Zfp312 is required for subcortical axonal projections and dendritic morphology of deep-layer pyramidal neurons of the cerebral cortex

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Pyramidal neurons of the cerebral cortex display marked layer- and subtype-specific differences in their axonal projections and dendritic morphologies. Here we show that transcription factor *Zfp312* is selectively expressed by layer V and VI subcortical projection pyramidal neurons and their progenitor cells. Knocking down *Zfp312* with small interfering RNAs dramatically reduced the number of subcortical axonal projections from deep-layer pyramidal neurons and altered their dendritic morphology. In contrast, misexpression of *Zfp312* in cortically projecting pyramidal neurons of layers II and III induced the expression of Tbr1, a transcription factor enriched in deep-layer neurons, and the formation of ectopic subcortical axonal projections. Thus, our results indicate that transcription factor Zfp312 plays a critical role in layer- and neuronal subtype-specific patterning of cortical axonal projections and dendritic morphologies.

development | neocortex | transcription factor

The development of the cerebral cortex requires the correct molecular specification of neuronal identity and the proper formation of neuronal connections. The majority of cortical neurons are pyramidal neurons, which extend long axonal projections both within and beyond the cortex (1–3). Pyramidal neurons display marked layer- and subtype-specific differences in their axonal projections and dendritic morphologies (1–6). The axons of pyramidal neurons in layers II and III form synaptic connections solely with other cortical neurons. In contrast, the majority of layer V and VI pyramidal neurons project axons to subcortical targets, comprising the collective output of the cortex. Layer VI pyramidal neurons project to the thalamus, whereas other subcortical regions, including the brainstem and spinal cord, receive cortical projections mainly from layer V pyramidal neurons.

Cortical progenitor cells give rise to pyramidal neurons in an inside-first, outside-last sequential manner (3, 6–8). Deep-layer neurons originate from early progenitors in the ventricular zone (VZ), whereas upper-layer neurons arise from late progenitors. Although laminar position is normally correlated with the type of axonal projection, it is not laminar position but the timing of neuron generation that determines the axonal target (3, 6–8). Cortical progenitors at the earliest stage of neurogenesis are multipotent, exhibiting the ability to generate multiple types of pyramidal neurons (8). Later in neurogenesis, the developmental potential of these progenitors becomes progressively restricted to the generation of only upper-layer neurons (8). It has been proposed that genetic programs control this restriction process. However, active molecular determinants for the generation and differentiation of deep-layer neurons remain unknown.

Layer- and neuronal subtype-specific molecular markers, many of which are transcription factors, have been identified in the cerebral cortex (3, 9–12). To explore the molecular development of deep-layer pyramidal neurons, we started with genome-wide expression analysis and identified Zinc finger protein 312 (Zfp312; also known as Fezl and Fez1) as a transcription factor selectively expressed by layer V and VI subcortical projection pyramidal neurons and their progenitors. Through a series of molecular manipulations in mouse embryos, we demonstrate that *Zfp312* is necessary for the normal development and sufficient for the ectopic formation of subcortical axonal projections of pyramidal neurons.

Methods

Animals. Experiments were carried out with CD1 mice in accordance with a protocol approved by the Committee on Animal Research at Yale University. The morning of a detectable vaginal plug and the first neonatal day were considered to be embryonic day 0.5 (E0.5) and postnatal day 0 (P0), respectively.

Affymetrix Microarrays and RT-PCR Analysis. Total RNA was isolated from freshly dissected brain tissue by using the TRIzol reagent and cDNA was synthesized by using SuperScript (Invitrogen). Messenger RNA expression profiles were determined by the Affymetrix GeneChip Mouse Expression Set 430 (for detailed procedure and results, see *Supporting Text*, which is published as supporting information on the PNAS web site). For quantitative real-time RT-PCR, predesigned primer and probe sets were obtained from Applied Biosystems or generated by using PrimerExpress. Thermocycling was carried out by using the Applied Biosystems 7900 system and monitored by TaqMan 5′ exonuclease assay or SYBR Green I dye detection. *Gapdh* levels were used for normalization.

***In Situ* Hybridization (ISH).** Adult mice were perfused with 4% paraformaldehyde (PFA). Embryos were fixed by immersion in PFA for 24 h. Brains were cryoprotected in graded sucrose solutions and frozen. ISH was performed on cryosections (40 μm) or whole mounts by using digoxigenin (DIG)-labeled riboprobe corresponding to nucleotides 1241–1734 of mouse *Zfp312* (GenBank accession no. NM_080433). Hybridization was performed overnight at 60°C, and the signal was detected with an alkaline phosphatase-conjugated anti-DIG antibody and NBT/BCIP (Roche).

DNA Cloning and Constructs. Detailed information on the generation of the Zfp312-GFP, CLEG-Zfp312, Zfp312-siRNAs, and the appropriate control constructs is published in *Supporting Text* and Table 1, which are published as supporting information on the PNAS web site.

N2a Cells and Immunoblot Analysis. N2a cells were cultured and transfected by using FuGENE 6 (Roche Diagnostics) as described (13). Two days after transfection, cells were either examined directly under a fluorescent microscope or sorted by FACS for GFP-positive cells for Western blotting (13).

Conflict of interest statement: No conflicts declared.

Abbreviations: VZ, ventricular zone; E*n*, embryonic day *n*; P*n*, postnatal day *n*; ISH, *in situ* hybridization.

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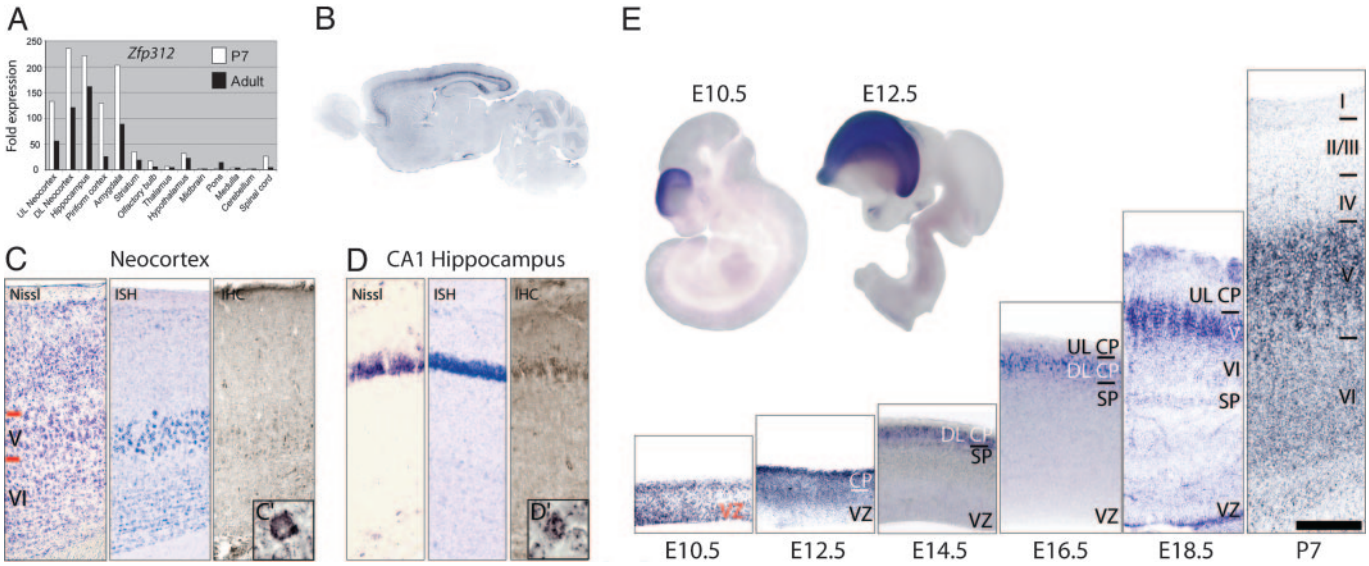

Fig. 1. *Zfp312* expression in early VZ progenitors and deep-layer pyramidal neurons. (*A*) Quantitative real-time RT-PCR analysis of *Zfp312* expression. (*B*) Sagittal section of adult brain hybridized with *Zfp312* riboprobe. (*C* and *D*) Nissl staining and *Zfp312* ISH and immunohistochemistry in neocortex and CA1 hippocampus. (*Insets*) Nuclear localization of Zfp312 in neocortical layer V (*C*′) and hippocampal (*D*′) pyramidal neurons. (*E*) *Zfp312* ISH in whole-mount embryos and sagittal sections of embryonic neocortical wall and P7 neocortex. *Zfp312* is expressed by early ventricular zone (VZ) progenitors (E10.5 and E12.5). From E14.5, *Zfp312* is down-regulated by VZ progenitors, and *Zfp312*-expressing neurons are localized solely in the subplate (SP), deep-layer cortical plate (DL CP), and layers V and VI and absent in the upper-layer CP (UL CP) and layers II to IV. (Scale bar, 275 μm in *C*, 140 μm in *D*, and 200 μm in *E*.)

***In Utero* Electroporation.** For *in utero* gene transfer by electroporation, 1–2 μl of DNA solution (4 μg/μl) was injected into the lateral ventricle and electroporated (five 50-ms pulses of 40 V with 950-ms intervals) as described (14).

Immunohistochemistry (IHC). The custom anti-mouse Zfp312 antibody was raised in rabbits against the peptide SVGPTATPSAKDLARTVQS (Alpha Diagnostic). Primary antibodies used were αZfp312 (1:100), αGFP (1:3,000; A11122, Molecular Probes), αTbr1 (1:1,000; ref. 3), and αPou3f3 (1:2,000; sc6028, Santa Cruz Biotechnology). IHC was performed as described (15).

