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Sp8 and COUP-TF1 Reciprocally Regulate Patterning and Fgf Signaling in Cortical Progenitors

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To gain new insights into the transcriptional regulation of cortical development, we examined the role of the transcription factor Sp8, which is downstream of Fgf8 signaling and known to promote rostral cortical development. We have used a binary transgenic system to express Sp8 throughout the mouse telencephalon in a temporally restricted manner. Our results show that misexpression of Sp8 throughout the telencephalon, at early but not late embryonic stages, results in cortical hypoplasia, which is accompanied by increased cell death, reduced proliferation, and precocious neuronal differentiation. Misexpression of Sp8 at early developmental stages represses *COUP-TF1* expression, a negative effector of Fgf signaling and a key promoter of posterior cortical identity, while ablation of Sp8 has the opposite effect. In addition, transgenic misexpression of *COUP-TF1* resulted in downregulation of *Sp8*, indicating a reciprocal cross-regulation between these 2 transcription factors. Although Sp8 has been suggested to induce and/or maintain *Fgf8* expression in the embryonic telencephalon, neither *Fgf8* nor *Fgf15* was upregulated using our gain-of-function approach. However, misexpression of Sp8 greatly increased the expression of Fgf target molecules, suggesting enhanced Fgf signaling. Thus, we propose that Sp8 promotes rostral and dorsomedial cortical development by repressing *COUP-TF1* and promoting Fgf signaling in pallial progenitors.

Keywords: corticogenesis, Fgf signaling, neurogenesis, patterning, proliferation, Sp8

Introduction

The complexity of functions characteristic of the vertebrate cerebral cortex are a consequence of the formation of specialized cortical areas, which are established during development. Secreted molecules expressed at patterning centers establish a graded expression of transcription factors, which initiate the formation of these functional areas of the cerebral cortex (Grove and Fukuchi-Shimogori 2003; O'Leary et al. 2007). Fgf8 and 17 signaling promote the formation of rostral cortical regions (Fukuchi-Shimogori and Grove 2001; Cholfin and Rubenstein 2007). Downstream of Fgf signaling, rostral versus caudal cortical identity is regulated by the transcription factors Pax6 and Emx2, respectively (Bishop et al. 2000, 2002; Hamasaki et al. 2004).

The COUP-TF1 (Nr2f1) transcription factor is enriched in caudoventral portions of the developing cerebral cortex (Liu et al. 2000). Conditional *COUP-TF1* knockouts are known to have reduced caudal cortical areas and a concomitant expansion of rostral areas (Armentano et al. 2007). Additionally, *COUP-TF1* overexpression under the *D6* promoter results in reduced levels of *Pax6* along the dorsoventral axis (Faedo et al. 2008). There is evidence that COUP-TF1 regulates cortical patterning by repressing MAPK/ERK signaling, which is likely downstream of Fgf signaling (Faedo et al. 2008).

The zinc finger transcription factor *Sp8* is expressed in a complementary pattern to *COUP-TF1* with high levels in the rostromedial cortical regions (Sahara et al. 2007). Accordingly, loss-of-function studies have indicated that Sp8 is required for the specification of rostral cortical identity (Sahara et al. 2007; Zembrzycki et al. 2007). *Sp8* appears to be induced by Fgf signaling and has been suggested to subsequently maintain *Fgf8* expression within the rostral cortical signaling center (Sahara et al. 2007). Thus, it remains unclear whether Sp8 plays a direct role in the specification of rostral cortical identity or if its role is indirect, though the regulation of *Fgf* expression. If it is direct, the complementary expression of *Sp8* and *COUP-TF1* might indicate a cross-repressive mechanism intrinsic to the cortical progenitors.

To gain further insights into the molecular mechanisms underlying the patterning and growth of the cerebral cortex, we focused our studies on Sp8. Using a binary transgenic approach to misexpress Sp8 temporally throughout the telencephalon, we examined the role of this transcription factor in rostrocaudal and dorsoventral patterning of the developing cortex. Moreover, our gain-of-function approach allowed us to examine the role of Sp8 in the proliferation, survival, and differentiation of telencephalic progenitors and, importantly, its relationship to Fgf signaling, allowing us to identify a novel molecular mechanism that couples patterning and growth of the developing cortex.

Materials and Methods

Animals

To generate the *tetO-Sp8-IRES-EGFP* (*IE*) mice a 1.3-kb cDNA PCR fragment, containing the entire coding sequence of Sp8, was cloned into the *Bam HI* site of the ptetO$_7$-IRES-EGFP vector using compatible *Bgl II* ends that were designed into the primers used to produce the Sp8 amplicon. The 3.7-kb TetO$_7$-Sp8-IRES-EGFP fragment was released from the construct with *AbdI* and *AscI* and gel purified. Pronuclear injections were done at the transgenic core at Cincinnati

Children's Hospital Medical Center (CCHMC) and produced 3 founder lines. Two of these founder lines robustly expressed *Sp8* and *EGFP* throughout the developing telencephalon when crossed to *Foxg1*^{tTA} mice (Hanashima et al. 2002). The third founder also expressed *Sp8* and *EGFP* but not as robustly as the other 2 lines. For experiments in this article, we have used the lines that showed strongest and most consistent expression of the *Sp8* and *EGFP*. We used the *Foxg1*$^{tTA/+}$ and the *tetO-Sp8-IE* single transgenic embryos as controls because they did not show any obvious patterning defects. Moreover, these 2 single transgenic lines were no different from wild-type embryos. To repress transgene expression in *Foxg1*$^{tTA/+}$*; tetO-Sp8-IE* embryos, doxycycline hyclate (Dox; Sigma) was administered to the pregnant mouse, at 0.02 mg/mL in the drinking water, between embryonic day E8–E11. This represents a slight modification from our previous studies in which Dox was added from E7 to E9, and transgene expression was delayed until approximately E13 (Waclaw et al. 2009; Pei et al. 2011).

D6/COUP-TF1 mice were maintained and genotyped as described (Faedo et al. 2008). *Foxg1*^{tTA} mice were genotyped as described (Waclaw et al. 2009). *Foxg1*^{cre} (Hébert and McConnell 2000), *Emx1*^{cre} (Gorski et al. 2002), and *Sp8*$^{fl/fl}$ mice (Waclaw et al. 2006) were maintained and genotyped as described (Waclaw et al. 2006, 2009, 2010). To account for the observed haploinsufficiency of the Foxg1$^{Cre/+}$ line (Eagleson et al. 2007; Shen et al. 2006; Storm et al. 2006; Siegenthaler et al. 2008), we used Foxg1$^{cre/+}$;Sp8$^{fl/+}$ embryos as a control for Foxg1$^{cre/+}$;Sp8$^{fl/fl}$ embryos. Mouse colonies were maintained at the University of California, San Francisco (UCSF) or CCHMC, in accordance with National Institutes of Health, CCHMC, and UCSF guidelines.

Histology

Pregnant females were euthanized with CO_2 followed by cervical dislocation. Noon on the day of the vaginal plug was considered as E0.5. The embryos were dissected and fixed overnight by immersion in a 4% PFA in phosphate-buffered saline (PBS) at 4°C. The tissue was cryoprotected by immersion in 30% sucrose/PBS, embedded in OCT (Tissue-Tek), and cryostat sectioned (10–20 μm).

In situ RNA hybridization on cryostat sections was performed as previously described (Borello et al. 2008). cRNA Probes (sources in parentheses) against *COUP-TF1* (M-J Tsai), *Emx2* (A. Simeone), *Erm* (A. Chotteau-Lelievre), *Fgf8* (G. Martin), *Fgf15* (J.R. McWhirter), *Lhx2* (S. Rétaux), *Mest* (R. Livesey) *Pax6* (P. Gruss), *Sp8* (S. Bell), and *Spry2* (G. Martin).

COUP-TF1 in situ hybridization signal (Supplementary Fig. S3) was quantified as described by Faedo et al. (2008).

Immunofluorescence on cryostat sections was performed as previously described (Borello et al. 2008; Waclaw et al. 2009). The antibodies used were as follows: mouse anti-βIII-tubulin (1:1000, Covance), rabbit anti-Foxg1 (NCFAB) (1:500, NeuroCell), rabbit anti-phospho-Histone H3 (1:500, Millipore), goat anti-Sp8 (1:7000, Santa Cruz), mouse anti-COUP-TF1 (1:5000, R&D Systems), rabbit anti-GFP-488 (1:500, Invitrogen), rabbit anti-Pax6 (1:1000, Covance), rabbit anti-pERK1/2 (1:500, Cell Signaling), and rabbit anti-Cleaved Caspase-3 (Asp175) (1:200, Cell Signaling). For the pERK1/2 and COUP-TF1 staining, an antigen retrieval step with citrate buffer was added at the beginning of our immunostaining protocol. The secondary antibodies for fluorescent staining were as follows: donkey anti-goat antibodies conjugated to Cy2, Cy3, or Cy5 (Jackson ImmunoResearch), donkey anti-mouse antibodies conjugated to Cy3 or Cy5 (Jackson ImmunoResearch), and donkey anti-rabbit antibodies conjugated to Cy3 (Jackson ImmunoResearch) or AlexaFluor 488 (Invitrogen) were used at a dilution of 1:200.

Comparisons of gene or protein expression patterns between brains of different genotypes were performed by matching the planes of sections, using multiple anatomical features. Whenever possible, this was performed for multiple planes of section for each gene, and from at least 3 brains for each genotype.

Cortical thickness of E12.5 embryos was measured using ImageJ software. Thickness of the ventrolateral and dorsomedial pallium were measured in double transgenic and control embryos as shown in Fig. 2*C,D*. Measurements were made from both hemispheres of matching midlevel sections from at least 3 animals of each genotype. Student's *t*-test was performed to determine statistical significance.

Cell counts for apoptosis and cell proliferation were performed by counting the total number of positive cells from designated levels (rostral areas were designated as those that only had the septum and lateral ganglionic eminence, midlevel sections were designated as those that had the lateral and medial ganglionic eminence or caudal levels were defined as those that had the caudal ganglionic eminence and within designated areas in the section—subpallium and lateral pallium). Caspase-3- or pH3-positive cells were counted separately from 2 hemispheres of 3 animals each for each genotype (*tetO-Sp8-IE* or *Foxg1*$^{tTA/+}$*; tetO-Sp8-IE*). Student's *t*-test was performed to assess statistical significance.

Results

Reciprocal Expression of Sp8 and COUP-TF1 in the Pallium

Toward understanding the role of Sp8 in the early phases of mouse cortical development, we have analyzed its expression along the rostrocaudal and dorsoventral axes between E9.5 and E12.5. We assessed these axes as many of the transcription factors that regulate cortical patterning are expressed in bidimensional gradients along these axes.

