 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 signalling, respectively. These changes in these signalling 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 guide posts 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 signalling and *Slit2* expression levels and provides new insights into the mechanisms underlying CC pathogenesis.

MATERIALS AND METHODS

Mice. The mutant mouse lines *Pdn*, τ*GFP*, *Slit2*, *Sprouty1* and *2* and mating strategies have been described previously (Basson MA et al., 2005; Naruse I *et al.*, 1990; Plump AS et al., 2002; Pratt T et al., 2000; Shim K et al., 2005; Simrick S 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, wildtype 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 analysed.

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

Immunohistochemical analysis was performed as described previously (Theil T, 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 anti-mouse 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 (170μm^2 for E16.5 and 297μm^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 Anova 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 D *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 72hrs, fixed with 4% PFA and processed for anti-GFP immunofluorescence as described above. For Fgf blocking experiments, slices were cultured in the presence of either DMSO or of 25µM or 100µM SU5402 (Calbiochem) for 48hrs, 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 PCR (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 Version1.2.3 running an absolute quantification protocol including background calibrations.

Quantification of Calbindin$^+$ and Calretinin$^+$ neurons

Calbindin$^+$ neurons in the induseum griseum (IG) were counted per section. For counting Calretinin$^+$ neurons, a box of 170µm^2 encompassing the IG was placed with its lower edge at the CSB and the proportion of Calretinin$^+$ neurons per area unit was calculated. For statistical evaluation, Bonferroni's Multiple Comparison Tests were performed.

RESULTS

CC midline guide-post cells are severely disorganized in *Pdn/Pdn* brains

Neurofilament, Tuj1 and L1 immunhistochemical stainings and cortical DiI labelling confirmed a previous description of CC malformation in *Pdn* mutants (Naruse I *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 guide-posts that are essential for callosal development (Paul LK *et al.*, 2007). In P0 control animals, the cingulate pioneer axons are immunopositive for Neuropilin-1 (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, Calretinin (CR) or Calbindin (CB) (Niquille M *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 M *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 glial wedge (GW), the indusium griseum glia (IGG) and the midline zipper glia (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 guide-post cells.

Given the severity of this disorganization, we started to study the origins of these midline defects by investigating the formation of 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 Figures 2A-L and 6P). 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 disorganised in *Pdn* mutants when callosal axons approach the CSB.

Agenesis of the corpus callosum in *Pdn* mutants is caused by CC midline defects

Since *Gli3* is widely expressed in progenitor cells which give rise to callosal neurons as well as to guidepost cells, agenesis of the 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 D *et al.*, 2010). Moreover, Satb2 upper layer callosal projection neurons are borne at E15.5 (Supplementary Figure 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 T *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 out of 9) (Fig. 2A). After transplantation of *Pdn/Pdn*;GFP^+ cortex into control cortex, *Pdn/Pdn* axons also migrated across the midline (n=7 out of 8) (Fig. 2B). However, control;GFP^+ callosal axons did not grow into *Pdn/Pdn*;GFP^- dorsomedial cortex (n=0 out of 7) (Fig. 2C) and only a few axons projected along the surface of the mutant host tissue (n=4 out of 7) (Fig. 2C). In contrast, corticofugal axons project into the lateral cortex and striatum under these conditions (Magnani D *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* homobox gene is lost in *Pdn* mutants (Kuschel et al., 2003). Moreover, *Emx1* mutants display ACC (Qiu M et al., 1996; Yoshida M 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 corpus callosum, the hippocampal and anterior commissures cross the midline at E16.5 (Moldrich RX et al., 2010). As these genes have important roles in forebrain and/or callosal development (Campbell CE et al., 2008; das Neves L et

