Mutual regulation between *Satb2* and *Fezf2* promotes subcerebral projection neuron identity in the developing cerebral cortex

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Generation of distinct cortical projection neuron subtypes during development relies in part on repression of alternative neuron identities. It was reported that the special AT-rich sequence-binding protein 2 (*Satb2*) is required for proper development of callosal neuron identity and represses expression of genes that are essential for subcerebral axon development. Surprisingly, *Satb2* has recently been shown to be necessary for subcerebral axon development. Here, we unravel a previously unidentified mechanism underlying this paradox. We show that SATB2 directly activates transcription of forebrain embryonic zinc finger 2 (*Fezf2*) and SRY-box 5 (*Sox5*), genes essential for subcerebral neuron development. We find that the mutual regulation between *Satb2* and *Fezf2* enables *Satb2* to promote subcerebral neuron identity in layer 5 neurons, and to repress subcerebral characters in callosal neurons. Thus, *Satb2* promotes the development of callosal and subcerebral neurons in a cell context-dependent manner.

Satb2 | Fezf2 | subcerebral neurons | cerebral cortex | cell fate

Projection neurons in the six-layered neocortex can be classified based on axonal projections. The corticocortical neurons send axons to other areas in the ipsilateral cortex or through the corpus callosum and into the contralateral cortex (i.e., callosal projection neurons). The callosal neurons are distributed throughout layers 2–6, but are most abundant in layers 2 and 3. The corticofugal neurons consist of corticothalamic and subcerebral neurons. The corticothalamic neurons are most abundant in layer 6, and send axons into the thalamus. The subcerebral neurons are located in layer 5, and project axons into the spinal cord, superior colliculus, pons, and other brain areas (1–4).

Much progress has been made toward understanding how corticofugal neurons are specified during development (1–4). The zinc-finger transcription factor *Fezf2* (also known as *Fezl* and *Zfp312*) is essential for specifying subcerebral neuron identity by repressing alternate corticothalamic and callosal fates (5–9). In $Fezf2^{-/-}$ mice, subcerebral neurons were lost; instead, mutant layer 5 neurons differentiated into corticothalamic or callosal neurons. Another zinc-finger transcription factor, *Ctip2* (also known as *Bcl11b*), is important for the development of subcerebral axons. The subcerebral axons defasciculated in $Ctip2^{-/-}$ mice and failed to reach the pyramidal decussation (8, 10). The T-box–containing transcription factor *Tbr1* promotes corticothalamic neuron identity by repressing subcerebral fate in layer 6 (9, 11–13). TBR1 binds to a 3′ region of the *Fezf2* gene and directly represses high-level *Fezf2* expression in layer 6 neurons (9, 11). Thus, the expression level of *Fezf2* is precisely regulated in the developing cortex for proper specification of subcerebral and corticothalamic neuronal fates (14, 15).

The special AT-rich sequence-binding protein 2 (SATB2) is involved in transcriptional regulation and chromatin remodeling (16–19). Human mutations in *Satb2* cause severe intellectual deficiency, language impairment, and behavioral defects (20–23). However, the function of *Satb2* in cortical neuron development remains incompletely understood. Previous studies using mouse models revealed that SATB2 was essential for specifying callosal neuronal fate by repressing subcerebral identity in these cells (17–19, 24). Intriguingly, a recent paper reported that SATB2 is also required for the development of subcerebral axons (25). However, it remains unknown how SATB2 performs two seemingly opposing functions. In this study, we investigate how SATB2 promotes subcerebral neuron identity despite repressing the expression of some subcerebral neuronal genes.

Results

SATB2 Is Expressed in Subcerebral and Corticothalamic Projection Neurons During Development. We determined whether SATB2 is expressed in corticofugal neurons by examining axons from SATB2-expressing neurons using the $Satb2^{lacZ}$ allele, in which a beta-galactosidase gene (*lacZ*) was inserted in the *Satb2* locus (16–18). In the postnatal day (P) 0 $Satb2^{lacZ/+}$ mice, $LacZ^{+}$ axons were observed in the internal capsule, cerebral peduncle, and thalamus; however, compared with the callosal axons, labeling of the corticofugal axons was less intense (Fig. S1 *A–C*).

To confirm that SATB2 is expressed in the corticofugal neurons, we combined cholera toxin β (CTβ) retrograde tracing from the cerebral peduncle or thalamus with SATB2 immunohistochemistry (Fig. 1). The specificity of the SATB2 antibody was confirmed by lack of staining in the neocortices of the $Satb2^{lacZ/lacZ}$ and *Satb2* conditional KO (*Satb2 cKO*) mice (Fig. S1 *D–K*). At P0, in general, SATB2 was expressed at higher levels in the upper layers and at lower levels in the deep layers (Fig. S1*D*). Low- or medium-level SATB2 expression was observed in 67% and 89% of

Significance

Mutations in special AT-rich sequence-binding protein 2 (*Satb2*) cause severe intellectual deficiency in humans. However, its function in brain development is not completely understood. Our study focuses on the function of *Satb2* in specifying cortical projection neuron fates. We find that, although *Satb2* activates the expression of some subcerebral neuronal genes, it also inhibits the expression of other genes that are expressed in subcerebral neurons. We report that *Satb2* promotes *Fezf2* and *Sox5* expression in subcerebral neurons, and that *Fezf2* in turn inhibits high-level *Satb2* expression. We show that the mutual regulation between *Satb2* and *Fezf2* is essential for *Satb2* to promote subcerebral neuron fate.