As shown for *Sp8* RNA (Sahara et al. 2007), Sp8 protein is expressed in a gradient of high rostral to low caudal at E9.5 (Fig. 1*A*). Along the dorsoventral axis of the pallium, Sp8 is expressed in a high-dorsal to low-ventral gradient, which is particularly obvious at E12.5 (Fig. 1*B*). Sp8 expression is complementary to that of *COUP-TF1* on both the rostrocaudal and dorsoventral axis (Fig. 1*A,B*). These expression gradients are maintained until at least E14.5, when the level of pallial *Sp8* expression is greatly decreased (Waclaw et al. 2006). The complementary expression patterns of *Sp8* and *COUP-TF1* suggest cross-repressive interactions.

Early Loss of Sp8 Function in the Nascent Cortex Leads to Increased COUP-TFI Expression in the Rostral Dorsomedial Pallium

To examine whether the complementary expression of *Sp8* and *COUP-TF1* in pallial progenitors reflects a cross-repressive interaction, we studied *COUP-TFI* expression in conditional *Sp8* mouse mutants. First, we inactivated *Sp8* at the earliest stages of telencephalic development (e.g., E8.5) using *Foxg1*$^{cre/+}$ mice (Hébert and McConnell 2000). *COUP-TFI* is normally expressed at high levels in the caudal and ventrolateral pallium (Fig. 1*C,E*), however, in the *Foxg1*$^{cre/+}$*;Sp8*$^{fl/fl}$ mutants *COUP-TFI* expression is expanded rostrally and dorsomedially within the pallium (Fig. 1*D,F*), supporting the hypothesis that Sp8 represses *COUP-TFI* in the rostral and dorsomedial pallium. These findings are consistent with those of Zembrzycki et al. (2007) showing that dorsomedial telencephalic patterning is disrupted in *Sp8* mutants.

Next, to test whether this cross-repression also functions at later telencephalic stages, we inactivated *Sp8* using the *Emx1*^{cre} allele (Gorski et al. 2002), which begins to delete the floxed *Sp8* allele around E10.5 (data not shown). We verified that the mutant lacked dorsal telencephalic *Sp8* expression at E12.5 (Supplementary Fig. S1 A,B). Interestingly, *COUP-TFI* expression at E12.5 in these conditional mutants (Fig. 1*H*) appeared normal when compared with the control (Fig. 1*G*). Therefore, unlike the *Foxg1*$^{cre/+}$*;Sp8*$^{fl/fl}$ mutants, *COUP-TFI* expression in the dorsomedial pallium of the *Emx1*$^{cre/+}$*;Sp8*$^{fl/fl}$ mutants was similar to the controls. Thus, it appears that Sp8

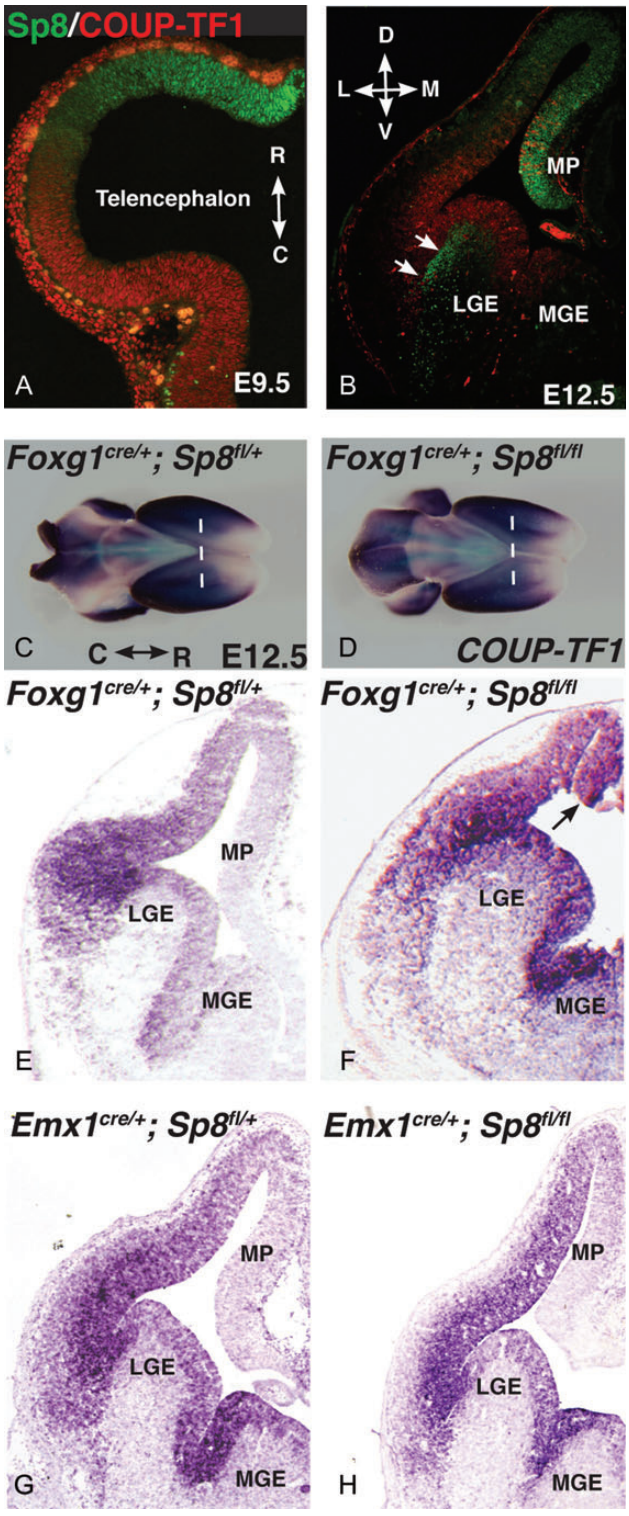

Figure 1. Sp8 and COUP-TF1 are expressed in complementary domains. As early as E9.5, Sp8 and COUP-TF1 form opposing gradients along the rostrocaudal (R-C) axis (*A*) and by E12.5 their opposing dorsoventral gradients are clearly established (*B*). Early deletion of *Sp8*, and not late deletion, results in the rostral and dorsal expansion of *COUP-TF1*. At E12.5 *COUP-TF1* is expressed in a caudoventral high, rostral-dorsal low gradient, as seen in whole mount in situ hybridization in control $Foxg1^{cre/+};Sp8^{fl/+}$ brains (*C*). In $Foxg1^{cre/+};Sp8^{fl/fl}$ animals, the expression of *COUP-TF1* is expanded dorsally and rostrally (*D*). The dotted lines in *C* and *D* provide landmarks from which to compare the change in *COUP-TF1* expression. Coronal section of E12.5 $Foxg1^{cre/+};Sp8^{fl/fl}$ mutant brains (*F*) show that *COUP-TF1* is expanded dorsally compared with control $Foxg1^{cre/+};Sp8^{fl/+}$ animals (*E*). Arrows in F indicate the dorsal expansion of *COUP-TF1* expression. Expression of *COUP-TF1* in E12.5 $Emx1^{cre/+};Sp8^{fl/fl}$ mutants (*H*) does not appear substantially changed from $Emx1^{cre/+};Sp8^{fl/+}$ control animals (*G*). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MP, medial pallium.

represses *COUP-TFI* pallial expression prior to E10.5. To explore this hypothesis, we made use of a doxycycline-regulated binary transgenic mouse model to spatially and temporally control *Sp8* expression within the embryonic cerebral cortex (Hanashima et al. 2002; Waclaw et al. 2009).

Misexpression of Sp8 Throughout the Developing Telencephalon

To misexpress Sp8 in the developing telencephalon, we generated a mouse line containing a *tetO-Sp8-IRES-EGFP* (*IE*) transgene and crossed it with $Foxg1^{tTA/+}$ mice (Hanashima et al. 2002) to induce *Sp8* and *EGFP* expression throughout the embryonic telencephalon (Fig. 2). To temporally repress transgene-derived *Sp8* and *EGFP* expression during specific intervals of embryonic development, doxycycline (Dox) was administered to the pregnant mothers between E8 and E11 (Fig. 2*A*,*B*).

Transgene (i.e., *Sp8* and *EGFP*) expression was detected in the pallium of $Foxg1^{tTA/+}$;*tetO-Sp8-IE* embryos as early as E10 (Supplementary Fig. S1*C*,*C*′); between E9.5 and E10.5, transgene expression was observed only in the basal ganglia (data not shown). We carried out the phenotypic analysis in double transgenic embryos at E11.5, E12.5, E14.5, E16.5, and E18.5 (see experimental design in Fig. 2*B*). The double transgenic embryos showed a uniform expression of *Sp8* and *EGFP* along the dorsoventral axis, excluding the cortical hem (Fig. 2*D*,*E*). This expression was distinct from the *tetO-Sp8-IE* or $Foxg1^{tTA/+}$ controls, which showed *Sp8* in the dorsomedial pallium and dLGE (Fig. 2*C* and data not shown). Additionally, transgene expression along the rostrocaudal axis of the cortex in double transgenic embryos appeared uniform (Fig. 2*G*, inset Fig. 2*E*, and data not shown). Using this system, we found that Sp8 is overexpressed by about 46-fold over that observed in the control cortex (data not shown). Misexpression of Sp8 throughout the telencephalon beginning E9.5 reduced telencephalic surface area and thickness, particularly affecting the subpallium (Fig. 2*D*,*E*,*G*), as compared to the controls (Fig. 2*C*,*F* and data not shown). Quantification of the ventrolateral and dorsomedial pallial thickness at E12.5 showed that ventrolateral pallial thickness was reduced by ~20% ($P < 0.05$; $n = 3$) in the double transgenic (cyan dashed lines in Fig. 2*D*) compared with similar regions of control animals (cyan dashed lines in Fig. 2*C*). On the other hand, no significant change was detected in dorsomedial pallial thickness (yellow dashed lines in 2*C*,*D*).

Misexpression of Sp8 Alters Dorsoventral Patterning in the Pallium

Although the highest levels of *Sp8* and *COUP-TF1* in the pallium reside at the rostral and caudal extremes, at midtelencephalic levels, these 2 factors are enriched in the dorsal and ventral portions of the pallium, respectively (e.g., Fig. 1*B*). To gain insights into the role of Sp8 in dorsoventral patterning of the pallium, we examined the expression of genes that mark defined progenitor zones within the dorsal and ventral pallium.

