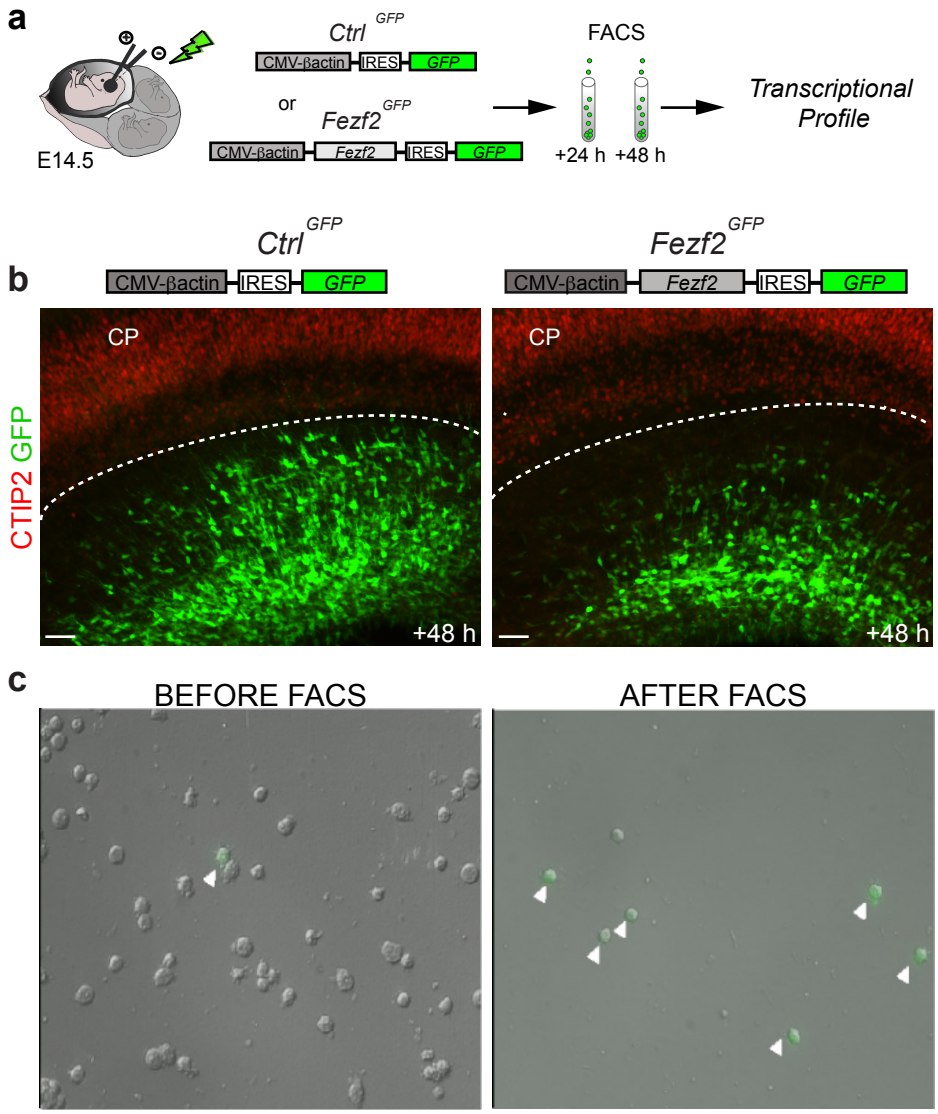
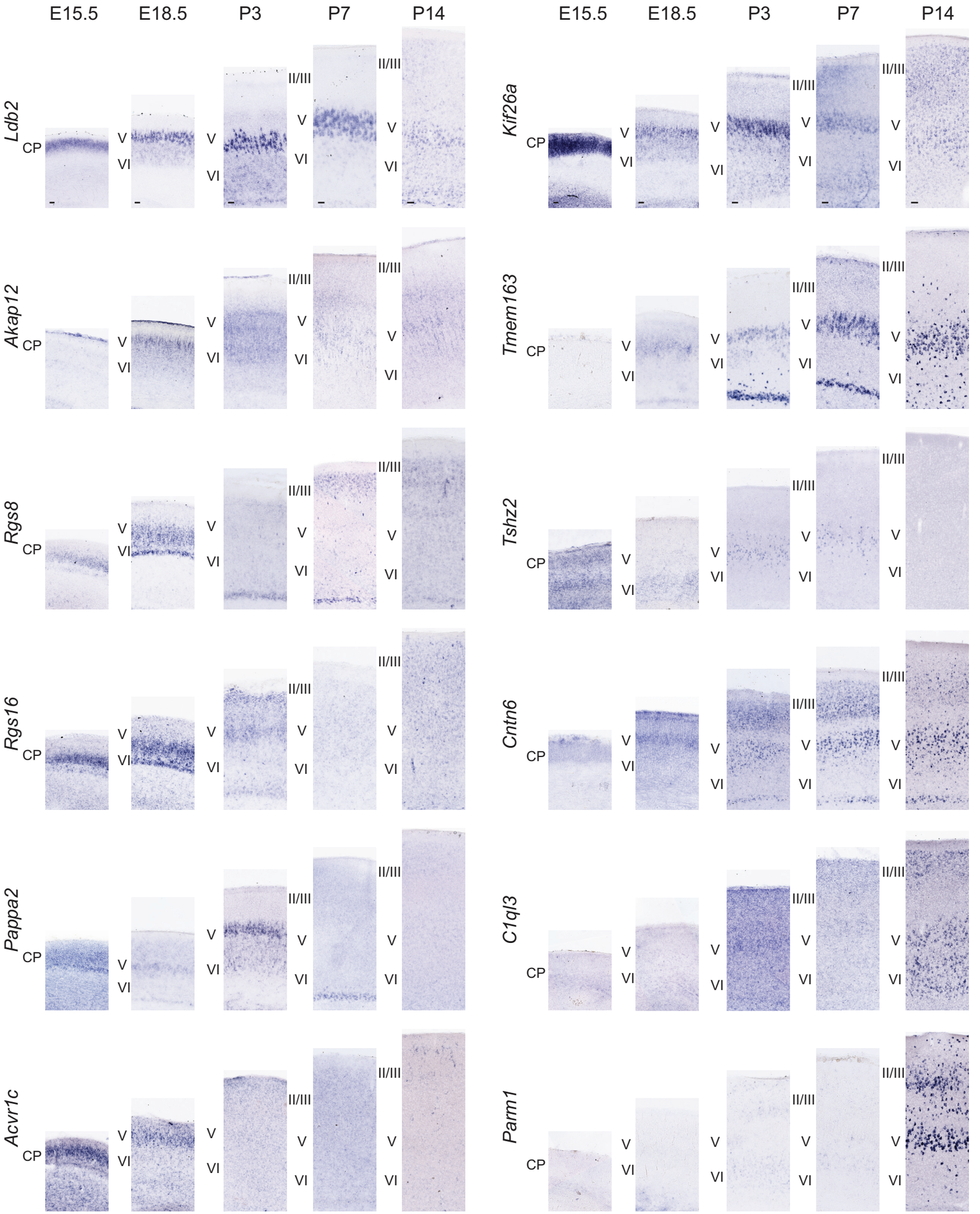
Lodato, Arlotta; Supplementary Figure 1



Supplementary Figure 1. **FACS-purification of cortical progenitors electroporated *in vivo* with *Fezf2*^{GFP} and *Ctrl*^{GFP} expression constructs at E14.5.**

(a) Schematic of the experimental approach. **(b)** Coronal sections of *Ctrl*^{GFP} and *Fezf2*^{GFP}-electroporated brains, 48 hours after electroporation, showing GFP-positive progenitors within the proliferative zones. CTIP2 immunohistochemistry (red) marks corticofugal neurons in the developing cortical plate. **(c)** Dissociated cortical cells before and after FACS purification; only a very small percentage of dissociated cells are electroporated (GFP-positive). FACS purification of GFP-positive cells results in virtually pure populations of labeled cells. CP, cortical plate. Scale bars, 100μm (b).

