The Fezf2–Ctip2 genetic pathway regulates the fate choice of subcortical projection neurons in the developing cerebral cortex

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Pyramidal neurons in the deep layers of the cerebral cortex can be classed into two major classes: callosal projection neurons and long-range subcortical neurons. We and others have shown that a gene expressed specifically by subcortical projection neurons, Fezf2, is required for the formation of axonal projections to the spinal cord, tectum, and pons. Here, we report that Fezf2 regulates a decision between subcortical vs. callosal projection neuron fates. Fezf2−/− neurons adopt the fate of callosal projection neurons as assessed by their axonal projections, electrophysiological properties, and acquisition of Satb2 expression. Ctip2 is a major downstream effector of Fezf2 in regulating the extension of axons toward subcortical targets and can rescue the axonal phenotype of Fezf2 mutants. When ectopically expressed, either Fezf2 or Ctip2 can alter the axonal targeting of corticocortical projection neurons and cause them to project to subcortical targets, although Fezf2 can promote a subcortical projection neuron fate in the absence of Ctip2 expression.

Fezf2 regulates a decision between subcortical and callosal projection neuron fates. Fezf2−/− mutants alter their fate and become callosal projection neurons, whereas Fezf2+/+ mice fail to extend axons into the spinal cord. Fezf2 expression is prominent in corticospinal motor neurons (CSMNs) (22). These neurons fail to extend axons into the spinal cord in Fezf2−/− mice (22). Fezf2 expression is detected in early forebrain progenitors and in their postmitotic progeny in cortical layers 5 and 6 (19–21, 23). In Fezf2−/− mice, deep-layer neurons are generated and migrate into appropriate positions but fail to express Ctip2 and other markers of layers 5 and 6 (19, 20). To assess axonal projections in mutants, we replaced the Fezf2 ORF with the axonal marker PLAP. These studies revealed that Fezf2−/− layer 5 neurons fail to form the CST and instead project aberrantly across the midline through the anterior commissure (19).

The extension of PLAP-labeled axons through the anterior commissure to the contralateral hemisphere in Fezf2−/− mice raises the possibility that mutant subcortical projection neurons may adopt an alternative fate. Callosal projection neurons normally reach the opposite hemisphere by traversing the corpus callosum. However, this commissure is missing in Fezf2 mutants (19), and the absence of the corpus callosum in Fezf2 mutants precludes axons from taking this route. The anterior commissure may serve as an alternative route by which axons can reach the opposite hemisphere. Here, we examine the hypotheses that Fezf2 regulates the choice between subcortical and callosal projection neuron fates and that Fezf2 acts upstream of Ctip2 and Satb2 in regulating this decision.

Results

Fezf2 Regulates a Fate Switch Between Subcortical and Callosal Projection Neurons. To examine the possibility that subcortical projection neurons in Fezf2 mutants alter their fate and become callosal projection neurons, we attempted to rescue corpus callosum development by generating aggregation chimeras. We predicted that if the formation of the corpus callosum could be restored by the presence of wild-type cells, axons from Fezf2 mutant neurons might extend across this structure.

Chimeric mice were generated by aggregating wild-type embryos with either Fezf2−/− or Fezf2+/− embryos. The resulting chimeric brains contained both wild-type cells and PLAP+ Fezf2+/- cells...
The restoration of callosal development in $\text{Fezf2}^{-/-}$ ↔ +/+ chimeras enabled us to ascertain whether $\text{Fezf2}^{-/-}$ neurons adopted a callosal identity. Consistent with this hypothesis, PLAP-labeled axons entered the callosum and crossed the midline into the contralateral hemisphere (Fig. 1b). We do not know whether these axons originate exclusively from $\text{Fezf2}^{-/-}$ neurons in layer 5 or are also derived from layer 6 (as seems likely in light of studies discussed below). Interestingly, although few PLAP-labeled axons were observed in the dorsal striatum in control chimeras (Fig. 1a), PLAP$^+$ axons formed patchy projections to the dorsal striatum in $\text{Fezf2}^{-/-}$ ↔ +/+ chimeras (Fig. 1b). Previous studies demonstrated that a subset of callosal projection neurons called intratelencephalic-type corticostriatal cells send contralateral projections to striatum and cortex (24). Because PLAP-labeled axons from $\text{Fezf2}^{-/-}$ cortical neurons do not form callosal projections, the observation that PLAP$^+$ axons in $\text{Fezf2}^{-/-}$ ↔ +/+ chimeras form both callosal and striatal projections suggests that some mutant cells adopt a callosal projection neuron fate and extend axon collaterals to the striatum.

