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Fezl Is Required for the Birth and Specification of Corticospinal Motor Neurons

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Summary

The molecular mechanisms controlling the differentiation of neural progenitors into distinct subtypes of neurons during neocortical development are unknown. Here, we report that *Fezl* is required for the specification of corticospinal motor neurons and other subcerebral projection neurons, which are absent from *Fezl* null mutant neocortex. There is neither an increase in cell death in *Fezl*$^{-/-}$ cortex nor abnormalities in migration, indicating that the absence of subcerebral projection neurons is due to a failure in fate specification. In striking contrast, other neuronal populations in the same and other cortical layers are born normally. Overexpression of *Fezl* results in excess production of subcerebral projection neurons and arrested migration of these neurons in the germinal zone. These data indicate that *Fezl* plays a central role in the specification of corticospinal motor neurons and other subcerebral projection neurons, controlling early decisions regarding lineage-specific differentiation from neural progenitors.

Introduction

During development of the neocortex, distinct classes of excitatory projection neurons are born from progenitors in the germinal zone of the dorsal telencephalon (Kriegstein and Noctor, 2004). Neurogenesis and radial migration of neuronal subtypes are precisely orchestrated such that deep layer neurons are born before upper layer neurons, resulting in an "inside-out" pattern of cortical development (Angevine and Sidman, 1961; Rakic, 1972, 1974). Different types of projection neurons are born in subsequent waves of asymmetric cell divisions (Kornack and Rakic, 1995; Luskin et al., 1988; Walsh and Cepko, 1988) as cortical progenitors undergo changes in fate potential (Desai and McConnell, 2000; McConnell and Kaznowski, 1991; Mizutani and Saito, 2005). Great progress has been made in identifying some of the genes that are important in controlling early specification of cortical progenitors (reviewed in Schuurmans and Guillemot, 2002). These include *Emx2*, *Pax6*, and *Lhx2*, which control some aspects of general cortical specification (Bulchand et al., 2001; Monuki et al., 2001; Muzio et al., 2002). Other studies have identified genes that are expressed in the ventricular zone or subventricular zone during the generation of either deep (*Otx1*; Weimann et al., 1999) or upper layer (*Cux2*; Nieto et al., 2004; Zimmer et al., 2004; and *Svet1*; Tarabykin et al., 2001) neurons. However, the molecular mechanisms by which neural progenitors produce individual subtypes of projection neurons are not known.

Among the different populations of cortical projection neurons, corticospinal motor neurons (CSMN), which are clinically important neurons that degenerate in amyotrophic lateral sclerosis (ALS) and are damaged in spinal cord injury, are an ideal model population for studying fate specification of individual neuronal subtypes in neocortex. CSMN and other subcerebral projection neurons are a readily identifiable and highly interrelated population of cortical projection neurons, located in layer V of cortex, that project to targets in the spinal cord and brainstem (Arlotta et al., 2005; O'Leary and Koester, 1993). During development, all subcerebral projection neurons are born at similar times and first extend axons toward the pyramidal tract before extending collaterals to other targets. Only later in development does area-specific pruning yield the adult pattern of connectivity (e.g., CSMN maintain projections to the spinal cord and caudal pons while corticotectal projection neurons maintain an axon to the superior colliculus and rostral pons) (O'Leary and Koester, 1993). Therefore, it is likely that the genes that control the early events of CSMN development will be shared by other subcerebral projection neurons.

In a previous study, we sought to identify transcription factors that might be involved in the earliest events of CSMN fate specification (Arlotta et al., 2005). One molecule, *Forebrain embryonic zinc finger-like* (*Fezl*; also referred to as *Zfp312*, *Fez1*, and *Fez*), which we found to be expressed in CSMN and other subcerebral projection neurons, is a particularly promising candidate for several reasons. It is a six zinc finger domain-containing transcription factor that is conserved from *Drosophila* to human (Hashimoto et al., 2000; Matsuo-Takasaki et al., 2000; Yang et al., 2001). In mouse, we found that *Fezl* is expressed at a constant level in CSMN and other subcerebral projection neurons from E18.5 to P14 (Arlotta et al., 2005). In addition, during earlier stages of development, *Fezl* is first expressed as early as E8.5 in the dorsal telencephalic wall (Hirata et al., 2004; Matsuo-Takasaki et al., 2000). Between E12.5 and E14.5, when subcerebral projection neurons are born, *Fezl* is detected in the ventricular zone in a pattern consistent with the location of neural progeni-

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tors and in the developing cortical plate (Hirata et al., 2004; Inoue et al., 2004), consistent with the position of newborn subcerebral projection neurons. Initial analysis of *Fezl* null mutants suggested that cortical layers, including the subplate, are generated normally but that the subplate exhibits abnormalities in maturation (Hirata et al., 2004). These studies did not investigate the development of CSMN or other subcerebral projection neurons. Therefore, it remained unclear if any neuronal populations of the cortex are affected in *Fezl*$^{-/-}$ mice.

Taken together, these data from our group and others led us to hypothesize that *Fezl* might be critically involved in the specification of CSMN and other subcerebral projection neurons. To test this hypothesis, we examined *Fezl* null mutant mice, employing a number of neuron type-specific markers to allow precise delineation of the development of different populations of cortical neurons. We find that, in the absence of *Fezl*, no subcerebral projection neurons are born and no cortical projections to the brainstem or the spinal cord ever develop. In contrast, other populations of neurons are unaffected. Gain-of-function experiments using electroporation of *Fezl* into the ventricular zone during embryonic development results in the production of subcerebral projection neurons, defined by molecular properties and anatomical connectivity. We conclude that in vivo *Fezl* is required for the initial fate specification of corticospinal motor neurons and the other closely related subcerebral projection neurons.

Results

Fezl Is Expressed in Subcerebral Projection Neurons of Layer V

We previously found that *Fezl* is expressed in CSMN and corticotectal projection neurons, two highly related subtypes of subcerebral projection neurons, throughout their development (Figure 1A and Arlotta et al., 2005), suggesting that this transcription factor could play a role in fate specification and maintenance of this neuronal lineage.

In order to determine whether *Fezl* is expressed in the ventricular zone during early stages of subcerebral projection neuron neurogenesis, we performed in situ hybridization for *Fezl* at E13.5, during peak neurogenesis of these neurons (Hevner et al., 2003; Polleux et al., 2001). Consistent with previous studies examining *Fezl* expression during development (Hirata et al., 2004; Inoue et al., 2004), we found that at this early stage *Fezl* is expressed in the ventricular zone and subventricular zone at low levels, and its expression is much higher in the developing cortical plate, where postmitotic neurons are positioned (Figures 1B and 1C). Additionally, the expression of *Fezl* in the cortical plate exhibits a high rostral/low caudal and high lateral/low medial gradient (Figures 1B and 1C), identical to the neurogenic gradient in the neocortex (Bayer and Altman, 1987). This temporally and spatially distinct expression profile indicates that *Fezl* is expressed early enough to play a role in the first stages of subcerebral fate specification and that higher levels of *Fezl* might be involved in differentiation into subcerebral neurons once migrating neuroblasts reach the cortical plate.

By retrograde labeling of CSMN combined with in situ hybridization for *Fezl*, we previously confirmed our microarray data and found that *Fezl* is highly expressed by CSMN (Arlotta et al., 2005). Intriguingly, the pattern of high levels of *Fezl* expression observed in layer V of neocortex (Figure 1D) is identical to the distribution of subcerebral projection neurons of layer V (Figure 1E), suggesting that *Fezl* is likely expressed at high levels in all subcerebral projection neuron populations of this layer (Arlotta et al., 2005; Hirata et al., 2004; Inoue et al., 2004). To test this hypothesis, we retrogradely labeled subcerebral projection neurons by injecting DiI into the pons at P1, followed by photoconversion of DiI to a permanent precipitate. We find that virtually all subcerebral projection neurons are also *Fezl* positive (Figure 1E′). Together, these prior data and our new results indicate that *Fezl* is expressed at the appropriate time

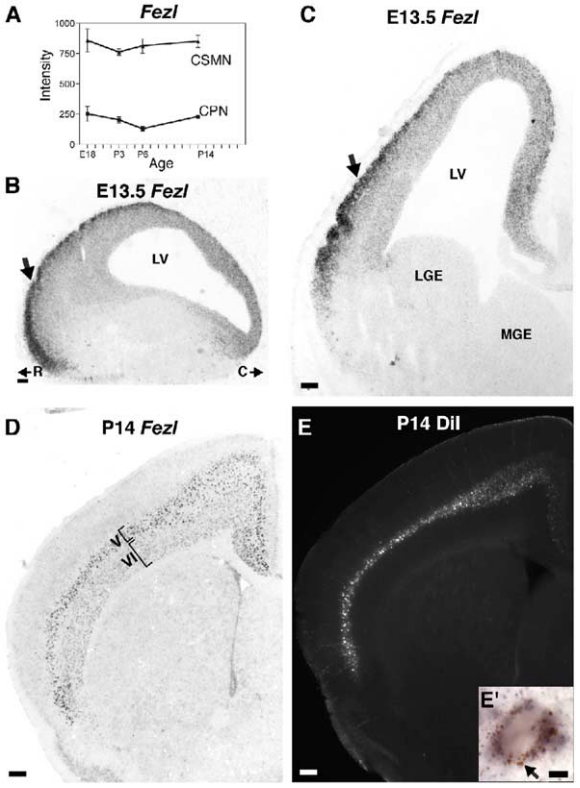

Figure 1. *Fezl* Is Expressed in Subcerebral Projection Neurons Throughout Development

(A) Microarray expression profiling reveals that *Fezl* is expressed at stable levels in CSMN throughout development (modified from Arlotta et al., 2005).

