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Patterning the Dorsal Telencephalon: A Role for Sonic Hedgehog?

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Division of the telencephalic vesicle into hemispheres and specification of the cerebral cortex are key stages in forebrain development. We investigate the interplay in these processes of Sonic hedgehog (Shh), fibroblast growth factors (Fgfs), and the transcription factor Gli3, which in its repressor form (Gli3R) antagonizes Shh signaling and downregulates expression of several *Fgf* genes.

Contrary to previous reports, Shh is not required for dorsal hemisphere separation. Mice lacking Shh develop a dorsal telencephalic midline, a cortical hem, and two cortical hemispheres. The hemispheres do not divide rostrally, probably because of reduced local *Fgf* gene expression, resulting from the loss of Shh inhibition of Gli3R. Removing one functional copy of *Gli3* substantially rescues *Fgf* expression and rostral telencephalic morphology.

In mice lacking Gli3 function, cortical development is arrested, and ventral gene expression invades the dorsal telencephalon. These defects are potentially explained by disinhibition of Shh activity. However, when both copies of *Shh* are removed from Gli3-null mice, dorsal telencephalic defects persist. One such defect is a large dorsal expansion of the expression of *Fgf* genes. *Fgf15* expression, for example, expands from a discrete ventral domain throughout the dorsal telencephalon. We propose that Fgf signaling, known to ventralize the telencephalon in a Shh-independent manner, suppresses cortical fate in the absence of Gli3. Our findings point away from Shh involvement in dorsal telencephalic patterning and encourage additional exploration of Fgf signaling and Gli3 repression in corticogenesis.

Key words: **sonic hedgehog; Gli3; fibroblast growth factors; telencephalon; cerebral cortex; roofplate; cortical hem**

Introduction

The mammalian telencephalon comprises the cerebral cortex and subcortical nuclei required for higher brain functions. Yet this complex structure is generated from a primitive neuroepithelium by the same molecular patterning mechanisms that operate generally in the embryo. This conceptual framework has simplified and accelerated investigation of telencephalic development (Wilson and Rubenstein, 2000; Ohkubo et al., 2002; Grove and Fukuchi-Shimogori, 2003).

Considerable evidence demonstrates that dorsoventral (D/V) patterning in the telencephalon shares mechanisms with D/V patterning of the spinal cord. Sonic hedgehog (Shh) signaling mediated by Smoothened (Smo) promotes ventral telencephalic structure (Chiang et al., 1996; Kohtz et al., 1998; Rallu et al., 2002; Fuccillo et al., 2004), and key features of dorsal development require bone morphogenetic protein (BMP) or Wingless-Int (Wnt) signaling (Lee et al., 2000; Hebert et al., 2002, 2003; Cheng et al., 2006), as in the spinal cord (Echelard et al., 1993; Ericson et al., 1995; Roelink et al., 1995; Liem et al., 1995, 1997; Backman et al., 2005).

Ventral telencephalic and spinal cord development is further regulated by antagonistic interactions between Shh signaling and the repressor form of Gli3 (Gli3R), a member of the Gli family of transcription factors (Litingtung and Chiang, 2000; Rallu et al., 2002). Shh inhibits the processing of Gli3 to Gli3R; thus, in *Shh* mutants, Gli3R is produced in excess. Illustrating the antagonism, ventral cell types are lost in both the spinal cord and telencephalon of mice deficient in *Shh* or *Smo*, but substantially rescued in compound mutants that also lack *Gli3* (Litingtung and Chiang, 2000; Rallu et al., 2002). These data support a model in which a still-unidentified factor ventralizes the neural tube, is functionally inhibited by Gli3R, and is disinhibited by Shh-mediated reduction of Gli3R.

Previous analyses of the telencephalon in *Shh*, *Gli3*, and compound mutant mice suggest that not all basic telencephalic patterning mechanisms are shared with other parts of the embryo. In the *Shh* mutant, the dorsal midline of the spinal cord is marked by formation of the roofplate; in contrast, the *Shh* mutant telencephalon reportedly lacks a dorsal midline and fails to divide into hemispheres (Chiang et al., 1996). How Shh induces the dorsal midline selectively in the telencephalon has been a longstanding mystery (Hayhurst and McConnell, 2003).

In mice lacking functional Gli3, no layered cortex develops, and gene expression characteristic of the ventrolateral telencephalon expands dorsally (Theil et al., 1999; Tole et al., 2000; Theil,

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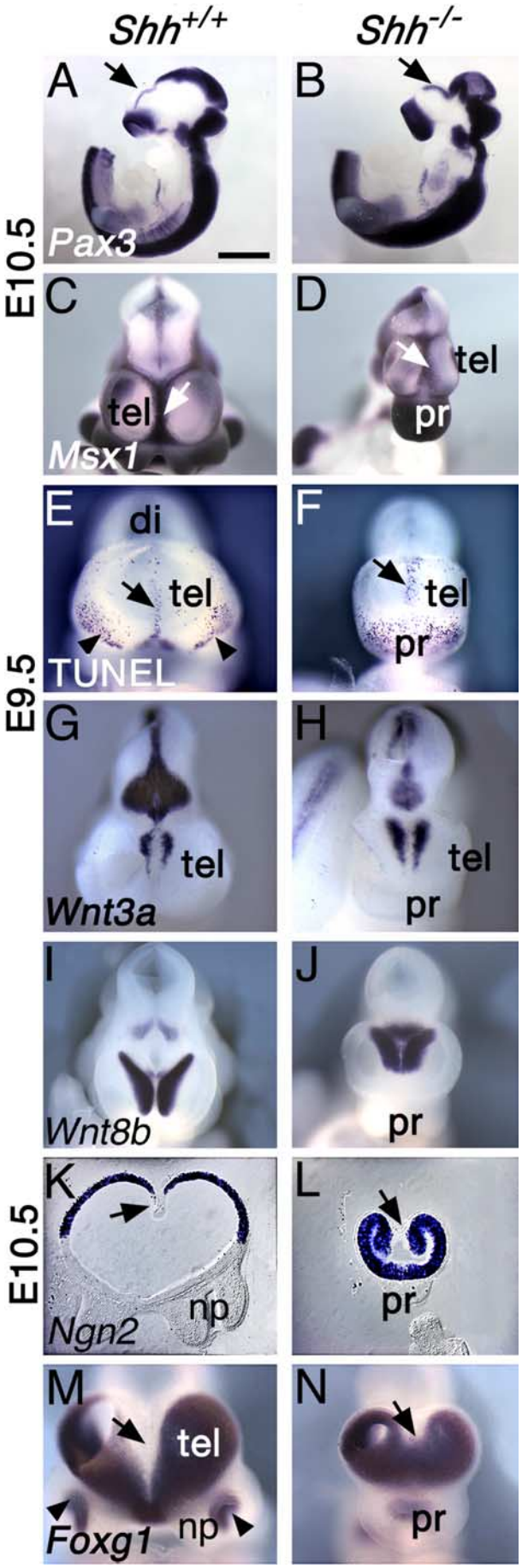