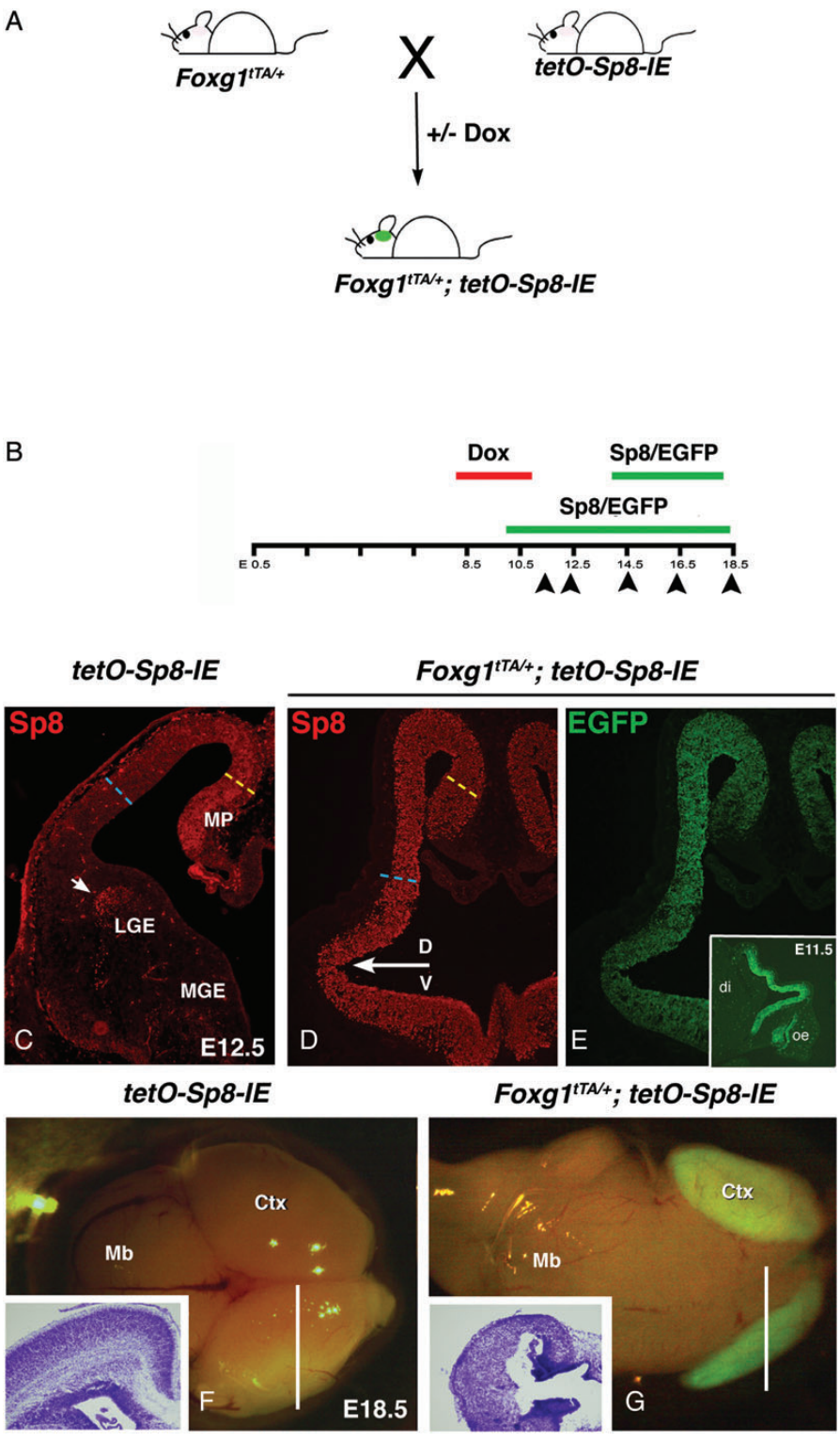

Figure 2. To misexpress Sp8 throughout the telencephalon, *Foxg1*$^{tTA/+}$ males were bred to *tetO-Sp8-IE* females to yield *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* progeny (*A*). *Foxg1*$^{tTA/+}$*; tetO-Sp8-IE* animals overexpress *Sp8* and *EGFP* starting at E9.5, and this expression can be delayed by 5 days by administering doxycycline (Dox) to the mother between Days E8 and E11 (*B*). *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* animals express the transgenes uniformly through the rostrocaudal extent of the telencephalon. Inset in *E*, shows *EGFP* expression in a sagittal section of an E11.5 animal, where the olfactory epithelium (oe) marks the rostral portion of the animals head and the diencephalon (di) indicates the caudal regions). Double transgenic animals also show uniform overexpression of *Sp8* and *EGFP* along the dorsoventral (D/V) axis of the telencephalon (*D* and *E*, respectively, arrow marks the D/V border) as compared to *tetO-Sp8-IE* controls (*C*) that only express *Sp8* in the medial pallium (MP) and the subventricular zone (arrow) of the LGE. Misexpression of *Sp8* leads to reduction in ventrolateral pallial thickness at E12.5 (cyan line in *D*) compared with control animals (cyan line in *C*); the thickness of the dorsomedial pallium (yellow lines in *C* and *D*) shows no significant changes. This phenotype is amplified by E18.5 resulting in a severe hypoplasia of the cortex (Ctx) in *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* animals (*G*) as compared to control *tetO-Sp8-IE* animals (*F*). Insets show Nissl-stained cross sections of the brains in *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* (*G*) and control *tetO-Sp8-IE* animals (*F*) at the level indicated by the white line. The cortex of double transgenic animals (*G*) has large ventricles, which lack a clear laminar organization compared with the brains of control animals (*F*). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MP, medial pallium; Ctx, cortex; Mb, midbrain; oe, olfactory epithelium; di, diencephalon.

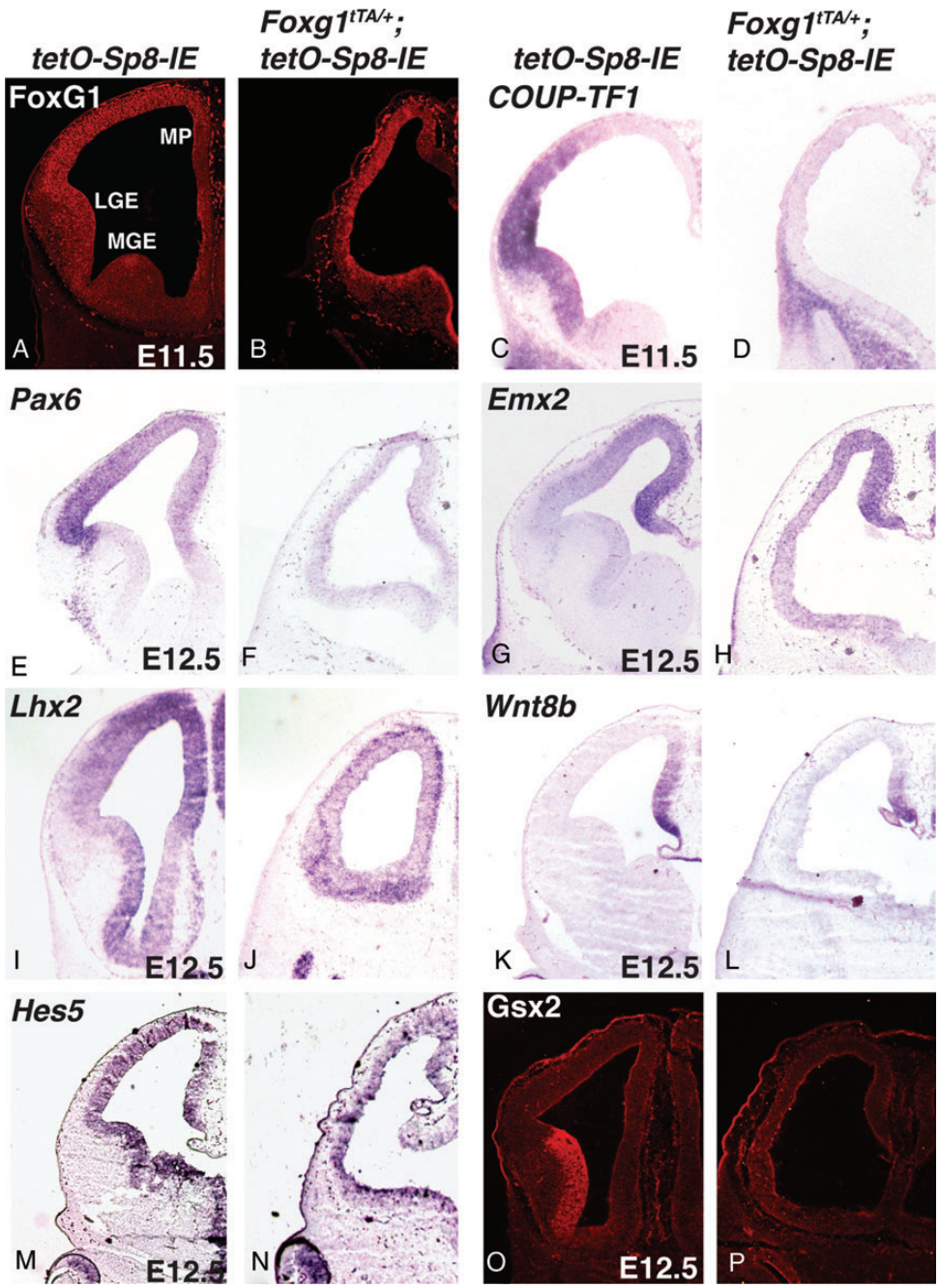

Figure 3. The brains of *Foxg1*$^{tTA/+}$;*tetO-Sp8-IE* maintain their telencephalic identity as indicated by expression of *Foxg1* (*B*). *Foxg1* expression in *tetO-Sp8-IE* controls is shown in panel (*A*). However, early dorsoventral telencephalic patterning is altered after Sp8 misexpression; there is repression of ventrally expressed transcription factors *COUP-TF*1 at E11.5 (*C* and *D*), and *Pax6* at E12.5 in *Foxg1*$^{tTA/+}$;*tetO-Sp8-IE* animals (*F*) as compared to *tetO-Sp8-IE* controls (*E*). On the other hand, the domain of expression of the dorsally expressed gene *Emx2* appears slightly increased in the lateral pallium in *Foxg1*$^{tTA/+}$;*tetO-Sp8-IE* animals (*H*) as compared to *tetO-Sp8-IE* control (*G*). On the other hand, *Lhx2* expression is downregulated in the embryos misexpressing Sp8 (*I* and *J*). Expression of the medial pallium and cortical hem marker, *Wnt8b* remains unchanged in *Foxg1*$^{tTA/+}$;*tetO-Sp8-IE* animals (*L*) as compared to *tetO-Sp8-IE* controls (*K*). *Hes5* expression, a marker for neuroepithelial progenitors and Notch signaling, is severely reduced in the *Foxg1*$^{tTA/+}$;*tetO-Sp8-IE* animals (*N*) as compared to *tetO-Sp8-IE* controls (*M*). Additionally, the expression of the subpallial transcription factor *Gsx2*, is severely reduced in *Foxg1*$^{tTA/+}$;*tetO-Sp8-IE* animals (*P*) as compared to *tetO-Sp8-IE* controls (*O*). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MP, medial pallium.

