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Gli3 is required in Emx1^{+} progenitors for the development of the corpus callosum

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ABSTRACT

The corpus callosum (CC) is the largest commissure in the forebrain and mediates the transfer of sensory, motor and cognitive information between the cerebral hemispheres. During CC development, a number of strategically located glial and neuronal guidepost structures serve to guide callosal axons across the midline at the corticoseptal boundary (CSB). Correct positioning of these guideposts requires the *Gli3* gene, mutations of which result in callosal defects in humans and mice. However, as *Gli3* is widely expressed during critical stages of forebrain development, the precise temporal and spatial requirements for *Gli3* function in callosal development remain unclear. Here, we used a conditional mouse mutant approach to inactivate *Gli3* in specific regions of the developing telencephalon in order to delineate the domain(s) in which *Gli3* is required for normal development of the corpus callosum. Inactivation of *Gli3* in the septum or in the medial ganglionic eminence had no effect on CC formation, however *Gli3* inactivation in the developing cerebral cortex led to the formation of a severely hypoplastic CC at E18.5 due to a severe disorganization of midline guideposts. Glial wedge cells translocate prematurely and *Slit1/2* are ectopically expressed in the septum. These changes coincide with altered Fgf and Wnt/β-catenin signalling during CSB formation. Collectively, these data demonstrate a crucial role for *Gli3* in cortical progenitors to control CC formation and indicate how defects in CSB formation affect the positioning of callosal guidepost cells.

Introduction

The corpus callosum (CC) is the largest transverse axon fibre tract in the forebrain and mediates the interhemispheric transfer of sensory, motor and cognitive information between the cerebral hemispheres (Paul et al., 2007; Richards et al., 2004). Malformations of the corpus callosum are amongst the most common brain anomalies found at birth and are thought to occur in up to 7/1000 of the total newborn population (Bedeschi et al., 2006). Complete or partial agenesis of the CC is associated with over 50 human congenital syndromes and is a cause of mental retardation having a wide range of cognitive, behavioural and neurological consequences (Paul et al., 2007; Richards et al., 2004). However, while the clinical implications of CC agenesis are known, the developmental mechanisms determining the formation of the CC are yet to be fully elucidated.

During development of the corpus callosum, callosal axons from each cortical hemisphere must cross the midline to reach the contralateral hemisphere. This crossing occurs at the corticoseptal boundary (CSB) which separates cortex and septum and involves complex interactions between callosal axons and several midline guidance structures. Glial cells of the glial wedge (GW) and the indusium griseum are located adjacent to callosal axons (Shu and Richards, 2001) and prevent them from migrating into the septum by producing the repellent axon guidance molecule Slit2 (Bagri et al., 2002; Shu et al., 2003a). In addition, these midline glial populations act in combination with several neuronal populations. GABAergic neurons derived from the medial ganglionic eminence populate the corpus callosum and channel axons across the midline (Niquille et al., 2009). Moreover, Calretinin+ and Calbindin+ neurons located in the indusium griseum and in the cingulate cortex express the Sema3c guidance factor which is required for callosal development (Niquille et al., 2009; Piper et al., 2009). Calretinin+ neurons are also detected within the corpus callosum where they delineate its dorsal and ventral components (Niquille et al., 2009). Taken together, these findings indicate that CC development is based on complex cellular and molecular interactions between callosal axons, midline glia and neuronal populations and guidance molecules produced by these cells. Moreover, these interactions require

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a precise spatial arrangement of the cellular guidance cues at the CSB but very little is known about the mechanisms by which these cells acquire their position.

Acrocallosal syndrome patients carry mutations in the *GLI3* gene and among other symptoms show complete absence of the CC (Elson et al., 2002). *Gli3* encodes a zinc finger transcription factor with crucial roles in early patterning of the dorsal telencephalon through controlling the expression of several signaling molecules (Fotaki et al., 2011; Grove et al., 1998; Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000). *Gli3*$^{Xt/Xt}$ mice which carry a *Gli3* loss of function allele have very severe cortical defects, precluding their use to study corpus callosum development. However, the *Gli3* hypomorphic mutant *Polydactyly Nagoya* (*Gli3*$^{Pdn/Pdn}$) displays corpus callosum agenesis (Naruse et al., 1990) and presents an interesting model to study *Gli3* function in callosal development. In these mutants, altered Fgf and Wnt/β-catenin signaling leads to the ectopic formation of glial fascicles which interfere with the growth of callosal axons and cause the formation of Probst bundles (Magnani et al., in press). Moreover, an ectopic expression of the chemorepellent axon guidance molecule Slit2 in the cortical midline also inhibits the migration of callosal axons and of guidepost neurons to their correct position at the CSB (Magnani et al., in press). However, while these findings clearly indicate an important role of *Gli3* in positioning of callosal guidepost cells at the midline, it remains unclear exactly when and where *Gli3* controls this process since it is expressed in both cortical and septal progenitor cells, i.e., on either side of the CSB, as well as in progenitors of the medial ganglionic eminence from which the GABAergic guidepost neurons are derived (Niquille et al., 2009). Moreover, *Gli3* is expressed in the forebrain from its induction at E8.5 till the end of cortical neurogenesis at E18.5 (Hui et al., 1994). This widespread and prolonged expression raises the possibility that *Gli3* could be required in dorsal and/or ventral telencephalic progenitor cells to control CC formation.

To address the spatial dependence of CC formation on *Gli3* expression, we employed a conditional knock-out approach to determine the effects of specific inactivation of *Gli3* in the ventral and dorsal telencephalon on the positioning of midline guideposts and on callosal development. We show that deletion of *Gli3* in progenitors of the septum and of the medial ganglionic eminence has no obvious defects on callosal development. In contrast, loss of *Gli3* function in the cortex using an *Emx1Cre* driver line results in severe disorganization of guidepost cells and in the formation of a severely hypoplastic CC. Examination of early developmental stages further showed that early changes in Wnt/β-catenin and Fgf8 signalling lead to the premature formation of ectopic glial fibres and to ectopic *Slit1/2* expression in the septum and that these alterations in the development of midline guideposts interfere with midline crossing of callosal axons. Collectively, these findings suggest that *Gli3* acts in *Emx1*$^+$ progenitors to control development of midline guidance cues and CC formation.

Materials and methods

Mice

Emx1Cre (Gorski et al., 2002), *Zic4Cre* (Rubin et al., 2010), *Nkx2.1Cre* (Kessaris et al., 2006), *Gli3*$^{fl/fl}$ (Blaess et al., 2008) and *ROSA26CAG dual stop EGFP reporter* (*RCE*) (Sousa et al., 2009) mice were kept on a mixed background, and were interbred. *Emx1Cre;Gli3*$^{fl/+}$, *Zic4Cre;Gli3*$^{fl/+}$ and *Nkx2.1Cre;Gli3*$^{fl/+}$ mice were mated with *Gli3*$^{fl/fl}$ mice to obtain *Emx1Cre;Gli3*$^{fl/fl}$ and *ZicCre; Gli3*$^{fl/fl}$ and *Nkx2.1Cre;Gli3*$^{fl/fl}$ conditional mutant embryos. Likewise, *Nkx2.1Cre;Gli3*$^{fl/+}$ mice were mated with *Gli3*$^{fl/fl}$;*RCE* females to obtain *Nkx2.1Cre;Gli3*$^{fl/fl}$;*RCE* conditional mutant embryos. *Emx1Cre;Gli3*$^{fl/+}$, *Zic4Cre;Gli3*$^{fl/+}$ and *Nkx2.1Cre;Gli3*$^{fl/+}$ embryos were used as controls. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. For each marker and each stage, 3–5 embryos were analysed.

In situ hybridization and immunohistochemistry

Antisense RNA probes for *Axin2* (Lustig et al., 2002), *Emx1* (Simeone et al., 1992), *Fabp7* (Genepaint. RNA probe 653), *Fgf8* (Crossley and Martin, 1995), *Gli3* (NM_008130, Genbank, 132–5113 bp), *Robo1* (Erskine et al., 2000), *Six3* (Oliver et al., 1995), *Slit1/2* (Erskine et al., 2000), *Sprouty2* (Minowada et al., 1999), *Wnt7b* (Parr et al., 1993) and *Wnt8b* (Richardson et al., 1999) were labelled with digoxigenin. In situ hybridisation on 10 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 antigens: Calbindin (CB) (1:1000, Swant); Calretinin (CR) (1:1000, CHEMICON); Glia Fibrillary Acidic Protein (GFAP) (1:1000, Dako-Cytomation); GFP (1:500, Abcam) Nf1a (1:1000, Active Motif); neural cell adhesion molecule L1 (1:1000, CHEMICON); Satb2 (1:50, Abcam); Tbr1 (1:2500, CHEMICON). Primary antibodies for immunohistochemistry were detected with Alexa- or Cy2/3-conjugated fluorescent secondary antibodies. For counter staining TOPRO-3 (1:2000, Invitrogen) was used.