2005), suggesting that Shh signaling activity is de-repressed and inhibits dorsal telencephalic development (Tole et al., 2000; Rallu et al., 2002). Consistent with this explanation, dorsal telencephalic defects appear rescued in *Gli3;Shh* double mutants (Rallu et al., 2002). These observations suggest a second part to the model above: a dorsalizing factor in the telencephalon is inhibited by Shh and disinhibited by Gli3-mediated suppression of Shh signaling (Rallu et al., 2002). Notably, however, this type of interaction between Shh and Gli3 is not general; that is, defects caused by *Gli3* deficiency at other embryonic sites, including the spinal cord, are not rescued by removing Shh or Smo function (Aoto et al., 2002; Litingtung et al., 2002; Persson et al., 2002).

In the present study, we investigate whether the dorsal telencephalon is patterned by specialized Shh/Gli3 activities. In doing so, we attempt to clarify the induction of the telencephalic dorsal midline, and, more broadly, specification of cerebral cortex in the dorsal telencephalon.

Materials and Methods

Mouse lines. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago, and mice were used according to National Institutes of Health guidelines. Noon of the day of vaginal plug detection was termed embryonic day 0.5 (E0.5).

Shh mutant mice (a gift from P. Beachy, Johns Hopkins University School of Medicine, Baltimore, MD) were obtained in a mixed C57BL/6/129 background used in the initial analysis of this mutant (Chiang et al., 1996; C. Chiang, personal communication). A background strain characterization by Charles River Laboratories (Wilmington, MA) confirmed the mixed background. To decrease variability, and potentially reduce exencephaly in *Shh*/*Gli3* compound mutants (see below), we increased the contribution of the C57BL/6 strain over five generations of additional crossbreeding. All *Shh* nulls from each generation showed similar gross abnormalities, including cyclopia, a facial proboscis, reduction of limb and tail bud, and loss of digits. Features of D/V forebrain development, described here, were qualitatively indistinguishable across generations. $Shh^{-/-}$ embryos of a given age were therefore pooled for analysis. No $Shh^{-/-}$ mice were excluded.

We found no evidence in our mice for a reduction in the deficits caused by Shh. Indeed, older $Shh^{-/-}$ embryos were recovered at lower rates than previously (Chiang et al., 1996), suggesting greater earlier lethality. Between E9.5 and E12.5, however, *Shh* mutants were recovered at Mendelian ratios (at E10.5, 51 $Shh^{+/+}$, 87 $Shh^{+/-}$, and 51 $Shh^{-/-}$).

Existing evidence indicates that the *extra-toes*^{J} (Xt^J) mutation is a loss-of-function mutation in the Gli transcription factor gene, *Gli3* (Buscher et al., 1998). Xt^J/Xt^J embryos can have a high incidence of midbrain exencephaly, with gross morphological changes that confound forebrain analysis. Following previous studies (Theil et al., 1999; Tole et al., 2000; Rallu et al., 2002; Kuschel et al., 2003; Theil, 2005), we restricted forebrain analysis to non-exencephalic Xt^J/Xt^J embryos. Xt^J mice maintained on a C57BL/6 background (gift from Y. Furuta, Program in Genes and Development University of Texas, Houston, TX) show decreased exencephaly; thus, we discarded only 1 of 34 Xt^J/Xt^J E10.5 embryos.

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Figure 1. Telencephalic dorsal midline development in the *Shh* null forms cerebral hemispheres. Whole-mount and coronal section (***K***, ***L***) *in situ* hybridization in wild-type ($Shh^{+/+}$; left column) and Shh-null ($Shh^{-/-}$; right column) embryos at E9.5 (***E–H***) and E10.5. Embryos are shown in lateral (***A***, ***B***), dorsal (***C***, ***D***, ***G–J***), and frontal views (***E***, ***F***, ***M***, ***N***). ***A–F***, *Pax3* and *Msx1* expression, and TUNEL-labeled apoptotic cells mark midline neuroepithelium (***A***, ***B***, ***E***, ***F***, arrows); *Msx1* is also expressed in mesenchyme invading between the hemispheres (***C***, ***D***, arrows). ***G–L***, Expression of *Wnt3a* and *Wnt8b* shows separation between two cerebral hemispheres (***G–J***), as does the lack of *Ngn2* expression at the dorsal midline (***K***, ***L***). *Foxg1* expression reveals rostral continuity of the hemispheres (compare ***M***, ***N***; arrows indicate interhemispheric fissure). Scale bar: ***A***, ***B***, 1.2 mm; ***C***, ***D***, ***I***, ***J***, 700 μm; ***E–H***, 540 μm; ***K–N***, 580 μm. Abbreviations: di, Diencephalon; tel, telencephalon; pr, proboscis; np, nasal primordia.

Table 1. Numbers of *Shh* mutant mice with dorsal midline features and dorsal but not rostral hemisphere separation

	Feature	Marker	Number of embryos	Age of embryos
Dorsal midline formation	Roofplate	*Pax3*	3 of 3	E10.5
		Noggin	3 of 3	E10.5
		Cell death	6 of 6	E9.5–E10.5
Dorsal hemisphere division	Deep interhemispheric fissure	Morphology	18 of 18	E12.5–E17.5
	Bilateral cortical hem	*Wnt3a*, *Lmx1a*, *Bmp4*	16 of 16	E9.5–E10.5
	Cortical neural progenitor zones separate dorsally	*Ngn2*, *Emx1*, *Emx2*, *Foxg1*	15 of 15	E10.5–E12.5
	Bilateral medial gene expression	*Wnt8b*	3 of 3	E10.5–E11.5
Lack of rostral hemisphere division	Absence of rostral interhemispheric fissure	Morphology	18 of 18	E12.5–E17.5
	Cortical neural progenitor zones fused rostrally	*Ngn2*, *Emx1*, *Emx2*, *Foxg1*	15 of 15	E10.5–E12.5

Mice heterozygous for both the Xt^J and *Shh* mutations were intercrossed to obtain *Shh;*Xt^J compound mutants. At E10.5, 194 embryos were recovered. Of 43 $Shh^{-/-}$;*Gli3*/Xt^J mice, only 1 or 2 were exencephalic; in contrast, 12 of 16 $Shh^{-/-}$;Xt^J/Xt^J mutants were exencephalic. Exencephalic mutants were not analyzed further. PCR genotyping for *Shh* and Xt^J mice was performed as described previously (Chiang et al., 1996; Maynard et al., 2002).

