Primary cilia control telencephalic patterning and morphogenesis via Gli3 proteolytic processing

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SUMMARY
Primary cilia have essential functions in vertebrate development and signaling. However, little is known about cilia function in brain morphogenesis, a process that is severely affected in human ciliopathies. Here, we study telencephalic morphogenesis in a mouse mutant for the ciliopathy gene Ftm (Rpgrip1L). We show that the olfactory bulbs are present in an ectopic location in the telencephalon of Ftm mutants and do not display morphological outgrowth at the end of gestation. Investigating the developmental origin of this defect, we have established that E12.5 Ftm mutants rescue both telencephalic patterning and olfactory bulb morphogenesis, despite the persistence of cilia defects. Together, our results show that olfactory bulb morphogenesis depends on primary cilia and that the essential role of cilia in this process is to produce processed Gli3R required for developmental patterning. Our analysis thus provides the first in vivo demonstration that primary cilia control a developmental process via production of the short, repressor form of Gli3. Moreover, our findings shed light on the developmental origin of olfactory bulb agenesis and of other brain morphogenetic defects found in human diseases affecting the primary cilium.

KEY WORDS: Primary cilium, Ftm (Rpgrip1L), Gli3, Telencephalon, Olfactory bulb, Mouse

INTRODUCTION
During vertebrate development, the brain becomes progressively subdivided into distinct regions with specific properties of cell specification, growth and morphogenesis. Telencephalic patterning relies on three main signaling centers: the anterior neural ridge (ANR), the ventral telencephalon and the dorsal telencephalic midline, which produce fibroblast growth factors (FGFs), sonic hedgehog (Shh) and Wnts/bone morphogenetic proteins (BMPs), respectively. These signaling centers interact with each other and set up a complex regulatory hierarchy of transcription factors (Hebert and Fishell, 2008). The zinc-finger transcription factor Gli3 plays a crucial role in telencephalic patterning (Aoto et al., 2002). The mouse extra-toes mutant (Xt), hereafter Gli3Xt, which behaves as a null Gli3 mutant (Büscher and Rüther, 1998), has a severely reduced dorsomedial telencephalon and lacks olfactory bulbs (ObS) (Hu and Joyner, 1993). Instead, it presents an expanded subpallium, in particular in the most anterior region (Theil et al., 1999; Tole et al., 2000; Aoto et al., 2002; Kuschel et al., 2003). In addition, some Gli3Xt embryos are exencephalic. Gli3 is an effector of the Hh pathway in vertebrates. In the absence of Hh ligand, Gli3 is cleaved in a ubiquitin/proteasome-dependent process into a short form (Gli3R) (Wang et al., 2000; Tempe et al., 2006). In the presence of Hh, the cleavage of Gli3 is inhibited and a full-length (Gli3A) is produced (Bai et al., 2004).

Recently, Gli3 functions were shown to depend on primary cilia (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005; Eggeschwiler and Anderson, 2007). Primary cilia are present on almost every cell during vertebrate embryogenesis. They consist of a specialized plasma membrane supported by a specific microtubule network, the axoneme, anchored to the basal body (Marshall, 2008; Gerdes et al., 2009). Cilio genesis and cilia function rely on a process called intraflagellar transport (IFT), which involves molecular motors and IFT protein complexes (Rosenbaum and Wittman, 2002). The study of IFT gene mouse mutants has revealed important functions for vertebrate primary cilia in signal transduction (Rosenbaum and Wittman, 2002; Gerdes and Katsanis, 2008; Wong and Reiter, 2008; Neugebauer et al., 2009). In particular, IFT mutants display neural tube patterning defects and polydactyly, resulting from perturbations in the Hh signaling pathway. Both genetic and in vitro studies have shown an essential role for cilia, downstream of the patched 1 (Ptc1, or Pch1) receptor and smoothened (Smo) co-receptor, in the formation of the full activator forms of Gli2 and Gli3. Interestingly, mouse mutants for a number of genes involved in anterograde and retrograde IFT and, more generally, in cilia maintenance, show an increased Gli3R/Gli3A ratio. This observation has led to the proposal that cilia are also required for the production of a short, repressor form of Gli3 in the absence of Hh signaling (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005; Huangfu and Anderson, 2006; Eggeschwiler and Anderson, 2007; Hoover et al., 2008; Tran et al., 2008; Cortellino et al., 2009). However, the demonstration that important aspects of cilia function are mediated by the control of Gli3R production has yet to be provided in vivo. The mouse telencephalon is an ideal structure with which to test
this hypothesis, given that Gli3 is a central effector in telencephalic patterning and acts mainly as a repressor in this structure (Rallu et al., 2002).

