Neuron
Article

Transcriptional Regulation of Enhancers Active in Protodomains of the Developing Cerebral Cortex

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http://dx.doi.org/10.1016/j.neuron.2014.04.014

SUMMARY

Elucidating the genetic control of cerebral cortical (pallial) development is essential for understanding function, evolution, and disorders of the brain. Transcription factors (TFs) that embryonically regulate pallial regionalization are expressed in gradients, raising the question of how discrete domains are generated. We provide evidence that small enhancer elements active in protodomains integrate broad transcriptional information. CreER^{T2} and GFP expression from 14 different enhancer elements in stable transgenic mice allowed us to define a comprehensive regional fate map of the pallium. We explored transcriptional mechanisms that control the activity of the enhancers using informatics, in vivo occupancy by TFs that regulate cortical patterning (*CoupTFI, Pax6*, and *Pbx1*), and analysis of enhancer activity in *Pax6* mutants. Overall, the results provide insights into how broadly expressed patterning TFs regulate the activity of small enhancer elements that drive gene expression in pallial protodomains that fate map to distinct cortical regions.

INTRODUCTION

At the core of cortical development lie transcriptional programs that orchestrate a sequence of processes beginning with specification of the cortical anlage and its regional subdivisions, or the protomap (Rakic, 2009; O'Leary et al., 2013). Ongoing work has identified a set of transcription factors (TFs) that control the size and areal identities of pallial subdivisions. These include *CoupTFI*, *Dmrta2 (Dmrt5)*, *Emx2*, *Lef1*, *Lhx2*, *Pax6*, and *Sp8* (Bishop et al., 2000; Galceran et al., 2000; Yun et al., 2001; Mallamaci and Stoykova, 2006; Armentano et al., 2007; Sahara et al., 2007; Faedo et al., 2008; Mangale et al., 2008; Chou et al., 2009; Konno et al., 2012; Borello et al., 2013; Saulnier et al., 2013). Each of these TFs is expressed in distinct gradients in progenitor cells of the pallial ventricular zone (VZ). For instance, *Pax6* is expressed in rostrocaudal and ventrodorsal gradients; *Pax6* loss of function in mice results in a respecification of cortical regions along both its rostrocaudal and ventrodorsal axes (Bishop et al., 2000; Yun et al., 2001). Despite the subdivision of the pallium into discrete structural/molecular units (e.g., the medial, dorsal, lateral, and ventral pallium [MP, DP, LP, and VP]; Puelles et al., 2000), to date the TFs that are known to control regional fate are expressed in gradients across these subdivisions, raising the intriguing question of how these gradients are interpreted in an integrative fashion to generate sharply delineated pallial subdivisions and later adult cortical regions.

One mechanism that could solve this conundrum would be that enhancer elements integrate TF expression to generate gene activation in distinct pallial subdivisions, much in the way that regional fate is generated in the cellular blastoderm of *Drosophila* embryos (Lagha et al., 2012). While this general paradigm had previously been supported through anecdotal reports of individual pallial enhancers identified in gene-centric studies (Kammandel et al., 1999; Theil et al., 2002; van den Bout et al., 2002; Ahituv et al., 2007; Colasante et al., 2008), a recent more comprehensive screen for forebrain enhancers that includes spatial activity data for ~145 human enhancers that are active in the embryonic day (E) 11.5 mouse telencephalon enables a rigorous and systematic search for enhancers involved in

prepatterning of the pallium (Visel et al., 2013). Here we present evidence that enhancers integrate information from TF gradients in the embryonic day E11.5 mouse pallium to generate distinct expression domains. Using a panel of 14 human enhancers carefully selected based on their in vivo activity patterns, we generated a set of stable mouse transgenic lines that express CreER^{T2} and GFP in distinct domains within the developing pallium. Leveraging this unique set of reporter mice, we derived fate maps that elucidate the embryonic origin of pallial subdivisions. Furthermore, we used a combination of bioinformatics, chromatin immunoprecipitation sequencing (ChIP-seq), and in vivo studies to elucidate the regulation of these enhancers by major pallial transcription factors including COUPTFI, PAX6, and PBX1. Overall, we propose that the enhancers defined through this study identify protodomains of the pallial neuroepithelium, which may be fundamental units of cortical development and evolution.

RESULTS

Pallial Protodomains Identified by Enhancer Activity Using Transient Transgenic Assay

To define enhancers potentially marking neuroepithelial subdivisions in the E11.5 pallium, we mined a previously described large collection of enhancers active in the developing telencephalon, assayed using transient transgenic mouse *LacZ* expression (Visel et al., 2013). We identified more than 40 enhancers that showed regional pallial expression, many of which showed intrapallial boundaries (Figures 1A–1C and Figure S1 available online). For instance, in the MP, several enhancer lines showed nested patterns of expression, varying between a small dorsocaudal domain (643), a domain in the ventral caudomedial telencephalon (653), a larger domain that includes the entire caudomedial telencephalon (192), and the entire dorsomedial and caudomedial region including the primordial septum (348) (Figure 1C). Regional patterns of activity were also observed for enhancers expressed in the DP, LP, and VP (Figures 1A and 1B). We mapped these expression limits onto a model schema of the E11.5 pallial neuroepithelium, from which we hypothesize the existence of a set of sharply delimited pallial progenitor domains or protodomains (A-I) (Figure 1D; Table S1).

Enhancer Activity of Pallial Enhancer CreER^{T2}-IRES-GFP Alleles

To test the idea that these human enhancers are active in protodomains that generate distinct pallial subdivisions, we produced stable transgenic mouse lines to characterize the properties of 14 enhancers that reproducibly exhibited boundaries in the E11.5 pallium (Figures 1A–1C and Figure S1; asterisks label the enhancers used to make stable lines).

We generated stable transgenic mouse lines that express CreER^{T2}-IRES-GFP and downstream of each one of the 14 selected "pallial" enhancers and a minimal Hsp68 promoter. We generated two to three founders for 10/14 of the lines; their expression domains were reproducible (Table S2). We further analyzed the properties of one founder for each enhancer.

To characterize the activity of each enhancer, we defined the GFP expression at E11.5 and compared the enhancer activity in the stable and transient transgenic assays. The stable lines showed enhancer activity patterns that closely resembled the transient transgenic assay (Table S2). We annotated the E11.5 expression domains on a flattened topologic representation of the embryonic pallium (right hemisphere), where stippled gray color indicates GFP expression (Figures 2I and 2I′ and Figures S2A–S2N). For instance, for enhancer 643, we observed progenitor GFP expression in the MP at E11.5 (Figures 2A–2H). On the other hand, enhancer 1,050 showed progenitor GFP expression in the DP and MP at E11.5 but was absent in the ventrolateral pallium (VLP) (Figures 2A′–2H′).

Next, we examined prenatal GFP expression at E12.5, E14.5, and E17.5 for all of the lines (Figures S2A–S2N and Table S2). In most cases, enhancer activity was strongest at E11.5 and was largely unchanged at E12.5 (Table S2). However, activity patterns of some of the enhancers were more dynamic. For instance, 636 was selectively active in the VLP at E10.5, but by E11.5, its activity was greatly reduced (Figure S2E). Activity of 12/14 enhancers decreased and/or became restricted to a smaller domain by E14.5 and E17.5 (Table S2). For instance, 218, 281, 653, and 1,318 activity was no longer detected in the pallium by E14.5. Three of the enhancers with MP expression (348, 643, and 1,006) were no longer active in the hippocampus but maintained activity in the hippocampal fissure, choroid plexus, and fimbrial area. The activity of 636, 840, and 1,172 became restricted to small populations of cells in the pallium at E17.5 (Figures S2E, S2I, and S2M). Enhancer 660, which was active in the caudoventral MP at E11.5, became active in the SVZ and superficial cortical layers of the DP at E17.5 (Figure S2H).

Fate Mapping Using Pallial Enhancer CreER^{T2}-IRES-GFP Alleles

To determine the identity of the cells whose progenitors have E11.5 enhancer activity, we performed fate map analyses by introducing the *Ai14 (tdTomato)* Cre reporter allele (Madisen et al., 2010) into the enhancer CreER^{T2}-IRES-GFP lines. We administered tamoxifen at E10.5 to induce CreER^{T2} translocation to the nucleus, where it activated tdTomato expression and then performed neuroanatomical analyses at later stages. Because of the ~24–36 hr window of tamoxifen action (Hayashi and McMahon, 2002), we assessed enhancer activity at both E11.5 and E12.5 to better interpret the results of E10.5 tamoxifen treatment (Figure S2 and Table S2). Since prenatal tamoxifen treatment frequently led to fetal death around the time of delivery, we obtained fate-mapping data at E17.5 for all enhancer lines. However, we also obtained postnatal fate maps (P30) for a subset of the enhancers (192, 348, 636, 643, 653, and 660; Figure S2 and Table S3). We chose these enhancers because of their activity in the hippocampus; the hippocampus matures later than the neocortex; thus, P30 data helped analysis of the hippocampal fate map.

We annotated the fate map domains on a flattened topological representation of the maturing/mature pallium (Figures 2S and 2S′ and Figure S2). Here we indicated anatomical locations containing tdTomato$^+$ cells using a graded rating scale of 1–4: 1 (red) high density to 4 (green) almost no tdTomato$^+$ cells (Figures 2S and 2S′). For instance, 643, which showed E11.5 activity restricted to the MP, fate mapped to the rostrodorsal CA fields,

