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Gli3 is required for the specification and differentiation of preplate neurons

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Abstract

During corticogenesis, the cerebral cortex develops a laminated structure which is essential for its function. Early born neurons of the preplate and its derivatives, the marginal zone (MZ) and the subplate (SP), serve as a framework during the cortical lamination process. Here, I report on defects in the generation and specification of these early born cortical neurons in *extra-toes* (Xt^J) mice which are defective for the Gli3 zinc finger transcription factor. The *Gli3* mutation dramatically disrupts early steps in the cortical lamination process. The MZ, SP and the cortical plate (CP) do not form layers but cortical neurons are arranged in clusters. These defects start to become evident at E12.5 when the cortex forms several protrusions and the ventricular zone becomes undulated. At this stage, cortical progenitor cells start to loose their apical/basal cell polarity correlating with an ectopic expression of *Wnt7b* in the ventricular zone. In addition, the cellular composition of the preplate is severely altered. Cajal-Retzius cells are reduced in numbers while early born Calretinin$^+$ neurons are overproduced. These results show that multiple aspects of corticogenesis including the organization of the venticular zone, the apical/basal cell polarity of cortical progenitors and the differentiation of early born cortical neurons are affected in the *Gli3* mutant.
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Keywords: Mouse; *Gli3*; *Reelin*; *Wnt7b*; Cerebral cortex; Lamination; Cajal-Retzius cells; Cell polarity; Proliferation

Introduction

The cerebral cortex as the main center for all higher cognitive functions unique to humans acquires a layered structure which is essential for its function. The development of this architecture represents a multistep process controlled by a complex genetic program. Several findings indicate that a tight control of neural progenitor cell proliferation and differentiation is an important aspect of cortical morphogenesis. In several human diseases (Walsh, 1999) and in a number of mouse mutants, cortical size and organization are affected due to altered proliferation and differentiation rates of cortical precursor cells. For example, the *Emx2* homeobox gene controls the proliferative characteristics of stem cells in the embryonic and adult cortex (Galli et al., 2002; Heins et al., 2001) and *Emx2*$^{-/-}$ mutants form cortical dysplasias due to an ectopic expression of *Wnt1* in the dorsomedial telencephalon (Ligon et al., 2003). Similarly, ectopic activation of the canonical Wnt signalling pathway results in cortical overgrowth and disorganization (Chenn and Walsh, 2002, 2003; Ligon et al., 2003). An impairment of cortical morphogenesis is also observed after dorsal forebrain specific inactivation of *Numb* and *Numblike*, asymmetrically distributed determinants of cell fate (Li et al., 2003; Petersen et al., 2004). Finally, disruption of the apical/basal cell polarity of neuroepithelial cells in the basal forebrain has profound affects on cell proliferation and differentiation (Klezovitch et al., 2004). Thus, during cortical morphogenesis, proliferation and differentiation rates are controlled by a variety of different biological processes.

In addition, the preplate and its derivatives, the MZ and the SP, play fundamental roles during the generation of cortical architecture by regulating neuronal migration, radial glia morphology, layer formation and axonal pathfinding (Super et al., 1998). These diverse functions are reflected by the presence of a variety of different cell types. SP neurons have been suggested to pioneer the first axon pathway from the cerebral cortex by establishing the first efferent axonal projections (Del Rio et al., 1997; McConnell et al., 1989).

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Another class of pioneer neurons is located within the MZ and consists of at least two neuronal populations defined by the differential expression of calcium-binding proteins. These neurons form dense clusters and send transient axonal projections into the nascent internal capsule (Meyer et al., 1998; Soria and Fairen, 2000). The second major cell population in the MZ is formed by subpial granule cells (Meyer and Goffinet, 1998) among which Cajal-Retzius (CR) cells represent the best characterized cell type. CR neurons are essential for cortical lamination as ablation of CR neurons causes disorders in cortical lamination (Super et al., 2000). This activity is mediated by the secreted extracellular matrix protein reelin (Tissir and Goffinet, 2003). Lack of *reelin*, as in the *reeler* mouse mutant (Curran and D'Arcangelo, 1998; D'Arcangelo et al., 1995; Ogawa et al., 1995) and in human congenital lissencephaly patients (Hong et al., 2000), or disturbance of the reelin signalling pathway (Howell et al., 1997; Sheldon et al., 1997; Trommsdorff et al., 1999) leads to inversions of cortical laminae. However, despite these fundamental roles of MZ and SP cells in cortical development, the molecular mechanisms underlying the generation and specification of these cells are largely unknown.

The zinc finger transcription factor Gli3 plays an important role in telencephalic development. *Extra-toes* (Xt^J) mouse mutants in which a deletion removes the 3' end of the *Gli3* gene (Briscoe and Ericson, 1999; Hui and Joyner, 1993; Maynard et al., 2002) lack the olfactory bulbs and the dorsomedial wall of the telencephalon and fail to develop the choroid plexus, the cortical hem and the hippocampal anlage, the dorsal most structures of the telencephalon (Franz, 1994; Grove et al., 1998; Johnson, 1967; Theil et al., 1999). Furthermore, the neocortex is misspecified correlating with a loss and/or severe reduction of *Emx* gene expression and becomes ventralized at later stages of development (Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000). In addition to these early regionalization defects, histological analysis suggested the lack of an overt morphological lamination (Franz, 1994; Theil et al., 1999). However, it was not determined to which extent the cellular organization of the cerebral cortex is disturbed and also the causes of these defects remained unknown.

This study presents a detailed analysis of the early steps of the cortical lamination process in Xt^J mice. These animals fail to develop a distinct MZ and subplate and neurons instead form aggregates of cells. This loss of the early cortical layers was found to be caused by several defects. The *Gli3* mutation leads to a defective organization of an undulated VZ and to a loss of apical–basal cell polarity of cortical precursors. These latter defects are preceded by an ectopic expression of *Wnt7b* in the VZ. In addition, preplate and MZ neurons are misspecified in *Gli3* mutants. In particular, the number of CR cells is reduced and these cells form abnormal clusters while the number of early born Calretinin$^+$ neurons is increased. This study therefore provides insights into the processes which control cell fate specification and differentiation of MZ and subplate neurons.

Material and methods

Mice

$Xt^J/^+$ animals were kept on a C57Bl6/C3H background. Embryonic (E) day 0.5 was assumed to start at midday of the day of the vaginal plug discovery. Xt^J/Xt^J embryos were readily distinguished from heterozygous and wild-type embryos by forebrain morphology (Johnson, 1967; Pellegrini et al., 1996; Theil et al., 1999).

In situ hybridization and immunohistochemistry

Antisense RNA probes for *Conductin* (Lustig et al., 2002) *Nscl2* (Krüger and Braun, 2002), *p73* (XM 131858; GenBank), *reelin* (D'Arcangelo et al., 1995), *SCG10* (Stein et al., 1988), *Tbr1* (Bulfone et al., 1995), *Wnt1* (Shimamura et al., 1994), *Wnt7b* (Parr et al., 1993) and *Wnt8b* (Richardson et al., 1999) were DIG labeled. In situ hybridization on paraffin sections were performed on 14 μm serial sections of mouse embryos as described (Christoffels et al., 2000).