$\text{Fezf2}^{-/-}$ Neurons Display the Electrophysiological Properties of Callosal Projection Neurons. Projection neurons in layer 5 can be categorized into several electrophysiological classes that correlate with their projection patterns. Callosal projection neurons exhibit strong spike frequency adaptation in response to intracellular current injections, whereas many neurons that extend axons to the spinal cord, thalamus, or trigeminal nucleus fire trains of single action potentials without adaptation, and corticotectal neurons fire in bursts (15). To ascertain whether $\text{Fezf2}$ regulates the physiological fates of layer 5 neurons, we blinded each animal’s genotype and recorded from individual layer 5 neurons in brain slices from $\text{Fezf2}^{-/-}$ and $\text{Fezf2}^{-/-}$ mice. In $\text{Fezf2}^{-/-}$ slices, neurons responded to depolarizing current injections in one of two manners: some showed spike frequency adaptation, whereas others were nonadapting (Fig. 2a). In slices from $\text{Fezf2}^{-/-}$ mice, all recorded layer 5 neurons demonstrated spike frequency adaptation in response to depolarizing current injections (Fig. 2b). To quantify these data, we plotted the number of neurons that exhibited a given adaptation ratio, where 1 is no adaptation and 0 is complete adaptation. This plot (Fig. 2c) revealed a bimodal distribution of layer 5 neurons in $\text{Fezf2}^{-/-}$ slices, consisting of neurons with either high ($\geq 0.75$) or low ($< 0.75$) adaptation ratios. $\text{Fezf2}^{-/-}$ neurons that showed little...
or no spike frequency adaptation had an average adaptation ratio of 1.05 ± 0.04 (n = 13), whereas the average ratio for adapting neurons was 0.48 ± 0.02 (n = 18). The distribution of adaptation ratios of Fezf2+/− neurons was significantly different from a unimodal distribution (P = 0.04; Hartigan’s statistic for unimodality). In contrast, all of the layer 5 neurons in slices from Fezf2−/− animals showed an adapting phenotype (average adaptation ratio 0.44 ± 0.02, n = 36), and the ratios showed a unimodal distribution (P = 0.45) (Fig. 2c). The adaptation ratios of mutant neurons were significantly different from those of nonadapting neurons in heterozygous controls (P = 1.61 × 10−13; Student’s t test) but were not significantly different from those of adapting neurons in control slices (P = 0.92; Student’s t test). Because we recorded at random from layer 5 neurons in the slices, it is unlikely that our sample of cells in Fezf2 mutants included only normal callosal projection neurons. These data indicate that many subcortical projection neurons in layer 5 of Fezf2−/− mice have undergone a physiological fate switch and have adopted a callosal phenotype.

Altered Dendritic Morphologies in Fezf2−/− Layer 5 Neurons. In rat, subcortical and callosal projection neurons exhibit distinct dendritic morphologies, with the apical dendrites of subcortical projection neurons giving rise to many branches and extending into layer 1, whereas those of callosal neurons contain fewer branches and terminate in or below layer 2/3 (2, 10, 13, 14, 16). Although it has been assumed that such differences also exist in mouse layer 5 neurons, the literature contains no definitive comparisons of the dendritic morphologies of layer 5 neurons with known long-distance projections. We examined the dendritic morphologies of biocytin-labeled layer 5 cells in slices from control Fezf2+/− mice (Fig. 2d), but we saw no significant differences in the heights or branching patterns of apical dendrites between adapting and nonadapting neurons (n = 10; data not shown). In the absence of clear differences between these two populations in Fezf2+/− slices, it was not surprising that the heights of the apical dendrites of Fezf2−/− layer 5 neurons in Fezf2 mutants (Fig. 2d) did not differ significantly from those in controls (n = 28, P = 0.38). However, the apical dendrites of mutant neurons showed significantly fewer terminal branches (average, 2.0 ± 1.7) than did those of adapting (9.5 ± 3.8) or nonadapting (11.0 ± 3.9) neurons in control slices (ANOVA, f = 44.899, P < 0.0001). Thus, although dendritic morphology may not differ clearly between adapting and nonadapting neurons in mouse, our data suggest that Fezf2 is required for their full elaboration in layer 5 neurons, consistent with results obtained by using an RNAi construct to knock down Fezf2 function (21).

