

Emx2 patterns the neocortex by regulating FGF positional signaling

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Molecular genetic studies implicate fibroblast growth factor 8 (FGF8), and the transcription factor Emx2, in development of the neocortical area map. Both are proposed to specify area position along the anterior-to-posterior axis of the cortical primordium. Whether FGF8 and Emx2 act independently or coordinately, or whether one controls the other, has not been determined. Here we report that Emx2, by regulating FGF8, has an indirect but vital role in area-map development. Using electroporation-mediated gene transfer in living mouse embryos, we found that overexpressing Emx2 altered the area map, but only when ectopic Emx2 overlapped the FGF8 source. Furthermore, we found that FGF8 levels were decreased by excess Emx2, and increased in mice lacking Emx2. Finally, cortical domain shifts that characterize Emx2 mutants were rescued by sequestering excess FGF8 with a truncated FGF receptor construct. These findings begin to clarify the signaling network that patterns the neocortical area map.

The fundamental organization of the mammalian cerebral cortex is its division into anatomically and functionally distinct areas. These areas form a map that is highly similar among individuals of the same species and has common features across species¹. In the classic ‘protomap’ model², a template of the area map is established in the proliferative layer of the cortical primordium and translated through orderly migration of neurons to the cortical plate. Several studies support such an early regionalization of the cortical primordium^{3–7}, but the underlying molecular mechanisms have been obscure. Recent observations support a model in which the area map is patterned by mechanisms similar to those used elsewhere in the embryo^{8–12}. That is, signaling molecules secreted from signaling centers establish positional information in the cortical primordium¹³. Such signaling sources have been identified in and near the cortical primordium and express characteristic embryonic patterning molecules, including bone morphogenetic proteins (BMPs) and FGF and Wingless-Int proteins^{12,14–19}. Elsewhere in the embryo, positional signals regulate the expression of transcription factors, leading to patterned tissue differentiation by a gene regulatory cascade²⁰. Reports that certain transcription factors are critical to cortical area patterning are therefore also consistent with the protomap model^{21–24}.

Two recent studies indicate that an anterior source of FGF8 in the cortical primordium controls anterior/posterior (A/P) position in the neocortical area map^{9,25}. The anterior pole is the site of the future prefrontal and orbital cortex; the posterior pole includes primary visual cortex. Robust evidence also supports a central role for the transcription factor Emx2 in area patterning along the A/P axis^{21–23}. A key finding is that FGF8 and Emx2 are closely related in patterning function. When endogenous FGF8 is augmented, or Emx2 depleted, A/P shifts in the area map are strikingly similar^{22,23,25}. Specifically, anterior domains of the neocortex are enlarged at the expense of more posterior domains, which are shrunk and shifted back^{22,23,25}.

Further, *Emx2* and *FGF8* show complementary expression patterns during early corticogenesis. At embryonic day 9 (E9), just before cortical neurogenesis begins, *Emx2* is expressed in a ‘highP/lowA’ gradient in the cortical primordium, with an anterior boundary abutting and partly surrounding the expression domain of *FGF8* (Fig. 1). The onset of telencephalic *Emx2* expression is E8.5 (ref. 26), around the time that *FGF8* begins to be expressed in anterior telencephalic neuroepithelium²⁷. By contrast, the related gene *Emx1*, which has no reported cortical patterning activity, is expressed later than *Emx2*, and does not extend as far toward the anterior pole.

Unlike the FGF8-expressing isthmus organizer, long known to pattern the midbrain and hindbrain^{28–31}, the FGF8 source in the anterior telencephalon has only recently been shown to play a patterning role^{9,25}. Consequently, the molecular mechanisms that are up- and downstream of the isthmus organizer are well studied^{30,31}, whereas interactions between telencephalic FGF8 and the transcription factors implicated in area patterning remain to be identified.

Investigation of this issue is aided by development of *in-utero* microelectroporation—a technique that makes the mouse embryo accessible for acute manipulations of gene function²⁵. We used this technique to examine the interplay between FGF8 and Emx2. Strongly similar changes in the area map after decreased Emx2 or increased FGF8 suggest an antagonistic relationship. Our observations indicate both that FGF8 downregulates Emx2 expression and that Emx2 constrains the boundaries of the FGF8 source. Further findings indicate that the primary relationship with respect to cortical area patterning is the latter: the regulation of FGF8 by Emx2.