For immunohistochemistry, embryos were fixed in 4% paraformaldehyde for 2–3 h, washed in PBS and transferred to 30% sucrose in PBS. After embedding in OCT, embryos were cryosectioned at 10–20 μm. Slides were air-dried, washed three times in PBS + 0.1% Triton-X-100 (PBST), incubated with PBST + 10% sheep serum and incubated with primary antibody overnight at 4°C. Slides were then washed 3× in PBST and the Cy2- and Cy3-conjugated secondary antibodies (Dianova) were applied for 4 h at room temperature in PBST with 1% sheep serum. Fluorescence imaging was carried out on a Leica TCS NT confocal microscope. Images were processed and mounted using Photoshop 6.0 (Adobe). The following antibodies were used: BrdU (1:20, Bio-Science), Calretinin (1:2000, Chemicon), β-catenin (1:1000) (Hülsken et al., 1994), Chondroitin sulfate CS56 (1:1000; Sigma), MAP2 (1:2000; Sigma), Numb (1:1000) (Zhong et al., 1996), Par-3 (1:500; Upstate), Pax6 (1:400; Covance), phospho-Histone H3 (1:800; Upstate), Sox2 (1:500) (Wilson et al., 2000), β-tubulin (1:1000, Sigma) and ZO-1 (1:1000; Santa Cruz).

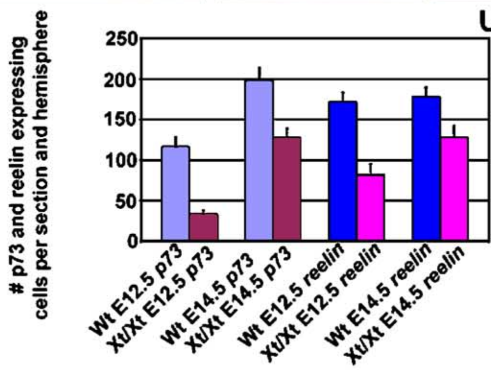
For BrdU labeling, pregnant females were intraperitonally injected with BrdU (10 mg/ml) and sacrificed 1 h later. For BrdU-immunocytochemistry, slides were incubated in 0.1 M $Na_4B_4O_7$ after denaturing with 2N HCl.

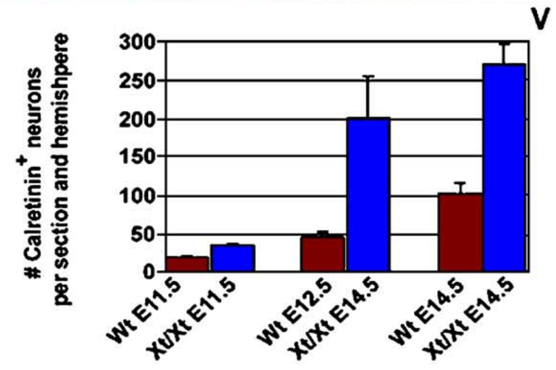
For each marker and each stage, 3–5 different embryos were analyzed at rostral, medial and caudal levels of the telencephalon. For quantification, the numbers of *reelin*$^+$, *p73*$^+$ and or Calretinin$^+$ cells on sections of a complete telencephalic hemisphere were counted. At least, 10 different sections were evaluated.

Fig. 1. Loss of early cortical layers in the *Gli3* mutant neocortex. Coronal sections through the brains of E14.5 wild-type (A–E, K–O) and Xt^J/Xt^J embryos (F–J, P–T). (A, F) Few *Dlx2* expressing cells were observed in the lateral neocortex of the *Gli3* mutant which might correspond to migrating interneurons (arrows). Note that F was turned at 90° to reveal the whole telencephalic section. (B, G) In wild-type embryos, Pax6 immunoreactivity is restricted to a smooth VZ. In Xt^J/Xt^J embryos, Pax6 staining reveals an undulated VZ. Note the Pax6 staining in differentiated neurons (arrowhead). BrdU (C, H) and pHH3 (D, I) immunohistochemistry further demonstrates the irregular structure of the VZ and the presence of ectopic mitotic cells (arrow in I). (E) *reelin* and (K) Calretinin expression is characteristic for the MZ covering the cortical surface of wild-type embryos. In Xt^J/Xt^J embryos, *reelin* expressing (arrows in J) and Calretinin$^+$ (P) neurons are arranged in clusters. (L) *Nscl2* expression in the MZ and in the IZ. (Q) Xt^J/Xt^J embryos display loss of *Nscl2* expression in the MZ and an undulated IZ. (M, N) Immunohistochemical analysis of MAP2 and CS56 expression in neurons of the MZ and the SP of wild-type embryos. (R) Blocks of MAP2$^+$ neurons and an irregular arrangement of CS56$^+$ cells (S) are observed in Xt^J/Xt^J embryos. (O) *Tbr1* is expressed in cortical neurons with higher expression levels in the CP. (T) The high level expression of *Tbr1* is lost and the remaining *Tbr1* expressing neurons form "u"-like structures. (U) Quantification of *reelin* and *p73* expressing cells, the numbers of which are reduced in the *Gli3* mutant. (V) Calretinin$^+$ neurons are produced in increased numbers in Xt^J/Xt^J embryos. Abbreviations: Ctx: cortex; D: diencephalons; IZ: intermediary zone; LGE: lateral ganglionic eminence; MZ: marginal zone; SP: subplate. The scale bars, also in Figs. 2–4, correspond to 200 μm.

Results

Previous histological analysis of cortical development in *Gli3* mutants indicated a loss of cortical lamination (Franz, 1994; Theil et al., 1999). However, the extent and the reasons for this defect remained elusive but are the focus of this study. For this purpose, only nonexencephalic Xt^J/Xt^J embryos were analyzed. Furthermore, the telencephalon of *Gli3* mutants becomes partially ventralized in a rostral dorsomedial region starting at E12.5 (Kuschel et al., 2003;





Tole et al., 2000) but the absence of ventralization in the caudolateral telencephalon suggests the existence of additional causes for the lack of cortical lamination. To identify these reasons and to avoid potential interferences through this ventralization, in situ hybridization or immunohistochemical analyses with ventral (*Dlx2*, *Mash1*) and dorsal specific markers (Pax6, *Ngn2*, *Tbr1*) were performed to define the nonventralized region of the dorsal telencephalon for each embryo. Only this area was further analyzed.