al., 1999; Lagutin OV et al., 2003; Piper M, RX Moldrich et al., 2009; Plachez C et al., 2008; Qiu M *et al.*, 1996; Shu T et al., 2003), 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. 4A,E). Nfia, *Nfi*b and *Nfix*, are expressed at high levels in the cortex and at lower levels in the dorsalmost septum (Plachez C *et al.*, 2008; Shu T *et al.*, 2003) (Supplementary Fig. 4B-D). In *Pdn* mutants, their cortical expression domains is lost while low level septal expression remains except for Nfia which is strongly expressed in the septum (Supplementary Fig. 4F-H). Taken together, these data indicate that the expression of several transcripton 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 signalling molecules in the telencephalon including *Shh*, *Bmp*/*Wnt* genes and *Fgf8* (Aoto K *et al.*, 2002; Grove EA *et al.*, 1998; Kuschel S *et al.*, 2003; Magnani D *et al.*, 2010; Theil T *et al.*, 1999; Tole S *et al.*, 2000). 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 signalling as judged by *Ptc1* expression remains confined to the septum and does not extend into the cortex (Supplementary Figure 5). Moreover, *Bmp7*, which is essential for callosal development (Sanchez-Camacho C et al., 2010), 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. 3A,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. 3C,D) while *Wnt7b* and *Wnt8b* expression is nearly absent from the *Pdn* dorsomedial telencephalon and *Wnt7b* transcription is increased in the septum (Fig. 3H,I). Consistent with reduced Wnt/βcatenin signalling, expression of the *Wnt* target gene *Axin2* is severely reduced in *Pdn* mutants (Fig. 3E,J).

Since telencephalic patterning is controlled by a balance between Bmp/ Wnt/βcatenin and Fgf signalling (Kuschel S *et al.*, 2003; Shimogori T et al., 2004; Theil T *et al.*, 1999) and since *Fgf8* is required for callosal development (Huffman KJ et al., 2004; Moldrich RX *et al.*, 2010), we also investigated *Fgf8* expression in *Pdn* mutants. In control embryos, *Fgf8* transcripts are confined to the commissural plate, but expands further dorsally in the E12.5 *Pdn* corticoseptal region (Fig. 3K,O) consistent with our previous whole mount expression analysis (Kuschel et al., 2003). Expression of *Sprouty2* and phospho-Erk (pErk), targets of Fgf signalling, 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 signalling is ectopically activated during patterning. We also analyzed *Fabp7* expression which in control embryos marks neurogenic radial glial cells (RGC) on the cortical side of the CSB (Fig. 3N) and which is increased upon up-regulation of Fgf signalling 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^{+} fibres which we observed at E16.5. Taken together, these analyses indicate severe changes in Fgf and Wnt/βcatenin signalling in the rostromedial telencephalon of *Pdn* mutants.

Sprouty1/2 **double mutants show agenesis of the corpus callosum**

To investigate the importance of these changes in Fgf and Wnt/βcatenin signalling for callosal development, we made use of *Sprouty1/2* double mutants. *Sprouty1 and Sprouty2* encode negative feedback regulators of Fgf signalling (Kim HJ and D Bar-Sagi, 2004). In the E12.5 rostromedial telencephalon of *Sprouty1/2* double mutants, Fgf signalling is up-regulated which in turn leads to a down-regulation of Wnt/βcatenin signalling (Faedo A et al., 2010) similar to the situation in *Pdn* mutants. We first determined the effects of these alterations to the signalling 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 which 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 fibres project towards 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 fibres in the ectopic axon bundles (Fig. 4I-N). Several $GFAP^+$ glia fibres 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 signalling is sufficient to induce callosal malformation.

Fgf signalling is reduced in the E16.5 *Pdn* cingulate cortex

A recent analysis had shown that Fgf signalling is required between E15.5 and E17.5 for the translocation of glial cells towards the indusium griseum (Smith KM et al., 2006). Since interfering with Fgf signalling 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 signalling 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 signalling 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 signalling plays an important part in the development of the *Pdn* callosal phenotype. However, the callosal phenotype of *sprouty1/2* embryos appears relatively mild compared to 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 this respect, we became interested in the *Slit2* gene which is critical for callosal development (Bagri A *et al.*, 2002) and which is already expressed in the commissural plate of E9.5 embryos (Yuan W 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 quantitative RT-PCR on rostromedial telencephic 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. 7B-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. 7P,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. 7L-N). Moreover, in contrast to *Pdn*/*Pdn* embryos, L1^{+} callosal axons progress through the cingulate cortex without disruption in double mutant embryos (Supplementary Fig. 7B-D;G-I;L-N). We also analyzed the positioning of guidepost cells in *Slit2*$^{-/-}$ embryos (Supplementary Fig. 7E,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 increase in the number of callosal axons reaching the midline region in *Slit2*$^{-/-}$ embryos as reported previously (Bagri A *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,B). 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, two large ectopic bundles of fibres were also found at either side of the corpus callosum as described previously (Bagri et al., 2002). Taken together, these analyses show a remarkable recovery of midline morphology in *Pdn*/*Slit2* double mutants.

