Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex

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During the development of the cerebral cortex, progenitor cells produce neurons that migrate to laminar positions appropriate for their birth dates, adopt specific neuronal identities, and form appropriate local and long-distance axonal connections. Here, we report that forebrain embryonic zinc-finger-like protein (*Fezl*), a putative zinc-finger transcriptional repressor, is required for the differentiation of projection neurons in cortical layer 5. In *Fezl*-deficient mice, these neurons display molecular, morphological, and axonal targeting defects. The corticospinal tract was absent in *Fezl*$^{-/-}$ mice, corticotectal and pontine projections were severely reduced, and *Fezl*-expressing neurons formed aberrant axonal projections. The expression of many molecular markers for deep-layer neurons was reduced or absent in the *Fezl*$^{-/-}$ cerebral cortex. Most strikingly, *Ctip2*, a transcription factor required for the formation of the corticospinal tract, was not expressed in the *Fezl*-deficient cortex. These results suggest that *Fezl* regulates the differentiation of layer 5 subcortical projection neurons.

axon guidance | cell fate | corticospinal tract | zinc-finger transcription factor

The mammalian cerebral cortex is organized into six layers, and neurons in the same layer tend to share similar functional properties and patterns of connectivity (1). Cells in layers 5 and 6 project to subcortical targets, with thalamic projections arising from layer 6 and projections to the midbrain, hindbrain, and spinal cord originating from layer 5, whereas neurons sending axons to other cortical areas are distributed throughout layers 2–6 (1). After their generation in the ventricular zone, the earliest-generated cortical neurons migrate away to form the preplate, which is split into the marginal zone and subplate during cortical plate formation (2). Early-born cortical plate cells populate the deepest layers, and later-generated neurons migrate past older cells and settle into progressively more superficial positions (3). Transplantation studies suggest that a neuron acquires a laminar identity, which specifies the layer to which it will migrate, by the time of its terminal mitotic division (4–7).

Transcription factors play a central role in the specification of cell fates and projection patterns. For example, in the developing spinal cord, neuronal fates are defined by unique combinations of transcription factors (TFs) (8, 9). In the developing cortex, in contrast, only a few TFs have been implicated in the formation of neuronal subtypes. For instance, Foxg1 is required constitutively to repress the Cajal–Retzius fate in deep-layer neurons (10), and COUP-TF1 and Tbr1 are each required for subplate neuron differentiation (11). Despite these examples, the molecular mechanisms that control cortical cell fates and laminar differences in connectivity remain largely unknown.

Reasoning that laminar identity genes may show layer-specific patterns of expression during development, we have focused on TFs expressed in distinct layers. The zinc-finger transcriptional repressor Fezl (forebrain embryonic zinc-finger-like protein) (12–14) is expressed in cortical layers 5 and 6 (15). In zebrafish, both ectopic expression and loss-of-function analyses suggest that *Fezl* regulates gene expression in the ventral forebrain (14) and the development of dopaminergic and serotonergic neurons (16). Based on the expression of *Fezl* in the deep layers of the cerebral cortex and functional data from zebrafish, we hypothesized that *Fezl* regulates the specification or differentiation of layer 5 and 6 neurons during mammalian cortical development.

Methods

The *Fezl* coding region was targeted with a cassette containing a 3-kb homology sequence upstream of the start codon, an *EGFP-IRES-PLAP* cassette (EGFP, enhanced GFP; IRES, internal ribosomal entry site; PLAP, human placental alkaline phosphatase), a floxed *PGK-neo* cassette, and a 4.3-kb homology sequence downstream of *Fezl* followed by a *RSV-TK* cassette (RSV-TK, Rous sarcoma virus promoter driving expression of thymidine kinase gene) (Fig. 6*A*, which is published as supporting information on the PNAS web site). The linearized construct was electroporated into E14a embryonic stem cells, which were subjected to positive (neo) and negative (TK) selection then screened by Southern analysis to select homologous recombinant clones. Heterozygous *Fezl*$^{+/-}$ mice were mated to β-actin-cre CD1 mice to excise the *pgk-neo* selection cassette, and these *Fezl*$^{+/-}$ mice were mated to generate *Fezl*$^{-/-}$ mutants.

In situ hybridization using ^{35}S-labeled probes was performed as described (17). PLAP activity was detected with AP staining buffer (0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate/1 mg/ml nitroblue tetrazolium in 100 mM Tris·Hcl, pH 9.5/100 mM NaCl). For anterograde axon tracing, three injections of biotinylated dextran amine (BDA) (10% in saline; Molecular Probes) were placed in the motor, somatosensory, or visual cortex of adult mice (see *Supporting Text*, which is published as supporting information on the PNAS web site). For retrograde labeling, rhodamine fluorescent latex microspheres (Lumafluor, New York) were injected bilaterally into the pyramidal decussation or superior colliculus of adult mice. Survival times were 2 weeks (BDA) or 1 week (microspheres). To visualize BDA, 45-μm coronal or sagittal sections were processed with ABC VectaStain Kit (Vector Laboratories) and diaminobenzidine. Each labeling experiment used three to six mutants and three to six littermate controls.

