Figure S1. Models of early axonogenesis *in vivo* and *in vitro*. Related to Figure 1

(A) Schematic of *in vivo* generation of cortical excitatory neurons. Modified from Sakakibara and Hatanaka 2015. MZ: marginal zone. CP: cortical plate. IZ: intermediate zone. SVZ: subventricular zone. VZ: ventricular zone. (B) Representative images of immature pre-axonogenesis neurons (DIV 1) and axon-generating neurons (DIV 3) in cultures.

Figure S2. RNA-Seq analysis of DIV 1 and DIV 3 neurons. Related to Figure 1 and Figure 2

Two biological replicates of immature pre-axonongeneis neurons (DIV1_1 and DIV1_2) and two biological replicates of axon-generating neurons (DIV3_1 and DIV3_2) are cross compared. X and Y axes show the value of $\log_2$(TPM+1). The red lines depict fitted regression. R^2 = 0.995 (A), 0.994 (B), 0.978 (C), 0.976 (D), 0.977 (E), and 0.978 (F). (G) Axonogenesis-associated gene lists identified via transcriptome comparison are assessed against gene ontology terms enriched in previously published axonogenesis-controlling genes. A scatter plot shows the Bonferroni-corrected P values of enriched Gene Ontology terms for axonogenesis-associated gene groups on the y axis vs previously published axonogenesis-controlling genes on the x axis. Dashed lines demark P value equal to 0.05 on the y axis. Group 1 (grey dots): 252 genes regulated at the gene and isoform level. Group 2 (red dots): genes regulated exclusively at the isoform level. Amino acids encoded by axonogenesis-associated exons in (H) CLIP2, (I) CAMSAP1, (J) ABI2, and (K) PPP3CA. For CAMSAP1, inclusion of the exon disrupts the domain. For others, inclusion of the exon is required for the domain.

Figure S3. SHTN1L promotes actin polymerization. Related to Figure 4

(A) A representative Coomassie blue-stained gel image demonstrating the purity of FLAG-tagged target proteins. The protein concentrations were obtained by densitometric analysis compared with 1:2 serial dilutions of recombinant BSA. (B-C) Cosedimentation experiments were performed using 0.15 mg/ml actin alone or with 1:2 serial dilutions of purified FLAG-SHTN1L and FLAG-SHTN1L$^{RRR>GGG}$ proteins starting from 286 nM for both. Mutation of three conserved arginine residues to glycine (R482G, R483G, and R484G; SHTN1L$^{RRR>GGG}$ mutant) disrupted F-actin binding.

Figure S4. PTBP2 regulate axonogenesis-associated exons. Related to Figure 5

(A) Enriched motif identified for down-regulated exons during early axonogenesis. (B) Axonogenesis-associated exons, control non-regulated exons, and constitutive exons are examined for PTBP2 CLIP-Seq signals at each nucleotide position. The percent of exons with PTBP2 CLIP signals are plotted. (C) PTBP2-regulated exons significantly overlap with axongenesis-associated exons. P value = 4.4×10^{-78}, hypergeometric test. (D) A 2x2 table shows the distribution of the overlapping exons regarding their responses to PTBP2 deletion and during early axonogenesis. (E) PTBP2-regulated exons determined by PTBP2 overexpression significantly overlap with axongenesis-associated exons. P value = 3.23×10^{-82}, hypergeometric test. (F) Splicing changes (increase or decrease) from pre-axonogenesis neurons (DIV 1) to axon-generating neurons (DIV 3) are mostly decelerated by gain of PTBP2. (G) PSI values of PTBP2-regulated exons genes at DIV 1, DIV3, and DIV 5 are determined by quantitative capillary electrophoresis. Data are represented as mean ± SEM of 3-4 animals. **, P-value < 0.01; *, P-value < 0.05; Student's t test.