We first analyzed the expression of *Foxg1* to examine whether cortical progenitors in the double transgenic embryos maintain telencephalic fate. Foxg1 protein expression persisted in the double transgenic embryos (Fig. 3*B*), albeit at a reduced level than in the controls (Fig. 3*A*), demonstrating that this feature of telencephalic fate was not significantly modified by the misexpression of Sp8.

To analyze dorsoventral patterning of the pallium, we looked at the expression of *COUP-TF1* and *Pax6*, markers that are expressed highly in the ventral portion the pallium (Fig. 3*C,E*), unlike *Sp8* (Fig. 1*B*). The earliest observed change in gene expression was the loss of *COUP-TF1* expression in the ventrolateral pallium at E11.5 (Fig. 3*D*). *COUP-TF1* is normally expressed in both progenitors and ventrolaterally

derived neurons. Thus, misexpression of *Sp8* in the ventrolateral portions of the pallium appeared to alter the identity of both progenitors and newborn neurons of this region. While *Pax6* expression was still present in the double transgenic pallium at E11.5 (data not shown), it was severely reduced by E12.5 (Fig. 3*F*). By contrast, expression of *Emx2*, a marker that is expressed highly in the dorsomedial cortical neuroepithelium, was maintained; its levels appeared to be slightly increased in ventrolateral pallium (cf. Fig. 3*G* and 3*H*). Unlike *Emx2*, *Lhx2* (which shows a similar dorsoventral gradient of pallial expression; Fig. 3*I*), was severely downregulated in the dorsomedial region of embryos misexpressing Sp8 (Fig. 3*J*). To analyze the properties of the dorsomedial pallium in the double transgenic embryos, we studied the expression of *Wnt8b*, a marker of the medial pallium and cortical hem. *Wnt8b* expression in this portion of the pallium appeared normal (cf. Fig. 3*K* and 3*L*), suggesting that some aspects of the molecular identity of these dorsal-most pallial regions remained intact in the embryos misexpressing Sp8. However, the repression of *Lhx2* in the dorsomedial pallium provides evidence that Sp8 has specific effects in that cortical domain.

Thus, telencephalic misexpression of Sp8 induces molecular patterning defects in the pallium. Initially it leads to a downregulation of *COUP-TF1* followed by other alterations in pallial gene expression, which disrupts ventrolateral pallium patterning. These changes in pallial gene expression were similar when analyzed at different positions along the rostocaudal axis (data not shown), likely reflecting the fact that *Foxg1*^{tTA} expression is fairly uniform along this dimension.

Although telencephalic size appeared normal at E11.5 in double transgenic embryos (data not shown), by E12.5 it was reduced (data not shown and Fig. 2), suggesting that *COUP-TF1* downregulation was not a secondary effect of the reduced cortical surface, and that by this stage the observed molecular patterning changes reduce progenitor maintenance and/or expansion. To test this idea, we performed in situ hybridization for *Hes5* at E12.5, to study expression of this marker of neuroepithelial progenitors and Notch signaling. Importantly, *Hes5* expression was reduced in the double transgenic embryos (Fig. 3*N*) as compared to controls (Fig. 3*M*), providing evidence that *Sp8* overexpression caused telencephalic hypoplasia by repressing neuroepithelial properties. Below, we show that the hypoplasia was also mediated by premature neurogenesis, reduced proliferation, and increased apoptosis. These data together demonstrate that Sp8 misexpression throughout the dorsal telencephalon suppresses the molecular identity of the ventrolateral pallium, while maintaining aspects of dorsomedial pallial identity.

Pax6 is required for dorsoventral patterning in the telencephalon, where it has a critical role in the ventrolateral pallium (Stoykova et al. 2000; Toresson et al. 2000; Yun et al. 2001). The loss of *Pax6* results in the dorsal expansion of subpallial markers such as Gsx2 and Dlx proteins. However, this is not the case when Sp8 is overexpressed in the double transgenic embryos, as expression of *Gsx2* and *Dlx* were severely reduced (Fig. 3*P* and data not shown), as compared to controls (Fig. 3*O* and data not shown), even though *Pax6* expression was suppressed (Fig. 3*E*,*F*). Despite the loss of ventral telencephalic markers, the molecular identity of the ventral portion of the double transgenic telencephalon does not appear to fully convert to pallial fates, because the pallial marker Tbr1 does not extend into the ventral-most regions (Supplementary Fig. S2).

Effects of Delayed Misexpression of Sp8

As mentioned earlier, *Sp8* is only expressed within pallial progenitors until around E15 (Waclaw et al. 2006). Thus, we wondered if the phenotype described above would be evident in pallial progenitors misexpressing Sp8 after E15. To test this, we delayed the overexpression of *Sp8* by administering Dox to the pregnant mice between E8 and E11 (Fig. 2*B*). This Dox treatment schedule delayed transgene activation until approximately E15 (data not shown). In contrast to the non-Dox-treated Sp8 misexpressing embryos, the morphology of the Dox-treated double transgenic embryos appeared similar to controls (cf. Fig. 4*A* and 4*B*). Moreover, *COUP-TF1* expression in the Dox-treated double transgenic embryos (Fig. 4*D*) appeared unchanged, similar to that in the control (Fig. 4*C*). These results indicate that delaying misexpression of Sp8 until E15 had no obvious effects on cortical development and supports an early role for Sp8 between E8 and E15 in dorsomedial pallial development.

Effect of COUP-TF1 Misexpression on Sp8 Expression

Our findings show that Sp8 negatively regulates the expression of the *COUP-TF1* and *Pax6* transcription factors that are expressed in ventrodorsal gradients of the pallium. Given that *Sp8* and *COUP-TF1* exhibit reciprocal pallial expression patterns (Fig. 1*A*,*B*), we wondered if COUP-TF1 might also function as a negative regulator of pallial *Sp8*

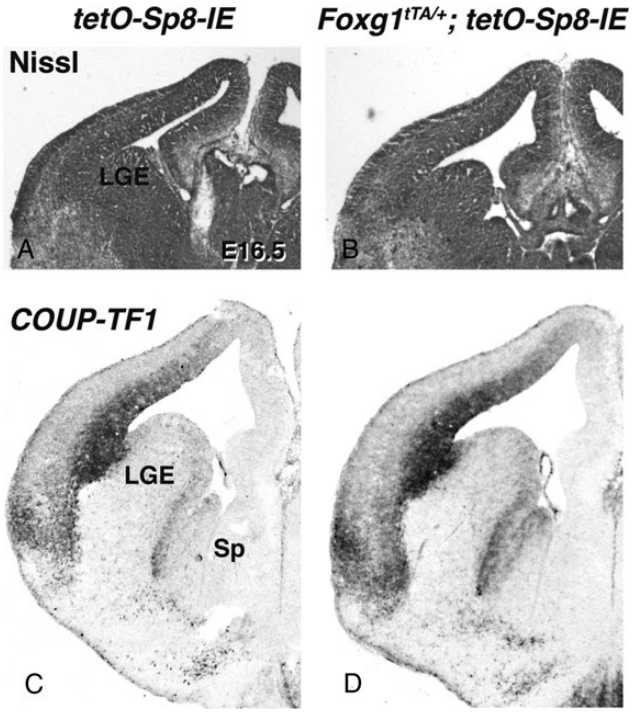

Figure 4. Misexpression of *Sp8* in late-stage telencephalic progenitor cells has no obvious effects on cortical development. *Sp8* misexpression was delayed until E14.5 by Dox administration. The effect of *Sp8* misexpression in late-stage telencephalic progenitors was analyzed at E16.5. Nissl-stained *tetO-Sp8-IE* and *Foxg1*$^{tTA/+}$*; tetO-Sp8-IE* cortices at E16.5 show similar morphology (*A* and *B*, respectively) and *COUP-TF1* expression appears similar in *tetO-Sp8-IE* and *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* cortices (*C* and *D*, respectively). LGE, lateral ganglionic eminence; Sp, septum.

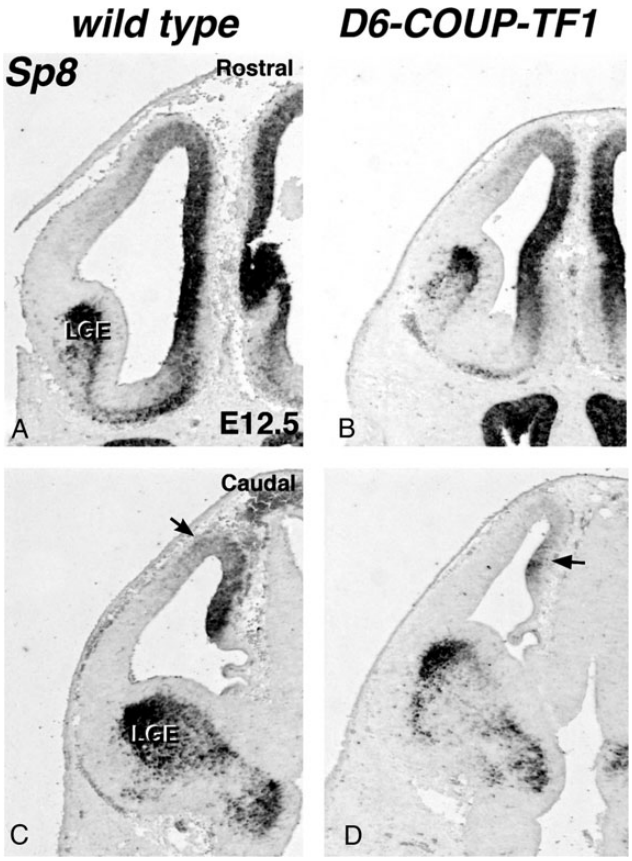

Figure 5. *COUP-TF1* overexpression in the medial pallium represses *Sp8* expression. Weak overexpression of *COUP-TF1* in the rostral pallium of *D6-COUP-TF1* animals (*B*) causes a mild reduction in *Sp8* expression as compared to wild-type controls at E12.5 (*A*). Strong (~4-fold) overexpression of *COUP-TF1* in the caudal pallium clearly reduces Sp8 expression in *D6-COUP-TF1* animals (*D*) as compared to controls (*C*). Arrows in *C* and *D* point to reduced *Sp8* expression in the dorsomedial cortex of the control and *D6-COUP-TF1* embryos. LGE, lateral ganglionic eminence.