Results

The *Fezl* ORF encodes a protein of 455 amino acids that contains six C_2H_2-type zinc-finger motifs and an eh1 domain, which is conserved in several transcriptional repressors (12, 14). To generate *Fezl*-deficient mice, we replaced the entire Fezl ORF with a promoterless *EGFP-IRES-PLAP* (EGFP, enhanced GFP; IRES, internal ribosomal entry site) cassette (18). Southern analysis and

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Abbreviations: BDA, biotinylated dextran amine; CSMN, corticospinal motor neuron; CST, corticospinal tract; PLAP, human placental alkaline phosphatase; TF, transcription factor; P*n*, postnatal day *n*; E*n*, embryonic day *n*.

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Table 1. Alterations in gene expression in *Fezl* mutant mice

Gene name	Class	Description	Pattern in *Fezl*$^{-/-}$
ER81	Layer 5 marker	ETS transcription factor	Reduced
Foxo1	Layer 5 marker	Forkhead transcription factor	Absent
Foxp2	Layer 6 marker	Forkhead transcription factor	Absent
Grg4	Layer 6, subplate	Groucho transcription factor	Reduced
Reelin	Strong in layer 5	Extracellular matrix	Reduced
PTP lambda	Strong in layers 2/3, 6	Protein tyrosine phosphatase	Reduced layer 6
Cadherin 13	CSMN general identity	Cadherin cell adhesion	Reduced
Ctip2	CSMN early development	Transcription factor	Absent
Clim1	CSMN early development	LIM domain-binding protein	Reduced
Riken 2010001O09	CSMN early development	Transmembrane domain	Absent
Synaptotagmin 9	CSMN early development	Vesicular trafficking protein	Absent
Crim1	CSMN intermediate development	Interacts with bone morphogenetic proteins	Reduced
Stk39	CSMN intermediate development	Protein kinase	Reduced
Netrin G1	CSMN late development	Extracellular matrix	Reduced
Neurofilament HC	CSMN-specific	Intermediate filament	Reduced
Lix1	Negative for CSMN	Unknown	Reduced
Diap3	CSMN vs. corticotectal	Cytoskeletal rearrangement	Reduced
S100a10	CSMN vs. corticotectal	Calcium binding	Reduced
Igfbp4	CSMN vs. corticotectal	Binds insulin-like growth factors	Expanded into 6
Mu-crystallin	CSMN intermediate development	Thyroid hormone-binding protein	Reduced

PCR genotyping confirmed germ-line transmission of the targeted allele (Fig. 6 *A* and *B*).

Consistent with a previous study of *Fezl* mutants (19), heterozygous *Fezl*$^{+/-}$ mice produced homozygous *Fezl*$^{-/-}$ progeny at a Mendelian ratio, and locomotor activity tests revealed that *Fezl*$^{-/-}$ mice were twice as active as littermate controls (control, 161.4 ± 64.3 counts per day; *Fezl*$^{-/-}$, 335.5 ± 126.7 counts per day, $P = 0.0039$). Mutant brains appeared grossly normal, with clear evidence of cortical lamination (Fig. 6 *C–F*). Although defects in subplate neurons and thalamocortical projections have been reported in *Fezl* mutants (19), subplate neurons were present in the mutants generated here, as revealed by cresyl violet staining (Fig. 6 *E* and *F*) and expression of *Tbr1* (Fig. 7 *C* and *D*, which is published as supporting information on the PNAS web site), and cytochrome oxidase staining revealed normal barrel fields in somatosensory cortex (Fig. 8, which is published as supporting information on the PNAS web site).

PLAP Recapitulates *Fezl* Expression in Mutant and Heterozygous Mice. To visualize *Fezl*-expressing neurons and their axons, we replaced the *Fezl* ORF with enhanced GFP (EGFP) and PLAP. For unknown reasons, EGFP could not be detected, but *PLAP* mRNA and protein activity were easily visualized. *In situ* hybridization revealed that the pattern of *PLAP* expression was essentially identical to that of *Fezl* mRNA (Fig. 9 *A* and *B*, which is published as supporting information on the PNAS web site) in both *Fezl*$^{+/-}$ and *Fezl*$^{-/-}$ brains, with expression in layers 5 and 6 (Fig. 9 *C–F*) and in the hippocampus and hypothalamus (data not shown) (15). These data suggest that deep-layer neurons were present in *Fezl* mutant brains, and that these cells continue to express the *Fezl* mutant allele. BrdUrd-labeling studies confirmed that layer 5 and 6 neurons were generated at the appropriate time, migrated normally, and survived into postnatal life (data not shown). However, layer 5 appeared thinner and more densely populated with smaller cells in *Fezl* mutants (Fig. 6 *C–F*).