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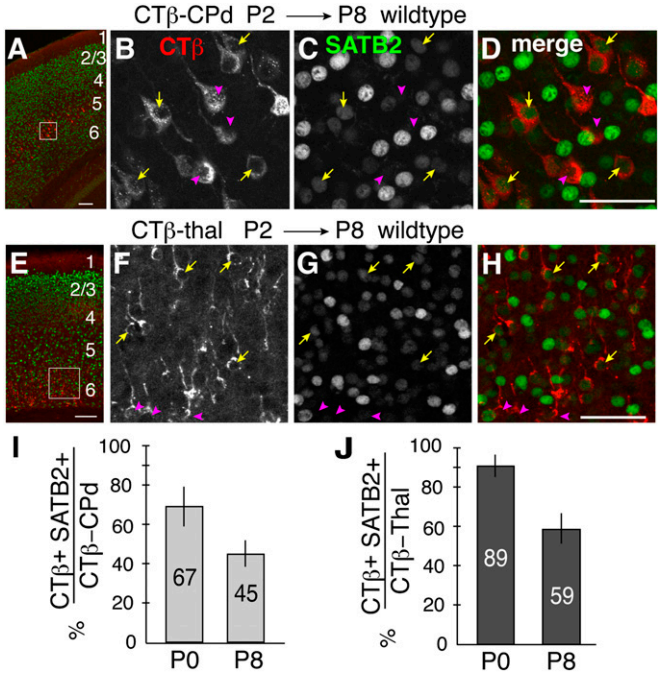

Fig. 1. SATB2 is expressed in subcerebral and corticothalamic neurons. Cortical neurons projecting to the cerebral peduncle (*A–D*) or the thalamus (*E–H*) were labeled by CTβ-Alexa Fluor retrograde tracing. The enlarged images of the boxed area in *A* are shown in *B* (CTβ), *C* (SATB2), and *D* (merge). The enlarged images of the boxed area in *E* are shown in *F* (CTβ), *G* (SATB2), and *H* (merge). The yellow arrows point to traced cells that expressed SATB2. The purple arrows point to traced cells not expressing detectable SATB2. (*I* and *J*) Percentages of traced subcerebral (*I*) and corticothalamic (*J*) neurons that expressed SATB2 at P0 and P8. Error bars indicate SD. (Scale bars: *A* and *E*, 100 μm; *B–D*, 50 μm; *F–H*, 50 μm.)

traced subcerebral and corticothalamic neurons, respectively (Fig. 1 *I–J*). At P8, 45% and 59% of the labeled subcerebral and corticothalamic neurons, respectively, expressed low- or medium-level SATB2 (Fig. 1 *I–J*). Thus, many corticofugal neurons express SATB2 at birth and early postnatal stages.

Misregulated Gene Expression in *Satb2*$^{lacZ/lacZ}$ Cortices. Previous studies showed that callosal and subcerebral axons failed to reach their targets in the *Satb2* mutant mice (16–18, 25). To investigate the molecular underpinning of these defects, we performed gene expression analysis of *Satb2*$^{lacZ/lacZ}$ cortices by using RNA sequencing (RNA-seq). Compared with *Satb2*$^{+/+}$ mice, expression of 1,277 genes was increased to at least 1.2 fold and expression of 1,852 genes was decreased by more than 20% in P0 *Satb2*$^{lacZ/lacZ}$ cortices (adjusted $P < 0.1$; Fig. S2*A*). We examined the expression patterns of some of the misregulated genes at neonatal stages [embryonic day (E) 18.5 and P4] by using the online in situ hybridization database generated by Allen Brain Institute (Allen Brain Atlas). Consistent with previous reports that callosal neurons in *Satb2* mutant mice showed molecular characteristics of subcerebral neurons (16–18), expression of many genes that are normally expressed in the deep layers was increased in the *Satb2*$^{lacZ/lacZ}$ cortices (Fig. S2*A* and Table S1). In addition, we found that genes that are specifically expressed in upper-layer neurons in normal mice showed reduced expression in the mutant mice (Fig. S2*A* and Table S2). CUX1 (26) was expressed in layers 2–4 in control mice (Fig. 2*G*). In the *Satb2 cKO* mice, CUX1 expression was significantly reduced (Fig. 2*J*). Thus, in addition to repressing deep-layer gene expression in upper-layer neurons, *Satb2* promotes callosal neuron identity through activating upper-layer gene expression.

Surprisingly, whereas some deep layer-specific genes showed increased expression, expression of other deep-layer genes was reduced in *Satb2*$^{lacZ/lacZ}$ brains (Fig. S2*A* and Table S1). The reduced expression of these genes was confirmed by immunohistochemistry (Fig. S2 *B–K*), suggesting that SATB2 is required for the development of deep-layer neurons.