Carbocyanine dye injection and analysis

P7 pups were perfused transcardially with 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS). For callosal labelling, single crystals of the lipophilic tracer DiI were placed into the cortex of whole brains using pulled glass capillaries. Dyes were allowed to diffuse at 37 °C for 5–6 weeks in 4% (w/v) PFA in PBS. Brains were rinsed in PBS, embedded in agarose and sectioned coronally on a vibratome at 100 μm. Sections were cleared in 9:1 glycerol:PBS solution containing the nuclear counter-stain TOPRO3 (0.2 μM) overnight at 4 °C.

Western blotting

Protein was extracted from the dorsal telencephalon of E12.5 *Gli3*$^{fl/+}$ (control) and *Emx1Cre;Gli3*$^{fl/fl}$ embryos as described previously (Fotaki et al., 2006). Equivalent amounts of protein were subjected to gel electrophoresis on a 3–8% gradient Tris-acetate gel (Invitrogen), and protein was transferred to a nitrocellulose membrane, which was incubated with rabbit polyclonal anti-Gli3 antibody (1:500; Abcam). After incubating with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2000; Dako), signal was detected using an ECL Plus detection kit (Amersham GE healthcare).

Statistical analysis

Analysis was performed on data collected from brains of at least 3 embryos of each genotype. Mann–Whitney test was used to compare the proportion of Satb2$^+$/Dapi$^+$ cells. To compare the density of Satb2$^+$ cells a normality test (Shapiro–Wilk) was performed first for 2-way analysis of variance and if failed statistical comparisons were made by Holm–Sidak (for more than 2-group comparisons). Independent *t*-test analysis (2 sample) was performed for cortical thickness measurements. For all statistical analyses SPSS software was used. Asterisks indicate $P < 0.05$.

Results

The corpus callosum forms normally following inactivation of Gli3 in the septum or MGE

We recently showed that *Gli3* is required for the correct positioning of guidepost cells around the CSB and hence for proper formation of the corpus callosum (Magnani et al., in press). However, as *Gli3* is expressed widely in the developing forebrain including both cortical and septal progenitors as well as in medial ganglionic eminence progenitors which give rise to GABAergic interneurons populating the corpus callosum, it remains unclear in which group of progenitor cells *Gli3* is required during callosal development. To address this issue, we conditionally inactivated *Gli3* using three different Cre driver lines. To remove *Gli3* expression from the septum and from the medial ganglionic eminence we used *Zic4Cre* (Rubin et al., 2010) and *Nkx2.1Cre* (Kessaris et al., 2006) driver lines, respectively. After mating these mice with *Gli3*$^{fl/fl}$ animals (Blaess et al., 2008), *Gli3* was specifically inactivated in progenitor cells in the expected regions, as shown by *Gli3* in situ hybridization (Supplementary Fig. 1).

To examine overall CC development in these conditional mutants we performed immunofluorescence analysis for the cell adhesion molecule L1, which is strongly expressed in callosal axons, on coronal sections of E18.5 *Zic4Cre;Gli3*$^{fl/fl}$ and *Nkx2.1Cre;Gli3*$^{fl/fl}$ embryos as well as *Zic4Cre;Gli3*$^{fl/+}$ and *Nkx2.1Cre;Gli3*$^{fl/+}$ control embryos. This analysis revealed callosal axons crossing the midline in embryos of all four genotypes and showed no obvious defects in callosal development at both rostral and caudal levels (Fig. 1A–L and Supplementary Fig. 2). To examine formation of the midline guidance structures in conditional mutant embryos, we performed immunofluorescence analysis for glial fibrillary acidic protein (GFAP) to reveal the glial wedge (GW), the indusium griseum glia (IGG) and the midline zipper glia (MZG) (Fig. 1A) (Shu et al., 2003a). Immunofluorescence stainings for Calbindin (CB), Tbr1 and Calretinin (CR) were used to label callosal guidepost neurons that transiently populate the CC (Fig. 1B–D) (Niquille et al., 2009). Tbr1$^+$, Calretinin$^+$ and Calbindin$^+$ neurons are located in the indusium griseum and Tbr1$^+$ and Calretinin$^+$ neurons are also found within the CC where they delineate its ventral and dorsal parts (Fig. 1D, H and L) (Niquille et al., 2009). No apparent malformations of the callosal guidepost cells were seen in *Zic4Cre;Gli3*$^{fl/fl}$ (Fig. 1E–H) or *Nkx2.1Cre;Gli3*$^{fl/fl}$ conditional mutants (Fig. 1I–L). Moreover, lineage tracing revealed that medial ganglionic eminence derived guidepost neurons are present as normal in the corpus callosum of conditional mutants (Supplementary Fig. 3). Therefore, these experiments indicate that CC formation is not affected after specific *Gli3* inactivation in either the septum or the medial ganglionic eminence.

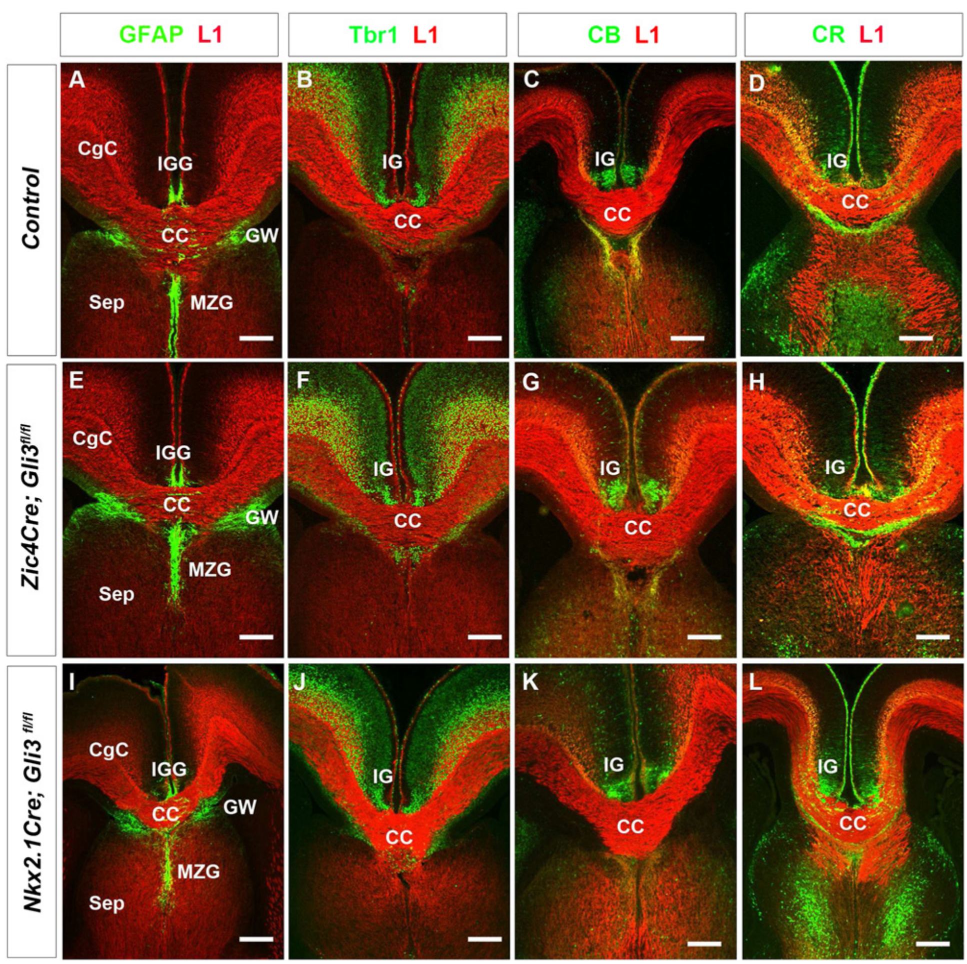

Fig. 1. Corpus callosum forms normally in *Zic4Cre;Gli3*$^{fl/fl}$ and *Nkx2.1Cre;Gli3*$^{fl/fl}$ conditional mutants. (A)–(L) Immunofluorescence analysis for L1 revealed that callosal axons of all three genotypes cross the midline (A)–(L). Midline glial structures labelled with GFAP (A), (E) and (I) and the callosal guidepost neurons labelled with Tbr1 (B), (F) and (J), Calbindin (C), (G) and (K) and Calretinin (D), (H) and (L) show no obvious malformations in mutant brains. Abbreviations: CC, corpus callosum; CgC, cingulate cortex; GW, glial wedge; HC, hippocampal commissure; IGG, indusium griseum glia; IG, indusium griseum; MZG, midline zipper glia; Sep, septum.