expression. A previous study demonstrated that *COUP-TF1* overexpression in the dorsomedial pallium, under the *D6* enhancer (Machon et al. 2002), ventralized the dorsomedial pallium, evidenced by the downregulation of *Emx2*, *Fez-l*, and *p75* expression and dorsal expansion of *FgfR3* and *Er81* in the dorsomedial pallium (Faedo et al. 2008). In line with this, *Sp8* pallial expression was also reduced in these *COUP-TF1* gain of function mutants; this phenotype was stronger in caudal regions (Fig. 5*D*) than rostral regions (Fig. 5*B*), consistent with the caudorostral gradient of the overexpressed *COUP-TF1* (Machon et al. 2002; Faedo et al. 2008; Supplementary Fig. S3). In fact, the overexpression of *COUP-TF1* driven by the *D6* enhancer was 4.75-fold higher than control in caudal regions, whereas it was only 2.1-fold higher in rostral regions (Supplementary Fig. S3). While this gain-of-function analysis showed that COUP-TF1 can repress *Sp8*, *COUP-TF1*$^{-/-}$ mutants do not show a change in *Sp8* expression (Faedo and Rubenstein, unpublished), which may reflect compensation by COUP-TFII. Taken together, our findings provide evidence that cross-repression between Sp8 and COUP-TF1 contributes to dorsoventral patterning of the developing pallium.

Misexpression of Sp8 Alters Survival, Proliferation and Differentiation of Cortical Progenitors

To understand the cellular mechanisms underlying the hypoplasia, resulting from Sp8 gain of function, we examined apoptosis, differentiation, and cell proliferation in the double transgenic at E11.5 and E14.5. Sp8 misexpression increased activated Caspase3 staining in both the lateral pallium and the subpallium of double transgenic embryos at E11.5 (Fig. 6*B*,*R*). There were no significant differences in the rostral to caudal distribution of these apoptotic cells (Fig. 6*Q*). By E14.5, activated Caspase3 staining was greatly increased in region of the ventral telencephalon; increased staining was also apparent in the lateral pallium of embryos misexpressing Sp8 as compared to control animals (cf. Fig. 6*C* and 6*D*). Thus, misexpression of Sp8 throughout the telencephalon increased apoptosis, which at later stages is particularly enhanced in the ventral telencephalon. This increase in apoptosis was, however, not due to a general cytotoxic effect of overexpressing Sp8 in telencephalic progenitors. When Sp8 overexpression in double transgenic embryos was delayed until E15 by Dox treatment between E8 and E11, there was no increase in cell death observed either at E16.5 or at E18.5 (i.e., 3–4 days of overexpression) when compared with control embryos (Fig. 6*E*,*F*,*G*,*H* respectively), despite robust transgene expression (see insets in Fig. 6*F*, *H*). Thus, the increased cell death after misexpression of Sp8 is specific to early stages (i.e., before E15).

In addition to apoptosis, Sp8 misexpression promoted precocious neuronal differentiation. There was a robust increase in βIII-tubulin expression throughout the neuroepithelial wall as early as E11.5 in the double transgenic embryos (Fig. 6*J*). At E14.5, expression of βIII-tubulin is evident within the ventricular zone region of the lateral pallium (arrows in Fig. 6*L*), whereas in controls staining is excluded from the ventricular zone, and is restricted to the mantle zone (Fig. 6*K*). Thus, misexpression of Sp8 leads to precocious neuronal differentiation of ventricular zone progenitors, particularly within the ventrolateral portion of the pallium. In conjunction, we observed a disruption of apical–basal polarity of ventricular zone progenitors within this portion of the pallium, based on β-catenin staining in the double transgenic embryos (data not shown).

Finally, we examined cell division by staining with phospho-Histone 3 (pH3), which marks cells in M-phase. Misexpression of Sp8 led to a reduction in pH3^{+} cells in the double transgenic telencephalon already at E11.5 (Fig. 6*N*) as compared to control animals (Fig. 6*M*). Along the rostrocaudal axis, proliferation was most significantly decreased in the mid and caudal levels, with reductions of 70% and 40%, respectively (Fig. 6*S*). Similarly, along the dorsoventral axis, proliferation was decreased by 63% in the subpallium and 42% in the dorsal pallium (Fig. 6*T*). The reduction in M-phase cells became more severe over time. By E14.5, the lateral portion of telencephalon in embryos misexpressing Sp8 was nearly devoid of pH3-expressing cells (Fig. 6*P*). Taken together, these results indicate that the misexpression of Sp8 leads to increased cell death, precocious neurogenesis, and reduced proliferation of telencephalic progenitors. These results are consistent with the reduction in *Hes5* expression in double transgenic animals (see Fig. 3*M*,*N*), suggesting that reduced Notch signaling may contribute to the reduced proliferation and precocious neuronal differentiation.

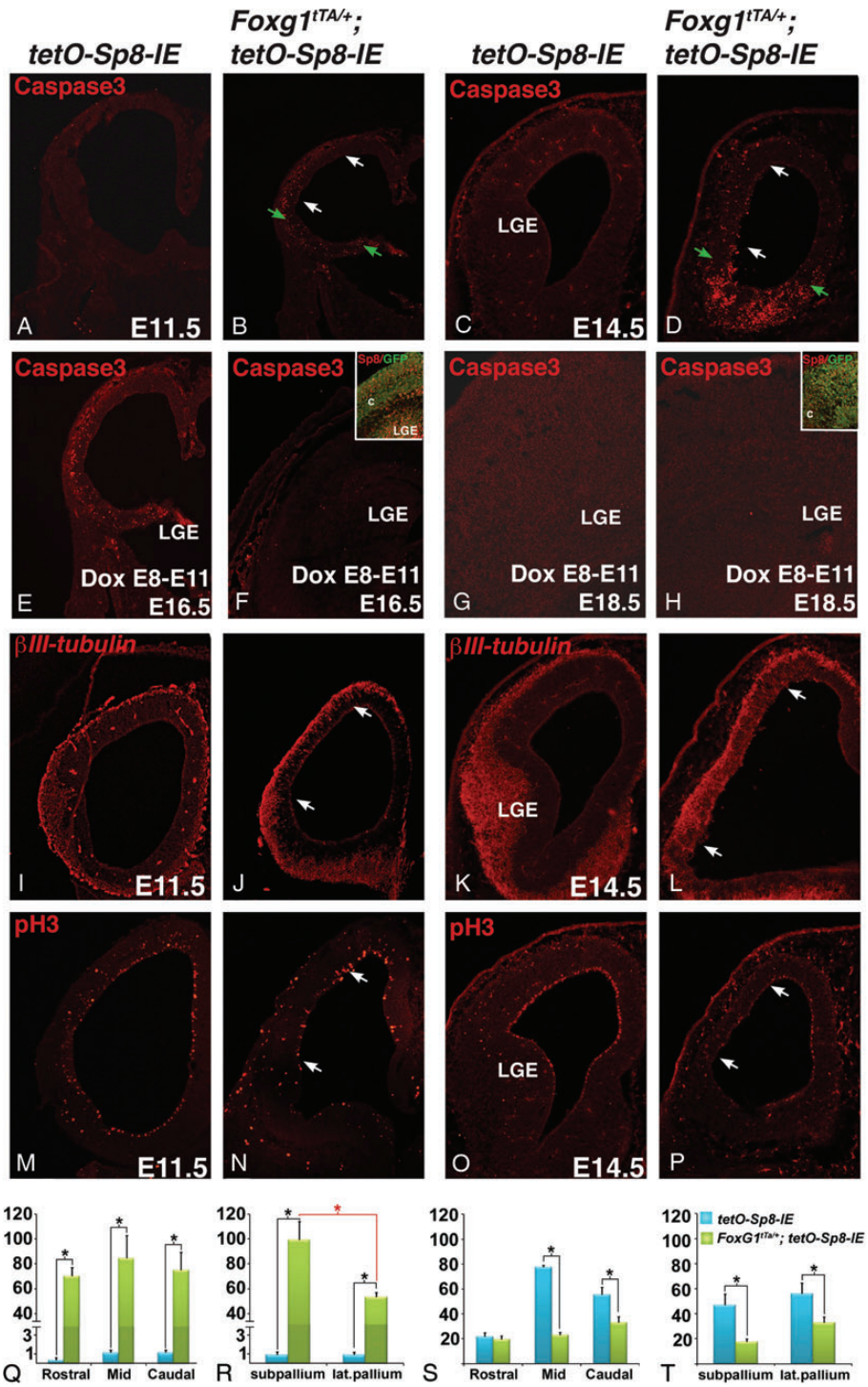

Figure 6. Misexpression of Sp8 alters cell death, proliferation, and differentiation of telencephalic progenitors. Cell death is strongly increased in the ventrolateral telencephalon of *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* animals (*B*) compared with *tetO-Sp8-IE* controls (*A*) as early as E11.5. These apoptotic cells are concentrated ventrally in the telencephalon (in the subpallium and ventrolateral pallium) (*R*); on the other hand, they appear to be evenly distributed along the rostrocaudal axis (*Q*). Cell death in these regions is further increased by E14.5 (*C* and *D*). Green arrows in *B* and *D* indicate the approximate position of the pallial/subpallial boundary; the white arrows indicate the ventrolateral pallium. However, no increase in apoptosis is observed in E16.5 or E18.5 Dox-treated animals, in which *Sp8* misexpression is delayed until E15 (cf. *E* with *F* and *G* with *H*). Insets in *F* and *H* show *EGFP* and *Sp8* overexpression throughout the telencephalon at E16.5 and E18, after Dox treatment between E8 and E11. Another early effect of Sp8 overexpression is an increase in neuronal differentiation, as marked by βIII-tubulin. As early as E11.5, βIII-tubulin is increased in *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* animals (*J*) compared with control *tetO-Sp8-IE* animals (*I*). βIII-tubulin immunoreactivity becomes even more pronounced by E14.5 (*K* and *L*) when the ventricular zone in lateral regions (arrows in *L*) of *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* cortex shows precocious neuronal differentiation compared with *tetO-Sp8-IE* control (*K*). Cell proliferation, as indicated by phospho-Histone 3 (pH3), can be seen along the ventricular surface of control brains (*M* and *N* and *O* and *P*, E11.5 and E14.5, respectively) but is severely reduced in the lateral pallium (arrows in *N* and *P*) and subpallium of *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* animals. The effects of Sp8 misexpression on cell proliferation are more pronounced at E14.5 (*O* and *P*) than at E11.5 (*M* and *N*). Proliferation is strongly reduced at mid and caudal levels of the telencephalon as shown in *S*; the reduction is not limited to either the lateral or ventral part of telencephalon (*T*). LGE, lateral ganglionic eminence, $^{*}P < 0.01$.