Lodato, Arlotta; Supplementary Figure 2

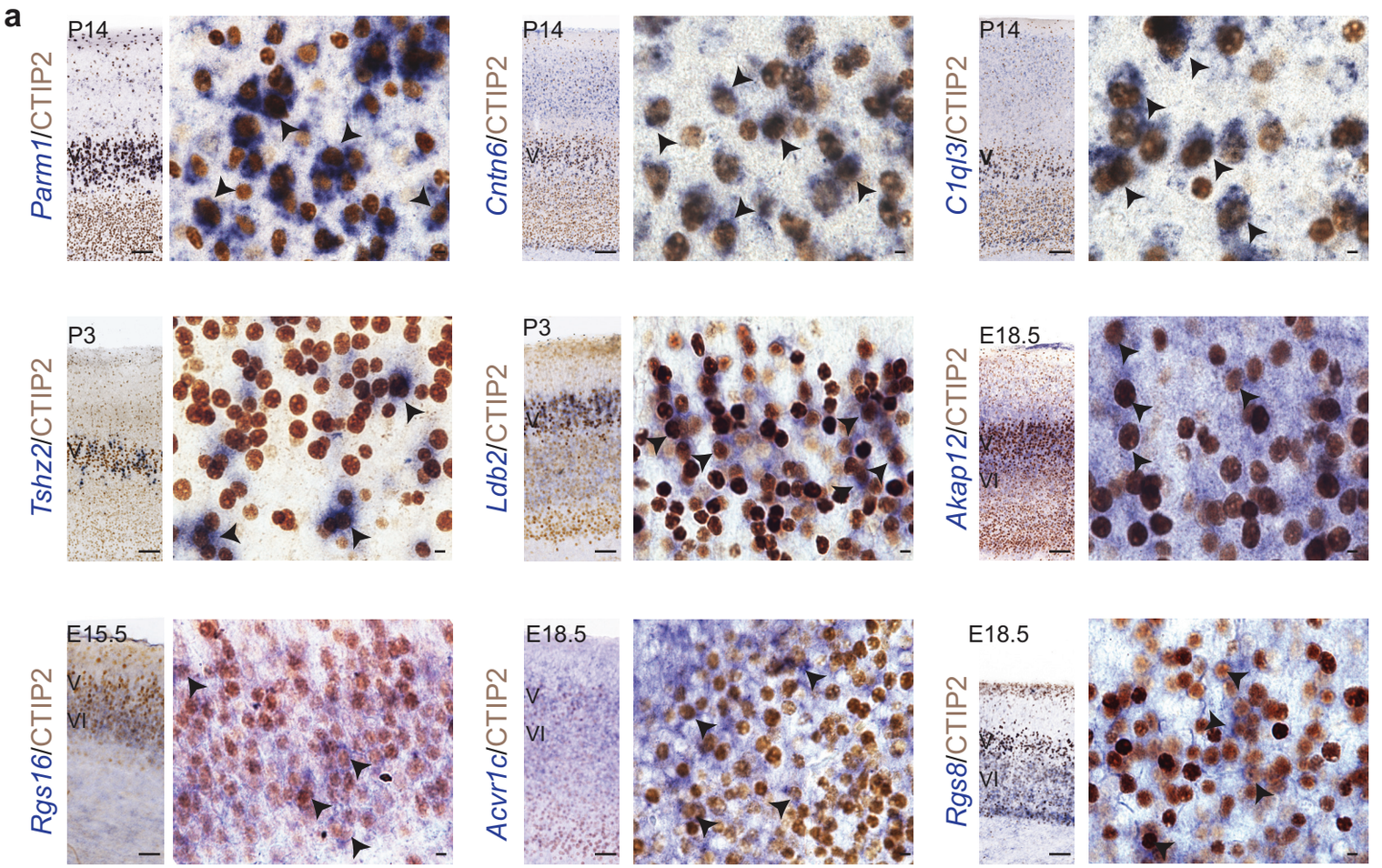


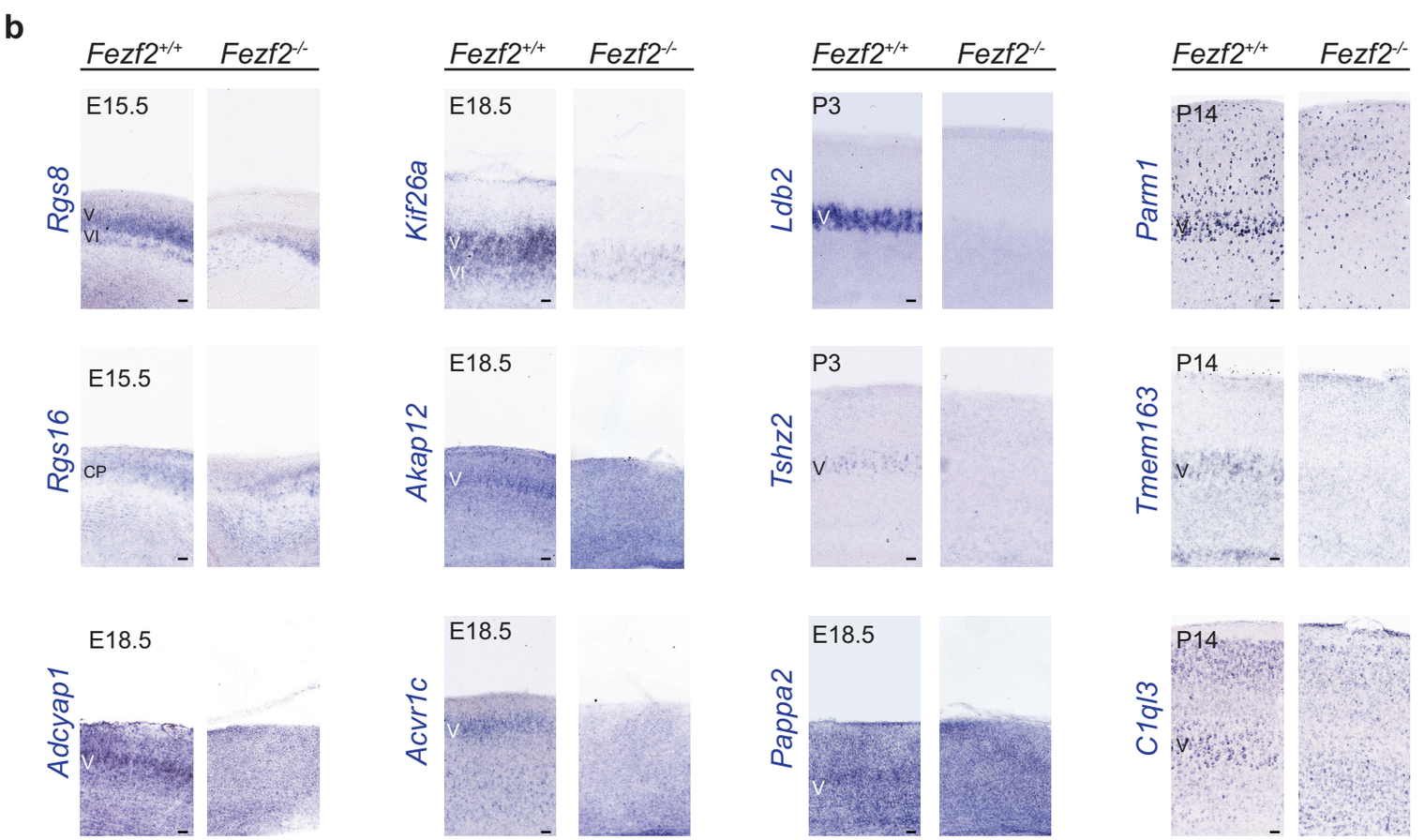
Supplementary Figure 2. Expression profiles of *Fezf2*-induced genes at multiple embryonic and postnatal stages.

Expression profiles of 12 genes up-regulated by *Fezf2* (shown in **Figure 1**) were examined by *in situ* hybridization in wild type cortices of E15.5, E18.5, P3, P7 and P14 wild type mice. CP, cortical plate. Scale bars, 50 μm.

(Related to Figure 1).

