

# Cis-regulatory control of corticospinal system development and evolution

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The co-emergence of a six-layered cerebral neocortex and its corticospinal output system is one of the evolutionary hallmarks of mammals. However, the genetic programs that underlie their development and evolution remain poorly understood. Here we identify a conserved non-exonic element (E4) that acts as a cortex-specific enhancer for the nearby gene *Fezf2* (also known as *Fez1* and *Zfp312*), which is required for the specification of corticospinal neuron identity and connectivity. We find that SOX4 and SOX11 functionally compete with the repressor SOX5 in the transactivation of E4. Cortex-specific double deletion of *Sox4* and *Sox11* leads to the loss of *Fezf2* expression, failed specification of corticospinal neurons and, independent of *Fezf2*, a *reeler*-like inversion of layers. We show evidence supporting the emergence of functional SOX-binding sites in E4 during tetrapod evolution, and their subsequent stabilization in mammals and possibly amniotes. These findings reveal that SOX transcription factors converge onto a *cis*-acting element of *Fezf2* and form critical components of a regulatory network controlling the identity and connectivity of corticospinal neurons.

The emergence and expansion of the neocortex in mammals has been crucial to the evolution of complex perceptual, cognitive, emotional and motor abilities<sup>1–3</sup>. The neocortex is organized into six layers based largely on the distinct subtypes of excitatory projection (or pyramidal) neurons and their patterns of connectivity<sup>4–9</sup>. Upper-layer (L2, L3 and L4) projection neurons form synaptic connections solely with other cortical neurons. By contrast, the majority of neurons in the deeper layers (L5 and L6) project to subcortical regions. Studies of laminar inversion in *reeler* mice lacking the reelin (RELN) protein<sup>10–14</sup> have shown that neuron identity and connectivity are determined by birth order rather than by laminar position, suggesting that neuronal specification and positioning are largely separately encoded.

The layer-specific pattern of connectivity is dependent on cortical areas. The long-range projections of L5 neurons in somatosensory-motor areas form the corticospinal (CS) system that directly connects the neocortex with various subcortical regions<sup>15–22</sup>. A major component of the system, the CS (or pyramidal) tract, descends through the brainstem and into the spinal cord to provide a high degree of direct control over the precise motor functions affected in many clinical conditions<sup>21,22</sup>. Despite these important functional implications, the genetic programs controlling CS system development and evolution remain unclear.

Phenotypic specification and evolution of neural circuits depend on precise regulation of the timing, location and level of gene expression<sup>23,24</sup>. Transcriptional control via *cis*-regulatory elements has emerged as a crucial mechanism<sup>25–29</sup>. The *cis*-regulatory mechanisms underlying the specification of distinct neuronal cell types and circuits, however, remain poorly understood. Specification of CS neurons and the formation of the CS tract critically depend on *Fezf2*, which encodes a zinc-finger transcription factor highly enriched in early cortical progenitor cells and their deep-layer neuron progenies<sup>30–35</sup>. Inactivation of *Fezf2* disrupts the molecular specification of deep-layer neurons and the formation of corticofugal projections, including the CS tract, without affecting the inside-out pattern of neurogenesis and lamination<sup>33–35</sup>. Misexpression of *Fezf2* in L2 or

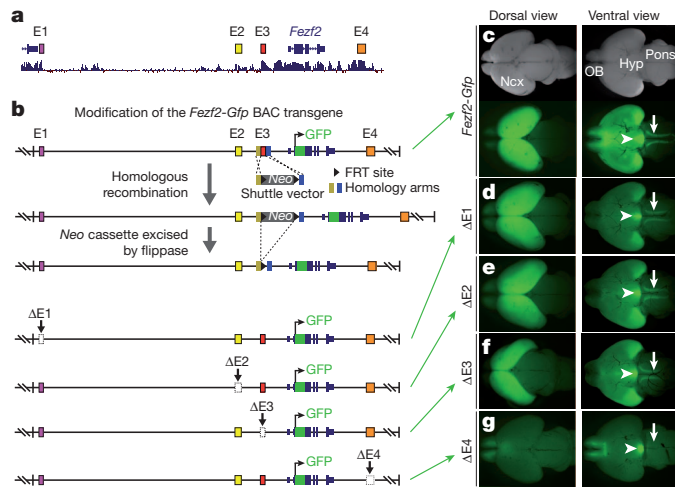
L3 cortico-cortical projection neurons<sup>35,36</sup> or striatal interneurons<sup>37</sup> alters their molecular profile and induces ectopic subcerebral projections. These findings indicate that the precisely regulated transcription of *Fezf2* is probably critical to the proper specification of distinct types of cortical projection neurons.

In this study, we used bacterial artificial chromosome (BAC) engineering and genetic inactivation in mice to identify and characterize a cortex-specific *Fezf2* enhancer and its *trans*-regulators. We show that three SOX transcription factors converge onto the *Fezf2* enhancer to control CS system development via functional binding sites that emerged in tetrapods. We also found that *Sox4* and *Sox11* are required for *Fezf2*-independent regulation of cortical RELN expression and laminar organization. Thus, these findings reveal novel developmental genetic programs that control layer formation and CS neuron identity, and the regulatory mechanisms by which they may have evolved.