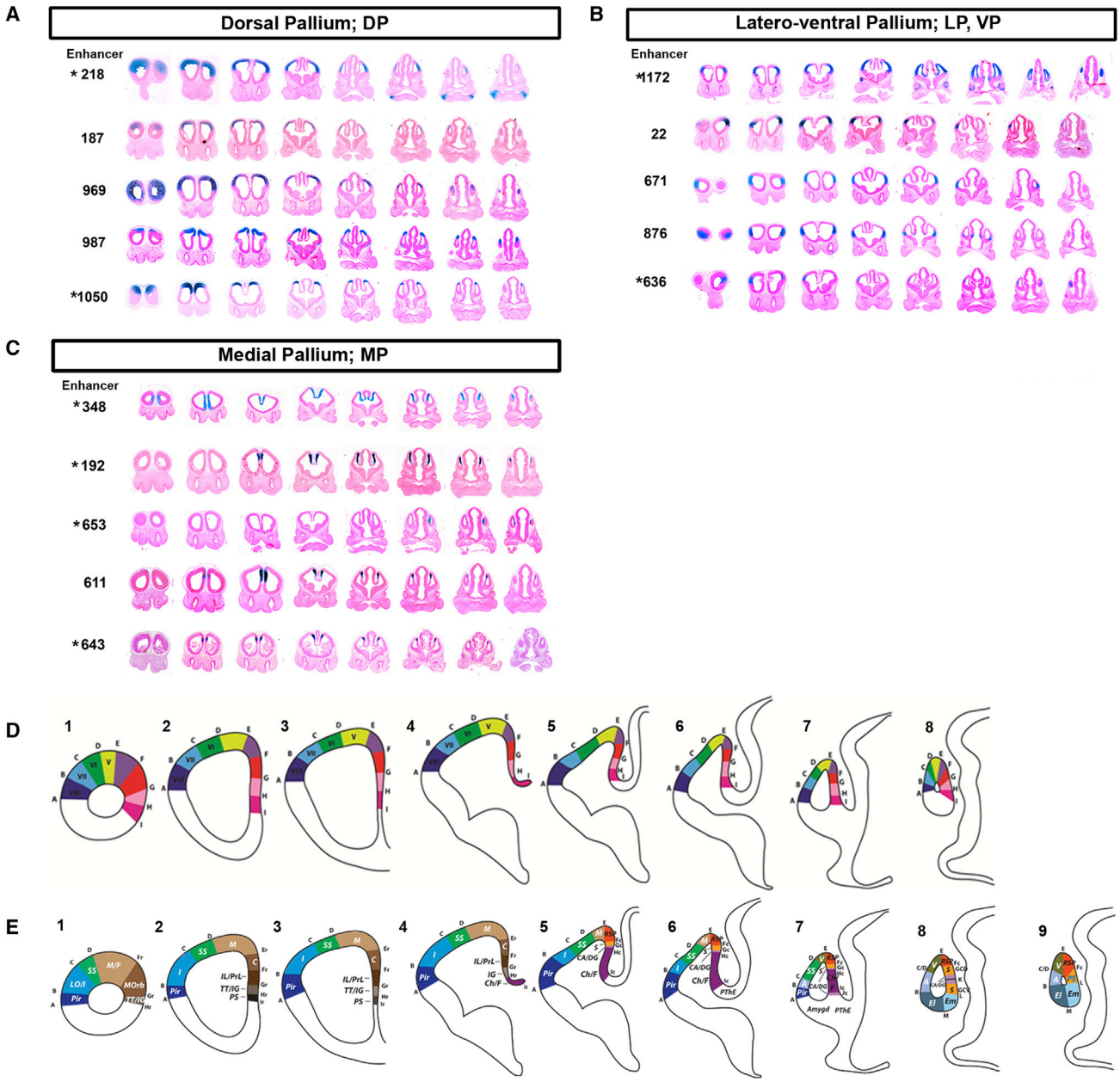


Figure 1. Enhancer Activity Assays at E11.5 of Transient Transgenics Expressing β-Galactosidase from the *LacZ* Gene

Asterisk indicates that stable transgenic lines were made using these enhancers. Coronal sections across the rostrocaudal telencephalon were studied for 15 different enhancers.

(A) Five enhancers with a nested pattern of *LacZ* expression in the dorsal pallium.

(B) Five enhancers with a nested pattern of *LacZ* expression in the lateroventral pallium.

(C) Five enhancers with a nested pattern of *LacZ* expression in the medial pallium.

(D) Schema of coronal sections across the rostrocaudal telencephalon showing progenitor domains and boundaries deduced from analysis of enhancer-driven expression patterns.

(E) Schema of coronal sections across the rostrocaudal telencephalon showing progenitor domains and boundaries (A-M) deduced from analysis of enhancer activity fate mapping (see subsequent figures). Some boundaries are specific to rostral (r), whereas other boundaries are specific to caudal (c) regions. See also Figure S1. For abbreviations, see legend to Figure 2.

dentate gyrus of the rostrodorsal hippocampus, the fimbrial area, and choroid plexus (Figures 2J–2R and 5B–5B‴). On the other hand, 1,050, which showed E11.5 activity restricted to DP and MP, was fate mapped to the neocortex and hippocampus and only weakly labeled the LP (insular cortex) and did not label the VP (piriform cortex) (Figures 2J′–2R′).

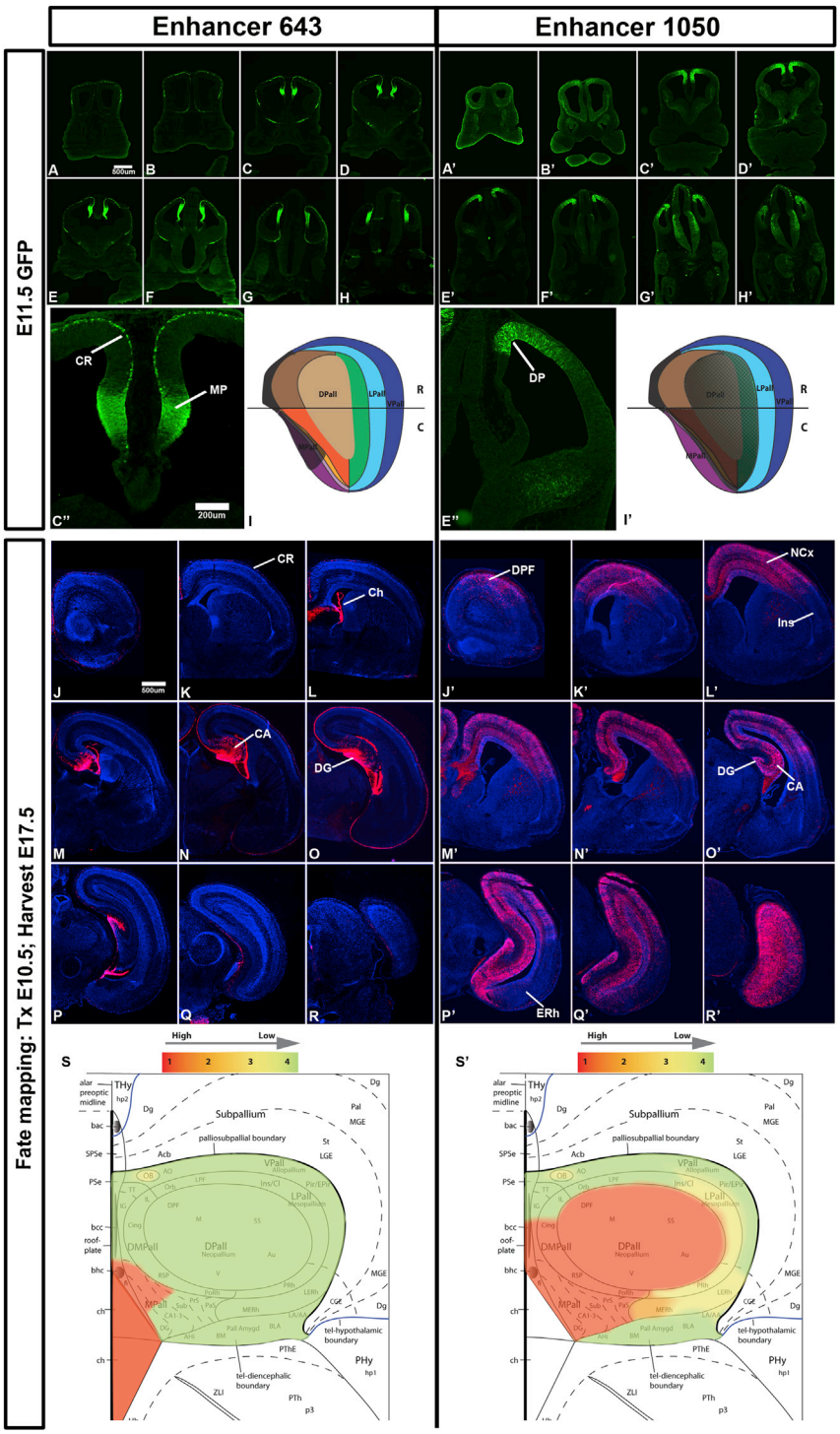


Figure 2. Analyses of Enhancers 643 and 1,050

(A–H′ and J–R′) Enhancer activity (GFP expression, E11.5; A–H′) and fate-mapping (Cre-induced tdTomato, E17.5; J–R′) assays of stable transgenics encoding enhancer 643 (left) or 1,050 (right). (C′) and (E″) show higher-magnification view of E11.5 expression.

(I and I′) Schemas showing approximate position of GFP expression (gray) within flattened view of E11.5 pallial progenitor zones of enhancers 643 (I) and 1,050 (I′).

(S and S′) Schemas showing approximate position of dtTomato expression within flattened view of E17.5 pallial subdivisions; color coded according to approximate density of tdTomato$^+$ cells of enhancers 643 (S) and 1,050 (S′).

Abbreviations according to region: ventral pallium (VPall, allopallium): AO, anterior olfactory nuclei; OB, olfactory bulb; Pir/EPir, piriform and ectopiriform; LERh, lateral entorhinal; MERh, medial entorhinal; lateral pallium (LPall, mesopallium): Ins/Cl, insula/claustrum; LO, lateral orbital; PRh, perirhinal; Orb, orbitofrontal; dorsal pallium (DPall; neopallium): AU (A), auditory; DPF, dorsal prefrontal; F, frontal; LPF, lateral prefrontal; M, motor; SS, somatosensory; V, visual; dorsomedial pallium (DMPall): Cing (C), cingulate gyrus; IL, infralimbic (and PrL, prelimbic); MOrb, medial orbital; RSP, retrosplenial; PoRh, postrhinal; medial pallium (MPall): CA1–3, CA fields 1–3; DG, dentate gyrus; fi (F), fimbria; IG, indusium griseum; Sub (S), subiculum; PaS, parasubiculm; PrS, presubiculum; TT, tenia tecta; dorsal midline: bac, brachium of the anterior commissure; bcc, brachium of the corpus callosum; bhc, brachium of the hippocampal commissure; ch, choroid plexus; PSe (PS), pallial septum; pallial amygdala (Pall Amygd): AA, anterior amygdala; Ahi, amygdalohippocampal area; BM, basomedial; BLA, basolateral; LA, lateral; subpallium: Acb, accumbens; CGE, caudal ganglionic eminence; Dg, Diagonal area; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Pal, pallidum; SPSe, subpallial septum; St, striatum; hypothalamus: hp1, 2, hypothalamic prosomere 1 and 2; PHy, peduncular; Thy, hypothalamus; diencephalon: Hb, habenula; p2, p3, prosomeres 2 and 3; Thy, terminal hypothalamus; PThE, prethalamic eminence; Th, thalamus.

Similar analyses were performed for all the enhancer lines; the data and analyses are compiled in Figures S2A–S2N. From these experiments, we have deciphered the embryonic origin of pallial subdivisions (see schema of pallial progenitor subdivisions in Figure 1E and Tables S3 and S4); we have organized these data into Figures 3, 4, and 5, which focus on the frontal cortex, ventrolateral cortex, and hippocampal structures, respectively.

