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Differential Regulation of Telencephalic Pallial–Subpallial Boundary Patterning by *Pax6* and *Gsh2*

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In the embryonic telencephalon, the pallial–subpallial boundary (PSB) separates the dorsal *Pax6*+ pallium from the ventral *Gsh2*+ subpallium. Previous studies have revealed that this region is a source of cells that will populate both the olfactory bulb and basal telencephalic limbic system. However, the level of progenitor cell heterogeneity and developmental genetic regulation of this progenitor region remains to be fully elucidated. In this study we carried out a comprehensive analysis of gene expression patterns at the PSB, in addition to an examination of the combinatorial function of *Pax6* and *Gsh2* in the specification of the PSB. First, we reveal that the PSB is comprised of a complex mix of molecularly distinct progenitor pools. In addition, by analysis of single *Sey*, *Gsh2*, and *Sey/Gsh2* double mutant mice, we demonstrate that both *Pax6* and *Gsh2* are directly required for major aspects of PSB progenitor specification. Our analysis also reveals that the establishment of the epidermal growth factor receptor positive lateral cortical stream migratory route to the basal telencephalon is *Pax6* dependent. Thus, in addition to their well-characterized cross-repressive roles in dorsal/ventral patterning our analyses reveal important novel functions of *Gsh2* and *Pax6* in the regulation of PSB progenitor pool specification and patterning.

Keywords: cortico-striatal border, development, gene expression, lateral cortical stream, mutant, small eye (*Sey*)

Introduction

The embryonic telencephalon can be parcellated into 2 broad progenitor zones, the pallium and subpallium, which are responsible for generating, in a highly precise and complex manner, telencephalic excitatory and inhibitory neurons, respectively (reviewed in Corbin et al. 2001; Marín and Rubenstein 2001; Wonders and Anderson 2006). The differential and temporally regulated expression patterns of pallial (e.g., *Pax6*, *Emx1*, *Ngn2*) and subpallial (e.g., *Gsh2*, *Mash1*, *Dlx* family members) genes define discrete progenitor domains that give rise to neuronal diversity in several telencephalic regions such as the cerebral cortex (Nery et al. 2002; López-Bendito et al. 2004; Xu et al. 2004; Butt et al. 2005; Flames et al. 2007; Fogarty et al. 2007; Miyoshi et al. 2007), olfactory bulb (OB) (Wichterle et al. 1999; Stenman, Toresson, et al. 2003; Kohwi et al. 2005; Vergaño-Vera et al. 2006; Waclaw et al. 2006; Kohwi et al. 2007), amygdala (Wichterle et al. 2001; Gorski et al. 2002; Nery et al. 2002; Remedios et al. 2007), and adult subventricular zone (SVZ) (De Marchis et al. 2007; Kohwi et al. 2007; Young et al. 2007). Emerging evidence also interestingly indicates that in a manner similar to the spinal cord, combinatorial codes of regionally expressed transcription factors endow telencephalic progenitor populations with the intrinsic potential to generate distinct neuronal cell types (reviewed in Wonders and Anderson 2006; Molyneaux et al. 2007).

From early stages of telencephalic development (by approximately embryonic day 10.0 [E10.0]), the pallium and subpallium are identified by the expression of the homeodomain containing genes *Pax6* and *Gsh2*, respectively. The pallial–subpallial boundary (PSB, also known as the cortico-striatal border) is the region where high *Pax6* expression (*Pax6* is also expressed at a low level in the dorsal-most aspect of the lateral ganglion eminence (LGE) [dLGE] ventricular zone [VZ]) abuts *Gsh2* expression near the cortico-striatal angle. The PSB is comprised of neural progenitor cells from both the ventral-most aspect of the pallium (VP) and dLGE (Puelles et al. 1999; Puelles et al. 2000; Yun et al. 2001; Stenman, Toresson, et al. 2003). In addition, previous studies have also revealed that progenitor cells of the PSB transiently express *Sfrp2*, *Dbx1* (VP) and *Sp8*, *mTsh1* (dLGE) that collectively mark this region as molecularly distinct from the rest of the telencephalon (Puelles et al. 2000; Kim et al. 2001; Caubit et al. 2005; Waclaw et al. 2006). Emerging evidence from our work (Carney et al. 2006) and that of others (Fernandez et al. 1998; Puelles et al. 1999; Puelles et al. 2000; Hirata et al. 2002; Stenman, Yu, et al. 2003; Bai et al. 2008) has revealed that a major target of lateral cortical stream (LCS) migrating PSB neurons (VP and dLGE) is the basal telencephalic limbic system, most prominently the amygdala and piriform cortex. Moreover, progenitors from the PSB generate one of two main subgroups of Cajal–Retzius neurons in the pallium (Bielle et al. 2005), as well as OB interneurons derived from the rostral migratory stream (RMS) (Waclaw et al. 2006; Kohwi et al. 2007). Thus, this region is an important source of telencephalic cell populations.

Previous studies have revealed that *Pax6* and *Gsh2* are required for proper positioning of the PSB by functioning to genetically cross repress each other (Toresson et al. 2000; Yun et al. 2001; Corbin et al. 2003; Stenman, Yu, et al. 2003; Yun et al. 2003). As such, the lack of *Pax6* in *Sey* (*Small eye*; Hill et al. 1991) mutant mice results in an expansion of subpallial markers such as *Dlx1*, *Dlx2*, *Gsh2*, *Mash1*, *Vax1* into the pallium (Stoykova et al. 1996; Corbin et al. 2000; Stoykova et al. 2000; Toresson et al. 2000; Yun et al. 2001; Kroll and O'Leary 2005). Conversely, in *Gsh2* mutant mice (Szucsik *et al.* 1997) pallial markers such as *Pax6*, *Ngn2*, *Math2*, *Tbr1* extend ventrally into the LGE (Corbin et al. 2000; Toresson et al. 2000; Toresson and Campbell 2001; Yun et al. 2001). Thus, in *Sey* mutant mice VP and lateral pallial (LP) progenitors acquire subpallial properties, whereas in *Gsh2* mutant mice the dLGE

is respecified to a pallial identity. Loss of VP markers, *Sfrp2*, *Dbx1*, and *Tgfα* in *Sey* mutant mice has suggested a direct requirement of *Pax6* for their expression (Kim et al. 2001; Yun et al. 2001; Assimacopoulos et al. 2003; Stenman, Yu, et al. 2003). Similarly, dLGE development and gene expression is severely affected in *Gsh2* mutants (Corbin et al. 2001; Yun et al. 2001; Waclaw et al. 2006). However, the interpretation of the genetic dependence of *Pax6* for PSB gene expression in *Sey* mutants in confounded by ectopic *Gsh2* expression in the VP and LP. Conversely, in *Gsh2* mutants, a direct function of *Gsh2* cannot be dissociated from ectopic *Pax6* expression in the dLGE.

The purpose of this study was 2-fold: First, we sought to systematically analyze the expression patterns of PSB-specific genes during key developmental time points of neurogenesis. Second, we wanted to comprehensively dissect the individual and combinatorial roles of *Pax6* and *Gsh2* in PSB patterning, thereby providing an in-depth understanding of the genetic requirement for the establishment of neural diversity in PSB-derived structures. Our expression studies reveal that the PSB is a highly complex and dynamic telencephalic progenitor zone, comprised of multiple molecularly distinct progenitor pools. Furthermore, gene expression analysis in single *Sey* and *Gsh2* mutant mice and *Sey/Gsh2* double mutant mice, reveals that in the absence of both *Pax6* and *Gsh2* all examined aspects of patterning of the PSB remain abnormal. This indicates that these genes, in combination, are required for correct positioning and expression of PSB-specific genes. Moreover, analysis of markers that individually label the VP and dLGE further reveals a complex and novel differential regulation of PSB progenitor pool specification by *Gsh2* and *Pax6*, which changes over time during development. Furthermore, we show that epidermal growth factor receptor positive (EGFR+) cells, that mark the LCS migratory population from the PSB, are also differentially regulated by *Pax6* and *Gsh2*. Thus, our study provides important insights into the genetic requirement for the generation of the PSB, a major progenitor region for the generation of OB and limbic system cell diversity.

Materials and Methods

Animal Use

All animals used in the study were maintained according to protocols approved by the Animal Welfare and use committee at Children's National Medical Center, Washington, DC, and adhering to all animal welfare laws. For staging of the embryos, midday of the day of vaginal plug detection was considered as E0.5. Previously published *Gsh2* (Szucsik et al. 1997) and *Pax6* (*Sey*) mutants (Hill et al. 1991) were used in this study. Adult $Gsh2^{+/-}$ and *Sey*/+ heterozygotes were maintained by crosses to Swiss Webster mice (Taconic, Albany, NY). In all cases, single $Gsh2^{-/-}$, *Sey/Sey* (referred to as *Sey* mutant), or *Sey/Sey*;$Gsh2^{-/-}$ double homozygous mutant (referred to as *Sey/Gsh2* double mutant) embryos were generated by heterozygous crosses as previously described (Corbin et al. 2000; Toresson et al. 2000; Yun et al. 2001; Waclaw et al. 2004, 2006). In some crosses, mice also carried the $Dlx2^{+/tauLacZ}$ allele, and in these cases embryos and brains were stained for X-gal to visualize Dlx2 (Corbin et al. 2000). For the *in situ* hybridization analyses, the $Gsh2^{-/-}$, *Sey/Sey*, and *Sey/Gsh2* double mutant genotypes were each processed independently with littermate heterozygote and wild-type mice used as controls to ensure correct RNA probe labeling. A minimum of 3 mutants and controls were used for all genotypes, *n* numbers are given in the figure legends.

Genotyping

Genomic DNA was isolated by phenol:chloroform extraction. Genotyping of *Gsh2* adult mice and embryos was carried out by PCR using previously described methods (Stenman, Wang, et al. 2003). PCR was performed using 2 primers (for adult genotyping) or 3 primers (for embryo genotyping) using a GC rich kit (Roche, Indianapolis, IN). *Sey* mutant embryos were identified by lack of eyes and abnormal craniofacial features (Hill et al. 1991). Additional confirmation of genotypes was carried out on sections by analysis of known, for example, *Dlx2* (RNA or X-gal staining of $Dlx2^{+/tauLacZ}$ sections) (Corbin et al. 2000) expression changes in *Gsh2* and *Sey* mutant embryos. *Sey/Gsh2* double mutant sections were confirmed by the lack of Pax6 and Gsh2 protein in addition to the lack of eyes and PCR for *Gsh2* mutant and wild-type alleles. Male heterozygous $Dlx5/6^{-cre\text{-}ires\text{EGFP}}$ (Stenman, Toresson, et al. 2003, herein referred to as Dlx5/6-GFP) mice were crossed with Swiss Webster females to generate Dlx5/6-GFP+ embryos. The adults were genotyped by PCR for Cre (CRE-F: GCGGTCTGGCAGTAAAAACTATC; CRE-R: GTGAAACAGCATTGCTGTCACTT) and the embryos were identified by GFP fluorescence using a dissecting microscope.