## E4 enhancer controls cortical *Fezf2* expression

Previously, it has been shown that the BAC transgenic mouse harbouring 200 kb of the mouse *Fezf2* locus and the *Gfp* reporter gene (*Fezf2-Gfp*; ref. 38) recapitulates the spatio-temporal expression of endogenous *Fezf2* (refs 39, 40). This indicated that the *cis*-regulatory elements required for cortical *Fezf2* expression are located within the BAC sequence. On the basis of the remarkable similarity in cortical expression pattern of *Fezf2* between mouse and human<sup>39,41</sup>, we proposed that the regulatory elements are also highly evolutionarily conserved. Comparative sequence analysis revealed several conserved non-exonic elements (CNEEs) within the *Fezf2-Gfp* BAC (Fig. 1a). To test whether the selected CNEEs regulate *Fezf2-Gfp* expression, we generated multiple lines of BAC transgenic mice in which one of the CNEEs within the *Fezf2-Gfp* BAC was deleted ( $\Delta E1$  to  $\Delta E4$ ; Fig. 1b and Methods). We analysed GFP expression in whole-mount brain preparations and tissue sections of transgenic mice at embryonic and postnatal ages (Fig. 1c–g and Supplementary Figs 1 and 2). We found no pronounced change in  $\Delta E1$ ,  $\Delta E2$  and  $\Delta E3$  mutants, which, like

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**Figure 1 | Identification of a cortex-specific *Fezf2* enhancer.** **a**, The locations of CNEEs (E1 to E4) analysed in this study are indicated. **b**, Deletion of each CNEE from the *Fezf2-Gfp* BAC and transgenesis. A positive-selection neomycin cassette (*Neo*) flanked by homology arms was inserted by homologous recombination, resulting in the deletion of each CNEE. After the removal of *Neo* by flippase, the modified BACs ( $\Delta E1$  to  $\Delta E4$ ) were used for transgenesis. **c–g**, Whole-mount brains of P0 control *Fezf2-Gfp* (**c**) and founder-mutant (**d–g**) transgenic mice.  $n \geq 3$  founders per mutant line. In *Fezf2-Gfp* mice (**c**), GFP was expressed in the neocortex (Ncx), olfactory bulb (OB), hypothalamus (Hyp) (arrowhead) and CS axons in the pons (arrow). The deletion of E4 (**g**), but not E1 to E3 (**d–f**), led to a specific loss of GFP expression in the neocortex and CS axons, but not in the olfactory bulb or hypothalamus.

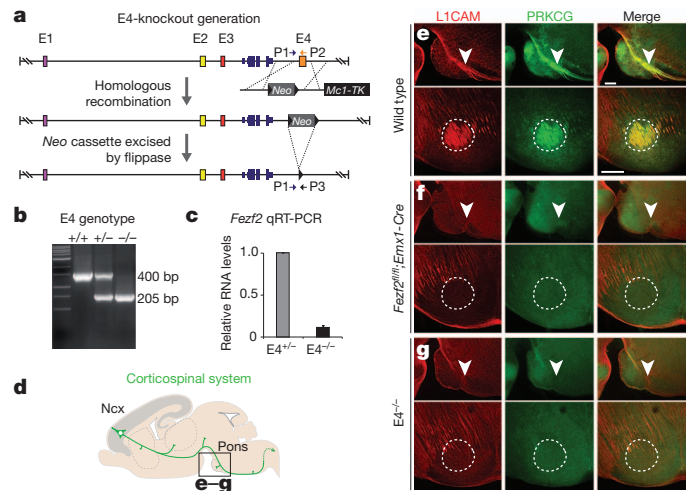
wild-type *Fezf2-Gfp* mice, expressed GFP in neocortex and pontine CS axons (Fig. 1d–f). By contrast, the deletion of CNEE E4 ( $\Delta E4$ ), which is located 7.3 kb downstream of the *Fezf2* transcription start site, resulted in a drastic loss of GFP expression in the cortex but not in the hypothalamus or the olfactory bulb (Fig. 1g). Moreover, in  $\Delta E4$  mice, GFP-positive CS axon fascicles were lost from the ventral surface of the pons (Fig. 1g). Consistent with this finding, E4 was identified as an *in vivo* binding site of the enhancer-associated protein EP300 in mouse embryonic forebrain<sup>27</sup>. Together, these results demonstrate that E4 is a *cis*-regulatory module acting as a cortex-specific enhancer of *Fezf2*.