Deletion of Gli3 in the dorsal telencephalon causes callosal defects

To determine whether *Gli3* is required in telencephalic progenitors dorsally to the CSB we used an *Emx1Cre* strain which drives Cre expression in the developing cortex from E9.5 (Gorski et al., 2002). We checked the efficiency and tissue-specificity of Cre-mediated deletion of *Gli3* by examining *Gli3* mRNA expression between E10.5 and E12.5 in *Emx1Cre;Gli3*$^{fl/+}$ (control) and *Emx1-Cre;Gli3*$^{fl/fl}$ conditional mutant brains using in situ hybridization (Fig. 2). In control brains, *Gli3* is expressed strongly in the ventricular zone of both the dorsal telencephalon and the lateral ganglionic eminence and at lower levels in the medial ganglionic eminence at all stages analysed (Fig. 2A–C). In contrast, in *Emx1Cre;Gli3*$^{fl/fl}$ embryos *Gli3* expression in the medial cortex is

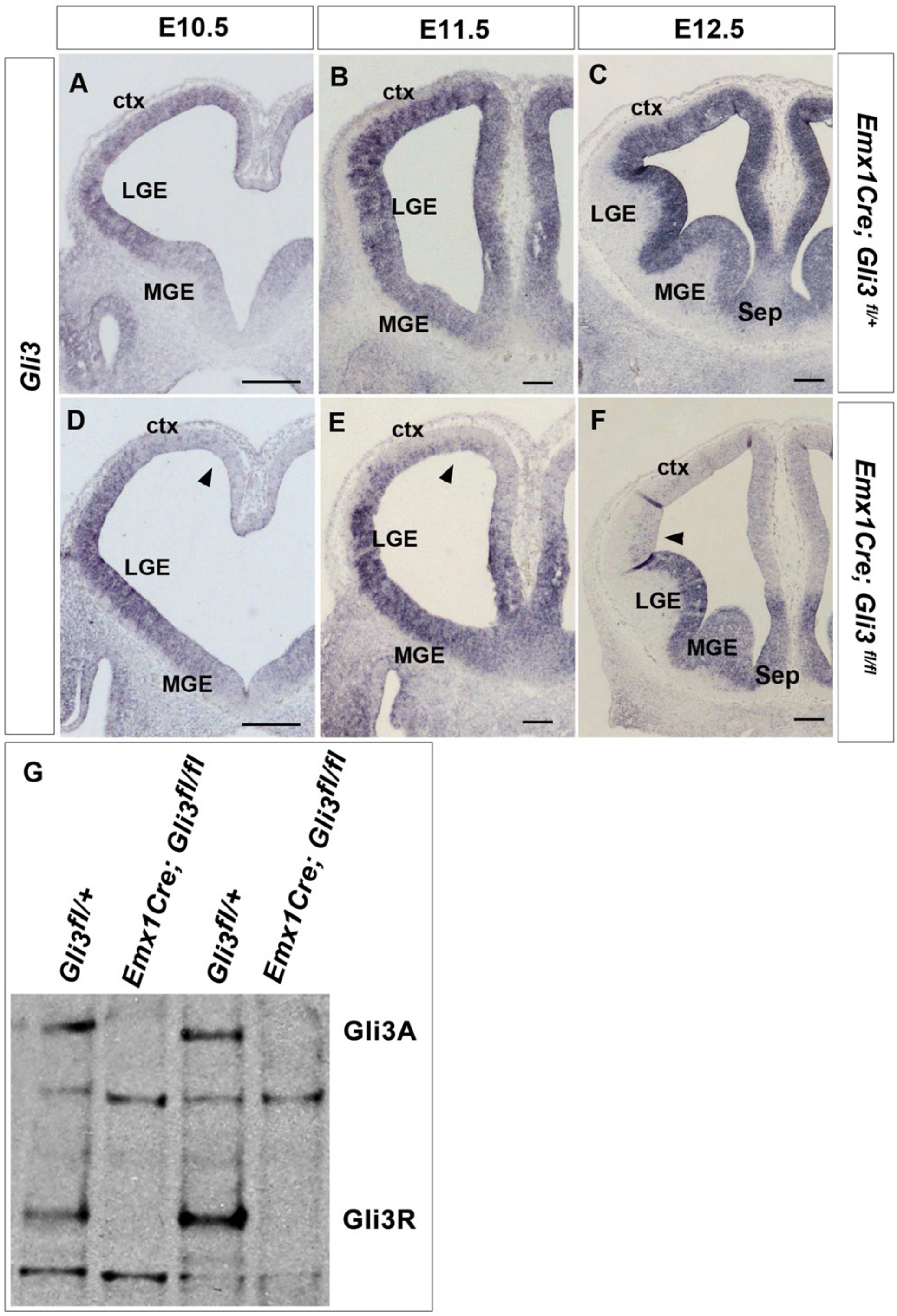

Fig. 2. *Gli3* is inactivated in the dorsal telencephalon of *Emx1Cre;Gli3*$^{fl/fl}$ conditional mutants. (A)–(C) In control brains, *Gli3* is expressed strongly in the ventricular zone of both the dorsal telencephalon and the LGE and at lower levels in the MGE at all stages analysed. (D)–(F) In E10.5 *Emx1Cre;Gli3*$^{fl/fl}$ mutant brains, *Gli3* mRNA expression is already lost in the medial cortex (arrowhead in D). This loss gradually expands to more lateral regions by E11.5 (arrowhead in (E)). At E12.5 (arrowhead in (F)) *Gli3* expression is lost from the dorsal telencephalon but remains unaffected in the septum, MGE and LGE. (G) In protein extracts from the E12.5 dorsal telencephalon of control embryos, Western blot analysis using a Gli3 *N*-terminal antibody showed two Gli3 forms of ca. 170 and 80 kDa, corresponding to the Gli3 activator (Gli3A) and repressor (Gli3R), respectively. In *Emx1Cre;Gli3*$^{fl/fl}$ conditional mutants, Gli3A and Gli3R are absent. Abbreviations: ctx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence.

abolished by E10.5. Loss of *Gli3* expression gradually expands to more lateral regions by E12.5 (Fig. 2E and F, arrowheads) while *Gli3* mRNA expression appears unaffected in the ventral telencephalon and the septum. Thus, *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants demonstrate a selective gradual deletion in the ventricular zone of the dorsal telencephalon starting as early as E10.5 (Fig. 2D–F).

Inactivation of the *Gli3* gene resulted in loss of Gli3 protein (Fig. 2G). In protein extracts from E12.5 dorsal telencephalon of control embryos, Western blot analysis using a Gli3 *N*-terminal antibody showed two Gli3 isoforms of about 170 and 88 kDa, corresponding to the Gli3 activator (Gli3A) and repressor (Gli3R) forms, respectively. Both forms are absent in extracts from *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants. Taken together these analyses confirmed that *Gli3* inactivation in the dorsal telencephalon is complete by E12.5.