SATB2 Promotes the Expression of *Fezf2*, a Subcerebral Cell-Fate Determinant. Among the genes that showed reduced expression in the *Satb2*$^{lacZ/lacZ}$ cortices was *Fezf2* (Fig. 2*C* and Table S1), a cell fate-determining gene of subcerebral neurons (5–8). In situ hybridization confirmed that *Fezf2* mRNA was severely reduced at E16.5 (Fig. S3 *A–F*) and P0 (Fig. 2 *A–B′* and Fig. S3 *G–J*) in the mutant mice. *Fezf2* expression was largely absent from layer 5 across the cortex except for the most medial regions. Its expression was detected in layer 6 but reduced in caudolateral areas.

To determine whether SATB2 directly regulates *Fezf2* expression, we performed ChIP and high-throughput DNA sequencing (ChIP-seq) by using SATB2 antibodies and dissected E15.5 cortices. We observed strong and specific binding of SATB2 to the highly conserved enhancer 434 at the 3′ end of the *Fezf2* gene (Fig. 2*D*) (27, 28). To test whether SATB2 regulates the enhancer 434, we cloned enhancer 434 in the luciferase reporter plasmid pGL4CP-TK (29) and performed the luciferase assay in Neuro-2A cells. Cotransfection with *Satb2* cDNA significantly decreased the activity of the luciferase in empty pGL4CP-TK plasmid (Fig. S4*A*). However, when enhancer 434 was cloned in pGL4CP-TK plasmid in either orientation, cotransfection with *Satb2* cDNA significantly increased the luciferase activity (Fig. 2*E* and Fig. S4*A*).

We have generated stable transgenic mouse lines that expressed a *lacZ* gene under the control of enhancer 434 and an hsp68 minimal promoter (*enhancer 434-lacZ* mice) (15). To test whether SATB2 regulates the activity of enhancer 434 in vivo, we generated *Emx1-Cre*; *Satb2*$^{+/+}$; *enhancer 434-lacZ* (control) and *Emx1-Cre*; *Satb2*$^{flox/flox}$; *enhancer 434-lacZ* (*Satb2 cKO*; *enhancer 434-lacZ*) mice. In P0 (Fig. S3*K*) and P4 (Fig. 2 *F* and *H* and Fig. S3*M*) control brains, although the strongest LacZ activity was present in the ventricular zone, many enhancer 434-LacZ$^+$ cells were detected in the cortical plate. The LacZ$^+$ cells consisted of projection neurons and few astrocytes, and were located throughout all cortical layers (Fig. 2 *F–H* and Fig. S3*K*). In the *Satb2 cKO*; *enhancer 434-lacZ* mice, the number of LacZ$^+$ cortical projection neurons was significantly reduced (Fig. 2 *I–L* and Fig. S3 *L* and *N*). These data indicate that SATB2 positively regulates the enhancer 434, and that SATB2 likely directly regulates *Fezf2* expression in cortical neurons.

SATB2 Positively Regulates *Sox5* Expression. Mutations in multiple genes affect the development of subcerebral neurons and axons (27, 28, 30, 31). *Sox5* is expressed at a high level in layer 6 corticothalamic neurons, and at a lower level in layer 5 subcerebral neurons (27, 30). However, its function in regulating the development of deep-layer cortical projection neurons has been controversial (27, 30). By using *Sox5*^{lacZ} and *Sox5*^{flox} alleles (27, 30), we confirmed that *Sox5* is essential for the development and likely fate specification of subcerebral neurons. Subcerebral neurons, marked by high-level expression of CTIP2 (9), were missing in layer 5 and the lateral cortical regions in the *Emx1-Cre*; *Sox5*$^{flox/flox}$ mice (Fig. S5). Accordingly, the corticospinal tract was present in the control *Emx1-Cre*; *Sox5*$^{lacZ/+}$; *RCE-GFP* mice, but severely reduced in the *Emx1-Cre*; *Sox5*$^{lacZ/flox}$; *RCE-GFP* brains (Fig. 3 *A* and *B*).

RNA-seq showed that expression of *Sox5* was reduced to 65% in the *Satb2*$^{lacZ/lacZ}$ cortices (Fig. 3*G* and Table S1). Immunohistochemistry confirmed that, in the *Satb2*$^{lacZ/lacZ}$ cortices, the number of SOX5$^+$ neurons was reduced, and SOX5 was no longer expressed in layer 5 (Fig. 3 *C–F*). ChIP-seq analysis showed that SATB2 bound specifically to a highly conserved region in an intron of *Sox5* gene (Fig. 3*H*). We cloned the