Nonradioactive Dioxygenin-Labeled RNA In Situ Hybridization

Whole heads (E12.5, E13.5) or isolated brains (E15.5) were fixed at 4 °C in 4% paraformaldehyde (PFA) (in 0.1 M phosphate buffer, pH 7.4) (4% PFA) overnight then cryoprotected in graded sucrose concentrations (10%, 20% then 30% overnight) before embedding in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA). Coronal sections at a thickness of 20 (E12.5, E13.5) or 30 μm (E15.5) were prepared using a cryostat (Microm HM505E, GMI, Inc., Ramsey, MN).

Section RNA *in situ* hybridization was carried out by slight modifications of previously described protocols (Schaeren-Wiemers and Gerfin-Moser 1993; Wilkinson and Nieto 1993). RNA probes were prepared using dioxygenin (DIG) RNA labeling kits (Roche). Air-dried cryostat sections were postfixed in 4% PFA for 10 min followed by 2 × 5 min rinses in phosphate-buffered saline (PBS). Proteinase K (Roche) digestion (20 μg/mL in PBS) was carried out for 6 min followed by 1 × 5 min rinse in PBS, refixing for 5 min in 4% PFA and another PBS rinse. The sections were acetylated for 10 min (2.2 g triethanolamine hydrochloride [Acros Organics, Geel, Belgium], 540 μL of 10 N NaOH [Fisher Scientific, Pittsburgh, PA], 300 μL of acetic anhydride [Sigma, St Louis, MO] in 60 mL of molecular grade water [Cellgro, Herndon, VA], prior to 3 × 5 min rinses in PBS). RNA probes, prepared at a dilution of 2 μL/mL of hybridization solution (50% formamide [Invitrogen, Carlsbad, CA], 10% dextran sulfate, 1% 100× Denhart's, 250 μg/mL yeast tRNA, 0.3 M NaCl, 20 mM Tris–HCl, pH8, 5 mM ethylene diaminetetraacetic acid [EDTA], 10 mM $NaPO_4$, 1% sarcosyl [all from Sigma except where indicated] in diethylpyrocarbonate-treated H_2O [Invitrogen]), were incubated at 80 °C for 2 min. Thereafter, 250 μL of the probe mix was applied to each slide, coverslipped with Hybri-slips (Sigma) and placed in a sealed box humidified with 50% formamide and H_2O and incubated at 55 °C overnight. The next day, the Hybri-slips were floated off by placing the slides in 5× saline-sodium citrate buffer (Cellgro), prior to a 30-min high stringency wash in prewarmed 50% formamide, 2× SSC at 65 °C. Next, the sections were rinsed in 3 × 10 min rinses in RNase buffer (0.5 M NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM EDTA), followed by RNaseA (Roche) treatment (20 μg/mL in RNase buffer) for 30 min and one 15 min rinse in RNase buffer, all at 37 °C. The high stringency washes were repeated twice for 20 min each at 65 °C, followed by a 15 min rinse in 2× SSC, then 0.1× SSC, both at 37 °C and a PBT (PBS + 0.1% Tween 20; Sigma) rinse for 15 min at RT. The sections were blocked with 10% goat serum in PBT for 1 h at RT, prior to a 3-h incubation with an alkaline phosphatase-coupled anti-DIG antibody (1:5000 in 1% goat serum in PBT; Roche) in a humidified chamber at RT. Then, the sections were rinsed extensively in PBT at RT (4 × 15 min rinses) and then underwent 2 × 10 min rinses in freshly prepared NTMT buffer (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20). The sections were then placed in a light-protected humidified chamber with approximately 400 μL of BM-purple AP substrate (Roche) containing ~0.25 mg/mL levamisol (Sigma) until satisfactory staining was achieved, typically overnight. Finally, the sections were rinsed twice in PBS, coverslipped using Crystal mount aqueous mounting media (Sigma) and photographed immediately.

The following probes were used in this study: *Dbx1* (Lu et al. 1992; Yun et al. 2001), *Dlx2* (Porteus et al. 1991), *Dlx5* (Eisenstat et al. 1999), *Er81* (Stenman, Toresson, et al. 2003), *Gsh1* (Toresson and Campbell, 2001), *Sfrp2* (Kim et al. 2001), *Sp8* (Bell et al. 2003; Waclaw et al. 2006), *Tgfα* (Vaughan et al. 1992; Assimacopoulos et al. 2003), and *mTsh1* (Caubit et al. 2005).

Immunohistochemistry

Sections prepared as described above for *in situ* hybridization analysis were air-dried and underwent microwave heat antigen retrieval (except for Dlx5/6-GFP+ brains which were fixed for 4 h and did not require antigen retrieval) in 10 mM sodium citrate (Fisher Scientific) pH 6.0 prior to several rinses in PBS. Subsequently, blocking of nonspecific binding sites was achieved using 10% normal serum diluted in PBS with 0.3% Triton-X (PBST; Sigma) to aid permeabilization. Primary antibodies, goat anti-β-galactosidase (β-gal) (1:200, Biogen, Cambridge, MA), sheep anti-EGFR (1:20; Millipore, Bedford, MA), rabbit anti-Gsh2 (1:2000; Toresson et al. 2000; kind gift of K. Campbell), mouse anti-Pax6 (1:2000; Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA), rabbit anti-Pax6 (1:1000; Covance Research Products, Berkeley, CA), mouse anti-RC2 (1:20; DSHB), rabbit anti-Tbr1 (1:1000; Hevner et al. 2001; kind gift of R. Hevner) were diluted in 1% normal serum in PBST and incubated overnight at 4 °C. Following 3 × 10 min rinses in PBS, the sections were incubated with the appropriate secondary Cy3- (1:200) or fluorescein isothiocyanate- (1:50) conjugated antibodies (serum and secondary antibodies were from Jackson Immunoresearch, West Grove, PA), diluted as for the primary antibodies, in the dark for 2 h at RT. After further rinses in PBS, nuclear counterstaining was performed by incubation in To-Pro-3 iodide (To-Pro-3; 1:100, Invitrogen) for 10 min at RT. Final rinses in PBS were carried out prior to coverslipping using Gel Mount aqueous mounting media (Sigma).

Data Analysis

Analysis of *in situ* hybridization experiments was performed using brightfield microscopy (Olympus BX51, Olympus, Center Valley, PA) and high-resolution digital images were captured under a ×4 objective using an Olympus D570 camera. For fluorescence, digital photographs were obtained from epifluorescence microscopy (Olympus BX61) and selected for further analysis using a Zeiss (Thornwood, NY) LSM 510 META confocal microscope. For confocal analysis, each fluorophore was scanned sequentially and Z-stacks of the images obtained were collapsed into a single projection image, or presented as individual optical sections using the LSM 510 software (Zeiss). To assess immunocolocalization, 3 sections from at least $n = 2$ brains (values given in figure legends) were examined. Figures were prepared using Adobe Photoshop CS software (Adobe Systems, San Jose, CA). Adjustments to contrast were applied across each image as a whole and equally to control and mutant brains.

Results

The PSB is defined as the location of the border between progenitor cells of pallial (*Pax6*+) and subpallial (*Gsh2*+) identity (Yun et al. 2001; Corbin et al. 2003; Stenman, Yu, et al. 2003; Yun et al. 2003). To examine the regulation of PSB patterning by *Gsh2* and *Pax6*, we used a series of well-characterized *in situ* hybridization probes that mark the VZ and/or SVZ progenitor cell compartments of either both the pallial and subpallial aspects of the PSB (*Er81*), dLGE (*Sp8*, *mTsh1*), or the VP (*Sfrp2*, *Dbx1*, *Tgfα*). These analyses were carried out at 2 developmental time points, E13.5 and E15.5, which represent the peak of severity of patterning defects in *Gsh2* and *Sey* mutants (Corbin et al. 2000; Toresson et al. 2000; Yun et al. 2001; and data shown here) and partial recovery of the striatal phenotype in *Gsh2* mutants (Corbin et al. 2000; Toresson and Campbell 2001; Yun et al. 2001; Yun et al. 2003), respectively. As the expression of *Pax6* occurs in a high-rostral to low-caudal gradient (Walther and Gruss 1991; Stoykova and Gruss 1994), we carried out this analysis at 2 rostro-caudal levels for each marker.

Pax6 and Gsh2 are Required for the Correct Expression of Er81 at the PSB

Er81 is a member of the ETS transcription factor family and is highly expressed in the dLGE SVZ, VP VZ, and other pallial and subpallial regions (Fig. 1*A,E,I,M*) (Stenman, Wang, et al. 2003; Flames et al. 2007). In *Gsh2* mutants at both E13.5 and E15.5, *Er81 e*xpression in the dLGE SVZ is missing and there is an expansion of the domain of VZ expression at E13.5 (Fig. 1*B,F,J,N*). In *Sey* mutants at both ages, *Er81* expression is absent in the VZ of the VP that is combined with an expanded expression in the SVZ in both the mutant dLGE and VP domains (Fig. 1*C,G,K,O*). In *Sey/Gsh2* double mutants, *Er81* expression remains abnormal and, although slightly less severe, closely resembles that observed in single *Sey* mutants at both E13.5 and E15.5 (Fig. 1*D,H,L,P*). Interestingly, *Er81* expression reveals nascent paraventricular ectopia as previously described in *Sey* mutant pallium (Kroll and O'Leary 2005), which are also observed in the double mutant.

Differential Regulation of dLGE Sp8 and mTsh1 Expression by Gsh2 and Pax6

Next, we examined 2 zinc finger transcription factors, *Sp8* and *mTsh1*, which are expressed in the SVZ of the dLGE aspect of the PSB. Recent studies have revealed that *Sp8* is required for the specification, migration and survival of OB interneurons (Waclaw et al. 2006) and plays a role in cortical arealization (Sahara et al. 2007; Zembrzycki et al. 2007). The mouse teashirt family of genes (*mTsh1-3*) encode zinc finger DNA binding proteins that are also expressed in the developing brain (Caubit et al. 2005).

At E13.5, *Sp8* is expressed at very high levels in the SVZ of the dLGE and at lower levels in the progenitor zones of the dorsolateral and dorsomedial cortex (Fig. 2*A,E*) (Waclaw et al. 2006). It has previously been shown that in *Gsh2* mutant mice Sp8 protein is missing at E12.5, with a partial recovery of this expression by E16.5 (Waclaw et al. 2006). Consistent with this observation, we find a striking reduction in *Sp8* expression at E13.5 in *Gsh2* mutants (Fig. 2*B,F*). In contrast, in *Sey* mutant mice, there is an expansion of *Sp8* mRNA into the *Sey* mutant pallium at E13.5 (Fig. 2*C,G*). In *Sey/Gsh2* double mutants, although there appears to be some improvement of ectopic *Sp8* expression, in general *Sp8* expression remains abnormal (Fig. 2*D,H*).

Similar to *Sp8*, *mTsh1* expression marks the dLGE SVZ. However, in contrast to *Sp8*, *mTsh1* expression persists in presumptive migrating cells that emanate from the dLGE and form the LCS migratory route to the basal telencephalon (Fig. 2*I,M*). The expression of *mTsh1* is maintained in the dLGE and cells of the presumptive LCS of *Gsh2* mutants at E13.5 (Fig. 2*J,N*), which is in surprising contrast to the loss of *Sp8* expression (Fig. 2*B,F*) thereby indicating a differential regulation of *Sp8* and *mTsh1* by *Gsh2*. In the *Sey* mutant telencephalon at E13.5, *mTsh1* expression expands into the pallium (Fig. 2*K,O*). In addition, there also appears to be ectopic expression along the outer ventral telencephalon in the area of the presumptive piriform cortex. Similar to *Sp8* expression in *Sey/Gsh2* double mutant mice, although partially recovered, *mTsh1* expression remains abnormal (Fig. 2*L,P*).