To examine CC development in control and *Emx1Cre;Gli3$^{fl/fl}$* mutants, we first carried out immunofluorescence analysis for L1 on coronal sections of E18.5 brains. L1^{+} callosal axons cross the midline normally in control brains (Fig. 3A–C). In *Emx1Cre;Gli3$^{fl/fl}$* mutants, however, the path of callosal axons is severely disrupted and ectopic axonal bundles are formed at several positions. Nonetheless, some callosal axons in the conditional mutants approach the midline but form a highly abnormal structure (Fig. 3D–F) at both rostral and caudal levels (Supplementary Fig. 2). Tbr1^{+} and Calbindin^{+} neurons are positioned dorsally to the CC in the

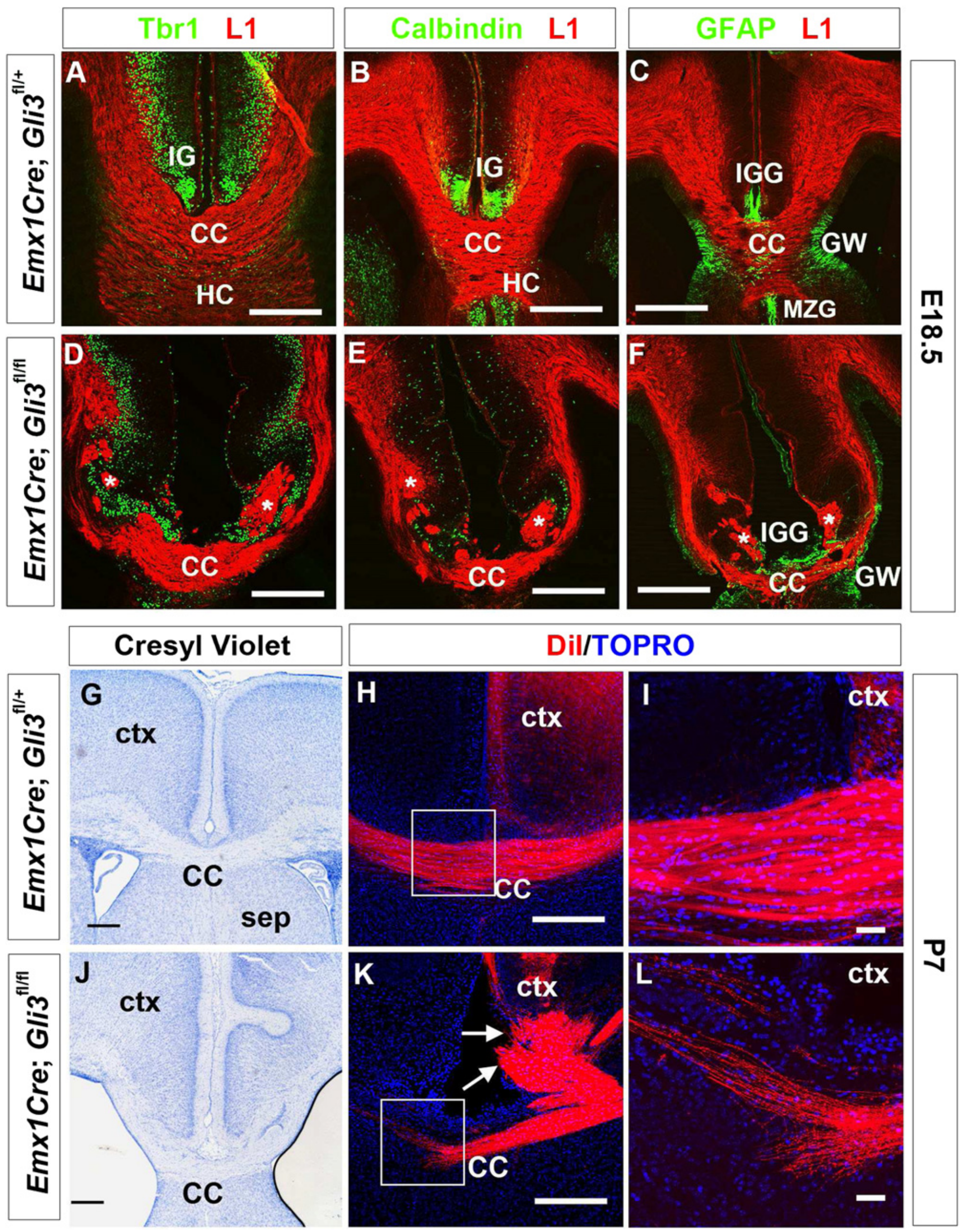

Fig. 3. Callosal defects and severe disorganization of midline structures in *Emx1Cre;Gli3$^{fl/fl}$* conditional brains (A)–(C) L1^{+} callosal axons cross the midline in E18.5 control brains. (D)–(F) In *Emx1Cre;Gli3$^{fl/fl}$* mutants, the path of callosal axons is disrupted at several positions and ectopic axon bundles (asterisks) form at several positions. (A)–(B) Tbr1^{+} and CB^{+} midline neurons are located dorsally to the CC in control brains. (D)–(E) In *Emx1Cre;Gli3$^{fl/fl}$* mutants, some Tbr1^{+} and CB^{+} neurons are scattered in the dorsomedial cortex but are closely associated with axon bundles. (C) GFAP immunofluorescence labels the glial wedge (GW), the indusium griseum glia (IGG) and the midline zipper glia (MZG) in control brains. (F) In *Emx1Cre;Gli3$^{fl/fl}$* mutants, the IGG is located at its correct position dorsal to the CC, but is expanded. The GW is present on each side of the CC (G) and (J) P7 control brains are stained with cresyl violet to visualize general midline morphology. (J) In P7 *Emx1Cre;Gli3$^{fl/fl}$* mutants, the CC is present but largely hypoplastic. Note the largely increased ventricles. (H)–(I) Cortical placements of DiI crystals identify the trajectory of callosal axons in control brains with large axon bundles crossing the midline. (K)–(L) In P7 *Emx1Cre;Gli3$^{fl/fl}$* mutants, most DiI labelled callosal axons form Probst bundles and only a few axons cross the midline.

indusium griseum of control brains (Fig. 3A and B). In *Emx1Cre;-Gli3$^{fl/fl}$* mutants, some Tbr1$^+$ and Calbindin$^+$ guidepost neurons are found scattered in the dorsomedial cortex, where they associate with the abnormal axon bundles and the hypoplastic CC (Fig. 3D and E). Finally, GFAP labels the indusium griseum glia which is located in its normal position dorsal to the CC but appears to be expanded and associated with the underlying axons in mutant embryos while the glial wedge is present on each side of the CC (Fig. 3F). Thus, the CC is severely abnormal in E18.5 *Emx1Cre;Gli3$^{fl/fl}$* mutant brains and midline guidance cues occupy highly aberrant positions.

The corpus callosum continues to develop postnatally. As *Gli3*$^{Pdn/Pdn}$ mutants die perinatally, these mutants could not be used to determine a role for *Gli3* in later aspects of callosal development. In contrast, *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants are viable therefore offering the opportunity to study postnatal CC development in a *Gli3* mutant background. We therefore analyzed CC formation in postnatal day 7 (P7) conditional brains. Cresyl violet staining revealed the overall midline morphology and the CC which is hypoplastic in the *Emx1Cre;Gli3$^{fl/fl}$* mutants (Fig. 3G and J) although it is enlarged compared to E18.5 mutant brains (compare Fig. 3D–F and Fig. 3J). To confirm whether callosal axons cross the midline, DiI crystals were placed in control and mutant rostromedial cortex (Fig. 3H, I, K and L). In P7 control brains, the CC contains thick axon bundles (Fig. 3H and I). In contrast, in *Emx1Cre;Gli3$^{fl/fl}$* mutants, although callosal axons do reach the midline, most of them form Probst bundles (Fig. 3K) and only a few cross the midline (Fig. 3L). Taken together, our results indicate that even though a number of axons are able to cross the midline in postnatal *Emx1Cre;Gli3$^{fl/fl}$* conditional brains most axons form Probst bundles.

Midline abnormalities are found in Emx1Cre;Gli3$^{fl/fl}$ conditional mutants at E12.5

Gli3 is expressed in progenitor cells which will give rise to both callosal neurons and to midline structures. Since *Emx1Cre* is active in both these groups of progenitor cells, the CC defects in *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants could result from misspecification of the callosal projection neurons or from defective formation of the midline guidance structures. To test the first possibility, we characterized cortical development. Rostrally, the thickness of the cerebral cortex is not altered in *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants but these embryos have a thinner cortex at caudal levels (Supplementary Fig. 4). Moreover, immunofluorescence analyses for the callosal neuron determinant Satb2 (Alcamo et al., 2008; Britanova et al., 2008) revealed that the proportion of Satb2$^+$ neurons to the total number of neurons is not affected in mutant embryos, however, their distribution in the cortical plate is slightly altered. At rostral levels, fewer Satb2$^+$ neurons were detected in the lower cortical plate of *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants while more Satb2$^+$ neurons had already reached their final position in the upper cortical plate. In contrast, significantly more Satb2$^+$ neurons were found in the lower cortical plate of mutant embryos caudally suggesting a delay in cortical layering at this level (Supplementary Fig. 4). Finally, expression of the *Robo1* receptor which has an important role in CC formation (Andrews et al., 2006) is not affected in *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants (Supplementary Fig. 5). These results, together with our previous finding that a number of axons cross the midline despite the abnormal positioning of the midline structures, suggest that callosal neuron specification is not affected and that the CC defects are primarily caused by midline defects.