Formation of a layered MZ, subplate and CP is disturbed in Xt^J/Xt^J embryos

It was previously shown that the Xt^J/Xt^J cortex starts to degenerate at E15.5 for unknown reasons and is completely lost at birth (Theil et al., 1999). Therefore, it was not possible to analyze cortical lamination at birth but Xt^J/Xt^J mutants allowed to study *Gli3* function during the formation of early cortical layers—the MZ, CP and subplate. To first define whether these layers are properly formed in Xt^J/Xt^J embryos, coronal sections of E14.5 embryos were analyzed with various layer specific markers. Consistent with previous observations, analysis of *Dlx2* and Pax6 expression revealed that the dorsolateral cortex of Xt^J/Xt^J embryos is not ventralized (Kuschel et al., 2003 and Figs. 1A, B, F, G), but this analysis showed an undulation of the cortical VZ. As reported previously, additional $Pax6^+$ cells were found outside the VZ and likely present septal neurons (Kuschel et al., 2003). Disturbances of VZ structure were also confirmed by BrdU pulse labeling experiments (Theil et al., 1999 and Figs. 1C, H). Unlike in wild-type embryos, where $BrdU^+$ S-phase cells are regularly arranged at the upper side of the VZ, these cells form massive clumps protruding to the pial surface of the Xt^J/Xt^J cortex. In addition, M-phase cell nuclei cells which are labeled by the antiphosphorylated histone H3 antibody and which occupy the ventricular apical surface in wild-type embryos were also detected in the basal part of the VZ (Figs. 1D, I) further indicating a disorganized VZ. In addition, this disorganization coincides with a thickening of the *Gli3* mutant cortex.

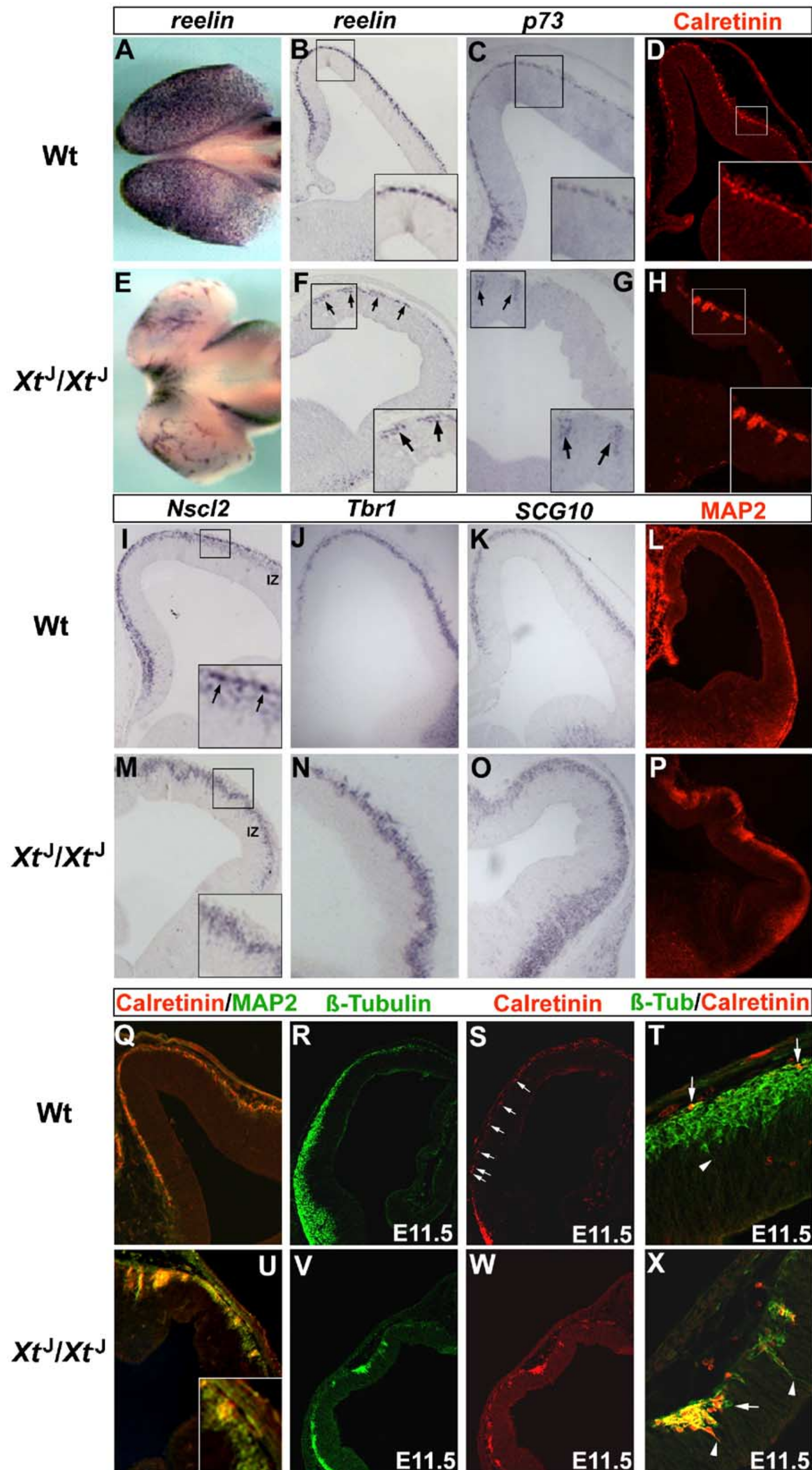
To study the generation of neuronal cell layers in the Xt^J/Xt^J cortex, the development of Cajal-Retzius (CR) cells was analyzed which play an important role in orchestrating the cortical lamination process (Tissir and Goffinet, 2003). CR cells expressing *reelin* and *p73*, which represents one of the earliest markers of CR cells in the neocortex and archicortex (Meyer et al., 2002) and which is required for CR cell development (Yang et al., 2000), form a well-organized layer throughout the entire cerebral cortex of E14.5 wild-type embryos (Fig. 1E and data not shown). In Xt^J/Xt^J embryos, *reelin* expressing cells were slightly reduced in numbers, had accumulated and formed clusters separated by gaps (Figs. 1J, U). In addition, the *reelin* expression levels were reduced. Similar accumulation of *p73* expressing cells was detected dorsomedially but were absent in dorsolateral regions of the Xt^J/Xt^J cortex (data not shown). To further examine MZ development in Xt^J/Xt^J embryos, Calretinin expression was determined which is a marker for CR cells and pioneer neurons. Interestingly, large numbers of $Calretinin^+$ cells had also aggregated in the Xt^J/Xt^J cortex, while these cells formed a single layer of cells on the surface of the developing cerebral cortex of wild-type embryos (Figs. 1K, P, V). Furthermore, the bHLH gene *Nscl2* is expressed in the intermediate zone (IZ) and at higher levels in the MZ (Krüger and Braun, 2002; Theodorakis et al., 2002). In situ hybridization indicated the absence of *Nscl2* expression in the MZ of Xt^J/Xt^J embryos while the IZ formed a wavy irregular structure (Figs. 1L, Q). These analyses therefore suggest defects in MZ development and a misspecification of CR neurons.

To assess whether other layers are affected in the Xt^J/Xt^J cortex, immunohistochemical analysis of MAP2 and of the chondroitin sulfate proteoglycan CS56 was performed. In the wild-type cortex, MAP2 and CS56 label the MZ and the subplate (Figs. 1M, N). This pattern was completely disrupted in Xt^J/Xt^J embryos, where clusters of $MAP2^+$ cells became evident similar to those observed for Calretinin (Figs. 1R, S). Finally, *Tbr1* marks postmitotic cortical neurons with higher expression levels in CP and CR neurons of E14.5 wild-type embryos (Hevner et al., 2001). This high level expression domain was absent in Xt^J/Xt^J embryos while cells expressing low *Tbr1* levels formed U-like structures with cells in the center of the U completely lacking *Tbr1* transcripts (Fig. 1T). Interestingly, this pattern appeared to be complementary to the blocks of *reelin* expressing cells and to the $Calretinin^+$ and/or $MAP2^+$ neurons. Taken together, these analyses indicate a dramatic disorganization of the E14.5 Xt^J/Xt^J neocortex with a disruption of its normal layered structure. While the VZ is disorganized and undulated, neurons are arranged in clusters of cells instead of forming distinct MZ, CP and subplate layers.

