The protomap is propagated to cortical plate neurons through an *Eomes*-dependent intermediate map

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The cortical area map is initially patterned by transcription factor (TF) gradients in the neocortical primordium, which define a "protomap" in the embryonic ventricular zone (VZ). However, mechanisms that propagate regional identity from VZ progenitors to cortical plate (CP) neurons are unknown. Here we show that the VZ, subventricular zone (SVZ), and CP contain distinct molecular maps of regional identity, reflecting different gene expression gradients in radial glia progenitors, intermediate progenitors, and projection neurons, respectively. The "intermediate map" in the SVZ is modulated by Eomes (also known as Tbr2), a T-box TF. *Eomes* inactivation caused rostrocaudal shifts in SVZ and CP gene expression, with loss of corticospinal axons and gain of corticotectal projections. These findings suggest that cortical areas and connections are shaped by sequential maps of regional identity, propagated by the Pax6 → Eomes → Tbr1 TF cascade. In humans, *PAX6*, *EOMES*, and *TBR1* have been linked to intellectual disability and autism.

patterning | arealization | Bcl6 | Bhlhb5

Areas of the cerebral cortex exhibit distinct laminar structure, gene expression, and axonal connections. The development of cortical areas is regulated by both intrinsic genetic factors and extrinsic influences (e.g., thalamic innervation). Early in development, a "protomap" of regional identity (1) is established in cortical progenitor cells. Signaling centers around the cortex produce diffusible morphogens that modulate transcription factor (TF) gradients in the ventricular zone (VZ) (2). Neuroepithelial cells and radial glia (RG) in the VZ express graded levels of TF genes, including *Pax6*, *Sp8*, *COUP-TFI*, and *Emx2*, along the rostrocaudal and mediolateral axes (2). These TF gradients, which have been observed in embryonic humans and other species (3), ultimately control the layout of cortical areas.

During subsequent differentiation, it is unclear how regional identity of the protomap is propagated to intermediate progenitors (IPs) in the subventricular zone (SVZ) and neurons in the cortical plate (CP). One possibility is that regional identity may be maintained by different sets of TF gradients in each zone. Indeed, recent studies have shown that TF gradients in the CP, including high rostral *Tbr1* and high caudal *Bhlhb5* (also known as *Bhlhe22*), regulate neuronal acquisition of regional identity (4, 5). Whereas the majority of CP neurons are produced from IPs (6–9), regionalization in the CP may depend on antecedent IP regionalization. IPs are derived from RG but exhibit distinct TF profiles (7, 10). Furthermore, differentiation from RG → IPs → neurons is linked to sequential Pax6 → Eomes → Tbr1 expression (11). Pax6 directly binds and activates the *Eomes* gene (12); in turn, Pax6 and Eomes are required for *Tbr1* expression (13–16). Because *Pax6* and *Tbr1* regulate regional identity in the VZ and CP, *Eomes* might function similarly in the SVZ. Indeed, *Eomes* shows graded expression (high rostrolateral) in embryonic mouse and human SVZ (3, 5, 17).

To investigate the hypothesis that *Eomes* influences regional identity in IPs, we examined gene expression patterns, and afferent and efferent axon projections, in the embryonic cortex of control and *Eomes* conditional KO (cKO) mice. We also compared gene expression changes with those in *Pax6* and *Tbr1* null mice. Changes in regional gene expression were studied anatomically by in situ hybridization (ISH) and immunohistochemistry and quantitatively by transcriptome profiling with microarrays. We found that RG, IPs, and CP neurons contain numerous gene expression gradients that define not only the VZ protomap but also an SVZ "intermediate map" dependent on *Eomes* and a CP map linked to area-specific subcerebral axon projections. These results have implications for disorders such as autism, in which cortical patterning is frequently abnormal (18).

Results

Gene Expression Gradients in the SVZ. To investigate the possibility that regional identity is encoded by molecular expression gradients in IPs, we searched for genes with graded expression in the SVZ or in the VZ and SVZ. The developing cortex contains two types of IPs, distinguished by location in the SVZ (svz-IP) or VZ (vz-IP); by multipolar or short radial morphology in SVZ and VZ, respectively; and by distinct but overlapping molecular expression profiles (7, 9). Genes expressed in both types of IPs, such as *Eomes*, are detected in SVZ and VZ, whereas genes expressed in svz-IPs only are detected mainly in SVZ (7). Few genes are specific for vz-IPs only (7).

Previous studies have found that *Eomes* exhibits high rostral expression in the SVZ and VZ, whereas *Nhlh1*, a basic helix–loop–helix (bHLH) TF, exhibits high caudal expression in the SVZ (5, 17, 19). To find additional genes with graded expression, we searched open online databases including Genepaint, St. Jude Brain Gene Expression Map, and Allen Developing Mouse Brain Atlas (*Materials and Methods*). Focusing on genes implicated in cortical development, we studied the rostrocaudal expression patterns of >2,000 genes present in embryonic day (E)14.5 and E15.5 cortex. From these, 89 genes were identified with graded expression within a zone. These genes with graded zonal expression included 8 rostral and 16 caudal SVZ genes; 13 rostral and 7 caudal VZ genes; and 26 rostral and 19 caudal CP genes (Figs. S1–S3; Tables S1 and S2).