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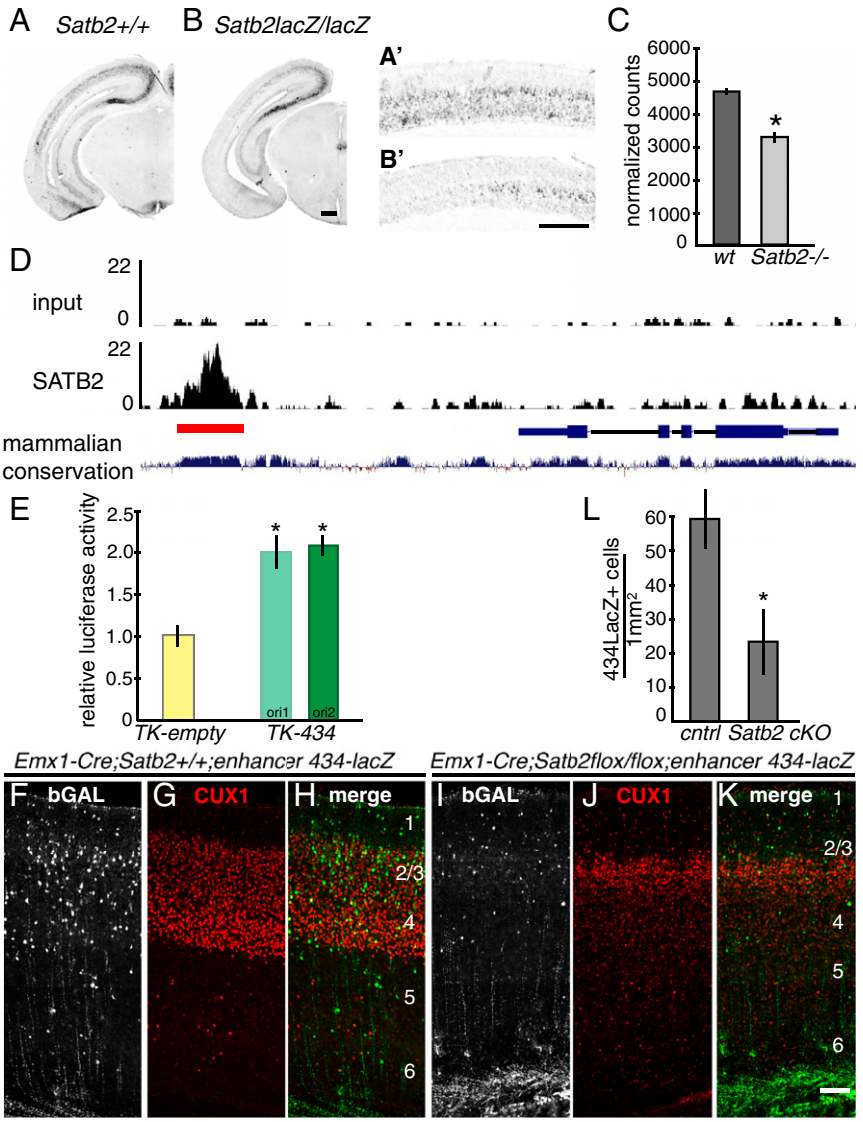

Fig. 2. SATB2 regulates *Fezf2* expression. (*A* and *B*) In situ hybridization showed that *Fezf2* mRNA expression was reduced in P0 $Satb2^{lacZ/lacZ}$ cortices. (*A′* and *B′*) Enlarged views of the cerebral cortex showing *Fezf2* mRNA expression in $Satb2^{+/+}$ (*A′*) and $Satb2^{lacZ/lacZ}$ (*B′*) cortices. (C) The normalized expression level of *Fezf2* mRNA in P0 $Satb2^{+/+}$ and $Satb2^{lacZ/lacZ}$ cortices revealed by RNA-seq (*$P < 0.001$). (*D*) ChIP-seq revealed that SATB2 bound to the *enhancer 434* (red) of the *Fezf2* gene (blue). (*E*) Luciferase assay showed that cotransfection of *Satb2* cDNA increased the activities of luciferase regulated by enhancer 434 and an HSV-TK promoter in Neuro-2A cells (*$P < 0.001$). (*F–H*) Immunohistochemistry for β-gal (green) and CUX1 (red) in P4 *Emx1-Cre*; $Satb2^{+/+}$; *enhancer 434-lacZ* mice. (*I–K*) The number of β-gal^+ neurons was reduced in P4 *Emx1-Cre*; $Satb2^{flox/flox}$; *enhancer 434-lacZ* mice. (*F–H* and *I–K*) Enlarged images of the boxed areas in Fig. S3 *M* and *N*, respectively. They were generated by merging three panels each. (*L*) Quantification of β-gal^+ neurons in P4 control and *Satb2 cKO* cortices (*$P = 0.013$; $n = 4$ mice per genotype from three litters, unpaired *t* test). Error bars in *C*, *E*, and *L* indicate SD. (Scale bars: *A* and *B*, 500 μm; *A′* and *B′*, 500 μm; *F–K*, 100 μm.)

SATB2 binding sequence into the pGL4CP-TK plasmid (29) and performed luciferase assays (Fig. 3*I* and Fig. S4*B*). Cotransfection with *Satb2* cDNA reduced the luciferase activity in the pGL4CP-TK plasmid (Fig. S4*B*). When the SATB2 binding sequence was cloned into the pGL4CP-TK plasmid, luciferase activity was still reduced when *Satb2* cDNA was cotransfected (Fig. S4*B*). However, compared with the empty pGL4CP-TK plasmid, contransfection of *Satb2* cDNA increased the activities of the luciferase when the SATB2 binding sequence was cloned into the pGL4CP-TK plasmid in either direction (Fig. 3*I*). These results suggest that, in addition to regulating *Fezf2*, SATB2 may promote the development of subcerebral neurons through the regulation of *Sox5* expression.