Consistent with the broad distribution and multiple functions of cilia, a large group of human diseases has been linked to primary cilium dysfunction. These pleiotropic and genetically heterogeneous diseases have been collectively termed ciliopathies (Badano et al., 2006; Sharma et al., 2008). We and others recently reported mutations in the human Ftm orthologue RPGRIP1L in Meckel (MKS; OMIM #611561) and Joubert type B (OMIM #611560) syndromes, which are autosomal recessive multisystem ciliopathies (Arts et al., 2007; Delous et al., 2007). The Ftm protein is widely expressed during embryogenesis, is mainly found at the base of primary cilia and is required for normal ciliogenesis in the mouse node, limb buds and spinal cord (Arts et al., 2007; Delous et al., 2007; Vierkotten et al., 2007). Mice with a targeted inactivation of the Ftm gene die around birth and recapitulate most malformations observed in MKS fetuses, including exencephaly, and a strong reduction in motoneuron and V3 interneuron patterning and morphogenesis. We show that non-exencephalic Ftm−/− embryos are affected in the developing brain of Ftm−/− mutants, and the Gli3-190:Gli3-83 ratio is increased.

In this study, we analyze the role of Ftm in telencephalic patterning and morphogenesis. We show that non-exencephalic Ftm−/− fetuses display a mislocalized OB-like (OBL) structure, which does not show morphological outgrowth. We next investigate the developmental origin of this defect in the telencephalon of E12.5 Ftm−/− embryos and observe that most telencephalic neuroepithelial cells lack normal primary cilia. Furthermore, in these mutant embryos, the subpallium is expanded at the expense of the pallium, a phenotype reminiscent of Gli3 mutant mice, and the Gli3-190:Gli3-83 ratio is increased. Interestingly, the introduction of one or two alleles of Gli3−/−, which produces only a short form of Gli3 (Böse et al., 2002), into the Ftm−/− background leads to the rescue of telencephalic patterning and OB morphogenesis, whereas cilia are not restored. Our work thus provides the first functional evidence that Ftm and cilia are essential for OB morphogenesis and that their function in this process is mediated by the control of Gli3R production.

MATERIALS AND METHODS

Mouse strains

Ftm, Gli3−/−, Gli3−/− and Gli3−/− mutant mice were maintained in a C57Bl6/J background. Double heterozygous Ftm−/− Gli3+/− mice were obtained and increased in order to study the phenotype of compound mutant fetuses. PCR genotyping was performed as previously described (Büscher and Rüther, 1998; Böse et al., 2002; Kuschel et al., 2003; Vierkotten et al., 2007). Embryonic day (E) 0.5 was defined as noon on the day of vaginal plug detection.

In situ hybridization, immunofluorescence and EdU staining

For histology and in situ hybridization (ISH), embryos were dissected in cold phosphate-buffered saline (PBS) and fixed overnight in 60% ethanol, 30% formaldehyde and 10% acetic acid. Embryos were embedded in paraffin and sectioned (8 μm). Cresyl thionin staining and ISH were performed as previously described (Anselme et al., 2007). For immunofluorescence, fetuses were perfused with 4% paraformaldehyde. Immunofluorescence stainings were performed on 14 μm serial cryostat sections as described (Anselme et al., 2007), with antibodies against Arl13b (Caspari et al., 2007), acetylated tubulin (Sigma), GABA (Sigma), γ-tubulin (Sigma), Pax6 (Covance), Tbr1 (Chemicon), Tbr2 (Chemicon), Omp (Wako) and tyrosine hydroxylase (Chemicon). Nuclei were counterstained with DAPI. For S-phase cell detection, intraperitoneal injections of pregnant mice were performed with 100-200 μg 5-ethyl-2′-deoxyuridine (EdU) in PBS and embryos were harvested 1 hour later (Salic and Mitchison, 2008). Anti-EdU staining was performed according to the manufacturer’s recommendations (Click-iT EdU Alexa Fluor Imaging Kit, Invitrogen).

Scanning electron microscopy

Embryos were dissected in 1.22× PBS (pH 7.4) and fixed overnight with 2% glutaraldehyde in 0.61× PBS (pH 7.4) at 4°C. Heads were then sectioned to separate the dorsal and ventral parts of the telencephalon, exposing their ventricular surfaces. Head samples were washed several times in 1.22× PBS and postfixed for 15 minutes in 1.22× PBS containing 1% OsO4. Fixed samples were washed several times in ultrapure water, dehydrated with a graded series of ethanol and prepared for scanning electron microscopy using the critical point procedure (CPD7501, Polaron). Their surfaces were coated with a 20 nm gold layer using a gold sputtering device (Scancoat Six, Edwards). Samples were observed under a Cambridge S260 scanning electron microscope at 10 keV.