Genes in each zonal-rostrocaudal category shared principal features of expression (e.g., rostral SVZ), but many genes exhibited unique details of expression. Some SVZ genes were highly restricted to the SVZ, such as *Dusp14* (Fig. S1*N*) and *Nhlh1* (Fig. S1*R*); some spanned the SVZ and VZ, such as *Eomes* (Fig. S1*D*)

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and *Sema5a* (Fig. S1*H*); and others spanned the SVZ and lower intermediate zone (IZ), such as *Prex1* (Fig. S1*F*) and *Robo2* (Fig. S1*G*). The zonal differences can be related to stages of cellular maturation or transitions between cell types. Gradients also varied in shape along the rostrocaudal axis. For example, some SVZ genes showed smooth gradations throughout the neocortex, such as *Cdh10* (Fig. S1*B*) and *Dusp4* (Fig. S1*O*); some showed sharp rostral boundaries, such as *Cdkn1c* (Fig. S1*L*) and *Nxph4* (Fig. S1*T*); and others showed sharp caudal boundaries, such as *Abcd4* (Fig. S1*J*) and *Htra1* (Fig. S1*Q*). These variations suggest that gene expression profiles differ markedly among cells in the developing cortex in relation to both regional and zonal position. These data also imply that distinct molecular maps of regional identity are manifest in VZ, SVZ, and CP.

***Eomes* Is Necessary for Regional Patterning in the Intermediate Map.** Pax6 regulates regional patterning of progenitors in the VZ (20, 21), and Tbr1 regulates regional patterning of neurons in the IZ and CP (5). In the Pax6 → Eomes → Tbr1 cascade (11), *Eomes* appears to be directly regulated by Pax6 (12). *Eomes* also exhibits graded, high rostral expression in the SVZ and VZ (Fig. S1*D*) (5). To investigate the hypothesis that *Eomes* regulates regional identity in the SVZ, we studied *Eomes* cKO mice (*Eomes*$^{flox/flox}$; *Nes11-Cre*), in which *Eomes* was inactivated in the developing nervous system on E11. In these cKO mice, *Eomes* mRNA and protein were undetectable in neocortex from E11.5 (Fig. S4).

We compared the expression of rostral and caudal marker genes in *Eomes* cKO and littermate control cortex, using ISH or immunohistochemistry to examine gene expression gradients anatomically and Affymetrix microarrays to assay gene expression quantitatively. Most genes with rostral enrichment in SVZ and CP were markedly down-regulated in *Eomes* cKO neocortex (Fig. 1). For example, *Cdh8, Robo2,* and *Plxna4* were down-regulated in E14.5 rostral SVZ and *Bcl6* and *Fat3* in the rostral CP (Fig. 1 *A–K*). Tbr1 protein, a rostral CP marker, was also decreased in *Eomes* cKO cortex, consistent with previous reports (15, 16). Rostral gene expression remained low in neonatal *Eomes* cKO cortex, as documented for *Rorb, Bcl6, Fat3, Robo2, Plxna4*, and *Etv5* (Fig. 1 *L–W*). Whereas the latter genes are expressed in the CP, it is unlikely they are direct targets of Eomes transcriptional control; their down-regulation may instead be ascribed to deficient propagation of regional identity from SVZ to CP.