### Loss of *Fezf2* expression and CS axons in $E4^{-/-}$ mice

To test the function of the E4 enhancer directly, we deleted the E4 region, while leaving intact the entire *Fezf2* coding and proximal promoter regions, using homologous recombination in embryonic stem cells (Fig. 2a, b). Mice lacking E4 ( $E4^{-/-}$ ) were viable, fertile and without overt behavioural or motor phenotypes. Quantitative reverse-transcription PCR (RT-PCR) analysis revealed that *Fezf2* expression is drastically downregulated in neocortex of these mice (Fig. 2c). Immunostaining of tissue sections of E4-null mice for PRKCG and LICAM, two proteins expressed by CS neurons and their axons<sup>11</sup> (Fig. 2d, e), revealed that CS axons were absent from the pons (Fig. 2g), similar to mutant mice with a cortex-specific deletion of *Fezf2* (*Fezf2<sup>fl/fl</sup>;Emx1-Cre*) (Fig. 2f). Furthermore, the expression of *Bcl11b* (*Ctip2*), a gene functioning downstream of *Fezf2* (refs 33, 36), was also downregulated in the E4-null neocortex (Supplementary Fig. 3). Taken together, these results indicate that the E4 enhancer is required for neocortical *Fezf2* expression, molecular specification of L5/6 neurons and the formation of CS tract.

### SOX4 and SOX11 bind and activate the E4 enhancer

We have previously shown that SOX5, a SOXD member of the large family of SOX transcription factors, binds to and represses the transcriptional activity of the E4 enhancer<sup>39</sup>. Unexpectedly, SOX5 itself is also required for CS tract formation independent of its repression of



**Figure 2 | Loss of neocortical *Fezf2* expression and CS axons in  $E4^{-/-}$  mice.** **a**, Generation of  $E4^{-/-}$  mice using the *pGK-Neo/Mc1-TK* selection cassette. **b**, Duplex PCR genotyping of wild-type and mutant alleles using primers P1, P2 and P3. **c**, Analysis of neocortical *Fezf2* expression by quantitative RT-PCR (qRT-PCR). Normalized to *Gapdh*, *Fezf2* messenger RNA levels were significantly reduced in the  $E4^{-/-}$  mice compared with heterozygous littermate controls ( $E4^{+/-}$ ).  $P = 2.1 \times 10^{-6}$ ; one-tailed Student's *t*-test;  $n = 3$  per genotype. Error bars; s.e.m. **d**, Schematic depiction of the CS system. **e–g**, Sagittal (top row) and coronal (bottom row) sections of the pons from wild-type (**e**), cortex-specific *Fezf2*-knockout (**f**) and  $E4^{-/-}$  (**g**) mice immunostained for axon marker LICAM (red) and PRKCG (green). The near-complete loss of CS axons (arrowheads and dashed outlines) in the  $E4^{-/-}$  mice is a phenocopy of cortex-specific *Fezf2* deletion. Scale bars, 200  $\mu\text{m}$ .

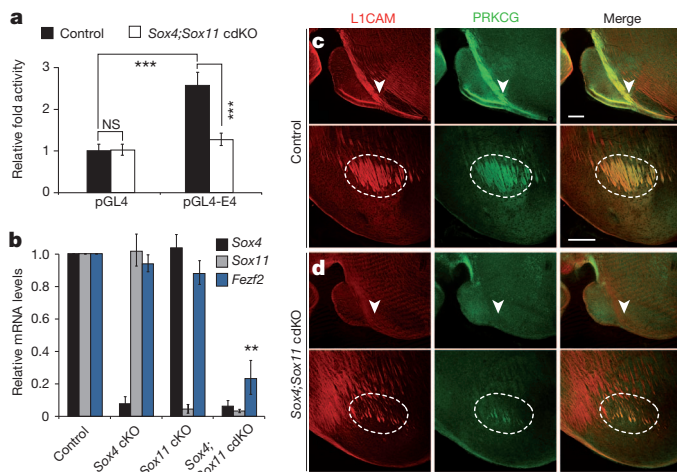
*Fezf2* (ref. 39). Because different SOX transcription factors are known to compete for a common motif to mediate both activation and repression of regulatory elements<sup>42–44</sup>, we proposed that other SOX members may activate the E4 enhancer, perhaps acting competitively against SOX5-mediated repression. Consistent with this hypothesis, our sequence analysis using MATINSPECTOR (Genomatix) revealed eight putative SOX-binding sites in E4. To prioritize which SOX transcription factors may be good candidates for potential *trans*-regulators of the E4 enhancer, we searched for those that are most highly correlated in their spatio-temporal expression pattern with *FEZF2*, using the Human Brain Transcriptome database<sup>45</sup> (<http://www.humanbrain-transcriptome.org>). The highest-correlated SOX genes were three members of the SOXC group (*SOX4*, *SOX11* and *SOX12*) and *SOX5* (Supplementary Fig. 4). The transcription factors encoded by these SOX genes are crucial in regulating cell fate and differentiation<sup>42–44,46</sup>, and a *de novo* deletion of *SOX11* was described in a patient with autism and intellectual disability<sup>47</sup>. Moreover, *Sox4* and *Sox11* act as transcriptional activators<sup>45</sup> and their expression patterns overlap with that of *Fezf2* in developing cortex (Supplementary Fig. 5).

To determine whether SOX4 or SOX11 binds E4, we performed chromatin immunoprecipitation (ChIP)-PCR assays in Neuro-2a cells transiently expressing V5-tagged SOX4 or SOX11 (Methods). Anti-V5 antibodies precipitated E4 DNA, but not E1, E2 or E3 DNA, confirming binding of SOX4 and SOX11 to the E4 enhancer (Fig. 3a). Moreover, recruitment of RNA polymerase II to E4 occurred in the presence of SOX4 or SOX11, which is consistent with increased transcriptional activity. To test the functional consequence of *SoxC*-gene expression on E4, we expressed a luciferase reporter under the control of E4 (pGL4-E4) in Neuro-2a cells (Fig. 3b). Luciferase activity driven by the E4 enhancer was significantly increased by co-transfected *Sox4* or *Sox11*, but not *Sox12*.