Up-regulation of Fgf signalling controls cortical *Slit2* expression and is required for RGC clustering in *Pdn* mutants

Taken together, our analyses demonstrate roles for Fgf signalling 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 signalling centre and maintained these sections in culture for 48h in the presence of DMSO or various concentrations of SU5402, which selectively inhibits Fgf signalling. In control experiments, 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. 7A,B). While the addition of 100µM SU5402 severely disrupted tissue morphology (data not shown), *sprouty2* expression was abolished in the presence of 25µM SU5402 (Fig. 7C) indicating that this concentration is sufficient to block Fgf signalling 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. 7D), but *Slit2* expression expands into the cortex and into the ventral most septum on *Pdn*/*Pdn* sections (Fig. 7E). 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. 7F) suggesting that up-regulated Fgf signalling 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 signalling 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. 7G,H) similar to our in vivo findings (compare with Fig. 3N,R). In contrast, addition of 25μM SU5402 nearly completely abolished the formation of RGC clusters on *Pdn*/*Pdn* sections (Fig. 7I) strongly suggesting that their formation depends on up-regulated Fgf signalling.

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 fibres which 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 EA et al., 2008; Britanova O et al., 2008) and the birthdate of upper layer callosal neurons are not affected in *Pdn* embryos (Magnani D *et al.*, 2010 and Supplementary Figure 3). 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 signalling pathways and of changed expression patterns of several transcription factors emphasizing this link between patterning and callosal development. First, several transcription factors with important 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 DE et al., 1999) and to severe truncations of the prosencephalon (Lagutin OV *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 M *et al.*, 1996; Yoshida M *et al.*, 1997). Furthermore, Nfia, *Nfib* and *Nfix* have high expression level domains dorsally to the CSB (Campbell CE *et al.*, 2008; Plachez C *et al.*, 2008; Shu T *et al.*, 2003) 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 (Piper M, RX Moldrich *et al.*, 2009; Shu T *et al.*, 2003; Steele-Perkins G et al., 2005). Our data suggest that these factors have an earlier patterning role which might be obscured by redundancy between these factors.

Secondly, we identified altered Fgf signalling and Wnt/βcatenin signalling at the CSB in E12.5 *Pdn* mutants as important regulators of callosal development. In fact, *Sprouty1/2* double mutants, in which increased Fgf signalling down-regulates Wnt/βcatenin signalling in the rostromedial telencephalon (Faedo A *et al.*, 2010), display agenesis of the corpus callosum. 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 signalling 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 signalling 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 signalling underlies a mislocalisation of glutaminergic guidepost neurons (Benadiba C et al., 2012). Consistent with recent findings on a regulatory role of Fgf signalling in RGC development (Kang W et al., 2009; Sahara S and DD O'Leary, 2009), we also show here that up-regulating Fgf signalling 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 signalling at this state. This lack is likely to result in a failure of ectopic glial cells to translocate (Smith KM *et al.*, 2006). Taken together, these findings indicate two phases for Fgf signalling in callosal

development. During a newly identified, early patterning phase Fgf signalling sets the CSB and positions glial and neuronal guidepost cells. In a second phase, Fgf signalling is required for glial cell translocation as described previously (Smith KM *et al.*, 2006). These data also demonstrate that a reduction as well as an increase in Fgf signalling 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 Y 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/Ca^{2+} signalling in promoting the escape of callosal axons from the midline into the contralateral hemisphere (Hutchins BI et al., 2011; Keeble TR et al., 2006). Moreover, the meninges and neurons of the cingulate cortex use a cascade of signals including *Wnt3* to regulate midline crossing of cingulate pioneer axons (Choe Y et al., 2012). In contrast, *Wnt8b* mutant mice show normal callosal development probably due to redundancy with other Wnt molecules (Fotaki V et al., 2009). However, *Wnt7b*/*8b* expression is already down-regulated before the onset of ectopic *Fgf8* expression in the E9.0 *Pdn* telencephalon (Ueta E et al., 2008). This and the reduced Wnt/βcatenin signalling in the *sprouty1/2* double mutants (Faedo A *et al.*, 2010) suggest an antagonistic interaction between Fgf and Wnt/βcatenin signalling 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 cortical axons towards the midline and in midline organization suggesting two, 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 A *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 M *et al.*, 2009) similar to its effect on the migration of LGE guidepost cells (Bielle F 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. Currently, we cannot distinguish between these scenarios, but our 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 signalling given its co-expression with *sprouty2* (Yuan W *et al.*, 1999) and its down-regulation in the septum of *Fgfr1* mutant mice (Tole S et al., 2006) and after blocking Fgf signalling 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/βcatenin signalling 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 E 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 JL and PL Beales, 2009). Therefore, our findings provide a framework for understanding the defective processes underlying the ACC in Acrocallosal syndrome and in ciliopathies.