Next, we set out to investigate the underlying causes for the abnormal positioning of the midline guidance cues. Previously, we showed that altered Fgf/Wnt/β—-catenin signalling in E12.5 *Gli3*$^{Pdn/Pdn}$ mutants caused malformation of the CSB and subsequently a mispositioning of the midline guidance structures (Magnani et al., in press). We therefore investigated the possibility that similar abnormalities might be present in E12.5 *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants. Anatomically, E12.5 *Emx1Cre;Gli3$^{fl/fl}$* mutant brains show an elongated and thinner midline accompanied by enlarged ventricles (Fig. 4) which persists throughout development. Next, we analyzed Wnt/β-catenin and Fgf signalling. In control embryos, high levels of *Wnt7b* and *Wnt8b* expression are confined to the region dorsal to the CSB and gradually decline in the neocortex (Fig. 4A and B). In *Emx1Cre;Gli3$^{fl/fl}$* mutants, *Wnt7b* expression is clearly reduced in the dorsomedial telencephalon with only a restricted region dorsal to the CSB still expressing *Wnt7b* (Fig. 4F, arrow) while *Wnt8b* shows no obvious expression changes (Fig. 4G). The *Wnt* target gene *Axin2*, expressed at the CSB, showed no obvious differences between control (Fig. 4C) and *Emx1Cre;Gli3$^{fl/fl}$* mutant brains (Fig. 4H). Moreover, in both control and conditional mutant brains, *Fgf8* expression and that of its target gene *sprouty2* are confined to the septal region with no obvious differences (Fig. 4D, E, I and J). Thus, with the exception of *Wnt7b*, no apparent expression changes of signalling molecules were found.

In E12.5 *Gli3*$^{Pdn/Pdn}$ mutants, RGCs form clusters in the dorsomedial telencephalon, presaging the formation of ectopic glial clusters at later stages (Magnani et al., in press). To determine whether this also happens in the *Emx1Cre* conditional mutants, we examined expression of the neurogenic RGC marker *Fabp7*. In control brains, RGCs in the dorsomedial cortex and the MGE express *Fabp7* (Fig. 4K). In *Emx1Cre;Gli3$^{fl/fl}$* mutant brains, *Fabp7* expression in the dorsomedial cortex is severely reduced (Fig. 4M, arrow).

Finally, we examined the expression of the chemorepellent axon guidance molecule *Slit2* which is already up-regulated in E12.5 *Gli3*$^{Pdn/Pdn}$ mutants causing a disorganization of midline guideposts (Magnani et al., in press). In control brains, *Slit2* is expressed in the septum (Fig. 4L) and no obvious differences were found in *Emx1Cre;Gli3$^{fl/fl}$* mutants (Fig. 4N). Taken together these analyses show a relatively normal development of the CSB except for reduced *Wnt7b* and *Fabp7* expression in medial cortical tissue.

Expression of signalling molecules is altered in Emx1Cre;Gli3$^{fl/fl}$ conditional mutants at E14.5

Given that only subtle changes were found in the dorsomedial cortex of conditional mutant brains at E12.5, we next examined CSB formation closer to the time at which callosal axons cross the midline. The regions of the commissural plate where the corpus callosum, the hippocampal and the anterior commissures normally cross the midline are delineated by the expression domains of several transcription factors, including *Six3*, *Emx1* and Nf1a, (Moldrich et al., 2010). *Six3* is an important regulator of early forebrain development and mice mutant for *Emx1* or *Nfia* lack the corpus callosum (Qiu et al., 1996; Shu et al., 2003a). We examined the expression of these transcription factors in the midline of *Emx1Cre;Gli3$^{fl/fl}$* mutant brains, to determine whether changes in their expression might contribute to the conditional mutant phenotype. However, no obvious changes in their expression patterns were found (Supplementary Fig. 6).

Next, we examined the expression of signalling molecules at the CSB. In E14.5 control embryos, *Wnt7b* and *Wnt8b* expression and that of the Wnt/β—catenin target gene *Axin2* are confined to the dorsomedial telencephalon (Fig. 5A–C). In *Emx1Cre;Gli3$^{fl/fl}$* mutant brains, *Wnt7b* and *Wnt8b* show patchy expression in a broader expression domain (Fig. 5D and E) while *Axin2* expression was detected in the dorsomedial cortex of *Emx1Cre;Gli3$^{fl/fl}$*

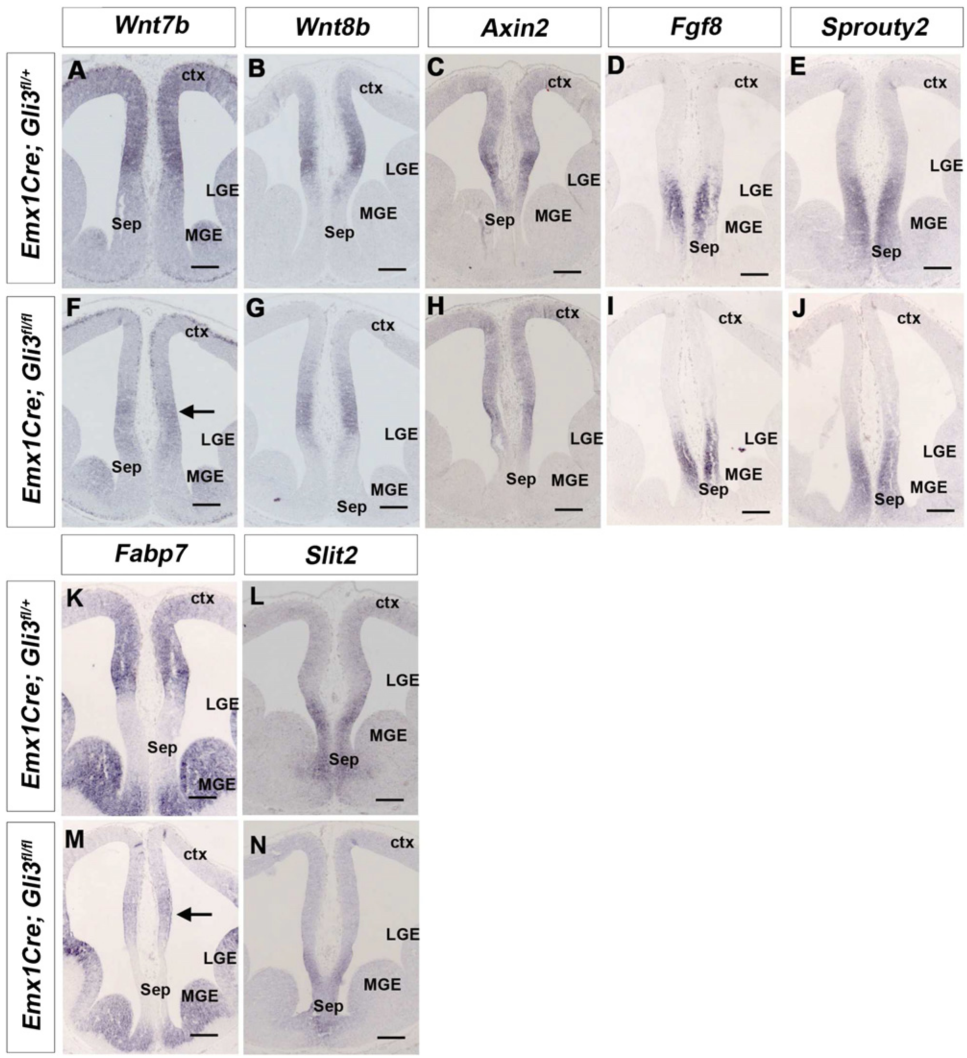

Fig. 4. *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants display subtle patterning defects in the E12.5 rostromedial telencephalon. (A), (B), (F) and (G) *Wnt7b* is expressed in cortical progenitors in a medial to lateral gradient and in preplate neurons. In *Emx1Cre;Gli3$^{fl/fl}$* mutants, *Wnt7b* expression is severely reduced in cortical progenitors but shows weak expression in septal progenitors (arrow in (F)) while its preplate expression is not affected. (B) and (G) *Wnt8b* expression is confined to dorsomedial cortical progenitors in control brains and in *Emx1Cre;Gli3$^{fl/fl}$* embryos (C) and (H). *Axin2* expression in the midline of control and mutant brains showed no obvious differences. (D), (E), (I) and (J) *Fgf8* and *sprouty2* expression in the septum shows no obvious defect in *Emx1Cre;Gli3$^{fl/fl}$* embryos. (K) and (M) Control embryos express *Fabp7* in the dorsomedial cortex and in the MGE. In *Emx1Cre;Gli3$^{fl/fl}$* mutants, *Fabp7* expression is reduced in the dorsomedial cortex (arrow in M). (L) and (N) *Slit2* expression in the septum shows no obvious difference in *Emx1Cre;Gli3$^{fl/fl}$* mutants.

mutants (Fig. 5F). Similarly, *Fgf8* and *sprouty2* expression are mainly confined to the commissural plate in the caudal septum and *sprouty2* transcripts were also identified in the ventral most part of the cortex (Fig. 5G–J). In contrast, at rostral levels of *Emx1Cre;Gli3$^{fl/fl}$* mutant brains *Fgf8* (Fig. 5M) and *sprouty2* (Fig. 5O) are strongly expressed and their expression even extends into the cortex where very little *Fgf8* and *sprouty2* expression was detected in control embryos. This up-regulation is even more prominent at more caudal levels (Fig. 5N and P). These analyses therefore indicate severe changes in Fgf and Wnt/β—catenin signalling in the dorsomedial telencephalon of E14.5 *Emx1Cre;Gli3$^{fl/fl}$* mutants.