Figure S5. *Ptbp2* expression in various tissues profiled in the ENCODE project. Related to Figure 5

TPM expression values of *Ptbp2* transcripts in the developing forebrain, hindbrain, and midbrain at E10.5, E11.5, E12.5, E13.5, E14.5, E15.5, E16.5, PND0, and in adult cerebellum, cortical plate, frontal cortex, colon, duodenum, gonadal fat pad, heart, kidney, intestine, liver, lung, mammary gland, small intestine, stomach, subcutaneous adipose tissue, and thymus. Each sample has two replicates.

Figure S6. PTBP2 direct targets are confirmed by PTBP2 CLIP-Seq. Related to Figure 5

(A-J) Browser tracks of PTBP2 CLIP-Seq in embryonic day 18 brains overlay the gene structure of PTBP2 targets. Red boxes indicate exons mis-regulated in *Ptbp2*$^{-/-}$ cortical neurons.

Figure S7. Mini Gene analysis of PTBP2-controlled *Clip2* Exon 9 and *Plekha5* Exon 10. Related to Figure 5

(A, E) Diagram of mini gene structures for *Clip2*-E9-pFlareA and *Plekha5*-E10-pFlareA. Blue box indicates the interested exon; bold blue horizontal lines flanking the blue box represent the flanking targeted introns; vertical lines within introns indicate the deletions of PTBP2 binding motifs in mutant minigenes for *Clip2* Exon 9 and *Plekha5* Exon 10. Exon inclusion results in red fluorescence and exon skipping leads to green fluorescence in transfected neurons. (B-D) Expression of WT or mutant *Clip2* Exon 9 mini genes when transfected in primary cortical neurons. (F-H) Expression of WT or mutant *Plekha5* Exon 10 mini genes when transfected in primary cortical neurons. Neurons were transfected at DIV 0 (after plating), fixed and imaged at DIV 2. Images were acquired under the same settings across experiments. Scale bar: 50 μm, applied to all images.

Figure S8. PTBP2 expression rescues axon growth of *Ptbp2*$^{-/-}$ neurons. Related to Figure 6

(A) Immunostaining images of WT and *Ptbp2*$^{-/-}$ (KO) neurons infected with GFP-expressing lentivirus (GFP LV) or GFP- and PTBP2-coexpressing lentivirus (PTBP2 LV). GFP indicates the infected neurons. (B) PTBP2 expression rescue the shortened axon phenotype in *Ptbp2*$^{-/-}$ neurons. The longest protrusion of each neuron was measured by GFP. N = 82 (WT + GFP LV), 82 (KO + GFP LV), and 131 (KO + PTBP2 LV). Neurons were infected at DIV0 and fixed at DIV3. Data are represented as mean ± SD of all neurons from 2 biological replicates (litters). *$p<0.05$, **$p<0.01$, Student's t-test.

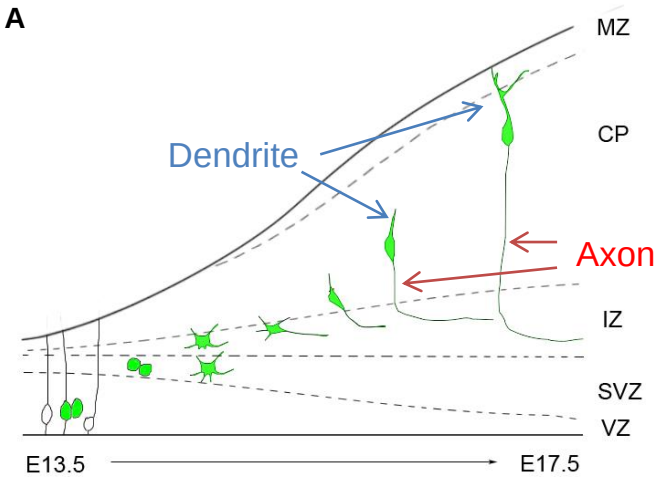
Figure S9. Axonal defects in *Ptbp2*$^{-/-}$ cortices. Related to Figure 7