RESULTS

FGF8 regulates the Emx2 expression gradient

To determine whether FGF8 regulates the Emx2 gradient in mouse cortical primordium, we altered levels of FGF8 using *in-utero*

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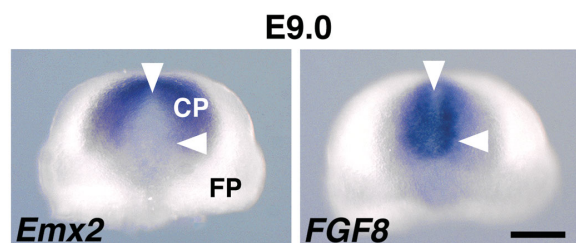


Figure 1 *Emx2* and *FGF8* are expressed in complementary patterns at E9. CD-1 forebrains viewed from the anterior pole just after closure of the telencephalic vesicle. Dorsal is up. Expression of *Emx2* (left) partially surrounds the anterior domain in which *FGF8* (right) is expressed (white arrowheads). FP, facial primordium; CP, cortical primordium. Scale bar, 0.25 mm.

microelectroporation. To raise FGF8 levels, an *FGF8*-containing expression plasmid was electroporated into the anterior cortical primordium at E11.5. To reduce FGF8 levels, FGF8 was sequestered by electroporation of a plasmid containing a cDNA encoding a soluble, truncated form of a high-affinity FGF8 receptor, sFGFR3c²⁵. Augmenting anterior FGF8 shifted the gradient of *Emx2* expression posteriorly, analyzed at E13.5; reduced FGF signaling shifted the *Emx2* gradient anteriorly (Fig. 2, right column). The latter shift is also seen in FGF8 hypomorphic mice⁹. Endogenous FGF8 could therefore contribute to generating or maintaining the normal lowA/highP *Emx2* gradient³². Pronounced shifts were also seen in expression gradients of other genes, such as those encoding the transcription factor COUP-TF1, which is also implicated in area patterning³³, and the FGF receptor FGFR3 (Fig. 2)⁹.

It has been proposed that area fates are specified by *Emx2* in combination with other transcription factors, most notably Pax6, expressed in an opposing gradient²¹. Changing the relative level of any one of these transcriptional regulators should therefore disturb cortical area pattern. That is, if the *Emx2* gradient indeed promotes different area fates along the A/P axis, then creating ectopic *Emx2* peaks in the gradient should alter the area map. To test this possibility, we used *in-utero* electroporation to generate a local surplus of *Emx2* in different positions in the cortical primordium.

Endogenous *Emx2* expression begins in the telencephalon at E8.5. We found that electroporation of *Emx2* at E11.5, our previous standard age for this procedure²⁵, was too late to affect patterning. We therefore electroporated embryos at E10.5, first confirming that electroporation can be regionally targeted at this age by assaying the embryos 1 d later (Fig. 3b–d). To check the electroporation site *post hoc* at P6, when brains were analyzed in this experiment, *Emx2* and *Cre* (encoding Cre recombinase) were co-electroporated into E10.5 embryos from the R26R reporter mouse line³⁴. Expression of β -galactosidase, visualized with X-gal histochemistry, then permanently marked the region of electroporation.

Emx2 misexpression has variable effects on area pattern

A caveat is that *Emx2* may regulate different area fates at different expression levels, but reach a ceiling in the posterior cortex, above which extra *Emx2* has no further effect. We therefore focused on analyzing mice in which the site of electroporation included anterior and central, but not extreme posterior, regions (Fig. 3j). At sites at which *Emx2* expression does not reach its highest endogenous level, increasing *Emx2* is most likely to alter the area map.

Figure 2 Gradients of gene expression in the early cortical primordium respond to FGF8 manipulations. Sagittal sections through E13.5 CD-1 mouse brains; anterior is to the left. Brains were electroporated at E11.5 in anterior cortical primordium. Control sections were taken from non-electroporated hemispheres. Note that the gradient of *COUP-TF1* (left column) is shifted posteriorly by anterior *FGF8* electroporation, and anteriorly by sFGFR3c. Shifts in the gradients of *FGFR3* and *Emx2* were similar. Scale bar, 0.65 mm.

To detect abnormalities in the postnatal area map, we monitored the somatosensory barrel fields. These fields form a substantial part of the mouse neocortex, reflect a function of primary somatosensory cortex (S1), and are readily seen in layer 4 of postnatal cortex by cytochrome oxidase (CO) histochemistry (Fig. 3e)³⁵.