In situ *hybridization, tissue processing, and imaging.* Embryos were fixed and processed for *in situ* hybridization (Grove et al., 1998). Some brains were labeled with terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) to identify apoptotic cells using an ApopTag kit (Chemicon, Temecula, CA) followed by an alkaline phosphatase nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color reaction. Processed tissue was imaged using a Leica (Nussloch, Germany) dissecting microscope for whole-mount embryos, and a Zeiss (Oberkochen, Germany) Axioskop for sections, and photographed using Zeiss Axiovision software.

Results

Expression of at least some genes associated with the dorsal telencephalon, *Bmp2*, *Bmp7*, *Msx1*, and *Msx2*, has been reported at increased levels in the *Shh* mutant telencephalon (Ohkubo et al., 2002). Here, a systematic study of the mutant telencephalon at a wide range of embryonic ages revealed an array of characteristic dorsomedial features.

The dorsal midline forms and hemispheres separate in the absence of Shh

As previously observed, the *Shh* mutant forebrain, like the entire CNS, is much smaller than in wild type. This reflects loss of the proliferative Shh signal, with consequent repression by Gli3R of Wnt/β-catenin mitogenic signaling (Ishibashi and McMahon, 2002; Ulloa et al., 2007). Nonetheless, a dorsal midline is identifiable in the *Shh* mutant telencephalon.

By E9.5–E10.5 in control and $Shh^{-/-}$ mice, the roofplate is marked by gene expression of the transcription factor Pax3, locally increased cell death, and expression of *Msx* genes associated with apoptosis (Fig. 1*A–F*; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The cerebral hemispheres separate along the dorsal midline (Fig. 1*C–N*), and a dorsal interhemispheric fissure persists to birth (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The *Shh* mutant telencephalon is therefore not an undivided holosphere as initial observations suggested (Chiang et al., 1996). Dorsal separation is unmistakable in frontal views of the *Shh* mutant forebrain processed for the telencephalic marker gene *Foxg1* (Tao and Lai, 1992) (Fig. 1*M*,*N*). In the same views, it is also clear that the rostralmost telencephalon does not divide. The dorsal midline is further marked in mutant and control mice by the absence of *Ngn2* expression (Fig. 1*K*,*L*, arrows). Features indicating formation of the dorsal midline and interhemispheric fissure, with a missing rostral fissure, were consistent across all *Shh* mutants assessed (Table 1).

Dorsomedial structures appear on both sides of the midline

The roofplate, defined as a band of tissue along the midline of the telencephalic vesicle, is required for development of the cortical hem (Chizhikov and Millen, 2005; Cheng et al., 2006), a second telencephalic signaling center at the dorsomedial edge of each hemisphere (Furuta et al., 1997; Grove et al., 1998). Between E9.5 and E10.5, expression of *Wnt3a*, *Bmp4*, and *Lmx1a* indicates that a hem has formed in both hemispheres in *Shh* mutants as in controls (Fig. 1*G*,*H*; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). By E10.5, *Wnt3a* and *8b* expression patterns give the impression that the hem in each hemisphere has fused across the caudal midline (Figs. 1*I*,*J*, 4*A*,*E*). These patterns reflect, instead, an upregulation of *Wnt* gene expression in the diencephalon (B. G. Rash and E. A. Grove, manuscript in preparation), which, because of reduced cell proliferation, is almost unidentifiable (Chiang et al., 1996; Ishibashi and McMahon, 2002).

Additional observations demonstrate that, like the roofplate, the cortical hem is functional in *Shh* mutant mice. The cortical hem provides Wnt3a, a required cell proliferation signal for hippocampal growth (Lee et al., 2000), and is the origin of most Cajal–Retzius (C–R) cells for the hippocampus and neocortex (Bielle et al., 2005; Yoshida et al., 2006). At E10.5, *Wnt8b* expression marks the hem and hippocampal primordium in wild-type mice (Lee et al., 2000); similar broad bands of *Wnt8b* expression appear in the *Shh* mutant telencephalon (Fig. 1*I*,*J*, Table 1). At E17.5, presumptive hippocampal field CA3 expresses *KA1*/*Grik 4*, which encodes a glutamate receptor subunit, whereas CA1 and neocortical primordium express the POU domain gene, *SCIP*/*Pou3f1* (Fig. 2*A*,*C*) (Tole et al., 1997). In the *Shh* mutant, complementary expression of *KA1*/*Grik 4* and *SCIP*/*Pou3f1* indicates medial telencephalic differentiation reminiscent of the wild-type hippocampus. Indeed, the distinct, medial domain of *KA1*/*Grik 4* in the mutant strongly suggests a nascent CA3 field (Fig. 2*B*,*D*). Further consistent with a functional cortical hem (Yoshida et al., 2006), *Reelin*-expressing, putative C–R cells populate the cortical marginal zone in the *Shh* mutant ($n = 6$ of 6) (Figs. 2*G*,*H*, 3*F*,*J*). Thus, substantial development of dorsomedial structures appears in each hemisphere of the *Shh* mutant mouse.

Cortical primordium develops both dorsally and ventrally in Shh nulls

Patterns of gene expression characteristic of the cortical ventricular zone, including *Emx1*, *Ngn2*, and *Pax6*, extend ectopically into the ventral telencephalon (Chiang et al., 1996; Rallu et al., 2002) (Fig. 1*L*). As might be predicted, these altered patterns of