Axonal Tracing. The retrograde tracer BDA-3k (Dextran-3000 MW conjugated to tetramethylrhodamine; Molecular Probes) was injected through a Hamilton syringe into designated regions of adult mice in a Kopf stereotaxic instrument. After recovery and survival of 14 days, brains were fixed by perfusion and cryosections were processed for *Zfp312* ISH as described above. The expression of *Zfp312* was examined for colocalization with BDA-3k under a fluorescent microscope.

Somato-Dendritic Analysis. The cell body size, dendritic arborization, and spines of diaminobenzidine-immunolabeled GFP$^+$ neurons in the primary motor cortex were quantified by 3D reconstruction using the Neurolucida system (Microbrightfield). To determine laminar distribution, sections through the primary somatosensory cortex were divided into 10 horizontal bins from superficial to deep, and the percentage of GFP$^+$ neurons in each bin was calculated.

Results

***Zfp312* Expression in Subcortically Projecting Neurons and Their Progenitors.** To identify transcription factors that may regulate the identity and connectivity of subcortically projecting neurons, we searched for transcripts selectively expressed in deep layers of the developing neocortex. We used the Affymetrix GeneChip Mouse Expression Set 430 to compare global gene expression in neocortical layers I–IV, layers V and VI, hippocampus, and striatum microdissected from P7 mice. Of the 16,930 genes and ESTs present in the neocortex and hippocampus, 56 with neocortical expression enriched in the deep layers (Fig. 7, which is published as supporting information on the PNAS web site) and were chosen for validation and secondary screening using quantitative real-time RT-PCR and ISH. One of the candidate transcripts, *Zfp312*, was highly enriched in neocortical layer V, and to a lesser extent in layer VI, the CA fields of the hippocampus, the piriform cortex, and the amygdala of the P7 and adult mouse brain (Fig. 1 *A* and *B*). Recently, *Zfp312* has also been identified by other groups as a marker of deep-layer neocortical neurons (11, 12, 16, 17, 27, ‡). The mouse *Zfp312* gene is a homolog of the *Xenopus* and zebrafish *Forebrain-specific embryonic zinc finger* gene (18–21), which contains six C_2H_2-type zinc-finger motifs, suggesting that it functions as a transcription factor. Consistent with this, Zfp312 localizes to nuclei of pyramidal neurons in layers V and VI and hippocampus (Fig. 1 *C* and *D*) as well as the nuclei of N2a cells when misexpressed (Fig. 3*A*). The zebrafish homolog of *Zfp312* is required for the development and function of forebrain monoaminergic neurons (21). Mice lacking *Zfp312* exhibit hyperactive behavior and have a reduced number of subplate neurons as well as diminished connections between the cortex and thalamus (16). The restricted expression pattern of this transcription factor and its involvement in brain development make it a promising candidate for controlling the identity and connectivity of layer V and VI pyramidal neurons.

To explore the possible role of *Zfp312* in the development of deep-layer projection neurons, we examined its expression using ISH in embryonic and postnatal mouse brains (Fig. 1*E*). Consistent with recent reports (16–18, 22, 27, ‡), *Zfp312* was expressed by early cortical VZ progenitor cells, which generate neurons destined for the subplate zone and deep layers of the neocortex. At E12.5, *Zfp312* transcripts were highly enriched in the postmigratory pyramidal neurons forming the cortical plate situated beneath the pial surface. During late embryonic and early postnatal development, *Zfp312* expression disappeared from cortical progenitors and was

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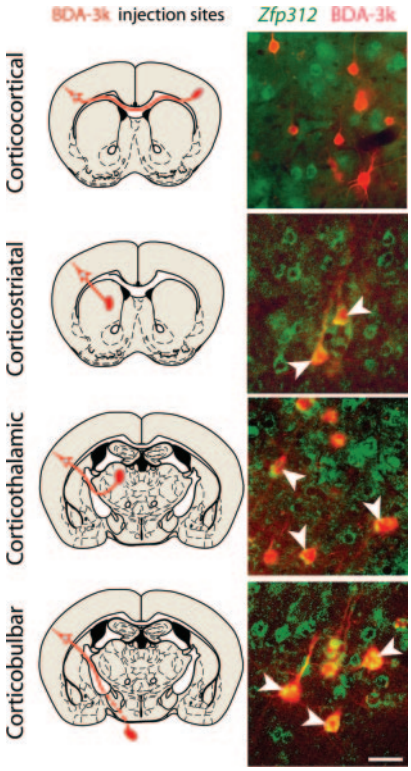

Fig. 2. *Zfp312* is exclusively expressed by subcortically projecting neurons. (*Left*) Schematic depictions of stereotaxical injection sites of retrograde tracer BDA-3k and expected projection routes (red). (*Right*) Representative images of neurons in the adult primary motor neocortex retrogradely labeled with BDA-3k (red) and colabeled by *Zfp312* ISH (green). *Zfp312*$^+$ neurons project to the striatum, thalamus, and brainstem (arrowheads). (Scale bar, 50 μm.)

restricted to the subplate and the prospective layer V and VI pyramidal neurons.

Cortical layers V and VI contain pyramidal neurons that extend either subcortical or cortical axons (1–3, 5, 10). To determine the projection targets of *Zfp312*-expressing neurons, we combined BDA-3k retrograde axonal tracing with ISH for *Zfp312* (n = 12 animals). *Zfp312* transcripts colocalized with a vast majority of retrogradely labeled deep-layer neurons with axonal projections to the striatum (84 ± 2%), thalamus (96 ± 2%), and brainstem (98 ± 2%), but not with the neurons that have callosal projections to the contralateral neocortex (0%) (Fig. 2). These results demonstrate that Zfp312 is a developmentally regulated transcription factor selectively expressed by the subcortically projecting deep-layer pyramidal neurons and their VZ progenitor cells.

***Zfp312* Is Required for the Formation of Subcortical Axonal Projections.** The specific expression of *Zfp312* in early VZ progenitor cells and the postmitotic deep-layer pyramidal neurons suggests that *Zfp312* may play a role in their generation, migration, or differentiation. To examine these possibilities, we used small interfering RNA (siRNA) to knock down *Zfp312* activity. Two different siRNA sequences, each targeting a specific region of the mouse *Zfp312* transcript, and corresponding control scrambled (scr) sequences were cloned into pLVTH or pCRLH, siRNA vectors that coexpress GFP or RFP, respectively (Fig. 8, which is published as supporting information on the PNAS web site). The ability of siRNAs to knock down Zfp312 expression was confirmed in N2a cells coexpressing Zfp312-GFP and *Zfp312* siRNAs or scr siRNAs. Compared with control scr siRNAs, *Zfp312* siRNAs dramatically reduced mRNA levels (data not shown), GFP fluorescence (Fig. 3*A*), and protein levels (Fig. 3*B*) of the Zfp312-GFP construct,

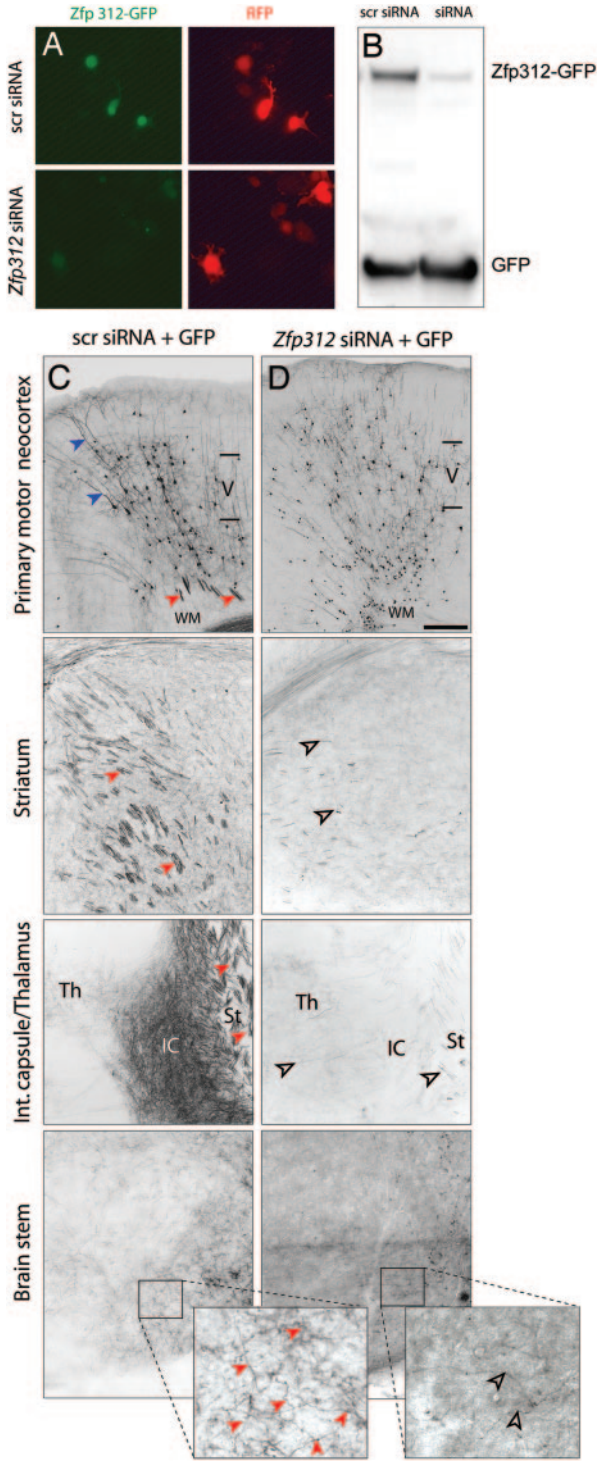

Fig. 3. *Zfp312* is required for subcortical projections and axonal fasciculation. (*A* and *B*) *Zfp312* siRNAs dramatically decreased the expression and nuclear localization of Zfp312-GFP in N2a cells. N2a cells were transfected with *Zfp312-GFP* in the presence of 10-fold excess of pCRLH (*A*) or pLVTH (*B*) expressing either scr siRNAs or *Zfp312* siRNAs. GFP from pLVTH was used as an internal control (*B*). pLVTH expressing either scr siRNA (*C*) or *Zfp312* siRNAs (*D*) was delivered by *in utero* electroporation to cortical VZ progenitors at E12.5 and analyzed at P14. GFP$^+$ apical dendrites (blue arrowheads) and axons (red arrowheads) were present in the control cortex. In control brains, numerous GFP$^+$ axons were present in the white matter (WM), striatum (st) internal capsule (IC), and thalamus (Th) and formed axonal fascicles (red arrowheads). GFP$^+$ subcortical axons were dramatically reduced in brains expressing *Zfp312* siRNAs and do not form fasciculated bundles (open arrowheads). (Scale bar, 300 μm in *C* and *D*.)