Disruption of Molecular Markers for Deep Cortical Layers in *Fezl* Mutant Mice. To explore the fates of layer 5 and 6 neurons in *Fezl*$^{-/-}$ brains, we performed *in situ* hybridization at embryonic day (E)14 and postnatal day (P)0 and P15 for genes that distinguish these neurons from those in other layers. The expression of some deep-layer genes, such as *Otx1* (20) and *Tbr1* (11), was unaffected in the *Fezl*$^{-/-}$ cortex (Fig. 7 *A–D*), suggesting that *Fezl*-deficient mice produce layer 5 and 6 neurons [including corticospinal motor neurons (CSMNs), which express *Otx1* (20)]; however, the expression of other deep-layer markers was severely disrupted (Table 1). Two mRNAs expressed in layer 5, *ER81* and *Foxo1*, were greatly reduced or completely absent in the *Fezl*$^{-/-}$ cortex (Fig. 1 *A–D*). Similarly, the expression of the layer 6 markers *Grg4* and *Foxp2* was severely reduced or abrogated in mutants (Fig. 1 *E–H*). Although expression of these genes was disrupted in the deep layers of cortex, expression in other brain regions was unaffected (e.g., *Foxp2* in striatum; Fig. 1*F*).

The altered expression of deep-layer markers suggested that *Fezl* mutant mice might have defects in the specification or differentiation of neurons in layers 5 and 6. To ascertain whether these cells acquired alternative laminar fates, we performed *in situ* hybridization for seven genes normally expressed in other cortical layers. All were expressed normally in *Fezl* mutants (Fig. 7 *E–H*; Table 2, which is published as supporting information on the PNAS web site), suggesting that layer 5 and 6 neurons did not adopt upper-layer identities.

Defective Axonal Projections Revealed by PLAP Staining. The PLAP marker targeted to the *Fezl* locus was used to visualize the axons of *Fezl*-expressing mutant neurons at P0, P8, and P15. In *Fezl*$^{+/-}$ and *Fezl*$^{-/-}$ animals, PLAP marked the cell bodies and dendrites of neurons in the cortical plate and hippocampus (Fig. 2). In addition, major axon tracts associated with layer 5 and 6 subcortical projections were clearly labeled and showed dramatic changes in *Fezl*$^{-/-}$ brains (Fig. 2 *K–R*). Layer 5 and 6 neurons project subcortical axons by growing through the internal capsule, then diverging into the dorsal thalamus (layer 6) or toward the spinal cord (layer 5). The fasciculation of PLAP-labeled axons in the internal capsule was disorganized in *Fezl*$^{-/-}$ brains (Fig. 2 *K* and *L*), and it appeared that a greater number of labeled axons entered the thalamus in mutants (Fig. 2 *B*, *E*, *M*, and *N*). However, because PLAP reveals fasciculated axons and not more diffuse projections, it was difficult to discern whether thalamic targeting was altered.

The axons of layer 5 neurons project to a variety of subcortical

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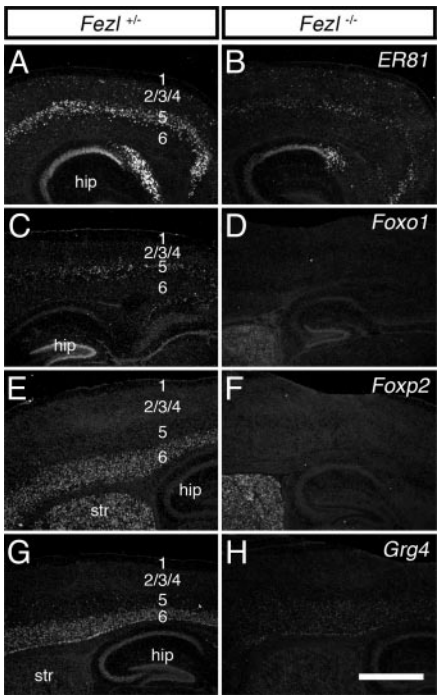

Fig. 1. *In situ* hybridization for genes normally expressed by deep layer neurons at P15. (*A* and *B*) *ER81* is normally expressed in layer 5 and hippocampus, but expression in layer 5 is severely reduced in *Fezl* mutants. (*C* and *D*) Expression of *Foxo1* in layer 5 is reduced in *Fezl*$^{-/-}$ mice. (*E* and *F*) *Foxp2*, which is normally expressed in layer 6 and striatum, is lost from layer 6 in *Fezl*$^{-/-}$ brains. (*G* and *H*) The expression of *Grg4* in layers 5 and 6 is severely reduced in mutants. (Scale bar, 0.5 mm.)

