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The determination of projection neuron identity in the developing cerebral cortex

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Here we review the mechanisms that determine projection neuron identity during cortical development. Pyramidal neurons in the mammalian cerebral cortex can be classified into two major classes: corticocortical projection neurons, which are concentrated in the upper layers of the cortex, and subcortical projection neurons, which are found in the deep layers. Early progenitor cells in the ventricular zone produce deep layer neurons that express transcription factors including Sox5, Fezf2, and Ctip2, which play important roles in the specification of subcortically projecting axons. Upper layer neurons are produced from progenitors in the subventricular zone, and the expression of Satb2 in these differentiating neurons is required for the formation of axonal projections that connect the two cerebral hemispheres. The Fezf2/Ctip2 and Satb2 pathways appear to be mutually repressive, thus ensuring that individual neurons adopt either a subcortical or callosal projection neuron identity at early times during development. The molecular mechanisms by which Satb2 regulates gene expression involves long-term epigenetic changes in chromatin configuration, which may enable cell fate decisions to be maintained during development.

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Introduction

The nervous system is populated by an enormous variety of neurons that show distinct dendritic morphologies, local and long-distance axonal connections, neurotransmitter phenotypes, and patterns of gene expression. The generation of these diverse phenotypes from mitotically active progenitor cells utilizes a range of cellular and molecular strategies. In general, cues derived from the early regionalization of the neural tube act in conjunction with intercellular signals, temporally regulated factors, and cell-intrinsic cues to progressively determine the fates and identities of specific classes of neurons. Recent studies of the developing cerebral cortex have elucidated some of the mechanisms that underlie the production of discrete types of projection neurons. Here we review progress in understanding the strategies by which cortical neurons are assigned layer-specific fates and elaborate their long-distance projections.

The cerebral cortex is organized into layers that are generated sequentially over developmental time

Ever since the time of Cajal, scientists have appreciated that the cerebral cortex is organized in layers that are defined by the densities and morphologies of their constituent neurons. The advent of retrograde tracing techniques and intracellular dye injections revealed that, as a general rule, neurons in the upper layers 2 and 3 tend to form corticocortical connections, including projections to the contralateral hemisphere across the corpus callosum, whereas neurons in layers 5, 6, and the subplate are the source of subcortical projections to targets that include the spinal cord, pons, midbrain, and thalamus. Neurons in layers 1 and 4 extend axons locally within the cortex. Although it was long assumed that the interneurons of the cortex arise from the same progenitor population like those that generate projection neurons, cell labeling and genetic studies revealed that the bulk of these neurons derive from the ganglionic eminence, which serves as the progenitor pool for the striatum and basal ganglia as well. The regulation of interneuron fates has been reviewed recently and will not be discussed here [1,2].

Cortical projection neurons are derived from progenitor cells that line the dorsal aspect of the lateral ventricles in the forebrain. Mitotically active cells are found in the ventricular zone (VZ), immediately adjacent to the ventricles, and, at later stages, in the subventricular zone (SVZ), which forms between the VZ and the overlying intermediate zone (IZ). Classic ^{3}H-thymidine 'birthdating' studies have revealed that neurons of different layers

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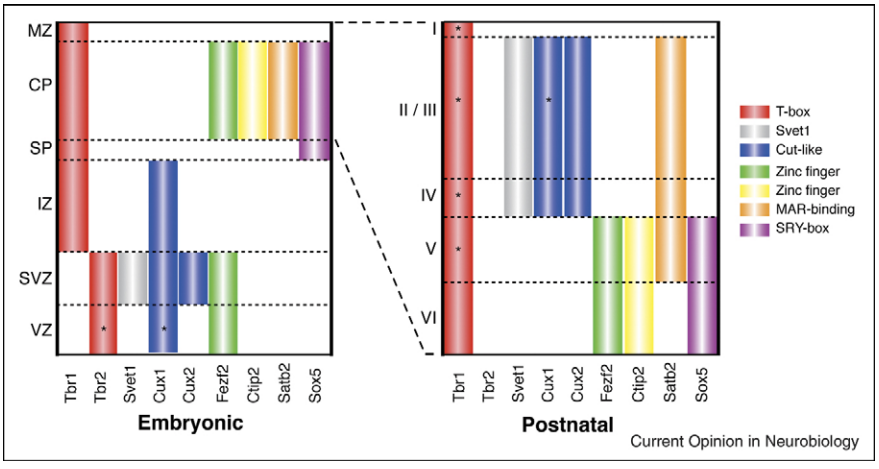
are generated in a stereotypic temporal sequence during development [3]. The first neurons to exit the cell cycle and migrate out of the VZ occupy the preplate, which is subsequently split into two zones by the arrival of neurons that form the cortical plate. The upper preplate, which is known during development as the marginal zone, is populated by Cajal–Retzius neurons, of which many or most are derived from a region known as the cortical hem (located near the hippocampal anlage) and then migrate tangentially to the populated neocortex [4,5]. The deeper domain of the preplate becomes the subplate, a largely transient zone of neurons that plays important roles in axon targeting during development [6]. Within the cortical plate, neurons of the deepest layers (6 and 5) are generated at the earliest stages, followed by neurons of layers 4, 3, and 2.