Lodato, Arlotta; Supplementary Figure 3



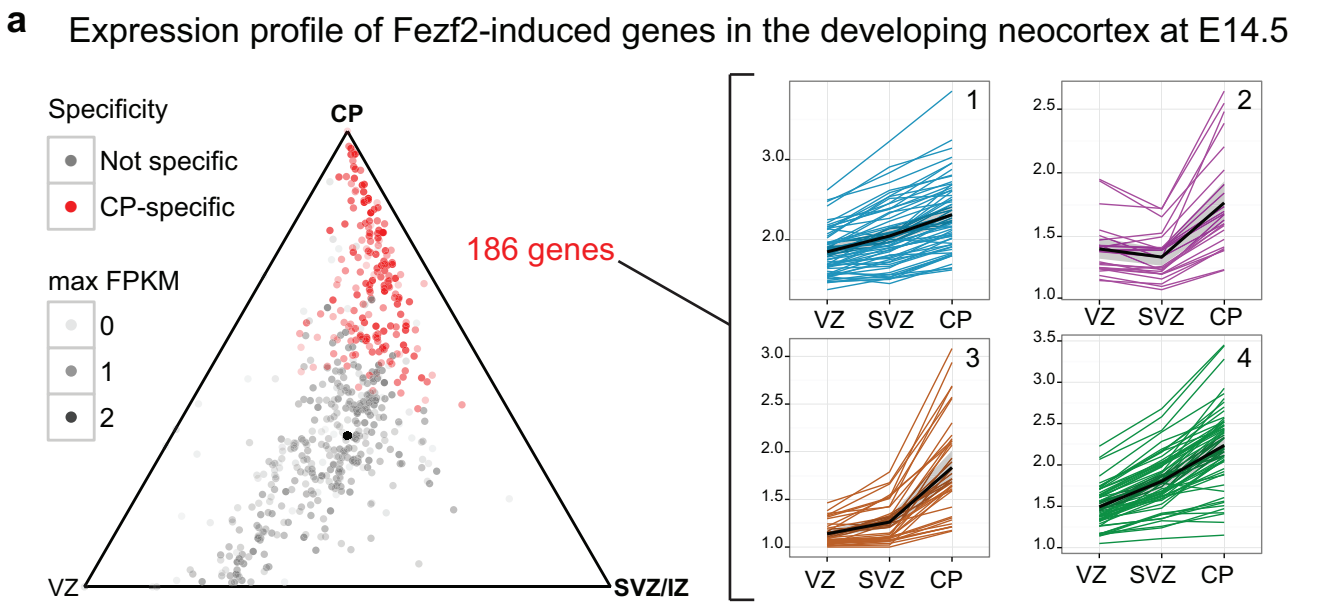


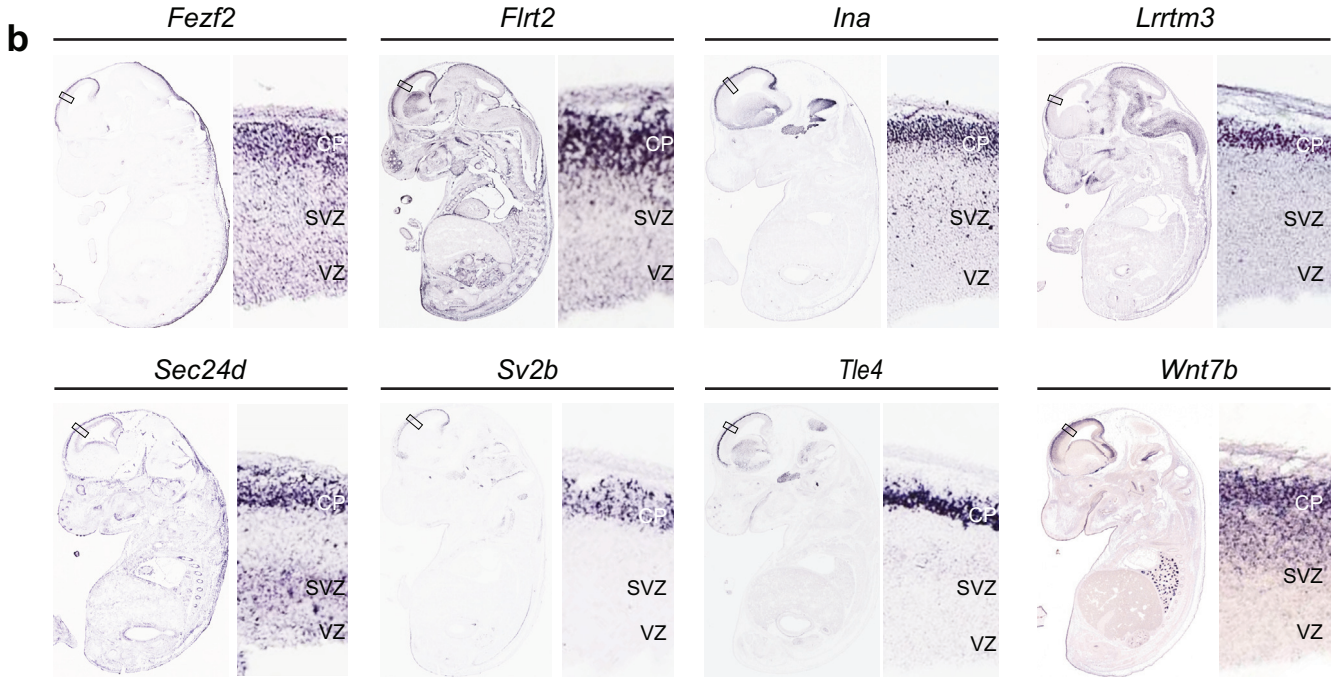
Supplementary Figure 3. Expression of *Fezf2* target genes in Layer Vb subcerebral projection neurons is dependent on *Fezf2* expression.

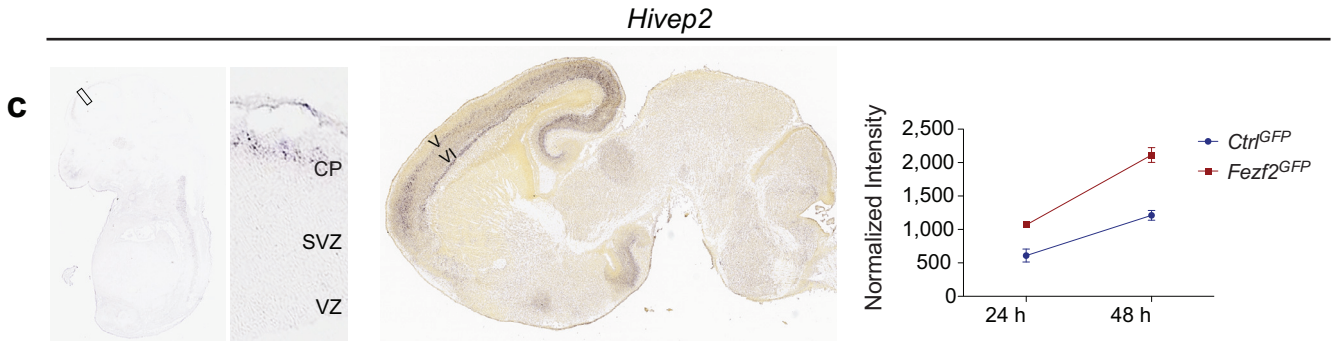
(a) *In situ* hybridizations for selected *Fezf2*-induced genes (blue signal) followed by immunocytochemistry for CTIP2 (brown signal) were performed on coronal sections of wild type mouse brains. **(b)** *In situ* hybridizations of selected *Fezf2*-induced genes on coronal sections of *Fezf*$^{-/-}$ brains (right) and wild type littermate controls (left). CP, cortical plate. Scale bars, 50 μm **(a)** left panels and **(b)**, and 20 μm **(a)** right panels.

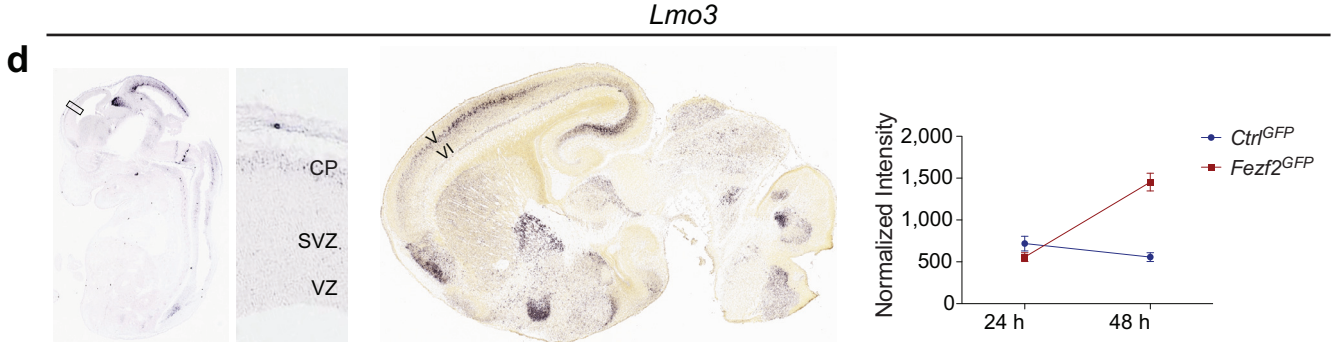
(Related to Figure 2).