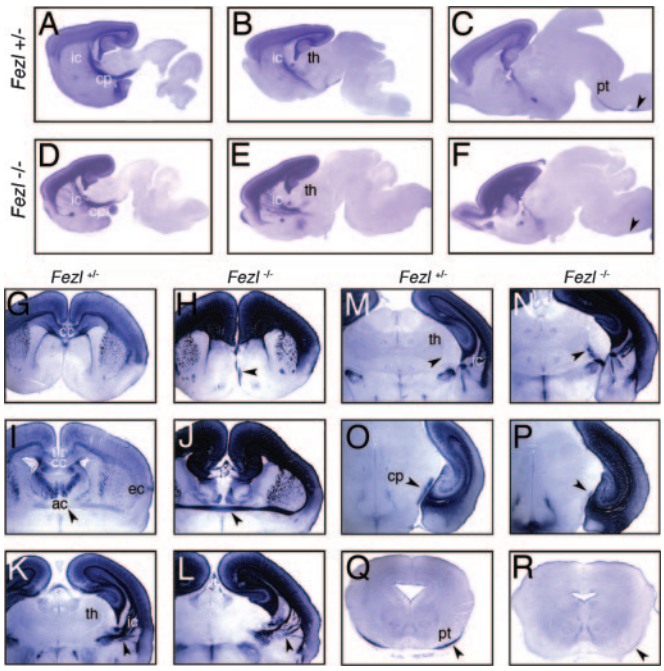

Fig. 2. PLAP staining reveals defective axonal projections in *Fezl*$^{-/-}$ brains at P0. (*A–F*) Sagittal sections showing PLAP staining in *Fezl*$^{+/-}$ brains (*A–C*) and *Fezl*$^{-/-}$ brains (*D* and *E*). (*A* and *D*) PLAP stains axons in the internal capsule and cerebral peduncle of control and mutant brains. PLAP activity is strong in the anterior commissure in *Fezl*$^{-/-}$ brains (*D*) but much weaker in heterozygotes (*A*). (*B* and *E*) A subset of axons in both controls and mutants leaves the internal capsule and enters the thalamus (th). (*C* and *F*) PLAP prominently marks the pyramidal tract in heterozygotes (arrowhead), but this labeling is absent in *Fezl* mutants. (*G–P*) Coronal sections showing PLAP staining in *Fezl*$^{+/-}$ (*G*, *I*, *K*, *M*, *O*, and *Q*) and *Fezl*$^{-/-}$ brains (*H*, *J*, *L*, *N*, *P*, and *R*). (*G–J*) The corpus callosum is apparent in heterozygotes (*G* and *I*) but absent in *Fezl* mutants (*H* and *J*). Some axons in *Fezl*$^{-/-}$ mice take an aberrant pathway into the septum (arrowhead in *H*). Mutants show increased PLAP in the external capsule and posterior limb of the anterior commissure (*J*, arrowhead) compared with controls (*I*). (*K* and *L*) PLAP marks axons in the internal capsule (arrowhead), but these axons appear disorganized in mutants. (*M* and *N*) More PLAP positive axons in the cerebral peduncle turn dorsally into the thalamus (arrowhead) in *Fezl*$^{-/-}$ brains than in controls. (*O* and *P*) PLAP activity in the cerebral peduncle (arrowhead) is dramatically reduced in *Fezl*$^{-/-}$ brains. (*Q* and *R*) As axons approach the pyramidal decussation, PLAP marks the pyramidal tract (arrowhead) in controls, but little or no staining is visible in mutants. ic, internal capsule; cp, cerebral peduncle; pt, pyramidal tract; th, thalamus; cc, corpus callosum; ac, anterior commissure; ec, external capsule.

targets, including the superior and inferior colliculi, pons, deep cerebellar nuclei, and spinal cord (1). Extensive defects were seen in these projections in *Fezl* mutants. In control brains at P0, PLAP-positive axons descended through the cerebral peduncle and pyramidal tract to the pyramidal decussation, where they formed the corticospinal tract (CST) (Fig. 2 *A–C*) (1). In *Fezl*$^{-/-}$ mutants, PLAP-labeled axons were present in the cerebral peduncle, but staining decreased dramatically at caudal levels (Fig. 2 *O* and *P*), with little or no PLAP activity apparent in the pyramidal tract (Fig. 2*R*). Given that *Fezl*$^{-/-}$ mice have two copies of *IRES-PLAP* at the *Fezl* locus rather than one, the absence of PLAP labeling in the CST of *Fezl*$^{-/-}$ mice suggested that layer 5 axons failed to develop normally.

The absence of CST axons raised the possibility that layer 5 neurons innervated abnormal targets. Indeed, PLAP revealed axon pathways in mutant brains that were not prominently labeled in heterozygotes. The external capsule and anterior commissure showed light PLAP labeling in *Fezl*$^{+/-}$ mice (Fig. 2*I*). However, even after taking into account the dosage of the mutant allele, much stronger PLAP activity was detected in these regions in *Fezl*$^{-/-}$ brains (Fig. 2*J*), suggesting that axons from *Fezl*-expressing neurons that normally extend through the internal capsule were diverted onto alternative inappropriate pathways.

The corpus callosum, which showed only faint PLAP staining in heterozygotes, was completely absent in *Fezl*$^{-/-}$ mice (Fig. 2 *G–J*). PLAP-positive axons failed to cross the midline and instead accumulated into Probst bundles (Fig. 2 *H* and *J*) or turned ventrally into the septum (Fig. 2*H*, arrowhead). It is not clear why the callosum is missing in Fezl-deficient brains, but this suggests that early *Fezl* expression may be required for midline development.

