The Ciliogenic Transcription Factor RFX3 Regulates Early Midline Distribution of Guidepost Neurons Required for Corpus Callosum Development

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Abstract

The corpus callosum (CC) is the major commissure that bridges the cerebral hemispheres. Agenesis of the CC is associated with human ciliopathies, but the origin of this default is unclear. Regulatory Factor X3 (RFX3) is a transcription factor involved in the control of ciliogenesis, and Rfx3–deficient mice show several hallmarks of ciliopathies including left–right asymmetry defects and hydrocephalus. Here we show that Rfx3–deficient mice suffer from CC agenesis associated with a marked disorganisation of guidepost neurons required for axon pathfinding across the midline. Using transplantation assays, we demonstrate that abnormalities of the mutant midline region are primarily responsible for the CC malformation. Conditional genetic inactivation shows that RFX3 is not required in guidepost cells for proper CC formation, but is required before E12.5 for proper patterning of the cortical septal boundary and hence accurate distribution of guidepost neurons at later stages. We observe focused but consistent ectopic expression of Fibroblast growth factor 8 (Fgf8) at the rostro commissural plate associated with a reduced ratio of GLIoma-associated oncogene family zinc finger 3 (GLI3) repressor to activator forms. We demonstrate on brain explant cultures that ectopic FGF8 reproduces the guidepost neuronal defects observed in Rfx3 mutants. This study unravels a crucial role of RFX3 during early brain development by indirectly regulating GLI3 activity, which leads to FGF8 upregulation and ultimately to disturbed distribution of guidepost neurons required for CC morphogenesis. Hence, the RFX3 mutant mouse model brings novel understandings of the mechanisms that underlie CC agenesis in ciliopathies.

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Introduction

The Corpus Callosum (CC), the major commissure of the brain, is composed of millions of axons that connect the two brain hemispheres [1,2]. Malformation of the CC is one of the most frequent brain anomalies found at birth, and may occur in as much as 7/1000 of the total newborn population. The most severe form of CC malformation is its complete absence also called callosal agenesis.

In mouse, callosal axons first start to cross the midline during late gestation at E16.5 [3,4]. Callosal axons are directed through the Cortical Septal Boundary (CSB) by several guidepost cell populations expressing guidance cues. Glial cell populations were first described to be involved in CC formation in this region [1,5]. Guidepost glial cells are found at the glial wedge (GW) of the lateral ventricles (initially described as the cortical septal plate [5]), in the induseum griseum (IG) of the medial pallium and in the so-called sling at the CSB [5–10]. More recently, GABAergic (γ-aminobutyric acidergic) neurons and glutamatergic neurons that populate transiently the CSB have also been shown to be involved in guiding callosal axons at the midline [11]. These glial and neuronal guidepost populations are also observed in the human foetal CC [2,12].

In humans, malformations of the CC have been found to be associated with a variety of syndromes [13,14]. In particular, a reduction or a complete absence of the CC has been found to be associated with several human syndromes recently recognized as ciliopathies [15,16]. However, it is not known where and at what stage of embryonic development cilia are required for proper CC formation. Several mouse models defective in cilia formation or
function have been described in the literature, but only few have been shown to be associated with CC malformations and none of them has so far been used to explore the molecular mechanisms that underlie CC development. One reason is that most mouse mutants for ciliary genes die early during embryogenesis and that the surviving mutants present severe brain malformations that preclude the study of late defects such as CC formation.

RFX transcription factors have been shown to play fundamental roles in the control of ciliogenesis by regulating many genes involved in cilia assembly or function [17]. Rfx3 deficient mouse mutants exhibit several hallmarks of ciliopathies and in particular left-right asymmetry defects and hydrocephalus [18,19]. We show here that Rfx3 deficient mice also harbour marked defects in CC development leading in most cases to agenesis of the CC. RFX3 is first expressed throughout the developing CC and cortex [18,19]. Expression of RFX3 in glutamatergic neurons of the developing CC and cortex contributes to the IG, the GW and the cerebral cortex (Figure S2E, S2G and S2I). From E16.5 to E18.5, callosal axons have crossed the midline and formed dense axonal bundles called Probst Bundles (PB) (arrowheads, Figure 1 and Figure S1). In some animals there was a relatively mild phenotype in which the two cerebral hemispheres fused correctly, and a few callosal axons still crossed the midline but, instead of crossing it, accumulated on both sides of the midline and formed dense axonal bundles called Probst Bundles (PB) (arrowheads, Figure 1 and Figure S1). In contrast, Rfx3−/− mice did not exhibit any obvious callosal defects (n = 3/11). In Rfx3−/− brains, many callosal axons reached the midline but, instead of crossing it, accumulated on both sides of the midline and formed dense axonal bundles called Probst Bundles (PB) (arrowheads, Figure 1 and Figure S1). In some animals there was a relatively mild phenotype in which the two cerebral hemispheres fused correctly, and a few callosal axons still crossed the midline albeit with abnormal trajectories (Figure 1B, 1F and Figure S1B). The most severe phenotype that we observed was a complete agenesis of the CC with no callosal axons crossing the midline. In these Rfx3−/− mice, the two cerebral hemispheres did not fuse correctly and displayed a large bulge along the inter-hemispheric fissure where callosal axons approach the midline (Figure 1D and Figure S1C, symbol O). Additionally, we observed in these embryos strong defects in the formation of the hippocampal commissure but not of the anterior commissure (Figure 1, Figure S1 and not shown). Thus Rfx3 contributes to the formation of the CC and the hippocampal commissure.

Expression of RFX3 in glutamatergic neurons of the developing CC and cortex

To understand how RFX3 is involved in CC formation, we analysed Rfx3 mRNA expression in coronal sections of the developing mouse telencephalon prior to and during CC formation. From E8 to E10.5, Rfx3 was uniformly expressed in the entire neuroepithelium (not shown). From E11.5 to E16.5, Rfx3 expression became progressively restricted to specific rostro-caudal levels in the telencephalon (Figure 2 and Figure S2). Rfx3 hybridization signal was strong at the CSB (*) where the CC will form, and in the cingulate cortex (CCi) that contains pioneer callosal projection neurons [3,4] (Figure 2B–2D). In addition, Rfx3 was expressed in the primordium of the IG and the ventricular zone of the GW at the border of the lateral ventricles (Figure 2B1, 2C1, 2D1–D2). Both regions surround the CSB and are known to be important for CC formation [1,7,8]. High Rfx3 expression was also observed at more rostral levels in the retrobulbar region and at caudal levels in the cortical hem (CH), the choroid plexus, the ventral pallium (VP) laterally, as well as, in the preoptic area (POA) (Figure 2B3, 2C3 and 2D2–D3, open arrows). From E16.5 to birth, Rfx3 expression in the rostral telencephalon was restricted to the IG, the GW and the cerebral cortex (Figure 2E2, 2G and Figure S3C, S3E to S3H).