Transmission electron microscopy

Tissues were fixed for 1 hour with 3% glutaraldehyde, post-fixed in 1.22× PBS containing 1% OsO4, then dehydrated with a graded ethanol series. After 10 minutes in a 1:2 mixture of propane:epoxy resin, tissues were embedded in gelatin capsules with freshly prepared epoxy resin and polymerized at 60°C for 24 hours. Sections (80 nm) obtained using an ultramicrotome (Reichert Ultracut S) were stained with uranyl acetate and Reynold’s lead citrate and observed with a Philips CM10 transmission electron microscope.

Western blotting and quantitative real-time RT-PCR

For western blots, dissected E12.5 telencephalons were frozen in liquid nitrogen and pooled as three or four of the same genotype. Protein extraction and immunoblot were performed as described (Wang et al., 2000) using mouse 6F5 and Gli3 (Genentech; 1:500) or mouse anti-actin (Sigma; 1:3000) antibodies. Statistical significance was evaluated by applying Student’s t-test to the individual relative expression values from five or six independent experiments.

RESULTS

Ftm is required for ciliogenesis in the telencephalon

The Ftm protein is present at the apical side of E12.5 telencephalic neuroepithelial cells, as a single dot distal to the basal body in control (Fig. 1A,C,D) but not Ftm−/− (Fig. 1B) mouse embryos. We investigated whether cilia were affected in the developing brain of Ftm−/− embryos. Staining with an antibody against the cilary protein Arl13b (Caspari et al., 2007) showed the presence of cilia at the apical surface of control but not Ftm−/− brains (Fig. 2A,B).

To confirm this observation, we used scanning electron microscopy to observe the cilia protruding into the telencephalic ventricles. In control embryos (n=11), almost all neuroepithelial cells had a single primary cilium, ~1 μm long (Fig. 2C-F). By contrast, in Ftm−/− embryos (n=6), most cells displayed an abnormal, short and bloated cilium-like protrusion (Fig. 2G-J). Transmission electron microscopy (Fig. 2K-T) confirmed the defect in cilium formation.
An olfactory bulb-like structure forms in Ftm\(^{-/-}\) fetuses

Ftm\(^{-/-}\) fetuses display severe brain abnormalities, including exencephaly, reduced dorsomedial telencephalic structures and OB agenesis (Delous et al., 2007). In the present study, 60% of Ftm\(^{-/-}\) fetuses were not exencephalic and all showed bilateral OB agenesis (n=84) (Fig. 3A). We investigated whether this agenesis reflected an absence of OB cell type specification. The OB has a laminar structure and is composed of two main neuronal types: projection neurons (mitral and tufted cells) and local interneurons (Long et al., 2003). At E18.5, the T-box transcription factor Tbr2 (Eomes – Mouse Genome Informatics) is present at high levels in differentiated OB projection neurons (Fig. 3C,E) and, at a lower level, in OB interneurons in the granular layer and in cortical neuronal progenitors undergoing radial migration and differentiation (Englund et al., 2005; Brill et al., 2009). We found that the pallium of Ftm\(^{-/-}\) fetuses contained an ectopic group of Tbr2-positive cells, distinct from the cortical progenitors (Fig. 3D,F). In this group, many Tbr2-positive cells had a large and round nucleus, a feature characteristic of mitral cells (Fig. 3E,F, insets). However, these cells were localized more dorsally and laterally than in control embryos, and were not properly laminated (Fig. 3D,F). The presence of mitral cells in this ectopic location was confirmed by in situ hybridization (ISH) on brain sagittal sections for Tbr2 (in Ftm\(^{-/-}\) and control mice) (Faedo et al., 2002). We named this ectopic site of mitral cell differentiation an OB-like (OBL) structure (Fig. 3B), in reference to a similar structure described in Pax6 (small eye, Sey) mutant mice (Jimenez et al., 2000). In this ectopic structure, we assessed the presence of OB interneurons, which are born in the dorsal part of the lateral ganglionic eminence (dLGE) and migrate towards the OB via the rostral migratory stream. Both \(\gamma\)-amino butyric acid (GABA)-positive and tyrosine hydroxylase (TH)-positive interneurons were present in the OBL and were not properly laminated, as with the projection neurons (Fig. 3I-L).

Our study of OB cell types at the end of gestation thus shows that OB agenesis in Ftm\(^{-/-}\) fetuses does not result from a cell specification defect, but from abnormal morphogenesis, possibly linked to the aberrant localization of the OBL.