To dissect which sequences within E4 drive *Fezf2* expression, we generated four truncated versions of the E4 sequence (E4F1 to E4F4; Fig. 3c). The luciferase activity of the E4F2 fragment was increased by SOX4 and SOX11, but not by SOX12 (Fig. 3d). Using







**Figure 4 | Sox4 and Sox11 are required for Fezf2 expression and CS tract formation.** **a**, The requirement of SOX4 and SOX11 for E4 transactivation was determined in neurons cultured from E14.5 heterozygous littermate control mice and *Sox4;Sox11* cdKO mice, and transfected with the E4 luciferase construct. The activity of E4 in double-knockout neurons was not significantly above background (1.1-fold). **b**, Analysis of neocortical *Fezf2* expression by quantitative RT-PCR in cortex-specific *Sox4* and/or *Sox11* mutants. Normalized to *Gapdh*, *Fezf2* messenger RNA (mRNA) levels were drastically reduced in cdKO mice but not in *Sox4* or *Sox11* mutants. **c**, **d**, Sagittal (top row) and coronal (bottom row) sections of the control and cdKO pons immunostained for L1CAM (red) and PRKCG (green). The drastic loss of L1CAM- and PRKCG-positive CS tract axons in the cdKO mice (arrowhead and dashed outline) is similar to that in the cortex-specific *Fezf2* mutant. One-tailed Student's *t*-test;  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ ;  $n = 4$  per genotype. Errors bars; s.e.m.; scale bars, 200  $\mu$ m.

Next, using quantitative RT-PCR, we found a significant reduction in neocortical *Fezf2* expression in cdKO littermates compared with control or single cKO littermates at postnatal day 0 (P0) (Fig. 4b). In addition, immunostaining for L1CAM and PRKCG revealed a drastic loss of CS axons in *Sox4;Sox11* cdKO mice but not in single cKO littermates at P0 and P6 (Fig. 4c, d and Supplementary Fig. 8), whereas the organization of other brainstem tracts was not affected. Because the absence of L1CAM and PRKCG labelling could reflect their down-regulated expression, as opposed to loss of CS axons, in the cdKO mice, we confirmed the absence of the CS tract using the CRE-responsive *CAG-Cat-Gfp* transgenic line to express GFP in all cortical projection neurons and their axons<sup>39,40</sup> (Supplementary Fig. 9).

Two additional observations indicate that the loss of *Fezf2* and CS axons in these mice was not due to an absence of L5 neurons. First, only a moderate increase in cell death was detected in the somatosensory-motor areas, in which CS axons originate (Supplementary Fig. 10). Second, many BCL11B-immunopositive L5 neurons were present in the somatosensory-motor areas of cdKO mice, although lightly immunostained (Supplementary Fig. 11), indicating that neurons that would normally give rise to the CS tract were present. Analysis of additional laminar markers further revealed an inversion of cortical layers similar to that of the *reeler* mutant mouse<sup>10–12</sup> (Supplementary Fig. 11), indicating that *Sox4* and *Sox11* are also required for proper laminar positioning of neurons. Previous studies have shown that the CS tract is present in *reeler* mice<sup>12</sup>, indicating that this laminar phenotype occurs independently of *Fezf2* and probably in response to defects in the RELN signalling pathway<sup>13,14</sup>. In support of this possibility, RELN was absent from cdKO mouse neocortex (Supplementary Fig. 12). Thus, the combined deletion of *Sox4* and *Sox11* leads to defects in the laminar position of neurons and the molecular specification and connectivity of CS neurons.

### Functional and evolutionary implications of E4 sequence variations

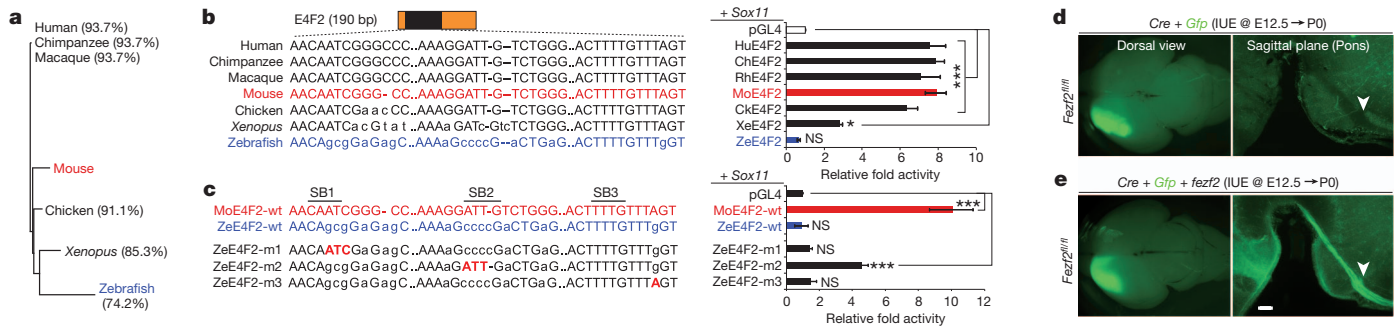
The differences in SOXC-mediated transcription between mouse E4F2 and zebrafish E4F2 suggest that species differences in the