Lodato, Arlotta; Supplementary Figure 4







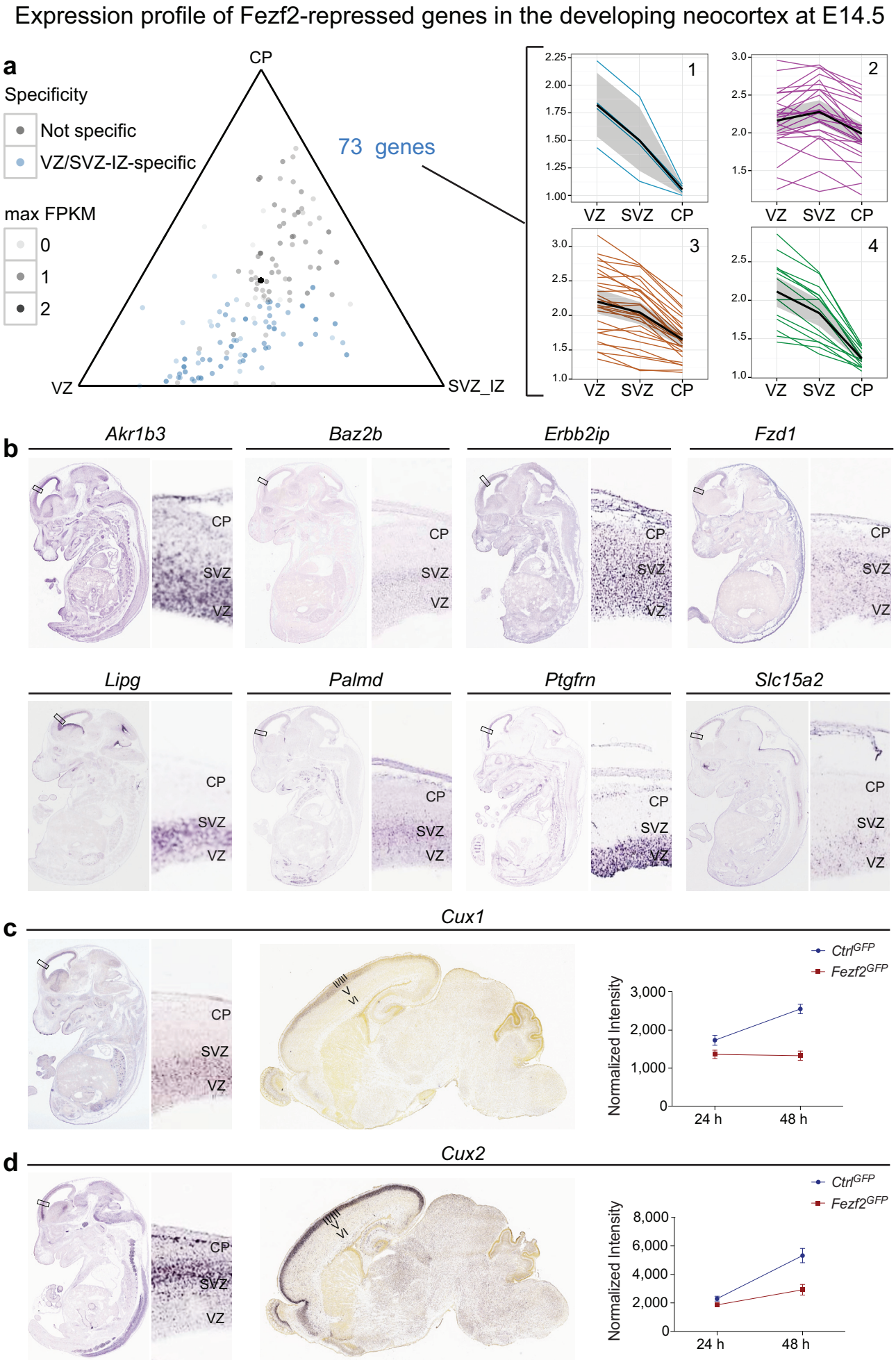


Supplementary Figure 4. *Fezf2*-induced genes are expressed in nascent CSMN.

(a) Left, ternary plot of gene expression probabilities in VZ, SVZ/IZ and CP for *Fezf2*-induced targets shows enrichment in the cortical plate. Right, cluster representation of 186 genes induced by *Fezf2* and also significantly enriched in the E14.5 CP. Y axis, FPKM (Fragments Per Kilobase RNA per Million mapped reads). Complete gene list is available in **Supplementary Table 2**. **(b)** Upper most left panel) *In situ* hybridization for *Fezf2* on an E14.5 embryo shows the position of the cortical plate (CP). *In situ* hybridizations (www.*genepaint.org*) for prototypical genes induced by *Fezf2* and enriched in the E14.5 CP (insets, enlarged from boxed areas). **(c-d)** *In situ* hybridizations for *Hivep2* and *Lmo3* at E14.5 (left panels) and at P4 (middle panels**,** *www.developingmouse.brain-map.org***)** show specific expression in the developing CP and in layer V CSMN, confirming that *Fezf2* induces expression of CSMN genes from early stages of development. Right panels, expression levels of *Hivep2* **(c)** and *Lmo3* **(d)** in $Ctrl^{GFP}$ (blue line) and $Fezf2^{GFP}$ samples (red line) at 24 hours and 48 hours. Error bars indicate standard errors of the mean (SEM). CP, cortical plate; SVZ, subventricular zone; VZ, ventricular zone; IZ intermediate zone. Images shown in **b; c** and **d** left 2 panels are from the Genepaint database and **c** and **d** middle panels are from the Allen Brain Atlas database.

(Related to Figure 2).

Lodato,Arlotta; Supplementary Figure 5

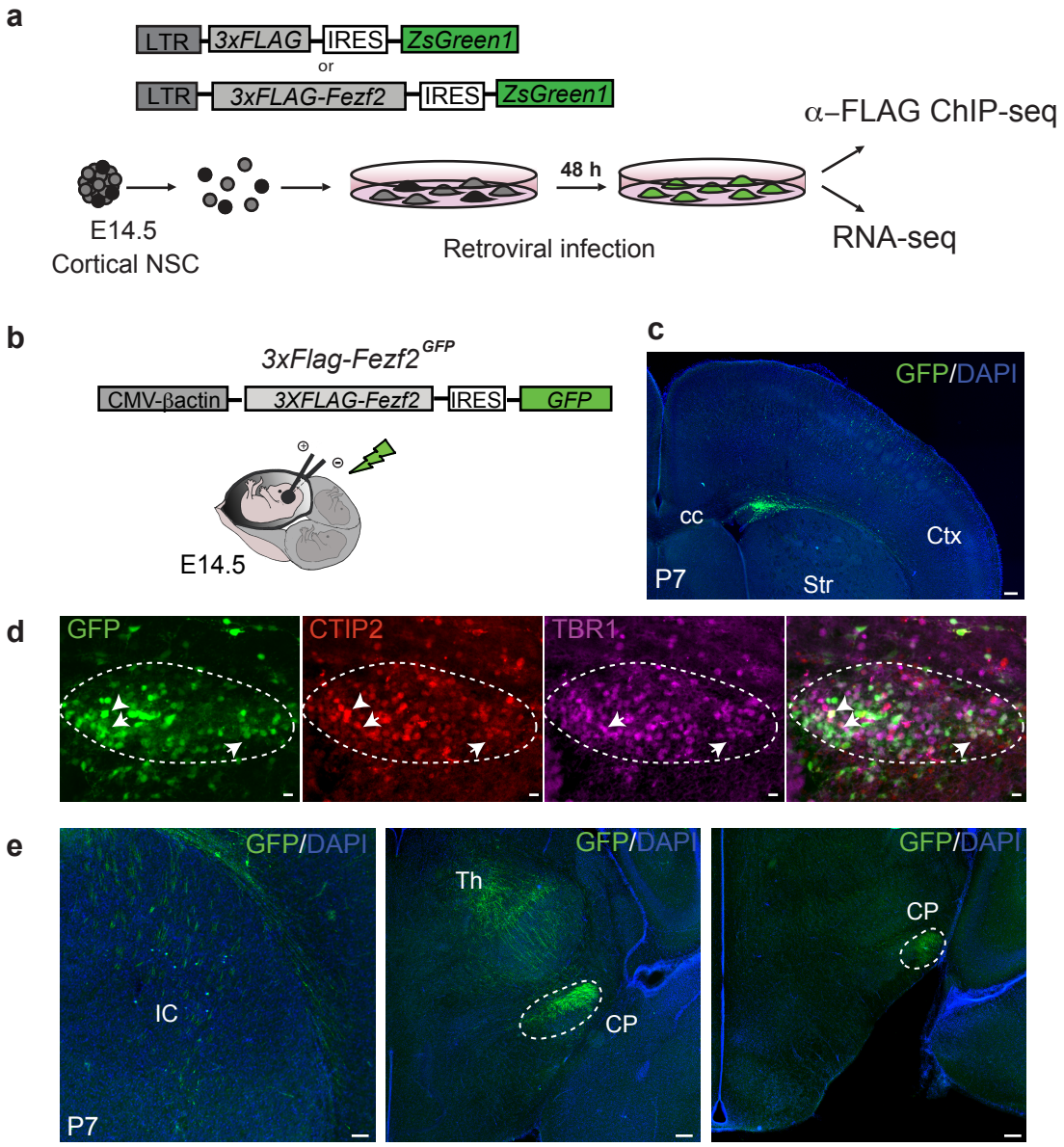


Supplementary Figure 5. *Fezf2*-repressed genes are expressed in E14.5 VZ and SVZ/IZ cortical progenitors and in their progeny.

(a) Left, ternary plot of expression profiles collapsed to probability distributions in VZ, SVZ/IZ and CP for *Fezf2*-repressed genes shows enrichment in the VZ and the SVZ/IZ. Right, cluster representation of 73 genes repressed by *Fezf2* and also significantly enriched in the E14.5 VZ-SVZ/IZ. Y axis, FPKM (Fragments Per Kilobase RNA per Million mapped reads). Complete gene list is available in **Supplementary Table 2**. **(b)** *In situ* hybridizations (www.genepaint.org) for prototypical genes repressed by *Fezf2* and preferentially expressed in the E14.5 VZ-SVZ/IZ (insets, enlarged from boxed areas). **(c,d)** *In situ* hybridizations for *Cux1* and *Cux2* at E14.5 (left panels) showing specific expression in cortical progenitors. Middle panel, *in situ* hybridizations for *Cux1* and *Cux2* P4 showing specific expression in mature upper layer CPN (www.developingmouse.brain-map.org). Far right**,** expression levels of *Cux1* and *Cux2* in $Ctrl^{GFP}$ (blue line) and $Fezf2^{GFP}$ samples (red line) are shown at 24 hours and 48 hours. Error bars indicate standard errors of the mean (SEM). CP, cortical plate; SVZ, subventricular zone; VZ, ventricular zone; IZ, intermediate zone. Images shown in **b; c** and **d** left 2 panels are from the Genepaint database and **c** and **d** middle panels are from the Allen Brain Atlas database.

(Related to Figure 2).