Enhancers Active in Primordia of Distinct Frontal Cortex Subdivisions

The analysis of E11.5 expression and CreER^{T2} fate-mapping experiments from 11 enhancer transgenic lines demonstrated which progenitor domains generated cells that populated different subdivisions of the prefrontal cortex (PFC) (Figure 3 and Figure S2; Tables S3 and S4). Fate mapping of enhancer lines (192, 348, 1,056) with E11.5 activity in the rostral-most E11.5 MP resulted in labeled cells in the medial PFC (MPFC). Enhancer 192

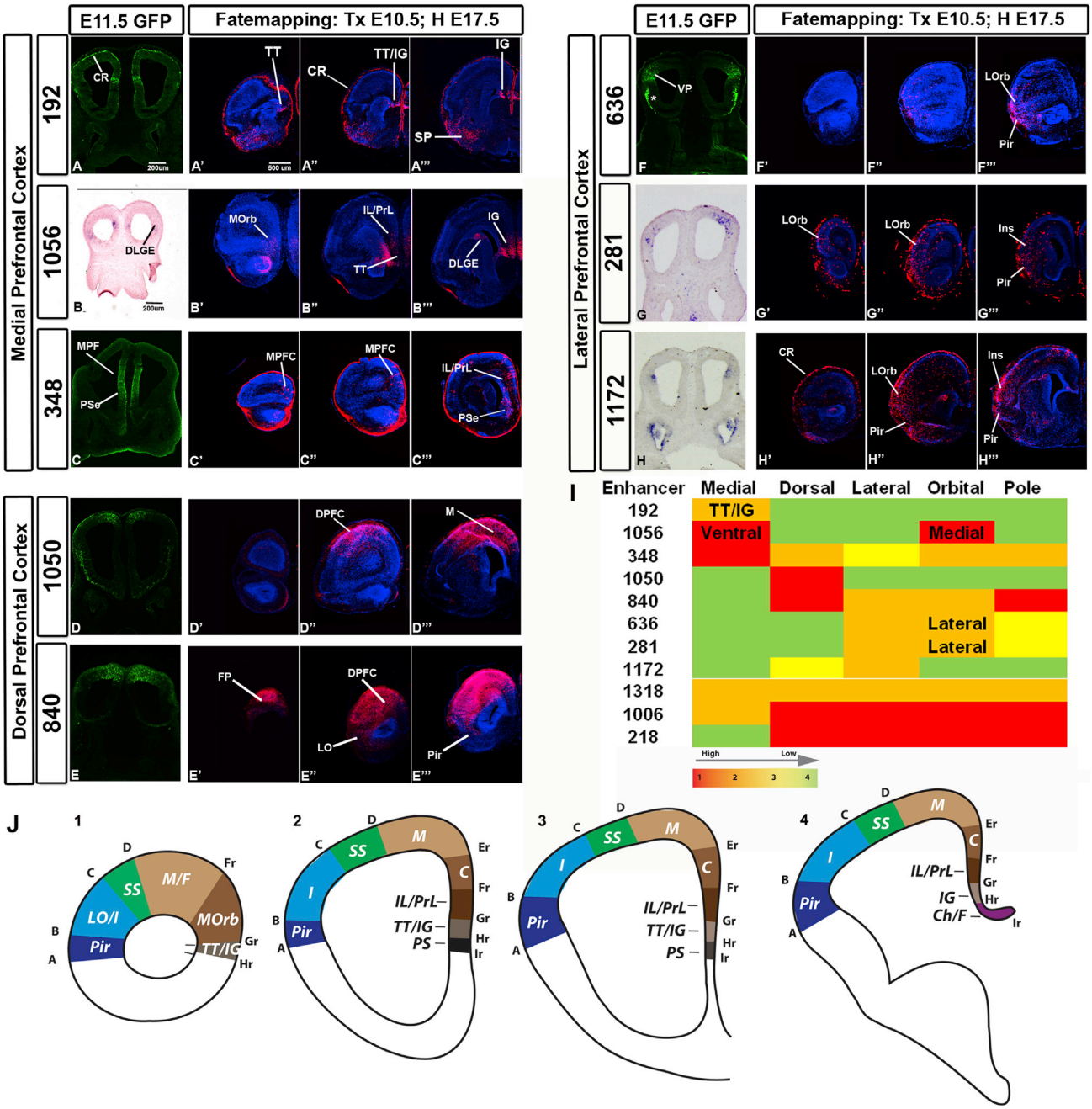


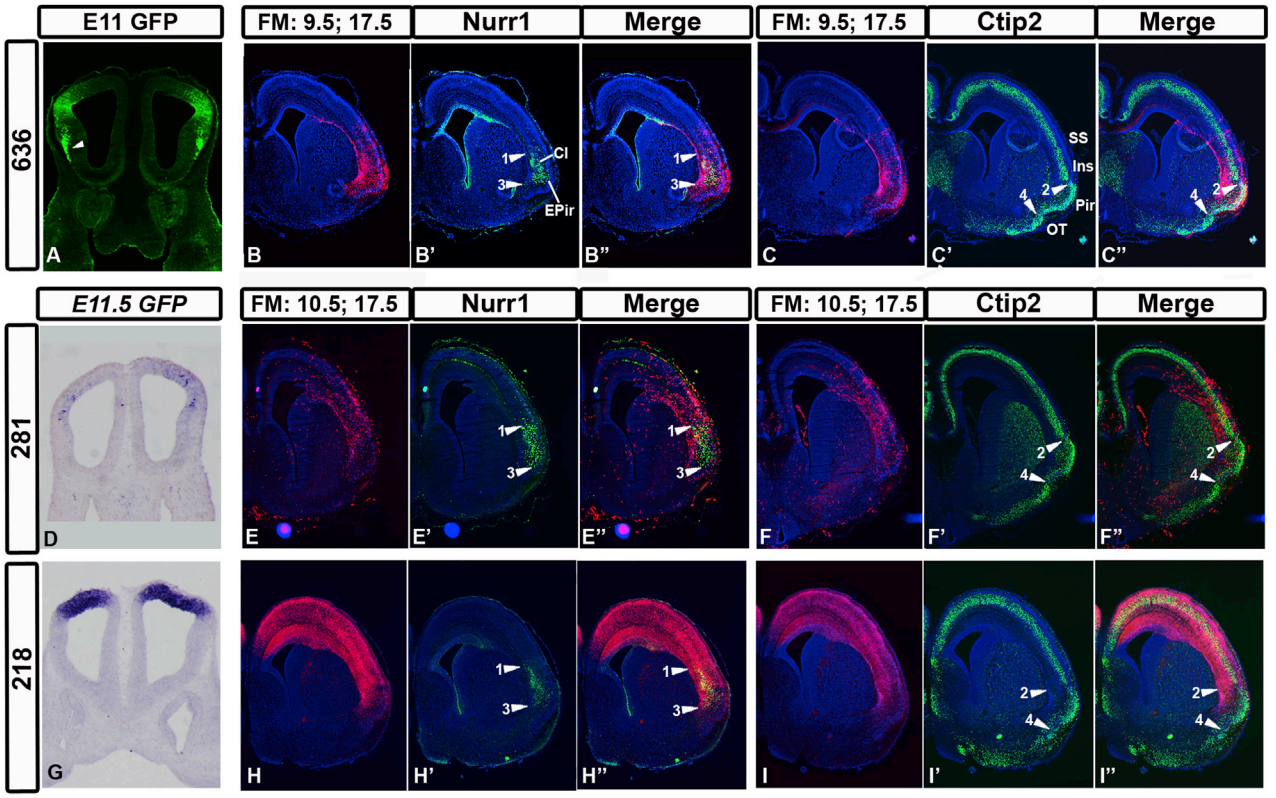
Figure 3. Frontal Cortex Analysis

(A–H‴) Eight enhancers with activity in pallial progenitors that fate map to prefrontal cortex subdivisions: medial: 192, 1,056, 348; dorsal: 1,050, 840; and lateral: 636, 281, 1,172. Coronal sections through prefrontal cortex are shown: left column shows GFP expression at E11.5 in situ or immunohistochemistry. Right columns show fate mapping with tdTomato expression in an E17.5 rostrocaudal series. See Figure S2 for additional E11.5 and E17.5 sections.

(I) Annotation of fate-mapping results from selected enhancers (y axis) in five regions of the frontal cortex (x axis). Different levels of density of tdTomato expression are estimated and described as high density (red), medium density (orange), low density (yellow), and negligible density (green). In some cases, we note subdomain expression.

(J) Deduced progenitor domain organization of the rostral E11.5 pallium. For abbreviations, see legend to Figure 2 and the following: CR, Cajal Retzius cells; DPFC, dorsal prefrontal; DLGE, dorsal LGE; FP, frontal pole; MPFC, medial prefrontal; SP, subpallium.

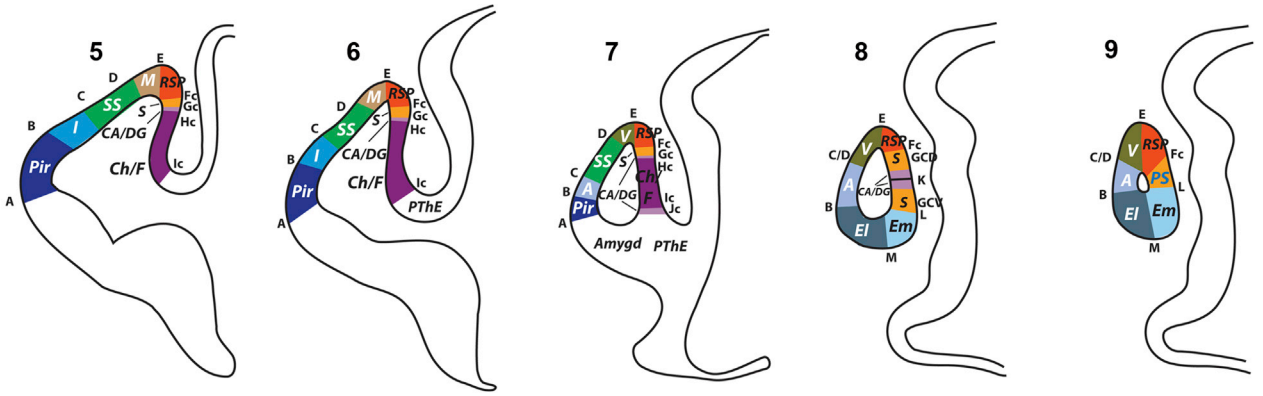
activity generated tissue probably representing the indusium griseum and taenia tecta (Figures 3A–3A‴ and Figure S2A); 1,056 generated the ventromedial PFC (including the medial orbital cortex) (Figures 3B–3B‴ and Figure S2L); 348 generated most regions of the MPFC (Figures 3C–3C‴ and Figure S2D).



J

Enhancer	Rostral			Medial				Caudal	
	Neo	Insula	Piriform	Neo	Insula	Piriform	P Amgy.	Neo	ERh
1318									
840									
218									
1050									
1006									
281									
1172									
636									

K



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Fate mapping of enhancer lines (218, 840, and 1,050) with E11.5 activity in the rostral-most DP (Figure 3, Figure S2, and Table S4) resulted in labeling of the dorsal PFC (DPFC). For instance, 1,050 only generated cells in the DPFC (Figures 3D–3D‴ and Figure S2K).

Fate mapping of enhancer lines (281, 636, and 1,172) that showed expression in the rostral-most E11.5 VLP (Figure 3 and Figure S2) resulted in cells of the lateral PFC (LPFC). This included the anterior insular cortex and the lateral orbital PFC (Figure 3 and Figure S2). Finally, fate mapping of 1,318 and 1,006 activities, which were similar in the rostral telencephalic pole at E11.5, resulted in cells that populate the entire PFC (Figure 3I and Figures S2J and S2N). These data are summarized in Figure 3I, Figure S2, and Tables S3 and S4.