Fig. 2. Defective differentiation of the preplate in Xt^J/Xt^J embryos. Dorsal view of wild-type (A) and Xt^J/Xt^J (D) E12.5 brains processed for *reelin* in situ hybridization and transverse (J, N) and coronal sections of wild-type (A–D, I–L, Q–T) and *Gli3* mutant (E–H, M–P and V–X) telencephali. (A–Q, U) E12.5 and (R–T, V–X) E11.5. (A, B, E, F) Xt^J/Xt^J embryos display reduced *reelin* expression levels and a reduced number of *reelin* expressing cells which form aggregates (arrows). (C, G) *p73* is expressed in CR neurons and in VZ cells of the cortical hem. In Xt^J/Xt^J embryos, *p73* positive cells form dense cluster (arrows) in the dorsomedial telencephalon but are absent dorsolaterally. (D, H) Groups of $Calretinin^+$ cells are detected in the whole preplate of Xt^J/Xt^J embryos. (I, M) *Nscl2* expression is lost from the MZ (arrows in I) while expression is still evident in an undulated IZ. (J, N) *Tbr1* expression is unaffected in the preplate of Xt^J/Xt^J embryos. The rostromedial area lacking *Tbr1* expression corresponds to the ventralized region of the Xt^J/Xt^J telencephalon. *SCG10* expression (K, O) and MAP2 immunoreactivity (L, P) reveal a continuous neuronal cell layer in the dorsal telencephalon of Xt^J/Xt^J embryos. (Q, U) Double immunostaining for Calretinin (red) and MAP2 (green) showing a clustering of $Calretinin^+$ neurons in the MZ. (R–T) In E11.5 wild-type embryos, β-$tubulin^+$ neurons are present throughout the cortex and only few $Calretinin^+$ neurons (arrows in S, T) are formed. (V–X) In contrast, the Xt^J/Xt^J neocortex produces locally neurons while the number of $Calretinin^+$ neurons is strongly increased and only few β-$tubulin^+$/$Calretinin^-$ cells are detected (arrow in X). The arrowheads in panels T and X point to axons extending towards the apical surface of the VZ.

Misspecification of CR cells in Xt^J/Xt^J embryos

To obtain insights into the development of the architectural defects in the cerebral cortex of *Gli3* mutants, corticogenesis was analyzed at E12.5. At this stage, the rostromedial dorsal telencephalon of Xt^J/Xt^J embryos is ventralized while caudal structures are unaffected (Tole et al., 2000; Kuschel et al., 2003 and Figs. 3F, G). In a first set of experiments, *reelin* expression was analyzed as a marker for CR neurons. In wild-type embryos, *reelin* expressing cells cover the whole surface of the developing cerebral cortex as a single layer of cells (Figs. 2A, B). In contrast, the number of *reelin* expressing cells and also the



reelin expression level were reduced in E12.5 Xt^J/Xt^J embryos (Figs. 1U and 2E, F). Moreover, the remaining, weakly *reelin* expressing cells often formed aggregates which consist of several cell layers and which are separated by gaps. To further analyze CR cell development, the expression of *p73* was analyzed. In wild-type embryos, *p73* transcripts were detected in CR cells throughout the entire cerebral cortex and in dorsomedial VZ cells consistent with their potential origin from the cortical hem (Meyer et al., 2002). In contrast, *p73* expressing cells formed clusters in the dorsomedial telencephalon of Xt^J/Xt^J embryos. Unlike the *reelin* expression pattern, however, *p73* expression was not detected in dorsolateral regions (Fig. 2G). These groups of *reelin* and *p73* expressing neurons were reminiscent of the neuronal aggregates observed at E14.5 and were also found for Calretinin$^+$ neurons (Figs. 2D, H). In addition, *Nscl2* transcripts were specifically lost in the preplate but were present in the IZ (Figs. 2I, M). Thus, the loss of a layered cortical structure observed at E14.5 is already preceded by alterations in the differentiation of preplate neurons at E12.5.

To further characterize preplate development in Xt^J/Xt^J embryos, the *Tbr1* expression pattern was determined which is essential for preplate differentiation (Hevner et al., 2001). However, in situ hybridization analysis revealed a continuous distribution of *Tbr1* expressing cells in the preplate of Xt^J/Xt^J embryos which appeared undulated especially in the caudal and lateral parts of the cortex (Figs. 2J, N). The presence of a continuous neuronal cell layer was also confirmed by the expression patterns of the panneuronal marker *SCG10* and by MAP2 but both markers also revealed the irregular shape of this layer (Figs. 2K, L, O, P). Moreover, double immunofluorescence staining with anti-Calretinin and anti-MAP2 antibodies showed that clusters of Calretinin$^+$Map2$^+$ cells were separated by MAP2$^+$ Calretinin$^-$ positive cells (Figs. 2Q, U). Interestingly, these clusters of Calretinin$^+$ neurons were already observed at E11.5. At this stage, neurogenesis occurs in the wild-type neocortex with a lateral to medial gradient and only few Calretinin$^+$ neurons are present (Figs. 2R–T). In contrast, in Xt^J/Xt^J embryos, β-tubulin$^+$ neurons were only locally produced. The majority of these neurons expressed Calretinin leading to a strong increase in the number of Calretinin$^+$ neurons (Figs. 1U and 2V–X). Moreover, these neurons started to form clusters and extended axons towards the luminal surface of the VZ. Taken together, these results suggest that in *Gli3* mutants a preplate is formed but its differentiation and cellular composition are severely altered.

Disorganization of the cortical VZ in Gli3 mutants

In addition to the defects in SP and MZ formation, the VZ also appeared disorganized at E12.5. Pax6 immunoreactivity was found in the VZ of wild-type embryos with higher expression levels in lateral parts of the cortex (Fig. 3B). This graded Pax6 expression is lost in Xt^J/Xt^J embryos and the VZ showed the formation of bulges protruding towards the pial surface (Fig. 3G). A wavy structure of the VZ was also found in BrdU pulse labeling experiments and with immunostainings for the proliferative antigen Ki67 (Figs. 3C, H and data not shown). In addition, ectopic mitoses were detected in the VZ of Xt^J/Xt^J embryos and in some regions, stripes of pHH3$^+$ cells extended towards the pial surface (Figs. 3D, I). Finally, to examine the relationship between the bulged VZ and the neuronal clusters in the preplate, double immunohistochem-