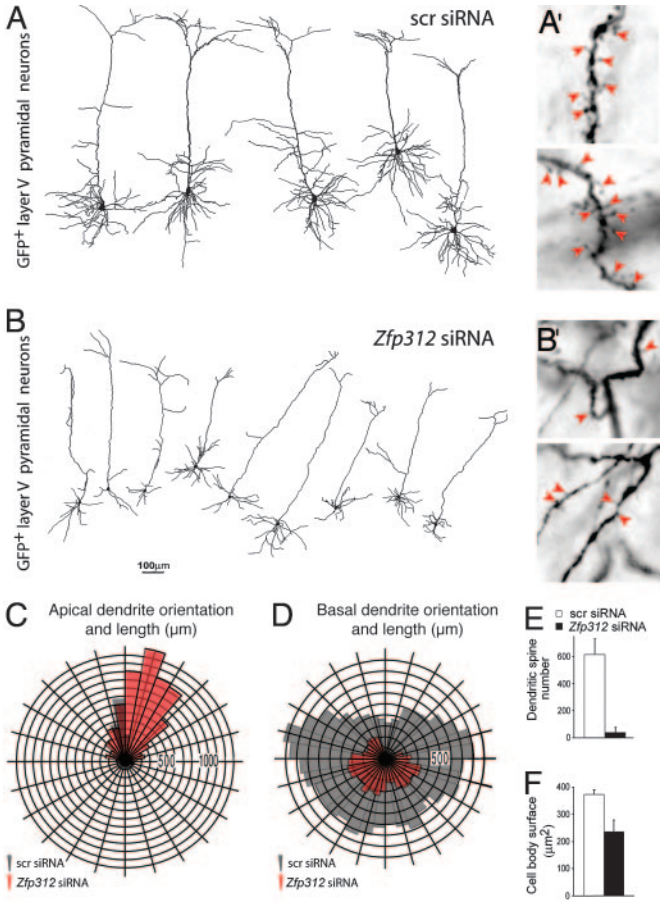

Fig. 4. *Zfp312* regulates pyramidal morphology, dendritic patterning, and spine number. (*A* and *B*) Neurolucida 3D reconstructions of layer V neurons in the primary motor cortex electroporated at E12.5 with pLVTH expressing either control (scr) siRNAs or *Zfp312* siRNAs and analyzed at P14. *Zfp312* siRNAs significantly reduced basal dendritic tree complexity (*D*; $P = 0.001$), spine number (arrowheads in *A′* and *B′*, quantification in *E*; $P < 0.001$), and soma size (*F*; $P < 0.001$) and affected the vertical orientation of apical dendrites (*C*; $P = 0.027$).

confirming that siRNA induced robust knockdown of Zfp312 expression.

To knock down *Zfp312* expression *in vivo*, we performed *in utero* electroporation to codeliver two *Zfp312* siRNAs (1:1) into neocortical VZ progenitors at E12.5, when layer V and VI neurons are generated (3, 6, 23, 24). Electroporated brains were analyzed at P14 by using diaminobenzidine immunohistochemistry to enhance the detection of GFP. Cells expressing siRNAs were reliably identified by the coexpression of GFP, which can be used to reveal complete dendritic arborizations and axonal projections. TUNEL analysis of DNA fragmentation revealed no difference in cell death between GFP^+ neurons expressing *Zfp312* siRNAs and scr siRNAs at P0 ($n = 1$; Fig. 9, which is published as supporting information on the PNAS web site), indicating that *Zfp312* inactivation did not affect neuronal survival. GFP^+ pyramidal neurons expressing scr siRNAs or *Zfp312* siRNA were found in all cortical layers ($n = 2$) (Figs. 3 *C* and *D*, and 5*A*), because the electroporation transfected early VZ progenitor cells, which eventually gave rise to pyramidal neurons of all layers. This finding indicates that the expression of *Zfp312* siRNAs did not interfere with the generation and migration of pyramidal neurons. Layer V and VI pyramidal neurons expressing scr siRNA exhibited typical pyramidal morphology and sent descending fasciculated bundles of GFP^+ axons to the white matter, striatum, and internal capsule (Fig. 3*C*, red arrowheads). In striking contrast, neurons expressing *Zfp312* siRNAs sent a dramatically reduced number of GFP^+ axonal projections to subcortical structures (Fig. 3*D*). Furthermore, the remaining subcortical GFP^+ axons lacked the typical tightly bundled organization (Fig. 3*D*, open arrowheads). Taken together, these results indicate that *Zfp312* is not required for the generation, migration, or survival of neurons in layers V and VI. However, *Zfp312* is necessary for the formation and fasciculation of subcortical axonal projections.

***Zfp312* Is Required for the Dendritic Development of Deep-Layer Pyramidal Neurons.** To assess whether somato-dendritic development was affected by *Zfp312* silencing, we performed 3D reconstruction and analysis of large GFP^+ pyramidal neurons in layer V of the primary motor cortex from serial sections of P14 brains using the Neurolucida system ($n = 7$ scr siRNAs; $n = 10$ *Zfp312* siRNAs). As expected, GFP^+ neurons expressing scr siRNAs displayed normal apical dendrites extending toward the pial surface and multiple basal dendrites extending laterally from the base of the pyramidal cell body (Figs. 3*C* and 4*A*). In contrast, GFP^+ deep-layer pyramidal neurons expressing *Zfp312* siRNAs had significantly smaller cell bodies (Fig. 4*F*) and showed a decrease in the complexity of basal dendrites (Fig. 4 *B* and *D*). Most strikingly, the total number of dendritic spines (Fig. 4 *B′* and *E*) and the radial length of basal dendrites were severely reduced (Fig. 4*D*). In addition, we found that the radial orientation, but not the length, of apical dendrites (main shaft) was disrupted (Fig. 4*C*). Additional dendritic quantifications are presented in Fig. 10, which is published as supporting information on the PNAS web site. Thus, in addition to its role in the formation of subcortical projections, *Zfp312*

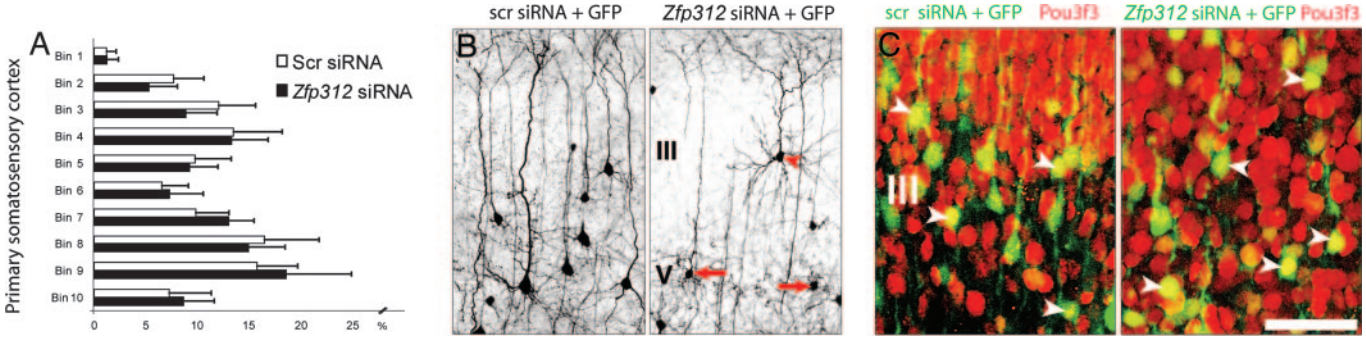

Fig. 5. *Zfp312* siRNAs do not alter the generation, migration, or development of upper-layer pyramidal neurons in the primary somatosensory cortex electroporated at E12.5 with pLVTH. (*A*) Quantification of laminar distribution of GFP^+ neurons at P14 showing no differences between neurons expressing scr siRNA and *Zfp312* siRNA ($P > 0.05$ for each bin). Error bars indicate SD. (*B*) *Zfp312* siRNAs alter morphology of layer V (arrows) but not layer III pyramidal neurons (arrowhead) at P14. (*C*) *Zfp312* siRNAs do not affect the expression of upper-layer marker Pou3f3 (red) at P0 (arrowheads). (Scale bar, 200 μm in *B* and 400 μm in *C*.)

controls the development of dendritic arborization and spines of large layer V pyramidal neurons.