Lodato, Arlotta; Supplementary Figure 6

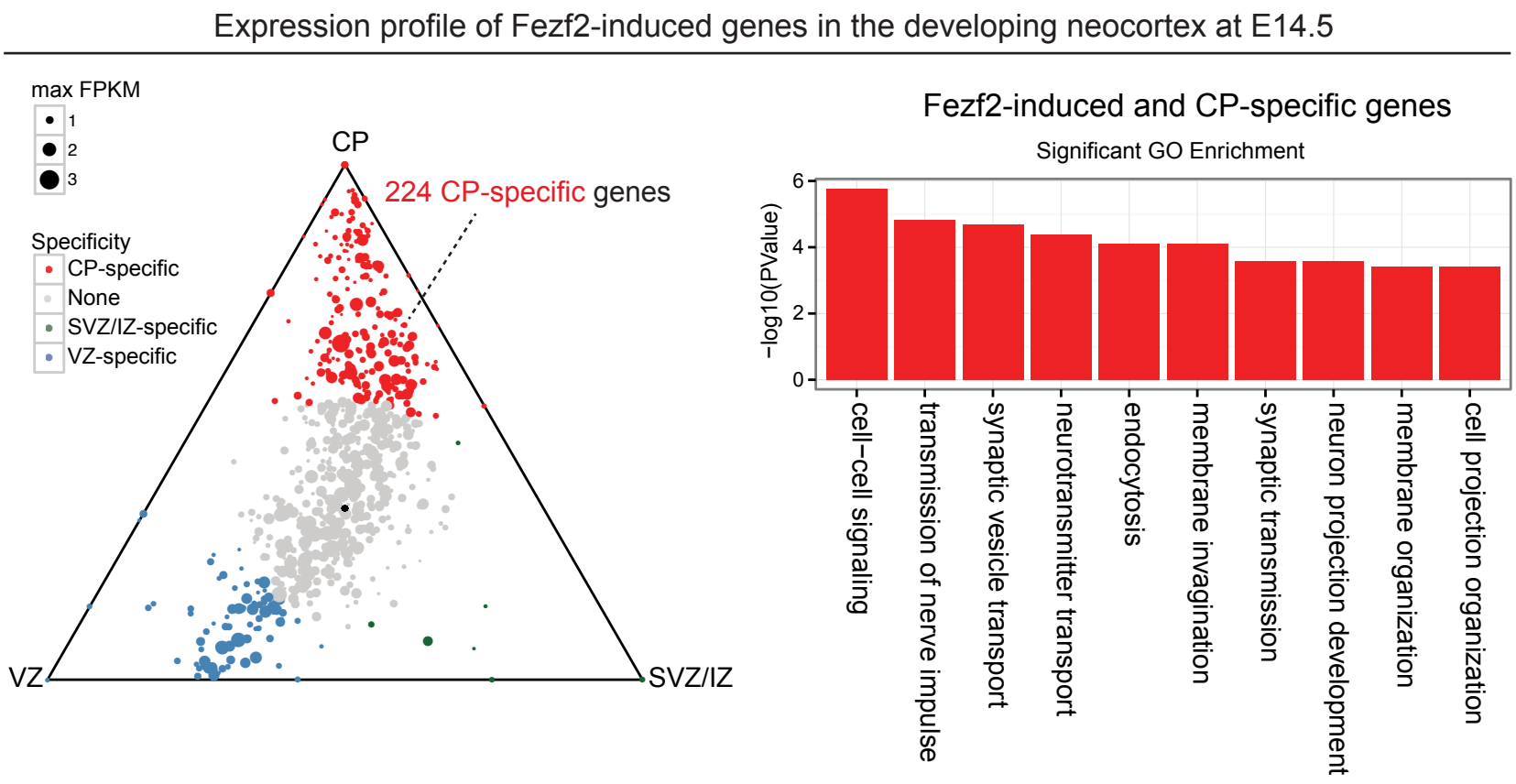


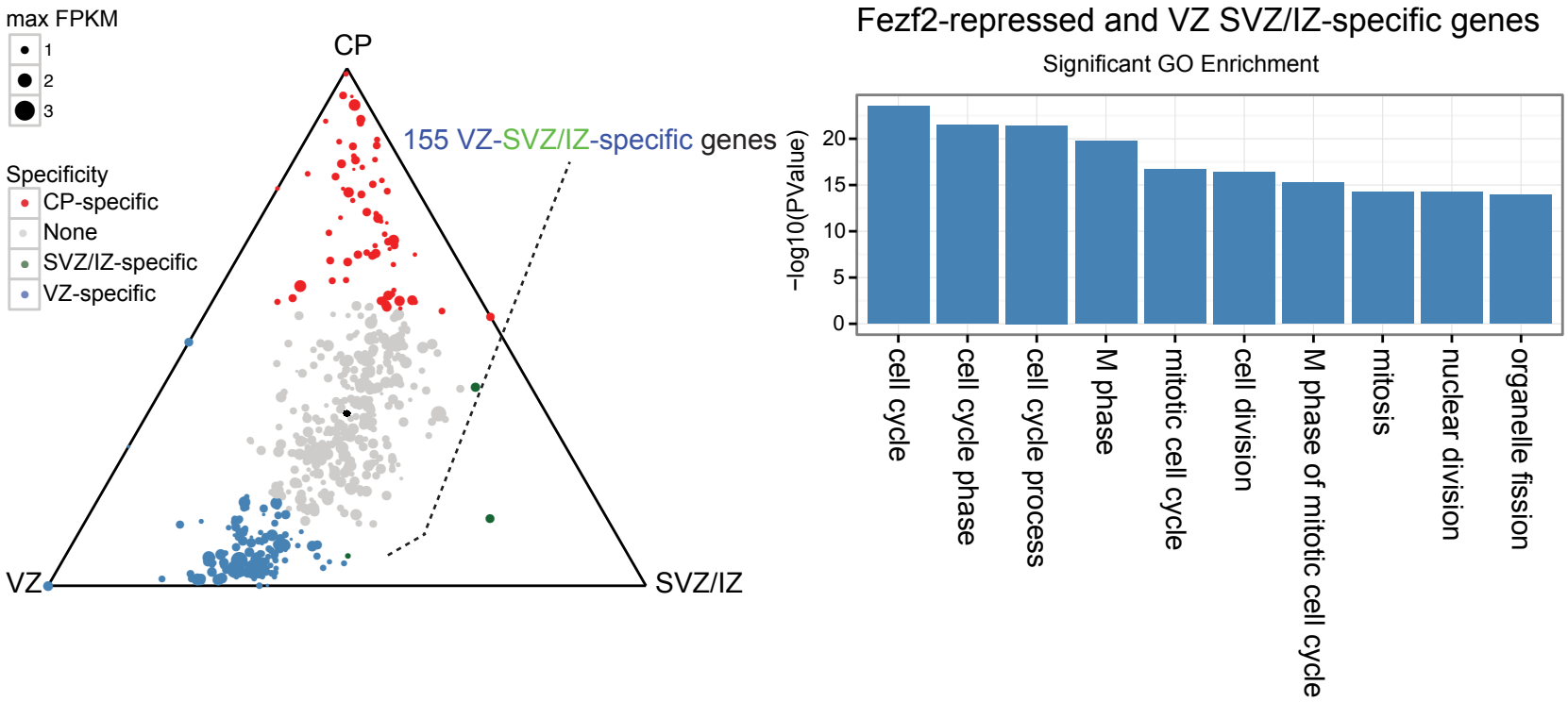
Supplementary Figure 6. 3xFLAG-*Fezf2* recapitulates *Fezf2* overexpression phenotype *in vivo*.

N-terminally FLAG-tagged *Fezf2*, employed for ChIP-seq and RNA-seq experiments, retains the ability to instruct the molecular identity and connectivity of deep layer projection neurons when ectopically expressed in progenitors of the upper layers at E14.5

(a) Experimental design of the ChIP-seq and RNA-seq approach. **(b)** Schematic of the experimental approach. **(c)** 3xFLAG-*Fezf2* overexpression results in ectopic GFP+ cell aggregates below the corpus callosum, phenocopying untagged *Fezf2* overexpression. **(d)** Immunohistochemistry for GFP, CTIP2 and TBR1 in electroporated brains harvested at P7 shows that 3xFLAG-*Fezf2* is sufficient to induce a switch of fate to CTIP2+ and TBR1+ corticofugal projection neurons. **(e)** Immunohistochemistry for GFP shows axonal projections through the internal capsule (IC) (left panel), toward the thalamus (Th) (middle panel) and through the cerebral peduncle (CP) (right panel) upon 3xFLAG-*Fezf2* overexpression. Ctx, cortex; Str, striatum; cc, corpus callosum. Scale bars, 100 μm (b, d), and 20 μm (c). (Related to Figure 3).

Lodato, Arlotta; Supplementary Figure 7



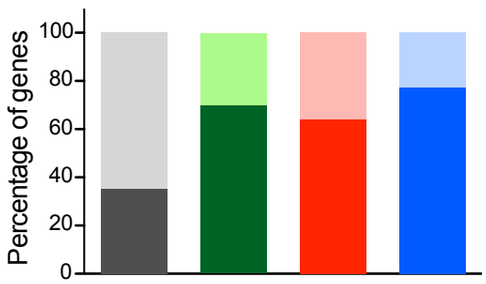


Supplementary Figure 7. Overexpression of *Fezf2* in neurosphere-derived neurons *in vitro* directs transcriptional changes toward the nascent CSMN state.