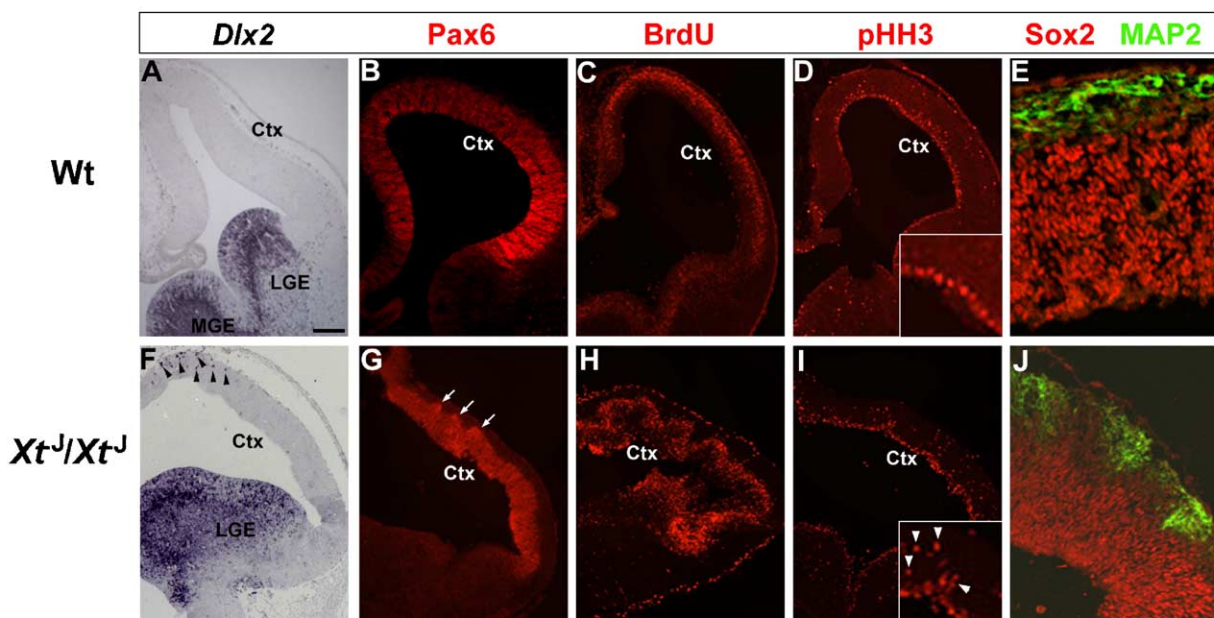


Fig. 3. The organization of the cortical VZ is disturbed in E12.5 Xt^J/Xt^J embryos. In situ hybridization and immunohistochemical analysis on coronal sections of wild-type (A–E) and Xt^J/Xt^J embryos (F–J). (A, E) Ectopic *Dlx2* expression in the dorsal telencephalon of Xt^J/Xt^J embryos is confined to rostromedial areas (arrows). (B, G) Immunostaining for Pax6 revealed protrusions of the VZ (arrows). (C, H) BrdU pulse labeling also showed its irregular, undulated structure. (D, I) While pHH3$^+$ cells line the luminal side of the VZ of wild-type embryos, ectopic pHH3$^+$ cells are observed in the upper part of the VZ (arrowheads in I). (E, J) Double immunostaining for Sox2 (red) and MAP2 (green) showing an increased number of neurons in the troughs of the undulated VZ. Abbreviations: MGE, medial ganglionic eminence.

ical analyses for MAP2 and Sox2 were performed which are specifically expressed in differentiated neurons and in neural progenitor cells, respectively (Pevny et al., 1998; Uwanogho et al., 1995). These analyses revealed the formation of neuronal aggregates in the troughs of the undulated VZ (Figs. 3E, J). Thus, the VZ starts to become disorganized around E12.5 and forms several bulges leading to the undulated shape of the VZ.

Ectopic expression of Wnt7b in the VZ of Xt^J/Xt^J embryos

Next, I was interested in identifying the molecular mechanisms underlying the misspecification of preplate neurons and the disorganization of the cortical VZ in Xt^J/Xt^J embryos. Interestingly, similar but less severe malformations in the cerebral cortex of *Emx2*$^{-/-}$ embryos leading to the formation of cortical dysplasias were attributed to ectopic expression of *Wnt1* in the dorsal midline of the telencephalon (Ligon et al., 2003). In contrast to *Emx2*$^{-/-}$ embryos, however, neither wild-type nor Xt^J/Xt^J E12.5 embryos showed *Wnt1* expression in the telencephalon while transcripts were readily detectable in the midbrain roof plate of both genotypes (Figs. 4A, F) suggesting that an ectopic *Wnt1* activation does not lead to the defects in the *Gli3* mutant VZ. I therefore started to analyze the expression of other *Wnt* family genes in the *Gli3* mutant telencephalon. Previous analysis had already shown that the expression of *Wnt* genes (*Wnt2b*, *Wnt3a* and *Wnt5a*) with specific expression in the cortical hem is completely disrupted (Grove et al., 1998; Theil et al., 2002). In addition, the expression of *Wnt7a* was unaffected in Xt^J/Xt^J embryos (Grove et al., 1998) while residual transcription of *Wnt8b* was confined to the medial cortex and does not expand into more lateral regions (Figs. 4B, G). In contrast, *Wnt7b* showed a dramatically altered expression in the dorsal telencephalon of Xt^J/Xt^J embryos. In the VZ of wild-type embryos, *Wnt7b* expression is confined to the cortical hem and to dorsomedial cortex. In the dorsolateral telencephalon, *Wnt7b* is weakly expressed in the VZ but shows strong expression in differentiated neurons (Kim et al., 2001). Xt^J/Xt^J embryos, however, displayed a strong expression of *Wnt7b* in dorsolateral cortical progenitor cells (Fig. 4H). This ectopic expression, which was not observed in *Emx2* mutants (T.T. unpublished data), appeared discontinuous and occurred in stripes corresponding to the bulges and protrusions of the VZ. Interestingly, ectopic *Wnt7b* expression was already detected at E11.5 in stripes of cells before the disorganization of the VZ became evident (Figs. 4D, I).

Next, I analyzed whether the ectopic *Wnt7b* expression resulted in the stimulation of the canonical Wnt signaling pathway in the Xt^J/Xt^J cortex. Recently, expression of *Conductin*/*Axin2* has been identified as a direct target gene of canonical Wnt signaling (Jho et al., 2002; Lustig et al., 2002). Indeed, strong *Conductin* expression was found in the dorsomedial telencephalon and gradually expands into more lateral regions of wild-type embryos (Fig. 4E). In contrast, *Conductin* expression was abolished from the dorsal telencephalon of Xt^J/Xt^J embryos (Fig. 4J) strongly suggesting that the canonical Wnt signaling pathway is inactive in the mutant cortex.