Subcortical Projection Defects Revealed by Retrograde Labeling. PLAP effectively reveals axon bundles but lacks the sensitivity to detect individual axons. To confirm the changes in axonal projections, we retrogradely labeled cortical neurons from the pyramidal decussation or superior colliculus with fluorescent latex microspheres. As expected, injections into the pyramidal decussation of control mice marked layer 5 neurons throughout the motor and somatosensory areas, but not the visual cortex (Fig. 3 *A* and *B*). However, in *Fezl*$^{-/-}$ mutants, only a very small number of layer 5 neurons, found exclusively in the somatosensory cortex, extended axons to the pyramidal decussation (Fig. 3 *C* and *D*). Injections into the superior colliculus of controls labeled layer 5 neurons in visual and somatosensory cortex (Fig. 10 *A* and *B*, which is published as supporting information on the PNAS web site). In *Fezl*$^{-/-}$ mice, very few layer 5 neurons were labeled, and these cells were confined to caudal visual cortex (Fig. 10 *C* and *D*). These experiments confirmed that most layer 5 cortical neurons in *Fezl*$^{-/-}$ mice failed to extend axons to subcortical targets.

Anterograde Tracing Reveals Defects in Axon Targeting. PLAP labeling suggested that *Fezl*-expressing neurons form aberrant axonal projections in *Fezl* mutants. To further explore this issue, we

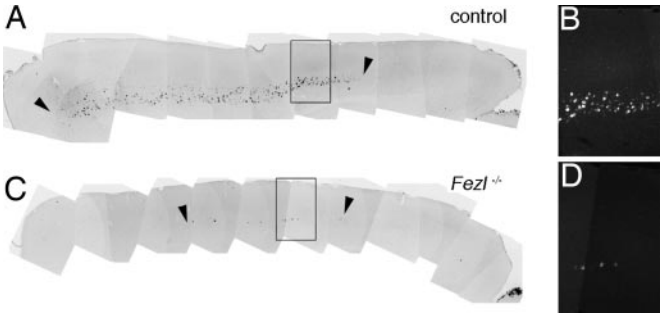

Fig. 3. Defects in layer 5 projections to the pyramidal decussion in *Fezl*$^{-/-}$ mutants. Sagittal sections showing retrograde labeling from the pyramidal decussation. The extent of retrograde labeling is indicated by arrowheads. Fluorescence images have been inverted in *A* and *C*. (*A*) Retrograde labeling normally marks layer 5 neurons throughout the motor and somatosensory areas. (*B*) High-power view of the region delineated by the box in *A*. (*C*) In *Fezl*$^{-/-}$ mutants, only layer 5 neurons within somatosensory cortex (arrowheads) retain projections to the spinal cord. (*D*) High-power view of the boxed region in *C*, showing sparse labeling in the mutant.