(B and C) In situ hybridization for *Fezl* at E13.5 in sagittal (B) and coronal (C) section, showing highest expression in the cortical plate (arrow).

(D and E) In situ hybridization for *Fezl* at P14 (D) indicates that *Fezl* is expressed at high levels in layer V in the same distribution as subcerebral projection neurons, identified by DiI retrograde labeling (E). (E′) In situ hybridization for *Fezl* (purple in situ signal) after retrograde labeling of subcerebral projection neurons with DiI (brown precipitate; arrow) confirms expression in subcerebral projection neurons. R, rostral, to C, caudal, indicated in (B). LV, lateral ventricle; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence.

Scale bars, 100 μm (B and C), 250 μm (D and E), 5 μm (E′).

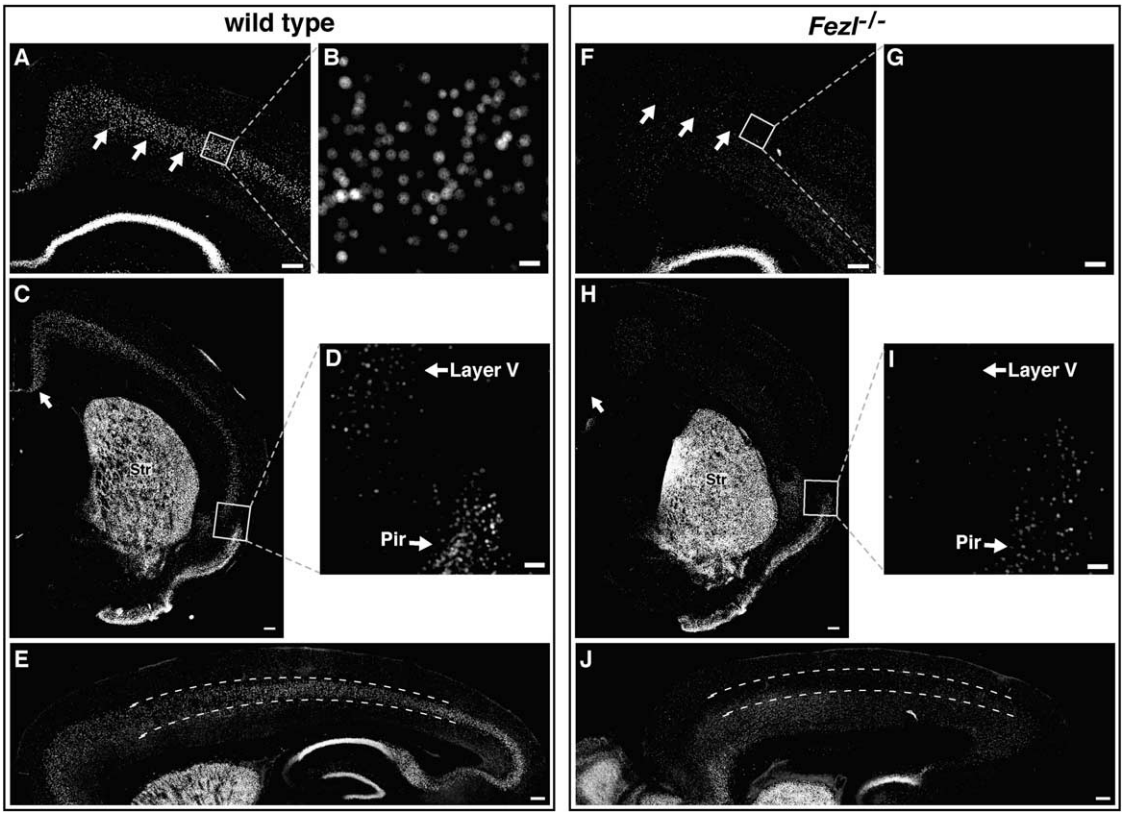

Figure 2. *Fezl*$^{-/-}$ Cortex Lacks All CSMN and Subcerebral Projection Neurons, Indicated by CTIP2 Expression

(A and E) Wild-type neocortex in coronal (A) and sagittal (E) sections, showing subcerebral projection neurons in layer V (arrows in [A] and dotted lines in [E]) intensely expressing CTIP2.

(B) Boxed area in (A).

(F and J) Coronal (F) and sagittal (J) sections matching those in (A) and (E) from a *Fezl*$^{-/-}$ brain showing absence of all CTIP2-positive neurons from layer V (arrows in [F]; dotted lines in [J]).

(G) Boxed area in (F).

(C) Coronal section of wild-type brain showing normal CTIP2 staining across the entire mediolateral extent of layer V, in piriform cortex (Pir), and in striatum (Str).

(D) Boxed area in (C).

(H) Section matching that in (C) from a *Fezl*$^{-/-}$ brain showing lack of subcerebral projection neurons from layer V, while CTIP2-expressing neurons in piriform cortex and striatum are not affected.

(I) Boxed area in (H).

Scale bars, 200 μm (A, C, E, F, H, and J), 20 μm (B and G), 50 μm (D and I).

and place to be involved in the early fate specification of CSMN and other subcerebral projection neurons.

Loss of the *Fezl* Gene Results in Absence of CSMN and Other Subcerebral Projection Neurons

Our *Fezl* expression data motivated us to examine *Fezl* null mutant mice to determine whether *Fezl* might play a role in the specification of subcerebral projection neurons. We used CTIP2, a transcription factor that we previously showed to be a specific marker of layer V subcerebral projection neurons (Arlotta et al., 2005), to visualize them. We find that, in the absence of *Fezl*, there is a striking absence of the entire population of subcerebral projection neurons, normally located in layer V and identified by high-level CTIP2 expression (Figures 2A, 2B, 2F, and 2G). This subtype-specific neuron deficiency occurs throughout the entire rostrocaudal extent of layer V (Figures 2E and 2J), and it includes CSMN and corticotectal projection neurons. In contrast, CTIP2 expression in the striatum remains unchanged, as it does in piriform cortex, strongly supporting the hypothesis that *Fezl* effects are neuronal subtype specific (Figures 2C, 2D, 2H, and 2I).

To rigorously investigate whether the loss of CTIP2 expression represents a true absence of the population of subcerebral projection neurons or simply a *Fezl*-mediated lack of expression of CTIP2, we performed careful histological analysis of *Fezl*$^{-/-}$ neocortex. Even at low magnification, it is apparent that layer V projection neurons are entirely missing in *Fezl*$^{-/-}$ mice (Figures 3A and 3D). Higher-magnification analysis confirms that all large neurons with pyramidal morphology are missing from layer V of the *Fezl*$^{-/-}$ mutants (Figures 3B, 3C, 3E, and 3F), with complete loss of all neurons greater than ~20 μm in diameter.

To further investigate the molecular phenotype of the absent cells, we took advantage of our recent identification of genes that define CSMN, *Diap3* and *Crim1*,

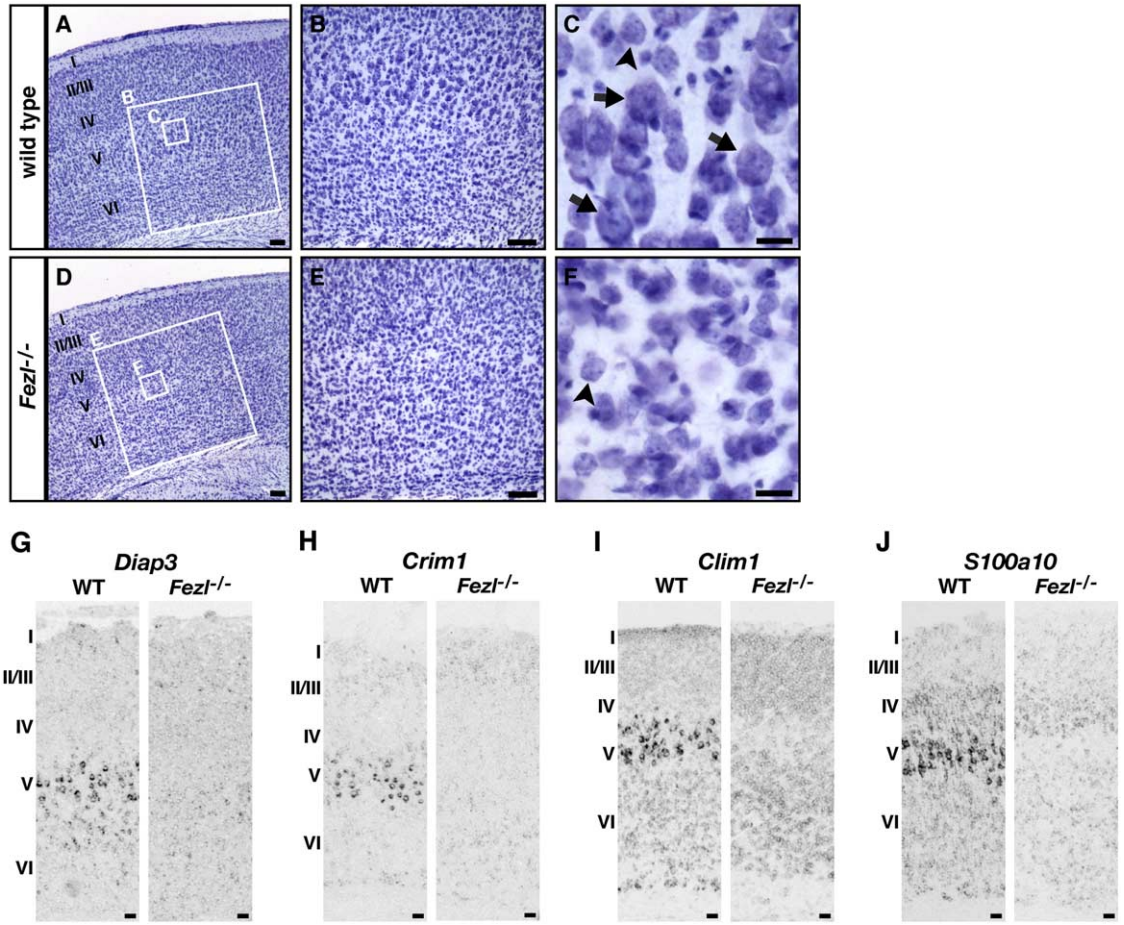

Figure 3. Large CSMN and Other Subcerebral Projection Neurons Are Missing in *Fezl*$^{-/-}$ Cortex by Morphological Criteria and Molecular Markers

(A and D) Nissl staining of cortex in sensorimotor area in (A) wild-type and (D) *Fezl*$^{-/-}$ mice.