Upper-Layer Pyramidal Neurons Develop Normally with *Zfp312* siRNA. We next examined whether the knockdown of *Zfp312* in early progenitor cells and deep-layer neurons would affect the migration and development of later-born upper-layer pyramidal neurons that normally project to other cortical areas. Analysis at P14 of the laminar distribution of GFP$^+$ neurons expressing *Zfp312* siRNAs showed no apparent disruption of neuronal migration (Figs. 3*D* and 5*A*). These neurons also developed normal pyramidal-shape morphology and dendritic arborization (Fig. 5*B*). In addition, numerous GFP$^+$ axons projected via the corpus callosum to the contralateral cortical hemisphere, suggesting that these neurons form normal axonal projections. Pyramidal neurons in layers II and III expressing *Zfp312* siRNAs were immunolabeled for transcription factor Pou3f3 (Fig. 5*C*) (also known as Brn1), a marker of upper-layer neurons (9). These results indicate that *Zfp312* inactivation in early VZ progenitors and deep-layer pyramidal neurons does not affect the generation, migration, or development of upper-layer cortically projecting neurons.

Misexpressing *Zfp312* in Upper-Layer Callosal Neurons Induces Ectopic Subcortical Projections. We next investigated whether the misexpression of *Zfp312* in layer II and III cortically projecting neurons, which normally do not express *Zfp312*, would induce the formation of ectopic subcortical axonal projections. The coding region of mouse *Zfp312* was cloned into pCLEG, a retroviral vector containing *GFP* (Fig. 8). Control pCLEG or pCLEG-*Zfp312* plasmids were delivered into cortical VZ progenitor cells by *in utero* electroporation at E17, when the last of the callosal pyramidal neurons destined for the upper part of layer III and layer II are generated (3, 6, 23, 24). Previous studies have shown that the production of corticospinal pyramidal neurons continues until E15.5 (23). Our own analysis of pyramidal neurons electroporated *in utero* with control pCLEG confirmed that the last subcortically projecting cortical neurons are generated at E15.5 (Fig. 11, which is published as supporting information on the PNAS web site). Therefore, undertaking electroporation at E17 ensures that *Zfp312* is introduced only into late cortical progenitors and cortically projecting layer II and III neurons normally not expressing *Zfp312*. Brains of electroporated animals were examined at P14 for the presence of ectopic GFP$^+$ axons in the internal capsule and subcortical targets. In control brains ($n = 16$), GFP$^+$ neurons were present at the uppermost part of the neocortex corresponding to layers II and III (Fig. 6*A*) and GFP$^+$ axons were present only in the white matter around the striatum, corpus callosum, and cortex. Pyramidal neurons misexpressing *Zfp312* migrated to layers II and III (Fig. 6*B*). Remarkably, the upper-layer neurons misexpressing *Zfp312* ($n = 36$ animals) showed numerous GFP$^+$ axons in the striatum, internal capsule, thalamus, and brainstem, in addition to their normal callosal projections (Fig. 6*B*). The induction of ectopic subcortical projections by *Zfp312* was confirmed by BDA-3k retrograde axonal tracing ($n = 6$ animals) from the pons (Fig. 6 *C* and *D*). Collectively, these results indicate that *Zfp312* is necessary for the formation and sufficient for the induction and direction of ectopic, subcortical projections.

Upper-Layer Neurons Misexpressing *Zfp312* Express Tbr1. Because Zfp312 is a transcription factor, we investigated whether its misexpression would alter the molecular identity of upper-layer pyramidal neurons. P14 brains electroporated with either control pCLEG or pCLEG expressing *Zfp312* at E17 were immunostained with antibodies against T-box brain gene 1 (Tbr1), a transcription factor with enriched expression in subplate and layer VI neurons (refs. 3 and 24; see also Fig. 7*B*). Functionally, *Tbr1* is necessary for the development of subplate neurons and deep-layer pyramidal neurons and their corticofugal connections (24). In control GFP$^+$ neurons, 15 ± 2% of upper-layer neurons were immunolabeled for Tbr1 (Fig. 6*E*). In contrast, virtually all *Zfp312*-misexpressing upper-layer neurons (98 ± 2%) were immunolabeled for Tbr1 (Fig. 6*F*). Based on this finding, we conclude that *Zfp312* misexpression can respecify upper-layer cortically projecting neurons to adopt certain molecular properties of deep layer subcortically projecting pyramidal neurons.

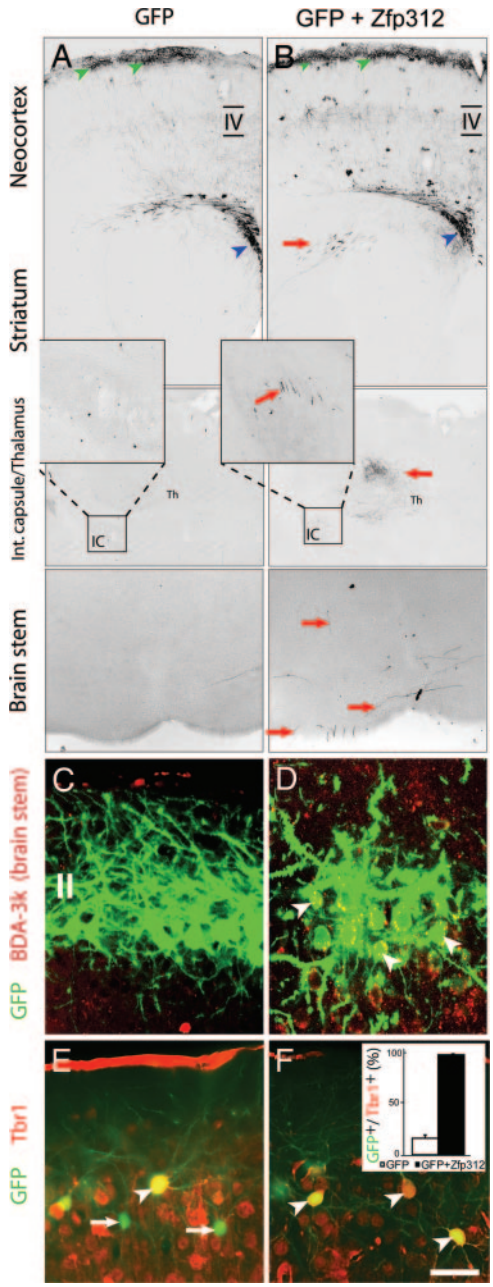

Fig. 6. *Zfp312* misexpression in layer II/III callosal neurons induces Tbr1 and ectopic subcortical axonal projections. Neocortical VZ progenitors were electroporation *in utero* at E17 with pCLEG or pCLEG-*Zfp312* and analyzed by GFP immunohistochemistry at P14. (*A*) Control GFP$^+$ neurons in the uppermost layer (green arrowheads) send axons through the white matter and corpus callosum (blue arrowheads). *Zfp312*-misexpressing but not control GFP$^+$ neurons have ectopic subcortical axons (*A* and *B*; red arrows) and are retrogradely labeled by BDA-3k injected into the pons (*C* and *D*; arrowheads). (*E* and *F*) A small number of neurons electroporated with control pCLEG expressed Tbr1 (*E*), whereas the vast majority of neurons misexpressing *Zfp312* coexpressed Tbr1 (*F*) (arrowheads). (Scale bar, 500 μm in *A* and *B*, 50 μm in *C* and *D*, and 90 μm in *E* and *F*.)

Discussion

A Dual Role of *Zfp312* in the Specification of Early Progenitors and the Postmitotic Development of Subcortically Projecting Neurons. We show here that the mouse transcription factor Zfp312 is required for the development of axonal projections and dendritic morphology of subcortically but not cortically projecting pyramidal neurons. *Zfp312* is a highly specific marker for deep-layer pyramidal neurons in all areas of the neocortex, and this specificity begins with the VZ progenitor cells that generate these neurons. The absence of *Zfp312* transcripts in late cortical progenitors suggests that *Zfp312* expression is actively shut off when upper-layer neurons are generated, which is consistent with the possibility that Zfp312 acts as a specific molecular determinant of early VZ progenitor cells.

Our study demonstrates that the silencing of *Zfp312* in early cortical progenitor cells and deep-layer neurons does not affect their generation, migration, or survival. However, it does disrupt the formation of axonal projections and dendritic arborizations, which occur postmitotically. Additionally, *Zfp312* is required for the development of the soma and dendritic spines of large layer V pyramidal neurons. Taken together, our data show that, in addition to a possible role in the specification of early VZ progenitor cells, *Zfp312* also has multiple functions in the postmitotic development of their progenies, the deep layer pyramidal neurons.

Putative Mechanisms by Which Zfp312 Functions. Mechanistically, Zfp312 may be involved in the specification of neuronal identity via the regulation of other transcription factors. Our results showed that *Zfp312* misexpression induced the expression of transcription factor Tbr1, which is required for the development of corticofugal projections (24). Alternatively, *Zfp312* may directly regulate the expression of guidance molecules that control the response of neuronal processes to external cues. The peak of *Zfp312* expression occurred in postmitotic neurons during the formation and refinement of axonal projections and synapses, which is consistent with the latter possibility.