(a) Left, ternary plot of expression profiles collapsed to probability distributions in VZ, SVZ/IZ and CP for *Fezf2*-induced genes (identified from RNA-seq analysis) shows enrichment in the CP. Genes are classified as CP-specific (red), VZ-specific (blue), SVZ-specific (green), or non-specific (grey) using a specificity score threshold of 0.65 (detailed in Methods). Right, top 10 Gene Ontology categories from GO analysis of 224 genes up-regulated by *Fezf2* i*n vitro*. **(b)** Left, ternary plot of expression profiles collapsed to probability distributions in VZ, SVZ/IZ and CP for *Fezf2*-repressed genes (identified from RNA-seq analysis) shows enrichment in the VZ- SVZ/IZ. Right, top 10 Gene Ontology categories from GO analysis of 155 genes down-regulated by *Fezf2 in vitro* and specifically expressed in E14.5 VZ or SVZ/IZ. CP, cortical plate; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone. (Related to Figure 3).

Lodato, Arlotta; Supplementary Figure 8

a

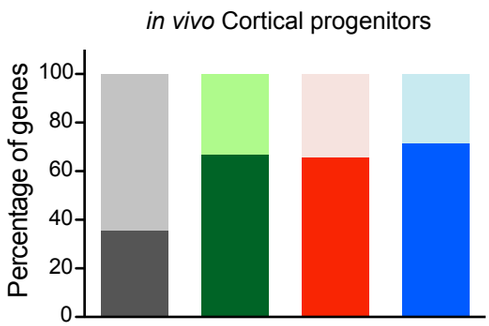


b

	Total # of genes	# genes bound by FEZF2	%	p-value Hypergeometric test	p-value Bootstrap method
All genes	36439	12860	35.3%		
Fezf2-regulated genes	1295	903	69.7%	<5.07E-110	<1.00E-04
Fezf2-induced genes	747	479	64.1%	<2.09E-42	<1.00E-04
Fezf2-repressed genes	548	424	77.4%	<1.14E-73	<1.00E-04

All genes and bound at TSS
All genes and not bound
Fezf2-regulated and bound at TSS
Fezf2-regulated and not bound
Fezf2-induced and bound at TSS
Fezf2-induced and not bound
Fezf2-repressed and bound at TSS
Fezf2-repressed and not bound

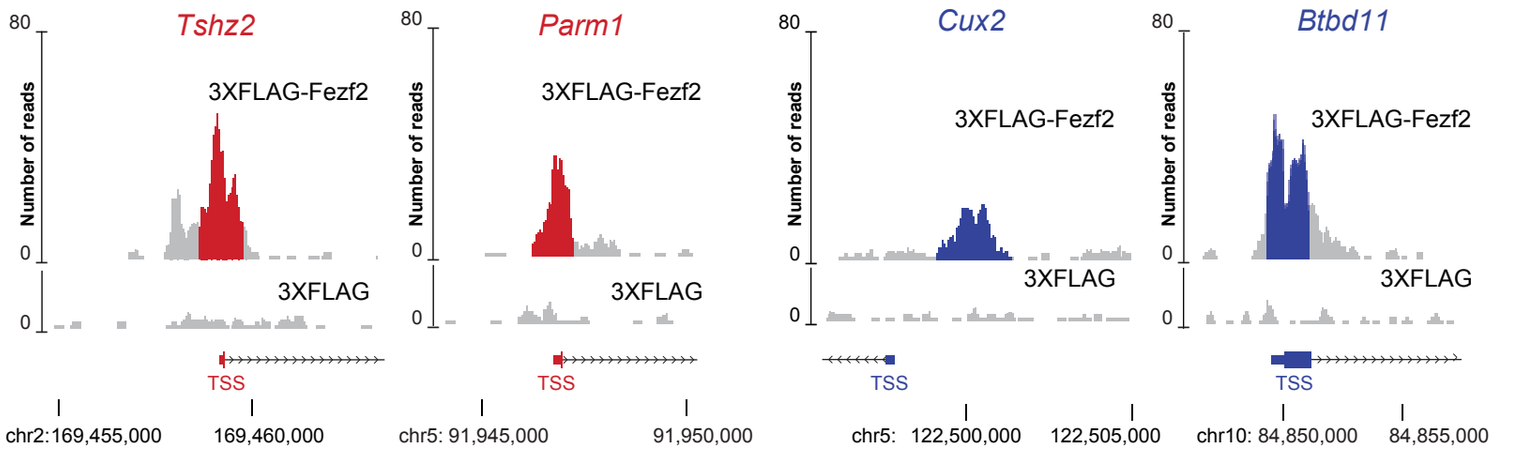
c



d

	Total # of genes	# genes bound by FEZF2	%	p-value (x-square test)
All genes	36439	12860	35.3%	
Fezf2-induced genes at 24h	263	164	62.4%	<4.12E-20
Fezf2-induced genes at 48h	441	288	65.3%	<1.01E-39
Fezf2-induced genes at 24h and 48h	95	69	72.6%	<2.62E-14
Fezf2 -induced genes at 24h and/or 48h	589	387	65.7%	<8.12E-54
Fezf2-repressed genes at 24h	90	58	64.4%	<7.15E-18
Fezf2-repressed genes at 48h	89	65	73.0%	<9.28E-14
Fezf2-repressed genes at 24h and 48h	22	18	81.8%	<4.96E-06
Fezf2 -repressed genes at 24h and/or 48h	153	109	71.2%	<1.34E-20

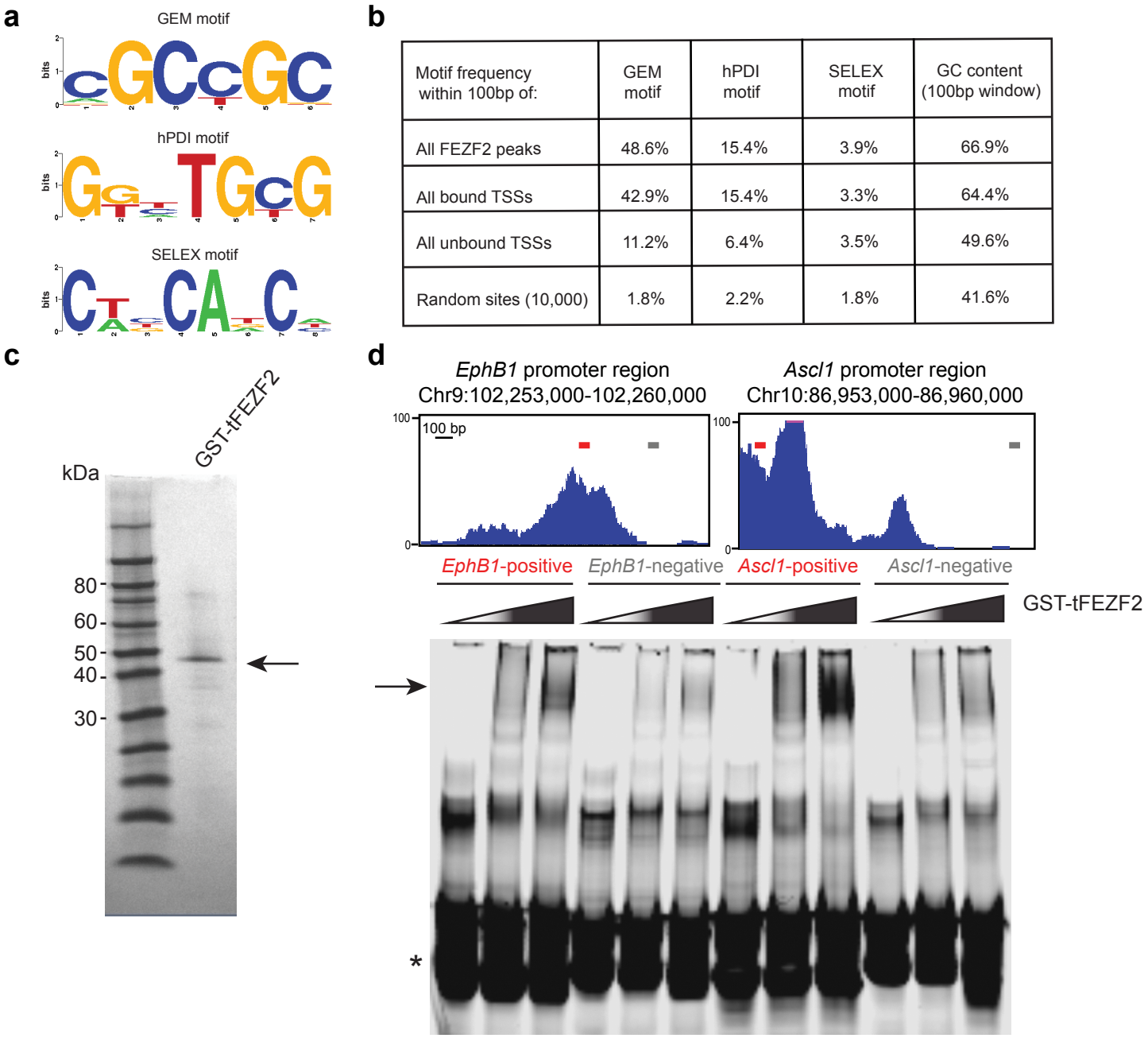
e



Supplementary Figure 8. FEZF2 directly controls expression of its transcriptional targets *via* association with their promoter regions

(a) Bar plots showing the percentage of FEZF2-bound genes among all genes (grey), *Fezf2*-regulated genes (green), *Fezf2*-induced genes (red), and *Fezf2*-repressed genes (blue) in *in vitro* cortical progenitors. **(b)** Table of gene counts, percentages of total, and p-values (hypergeometric test and bootstrap method) showing significant enrichment for FEZF2-bound genes in both the *Fezf2*-induced and *Fezf2*-repressed significant gene sets from in vitro cortical progenitors. **(c)** Bar plots showing the percentage of FEZF2-bound genes among all genes (grey), *Fezf2*-regulated genes (green), *Fezf2*-induced genes (red), and *Fezf2*-repressed genes (blue) in *in vivo* purified cortical progenitors. **(d)** Table of gene counts, percentages of total, and p-values (χ^2-test) demonstrating significant enrichment for FEZF2-bound genes in both the *Fezf2*-induced and *Fezf2*-repressed significant gene sets from i*n vivo* purified E14.5 cortical progenitors collected at 24h and 48h after *Fezf2* over-expression. **(e)** Examples of 3xFLAG-*Fezf2* peaks at the proximal promoters of CSMN-specific, *Fezf2*-induced genes (red peaks) and CPN-specific, *Fezf2*-repressed genes (blue peaks). Source data is shown in **Supplementary Table 6**. (Related to Figure 3).