(B and C) Areas boxed in (A), showing distinct large somas of CSMN (arrows) in layer V and smaller size neighboring neurons (arrowhead) in wild-type mice.

(E and F) Areas boxed in (D), showing the absence of all large CSMN somas while smaller neurons are unaffected (arrowhead) in *Fezl*$^{-/-}$ mice.

(G–J) In situ hybridization on coronal sections for four subcerebral projection neuron-specific markers, *Diap3*, *Crim1*, *Clim1*, and *S100a10*, showing absence of expression in *Fezl*$^{-/-}$ mice compared to wild-type mice (wt). Ages: Nissl, P28; *Diap3* and *Crim1*, P14; *Clim1* and *S100a10*, P6.

Scale bars, 100 μm (A, B, D, and E), 20 μm (C and F), 50 μm (G–J).

or that delineate subcerebral projection neurons from callosal projection neurons, *Clim1* and *S100a10* (Arlotta et al., 2005). Using these four molecular markers, we found that there is a striking absence of expression of each of these markers in layer V, confirming that CSMN and the rest of the population of subcerebral projection neurons are absent (Figures 3G–3J).

Lack of CSMN and Other Subcerebral Projection Neurons Is Not Due to Cell Death or Abnormal Neuronal Migration in *Fezl*$^{-/-}$ Mice

The absence of subcerebral projection neurons suggests that *Fezl* might be required for the initial fate specification of this neuronal lineage. However, two alternative possibilities exist: (1) *Fezl* might control subcerebral projection neuron survival; or (2) subcerebral projection neurons could be initially born but fail to migrate to the appropriate laminar location.

We investigated both of these possibilities directly and found neither to be the case. To assess the possibility of early death of newborn subcerebral projection neurons, we performed FluoroJade and TUNEL staining at E14.5 (n = 2 wt and n = 2 *Fezl*$^{-/-}$), when subcerebral projection neurons have just been born, and at P0 (n = 2 wt and n = 2 *Fezl*$^{-/-}$), when they have migrated to the cortical plate. We find no difference in FluoroJade-positive or TUNEL-positive cells in the ventricular zone or in any part of the developing cortical plate (Figures 4A and 4B and data not shown). In addition, similar to our findings at P7, cells expressing high levels of CTIP2 are absent from the developing cortical plate at E16.5 (Figures 4C, 4C′, 4D, and 4D′) and from layer V at P0 (data not shown), further supporting the hypothesis

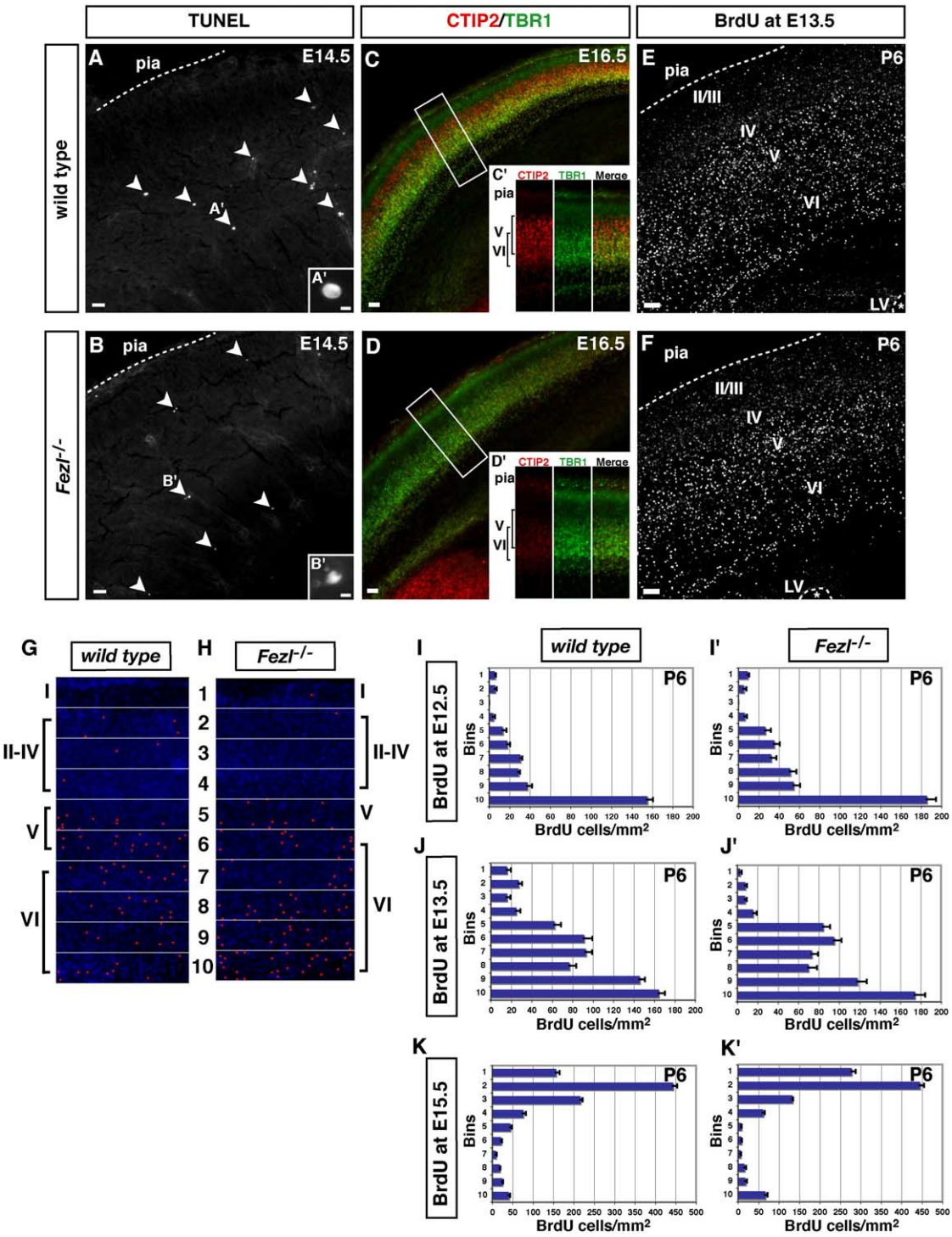

Figure 4. *Fezl*$^{-/-}$ Progenitors Do Not Exhibit Increased Cell Death or Migratory Abnormalities

(A and B) TUNEL staining of cortex at E14.5 reveals comparable rates of cell death in wild-type (A) and *Fezl*$^{-/-}$ (B) mice. (A′ and B′) Images of single TUNEL-positive cells in wild-type (A′), and *Fezl*$^{-/-}$ (B′) mice.

(C and C′) In E16.5 wild-type mice, CTIP2-positive subcerebral projection neurons (red) are seen during migration through layer VI (TBR1 in green).

(D and D′) CTIP2-positive subcerebral projection neurons are absent from *Fezl*$^{-/-}$ cortex, while TBR1 and low-level CTIP2-expressing neurons of layer VI are unaffected.

(E and F) BrdU labeling of P6 cortex after a single pulse of BrdU at E13.5, showing no difference in the distribution and migration of labeled neurons between wild-type (E) and *Fezl*$^{-/-}$ (F) mice.

(G and H) Cells scored as BrdU positive (red) in matched 500 μm wide sections of P6 cortex (BrdU labeled at E13.5). 1–10, bins of equal depth spanning the thickness of cortex.

(I–K) Quantification of BrdU-positive cells born at E12.5 (I and I′), E13.5 (J and J′), and E15.5 (K and K′) showing no differences between wild-type (I–K) and *Fezl*$^{-/-}$ (I′–K′) mice.

Error bars indicate SEM. LV, lateral ventricle (asterisk). Roman numerals indicate cortical layers. Scale bars, 20 μm (A and B), 2 μm (A′ and B′), 50 μm (C and D), 100 μm (E and F).

that subcerebral projection neurons are absent from the earliest stages of cortical development. Therefore, the lack of subcerebral projection neurons in *Fezl*$^{-/-}$ mice is not due to early death of this neuronal subtype.

To assess the second possibility, of neurons failing to migrate to layer V from their birthplace in the ventricular zone, we assessed laminar positioning of subcerebral projection neurons by birthdate analysis via injections of BrdU at three stages of cortical projection neuron neurogenesis: (1) E12.5, the time of peak production of layer VI neurons; (2) E13.5, the time of peak production of subcerebral projection neurons; and (3) E15.5, the time of peak production of layer II/III neurons. We find no qualitative difference in neuronal migration behavior between *Fezl*$^{-/-}$ and wild-type mice at any of these birthdays; e.g., there is no population of cells that does not enter the cortical plate (Figures 4E and 4F [E13.5] and data not shown [E12.5 and E15.5]). To determine whether the normal pattern of inside-out cortical neurogenesis is preserved in the *Fezl* mutants, we quantified the number of BrdU-positive cells located at a range of depths covering all cortical layers of P6 mice that were labeled by BrdU injection at E12.5 (n = 3 wt; n = 3 *Fezl*$^{-/-}$), E13.5 (n = 3 wt; n = 3 *Fezl*$^{-/-}$), or E15.5 (n = 3 wt; n = 3 *Fezl*$^{-/-}$). Even at this fine level of spatial resolution, we find no difference between *Fezl*$^{-/-}$ and wild-type control mice in the final distribution of neurons into cortical layers (Figures 4G–4K′). Together, these data demonstrate that neither neuronal death nor a migrational abnormality accounts for the absence of subcerebral projection neurons and strongly support the hypothesis that *Fezl* is involved in the initial fate specification of these neurons.