Brains that revealed effective electroporation by abundant X-gal staining were analyzed at P6 ($n = 25$). In all cortices in which the transfection site included the anterior pole, the barrel fields were shifted anteriorly (Fig. 3g,i; $n = 9$). This finding fits the suggestion that higher levels of *Emx2* promote an increasingly posterior identity: excess anterior *Emx2* transforms frontal cortex into the normally more central barrel fields. By contrast, overexpressing *Emx2* at anterior central sites ($n = 7$), or at more posterior central sites that did not include the anterior pole but overlapped S1 ($n = 9$), had no detectable effect on the position, morphology or orientation of the barrel fields (Fig. 3f,h). The latter findings do not support a direct role for the *Emx2* gradient in specifying area fate. Presumptive S1 lies roughly in the middle of the endogenous *Emx2* gradient. Excess *Emx2* at these central sites should promote a more posterior area fate, or disrupt an identifiable fate, forcing S1 to shift or disappear.

We previously found that the barrel fields are shifted anteriorly when FGF8 is sequestered by a soluble, truncated form of the high-affinity FGF8 receptor sFGFR3c^{25,36}. Thus, our present observations could be explained if *Emx2* negatively regulates FGF8. *Emx2* electroporation would then shift the barrel fields only if the electroporation site impinges on the anterior FGF8 source—exactly what we observed. Consequently, we tested the hypothesis that *Emx2* regulates the size and signaling capacity of the FGF8 source in the anterior cortical primordium.

Emx2 regulates expression of FGF8 and FGF17

The anterior pole of the cortical primordium expresses not only *FGF8*, but also *FGF17* and *FGF18*^{14,16,37}, encoding members of the FGF8 subfamily of FGFs with similar receptor affinities and function in other systems^{37–39}. *In-utero* electroporation of *FGF17* has similar effects on neocortex to that of *FGF8* (data not shown), and the area shifts caused by sFGFR3c are likely to be due to sequestration of both

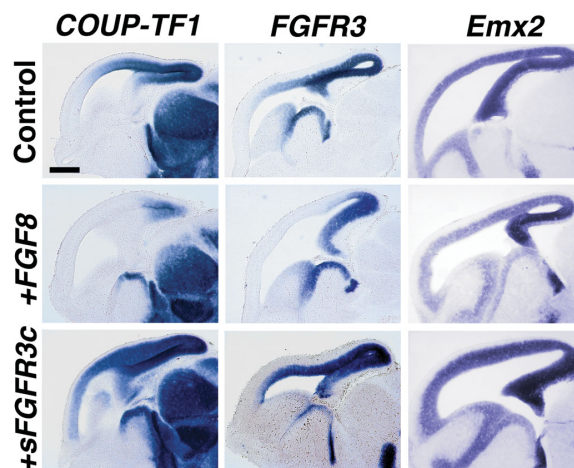
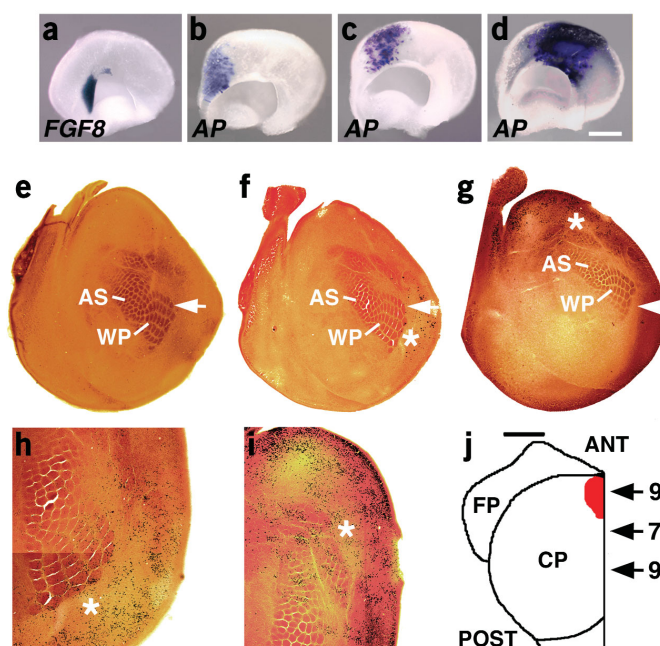