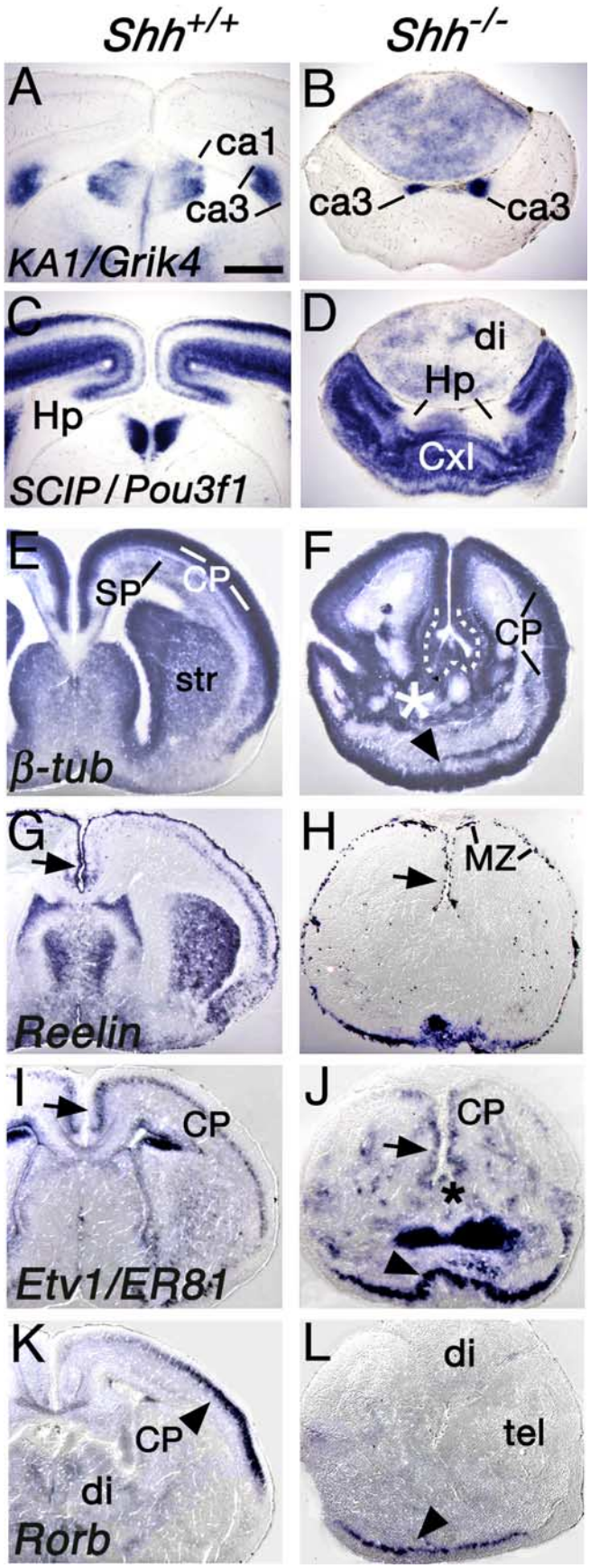

Figure 2. A rudimentary hippocampus and ventral cortical primordium in the *Shh* mutant. Coronal sections of E17.5 wild-type (***A***, ***C***, ***E***, ***G***, ***I***, ***K***) and Shh-null (***B***, ***D***, ***F***, ***H***, ***J***, ***L***) embryos processed for *in situ* hybridization. ***A–D***, The presumptive hippocampal CA3 region is *KA1*/Grik4-positive; *SCIP*/*Pou3f1*-negative in a wild-type mouse (***A***, ***C***). The approximate boundaries of CA1 and CA3 are indicated in ***A*** (ca1, ca3). Similar complementary expression of *KA1*/*Grik4* and *SCIP*/*Pou3f1* is seen in the medial telencephalon in a Shh $^{-/-}$ embryo (regions marked ca3 and Hp in adjacent sections; ***B***, ***D***). ***E–L***, The β-*tubulin*-expressing, neuron-dense cortical plate reaches across the ventral midline in *Shh* nulls (***F***, arrowhead) and envelops disorganized deep structures (***F***, asterisk). Cortical primordium in both control and *Shh* mutant mice expresses genes that mark postnatal neocortical layer 1 (*Reelin*; ***G***, ***H***), layer 5 (*Etv1*/*Er81*; ***I***, ***J***), and layer 4 (*Rorb*; ***K***, ***L***). *Etv1*/*Er81* and *Rorb* expression crosses the ventral midline in the *Shh* mutant (***J***, ***L***, arrowheads). Note the interhemispheric fissure (***G–J***, arrows), especially well marked by *reelin* expression (***G***, ***H***). Scale bar: ***A***, ***C***, 375 μm; ***E***, ***G***, ***I***, ***K***, 600 μm; ***B***, ***D***, ***F***, ***H***, ***J***, ***L***, 200 μm. Abbreviations: CP, Cortical plate; Cxl, cortex-like; di, diencephalon; Hp, hippocampus; MZ, marginal zone; sp, subplate; str, striatum; tel, telencephalon.

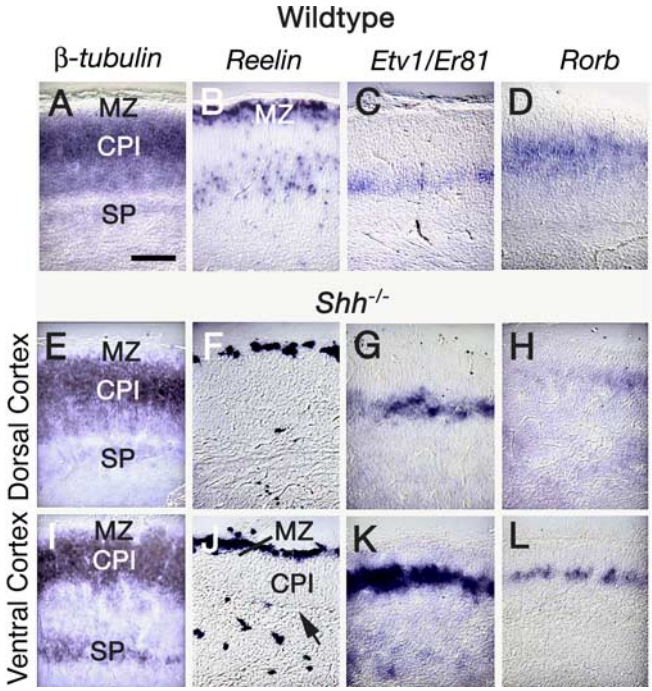

Figure 3. Layering in dorsal and ectopic ventral cortical primordium of *Shh* mutant mice. ***A–D***, High magnification, coronal sections through E17.5 wild-type cortex processed with *in situ* hybridization to show expression of β-*tubulin* (***A***), a pan-neuronal marker, and genes associated with particular cortical layers (see text) (***B–D***). *Reelin* is expressed in the marginal zone (MZ), the future layer I of neocortex (***B***). ***E–L***, By the same gene expression markers, *Shh* mutants display a cortical plate, and possibly a subplate in both dorsal and ventral telencephalon (***A***, ***E***, ***I***). The CP can be seen by morphology in ***J*** (arrow indicates lower boundary of the CP). Cells expressing *Reelin* are sparser in the *Shh* mutant but mark the MZ (***B***, ***F***, ***J***). *Etv1* and *Rorb* are expressed in dorsal (***G***, ***H***) and ventral (***K***, ***L***) cortical primordium in the *Shh* mutant. The three layer selective markers appear in approximately similar positions in the depth of the CP in wild type and mutant (***A–H***). Scale bar, 100 μm. Abbreviations: CPl, Cortical plate; MZ, marginal zone; SP, subplate.

gene expression are followed by structural transformations. At E17.5, a cortical plate and marginal zone form a distinct cortical primordium in both control mice and *Shh* mutants (Figs. 2*E–H*, 3*E*,*F*,*I*,*J*). However, in the mutants, the cortical primordium reaches far into the ventral telencephalon (Fig. 2*F*, arrowhead).