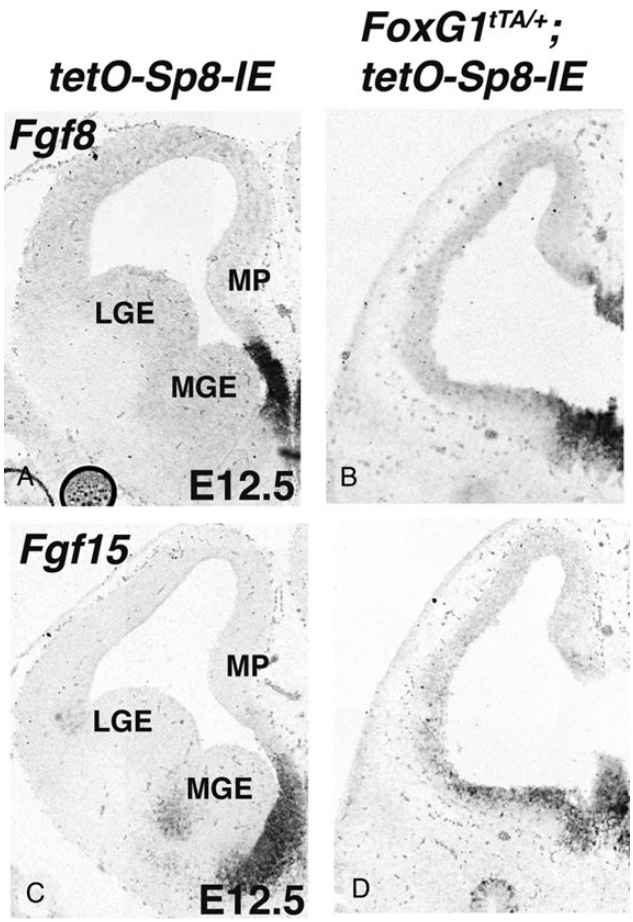

Figure 7. Coronal sections of E12.5 brains show expression of *Fgf8* (*A* and *B*) and *Fgf15* (*C* and *D*). *Fgf8* and *Fgf15* expression persist in *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* animals (*B* and *D*) compared with *tetO-Sp8-IE* controls (*A* and *C*). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MP, medial pallium.

Expression of Fgfs and Downstream Effectors in Embryos Misexpressing Sp8

A previous gain-of-function study using *Sp8* electroporation (Sahara et al. 2007) suggested that Sp8 is sufficient to induce and/or maintain Fgf8 expression within the telencephalon. However, *Sp8* loss-of-function studies show that, although reduced, *Fgf8* remains expressed in the *Sp8* null mutant telencephalon (Bell et al. 2003; Zembrzycki et al. 2007). To determine whether misexpression of Sp8 alters the expression domain of *Fgf* genes, we examined the expression of *Fgf8* and *Fgf15*. In the double transgenic telencephalon, *Fgf8* expression was confined to the rostroventral midline (Fig. 7*B*), similar to that in the control (Fig. 7*A*). On the other hand, *Fgf15* expression was present in much of the ventral/subpallial portion of the double transgenic telencephalon; however, it was not ectopically expressed in the pallial domain of Sp8 misexpression (Fig. 7*D*). Therefore, misexpression of Sp8 using our binary transgenic approach did not induce *Fgf8* or *Fgf15* expression in pallial progenitors.

Sp8 expression is downstream of Fgf8 and Fgf17 function based on loss-of-function (Storm et al. 2006; Cholfin and Rubenstein 2008) and gain-of-function (Sahara et al. 2007) experiments. Therefore, we reasoned that Sp8 may participate in transducing Fgf signaling. To examine this possibility, we analyzed the expression of the Fgf effectors like *Spry2* (Minowada et al. 1999; Storm et al. 2003; Kim and Bar-Sagi 2004; Borello et al. 2008; Cholfin and Rubenstein 2008; Faedo et al. 2010), *Erm* (Roehl and Nusslein-Volhard 2001; Shimogori et al. 2004; Cholfin and Rubenstein 2008), and *Mest* (Sansom et al. 2005; Borello et al. 2008). In double transgenic embryos at E11.5 and E12.5, we observed ectopic expression of *Spry2* throughout the telencephalon (Fig. 8*B* and data not shown), as compared to the control where *Spry2* expression matched closely the expression of *Fgf8* and *Fgf15* (cf. Fig. 8*A* with 7*A* and 7*C*). This was also true for *Erm* and *Mest*, which were ectopically expressed, albeit to a lesser extent than *Spry2*, throughout the telencephalon of E12.5 embryos misexpressing Sp8 (Fig. 8*D*,*F*).

Fgfs signal through the MAPK cascade ultimately leading to phosphorylation of ERK (Powers et al. 2000; Mason 2007). To assess putative Fgf signaling, we examined phospho(p)-ERK expression in the embryos misexpressing Sp8. Interestingly, the double transgenic embryos at E11.5 and E12.5 showed increased pERK staining particularly within the ventrolateral pallial regions (Fig. 8*H* and data not shown). Therefore, our results provide evidence of a feedback loop between Sp8 and Fgf signaling effectors (Fig. 9).

Discussion

In this study, we have examined the role of the transcription factor Sp8 on cortical patterning and differentiation. While the role of Sp8 on cortical arealization has been reported in other studies (Sahara et al. 2007; Zembrzycki et al. 2007), here we focus on the earlier cortical functions of Sp8. We concentrated on the early phenotype of Sp8 gain-of-function mutants to identify the first changes in gene expression and cell behavior (e.g., survival, proliferation, and differentiation) due to Sp8 misexpression.

Using a unique and powerful binary transgenic approach to misexpress Sp8 temporally throughout the telencephalon, we examined the role of this transcription factor in rostrocaudal and dorsoventral patterning of the developing cortex. Moreover, our gain-of-function approach allowed us to examine the role of Sp8 in the proliferation, survival, and differentiation of telencephalic progenitors. In our study, we found that Sp8 exerts its activity on regional patterning and progenitor cell differentiation and survival during a precise time window in the embryonic cortical anlage. We also provide evidence that Sp8 regulates these processes by promoting Fgf signaling. Because of the severe effects of Sp8 misexpression on early cortical development, analysis of later cortical properties, such as arealization, was not possible.

Our findings provide evidence that Sp8 and another transcription factor, COUP-TF1, mutually repress each other's cortical neuroepithelial expression along the anterior–posterior and dorsoventral axes. The expression of these factors is downstream of Fgf signaling: *Sp8* is positively regulated by Fgf8 (Storm et al. 2006; Sahara et al. 2007), whereas *COUP-TF1* is repressed (Garel et al. 2003; Storm et al. 2006; Borello et al. 2008). Our data suggest that Sp8 mediates its effects, at least in part, by potentiating Fgf signaling, whereas previously COUP-TF1 was proposed to repress Fgf signaling. Therefore, we propose a novel molecular mechanism that couples cortical patterning and growth through a regulatory circuit consisting of a feedback loop between Fgf signaling and its transcription factor effectors Sp8 and COUP-TF1.

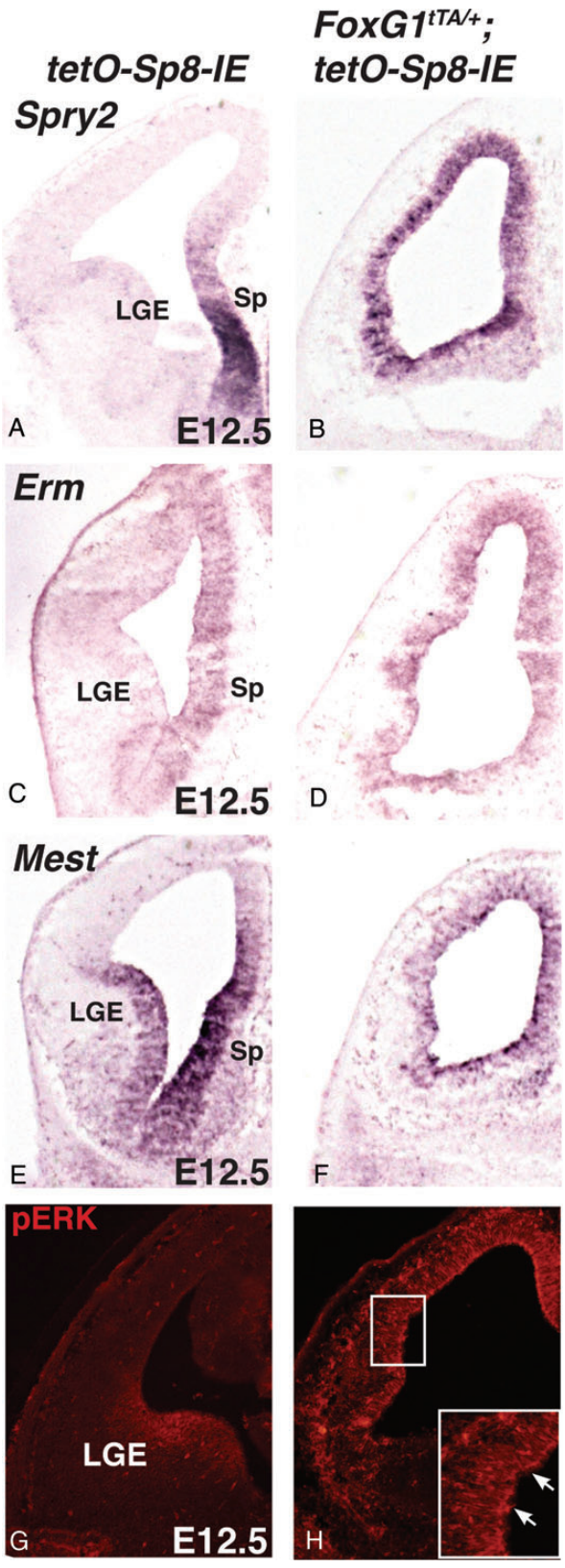

Figure 8. Misexpression of Sp8 increases the expression of Fgf effector molecules within the telencephalon. Expression of *Spry2* (*A* and *B*), *Erm* (*C* and *D*), and *Mest* (*E* and *F*) and immunoreactivity of pERK (*G* and *H*) are increased in *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* (*B*, *D*, *F*, and *H*) brains as compared to *tetO-Sp8-IE* controls (*A*, *C*, *E*, and *G*). Inset in H is a higher magnification image showing increased pERK staining (arrows) in the ventrolateral pallium of the *Foxg1*$^{tTA/+}$*;tetO-Sp8-IE* animals. LGE, lateral ganglionic eminence; Sp, septum.