E4F2 sequence have functional implications. Analysis of SB1, SB2 and SB3 sites within the E4F2 sequence in 23 species revealed that SB2 is conserved in all analysed mammals and the two available non-mammalian amniotes (chicken and lizard) (Supplementary Fig. 13). To investigate the functional consequences of species differences, we used luciferase assays in Neuro-2a cells to analyse the activity of E4F2 sequence from different vertebrates (Fig. 5a, b) in response to SOX11, a more powerful transactivator than SOX4 (Fig. 3). Of the constructs containing an E4F2 sequence of a mammal (human, chimpanzee, macaque, or mouse) or non-mammalian amniote (chicken), which have conserved SB1–3 sequences, the luciferase reporter activity was strongly increased following co-transfection with *Sox11*. The reporter activity of a non-amniote tetrapod (*Xenopus*) E4F2 construct was moderately increased compared with the empty plasmid, but was not as high as that of the mammalian constructs ( $P = 3.9 \times 10^{-16}$ ; one-tailed Student's *t*-test; Fig. 5b). By contrast, the reporter construct containing the zebrafish E4F2 sequence, the SB2 sequence of which is highly divergent from those of mammals, exhibited only basal level activity similar to the empty control luciferase plasmid. To confirm that this is dependent on sequence variations between species, we mutated zebrafish E4F2 by 'murinizing' its SB1, SB2 or SB3 sequence (ZeE4F2-m1, -m2 or -m3, respectively). Reporter activity of the murinized zebrafish SB2 (ZeE4F2-m2), but not SB1 or SB3, was robustly induced by SOX11 (Fig. 5c). ZeE4F2-m2 murinization, however, was insufficient to restore the level of expression observed in the wild-type mouse sequence, suggesting the potential contribution of additional sequences. Thus, evolutionary differences in the E4F2 sequence, especially within SB2, are directly related to functional differences in the ability of SOX11 to activate this regulatory element.

Next, to investigate the functional consequences of species differences in the FEZF2 coding sequence, we tested the ability of zebrafish *fezf2* to rescue the effects of mouse *Fezf2* deficiency. We electroporated *in utero* the neocortical wall of the floxed *Fezf2* (*Fezf2<sup>fl/fl</sup>*) mice<sup>40</sup> with Cre and CRE-responsive *Gfp*-expressing constructs with or without *fezf2* at embryonic day 12.5 (E12.5) (Fig. 5d). Analysis of electroporated mice at P0 revealed that *fezf2* was sufficient to rescue the formation of the CS tract in *Fezf2*-null mouse neurons (Fig. 5e). Taken together, these results suggest that sequence variations in the E4 enhancer, but not the *Fezf2* coding region, gave *Fezf2* an essential role in CS tract evolution.

### Discussion

Our data provide critical insight into the genes and regulatory components controlling CS system development, centring on the *cis*-regulation of *Fezf2* by SOX4 and SOX11. We show that genetic inactivation of the E4 *cis*-regulatory module results in compartmentalized phenotypic effects largely limited to the loss of cortical *Fezf2* expression and CS system. The spatio-temporal dynamics of cortical *Fezf2* is further controlled by SOX5 and TBR1, two transcription factors expressed post-mitotically in projection neurons<sup>39,40,46,48,49</sup>. On its initial activation in early cortical progenitors, *Fezf2* is highly expressed in deep-layer neurons during early corticogenesis but subsequently repressed in L6 neurons by SOX5 and TBR1 to create a postnatal L5-enriched pattern. The earlier expression of SOX4 and SOX11 is consistent with their role in *Fezf2* activation before the L6 upregulation of SOX5, which is a functional competitor. Despite what is known about the function and regulation of *Fezf2*, a number of key questions have yet to be addressed. First, additional, as yet untested, *trans*-regulators and regulatory elements probably contribute to *Fezf2* regulation in a context-dependent manner. These include the mediators of *Fezf2* repression in L2, L3 and L4 projection neurons. Second, the distinct, and seemingly incongruous, effects of SOX5 in repressing *Fezf2* but being independently required for CS tract formation<sup>39</sup> have not been resolved. Third, the direct transcriptional targets of FEZF2 in CS neurons remain largely unknown.