Cellular studies of cell fate determination suggest a progressive restriction in developmental potential

Transplantation experiments have probed the process by which neurons become committed to the laminar fate that is typical of their time of origin. These studies have demonstrated that by the time a young neuron has progressed through its final mitotic division and is ready to initiate migration, the cell has acquired the information needed to migrate to the layer typical of its birthday, even in an environment in which host neurons are destined for other layers [7–9]. However, at earlier stages of differentiation, cells can show a broader developmental potential and adopt alternative fates. Transplantation experiments revealed that early progenitors, which normally produce deep layer neurons, are multipotent: these cells can directly produce upper layer neurons when transplanted into an older brain environment [7]. Interestingly, the competence of progenitors to respond to fate-inducing cues changes over developmental time. The progenitors of layer 4 neurons retain the ability to differentiate into later-generated types when transplanted into older hosts, but have lost the ability to form layer 6 neurons if transplanted into younger brains [9]. The progenitors of layer 2/3 neurons are even more restricted; when transplanted into younger hosts, these cells generate only upper layer neurons, even if they divide again in the new environment [8]. These transplantation experiments addressed the laminar positions and connections of cortical neurons, but did not track the expression of layer-specific molecular markers. However, clonal analyses of cortical progenitor cells in culture have relied on such markers, and suggest that both the sequential production of distinct laminar phenotypes and the progressive loss of competence to produce early-generated deep layer neurons can occur in isolation [10••]. This suggests that cortical progenitor cells may employ a cell-intrinsic clock as part of the mechanism by which to time the production of distinct laminar phenotypes, or that feedback is required only within an individual lineage in order to produce an appropriate sequence of cell types over time.

Specific patterns of gene expression and cell behavior correlate with the changes in cortical progenitor cells, and in their progeny, over time (Figure 1). For example, progenitors in the VZ at early stages of corticogenesis express a number of transcription factors (such as Fezf2, Otx1, Sox2, and Emx2) that are maintained by their progeny as young neurons populate the deep layers

Figure 1



Gene expression patterns in the developing cerebral cortex during mid-neurogenesis and early postnatal life. Summary of the expression patterns of the T-box transcription factors *Tbr1* and *Tbr2*, the noncoding RNA *Svet1*, the cut-like transcription factors *Cux-1* and *Cux-2*, the zinc-finger transcription factors *Fezf2* and *Ctip2*, the chromatin remodeling protein *Satb2*, and the SRY-box transcription factor *Sox5* during embryonic and early postnatal development in the mouse. Note that many of these genes show dynamic expression patterns (particularly in postnatal life) that are not fully summarized here, and that the protein expression patterns of some are more restricted compared to those of the corresponding mRNAs. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

[11,12••,13,14]. However, at middle stages of neurogenesis, as progenitors begin to produce the neurons of layers 4 through 2, the cells undergo a series of interesting cellular and molecular alterations. The traditional view of cortical neurogenesis held that all neurons are derived from the VZ, and that the SVZ was the source of glial cells [15]. However, this view has been challenged first based on histological studies [16], and subsequently by the observation that SVZ cells show patterns of gene expression that are echoed by those in upper layer neurons. For example, the noncoding RNA *Svet1* and several homodomain genes, including *Cux1* and *Cux2* are expressed both in the SVZ and in layers 2–4 [17,18•]. These corresponding patterns of gene expression suggested that upper layer neurons might be derived from the SVZ. Indeed, in the *Pax6/small* eye mutant, *Svet1* expression is lost in both the SVZ and the upper layers, providing indirect evidence that the SVZ is necessary for the generation of upper layer neurons [18•].