Next, we analyzed the consequences of these signalling changes. In *Gli3*$^{Pdn/Pdn}$ mutants, up-regulation of Fgf signalling underlies a clustering of RGCs marked by *Fabp7* expression (Magnani et al., 2012). Interestingly, while overall expression of the neurogenic RGC marker *Fabp7* was dramatically reduced at the midline of E14.5 conditional mutant brains, clusters of cells expressing high levels of *Fabp*7 were found similar to those seen in *Gli3*$^{Pdn/Pdn}$ embryos (Fig. 5Q, arrows). Fgf signalling also regulates the expression of the chemorepulsive molecule Slit2 in the septum and the cingulate cortex (Magnani et al., in press). In mutant brains, we detected an expansion of *Slit2* expression in the dorsomedial telencephalon and an area of ectopic *Slit2* expression at the centre of the septum (Fig. 5L and R). Taken together, these results indicate major changes in the expression and activity of several signalling molecules, a clustering of radial glial cells and misexpression of the axon guidance molecule *Slit2*.

Emx1Cre;Gli3$^{fl/fl}$ mutants show midline defects at early stages of CC development

To analyze whether these changes during CSB formation affect the positioning of callosal guidepost cells, we performed immunofluorescence analyses in E16.5 control and mutant embryos. At this stage, L1^{+} callosal axons start to cross the midline at the CSB (Rash and Richards, 2001) (Fig. 6A–D and I–L). In contrast, in *Emx1Cre;Gli3$^{fl/fl}$* mutant brains axons reached the cingulate cortex but their path is disrupted and some axons form bundles close to the midline (Fig. 6E, G and O arrow) while others abnormally

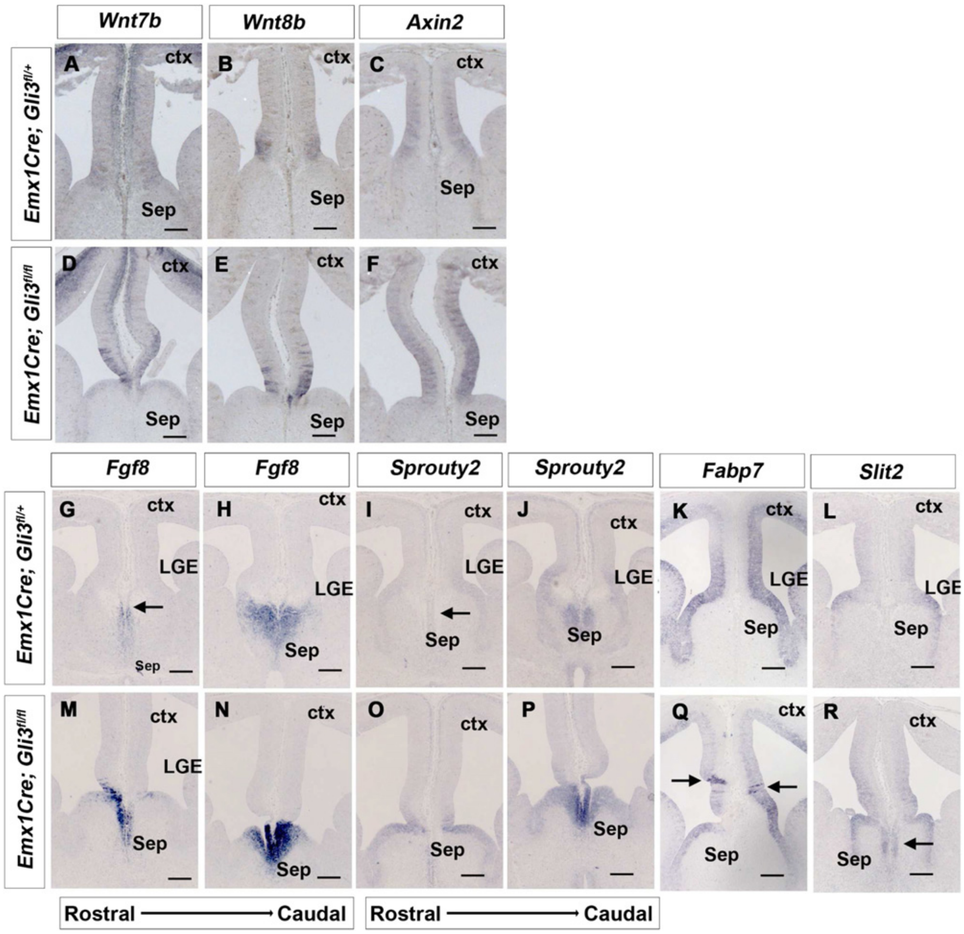

Fig. 5. Patterning defects in the rostromedial telencephalon of E14.5 *Emx1Cre;Gli3$^{fl/fl}$* conditional mutants. (A)–(C) *Wnt7b*/*Wnt8b* expression and Wnt signalling are confined to the dorsomedial telencephalon of control embryos. (D)–(F) In *Emx1Cre;Gli3$^{fl/fl}$* mutant brains, *Wnt7b* and *Wnt8b* show patchy expression while normal *Axin2* expression was detected in the elongated dorsomedial cortex (F). (G)–(J) In control brains, *Fgf8* expression and that of its target gene *sprouty2* are confined to the commissural plate (cp) and only show weak expression at rostral levels (arrow in G and I) (M)–(P) *Emx1Cre;Gli3$^{fl/fl}$* mutant brains show rostrally expanded and increased *Fgf8* and *sprouty2* expression. (K) *Fabp7* is expressed at high levels in the dorsomedial cortex and the septum of control brains. (Q) *Emx1Cre;Gli3$^{fl/fl}$* brains show clusters of *Fabp7* expressing cells whereas *Fabp7* expression is otherwise strongly reduced in rostromedial cortex. (L) *Slit2* is expressed in the septum and cingulate cortex of control brains. (R) In conditional mutants, *Slit2* expression is expanded in both tissues. Note the ectopic *Slit2* expression in the septal midline (arrow).

enter the septum without crossing the midline (Fig. 6E, G and M). In control brains, the Tbr1$^+$, Calbindin$^+$ and Calretinin$^+$ guidepost neurons are located within the cingulate cortex acquiring the correct position in the prospective indusium griseum and channelling the axons to the contralateral side (Fig. 6A–D, I and J). Also, L1$^+$ axons appear to contact both Calretinin$^+$ and Tbr1$^+$ neurons located within the path of callosal axons (Fig. 6B and J). In *Emx1Cre;Gli3$^{fl/fl}$* mutants, the callosal guidepost neurons are present but Tbr1$^+$ neurons do not intermingle with the L1$^+$ axons (Fig. 6E, F, M and N). Moreover, a few Calbindin$^+$ neurons form bundles on each side of the cingulate cortex (Fig. 6G and H). Finally, at this stage in control embryos fibres of nascent glial wedge cells which express GFAP start to translocate towards the pial surface to form the IGG (Fig. 6K and L). In *Emx1Cre;Gli3$^{fl/fl}$* mutants, ectopic GFAP$^+$ fibres were observed in the cingulate cortex and glial projections from the ventricular to the pial surface appeared to interrupt the path of callosal axons (Fig. 6O and P). Thus, the midline guidance structures are severely defective in E16.5 *Emx1Cre;Gli3$^{fl/fl}$* mutant brains.