Satb2 Is Up-Regulated in the Deep Layers of Fezf2−/− Mice. The above results are consistent with the hypothesis that Fezf2 specifies the fates of subcortical projection neurons and that at least some of these cells in Fezf2−/− mice adopt a callosal projection neuron identity. To explore this notion at a molecular level, we assessed the expression of Satb2, a DNA-binding protein required for the differentiation of callosal projection neurons (17, 18). Sections of Fezf2 mutant brains stained for Satb2 and Ctip2 showed the expected decrease in Ctip2 expression in layer 5, although some immunostaining was still detected in layer 6 (Fig. 3 e and f). Satb2 staining, in contrast, revealed similar numbers of Satb2+ neurons in the upper layers of cortex, whereas increased staining was apparent in layers 5 and 6 (Fig. 3 a, c, d, and f). To quantify this result, we delineated 200-μm-wide regions of the cortical plate in posterior and anterior–medial regions of cortex, divided each region into 67-μm-thick bins extending from layer 2/3 to subplate, then assessed the density of Satb2+ neurons at each depth. Our results revealed significant increases in the density of Satb2+ expressing cells in the deep layers of Fezf2−/− mice compared with controls, whereas the density of Satb2+ neurons in the upper layers was comparable [Fig. 3g and supporting information (SI) Fig. S1]. These results suggest that many subcortical projection neurons become Satb2+ callosal projection neurons in the absence of Fezf2 function. These data further suggest that the cell fate transformations include neurons in layer 6 and those in layer 5 because Satb2 expression is altered in both layers.

Ctip2 Is a Major Downstream Effector of Fezf2 in Regulating CST Formation. To explore the mechanisms by which Fezf2 regulates the development of subcortical projection neurons, we focused on the zinc finger transcription factor Ctip2. Ctip2 expression is perturbed in Fezf2 mutant mice (19), and Ctip2−/− mice show profound defects in the formation of the CST (22). To test the hypothesis that Ctip2 is an essential effector of Fezf2, we asked whether restoring Ctip2 expression in Fezf2−/− layer 5 neurons is sufficient to rescue their axonal phenotype. To this end, expression plasmids encoding Ctip2 were electroporated into the cerebral hemispheres of Fezf2−/− embryos at embryonic day (E) 13.5, when layer 5 neurons are generated. As a positive control, we found that electroporation of a plasmid encoding Fezf2 rescued the Fezf2 mutant phenotype: PLAP-labeled axons failed to extend into the CST after electroporation of control vector (Fig. 4d), but PLAP+ axons populated the CST after electroporation of Fezf2 (Fig. 4e). Electroporation of Ctip2 also restored the Fezf2−/− phenotype: expression of Ctip2 in Fezf2−/− neurons resulted in the extension of PLAP-labeled axons into the CST (Fig. 4f and Fig. S2). These data indicate
that Ctip2 is a major downstream effector of Fezf2 in regulating CST development.

To explore further the relationship between Fezf2 and Ctip2, we asked whether electroporating Fezf2 into Fezf2−/− progenitors at E13.5 could restore Ctip2 expression. Intriguingly, even though electroporating Fezf2 rescued axon extension along the CST, Ctip2 protein was not detected in electroporated neurons at any time examined before postnatal day (P) 5 (E15.5, E16.5, E17.5, E18.5, or P0) (Fig. S3e). These data suggest that Fezf2 is required in progenitor cells before E13.5 to enable normal Ctip2 expression in postmitotic neurons. Collectively, the results of rescue experiments indicate that Ctip2 is sufficient to replace Fezf2 function but that Fezf2 can promote CST formation by affecting downstream components that are independent of Ctip2. Although the identity of these effectors is unknown, numerous genes show altered expression in Fezf2 mutant brains (19), and the functions of most remain to be tested.

Ectopic Expression of Fezf2 or Ctip2 in Layer 2/3 Neurons Alters Axon Targeting. Previous work and the present experiments suggest that the Fezf2–Ctip2 genetic pathway is necessary for specification and differentiation of subcortical projection neurons. To ascertain whether this pathway is sufficient for specifying this fate, we ectopically expressed either Fezf2 or Ctip2 in layer 2/3 neurons of wild-type mice because these neurons normally form connections with ipsilateral and contralateral cortical areas but do not extend axons to the thalamus or CST. Expression plasmids encoding either Fezf2 or Ctip2 were electroporated into wild-type embryos at E15.5, when upper-layer neurons are generated. To label the axons of electroporated layer 2/3 cells, we coelectroporated a plasmid encoding green fluorescence protein (EGFP). Brains that contained labeled cells in layers 4, 5, or 6 were excluded from further analysis.