Mutual Regulations Between *Fezf2* and *Satb2* in Deep-Layer Neurons. Although the aforementioned data reveal that SATB2 is required to promote *Fezf2* expression in layer 5 neurons, we previously reported that *Fezf2* represses high-level SATB2 expression: SATB2 expression was significantly increased in the deep layers of $Fezf2^{-/-}$ cortices (8). These results implicate a mutual regulatory relationship between *Satb2* and *Fezf2*. To further investigate this mutual regulation, we analyzed Satb2-LacZ and Fezf2- placental alkaline phosphatase (PLAP) expressions in *Satb2 cKO* mice (Fig. 4). As the LacZ and PLAP reporters were knocked into endogenous *Satb2* and *Fezf2* genes, respectively (6, 16), the expression of Satb2-LacZ and Fezf2-PLAP likely reflects that of *Satb2* and *Fezf2* loci. In the $Satb2^{lacZ/+}$; $Fezf2^{PLAP/+}$ brains, Fezf2-PLAP was expressed in layers 5 and 6. Consistent with *Fezf2* inhibiting high-level *Satb2* expression, the Satb2-$LacZ^+$ domain consisted of a

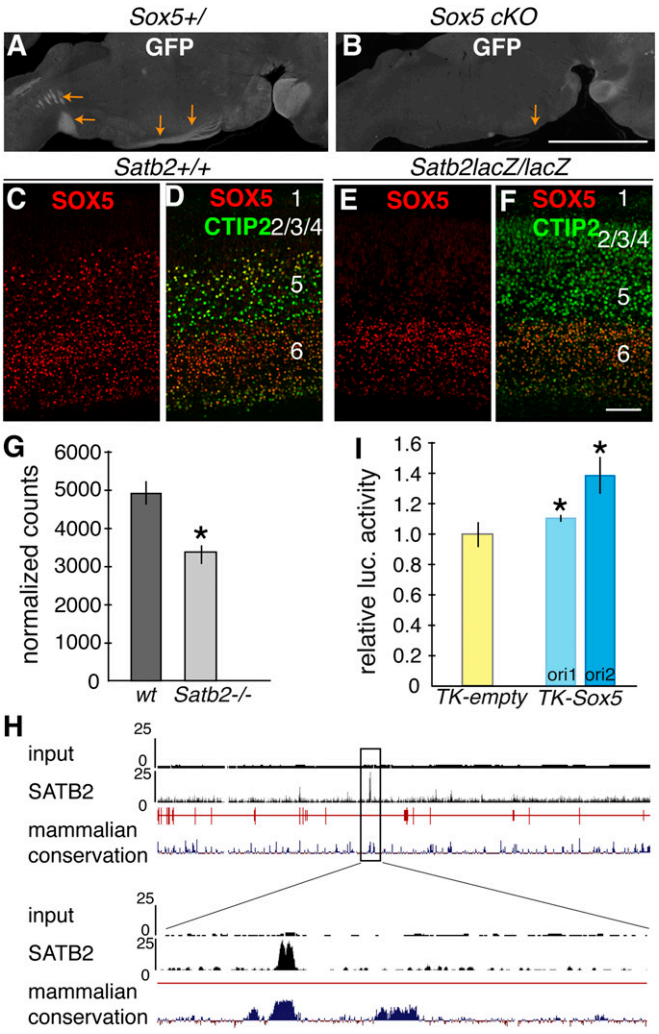

Fig. 3. SATB2 regulates expression of *Sox5*, a gene required for the development of subcerebral axons. (*A* and *B*) Corticospinal tract axons are missing in the *Sox5* mutant mice (*B*). Arrows in *A* point to the GFP$^+$ corticospinal axons in *Emx1-Cre*; *Sox5*$^{+/lacZ}$; *RCE-GFP* mice, which are missing in *Sox5 cKO* (*Emx1-Cre*; *Sox5*$^{lacZ/flox}$; *RCE-GFP*) mice. (*C–F*) SOX5 staining (red) is reduced in layer 5 neurons in *Satb2*$^{lacZ/lacZ}$ mice. CTIP2 staining is shown in green. (*G*) The normalized expression level of *Sox5* mRNA in P0 *Satb2*$^{+/+}$ and *Satb2*$^{lacZ/lacZ}$ cortices revealed by RNA-seq (*$P < 0.001$). (*H*) SATB2 binds to conserved intronic sequences of *Sox5* gene (red line, *Top*). (*Bottom*) Close-up view of the boxed SATB2 binding peak. (*I*) Luciferase assay showed that, compared with the empty luciferase reporter plasmid, cotransfection of *Satb2* cDNA increased the activities of luciferase in the reporter plasmids containing the SATB2 binding sequence in the *Sox5* intron in either orientation (*$P < 0.001$). Error bars in *G* and *I* indicate SD. (Scale bars: *A* and *B*, 500 μm; *C–F*, 100 μm.)

Satb2-LacZ^{high} domain in layers 2–4 and a Satb2-LacZ^{low} domain in deep layers (Fig. 4 *A–C*). The Satb2-LacZ expression was lowest in layer 5, where high-level *Fezf2* expression was located. In the *Emx1-Cre; Satb2*$^{lacZ/flox}$; *Fezf2*$^{PLAP/+}$ mice, Fezf2-PLAP expression was absent in layer 5, and the Satb2-LacZ^{high} domain expanded from upper layers into layer 5, and stopped where *Fezf2* expression persisted (Fig. 4 *D–F*). This result confirms that, although *Satb2* is required for *Fezf2* expression in layer 5 neurons, *Fezf2* in turn represses *Satb2* expression and reduces its expression level in deep-layer neurons.