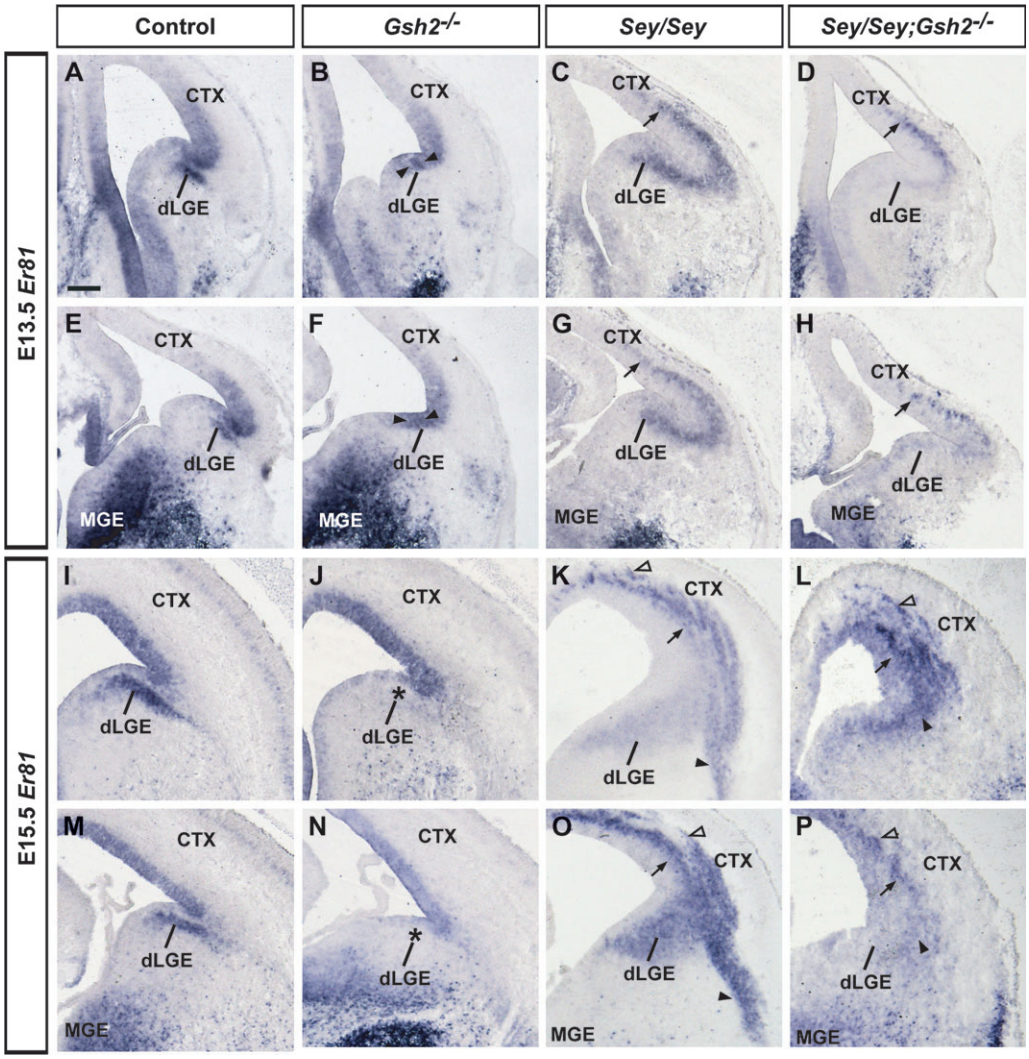

Figure 1. Expression of *Ets* transcription factor *Er81* at E13.5 and E15.5. *Er81* is expressed in the SVZ of the dLGE and VZ of the VP as shown at E13.5 in controls (*A*, *E*). VZ expression expands ventrally in *Gsh2* mutant mice (*B*, *F*, arrowheads). In *Sey* mutants, SVZ expression extends ectopically into the cerebral cortex (CTX, also termed the pallium) (*C*, *G*, arrows). In *Sey/Gsh2* double mutants, ectopic pallial expression of *Er81* resembles the *Sey* mutant phenotype, but is weaker (*D*, *H*, arrows). At E15.5, the dLGE SVZ expression of *Er81* in controls (*I*, *M*) is missing in the *Gsh2* mutant (*J*, *N*, asterisks). Conversely, in *Sey* single mutants (*K*, *O*) and *Sey /Gsh2* double mutants (*L*, *P*), *Er81* is ectopically expressed in the pallium (arrows) and forms nascent paraventricular ectopia (*K*, *L*, *O*, *P*, empty arrowhead). In the *Sey* mutant, a basal stream of *Er81* expression is also observed (*K*, *O*, arrowheads), which is not present in the double mutant (*L*, *P*, arrowhead). *n* numbers are as follows: controls, $n = 5$ [E13.5], $n = 5$ [E15.5]; $Gsh2^{-/-}$, $n = 6$ [E13.5], $n = 5$ [E15.5]; *Sey /Sey*, $n = 5$ [E13.5], $n = 5$ [E15.5]; *Sey /Sey* ;$Gsh2^{-/-}$, $n = 5$ [E13.5], $n = 5$ [E15.5]. Scale bar: 200 μm.

At E15.5, *Sp8* is still strongly expressed in the dLGE SVZ (Fig. 3*A*,*E*), which shows partial recovery of *Sp8* expression in the dLGE of *Gsh2* mutants (Fig. 3*B*,*F*) as compared with E13.5 (Fig. 2*B*,*F*), similar to that observed at E16.5 (Waclaw et al. 2006). In contrast, the *Sey* mutant telencephalon continues to exhibit robust ectopic *Sp8* expression in the pallium (Fig. 3*C*,*G*), and at medial telencephalic levels an ectopic basal stream of *Sp8* expression is observed (Fig. 3*G*). This domain of ectopic pallial *Sp8* expression, although less severe, persists in *Sey/Gsh2* mutants at E15.5 (Fig. 3*D*,*H*). *Sp8* expression reveals paraventricular ectopia in *Sey/Gsh2* double mutants (Fig. 3*D*,*H*) as in the case of the *Sey* single mutant (Kroll and O'Leary, 2005; this study).

At E15.5, similar to E13.5, *mTsh1* is expressed in the dLGE SVZ and in the LCS migratory route (Fig. 3*I*,*M*). Interestingly, although *mTsh1* expression in *Gsh2* mutants was normal at E13.5, at E15.5 expression in the dLGE and LCS is markedly reduced (Fig. 3*J*,*N*), revealing that *mTsh1* expression is temporally regulated by *Gsh2* (Fig. 3*J*,*N*).

Ectopic pallial and basal expression of *mTsh1* persists at E15.5 in the *Sey* mutant (Fig. 3*K*,*O*). Moreover, double mutant analysis shows that the loss of ectopic *Gsh2* in *Sey* mutants does not significantly rescue the abnormal phenotype (Fig. 3*L*,*P*). The formation of *mTsh1*+ paraventricular ectopia shows that dorsal telencephalic progenitors in the *Sey* mutant pallium are respecified to not only an *Sp8* lineage (Kroll and O'Leary 2005; this study), but to *mTsh1* and *Er81* lineages. Furthermore, removal of *Gsh2* from the *Sey* mutant pallium is not sufficient to rescue the respecification of dorsal telencephalic precursors to these dLGE identities.

In summary, this analysis reveals that 1) *Sp8* and *mTsh1* appear to be differentially regulated by *Gsh2* in a temporally dependent manner and 2) altered expression of *Sp8* and