Electroporating EGFP alone into upper-layer neurons revealed their normal cortical projections (data not shown) and relatively small numbers of axons in the internal capsule and striatum (Fig. 5a and b). No labeled axons were visible in the thalamus, pons, or CST in any animal examined (Fig. 5c and d). However, electroporating expression constructs encoding either Fezf2 or Ctip2 into upper-layer neurons produced marked changes in the behavior of EGFP-labeled axons. Both constructs resulted in increased numbers of labeled axons in the internal capsule (Fig. 5e, f, i, and j) and in regions normally targeted by deep layer neurons, including the thalamus (Fig. 5g and k) and the CST (Fig. 5h and l). These striking alterations in axon trajectories suggest that the Fezf2–Ctip2 genetic pathway is sufficient to specify the subcortical projection neuron fate. Interestingly, even though the axonal trajectories of electroporated neurons were markedly altered, EGFP+ neurons electroporated with Fezf2 migrated into normal positions in layer 2/3 (Fig. 5d and g), although some neurons electroporated with Ctip2 failed to migrate into the cortical plate (Fig. S4a and c).

Although ectopic expression of either Fezf2 or Ctip2 altered the axonal targeting of upper-layer neurons, the neurons transfected with Fezf2 did not express Ctip2 (Figs. S4d–f and S5). As a positive control, Ctip2-transfected neurons did express Ctip2 protein (Fig. S4a–c). Thus, even though Fezf2 function is necessary for Ctip2 expression in the deep layers, Fezf2 expression in upper-layer neurons is not sufficient to induce Ctip2. This may contribute to the apparently lower efficacy of Fezf2 in altering axon targeting of layer 2/3 neurons after electroporation (Fig. 5g, h, k, and l).}

Many upper-layer neurons normally express Satb2, which inhibits Ctip2 expression by altering chromatin structure at the Ctip2 locus (17, 18). The lack of Ctip2 expression in layer 2/3 neurons electroporated with Fezf2 led us to ascertain whether Fezf2 alters Satb2 expression in these neurons. Interestingly, neurons electroporated with Fezf2 showed no obvious loss of Satb2 (Figs. S4i–l and S5), suggesting that Satb2 continues to repress Ctip2 expression in these cells. These data further emphasize the finding that Fezf2 can promote CST formation in a manner independent of Ctip2.
Discussion

Fezf2 is required for the differentiation and axon targeting of layer 5 subcortical projection neurons (19–21). Here, we show that Fezf2 regulates a choice between subcortical projection neuron and callosal projection neuron fates. In the absence of Fezf2, mutant neurons not only adopt the axonal targeting and the physiological properties of callosal projection neurons, but they also acquire expression of the callosal marker Satb2. Our data indicate that Ctip2 is a major downstream effector of Fezf2 and can rescue the axonal phenotype resulting from mutation of Fezf2. Finally, we show that ectopic expression of either Fezf2 or Ctip2 in upper-layer neurons is sufficient to redirect their axons subcortically, and Fezf2 can promote CST formation without inducing Ctip2 expression in these cells.

Fezf2 Regulates the Choice Between Subcortical and Callosal Projection Neuron Fates. Projection neurons in layer 5 of the rodent cerebral cortex fall into two major classes that can be distinguished on the basis of their axonal projections, morphologies, and physiological properties in the adult (2, 10, 12). Interestingly, in mouse, electrophysiological differences between subcortical and callosal projection neurons are similar to those in rat; however, we did not observe clear morphological differences between these two classes of neurons. Thus, we focused our study on the long-distance axonal projections and electrophysiological characteristics that clearly distinguish these neurons in mouse.

The fact that layer 5 subcortical and callosal projection neurons are produced by progenitors at the same time and develop side-by-side in the same cortical layer has raised the question of how their distinct fates are determined. Defects in the layer 5 subcortical projection neurons in Fezf2−/− mice, including the absence of the CST and other subcortical projections, along with misregulation of gene expression in layers 5 and 6, have suggested that Fezf2 plays an essential role in deep-layer neuronal development (19, 20). However, it was unclear whether mutation of Fezf2 simply blocked the differentiation of subcortical projection neurons or caused them to adopt distinct fates.