Figure 3 Anterior but not central overexpression of *Emx2* shifted the barrel fields in primary somatosensory cortex. (a–d) E11.5 CD-1 mouse hemispheres, medial view; anterior is to the left. The *FGF8* domain is anterior and medial (a). One day after targeted *in-utero* electroporation of alkaline phosphatase (AP) at E10.5, expression of AP protein overlaps the *FGF8* domain completely (b), partially (c), or not at all (d). Electroporated cells are densely packed soon after electroporation (b–d), but not by P6 (h,i), reflecting growth of the hemisphere. (e–i) Tangential sections through layer 4 of flattened P6 cortices from R26R mice, processed for CO histochemistry. Anterior, top; medial, right. White arrows indicate the A/P midpoint of the neocortex. (f–i) Sections counterstained with X-gal histochemistry to indicate the region of electroporation (see text). (h,i) X-gal labeled cells in f and g at higher magnification (white asterisks mark comparable positions in each pair). In a control (e), the main whisker pad (WP) and anterior snout (AS) somatosensory subfields are centrally positioned along the A/P axis. (j) Tangential section drawing through E10.5 cortical primordium showing the anterior and posterior poles (ANT, POST), *FGF8* source (red), and number of replications targeted to different sites. Overexpression of *Emx2* focused near the WP subfield (f,h)—similar to the site shown in d—failed to shift the barrel fields. By contrast, *Emx2* overexpression in anteromedial cortex, including the anterior pole (g,i) and reaching to the A/P midpoint (white arrowhead in g)—similar to the sites in b and c combined—draws the fields into the anterior half of the neocortex. Scale bar in d is 0.4 mm for a–d. Scale bar in j is 1.6 mm for e–g and 0.7 mm for h, i.



FGF8 and *FGF17*, suggesting that a cocktail of FGF ligands is involved in A/P neocortical patterning. In the following experiments, both *FGF8* and *FGF17* were analyzed.

We first investigated whether *Emx2* regulates expression of *FGF8* and *FGF17* in explant culture. E10.5 embryos were removed from the

uterus and electroporated. The better view of the embryo obtained outside the uterus allowed uniformly consistent anterior placement of *Emx2* and a fluorescent marker gene, *DsRed*. Explants of the entire dorsal forebrain were analyzed after two days *in vitro* (2 d.i.v.). When *DsRed* was transfected alone, *FGF8* and 17 were strongly expressed near the anterior pole of the cortical primordium (Fig. 4c,e; $n = 9$), as *in vivo*. Co-electroporation of *Emx2* and *DsRed* at sites overlapping the FGF domain, however, severely downregulated *FGF8* and *FGF17* expression (Fig. 4d,f; $n = 12$).

To determine whether loss of *Emx2* increases *FGF8/17* expression, we analyzed a mouse line engineered to lack *Emx2*⁴⁰. At E10.5, *FGF8* and *FGF17* expression domains in all *Emx2* homozygote mutants examined ($n = 8$) were larger in absolute size than those in littermate control mice ($n = 8$) (Fig. 4g–j). Proportionally, the *FGF* domains were still larger in *Emx2* homozygotes because of the smaller overall size of the mutant cerebral hemispheres.

Emx2 regulates functional FGF activity

A crucial issue is whether *Emx2* regulates functional signaling by *FGF8* and *FGF17*. We therefore identified a read-out of *FGF8/17* protein activity in mouse cortical primordium. In zebrafish, expression of genes encoding members of the Pea3 subfamily of ETS (E-twenty-six) domain transcription factors is tightly controlled by *FGF8* (ref. 41). We found that the mouse homologs of *Pea3*, *ERM* and *ER81* were expressed in the anterior half of the cortical primordium at E13.5 (Fig. 5, top row). The three expression domains have different

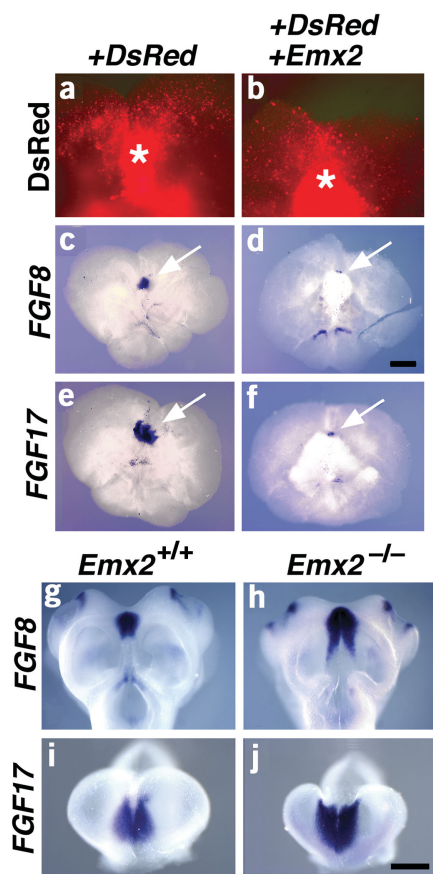


Figure 4 *Emx2* regulates *FGF8/17* expression in the anterior cortical primordium. (a–f) E10.5 CD-1 dorsal forebrain explants maintained for 2 d.i.v.; anterior is to the top. (a,b) *DsRed* fluorescence indicates, at double magnification, electroporation sites in c and d; asterisks indicate the position of the *FGF8/17* source. (c–f) *In-situ* hybridization reveals *FGF8* and *FGF17* expression domains. (g–j) E10.5 embryo heads in dorsal view with anterior up (g,h) or frontal view with dorsal up (i,j). *FGF8* and *FGF17* expression domains extend further laterally, dorsally and posteriorly in the *Emx2* mutant cortical primordium compared with controls. Scale bar in d is 0.5 mm for c–f and 0.25 mm for a and b. Scale bar in j is 0.38 mm for g–j.