To clarify the nature of the embryonic midline cells expressing RFX3, we performed co-labelling experiments with markers for different cell types. Glutamatergic guidepost neurons colonizing the forming CC express the calcium binding protein calretinin as well as several transcription factors known to promote the glutamatergic fate such as empty spiracles homolog 1 (EMX1)
Figure 1. Abnormal callosal axon pathfinding in Rfx3−/− mice. (A–F) Immunohistochemistry for calretinin and Npn-1 (A1–A3 and B1–B3), for calbindin and Npn-1 (C1–C3 and D1–D3), and for GFAP and L1 (E1–E3 and F1–F3) in coronal CC sections from E18.5 WT (A1–A3, C1–C3 and E1–E3) or Rfx3−/− (B1–B3, D1–D3 and F1–F3) mice. A2, B2, C2, D2, E2 and F2 are higher magnifications of the lateral CC seen in A1, B1, C1, D1, E1 and F1, respectively. A3, B3, C3, D3, E3 and F3 are higher magnifications of the medial CC seen in A1, B1, C1, D1, E1 and F1, respectively. (A1–A3, C1–C3 and E1–E3) At E18.5, the hemispheres of WT brains have fused. Callosal fibres (in red) cross the midline and project into the contralateral cortex. (B1–B3, D1–D3 and F1–F3) Aberrant callosal axon bundles are observed in Rfx3−/− embryos (arrowheads). (B1–B3 and F1–F3) While the hemispheres have fused, most of callosal fibres do not cross the midline and form large ectopic bundles on the CC border, reminiscent of Probst bundles (PB). (D1–D3) Some Rfx3−/− embryos exhibit a more severe phenotype with an absence of midline hemispheric fusion and absolutely no callosal axons crossing the midline. In this case, a large bulge is observed along the inter-hemispheric fissure at the location where the callosal axons approach the midline (O). In all mutants, axonal defects are accompanied by cellular mis-positioning through the CC and the IG (open arrowheads). While calretinin and calbindin are expressed by glutamatergic neurons, as well as, GFAP+ glia, are still present in the CC and the IG of Rfx3−/− mice, there is a midline disorganization and a lateral shift of these cell populations. Bar = 435 µm in A1, B1, C1, D1, E1, F1; 220 µm in A3, B3, C3, D3, E2, F2 and 110 µm in A2, B2, C2, E3, F3.

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Moreover, Rfx3-positive cells populating the ventricular zone in the GW region from E14.5 to E18.5 are radial glial cells, labelled for Nestin, GFAP and GLAST (Figure S3D–S3F). However, no such overlap can be observed in the IG region, confirming that Rfx3-positive cells are not glial cells in the IG (Figure S3F1).

In addition to Rfx3 expression in the midline, we also observed at E12.5, that Rfx3 mRNA was detected in pioneer calretinin+ glutamatergic cortical neurons of the preplate (Figure 2C). From E15.5 to E16.5, Rfx3 mRNA was found in calretinin+ glutamatergic neurons in all layers of the developing cortex (Figure 2D and Figure S2B–S2E, S3E) and at E18.5 it was found in the projection neurons of the upper cortical layers labelled for COUP-TF (Figure 2H). By contrast, we never detected any Rfx3 hybridization signal in the reelin+ Cajal Retzius cells or calbindin+ neurons of the cortical MZ (Figure S2A, S2F).

The presence of Rfx3 transcripts at the midline in the corticoseptal region and in the cerebral cortex is consistent with the importance of this gene in CC formation. Given the large distribution of RFX3 in the embryonic brain, it might contribute to the proper development of the cortex or of midline structures. We, thus, examined if these different regions are affected by Rfx3 inactivation.
RFX3 is not required in callosal axons for proper CC formation

We first analysed the cerebral cortex of Rfx3 mutants. In Rfx3−/− cortex, the laminar distribution of SATB2+ and CUX1+ (Cut-like homeobox 1) callosally projecting neurons [20–26] was normal (Figure S4A–S4D). In addition, CTIP2+ cortical layer V and TBR1+ cortical layers V–VI which contain about 20% of the callosally projecting neurons, were similar in mutant and WT brains (Figure S4I–S4J).

To study if RFX3 expression in cortical neurons is necessary for axonal growth in the CC, we investigated whether the targeted inactivation of Rfx3 in pyramidal cortical neurons results in pathfinding defects. Using a Ngn2-CreER driver line, we induced recombination of a Rfx3 floxed allele in neurogenin 2 (NGN2)-derived glutamatergic projection neurons of the cortex by tamoxifen application at E13.5 [27,28]. While Rfx3 was not any more expressed in the cerebral cortex of Rfx3f/f; Ngn2-CreERTm+/- mice, (compare Figure S4I–S4J), the SATB2+ callosally projecting neurons and the CC still formed normally (n = 7/7; Figure S4K–S4L). This result shows that the loss of Rfx3 in cortical pyramidal neurons is not responsible for callosal axon guidance defects.
RFX3 is required for proper distribution of guidepost neurons in the CSB

To determine if RFX3 was required for the development of the CSB we followed the organization of guidepost cells in mutant CSB compared to WT. We first followed the distribution of CC guidepost glutamatergic neurons in Rfx3<sup>−/−</sup> mice at E18.5, after callosal axons have crossed the midline. Glutamatergic neurons of the CC, labelled for TBR1 and calretinin were shifted laterally, leaving a large portion of the CC devoid of neurons (not shown and Figure 1A and 1B, open arrowheads). Calretinin<sup>+</sup> and calbindin<sup>+</sup> glutamatergic neurons were both severely disorganized through the IG (Figure 1A–1D, open arrowheads). In addition, a progressive disorganization of CC glial cells was noticed in Rfx3<sup>−/−</sup> CC regions. The GFAP-positive astrocyte-like cells of the IG and of the midline were disorganized and the curvature of the radial glial processes was increased (Figure 1E and 1F, open arrowheads). Because this disorganization could be a secondary effect of callosal misrouting, we also looked at the distribution of guidepost cells before callosal axons cross the midline.

As early as E14.5, glutamatergic guidepost neurons labelled for reelin, calretinin and TBR1 failed to form a well organized band of neurons at the CSB of Rfx3<sup>−/−</sup> mice and instead accumulated ectopically on both sides of the midline (Figure 3A–3D; open arrowheads). In addition, Reelin<sup>+</sup> Cajal Retzius and calretinin<sup>+</sup> neurons lost dramatically their tangential distribution in the MZ layer and are more broadly distributed in the cortico-septal region (Figure 3A–3D and Figure 4A–4D). In addition, they lost their fusiform/bipolar shape. For both neuronal populations, given the density of the cells, the number of neurons in the corticoseptal region and the MZ was difficult to quantify. Similarly, from E14.5 to E16.5 glutamatergic neurons labelled for calbindin were mislocalised in the cortical MZ and IG of Rfx3<sup>−/−</sup> brains (Figure 3E–3F and Figure 4E and 4F). Moreover, some calbindin<sup>+</sup> neurons were found to accumulate within the CC white matter (Figure 4E and 4F). The midline neuronal defects were accompanied, at E16.5, by pathfinding errors of pioneer callosal axons that failed to cross the midline and formed ectopic bundles (Figure 4A–4F, white arrowheads).