A previous study showed that, in a genetic knockout of *Fezl* (*Zfp312*), thalamocortical axons were reduced in numbers and exhibited aberrant projections (16). Because *Zfp312* expression is absent from the dorsal thalamus, defects in thalamocortical projections are likely non-cell-autonomous. According to the handshake hypothesis, thalamocortical axons grow in the direction opposite to, but associate with, corticofugal axons from the deep layers of the cortex (25). Therefore, the aberrant thalamocortical projections in *Zfp312* knockouts may occur via the disruption of corticothalamic projections, which we observed in our study. We did not find a reduction of the subplate as described in *Zfp312* mutant mice (16), possibly because of the timing of our experiments on or after E12.5, when most subplate neurons had been generated (3, 6, 23, 24). No other cortical abnormalities were reported (16), suggesting that our experimental approach may uncover additional roles for *Zfp312* in cortical development. Our RNA interference strategy offers possible advantages compared with genetic knockouts, by allowing for gene inactivation within a specific developmental period and in a mosaic to facilitate the analysis of cell autonomous effects. Importantly, possible compensatory mechanisms are minimized. Analysis of the mouse genome revealed that *Zfp312* has a highly similar homolog BC049157, or *Zfp312-like*, which is also expressed by pyramidal neurons (Fig. 12, which is published as supporting information on the PNAS web site). The zinc-finger binding domains of Zfp312 and Zfp312-like are 95.7% identical, indicating that these two proteins very likely bind the same DNA sequences and may functionally compensate for each other.

Implications for the Evolution and Development of the Neocortex. The six-layered structure of the neocortex is unique to mammals. Evolutionary differences in *Zfp312* may underlie phylogenetic differences in the development of subcortical projections (2, 25, 26). Thus, we investigated whether *Zfp312* might have undergone adaptive changes during the evolution of the cerebral cortex. *In silico* amino acid sequence analyses of Zfp312 orthologs in six mammalian and four nonmammalian species revealed that Zfp312 is remarkably well-conserved in mammals (92.2% identity) (Figs. 12 and 13, which are published as supporting information on the PNAS web site). Between mammalian and nonmammalian species, the conservation is very high within the zinc finger region but lower in other regions. Additionally, several regions in the protein are completely conserved within mammals but are absent from nonmammals. These include a polyglycine repeat region, which may function as a flexible hinge to facilitate protein interactions. These observations indicate that significant amino acid changes occurred during the divergence of the mammalian lineage, rapidly arose to fixation, most likely due to strong positive selection, and have remained virtually unchanged since. This finding is consistent with the possibility that *Zfp312* played a role in the evolution of long-range subcortical axonal projections of the neocortex.

Note. After the completion of these experiments, Molyneaux *et al.* (27) reported that *Fezl* (*Zfp312*) is required for the birth and specification of corticospinal motor neurons.

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Supporting Information

10.1073/pnas.0509032102.

Jie-Guang Chen, Mladen-Roko Rašin, Kenneth Y. Kwan, and Nenad Šestan

"Zfp312 is required for subcortical axonal projections and dendritic morphology of deep-layer pyramidal neurons of the cerebral cortex"

Supporting Text

To compare the expression profiles among different regions of the mouse forebrain, CD1 mice were killed at the postnatal day 7 (P7) and their brains were immediately removed, stripped of meninges, and cut into 300 μm-thick coronal sections using a tissue chopper (McIlwain Instruments, Gomshall, Surrey, UK). Sections were then placed on 0.45 pm Biopore membrane inserts (Collaborative Research, Bedford, MA) and dissected in cold HBSS supplemented with 0.5% (wt/vol) d-glucose and 25 mM Hepes buffer (Invitrogen) under a dissecting microscope. Four forebrain regions (n = 3 animals) were isolated with a microdissecting knife (Biomedical Research Instruments, Malden, MA): (*i*) the upper layers of the neocortex (i.e., II-IV); (*ii*) the deep layers of the neocortex (i.e., V-VI); (*iii*) the hippocampus (archiocortex with a single pyramidal layer); and (*iv*) the striatum (a control forebrain region that does not contain excitatory projection neurons). The dissected tissue was immediately immersed in TRIzol (Invitrogen) on ice for isolation of total RNA according the manufacturer's protocol. The quality of total RNA was evaluated by A260/A280 ratio (>1.9), and by electrophoresis. Labeled cRNA for hybridization were synthesized according to the recommended protocol from Affymetrix. Briefly, first-strand cDNA was synthesized from isolated total RNA with an oligo(dT) primer containing the T7 promoter. After synthesis of the second strand, the cDNA was used as template for *in vitro* transcription of cRNA using the Bioarray High Yield RNA Transcription Labeling kit (Enzo Diagnostics). Biotin-labeled cRNA was purified, fragmented, and hybridized to the Affymetrix Mouse GeneChip 430 (Chip A and B contains 39,000 probe sets in total) at the Affymetrix Core Facility of the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). Data were analyzed by using the microarray suite software (Affymetrix) to obtain absolute and comparison data files.

Plasmid Construction

The replication-defective retroviral vector pCLEG was used for gain-of-function experiments. pCLEG was derived from pCLE (kindly provided by Gord Fishell and Nicholas Gaiano) by replacing the IRES-AP with IRES2-EGFP cassette (CLONTECH). The vector contains a CMV/MLV promoter and an EF1α enhancer for efficient expression in the embryonic brain and an internal ribosome entry site (IRES) for the translation of GFP as a reporter. The full-length mouse *Zfp312* was amplified by RT-PCR from E12.5 mouse brain, cloned into pCRII-TOPO, and subcloned into the pCLEG plasmid using unique restriction sites. The final construct was verified by sequence analysis. For loss-of-function experiment, siRNA sequences targeting *Zfp312* were designed using siRNA Target Finder (Ambion). A template for hairpin siRNA synthesis was made by annealing a pair of oligonucleotides representing the sense and antisense strands of the target sequence. The annealed template was cloned into the HindIII and BglII sites of pSUPER (OligoEngine). The siRNAs were subcloned from pSUPER into pLVTH or pCRLH. Two constructs with siRNA sequences targeting different regions of the gene were combined to

achieve robust inhibition of *Zfp312* expression. Scrambled control sequences were designed by mutating three nucleotides in the siRNA sequence. More cloning and sequence information are included in Table 1.

The Zfp312-GFP fusion construct was created by in-frame ligation of mouse *Zfp312* with the GFP reporter gene in the pEGFP-N3 vector (Clontech), using XhoI and KpnI sites introduced by PCR. The Zfp312-GFP fusion protein was expressed under a CMV promoter.

Table 1. DNA constructs used in this study

Name	Cloning primers or RNAi targeting sequences (5' to 3')	Insert size or position	Vector
TA-Zfp312	GCCATGGCCAGCTCAGCTTCC AGAAATAAGTTTATACGTGAGATCTGC	1430 bp	pCRII-TOPO
CLEG-Zfp312	Xho I/BamH I fragment from TA-Zfp312	1430 bp	pCLEG
Zfp312-GFP	CCTCTAGATGCATGCTCGAGCG GGTGGCGGTGGGTACCCACG	1378 bp	pEGFP-N3
TA-Zfp312-like	TCAACCCCGGCTGACCGACGC ATGTAGAGGCATCCGGTCCTCC	1609 bp	pCRII-TOPO
Zfp312 siRNA-1	GGAGAACTCGGCCTTGACA	775-793 (position)	pSUPER pLVTH pCRLH
Zfp312 siRNA-2	GGTGTTCAATGCTCACTAT	886-904 (position)	pSUPER pLVTH pCRLH
scrambled siRNA-1	GGAGAAGTGGCCTTGACA	N/A	pSUPER pLVTH pCRLH
scrambled siRNA-2	GGTGTTGAATCCTGACTAT	N/A	pSUPER pLVTH pCRLH

A

Nctx layers II-IV	P				P	P		P	A	A	A	P	P	P	A	P	A	P	P	P	A
Nctx layers V-VI	P			P		P	P	P	A	A	P	A	P	P	P	A	P	A	P	P	A
Hippocampus	P		P				P	P	A	P	A	A	P	A	P	P	P	P	A	A	A
Striatum	P	P							P	A	A	A	A	A	A	A	P	P	P	P	P
430A	11289	13431	12444	12567	12818	12015	11653	11425	444	168	114	129	94	74	40	53	103	203	305	112	152	127
430B	5049	6232	6657	7254	6618	6075	6023	5505	188	250	381	216	349	219	184	73	180	52	201	109	45	66
Total	16338	19663	19062	19821	19438	17745	17676	16930	632	268	145	345	440	293	224	126	283	255	506	221	197	193

B

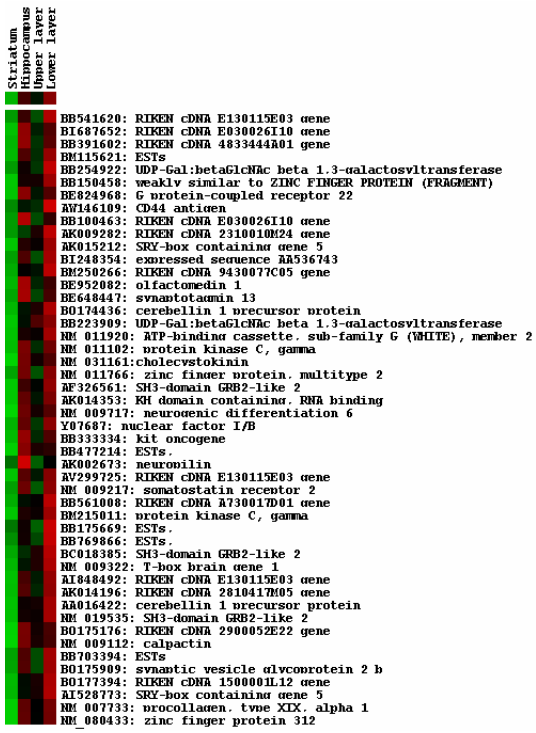


Fig. 7. (*A* and *B*) Regional distribution of gene expression in postnatal day 7 (P7) mouse brain and genes selectively expressed in deep layers of the neocortex. Summary of Affymetrix GeneChip analysis of gene expression in the P7 mouse brain. The upper layers and deep layers of the neocortex, the hippocampus, and the striatum were analyzed. The presence (P) or absence (A) of particular transcripts was determined by using the microarray suite software. Comparison data files were input into access (Microsoft) for grouping and crossover. Of the 16,930 genes and ESTs present in the neocortex and hippocampus, 56 were selected for their expression in the deep layers of the neocortex and hippocampus, both of which are regions containing neurons with subcortical projections. These genes were also much more highly expressed in these regions compared with the striatum (Signal Log Ratio >0.6), which is a control region without pyramidal neurons. Therefore, these are good candidates for genes selectively expressed in pyramidal neurons with subcortical projections. The tree view of relative expression was generated by using genecluster and treeview programs (Michael Eisen, Stanford University).