**Figure 5 | Functional analysis of species differences in E4 sequence.**

**a**, Hierarchical clustering of E4F2 sequences from seven vertebrates. Percentage nucleotide identity relative to mouse E4F2 is indicated in parentheses. **b**, Analysis of species differences in *Sox11* activation of E4F2. *Sox11* transactivated the E4F2 sequence of four mammals and chick ( $\geq 6.3$ -fold) and *Xenopus* (2.8-fold). By contrast, the activity of zebrafish E4F2, which is divergent in SB1 and SB2, was not activated by *Sox11*. **c**, Murinization (red uppercase nucleotides) of zebrafish SB2 (ZeE4F2-m2) partly rescued the loss of

The laminar position and radial organization of projection neurons are key features of cortical development, which rely on RELN signaling<sup>10–14</sup>. We also show that SOX4 and SOX11 are crucial in regulating RELN expression and the inside-out pattern of cortical layer formation, independent of E4 or *Fezf2* and probably involving interactions with distinct regulatory elements. Moreover, SOX4 and SOX11 have additional roles, as in mice lacking both genes, the cortex and olfactory bulb are smaller and cell death is increased. Thus, SOX4 and SOX11 have pleiotropic functions, which are probably mediated by distinct regulatory elements and downstream target genes that are involved in multiple developmental processes.

Our results indicate that, following their emergence in tetrapods, functional SOX-binding sites have retained high conservation through purifying selection in mammals and some amniotes, thus directly linking species variations in regulatory sequences to functional outcomes. E4 sequence substitutions in SB2 may constitute an evolutionary turning point for *Fezf2* function during forebrain development, possibly facilitating the formation of descending telencephalic pathways including the CS system. Whereas minor projections from the ventral (subpallial) telencephalon to the spinal cord are present in amphibians<sup>2</sup>, direct dorsal telencephalo-spinal projections resembling the CS tract have been reported only in mammals and some birds<sup>2,50</sup>. We propose that the concurrent emergence of the described regulatory mechanisms and direct telencephalo-spinal projections in early amniotes, together with subsequent changes in genetic programs driving the patterning and expansion of a six-layered dorsal pallium, made possible the evolution of the CS system in mammals.

## METHODS SUMMARY

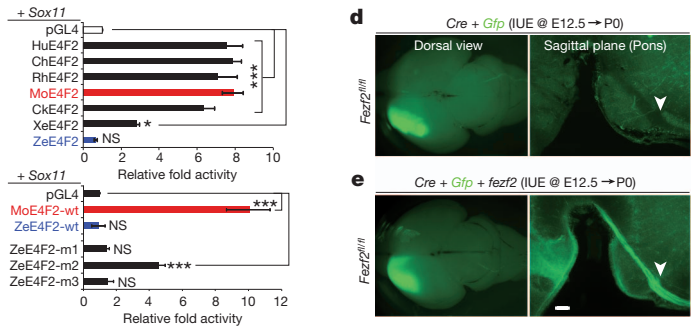
Selected CNEE sequences were deleted from a *Fezf2-Gfp* BAC by recombination-mediated genetic engineering, and GFP expression from modified BACs was analysed by transgenesis. To confirm the requirement of E4 enhancer for *Fezf2* expression, a germline E4 deletion mutant was generated. Putative E4 transactivators SOX4 and SOX11, identified by E4 sequence and co-expression analyses, were tested using ChIP and luciferase reporter assays. To analyse the requirement of *Sox4* and *Sox11* for *Fezf2* expression and cortical development, we generated cortex-specific single and double *Sox4* and *Sox11* null mice. Complete materials and methods are described in Supplementary Information.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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transactivation of wild-type zebrafish E4F2 (*ZeE4F2-wt*) by *Sox11*. **d**, **e**, Cell-autonomous rescue of mouse *Fezf2* loss of function by zebrafish *fezf2*. *In utero* electroporation (IUE) of *Fezf2*<sup>fl/fl</sup> neocortical wall at E12.5 with *Cre* and *Cre*-responsive *Gfp* plasmids. *Fezf2*-deficient L5 neurons do not form CS tract at P0 (**d**). Co-electroporation of an *fezf2* plasmid cell-autonomously rescued the formation of CS tract by *Fezf2*-deficient neurons (**e**). One-tailed Student's *t*-test; \* $P < 0.05$ , \*\*\* $P < 0.001$ ;  $n = 4$  per condition. Errors bars, s.e.m.; scale bar, 200  $\mu\text{m}$ .

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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## METHODS

**Animals.** All experiments were performed in accordance with a protocol approved by Yale University's Committee on Animal Research. The generation of mice with the floxed *Sox4* and *Sox11* alleles (*Sox4<sup>fl/fl</sup>* and *Sox11<sup>fl/fl</sup>*, respectively) was described elsewhere<sup>44,51</sup>. The *CAG-Cat-Gfp* and *Emx1-Cre* P1 artificial chromosome transgenic mice were a gift from Melissa Colbert<sup>52</sup> and Takuji Iwasato<sup>53</sup>, respectively. The *Fezf2-Gfp* BAC transgenic mouse was generated by the GENSAT project<sup>38</sup>.