Figure 5 FGF8/17 signaling regulates *Pea3* gene subfamily expression in the cortical primordium. E13.5 CD1 cortical hemispheres viewed from the medial face; anterior is to the left. Hemispheres were electroporated at E11.5 with *FGF8* or *sFGFR3c*, and processed with *in-situ* hybridization 2 d later to reveal *Pea3* gene subfamily expression. Scale bar, 0.65 mm.

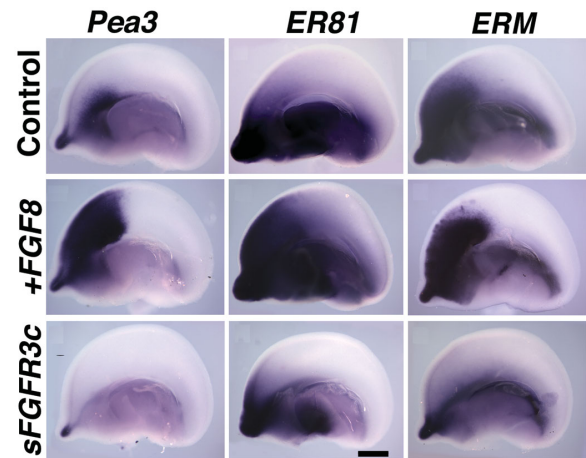
posterior boundaries, forming a nested set. Expression of each *Pea3* subfamily member was upregulated and/or expanded posteriorly by anterior electroporation of *FGF8* (Fig. 5, middle row). Conversely, expression domains shrank when FGF8 was sequestered by *sFGFR3c* (Fig. 5, bottom row). Thus, expression of the *Pea3* subfamily of *ETS* genes is regulated by FGF signaling in mouse forebrain.

Anterior overexpression of *DsRed* and *Emx2* in explants ($n = 17$) diminished expression of all three *Pea3* subfamily genes, indicating that functional FGF8 signaling was lower than in control explants ($n = 20$) (Fig. 6a–c,e–g). In the absence of *Emx2*, functional FGF8 signaling was increased. In all *Emx2* homozygote mutant mice examined ($n = 13$), expression of the *Pea3* subfamily genes expanded (Fig. 6i–n; littermate control, $n = 15$).

Excess *Emx2* additionally pulled the expression boundaries of *FGFR3* (Fig. 6d,h) and *COUP-TF1* (data not shown) toward the anterior limit of the explant. Importantly, *Emx2* did not show a cell-autonomous effect in promoting a posterior identity. Neither gene was upregulated at the anterior site of electroporation (site position confirmed by *DsRed*). Instead, *Emx2* seemed to act globally to shift gene expression boundaries, as does a decrease in FGF8/17.

Reducing FGF8/17 rescues cortical domain shifts in *Emx2* mutant

These findings suggest a new hypothesis: domain shifts described in the neocortex of *Emx2* mutant mice^{21,23} could be caused by excess FGF8/17. To test this possibility, we electroporated *sFGFR3c* into the anterior cortical primordium of *Emx2* mutants at E11.5 ($n = 13$). As noted, *sFGFR3c* should sequester both FGF8 and 17, preventing them from binding to their endogenous receptors^{36,38}. Depending on the levels of truncated receptor protein generated, the *Emx2* mutant cortex should receive at least partial relief from excess FGF8/17.



Emx2 null mice do not live beyond birth because of defects outside the cerebral cortex^{40,42}. Therefore, we examined gene expression patterns that show regional expression at E18.5^{12,21,43}. Genes encoding the orphan nuclear receptor *RORβ* or the classic Cadherins 6 and 8 (*Cdh6*, *Cdh8*) are expressed in regional domains in wild-type E18.5 cerebral cortex (Fig. 7a,d,g). In *Emx2* homozygote mutant mice, anterior cortical domains are expanded at the expense of more posterior domains^{21,23}. Thus, the anterior boundaries of *Cdh6* and *RORβ* are shifted posteriorly (Fig. 7b,h), whereas the entire anterior domain of *Cdh8* expression is greatly expanded posteriorly (Fig. 7e)^{21–23}. After electroporation of *sFGFR3c* in *Emx2* mutants, expression boundaries of each gene returned approximately to their wild-type positions (Fig. 7c,f,i). The proportion of mutant mice rescued was as expected (7/13), given the predicted rate of effective *in-utero* electroporation (see Methods)²⁵. Rescue was more complete in lateral than medial cortical primordium, away from the strongest FGF8/17 signal, suggesting that the truncated receptor did not sequester all excess FGF ligand.