By contrast, GAD67-expressing GABAergic neurons were properly positioned through the lateral part of the CC at E16.5 in Rfx3<sup>−/−</sup> mutant (Figure 5A and S2B). Finally, immunohistochemistry with several markers for astrocytes (nestin, GLAST and GFAP), indicated that the position and organization of the guidepost glial cell populations of the GW and of the midline glial zipper was indistinguishable in WT and Rfx3<sup>−/−</sup> mice, suggesting that their development is not sensitive to the loss of Rfx3 (Figure 4G–4H and Figure S5).

Altogether, these experiments indicate that RFX3 is necessary for the proper positioning of multiple corticoseptal neuronal populations but not of glial cell populations at the midline early in development.

Abnormal development of the CC midline in Rfx3<sup>−/−</sup> mutant mice is responsible for callosal axon pathfinding defects

To verify if CC guidance defects were caused by altered development in the midline region, we performed transplantation experiments as previously described [11]. Midline structures comprising the CC were transplanted into telencephalic slices at E16.5, using different combinations of wild type and Rfx3<sup>−/−</sup> embryos (Figure 5). When midline explants from Rfx3<sup>+/+</sup> mice were transplanted into Rfx3<sup>−/−</sup> slices, DiI-labelled callosal axons crossed the midline (Figure 5A; n = 7 slices with crossing axons out of 10), thereby reproducing the in vivo behavior of callosal axons. By contrast, with Rfx3<sup>−/−</sup> midline explants transplanted in Rfx3<sup>−/−</sup> slices, DiI-labelled callosal axons failed to cross the midline (Figure 5B; n = 0 slices with crossing axons out of 3). Similarly, transplantation of midline from Rfx3<sup>−/−</sup> mice into
Figure 4. Abnormal neuron localization and aberrant callosal axon pathfinding at the onset of CC formation. (A–H) Immunohistochemistry for calretinin and reelin (A1–A2 and B1–B2), for calretinin and neuropilin-1 receptor (Npn-1) (C1–C2 and D1–D2), for calbindin and L1 receptor (E1–E2 and F1–F2) and for GFAP and L1 receptor (G1–G2 and H1–H2), in coronal CC sections from WT (A1–A2, C1–C2, E1–E2 and G1–G2) and Rfx3<sup>+/−</sup> (B1–B2, D1–D2, F1–F2 and H1–H2) mice. A2, B2, C2, D2, E2, F2, G2 and H2 are higher magnifications of the midline seen in A1, B1, C1, D1, E1, F1, G1 and H1. (A1–A2 to D1–D2) From E15.5 to E16.5, calretinin<sup>+</sup> guidepost neurons fail to form a well organized band of neurons at the CSB (*) and are dispersed in the septum of Rfx3<sup>−/−</sup> mice (B2 and D2, white open arrowheads). Reelin<sup>+</sup> and calretinin<sup>+</sup> neurons lose their tangential organization through the cortical marginal zone (MZ) (compare B2 to A2, red open arrowheads). (E1–E2 and F1–F2) At E16.5, calbindin<sup>+</sup> neurons (green) do not organize appropriately within the indusium griseum (IG) and accumulate at the CC midline in Rfx3<sup>−/−</sup> mice (compare F2 to E2, open arrowheads). (G1–G2 and H1–H2) At E16.5, the organization of GFAP<sup>+</sup> glial cell populations within the CC is indistinguishable between WT and Rfx3<sup>−/−</sup>.
Rfx3 inactivation in midline guidepost cells is not responsible for CC developmental defects

To study if RFX3 expression in guidepost glia or neurons is required for callosal axon growth, we investigated whether the targeted inactivation of Rfx3 in both cell type progenitors might result in cell differentiation defects that could have an impact on axonal guidance. We thus inactivated Rfx3 in GFAP-positive radial glia precursors by mating Rfx3<sup>m</sup> and hGfap-Cre<sup>Cre<sup>+</sup></sup> mice [29]. According to Zhuo et al., 2001, these mice start to express Cre recombinase in the forebrain at E13.5 [29]. We observed that while Rfx3 was already inactivated at E15.5 in CC guidepost glia and neurons of Rfx3<sup>m</sup>; hGfap-Cre<sup>Cre<sup>+</sup></sup> mice (Figure 6A and 6B), the CC still formed normally (Figure 6C–6F, n = 9/9). This result shows that the loss of Rfx3 in midline neuronal and glial guidepost cells as early as E15.5 is not responsible for CC agenesis.

However, we cannot exclude that RFX3 is needed in the glutamatergic guidepost neurons that invade the CSB region from E12.5 to E14.5. To test this possibility, we induced recombination of Rfx3 floxed allele specifically in guidepost neurons, as early as E12.5, by mating Rfx3<sup>m</sup> and Emx1-Cre<sup>Cre<sup>+</sup></sup> mice [30]. Inactivation of Rfx3 in Emx1<sup>+</sup> precursors of Rfx3<sup>f/f</sup>; Emx1-Cre<sup>Cre<sup>+</sup></sup> mice led to loss of Rfx3 in all the CSB anlage at E12.5 and in midline postmitotic glutamatergic neurons at E14.5 (Figure 6G and 6H). While Rfx3 was already inactivated at E12.5 in the CSB region of Rfx3<sup>m</sup>; Emx1-Cre<sup>Cre<sup>+</sup></sup>, we did not observe any callosal pathfinding defects (Figure 6I–6L, n = 6/6). These results also sustain the conclusion that Rfx3 is not required in CC neurons. Finally, Rfx3 was inactivated in GABAergic neurons originating from the ventral telencephalon by using the Nkx2.1-Cre<sup>Cre<sup>+</sup></sup> mouse [31]. In accordance with the absence of Rfx3 expression in CC guidepost GABAergic neurons, Rfx3<sup>m</sup>; Nkx2.1-Cre<sup>Cre<sup>+</sup></sup> mice did not present any CC defects (not shown).

Taken together, these results demonstrate that callosal pathfinding defects observed in Rfx3<sup>m</sup>; mice are not due to a cell autonomous function of RFX3 in CC guidepost cells suggesting a requirement for RFX3 for proper CC development, at early embryonic stages, during midline specification.

Midline specification is affected in Rfx3<sup>m</sup> mice

To understand how RFX3 governs CC midline structure formation before E12.5, we looked at early patterning defects that could affect Rfx3<sup>m</sup> mice. Telenchephalic patterning relies on the interaction of well-described dorsal, rostral and ventral signalling centres in the forebrain that produce secreted signalling molecules [32]. We first analysed the expression of genes characteristic for the dorsal signalling centres in Rfx3<sup>m</sup> mice by in situ hybridization. Genetic evidences show that Bone morphogenic 4 (Bmp4) is essential for roof plate formation in the mouse forebrain [33]. BMP4 is expressed in the telencephalic midline at E10.5 and in the entire forebrain midline at E12.5. Moreover, several WNT proteins are expressed in the cortical hem [34] which is crucial for dorsal midline development. However, our analyses did not reveal any differences in Bmp4 and Wnt2b expression between E12.5 WT and Rfx3<sup>m</sup> embryos (n = 2/2, Figure S6A–S6D). Thus no major defects could be observed in dorsal midline markers in Rfx3<sup>m</sup> mice.