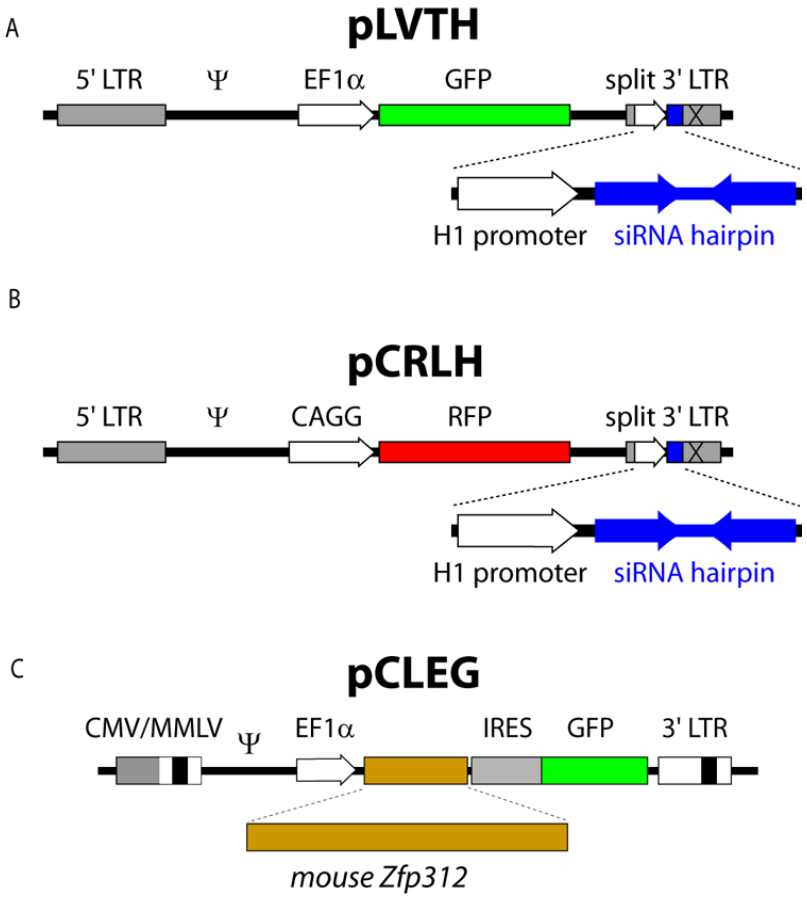


Fig. 8. Schematic representation of plasmids for siRNA hairpins and gene overexpression. (*A*) pLVTH lentivirus-based vector allows for the simultaneous expression of siRNA and reporter GFP. The expression of hairpin siRNA is driven by the human H1 promoter cassette, and the expression of GFP is driven by the Xenopus EF1 enhancer/promoter for improved expression in neural cells. (*B*) pCRLH retroviral vector allows for the simultaneous expression of siRNA hairpins and reporter RFP. The expression of hairpin siRNA is driven by the human H1 promoter cassette, and the expression of RFP is driven by the CAGG promoter. (*C*) The pCLEG retrovirus vector contains a modified 5' long terminal repeat (CMV/MMLV) to obtain higher titers, as well as the EF1 enhancer/promoter to improve vector expression in neural cells. The pCLEG retroviral vector is a bicistronic construct that utilizes an internal ribosomal entry sequence (IRES) to drive translation of GFP.

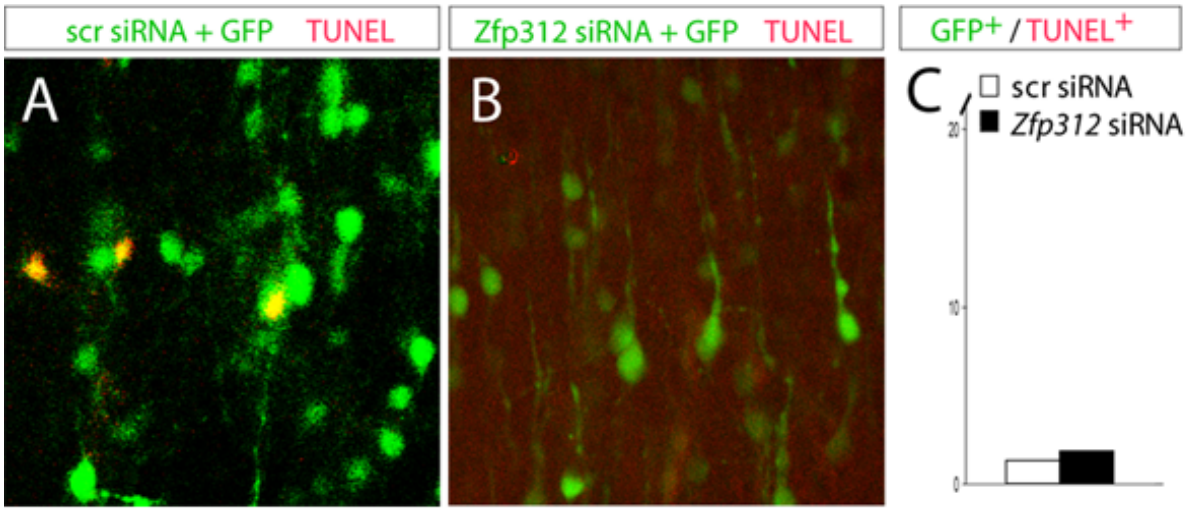


Fig. 9. *Zfp312* siRNAs do not increase neuronal cell death. (*A* and *B*) Scr siRNAs and *Zfp312* siRNAs were electroporated *in utero* into cortical VZ progenitors at E12.5. TUNEL staining was carried out at P0 according to the manufacturer's protocol (Roche Diagnostics) and quantified in *C*. There was no significant difference in TUNEL signal (red) between GFP^+ neurons expressing *Zfp312* siRNAs and those expressing control scr siRNAs ($P > 0.05$).