Enhancers Active in Primordia of Distinct VP and LP Subdivisions

The analysis of E11.5 expression and CreER^{T2} fate-mapping experiments from eight enhancer transgenic lines identified progenitor domains generating the VP and LP, which contain cortical domains superficial to pallial nuclei (Puelles, 2014) (Figures 4K and Figure S2). To systematize the E17.5 fate-mapping analyses, we compared the td-Tomato expression with the expression of two proteins that have boundaries in the VP and LP domains: NURR1 (NR4A2) and CTIP2 (BCL11B) (Figure 4). NURR1 was expressed dorsally in the claustrum, a nucleus lying deep to the insular cortex (LP) and more ventrally in the dorsal endopiriform nucleus, which is deep to the piriform cortex (VP). We defined boundary 1 as the dorsal limit of the claustrum and boundary 3 as the ventral limit of the endopiriform nuclei (Figures 4B–4B‴, 4E–4E‴, and 4H–4H‴). CTIP2 was expressed in the superficial corticoid strata of these two pallial regions; we defined boundary 2 as the limit between the insular cortex and the piriform cortex and boundary 4 as the ventral limit of the VP with the subpallium (Figures 4C–4C‴, 4F–4F‴, and 4I–4I‴).

Fate maps from the ventrolateral enhancers (Figures 4 and S2) showed different tdTomato^{+} cell distributions in the ventrolateral cortices. Rostrally, enhancers 1,050, 1,006, 218, 281, and 636 showed progressively more ventral boundaries. Cells marked by 1,050 activity were restricted to the DP (ending before the LP), 1,006 ended roughly at the DP/LP boundary, 218 ended roughly at the LP/VP boundary, and 281 and 636 extended to the pallial-subpallial boundary (Figures 4A–4F‴ and Figures S2C, S2E, and S2J). Enhancer 636 was most active in the VLP, with little activity in the DP (Figures 4A–4C‴ and Figure S2E).

Figure 4J shows enhancer fate map annotation along the dorsoventral axis in separate rostral, middle, and caudal regions. Some enhancers had clear rostrocaudal differences in the dorsoventral position of their respective fate maps (281, 636, 1,172, and 1,318). For instance, 1,172 labeled a domain that rostrally was largely ventrally restricted, whereas caudally it extended into the DP and MP (Figure S2M). Note that enhancer 1,172 maps to a genomic region ~100 kb away from *CoupTFI*, which shows a very similar expression pattern (Figure S2M, Table S2; Armentano et al., 2007; Faedo et al., 2008).

Enhancers Active in Primordia of MP Subdivisions: Hippocampal Complex and Adjacent Structures

E11.5 expression and fate-mapping experiments from six enhancer transgenic lines demonstrate the progenitor domains that generate different MP derivatives; these enhancers were active either in the rostrodorsal or caudoventral hippocampal fields (Figures 5, 6F, and 6G and Figure S2). Note that the dorsoventral adult hippocampal topography corresponds topologically to the embryonic rostrocaudal axis, the ventral tip next to the amygdala being caudalmost. The hippocampal region is topologically dorsal; the choroid plexus (Ch) is the dorsalmost component.

CreER^{T2} fate mapping from enhancers 192, 218, 348, and 643 generated a nested pattern of derivatives within the hippocampal complex. Enhancer 192 activity was the most restricted; its derivatives contributed to the choroid plexus, fimbrial area (F or hem), and hem-originated Cajal Retzius (CR) cells, with very sparse labeling of the dentate gyrus and CA fields (Figures 5C–5C‴ and Figure S2A). Enhancer 643 was active in the progenitors of the Ch, F, CR cells, dentate gyrus (DG), and CA fields (strongest in CA1) (Figures 5B–5B‴ and Figure S2F). Its activity was restricted to the rostrodorsal MP. Likewise, 218 was active in progenitors of the rostrodorsal MP but was weak in caudoventral MP progenitors (Figure S2B). Enhancer 348 was active in the entire MP but with stronger activity in its rostrodorsal components (Figures 5A–5A‴ and 5D–5D″ and Figure S2D).

By contrast with these rostrodorsal MP enhancers, we identified two MP enhancers that were almost exclusively restricted to the caudoventral MP: 653 and 660. These were active in progenitors that produced cells in the caudoventral DG and CA fields (Figures 5E–5E‴ and Figures S2G and S2H).

Enhancer 643 Marks the Formation of the Hippocampal Field

Based on the nested activity of enhancers 192, 643, and 348 within the hippocampal primordia (GFP expression and fate maps), we studied its ontogenesis in detail by examining enhancer MP activity at E11.5 (Figure 6). We compared the expression of GFP and *Lmx1a* RNA, a marker of the F and Ch (Chizhikov et al., 2010). Histochemical analysis at E11.5 showed that GFP expression from enhancers 192 and 643 and *Lmx1a*

Figure 4. Four Enhancers with Activity in Pallial Progenitors that Fate Map to Ventrolateral Cortex Subdivisions

(A–I″) Left column shows GFP protein (green fluorescence) or RNA (purple in situ) expression at E10.5 (636; arrowhead: migrating neurons) or E11.5 (281, 218). Right columns show fate mapping with tdTomato expression at E17.5. To map the fate map boundaries, we performed double immunofluorescnece to detect tdTomato (red) and either Nurr1 or Ctip2 (green). Nurr1 or Ctip2 expression was used to define boundaries 1, 2, 3, and 4 (see Results), which distinguished the limits of the fate maps of 636, 281, and 218.

(J) Annotation of fate-mapping results from selected enhancers (y axis) in nine regions of the ventrolateral cortex (x axis). Different levels of density of tdTomato expression are estimated and described as high density (red), medium density (orange), low density (yellow), and negligible density (green).

(K) Deduced progenitor domain organization of middle-to-caudal regions of the E11.5 pallium. See Figure S2 for additional E11.5 and E17.5 sections. For abbreviations, see legend to Figure 2 and the following: Cl, claustrum; EPir, endopirifom; OT, olfactory tubercle; Neo, neocortex; P Amgy, pallial amgydala.

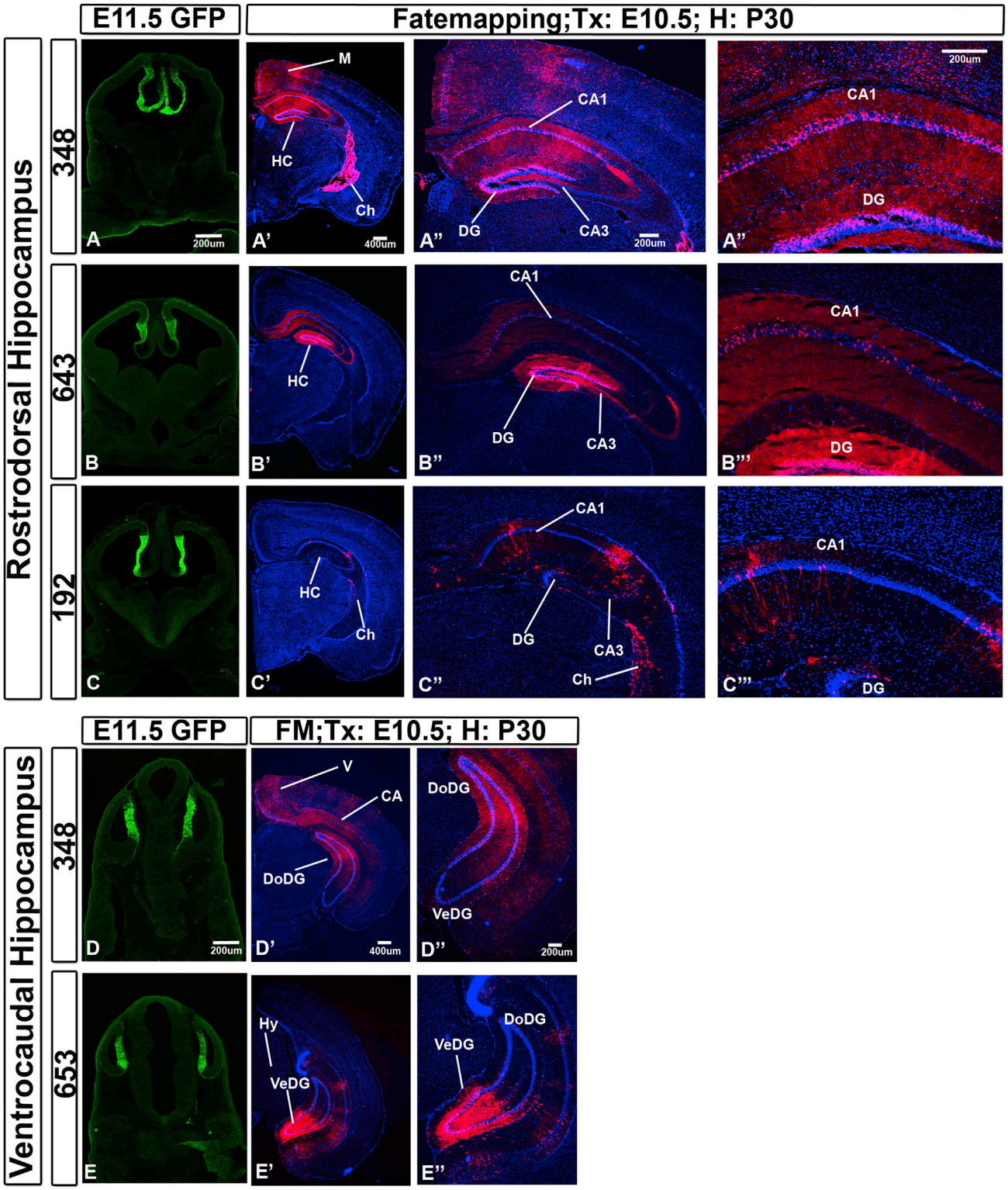

Figure 5. Four Enhancers with Activity in Medial Pallial Progenitors that Fate Map to Hippocampal Subdivisions

Coronal sections show GFP expression at E11.5 and fate mapping with tdTomato expression at P30. Two enhancers show activity and fate map to the rostrodorsal hippocampus (348 and 643); P30 fate map pictures are shown at 2× (A′, B′, and C′), 4× (A″, B″, and C″), and 10× (A‴, B‴, and C‴) magnifications. Enhancer 192 fate maps to the fimbria and choroid plexus. One enhancer (653) shows activity and fate maps to the caudoventral hippocampus and choroid

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RNA expression were nearly identical; they shared a sharp boundary (arrowhead; Figures 6A–6A‴ and 6B–6B‴). Likewise at E12.5, enhancer 192 activity (GFP) and *Lmx1a* RNA expression remained nearly identical (arrowhead; Figures 6C–6C″), whereas enhancer 643 GFP expression spread into the adjacent pallial neuroepithelium (between arrowhead and arrow; Figures 6D–6D″). Recall that enhancer 192 fate mapping labeled very little of the hippocampus, whereas enhancer 643 fate mapping labeled the DG and the CA fields of the rostrodorsal hippocampus (Figures 5C–5C‴, arrows, and 6C). Thus, the hippocampal field is first detectable between E11.5 and E12.5, concomitant with the expansion of enhancer 643 activity (Figure 6E).