The present experiments suggest that in the absence of Fezf2, many deep layer subcortical projection neurons adopt a callosal projection neuron fate. First, electrophysiological recordings of layer 5 neurons in mutant mice revealed that all recorded neurons exhibited strong spike frequency adaptation to current injection, a characteristic of callosal projection neurons, linking the electrophysiological identity of a cortical pyramidal neuron to the expression of a particular transcription factor. Second, instead of projecting in the CST or to midbrain targets, many PLAP+ axons in Fezf2−/− mice crossed the midline through the anterior commissure, as if trying to reach the contralateral hemisphere through the closest available commissure. Aggregating chimeric mice containing Fezf2−/− and wild-type cells restored callosal development, and many PLAP+ axons crossed the corpus callosum. Finally, deep layer neurons in Fezf2−/− mice exhibited dramatic increases in the expression of Satb2, a DNA-binding protein that represses Ctip2 expression and specifies callosal neuron identity (17, 18). The density of Satb2+ cells increased in both layers 5 and 6 of Fezf2 mutants, suggesting that some layer 6 neurons also contributed PLAP+ callosal axons in Fezf2−/−/+/+ chimeras. Our data are consistent with the possibility that Fezf2 normally inhibits Satb2 expression in subcortical projection neurons, thus enabling Ctip2 expression in these cells. Collectively, these studies suggest the hypothesis that callosal (corticocortical) and subcortical projection neuron identities involve mutually repressive pathways, each of which confines the specification of distinct fates onto cortical neurons.

Fezf2 and Ctip2 Can Convert the Axon Targeting of Upper-Layer Cortical Projection Neurons. Not only are Fezf2 and Ctip2 required for subcortical projection neuron development, but these molecules appear also to be sufficient for forming subcortical axon projections in other cortical cell types. Whereas normal layer 2/3 neurons form corticocortical projections and do not extend axons into the thalamus or CST, ectopic expression of Fezf2 or Ctip2 in layer 2/3 cells caused their axons to project subcortically. We note that not all GFP+ axons altered their projections in these experiments; some axons still projected across the corpus callosum (data not shown). We do not know whether these axons originated from a distinct subset of layer 2/3 neurons (such as those with lower Fezf2 or Ctip2 expression) or from cells that also formed subcortical projections. Interestingly, the migration of electroporated neurons was largely normal, with neurons still populating their normal superficial positions despite altered connectivity. These results suggest that the Fezf2–Ctip2 pathway controls specific aspects of fate determination related more to axon targeting than to cell body positioning or layer formation.

Previous transplantation experiments (6) and lineage analysis of cortical progenitors in vitro (25) have shown that late progenitor cells are restricted to generating upper-layer neurons. Neither Fezf2 nor Ctip2 is normally expressed by layer 2/3 neurons or their progenitors (19, 21, 23). We speculate that transplanting these cells into a younger brain environment is not sufficient to induce the expression of either gene. However, although misexpressing Fezf2 or Ctip2 in layer 2/3 neurons caused many axons to project subcortically, GFP+ axons were still visible in the corpus callosum, and expression of the callosal determinant Satb2 was not inhibited in electroporated neurons. This incomplete fate switch may be the result of the restricted developmental potential of late cortical progenitors.

In addition to their expression in layer 5, Fezf2 and Ctip2 are also expressed in layer 6 neurons, the majority of which normally project to the thalamus. Interestingly, electroporation of either Fezf2 or Ctip2 into layer 2/3 neurons resulted in the extension of EGFP+ axons into the thalamus, suggesting that Fezf2 and Ctip2 normally regulate axon targeting in both layer 5 and 6 neurons. In postnatal Fezf2−/− mice, cortical projections to the thalamus appear grossly normal (19), but at earlier stages the axons exhibit a transient delay in reaching this target (26). These data raise the question of how Fezf2 and Ctip2 regulate the development of multiple fates in the deep layers. Recent studies suggest that the precise levels of these proteins, in conjunction with the expression of other factors such as Sox5, can determine the identities of distinct subtypes of subcortical projection neurons in layers 5 and 6 (27).