Lodato, Arlotta; Supplementary Figure 9

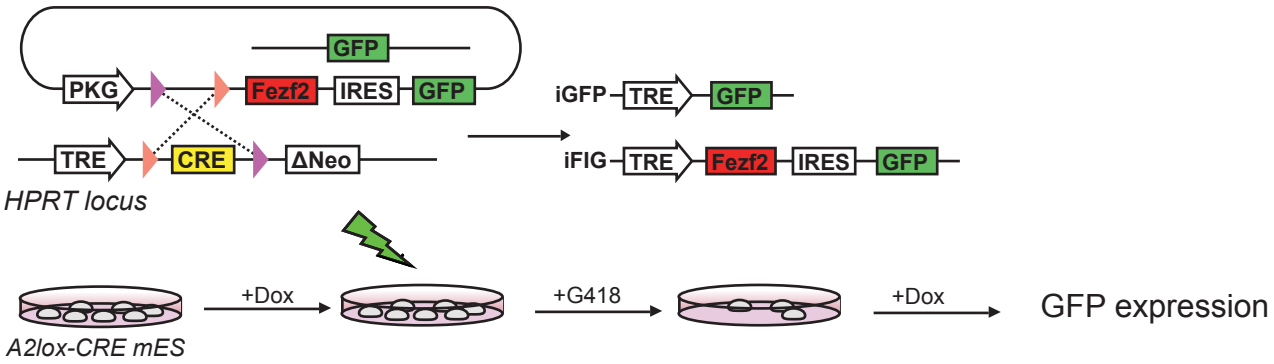


Motif frequency within 100bp of:	GEM motif	hPDI motif	SELEX motif	GC content (100bp window)
All FEZF2 peaks	48.6%	15.4%	3.9%	66.9%
All bound TSSs	42.9%	15.4%	3.3%	64.4%
All unbound TSSs	11.2%	6.4%	3.5%	49.6%
Random sites (10,000)	1.8%	2.2%	1.8%	41.6%

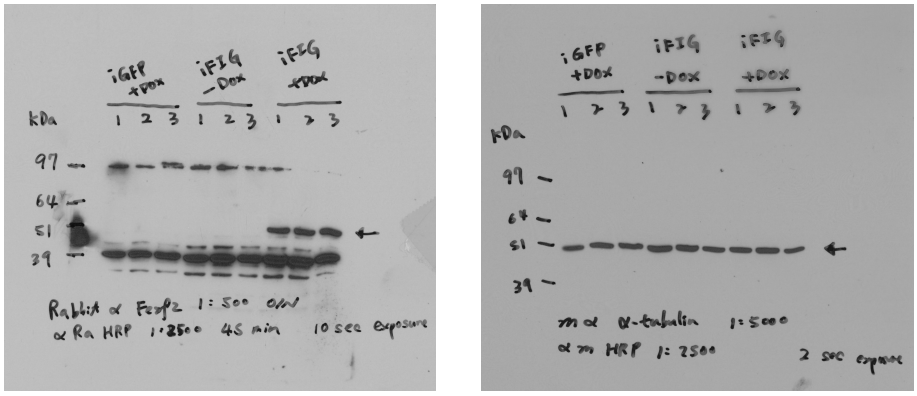
Supplementary Figure 9. FEZF2 directly associates with a CG-rich consensus sequence.
(a) The CG-rich FEZF2 consensus motif identified by GEM differs from two previously predicted FEZF2 motifs (SELEX and hPDI). **(b)** The motif-independent approach GPS found the GEM-defined motif more represented at the FEZF2-bound sites compared to the two previously defined motifs. FEZF2-bound Transcriptional Start Sites (TSSs) were also found preferentially associated with the GEM-defined motif compared to unbound TSSs. **(c)** Coomassie blue staining of purified GST-tFEZF2 protein (see Methods). The expected molecular weight of the GST-fusion truncated protein is 43.5 kDa, (arrow). **(d)** Electrophoretic mobility shift assay of probes for regions within the promoters of *Ascl1* and *EphB1* in the presence of GST-tFEZF2. Probes containing FEZF2 consensus motifs (*EphB1*-positive and *Ascl1*-positive) showed specific interaction with GST-tFEZF2 compared to probes without the consensus motifs in neighboring region of the same promoters (*Ephb1*-negative and *Ascl1*-negative). Arrow, probes bound to GST-tFEZF2; Asterisk, unbound probes.
(Related to Figure 3).

Lodato, Arlotta; Supplementary Figure 10

a



b

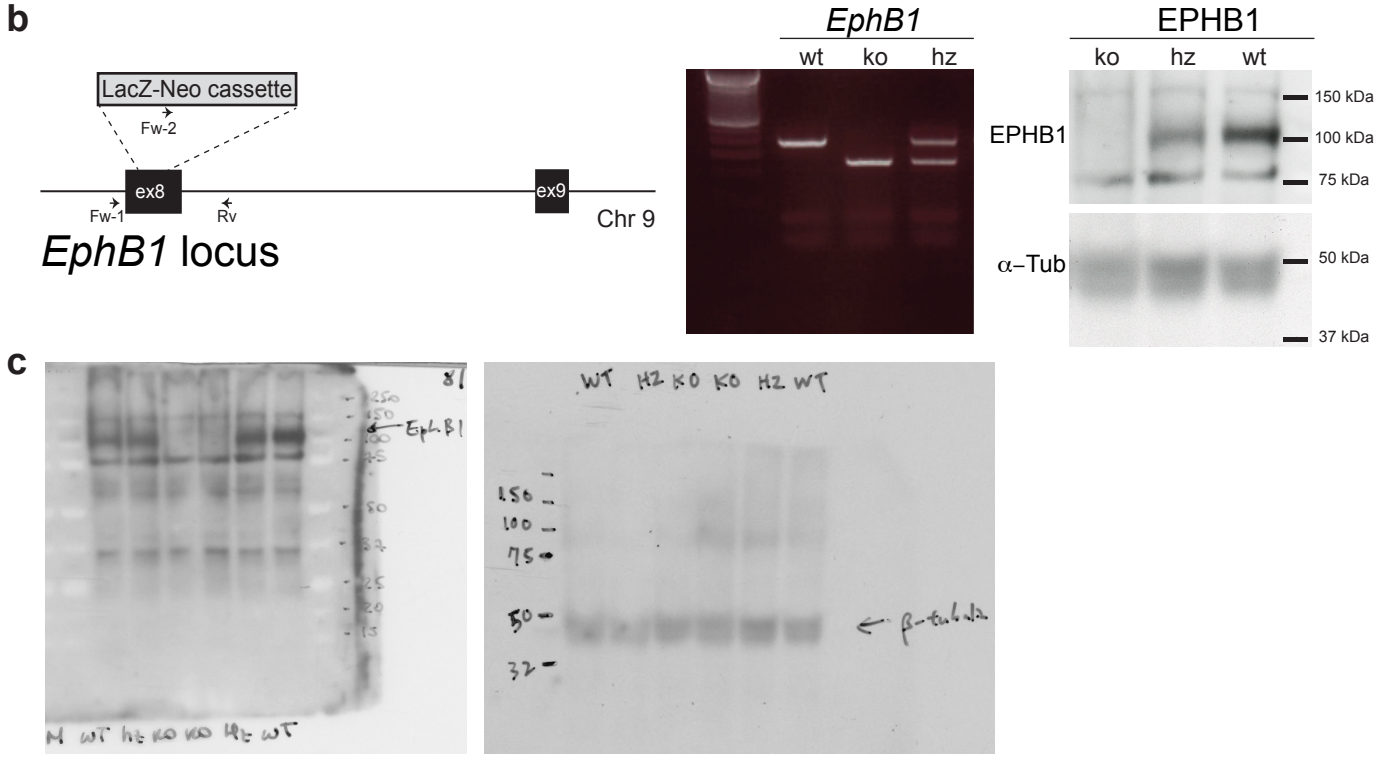


Supplementary Figure 10. Generation of inducible Fezf2 mouse ES cell lines. **(a)** Strategy used for derivation of TET-ON Fezf2 mouse ES cell lines using the inducible cassette exchange method (see Methods). **(b)** Uncropped original immunoblots of Figure 4c.