(A) Representative images of single neurons from E17.5 WT and *Ptbp2*$^{-/-}$ (KO) neocortices after *in utero* electroporation (IUE) with CAG-GFP plasmid at E13.5. Arrows point to primary trailing processes (axons) of the migrating neurons. Scale bar: 20 μm. (B) *Ptbp2*$^{-/-}$ cortical neurons contain a higher percentage of surplus axons than WT neurons. P value = 0.0078, Fisher's exact test. Error bars represent 95% confidence intervals. N = 86 (WT) and 103 (KO) from 2 litters for each genotype. Only clearly traceable neurons were included and primary trailing processes longer than 10 μm were considered. (C-D) Representative images of the reconstructed neurons show normal neuronal migration and dendritic orientation with defective axon morphology in *Ptbp2*$^{-/-}$. CP: cortical plate. IZ: intermediate zone.

Figure S10. Normal neurogenesis, cortical lamination, and neuronal numbers in *Ptbp2*$^{-/-}$ cortices. Related to Figure 7

(A-D) Cortical lamination of (A, C) WT control and (B, D) Emx1-Ptbp2cKO brains shown by layer markers (A, B) SATB2, TBR1, (C, D) CUX1, and CTIP2 at E17.5. A double arrow marks the gap between the cortical plate and the ventricle zone in each case. Scale bars are 50μm. (E) Quantification shows cortical lamination of Emx1-Ptbp2cKO brains is normal. 25-30 slices from 2 brains of each genotype were analyzed. Data are represented as the mean ± SEM from all slices. n.s, $p>0.05$, Student's t-test.