Layer VI Is Expanded in *Fezl*$^{-/-}$ Mice

To determine whether there is a change in total cortical thickness or in the thickness of individual layers in the absence of *Fezl*, we measured the thickness of the cortex and individual lamina (Figures 5A–5C). Interestingly, despite the absence of layer V subcerebral neurons and an accompanying reduction in layer V thickness by 44% (mean layer V thickness: wt, 242 ± 5 μm, n = 11; *Fezl*$^{-/-}$, 136 ± 4 μm, n = 9; $p = 3 \times 10^{-12}$), we find that the total cortical thickness is identical in wild-type and *Fezl*$^{-/-}$ cortex (mean cortical thickness: wt, 1075 ± 26 μm, n =11; *Fezl*$^{-/-}$, 1031 ± 23 μm, n = 9; p = 0.24). The decrease in layer V thickness appears to be compensated for by a concomitant 23% increase in the thickness of layer VI in *Fezl*$^{-/-}$ cortex (mean layer VI thickness: wt, 456 ± 20 μm, n = 11; *Fezl*$^{-/-}$, 563 ± 11 μm, n = 9; $p = 3 \times 10^{-4}$). In contrast, the thickness of layers II-IV is identical in wild-type and *Fezl*$^{-/-}$ cortex (mean II-IV thickness: wt, 288 ± 11 μm, n = 11; *Fezl*$^{-/-}$, 276 ± 12 μm, n = 9; p = 0.45). The reduction of layer V and increase of layer VI thickness suggests the intriguing possibility that lack of *Fezl* might result in a fate switch whereby neural progenitors continue generating layer VI neurons instead of producing layer V subcerebral projection neurons.

To further investigate this possibility, we analyzed more precisely the cellular identity of layer VI neurons in *Fezl*$^{-/-}$ cortex. Although there is an absence of CTIP2 expression in layer V of *Fezl*$^{-/-}$ mice, there was no difference in layer VI neurons with low-level CTIP2 expression in *Fezl*$^{-/-}$ compared to wild-type mice (Figure 5D), supporting the interpretation that layer VI neurons are born normally and migrate appropriately to the correct laminar location. However, when we examined layer VI neurons using TBR1, a transcription factor expressed at high levels in neurons of layer VI (Figure 5E) (Bulfone et al., 1995; Hevner et al., 2003), we observed a layer of neurons expressing unusually high levels of TBR1 at the interface between layers V and VI in *Fezl*$^{-/-}$ cortex but not in wild-type cortex (Figures 5E and 5F). Importantly, these neurons are molecularly distinct from the population of neurons expressing TBR1 at lower levels that are normally found in layer VI, many of which coexpress CTIP2 and TBR1 (Figure 5F). The vast majority of these ectopic neurons are only positive for TBR1 and negative for CTIP2 (Figures 5F′–5F‴). Although the level of TBR1 expression in these abnormal neurons is clearly higher than that normally found in nonsubcerebral layer V neurons, the molecular phenotype of these heterotopic neurons is more like that of CTIP2 negative/low-level expressing TBR1 cells that are normally dispersed through layer V and intermixed with subcerebral projection neurons (Figure 5F′). These results demonstrate that, in the absence of *Fezl* function, an unusual population of neurons that are TBR1 high expressing and mostly CTIP2 negative accumulates in a tightly packed band at the border of layer V and VI and strongly indicate that in the absence of *Fezl* a fate switch has occurred at the level of progenitor cell specification and differentiation to produce extra, but abnormal, layer VI neurons.

Layer VI Neurons Fail to Mature in *Fezl*$^{-/-}$ Mice

In addition to the high level expression of *Fezl* in CSMN and other subcerebral projection neurons of layer V, *Fezl* is also expressed at much lower levels in layer VI neurons (Figure 1D). Together with the abnormalities we observed in layer VI thickness, this motivated us to further investigate layer VI neurons to examine whether their maturation is affected by the absence of *Fezl* function. While the early specification and migration of layer VI neurons appear normal in *Fezl*$^{-/-}$ mice, layer VI is disorganized (Figures 3A, 3D, 5A, and 5B), and we observe distinct molecular abnormalities at later stages of development. We used four different molecular markers that identify these neurons at mid and late stages of development. We used *T cell receptor* β (*Tcr*β), known to be expressed in layer V and VI cortical neurons (Syken and Shatz, 2003), which we recently found to be expressed in deep layer V callosal projection neurons (overlapping with nominal layer VI in mouse) (B.J.M. et al., unpublished data). A second marker, DARPP-32, though typically associated with medium spiny neurons in the striatum, has also been localized to cortical layer VI (Ouimet et al., 1984), where we find that it colocalizes with low-level CTIP2-expressing neurons, most likely corticothalamic neurons (B.J.M. et al., unpublished data). A third marker, *Crystallin-mu*, a gene that we recently found to be expressed at high levels in layer V neurons, including CSMN, also labels deep layer VI neurons at lower levels (Arlotta et al., 2005). Finally, *Foxp2*, a transcription factor that labels layer VI neu-

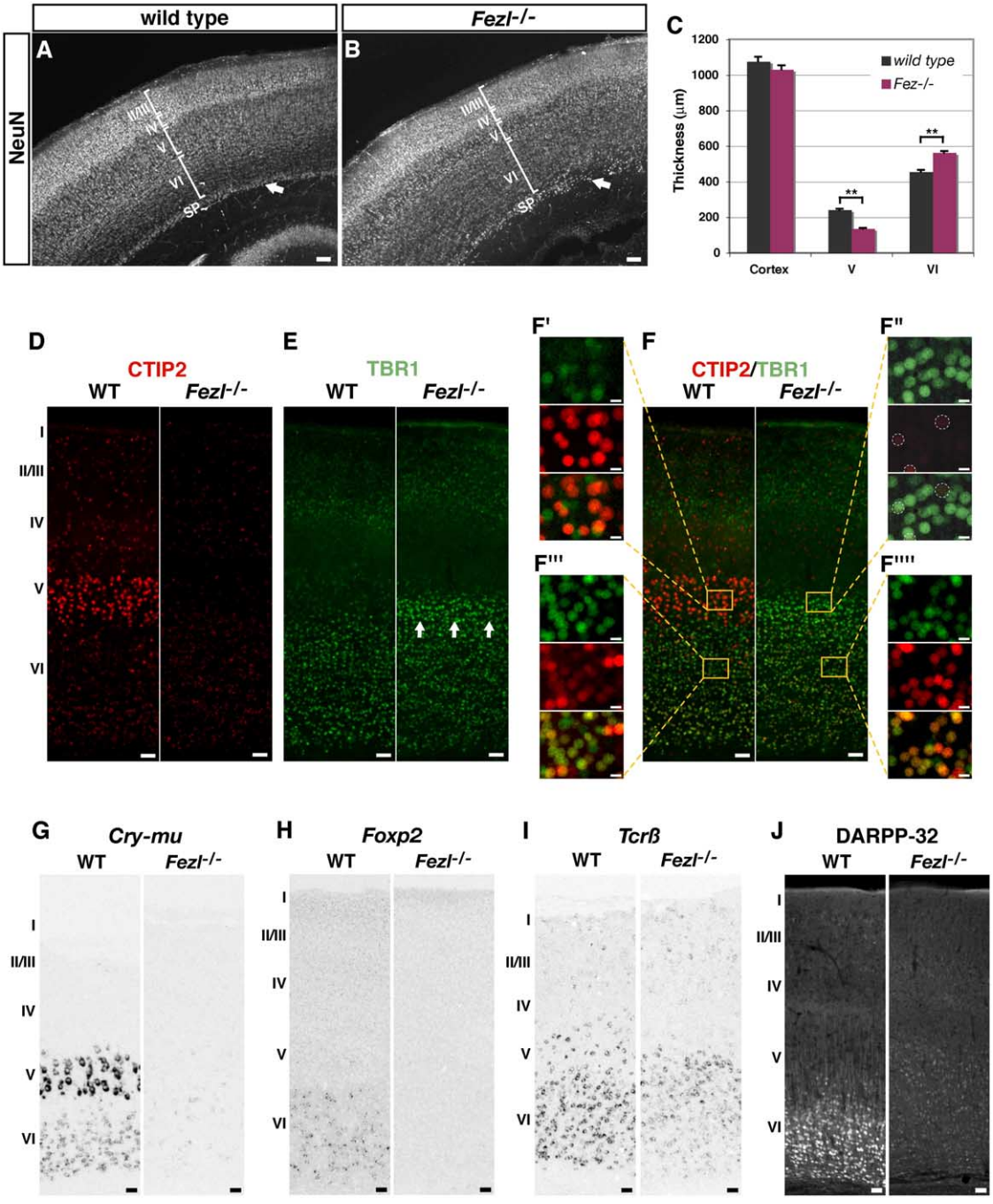

Figure 5. Layer VI Neurons Are Born Correctly, but Show Late Differentiation Abnormalities in *Fezl*$^{-/-}$ Mice

(A and B) NeuN immunocytochemistry on (A) wild-type and (B) *Fezl*$^{-/-}$ cortex reveals a decrease in layer V thickness and an increase in layer VI thickness in *Fezl*$^{-/-}$ cortex as well as disorganization of the subplate in *Fezl*$^{-/-}$ cortex (arrow).