Discussion

In this study, we have identified a mechanism by which *Satb2* promotes the development of subcerebral and callosal neurons in a cell context-dependent manner (Fig. 5).

SATB2 Promotes Callosal Neuron Identity by Repressing Expression of Subcerebral Neuronal Genes and Promoting Expression of Upper-Layer Neuronal Genes. *Satb2* was reported to promote the development of callosal neurons through repressing expression of subcerebral neuron genes such as CTIP2 (17–19, 24). Our study confirmed this, and identified many more deep-layer genes that were up-regulated in the *Satb2* mutant mice (Fig. S2*A* and Table S1). Further, we found that expression of upper-layer neuronal genes was reduced in the *Satb2*$^{lacZ/lacZ}$ cortices (Table S2), suggesting that *Satb2* is required to promote their expression. Thus, our study demonstrates that *Satb2* promotes callosal neuron development not only through repressing expression of deep-layer genes in upper cortical layers, but also by promoting expression of upper-layer neuronal genes (Fig. 5*G*).

Corticothalamic Neurons and Axons Are Present in the *Satb2* Mutant Mice. The expression of *Satb2* in the corticothalamic neurons (Fig. 1 *E–H* and *J*) suggests that it may regulate the development of these neurons. Indeed, a recent study reported increased Satb2-LacZ$^+$ axons in the thalamus of the *Satb2 cKO* mice (25). We used multiple methods to investigate the corticothalamic axons, and discovered that the innervation pattern of the thalamus by corticothalamic axons was mostly normal in the *Satb2* mutant brains (Fig. 5*F*, *SI Results*, and Fig. S6). The increased Satb2-LacZ$^+$ axons observed in the *Satb2 cKO* mice (Fig. S6 *A* and *B*) (25) were a result of increased expression of *Satb2-LacZ* in the layer 5 and upper layer 6 neurons that normally express low-level SATB2 and project axons into the thalamus (Figs. 1 *E–H* and 5 *A–F* and Fig. S6). Thus, SATB2 appears to have a limited role in the initial fate specification and axon projection of corticothalamic neurons.

Mutual Regulation Between *Satb2* and *Fezf2* Enables *Satb2* to Promote the Development of Subcerebral Neurons. RNA-seq analysis indicated that SATB2 activates the expression of deep-layer neuronal genes that are essential for specifying subcerebral neuronal fate (e.g., *Fezf2* and *Sox5*) and represses the expression of other deep-layer neuronal genes that are necessary for the development of subcerebral axons (e.g., *Ctip2*; Fig. S2*A* and Table S1). Then how does *Satb2* promote the development of subcerebral neurons?

Although SATB2 expression has been generally associated with callosal neurons (8, 17, 18), our retrograde tracing experiment showed that most corticofugal neurons express low- or medium-level SATB2 at birth (Fig. 1). The dynamic expression

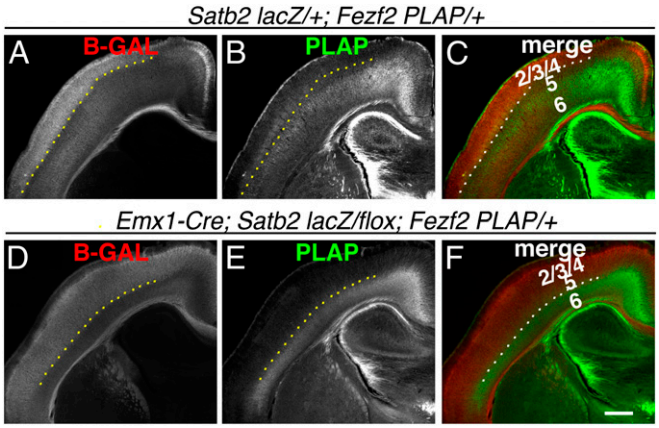

Fig. 4. *Satb2* and *Fezf2* mutually regulate the expression of each other. Immunohistochemistry using β–gal (red) and PLAP (green) antibodies of P7 brains. (*A–C*) A section of a *Satb2*$^{lacZ/+}$; *Fezf2*$^{PLAP/+}$ brain. (*D–F*) A section of an *Emx1-Cre*; *Satb2*$^{lacZ/flox}$; *Fezf2*$^{PLAP/+}$ brain. Dotted lines indicate the boundary between Satb2-LacZ^{high} and Satb2-LacZ^{low} domains. (Scale bar: 500 μm.)