Computational Identification of Transcriptional Drivers of Region-Specific Enhancer Activity

To explore the molecular mechanisms controlling enhancer activity in subregions of the pallium, we compared the sequences of enhancers with activity largely restricted specific pallial domains. We were most successful when we compared MP enhancer sequences (N = 9; 192, 348, 480, 611, 622, 643, 653, 660, 1,006) to the sequence of enhancers active in DP, LP, and VP (N = 15; 22, 200, 218, 488, 595, 619, 632, 636, 671, 876, 957, 978, 987, 1,025, 1,050). We searched for nucleotide motifs that distinguished these groups using two models. Model 1 was trained to distinguish sequence motifs between 9 MP enhancers, 15 non-MP enhancers, and 480 random genomic sequences. Model 2 was trained to distinguish sequence motifs between 9 MP enhancers, 15 non-MP enhancers, a set of background sequences consisting of 480 random genomic sequences, and 765 sequences from the VISTA Enhancer Browser that were negative for enhancer activity (see Supplemental Experimental Procedures). This approach generated motifs that were enriched in MP enhancers compared to non-MP enhancers. The top 20 de novo motifs for each set of enhancers (MP and non-MP) were mapped to the Transfac and JASPAR database to identify TFs that have similar binding sites (Figure S3; see Tables S5 and S6).

Using the list of TF binding motifs preferentially identified in MP enhancers or in non-MP enhancers, we scrutinized the E11.5 expression of the top 40 TFs using the Allen Brain Atlas (http://developingmouse.brain-map.org/). Five TFs (*Lhx5*, *AR*, *Nr4a2* [nuclear receptor family], *Lmx1a*, and *Foxj1*) were only expressed in the Ch/F domain, and five TFs showed expression in the DP, but not in the MP, and especially in Ch/F, expression was either low or not detectable (Figures S3 and S4 and Tables S5 and S6). Thus, the method successfully selected for TFs that were either expressed within or excluded from Ch/F. Of note, the Ch is perhaps the most distinct region of the pallium, because its derivative, the choroid plexus, is a nonneural structure.

Transcriptional Mechanisms Regulating Enhancer Function: In Vivo Binding by PAX6, COUPTFI, and PBX1

Next, we directly screened the enhancers for binding sites for TFs known to regulate pallial pattering. We found binding sites for PAX6, COUPTFI (NR2F1), and PBX1 in pallial enhancers (Genomatix); each of these TFs regulates patterning of the pallial primordium (Bishop et al., 2000; Yun et al., 2001; Mallamaci and Stoykova, 2006; Armentano et al., 2007; Faedo et al., 2008; O.G. and J.L.R.R., unpublished data). We then tested whether these enhancers were bound in vivo by these TFs using chromatin immunoprecipitation and DNA sequencing (ChIP-seq). We performed ChIP-seq with antibodies to PAX6 (n = 3), COUPTFI (n = 1), and PBX1 (n = 1) on dissected E12.5 mouse pallium; information about the quality of the sequence mapping and peak calling are reported in Table S7. We surveyed the genome for binding to the 44 enhancers assayed in Figure 1 and Figure S1. The results are organized according to the regional activity of the enhancers, MP (n = 11), DP+MP (n = 8), DP (n = 2); LVP+DP; LVP (n = 12), and MP+DP+LVP (n = 6) (Table S8). Then, we annotated ChIP-seq binding to each enhancer by PAX6, COUPTFI, and PBX1.

PAX6 bound to all of the enhancers that were globally expressed (MP+DP+LVP) in the pallium (6/6). As the enhancers became more restricted in their regional activity, PAX6 binding frequency reduced, particularly if LVP activity was absent. PAX6 and COUPTFI (NR2F1) bound to few MP enhancers, and PBX1 bound none (Figure 7A and Table S8). We show an example of PAX6 peaks over enhancer 840 and 636 (Figure 7C), and PAX6, COUPTFI, and PBX1 binding over the other enhancers are shown in Figure S5.

We then focused on *Pax6* regulation of some of the enhancers that had in vivo binding sites. We used transient transfection luciferase assays to study whether *Pax6* cotransfection modulated activity of 636, 643, 840, and 1,172 (n = 4). In each case, we observed >5-fold activation of luciferase expression (Figure 7B). Of note, PAX6 activation declined in enhancers with MP activity (840, 643) or that were expressed in a caudorostral gradient (1,172; note: *Pax6* is expressed in a rostocaudal gradient).

Finally, we tested *Pax6* in vivo regulation of enhancer activity by introducing the 636, 643, and 840 *enhancer-CreER^{T2}-GFP* alleles into mice harboring a *Pax6* null allele (*Sey*). We generated E11.5 embryos and found that *Pax6*$^{-/-}$ mutants had reduced GFP expression from enhancers 636 and 840 in pallium (Figures 7D, 7D′, 7F, and 7F′). On the other hand, enhancer 643 continued to express GFP in the *Pax6*$^{-/-}$ mutant, although the ventral boundary was less sharp (Figure S5).

DISCUSSION

We generated stable transgenic mouse lines that express CreER^{T2} and GFP from 14 different enhancer elements with activity in distinct domains within the E11.5 pallium. These enhancer-CreER^{T2}-GFP lines have obvious broad utility for experimental manipulation of gene expression in specific domains and at specific times, including Cre-mediated gene

plexus; results are compared with the rostrodorsal hippocampal enhancer (348); P30 fate map pictures are shown at 2× (D′ and E′) and 4× (D″ and E″) magnifications. See Figure 6 for fate-mapping annotation and Figure S2 for additional E11.5 and E17.5 sections. For abbreviations, see legend to Figure 2 and the following: CA1 and CA3, hippocampal pyramidal cell fields; DG, dentate gyrus (Do, dorsal; Ve, ventral); HC, hippocampus; Hy, hypothalamus.

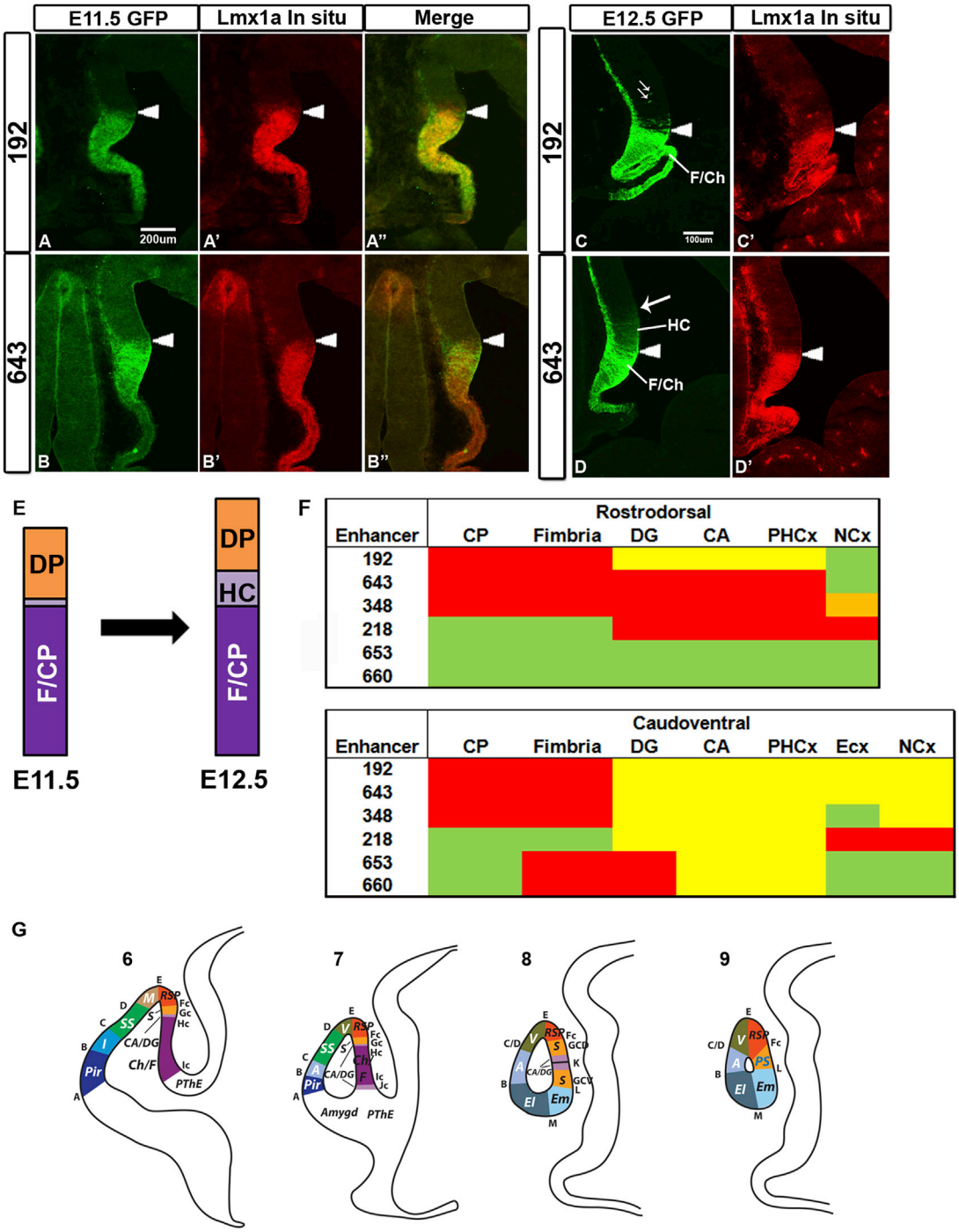

Figure 6. Expansion of the Hippocampal Primordium at E12.5

(A–D′) Comparison of the activity of MP enhancers 192 and 643 at E11.5 and E12.5. Enhancer activity (A and B: GFP expression) is compared with *Lmx1a* RNA expression (A′ and B′) using double immunohistochemistry (GFP)/in situ hybridization (A′′ and B′′: *Lmx1a*). *Lmx1a* marks the F/Ch domain; at E11.5, both 192 and 643 have nearly identical patterns, sharing a common boundary (arrowhead). However, by E12.5, enhancer 643 (C and C′) activity expands beyond the *Lmx1a*/enhancer 192 (D and D′) boundary (arrowhead) into the neuroepithelium that generates the dentate gyrus and CA fields (arrow; see Figures 5C′–C′′′); note that 192 activity is present in a few scattered hippocampal progenitors (C, arrows).

(E) Schema summarizing results in (A–D′), showing the expansion of the hippocampus (HC) between E11.5 and E12.5.

(F) Annotation of fate-mapping results from selected enhancers (y axis) in 13 regions of the medial pallium (x axis). Different levels of density of tdTomato expression are estimated and described as high density (red), medium density (orange), low density (yellow), and negligible density (green).

(G) Deduced progenitor domain organization of E11.5 medial pallium and other regions of the caudal pallium. For abbreviations, see legend to Figure 2.