The rostral signaling center is specified at E8.5 as the anterior neural ridge (ANR) at the anterior border between the eктoderm and neuroectoderm and will give rise to the commissural plate at later stages. Both the ANR and the commissural plate express FGFR8 that has been shown to be important to induce ventral and rostromdorsal cell fates [35]. We determined the telencephalic rostral expression profile of Fgf8 in wild type and Rfx3<sup>m</sup> embryos. As observed in Figure 7, at E12.5, Fgf8 expression was restricted to the commissural plate of wild type embryos. Remarkably, we observed an extension of Fgf8 expression into the rostromedial pallium in Rfx3<sup>m</sup> embryos on both coronal and sagittal sections (n = 6/6, Figure 7A, 7B and Figure S7A, S7B). These data suggest that RFX3 is necessary to restrict Fgf8 expression to the commissural plate. FGFR8 has been shown to induce Sprouty2 gene expression which in turns negatively regulates FGFR8 signalling [36,37] and we observed a small expansion of Sprouty2 in the rostromedial pallium consistent with an increase in FGFR8 signalling in the midline (n = 7/7, Figure 7C, 7D and Figure S7C, S7D).

Several key transcription factors have been associated with the specification of the commissural plate in mouse, including: SIX3, nuclear factor I/A (NFIa) and EMX1 [38]. We precisely analyzed the expression of these markers but did not observe extensive variations in the expression of Emx1 (n = 3/3), Six3 (n = 3/3), and Nfia (n = 4/4) between WT and Rfx3<sup>m</sup> mutant mice (Figure S6E–S6H and not shown) suggesting that ectopic FGFR8 in Rfx3<sup>m</sup> mice is not due to uncontrolled changes in the expression of these key transcription factors.

It has been shown that proper Sonic Hedgehog (SHH) signalling is required to maintain FGFR8 signalling at the rostral midline. In addition, defects in ciliary proteins lead to defective SHH signalling in many tissues and organs (for review see [39]) and also in the telencephalon [40,41]. In comparison with Rfx3<sup>m</sup> embryos, we did not observe any differences in Shh expression in the ventral telencephalon of Rfx3<sup>m</sup> mutants (n = 3/3, Figure S6I and S6J). However, we observed that SHH signalling is likely to be affected since the Shh target genes Patched1 (Ptc1) (n = 3/3) and Gli1 (n = 2/3), were both slightly down-regulated in the Rfx3<sup>m</sup> ventral telencephalon (Figure S6K–S6N, arrows). Taken together, these findings suggest that the up-regulation of Fgf8 expression does not coincide with an up-regulation of SHH signalling in the ventral telencephalon.

Interestingly, it has been shown that Gli3<sup>m</sup> embryos present an abnormal development of the prosencephalic midline with a similar ectopic expansion of Fgf8 expression into the dorsal midline [42–44]. Since GLI3 processing has been shown to require cilia [45] and that RFX3 regulates ciliogenesis in several mouse cell types, we hypothesized that the FGFR8 expression defects could
Figure 5. Midline integrity is necessary for pathfinding by callosal axons. (A1) Experimental paradigm used to confirm the growth of E16.5 Rfx3+/+ control callosal axons in midline structure transplants from Rfx3+/+ control mice. (A2–A3) Dil labeling showing that WT callosal axons grow normally and cross the midline when they are confronted to a WT environment. (B1) Experimental paradigm used to confirm the growth defects of Corpus Callosum Development Requires RFX3.
result from abnormal function of GLI3 in Rfx3−/− embryos. GlI3 mRNA expression did not appear to be affected in Rfx3−/− telencephalon, as observed by in situ hybridization on coronal sections (Figure S6O and S6P). Thus, RFX3 does not seem to act on GlI3 transcription. GlI3 produces two antagonistic protein isoforms: the full-length activator form (GlI3A) and the proteolytically cleaved repressor form (GlI3R) [46–48] with the ratio between GlI3A and GlI3R being an important determinant of patterning for various tissues. We thus investigated GLI3 proteolytic processing by western blot in wild type or Rfx3−/−, Rfx3+/−, and Rfx3−/−/− mice. Western blot analysis showed that WT callosal axons are misrouted and do not cross the midline of Rfx3−/− mice. Moreover, when Rfx3−/− callosal pathfinding was examined, the continued presence of Rfx3+/− axons was found to strongly support proper pathfinding. Thus, RFX3 does not seem to act on GlI3 transcription and is not required for callosal pathfinding in Rfx3−/− mice. (B2–B3) DiI labeling showing that WT callosal axons are misrouted and do not cross the midline of Rfx3 mutant mice. (C1) Experimental paradigm used to study the growth defects of E16.5 control Rfx3+/+ callosal axons in transplants of midline structures from Rfx3−/− mice. (C2–C3) DiI labeling showing that WT callosal axons are misrouted and do not cross the midline of Rfx3 mutant mice. (D1) Experimental paradigm used to test whether the midline integrity is necessary and sufficient to direct the growth of callosal axons. To this end, control Rfx3+/− midline regions are transplanted in Rfx3−/− mice. (D2–D3) DiI labeling showing the complete restoration of Rfx3−/− callosal axon pathfinding. Dashed lines outline the CC transplant localizations. Brain slices in A2–A3, B2–B3, C2–C3 and D2–D3 were counterstained with Hoechst. Bar = 435 μm in A2, B2, C2, D2 and 220 μm in A3, B3, C3 and D3.

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Discussion

Ectopic FGF8 signalling at the CSB leads to altered organisation of CC guidepost neurons

To study whether ectopic FGF8 signalling could be responsible for the disorganisation of guidepost neurons at the CSB, we performed ex-vivo cultures of brain slices at E12.5 in the presence or absence of ectopic FGF8 (Figure 8). Ectopic FGF8 sources were provided by bath application (Figure 8B) or by implanting FGF8-coated beads into the rostral medial pallium (Figure 8E), where the extension of Fgf8 expression was observed in Rfx3−/− embryos. We followed the distribution of guidepost neurons 2–3 days later by immunostaining for Calretinin. Remarkably, under both conditions, we observed drastic consequences of ectopic FGF8 on guidepost distribution at the midline in treated explants (n = 10/10 after FGF8 bath application and n = 6/6 with FGF8-coated beads) compared to control (n = 11/11 without FGF8 bath application and n = 5/5 with control-coated beads). The observed phenotypes were similar to what is observed in Rfx3−/− brains. The tangential distribution of Calretinin+ neurons through the cortical MZ was severely reduced and neurons were broadly distributed at the CSB and in cortical layers. No MZ could be clearly distinguished. We also noticed a marked thinning of the commissural plate as it was observed in several Rfx3 mutant mice compared to WT mice (Figure 7B and white arrowheads in Figure S6). These results support the hypothesis that FGF8 controls guidepost neuronal distribution at the CSB. Hence, in Rfx3−/− brains, the observed increase in Fgf8 expression can be indirectly responsible at early stages for disturbed guidepost neuron distribution by acting on CSB patterning, but also directly responsible at later stages for the positioning of guidepost neurons at the CSB. Therefore, loss of CC in Rfx3−/− mice likely results from the perturbed processing of GLI3 which controls Fgf8 expression early during development.