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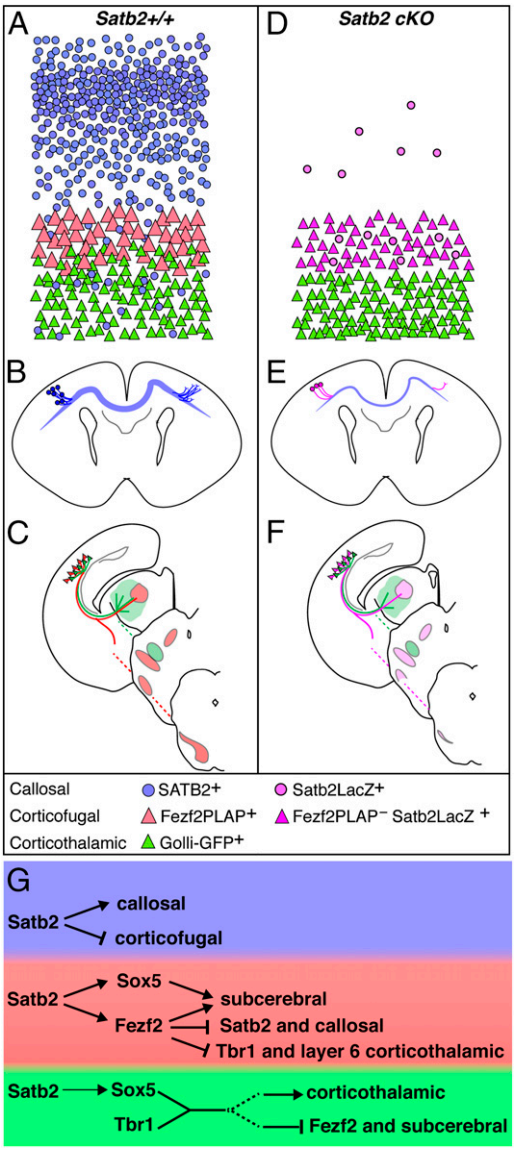

Fig. 5. SATB2 regulates cortical projection neuron subtype identities in a cell context-dependent manner. (*A*–*C*) Summary of cortical projection neuron subtypes and projections in *Satb2*$^{+/+}$ mice. (*A*) Distribution of SATB2^{+}callosal (blue circle), subcerebral (Fezf2-PLAP^{+}; pink triangle), and corticothalamic (Golli-GFP^{+}; green triangle) neurons in the *Satb2*$^{+/+}$ cortices. (*B*) Callosal neurons project axons through corpus callosum and into the targets in contralateral hemisphere. (*C*) Layer 6 corticothalamic neurons project axons into the thalamus, and layer 5 Fezf2-PLAP^{+} neurons project axons into cerebral peduncle, pyramidal tract, and thalamic nuclei. (*D* and *E*) Summary of cortical projection neuron subtypes and projections in the *Satb2 cKO* mice. (*D*) Fewer Satb2-LacZ^{+} callosal neurons were present in the mutant mice. Most upper-layer neurons show mixed identity and do not project axons into the corpus callosum and into the contralateral hemisphere. Subcerebral neurons were absent. Instead, Satb2-LacZ expression is increased in layer 5 neurons. There is a mild decrease of layer 6 Golli-GFP^{+} corticothalamic neurons. (*E*) Fewer Satb2-LacZ^{+} neurons extend axons through the corpus callosum and into the contralateral hemisphere. (*F*) Golli-GFP^{+} axons from layer 6 neurons project into the thalamus, and Fezf2-PLAP^{+} axons fail to project into the cerebral peduncle, pyramidal tract, or the thalamic nuclei innervated by layer 5 neurons. Instead, Satb2-LacZ^{+} axons innervated the thalamic nuclei that normally receive inputs from layer 5 neurons. Satb2-LacZ^{+} axons do not project into the pyramidal tract. (*G*) SATB2 regulates cortical projection neuron fates in a cell context-dependent manner.

of SATB2 in corticofugal neurons is further supported by a recent gene-expression study of different cortical projection neuron subtypes (32). Although the authors used high SATB2 expression as a criteria to sort callosal neurons, SATB2 was not among the top 25 most specific markers to distinguish callosal neurons from corticofugal neurons. During cortical neurogenesis, SATB2 is expressed in the newly generated projection neurons in the intermediate zone (33). This expression of SATB2 ensures the expression of *Fezf2* and *Sox5* in newly generated subcerebral neurons. Indeed, reduced *Fezf2* mRNA was already evident in the E16.5 *Satb2*$^{lacZ/lacZ}$ mice (Fig. S3). However, SATB2 also represses the expression of *Ctip2* (16–18), which is necessary for the development of subcerebral axons (10). How do the developing subcerebral neurons overcome the inhibition of *Ctip2* by SATB2? The repression of high-level SATB2 by *Fezf2* is likely the key. Once *Fezf2* is expressed in subcerebral neurons, it inhibits high-level SATB2 expression (8), so that high-level CTIP2 is maintained in these cells. Indeed, although most newly generated CTIP2^{+} deep-layer neurons express SATB2 at E13.5 (18), by E15.5, high-SATB2^{+} and high-CTIP2^{+} neurons start to segregate into distinct populations (18, 33).

We propose that *Satb2* promotes the identities of subcerebral and callosal neurons in a cell context-dependent manner (Fig. 5*G*). In the presumptive subcerebral neurons, SATB2 activates the expression of *Fezf2*, *Sox5*, and other subcerebral neuronal genes. The high-level *Fezf2* in subcerebral neurons in turn represses *Satb2* expression. Reduced SATB2 ensures the expression levels of subcerebral neuronal genes such as *Ctip2* remain high in these cells, which further promotes a subcerebral neuron identity. In addition, reduced SATB2 in subcerebral neurons prevents expression of callosal neuron genes that are positively regulated by SATB2. In the developing callosal neurons, SATB2 does not activate *Fezf2*, and SATB2 level remains high. High SATB2 promotes the expression of upper-layer genes that are required for callosal neuron development and prevents expression of deep-layer neuron genes such as CTIP2, which may lead to corticofugal axon development (8).