Direct evidence that the SVZ is a neurogenic zone has been derived from a recent series of elegant imaging experiments [19,20] in conjunction with related work from other labs [21–23]. Noctor and colleagues used time-lapse confocal microscopy to track the behavior of individual GFP-labeled mitotic progenitors in the VZ and SVZ, and then to follow the behavior and fates of their daughter cells [19,24•,25••]. These studies revealed that cell divisions within the VZ are primarily symmetric during early development before the onset of neurogenesis, when the progenitor pool is expanding. At the onset of neurogenesis, most cell divisions in the VZ are asymmetric, with one daughter cell remaining in the VZ and the other cell exiting this zone to differentiate. Several imaging studies suggest that daughter cell fates may be regulated by the asymmetric inheritance of determinants associated with the apical membrane of the progenitor cell, such that the daughter cell that inherits this domain retains a progenitor cell fate [24•,25••,26,27]. At mid-to-late stages of neurogenesis, the outcomes of VZ cell divisions undergo a fundamental alteration. Although the cell divisions remain asymmetric, the daughter cell that exits the VZ does not differentiate directly into a neuron, but rather moves into the SVZ and undergoes one or more symmetric neurogenic divisions, thus serving as a transit amplifying cell [23,24•,25••,28]. It is these divisions that appear to be primarily responsible for generating the neurons that migrate into the upper layers.

Collectively, these studies suggest that the early-generated neurons of the deep layers are derived from progenitors in the VZ, whereas late-generated upper layer neurons are produced primarily from SVZ cells, which serve as intermediate progenitors. The corresponding patterns of gene expression between early VZ cells and the deep layers, and SVZ cells and upper layer neurons, suggest that the fates of young neurons are determined at the time of their generation. Below we review the roles of specific genes in the progressive establishment of laminar identity and patterns of connectivity among subtypes of cortical projection neurons.

Fezf2 and *Ctip2* define the fates of subcortical projection neurons

VZ cells at early stages of cortical neurogenesis express a number of transcription factor genes that have the potential to determine or influence the fates of their daughter cells. Some of these (e.g. Otx1) show a clear correlation in expression between early progenitors and deep layer neurons, but have no obvious functional role in the establishment of neuronal fates or identities [13,29]. However, others do appear to play important roles in fate determination. For example, the zinc-finger transcription factor *Fezf2* is expressed in telencephalic progenitor cells starting at E8.5, and its expression is retained by deep layer neurons during corticogenesis [12••,30,31,33••,34••]. Layer 5 and 6 neurons subsequently acquire the expression of a second zinc-finger transcription factor, *Ctip2*, during postmitotic differentiation [12••,33••,35••]. The expression domain of *Fezf2* (which is most prominent in cortex and hippocampus, but is also found in a subset of hypothalamic cells) is much more limited than that of *Ctip2*, which is broadly expressed in the striatum, olfactory bulb, and other regions. However, within the cortex, both *Fezf2* and *Ctip2* are expressed by subcortically projecting neurons in layer 5 and in the corticothalamic projection neurons of layer 6 [12••,33••,35••]. Importantly, *Ctip2* expression is lost in the cortices of *Fezf2* mutant mice (but not in other brain regions) [12••,33••], whereas the converse is not the case: *Ctip2* mutants continue to express *Fezf2* (JD Macklis, unpublished data). Thus, the timing of *Fezf2* and *Ctip2* expression is consistent with the suggestion that *Fezf2* acts upstream of *Ctip2* during cortical development.

Genetic knockouts targeting either the *Fezf2* or *Ctip2* locus in mice have revealed striking similarities in the phenotypes of layer 5 neurons. In both cases, the development of subcortically projecting axons was highly abnormal, with the axons of corticospinal motor neurons (CSMNs) failing to extend into the corticospinal tract (CST) [12••,33••,34••]. In *Fezf2* mutant mice, defects have also been noted in projections to other subcortical targets, including the midbrain and pons [12••]. Because mutation of *Ctip2* causes lethality at about the time of birth [33••], it has been difficult to conduct extensive studies of the fates of CSMNs in these mutants. Such experiments have been feasible in *Fezf2* mutant mice, which survive into adulthood, and particularly in a mouse line in which the *Fezf2* coding sequence was replaced with placental alkaline phosphatase (PLAP), which marks the axonal projections of neurons that normally express *Fezf2* [12••]. Unpublished evidence suggests that *Fezf2* regulates a fate switch: in its absence, many layer 5 and 6

neurons acquire the phenotypes of callosal projection neurons [32••]. Conversely, the ectopic expression of *Fezf2* in neurons that normally form corticocortical and callosal projections is sufficient to redirect their axons to subcortical targets [33••,34••].

