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Pyramidal Neurons Grow Up and Change Their Mind

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The precise stereotypic projections of pyramidal neurons within the six-layered cortex of mammals are key in allowing this structure to attain its high level of function. Recent studies have provided the first indications that postmitotic transcription factors are required for the formation and maintenance of both corticofugal and intracortical pyramidal cell populations. Here, we discuss these new findings in the context of our present understanding of cortical cell specification.

Anyone who has admired Cajal's drawings of pyramidal neurons from the human brain can't help but wonder how such precise cellular architecture is achieved. Despite the ultimate complexity of the mammalian neocortex, it, like the rest of the central nervous system, arises from a simple neuroepithelium. The elaborate organization of the mature neocortex appears in stages, with the sequential formation of the marginal zone, the intermediate zone, and the subventricular zone (Boulder Committee, 1970). Finally, with the splitting of the preplate, the cellular intricacy of the neocortex begins to emerge. Despite their obvious diversity, pyramidal cells are often treated experimentally as a homogenous population. Indeed, prior to the advent of molecular markers, a pyramidal neuron's position within the six-layered neocortex was the only available method to easily distinguish different subtypes. Recent molecular, anatomical, and physiological methods have transformed our ability to study pyramidal cells (Arlotta et al., 2005; Molnár and Cheung, 2006; Nelson et al., 2006; Molyneaux et al., 2007; Migliore and Shepherd, 2005). These advances, including those described below, are finally allowing us to explore the neocortex at a level of complexity that begins to do justice to its intricate function.

The Progressive Specification of Laminar Projection Neurons

The classic birthdating study of Angevine and Sidman (1961) demonstrated that neurons in distinct cortical layers are born from the inside out over time and provided the first clue to the origins cortical pyramidal neuron diversity. The advent of the protomap hypothesis by Pasko Rakic (Rakic, 1988, 1991) provided a structural framework to explain how cortical neuronal diversity was generated. This protomap theory posited that within cortical progenitor zones, a "map" composed of two orthogonal axes is transposed through migration to form the layers and columns of the mature cortex. Physical evidence for this proposal came from lineage-tracing experiments using replication-incompetent retroviruses, to show that the descendants of a cortical progenitor cell can span over multiple neuronal layers and across cortical regions (Walsh and Cepko, 1988, 1993). These studies suggested that neocortical progenitors undergo asymmetric cell divisions, resulting in the sequential production of deep to superficial layer cortical neurons. Pioneering heterochronic transplant experiments by Susan McConnell (McConnell, 1985, 1988) explored mechanisms by which this mode of division produced neurons destined for different cortical layers. She demonstrated that cortical neurons were generated by a ratchet mechanism, whereby the progenitors of early-born neurons could be coaxed to populate more superficial layers of cortex by transplanting them to more mature cortex. Moreover, these cells exhibited laminar plasticity until the time of their final mitosis (McConnell and Kaznowski, 1991). However, neither late-born neurons nor their progenitors could be induced to populate deep layers when moved to younger cortex (Frantz and McConnell, 1996; Desai and McConnell, 2000). These experiments provided compelling evidence for the progressive restriction of cortical progenitors.

Specification of Axon Projection Patterns in the CNS: Insights from Spinal Cord

Although the above studies provided a general cellular framework of how cortical lamination is achieved, continued progress in understanding the nuances of cell-fate specification in the cortex required better molecular markers for specific pyramidal cell subtypes. Further progress had to wait a decade for the tools needed to distinguish different subtypes of pyramidal neurons at the molecular level (Figure 1). In the interim, work by numerous groups demonstrated that neuronal diversity in the caudal CNS was specified in a series of steps wherein progenitors are first subdivided into a set of cardinal groups restricted to giving rise to a prescribed set of interneuron or motor neuron subtypes (reviewed in Jessell, 2000). With regards to their subsequent specification, the motor neurons of spinal cord are by far the best understood. Recent studies by the Jessell laboratory and a number of their colleagues (Dasen et al., 2005; Song and Pfaff, 2005) have demonstrated that the progressive specification of motor neurons is controlled by a wide array of homeobox transcription factors. As a result of the action of these transcriptional effectors, the cardinal group of motor neuron precursors is subdivided into columns and pools, ultimately producing subsets of neurons that innervate specific muscles (Shirasaki and Pfaff, 2002).