**Ftm\(^{-/-}\) olfactory sensory neurons differentiate but do not contact mitral cells**

OB lamination and outgrowth depend on the arrival of olfactory sensory neuron (OSN) axons (Gong and Shipley, 1995; Hirata et al., 2006). OSNs differentiate in the olfactory epithelium (OE). Their axons enter the anterior telencephalon at ~E11.5 in the mouse, and then make contacts with mitral cell dendrites in glomeruli (Whitesides and LaMantia, 1996; Blanchart et al., 2006). We analyzed both cell differentiation in the OE and the trajectories of OSN axons in Ftm mutants. Differentiated OSNs were present in the OE of Ftm\(^{-/-}\) fetuses (data not shown) and OSN axons positive for the olfactory marker protein (Omp) reached the ventral telencephalon in Ftm\(^{-/-}\) as well as in control fetuses (Fig. 3M-O). However, instead of invading the OB as in controls (Fig. 3M), the mutant OSN axons remained together close to the ventral telencephalon (Fig. 3O) and did not enter the ectopic OBL (Fig. 3N). This lack of connection between OSN axons and mitral cells might contribute to the absence of OBL lamination and outgrowth.

**The OB primordium is specified in an ectopic position in the telencephalon of Ftm\(^{-/-}\) embryos**

In order to trace back the origin of OB agenesis, we investigated earlier stages of telencephalic development. The OB primordia (OBp) originate in the anterior part of the ventral pallium (Cobos et al., 2001; Marin and Rubenstein, 2003). At E13.5 the OBp are morphologically distinguishable as anterior buddings of the neuroepithelium in each telencephalic hemisphere, and express the transcription factor genes Er81 and AP2e (Etv1 and Tcfap2e – Mouse Genome Informatics) (Fig. 4A,C) (Fukuchi-Shimogori and Grove, 2003; Stenman et al., 2003; Feng et al., 2009). In E13.5 Ftm\(^{-/-}\) embryos, we observed Er81- and AP2e-expressing cells in a dorsolateral domain of the telencephalon, although we could not detect any budding of the OBp (Fig. 4B,D). This shows that the OBp is specified in an aberrant position and that OB initial outgrowth is impaired in Ftm\(^{-/-}\) embryos. As OB budding correlates with a sudden reduction in the rate of cell proliferation in the OBp ventricular zone (Gong and Shipley, 1995; Hebert et al., 2003), we analyzed cell proliferation in E12.5 Ftm\(^{-/-}\) embryos. By counting the proportion of neuroepithelial cells in S phase in
different telencephalic domains by EdU incorporation, we found that the proportion of EdU-positive cells in Ftm–/– embryos was similar in the cortex and in the Er81-positive region (the OBL), in contrast to control siblings (Fig. 4E). Thus, although the OBp is present in the Ftm–/– telencephalon, its specific cell cycle properties are impaired.

**Ventral structures are expanded dorsally in the anterior telencephalon of Ftm–/– embryos**

The dorsolateral position of the OBp in E12.5 Ftm–/– embryos could suggest dorsoventral (DV) telencephalic patterning defects. In order to test this hypothesis, we analyzed the expression of Ngn2 (Neurog2 – Mouse Genome Informatics) and Pax6 as dorsal markers and Dlx2 and Gsx2 (Gsh2) as ventral markers. Dlx2 and Ngn2 are expressed in mutually exclusive ventral (subpallial) and dorsal (pallial) domains and present a common limit at the pallial-subpallial boundary, whereas Pax6 and Gsx2 expression domains overlap in the dorsal subpallium (the dLGE) (Yun et al., 2001). The parasagittal section plane we used allowed us to analyze their relationships in the most anterior telencephalon. In control embryos, Dlx2 and Ngn2 were expressed in mutually exclusive areas in the most anterior telencephalon (Fig. 4F,H) and the OBp formed in the dorsal Ngn2-positive domain, just above this gene expression boundary (Fig. 4A,C,F,H). The anterior expression limit of Gsx2 was similar to that of Dlx2 (Fig. 4H,L), and the Pax6 domain partially overlapped with that of Gsx2 and Dlx2 (Fig. 4H,L). In Ftm–/– embryos, dorsal Ngn2 and Pax6 expression was markedly reduced in the anterior telencephalon (Fig. 4G,K), whereas the Dlx2 and Gsx2 expression domains were expanded anteriorly and dorsally to span the most anterior telencephalon (Fig. 4L,M). Interestingly, Er81-expressing cells were located in the Ngn2-positive dorsal domain, just above the ectopic Ngn2/Dlx2 boundary (Fig. 4B,D,G,I). The analysis of E14.5 telencephalic coronal sections confirmed the dorsal expansion of ventral structures in the anterior Ftm–/– telencephalon and revealed milder defects in more caudal regions of the telencephalon, where the pallial-subpallial boundary was less distinct in Ftm–/– than in control fetuses (see Fig. S1 in the supplementary material). Thus, the dorsal expansion of ventral structures is limited to the most anterior part of the telencephalon. A similar, although milder, patterning defect was observed in the anterior telencephalon of E10.5 Ftm–/– embryos (see Fig. S2 in the supplementary material).