Fezf2 encodes a zinc-finger protein that contains a putative repressor domain similar to that found in the Engrailed protein [36,37], and Ctip2 is known to repress transcription of the *p57KIP2* locus [38]. Thus, it seems plausible that Fezf2 and Ctip2 function as transcriptional repressors in the regulation of deep layer neuron fates. However, some zinc-finger transcription factors can confer both activator and repressor activities [37], and in addition, Ctip2 has been implicated in chromatin remodeling at the *HIV1* locus, where it recruits histone deacetylases and methylases to repress transcription [39]. The latter result suggests that the Fezf2–Ctip2 pathway has the potential to modify chromatin configuration; however, the detailed biochemical mechanisms by which these two proteins function in cortical neurons remain to be explored.

A role for *Sox5* in the specification of distinct subtypes of deep layer neurons

Although substantial progress has been made in unravelling the genetic and molecular pathways that control deep layer neuronal identity, little is yet known about the pathways that act upstream of *Fezf2* and *Ctip2*. Recently, however, the SRY-box gene *Sox5* has been implicated in regulating the timing of deep layer differentiation [40••]. *Sox5* is normally expressed by subcortically projecting neurons in layers 5, 6, and the subplate, and its expression are largely excluded from callosal projection neurons [40••]. In *Sox5*$^{-/-}$ mutants, neurons of layers 5 and 6 show aberrant migration patterns and fail to segregate into distinct layers, with cells expressing high levels of *Fezf2* or *Ctip2* occupying the deepest region of the cortical plate rather than their normal positions in layer 5. Cells expressing Ctip2 were also scattered throughout the upper layers, and many of these extended axons subcortically because they could be labeled retrogradely from the cerebral peduncle. Some of these neurons were likely to be mispositioned subplate cells; although mutant brains showed reduced expression of subplate-specific markers, BrdU labeling studies demonstrated that the earliest-generated neurons failed to form a subplate layer and instead were distributed throughout the cortical plate. Lai *et al.* [40••] argued that these subplate neurons acquired a novel fate, because many earliest generated neurons acquired Ctip2 expression, which is normally absent from the subplate. Interestingly, double mutant mice lacking both *Sox5* and *Ctip2* function showed a partial rescue of the migration defects seen in *Sox5* mutants. These data suggest that *Ctip2* expression by subplate neurons may prevent normal preplate splitting as the neurons of the cortical plate migrate into position, and thus lead to defects in lamination similar to those seen in *reeler* mice. Finally, *Sox5* mutants displayed striking defects in the formation of subcortical projections, with axons extending along novel trajectories and apparently failing to form a coherent CST in caudal regions [40••].

The ectopic expression of *Sox5* in upper layer neurons prevented them from extending axons across the corpus callosum and stimulated the extension of corticofugal axons, suggesting that *Sox5* actively promotes the differentiation of subcortical projection neurons. In light of the normal expression of this gene in three distinct types of deep layer neurons (layer 5 subcortical, layer 6 corticothalamic, and subplate neurons), Lai and colleagues hypothesized that varying levels of Sox5 act in combination with distinct levels of Tbr1 and Ctip2 to control the specific identities of subplate and deep layer neurons. For example, subplate neurons are characterized by high levels of Tbr1 expression, little or no Ctip2, and an intermediate level of Sox5, whereas layer 6 neurons normally express high levels of both Sox5 and Tbr1 and an intermediate level of Ctip2. As a known repressor of transcription, it is conceivable that Sox5 may directly repress *Ctip2* expression in subplate neurons.