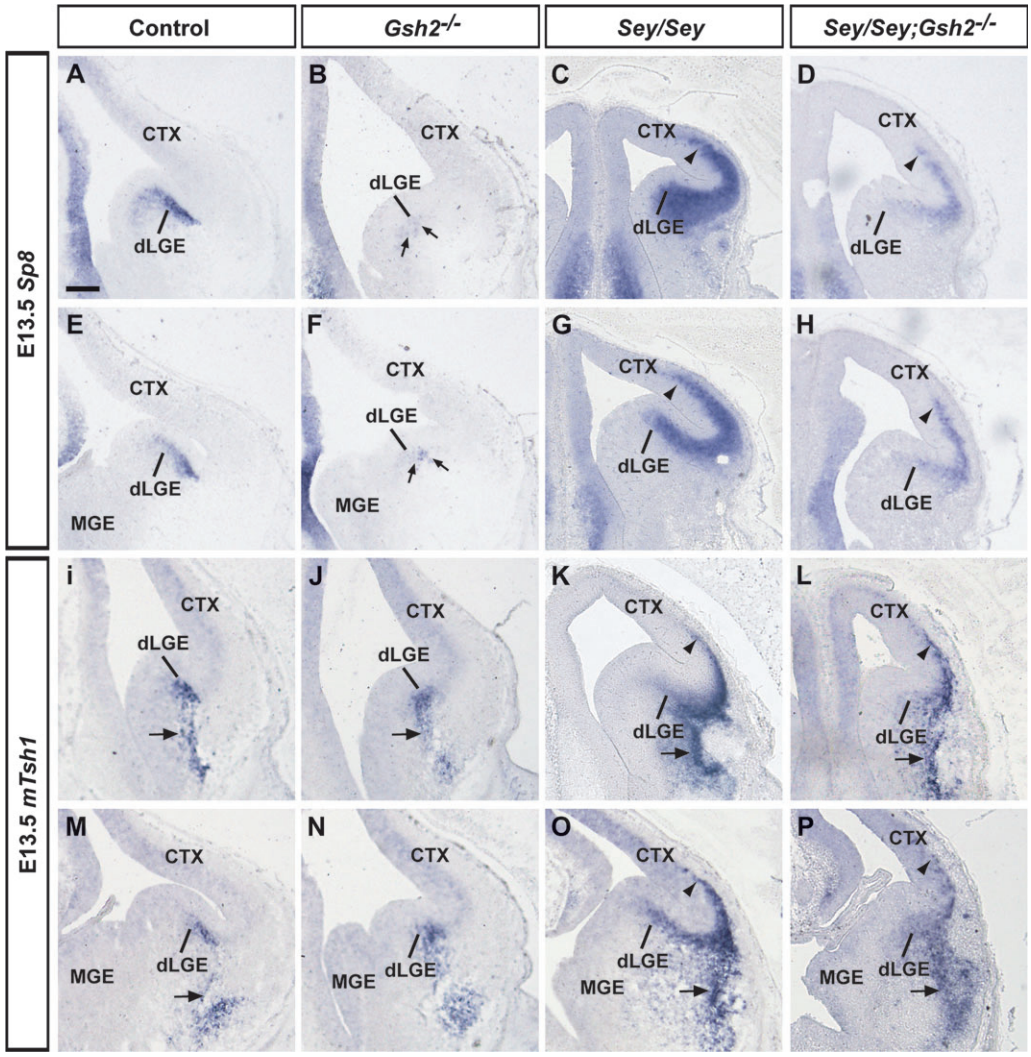

Figure 2. Expression of dLGE markers *Sp8* and *mTsh1* at E13.5. *Sp8* is strongly expressed in the dLGE SVZ in controls at E13.5 (*A*, *E*). The level of expression is markedly diminished in *Gsh2* mutants (*B*, *F*, arrows). The loss of *Pax6* in *Sey* mutants results in ectopic pallial expression of *Sp8* (C, G, arrowheads). In *Sey/Gsh2* double mutant mice, this ectopic expression, although improved over the *Sey* single mutant phenotype, persists (*D*, *H*, arrowheads). At E13.5, *mTsh1* is expressed in the dLGE SVZ and also the putative LCS migratory route (*I*, *M*, arrows). This expression is generally unaffected in *Gsh2* mutants (*J*, *N*, arrows). Conversely, in the *Sey* mutant, ectopic *mTsh1* expression is observed in both the pallium (*K*, *O*, arrowheads) and basally (*K*, *O*, arrows).This mispatterning is generally similar in the *Sey/Gsh2* double mutants (*L*, *P*, arrows, arrowheads). Abbreviation: *n* numbers for each probe are as follows: controls, $n = 4$ [*Sp8*], $n = 6$ [*mTsh1*]; $Gsh2^{-/-}$, $n = 4$ [*Sp8*], $n = 4$ [*mTsh1*]; *Sey/Sey*, $n = 4$ [*Sp8*], $n = 5$ [*mTsh1*]; $Sey/Sey;Gsh2^{-/-}$, $n = 5$ [*Sp8*], $n = 5$ [*mTsh1*]. Scale bar: 200 μm.

mTsh1 in *Sey/Gsh2* double mutants broadly resembles that of single *Sey* mutants.

Differential Regulation of Ventral Pallial Markers Sfrp2 and Dbx1 by Pax6 and Gsh2

Sfrp2, an inhibitor of the WNT secreted growth factor protein is expressed in the VZ of the VP, as shown at E13.5 and E15.5 (Figs 4 and 5*A*,*E*). In *Gsh2* mutants at E13.5, the expression domain of *Sfrp2* expands ventrally (Fig. 4*B*,*F*). This expansion remains present at E15.5 (Fig. 5*B*,*F*). In agreement with previous findings at E13.5 (Kim et al. 2001; Muzio et al. 2002), we observe that the telencephalic expression of *Sfrp2* at the PSB is missing in *Sey* mutant mice at E13.5 and E15.5 (Figs 4 and 5*C*,*G*). At E13.5 and E15.5, lack of both *Pax6* and *Gsh2* in *Sey/Gsh2* double mutant mice does not restore *Sfrp2* expression at the PSB at rostral or medial levels (Figs 4 and 5*D*,*H*). Thus, we reveal that the *Pax6*-regulated expression of *Sfrp2* is independent of *Gsh2*.

Dbx1 is a homeodomain gene that, similar to *Sfrp2*, is expressed in the VZ of the VP aspect of the PSB (Yun et al. 2001; Medina et al. 2004; Bielle et al. 2005). The VP generates cells destined for the pallium (Bielle et al. 2005) and the basolateral and lateral amygdala (Stenman, Yu, et al. 2003; Carney et al. 2006). We analyzed the expression of *Dbx1* at E12.5, after which VP expression is downregulated (Fig. 4*I*). In *Gsh2* mutants, *Dbx1*, similar to *Sfrp2* at E13.5, is ectopically expressed in the dLGE (Fig. 4*J*). We further observe that *Sey* mutants lack *Dbx1* expression at the PSB (Fig. 4*K*). Interestingly, in contrast to *Sfrp2*, removal of ectopic *Gsh2* in the *Sey* mutant pallium re-initiated expression of *Dbx1* in the LGE of *Sey/Gsh2* double mutants (Fig. 4*L*). However, this expression appears to be ectopically localized to the SVZ. This reveals that *Pax6* is required for *Dbx1* expression at the VP and *Gsh2*

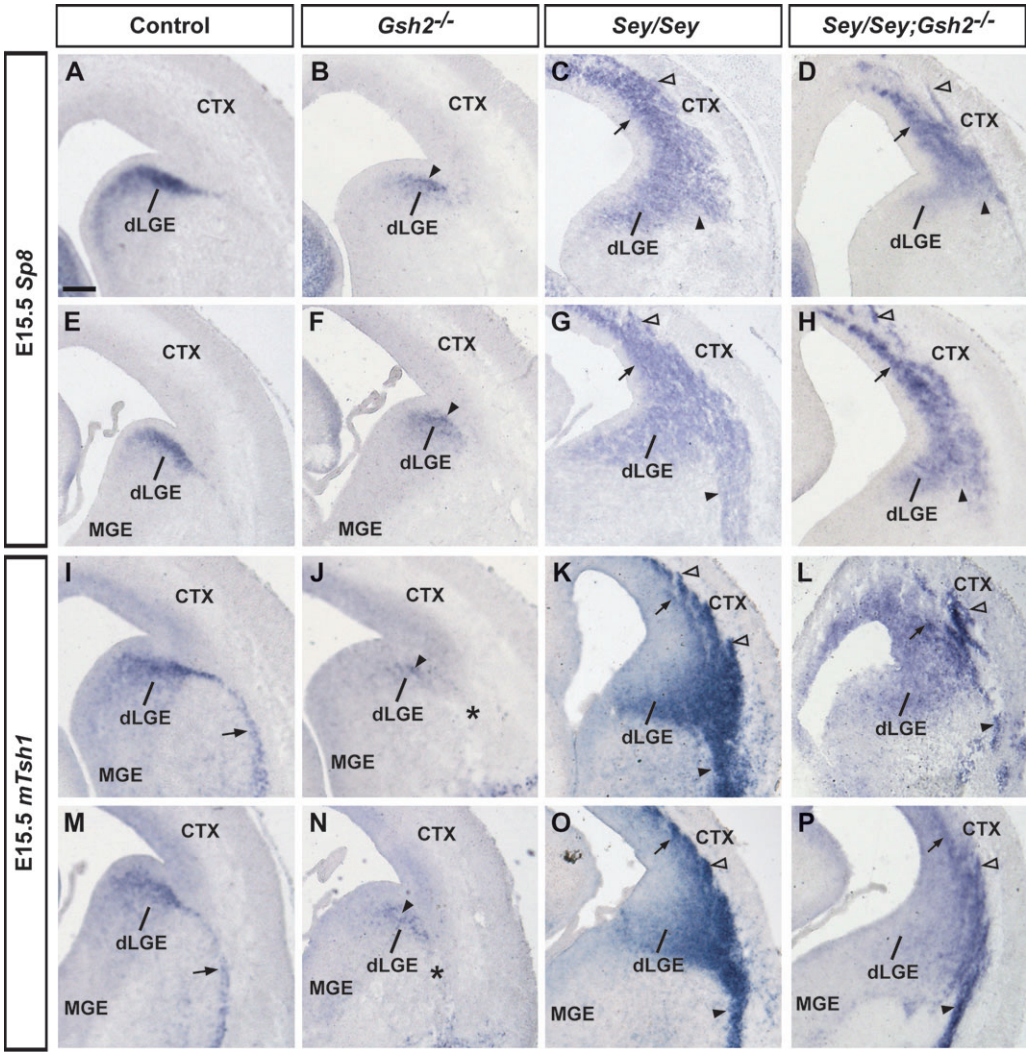

Figure 3. Expression of dLGE markers *Sp8* and *mTsh1* at E15.5. At E15.5, *Sp8* strongly marks the dLGE SVZ (*A*, *E*). In *Gsh2* mutants, this expression pattern is largely normal (*B*, *F*, arrowheads).*Sey* mutants display significant ectopic expression of *Sp8* in the pallium (*C*, *G*, arrows), with an enlarged basal stream at more medial levels (*G*, arrowhead) that is not observed more rostrally (*C*, arrowhead). *Sey/Gsh2* double mutants display ectopic pallial expression, though at diminished levels from the *Sey* mutants (*D*, *H*, arrows), with nascent paraventricular ectopia (*D*, *H*, empty arrowheads). *Sey/Gsh2* double mutants also do not display a basal stream of *Sp8* expression (*D*, *H*, arrowheads). *mTsh1* is expressed in the dLGE SVZ and putative LCS (arrows) (*I*, *M*). In *Gsh2* mutants, there is diminished *mTsh1* in the dLGE SVZ (arrows) with a lack of expression in the putative LCS (*J*, *N*, asterisk). In the *Sey* mutant, ectopic *mTsh1* expression is found in the pallium (arrows) and forms paraventricular ectopia (empty arrowheads) (*K*, *O*). Basal-directed putative migration is observed at rostral (*K*, arrowheads) and medial (*O*, arrowheads) levels. *Sey/Gsh2* double mutants also exhibit pallial ectopic *mTsh1* expression (arrows) with paraventricular ectopia (empty arrowheads) and putative basal-directed migration (arrowheads) (*L*, *P*). *n* numbers for each probe are as follows: controls, $n = 6$ [*Sp8*], $n = 6$ [*mTsh1*]; *Gsh2*$^{-/-}$, $n = 5$ [*Sp8*], $n = 5$ [*mTsh1*]; *Sey/Sey*, $n = 5$ [*Sp8*], $n = 6$ [*mTsh1*]; *Sey/Sey;Gsh2*$^{-/-}$, $n = 4$ [*Sp8*], $n = 5$ [*mTsh1*]. Scale bar: 200 μm.

functions to repress ectopic *Dbx1* expression in the LGE. Thus, similar to the above differential regulation of *mTsh1* and *Sp8 by Gsh2 and Pax6, Sfrp2,* and *Dbx1* are also regulated in a complex manner by *Gsh2* and *Pax6.*

Regulation of Tgfα Expression by Gsh2 and Pax6 at E13.5 and E15.5

Next, we examined expression of the secreted factor *Tgfα*, which is a ligand for EGFR in the developing forebrain (Kornblum et al. 1997). At E13.5 and E15.5, *Tgfα* is strongly expressed in the VP aspect of the PSB (Fig. 6*A*,*E*,*I*,*M*) (Assimacopoulos et al. 2003). In *Gsh2* mutants at E13.5, *Tgfα* expression ectopically extends into the LGE (Fig. 6*B*,*F*), similar to other VP markers, *Sfrp2* and *Dbx1* (Fig. 4*B*,*F*,*J*). At E15.5 in *Gsh2* mutants, *Tgfα* expression remains slightly expanded (Fig. 6*J*,*N*) along with ectopic expression along the route of the putative LCS (Fig. 6*N*). In agreement with previous findings (Assimacopoulos et al. 2003), *Tgfα* expression is absent in the VP in *Sey* mutants at both ages (Fig. 6*C*,*G*,*K*,*O*). Similar to *Sfrp2*, but in contrast to the VP marker *Dbx1*, expression of *Tgfα* is not restored at the PSB in the *Sey/Gsh2* double mutants at either age (Fig. 6*D*,*H*,*L*,*P*).