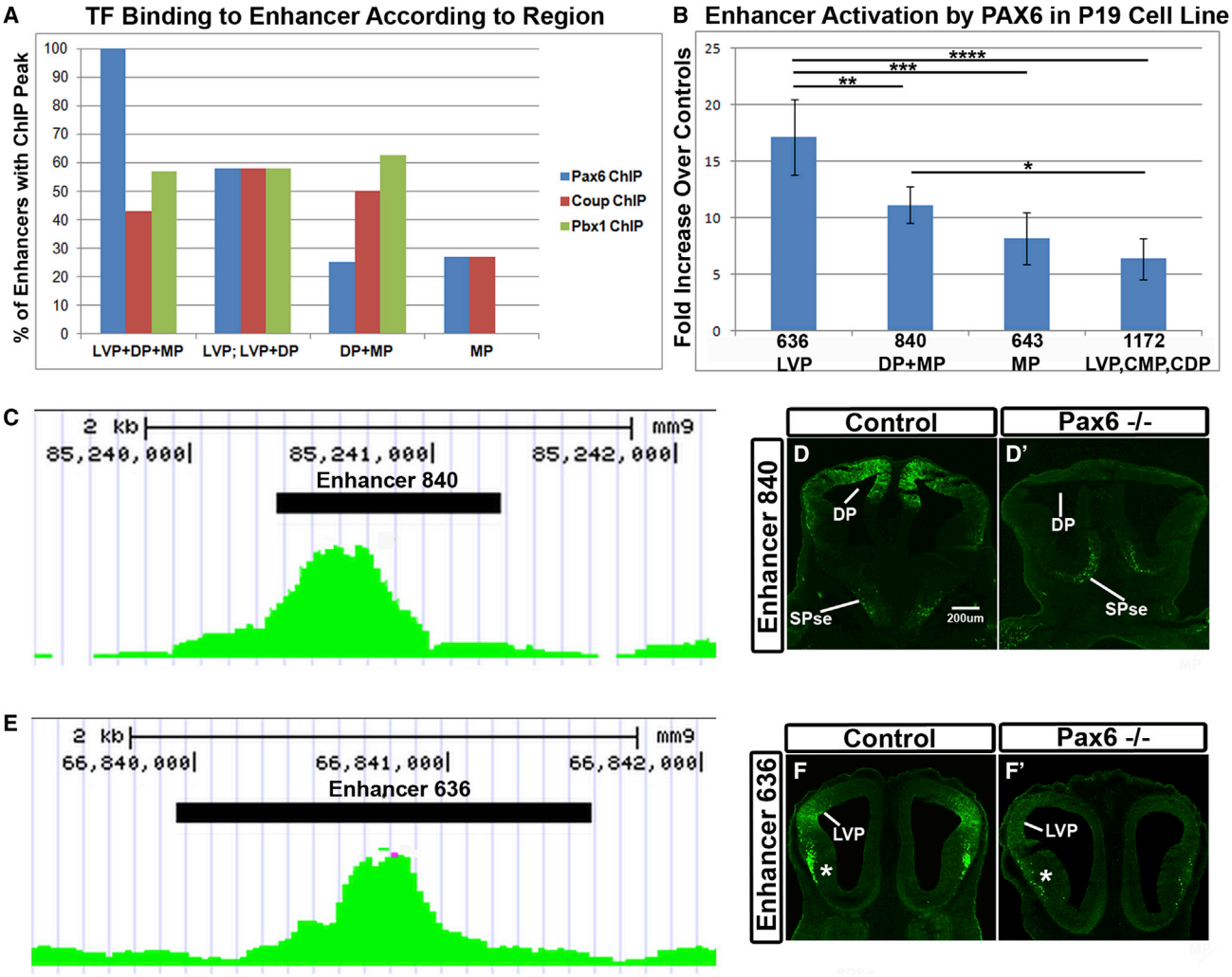

Figure 7. Transcription Regulation of Pallial Enhancers

(A) Percentage of enhancers with ChIP-seq peaks for PAX6, COUPTFI, and PBX1 on LVP+DP+MP, LVP; LVP+ DP, DP+MP, and MP enhancers.

(B) Transcription assays in transfected P19 cells (2 days) measuring luciferase expression driven by PAX6 activation of enhancers 636, 840, 643 and 1,172. Error bars were generated using a one-way ANOVA with a Tukey post hoc test.

(C) PAX6 ChIP-seq analysis from E12.5 cortex showing a peak directly over endogenous enhancer 840 (black bar).

(D) GFP pallial expression driven by enhancer 840 in E11.5 cortex.

(D′) Reduced pallial GFP expression in *Pax6*$^{-/-}$.

(E) PAX6 ChIP-seq analysis from E12.5 cortex showing a peak directly over endogenous enhancer 636 (black bar).

(F) GFP pallial expression driven by enhancer 636 in E11.5 cortex.

(F′) Reduced pallial GFP expression in *Pax6*$^{-/-}$ (asterisk labels migrating VP neurons). See also Figure S5. For abbreviations, see legend to Figure 2 and the following: CDP and CMP, caudal DP and MP; SPSe, subpallial septum.

deletion. Herein, using these unique tools, we (1) determined comprehensive regional fate map of the mouse pallium, which includes evidence for a set of progenitor domains defined by the activity of the enhancers (Figure 1E), and (2) began to decipher transcriptional mechanisms that control the enhancers using informatics, in vivo occupancy by TFs that regulate cortical patterning (PAX6, COUPTFI, and PBX1), and analysis of enhancer activity in *Pax6* mutants. Below we elaborate on these discoveries and their implications for understanding cortical development, evolution, and disorders.

Dynamic Temporal Activity of Cortical Enhancers

Previously published transient transgenic analysis of cortical enhancers (Visel et al., 2013) interrogated only one developmental time point, E11.5. Using the stable enhancer lines described in this paper, we analyzed enhancer activity at different developmental ages. The majority of the enhancers maintained similar patterns of activity between E11.5 and E13.5; however, by E14.5, the activity of most of the enhancers decreased and/or became restricted to a smaller domain (Figure S2; Table S2). Thus, the set of enhancers we studied were

primarily active during stages when regional patterning of pallium takes place (~E9.5–E12.5) and at early stages of neurogenesis (~E11–E13.5), suggesting that other enhancers have roles at later stages to drive gene expression for later developmental processes. Recently, Nord et al. (2013) provided evidence for distinct cohorts of enhancers that are active at different stages of brain development. In addition, some enhancers can be active at different stages. For example 660 expression, as its E11.5–E13.5 activity in the caudoventral cortex wanes, begins at ~E13.5 in the neocortical subventricular zone (Figure S2). The *Dlx12b* enhancer is active both in subpallial subventricular zone progenitor cells and in maturing and mature GABA neurons (Ghanem et al., 2007; Vogt et al., 2014).

Fate-Mapping Analyses Define the Regional Derivatives from Distinct Pallial Progenitor Domains

The transient transgenic analysis of enhancer E11.5 activity led us to hypothesize subdivisions of the pallial progenitors (Figure 1D). The stable transgenic analysis of E11.5 enhancer activity (GFP expression), and CreER^{T2} fate analyses at E17.5 and P30, supported many aspects of our initial model (Figure 1D) and importantly enabled us to describe the regional fates of each proposed progenitor domain (Figure 1E and Table S2). Among the important observations, we discovered E11.5 progenitor domains that produce distinct subdivisions of the frontral cortex, providing information about where these distinct regions originate (Figure 3).

Previous fate mapping of pallial regions have used transplantation (chick-quail; Garcia-Lopez et al., 2009) and Cre recombination methods, in which a constitutive Cre was driven from a gene locus. Thus, unlike our study, the previous Cre fate mapping did not obtain temporal-specific data, since generally the alleles were active over long periods of pallial development. Emx1-Cre and Foxg1 fate mapping showed that expression from these loci covers most of the pallium (Gorski et al., 2002; Hébert and McConnell, 2000). Wnt3a-Cre fate mapping labeled the cortical hem and derived Cajal-Retzius cells (Yoshida et al., 2006). Dbx1-Cre fate mapping showed that the ventral pallium is another source for Cajal Retzius cells and that it contributes glutamatergic neurons to specific nuclei in the amygdala and ventral cortical structures (Bielle et al., 2005; Hirata et al., 2009; Teissier et al., 2010; Waclaw et al., 2010). Thus, while these studies provide important information about the fates of pallial regions that express Cre over the course development, they do not provide a comprehensive fate map from multiple pallial progenitor domains from temporally restricted Cre activity.

The fate maps obtained using the 14 enhancer lines illuminated unexpected facets of the E11.5 expression domains. There was a rostrocaudal discontinuity in the properties of dorsomedial progenitor domains (between coronal planes 4 and 5 of schema; Figure 1E); rostrally, next to the septocommissural region, the dorsomedial progenitors generate the motor, cingulate, and prefrontral cortex; caudally, next to the choroid plexus, the dorsomedial progenitors generate the hippocampal complex and fimbrial area (hem). Within the rostral domain, we observed other rostrocaudal discontinuities, such as the restriction of the pallial septum, IL, and PrL domains within coronal planes 2 and 3 (Figure 3J).

Topographic discontinuities in caudal progenitor domains include the restriction of the Ch and F to the regions illustrated in coronal planes 4–7, the joining of the caudoventral (caudal) and rostrodorsal hippocampal domains in coronal plane 8, and the end of the hippocampal domain in coronal plane 9. Remarkably, we identified enhancers only active in the rostrodorsal (218, 348, and 643) or caudoventral (653 and 660) hippocampal primordia at E11.5.

The relative sizes of some progenitor domains were disproportionate to the size of their derived regions at E17.5 and P30, such as the size of Ch/F compared to the rest of the hippocampal complex (see sections 5–7; Figures 1E and 6G). Thus, there was not a 1:1 proportional matching of the sizes of E11.5 progenitor and mature domains, providing evidence that the timing and relative distribution of regional growth is not uniform.

We provided evidence that the hippocampal primordium begins to expand at E12.5, based on the likewise expanded activity of enhancer 643 (Figure 6). Furthermore, the activity of enhancer 1,050 becomes progressively focused in the hippocampal region between E11.5 and E14.5 (Figure S2K). Thus, further investigations into the transcriptional process that drives hippocampal development will be aided by understanding the transcription mechanisms that drive enhancer 643 and enhancer 1,050 activities in the hippocampal primordium.

While the aim of our anatomical analyses was to derive a pallial fate map, we made some observations about regional histogenesis and cell-type generation. Tamoxifen induction of recombination at E10.5 generally resulted in radial clones of cells that spanned the cortical plate, providing evidence that this set of enhancers is not lineage restricted with regard to subsequent laminar fate. This adds evidence for the model that intrinsically produced neurons for each cortical layer are sequentially generated from the same neuroepithelial progenitor (Leone et al., 2008; Guo et al., 2013), although it does not eliminate the possibility that enhancers will be discovered that show more restricted fate properties. Indeed, as has already been elucidated, excitatory neurons of layer 1 (Cajal Retzius cells) are generated from specific domains at the pallial perimeter (Bielle et al., 2005; Yoshida et al., 2006; Puelles, 2011); several of our enhancers (192, 348, and 643) provide additional evidence for this process (Figures S2A, S2D, and S2F). Furthermore, because the enhancers drive CreER^{T2}, tamoxifen induction of Cre activity at later time points can be used to study later stages of neuro- and gliogenesis with enhancer lines that maintain progenitor cell activity after E11.5 (Table S2).