Finally, it is clear that *Tbr1* plays an important role in cortical development, particularly in regulating the differentiation of subplate neurons. *Tbr1* is expressed in the preplate and layer 6 during early corticogenesis [41], and *Tbr1*-deficient mice show defects in preplate splitting and in the positioning of early-born neurons (similar to the migration defects observed in *reeler* mice) [42]. Indeed, in the absence of *Tbr1*, the expression of reelin by Cajal–Retzius cells in the marginal zone is reduced [42]. *Tbr1* knockouts also exhibit a variety of axon projection defects, with corticothalamic projections growing only as far as the internal capsule, callosal projections mostly terminating in Probst bundle without crossing the midline, and thalamocortical projections reaching the internal capsule, but then turning away from the cortex and extending into the external capsule and amygdala. These complex phenotypes highlight the difficulty in teasing apart nonautonomous gene functions in regulating processes such as migration, from possible cell-autonomous alterations in neuronal identity and connectivity. Clearly, much remains to be learned about the function of *Tbr1* in cortical neurogenesis.

Mechanisms that direct upper layer neuronal identity

Although our understanding of the mechanisms that produce distinct subtypes of subcortical projection neurons in the deep layers is growing, much less is known about how the brain produces the classes of neurons that populate the upper layers. As with VZ cells and their deep layer progeny, gene expression patterns in the SVZ are

correlated with those of neurons in layers 2–4. However, the functional roles of these genes remain poorly understood. For example, the T-box gene *Tbr2* is expressed prominently in the SVZ [41,43], but *Tbr2* knockout mice die early at the blastocyst stage [44], precluding an analysis of *Tbr2* function in cortical development.

The homeodomain genes *Cux1* and *Cux2* are expressed in the developing brain [45], particularly in SVZ cells and their progeny in layers 2–4 [46]. The generation and analysis of *Cux2* knockout animals has revealed that *Cux2* promotes the exit of SVZ cells from the cell cycle, thereby limiting the numbers of upper layer neurons that are generated from this zone. *Cux2*$^{-/-}$ mice experience an increase in the proliferation of SVZ cells and a moderate increase in the densities of cells in layers 2–4 [47••]. Birthdating experiments suggest that *Cux2*-deficient SVZ progenitors reenter the cell cycle at a higher frequency than in wild-type controls, leading to an increase in the size of the SVZ progenitor pool.

The POU domain transcription factors *Brn-1* and *Brn-2* also regulate the generation of upper layer neurons. Both genes are expressed in the VZ and SVZ starting at mid-neurogenesis [48]. Although single mutations in either *Brn-1* or *Brn-2* yield phenotypes in only limited areas of the brain, double mutants show severe proliferation defects in VZ and SVZ cells after E14.5, with no obvious changes in the proliferation of progenitors at earlier times [48]. Double mutant animals also show striking decreases in the numbers of layer 4 neurons, and the brains of surviving *Brn-1/2* double knockouts seem to lack the upper layers as the thickness of the cortex was significantly reduced. This suggests a cell-autonomous role for *Brn* genes in upper layer neurogenesis. Interestingly, although the numbers of deep layer neurons did not appear to be affected in mutants, their migration was perturbed. Analysis of lamination using layer-specific markers revealed that layer 5 neurons (which normally reside above layer 6) were positioned below layer 6 in the mutant cortex. The authors further provide evidence that the defects in migration could be because of a reduction in mDab1, a protein that acts downstream of reelin signaling. Although the two phenotypes (loss of upper layer cortical progenitors and defects in migration) might be independent of each other, further analysis is required to unravel the precise role of *Brn* genes in upper layer generation.

Satb2 and the determination of callosal projection neuron identity

Neurons that extend axons across the corpus callosum to the opposite cerebral hemisphere are a subtype of neurons that form corticocortical connections. Callosal projection neurons are particularly prominent in the upper layers, although they are present in the deep layers. Recent work has revealed that callosal projection neurons require the chromatin remodeling protein Satb2 for the formation of their normal projections, and that in the absence of *Satb2*, these cells extend axons toward subcortical targets.