Interneuron markers Dlx2 and Dlx5

The *Dlx* family of homeobox transcription factors comprises 6 genes, 4 of which are expressed in the developing forebrain (Bulfone, Puelles, et al. 1993; Simeone et al. 1994; Anderson et al. 1997; Liu et al. 1997). *Dlx1* and *Dlx2* are expressed in the subpallial VZ and SVZ, whereas *Dlx5* and *Dlx6* are strongly expressed in the subpallial SVZ and postmitotic regions (Eisenstat et al. 1999). We have previously shown that in the absence of *Gsh2*, there is a lack of Dlx2+ cells along the LCS

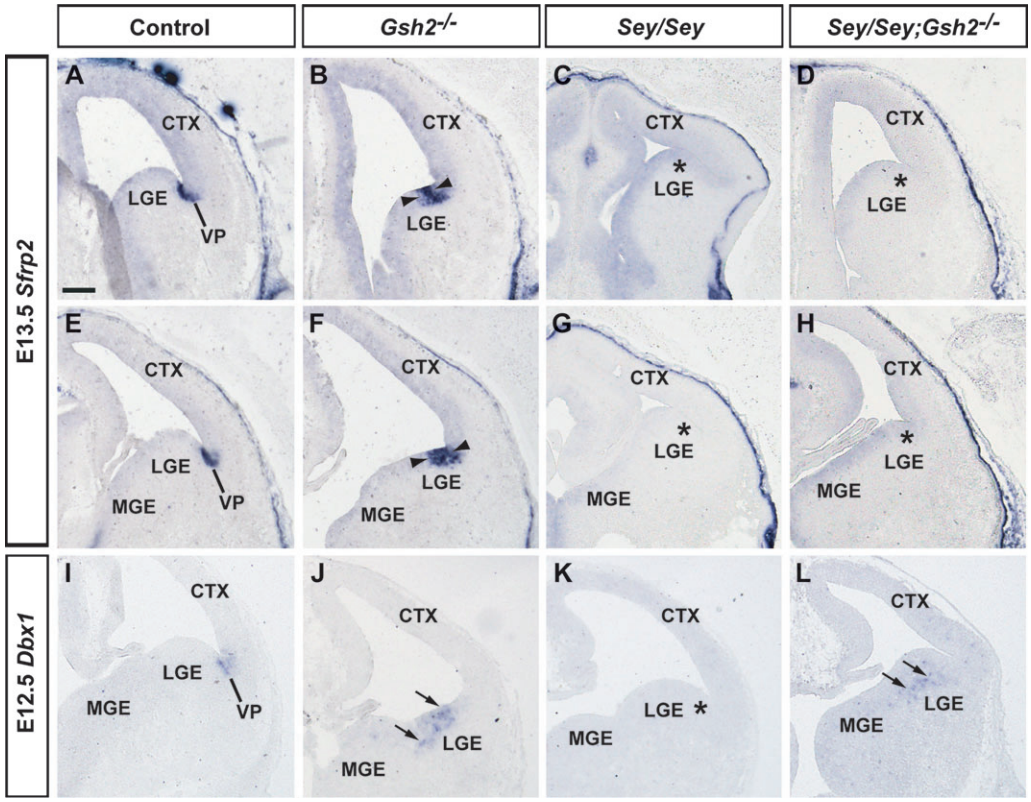

Figure 4. Expression of VP markers *Sfrp2* and *Dbx1* at E13.5 and E12.5. *Sfrp2* is expressed in the VP as shown at E13.5 (*A*, *E*). This expression domain is expanded ventrally in *Gsh2* mutants (*B*, *F*, arrowheads). *Sfrp2* expression is absent at the PSB (asterisk) in the *Sey* mutant (*C*, *G*, asterisks). In *Sey/Gsh2* double mutants expression is not restored (*D*, *H*, asterisks) *Dbx1* is expressed in the VP at E12.5 (*I*). Loss of *Gsh2* results in ectopic *Dbx1* expression in the LGE (*J*, arrows). In *Sey* mutants, *Dbx1* is missing in the VP (*K*, asterisk). In *Sey/Gsh2* double mutants *Dbx1* expression is re-expressed, but mislocalized in the dLGE SVZ (*L*, arrows). *n* numbers for each probe are as follows: controls, $n = 6$ [*Sfrp2*], $n = 3$ [*Dbx1*]; *Gsh2*$^{-/-}$, $n = 4$ [*Sfrp2*], $n = 3$ [*Dbx1*]; *Sey/Sey*, $n = 4$ [*Sfrp2*], $n = 2$ [*Dbx1*]; *Sey/Sey;Gsh2*$^{-/-}$, $n = 4$ [*Sfrp2*], $n = 2$ [*Dbx1*]. Scale bar: 200 μm.

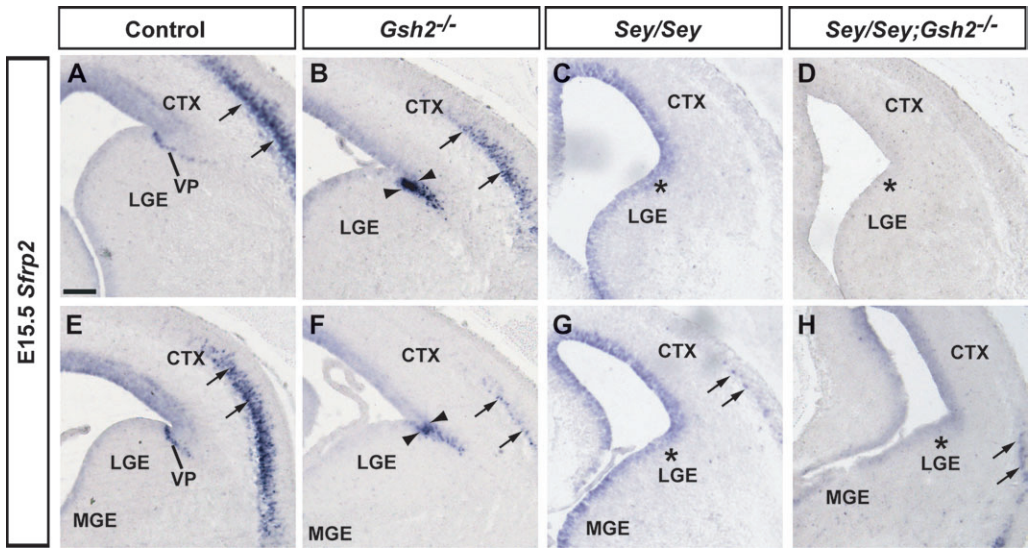

Figure 5. Expression of *Sfrp2* at E15.5. *Sfrp2* is expressed in the VP and layer V neurons of the cortex (*A*, *E*, arrows). In *Gsh2* mutants, the VP expression domain is expanded (*B*, *F* , arrowheads) but reduced in the lateral cortex (*B*, *F*, arrows). In *Sey* mutants, VP expression of *Sfrp2* is missing (asterisks) and layer V expression is drastically reduced (arrows) (*C*, *G*). In *Sey/Gsh2* double mutants, *Sfrp2* expression is not restored in the VP (asterisks), and is severely reduced in the lateral cortex (arrows) (*D*, *H*). *n* numbers are as follows: controls, $n = 6$; *Gsh2*$^{-/-}$, $n = 4$; *Sey/Sey*, $n = 4$; *Sey/Sey;Gsh2*$^{-/-}$, $n = 4$. Scale bar: 200 μm.

migratory route to the basal telencephalon (Carney et al. 2006). Therefore, the presence of *mTsh1*-positive cells along the putative LCS in the *Gsh2* mutant in this study is surprising (Fig. 2*J*,*N*). To this end, we sought to examine the expression patterns of *Dlx2* and *Dlx5*, which in addition to marking subpallial cells, demonstrate migrating cells of the LCS. At

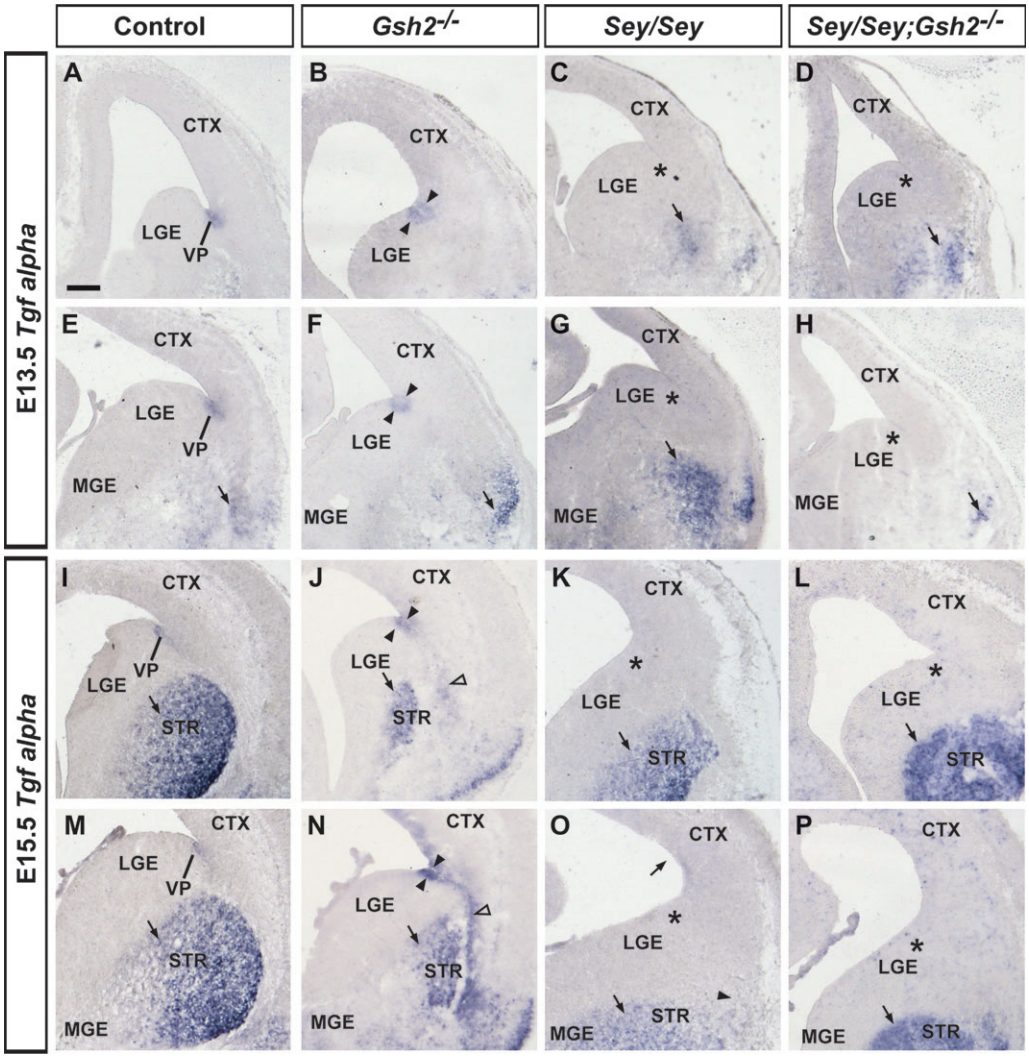

Figure 6. Expression of secreted factor *Tgfα* at E13.5 and E15.5. *Tgfα* is expressed in the VP and basal telencephalon at E13.5 (*A*, *E*, arrow). In *Gsh2* mutants, VP expression is expanded ventrally (arrowheads) and basal expression is maintained (arrow) (*B*, *F*). In *Sey* mutants, VP expression is missing (asterisks) whereas basal expression is unaffected (arrows, *C*, *G*). VP expression remains absent in *Sey/Gsh2* double mutants (asterisks), though basal expression is present (arrows) (*D*, *H*). At E15.5, expression is observed in the VP and differentiating striatum (STR, arrows) (*I*, *M*). In *Gsh2* mutants, VP expression is expanded (arrowheads), and a putative ectopic basal migration is observed (empty arrowheads). A smaller domain of expression is observed in the striatum (*J*, *N*, arrows). In *Sey* mutants, VP expression is missing (asterisks), although striatal expression remains (arrows) (*K*, *O*). In *Sey/Gsh2* double mutants, expression at the VP is not restored (asterisks), and remains present in the striatum (arrows) (*L*, *P*). *n* numbers are as follows: controls, *n*= 4 [E13.5], *n*= 4[E15.5]; *Gsh2*$^{-/-}$, *n*= 3 [E13.5], *n*= 3 [E15.5]; *Sey/Sey*, *n*= 3 [E13.5], *n*= 4 [E15.5]; *Sey/Sey;Gsh2*$^{-/-}$, *n*= 3 [E13.5], *n*= 3 [E15.5]. Scale bar: 200 μm.