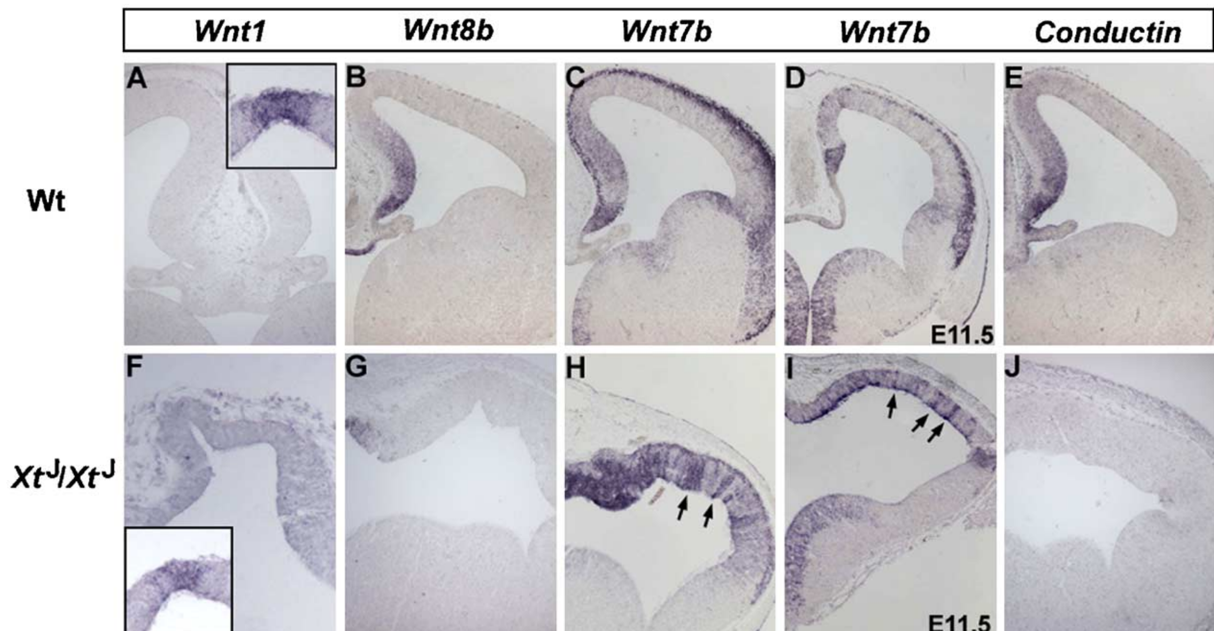


Fig. 4. Ectopic *Wnt7b* expression in the VZ of Xt^J/Xt^J embryos. Coronal sections through the brains of E12.5 and E11.5 (D, I, N), wild-type (A–E) and Xt^J/Xt^J (F–J) embryos. (A, F) Xt^J/Xt^J embryos do not show an ectopic *Wnt1* expression in the dorsomedial telencephalon while *Wnt1* expression is readily detectable in the roof plate at rostral midbrain levels (insets in A and F). (B, G) *Wnt8b* expression remains restricted to the dorsomedial telencephalon of Xt^J/Xt^J embryos. Note the smaller *Wnt8b* expression domain in Xt^J/Xt^J embryos due to the absence of the cortical hem. (C, D) *Wnt7b* expression is detected in cortical neurons and in the VZ of the cortical hem and the dorsomedial telencephalon of wild-type embryos. (H, I) In contrast, Xt^J/Xt^J embryos show an ectopic, striped *Wnt7b* expression in the dorsolateral telencephalon which can already be detected in E11.5 (arrows). (E, J) *Conductin* expression reveals activation of the canonical Wnt signaling pathway in the dorsomedial telencephalon of wild-type embryos while the Xt^J/Xt^J neocortex lacks *Conductin* expression.

Loss of cell polarity in the dorsal telencephalon of Gli3 mutants

To further analyze whether canonical Wnt signalling is activated upon ectopic *Wnt7b* expression, the subcellular distribution of β-catenin protein was determined. Upon canonical Wnt signaling, β-catenin becomes stabilized and translocates to the nucleus where it regulates the transcription of Wnt target genes (Behrens et al., 1996; Willert et al., 2002). Interestingly, nuclear distribution of β-catenin could not be detected either in the dorsomedial or in the dorsolateral telencephalon of wild-type embryos nor in the dorsal telencephalon of Xt^J/Xt^J embryos (Figs. 5A, B). While the absence of nuclear β-catenin localization in the cortical hem of wild-type embryos does not allow to draw further conclusions on the activation of canonical Wnt signalling upon the ectopic *Wnt7b* expression, confocal analysis of β-catenin distribution demonstrated several differences between wild-type and Xt^J/Xt^J embryos which were confined to the extra protrusions of the VZ. Whereas in wild-type embryos, β-catenin is evenly distributed in adherens junctions, neuroepithelial cells of Xt^J/Xt^J mutants showed an upregulation in certain surface regions (Figs. 5A, B). In addition, these cells attach to the ventricular surface by their apical ends which are enriched for β-catenin protein while in Xt^J/Xt^J mutants, this apical localization is abolished. Distribution of E-cadherin, a main membrane component of adherens junctions which also localizes to the apical surface, was similarly affected in *Gli3* mutants (data not shown). The apical localization of other proteins, including ZO-1 and Par-3, was also disrupted (Aaku-Saraste et al., 1996; Izumi et al., 1998; Lin et al., 2000). Also, the web-like expression pattern of these proteins characteristic for their localization to adherens junction was more diffuse and in some regions even absent in Xt^J/Xt^J embryos (Figs. 5C–F). Phalloidin staining revealed the distribution of F-actin in adherens junctions and the presence of an actin-rich structure at the ventricular surface (Chae et al., 2004) (Fig. 5G). Instead of being confined to the ventricular surface, this actin-rich structure formed a web-like structure in Xt^J/Xt^J embryos (Fig. 5H). Finally, the apical/basal complex has been involved in the apical localization of the cell fate determinants such as Numb in flies and worms (Cai et al., 2003; Yu et al., 2003). In contrast to wild-type embryos (Zhong et al., 1996, 1997), however, few cortical progenitor cells displayed an apical Numb localization in Xt^J/Xt^J embryos and this distribution appeared to be more diffuse (Fig. 5J). Taken together, these analyses showed altered adhesive properties of neuroepithelial cells and a partial loss of apical basal cell polarity in the dorsal telencephalon of Xt^J/Xt^J embryos.