Bands of gene expression selective to particular neocortical layers (Miyashita-Lin et al., 1999; Rubenstein et al., 1999; Garel et al., 2003; Hevner et al., 2003) also appear in the *Shh* mutant (Figs. 2*I–L*, 3*C*,*D*,*G*,*H*,*K*,*L*). Within the dorsal cortical primordium, and to a lesser extent ventrally, these genes are expressed in a pial to ventricular pattern similar to wild type (Fig. 3*C*,*D*,*G*,*H*). Dorsalization shifts the regional expression of these genes ventrally,

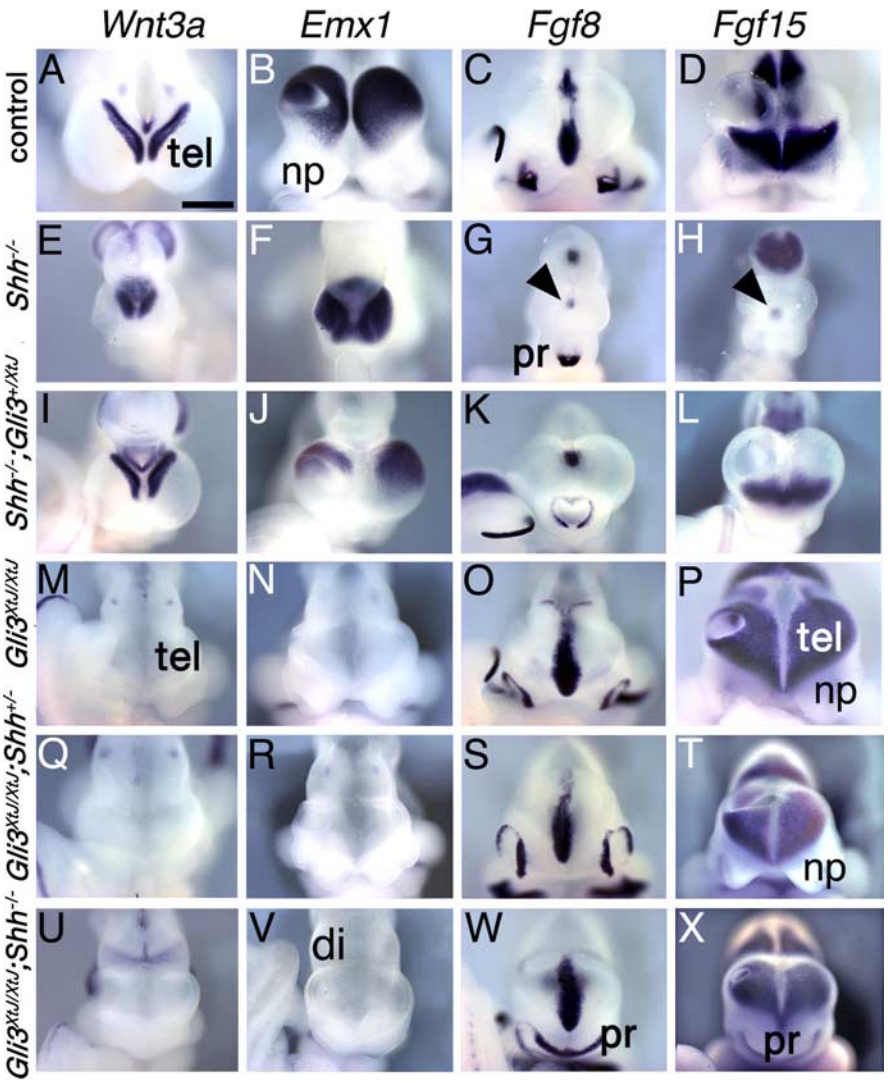

Figure 4. Shh and Gli3 do not show reciprocal effects on dorsal–ventral patterning. Whole-mount *in situ* hybridization of E10.5 control and *Shh;Xt*^{J} compound mutant embryos. Brains viewed dorsally (***A***, ***E***, ***F***, ***I***, ***J***, ***M***, ***Q***, ***U***) or frontally (all other panels). ***A–D***, In control telencephalon, *Wnt3a* is expressed in the cortical hem, *Emx1* in the cortical primordium, *Fgf8* rostrally, and *Fgf15* ventrally. ***E–H***, In *Shh* mutants, *Wnt3a* expression marks the cortical hem and *Emx1* is expressed broadly in the telencephalon; *Fgf8* and *Fgf15* expression is greatly reduced (***G***, ***H***, arrowheads). Indeed, the spot of *Fgf15* expression seen in ***H*** is in the underlying eye field, not in the telencephalon. ***I–L***, Partial rescue of *Emx1* and *Fgf8/15* expression patterns occurs after removing one functional copy of *Gli3* from Shh-null mice. ***M–P***, In *Gli3*$^{Xt^{J}/Xt^{J}}$ mice, loss of *Wnt3a* and *Emx1* expression is accompanied by caudal and dorsal overexpression of *Fgf8/15*, particularly *Fgf15* (***P***). These gene expression patterns in *Gli3*$^{Xt^{J}/Xt^{J}}$ mice are unaffected by loss of one (***Q–T***) or both (***U–X***) *Shh* alleles. Scale bar, 600 μm. Abbreviations: tel, telencephalon; pr, proboscis; np, nasal primordia.

resulting in bands of expression that cross the ventral midline (Figs. 2*J*,*L*, 3*K*,*L*) ($n = 6$ of 6).

Thus, early dorsal-to-ventral gene expression shifts in the *Shh* mutant telencephalon reflect a respecification of the ventral telencephalon to a dorsal fate. In consequence, the ventral part of the telencephalon differentiates as layered cortex.

Rostral continuity of the hemispheres and loss of Fgf signaling

Although the dorsal midline is evident in *Shh* mutants, the rostral interhemispheric fissure and adjacent septal nuclei do not form. Similar defects occur in mice deficient in telencephalic Fgf signaling (Meyers et al., 1998; Gutin et al., 2006; Storm et al., 2006; Tole et al., 2006); conversely, Fgf8 can induce an ectopic sulcus in the chick telencephalon that resembles the rostral sulcus (Crossley et al., 2001). Strongly suggesting that deficient Fgf signaling contributes to the *Shh* mutant phenotype, *Fgf8* gene expression in the mutant telencephalon is weak and transient (Aoto et al., 2002; Ohkubo et al., 2002). The *Shh* mutant further shows loss or near loss of expression of *Fgf3*, *15*, *17*, and *18* (Fig. 4*H*) (data not shown). In wild-type mice, *Fgf8*, *17*, and *18* expression overlaps at the rostral pole of the telencephalon (Heikinheimo et al., 1994; Crossley and Martin, 1995; Bachler and Neubuser, 2001); in a mostly separate domain in the ventral telencephalon, *Fgf3* and *Fgf15* are expressed (Fig. 4*C*,*D*; supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Thus, the *Shh* mutant loses expression of *Fgf* genes, encoding multiple Fgf ligands, in a sizeable rostroventral region of the telencephalon.