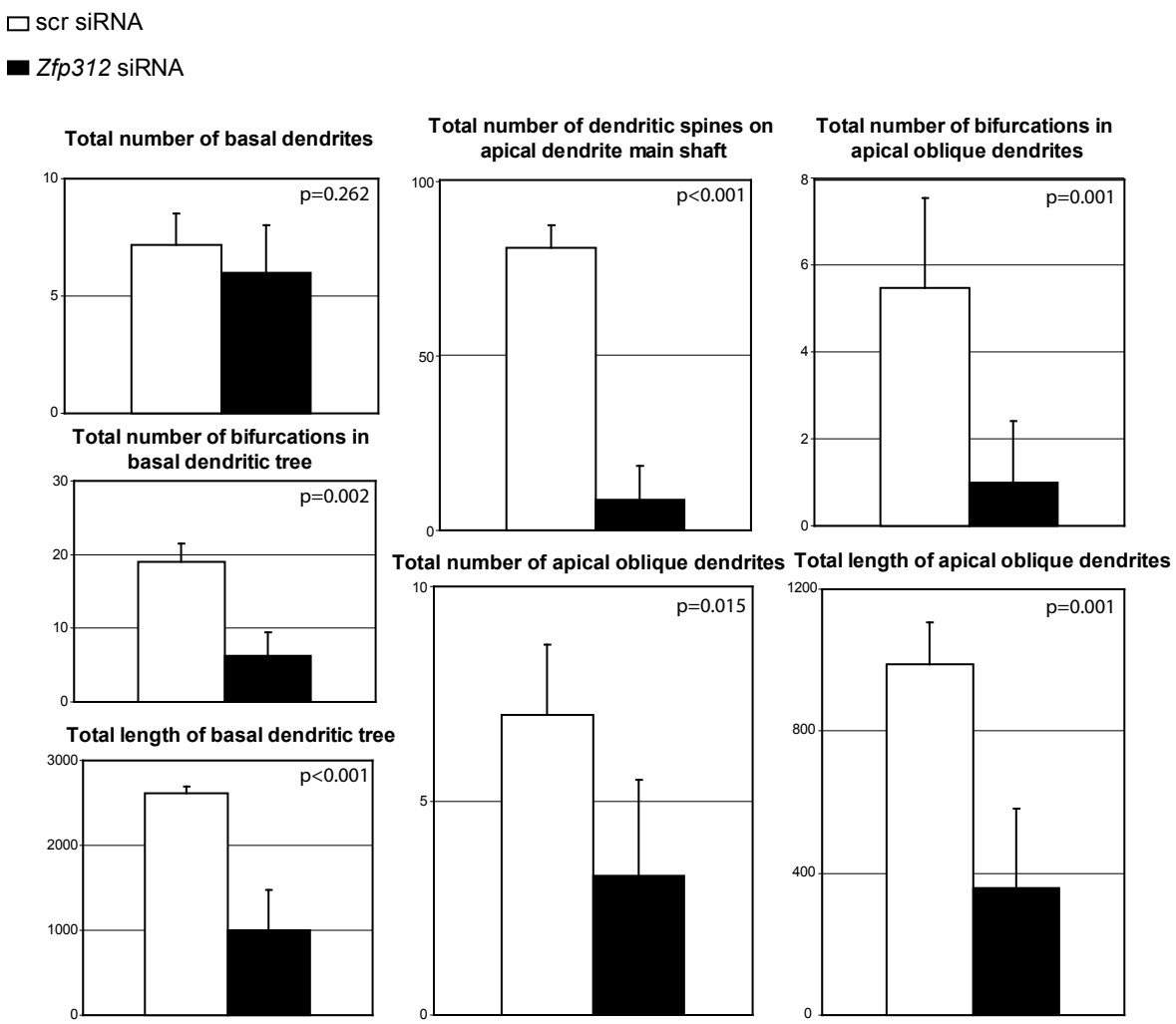


Fig. 10. Quantitative analysis of basal and apical oblique dendrites of layer V pyramidal neurons in the primary motor cortex at P14 (Fig. 4). Apical and basal dendrites were analyzed by 3D reconstruction using the Neurolucida System (n = 7-10 neurons per condition). Although the number of basal dendrites was not significantly altered, knocking down *Zfp312* significantly reduced the number of bifurcations and the total lengths of both basal and apical oblique dendrites. Most significantly, the total number of spines on the main shaft of the apical dendrite was dramatically reduced by *Zfp312* knockdown. Error bars indicate SD.

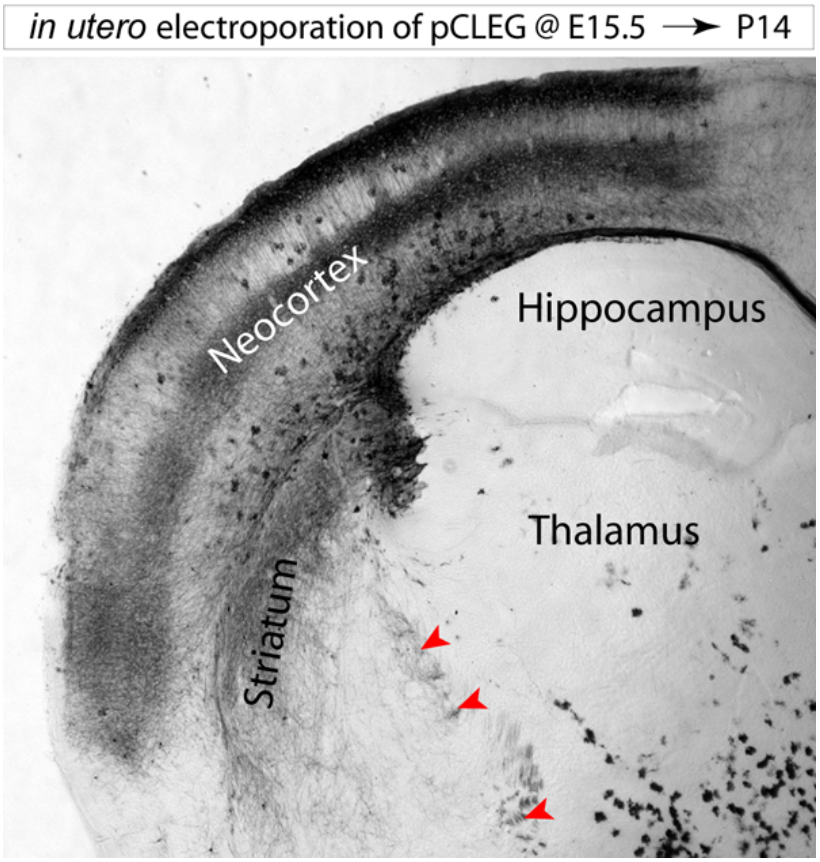


Fig. 11. Corticospinal neurons are generated up to E15.5 in mice. The pCLEG plasmid electroporated *in utero* at E15.5 and analyzed at P14 by DAB IHC for GFP. Numerous GFP^+ axonal fascicles from necortical neurons electroporated *in utero* with pCLEG are present in the interanal capsule (arrowheads) and brainstem. GFP^+ axons were absent from the dorsal thalamus.

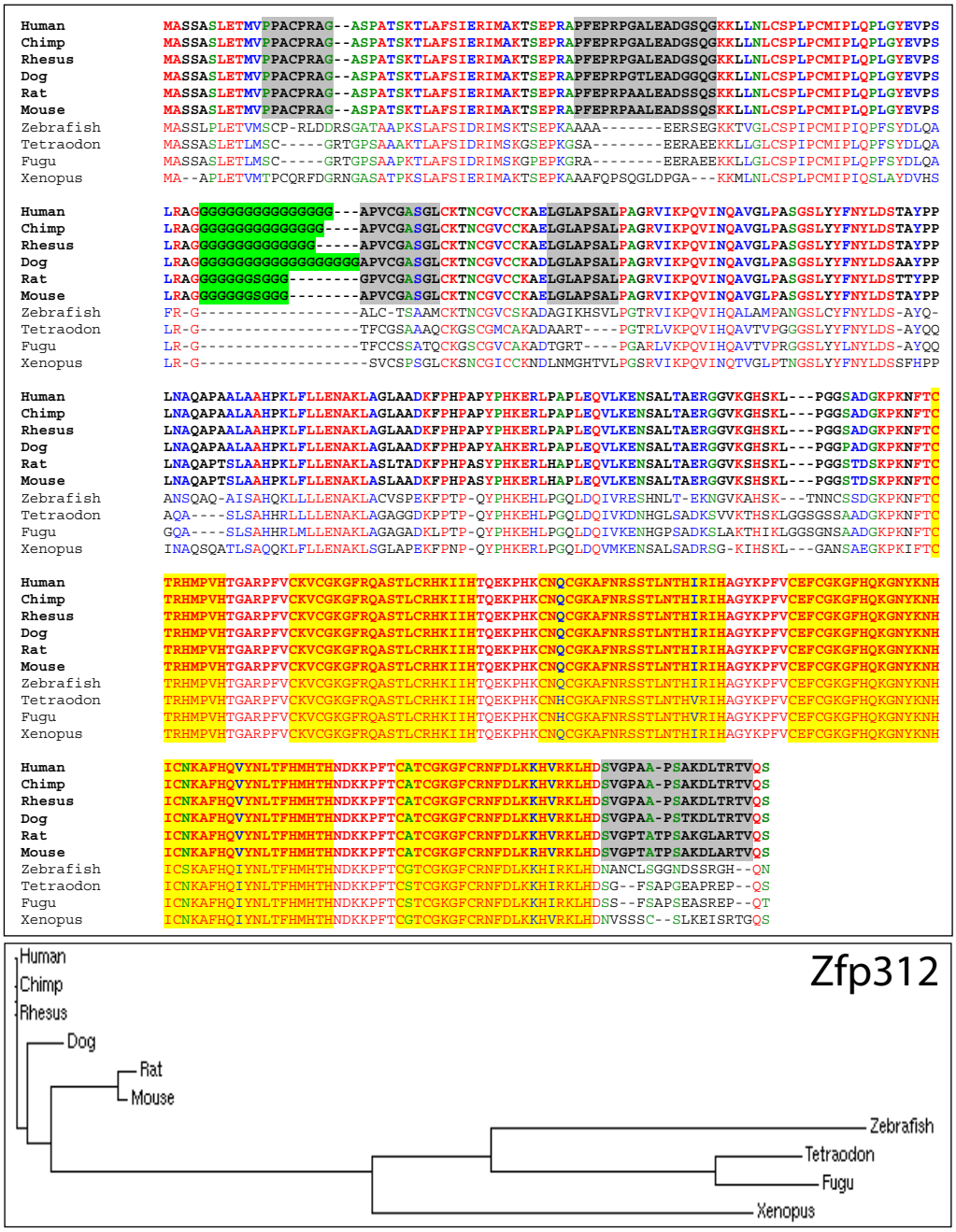
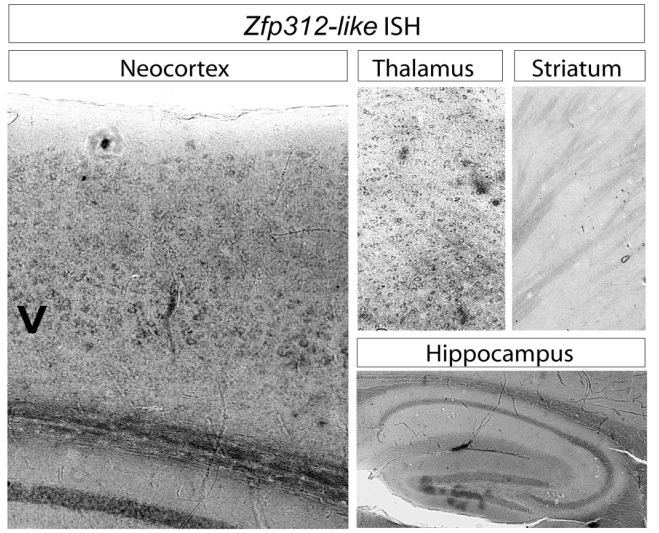


Fig. 12. Alignment of amino acid sequences from mammalian and nonmammalian orthologs of Zfp312. Orthologs of Zfp312 were obtained from Genome Browser (http://genome.ucsc.edu). Mammalian sequences are shown in bold. Residues that share identity are denoted in red-colored text and residues that share similarity are in blue and green text. The zinc-finger domains are colored in yellow background. Green background denotes the polyglycine region uniquely conserved in mammals, and gray background denotes other parts of the protein uniquely conserved in mammals. The clustering of species is summarized in the dendrogram. Amino acids alignments were performed by using the ClustalW algorithm available from DeCypher (http://coe02.ucalgary.ca), and minor corrections were made manually.