For years it has remained unclear whether a similar strategy of progressive restrictions also occurs in the developing cortex or whether a cell's fate is specified in a single "fell swoop" step as

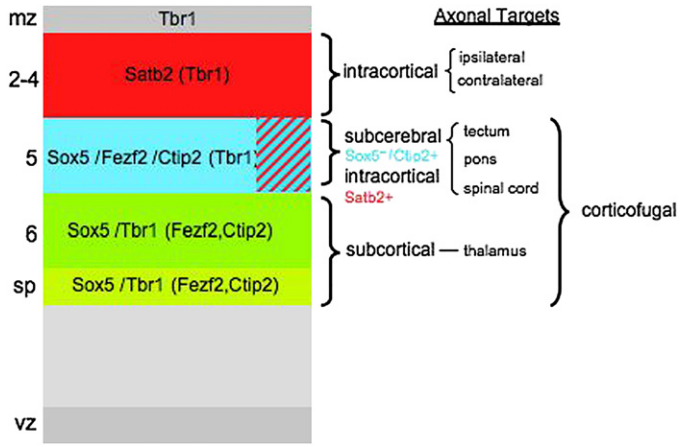

Figure 1. The Expression Patterns of Some Transcription Factors Currently Known to Be Involved in the Specification of the Identities of Projection Neurons (Genes Indicated in Parentheses Are Expressed at Low Levels)
mz, marginal zone; sp, subplate; vz, ventricular zone.

a neuron becomes postmitotic. The rub here hinges on what one means by the word "specification." Specified to precisely what? The McConnell work described above focused solely on laminar position, but even within a single layer, pyramidal neurons can display distinct properties, including marked differences in their axonal targets. One of the clearest and most compelling demonstrations of this came from the beautiful work of Koester and O'Leary (1993), which showed that within layer V, callosal and subcortically projecting neurons are born simultaneously and migrate into the same layer. Despite being intermixed and experiencing the same environment since birth, they execute completely divergent developmental programs, either by sensing distinct sets of environmental cues or by interpreting the same cues completely differently. To do so, different newborn neurons must maintain distinct intrinsic programs as soon as they become postmitotic.

Transcriptional Control of Projection Patterning in the Neocortex

When and how then is the target selection of pyramidal neurons in the neocortex specified? Insights into the molecular mechanisms controlling this process have grown rapidly over the past several years. From a number of efforts that allowed specific subpopulations of cortical pyramidal neurons to be isolated, several genes have been identified that not only provide markers for specific subsets of pyramidal neurons but directly act in the specification of both corticofugal and upper-layer projection neurons (Arlotta et al., 2005; Britanova et al., 2005). In one particularly successful screen (Arlotta et al., 2005), subclasses of corticospinal, corticotectal, and callosal projection neurons were individually purified by labeling each of these populations through the introduction of axonal tracers into their respective targets, then FACS purifying the retrogradely labeled populations from the cortex. Subsequent to their isolation, microarray analysis was performed, and genes enriched in each of these populations were identified. Among the genes implicated in this screen was *Fezf2*, a gene required for the formation of corticospinal motor neurons (Molyneaux et al., 2005; Chen et al., 2005a, 2005b). In contrast to the postmitotically acting genes described below, Fezf2 is expressed within progenitor cells, as well as their neuronal progeny. In line with the notion that pyramidal precursors like their spinal cord brethren are progressively restricted, both recent (Molyneaux et al., 2005; Chen et al., 2005a, 2005b) and soon-to-be-published work (S. McConnell, personal communication) suggests that Fezf2 acts early in the specification process compared to the postmitotically expressed genes described below. It achieves this by activating later expressed downstream effectors, likely including *Ctip2* to promote deep-layer fates. Indeed, Fezf2 controls several facets of lineage specification of subcerebral projection neurons, such as intrinsic physiological properties, dendritic morphology, and axonal targeting and thus appears to specify many aspects of the pyramidal neuron subtype.

Postmitotic Modulators of Pyramidal Neuron Projections

The identification of Sox5 (Arlotta et al., 2005) and Satb2 (Britanova et al., 2005) are a testament to the power of modern gene expression analysis. In the last issue of *Neuron*, a manuscript by Macklis and colleagues examined the function of Sox5 in cortical development. In this issue, both a three-way collaboration between the McConnell, Grosschedl and Fariñas laboratories, as well as a separate study from the Tarabykin group report on the role of *Satb2* in cortical callosal projecting neurons. These papers provide us with a first glimpse of the mechanisms by which the divergent behavior of postmitotic pyramidal neurons is controlled. *Sox5* and *Satb2* are expressed in distinct subsets of pyramidal neurons, with *Sox5* expression confined to deep-layer corticofugal populations of neurons (Figure 1), whereas *Satb2* is found in callosally projecting cells of layers II–V (Figure 1). Each of these genes is expressed only after cells have exited the cell cycle, and analysis of their function show that while central aspects of pyramidal neuron fate are determined by the time a neuron has been generated, others are dependent on postmitotic gene expression.