Taken together, our data point to a dorsal expansion of ventral structures in the anterior telencephalon of Ftm mutants, and strongly suggest that the aberrant localization of the OBp results from this DV patterning defect. This phenotype is reminiscent of that of Gli3Xt and Gli3Pdn mutants, in which Dlx2 expression is expanded dorsally in the anterior telencephalon at E12.5 (Kuschel et al., 2003). Notably, Gli3Xt mutants lack OBs (Johnson, 1967; Balmer and LaMantia, 2004). We compared the Gli3 and Ftm mutant phenotypes in greater detail and found that both Gli3Xt and Gli3Pdn/Pdn embryos had an ectopic, more dorsal OBp in the E13.5 telencephalon and that the anterior Ngn2/Dlx2 boundary was shifted dorsally in both mutants (see Fig. S3 in the supplementary material). The phenotype of the Gli3Pdn/Pdn telencephalon was very similar to that of Ftm–/–, whereas Gli3Xt embryos were more

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**Fig. 2. Impaired ciliogenesis in the telencephalon of Ftm–/– embryos.**

(A,B) Immunofluorescence on telencephalic sections of control (labeled Ftm+/+ or FtmX+/x or Ftm+/+) (A) and Ftm–/– (B) mouse embryos with antibodies to Arl13b (cilium) and γ-tubulin (basal body). Nuclei are counterstained with DAPI (blue).

(C-J) Scanning electron microscopy of the ventricular surface of the subpallium (C-E,G-I) and pallium (F,J) of control (C-F) and Ftm–/– (G-J) E12.5 embryos. In H, the arrowheads point to rounded and short cilia and the arrow points to a normal cilium.

(K-T) Ultrastructure of telencephalic neuroepithelial cells and primary cilia by transmission electron microscopy of control (K-O) and Ftm–/– (P-T) E12.5 embryos. (K,L,P-R) Longitudinal sections through cilia; white arrowheads point to basal bodies (bb). Black arrows (Q,R) point to small vesicles in a ciliary compartment in Ftm–/– embryos. (M) Transverse section through the axoneme of a primary cilium. (N,S) Transverse sections through basal bodies. tj, tight junction. Scale bars: 1 mm in C,F,G,J; 10 μm in A,B; 1 μm in D,E,H,I,O,T and insets in F,J; 200 nm in K,P-R; 50 nm in L,N,S.
severely affected. Nevertheless, an OBL was present in the E18.5 Gli3<sup>Xt/Xt</sup> dorsal telencephalon (see Fig. S3 in the supplementary material).

**Gli3 processing and function are impaired in the Ftm<sup>+/−</sup> telencephalon**

The similarity between Ftm<sup>−/−</sup> and Gli3<sup>Pdn/Pdn</sup> telencephalic phenotypes suggests that the absence of Ftm could lead to decreased Gli3 expression levels and/or impaired Gli3 protein activity in the telencephalon. Neither RT-PCR nor ISH on E12.5 coronal sections detected any significant change in Gli3 mRNA levels in Ftm<sup>−/−</sup> telencephalon as compared with control littermates (Fig. 5A-C). Thus, the DV patterning defect is not caused by a reduction in Gli3 expression levels.

An altered Gli3-190:Gli3-83 ratio has been described in a number of IFT mutant mice (Haycraft et al., 2005; Liu et al., 2005; May et al., 2005). Consistent with the IFT-like phenotype of Ftm mutants, an increased Gli3-190:Gli3-83 ratio was described for E11.5 Ftm<sup>−/−</sup> whole embryos (Vierkotten et al., 2007). Since cilia function may have different effects on Gli transcription factors depending on the tissue and stage (Huangfu and Anderson, 2005; Eggenschwiler and Anderson, 2007), we examined the Gli3-190:Gli3-83 ratio by western blot on E12.5 telencephalic extracts (Fig. 5D). In control telencephalic extracts, Gli3-83 was 5-fold more abundant than Gli3-190 (Fig. 5D,E). In the telencephalon of Ftm<sup>−/−</sup> fetuses, although the total amount of Gli3 protein was similar to that in the control, the level of Gli3-190 was elevated and Gli3-83 was lowered, leading to an inverted ratio, with Gli3-190 being 8.5-fold more abundant than Gli3-83 (Fig. 5D,E). This reduction in the abundance of Gli3-83 correlated with a reduction in Gli3R activity, as dorsomedial telencephalic structures, which depend on Gli3 for their formation (Theil et al., 1999; Tole et al., 2000; Kuschel et al., 2003), were reduced in size and were misshapen (see Fig. S4 in the supplementary material). These data demonstrate that, in the telencephalon, Ftm is required for the proper processing of Gli3 into its repressor form.