(C) Quantification of layer and cortical thickness in wild-type (gray) and *Fezl*$^{-/-}$ (purple) mice (error bars indicate SEM).

(D and E) While high-level CTIP2-expressing subcerebral projection neurons are absent from layer V of *Fezl*$^{-/-}$ mice, low-level CTIP2-expressing (D) and TBR1-expressing (E) neurons of layer VI form normally in *Fezl*$^{-/-}$ mutant mice. However, layer VI is expanded, with an abnormal, compact population of intensely TBR1-positive cells present in *Fezl*$^{-/-}$ mice (arrows in [E]).

(F) The vast majority of the intensely TBR1-positive cells are CTIP2 negative (F″), as are TBR1-positive cells in layer V of wild-type neocortex (F′). This is in contrast to the TBR1-positive cells of layer VI, most of which are CTIP2 positive (F‴–F⁗). Dotted circles in (F″) indicate low-level CTIP2-expressing cells. Electronic gain in (F‴) and (F⁗) is increased approximately 3-fold compared to (F′) and(F″).

(G–J) In situ hybridization on P14 coronal sections (*Cry-mu*, *Foxp2*, *TCR*β) and immunocytochemistry on P28 coronal sections (DARPP-32) shows that expression is dramatically downregulated in layer VI of *Fezl*$^{-/-}$ mice compared to wild-type (wt) mice.

Scale bars, 100 μm (A and B), 50 μm (D–J), 10 μm (F′–F⁗).

rons throughout development (Ferland et al., 2003), was also used to identify layer VI neurons.

By using this panel of molecular markers, we find that, in contrast to the normal expression of CTIP2 and TBR1 in deep layer VI neurons, all four markers of more mature layer VI neurons are abnormal in *Fezl*$^{-/-}$ mice. Analysis of *Crystallin-mu* and *Foxp2* expression reveals an almost total elimination of expression of these markers in layer V and VI for *Crystallin-mu* (Figure 5G) and layer VI for *Foxp2* (Figure 5H). Analysis of *Tcr*β and DARPP-32 reveals a decreased level of expression, most pronounced in deep layer VI (Figures 5I and 5J).

In a fashion analogous to the subtly abnormal generation of layer VI neurons that we observed, Hirata et al. (2004) reported that subplate neurons are generated normally, but that GAP-43 (labeling both cell bodies and passing axons) and CSPG (labeling extracellular matrix) expression in the subplate are abnormal at E16.5, suggesting that the subplate is not organized properly. To examine subplate neurons at a later stage of development, when they can be better distinguished from neuroblasts migrating to more superficial cortical layers, we labeled for NeuN at P6, which allows the clear identification of typical tightly packed and highly organized subplate neurons in wild-type cortex (Figure 5A). We find that subplate neurons in *Fezl*$^{-/-}$ cortex are extremely disorganized, but distinctly present (Figure 5B).

Taken together, these results demonstrate that loss of *Fezl* function not only results in a total elimination of the large subcerebral projection neurons in layer V, which express *Fezl* at high levels, but also results in more subtly abnormal development of the population of deep layer V and VI neurons that express *Fezl* at lower levels.

Upper Layer Neurons Develop Normally in *Fezl*$^{-/-}$ Mice

In order to determine whether lack of *Fezl* affects cortical layer II/III and IV neurons, we investigated the phenotype of upper layer neurons in *Fezl*$^{-/-}$ and wild-type mice. In striking contrast to the complete absence of subcerebral projection neurons in layer V and the abnormal deep layer VI neurons in *Fezl*$^{-/-}$ mice, we find that the differentiation of superficial layers in *Fezl*$^{-/-}$ mice appears entirely normal histologically and by a variety of molecular markers. *Cux2*, a recently identified marker of layer II-IV (Nieto et al., 2004; Zimmer et al., 2004), shows no difference in laminar distribution between *Fezl*$^{-/-}$ and wild-type mice (Figure 6A). Similarly, BRN2 (McEvilly et al., 2002; Sugitani et al., 2002) and *Igfbp4* (Arlotta et al., 2005) also exhibit comparable distribution and intensity of expression in layers II/III in *Fezl*$^{-/-}$ and wild-type mice. In contrast, both BRN2-expressing and *Igfbp4*-expressing neurons are completely absent from layer V of *Fezl*$^{-/-}$ cortex (Figures 6B and 6C). Additionally, we find that there is no difference between wild-type and *Fezl*$^{-/-}$ mutants with regard to the distribution and expression levels of RORβ (Figure 6D), a marker of layer IV neurons (Schaeren-Wiemers et al., 1997). Finally, *Plexin-D1*, a recently identified gene (van der Zwaag et al., 2002) that we find labels a subpopulation of superficial layer callosal projection neurons in layers II/III and Va (B.J.M. et al., unpublished data), exhibits unaltered expression in wild-type and *Fezl*$^{-/-}$ cortex (Figure 6E). Together, these data showing unperturbed upper cortical layer development argue strongly that *Fezl* is not involved simply in basic aspects of early cortical differentiation but rather is specifically involved in the specification and differentiation of deep layer projection neurons, most centrally with subcerebral projection neurons.

While molecular marker analysis indicates that upper layer neurons are born and develop normally in the absence of *Fezl*, we observed defects in *Fezl*$^{-/-}$ brains in the formation of the corpus callosum, through which many of these neurons project. Axons in the *Fezl*$^{-/-}$ mutants form Probst bundles (Figures 6F), structures typically observed in genetic mutants with callosal agenesis (Richards et al., 2004). Prior studies have found that these generally have normal projection neurons, while the defect is typically either the result of lack of midline fusion or abnormal signaling at the midline (Ozaki and Wahlsten, 1993; Richards et al., 2004). Early arriving axons tend to form Probst bundles, while some later arriving axons are able to cross to the contralateral hemisphere through an enlarged hippocampal commissure (Ozaki and Wahlsten, 1993). To determine whether *Fezl* null mutants have a similar phenotype, we injected DiI into the hippocampal commissure and found that projection neurons throughout neocortex are labeled (data not shown). Because callosal projection neurons have normal molecular profiles (Figures 6A–6E) and extend projections, the absence of the corpus callosum in *Fezl* null mutants is likely not due to abnormalities in callosal projection neurons. Rather, callosal agenesis is likely due to abnormal guidance cues at the midline or due to abnormalities in pioneering axons from neurons of the cingulate cortex, as suggested by the absence of CTIP2-expressing neurons of the cingulate cortex all the way to the midline (Figure 2F and arrow in 2H).

Complete Absence of Subcerebral and Spinal Cord Axonal Projections in *Fezl*$^{-/-}$ Mice

In order to conclusively determine whether the subcerebral projection neuron population is truly absent, it is critical to assess whether long-distance connections are formed by these neurons. While no subcerebral projection neuron somas exist in layer V, as assessed by Nissl staining or by eight independent molecular markers, there existed the theoretical possibility that an alternate population of neurons might send atypical subcerebral projections or that subcerebral projection neurons might exist in an altered form or ectopic location in the neocortex. To rigorously investigate these possibilities, we performed in vivo DiI anterograde tracing in an attempt to detect any subcerebral axonal projections; injections of DiI into sensorimotor cortex were performed at P3. Using these methods, thalamic projections were clearly visible, but no projections were present beyond the thalamus to the brainstem or spinal cord in *Fezl*$^{-/-}$ brains (n = 8) (Figure 7D); such projections were always distinctly present in wild-type and *Fezl*$^{+/-}$ brains (n = 9) (Figure 7A).

To determine whether even a small number of subcerebral projections might be detected at later stages of development, we examined a separate set of *Fezl*