**Generation of BAC transgenic mouse.** To generate BAC reporter constructs harbouring deletions of putative enhancer elements (E1–E4) in the mouse *Fezf2-Gfp* BAC (RP23-141E17-*Gfp*; the GENSAT project<sup>38</sup>), homology arms A and B flanking the sequences targeted for deletion (E1, Chr14: 13,204,475–13,204,944, 470 bp; E2, Chr14: 13,183,314–13,183,991, 678 bp; E3, Chr14: 13,180,797–13,181,283, 487 bp; E4, Chr14: 13,170,100–13,170,960, 861 bp) were amplified by PCR and cloned into the PL451 shuttle vector. SW102 *Escherichia coli* transformed with the *Fezf2-Gfp* BAC was induced for recombination-mediated genetic engineering (recombineering) at 42 °C and electroporated with the linearized shuttle vector. After neomycin selection, *Flpase* expression was induced by arabinose to excise the neomycin cassette<sup>54</sup>. Founder lines were confirmed by PCR using primers corresponding to sequences from E1–E4 (Supplementary Table 1). *Fezf2-Gfp* lines were maintained on a C57BL6/J background and all animals were housed under identical conditions. The intactness of the integrated BACs was confirmed by PCR and deep sequencing of genomic DNA.

**Generation of E4-null mice.** The targeting vector for the deletion of the E4 enhancer was constructed by a recombineering-based method with an unmodified BAC clone (RP23-141E17). A 6,627-bp fragment containing the E4 enhancer was retrieved from the BAC and inserted into the PL253 vector by recombination in SW102 bacteria. The removal of the E4 sequence was achieved using the strategy described above for the generation of the  $\Delta$ E4 BAC transgenic mouse. ES cells (C57BL6/J) were electroporated with the resulting targeting construct and expanded under positive (*pGK-Neo*, from the PL451 vector) and negative (*Mcl-TK*, from the PL253 vector) selection. Correctly recombined clones were identified by long-distance PCR, confirmed by sequencing, and used for blastocyst injection and subsequent generation of mice with the targeted allele. The neomycin cassette was subsequently removed by flippase-mediated recombination by breeding with *Flp* mice. The mice were genotyped using the P1/P2 primer set (400 bp) for the wild-type allele and the P1/P3 primer set (205 bp) the E4 deletion allele (Supplementary Table 1).

**Immunohistochemistry.** P0 and P6 brains were fixed by immersion in 4% paraformaldehyde overnight at 4 °C and sectioned using a vibratome (Leica). Immunostaining was performed as previously described<sup>20</sup>. The following primary antibodies were used: anti-L1CAM (rat, 1:300; Millipore), anti-PRKCG (rabbit, 1:300; Santa Cruz), anti-GFP (chicken, 1:3,000; Abcam), anti-CUX1 (rabbit, 1:150; Santa Cruz), anti-SATB2 (mouse, 1:200; Genway), anti-BCL11B (rat, 1:250; Santa Cruz), anti-ZFPM2 (rabbit, 1:250; Santa Cruz), anti-RELN (mouse, 1:300; Millipore) and anti-CASP3, active (rabbit, 1:200; Millipore). We tested a total of 12 commercially available anti-SOX4 antibodies (Abcam, ab52043, ab86809, ab90696; Abgent, AP2045a, AP2045c; LS Bioscience, LS-B3520; Millipore, AB5803, AB10537; Pierce, PA1-38638, PA1-38639; Santa Cruz Biotechnology, sc-17326; Sigma, HPA029901) and 10 commercially available anti-SOX11 antibodies (Abcam, ab42853; Aviva Systems Biology, ARP33328, ARP38235; LS Bioscience, LS-B1567, LS-C10306; Millipore, AB9090; Pierce, PA5-19728; Santa Cruz Biotechnology, sc-20096, sc-17347; Sigma, HPA000536) on western blots and immunostaining. Three antibodies for each of SOX4 and SOX11 recognized a single band of the expected size on western blots but none of them were suitable for immunostaining or ChIP assay. In addition, a few antibodies showed nuclear immunostaining but also had strong background staining of tissue sections of the knockout brain, so we concluded that they are not specific.

**Plasmids.** For expression of *SoxC* genes and *Fezf2*, full-length complementary DNAs (mouse *Sox4*, BC052736; mouse *Sox11*, BC062095; mouse *Sox12*, BC067019, zebrafish *fezf2*, BC085677) were inserted into pCAGEN. For ChIP and electrophoretic mobility shift assay, PCR-amplified products (for mouse *Sox4* and *Sox11* full-length complementary DNA and *Sox11(1–276aa)*) were inserted into pcDNA3.1/V5-His TOPO vector (Invitrogen). For luciferase reporter plasmids, PCR-amplified products (for E1 to E4, E4F1 to E4F4 of mouse and E4F2 of additional species) and annealed, 190-bp complementary synthetic oligonucleotides (for MoE4F2-m1-3 and ZeE4F2-m1-3) were inserted into pGL4 (Promega). The sequences of the PCR primers and synthetic oligonucleotides are listed in Supplementary Table 1.