Figure S1



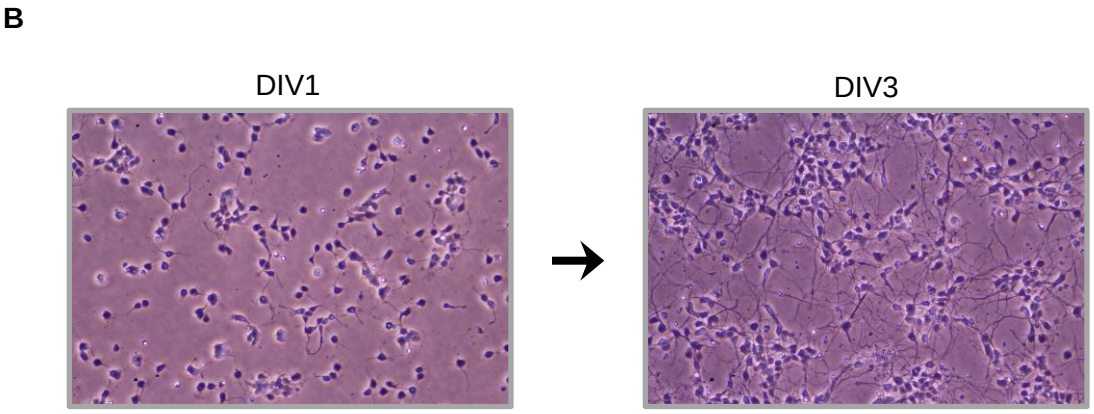
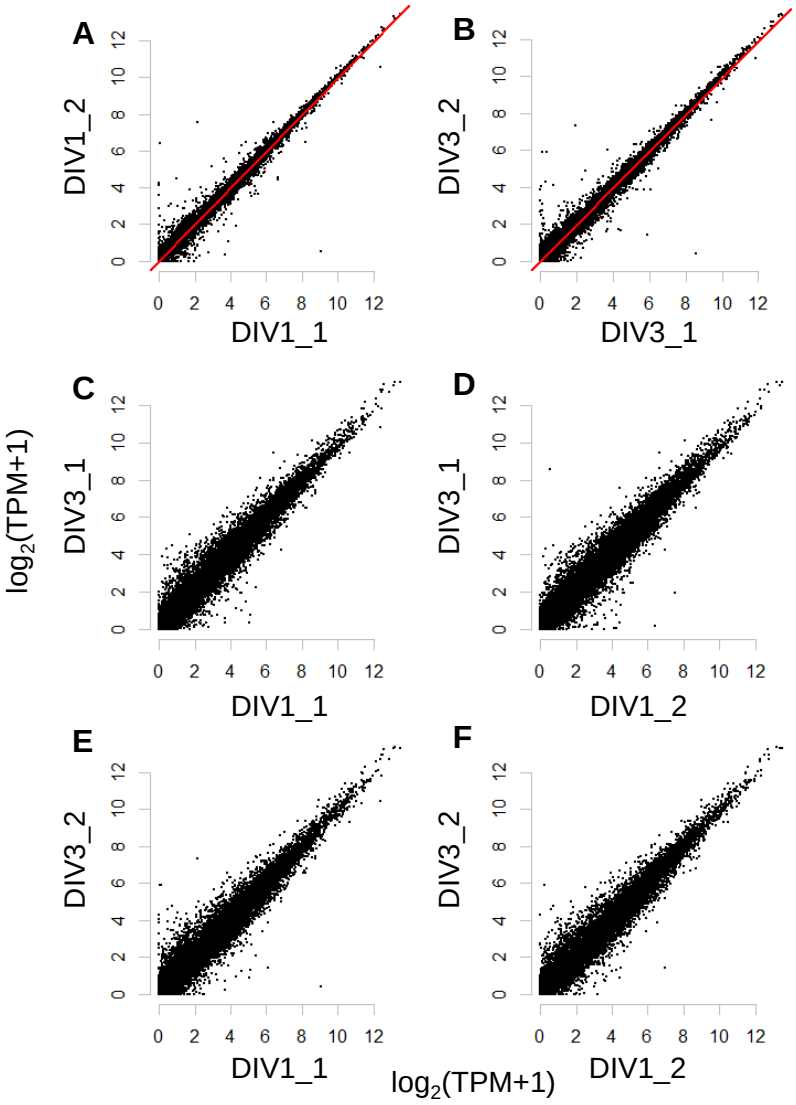
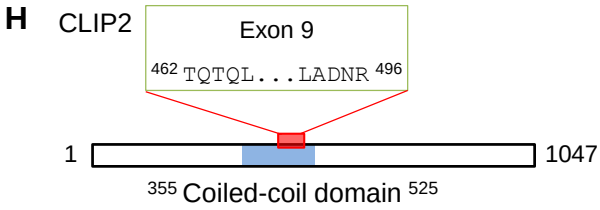
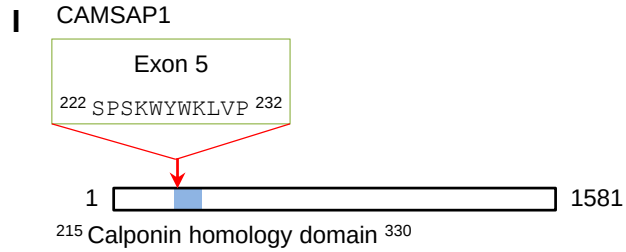
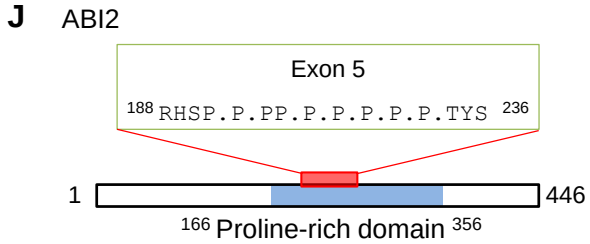


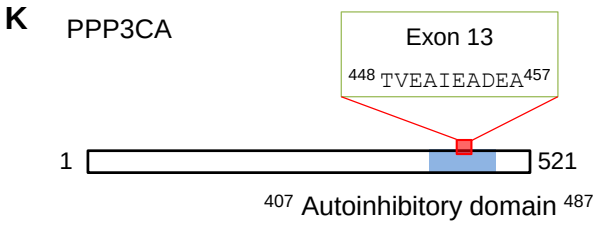
Figure S2


A
DIV1_2
DIV1_1
B
DIV3_2
DIV3_1
C
DIV3_1
DIV1_1
D
DIV3_1
DIV1_2
E
DIV3_2
DIV1_1
F
DIV3_2
DIV1_2
log2(TPM+1)
log2(TPM+1)