These observations indicate that FGF8/17 can regulate the position of neocortical gene expression boundaries in the absence of

Emx2. Although *Emx1* is still expressed in the *Emx2* mutant, it is unlikely to substitute for *Emx2* in cortical patterning. Unlike *Emx2*, *Emx1* expression does not respond to electroporation of *FGF8* (T.F.-S., unpublished observations). Furthermore, *Emx1* null mice have no area map defects, and

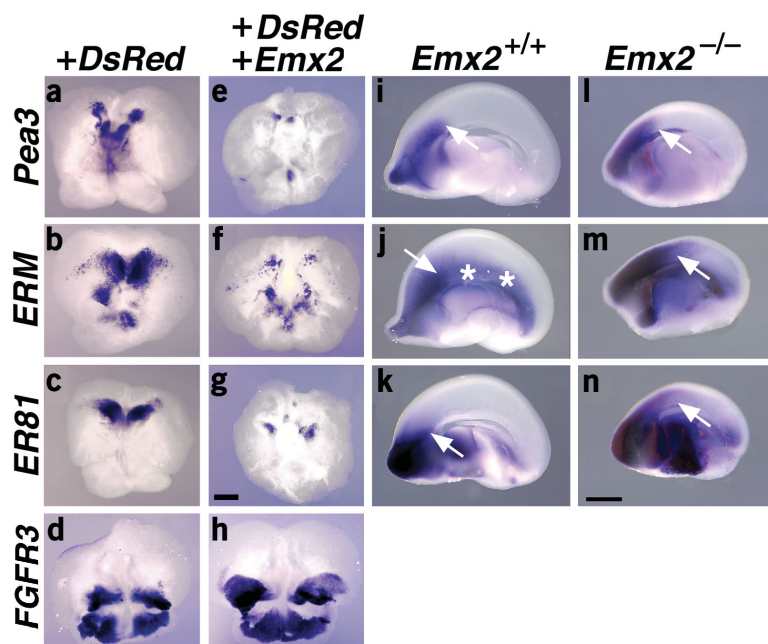
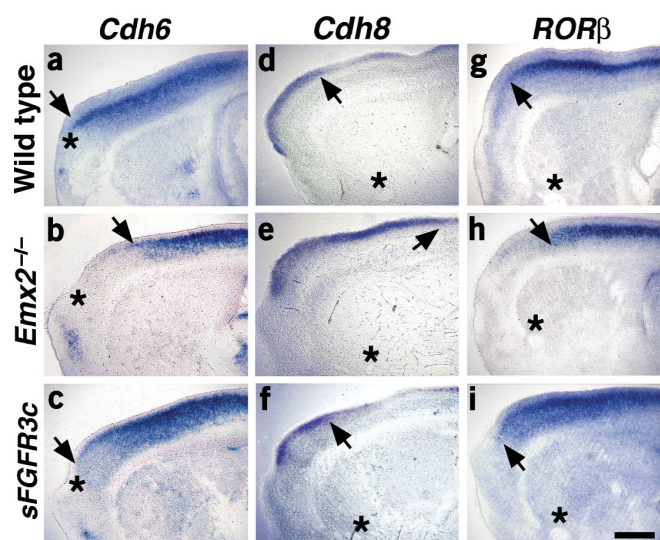


Figure 6 *Emx2* regulates functional FGF8/17 signaling in the cortical primordium. (a–h) In explant cultures, *Pea*, *ERM* and *ER81* expression was robust after electroporation of *DsRed* alone, but reduced by co-electroporation of *Emx2*. By contrast, the expression domain of *FGFR3* expanded anteriorly (h). (i–n) The domains of strong expression of each *ETS* gene are markedly expanded along the A/P axis in *Emx2* mutants compared with littermate controls (white arrows). For example, the *ERM* expression domain roughly doubles in absolute length. (Note that the curving band of *ERM* expression marked by asterisks in j is in the opposite, lateral wall of the hemisphere.) Scale bars, 0.5 mm for explants (g) and 0.65 mm for hemispheres (n).