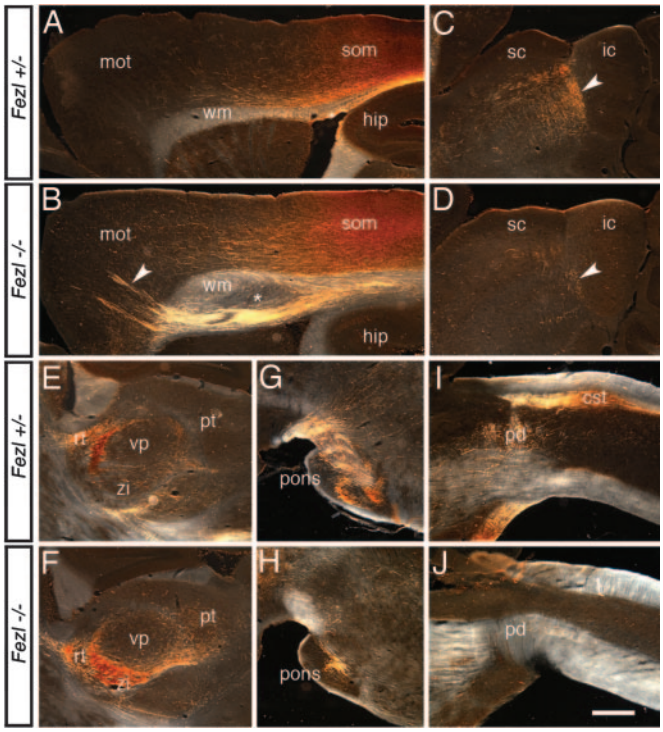

Fig. 4. Abnormal axon projections from the somatosensory and motor cortices of *Fezl*$^{-/-}$ mice. Sagittal sections showing BDA-labeled axons traced from somatosensory (*A–F*) or motor cortex (*G–J*) of adult mice. (*A* and *B*) BDA injections into somatosensory cortex normally label axons that innervate neighboring cortical areas. Axons in *Fezl*$^{-/-}$ mice fail to cross the corpus callosum and instead form Probst bundles (asterisk), and some labeled axon bundles enter rostral motor areas (arrowhead). (*C* and *D*) Control axons from somatosensory cortex project to the superior colliculus, which contains many fewer labeled axons in *Fezl* mutants. (*E*) Somatosensory axons innervate the reticular nucleus, the lateral part of the ventral posterior nucleus and the posterior nucleus of the thalamus. (*F*) Thalamic projections in *Fezl*$^{-/-}$ mice show a grossly normal pattern, but more axons appear to innervate the medial ventral posterior nucleus, pretectum, and zona inserta. (*G* and *H*) BDA-labeled axons from motor cortex normally branch in the central pons, which contains many fewer axons in mutants. (*I*) Axons from motor cortex extend through the pyramidal tract, cross in the pyramidal decussation, then descend in the CST. (*J*) No axonal labeling was visible in the pyramidal decussation or CST of *Fezl*$^{-/-}$ mutants. mot, motor cortex; wm, white matter; som, somatosensory cortex; hip, hippocampus; sc, superior colliculus; ic, inferior colliculus; rt, reticular thalamic nucleus; vp, ventral posterior nucleus; pt, pretectum; and zi, zona inserta. (Scale bar, 0.5 mm.)

injected the anterograde tracer BDA into one of three cortical areas (motor, somatosensory, or visual), then visualized axon projections in brain sections (Fig. 4; Fig. 11, which is published as supporting information on the PNAS web site). BDA labeling in *Fezl* mutants was distinct from that in control brains. In addition to callosal defects (Figs. 4*B* and 10*B*), neurons from somatosensory and visual cortex extended fewer axons into the superior colliculus (Figs. 4 *C* and *D* and 11 *E* and *F*). Dramatic changes were seen for motor and somatosensory cortex projections to the hindbrain and spinal cord; no labeled axons were visible in the pyramidal decussation or CST of *Fezl*$^{-/-}$ mutants (Fig. 4 *G–J*).

The BDA-labeling patterns support the view that the axons of layer 5 neurons adopted alternative trajectories. As seen with PLAP, *Fezl* mutants showed an increased number of axons extending through the external capsule and anterior commissure, with a small number then extending dorsally into the contralateral cortex (Fig. 11*D*). A second projection was observed in the ipsilateral cortex, where BDA injections into somatosensory cortex of mutants labeled axon bundles extending into motor cortex (Fig. 4 *A* and *B*). Collectively, these experiments both confirmed the targeting defects revealed by PLAP and suggested that axons were redirected along inappropriate trajectories to novel targets.

We also examined thalamic projections after BDA injections. Regardless of the area injected, labeled axons appeared to choose appropriate thalamic targets in *Fezl*$^{-/-}$ mice. For example, somatosensory axons were present in the reticular nucleus, the lateral part of the ventral posterior nucleus, and the pretectum in both control and mutant animals (Fig. 4 *E* and *F*), although stronger labeling was seen in the zona inserta of *Fezl*$^{-/-}$ mice.

***Fezl* Regulates the Expression of Several Genes Expressed in CSMNs.** To investigate the molecular mechanisms underlying the defects observed in the subcortical projections of layer 5 neurons, we used *in situ* hybridization to examine the expression of 125 genes involved in forebrain patterning, axon guidance, and the development of CSMNs. Mutations in genes such as *netrin1* (21), *DCC* and *Unc5h3* (22), and *slit1/2* (23) cause defects in corpus callosum and pyramidal tract development; thus, we examined the expression of a variety of axon guidance molecules, including netrins and their receptors, Robo and slit family members, and semaphorins and plexins. Of 36 genes tested, no obvious differences in expression were detected at E14, E15, E17, or P0 (Fig. 12 and Table 2, which are published as supporting information on the PNAS web site), suggesting that most major axon guidance molecules were expressed normally in *Fezl*-deficient brains. A second explanation for the altered axon trajectories in *Fezl* mutants is that *Fezl* is required for patterning the environment through which axons migrate. However, examination of markers that distinguish the major dorsal and ventral domains of the forebrain revealed no differences between controls and mutants (data not shown; see Table 2 for list of probes).

Another explanation for the defective projections in *Fezl*$^{-/-}$ mice is a failure in the specification or differentiation of subcortical projection neurons. Recent gene profiling experiments identified several genes, including *Fezl*, that are expressed in specific subtypes of layer 5 neurons (24). We explored the expression patterns of 35 of these genes and found that many, including *Ctip2*, Riken *2010001O09*, *Crim1*, *NFH*, *Lix1*, and *Synaptotagmin 9*, showed a loss or reduction of expression in *Fezl*$^{-/-}$ animals (Fig. 5, Table 1). However, not all CSMN genes were lost in mutants. Expression of the CSMN general identity gene *Contactin 6* (Fig. 5 *O* and *P*) and the later-expressed gene *Netrin G1* (data not shown) was maintained in *Fezl*-deficient layer 5 neurons, indicating that these neurons still occupy their correct positions and retain at least some aspects of CSMN differentiation. In addition, the expression of the