Both FGF signaling from the ANR and Hh signaling from the ventral telencephalon have been implicated in the specification of ventral telencephalic fates (Hebert and Fishell, 2008). Moreover, alien (Tie2<sup>ib</sup>) mutants affected in retrograde IFT display a ventralized telencephalon caused by increased Hh expression and signaling (Stottmann et al., 2009). The telencephalic phenotype of...
**Fig. 4. OB primordium formation and telencephalic dorsoventral patterning at E12.5-E13.5.** (A-D) ISH on parasagittal sections of E13.5 control (A,C) and Ftm−/− (B,D) mouse brains with Er81 (A,B) and AP2e (C,D) probes, showing early cell differentiation in the OB primordium (OBp, arrow in A). Insets in B and D are higher magnifications of the OBp-specific cells that are present in an ectopic position in the Ftm−/− telencephalon. (E) Quantification of EdU incorporation, which marks cells in S phase, in different telencephalic domains: cortex versus OB or OBL regions in E12.5 control (Ctl) and Ftm−/− embryos. Error bars indicate mean relative expression values ± s.e.m. (F-M) ISH on parasagittal sections of E12.5 control (F,H,I,L) and Ftm−/− (G,I,K,M) embryos with Ngn2 (F,G), Dlx2 (H,I), Pax6 (I,K) and Gsx2 (L,M) probes. Dashed lines indicate the anterior boundaries of the gene expression domains. These boundaries are shifted dorsally in Ftm−/− embryos (G,I,K,M). Scale bars: 500 μm.

Ftm mutants could therefore result from increased FGF or Hh pathway activity. Fgfl8 expression and signaling from the ANR were not obviously altered in Ftm−/− embryos from E8.5 to E12.5 (see Fig. S5 in the supplementary material; data not shown). We tested Hh pathway activity by examining the expression of four target genes, Shh, Gli1, Ptc1 and Nkx2.1, that are known to respond differentially to Hh signaling in the telencephalon (Rallu et al., 2002). At E9.5-E12.5, all four genes were expressed in the ventral telencephalon with distinct patterns (Fig. 5F-M; data not shown).

In the E9.5 telencephalon, Shh and Nkx2.1 expression was similar in Ftm−/− and controls (data not shown). At E12.5, telencephalic expression of Shh, Gli1 and Nkx2.1 was maintained or slightly reduced (Fig. 5G,I,K) and Gli1 expression was more scattered (Fig. 5I) in Ftm−/− than in control embryos. By contrast, Ptc1 was expanded dorsally (Fig. 5M). The upregulation of Ptc1 expression was confirmed by quantitative RT-PCR from Ftm−/− telencephalic extracts (Fig. 5N). Interestingly, we detected a similar Ptc1 expansion in Gli3Xt/Xt embryos (data not shown), confirming that Ptc1 does not require Gli3A for its activation (Rallu et al., 2002). These results argue against an increase in the Hh pathway and/or Gli3A activity in the Ftm−/− telencephalon. The dorsal expansion of the Ptc1 expression domain and the dorsal shift of the anterior telencephalic DV boundary might therefore result from a reduction in Gli3R activity. Together, these data strongly suggest that the PVN patterning defect of the Ftm−/− anterior telencephalon is due to a reduction in Gli3R production.

**Gli3Xt/Xt rescues telencephalic patterning and OB morphogenesis defects in Ftm−/− fetuses**

If, as we propose, the telencephalic patterning defects in Ftm−/− embryos result from a reduction in Gli3R levels, it should be possible to rescue these defects by introducing a constitutively active repressor form of Gli3 into the Ftm mutant background. To test this, we used Gli3Xt/Xt mice, obtained by the knock-in of a mutation identified in human Pallister-Hall syndrome into the Gli3 locus (Böse et al., 2002). The Gli3Xt allele exclusively produces a short (88 kDa) form of Gli3 (Fig. 5D) that lacks the transactivation domain (Böse et al., 2002; Hill et al., 2007). We first analyzed telencephalic development in Gli3Xt/Xt mice, which had not been described previously. Telencephalic DV patterning was unaffected in E12.5 Gli3Xt/Xt embryos: expression of Ngn2 (Fig. 6D) and Dlx2 (Fig. 6G) was similar to that of control embryos (Fig. 4F,H). Moreover, in these mice, the OBp formed at the correct position, as shown by Er81 expression, and presented normal outgrowth (compare Fig. 6A with Fig. 4A). At E18.5, the OB was formed normally, the different OB cell types were correctly specified and laminated, and OSN axons reached and entered the OB nerve layer (Fig. 7A,C,E,G,I). This demonstrates that the activator form of Gli3 is dispensable for telencephalic patterning and OB morphogenesis and that the telencephalic defects described in Gli3Xt/Xt mutants result exclusively from the loss of Gli3R.