H
CLIP2
Exon 9
462 TQTQL...LADNR 496
1
1047
355 Coiled-coil domain 525


I
CAMSAP1
Exon 5
222 SPSKWYWKLVP 232
1
1581
215 Calponin homology domain 330


J
ABI2
Exon 5
188 RHSP.P.PP.P.P.P.P.P.TYS 236
1
446
166 Proline-rich domain 356


K
PPP3CA
Exon 13
448 TVEAIEADEA457
1
521
407 Autoinhibitory domain 487

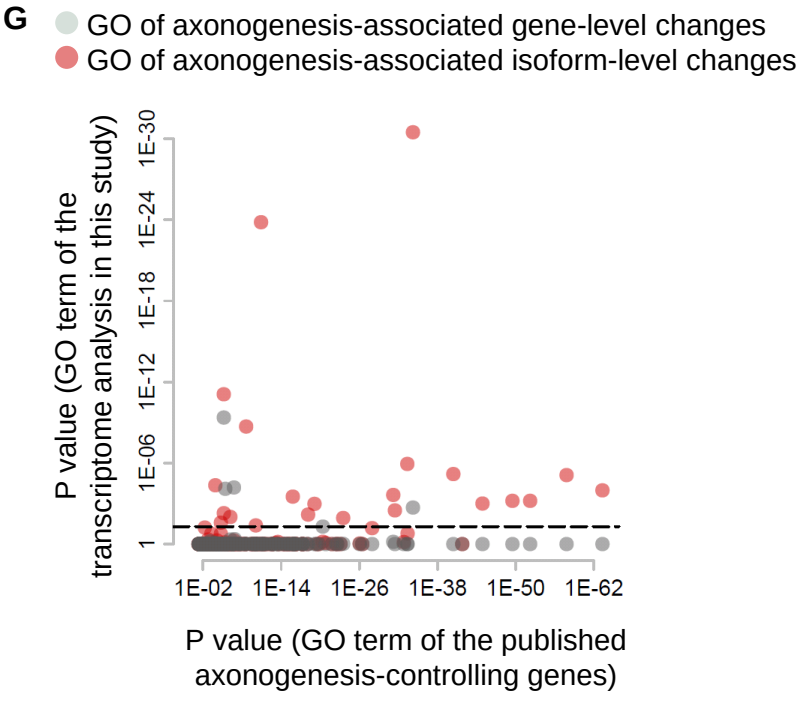

G
GO of axonogenesis-associated gene-level changes
GO of axonogenesis-associated isoform-level changes
P value (GO term of the transcriptome analysis in this study)
1E-30
1E-24
1E-18
1E-12
1E-06
1
1E-02
1E-14
1E-26
1E-38
1E-50
1E-62
P value (GO term of the published axonogenesis-controlling genes)