Depleting *Gli3* restores *Fgf* gene expression and rostroventral patterning, without fully inhibiting dorsal fate

Replacing one or both functional *Gli3* alleles in *Shh* or *Smo* mutants with the *extratoes* mouse mutant (*Xt*^{J}) allele partially restores *Fgf8* expression at multiple sites in the embryo (Aoto et al., 2002; Kuschel et al., 2003), and rostroventral telencephalic expression of *Fgf3*, *Fgf15*, *Fgf17*, and *Fgf18* (Fig. 4*K*,*L*, Table 2) (data not shown). Upregulated *Fgf* gene expression correlates with restoration of the rostral sulcus and septum (Fig. 5*D–F*).

Although ventral structures, such as the striatum, are substantially rescued in the compound mutant (Litingtung and Chiang, 2000; Rallu et al., 2002), ectopic cortical primordium extends nearly as far ventrally as in mice lacking Shh alone (Fig. 5*D–F*). What accounts for the misalignment of ventral and dorsal domains? The ability of ectopic Gli3R to induce ectopic intermediate/dorsal cell types in the spinal cord (Meyer and Roelink, 2003) suggests that Gli3R could also induce dorsal structure in the telencephalon. One possibility, therefore, is that levels of Gli3R in the compound mutant telencephalon are insufficient to block ventral structure, but sufficient, in the absence of Shh, for ectopic specification of cortex. This model gives Gli3 an active role in cortical specification. Misalignment is also consistent, however, with the model in which a telencephalic dorsalizing factor is inhibited by Shh and disinhibited by Gli3 (Rallu et al., 2002). In the absence of Shh, reduction of Gli3 has no effect; the dorsalizing factor prevails and ectopic cortex is specified.

In the latter influential model of D/V patterning in the telencephalon, D/V patterning of the telencephalon requires ventral repression of Gli3 function by Shh, and dorsal repression of Shh signaling by Gli3. In the absence of both Shh and Gli3, other factors specify dorsal and ventral identities unimpeded, resulting

in a near-normal D/V pattern (Rallu et al., 2002). The specification of ventral cell identities in mice deficient in both Shh and Gli3 has been demonstrated repeatedly (Fuccillo et al., 2006). Here, we test the second part of the model, which predicts that the dorsal telencephalon is also specified near-normally in *Gli3/Shh* compound mutant mice.

We removed one or both functional copies of *Shh* from Xt^J/Xt^J mutants, and assessed the foremost defects of the Xt^J/Xt^J dorsal telencephalon, namely, a defective cortical primordium, reduction of the cortical hem, and dorsal expansion of normally rostroventrally expressed genes (Theil et al., 1999; Tole et al., 2000; Rallu et al., 2002; Kuschel et al., 2003; Theil, 2005). These features, represented by patterns of *Emx*, *Wnt*, and *Fgf* gene expression, are all apparent in Xt^J/Xt^J mutant mice by E10.5 (Fig. 4*M–P*).

In the Xt^J/Xt^J mouse, *Wnt3a* and *Emx1* expression are lost, and *Fgf8* and *Fgf15* expression expands (Fig. 4*M–P*). The enormous expansion of *Fgf15* expression from a ventral domain to virtually the entire telencephalon is a particularly striking example of "ventralization" in the Xt^J/Xt^J telencephalon. Moreover, increased dorsal expression of *Fgf15* is evident earlier than expanded expression of other genes, such as *Dlx2*, which is apparent only after E10.5 (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

Each of the defects described in E10.5 Xt^J/Xt^J brains persists when one copy of *Shh* is removed (Fig. 4*Q–T*). The Xt^J/Xt^J; $Shh^{-/-}$ genotype was rare (four non-exencephalic E10.5 embryos recovered from >20 litters), but the sample is consistent: dorsomedial features are lost, and rostroventral features are enhanced. The presence of a proboscis instead of nasal primordia indicates complete loss of Shh in these embryos (Fig. 3*W*,*X*) and alters the shape of the rostral forebrain, but the telencephalic phenotype is the same as in Xt^J/Xt^J mutants (Fig. 4*U–X*). Our findings demonstrate that the dorsal telencephalon remains highly abnormal in *Gli3/Shh* compound mutant mice: basic D/V pattern is not recovered.

A shared finding in this and a previous study is that the dorsal midline is disrupted in both Xt^J/Xt^J and $Shh^{-/-}$;Xt^J/Xt^J mutants (Rallu et al., 2002; this study), but this is likely attributable to the dominance of the Xt^J phenotype, rather than the loss of *Shh* itself. Discrepancies between the two studies are likely to be attributable to the ease with which specific phenotypic features can be identified as present or absent in the small double-null mutant forebrain. We assessed particularly conspicuous features, including expression of *Emx1* in the cortical primordium. At E10.5, *Emx1* is strongly expressed in wild-type mice, but lost in Xt^J/Xt^J mice. We also analyzed expression of *Fgf15*, a normally ventrally expressed gene that shows particularly marked dorsal expansion in the Xt^J/Xt^J mutant by E10.5. In contrast, gene expression markers previously used to establish ventralization in the Xt^J/Xt^J mutant (Tole et al., 2000; Rallu et al., 2002), such as the expansion of *Dlx2* expression into the rostrodorsal telencephalon, are readily apparent at E12.5, but not E10.5 (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), the stage at which analysis of double-null mice is practicable (Rallu et al., 2002; this study). Thus, at E10.5, it may not be feasible to distinguish normal from rescued expression of *Dlx2* in double nulls. In summary, the persistence of Xt^J/Xt^J mutant D/V patterning defects in double mutants may have been easier to detect in our study.

Table 2. Activities of Gli3 in the doral telencephalon are Shh independent

	Medial dorsal		Rostral ventral	
	Wnt3a; *Emx1*	Number	*Fgf8*; *Fgf15*	Number
Control embryo expression	+	38/38	+	17/17
$Shh^{-/-}$	+	7/7	−	8/8
$Shh^{-/-}$;$Gli3^{+/Xt^J}$	+	8/8	+	6/6
$Gli3^{Xt^J/Xt^J}$	−	7/7	++	8/8
$Gli3^{Xt^J/Xt^J}$;$Shh^{+/-}$	−	8/8	++	7/7
$Gli3^{Xt^J/Xt^J}$;$Shh^{-/-}$	−	2/2	++	2/2

Rostral and ventral identity is partially rescued in the *Shh* mutant by loss of one functional copy of *Gli3*. In contrast, dorsomedial and rostroventral defects in $Gli3^{Xt^J/Xt^J}$ mice are not rescued by loss of one or both copies of *Shh*.