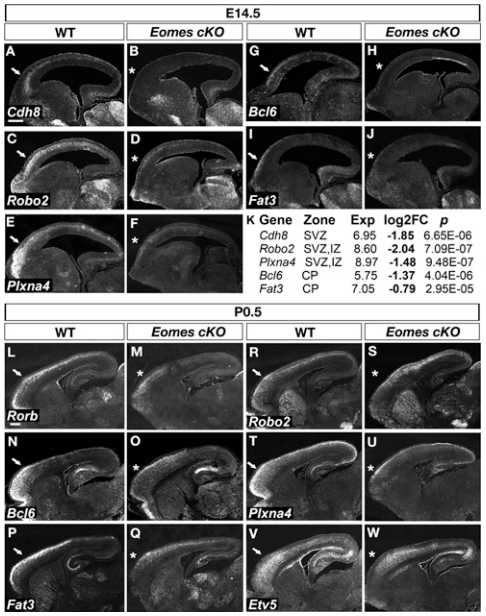

Fig. 1. Down-regulation of rostral enriched genes in *Eomes* cKO cortex. (*A–J*) Expression of *Cdh8* (*A* and *B*), *Robo2* (*C* and *D*), *Plxna4* (*E* and *F*), *Bcl6* (*G* and *H*), and *Fat3* (*I* and *J*) in sagittal sections (rostral left) of E14.5 control (*A, C, E, G,* and *I*) and *Eomes* cKO (*B, D, F, H,* and *J*) cortex. (*K*) Microarray results documenting down-regulation of rostral genes in E14.5 *Eomes* cKO cortex. Log_2FC is log_2 of the fold change (FC) of gene expression in *Eomes* cKO compared with control cortex. (*L–W*) Expression of *Rorb* (*L* and *M*), *Bcl6* (*N* and *O*), *Fat3* (*P* and *Q*), *Robo2* (*R* and *S*), *Plxna4* (*T* and *U*), and *Etv5* (*V* and *W*) in sagittal sections (rostral left) of P0.5 control (*L, N, P, R, T,* and *V*) and *Eomes* cKO (*M, O, Q, S, U,* and *W*) cortex. Arrows, high rostral gene expression in normal cortex; asterisks, gene down-regulation in rostral *Eomes* cKO cortex. (Scale bars: 200 μm for *A–J* and 400 μm for *L–W*.)

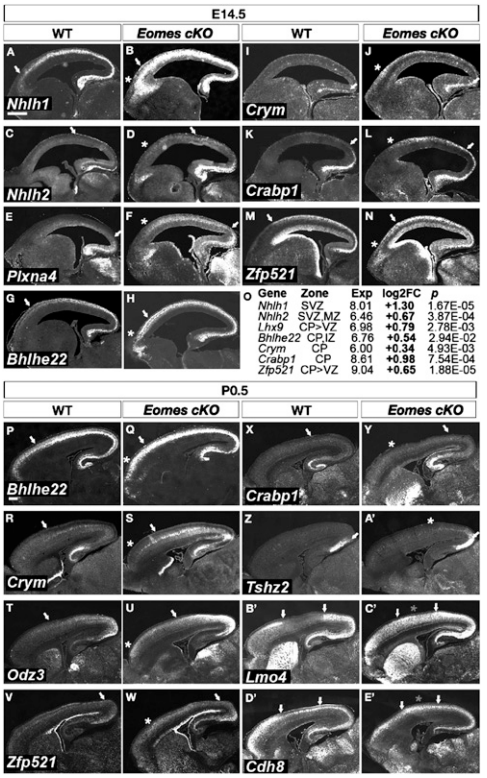

Fig. 2. Up-regulation and rostral shift of caudal enriched genes in *Eomes* cKO cortex. (*A–N*) Expression of *Nhlh1* (*A* and *B*), *Nhlh2* (*C* and *D*), *Lhx9* (*E* and *F*), Bhlhb5 protein (*G* and *H*), *Crym* (*I* and *J*), *Crabp1* (*K* and *L*), and *Zfp521* (*M* and *N*) in sagittal sections (rostral left) of E14.5 control (*A, C, E, G, I, K,* and *M*) and *Eomes* cKO (*B, D, F, H, J, L,* and *N*) cortex. Arrows, rostral limits of gene expression in normal cortex; asterisks, rostrally shifted limits of up-regulated genes in *Eomes* cKO cortex. (*O*) Microarray results confirmed up-regulation of caudal genes in E14.5 *Eomes* cKO cortex. (*P–E′*) Expression of Bhlhb5 (*P* and *Q*), *Crym* (*R* and *S*), *Odz3* (*T* and *U*), *Zfp521* (*V* and *W*), *Crabp1* (*X* and *Y*), *Tshz2* (*Z* and *A′*), *Lmo4* (*B′* and *C′*), and *Cdh8* (*D′* and *E′*) in sagittal sections (rostral left) of P0.5 control and *Eomes* cKO cortex. Arrows, rostral boundaries of gene expression in normal cortex; asterisks, up-regulation and rostral shift in *Eomes* cKO cortex. Scale bar: 200 μm for *A–N*, 400 μm for *P–E′*.

Conversely, many caudal marker genes were up-regulated and rostrally shifted in the developing *Eomes* cKO cortex (Fig. 2). *Nhlh1* and *Nhlh2* were up-regulated and shifted in E14.5 SVZ, and *Lhx9,* Bhlhb5, *Crym, Crabp1,* and *Zfp521* in CP (Fig. 2 *A–O*). Caudalization persisted through birth, as indicated by Bhlhb5, *Crym, Odz3, Zfp521, Crabp1, Tshz2, Lmo4,* and *Cdh8* (Fig. 2 *P–E′*). Up-regulation of Bhlhb5 was noteworthy because *Bhlhb5* is known to function in the acquisition of CP area identity (4). Together, these results suggested that *Eomes* is necessary to promote rostral and suppress caudal identity in the SVZ and in IP-derived postmitotic neurons in the IZ and CP. Some regional genes in the SVZ may be directly regulated by Eomes; indeed, overexpression

of Eomes repressed *Nhlh1* in the SVZ (Fig. S5). However, further studies will be necessary to determine whether Eomes binds *Nhlh1* gene regulatory sequences to directly regulate this putative target gene.