With regards to its presence in cortex, Sox5, a member of the SRY gene family (Lefebvre, 2002), was found in the same screen that identified Fezf2 in corticospinal motor neurons (Arlotta et al., 2005). Interestingly, loss of *Sox5* gene function differentially affects each of the three main corticofugal neuron subclasses: i.e., layer V subcortically projection neurons, layer VI corticothalamic projection neurons, and subplate (SP) neurons (Figure 2). Lai et al. (2008) demonstrate that in *Sox5*$^{-/-}$ mice, SP neurons are reduced in number and fail to differentiate properly, as evidenced by the loss of their normally distinct subplate neuron morphology and the acquisition of aberrant axonal projections. Layer V subcortically projecting neurons maintain their normal axonal projection to the spinal cord but are generated at abnormal times and are ectopically located in both layer VI and the superficial layers. In contrast to SP and layer V corticospinal neurons, layer VI corticothalamic neurons are largely preserved in terms of their numbers, projection patterns, and laminar position.

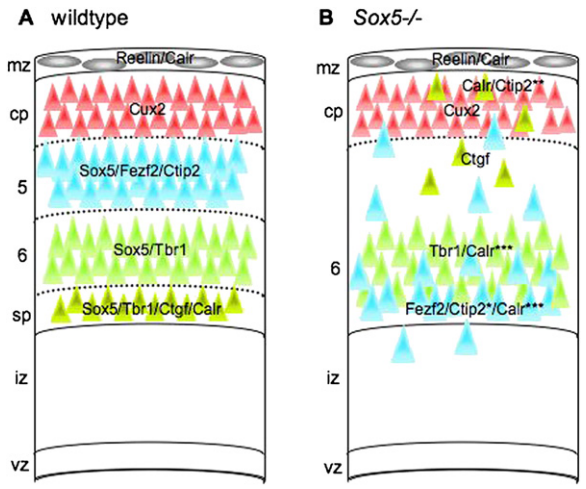

Figure 2. The Expression Pattern of Layer-Specific Markers in Wild-Type and *Sox5*$^{-/-}$ Mutants

In *Sox5*$^{-/-}$ mutants, upper-layer (as indicated by Cux2^{+} neurons) and layer 6 neurons (as indicated by Tbr1^{+} Ctgf^{-} cells) maintain their normal layer identity. In contrast, subcerebral projection neurons (Fezf2/Ctip2-positive neurons shown in blue) do not migrate to the appropriate laminar position. Subplate neurons are abnormal both in terms of their location (as indicated by Ctgf-expressing cells) and as indicated by their ectopic expression of Ctip2. Genes that are expressed at low levels (Fezf2 and Ctip2 expression in layer 6 and subplate, Tbr1 in layer 5 and upper layers in wild-type) are not included in the diagram. Calr, Calretinin. *Fezf2/Ctip2 expression is restricted to the medial neocortex in *Sox5*$^{-/-}$. **Whether Ctgf/Tbr1 is coexpressed in Calr/Ctip2 expressing cells is unknown. ***Whether calretinin and Fezf2 are coexpressed in these cells is unknown.

Previous work has shown that the transcriptional regulator Ctip2 is required for the formation of descending cortical projections (Arlotta et al., 2005). Lai et al. (2008) suggest that the defects in *Sox5*$^{-/-}$ mice in both SP and layer V corticospinal neurons may be a result of misregulation of *Ctip2*, a critical determinant of corticospinal motor neuron identity (Arlotta et al., 2005). In the case of SP neurons, the ectopic expression of *Ctip2* alters laminar fate and likely causes extension of axons towards the spinal cord rather than the thalamus. Interestingly, these authors speculate that Sox5 may have been co-opted through evolution as a means to prolong the production of SP neurons and delay the production of corticospinal neurons in higher mammals. It is also intriguing that the layer VI corticothalamic projection population is relatively spared in the *Sox5* null animal. The authors suggest that this indicates a divergence in the lineages giving rise to SP versus corticothalamic neurons, a notion supported by their demonstration that these two cell types have quite distinct profiles of gene expression. Sox5 also appears to delay the onset of *Ctip2* expression in layer V corticospinal neurons, which allows them to migrate to their correct laminar position before initiating their *Ctip2*-dependent projections to the spinal cord. In the absence of *Sox5*, *Ctip2* expression is initiated precociously, arresting the migration of corticospinal neurons prior to their integration into layer V.