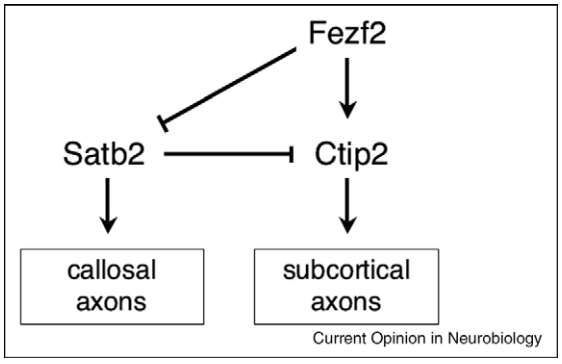
Satb2 is expressed by a subset of neurons throughout the cortical layers, but its expression is most prominent in layers 2–4 [18•,49,50••,51••]. Targeting of the *Satb2* locus with a *lacZ* gene revealed that *Satb2* expressing neurons normally extend axons across the corpus callosum [50••]. Strikingly, these axons fail to cross the corpus callosum in *Satb2* mutant brains [50••,51••], although some β-galactosidase-labeled axons do cross the midline, suggesting that the corpus callosum itself is intact [50••]. Instead, β-galactosidase-labeled axons assume an alternate trajectory and extend subcortically along the CST. In addition to adopting the axon trajectory of deep layer neurons, *Satb2* mutants display alterations in the expression of several axon guidance molecules and a dramatic expansion of Ctip2 expression into the upper layers and within the deep layers, although the expression of *Fezf2* was not obviously affected [50••,51••]. These data suggest that Satb2 functions to repress the expression of *Ctip2* in callosal projection neurons. Indeed, the ectopic expression of *Satb2* in neurons markedly reduces the fraction of cells that express Ctip2 [50••,51••], and alters the projections of deep layer neurons, such that the axons of electroporated cells fail to extend past the cerebral peduncle [51••]. This axonal phenotype is reminiscent of that observed in *Ctip2*$^{-/-}$ brains [35••], suggesting that the ectopic expression of *Satb2* might alter the fate of deep layer neurons by regulating *Ctip2* expression.

Collectively, these data suggest that *Satb2* promotes a callosal projection neuron identity in the cortex by repressing *Ctip2* expression. Indeed, the repression of *Ctip2* by Satb2 appears to be direct: Satb2 binds directly to matrix attachment regions (MARs) in the *Ctip2* locus, where it recruits histone deacetylases [51••,52•] and modifies chromatin configuration to assume a less activated state [50••,52•]. It is not yet clear whether this repression is mutual, because *Satb2* expression in *Ctip2* mutant brains has not yet been reported. However, the expression of Satb2 is significantly upregulated in the deep layers of *Fezf2* mutants [32••], suggesting that Fezf2 can repress *Satb2* expression in subcortical projection neurons. It remains to be determined whether this effect is direct or indirect, and whether Fezf2 can also directly promote the expression of *Ctip2* in deep layer neurons.

Conclusions

Collectively, the studies of the roles of *Sox5*, *Fezf2*, *Ctip2*, and *Satb2* during cortical development suggest that an elegant genetic mechanism exists to control the identity of a subcortical versus callosal projection neuron

Figure 2



A working model for the specification of callosal versus subcortical projection neuron identity during the development of the cerebral cortex. This model is based on genetic (rather than biochemical) studies, which suggest that *Fezf2* acts upstream of *Ctip2* and that both play essential roles in the specification of subcortical projection neuron fates. *Satb2* is required for the development of callosal projection neurons and represses the expression of *Ctip2* in these cells. In the absence of *Satb2*, callosal projection neurons extend axons subcortically. Conversely, in the absence of *Fezf2*, *Satb2* expression is derepressed, enabling cells to take on a callosal projection neuron fate.

(Figure 2). Early in development, when deep layer neurons are generated, *Fezf2* expression in VZ cells may promote the expression of *Ctip2* in young neurons, and together these genes confer a subcortical projection neuron fate during differentiation. Differences in the levels of expression of Sox5, Tbr1, and Ctip2 can further confer specific identities onto subtypes of deep layer and subplate neurons. At the same time, Fezf2 appears to repress *Satb2* (directly or indirectly) thereby repressing callosal identity. At later times during corticogenesis, when upper layer neurons are generated, Fezf2 expression is absent, which relieves the repression of *Satb2*. This, in turn, enables Satb2 to actively repress *Ctip2* expression and promote the adoption of a callosal or corticocortical projection neuron identity. Interestingly, these two pathways also operate within a layer: for example, both subcortical and callosal projection neurons coexist within layer 5 [53], where Satb2 and Ctip2 are coexpressed by individual neurons during a brief window of time, just as layer 5 neurons are settling into their final positions within the cortical plate [50••,51••]. However, this period of coexpression quickly resolves, with the vast majority of layer 5 cells expressing either Satb2 or Ctip2, but not both. At this point, we do not understand what factors within the cell lead it to choose one genetic pathway over the other, but it is clear that the choice leads to fundamental differences in neuronal fate determination.

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