Pax6, Eomes, and Tbr1 Implement Regional Identity Sequentially. To compare the effects of *Eomes* deficiency with those of *Pax6* and *Tbr1* deficiency, we studied quantitative changes in the expression of rostral and caudal marker genes in all three mutants using microarray data from this and previous studies (5, 13). Overall, rostral gene expression decreased and caudal gene expression increased in *Pax6*, *Eomes*, and *Tbr1* mutant cortices (Fig. 3). However, gradients in the VZ, SVZ, and CP were affected differentially in each mutant. *Pax6* was required for rostral gene expression in the SVZ and CP, but effects on rostral gene expression in the VZ appeared mixed (Fig. 3*A1*). Caudal genes were modestly increased in the SVZ of *Pax6* mutants, slightly increased in the CP, and essentially unchanged in the VZ (Fig. 3*B1*). Changes in SVZ and CP gene expression in the *Pax6* mutant cortex were attributed in part to decreased *Eomes* and *Tbr1* expression (Fig. 3*A1*) (13, 14). These results suggested that Pax6 primarily promotes rostral identity and, to a lesser extent suppresses caudal identity, in IPs. Although Pax6 is not expressed in neurons, the CP map was perturbed in *Pax6* mutants, suggesting that IP regionalization is necessary for the acquisition of regional identity in neurons. These experiments may underestimate the role of *Pax6* in cortical regionalization, because patchy transformation from pallial to subpallial identity (22, 23) may affect the detection of changes in pallial gene expression.

Regional gene expression was perturbed even more in *Eomes* cKO than in *Pax6* mutant cortex. Rostral genes were markedly decreased in *Eomes* cKO SVZ and CP (Fig. 3*A2*), whereas caudal genes were markedly increased (Fig. 3*B2*). Reduced *Tbr1* expression presumably contributed to the loss of rostral and gain of caudal identity in *Eomes* cKO CP (Fig. 3*A2*). Regional markers in the VZ were affected little or not at all in *Eomes* cKO cortex (Fig. 3 *A2* and *B2*; Fig. S6).

Tbr1 mutants evinced loss of rostral and gain of caudal gene expression primarily in the CP, with smaller changes in the SVZ (Fig. 3 *A3* and *B3*). These results suggest that Tbr1 regulates regional identity not only in the CP, but also, to a lesser extent, in the intermediate map. Indeed, Tbr1 is detected in rare mitotic IPs. Interestingly, although Tbr1 is not detectable in the VZ, *Tbr1* mutants had increased expression of several rostral VZ genes, including *Mest*, *Elk3*, *Fgfbp3*, and others (Fig. 3*B3*). This observation suggests that rostral CP neurons may generate feedback to suppress rostral VZ identity (5). This effect might involve FGF signaling, as *Mest* and *Spry2* (both increased in *Tbr1* KO cortex; Fig. 3*A3*) are induced by FGF signaling (24, 25). Alternatively, these rostral VZ genes might be ectopically expressed in *Tbr1* KO IPs or neurons due to impaired neuronal differentiation.

The microarray comparisons suggest that Pax6 promotes the acquisition of regional identity in IPs; Eomes maintains regional identity in IPs and promotes its acquisition in neurons; and Tbr1 implements regional identity from IPs to postmitotic neurons. This interpretation agrees with the sequential expression of these TFs in cortical projection neuron lineages (26). Pax6, Eomes, and Tbr1 appear to act in concert with other TFs in a sequential