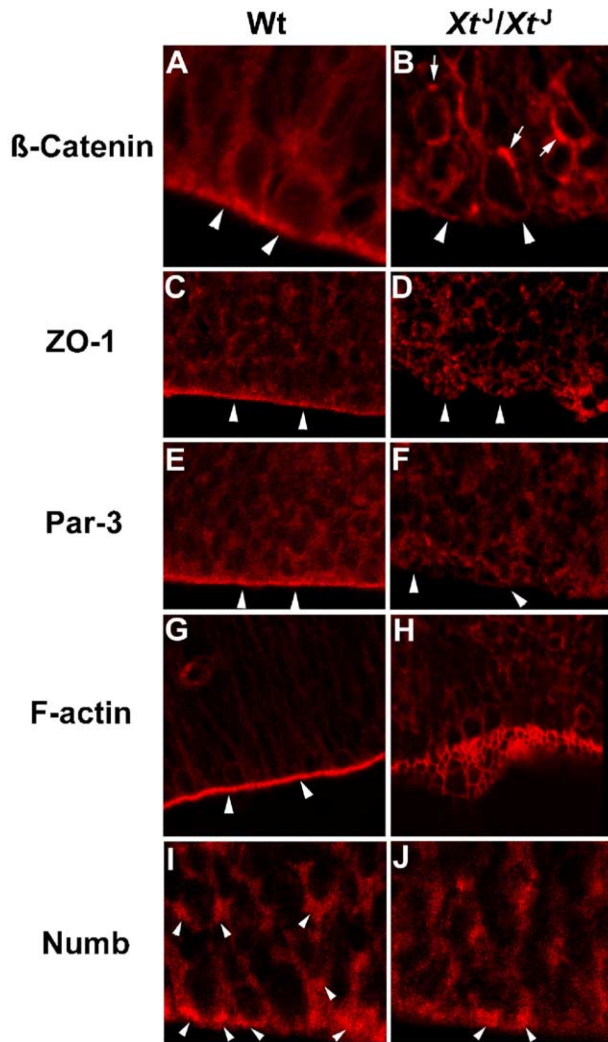


Fig. 5. Abnormalities of the ventricular neuroepithelium in the dorsolateral neocortex of Xt^J/Xt^J embryos. Immunostaining for apically localized proteins in coronal sections of E14.5 wild-type (A, C, E, G, I) and Xt^J/Xt^J embryos (B, D, F, H, J). (A, B) Lack of β-catenin enrichment at the apical surface of the neuroepithelium in Xt^J/Xt^J embryos (arrowheads) which show an upregulation in parts of the cell surface (arrows). Note the absence of nuclear β-catenin localization in both genotypes. (C–F) Immunoreactivity for Par-3 and ZO-1 concentrates apically in wild-type embryos but this apical localization is absent in Xt^J/Xt^J embryos (arrowheads). The localization of both proteins to adherens junction is more diffuse and in some regions even absent in Xt^J/Xt^J embryos. (G) Rhodamine-phalloidin staining revealed the distribution of F-actin in adherens junctions and the presence of an actin-rich structure (arrowheads) which formed a web-like structure at the ventricular surface of Xt^J/Xt^J embryos (H). (I, J) Numb protein is apically localized in the wild-type cortex (arrowheads). Whereas only few cortical progenitors display an apical Numb localization in Xt^J/Xt^J embryos (arrowheads), a more diffuse Numb distribution is observed in the majority of cells. Scale bars correspond to 20 μm (A, I) and 10 μm (C, E, G).

Discussion

Defective MZ and SP development in Xt^J/Xt^J embryos

In this study, I report on the effects of the *Gli3* mutation on the generation of the MZ, CP and SP-early cortical layers which serve as framework for the development of a six layered cerebral cortex. A major finding of this study corresponds to the dramatic disorganization of these structures which have lost their layered organization and corresponding neurons were instead found to be arranged in clusters. This altered arrangement already starts to become evident at around E11.5 when the lateral/medial gradient of neurogenesis is lost and neurons are only locally produced. The presence of an

irregularly shaped, undulated preplate 1 day later in development, however, argues against a general defect in neuronal differentiation but rather suggests a delay. In addition, the subsequent differentiation of the preplate into its derivatives, the MZ and the SP, appeared to be affected in the *Gli3* mutant. Already at E11.5, Calretinin$^+$ and 1 day later, also *reelin* or *p73* expressing cells did not distribute over the cortical surface but formed small clusters. The loss of these early cortical layers and the rearrangement of neurons into groups of cells distinguish *Gli3* mutants from previously described mouse mutants with cortical lamination defects and raise questions concerning the mechanisms underlying this defect. The previously described partial ventralization of the dorsal telencephalon (Kuschel et al., 2003; Tole et al., 2000; this paper) is unlikely to play a role as it is confined to rostromedial areas. Also, these early layering defects become obvious several days before the onset of the degeneration of the *Gli3* mutant neocortex. Furthermore, the altered neuronal arrangement cannot solely be attributed to a failure in preplate splitting as in *reeler* mutants cortical laminae are present but inverted (Caviness, 1982). In addition, $p73^{-/-}$ mice do not display cortical lamination defects despite reduced *reelin* expression levels (Meyer et al., 2002; Yang et al., 2000). Also, *Gli3* mutants show an unaltered expression of *Tbr1* at E12.5 which is essential for the differentiation of the preplate and layer 6 neurons (Hevner et al., 2001) indicating the existence of additional, *Tbr1* independent pathways controlling preplate differentiation. Finally, the cortical defects in Xt^J/Xt^J embryos are unlikely to be due to a severe reduction in *Emx* gene expression as *Emx1/2* double mutants show a disruption of cortical lamination but the VZ and an overlying neuronal cell layer can still be distinguished (Bishop et al., 2003; Shinozaki et al., 2002). Interestingly, the thickenings of the preplate inversely correlate with the undulated structure of the underlying VZ suggesting that a local increase in the production of neurons from the VZ might contribute to this phenotype. Alternatively, the reorganization might be indicative of changes in the adhesiveness of MZ neurons so that they do not spread over the cortical surface. In this regard, it is worth mentioning that CR neurons express specific cell adhesion molecules although the exact role of these molecules in CR cell development is unknown (Seki and Arai, 1991; Tsuru et al., 1996). Finally, MZ and SP neurons which serve as a framework for developing a properly laminated cortex may be misspecified due to defects in cortical progenitor cells. According to this scenario, defective specification of cell fates within the VZ would lead to abnormal generation and/or differentiation of MZ and SP neurons and subsequently to the layering defects in the Xt^J/Xt^J cortex. While the reduction in CR cell number is likely to be the result of the absence of the cortical hem (see below), Calretinin$^+$ neurons are overproduced and form aggregates reminiscent of the clusters of pioneer neurons (Meyer et al., 1998; Soria and Fairen, 2000) suggesting a potential overproduction of pioneer neurons at the expense of other preplate and MZ cell types. Such a cell fate switch could have been caused by the severe reduction in *Emx* gene expression and/or by the altered distribution of cell fate determinants as indicated by the altered subcellular localization of Numb. Given the known role of *Wnt* genes in cell fate regulation, the ectopic *Wnt7b* expression could also be responsible for altering the differentiation program of MZ and subplate neurons. Clearly, future experiments are needed to explore these intriguing possibilities. Regardless of the exact molecular mechanisms, the defective generation of the MZ and the subplate is highly likely to underlie the layering defects in the Xt^J/Xt^J cortex.