We then crossed Ftm mice with Gli3Xt/Xt mice and analyzed telencephalic development in Ftm−/−; Gli3Xt/Xt and Ftm−/−; Gli3Xt/Xt embryos. At E12.5, we observed complete rescue of the forebrain DV patterning defects: Ftm−/−; Gli3Xt/Xt embryos showed the correct expression pattern of Ngn2 (Fig. 6E), Dlx2 (Fig. 6H) and Er81 (Fig. 6J). Moreover, the OBp bulged out anteriorly as in control embryos, suggesting that initial OB morphogenesis was also restored. A partial rescue was observed in Ftm−/−; Gli3Xt/Xt embryos, with a slightly more dorsal position of the Dlx2/Ngn2 boundary and of the OBp (Fig. 6C,F,I) and incomplete OBp bulging (Fig. 6C). Strikingly, primary cilia were totally absent from the telencephalon of E12.5 Ftm−/−; Gli3Xt/Xt embryos (Fig. 6J,K).

We next analyzed in greater detail the extent of OB morphogenesis in E18.5 fetuses. We did not obtain Ftm−/−; Gli3Xt/Xt fetuses at this stage. In contrast to Ftm−/− fetuses (Fig. 3), Ftm−/−; Gli3Xt/Xt fetuses displayed OB outgrowth in the expected rostral position (Fig. 7). This OB contained specific layers...
of projection neurons (cells positive for Tbr1, Tbr2 and Er81) (Fig. 7B,D; data not shown) and mature interneurons (cells positive for Tbr1, Tbr2 and Er81) (Fig. 7C). Cilia shape the telencephalon via Gli3R function of the primary cilium in telencephalic patterning and morphogenesis. Indeed, Ftm–/– fetuses display a severe phenotype that resembles that of IFT mutants and has significant similarities with human MKS, the most severe ciliopathy (Delous et al., 2007; Vierkotten et al., 2007). Moreover, a large proportion of Ftm homozygous mutants survive until birth, thus allowing brain morphological and anatomical analyses. In this paper, we show that OB agenesis in Ftm mutants originates from an early absence of OB outgrowth that is associated with DV patterning defects in the anterior telencephalon. Moreover, we demonstrate that a reduction in Gli3R levels is the sole cause of the observed telencephalic defects.

**DISCUSSION**

Ftm mutant mice are an excellent model with which to study the function of the primary cilium in telencephalic patterning and morphogenesis. Indeed, Ftm–/– fetuses display a severe phenotype that resembles that of IFT mutants and has significant similarities with human MKS, the most severe ciliopathy (Delous et al., 2007; Vierkotten et al., 2007). Moreover, a large proportion of Ftm homozygous mutants survive until birth, thus allowing brain morphological and anatomical analyses. In this paper, we show that OB agenesis in Ftm mutants originates from an early absence of OB outgrowth that is associated with DV patterning defects in the anterior telencephalon. Moreover, we demonstrate that a reduction in Gli3R levels is the sole cause of the observed telencephalic defects.

The function of cilia in the mouse spinal cord has been extensively studied, whereas only a couple of previous studies have addressed cilium function in the developing brain, and the mechanisms of OB morphogenesis have not been examined. Cilia are required after birth for Hh-dependent proliferation of granule neuron precursors in the cerebellum and dentate gyrus (Chizhikov...
et al., 2007; Han et al., 2008; Spassky et al., 2008). Ift172 null (Sib) mouse mutants lack telencephalic cilia and present a severe reduction in forebrain size that is attributed to an abnormal specification of the anterior mesendoderm during gastrulation (Gorivodsky et al., 2009). Sib mutants die at E12.5, precluding later analysis of brain morphogenesis. Ifb88 hypomorphic (cobblestone, Cbs) mouse mutants show an abnormal morphology of dorsomedial telencephalic structures with rosette-like heterotopias, and present a fuzzy pallial-subpallial boundary (Willaredt et al., 2007). This latter feature is shared by both Dnchc2 (Dyne2h1 – Mouse Genome Informatics) mutants, which are affected in retrograde IFT (May et al., 2005), and Ftm mutants (present study). Interestingly, alien mutant mice defective in retrograde IFT show telencephalic DV patterning defects and OB agenesis similar to Ftm mutants, and this has been associated with enhanced Hh signaling (Stottmann et al., 2009). Contrary to Ftm mutants, Cbs and alien embryos display telencephalic cilia, suggesting that some ciliary functions are still present in these mice. Our results therefore advance our understanding of the role of primary cilia in the patterning and morphogenesis of the anterior telencephalon.