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FIGURE LEGENDS

Figure 1: Disorganization of midline structures in P0 *Pdn/Pdn* brains. **(A-D)** Neuropilin-1 (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,D). **(A, B)** Calretinin (CR) labels glutamatergic guidepost neurons. **(C,D)** In *Pdn/Pdn* brains, CR^+ neurons are disorganized and clusters of CR^+ neurons are associated with Probst bundles (arrowhead in D). **(E,F)** Calbindin (CB) labels guidepost neurons located in the indusium griseum (IG). **(G,H)** CB^+ neurons abnormally cluster with $Tuj1^+$ callosal axons in the *Pdn/Pdn* cortex (arrowheads in H). **(I-J)** GFAP immunofluorescence labels the glial wedge (GW), the indusium griseum glia (IGG) and the midline zipper glia (MZG) in control brains. **(K,L)** In *Pdn/Pdn* brains, $GFAP^+$ fascicles are ectopically formed within the CiC (arrowhead in L) and transect the path of callosal axons. The midline zipper glia expands into more ventral regions of the septum.

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 migration of GFP^+ callosal axons across the midline (n=8 out of 9). **(B)** *Pdn/Pdn*; GFP^+ cortex transplantion into control cortex also shows crossing callosal axons (n=7 out 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 out of 7), only few axons project along the surface of the mutant tissue (arrowhead) (n=4 out of 7). **(E)** 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 72h of culture.

Figure 3: Altered Wnt/βcatenin and Fgf signalling in the E12.5 *Pdn* rostromedial telencephalon. (**A,B,F,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,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,J)** *Axin2* expression is severely reduced in the *Pdn* dorsomedial cortex (arrow in J). **(K-M,O-Q)** *Fgf8, Sprouty2* and phospho-Erk (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,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).

Figure 4: *Sprouty1-2* double mutants lack the corpus callosum. **(A,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,D)** Unlike control embryos, CB^+ guidepost neurons were not detected at the CSB of *Sprouty1/2* double mutants at E14.5. **(E,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,P)** Neurofilament and Tuj1 staining reveal agenesis of the CC in E18.5 *sprouty1-2* mutants. Callosal fibres 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 fibre bundles within the Probst bundles **(O,P)** GFAP immunofluorescence reveals abnormally formed midline glia

populations. Several GFAP$^+$ glia fibres abnormally cluster at the CSB (arrowheads in J), while the IGG can not be identified.

Figure 5: *Slit2* expression expands into the rostromedial cortex of *Pdn*/*Pdn* embryos. (**A,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 midlineof the septum which is enlarged in E14.5 *Pdn*/*Pdn* embryos (arrowheads in F,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).

Figure 6: Corpus callosum development in E18.5 *Pdn*/*Slit2* double mutants. **(A,F,K)** Immunostaining on control brain sections revealing L1$^+$ callosal axons, CR$^+$ and CB$^+$ guidepost neurons and GFAP$^+$ midline glia cells. **(B,G,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,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,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,I)** *Pdn*/*Pdn*;*Slit2*$^{+/-}$ and in *Pdn*/*Pdn*;*Slit2*$^{-/-}$ CR$^+$ sling neurons are normally localized in the cingulate cortex. **(M,N)** In *Pdn*/*Slit2* double mutants, the IGG is absent and ectopic glial fascicles are formed at the CSB. (**E,J,O**) Formation of guide-post 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 fibres intermingling with the callosal axons crossing the midline (**O**). (**P,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).

Figure 7: Effects of blocking Fgf signalling 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 25µM 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 25µM 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 signalling is required for RGC cluster formation. (G) Blbp marks RGCs in the cortex dorsal to the CSB (arrow). (H,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 25µM SU5402 (I).