E13.5, Dlx mRNA and protein expression is confined to the subpallial progenitor zones and postmitotic cells undergoing tangential migration (Supplemental Figs 1 and 2*A*,*E*) (Porteus et al. 1991; Bulfone, Kim, et al. 1993; Porteus et al. 1994; Puelles et al. 2000; Nery et al. 2003; Carney et al. 2007; Flames et al. 2007). At E15.5, *Dlx2* and *Dlx5* streams are clearly observed along the LCS (Supplemental Figs 1 and 2*I*,*M*). In *Gsh2* mutant mice, *Dlx2* and *Dlx5* expression is truncated along the emanating LCS (Supplemental Figs 1 and 2*J*,*N*) (Szucsik et al. 1997; Corbin et al. 2000; Nery et al. 2002; Carney et al. 2006). In the *Sey* mutant, there appears to be a large ectopic stream of LCS cells destined for the basal telencephalon that is also consistent with other findings (Supplemental Figs 1 and 2*K*,*O*) (Caubit et al. 2005). This abnormal putative migratory stream appears to persist in *Sey/Gsh2* double mutants (Supplemental Fig. 1*L* and Supplemental Fig. 2*L*,*P*).

In addition to putative migratory defects along the LCS as defined by *Dlx2* and *Dlx5* expression in both *Sey* and *Sey/Gsh2* double mutants, we also observe alterations in cortical patterning that is generally consistent with previous observations. At both E13.5 and E15.5, in the *Sey* mutants, there is a large ectopic expression of *Dlx2* and *Dlx5* in the cerebral cortex indicative of a broad ventralization of the dorsal telencephalon (Supplemental Figs 1 and 2*C*,*G*,*K*,*O*). In *Sey/Gsh2* double mutants this phenotype is slightly improved (Supplemental Figs 1 and 2*D*,*H*) (Toresson et al. 2000).

Alterations in LCS cell migration in Gsh2, Sey, and Sey/Gsh2 double mutants at E15.5

To examine defects in cell migration along the LCS in *Gsh2*, *Sey*, and *Sey/Gsh2* double mutant mice, we carried out immunohistochemical analysis for EGFR, which is expressed

at the PSB and in migrating cells of the LCS (Caric et al. 2001). First, we further characterized the cell types of the PSB that express EGFR. To this end, we performed dual immunolabeling experiments to label VZ and SVZ/mantle cells that are pallial-derived (Pax6+ and Tbr1+) and subpallial-derived (Dlx2+ and Dlx5/6+) at E15.5, which represents the time point of robust LCS cell migration of both populations (Carney et al. 2006). We observe that many EGFR+ cells at the PSB also express Pax6 (Fig. 7*A,B*) and a minority colocalize with the pallial mantle marker Tbr1 (Fig. 7*C,D*). To analyze whether the subpallial progenitors also coexpress EGFR, EGFR immunolabeling was combined with β-gal immunohistochemistry in sections from *Dlx2*$^{+/tauLacZ}$ mice or colocalization with endogenous GFP from Dlx5/6-GFP mice. We found that many EGFR+ cells at the PSB are also β-gal+, indicating that these cells are Dlx2+ (Fig. 7*E,F*). In contrast, Dlx5/6-GFP+ cells do not express EGFR (Fig. 7*G,H*). Although this was somewhat surprising, it has been previously shown that not all Dlx2+ cells also express Dlx5 (Eisenstat et al. 1999), thus the Dlx2+/EGFR+ population may be a subset of this subpallial Dlx2+/Dlx5/6- population. Alternatively, this EGFR+ population may also be part of the subset of Dlx2+ cells at the PSB that are also Pax6+ (Carney et al. 2006). Taken together, these data suggest that EGFR+ cells of the LCS arise from progenitors of both the pallial and subpallial aspect of the PSB.

We next examined the status of the EGFR+ LCS population and the radial glial scaffold in *Gsh2*, *Pax6*, and *Sey/Gsh2* mutants by dual immunolabeling for EGFR and RC2 at E15.5. In controls, strong RC2 expression defines the radial glial scaffold of the LCS migratory route to the basal telencephalon, and EGFR+ cells are observed at the PSB and along the LCS (Fig. 7*I,M*). In *Gsh2* mutant mice, the RC2+ radial glia scaffold appears to occupy a larger domain at the PSB, concomitant with a wider domain of EGFR+ cells in the LGE and the LCS (Fig. 7*J,N*). Conversely, in *Sey* mutants, the fasciculated RC2+ processes of the LCS radial glia scaffold are missing, and RC2 labeling appears diffuse (Fig. 7*K,O*) (Stoykova et al. 1997). Also, there is a striking absence of EGFR expression both at the PSB and in the LCS in *Sey* mutants (Fig. 7*K*). In *Sey/Gsh2* double mutants, the RC2+ immunolabeling also appears diffuse, similar to the *Sey* mutant. Interestingly, EGFR expression is restored in the double mutant, although EGFR+ cells appear scattered in the LGE and LCS (Fig. 7*L,P*). This observation is similar to the restoration of *Dbx1* expression in the LGE in *Sey/Gsh2* mutants (Fig. 4), and reveals that the loss of EGFR expression at the PSB and LCS in *Sey* mutant mice is a consequence of ectopic *Gsh2* expression.

Gsh1 expression in single Sey, Gsh2, and Sey/Gsh2 double mutants

Previously it has been shown that *Gsh1* can rescue many, but not all, of the subpallial patterning defects in found in *Gsh2* mutants starting at around E14 (Toresson and Campbell 2001; Yun et al. 2001, 2003). As described previously (Toresson and Campbell 2001), and shown here at E13.5 and E15.5, *Gsh1* is normally expressed at high levels in the medial ganglionic eminence (MGE) and ventral LGE, with intense expression in the VZ and in scattered cells in the SVZ (Fig. 8*A,E,I,M*). In *Gsh2* mutant mice at E13.5, high levels of expression extend to the PSB, most prominent at rostral levels (Fig. 8*B,F*). At E15.5 in *Gsh2* mutant mice, high levels of *Gsh1* expression extend into the VP (Fig. 8*J,N*). In contrast, in the *Sey* mutant, this dorsal limit of expression of *Gsh1* is located much more ventrally at both rostral and medial levels at E13.5, and at rostral levels at E15.5 (Fig. 8*C,G,K*). In contrast, at medial levels at E15.5, a low level of *Gsh1* expression expands into the pallium in *Sey* mutants (Fig. 8*O*). In *Sey/Gsh2* double mutants at E13.5 and E15.5, the dorsal limit of *Gsh1* expression resembles that of *Gsh2* mutants (Fig. 8*D,H,L,P*). Thus, *Pax6* and *Gsh2* act combinatorially to repress *Gsh1* expression in the ventral and lateral pallium.

Discussion

Gene expression studies have revealed that the embryonic telencephalon can be parcellated into refined molecular maps (Puelles et al. 2000; Medina et al. 2004; Flames et al. 2007). Combined with *in utero* transplantation and genetic fate mapping studies this work has unraveled the correlation between specific subpallial progenitor pools and the diversity of cortical interneurons in the adult brain (Nery et al. 2002; Butt et al. 2005; Flames et al. 2007; Fogarty et al. 2007; Wonders et al. 2008, Xu et al. 2008). This restricted potential of neural progenitors is also maintained in adulthood (Merkle et al. 2007). Moreover, to a large extent major aspects of the function of both intrinsic and extrinsic key players that pattern the telencephalon have been elucidated. However, our understanding of the genetic mechanisms that pattern specific telencephalic progenitor zones, such as PSB, remain unknown. In this study, we examined the development of the PSB, an important progenitor domain for the OB, amygdala and early cerebral cortical Cajal–Retzius populations. Our studies reveal that the PSB is a highly complex and dynamic telencephalic progenitor zone, comprised of multiple molecularly distinct progenitor pools. We also provide novel insight into the genetic regulation of their specification by *Pax6* and *Gsh2*.

Combinatorial Codes of Gene Expression Define Unique Progenitor Domains at the PSB

The importance of intrinsic specification for neuronal output in the cerebral cortex was first suggested by the "protomap" theory (Rakic 1988). Furthermore, the parcellation of progenitor domains and the generation of restricted neuronal diversity were first appreciated in the spinal cord (reviewed in Jessell 2000). Collectively, these studies defined a paradigm in which regional expression of key genes (typically transcription factors) instruct cell fate decisions in a cell-autonomous manner, before or at the last cell division (reviewed in Dehay and Kennedy 2007). A major prediction of these studies is that progenitor pools express combinations of transcription factors, which endow these cells with unique fate potentials. Based on this idea, recent studies have comprehensively explored gene expression patterns of putative instructive factors in the subpallial proliferative zones. One study defined at least 18 molecular distinct progenitor domains in the embryonic subpallium (Flames et al. 2007). Moreover, at embryonic stages, postmitotic subpallial-derived cells express genes which encode proteins of a characteristically mature neuronal phenotype as early as E13.5 (Batista-Brito, Machold, et al. 2008). Collectively, these analyses have provided both an important reference and context to consider the spatio-temporal and genetic origins of neural diversity. However, to a large extent such studies have focused on the MGE, which generates the majority of cortical interneurons (Sussel et al. 1999).