Origin of cortical CR neurons

Despite the fundamental importance of CR neurons for cortical lamination and the considerable progress in characterizing the reelin signaling pathway, much less is known about their generation and specification. Importantly, the site of origin of these cells has been highly debated. Both, the neocortex itself as well as extracortical sites such as the medial ganglionic eminence (MGE), the retrobulbar complex and the cortical hem have been suggested as potential sources of CR cells (Lavdas et al., 1999; Meyer and Goffinet, 1998; Meyer and Wahle, 1999; Meyer et al., 2000, 2002; Takiguchi-Hayashi et al., 2004). In particular, recent gene expression analysis and cell labeling studies have emphasized a role for the caudomedial wall of the telencephalon including the cortical hem in the generation of these neurons; however, experimental proof for such a role is still missing (Takiguchi-Hayashi et al., 2004). The finding that the loss of the cortical hem and of the olfactory bulbs in Xt^J/Xt^J embryos coincides with a substantial reduction in the number of CR neurons therefore provides evidence for an important role of these structures in CR cell formation. As the *Gli3* mutation, however, does not completely abolish the generation of CR neurons, additional sources of CR cell development must exist. In contrast to *Pax6* mutants (Stoykova et al., 2003), the number of tangentially migrating *Lhx6* positive neurons appears to be unchanged in Xt^J/Xt^J embryos (T.T. unpublished data). As development of the MGE is also unaffected in these embryos (Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000), the MGE represents an unlikely source of the remaining CR neurons. On the other hand, the loss and/or severe reduction of *Emx* gene expression in Xt^J/Xt^J embryos and the complete loss of CR neurons in *Emx*1/2 double mutants support a role of the neocortex in the generation of the remaining CR neurons (Shinozaki et al., 2002). The reduced *Emx2* expression in Xt^J/Xt^J embryos might also contribute to the misspecification of these cells as the *Emx2* homeobox gene is required for the maintenance of the CR neuronal phenotype (Mallamaci et al., 2000). Taken together, the reduced CR neuron number is likely to be the result of the loss of the caudomedial wall of the telencephalon and the misspecification of the neocortex. This view is also supported by our finding that *reelin* and *p73* expression is affected to different extents suggesting the existence of different *reelin*+ CR cell populations with potential different origins. However, as the cortical hem plays a major role in both CR cell and cortical development, it will be difficult to determine the

relative contributions of both structures to CR cell generation by analyzing animals lacking the hem.

The organization of the cortical VZ is severely disturbed in Xt^J/Xt^J embryos

Besides defective preplate differentiation, Xt^J/Xt^J embryos show severe abnormalities in the organization of the neuroepithelium. At E14.5, the cortex is thickened and from E12.5 onwards, the VZ becomes disorganized and shows ectopic mitoses. In addition, the neocortex obtained an irregular undulated shape with the VZ forming protrusions which might be caused by altered adhesive properties of VZ cells as indicated by the altered subcellular distribution of β-catenin. Similarly, the integrity of the neuroepithelial VZ is disturbed by mutations in zebrafish N-cadherins or by blocking of cadherins by specific antibodies (Ganzler-Odenthal and Redies, 1998; Lele et al., 2002). Alternatively, as these protrusions resembled bulges in the developing hindbrain which are caused by differences in the proliferation rates of VZ cells (Lumsden and Keynes, 1989), local differences in the proliferation and/or differentiation characteristics of cortical precursors might contribute to this phenotype. Similar, but less severe malformations were observed in $Emx2^{-/-}$ mutants and were attributed to an ectopic *Wnt1* expression in the dorsomedial telencephalon (Ligon et al., 2003). While ectopic *Wnt1* expression could not be detected in the Xt^J/Xt^J forebrain, these embryos showed an ectopic expression of *Wnt7b* in the cortical VZ which preceded the occurrence of bulge formation and was stronger in the center of the bulges. In addition, a dorsomedially restricted ectopic expression of *Wnt7b* in the telencephalon of a *Gli3* hypomorphic mutant correlates with defects in hippocampal development (T.T. unpublished data). A potential role for *Wnt7b* in controlling proliferation in the Xt^J/Xt^J telencephalon is further supported by the recent finding that retroviral mediated expression of *Wnt7b* stimulates the proliferation of cortical progenitors (Viti et al., 2003). Moreover, canonical Wnt signaling is known to control the proliferation/differentiation of progenitor cells in the developing cerebral cortex (Chenn and Walsh, 2002, 2003; Lee et al., 2000; Megason and McMahon, 2002). In contrast, ectopic *Wnt7b* expression in the caudal neural tube had no effects on the proliferation of neural progenitor cells (Megason and McMahon, 2002). Furthermore, the response of cortical progenitors to Wnt signalling changes with time (Hirabayashi et al., 2004). Therefore, the exact role of the ectopic *Wnt7b* expression in the *Gli3* mutant cortex remains to be determined and requires the analysis of cortical development in *Gli3*/*Wnt7b* double mutants. Also, the signaling pathway by which *Wnt7b* is acting during cortical development in Xt^J/Xt^J embryos and in other developmental contexts is unclear (Shu et al., 2002). The lack of *Conductin* expression provides evidence that canonical Wnt signalling is not activated in response to the ectopic *Wnt7b* expression in the *Gli3* mutant cortex. Therefore, the possibility remains that *Wnt7b* influences noncanonical Wnt signaling in the developing neocortex similar to its role in controlling dendritic branching in cultured hippocampal neurons (Rosso et al., 2005).

In addition, immunostaining for β-catenin and other apically localized proteins revealed marked abnormalities of the normally polarized structure of the VZ. Specifically in the protrusions of the VZ, the formation of adherens junctions is disturbed in the Xt^J/Xt^J cortex and neuroepithelial cells do not show the characteristic web-like distribution of cell adhesion molecules suggesting differences in adhesive properties. The disturbance of the apical localization of these proteins might be a consequence of altered proliferative characteristics as tumor cells often display such defects. Conversely, loss of apical/basal cell polarity in *Lgl1* mutant mice leads to an over-proliferation in the basal forebrain and to the formation of neuroepithelioma-like structures (Klezovitch et al., 2004) suggesting that the loss of apical/basal cell polarity may lead to an altered proliferation of cortical progenitors in the Xt^J/Xt^J forebrain. The disturbance of apical/basal cell polarity is also paralleled by a more diffuse distribution of the cell fate determinant Numb which is involved in the proliferation and differentiation control of cortical progenitors (Li et al., 2003; Shen et al., 2002; Zhong et al., 1996, 1997, 2000). The detection of correct Numb localization in few cortical progenitor cells, however, suggests that some aspects of apical/cell polarity are maintained in Xt^J/Xt^J embryos and that Numb localization is controlled by redundant pathways as reported before (Cai et al., 2003; Chae et al., 2004; Yu et al., 2003). Nevertheless, given the roles for β-catenin, adherens junctions and other apical proteins in the control of cell fate and proliferation, disturbance of their normal localization could cause defects in cell fate and growth during cortical development (Chae et al., 2004).

Interestingly, *Gli3* represents the first transcription factor to be involved in the control of apical–basal cell polarity. The molecular mechanisms of this control are unknown but could involve the direct transcriptional regulation of genes involved in establishing or maintaining cell polarity. Alternatively, *Gli3* could affect the apical localization of proteins indirectly. In this regard, *Gli3* genetically interacts with *Scrib1* (Rachel et al., 2002) which regulates the apical/basal cell polarity of epithelial cells in flies (Bilder and Perrimon, 2000) and also functions as a planar cell polarity (PCP) gene in mice (Montcouquiol et al., 2003; Murdoch et al., 2003). As the apical/basal polarity of precursor cells is an important determinant of stem cell characteristics, it is also interesting that Hedgehog/Gli signaling was recently reported to regulate the behavior of cells with stem cell properties in the developing neocortex and in the adult brain (Palma and Ruiz i Altaba, 2004; Palma et al., 2005). Therefore, future studies will have to address the interesting relationship between *Gli3*, cell polarity and stem cell characteristics during normal development and in the development of the *Gli3* telencephalic phenotype.

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