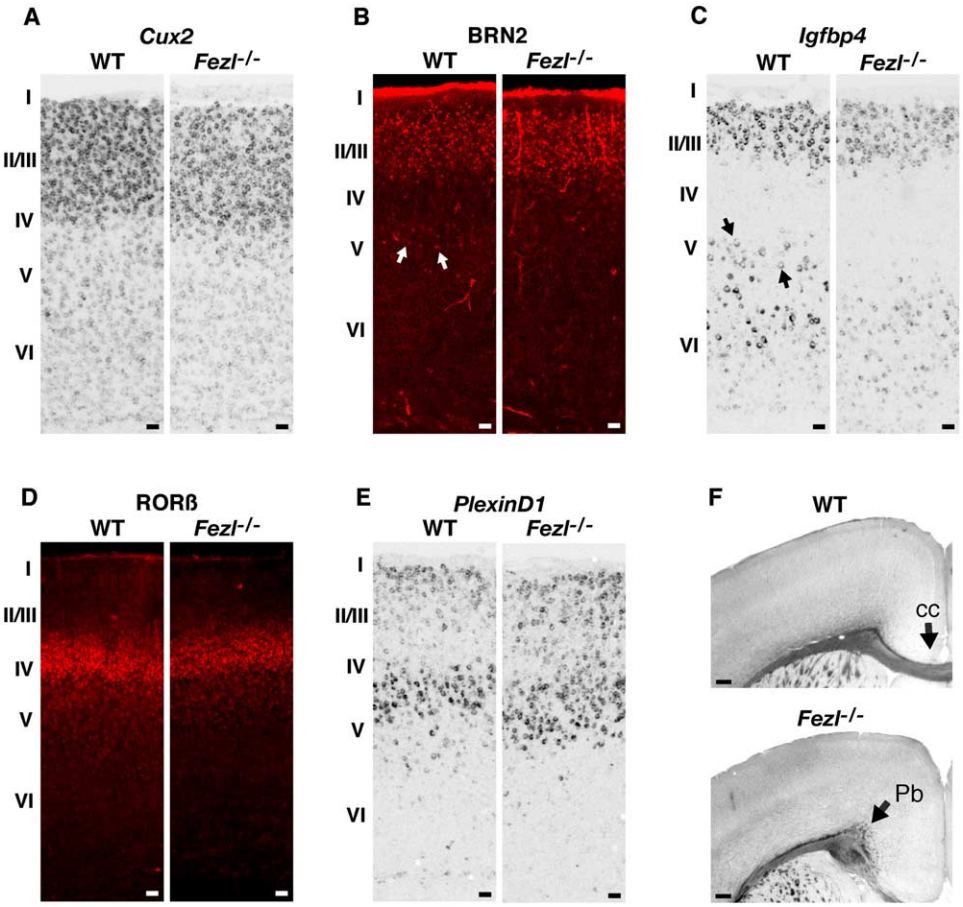

Figure 6. Upper Layers II/III and IV Develop Normally in *Fezl*$^{-/-}$ Mice

(A–E) In situ hybridization at P14 (A, C, and E) and immunocytochemistry at P7 (B and D) on coronal sections of neocortex, showing comparable expression of layer II/III (A, B, C, and E), layer IV (D), and layer Va (E) markers in *Fezl*$^{-/-}$ and wild-type (wt) mice. (A) *Cux2.* (B and C) BRN2 (B) and *Igfbp4* (C) exhibit comparable expression in layers II/III in *Fezl*$^{-/-}$ and wild-type mice, while BRN2-expressing and *Igfbp4*-expressing neurons normally present in layer V are absent from *Fezl*$^{-/-}$ cortex (arrows). (D) RORβ. (E) *Plexin-D1*.

(F) Coronal section of cortex at P28 showing Probst bundles (Pb) and aborted formation of the corpus callosum (cc) in *Fezl*$^{-/-}$ mice.

Scale bars, 50 μm (A–E), 200 μm (F).

mutants injected with DiI at P6, when all axons have normally reached the spinal cord in wild-type mice (Polleux et al., 2001). Again, no cortical axons projected to the spinal cord or brainstem of this independent group of *Fezl* null mutants (n = 5), while all wild-type and *Fezl*$^{+/-}$ mice (n = 5) display a normal corticospinal tract (Figures 7B and 7E). The striking and absolute absence of the corticospinal tract from *Fezl*$^{-/-}$ spinal cords is so distinct that it can even be detected by oblique coherent contrast imaging without anterograde labeling (Figures 7B and 7E).

To even further exclude the possibility that an extremely small number of cortical neurons in *Fezl*$^{-/-}$ mutant mice might project to the spinal cord but not be detected by anterograde tracing, we performed retrograde labeling from the cervical spinal cord to label all corticospinal projections. We injected fluorescent microspheres in both mutant and wild-type mice and examined the cortex for any retrogradely labeled neurons at P6. There were no retrogradely labeled neurons in the neocortex of the *Fezl*$^{-/-}$ mice (n = 5), while all wild-type mice (n = 4) exhibited typical CSMN labeling (Figures 6C and 6F). Together with the prior data regarding the loss of the large layer V projection neuron population with the molecular characteristics of subcerebral projection neurons, these results definitively establish that CSMN and all other subcerebral projection neurons are entirely absent in *Fezl*$^{-/-}$ mice.

Overexpression of *Fezl* Induces the Generation of CTIP2-Positive Neurons

The loss-of-function experiments described above demonstrate that *Fezl* is necessary for the specification of CSMN and other subcerebral projection neurons in vivo. In order to investigate whether *Fezl* is sufficient to specify neural progenitors to the subcerebral projection neuron lineage, we performed gain-of-function experiments by overexpressing *Fezl* via in vivo electroporation. This allows for precise control over the timing and location of overexpression in an otherwise wild-type environment (Saito and Nakatsuji, 2001). We expressed *Fezl* under the control of a constitutively active

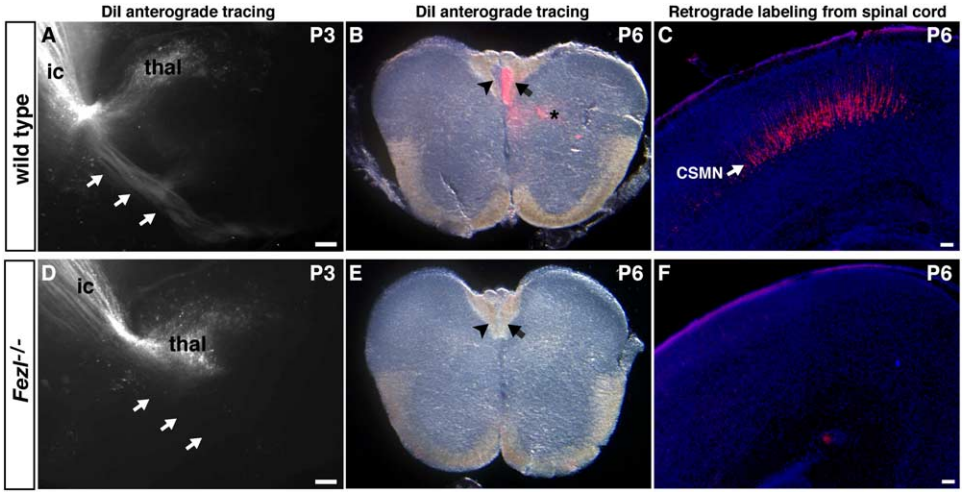

Figure 7. Absence of the Corticospinal Tract and Other Subcerebral Projections in *Fezl*$^{-/-}$ Mice

(A and D) Anterograde DiI-labeled projections showing absence of any subcerebral axonal tract in *Fezl*$^{-/-}$ brain ([D], arrows), compared to well-defined axon tracts in wild-type brain ([A], arrows). Both thalamic projections via the internal capsule (ic) and retrogradely labeled neurons in the thalamus (thal) are present in *Fezl*$^{-/-}$ mice.

(B and E) Cross-sections of P6 cervical spinal cord, showing the corticospinal tract in wild-type dorsal funiculus (B) by both DiI tracing of CSMN axons from the cortex (arrow in [B] points at DiI-labeled corticospinal tract; asterisk indicates fibers growing into dorsal horn), and oblique coherent contrast imaging (arrowhead in [B]), while no corticospinal fibers are detected in *Fezl*$^{-/-}$ mice ([E]; arrow, arrowhead).

(C and F) Retrograde labeling of CSMN reveals normal location and numbers in wild-type P6 motor cortex (C), while no neurons are labeled in *Fezl*$^{-/-}$ cortex (F). DAPI staining in blue shows underlying cellular structure.

Scale bars, 100 μm (A, C, D, and F).

CMV/β actin promoter with an IRES-GFP element for identification of electroporated cells (*Fezl*^{GFP}; Figure 8A). We electroporated this construct into the neocortical ventricular zone of developing E13.5 embryos. A control vector containing only GFP (*control*^{GFP}; Figure 8A) was electroporated in parallel experiments to generate control mice. Electroporated embryos were recovered at two developmental stages: (1) E17.5 (during late-stage embryonic corticogenesis) and (2) P4 (when postmitotic neurons are already positioned in cortical layers).

Electroporation with *control*^{GFP} at E13.5 results in the transfection of cells that give rise to neurons predominantly of layer IV, as well as fewer cells scattered in deeper layers (Figures 8B and 8H). In striking contrast, we find that cells overexpressing *Fezl* form a new, heterotopic layer of neurons in the germinal zone beneath the corpus callosum and fail to migrate to layer IV (Figures 8E and 8K; n = 11 *Fezl*^{GFP}, n = 8 *control*^{GFP}). Only cells expressing *Fezl*^{GFP} at a much lower level migrate into the cortex. This premature arrest of migration is consistent with the finding that *Fezl* expression increases dramatically postmigrationally during normal development. Therefore, *Fezl* overexpression might result in premature differentiation and a failure to migrate out of the germinal zone.

Remarkably, essentially all *Fezl*^{GFP} electroporated neurons in this heterotopic layer express CTIP2 at levels similar to those seen in layer V of cortex at both E17.5 and P4 (Figures 8E–8G, 8K–8M, and 8Q–8S). In contrast, in mice electroporated with *control*^{GFP}, in which the bulk of GFP-positive neurons are found in layer II-IV, only a small percentage of GFP-positive neurons express CTIP2 (0.2% of layer II-IV GFP^{+} neurons; ~3% of total GFP^{+} neurons), (Figures 8B–8D, 8H–8J, and 8N–8P). These data strongly indicate that overexpression of *Fezl* is sufficient to induce expression of CTIP2 in neurons that would not normally express this transcription factor.

In order to further characterize the population of heterotopic neurons, we examined *Fezl*^{GFP}-overexpressing cells for TBR1 expression and for potential axonal projections. TBR1 is expressed at high levels in layers VI and II/III and is expressed at lower levels in a subpopulation of neurons in layer V (Bulfone et al., 1995; B.J.M. et al., unpublished data). The majority of *control*^{GFP} electroporated neurons in layer IV do not express TBR1 or express TBR1 at very low levels (see Figures S1A–S1C and S1G–S1I in the Supplemental Data available online). In contrast, we find that the majority of the cells overexpressing *Fezl* express TBR1 (Figures S1D–S1F and S1J–S1L), although the level of TBR1 expression varies, consistent with the variation of TBR1 expression seen in wild-type CTIP2-expressing neurons of the cortex. Together with the expression of CTIP2, the expression of TBR1 indicates that *Fezl* overexpression induces a deep layer cortical projection neuron phenotype.