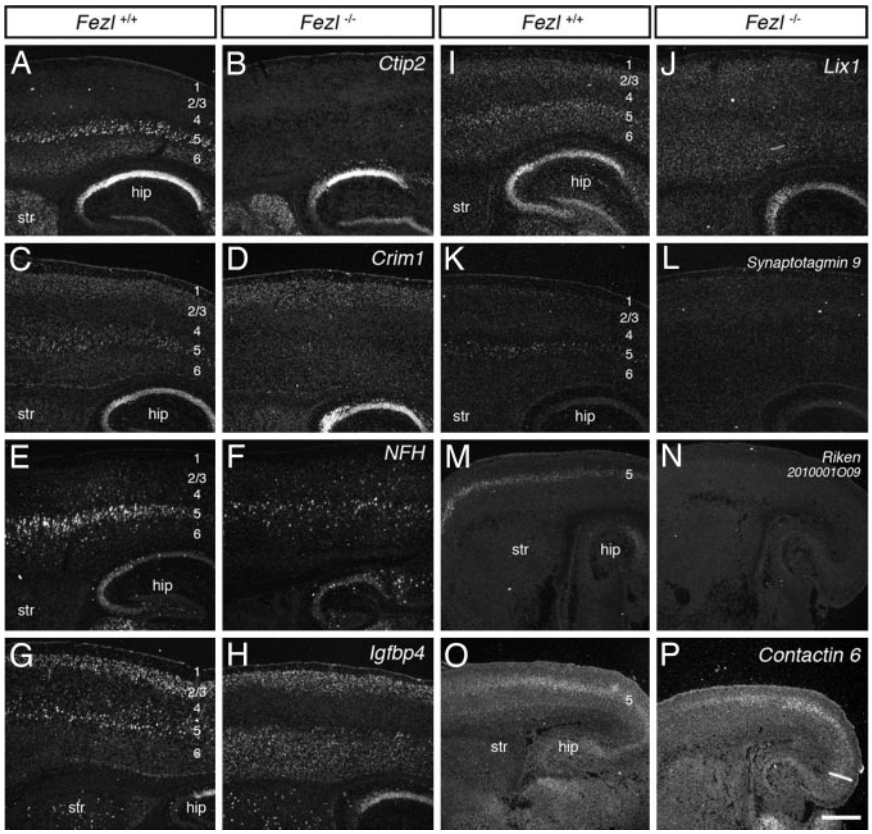

Fig. 5. Reduced expression of CSMN genes in *Fezl* mutant mice. Sagittal sections from P15 (*A–L*) or P0 (*M–P*) brains. (*A* and *B*) *Ctip2* is normally expressed in layers 5 and 6 of cerebral cortex, hippocampus (hip) and striatum (str), but cortical expression is lost in *Fezl*$^{-/-}$ mice. (*C* and *D*) *Crim1* is expressed in layers 2, 3, and 5 in control brains, but layer 5 expression is lost in mutants. (*E* and *F*) *Neurofilament heavy chain* (*NFH*) in layer 5 is severely reduced in mutants. (*G* and *H*) Expression of *Igfbp4* expands into layer 6 in *Fezl* mutants. (*I* and *J*) The expression of *Lix1* in layer 5 is severely reduced in mutants. (*K* and *L*) Layer 5 cells express *Synaptotagmin 9* in controls but not in *Fezl* mutants. (*M* and *N*) Expression of *Riken 2010001O09* in layer 5 at P0 is lost in the *Fezl* mutants. (*O* and *P*) The CSMN general identity gene *Contactin 6* is expressed normally in mutants. (Scale bar, 0.5 mm.)

CSMN-specific gene *Igfbp4* was expanded into layer 6 in mutants (Fig. 5 *G* and *H*); thus, although the projections of layer 6 neurons appeared normal, their molecular identities were substantially altered.

The most striking loss of expression in *Fezl*$^{-/-}$ mice involved the CSMN early development gene *Ctip2* (24), which encodes a zinc-finger TF. In control brains, *Ctip2* was expressed in layer 5, weakly in layer 6, and in hippocampus and striatum (Fig. 5*A*). In *Fezl*$^{-/-}$ mice, *Ctip2* expression in striatum and hippocampus was maintained, but expression of *Ctip2* in the cortex was completely abrogated (Fig. 5*B*). Previous work revealed defects in CST development in *Ctip2*$^{-/-}$ mice, which show a phenotype remarkably similar to that of *Fezl* mutants, with CST axons failing to extend past the pons in both cases (24). That both *Fezl* and *Ctip2* are expressed in layer 5 CSMNs, the similarity in their mutant phenotypes, and the absence of *Ctip2* expression (as well as that of other deep layer-specific genes) in *Fezl* mutants suggest that *Fezl* acts upstream of *Ctip2* to regulate multiple aspects of subcortical projection neuron differentiation (24).