Mechanisms of Fezf2 Function. Because Fezf2 encodes a putative DNA-binding protein, it likely controls cell fates by regulating the expression of its target genes. Fezf2−/− mice exhibit defects in the cortical expression patterns of many transcription factors, including ER81, Grg4, Fox1, and Foxp2 (19). Several of these genes are involved in fate determination and axon targeting in the spinal cord (28–31); however, their roles in cortical development are largely unknown. Here, we focused on the zinc finger transcription factor Ctip2 as an effector of Fezf2. The phenotype of Ctip2 mutants is reminiscent of that of Fezf2-deficient mice in that CST axons fail to reach the spinal cord (22). Fezf2 is expressed at a developmentally earlier stage than Ctip2 and is expressed in both cortical progenitors and subcortical projection neurons, whereas Ctip2 expression occurs later, in postmitotic neurons (19). In conjunction with the observation that Ctip2 expression is abrogated in the Fezf2−/− cortex (19, 20), these data suggest that Fezf2 acts upstream of Ctip2 in deep layer neurons. Indeed, restoration of Ctip2 expression in Fezf2−/− subcortical projection neurons was sufficient to rescue the targeting of PLAP-labeled axons to the spinal cord. Collectively, these observations suggest that Ctip2 is a major downstream effector of Fezf2 in regulating axon targeting.

Two lines of evidence, however, suggest that Ctip2 is unlikely to be the sole effector of Fezf2 (Fig. S6). First, although electroporating Fezf2 into E13.5 Fezf2−/− brains restored the extension of
PLAP+ axons into the CST, Ctip2 protein expression was not detected in electroporated neurons. This suggests that the timing or level of Ctip2 expression may be critical in regulating Ctip2 expression. Second, ectopic expression of Ctip2 in wild-type layer 2/3 neurons was sufficient to promote axon extension to the CST and thalamus, but did not induce Ctip2 expression in these cells. In the latter case, it is possible that cofactors required for Ctip2 expression are absent in layer 2/3 neurons or that these cells actively repress Ctip2. Indeed, upper-layer neurons expressed Satb2 even after electroporation with Ctip2, suggesting that Ctip2 is not sufficient to repress Satb2 expression and that Satb2 continued to repress the Ctip2 locus in these cells.

How might Ctip2 promote the formation of subcortical projections, apart from (and in addition to) using Ctip2+? Recent studies have identified several transcription factors that regulate the development of cortical projection neuron subtypes. For example, Tbr1 is required for subplate formation (32), whereas Sox5 controls the temporal sequence of differentiation and the identity of subplate, corticothalamic, and layer 5 subcortical projection neurons (27). We speculate that Ctip2 regulates the expression of other genes, in addition to Ctip2, that regulate the development of CSMNs (Fig. S6). Parallel pathways are commonplace in genetics, and the fact that Ctip2 is not an obligate target of Ctip2 in specifying subcortical connectivity is both important and interesting. We also note that the elaboration of subcortically directed axons in Ctip2 knock-out mice appears more extensive than that observed in Ctip2 knockout mice, because CST axons extend at least to the level of the pons in Ctip2−/− brains (20). The identification of additional genes that show altered expression in Ctip2 mutants may provide further insight into the molecular mechanisms by which Ctip2 regulates the acquisition of subcortical projection neuron fates.

Methods

Animals. Generation of Fezf2−/− mutant mice was described in ref. 19. Chimeric mice were generated using established procedures (33). Experiments were carried out in accordance with protocols approved by the Administrative Panel for Laboratory Animal Care (APLAC) at Stanford University and at University of California at Santa Cruz.

Electrophysiology and Dendritic Analysis. Recording from individual layer 5 neurons and dendritic analysis in Fezf2−/− or Fezf2+/− mice were performed as described in ref. 15. Details are available in SI Methods.

PLAP Staining, Immunohistochemistry, and Cell Counts. PLAP staining was performed as described in ref. 19. Immunohistochemistry staining was carried out by using standard protocol.

Quantitation of Satb2-expressing cells used three anatomically matched brain sections from each of four control (wild-type or Fezf2+/−) or Fezf2−/− mice at P0 and at P4. Sections were stained with antibodies against Ctip2 and Satb2 and visualized by using confocal fluorescence microscopy. Satb2+ cells were counted in a 200-μm-wide column through the cortical plate. The cortical plate was divided into bins of 67-μm thickness from layer 2/3 at the top to the subplate at the bottom. The density of Satb2+ cells in each bin was calculated by dividing the number of Satb2+ cells in that bin by its area (200 μm × 67 μm). Cell densities in mutant and control brains were compared statistically by using the Student’s t test.

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