Most remarkably, *Fezl*-overexpressing/CTIP2-positive neurons are able to extend axons via the internal capsule toward subcerebral targets despite their location beneath the corpus callosum, where they would not be exposed to the normal guidance cues of the cortex. Distinct, brightly GFP^{+} axonal bundles are clearly visible in the internal capsule coursing through the striatum (Figures 8T and 8U) and can be traced back to the heterotopic *Fezl*-overexpressing neurons. Axons were

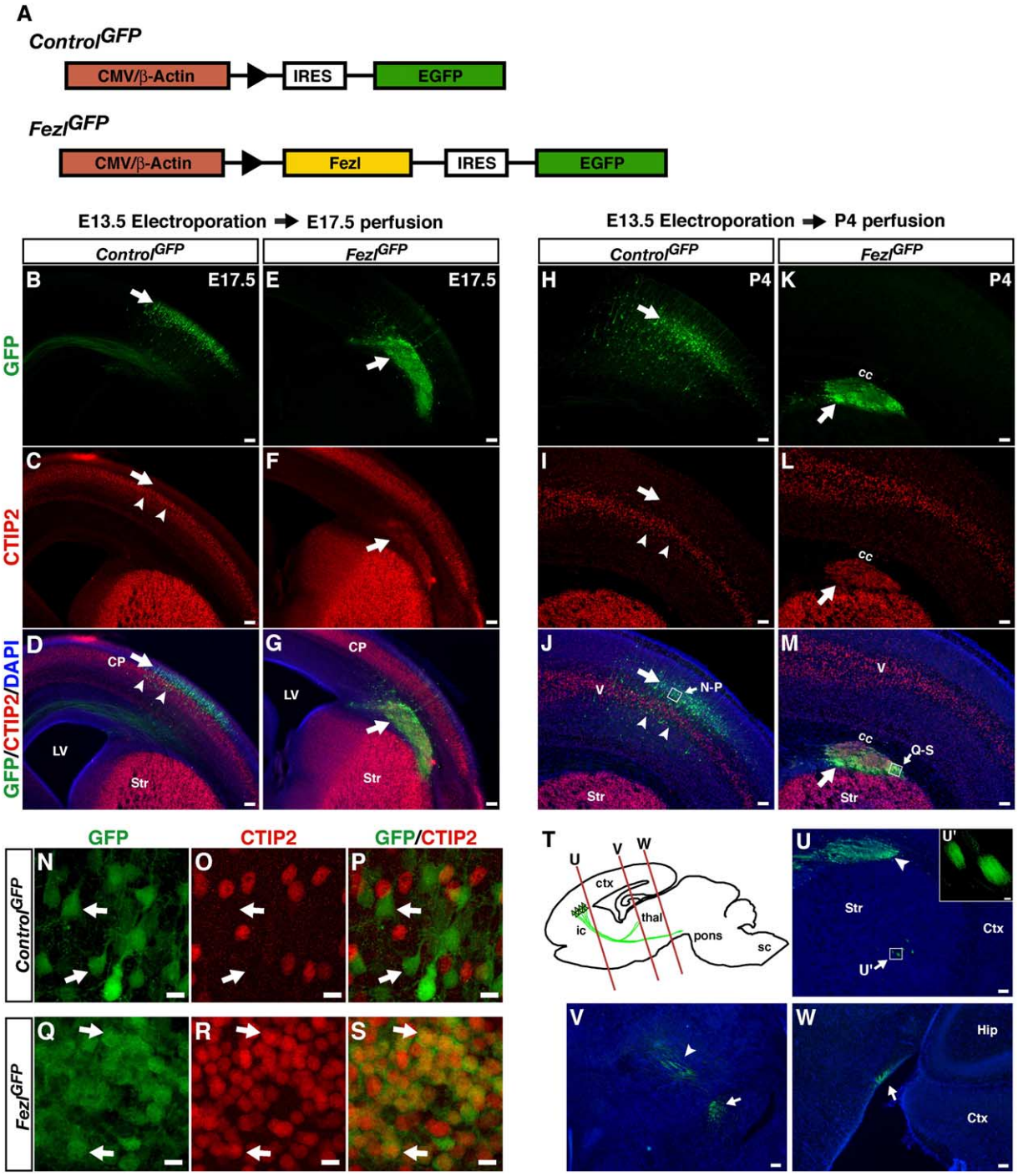

Figure 8. Overexpression of *Fezl* Produces CTIP2-Expressing Subcerebral Projection Neurons

(A) *Control*^{GFP} and *Fezl*^{GFP} constructs. (B–D and H–J) Coronal sections from brains after electroporation of *Control*^{GFP} at E13.5 and perfusion at E17.5 (B–D) or P4 (H–J). GFP-expressing neurons from the controls are primarily located in layer IV ([B and H], arrows), superficial to CTIP2-labeled subcerebral projection neurons ([C, D, I, and J], arrowheads). (E–G and K–M) Coronal sections after electroporation of *Fezl*^{GFP} at E13.5 and perfusion at E17.5 (E–G) or P4 (K–M). *Fezl*- and GFP-expressing neurons accumulate overwhelmingly in the germinal zone (arrow) beneath the corpus callosum (cc), and virtually all of them express CTIP2 (arrows, [F, G, L, and M]). Boxed areas (J and M) enlarged in (N)–(P) and (Q)–(S). (N–P) Confocal images of neurons in layer IV electroporated with *Control*^{GFP} showing GFP (N), CTIP2 (O), and merged image (P). No *Control*^{GFP} electroporated neurons in layer IV (green, arrows) express CTIP2 (red). (Q–S) Confocal images of neurons electroporated with *Fezl*^{GFP} showing GFP (Q), CTIP2 (R), and merged image (S). Essentially all *Fezl*^{GFP} electroporated neurons (green, arrows) express CTIP2 (red). (T) Schematic sagittal view of mouse brain following electroporation of *Fezl*^{GFP} at E13.5 and perfusion at P4, showing position of coronal sections shown in (U)–(W). (U) Distinct, brightly GFP^{+} axonal fascicles from neurons electroporated with *Fezl*^{GFP} (arrowhead) are clearly visible descending through the internal capsule within the striatum. (U′) Image of boxed area in (U) showing many *Fezl*$^{+}$/GFP^{+} axons tightly organized in each fascicle. (V and W) Some fibers turn to innervate the thalamus (arrowhead in [V]), while others continue toward the cerebral peduncle (arrow in [V] and [W]). Scale bars, 100 μm (B–M and U–W), 10 μm (N–S and U′). Cortical plate, CP; cortex, Ctx; lateral ventricle, LV; striatum, Str; layer V, V; thalamus, Thal; hippocampus, Hip.

also found to extend across the midline along the ventral surface of the corpus callosum (data not shown). More caudally, while some axons turn to innervate the thalamus (Figure 8V), others continue into the cerebral peduncle as entirely new subcerebral projections (Figure 8W).

Therefore, *Fezl* overexpression induces the expression of CTIP2 and TBR1 in neurons that would not normally express these transcription factors, directs the extension of axons through the internal capsule toward subcerebral targets, and arrests the migration of these neurons. These gain-of-function data indicate that *Fezl* expression is sufficient to specify the differentiation of subcerebral projection neurons from neural progenitors.

Discussion

We report that the transcription factor *Fezl* is required for the specification of corticospinal motor neurons and all other subcerebral projection neurons during neocortical development. CSMN and other subcerebral projection neurons are specifically absent from *Fezl*$^{-/-}$ neocortex, as assessed by morphological, anatomical, and molecular analysis with eight cell type-specific genes. Neither death of an early born population nor ectopic positioning of subcerebral projection neurons account for the absence of these neurons, and *Fezl*$^{-/-}$ mice lack all corticospinal and other subcerebral axonal projections, unequivocally confirming that this specific population of neurons never develops in the absence of *Fezl*. In gain-of-function experiments, overexpression of *Fezl* is sufficient to induce the formation of cortical projection neurons expressing CTIP2 (a molecular marker of all subcerebral projection neurons) and extend projections through the internal capsule and descending tracts. Together, the loss-of-function and gain-of-function data indicate that *Fezl* is a critical regulator controlling the fate specification of subcerebral projection neurons from early cortical progenitors.

The role of *Fezl* in the fate specification of a distinct neuronal population is supported by recent studies in zebrafish in which *Fezl* function was found to be required for the birth of serotonergic and dopaminergic neurons of the hypothalamus, although in a non-cell-autonomous fashion (Levkowitz et al., 2003). In contrast to these findings in zebrafish hypothalamus, subcerebral projection neurons in the mouse telencephalon express *Fezl* throughout their development, and *Fezl* overexpression induces CTIP2 expression and projection neuron differentiation, indicating that *Fezl* plays a cell-autonomous role in their fate specification.

Lineage-Restricted Effects of *Fezl*

Importantly, the absence of *Fezl* function does not affect the ability of cortical progenitors to form other types of neocortical projection neurons. Layer VI neurons, which are born before subcerebral projection neurons and express *Fezl* at lower levels, are born normally in the absence of *Fezl*, although they exhibit abnormalities in their maturation. Neurons of layers II/III, IV, and Va, which are born after subcerebral projection neurons, are normal in *Fezl* null mice. These data confirm that *Fezl* is not required for neurogenesis of all cortical projection neurons, but only for the specification of the lineage of subcerebral projection neurons.

Early in development, we find that layer VI neurons are born at the correct time and migrate to the appropriate laminar position in *Fezl* null mice. However, we observed an expansion of layer VI thickness in *Fezl*$^{-/-}$ cortex accompanying the distinct reduction in layer V thickness. Upon close examination of layer VI neurons, we find that a new population of neurons exists in the *Fezl*$^{-/-}$ cortex at the upper border of layer VI, supporting the model that absence of *Fezl* results in a substitution for missing subcerebral projection neurons by a switch to production of layer VI neurons.