Figure 7 Reducing excess FGF8/17 rescues gene expression shifts in the *Emx2* mutant. Sagittal sections of E18.5 cerebral hemispheres; anterior is to the left. Left column, asterisks mark the olfactory bulb as an A/P landmark; right column, asterisks mark the anterior commissure. Left column, *Cdh6* expression is shifted posteriorly in an *Emx2* mutant mouse by comparison with a wild-type littermate. Anterior electroporation of *sFGFR3c* into the cortical primordium of an *Emx2* mutant restores the anterior boundary of *Cdh6* expression. Middle column, an anterior domain of *Cdh8* expression is enlarged in the *Emx2* mutant and reduced by electroporation of *sFGFR3c*. Right, *RORβ* expression shows a similar shift and rescue to *Cdh6*. Scale bar, 0.5 mm.



double *Emx1/2* mutant mice do not show greater defects than mice lacking *Emx2* alone²². An implication of the latter finding is that *Emx1* and *Emx2* are not interchangeable: *Emx1* does not partially rescue area pattern in the *Emx2* mutant.

DISCUSSION

Strong evidence indicates that an anterior telencephalic source of FGF8/17 imparts positional information, regulating the A/P axis of the cortical primordium and directing the development of the cortical area map²⁵. For example, introducing a new posterior source of FGF8 locally reverses A/P polarity, eliciting partial duplications of a central area, the S1 barrel fields²⁵. Augmenting or diminishing the endogenous source distorts the area map^{9,25}. These observations imply that normal development of the map requires careful control of the FGF8/17 source.

At the midbrain/hindbrain isthmus organizer, a complex set of transcription factors and signaling proteins induce FGF8 and control the boundaries of the FGF8 domain³¹. Our results support a role for *Emx2* in a similar regulatory network controlling FGF8/17 in the anterior telencephalon. *Emx2* expression abuts and surrounds the domain of *FGF8* expression in early corticogenesis, and FGF8/17 signaling is enhanced in the absence of *Emx2*, revealing the importance of endogenous *Emx2* in restricting the FGF8/17 source. However, *FGF8/17* expression does not spread throughout the cortical primordium in the *Emx2* null mouse, indicating that *Emx2* interacts with other factors to control the source. Analyses of mice deficient in the zinc finger transcription factor, *Gli3*, suggest that *Gli3* is part of this regulatory network. In the absence of *Gli3*, mice show both reduced *Emx2* expression and an enlarged *FGF8* expression domain in the anterior telencephalon^{44–46}.

Although we focus here on a new interpretation of *Emx2* function, some of our findings seem consistent with the traditional view that *Emx2* promotes the orderly development of anterior-to-posterior regional fates. For example, *Emx2* could restrict the *FGF8/17* domain as part of a role in suppressing anterior regional identity. However, two main observations prompt radical reinterpretation of *Emx2* function. First, the chief support for the idea that a gradient of *Emx2* regulates area patterning comes from analyses of the *Emx2* mutant mouse^{21–23}. But, given previous demonstrations of the effects of FGF8 signaling on the neocortical area map, increased FGF8/17 function in the *Emx2* mutant is sufficient to explain the mutant's cortical patterning shifts. Second, a gain of function experiment indicates that *Emx2* does not act generally to suppress anterior cortical area fates or promote posterior fates. Electroporation of *Emx2* involving the central cortical primordium, where *Emx2* expression is submaximal, has no obvious effect on area pattern. Thus, the *Emx2* gradient may be neither necessary nor sufficient to regulate area patterning directly.

Emx2 regulates *FGF8/17* expression and, in turn, FGF8 regulates *Emx2* expression. We propose that the latter represents a feedback

mechanism. The FGF signaling source inhibits negative regulation, so that the source itself is not obliterated. Alternatively, this two-way suppression may again suggest a version of the original model of *Emx2* function^{21–23}. In this scenario, *Emx2* and FGF8 antagonize one another to create the area map: *Emx2* promotes posterior fates and suppresses anterior fates; FGF8 does the reverse. However, findings to date do not strongly support comparable, but opposing roles for *Emx2* and FGF8. For example, there is no direct evidence, as yet, that *Emx2* can transform area fate. By contrast, FGF8 can induce specific area fates. A second, ectopic FGF8 source transforms presumptive posterior mouse cortex into a central area, S1, revealed by duplicate whisker barrels²⁵.

In conclusion, previous studies indicate that *Emx2* is involved in many aspects of cerebral cortical development^{26,42,47}, including the generation of the area map^{21–23}. We provide evidence that when altered *Emx2* levels change cortical pattern, there is a concomitant change in FGF8/17 signaling levels. By contrast, FGF8/17 manipulations can shift area pattern in the absence of *Emx2*. Taking present and previous^{21,23–25} findings together, the most parsimonious conclusion is that *Emx2* is crucially involved in neocortical area patterning by controlling the extent of a primary neocortical signaling source. The cortical domain shifts in the *Emx2* null mouse therefore reveal the effects of deregulation of this source.