Rfx3−/− mouse mutant provides a valuable tool for dissecting the relative contributions of early brain patterning mechanisms in the organization of the CSB and in CC formation. We show that Rfx3−/− mice have mild and focussed FGF8 over expression that is restricted to the CSB and that severe CC defects coincide with this early patterning anomaly. In turn, this patterning defect is responsible for dramatic changes in the distribution of guidepost neurons but not of glial cells at the corticoseptal midline in Rfx3−/− mice. In addition, our transplantation experiments support the conclusion that proper guidepost neuronal network organization mediated by FGF8 signalling at the midline is essential for proper CC formation.

RFX3 and guidepost cells of the corticoseptal boundary

RFX3 is strongly expressed in corticoseptal guidepost cells from E14.5 to E16.5. However, we did not observe any consequences of Rfx3 loss of function on CC formation by Emx1-Cre or hGFAP-Cre induced Rfx3 inactivation in these cells, suggesting that RFX3 function in guidepost cells is not required for commissural axonal formation or that RFX3 function in these cells is masked by a redundant function of other RXF transcription factors. Indeed, 7 RXF proteins are present in the mouse genome and at least one, RFX4, has already been shown to play major functions in brain patterning and to be widely expressed in the telencephalon [49,50]. However, no precise description of RFX4 expression in guidepost neurons or glial cells have been described and CC callosal defects have not been precisely investigated in RFX4 mutants. Hence, the function of RXF proteins in guidepost cells still remains to be deciphered. Nevertheless, our work shows that RFX3 plays a crucial function in early patterning of the CSB.

Increased FGF8 signalling disturbs distribution of guidepost neurons required for CC formation

We show that RFX3 is required early to restrict FGF8 expression in the telencephalic rostral midline. Our results suggest that a small increase in Fgf8 signalling is sufficient to disorganize the CSB and hence guidepost neuron distribution at early stages of embryonic development. This is supported by in-vivo observations on Rfx3 mutant brains. On the other hand, our ex-vivo explant experiments support the hypothesis that FGF8 also has a direct action on guidepost neuron distribution at later stages. Altogether, these observations show that FGF8 plays a critical function for the distribution of guidepost neurons at the CSB. Previous data in the literature indicated that reducing FGF8 signalling in the telencephalon leads to severe brain phenotypes, and in particular to holoprosencephaly [35,51,52] and Fgf8 hypomorphic mutants show corpus callosum defects [51]. Moreover Fgf8 inactivation by Emx1-Cre induced recombination has been shown to induce CC defects at E18.5, suggesting a key role for FGF8 in CC formation [38]. In all these studies, while FGF8 signalling defects were associated with severe alterations of the dorsal signalling centres and possible defects at the CSB, the distribution of guidepost neurons was not examined. In Rfx3−/− mice, unbalanced FGF8 expression in the rostral telencephalon is not associated with alterations of the dorsal signalling centre but is nevertheless sufficient to disturb the distribution of guidepost neurons, leading to the conclusion that FGF8 signalling is primarily responsible for
Figure 6. Mice carrying a conditional deletion of *Rfx3* in guidepost cells have a normal corpus callosum. (A–B) *Rfx3* mRNA (red) and GLAST protein (green) expression on coronal brain sections of control *Rfx3*+/−;hGfap-Cre+/− (A1–A2) and *Rfx3*+/−;hGfap-Cre+/− (B1–B2) embryos at E15.5. A2 and B2 are higher magnifications of the glial wedge (GW) seen in A1 and B1, respectively. (A1–A2) In control *Rfx3*+/−;hGfap-Cre+/− mice, *Rfx3* is strongly expressed through the cortex, the induseum griseum (IG) and the CSB, as well as, the GW. (B1–B2) No more *Rfx3* mRNA is detected in guidepost glia and neurons of *Rfx3*+/−;hGfap-Cre+/−. (C–F) Immunohistochemistry for GFAP and L1 receptor (C and D) and for calretinin and Npn-1 receptor (E and F) in coronal CC sections from E18.5 control *Rfx3*+/−;Emx1-Cre+/− (C and E) and *Rfx3*+/−;hGfap-Cre+/− (D and F) mice. In mice where *Rfx3* is inactivated after E14.5 in midline neurons and glia, the CC and callosal axons develop normally. (G–H) *Rfx3* mRNA (red) and calretinin protein (green) expression on coronal brain sections of control *Rfx3*+/−;Emx1-Cre+/− (G1–G2) and *Rfx3*+/−;Emx1-Cre+/− (H1–H2) embryos at E14.5. G2 and H2 are higher magnifications of the corticoseptal boundary (CSB, *) seen in G1 and H1, respectively. (G1–G2) In control *Rfx3*+/−;Emx1-Cre+/− mice, *Rfx3* is strongly expressed through the calretinin+ glutamatergic neurons of the cortex and of the CSB. (H1–H2) In *Rfx3*+/−;Emx1-Cre−/− brains, no more *Rfx3* mRNA is detected in midline glutamatergic guidepost neurons of the CSB. (I–L) Immunohistochemistry for GFAP and L1 (I and J) and for calretinin and Npn-1 (K and L) in coronal CC sections from E18.5 control *Rfx3*+/−;Emx1-Cre−/− (I and K) and *Rfx3*+/−;Emx1-Cre−/− (J and L) mice. *Rfx3* inactivation after E12.5 in guidepost glutamatergic neurons of the CSB does not affect callosal axon navigation. Bar = 435 μm in A1, B1, G1, H1; 220 μm in C, D, E, F, I, J, K, L; 110 μm in G2, H2 and 40 μm in A2, B2.

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guidepost neuron organization at the CSB and hence CC formation.

Our work also indicates that RFX3 acts on FGF8 signalling by regulating GLI3 activity in the telencephalon. Indeed, in Shh mutants Fgf8 expression is lost, whereas in Gli3 mutants Fgf8 expression is expanded [42–44]. Moreover, in Shh; Gli3 double mutants, Fgf8 expression is also expanded suggesting that this expansion occurs independently of Shh in a Gli3 mutant background [44]. Last, loss of Gli3 rescues ventral patterning defects in Shh mutants but not in Fgf1;Fgf2 mutants, placing FGF signalling downstream of Gli3 [53]. This also explains why the upregulation of Fgf8 expression likely occurs in Rfx3−/− brains despite an observed downregulation of Shh signalling. Our observations and these data are consistent with the conclusion that Rfx3 acts on GLI3 activity and consequently on FGF8 signalling at the rostral telencephalic midline. Interestingly, our work brings a mechanistic interpretation to the observation that Gli3 mutations have been found in human patients suffering from Acrocallosal syndrome [54]. In addition, hypomorphic Gli3Pdn mutant mice show CC defects [55] with a similar but more severe increase in FGF8 expression in the rostral midline [42]. Consistent with our observations, Gli3Pdn mutants also show strong alterations of guidepost neuronal organization (D. Magnani and T. Theil, unpublished).