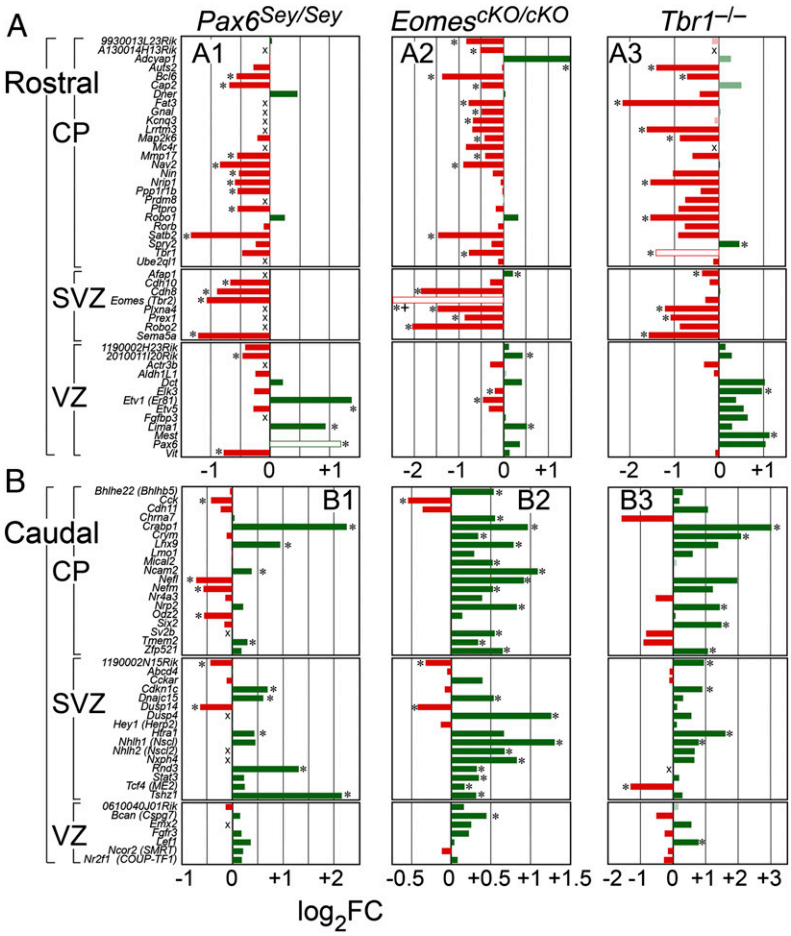

Fig. 3. Quantitative microarray analysis of rostral and caudal genes in *Pax6*, *Eomes*, and *Tbr1* mutant cortex. (*A*) Rostral genes organized according to predominant expression in CP, SVZ, or VZ. (*A1*) *Pax6* null cortex. (*A2*) *Eomes* cKO cortex. (*A3*) *Tbr1* KO cortex. (*B*) Caudal genes organized according to predominant expression in CP, SVZ, or VZ. (*B1*) *Pax6* null cortex. (*B2*) *Eomes* cKO cortex. (*B3*) *Tbr1* null cortex. Genes are listed (in the same order as they appear in this figure) in Tables S1 and S2. Transparent bars (50% opacity) indicate genes with signal below threshold for reliable detection on microarray. Open bars indicate mutated genes. x, genes not represented on microarray platform; +, $\log_2$FC off scale of graph (−3.75 for *Eomes* expression in *Eomes* cKO). *$P < 0.05$.

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transcriptional network that coordinates regional and laminar identity (2, 4, 5).

Defective Thalamocortical Innervation and Cortical Modules in *Eomes* cKO Cortex. Perturbations of regional gene expression are frequently associated with abnormalities of thalamocortical innervation, such as topographic shifts (2), defective formation of thalamocortical modules (4, 21), or deficient growth of thalamic axons into cortex (5, 27). A previous study of *Eomes* cKO mice, generated by *Sox1*-Cre–mediated recombination, described topographically correct but quantitatively deficient thalamocortical innervation and abnormal cytoarchitectonic organization of somatosensory barrel cortex (15). To further study these phenotypes, we studied thalamocortical innervation and barrel formation in *Eomes* cKO mice produced by *Nes11*-Cre–mediated recombination.

Because barrel modules develop postnatally, we extended our analysis of rostrocaudal gene expression patterns to postnatal day (P)7. As expected, gene expression changes indicated that rostral identity was persistently deficient, and caudal identity persistently excessive, in P7 *Eomes* cKO cortex (Fig. 4 *A–H*). However, thalamocortical innervation was not topographically shifted, but was abnormally diffuse and overall reduced (Fig. 4 *I–L′*). Also, the barrel pattern was distorted and indistinct; some barrels did not form; and cortical neurons did not organize in barrel walls (Fig. 4 *K–L″*). These abnormalities resembled barrel phenotypes in *Bhlhb5* null and *Pax6* cKO mice (4, 21). Such similarities support the conclusion that *Eomes*, *Pax6,* and *Bhlhb5* participate in the same genetic network controlling regional identity. Also, preserved thalamocortical topography suggests that subplate (SP) regional identity was not shifted in *Eomes* cKO cortex (*Discussion*). However, SP regionalization could not be further assessed due to lack of SP-specific rostral and caudal markers.