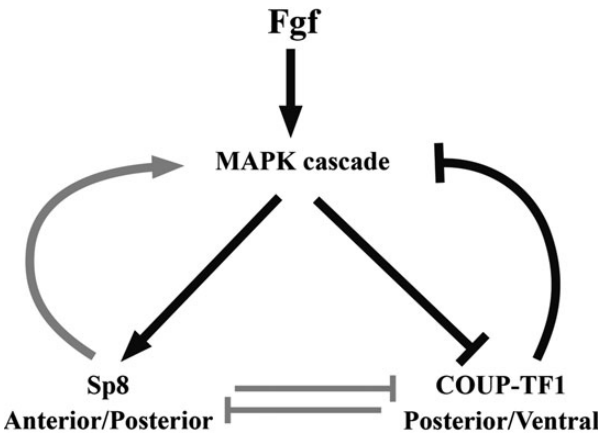

Figure 9. Model of the Sp8 regulated gene network involved in cortical development. Fgfs produced at the cortical patterning center act on *Sp8* and *COUP-TF1* through the MAPK cascade. MAPK signaling upregulates *Sp8* and downregulates *COUP-TF1*. *COUP-TF1* in-turn downregulates Fgf signaling. Additionally, we show that *COUP-TF1* and *Sp8* cross-repress each other and that *Sp8* can enhance MAPK signaling in cortical progenitor cells. A balance between the various branches of this gene network controls rostral-caudal and dorsoventral patterning of the cortex.

Sp8 Regulates Cortical Patterning

To analyze the role of Sp8 during forebrain development, we focused on the effects of cortical Sp8 gain of function, using a method that allows for temporal and spatial control of *Sp8* expression. Early misexpression of Sp8 throughout the telencephalon (from E8.5 onward) using the *Foxg1tTA* driver mice, causes severe telencephalic hypoplasia. Additionally, this misexpression alters the expression gradients of cortical patterning genes. We observed the most severe alterations in ventrolateral pallial identity, with the earliest change being a loss of *COUP-TF1* followed by a loss of *Pax6* expression. Medial pallium identity was less affected by Sp8 misexpression; expression of *Emx2* and *Wnt8b* was maintained, while there was a clear reduction in *Lhx2* expression. It is interesting to note that reduced *Lhx2* may contribute to forebrain hypoplasia (Porter et al. 1997). Taken together, Sp8 appears to promote medial pallial identity through the repression of dorsolateral pallial specification, notably via the downregulation of *COUP-TF1* and *Pax6*.

Sp8 is also expressed in a rostral high/caudal low gradient (e.g., Fig. 1*A*,*C*) and its expression is downstream of the Fgf8 signaling pathway driven from the rostral patterning center (Storm et al. 2006). This suggests that Sp8 has a role on rostrocaudal as well as dorsoventral patterning of the developing cortex. Indeed, by repressing *COUP-TF1* expression and by promoting Fgf signaling, we propose that Sp8 promotes rostral properties.

Patterning and growth are generally linked. During corticogenesis, COUP-TF1 induces neuronal differentiation and downregulates progenitor markers such as *Pax6* (Faedo et al. 2008). One would, therefore, predict that the reduction of *COUP-TF1* expression in the double transgenic embryos misexpressing Sp8 would repress neurogenesis. However, this was not the case; on the contrary, we observed precocious neuronal differentiation in the cortex of these embryos. Thus, despite their opposing roles in ventrolateral and dorsomedial cortical patterning, COUP-TF1 and Sp8 both appear to promote neurogenesis, at least in overexpression transgenic

experiments. Thus, in synchrony with their distinct roles in cortical regional specification, Sp8 might promote the generation of neurons with dorsomedial pallial properties, whereas COUP-TF1 might promote the generation of neurons with ventrolateral pallial properties.

Finally, to gain insights into the temporal requirement of Sp8 activity during corticogenesis, we took advantage of our binary genetic system. We used Dox treatment to delay the misexpression of Sp8 in double transgenic embryos until roughly E15, and found no obvious effect on cortical development. Thus, cortical progenitors were only sensitive to Sp8 misexpression between E10 and E15.

Sp8 Modulates the Balance Between Proliferation and Differentiation of Cortical Progenitor Cells

By misexpressing Sp8, we provide evidence that this transcription factor promotes neuronal differentiation of cortical progenitor cells, particularly when ectopic in the dorsolateral pallium. One hypothesis is that precocious neuronal differentiation could be attributed to the loss of apical polarity within the epithelium of the cortical VZ, as demonstrated by the loss of the apical protein β-catenin (data not shown). Indeed, previous studies have shown that disruptions in apical–basal polarity promote neurogenic divisions (Gotz and Huttner 2005).

The early neural differentiation of the *Sp8* overexpressing progenitor cells was accompanied by a strong reduction in proliferation. This correlates well with evidence that there was reduced Notch signaling, based on the reduced *Hes5* expression. Therefore, an increase in Sp8 dosage shifts the proliferation/differentiation balance toward neural differentiation. This Sp8-induced alteration in the balance of proliferation/differentiation leads to a depletion of the early cortical progenitor cell pool, which we propose is the main cause of the cortical hypoplasia observed after Sp8 misexpression. The loss of progenitors would also deplete radial glia; this may underlie the observed lamination defect (Supplementary Fig. S4).

Sp8 Modulates Cell Survival

Sp8 misexpression induced apoptosis in the ventrolateral pallium and subpallium; higher levels of apoptosis were observed with the subpallium. In the wild-type subpallium, *Sp8* is expressed in the SVZ and not the VZ of the dLGE (Waclaw et al. 2006). When Sp8 is misexpresssed throughout the SVZ of the subpallium (including both the MGE and LGE), cell death is not increased (Madhavan and Campbell unpublished results). Moreover, Sp8 misexpression in telencephalic progenitors is not generally toxic because Dox-delayed misexpression of Sp8 between E15 and E18 did not increase cell death above levels seen in the controls. Therefore, the proapoptotic effect of high levels of Sp8 depends on regional, cell-type, and temporal variables. The role of Sp8 on progenitor cell survival will be the focus of future studies. It is worth noting, however, that Sp8 exerts its proapoptotic effect when Sp8 protein levels are increased as well as when they are decreased (Zembrzycki et al. 2007). This same trend for apoptosis and other effects, observed during corticogenesis in the 2 opposite conditions of the gain and loss of Sp8 function, suggests that Sp8 is a key molecule of a basic gene network regulating corticogenesis. When Sp8 protein concentration changes in progenitor cells, the main protein interactions of this network are not established properly and cortical patterning and growth are severely affected.

Sp8/Fgf Signaling Feedback Loop

Our study provides evidence that Sp8 misexpression promotes Fgf signaling in cortical progenitor cells. This could be through directly activating Fgf signaling in the cells and/or through increasing the receptivity to Fgf signaling.

Sp8 has been implicated in the induction and maintenance of *Fgf8* expression in the developing limb (Bell et al. 2003). Although *Fgf8* expression is present in the telencephalic midline of *Sp8* mutants at early stages (i.e., E9.5 and E10.5), it appears to be lost by E12.5 (Bell et al. 2003; Zembrzycki et al. 2007). This suggests that Sp8 is required for the maintenance but not the induction of *Fgf8* expression in the telencephalic midline. In contrast, a previous study showed that electroporation of *Sp8* in the lateral telencephalon resulted in ectopic expression of *Fgf8* (Sahara et al. 2007). We did not observe a similar induction of *Fgf8* in our Sp8 misexpression system. In fact, the expression domains of both *Fgf8* and *Fgf15* appeared similar to those in controls. The discrepancy in these results may come from the fact that the electroporation experiments may drive high levels of the *Sp8* expression in the SVZ and mantle zone (with lower levels in the VZ) of the lateral telencephalon, whereas our transgenic system drives high levels of *Sp8* throughout the telencephalic VZ. Thus, in line with the loss-of-function findings (Bell et al. 2003; Zembrzycki et al. 2007), it appears that Sp8 does not induce *Fgf8* expression in VZ progenitors, but rather appears to be important for the maintenance of its expression within the telencephalic midline.

Despite the observation that Sp8 does not increase *Fgf* gene expression, it appears that it increases the level of Fgf signaling, particularly in ventrolateral pallial progenitors. Indeed, we found that downstream effectors of Fgf signaling such as Spry and pERK were upregulated in the lateral pallium of embryos misexpressing Sp8.

Fgf signaling is most commonly associated with proliferation and patterning of cortical progenitors, however, it also plays a role in neuronal differentiation (Maric et al. 2007; Iwata and Hevner 2009). Our data suggest that Sp8, which is regulated in a dose-dependant manner by Fgf8 (Storm et al. 2006), enhances the differentiative effects of Fgfs in ventrolateral pallial progenitors. The increased Fgf signaling in this context promotes neural differentiation and represses proliferation. It is possible, however, that Sp8 misexpression sensitizes ventrolateral pallial cells to additional signals, because Spry and pERK are also downstream of other factors such as EGF and Ras (Mason et al. 2006). The increase in Fgf signaling could be a direct transcriptional effect of Sp8, and/or could be through Sp8-inducing molecules that increase the cell's sensitivity to Fgf signals. In either case, our findings have led us to propose a model for Sp8 function in cortical patterning. We suggest a feedback loop between Fgf signaling and Sp8 for regulating rostral and dorsomedial cortical patterning (Fig. 9). This feedback loop could be at the core of how Sp8 regulates the balance between cortical progenitor proliferation, survival, and differentiation.