OB agenesis is very uncommon in mouse mutants, suggesting that OB specification and budding are controlled by robust mechanisms. Only mutants displaying a very reduced pallium, such as Emx1/2 double knockout, Pax6<sup>628y</sup> and Gli3<sup>251</sup> mice show OB agenesis (Johnson, 1967; Anchan et al., 1997; Dellovade et al., 1998; LaMania, 1999; Bishop et al., 2003; Balmer and LaMania, 2004). The similarity of the OB phenotype in Ftm, Pax6<sup>628y</sup> and Gli3<sup>251</sup> mutants suggests that it is secondary to a patterning defect. Consistently, we observed DV patterning defects in the anterior telencephalon as early as E10.5, 2 days before OB budding.

Two non-exclusive hypotheses could account for the absence of OB budding in Ftm mutants and its rescue by Gli3<sup>3699</sup>. First, Ftm (and Gli3R) could be required within the OBp for morphogenesis. Second, the OBL could form in a region that is not permissive for budding. Conditional activation of Ftm in the OB will be required to discriminate between these two hypotheses. At the cellular and molecular levels, several mechanisms can be proposed to explain the OB outgrowth defect. First, the arrival of OSN axons has been proposed to be important for OB lamination and outgrowth (Gong and Shipley, 1995; Garel et al., 2003; Hebert et al., 2003; Stout and Graziaedi, 1980; Gong and Shipley, 1995; Whitesides and LaMania, 1996; Balmer and LaMania, 2005). In Ftm mutants, the OSN axons are correctly targeted towards the anterior telencephalon, but very few enter the brain, similar to what had been reported for Gli3<sup>3699</sup> mice (St John et al., 2003; Balmer and LaMania, 2004; Hirata et al., 2006; Watanabe et al., 2009). Thus, the absence of contact between mitral cells and OSN axons might participate in OB outgrowth defects. Second, the OB normally forms in an anterior region close to the ANR source of FGF signals and to the frontonasal mesenchymal source of retinoic acid (RA), which are both required for OB formation (Meyers et al., 1998; Garel et al., 2003; Hebert et al., 2003; Meyer and Roelink, 2003; Balmer and LaMania, 2005). In Ftm mutant mice, the OBL forms further away from the ANR, in a region that might not receive enough of these signals. It has been proposed that a reduction in the rate of proliferation is involved in OBp outgrowth (Gong and Shipley, 1995). In Fgrl1 mutants, the rate of cell division fails to decrease in the OBp, and this correlates with the delay in OB outgrowth (Hebert et al., 2003). Similarly, in Ftm mutants, we find that the proportion of S-phase nuclei fails to decrease in the OBp, and this correlates with the delay in OB outgrowth (Hebert et al., 2003). In Ftm mutant mice, opposite phenotypes are found in different regions of the central nervous system. In the spinal cord, Ftm mice, like many other ciliary mutants, display a reduction in ventral cell
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types. Conversely, in the anterior telencephalon, we show that subpallial (ventral) markers are expanded at the expense of pallial (dorsal) markers, whereas the most ventral (Nkx2.1-positive) cell types appear unaffected. We cannot exclude the possibility that these differences result from regional differences in the total amount of Gli3 or in the precise Gli3A:Gli3R ratio. However, we favor an alternative hypothesis, in which region-specific responses to cilium defects result from differences in the relative importance of the activator and repressor forms of Gli2 and Gli3 along the anteroposterior axis of the neural tube. In the spinal cord, ventral cell type specification is regulated by a balance between Gli2A (and, to a lesser extent, Gli3A) and Gli3R (Ding et al., 1998; Theil et al., 1999; Park et al., 2000; Tole et al., 2000; Bai et al., 2002; Motoyama et al., 2003). In the telencephalon, Gli3A is dispensable for global DV patterning, as judged by the absence of telencephalic defects in Gli3A/+ deleted embryos (present study). By contrast, Gli3R is a major player in telencephalic DV patterning, being essential for both the specification of dorsal fates and the repression of ventral fates (Theil et al., 1999; Tole et al., 2000; Aoto et al., 2002; Rallu et al., 2002; Kuschel et al., 2003). The function of cilia in Gli3R production is thus of prime importance in this process.

The last decade has revealed multiple functions for primary cilia, acting as cellular antennae in sensory perception and signaling. In this context, it is essential to understand how the cilia controls each developmental process. In particular, the developing telencephalon is under the influence of several signaling pathways emanating from different organizing centers. Our findings help to clarify how primary cilia constrain telencephalic morphogenesis by allowing Gli3R production and hence regulating downstream targets of this transcription factor. Moreover, the frequent observation of encephaloceles and the occurrence of OB agenesis in cases of MKS (Ahdab-Barmada and Claassen, 1990; Baala et al., 2007; Khaddour et al., 2007; Sharma et al., 2008) make our findings highly relevant for the physiopathology of this disease.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Meyer, N. P. and Roelink, H.
Marshall, W. F.

Huangfu, D. and Anderson, K. V.
Hui, C. C. and Joyner, A. L.


Whitmore, J. G., 3rd and LaMantia, A. S.