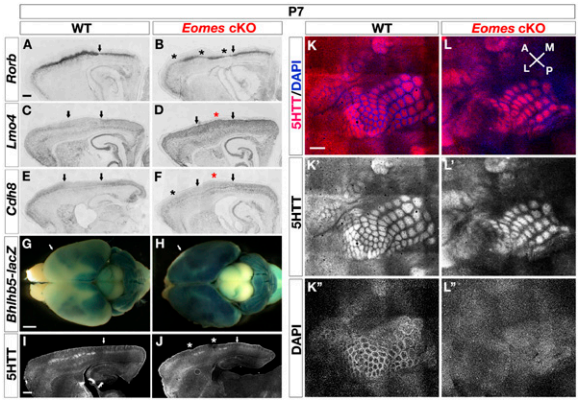

Fig. 4. Abnormal regionalization, thalamocortical innervation, and somatosensory barrels in P7 *Eomes* cKO cortex. (*A–F*) Expression of *Rorb* (*A* and *B*), *Lmo4* (*C* and *D*), and *Cdh8* (*E* and *F*) in control (*A, C,* and *E*) and *Eomes* cKO (*B, D,* and *F*) cortex (sagittal sections; rostral left). In *Eomes* cKO mutants, *Rorb* was decreased in layer 4 of somatosensory cortex (asterisks, *B*), but showed normal levels in layer 4 of occipital cortex (right of arrow, *B*). *Lmo4* and *Cdh8* were increased in *Eomes* cKO somatosensory cortex (red asterisks, *D* and *F*), equivalent to levels in caudal cortex. Also, *Cdh8* was reduced in *Eomes* cKO frontal cortex (black asterisk, *F*). (*G* and *H*) β-galactosidase (β-gal) activity driven by *Bhlhb5-lacZ* expression as seen in dorsal view (rostral left) of control (*G*) and *Eomes* cKO (*H*) brains. In controls (*G*), β-gal labeled somatosensory (S1) and visual (V1) but not motor (M) areas. In *Eomes* cKO cortex, β-gal activity was markedly increased in frontal cortex (left of arrows, *G* and *H*). (*I* and *J*) 5HTT immunoreactivity, a marker of thalamocortical axons, in control (*I*) and *Eomes* cKO (*J*) cortex (sagittal sections, rostral left). 5HTT immunoreactivity labeled barrel hollows in controls (patchy labeling left of arrow, *I*) but was decreased and disorganized in *Eomes* cKO somatosensory cortex (asterisks, *J*). (*K–L″*) 5HTT immunoreactivity (red, *K* and *L*; gray, *K′* and *L′*) and DAPI nuclear stain (blue, *K* and *L*; gray, *K″* and *L″*) in flattened tangential sections through the barrel field of control (*K–K″*) and *Eomes* cKO (*L–L″*) cortex. In *Eomes* cKO cortex, 5HTT immunoreactivity was overall reduced, and barrels were disorganized. Orientation for *K–L″*: A, anterior; L, lateral; M, medial; P, posterior. (Scale bars: 500 μm for *A–F* and *I–J*; 1 mm for *G* and *H*; and 250 μm for *K–L″*.)

***Eomes* Is Required for Correct Area-Specific Subcerebral Projections.** We next evaluated area-specific layer 5 efferent projections (28) in *Eomes* cKO mice. Using retrograde tracers, we labeled corticospinal motor neurons (CSMNs) and corticotectal projection neurons (CTPNs) and compared the rostrocaudal distributions of these neurons in control and *Eomes* cKO mice. CSMNs, located in motor and sensorimotor cortex of control mice, were reduced in the frontal cortex of *Eomes* cKO mice (Fig. 5 *A–H*). Conversely, CTPNs were restricted to occipital cortex in control mice, but occupied an expanded domain encroaching on parietal cortex in *Eomes* cKO mice (Fig. 5 *I–P*). These changes in efferent axon topography were broadly consistent with the apparent loss of rostral and gain of caudal identity in *Eomes* cKO mutants (Figs. 1–3). However, efferent connections were not simply shifted (as with protomap perturbations), but rather exhibited a combination of decreased rostral differentiation, increased caudal differentiation, and anomalous mixed differentiation of sensorimotor cortex.

Discussion

The present study demonstrates that, besides its role in IP genesis (15, 16), *Eomes* influences regional gene expression and efferent axon projections. These findings suggest that IPs and the intermediate map in SVZ are essential for implementing some aspects of arealization.

Dissociated Topographic Shifts in *Eomes* cKO Cortex. In contrast to the coherent shifts of areal topography (including gene expression gradients and thalamocortical innervation) induced by *Fgf8* overexpression (29) and other perturbations to the preneurogenic protomap (2), topographic shifts in *Eomes* cKO cortex were dissociated. Shifts of rostrocaudal gene expression were biased in the same direction but varied in magnitude for different genes (Figs. 1–3). Layer 5 efferent projections showed a paucity of CSMNs and expansion of CTPNs (Fig. 5). However, thalamocortical topography was normal in *Eomes* cKO barrel cortex (Fig. 4) (15). Thus, multiple elements of areal identity were dissociated in *Eomes* cKO cortex, and areal identity was not simply shifted, but anomalous. A similar phenotype was reported in cortex-specific *Pax6* cKO mutants, which likewise exhibited shifts of rostrocaudal gene expression but spared thalamocortical topography (21).

The preservation of thalamocortical topography in *Eomes* and *Pax6* cKO mutants suggests that these TFs are dispensable for the regionalization of SP neurons, which guide the initial topography of thalamocortical innervation (29, 30). Early overexpression of *Fgf8* before the genesis of SP and CP neurons caused a coherent shift of thalamocortical topography affecting all layers in register. However, later *Fgf8* overexpression (after SP neurons were born) caused out-of-register shifts between SP and CP layers, so that thalamocortical axons grew obliquely rather than radially from SP into CP (29, 30). Because SP neurons are the earliest generated cortical neurons (31–33), our results suggest that early-born SP neurons are patterned independently of *Eomes* and *Pax6*. This conclusion is compatible with the low abundance of IPs during SP neurogenesis (6, 9), the relatively late onset of cortical *Pax6* expression (~E12.5) (9), and the distinct origin of SP neurons from neuroepithelial cells lacking radial glia character (34).