Identification of Enhancers that Detect Pallial Subdivisions: Insights into the Transcription Networks Driving Pallial Regional Development and Evolution

In the cellular blastoderm of *Drosophila*, enhancer activities reveal developmental domains generated by the combinatorial activity of TFs that ultimately underlie body subdivisions, as exemplified by enhancers that drive gap-gene expression (Perry et al., 2011). Enhancer activity domains can be smaller and sharper than the expression domains of the TFs that drive their expression (Perry et al., 2011).

In the pallial primordium *CoupTFI*, *Dmrta2 (Dmrt5)*, *Emx2*, *Lhx2*, *Pax6*, *Pbx1*, and *Sp8*, TFs that control pallial regionalization, are

expressed in broad gradients (Bishop et al., 2000; Galceran et al., 2000; Yun et al., 2001; Mallamaci and Stoykova, 2006; Armentano et al., 2007; Sahara et al., 2007; Faedo et al., 2008; Mangale et al., 2008; Chou et al., 2009; Konno et al., 2012; Borello et al., 2013; Saulnier et al., 2013). Positional information appears to lie in these TF gradients, and in their combinatorial interactions.

Here, we provide evidence for a mechanism that can integrate this transcriptional information to generate discrete pallial subdivisions. Many of the enhancers show patterns of activity at E11.5 that are more discrete than the broadly expressed patterns of aforementioned patterning TFs. Thus, we suggest these enhancer activities reflect the integration of transcriptional activities that together pattern the pallium. Furthermore, it is possible that these and related enhancers are fundamental elements that have driven pallial evolution, as duplication and transposition of these distant-acting regulators have the potential to alter gene expression. Significantly, our gain-of-function transgenic assays show the ability of these enhancers to function in a variety of chromosomal locations.

Currently, we do not have definitive evidence for the gene(s) that each of these enhancers regulates. However, based on proximity, and similar expression profiles, we have some predictions for enhancer/gene pairs (Figure S2 and Table S2). For instance, the activity of enhancers 1,006, 1,050, and 1,172, which have genomic positions close to *Wnt8b*, *Lef1*, and *CoupTFI*, respectively, closely resembles the pallial expression of these genes (Figures S2J, S2K, and S2M; Visel et al., 2013). Future studies are required to test for enhancer/gene interactions using chromatin conformation methods (Clowney et al., 2012), as well as loss-of-function mutagenesis. While some enhancers are clearly required for gene expression (Shim et al., 2012), there is evidence that enhancer redundancy exists (Ahituv et al., 2007; Lagha et al., 2012).

Mechanisms that Regulate Enhancer Activity

We used informatic, biochemical, and genetic approaches to begin deciphering transcriptional mechanisms that control the activity of the enhancers. Informatic methods provide insights into candidate TFs that may regulate enhancer activities (Shim et al., 2012; Visel et al., 2013). We used a machine learning method to identify nucleotide signatures that may underlie regional differences in enhancer activities. Transcriptional binding sites that were enriched in enhancers with and without MP activity led us to identify TFs with expression either in, or excluded from, the Ch/F part of the E11.5 MP (Figures S3 and S4). This is interesting because histogenesis of the primordium of the choroid plexus (Ch) is distinct from the rest of the pallium, as the Ch is a nonneural tissue generated from the neural tube roof plate (Puelles, 2014), and the fimbrial area (cortical hem) represents the border between roof and alar plate tissues. As more pallial enhancers are defined, and as the binding sites for additional TFs are identified, it is likely that informatic approaches will gain power in defining sequences that control regional expression.

Next, we used ChIP-seq to test whether the enhancers under study were bound in vivo in the E12.5 mouse cortex by PAX6, COUPTFI, and PBX1, three transcription factors that regulate pallial patterning (Bishop et al., 2000; Yun et al., 2001; Mallamaci and Stoykova, 2006; Armentano et al., 2007; Sahara et al., 2007; Faedo et al., 2008; Borello et al., 2013; O.G. and J.L.R.R., unpublished data). Previous analyses of PAX6 binding in the developing pallium used ChIP-promoterChIP and thus did not examine PAX6 binding to the enhancers described herein (Sansom et al., 2009; Xie et al., 2013). Our ChIP-seq analyses showed that PAX6 (a general marker for the telencephalic pallium; Puelles et al., 2000) bound to all of the enhancers globally expressed in the pallium (Figure 7A and Table S8), suggesting that PAX6 may have a fundamental role coordinating pallial properties.

Enhancers with more restricted intrapallial regional activity had reduced frequencies of PAX6 binding, particularly when LVP activity was absent (Figure 7A and Table S8). In addition, COUPTFI and PBX1 bound few MP enhancers (Figure 7A and Table S8). Both *Pax6* and *CoupTFI* regulate dorsoventral patterning of the pallium; and they both promote ventral identity (Yun et al., 2001; Faedo et al., 2008). Consequently, our ChIP-seq analysis provides evidence that these two TFs may regulate dorsoventral pallial patterning by promoting activity of enhancers specifically active in the VP, LP, and DP.

This concept is consistent with that the fact that PAX6 has a potent role in patterning the ventrolateral cortex (Figure 7A; Yun et al., 2001). Furthermore, transcription assays in tissue culture showed that PAX6 strongly activated (~15-fold) 636, the enhancer with VP and LP activity (Figure 7B). On the other hand, PAX6-mediated activation was lower for enhancers with MP activity (840 and 643), suggesting that they have elements that antagonize activation by PAX6.

To test hypotheses generated by the ChIP-seq and transfection assays, we examined 636 and 840 enhancer activities in *Pax6* loss-of-function mutants. As predicted by their PAX6 ChIP-seq peaks and their activation by PAX6 in the cell culture transcription assay, 636 and 840 pallial activity was greatly reduced in E11.5 $Pax6^{-/-}$ (Figures 7D, 7D′, 7F, and 7F′).

Implications

The identification of human enhancers with restricted spatial and temporal activities in pallial protodomains demonstrates that the genome has relatively small (0.5–3 kb) regulatory elements that can integrate transcriptional information to generate highly specific patterns of gene expression, even in ectopic genomic loci (herein and Visel et al., 2013). Importantly, the enhancer activity patterns for the most part do not resemble the expression of single known TFs, highlighting the enhancers' roles as spatial integrators of regulatory information. This knowledge opens the door to deciphering the sequence-specific regulation of enhancer activity and how mutations alter their function and contribute to disease.

EXPERIMENTAL PROCEDURES

Generation and Characterization of Stable Enhancer Transgenic Mice

PCR-amplified human enhancer regions were subcloned into *Hsp68-CreERT2-IRES-GFP* (Visel et al., 2013) and used to generate stable transgenic mice. Founders were screened using CreERT2 specific primers. Enhancer transgenic embryos were examined for GFP expression. For fate mapping, enhancer lines were crossed to *Ai14* Cre-reporter mice (Madisen et al.,

2010). Tamoxifen was administered at E9.5 or E10.5; tdtomato was assayed at later stages. Stable transgenic mice were crossed to the *Pax6* mutant. Mice were used in accordance with National Institutes of Health and UCSF guidelines.

Histology

Immunohistochemistry was performed as in Flandin et al. (2010). RNA in situ hybridization and in situ/immunohistochemistry was performed as in Jeong et al. (2008).

Identification of Region Specific Motifs

See Supplemental Experimental Procedures for details.

Chromatin Immunoprecipitation

ChIP was performed using E12.5 or E13.5 cortex and Pax6 (Millipore), CoupTf1 (R&D systems), and Pbx1/2/3 (C-20, Santa Cruz Biotechnology) antibodies (McKenna et al., 2011). Libraries were prepared using an Ovation Ultralow DR Multiplex System (Nugen). Reads from ChIP, input, and negative control (IgG) libraries were mapped to the mouse genome (mm9) using BWA and peaks were called using MACS considering both input and IgG as the control sample with filtering to remove peaks in repeat regions.

Luciferase Assay

Enhancer activity was studied in P19 cells (Farah et al., 2000) cotransfected with *pCAGGs* (empty) or *pCAGGs-Pax6/CoupTf1/Pbx1*, and Promega *pGL4.23* luciferase reporter (empty) or containing an enhancer element upstream of the luciferase gene (pGL4.23-enhancer).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, fives figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.04.014.

AUTHOR CONTRIBUTIONS

K.P. and O.G. were the leaders of the project from the initiation of the experiments, generating the DNA constructs used to make the transgenic lines, analyzing the activity of the enhancers, and performing and analyzing the fate maps of the different enhancer lines. K.P. then led the analysis of transcription factor regulation of the enhancers using mouse genetic and luciferase assays. S.L. performed the ChIP-seq experiments, made possible by B.C. who provided critical technical supervision; the data were analyzed by K.P. and A.N. L.P. contributed to the analysis of the fate-mapping experiments and generated the topological maps of the flattened E11.5 and E17.5/adult pallium. D.Z. worked with K.P. in generating and analyzing the transgenic mice. R.H. generated the enhancer expression cassette and generated an enhancer line. S.S. contributed data from an enhancer line. L.T. performed the informatic analyses to identify candidate functional domains within the enhancers. H.Z. made possible the funding of part of the project from the Allen Institute for Brain Science. A.V. and L.A.P. initiated the identification of the enhancers and provided the DNA constructs used to generated the transgenic mice. J.L.R.R. conceived of and supervised the project and performed data analysis. All co-authors contributed to editing the paper; generation of the figures was performed by K.P.; writing was principally done by J.L.R.R. and K.P.

ACKNOWLEDGMENTS

Grant Support includes the following: J.L.R.R.: Allen Institute for Brain Science, Nina Ireland, Weston Havens Foundation, and NINDS R01 NS34661; O.G.: NARSAD; K.P.: NIH NIGMS MSTP T32 GMO7618; S.S.: T32 GM007449; R.H.: NIH NRSA; L.P.: Spanish Ministry of Science and Competitivity (BFU2008-01456), Seneca Foundation (04548/GERM/06-10891); L.T.: Intramural Program of NIH, NLM; A.S.N.: NIH/NIGMS NRSA F32 GM105202; A.V. and L.A.P. NIH R01NS062859A and R01HG003988, and DOE Contract DE-AC02-05CH11231.

Accepted: March 28, 2014
Published: May 8, 2014

REFERENCES

Ahituv, N., Zhu, Y., Visel, A., Holt, A., Afzal, V., Pennacchio, L.A., and Rubin, E.M. (2007). Deletion of ultraconserved elements yields viable mice. PLoS Biol. *5*, e234.

Armentano, M., Chou, S.J., Tomassy, G.S., Leingärtner, A., O'Leary, D.D., and Studer, M. (2007). COUP-TFI regulates the balance of cortical patterning between frontal/motor and sensory areas. Nat. Neurosci. *10*, 1277–1286.

Bielle, F., Griveau, A., Narboux-Nême, N., Vigneau, S., Sigrist, M., Arber, S., Wassef, M., and Pierani, A. (2005). Multiple origins of Cajal-Retzius cells at the borders of the developing pallium. Nat. Neurosci. *8*, 1002–1012.