Ciliary proteins and corpus callosum defects

In Rfx3−/− brains, we observed a reduced processing of GLI3 in its repressor form and a reduction in SHH signalling in agreement with the already described function of RFX3 in ciliogenesis in various cell types. Indeed, the function of cilia in regulating SHH signalling and GLI3 processing has been well documented in various cell types and organs [for review see [39]]. Interestingly, RFX proteins have been shown to control consistently the regulation of several IFT components, dynein retrograde motors and many basal body associated proteins such as MKS1 from C. elegans to mammals [17,56,57]. Many of these RFX target genes appear to be associated with overall reduced SHH signalling and reduced GLI3 processing when mutated in mouse. These observations suggest that RFX3 indirectly modu-

Figure 7. Disturbed expression of Fgf8 and of the ratio of GLI3 repressor/GLI3 activator forms in Rfx3−/− CSB. In situ hybridization for Fgf8 (A and B), Sprouty2 (C and D) mRNAs on coronal sections from E12.5 WT (A and C) and Rfx3−/− (B and D) mice at the CSB. Fgf8 expression domains is expanded into the rostromedial pallium in Rfx3−/− embryos. Interestingly, the frontier region between the septum and the cortex is reduced in the mutant compare to WT (arrowhead). In addition, Sprouty2 expression is slightly increased in the Rfx3 mutant. Bar = 500 μm in all figures. (E) Western blot analysis of E13.5 individual forebrains from Rfx3+/−, Rfx3−/− and Rfx3−/− embryos from a same litter. As control, extracts from bodies of Gli3+/+ or Gli3Xt/Xt embryos were included. No GLI3 protein is produced in Gli3Xt mutants allowing the identification of GLI3 specific bands. (F) Quantification of the Western blot shows that the ratio of GLI3 repressor form to the full-length form is reduced in Rfx3 deficient mice compared to heterozygotes and WT mice. doi:10.1371/journal.pgen.1002606.g007
lates GLI3 activity and SHH signaling in the anterior telencephalon by regulating the levels of several proteins involved in cilia associated transport and biogenesis.

In humans, several syndromes resulting from mutations in genes encoding ciliary proteins are associated with corpus callosum malformation of various severity [15]. Recently, mutations in the Kif7 gene involved in ciliogenesis and GLI3 processing have been found in human patients suffering from acrocallosal syndrome, characterized by Corpus Callosum and digit malformations [58]. Our work provides a first insight into the cellular mechanisms that are responsible for Corpus Callosum defects following GLI3 processing alterations. Our work demonstrates that small alterations in GLI3 processing is correlated with altered patterning of the CSB and aberrant distribution of guidepost neurons in this region and that this is sufficient to induce midline crossing defects of callosal axons. In mouse, only a few ciliary mutants with altered patterning of the telencephalon have been described. The cobblestone hypomorphic mutation of Ift88, and the ftm, and alien mutants all show severe morphological defects of the brain associated with dorsal ventral patterning defects of the telencephalon [40,41,59]. All three mutants are associated with an alteration in GLI3 processing, but with a more severe shift in the balance of GLI3 activator and repressor forms than in Rfx3 mutants. However, the CC was not precisely investigated in these mutants, probably because embryos die too early to allow for an analysis of CC development. Another interesting mutant is a Wnt1-Cre induced Kif3A-deleted mouse that shows craniofacial anomalies due to neural crest migration defects and is associated with agenesis of the CC [60]. Neural crest migration is required for the proper patterning of the telencephalon, in particular by acting on FGF8 rostral patterning centre [61,62]. The patterning of the telencephalon has not been described in Wnt1-cre; Kif3Afl/fl mice but it is tempting to hypothesize that dysregulation of FGF8 signalling could be sufficient to mis-pattern the commissural plate in this mutant. Hence, the Rfx3−/− mutant represents the first mouse model establishing a link between proper GLI3 processing
and the distribution of guidepost neurons at the CSB for CC formation.

In conclusion, the analysis of Rfx3−/− mice provides strong evidence for the important contribution of corticoseptal neuronal populations in CC formation and for the critical function of Fgf8 signalling in CSB patterning at early stages of CC formation. It provides new understanding of the cellular origins of CC defects in human ciliopathies.

Materials and Methods

Animals

All animal research has been conducted according to relevant national and international guidelines. Rfx3-deficient and floxed mice were generated and genotyped as previously described [18]. GAD67-GFP knock-in mice, hGfp-CreERT2/−, Ngn2-creERT2/−, Enx1-creERT2/−, Nkx2-1-creERT2/− mice used in this work have been previously described [27,29–31].

Histology

Brains were fixed in 4% PFA/PBS at 4°C until experimentation and then dehydrated in graded series of ethanol (25–100%). Brains were transferred into absolute butanol and substituted for paraffin in graded series of butanol/paraffin solutions. Sections of 10 μm were deparaffinized in Methylocyclohexan, rehydrated and stained with Hematoxylin following standard procedures before mounting in Eukitt.

Immunocytochemistry

Brain embryos were dissected and fixed overnight at 4°C in 4% paraformaldehyde (PFA) (Sigma P6148) in 1×PBS (Invitrogen). Brains were cryoprotected in 30% sucrose and cut in coronal 50 μm-thick frozen sections for staining. Mouse monoclonal antibodies were: Nestin (1/600) (BD bioscience). Rat monoclonal antibodies were: L1 (1/200) (Chemicon, Temecula, CA) and CTIP2 (1/500) (Abcam, Cambridge, UK). Rabbit polyclonal antibodies were: calbindin (1/2500) and calretinin (1/2000) (Swant, Bellinzona, Switzerland); CUX1 (1/200) (Santacruz, Heidelberg, Germany); EMX1 (1/250) (gift form A. Trembleau); GFAP (1/500) (DAKO, Carpinteria, CA); GFP (Molecular Probes, Eugene, OR); SATB2 (1/500) (gift from V. Tarabykin); RFX3 (1/100) [63], TBR1 (1/500) (Abcam, Cambridge, UK). Goat polyclonal antibody was Npn-1 (1/50) (R&D System, Minneapolis, Mn). GLAST guinea-pig polyclonal antibody was (1/2000) (Millipore, Billerica, MA). GFP chicken polyclonal antibody was (1/500) (AVES, Oregon, USA).