In addition to topographic shifts, the *Eomes* cKO neocortex showed evidence of anomalous, mixed rostral, and caudal differentiation. In E14.5 cortex, rostral and caudal genes that normally did not overlap in the SVZ—for example, rostral *Robo2* and caudal *Lhx9*—showed substantial overlap in the *Eomes* cKO SVZ (Figs. 1 *C* and *D* and 2 *E* and *F*). Likewise, in P0.5 cortex, rostral

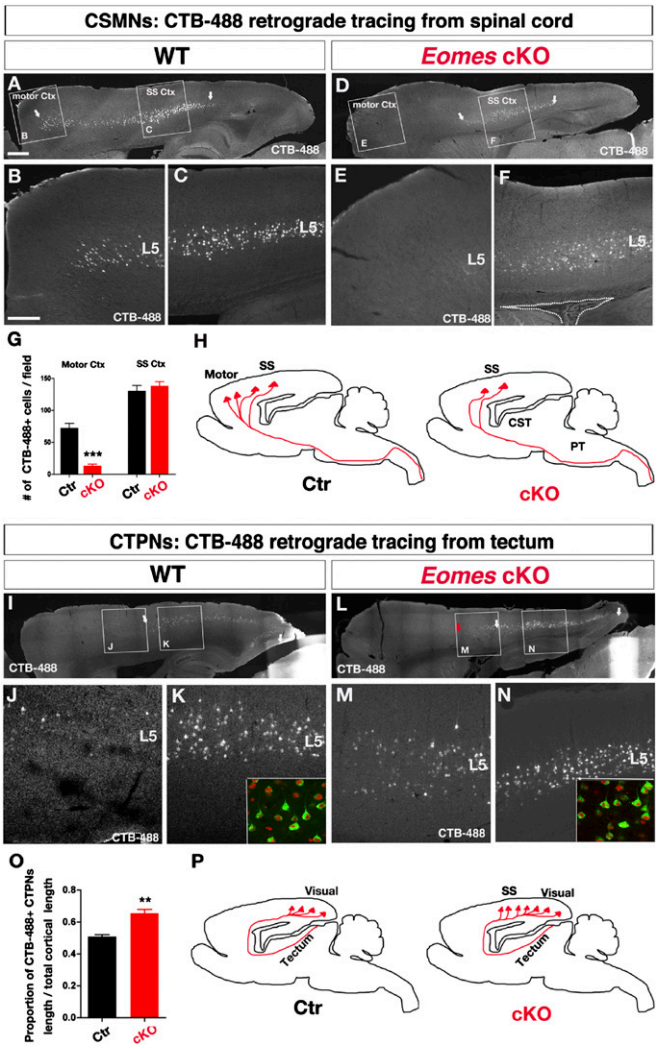

Fig. 5. Altered distribution of subcerebral projection neurons in *Eomes* cKO cortex. (*A–H*) CSMNs (retrogradely labeled from cervical spinal cord) were distributed in frontal motor and parietal somatosensory (SS) areas of control mice (*A–C*) but were markedly reduced in the frontal cortex of *Eomes* cKO (*D–F*) mice (sagittal sections; rostral left). Boxed regions in *A* and *D* are shown at higher magnification in *B* and *C* and *E* and *F*, respectively. (*G*) Quantification of CSMNs (mean ± SEM) in motor and SS cortex of control (Ctr) and *Eomes* cKO mice. ***$P < 0.001$. Arrows in *A* and *D* indicate rostral and caudal limits of CSMN domains. (*H*) Summary diagram of CSMNs in control and *Eomes* cKO mice. (*I–P*) CTPNs (retrogradely labeled from tectum) were located in occipital cortex of control mice (*I–K*) but occupied an expanded domain including parietal cortex of *Eomes* cKO (*L–N*) mice. Boxed regions in *I* and *L* are shown at higher magnification in *J* and *K* and *M* and *N*, respectively. *Insets* in *K* and *N* confirm expression of layer 5 corticofugal marker Ctip2 (red) in retrogradely labeled CTPNs (green). (*O*) Quantification of CTPNs (mean ± SEM) in control and *Eomes* cKO cortex. **$P < 0.005$. White arrows in *I* and *L*, limits of CTPN domains in control mice; red arrow in *L*, rostrally shifted limit of CTPNs in *Eomes* cKO mutants. (*P*) Summary diagram of CTPNs in control and *Eomes* cKO mice. L5, layer 5. (Scale bars: 500 μm for *A, D, I*, and *L* and 50 μm for *B, C, E, F, J, K, M*, and *N*.)

Fat3 and caudal *Zfp521* overlapped in *Eomes* cKO CP, but not in normal CP (Figs. 1 *P* and *Q* and 2 *V* and *W*). Also, some genes that were normally restricted to caudal archicortex, such as *Crym* on E14.5 and *Tshz2* on P0.5, shifted rostrally into the *Eomes* cKO neocortex (Fig. 2 *C, I, J*, and *A′*). Thus, the overall loss of rostral and gain of caudal identity in *Eomes* cKO cortex was complicated by noncoherent shifts of rostrocaudal gene expression within the CP, leading to a profound disturbance of areal differentiation and cortical-subcortical connections.