Interestingly, the ectopic expression of Sox5 in upper-layer neurons results in the loss of callosal projections and acquisition of projections directed subcortically. It remains to be determined whether this gain-of-function ability of Sox5 occurs through repression of Satb2 and/or activation of Ctip2, both of which are demonstrated to be crucial postmitotic modulators of projection neuron identities (Arlotta et al., 2005; Alcamo et al., 2008 [this issue of Neuron]; Britanova et al., 2008 [this issue of *Neuron*]). Given both its differential requirement in various corticofugal neurons and its ability to redirect the axonal projections of superficial neurons, it will be interesting to explore how *Sox5* interacts with other genetic regulators of cortical pyramidal cell fate.

Satb2 as Postmitotic Determinant of Callosal Projections

The discovery of *Fezf2*, *Ctip2*, and now *Sox5* has begun to clarify the molecular mechanisms that mediate the formation of different subclasses of corticofugal neurons. Much less is known regarding the mechanisms that control the fate and axonal targeting of intracortical projection neurons. Two studies in this issue (Alcamo et al., 2008; Britanova et al., 2008) demonstrate that the expression of the transcription factor Satb2 is essential for the establishment of callosal projection neurons. Like *Sox5* and *Ctip2*, *Satb2* is predominantly expressed in postmitotic projection neurons in the neocortex. Using LacZ expression directed from the *Satb2* locus and retrograde labeling, Alcamo et al. (2008) and Britanova et al. (2008), respectively, have demonstrated that *Satb2* is expressed in callosal projection neurons but excluded from the corticofugal population (Figure 3). Indeed, in a observation reminiscent of the Koester and O'Leary (1993) findings, those callosal projection neurons that express Satb2 within layer V of cortex are intermixed with *Ctip2*-expressing corticofugal neurons.

Strikingly, loss of *Satb2* results in a respecification of the target selection of callosal neurons. In *Satb2*$^{-/-}$ mice, callosal neurons send their projections subcortically through the internal capsule. These axons can extend as far as the ventral midbrain, where LacZ-expressing axons are seen in the vicinity of the substantia nigra. As a result the corpus callosum is severely reduced in these mutants (Figure 3). While problems in callosal development are often indicative of a general dorsal midline defects rather than a requirement for specific genes in callosal projection neurons, four lines of evidence strongly argue that *Satb2* acts cell autonomously within the callosal population. First, *Satb2* is not appreciably expressed in the dorsal midline. Second, in *Satb2* mutants misguided callosal fibers do not form Probst bundles adjacent to the midline, a hallmark of mutants where the corpus callosum is absent as a result of nonautonomous dorsal midline defects. Third, a small number of *Satb2*-negative axons are present within the corpus callosum of *Satb2*$^{-/-}$ mutants (suggesting a few *Satb2-ve* callosal cells persist Alcamo et al. [2008]). Fourth, the loss of *Satb2* predominantly affects callosal projections, as other commissural axons, including those of the hippocampal and anterior commissures, are unaffected or only mildly increased (Alcamo et al., 2008; Britanova et al., 2008).

How, then, does this dramatic switch in axonal targeting occur? The answer to this again appears to involve *Ctip2*. This gene becomes constitutively activated in callosal projection neurons in *Satb2* mutants, while it is normally only expressed transiently in the precursors of this population. In *Satb2*$^{-/-}$ mice, coexpression of LacZ and Ctip2 demonstrates that Ctip2 is