Fgf signalling and Slit2 expression are altered in the Emx1Cre;Gli3$^{fl/fl}$ mutant cingulate cortex at E16.5

After the initial control of RGC cell differentiation by Fgfs (Faedo et al., 2010; Magnani et al., in press), Fgf signalling is re-employed to control the translocation of glia from the GW to the IGG in E16.5 embryos (Smith et al., 2006). Given the premature glial translocation at E16.5 and the upregulation of Fgf signalling in E14.5 conditional mutant brains, we examined the expression of *Fgf8* and that of its target gene *sprouty2* in E16.5 control and *Emx1Cre;Gli3$^{fl/fl}$* mutant brains. In control brains, *Fgf8* and *sprouty2* are expressed in the indusium griseum and the glial wedge (Fig. 7A and B) and *sprouty2* expression extends into the cingulate cortex (Fig. 7B). In contrast, cells expressing *Fgf8* form an abnormal ventrally elongated triangular pattern which extends into the septum of *Emx1Cre;Gli3$^{fl/fl}$* mutants (Fig. 7E). Interestingly, the *sprouty2* expression pattern in this region closely matches that of *Fgf8*, as *sprouty2* expressing cells formed a similar triangular pattern (Fig. 7F). Moreover, *sprouty2* expression appeared to be patchy in the GW region with reduced expression in the cingulate cortex (Fig. 7F). Thus, Fgf signalling is altered with *Fgf8*/*Sprouty2* expressing cells forming an abnormal structure in the septum. Since *Slit2* expression closely resembles that of *Fgf8* throughout rostromedial telencephalic development (Yuan et al., 1999) we hypothesized that the *Slit2* expression pattern could be altered in E16.5 conditional brains as well. In control brains, *Slit2* is expressed by glial cells in the GW and the IG (Fig. 7C) but in *Emx1Cre;Gli3$^{fl/fl}$* mutants, *Slit2* expressing cells form a triangular pattern in the septum similar to that of *Fgf8* and *sprouty2* (Fig. 7G). Moreover, *Slit1* expression is also

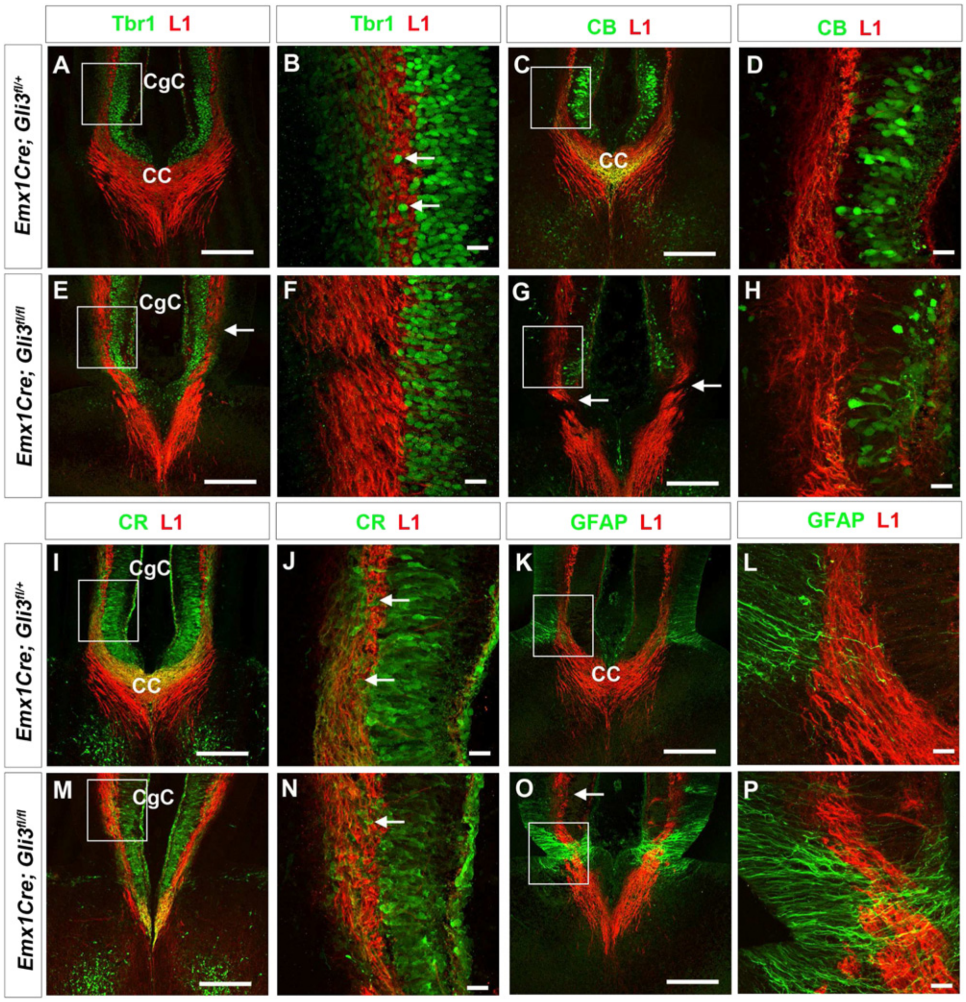

Fig. 6. E16.5 *Emx1Cre;Gli3*$^{fl/fl}$ mutant brains show midline defects. (A)–(D) and (I)–(L) In control brains, L1$^+$ axons start to cross the midline. (E)–(H) and (M)–(P) In contrast, in *Emx1Cre;Gli3*$^{fl/fl}$ mutant brains some L1$^+$ axons form bundles in the cingulate cortex close to the midline (arrows in (E), (G) and (O)) and do not cross the midline. (A)–(D), (I) and (J) Tbr1$^+$ (A), (B), CB$^+$ (C), (D) and CR$^+$ (I), (J) callosal guidepost neurons are positioned in the cingulate cortex and Tbr1$^+$ and CR$^+$ neurons intermingle with the L1$^+$ callosal axons (arrows in B,J). (E)–(H), (M), and (N) In *Emx1Cre;Gli3*$^{fl/fl}$ mutants, Tbr1$^+$, CB$^+$ and CR$^+$ callosal guidepost neurons are located in the cingulate cortex but Tbr1$^+$ neurons do not intermingle with the L1$^+$ axons (F) and fewer CB$^+$ neurons are located in the IG (H). (K) and (L) GFAP$^+$ radial glial cells at the GW start to translocate to the pial surface. (O) and (P) In *Emx1Cre;Gli3*$^{fl/fl}$ mutants, ectopic GFAP$^+$ fibres extend from the ventricular to the pial surface.

up-regulated in the septum of conditional mutants (compare Fig. 7D and H). This up-regulation of *Slit* gene expression could indicate a ventral expansion and/or displacement of the indusium griseum. We therefore compared *Slit2* expression on adjacent sections with that of GFAP and Calretinin which label glial cells and neurons of the indusium griseum, respectively, but this analysis revealed no overlap between *Slit2* and GFAP or Calretinin expression in the septal midline (Supplementary Fig. 7).

Discussion

Formation of the corpus callosum requires complex interactions between callosal axons and several glial and neuronal guidepost cells. Since malformation of these guidance structures underlies agenesis of the corpus callosum, it is important to understand the factors which control their development. The *Gli3* transcription factor plays a critical role in this process and here we have used a conditional knock-out approach to determine the spatial requirements for *Gli3* function in callosal development and to characterize molecular pathways controlled by *Gli3*. We show that *Gli3* is specifically required in Emx1$^+$ progenitor cells but not in the septum or in the medial ganglionic eminence for callosal development. Moreover, *Emx1Cre*;*Gli3*$^{fl/fl}$ embryos show a premature translocation of glial wedge cells and ectopic *Slit2* expression which coincide with altered Fgf and Wnt/β-catenin signalling during CSB formation.