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Human Zfp312          MASS-ASLETMVPPACPRAG------ASPATSKTLAFSIERIMAKTSEPRAPFEPRPGAL
Mouse Zfp312          MASS-ASLETMVPPACPRAG------ASPATSKTLAFSIERIMAKTSEPRAPFEPRPAAL
Xenopus Zfp312        MA---APLETVMTPCQRFDGR----NGASATPKSLAFSIERIMAKTSEPKAAAFQPSQGL
Human Zfp312-like     MDSSCHNATTKMLATAPARGN------MMSTSKPLAFSIERIMARTPEPKALPVPHFLQG
Mouse Zfp312-like     MDSSCLNATTKMLATAPARGN------VMSTSKPLAFSIERIMARTPEPKALPVPHFLQG
Xenopus Zfp312-like   MDSSLQHKTTKIFPSQASRDSLSNRVTMISGAKPLAFSIERIMSRTPEPKCLPVPSLLQG

Human Zfp312          EADGSQGKKLLNLCSP-LPCMIPLQPLGYEVPSKTLLSYSELWKSSLRAGGGGGGGGGGG
Mouse Zfp312          EADSSQSKKLLNLCSP-LPCMIPLQPLGYEVPSKTLLSYSEFWKSSLRAGGGGGGS---
Xenopus Zfp312        DPGA---KKMLNLCSP-LPCMIPIQSLAYDVHSKALLNYSELWKSSLRG----------
Human Zfp312-like     ALPKGEPKHSLHLNSS-IPCMIPFVPVAYDTSPKAGVTGSEPRKASLEAPAAPAAVP---
Mouse Zfp312-like     AVPKGDPKHSLHLNSS-IPCMIPFVPVAYDTNSKAGVNGSEPRKASLEVPAPPAVAP---
Xenopus Zfp312-like   SVPKGDQKQALHINSSSIPCMIPFVPVAYDHCPKIGISGAELRKSHLD-----------

Human Zfp312          GGGGGAPVCGASGLCKTNCGVCCKAELGLAPSALPAGRVIKPQVINQAVGLPASGSLYYF
Mouse Zfp312          --GGGAPVCGASGLCKTNCGVCCKAELGLAPSALPAGRVIKPQVINQAVGLPASGSLYYF
Xenopus Zfp312        ------SVCSPSGLCKSNCGICCKNDLNMGHTVLPGSRVIKPQVINQTVGLPTNGSLYYF
Human Zfp312-like     ------SAPAFSCSDLLNCALSLKGDLARDALPLQQYKLVRPRVVNHSSFHAMGA-LCYL
Mouse Zfp312-like     ------SAPAFSCSDLLNCALSLKGDLARDALPLQQYKLVRPRVVNHSSFHAMGA-LCYL
Xenopus Zfp312-like   ------SSPPFSCNDLLNCALTLKGDFPREALPLQQYKLVRPRVVNHSSFHAMGAAFCYF

Human Zfp312          NYLDSTAYPPSELLSGHLFPSGLLNAQAPAALAAHPKLFLLENAKLAGLAADKFPHPAPY
Mouse Zfp312          NYLDSTAYPPSELLGGHLFPSGLLNAQAPTSLAAHPKLFLLENAKLASLAADKFPHPASY
Xenopus Zfp312        NYLDSSFHPP-EILSGQLLSSSLINAQSQATLSAQQKLFLLENAKLSGLAPEKFPNP-QY
Human Zfp312-like     NRGDGPCHPA-AGVNIHPVASYFLSSP----LHPQPKTYLAERNKLVVPAVEKYPSG--V
Mouse Zfp312-like     NRGDGPCHPA-ASVNMHPVASYFLSSP----LHPQPKTYLAERNKLVVPAVEKLPSG--V
Xenopus Zfp312-like   NRGDSQCHPP-ASINIHPVASYFLGSP----LHQAPKSYLAERNKLVLPSVEKFSSG--V

Human Zfp312          PHKERLPAPLEQVLKENSALTAERGGVKGHSKLPGGSADGKPKNFTCEVCGKVFNAHYNL
Mouse Zfp312          PHKERLHAPLEQVLKENSALTAERGGVKSHSKLPGGSTDSKPKNFTCEVCGKVFNAHYNL
Xenopus Zfp312        PHKERLPGQLDQVMKENSALSADRSG-KIHSKLGANSAEGKPKIFTCEVCGKVFNAHYNL
Human Zfp312-like     AFKDLSQAQLQHYMKESAQLLSEKIAFKTS-DFSRGSPNAKPKVFTCEVCGKVFNAHYNL
Mouse Zfp312-like     AFKDLSQAQLQHYMKESAQLLSEKIAFKTS-DFSRGSPNAKPKVFTCEVCGKVFNAHYNL
Xenopus Zfp312-like   TFKDLSQAQFQHYMKEGAHSLSDKITFKTSAKFSSASPSNKPKVFTCEVCGKVFNAHYNL

Human Zfp312          TRHMPVHTGARPFVCKVCGKGFRQASTLCRHKIIHTQEKPHKCNQCGKAFNRSSTLNTHI
Mouse Zfp312          TRHMPVHTGARPFVCKVCGKGFRQASTLCRHKIIHTQEKPHKCNQCGKAFNRSSTLNTHI
Xenopus Zfp312        TRHMPVHTGARPFVCKVCGKGFRQASTLCRHKIIHTQEKPHKCNQCGKAFNRSSTLNTHI
Human Zfp312-like     TRHMPVHTGARPFVCKVCGKGFRQASTLCRHKIIHTQEKPHKCNQCGKAFNRSSTLNTHT
Mouse Zfp312-like     TRHMPVHTGARPFVCKVCGKGFRQASTLCRHKIIHTQEKPHKCNQCGKAFNRSSTLNTHT
Xenopus Zfp312-like   TRHMPVHTGARPFVCKICGKGFRQASTLCRHKIIHTQEKPHKCNQCGKAFNRSSTLNTHT

Human Zfp312          RIHAGYKPFVCEFCGKGFHQKGNYKNHKLTHSGEKQYKCTICNKAFHQVYNLTFHMHTHN
Mouse Zfp312          RIHAGYKPFVCEFCGKGFHQKGNYKNHKLTHSGEKQYKCTICNKAFHQVYNLTFHMHTHN
Xenopus Zfp312        RIHAGYKPFVCEFCGKGFHQKGNYKNHKLTHSGEKQYKCTICNKAFHQIYNLTFHMHTHN
Human Zfp312-like     RIHAGYKPFVCEFCGKGFHQKGNYKNHKLTHSGEKQFKCNICNKAFHQVYNLTFHMHTHN
Mouse Zfp312-like     RIHAGYKPFVCEFCGKGFHQKGNYKNHKLTHSGEKQFKCNICNKAFHQVYNLTFHMHTHN
Xenopus Zfp312-like   RIHAGYKPFVCEFCGKGFHQKGNYKNHKLTHSGEKQFKCNICNKAFHQIYNLTFHMHTHN

Human Zfp312          DKKPFTCATCGKGFCRNFDLKKHVRKLHDSVGPAA-PSAKDLTRTVQS------------
Mouse Zfp312          DKKPFTCATCGKGFCRNFDLKRHVRKLHDSVGPTATPSAKDLARTVQS------------
Xenopus Zfp312        DKKPFTCGTCGKGFCRNFDLKKHVRKLHDNVSSSC--SLKEISRTGQS------------
Human Zfp312-like     DKKPFTCPTCGKGFCRNFDLKKHVRKLHDSSLGLARTPAGEPGTEPPPPLPQQPPMTLPP
Mouse Zfp312-like     DKKPFTCPTCGKGFCRNFDLKKHVRKLHDSSLGLTRTPTGEPSSDPPPQLQQPPPAPLPP
Xenopus Zfp312-like   DKKPFTCPTCGKGFCRNFDLKKHVRKLHDSNTAAPHAIGGTGQEELLLPNREPSP-----

Human Zfp312          --------------------
Mouse Zfp312          --------------------
Xenopus Zfp312        --------------------
Human Zfp312-like     LQPPLPTPGPLQPGLHQGHQ
Mouse Zfp312-like     LQPTLPPPGPLPSGLHQGHQ
Xenopus Zfp312-like   ---TIQSPQLQKSGY-----
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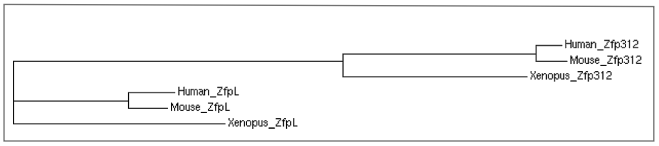

Fig. 13. *Zfp312-like* is a highly similar homolog of *Zfp312*, expressed by pyramidal neurons and thalamic neurons. Analysis of the mouse genome with Genome Browser (http://genome.ucsc.edu) revealed that *Zfp312* has a homolog, *Zfp312-like* (BC049157). *In situ* hybridization revealed that *Zfp312-like* is expressed by layer V pyramidal neurons. Additionally, it is expressed by layer II and III neurons, hippocampal pyramidal neurons, and thalamic neurons. The amino acid sequence of Zfp312-like is 47.9% identical (red) and 71.0% similar (blue and green) to that of Zfp312. Within the zinc-finger region, Zfp312 and Zfp312-like are 95.7% identical and 98.6% similar. Clustering is summarized in the dendrogram.