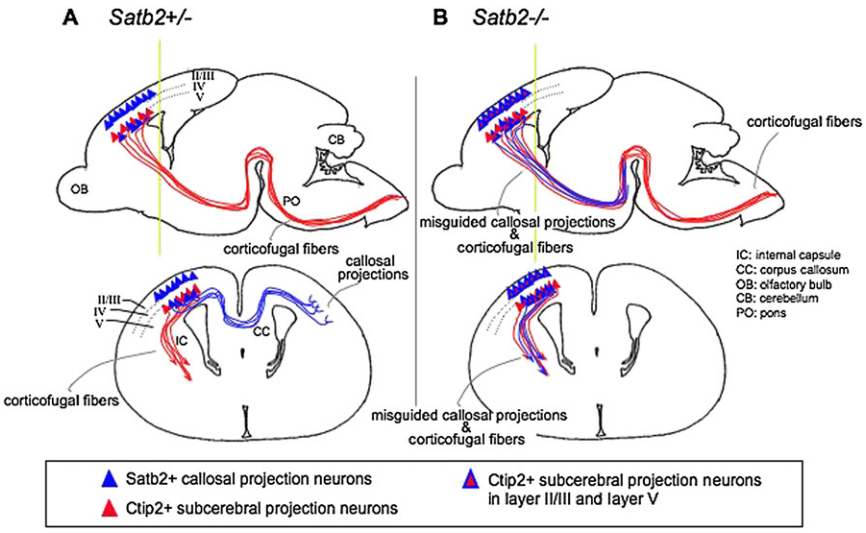

Figure 3. The Cortical Callosal Projection Abnormalities Observed in *Satb2*$^{-/-}$ Mice

In mutant mice, layer V callosal projection neurons are redirected subcortically. The sagittal sections in the top panels show the descending axonal projections of wild-type corticospinal motor neurons on the left and *Satb2*$^{-/-}$ mutant neurons on the right. Note that in mutant animals, callosal projection neurons travel with the corticospinal motor neurons to the level of the substantia nigra. In the bottom panels ,coronal sections taken at the level indicated by the yellow line in the top panel show that callosal projection neurons extend axons across the midline in wild-type mice but redirect their axons ventrally in *Satb2*$^{-/-}$ mutants (although a small Satb2-negative callosal projection persists in mutant animals; see text for details). Note that in both wild-type and mutant callosal neurons, LacZ expressed under the control of the Satb2 locus shown in blue indicate the extent of callosal projections (Alcamo et al., 2008).

strongly expressed in cells normally destined to become callosal neurons (Figure 3). Do upper-layer neurons therefore fully adopt deep-layer fates in *Satb2* mutants? While a number of upper layer markers, such as *Cdh10*, *Ptn*, and *Lmo4*, are lost or reduced in expression, other upper-layer markers such as *Brn2*, *Sip1*, *Svet-1*, *Cux2*, and *Satb2* itself are maintained in *Satb2*$^{-/-}$ mutants. With regards to upregulation of deep-layer markers in *Satb2*$^{-/-}$ mutants, in addition to *Ctip2*, genes restricted to layers V and VI, such as *Clim/Ldb2* and *Cdh13*, are expanded into the more superficial layers of cortex. However, other deep-layer marker genes such as *Fezf2* and *Er81* are not. This suggests that *Satb2*$^{-/-}$ callosal neurons, while taking on some of the characteristics of corticofugal populations, are not fully transfated.

Satb2 is known to be involved in chromatin modification through its ability to bind AT-rich DNA sequences known as matrix attachment regions (MARs) (Szemes et al., 2006; Britanova et al., 2006; Dobreva et al., 2006). It can, depending upon the locus, act directly to either repress or activate specific genes through acetylation or methylation of histone H3 (Britanova et al., 2005; Dobreva et al., 2006). Indeed, Britanova et al. (2008) show that Satb2 interacts with HDAC1 and MTA2, two histone deacetylases (Figure 4). What, then, are the targets of Satb2 in callosal projection neurons? Given the upregulation of *Ctip2* in the cortex of *Satb2*$^{-/-}$ mice, this gene is an obvious candidate for negative regulation by Satb2. Through extensive biochemical studies, both groups demonstrated that Satb2 protein interacts with *Ctip2* regulatory regions and induces changes in chromatin structure at this locus. Moreover, Alcamo et al. (2008) identify several MAR-binding sites in the *Ctip2* locus and demonstrate that Satb2 interacts differentially with each of these regions. Previous work has shown that the acetylation