Spatial and temporal requirements for Gli3 in callosal development

The CSB separates the cortex and septum and plays a critical role during callosal development. Several midline guidepost cells occupy strategic positions at this boundary allowing them to guide callosal axons across the midline. Malformation of the CSB is therefore predicted to affect the spatial organization of the guideposts and hence callosal development. Indeed, we recently showed that a reduction in *Gli3* function as in the *Gli3* hypomorphic mutant *Gli3*^{Pdn} causes severe defects in CSB formation which subsequently interfere with positioning of the guideposts and midline crossing of callosal axons (Magnani et al., in press). The importance of *Gli3* for CSB and CC formation is further emphasized by recent findings on *Rfx3* mutant mice in which defective Gli3 processing leads to a similar though less severe disorganization of neuronal guideposts and to agenesis of the corpus callosum (Benadiba et al., 2012). Since CC malformation is characteristic of Acrocallosal Syndrome which can be caused by

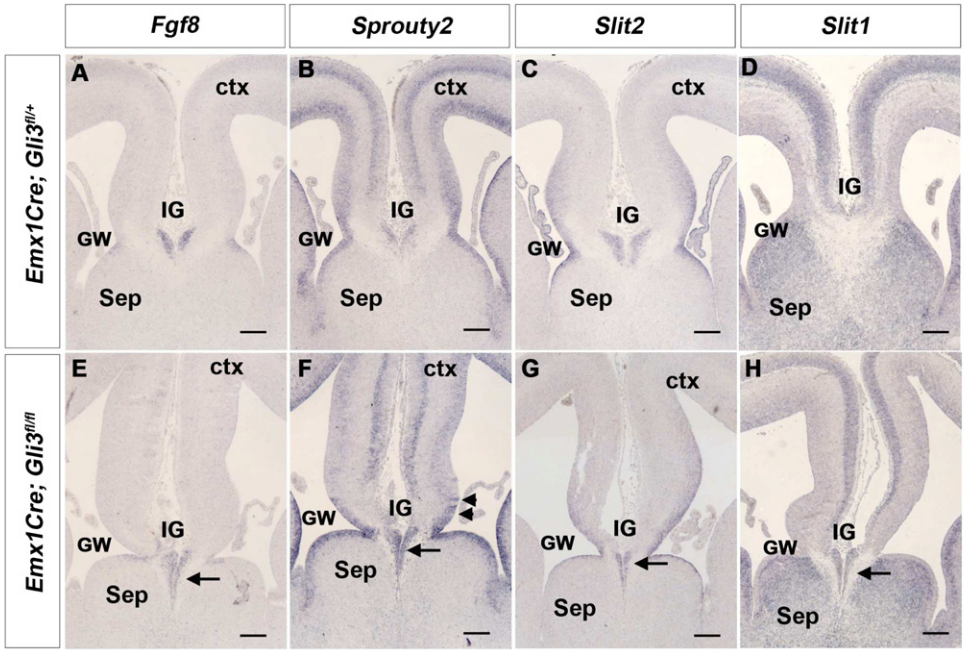

Fig. 7. Defective Fgf signalling and *Slit2* expression in the dorsomedial cortex of E16.5 *Emx1Cre;Gli3*$^{fl/fl}$ mutants. (A) In control brains, *Fgf8* expression is confined to the GW and the IG. (E) In *Emx1Cre;Gli3*$^{fl/fl}$ mutants, *Fgf8* expression is present in the GW but *Fgf8* expressing cells in the midline form a triangular pattern (arrow). (B) In control brains, *Sprouty2* is highly expressed in the GW, the IG and in the cingulate cortex. (F) *Sprouty2* expressing cells form a triangular pattern extending into the septum (arrow). Note the patchy *sprouty2* expression in the GW (arrowheads). (C) and (G) In control embryos, *Slit2* expression is confined to the IG and the GW (C) while it is protruding into the septum of *Emx1Cre;Gli3*$^{fl/fl}$ mutants (G). *Slit2* expression in the ventricular zone of the cingulate cortex appears unaffected. (D) and (H) *Slit1* expression is detected in cortical and septal neurons, in the indusium griseum and in the glial wedge of control embryos (D). In conditional mutants, an additional *Slit1* expression domain extends into the septum similar to that of *Slit2*.

mutations in *GLI3* and is also a hallmark of ciliopathies in which the structure and/or function of the primary cilium is affected, a better knowledge of the molecular mechanisms by which *Gli3* controls CSB formation is required. However, understanding this role is complicated by the widespread expression of *Gli3* in dorsal and ventral telencephalic progenitors on either side of the CSB and in progenitors of the medial ganglionic eminence which give rise to the GABAergic guidepost neurons in the callosal sling. By using *Gli3* conditional knock-out mice we demonstrated that *Gli3* in the septum and in the medial ganglionic eminence is dispensable for callosal development. In contrast, deletion of *Gli3* using an *Emx1Cre* driver line affects the development of the midline guideposts causing a severe malformation of the corpus callosum. Moreover, *NestinCre*;*Gli3*$^{fl/fl}$ embryos (Wang et al., 2011) also have a hypoplastic corpus callosum though this is due to apoptosis of callosal projection neurons at postnatal stages. Since *Gli3* is inactivated in *NestinCre*;*Gli3*$^{fl/fl}$ embryos after patterning is completed (Wang et al., 2011), our findings indicate a requirement for *Gli3* specifically in Emx1$^+$ progenitor cells at patterning stages to control CSB formation and callosal development and offer the possibility for investigating the molecular mechanisms by which *Gli3* controls this process.

Defective midline organization underlies the formation of the hypoplastic corpus callosum in Emx1Cre;Gli3$^{fl/fl}$ *animals*

Given the expression of *Gli3* in dorsal telencephalic progenitor cells, defective callosal development in *Emx1Cre*;*Gli3*$^{fl/fl}$ animals could be due to defects in callosal axons and/or in the midline environment guiding callosal axons across the midline. Although we cannot exclude that cell-autonomous defects may contribute to the callosal phenotype, we consider it to be highly unlikely. Callosal neurons are specified correctly and are formed in correct proportions. Although some Satb2$^+$ neurons are still in the deeper half of the caudal cortical plate at E18.5, they have acquired their correct laminar position by P7 (data not shown). In contrast to *NestinCre*;*Gli3*$^{fl/fl}$ mutants (Wang et al., 2011), their survival is also not affected in *Emx1Cre*;*Gli3*$^{fl/fl}$ animals. Moreover, some callosal axons are capable of midline crossing and form a hypoplastic corpus callosum despite a severe disorganization of the glial and neuronal guideposts which appears to be the primary cause of ACC in these mutants. Indeed, glial wedge cells start to translocate to the pial surface prematurely and their fibres from a barrier impermeable to callosal axons at this stage. In contrast to *Gli3*$^{Pdn/Pdn}$ embryos, however, Fgf signalling to the cortex which controls this translocation (Smith et al., 2006) is not disrupted in *Emx1Cre*;*Gli3*$^{fl/fl}$ embryos and glial translocation is completed by E18.5 opening up a corridor for callosal axons. Second, *Slit1* and *2* expressing cells form an ectopic triangular pattern in the midline region where callosal axons would normally cross. While we do not know the origin and nature of these *Slit* expressing cells in the septum, they are unlikely to be displaced indusium griseum cells (Unni et al., 2012) since we already detected this ectopic *Slit2* expression at E14.5 when the cells of the indusium griseum are just born (Shu et al., 2003b) and before their migration or translocation to their final position is completed (Smith et al., 2006). Moreover, in E16.5 *Emx1Cre*;*Gli3*$^{fl/fl}$ embryos, the *Slit2* expression in the septal midline does not overlap with that of GFAP and Calretinin which label the glial and neuronal components of the indusium griseum, respectively. Regardless of the exact nature of these cells, however, it is likely that this *Slit1/2* expression in the septum leads to the deflection of callosal axons away from the midline given the chemorepellent activity of Slit1/2 on callosal axons (Bagri et al., 2002).

Our further analyses strongly suggest that these midline abnormalities are caused by early defects during CSB formation. One of the earliest changes is the strong down-regulation of *Wnt7b* in E12.5 embryos followed by patchy expression two days later. At that time, *Fgf8* expression and Fgf signalling become drastically up-regulated similar to our previous findings in *Gli3*$^{Pdn/Pdn}$ and in *Rfx3* mutant embryos where alterations in these two signalling pathways are a major cause of the agenesis of the corpus callosum (Benadiba et al., 2012; Magnani et al., in press). Moreover, a down-regulation of *Fabp7* expression, a marker of neurogenic RGCs, indicates a potential delay in neuronal differentiation which is supported by the elongation of the rostral midline in *Emx1Cre*;*Gli3*$^{fl/fl}$ animals. Similar to *Wnt7b* expression, this initial down-regulation is followed by patchy *Fabp7* expression at E14.5 which presages the premature formation of GFAP^{+} fibres in the conditional mutants as well as in *Gli3*$^{Pdn/Pdn}$ mutants (Magnani et al., in press). Interestingly, Fgf signalling controls RGC differentiation (Kang et al., 2009; Sahara and O'Leary, 2009) and is required for the formation of RGC clusters in *Gli3*$^{Pdn/Pdn}$ embryos (Magnani et al., in press). Finally, Fgf signalling also controls *Slit2* expression in the septum (Magnani et al., in press; Tole et al., 2006). Therefore, these findings emphasize the importance of a *Gli3* controlled balance between Fgf and Wnt/β-catenin signalling for controlling CSB formation and hence for the positioning of midline guideposts and for midline crossing of callosal axons. Future studies will aim to identify the mechanism(s) by which *Gli3* controls this balance at the molecular level.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.02.001.

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