Fluorescence immunostaining: The primary antibodies were detected with secondary antibodies coupled with Cy3 or Alexa (Jackson ImmunoResearch and Molecular Probes; respectively). For RFX3 detection, we used an amplification system with secondary anti-rabbit IgG coupled to biotin (1/250) (Jackson Laboratory) and subsequent revelation with Streptavidin Fluoprobe 488 and 541 (gift from V. Tarabykin). Alexa 488 and 594 nm (green excitation for Alexa 549, Cy3 and Cy5), with a HeNe laser 633 nm (excitation for Alexa 647 and Cy5) and a Diode laser 405 nm (for Hoechst staining). Z-stacks of 10–15 plans were acquired for each CC coronal section in a multitrack mode avoiding crosstalk.

Images processing: all 3D Z stack reconstructions and image processing were performed with Imaris 6.0 software. To create real 3D data sets we used the mode “Surpass”. The colocalization between two fluorochromes was calculated and visualized by creating a yellow channel. Figures were processed in Adobe Photoshop CS2.

Western blot

Western blots were performed following standard procedures. Gl3 6F5 mouse monoclonal antibody was kindly provided by S. Scales [65]. Gl3XT and WT body samples were kindly provided by M. Willaredt and S. Schneider-Maunoury.

Slice culture experiments

We have used our previously developed in vitro model of CC organotypic slices [11]. Embryos were placed in ice cold dissecting medium (MEM Gibco ref 11012-044 with 15 mM glucose and 10 mM Tris pH 7–9). Brains were removed and embedded in 3% low-melting point agarose (Invitrogen). 250 μm thick coronal sections were then cut using a vibrotome filled with cold dissecting medium and slices at the level of the CC were collected in the same medium. CC slices were cultured on Millicell membranes in tissue dishes containing 1 ml of BME/HBSS (Invitrogen) supplemented with glutamine, 5% horse serum, and Pen/Strep [54]. In our slice assay, as in vivo, the callosal axons from dorso-lateral neocortex develop later and their growth cones enter after E16.5 the CC region in successive streams over a period of several days. Our slice assay performed at E16.5 allowed us to study: (1) the function of CSB cells, (2) the outgrowth properties of the majority of callosal axons that are growing through the CC after E16.5 and (3) the effects of transplantations on callosal axon navigations.

The transplantation assay was performed at E16.5 as previously described [11] to analyze the growth of either WT or Rfx3−/−-Dil-labelled callosal axons within midline structures of WT or Rfx3−/− slices. Small explants of E16.5 WT or Rfx3−/−-midline structure comprising were excised using tungsten needles and transplanted into the midline of WT or Rfx3−/− host slices. After

In situ hybridization combined with immunocytochemistry

Embryonic brains were treated as described for immunocytochemistry procedure and coronal 100 μm-thick sections were cut using a vibratome (Leica Microsystems). 100 μm free-floating vibratome sections were hybridized with digoxigenin-labeled cRNA probe as described before [64]. To combine in situ hybridization with immunocytochemistry, fast Red (Roche) was used as an alkaline phosphatase fluorescent substrate instead of NBT/BCIP solution. Slides were incubated in Fast Red (Roche) until the appearance of staining in dark chamber at RT. Thereafter, sections were fixed for 15 min in 4% PAF and immunostaining was performed.
incubation for 48 hours, the slices were fixed and axon trajectories through the various regions were analysed by confocal analysis. For FGF8 bath application: slices of E12.5 brains were cultured as above but FGF8 (FGF-8b isoform, R&D Systems, ref 429-F8) or BSA (control) was added in the culture medium at 1 µg/ml in PBS 1X. After two days of incubation, slices were processed for immunohistochemistry as described above. For FGF8-coated beads experiments: slices of E12.5 brains were cultured as above but FGF8-coated beads or control-coated beads were implanted in the rostromedial pallium. After three days of incubation, slices were processed for immunohistochemistry as described above. To make FGF8-soaked beads, 45 µm polystyrene beads (Polysciences) were rinsed in PBS, the beads were pelleted by 5-min centrifugation at 13,000 rpm and the supernatant was removed. They were incubated in 5 mg/ml heparin for 1 hour at room temperature, rinsed and then incubated with 250 µg/ml mouse FGF8 (R&D Systems) in 0.5% bovine serum albumin (BSA) in PBS overnight at 4°C. Control beads were incubated in 0.5% BSA in PBS. Before implantation, beads were rinsed three times for 10 minutes in PBS.

Atlas and nomenclature

The nomenclature for callosal development is based on the “Atlas of the prenatal mouse brain” [66].

Supporting Information

Figure S1 Defects of the CC and hippocampal commissure in Rfx3+/− brains at E18.5. (A-C) Haematoxylin-eosin staining performed at different rostro-caudal (R→C) levels on coronal brain sections of E18.5 wild type (A1–A2) or Rfx3+/− (B1–B2 and C1–C2) embryos. (A1–A2) At E18.5, the hemispheres of the WT brain have fused. The CC and the hippocampal commissure (HC) are already formed. (B and C) Around 70% of Rfx3+/− embryos show either a partial CC agenesis with few callosal axons crossing (B1–B2) or a complete CC agenesis with an absence of midline fusion (O) and no midline crossing (C1–C2). All callosal defects are associated with Probst Bundle (PB) formation (arrowheads). The hippocampal commissure (HC) development is also affected in most mutants either as a reduction or a complete loss of this commissure (C2). Bar = 600 µm in all. (TIF)

Figure S2 Numerous RFX3-positive neuronal populations are found within the embryonic cingulate cortex and at the corticoseptal boundary. (A-G) In situ hybridizations for Rfx3 (in red) combined with immunohistochemical staining for reelin (A1–A2), TBR1 (B1–B2), calretinin (C1–C2 to E1–E2) or calbindin (F1–F2 and G1–G2) (in green) on coronal brain sections in WT mice at E13.5 (C1–C2), E14.5 (A1–A2 and B1–B2), E15.5 (D1–D4 and F1–F2) and E16.5 (E1–E2 and G1–G2). A2, B2, C2, D2, D4, E2, F2 and G2 are high-power views of the midline region seen in A1, B1, C1, D1, E1, F1 and G1, respectively. (A1–A2) Rfx3+/− cells of the marginal zone (MZ) within the cingulate cortex (CCg) are not reelin+ Cajal Retzius cells. (A1) Reelin is expressed in the cingulate region (CSB, *). (B1–B2) Rfx3+/− cells residing in the CSB and in the cortex express TBR1. (B2) All neurons that contain high levels of cytosolic Rfx3 mRNAs express the nuclear TBR1 transcription factor (arrowheads). Therefore, Rfx3+/− cells of the CSB and of the cortex are glialmatagamic neurons. (C to E) Co-labeling experiments performed from E13.5 to E16.5, show that the majority of Rfx3+/−-expressing neurons in the cortical MZ, (C1–C2, D3–D4 and E2), as well as, in the CSB (D1–D2) express calretinin (arrowheads). After E16.5, while the cerebral hemispheres have fused and the CC is formed, Rfx3 expression persists in calretinin+ neurons of the cortex (E2; arrowheads) but stops in calretinin+ guidepost neurons (E1; arrow). (F1–F2 and G1–G2) From E15.5 to E16.5, the Rfx3+/− neurons of the IG express the calbindin. By contrast, Rfx3+/− neurons of the MZ do not express calbindin. Bar = 220 µm in A1, B1, C1, D1, E1, F1, G1; 110 µm in C2, D3; 60 µm in A2, G2 and 40 µm in B2, D2, D4, E2, F2. (TIF)