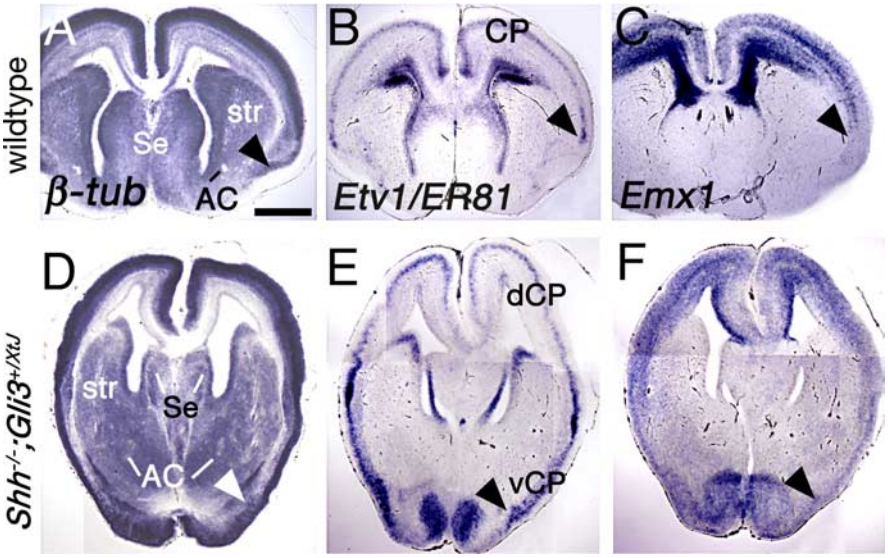

Figure 5. Removing one copy of *Gli3* from the *Shh* mutant partially rescues rostroventral structures, but not ectopic cortical primordium. E17.5 coronal section *in situ* hybridization. ***A–C***, The wild-type cortical primordium is confined dorsally, marked by a dense band of *β-tubulin* expression (***A***), and by expression of *Etv1*/*Er81* (***B***), and *Emx1* (***C***). ***D–F***, $Shh^{-/-}$;$Gli3^{+/Xt^J}$ embryos show rescued septal and striatal structures (compare Fig. 2), but retain a cortical plate that extends ventrally compared with wild type (***A–F***, arrowheads). Scale bar, 600 μm. Abbreviations: AC, Anterior commissure; dCP, dorsal cortical plate; Se, septum; str, striatum; vCP, ventral cortical plate.

Discussion

Our findings reconcile the functions of Shh and genetic interactions between *Shh* and *Gli3* in the telencephalon and elsewhere in the embryo. In Shh-null mice, the spinal cord develops a dorsal midline; this now appears true for the telencephalon as well (Fig. 6*A*). Furthermore, major defects in the dorsal telencephalon of *Gli3*-deficient mice do not reflect the loss of a brake on Shh activity (Fig. 6*B*,*C*). The latter finding is consistent with reports that defects attributable to Gli3 deficiency in the spinal cord, limb bud, and at *Fgf8*-expressing sites in Xt^J/Xt^J mutants are unaffected by the status of Shh (Aoto et al., 2002; Litingtung et al., 2002; Persson et al., 2002), and supportive of the hypothesis that similar early patterning mechanisms are at work in the limb bud and telencephalon (Ohkubo et al., 2002).

D/V patterning of the telencephalon thus requires ventral repression of Gli3 function by Shh signaling (Litingtung and Chiang, 2000; Rallu et al., 2002), but does not require dorsal repression of Shh signaling by Gli3 (this study). Dorsal telence-

phalic identities are specified incorrectly in the absence of both Shh and Gli3. A remaining role for Shh in the dorsal forebrain, as elsewhere in the neural tube, is to maintain normal levels of cell proliferation and tissue growth (Ishibashi and McMahon, 2002; Ulloa et al., 2007).

These results clear the way for additional investigation of dorsal telencephalic patterning. Shh is dispensable for induction of the *BMP*/*Wnt*-expressing dorsal midline, as, perhaps, is Fgf8, previously suggested to mediate dorsal midline induction by Shh. Expression of multiple *Fgf* genes, including *Fgf8*, is severely reduced in the *Shh* mutant; moreover, mouse lines engineered for defective Fgf signaling can develop a dorsal telencephalic midline (Garel et al., 2003; Gutin et al., 2006; Storm et al., 2006). Juxtaposed sources of Shh, Fgf8, and BMP/Wnt proteins regulate one another to pattern the telencephalon (Crossley et al., 2001; Ohkubo et al., 2002; Shimogori et al., 2004) but are not the only patterning cues. Given that dorsal epidermis induces roofplate in the spinal cord (Liem et al., 1995) and dorsal gene expression in the chick telencephalon (Gunhaga et al., 2003), dorsal telencephalic midline inductive signals seem likely to derive from adjacent mesenchyme and epidermis.

Initial description of the *Shh* mutant mouse forebrain as a holosphere suggested the mouse as a model for studying mechanisms underlying human holoprosencephaly (HPE) (Chiang et al., 1996), a developmental disorder in which the cerebral hemispheres separate incompletely or not at all (Muenke and Beachy, 2000; Hayhurst and McConnell, 2003). Approximately 20% of genetic lesions that have been associated with HPE involve genes encoding components of the Shh signaling pathway (Muenke and Beachy, 2000). We find that the *Shh* mutant forebrain, although not a holosphere, fits surprisingly well with features of classic human HPE (Takahashi et al., 2003, 2004). Magnetic resonance imaging studies of human infants with HPE show joined septal midline structures in the forebrain, but dorsal separation of the hemispheres. At the diencephalon–telencephalon boundary, additional defects may include obstructed flow of CSF through the forebrain ventricles (Takahashi et al., 2003, 2004). Similarly, in the Shh mutant mouse, the cerebral hemispheres are joined rostrally but separated dorsally, and as development proceeds, ventricular flow is blocked at the level of the third ventricle (B. G. Rash and E. A. Grove, manuscript in preparation). Analysis of primary and secondary effects of loss of Shh on the mouse forebrain may therefore continue to clarify the developmental mechanisms of human HPE.