METHODS

Mice were CD-1, *Emx2* mutant or R26R reporter mice (Jackson Laboratories), as indicated in each experiment. R26R mice carry a *neo* cassette, 5' to the *ROSA* locus, flanked by *loxP* sites. Cre recombination activates expression of β -galactosidase. All mice used in this study were bred and maintained in compliance with National Institutes of Health guidelines. Animal protocols were approved by the University of Chicago IACUC.

In-utero electroporation. *In-vivo* electroporation was as previously described²⁵, except that we used a CUY21 Electroporator (Nepagene), which enables monitoring of the passage of current. Laparotomies were performed at E10.5 or 11.5, and embryos were visualized *in utero* with a fiber optic light source. Expression plasmid DNA was mixed with fast green (Sigma) and injected into the cerebral ventricle via a glass capillary. Filling the ventricles with dye allowed better visualization of the cerebral vesicles so that a tungsten negative electrode and a platinum positive electrode could be positioned to electroporate at different sites. After electroporation, the surgical incision in the mother was closed and embryos developed *in utero* with 60% survival beyond birth. As previously described²⁵, 50% of

surviving mice showed effective electroporation, indicated by strong expression of a transgene, and in the case of FGF manipulations, shifts in regional gene expression or other area pattern changes as well. When E11.5 electroporation is successful, targeting of the transgene to the anterior cortical primordium is virtually 100% accurate (based on over 400 embryos analyzed 1–2 d after electroporation). Control mice included non-electroporated animals or mice with hemispheres electroporated with either *AP* or *DsRed*, as noted for specific experiments.

Verifying the electroporation site. The position of the electroporation site was determined in varying ways to suit the experiment. Expression is transient following electroporation. Thus, when brains were analyzed in tissue sections a week or less after electroporation, one series of sections per brain was processed for transgene expression. In explant cultures, examined after 2 d.i.v., the electroporation site was determined in each explant by the fluorescence of coelectroporated *DsRed*. With *Emx2* *in-vivo* electroporation experiments, location of the site was determined *post hoc* by co-electroporation of *Cre* into R26R mice, and consequent Cre recombination to activate β -galactosidase. (Note that co-electroporation of precisely the same cells by two vectors was not essential to mark the region of electroporation.)

Targeting different positions in the cerebral vesicle can be done *in utero* at E10.5 (Fig. 1), but is only 50% accurate (hence the need for *post hoc* confirmation, as above). By contrast, *ex-utero* targeting at E10.5 is virtually 100% accurate because the uterine wall no longer obscures the embryo. *Ex-utero* electroporation of the whole embryo was therefore used to determine the effect of *Emx2* overexpression on FGF8/17 expression and signaling. E10.5 CD1 embryos were removed from the uterus and electroporated with *DsRed* (Clontech), or with *DsRed* and *Emx2*. After electroporation, explants were dissected from the dorsal forebrain, placed on filters (Millicell CM), maintained *in vitro*, and assayed for gene expression with *in-situ* hybridization⁴⁸.

Rescue of *Emx2* mutant cortex. For rescue experiments, the *Emx2* mutant mouse line was maintained on a mixed CD1/C57BL/6 background. In comparison with mice on a C57BL/6 background, the addition of the CD1 outbred strain seemed to increase survival of electroporated embryos. Cortical gene expression shifts and enlarged FGF8/17 domains were the same as in the *Emx2* mutant maintained on a C57BL/6 background (data not shown). Mice were genotyped as previously described⁴⁰. In each experiment with *Emx2* homozygote mutants, comparisons were made with littermate wild-type controls.

Constructs and histology. The *AP*, *FGF8* and *sFGFR3c* electroporation constructs were those used previously²⁵. The *Cre* construct was generated from an *IRES-Cre* cassette kindly provided by K. Lee and T. Jessell (Columbia University). For *Emx2* electroporation, full-length coding sequence for *Emx2* was cloned into the expression vector pEFX⁴⁹. ERM cDNA was obtained from the IMAGE Consortium, GenBank number BF021513. Brains, whole embryos and explants were fixed in 4% paraformaldehyde, and, where needed, sectioned tangentially on a Leica sliding microtome. Section, whole-mount and explant *in situ* hybridization followed described procedures¹⁹, as did CO, X-gal and AP histochemistry^{35,50}.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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