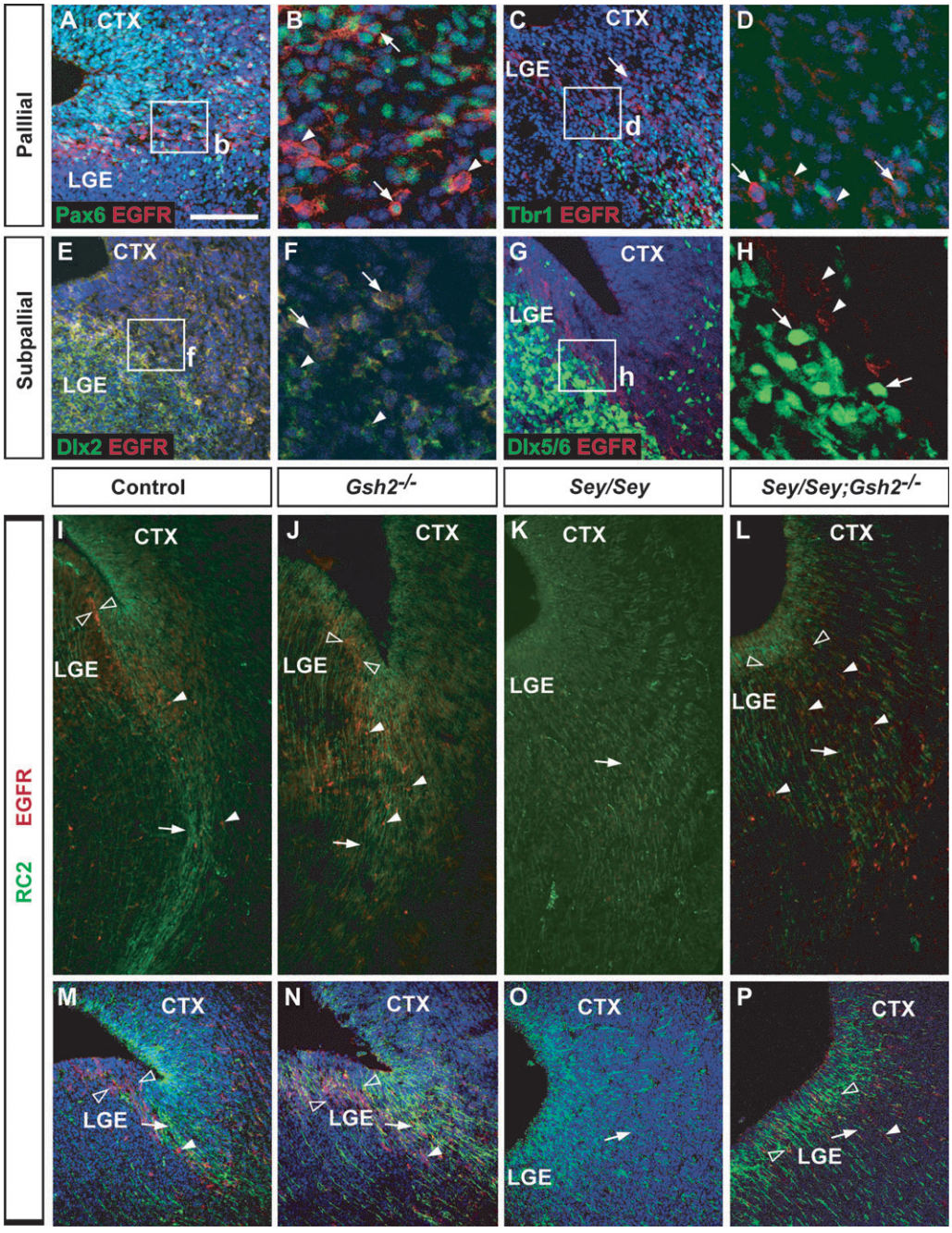

Figure 7. Alterations in LCS cell migration in *Gsh2*, *Sey,* and *Sey/Gsh2* double mutants at E15.5. Low magnification of dual immunolabeling for Pax6 (green, *A*), Tbr1 (green, *C*), Dlx2 (green, *E*) and EGFR (red) shows coexpression of a subset of cells at the PSB (*A*, *C*, *E*). Higher magnification of boxed regions in (*A*, *C*, *E*) show individual double EGFR+ cells (arrows) colocalized with Pax6 (*B*, arrows), Tbr1 (*D*, arrows), or Dlx2 (*F*, arrows) intermingled with EGFR+ cells not expressing any of these markers (arrowheads). In contrast, there is no colocalization of endogenous Dlx5/6-GFP labeling (green, arrows) and EGFR immunolabeling (red, arrowheads) (*G*, *H*). RC2 (green) and EGFR (red) dual immunolabeling reveals the LCS radial glial scaffold (arrows) and EGFR+ migratory cells (arrowheads) emanating from the PSB (empty arrowheads) (*I*, *M*). In *Gsh2* mutants, the VZ EGFR+ domain appears expanded (empty arrowheads), with a more diffuse RC2 immunolabeling (arrows) (*J*, *N*). EGFR+ cells (arrowheads), although disorganized, remain present along the LCS. In *Sey* mutants, EGFR+ cells are completely absent, and the RC2+ radial glia along the LCS migratory route is missing (*K*, *O*, arrows). In *Sey/Gsh2* double mutants, RC2+ radial glia (arrows) resemble *Sey* mutants, but EGFR+ cells are now present along the LCS (arrowheads) and occupy an expanded domain at the PSB (empty arrowheads) (*L*, *P*). To-Pro-3 counterstaining (blue) is shown in the majority of panels. *n* numbers are as follows: EGFR and Pax6 or Dlx2 or Tbr1, $n = 2$; EGFR/Dlx5/6-GFP, $n = 2$; EGFR/RC2: controls, $n = 3$; $Gsh2^{-/-}$, $n = 3$; *Sey/Sey*, $n = 4$; $Sey/Sey;Gsh2^{-/-}$, $n = 3$. Scale bar: *A*, *C*, *E*, *G*, *M–P*: 100 μm, *I–L*: 40 μm, *B*, *D*, *F*, *H*: 30 μm.

In this study, we sought to extensively examine the molecular code of gene expression of the PSB. Our analysis using a battery of markers that delineate the dLGE (*mTsh1*, *Sp8*), VP (*Sfrp2*, *Tgfα*, and *Dbx1*), or both (*Er81*) subdomains reveals that the PSB also contains highly regulated patterns of gene expression. These combinatorial expression patterns highlight the importance of the PSB as a unique and highly heterogeneous telencephalic progenitor domain. Moreover considering that the PSB is a major source of cells that generates neuronal cell diversity in the limbic system and OB,

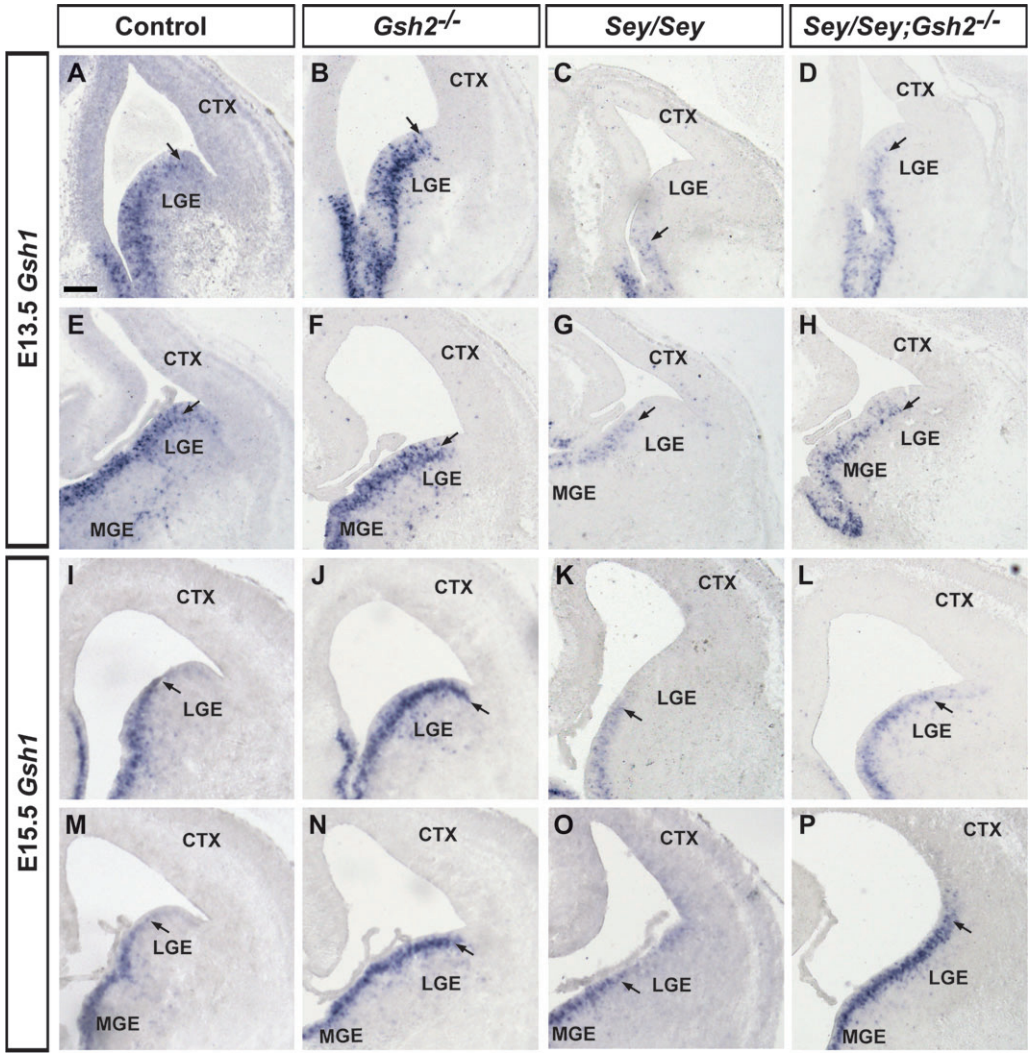

Figure 8. *Gsh1* expression in *Gsh2*, *Sey* single, and *Sey/Gsh2* double mutants *at E13.5 and E15.5. At E13.5 Gsh1* is expressed in the MGE and LGE, with highest expression more ventrally (*A*, *E*). Arrows mark the dorsal limit of expression (*A–P*). In *Gsh2* mutants, expression is similar to controls (*B*, *F*). In *Sey* mutants, expression is present in the MGE and ventral LGE only (*C*, *G*). In *Sey/Gsh2* double mutants, expression is similar to that of *Gsh2* mutants (*D*, *H*). In controls, at E15.5, expression is similar to E13.5 (*I*, *M*). In *Gsh2* mutants, expression expands to the PSB (*J*, *N*). In *Sey* mutants, expression is similar to that at E13.5 (*K*, *O*). In *Sey/Gsh2* double mutants, at rostral levels, the expression domain normal appears normal (*L*). However, at more medial levels, expression extends dorsally beyond the PSB into the pallium (*P*). *n* numbers are as follows: controls, $n = 4$ [E13.5], $n = 3$ [E15.5]; $Gsh2^{-/-}$, $n = 3$ [E13.5], $n = 3$ [E15.5]; *Sey/Sey*, $n = 3$ [E13.5], $n = 3$ [E15.5]; $Sey/Sey;Gsh2^{-/-}$, $n = 2$ [E13.5], $n = 3$ [E15.5]. Scale bar: 200 μm.

we speculate that this molecular diversity is directly related to the vast array of neuronal subtypes that populate these mature telencephalic structures.