Bishop, K.M., Goudreau, G., and O'Leary, D.D. (2000). Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science *288*, 344–349.

Borello, U., Madhavan, M., Vilinsky, I., Faedo, A., Pierani, A., Rubenstein, J., and Campbell, K. (2013). Sp8 and COUP-TF1 reciprocally regulate patterning and Fgf signaling in cortical progenitors. Cereb. Cortex. Published online January 10, 2013. http://dx.doi.org/10.1093/cercor/bhs412.

Chizhikov, V.V., Lindgren, A.G., Mishima, Y., Roberts, R.W., Aldinger, K.A., Miesegaes, G.R., Currle, D.S., Monuki, E.S., and Millen, K.J. (2010). Lmx1a regulates fates and location of cells originating from the cerebellar rhombic lip and telencephalic cortical hem. Proc. Natl. Acad. Sci. USA *107*, 10725–10730.

Chou, S.J., Perez-Garcia, C.G., Kroll, T.T., and O'Leary, D.D. (2009). Lhx2 specifies regional fate in Emx1 lineage of telencephalic progenitors generating cerebral cortex. Nat. Neurosci. *12*, 1381–1389.

Clowney, E.J., LeGros, M.A., Mosley, C.P., Clowney, F.G., Markenskoff-Papadimitriou, E.C., Myllys, M., Barnea, G., Larabell, C.A., and Lomvardas, S. (2012). Nuclear aggregation of olfactory receptor genes governs their monogenic expression. Cell *151*, 724–737.

Colasante, G., Collombat, P., Raimondi, V., Bonanomi, D., Ferrai, C., Maira, M., Yoshikawa, K., Mansouri, A., Valtorta, F., Rubenstein, J.L., and Broccoli, V. (2008). Arx is a direct target of Dlx2 and thereby contributes to the tangential migration of GABAergic interneurons. J. Neurosci. *28*, 10674–10686.

Faedo, A., Tomassy, G.S., Ruan, Y., Teichmann, H., Krauss, S., Pleasure, S.J., Tsai, S.Y., Tsai, M.J., Studer, M., and Rubenstein, J.L. (2008). COUP-TFI coordinates cortical patterning, neurogenesis, and laminar fate and modulates MAPK/ERK, AKT, and beta-catenin signaling. Cereb. Cortex *18*, 2117–2131.

Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., and Turner, D.L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development *127*, 693–702.

Flandin, P., Kimura, S., and Rubenstein, J.L. (2010). The progenitor zone of the ventral medial ganglionic eminence requires Nkx2-1 to generate most of the globus pallidus but few neocortical interneurons. J. Neurosci. *30*, 2812–2823.

Galceran, J., Miyashita-Lin, E.M., Devaney, E., Rubenstein, J.L., and Grosschedl, R. (2000). Hippocampus development and generation of dentate gyrus granule cells is regulated by LEF1. Development *127*, 469–482.

Garcia-Lopez, R., Pombero, A., and Martinez, S. (2009). Fate map of the chick embryo neural tube. Dev. Growth Differ. *51*, 145–165.

Ghanem, N., Yu, M., Long, J., Hatch, G., Rubenstein, J.L., and Ekker, M. (2007). Distinct cis-regulatory elements from the Dlx1/Dlx2 locus mark different progenitor cell populations in the ganglionic eminences and different subtypes of adult cortical interneurons. J. Neurosci. *27*, 5012–5022.

Gorski, J.A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J.L., and Jones, K.R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. J. Neurosci. *22*, 6309–6314.

Guo, C., Eckler, M.J., McKenna, W.L., McKinsey, G.L., Rubenstein, J.L.R., and Chen, B. (2013). Fezf2 expression identifies a multipotent progenitor for neocortical projection neurons, astrocytes, and oligodendrocytes. Neuron *80*, 1167–1174.

Hayashi, S., and McMahon, A.P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev. Biol. *244*, 305–318.

Hébert, J.M., and McConnell, S.K. (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.

Hirata, T., Li, P., Lanuza, G.M., Cocas, L.A., Huntsman, M.M., and Corbin, J.G. (2009). Identification of distinct telencephalic progenitor pools for neuronal diversity in the amygdala. Nat. Neurosci. *12*, 141–149.

Jeong, J., Li, X., McEvilly, R.J., Rosenfeld, M.G., Lufkin, T., and Rubenstein, J.L. (2008). Dlx genes pattern mammalian jaw primordium by regulating both lower jaw-specific and upper jaw-specific genetic programs. Development *135*, 2905–2916.

Kammandel, B., Chowdhury, K., Stoykova, A., Aparicio, S., Brenner, S., and Gruss, P. (1999). Distinct cis-essential modules direct the time-space pattern of the Pax6 gene activity. Dev. Biol. *205*, 79–97.

Konno, D., Iwashita, M., Satoh, Y., Momiyama, A., Abe, T., Kiyonari, H., and Matsuzaki, F. (2012). The mammalian DM domain transcription factor Dmrta2 is required for early embryonic development of the cerebral cortex. PLoS ONE *7*, e46577.

Lagha, M., Bothma, J.P., and Levine, M. (2012). Mechanisms of transcriptional precision in animal development. Trends Genet. *28*, 409–416.

Leone, D.P., Srinivasan, K., Chen, B., Alcamo, E., and McConnell, S.K. (2008). The determination of projection neuron identity in the developing cerebral cortex. Curr. Opin. Neurobiol. *18*, 28–35.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. *13*, 133–140.

Mallamaci, A., and Stoykova, A. (2006). Gene networks controlling early cerebral cortex arealization. Eur. J. Neurosci. *23*, 847–856.

Mangale, V.S., Hirokawa, K.E., Satyaki, P.R., Gokulchandran, N., Chikbire, S., Subramanian, L., Shetty, A.S., Martynoga, B., Paul, J., Mai, M.V., et al. (2008). Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. Science *319*, 304–309.

McKenna, W.L., Betancourt, J., Larkin, K.A., Abrams, B., Guo, C., Rubenstein, J.L., and Chen, B. (2011). Tbr1 and Fezf2 regulate alternate corticofugal neuronal identities during neocortical development. J. Neurosci. *31*, 549–564.

Nord, A.S., Blow, M.J., Attanasio, C., Akiyama, J.A., Holt, A., Hosseini, R., Phouanenavong, S., Plajzer-Frick, I., Shoukry, M., Afzal, V., et al. (2013). Rapid and pervasive changes in genome-wide enhancer usage during mammalian development. Cell *155*, 1521–1531.

O'Leary, D.D.M., Stocker, A.M., and Zembrzycki, A. (2013). Area patterning of the mammalian cortex. In Patterning and Cell Type Specification in the Developing CNS and PNS: Comprehensive Developmental Neuroscience, J. Rubenstein and P. Rakic, eds. (San Diego: Academic Press), pp. 61–86.

Perry, M.W., Boettiger, A.N., and Levine, M. (2011). Multiple enhancers ensure precision of gap gene-expression patterns in the Drosophila embryo. Proc. Natl. Acad. Sci. USA *108*, 13570–13575.

Puelles, L. (2011). Pallio-pallial tangential migrations and growth signaling: new scenario for cortical evolution? Brain Behav. Evol. *78*, 108–127.

Puelles, L. (2014). Development and evolution of the claustrum. In The Claustrum: Structural, Functional, and Clinical Neuroscience, J. Smythies, L. Edelstein, and V.S. Ramachandran, eds. (San Diego: Academic Press), pp. 119–176.

Puelles, L., Kuwana, E., Puelles, E., Bulfone, A., Shimamura, K., Keleher, J., Smiga, S., and Rubenstein, J.L.R. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J. Comp. Neurol. *424*, 409–438.

Rakic, P. (2009). Evolution of the neocortex: a perspective from developmental biology. Nat. Rev. Neurosci. *10*, 724–735.

Sahara, S., Kawakami, Y., Izpisua Belmonte, J.C., and O'Leary, D.D. (2007). Sp8 exhibits reciprocal induction with Fgf8 but has an opposing effect on anterior-posterior cortical area patterning. Neural Dev. *2*, 10.

Sansom, S.N., Griffiths, D.S., Faedo, A., Kleinjan, D.J., Ruan, Y., Smith, J., van Heyningen, V., Rubenstein, J.L., and Livesey, F.J. (2009). The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet. *5*, e1000511.

Saulnier, A., Keruzore, M., De Clercq, S., Bar, I., Moers, V., Magnani, D., Walcher, T., Filippis, C., Kricha, S., Parlier, D., et al. (2013). The doublesex homolog Dmrt5 is required for the development of the caudomedial cerebral cortex in mammals. Cereb. Cortex *23*, 2552–2567.

Shim, S., Kwan, K.Y., Li, M., Lefebvre, V., and Sestan, N. (2012). Cis-regulatory control of corticospinal system development and evolution. Nature *486*, 74–79.

Teissier, A., Griveau, A., Vigier, L., Piolot, T., Borello, U., and Pierani, A. (2010). A novel transient glutamatergic population migrating from the pallial-subpallial boundary contributes to neocortical development. J. Neurosci. *30*, 10563–10574.

Theil, T., Aydin, S., Koch, S., Grotewold, L., and Rüther, U. (2002). Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. Development *129*, 3045–3054.

van den Bout, C.J., Machon, O., Røsok, Ø., Backman, M., and Krauss, S. (2002). The mouse enhancer element D6 directs Cre recombinase activity in the neocortex and the hippocampus. Mech. Dev. *110*, 179–182.

Visel, A., Taher, L., Girgis, H., May, D., Golonzhka, O., Hoch, R.V., McKinsey, G.L., Pattabiraman, K., Silberberg, S.N., Blow, M.J., et al. (2013). A high-resolution enhancer atlas of the developing telencephalon. Cell *152*, 895–908.

Vogt, D., Hunt, R.F., Mandal, S., Sandberg, M., Silberberg, S.N., Nagasawa, T., Yang, Z., Baraban, S.C., and Rubenstein, J.L.R. (2014). Lhx6 directly regulates Arx and CXCR7 to determine cortical interneuron fate and laminar position. Neuron *82*, 350–364.

Waclaw, R.R., Ehrman, L.A., Pierani, A., and Campbell, K. (2010). Developmental origin of the neuronal subtypes that comprise the amygdalar fear circuit in the mouse. J. Neurosci. *30*, 6944–6953.

Xie, Q., Yang, Y., Huang, J., Ninkovic, J., Walcher, T., Wolf, L., Vitenzon, A., Zheng, D., Götz, M., Beebe, D.C., et al. (2013). Pax6 interactions with chromatin and identification of its novel direct target genes in lens and forebrain. PLoS ONE *8*, e54507.

Yoshida, M., Assimacopoulos, S., Jones, K.R., and Grove, E.A. (2006). Massive loss of Cajal-Retzius cells does not disrupt neocortical layer order. Development *133*, 537–545.

Yun, K., Potter, S., and Rubenstein, J.L.R. (2001). *Gsh2* and *Pax6* play complementary roles in dorsoventral patterning of the mammalian telencephalon. Development *128*, 193–205.