Figure S3 Expression of Rfx3 in radial glia precursors of the GW and in cortical pyramidal neurons. (A1–A2) Immunohistochemistry for calretinin and Emx1 in coronal sections from mice at E15.5. A2 is a higher magnification of the midline corticoseptal boundary (CSB, *) seen in A1. Calretinin+ neurons of the CSB are glialmatagamic since they express Emx1 (arrowheads in A2). (B1–B2) In situ hybridizations for Rfx3 (red) combined with immunohistochemical staining for Calbindin (green) on coronal brain sections in WT mice at E16.5. Calbindin+ neurons of the CSB are glialmatagamic since they express Emx1 (arrowheads in B2). (C1 and C2) Immunohistochemical staining for RFX3 in coronal sections from GAD67-GFP transgenic mice at E18.5 showing that RFX3 is not expressed by GABAergic interneurons. (D–H) In situ hybridizations for Rfx3 (red) combined with immunohistochemical staining for Nestin (D1–D2), GFAP (E1–E2 and F1–F2), SATB2 (G1–G2) or CTIP2 (H1–H2) (green) on coronal brain sections in WT mice at E14.5 (D1–D2), E16.5 (E1–E2 and E18.5 (F1–F2 to H1–H2). D2, E2, F2, G2 and H2 are high magnifications of D1, E1, F1 G1 and H1, respectively. (D1–D2 to F1–F2) Radial glial cells of the glial wedge (GW) labelled for Nestin and GFAP express high levels of Rfx3 (arrowheads). By contrast, astrocytes of the indusium griseum (IG) labelled for the same markers are devoided of Rfx3. (G1–G2 and H1–H2) At E18.5, Rfx3 is expressed by callosal pyramidal neurons (SATB2+) and by sub-cerebral projecting neurons (CTIP2+). (G2 and H2) High magnified views of the cortex showing that glialmatagamic pyramidal neurons expressing the nuclear transcription factors SATB2 or CTIP2 contain cytosolic Rfx3 mRNAs (arrowheads). Bar = 435 µm in A1, G1, H1; 220 µm in B1, D1, E1; 110 µm in C1, C2; 60 µm in A2, B2; 40 µm in D2, E2, F1, F2, G2, H2. (TIF)

Figure S4 Rfx3 inactivation in cortical pyramidal neurons is not responsible for callosal axon guidance defects. (A–H) Single immunohistochemistry for SATB2 (A and B), for CUX1 (C and D), for TBR1 (E and F) or for CTIP2 (G and H) in coronal sections from E18.5 WT (A, C, E and G) and Rfx3+/− (B, D, F and H) mice. SATB2+, CUX1+, TBR1+ and CTIP2+ cortical layers are not affected in the Rfx3+/−/. (I–J) In situ hybridizations for Rfx3 (in red) combined with immunohistochemical staining for SATB2 (in green) on coronal brain sections of control Rfx3+/−;Ngn2-CreERTm−/− (I1–I2) and Rfx3+/−;Ngn2-CreERTm−/−;Ngn2−/− (J1–J2) embryos at E18.5. (I1–I2) In control Rfx3+/−;Ngn2-CreERTm−/− mice, Rfx3 is strongly expressed through the glialmatagamic neurons of the cortex and of the indusium griseum (IG). (J1–J2) In Rfx3+/−;Ngn2-CreERTm−/− brains, Rfx3 hybridization signal is significantly decreased in all the cortical layers after induced recombination of Rfx3 floxed allele in Ngn2-derived cells. Rfx3 inactivation in the cortex does not affect the cortical distribution of SATB2+ callosal projecting neurons. (K–L) Double immunohistochemistry for calretinin and Npn-1 (K1–K2 and L1–L2) in coronal CC sections from E18.5 control Rfx3+/−;Ngn2-CreERTm−/−;Ngn2−/− mice. Rfx3+/−;Ngn2−/− (K1–K2) and Rfx3+/−;Ngn2−/− (L1–L2) mice. In brain sections of mice where Rfx3 is conditionally inactivated in Ngn2-derived cortical pyramidal neurons, callosal axons develop normally. Bar = 435 µm in I1, J1, J2, J3, K1, L1; 220 µm in K2, L2 and 110 µm in A, B, C, D, E, F, G, H. (TIF)
Figure S5  Localization of GABAergic interneurons in Rfx3 deficient mice. (A–B) In situ hybridization for Gad67 mRNAs on coronal CC slices of E16.5 Rfx3 deficient mice (B1–B2) in comparison with wild type (A1–A2). We observe a normal localization of Gad67 in telencephalon, and notably in the lateral CC. Expression of Rfx3 deficient mice (B1–B2) compared to wild type mice (A1–A2). (C–D) DAB staining for GLAST on coronal rostromedial slices from E16.5 WT (C) and Rfx3−/− (D) mice. We observe a normal localization and organization of guidepost glia of the midline zipper glia (MZG) and of the glial wedge (GW) of Rfx3 deficient mice (D) compared to wild type mice (C). Bar = 1200 μm in A1, B1, Bar = 300 μm in C, D, and 150 μm in A2, B2. (TIF)

Figure S6  Expression of dorsal and ventral markers in Rfx3−/− brains. In situ hybridization for Shh (A and B), Gli1/C and D, pc1/1 and F, Gli3 (G and H),bmp4 (I and J) and Wnt2b (K and L) mRNAs on coronal sections from E12.5 WT (A, C, E, G, I, K, M and O) and Rfx3−/− (B, D, F, H, J, L, N and P) mice. (A to D) All dorsal midline markers are normally expressed in the Rfx3−/− embryos. (E to P) While Shh and Gli3 were properly specified, Pcl1 receptor and Gli1 are down-regulated (black arrows) in Rfx3−/−. Interestingly, the frontier region between the septum and the cortex is reduced in some mutants compared to WT (empty arrows). Bar = 500 μm in all. (TIF)

Figure S7  Enlargement of the Fgf8 expression domain in Rfx3−/− embryos. In situ hybridization for Fgf8 (A and B) and Sprouty2 (C and D) mRNAs on sagittal midline sections from E12.5 WT (A and C) and Rfx3−/− (B and D) embryos at the CSB. Fgf8 and Sprouty2 expression domains are expanded rostrally and laterally in the pallium of Rfx3−/− embryos (arrows). Bar = 500 μm in all figures. (TIF)

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Author Contributions

Conceived and designed the experiments: TT CL BD. Performed the experiments: CB DM MN LM DV BO TT BD CL. Analyzed the data: CB DM MN LN BO TT BD CL. Contributed reagents/materials/analysis tools: AA-L WR J-PH. Wrote the paper: TT CL BD.

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Figure S3