Function of Pax6 and Gsh2 in the Specification of the PSB Progenitor Domains

In addition to gene expression analysis in wild-type animals, we have examined the role of the homeodomain encoding genes *Pax6* and *Gsh2*, both alone and in combination, in the specification of progenitor pools of the PSB. Overall, we find that *Pax6* and *Gsh2* function to regulate the expression of all PSB markers analyzed here. Previous analysis of the function of these genes from a number of groups has revealed that *Pax6* and *Gsh2* function as major regulators of dorsal/ventral patterning by acting in a genetic cross-repressive manner to specify proper cortical and striatal identity at either side of the PSB (Stoykova et al. 1996; Corbin et al. 2000; Stoykova et al. 2000; Toresson et al. 2000; Toresson and Campbell 2001; Yun et al. 2001). Consistent with this finding, the lack of both *Pax6* and *Gsh2* in mutant mice leads to a significant, but not complete, rescue of many of these dorsal/ventral patterning defects observed in single mutants (Toresson et al. 2000; Waclaw et al. 2004). However, because *Pax6* (*Sey*) mutants exhibit ectopic pallial *Gsh2* expression and *Gsh2* mutants display ectopic subpallial *Pax6* expression, a direct genetic requirement for each of these genes for PSB formation could not be differentiated from the consequences of ectopic expression of *Gsh2* and *Pax6*.

By an in-depth analysis of *Sey/Gsh2* double mutants, we reveal that both *Pax6* and *Gsh2* are required for the expression *Er81*, *Sp8*, *mTsh1*, *Sfrp2*, and *Tgfα*, as in the absence of both genes expression of these genes remains abnormal. In contrast, the regulation of *Dbx1* expression, which marks VP progenitors, and EGFR, which marks cells of the LCS, is more complex, as the loss of *Gsh2* on the *Sey* background rescues their expression. In addition, we demonstrate that dorsal telencephalic

Sey mutant progenitors may be respecified to several dLGE cell lineages other than *Sp8* as previously observed, and therefore importantly extend previous studies (Schuurmans et al. 2004; Kroll and O'Leary 2005; Gopal and Golden 2008), which have indicated differential patterning defects in the *Sey* cortex. Moreover, we show that this cortical misspecification also persists in the absence of both *Pax6* and *Gsh2* revealing a direct requirement for *Pax6* in repression of ectopic dLGE identity in the lateral cortex.

In addition to the combinatorial function of *Pax6* and *Gsh2* in patterning of PSB progenitor pools, our study reveals novel individual roles for *Gsh2* and *Pax6*. During embryogenesis, *Gsh2* is expressed in a graded pattern in the subpallium with the highest expression at the PSB (Corbin et al. 2000; Toresson et al. 2000; Yun et al. 2001). *Gsh2* mutant mice display cell proliferation defects in the SVZ of the LGE (Yun et al. 2001), as well as a loss of the dLGE marker *Sp8* at E12.5, though interestingly this expression is partially recovered by E16.5 (Waclaw et al. 2006). Consistent with this finding, our study reveals that re-expression of *Sp8* in the dLGE is initiated at E13.5, and is further improved by E15.5. This timing coincides with the increase in *Gsh1* expression in the dLGE. As *Gsh1* can partially rescue the striatal patterning defects observed in *Gsh2* mutants (Toresson and Campbell 2001), it is therefore possible that *Gsh1* performs a similar function in the restoration of *Sp8* expression in the mutant dLGE that is reinitiated from E13.5. Our analysis of expression of *mTsh1*, which also marks the dLGE SVZ, importantly reveals that *Sp8* and *mTsh1* are differentially regulated by *Gsh2*. In contrast to the changes in expression of *Sp8* in the absence of *Gsh2*, *mTsh1* expression was grossly unaffected at E13.5. Interestingly, this regulation by *Gsh2* is temporally dependent as *Sp8* expression is re-initiated at later developmental time points, whereas *mTsh1* expression is reduced. There are 2 non-mutually exclusive mechanisms by which this may be occurring: 1) the expression of *mTsh1* and *Sp8* in individual dLGE progenitor cells may be differentially regulated by *Gsh2*, or 2) *mTsh1* and *Sp8* mark separate progenitor subpopulations in the dLGE SVZ that are differentially regulated by *Gsh2* function. In support of the latter explanation, analysis of patterns of protein expression in the PSB SVZ has shown a significant amount of molecular heterogeneity. For example, a subset, but not all, Sp8+ cells colocalize with Pax6 or Dlx5/6-GFP (Waclaw et al. 2006). Similarly, only some *mTsh1*+ cells express *Dlx2* mRNA or DLX protein (as revealed by immunostaining with a pan-DLX antibody) (Caubit et al. 2005). Future genetic fate mapping analyses of both *Sp8* and *mTsh1* will be essential for unraveling the lineal relationships between these progenitor populations and their potential differential fates in the mature brain.

Previous analysis of *Pax6* mutant mice has revealed numerous important functions for *Pax6* in telencephalic development. These include cell-autonomous repression of ventral gene expression in the ventral and lateral pallium, the normal adhesiveness of cortical progenitor cells and their migration, axonal pathfinding, corticogenesis and arealization of the cerebral cortex (Schmahl et al. 1993; Stoykova et al. 1996, 1997, 2000; Götz et al. 1998; Estivill-Torrus et al. 2002; Jones et al. 2002; Jiménez et al. 2002; Talamillo et al. 2003; Tyas et al. 2003; Haubst et al. 2004; Holm et al. 2007; Manuel et al. 2007; Quinn et al. 2007). Consistent with previous studies, we find that the lack of functional Pax6 protein in the *Sey* mutant pallium results in the ectopic expression of subpallial markers (Stoykova et al. 1996, 2000; Chapouton et al. 1999; Corbin et al. 2000; Toresson et al. 2000; Yun et al. 2001; Kroll and O'Leary 2005), including several dLGE markers.

Based on the finding that both *Sp8* and *Er81* progenitors can give rise to calretinin-expressing OB cells (Waclaw et al. 2006; Allen et al. 2007), we hypothesize that ventralized dorsal telencephalic progenitors in the *Sey* mutant may produce a gamma-aminobutyric acidergic phenotype that derives from the ectopic expression of several PSB markers, such as *Sp8*, *Er81*, and perhaps *mTsh1* as shown here. Importantly, this ventralization is generally independent of ectopic pallial *Gsh2* expression, as the *Sey*/*Gsh2* double mutants display a similar phenotype (Toresson et al. 2000).

In addition, our analysis of the VP markers *Dbx1*, *Sfrp2*, and *Tgfα* in single *Gsh2* and *Sey* mutants and *Sey*/*Gsh2* double mutants reveals an interesting and novel regulation of their expression by *Pax6* and *Gsh2*. Previous studies of *Sey* and *Gsh2* mutants, as well as our data shown here, reveal that *Pax6* is required for the expression of each of these markers at the VP, whereas *Gsh2* is required to restrict their expression from the dLGE domain. Our analysis of *Sey*/*Gsh2* double mutants has uncovered an important differential regulation by *Pax6* and *Gsh2*. As *Sfrp2* and *Tgfα* expression remains absent in double mutants, this reveals that their expression is directly dependent on *Pax6* and not repressed by ectopic *Gsh2* pallial expression in *Sey* mutants. In contrast, *Dbx1* expression is rescued in the double mutants. However, this expression is mislocalized to the LGE SVZ. Therefore, although *Pax6* is required for *Dbx1* expression at the VP, *Gsh2* is also necessary to repress *Dbx1* expression in the LGE. Thus, similar to the differential regulation of the dLGE markers *mTsh1* and *Sp8* by *Gsh2*, individual VP markers are differentially regulated by *Pax6* (*Sfrp2*, *Tgfα*) or the combinatorial actions of both *Pax6* and *Gsh2* (*Dbx1*). This complex genetic regulation of PSB boundary formation by *Pax6* and *Gsh2* raises important questions regarding the spatial and functional heterogeneity of progenitor pools within the PSB.

Requirement of Pax6 and Gsh2 for Basal-Directed Migration

This analysis, along with previous studies, reveals that the PSB is a highly heterogeneous and complexly regulated progenitor region of the developing telencephalon. The PSB is the origin of the LCS and RMS, two major migratory streams in the developing telencephalon. The LCS populates the basal telencephalic limbic system, in particular the piriform and olfactory cortices and the amygdala (Hirata et al. 2002; Carney et al. 2006, Bai et al. 2008). The PSB also seeds the embryonic and postnatal SVZ stem cell niche and their subsequent progeny that migrate along the RMS to generate diverse subtypes of OB interneurons (Waclaw et al. 2006; Kohwi et al. 2007)

Our analysis of EGFR expression, a previously identified marker of LCS migratory cells (Caric et al. 2001), reveals that both the subpallial (Dlx2+) and pallial (Pax6+) subpopulations are both EGFR+. As *Tgfα*, a known ligand for EGFR, is expressed in the VP, it is possible that this signaling pathway is required for specification/migration of EGFR+ cells of the LCS. Indeed, the expansion of both *Tgfα* and EGFR in *Gsh2* mutants and their loss in *Sey* mutants supports this notion. However, the re-expression of EGFR in *Sey*/*Gsh2* double mutants, without rescue of *Tgfα* expression, reveals that this regulation is likely more complex, and may also/instead occur via *Dbx1*, as *Dbx1*

expression at the PSB is also restored in *Sey/Gsh2* double mutants. In addition, our previous studies (Carney et al. 2006), as well as data shown here, reveal that *Gsh2* is required for development of the Dlx2+ dLGE component of the LCS. Interestingly, as noted above, in this study we surprisingly reveal that at E13.5, the expression of *mTsh1* in the dLGE and in putative migratory cells along the LCS appeared normal. However, 2 days later, we show reduced expression of *mTsh1* in the dLGE SVZ and LCS. Thus, the complex *Gsh2* regulation of specification of dLGE progenitor pools has consequences for the generation of the migratory populations that arise from this region. Some migratory populations (e.g., Dlx2+) are *Gsh2*-dependent, whereas other populations (e.g., early *mTsh1*) populations are *Gsh2*-independent. This observation is reminiscent of the complex genetic regulation of OB neurogenesis in which specific subpopulations are *Dlx1/2*-dependent and others *Dlx1/2*-independent (Long et al. 2007; Batista-Brito, Close, et al. 2008). As the PSB is a major progenitor pool for both the LCS and the RMS/OB (Wichterle et al. 1999; Stenman, Toresson, et al. 2003; Carney et al. 2006; Waclaw et al. 2006; Allen et al. 2007; De Marchis et al. 2007; Kohwi et al. 2007) the output of this embryonic heterogeneity may be directly linked to the functional complexity of neurons that populate both the mature basal telencephalic limbic system and postnatal SVZ/OB.

Supplementary Material

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

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R.S.E.C. and J.C.G. devised the study and R.S.E.C. carried out the majority of experiments. L.A.C. and T.H. performed the *Tgfα* and *Dbx1 in situ* hybridization experiments, respectively, with tissue prepared and genotyped by R.S.E.C. K.M. provided further technical assistance. R.S.E.C. and J.C.G. analyzed the data and wrote the manuscript. All authors edited and approved the final manuscript. *Conflict of Interest*: None declared.

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