Discussion

Although much progress has been made in elucidating the mechanisms of neuronal fate determination in the vertebrate spinal cord (8, 9), little is known about the mechanisms that create specificity in higher brain regions such as the cerebral cortex. Here, we report that Fezl, a zinc-finger transcriptional repressor expressed in layers 5 and 6, regulates the differentiation and connectivity of layer 5 subcortical projection neurons. We found that in *Fezl*-deficient mice, *Fezl*-expressing deep-layer neurons failed to extend axons to the tectum, pons, and spinal cord and instead formed inappropriate projections. These defects could not be explained by alterations in the expression of axon guidance ligands or receptors or by changes in forebrain patterning. The generation and migration of deep-layer neurons appeared normal, because *PLAP* mRNA was robustly expressed in the deep layers of *Fezl* mutants, and many deep-layer markers (including many CSMN-specific genes) were expressed normally in mutants. These findings suggest that *Fezl* regulates specific features of deep-layer differentiation, including the expression of *Ctip2*, a TF that is essential for CST development (24).

The transcriptional control of cell fate decisions and differentiation plays a critical role in the elaboration of distinct neuronal phenotypes throughout the nervous system (8, 9). Among the genes affected by the *Fezl* mutation, several [such as the ETS gene *ER81* (25)] regulate the differentiation and connectivity of other neuronal subtypes. Another *Fezl* target, *Grg4*, is a member of the *Groucho* family of transcriptional corepressors, which are recruited by homeodomain TFs to regulate progenitors fates along the dorsal–ventral axis of the spinal cord (9). Interference with repression suggests that ventral neuron identities in the spinal cord are achieved through the repression of repressors (26). Interestingly, the recruitment of *Groucho* corepressors is mediated by the engrailed homology (eh1) domains of homeodomain proteins (27). Fezl also contains an eh1 motif (12), raising the possibility that *Groucho* family members may interact with *Fezl* to regulate differentiation in the developing cortex. However, our data suggest that *Fezl* does not actively suppress the acquisition of alternative laminar

identities during cortical development, because we saw no expansion of markers for upper layer neurons into the deep layers, nor did we detect an expansion of markers for layers 5 or 6 (apart from *Igfbp4*) into inappropriate layers.

Fezl mutant mice exhibit profound defects in the elaboration of subcortical axonal projections. The growth and refinement of subcortically projecting axons from layer 5 neurons in rodents have been studied extensively (1). In neonates, layer 5 neurons throughout the cortex extend simple unbranched axons into the spinal cord. The parental axons then branch and invade additional targets, including the pons, colliculi, and deep cerebellar nuclei. Later, axon branches are selectively eliminated in a manner appropriate for the areal position of each neuron (1). In *Fezl*- deficient mice, CST projections were not detected by PLAP or BDA labeling, although a very small number of cells could be retrogradely labeled from the pyramidal decussation. PLAP- and BDA-labeled axons were also observed in inappropriate regions, suggesting that *Fezl*-expressing neurons have diverted their axons to novel destinations (including the anterior commissure, rostral cortex, striatum, and dorsal thalamus) in *Fezl* mutants.

Although *Fezl* mutants displayed severe defects in the guidance and targeting of CST axons, molecules that regulate the corticospinal axon growth and guidance (1, 28–32), as well as related family members and receptors, were expressed normally in *Fezl*-deficient brains. Proper patterning of the basal forebrain and hypothalamus is also essential for pyramidal tract development (32), but examination of basal forebrain patterning in *Fezl* mutant mice revealed that ventral forebrain and hypothalamic markers were expressed normally. Thus, although we have not exhausted the list of all known axon guidance molecules, it is likely that the layer 5 subcortical projection defects are caused by a misregulation of guidance molecules that remain to be identified and/or the failure of axons to respond properly to known guidance cues.

The anatomical and morphological development of CSMNs has been studied extensively (33), but the molecular mechanisms that control their specification, differentiation, survival, and connectivity are largely unknown. The recent advent of gene expression profiling, coupled with the purification of layer 5 CSMN and corticotectal neurons, has enabled the identification of specific patterns of gene expression in CSMNs (24). This previous study showed that *Fezl* is expressed throughout CSMN differentiation, and that *Ctip2*, which is also expressed in CSMNs, is required for normal CST development. In *Ctip2*-deficient mice, corticofugal axons failed to extend past the pons to reach the spinal cord (24), a phenotype reminiscent of that in *Fezl*-deficient mice (although *Ctip2* mutant mice die perinatally, precluding an analysis of mature patterns of connectivity). The similar phenotypes of *Fezl*-deficient and *Ctip2* mutant mice suggest that these two genes may act in a common pathway to control CSMN development. The earliest expression of *Ctip2* is observed in postmitotic neurons in lateral cortex at E14, with expression spreading medially over time (24). *Fezl*, in contrast, is first detected in the forebrain ventricular zone at E8.5, and expression remains strong during the production and differentiation of deep-layer neurons (19). This timing suggests that *Fezl* acts upstream of *Ctip2* to control the differentiation of deep-layer neurons. Indeed, we found that in the absence of *Fezl* function, *Ctip2* expression in the cerebral cortex was completely abrogated, even though expression in hippocampus and striatum was normal. Collectively, our data suggest that *Fezl* is a key upstream regulator of corticospinal projection neuron differentiation.

Note. After the completion of these experiments, a report suggesting that *Fezl* is required for specification of CSMNs and the formation of corticospinal projections was published by Molyneaux *et al*. (34).

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