During cortical development, *Sp8* is expressed in the dorsomedial pallium together with *Emx2* and the 2 have been

shown to physically interact (Zembrzycki et al. 2007). *Emx2* keeps progenitor cells in an undifferentiated state and enlarges the pool of cortical progenitor cells by promoting symmetric cell division (Heins et al. 2001), whereas *Sp8* induces differentiation of cortical progenitor cells. In fact, *Sp8* expression is increased in the dorsomedial domain of the *Emx2* mutant cortex (Cholfin and Rubenstein 2008). *Emx2* mutants also have increased expression of the neuronal marker βIII-tubulin (Mallamaci et al. 2000) and the neurogenesis-promoting transcription factor *Ngn2* (Muzio et al. 2005). Therefore, one role for Emx2 may be to counteract and/or delay the neuronal differentiation effects of Sp8. When we overexpress *Sp8* in the medial pallium (which still expresses high levels of *Emx2*), the balance between proliferation and differentiation is largely unaffected. However, in the lateral pallium, which has lower levels of *Emx2*, misexpression of *Sp8* at high levels shifts the balance toward precocious neurogenesis. A similar scenario was proposed for *COUP-TF1* and *Pax6* in the ventrolateral pallium, where *COUP-TF1* promotes neuronal differentiation, while *Pax6* promotes progenitor cell proliferation (Faedo et al. 2008).

In summary, we propose a novel molecular mechanism to explain the link between patterning and growth of the cortex with the identification of a new regulative module consisting of feedback loops between the Fgf signaling and its effectors Sp8 and COUP-TF1, which together coordinates patterning and differentiation of the cerebral cortex (Fig. 9).

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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References

Armentano M, Chou SJ, Srubek Tomassy G, Leingartner A, O'Leary DD, Studer M. 2007. COUP-TFI regulates the balance of cortical patterning between frontal/motor and sensory areas. Nat Neurosci. 10:1277–1286.

Bell SM, Schreiner CM, Waclaw RR, Campbell K, Potter SS, Scott WJ. 2003. Sp8 is crucial for limb outgrowth and neuropore closure. Proc Natl Acad Sci USA. 100:12195–12200.

Bishop KM, Goudreau G, O'Leary DD. 2000. Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science. 288:344–349.

Bishop KM, Rubenstein JL, O'Leary DD. 2002. Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. J Neurosci. 22:7627–7638.

Borello U, Cobos I, Long JE, McWhirter JR, Murre C, Rubenstein JL. 2008. FGF15 promotes neurogenesis and opposes FGF8 function during neocortical development. Neural Dev. 3:17.

Cholfin JA, Rubenstein JL. 2008. Frontal cortex subdivision patterning is coordinately regulated by Fgf8, Fgf17, and Emx2. J Comp Neurol. 509:144–155.

Cholfin JA, Rubenstein JL. 2007. Patterning of frontal cortex subdivisions by Fgf17. Proc Natl Acad Sci USA. 104:7652–7657.

Eagleson KL, Schlueter McFadyen-Ketchum LJ, Ahrens ET, Mills PH, Does MD, Nickols J, Levitt P. 2007. Disruption of Foxg1 expression by knock-in of cre recombinase: effects on the development of the mouse telencephalon. Neuroscience. 148:385–399.

Faedo A, Borello U, Rubenstein JL. 2010. Repression of Fgf signaling by sprouty1-2 regulates cortical patterning in two distinct regions and times. J Neurosci. 30:4015–4023.

Faedo A, Tomassy GS, Ruan Y, Teichmann H, Krauss S, Pleasure SJ, Tsai SY, Tsai MJ, Studer M, Rubenstein JL. 2008. COUP-TFI coordinates cortical patterning, neurogenesis, and laminar fate and modulates MAPK/ERK, AKT, and beta-catenin signaling. Cereb Cortex. 18:2117–2131.

Fukuchi-Shimogori T, Grove EA. 2001. Neocortex patterning by the secreted signaling molecule FGF8. Science. 294:1071–1074.

Garel S, Huffman KJ, Rubenstein JL. 2003. Molecular regionalization of the neocortex is disrupted in Fgf8 hypomorphic mutants. Development. 130:1903–1914.

Gorski JA, Talley T, Qiu M, Puelles L, Rubenstein JL, Jones KR. 2002. Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. J Neurosci. 22:6309–6314.

Gotz M, Huttner WB. 2005. The cell biology of neurogenesis. Nat Rev Mol Cell Biol. 6:777–788.

Grove EA, Fukuchi-Shimogori T. 2003. Generating the cerebral cortical area map. Annu Rev Neurosci. 26:355–380.

Hamasaki T, Leingartner A, Ringstedt T, O'Leary DD. 2004. EMX2 regulates sizes and positioning of the primary sensory and motor areas in neocortex by direct specification of cortical progenitors. Neuron. 43:359–372.

Hanashima C, Shen L, Li SC, Lai E. 2002. Brain factor-1 controls the proliferation and differentiation of neocortical progenitor cells through independent mechanisms. J Neurosci. 22:6526–6536.

Hébert JM, McConnell SK. 2000. Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. Dev Biol. 222:296–306.

Heins N, Cremisi F, Malatesta P, Gangemi RM, Corte G, Price J, Goudreau G, Gruss P, Gotz M. 2001. Emx2 promotes symmetric cell divisions and a multipotential fate in precursors from the cerebral cortex. Mol Cell Neurosci. 18:485–502.

Iwata T, Hevner RF. 2009. Fibroblast growth factor signaling in development of the cerebral cortex. Dev Growth Differ. 51:299–323.

Kim HJ, Bar-Sagi D. 2004. Modulation of signalling by Sprouty: a developing story. Nat Rev Mol Cell Biol. 5:441–450.

Liu Q, Dwyer ND, O'Leary DD. 2000. Differential expression of COUP-TFI, CHL1, and two novel genes in developing neocortex identified by differential display PCR. J Neurosci. 20:7682–7690.

Machon O, van den Bout CJ, Backman M, Rosok O, Caubit X, Fromm SH, Geronimo B, Krauss S. 2002. Forebrain-specific promoter/enhancer D6 derived from the mouse Dach1 gene controls expression in neural stem cells. Neuroscience. 112:951–966.

Mallamaci A, Mercurio S, Muzio L, Cecchi C, Pardini CL, Gruss P, Boncinelli E. 2000. The lack of Emx2 causes impairment of Reelin signaling and defects of neuronal migration in the developing cerebral cortex. J Neurosci. 20:1109–1118.

Maric D, Fiorio Pla A, Chang YH, Barker JL. 2007. Self-renewing and differentiating properties of cortical neural stem cells are selectively regulated by basic fibroblast growth factor (FGF) signaling via specific FGF receptors. J Neurosci. 27:1836–1852.

Mason I. 2007. Initiation to end point: the multiple roles of fibroblast growth factors in neural development. Nat Rev Neurosci. 8:583–596.

Mason JM, Morrison DJ, Basson MA, Licht JD. 2006. Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. Trends Cell Biol. 16:45–54.

Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N, Krasnow MA, Martin GR. 1999. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development. 126:4465–4475.

Muzio L, Soria JM, Pannese M, Piccolo S, Mallamaci A. 2005. A mutually stimulating loop involving emx2 and canonical wnt signalling specifically promotes expansion of occipital cortex and hippocampus. Cereb Cortex. 15:2021–2028.

O'Leary DD, Chou SJ, Sahara S. 2007. Area patterning of the mammalian cortex. Neuron. 56:252–269.

Pei Z, Wang B, Chen G, Nagao M, Nakafuku M, Campbell K. 2011. Homeobox genes Gsx1 and Gsx2 differentially regulate telencephalic progenitor maturation. Proc Natl Acad Sci USA. 108:1675–1680.

Porter FD, Drago J, Xu Y, Cheema SS, Wassif C, Huang SP, Lee E, Grinberg A, Massalas JS, Bodine D et al. 1997. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. Development. 124:2935–2944.

Powers CJ, McLeskey SW, Wellstein A. 2000. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer. 7:165–197.

Roehl H, Nusslein-Volhard C. 2001. Zebrafish pea3 and erm are general targets of FGF8 signaling. Curr Biol. 11:503–507.

Sahara S, Kawakami Y, Izpisua Belmonte JC, O'Leary DD. 2007. Sp8 exhibits reciprocal induction with Fgf8 but has an opposing effect on anterior-posterior cortical area patterning. Neural Dev. 2:10.

Sansom SN, Hébert JM, Thammongkol U, Smith J, Nisbet G, Surani MA, McConnell SK, Livesey FJ. 2005. Genomic characterisation of a Fgf-regulated gradient-based neocortical protomap. Development. 132:3947–3961.

Shen L, Nam HS, Song P, Moore H, Anderson SA. 2006. FoxG1 haploinsufficiency results in impaired neurogenesis in the postnatal hippocampus and contextual memory deficits. Hippocampus. 16:875–890.

Shimogori T, Banuchi V, Ng HY, Strauss JB, Grove EA. 2004. Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. Development. 131:5639–5647.

Siegenthaler JA, Tremper-Wells BA, Miller MW. 2008. Foxg1 haploinsufficiency reduces the population of cortical intermediate progenitor cells: effect of increased p21 expression. Cereb Cortex. 18:1865–1875.

Storm EE, Garel S, Borello U, Hebert JM, Martinez S, McConnell SK, Martin GR, Rubenstein JL. 2006. Dose-dependent functions of Fgf8 in regulating telencephalic patterning centers. Development. 133:1831–1844.

Storm EE, Rubenstein JL, Martin GR. 2003. Dosage of Fgf8 determines whether cell survival is positively or negatively regulated in the developing forebrain. Proc Natl Acad Sci USA. 100:1757–1762.

Stoykova A, Treichel D, Hallonet M, Gruss P. 2000. Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. J Neurosci. 20:8042–8050.

Toresson H, Potter SS, Campbell K. 2000. Genetic control of dorsalventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development. 127:4361–4371.

Waclaw RR, Allen ZJ 2nd, Bell SM, Erdelyi F, Szabo G, Potter SS, Campbell K. 2006. The zinc finger transcription factor Sp8 regulates the generation and diversity of olfactory bulb interneurons. Neuron. 49:503–516.

Waclaw RR, Ehrman LA, Pierani A, Campbell K. 2010. Developmental origin of the neuronal subtypes that comprise the amygdalar fear circuit in the mouse. J Neurosci. 30:6944–6953.

Waclaw RR, Wang B, Pei Z, Ehrman LA, Campbell K. 2009. Distinct temporal requirements for the homeobox gene Gsx2 in specifying striatal and olfactory bulb neuronal fates. Neuron. 63: 451–465.

Yun K, Potter S, Rubenstein JL. 2001. Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. Development. 128:193–205.

Zembrzycki A, Griesel G, Stoykova A, Mansouri A. 2007. Genetic interplay between the transcription factors Sp8 and Emx2 in the patterning of the forebrain. Neural Dev. 2:8.