Figure S3

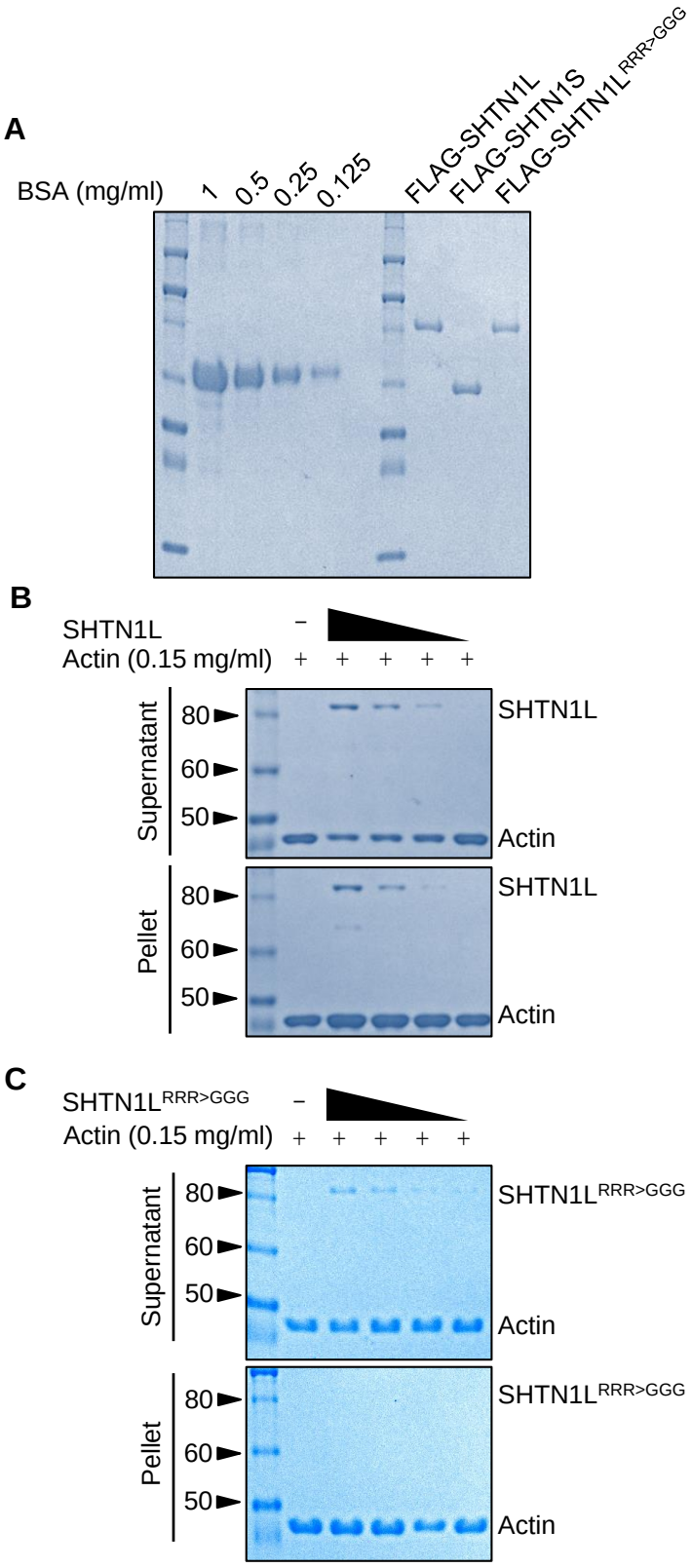
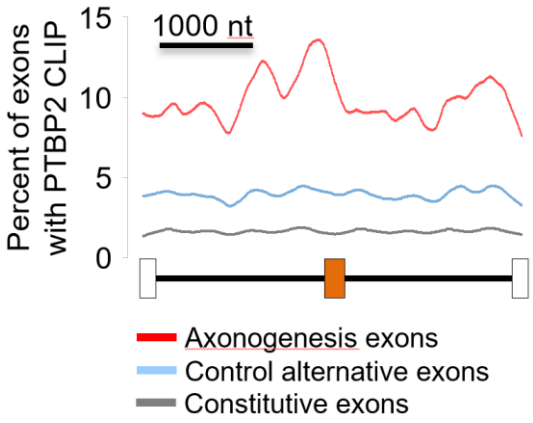

A
BSA (mg/ml)
1
0.5
0.25
0.125
FLAG-SHTN1L
FLAG-SHTN1S
FLAG-SHTN1L RRR>GGG
B
SHTN1L
Actin (0.15 mg/ml)
Supernatant
Pellet
80
60
50
SHTN1L
Actin
C
SHTN1L RRR>GGG
Actin (0.15 mg/ml)
Supernatant
Pellet
80
60
50
SHTN1L RRR>GGG
Actin