Lodato, Arlotta; Supplementary Figure 11

a

	Function	p-value	Molecules
Fezf2-induced genes at 24 h	Axonal Guidance Signaling	2.60E-02	PLCB4,STK36,NTRK2 SDCBP,NRP2,EFNA3 GNAZ,ADAMTS9,ROBO1, WNT5A,PRKCA,PTCH2
Fezf2-induced genes at 48 h	Axonal Guidance Signaling	6.29E-02	PAPPA2,UNC5A,BDNF, PIK3R1,UNC5B,PLCH2, ROBO1,SEMA6C,PTCH2 EPHB6,PAK1,**EPHB1**, WNT7A,WNT7B,PLXNB2, PIK3R2,EFNB3,FZD2 ADAMTS5,EPHA7,STK36 NRP2,SEMA5A,EFNA3, GNAZ,ADAMTS9,PLCB4, LINGO1,SEMA4G

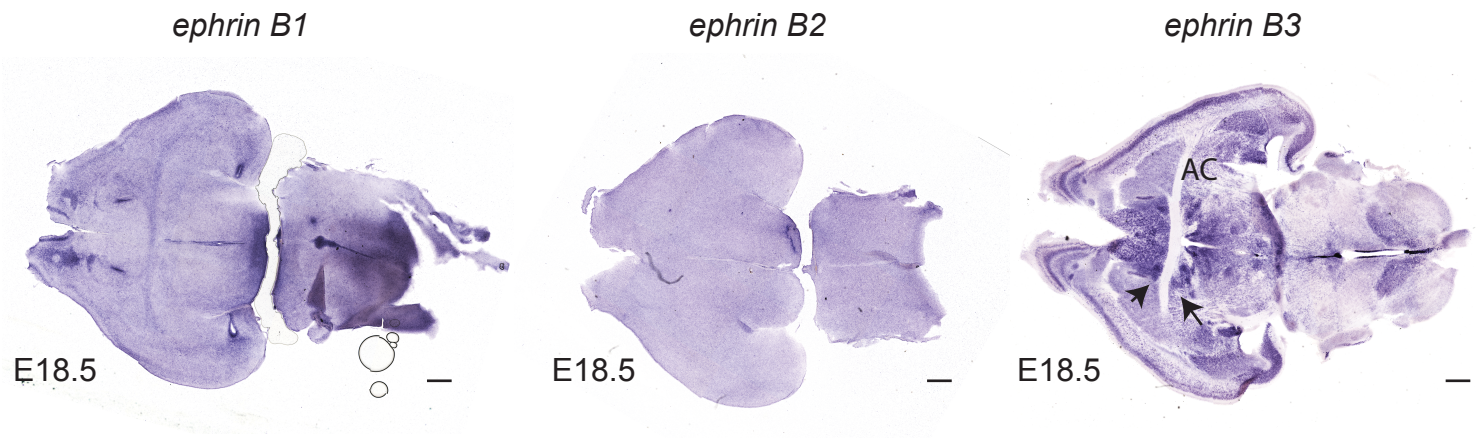


Supplementary Figure 11. Axon guidance signaling molecules are highly represented in the GO analysis of *Fezf2*-induced genes and include the tyrosine kinase receptor *EphB1*.

(a) GO analysis using *Fezf2*-induced genes at 24 hours and 48 hours identifies "axon guidance signaling" among the most significant categories (ranking as the most significant category at 48 hours). (**b**, far left panel) Schematic representation of the *EphB1* gene targeting strategy (Deltagen Inc.). Arrows show positions and orientations of the primers employed for genotyping PCR. (**b**, middle panel) Genotyping PCR using primers flanking the targeted region identifies a 350 bp product corresponding to the wild type allele and a 300 bp product corresponding to the $EphB1^{-/-}$ allele. (**b**, most right panel) Western blot of brain lysate from P1 wild type, $EphB1^{+/-}$ pups, and $EphB1^{-/-}$ P1 pups using anti-EPHB1 antibody shows absence of EPHB1 protein (expected MW=100 kDa) in the $EphB1^{-/-}$ sample and a reduction in the $EphB1^{+/-}$ sample compared to wild type. Anti-β-tubulin is used as loading control. (c) Uncropped original immunoblots shown in panel (**b**, most right panel).

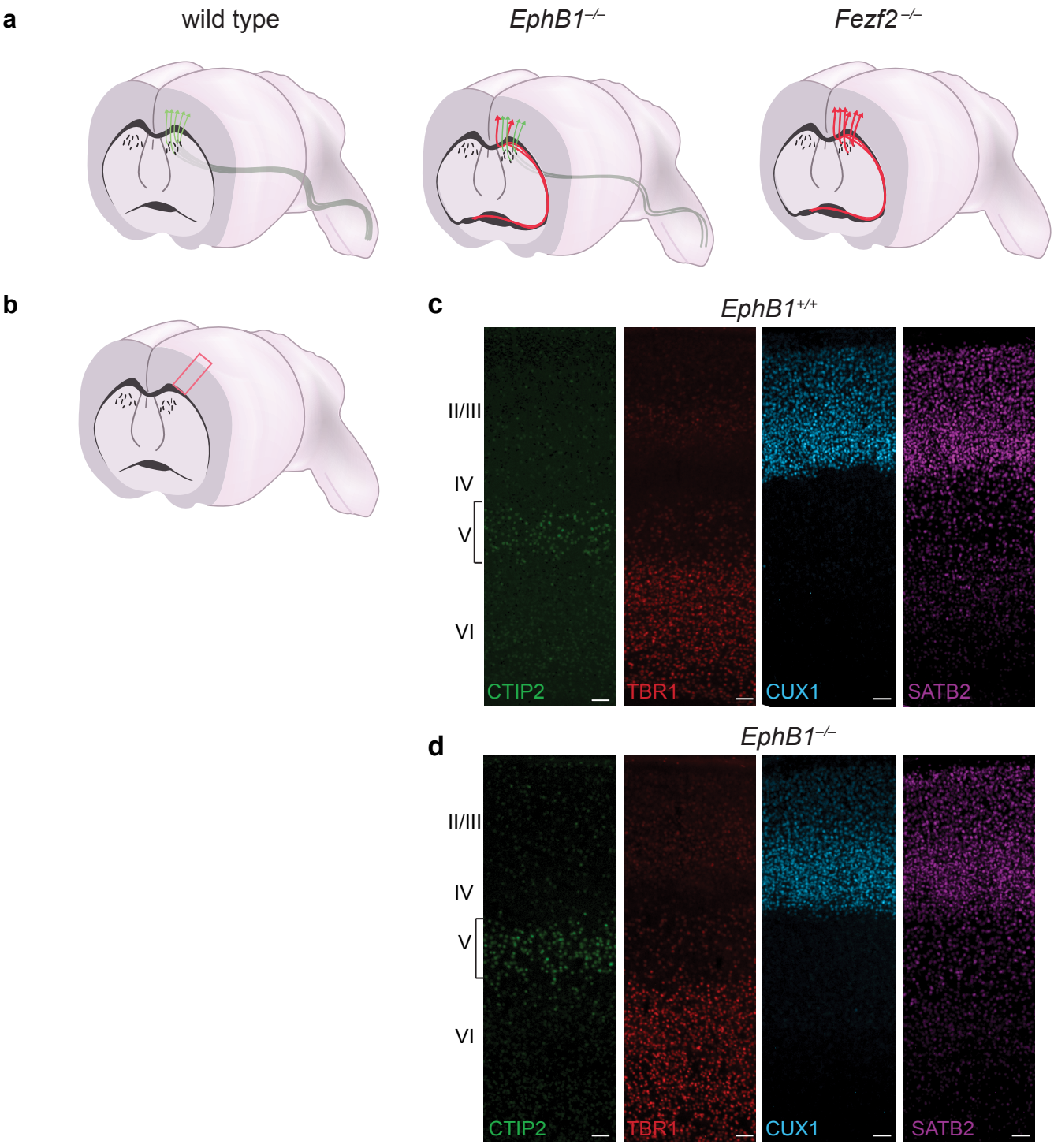
(Related to Figure 5).

Lodato, Arlotta; Supplementary Figure 12



Supplementary Figure 12. Expression patterns of *EphB* ligands in the mouse brain.
In situ hybridization for *ephrin B1*, *ephrin B2* and *ephrin B3* on horizontal wild type brain sections at E18.5 shows expression of *ephrin B3* at the ventral forebrain midline. AC, anterior commissure. Scale bars, 100 μm. (Related to Figure 7).

Lodato, Arlotta; Supplementary Figure 13



Supplementary Figure 13. Class-specific identity and distribution of cortical projection neurons is unaltered in *EphB1*$^{-/-}$ mice.

(a) Schematic representation of the axon pathfinding phenotypes observed in both *EphB1*$^{-/-}$ and *Fezf2*$^{-/-}$ mice. **(b)** Schematic drawing of a P7 brain showing the position of area imaged. **(c-d)** Immunocytochemistry for CTIP2, TBR1, CUX1 and SATB2 show no difference in the layer distribution of these markers in *EphB1*$^{-/-}$ **(d)** compared to wild type mice **(c)**. Scale bars, 100 μm. (Related to Figure 7).