Regional Identity Is Propagated Through Zonal Maps Linked to TF Cascades. The cortical map is initially patterned by morphogens secreted from signaling centers at the cortical periphery, which regulate TF gradients in the VZ before neurogenesis (2). Our data suggest that regional identity is thereafter propagated from RG → IPs → neurons by transformation of the VZ protomap → SVZ (intermediate) map → CP map (Fig. S7). These transformations are regulated by the Pax6 → Eomes → Tbr1 cascade, which overall promotes rostral and suppresses caudal identity. In this sequence, Pax6 is thought to directly transactivate *Eomes* expression (12). Consistent with this relation, defects of cortical gene expression and connections were remarkably similar between *Pax6* and *Eomes* cKO mutants. Changes in rostral and caudal marker gene expression were almost identical between these mutants (Fig. 3), as were effects on thalamocortical innervation and barrel development (Fig. 4) (13, 21). Efferent projections were not examined in cortex-specific *Pax6* cKO mice.

Regulation of the SVZ Intermediate Map. Like the VZ protomap and the CP areal map, it seems likely that the SVZ intermediate map may be regulated by opposing TF gradients, and possibly, by diffusible factors and thalamic innervation as well (1, 2, 4, 5). Several TFs exhibit high caudal to low rostral gradients in the SVZ, including *Hey1*, *Nhlh1*, *Nhlh2*, *Tcf4*, and *Tshz1* (Fig. S1; Table S1). Interestingly, *Nhlh1* was markedly up-regulated in *Pax6*, *Eomes*, and *Tbr1* mutants (Fig. 3*B*) and showed a clear rostral shift in E14.5 *Eomes* cKO cortex (Fig. 2*B*). Previous studies of *Nhlh1* null mutant mice found no deficiencies of cortical neurogenesis, although regionalization was not specifically evaluated (19). Further studies of *Nhlh1* and other TF genes will be necessary to better understand how the cortical map is regulated in the SVZ.

It is also possible that the intermediate map may be modulated by extrinsic factors acting on IPs. Thalamic axons are known to influence areal differentiation (1, 35) and, by their proximity to the SVZ, might affect IP differentiation. Also, some interneurons migrate into cortex through the SVZ and may interact with IPs. Recent studies have shown that IPs attract interneurons into the SVZ by producing SDF-1 (*Cxcl12*) (36). Signaling in the reverse direction, from interneurons to cortical IPs, hypothetically might occur as well. One potential means of interaction could involve *Cck* (cholecystokinin), expressed in migrating interneurons, and *Cckar* (cholecystokinin A receptor), expressed by IPs in a low rostral to high caudal gradient (Fig. S1*K*) (37). Also, several GABA receptors are expressed by progenitors in the VZ and SVZ and could potentially respond to GABA released from interneurons. Such interactions might impact regionalization and neurogenesis (8).

Abnormal Patterning in Autism and Intellectual Disability. In humans, the Pax6 → Eomes → Tbr1 cascade is important in the pathogenesis of autism and intellectual disability (ID). Mutations affecting *PAX6* or *EOMES* cause ID with cortical and cerebellar malformations (38–42). Recently, exonic de novo mutations of *TBR1* were associated with autism and ID, suggesting *TBR1* mutations contribute to the risk of these disorders (43, 44). Whereas cortical patterning is abnormal in autism (18), the present study reveals that defects of patterning can arise at several stages in neurogenesis and may exhibit diverse and complex features.

Materials and Methods

Animals, Histology, *Eomes* Overexpression, Microarrays, and Gene Expression Analysis. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seattle Children's Research Institute. Mice carrying the floxed *Eomes* allele (*Eomes*$^{flox/+}$) (45) were bred with *Nestin-Cre* (*Nes-Cre*) transgenic mice (stock 003771; Jackson Labs) (46) and with *Bhlhb5-lacZ* knockin mice (4). Controls were *Eomes*$^{flox/+}$. Histological methods and microarray (Affymetrix Mouse Gene 1.0) experiments were done as previously

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described (5). *Eomes* overexpression was done as described (5, 47). Markers of regional identity in the E14.5–E15.5 cortex were augmented from our previous study (5). Digital images were acquired with Zeiss AxioImager and LSM7 (confocal) microscopes and adjusted for contrast and brightness (Adobe Photoshop). Additional details are in *SI Materials and Methods*.

Axon Tracing. CSMNs and CTPNs were labeled retrogradely (32). The anteroposterior length of neocortex, and of the domain of layer 5 retrogradely labeled cells, was measured using Axiovision software (Zeiss) in sagittal sections through the plane shown (Fig. 4). Retrogradely labeled cells were counted in three nonadjacent sections per animal. Statistical comparisons were done using unpaired, two-tailed Student *t* test (Graphpad Prism 5.02).

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