**Gene expression analysis.** To show which genes in the SOX family are highly correlated with human *FEZF2*, we used the previously generated data<sup>45</sup> for

pairwise Pearson comparisons. This data set is generated by exon arrays and is available from the Human Brain Transcriptome database (<http://www.humanbraintranscriptome.org>) and the NCBI Gene Expression Omnibus under the accession number GSE25219. It covers 16 brain cortical regions over 15 periods, ranging in stage from embryonic development to late adulthood. The evaluation of gene expression in each region and each period was detailed in the study<sup>45</sup>. In this study, we averaged gene expression values of 11 neocortical regions in each of 15 developmental periods represented in the data set, and then performed pairwise Pearson correlation analysis for *FEZF2* and the members of the SOX family. In total, 18 SOX genes were represented in the database. The correlation analysis was shown by heatmap and the correlation coefficients were indicated in each cell (Supplementary Fig. 4). Mouse *Fezf2*, *Sox4*, *Sox5*, *Sox11*, and *Sox12 in situ* hybridization images were obtained from the Genepaint database<sup>55</sup> (<http://www.genepaint.org>).

**Chromatin immunoprecipitation.** After testing available anti-SOX4 and anti-SOX11 antibodies and finding none to be specific and suitable for ChIP assay, we performed ChIP assay with Neuro-2a cells that has been transfected with pcDNA3-*Sox4-V5* or pcDNA3-*Sox11-V5* plasmid using Lipofectamine 2000 (Invitrogen). At 36 h after transfection, cells were crosslinked in 1% formaldehyde for 10 min at 37 °C and processed for ChIP assay using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions. Capture of the DNA fragments was tested by PCR using primers 5'-TGGAGAGAAGGCCA ACAAAC-3' (E1, forward); 5'-GCTGGGGATGGAGAAGAATA-3' (E1, reverse); 5'-TCACCAAAGCGCCTTTTAT-3' (E2, forward); 5'-GTAAGCGG ACATGCCATTTT-3' (E2, reverse); 5'-TGACTTCCCCAGCCTTCTA-3' (E3, forward); 5'-CAACAGCTCACCCACACAAT-3' (E3, reverse); 5'-ATGCCTA GCCCAAAGAAAT-3' (E4, forward) and 5'-TTAAGTCCCCCTTGGC TCT-3' (E4, reverse).

**Electrophoretic mobility shift assay.** Double-stranded DNA probes were generated by annealing with complementary single-stranded oligonucleotides. Each DNA probe was biotin-end-labelled with Klenow enzyme and then purified using the G-50 Sephadex Column (Roche). The labelled and unlabelled (cold probe; 100-fold) probes were incubated with SOX4-V5 and SOX11(1–276aa)-V5 fusion proteins, which were produced using a TNT Quick coupled transcription/translation kit (Promega) in a DNA-binding buffer (75 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl (pH 7.5), 6% glycerol, 2  $\mu$ g BSA, 16 ng poly (dI-dC) and 0.1  $\mu$ g salmon sperm DNA) at 30 °C for 30 min. For supershift assay, anti-V5 antibody (Invitrogen) was added into the binding reaction and incubated for an additional 10 min. The shift assay was carried out in 6% polyacrylamide gel in  $\times 0.5$  TBE buffer. Subsequently, the labelled DNA was transferred on a nylon membrane (Amersham Biosciences). For detection, we used the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) according to manufacturers' recommendations. All primer sequences are described in Supplementary Table 1.

**Luciferase assays.** Neuro-2a cells were transfected using Lipofectamine 2000 (Invitrogen) with one of pCAG-*Sox5*, pCAG-*Sox4*, pCAG-*Sox11*, pCAG-*Sox12* or empty pCAGEN, together with one of the pGL4 (Promega) luciferase vectors generated with enhancer sequences as described above. A *Renilla* luciferase plasmid (pRL, Promega) was co-transfected to control for transfection efficiency. The luciferase assays were performed 48 h after transfection using the dual-luciferase kit (Promega) according to the manufacturer's instructions. Primary cortical neurons were prepared from E14.5 heterozygous littermate control (*Sox4<sup>fl/+</sup>; Sox11<sup>fl/+</sup>; Emx1-cre* and *Sox4<sup>fl/+</sup>; Sox11<sup>fl/+</sup>*) and *Sox4; Sox11* cdKO (*Sox4<sup>fl/fl</sup>; Sox11<sup>fl/fl</sup>; Emx1-cre*) cortices and transfected with pGL4 or pGL4-E4 with pRL using the AMAXA Mouse Neuron Nucleofector Kit (VPG-1001; Lonza). At 48 h after transfection, the luciferase assays were performed as described above.

**In utero electroporation.** Plasmid DNA (4  $\mu$ g  $\mu$ l<sup>-1</sup>) mixture (pCAGEN-Cre;pCALNL-Gfp or pCAGEN-Cre;pCALNL-Gfp;pCAGEN-fezf2) was injected into the lateral ventricles of embryonic mice at E12.5 and transferred into the cells of ventricular zone by electroporation (five 50-ms pulses of 40 V at 950-ms intervals) as described elsewhere<sup>35,39</sup>. Brains and tissue sections of electroporated animals were analysed for GFP expression after fixation with 4% paraformaldehyde at P0.

**Quantitative RT-PCR.** Neocortex was dissected from P0 brain and RNA was isolated using the RNeasy kit (Qiagen). Quantitative RT-PCR was performed using primers listed in Supplementary Table 1 and the SYBR FAST qPCR kit (KAPA Biosystems) according to the manufacturer's instructions. Gene expression levels were normalized to *Gapdh* expression.

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