Although this model can explain most of the molecular and axonal defects of the *Satb2* mutant mice, some important questions await further investigation. For example, what genes turn on *Satb2* expression in the newly generated projection neurons? Artegiani et al. recently showed that HMG group transcription factor Tox binds to *Satb2* gene and thus potentially regulates its expression (34). Dominguez et al. showed that electroporation of Brn1/2 at E12.5 led to precocious expression of *Satb2* in the cortical neurons (35). However, whether these genes directly activate *Satb2* transcription awaits further investigation. In addition, if SATB2 is expressed in presumptive subcerebral neurons and callosal neurons, why does it not activate *Fezf2* expression in callosal or upper-layer neurons? It is possible that other genes that are required to activate *Fezf2* expression are present only in corticofugal neurons, or that there are repressors in the callosal neurons that inhibits *Fezf2* expression. We emphasize that, although we have identified *Fezf2* and *Sox5* as likely direct targets of SATB2 in promoting subcerebral neuron differentiation, additional downstream targets of SATB2 may also be essential for the development of subcerebral axons. Further delineation of the functions of these genes may reveal novel regulatory mechanisms for subcerebral neuron fate specification.

Methods

All experiments on mice were carried out in accordance with the protocols approved by the institutional animal care and use committee at the University of California, Santa Cruz (UCSC), and institutional and federal guidelines. The day of vaginal plug detection was designated as E0.5. The day of birth was designated as P0.

The *Satb2*$^{lacZ/+}$, *Fezf2*$^{PLAP/+}$, *enhancer 434-lacZ*, *Emx1-Cre*, *Golli-GFP*, and *RCE-GFP*Cre reporter mice were described previously (6, 15, 16, 36–38). The *Satb2*$^{tm1a(KOMP)Wtsi/+}$ mice were obtained from the Knockout Mouse Project (KOMP) repository and bred with *Rosa26Sor*$^{tm2(FLP*)Sor}$ mice from the Jackson Laboratory (JAX number 007844) to generate the *Satb2*$^{flox(KOMP)/+}$ mice (referred as *Satb2*$^{flox/+}$ mice in this study).

Details of retrograde tracing, immunohistochemistry (9, 39), cell counting, and luciferase assays are described in *SI Methods*.

Gene Expression Analysis. RNA-seq was performed from P0 cortices of *Satb2*$^{+/+}$ and *Satb2*$^{lacZ/lacZ}$ mice ($n = 3$ for each genotype). The sequencing libraries were prepared following the TruSeq RNA sample preparation protocol (Illumina), and pair-end (50 bp per end) sequenced on an Illumina Genome Analyzer II platform. Approximately 40 million reads were obtained for each library. The reads were first mapped with Bowtie (40) against the set of RepeatMasker (www.repeatmasker.org, 1996–2010) elements in mouse to remove repetitive elements from further analysis. The remaining reads were mapped with TopHat (41) against the mouse (NCBI37/mm9) assembly. Only uniquely mapped and properly paired reads were kept. Potential PCR duplicates were removed with the SAMtools (42) "rmdup" command. The net count of remaining mappings for the six samples ranged from 27.8 million to 33.0 million. From these TopHat mappings, coverage of each gene in the set of UCSC Known Genes (43) for the mm9 assembly was determined. The read counts for each gene in each sample were used as input to DESEq. (44) for differential expression analysis. The resulting DESeq "sizeFactors" were used to normalize the read counts of individual replicates. Differentially expressed genes were defined as the ones that were decreased by 20% or increased by 20% in the *Satb2*$^{lacZ/lacZ}$ cortices, compared with the controls with DEseq, with a false discovery rate < 0.1.

ChIP-Seq Analysis. ChIP was performed on dissected E15.5 WT mouse cortices as previously described (9) by using an SATB2 antibody (ab34735; Abcam). The input DNA and SATB2 ChIPed DNA were pair-end (100 bp per end) sequenced on an Illumina Genome Analyzer II platform. Approximately 35 million and 33 million reads were obtained for the SATB2 ChIPed DNA and input DNA, respectively. The raw 100×100 bp paired-end reads were trimmed to 75×75 bp by discarding 5 bp from the 5′ end and 20 bp from the 3′ end of read1 and read2. The trimmed reads were first mapped with Bowtie (40) against the set of RepeatMasker elements to remove repetitive elements from further analysis. The remaining reads were mapped with Bowtie (40) to the mouse (NCBI37/mm9) assembly. Only uniquely mapped and properly paired reads were kept. Fewer than 2% of the mappings were found to be potential PCR duplicates, so they were not removed. The net count of remaining mappings was approximately 21 million for both the SATB2 ChIPed DNA and input DNA. Peak calling was done with the MACS 1.4 tool (45). For input to MACS, only read1 of the mapped reads was used, and the MACS "shiftsize" parameter was determined from the genomic length of the paired-end Bowtie mappings of the ChIPed sample.

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