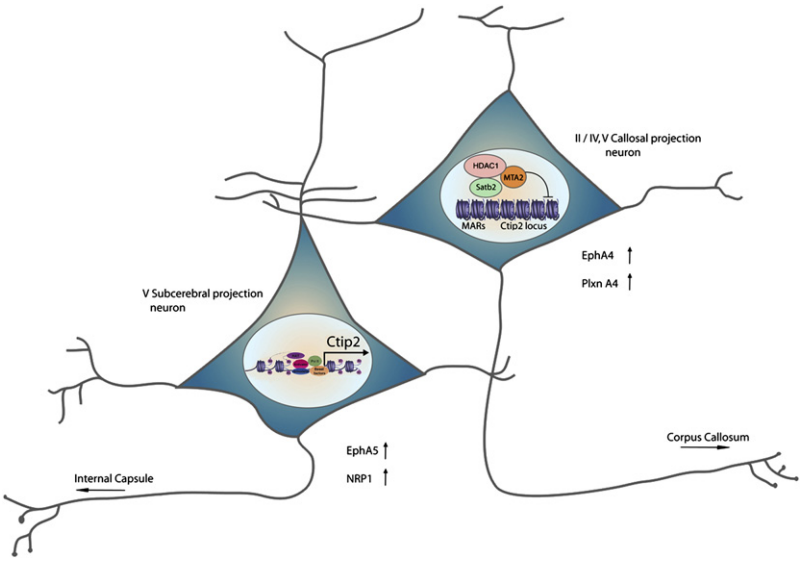

Figure 4. Satb2 Represses Ctip2 Expression in Cortical Callosal Projection Neurons

Ctip2 is expressed in layer V subcortically projecting neurons (as shown in the neuron on the left) but is repressed through the actions of Satb2 containing NuRD complex in the callosal projection neurons (shown on the right). This NuRD complex contains HDAC1 (histone deacetylase 2) and MTA2 (metastasis-associated protein 2). Different axonal guidance molecules are expressed in corticospinal motor neurons (EphA4, Nrp1) versus callosal projection neurons (EphA5, PlxnA4).

or methylation at K4 of histone H3 activates transcription, whereas methylation of histone H3 at K9 represses transcription (reviewed in An, 2007). Alcamo et al. (2008) demonstrate that in the absence of Satb2 a number of MAR sites in the *Ctip2* locus showed increases in both acetylation and methylation of K4 of histone H3, alterations that likely permit the ectopic activation of *Ctip2* in *Satb2*$^{-/-}$ mutant callosal neurons (Figure 4).

Gain-of-function experiments by both groups directly confirmed that expression of Satb2 could repress *Ctip2* expression. To do so, Alcamo et al. (2008) used cortically derived neural stem cells and Britanova et al. (2008) examined *Ctip2* expression in deep-layer neurons after in vivo electroporation of *Satb2*. In the latter experiment, the authors were also able to show that deep-layer neurons overexpressing *Satb2* failed to extend their axons into the cerebral peduncle, thus resembling the phenotype observed in *Ctip2*$^{-/-}$ mutants (Arlotta et al., 2005).

What Does It Take to Change a Pyramidal Neuron's Mind?

As discussed above, from their outset, callosal and corticofugal neurons show decisively different patterns of axon extension (Koester and O'Leary, 1993). The evidence for transcriptional repression of *Ctip2* by Satb2 demonstrated in the current issue (Alcamo et al., 2008; Britanova et al., 2008) clearly provides molecular insight as to how this segregation occurs in vivo. Moreover, both the reports on Satb2 and Sox5 revealed that the identity of pyramidal neurons does not become immutable at the progenitor stage but rather depends on postmitotic expression of transcriptional modulators. These findings are a significant step forward toward understanding the molecular pathways by which distinct projection identities emerge in the neocortex.

Much, however, still remains to be learned about the means by which pyramidal neurons direct their axons. The realization that *Ctip2* is both necessary and (largely) sufficient to direct pyramidal neurons to extend a corticofugal projection raises a number of interesting issues. As shown by Lai et al. (2008), the ability of Sox5 to differentially contribute to the timing and levels of *Ctip2* expression in different corticofugal populations appears to provide part of the answer. However, with regards to their ultimate target selection, clearly *Ctip2* does not provide the whole story (for instance, *Ctip2* functions quite distinctly in the striatum, see Arlotta et al. [2008]). Most pressing is the need to identify a factor that positively regulates intracortical neuron projections, in particular callosal projections, as it seems highly unlikely that such projections will comprise a pyramidal cell ground state. One obvious place to look for such factors is in identifying additional genes that are positively regulated by Satb2. The Alcamo et al. (2008) study, through its in situ hybridization analysis of gene expression in wild-type versus *Satb2*$^{-/-}$ cortex, clearly takes a first step in this direction. Indeed, their demonstration that numerous axonal guidance receptors are both up (e.g., *EphA5*, *NRP1*) and downregulated (*EphA4*, *PlxnA4*) in *Satb2*$^{-/-}$ cortex provides a starting point to identify signals that direct callosal versus corticofugal projections. However, while the inclinations of different classes of young pyramidal neurons are beginning to be revealed, the puzzle of how they form functional cell assemblies is, like the neurons in these studies, in its nascence.

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