How the dorsal telencephalon is specified to develop as the intricately organized layered cerebral cortex remains an open question. In the Xt^J/Xt^J mouse, anomalies appear in the dorsal telencephalon by E9.5; *Gli3* itself is expressed from E8.0 in the dorsal headfold, and in the forebrain anlage from E9.0 (Aoto et al., 2002). Gene expression and morphology indicate that the early progenitor cells of the cortical ventricular zone (VZ) are disorganized, losing their apical/basal cell polarity, and that postmitotic neurons form clusters, rather than layers, beginning with the failure of the earliest born cortical neurons to form a preplate (Theil et al., 1999; Tole et al., 2000; Theil, 2005). Cortical VZ progenitors express transcription factor genes associated with cortical development, such as *Ngn2*, *Pax6*, and *Emx2*, but *Emx1* expression, initiated later in the telencephalon than *Emx2*, does not upregulate on schedule in the mutant (Simeone et al., 1992; Theil et al., 1999; Tole et al., 2000). In brief, several features of the Xt^J/Xt^J telencephalon indicate that, in the absence of Gli3, the earliest differentiation of the cortex, and perhaps its specification, is interrupted.

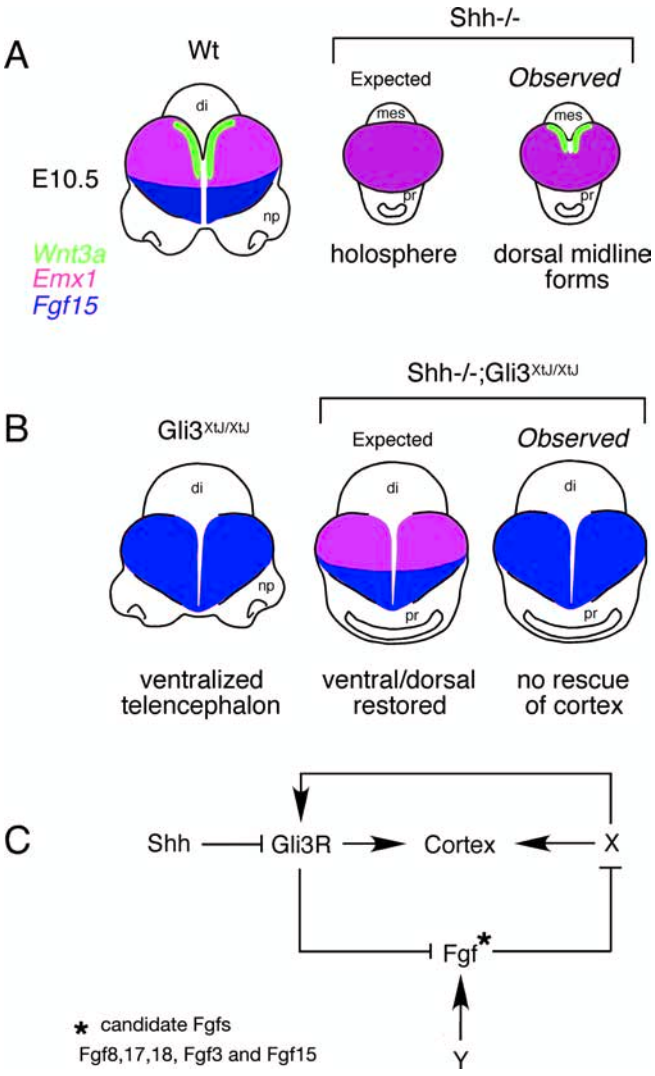

Figure 6. Summary of dorsal telencephalic patterning. Schematics in ***A*** and ***B*** represent frontal views of the developing brain at E10.5. In wild-type mice, the nasal primordia (np) and diencephalon (di) are adjacent to the telencephalon. Three gene expression patterns were selected to indicate the dorsal (*Emx1*; purple) and ventral (*Fgf15*; blue) telencephalon and the cortical hem (*Wnt3a*; green). ***A***, The telencephalon in the Shh-null mouse was expected (see text) to form a holosphere, without midline division. The mutant telencephalon, as observed, divides dorsally into cortical hemispheres with an intervening cortical hem. ***B***, In the Xt^J/Xt^J mouse, the telencephalon is ventralized. Additional removal of both copies of *Shh* was expected (see text) to restore the dorsal telencephalon. Instead, no rescue occurs; the telencephalic phenotype of the Xt^J/Xt^J appears independent of the status of Shh. ***C***, X represents factors that specify the cerebral cortex and direct its early differentiation. Y represents Shh-independent inducers of *Fgf* gene expression, revealed by persistent expression of *Fgf8* and *Fgf15* in double *Gli3*/*Shh* mutants (see text). Gli3R is a component of at least one major pathway downstream of X. Gli3R may actively induce layered cortex (see text) or promote cortical development indirectly. An instance of the latter is the inhibition of *Fgf* gene expression by Gli3R. This inhibition protects the dorsal telencephalon from excess Fgf signaling that might otherwise promote ventral, and inhibit dorsal, identities (see text).

Emx2 and *Pax6* together have been suggested as "selector" genes for cortical fate (Muzio et al., 2002). In mice lacking both, dorsal tissue forms in the telencephalon but develops as basal ganglia rather than cortex (Muzio et al., 2002). Although both *Pax6* and *Emx2* are substantially expressed in the Xt^J/Xt^J dorsal telencephalon (Theil et al., 1999; Tole et al., 2000; Theil, 2005), they are not sufficient to drive structural formation of the cortex, perhaps because their pattern of expression becomes increasingly

disorganized, or because Gli3 is responsible for critical steps downstream of Pax6 and Emx2.

Contributing to this disruption may be the excess of Fgf signaling in the Xt^J/Xt^J dorsal telencephalon (this study). Fgf signaling promotes ventral and suppresses dorsal identities in the telencephalon (Kuschel et al., 2003; Gutin et al., 2006); for example, beads coated with Fgf8 upregulate *Dlx2* and downregulate *Emx1* expression in dorsal telencephalic explants (Kuschel et al., 2003). A close correlation has been noted in the Xt^J/Xt^J telencephalon between the selectively rostrodorsal overexpression of ventral genes such as *Dlx2* and the expanded domain of *Fgf8* expression, but the latter seems too limited to explain widespread loss of cortical histology and gene expression (Kuschel et al., 2003). We find, however, that expression of other *Fgf* genes, including *Fgf17*, and, most prominently, *Fgf15*, are upregulated extensively in the Xt^J/Xt^J dorsal telencephalon, with the potential for disrupting cortical development.

Whether Gli3R permits cortical specification or differentiation, or both, by inhibiting Fgf signaling remains to be tested, but fits with correlations among *Gli3* gene dosage, levels of telencephalic *Fgf* gene expression, and the D/V spread of cortical primordium (this study). We propose a provisional model, similar in outline to that described above (Rallu et al., 2002). That is, unidentified factors promote cortical specification in the dorsal telencephalon, are inhibited by Fgf activity, and are disinhibited by Gli3R-mediated inhibition of *Fgf* gene expression (Fig. 6*C*).

Note added in proof. While this paper was in press, a paper reporting evidence for a roofplate in the telencephalon of $Shh^{-/-}$ mouse embryos was published by Fernandes et al. (2007).

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