In contrast to layer V, upper layer cortical neurons are all born normally from *Fezl*$^{-/-}$ progenitors after these progenitors have failed to make subcerebral projection neurons. These findings are consistent with other work showing that the failure of cortical progenitors to form deeper layer neurons does not affect their ability to respond to later environmental cues for the production of later classes of projection neurons (Frantz and McConnell, 1996; Mizutani and Saito, 2005).

Role of *Fezl* in Specification of Subcerebral Neurons

During normal development, *Fezl* expression increases soon after immature neurons arrive in the cortical plate, and CTIP2 expression increases dramatically at approximately the same time (Arlotta et al., 2005). In our overexpression experiments, we expressed high levels of *Fezl*, which induced CTIP2 expression prematurely, while neurons were still in the germinal zone. Their arrest of migration at the time of upregulation of CTIP2 expression suggests that the increase in *Fezl* expression normally observed upon arrival at the cortical plate might play a role in the switch of neuroblasts from a migrational to a differentiation state. These overexpression experiments establish that *Fezl* plays a specific role in the specification of CSMN and other subcerebral projection neurons because the overexpression of *Fezl* is sufficient to induce CTIP2 expression and direct subcerebral projection neuron differentiation, including axon extension subcortically. It also suggests the possibility that *Fezl* might be a direct regulator of CTIP2 expression, a hypothesis that will be interesting to test in the future.

Finally, the apparent involvement of *Fezl* in the transition from migratory to differentiating neuroblast, and the seeming linkage between premature differentiation and migratory arrest, suggest a novel mechanism for the formation of neuronal heterotopias. The heterotopic layer of subcerebral projection neurons that results when *Fezl* is expressed at high levels in progenitors of the germinal zone is reminiscent of human periventricular heterotopias caused by mutations in genes such as *FilaminA*, which is involved in cytoskeletal dynamics (Eksioglu et al., 1996; Lu and Sheen, 2005). Premature expression of genes such as *Fezl*, due to defects in the regulation of gene expression, could result in premature differentiation of particular subtypes of neuroblasts and the subsequent failure of those neurons to migrate into the cortical plate, resulting in heterotopia formation and disorganized cortical circuitry.

Mechanism of Subcerebral Projection Neuron Specification from Cortical Progenitors

Evidence from a number of different systems indicates that neural cell fate determination relies on both extrinsic signals from the environment and intrinsic controls that act cell-autonomously in progenitor cells. Together, these different controls define the competence state of progenitors (Edlund and Jessell, 1999). In several regions of the nervous system, including the retina (Livesey and Cepko, 2001) and the cerebral cortex (Desai and McConnell, 2000; Luskin et al., 1988; Walsh and Cepko, 1988), progenitors become progressively restricted in their competence state over time and are able to give rise to different subtypes of neurons at different times during development.

In the cerebral cortex, early progenitors normally fated to form layer VI neurons are multipotent and can generate later-born neurons of upper layer II/III, if exposed to signals inducing these later fates (McConnell and Kaznowski, 1991). In contrast, later progenitors normally fated to give rise to layer II/III neurons no longer retain the ability to acquire earlier fates (Frantz and McConnell, 1996). However, our experiments demonstrate that the overexpression of one intrinsic factor, *Fezl*, is sufficient to at least partially override this restriction and induce later-stage progenitors to produce neurons with molecular and anatomical features of earlier-born neurons. This indicates that later-stage progenitors might be more plastic than previously suspected, if manipulated by appropriate control molecules.

Insight into the potential mechanisms of this plasticity is provided by the field of *Drosophila* motor neuron development, in which progenitor cells have been found to express a sequential series of transcription factors during neurogenesis and their progeny maintain expression of the transcription factor that is present at the time of birth (Isshiki et al., 2001). In this system, expression of *Hunchback*, which is normally expressed in early progenitors and their neuronal progeny, is sufficient to allow later progenitors to generate neurons with an early phenotype. However, this plasticity decreases over time so that progenitors at advanced stages of development are resistant to *Hunchback* expression and do not revert to an earlier phenotype (Pearson and Doe, 2003). In an analogous fashion in the cortex, we find that *Fezl* is expressed in the ventricular zone during the generation of deep layer neurons and its expression is maintained in postmitotic neurons of layers V and VI. Overexpression of *Fezl* in progenitors soon after the generation of layer V and VI is completed (i.e., in progenitors that give rise to layer IV neurons) results in the production of additional neurons with a deep layer phenotype. If cortical projection neurons are specified in a similar fashion as *Drosophila*, this plasticity might be lost at later stages of cortical development.

The precise code of transcription factors expressed in primitive progenitors during cortical development has not been identified. Interestingly, *Cux2* and *Svet1* are expressed in the subventricular zone within intermediate progenitors that might give rise to upper layer neurons (Nieto et al., 2004; Tarabykin et al., 2001; Zimmer et al., 2004). However, the lineage relationship between deep layer and upper layer progenitors is still undefined. The identification of additional genes with restricted patterns of expression like *Fezl*, *Cux2*, *Svet1*, and *Otx1* and, ideally, genes restricted to individual layers and individual subtypes of neurons within the layers will be needed to decipher the sequence and diversity of neocortical progenitors as well as the molecular programs that control cortical neurogenesis.

Fezl is now identified as being required for the specification of a distinct population of neurons in the cerebral cortex. This is in contrast to genes such as *Emx2*, *Pax6*, and *Lhx2* that are involved more broadly in specifying dorsal telencephalic identity or arealization of the neocortex (Bishop et al., 2000; Bulchand et al., 2001; Mallamaci et al., 2000; Monuki et al., 2001; Muzio et al., 2002). In order to truly understand how the identity of CSMN is specified, it will be important to determine how *Fezl* interacts with these genes.

As a first step in this direction, it will be critical to investigate precisely when during progenitor differentiation absence of *Fezl* causes deletion of subcerebral projection neurons. The identification and characterization of the mechanisms by which *Fezl* functions to specify the population of corticospinal motor neurons and other subcerebral projection neurons will provide further insight into the basic mechanisms of the development of neuronal diversity in the cerebral cortex and may allow directed differentiation of neural precursors toward cellular repair of diseased neocortical output circuitry.

Experimental Procedures

Generation of *Fezl*$^{-/-}$ Mice

Fezl$^{-/-}$ mice were generated by Hirata and colleagues (Hirata et al., 2004) (*Fezl* GenBank accession number: AB042399). The day of vaginal plug was designated embryonic day 0.5 (E0.5). The day of birth was designated postnatal day 0 (P0). All mouse studies were approved by The Massachusetts General Hospital IACUC and were performed in accordance with institutional and federal guidelines.

Immunocytochemistry and Nissl Staining

Brains for immunocytochemistry and Nissl staining were processed as previously described (Arlotta et al., 2005; Macklis, 1993). Primary antibodies and dilutions are described in the Supplemental Experimental Procedures. Appropriate secondary antibodies were from Molecular Probes Alexa series. TUNEL staining was performed using the DeadEnd Fluorometric TUNEL system (Promega), following the instructions of the manufacturer. Fluoro-Jade B staining was performed according to Schmued and Hopkins (2000).

BrdU Birthdating and Quantification of Layer Thickness

Timed pregnant females received a single intraperitoneal injection of BrdU (100 mg/kg) at E12.5, E13.5, or E15.5. Pups were allowed to develop to P6 and then were perfused and processed for BrdU immunocytochemistry (Magavi et al., 2000). Quantification of newborn cells and distribution within cortical layers was analyzed according to (Hevner et al., 2001) and is described in detail in the Supplemental Experimental Procedures.

In Situ Hybridization and DiI Photoconversion

Nonradioactive in situ hybridization was performed using reported methods (Berger and Hediger, 2001). Riboprobes were generated as previously described (Arlotta et al., 2005). cDNA clones used for riboprobes for *Fezl*, *Diap3*, *Crim1*, *Clim1*, *S100a10*, *Crystallin-mu*, and *Igfbp4* were previously described (Arlotta et al., 2005). cDNA clones for *Cux2*, *Tcr*β, *PlexinD1*, and *Foxp2* were generated by RT-PCR using primers detailed in the Supplemental Experimental Procedures. DiI photoconversion combined with in situ hybridization

was performed following published protocols (Arlotta et al., 2005; Fujimori et al., 2000).

Anterograde and Retrograde Labeling

Anterograde DiI tracing in vivo was performed as previously described (Arlotta et al., 2005; O'Leary and Terashima, 1988). CSMN in sensorimotor and lateral sensory cortex were retrogradely labeled via injections of red fluorescent microspheres (Lumafluor) into the dorsal funiculus at the C4 level of spinal cord. For DiI labeling of subcerebral projection neurons, DiI was injected into the pons at P0 under ultrasound guidance (Vevo 660, VisualSonics; Arlotta et al., 2005) and mice were perfused at P14. Additional details are included in the Supplemental Experimental Procedures.

Electroporation

For control experiments, a vector containing IRES-EGFP under the control of a constitutively active CMV/β actin promoter was used (*control*^{GFP}; Figure 8A; generous gift of C. Lois, MIT). *Fezl* was cloned into this vector to create the construct *Fezl*^{GFP} for overexpression (Figure 8A). 750 nl of purified DNA (0.5–1.0 μg/μl) mixed with 0.005% Fast Green was injected in utero into the lateral ventricle of CD1 embryos at E13.5 under ultrasound guidance (Vevo 660, VisualSonics) and electroporated into the neocortical ventricular zone essentially according to Saito and Nakatsuji (2001) as detailed in the Supplemental Experimental Procedures.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/47/6/817/DC1/.

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