Figure S4

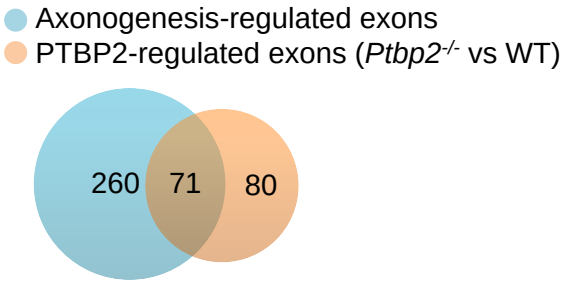
A

Motif	E value	Region
	9.50E-06	2

B



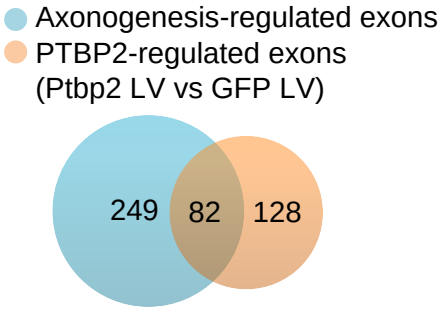
C



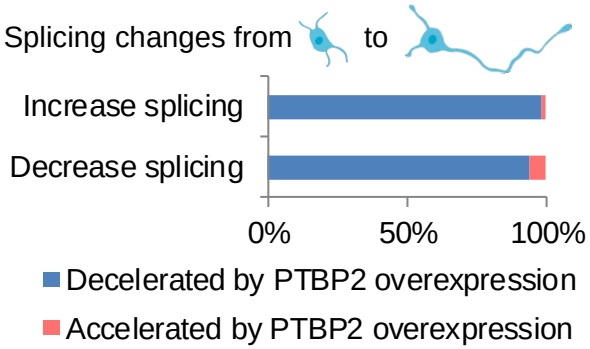
D

Exon number		$PSI_{Ptbp2^{-/-}} - PSI_{WT}$	
		< -10	> 10
$PSI_{DIV3} - PSI_{DIV1}$	< -10	16	3
	> 10	1	51

E



F



G

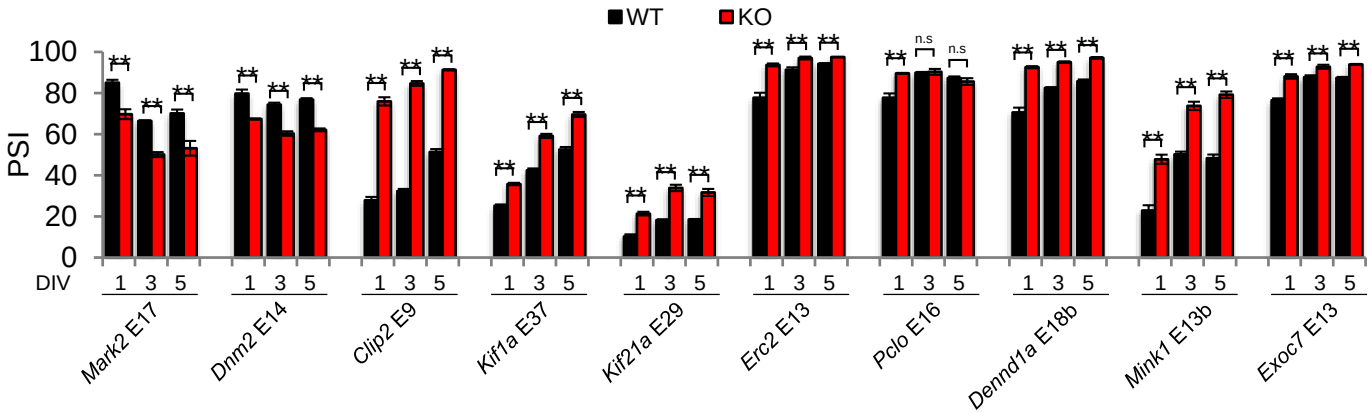


Figure S5

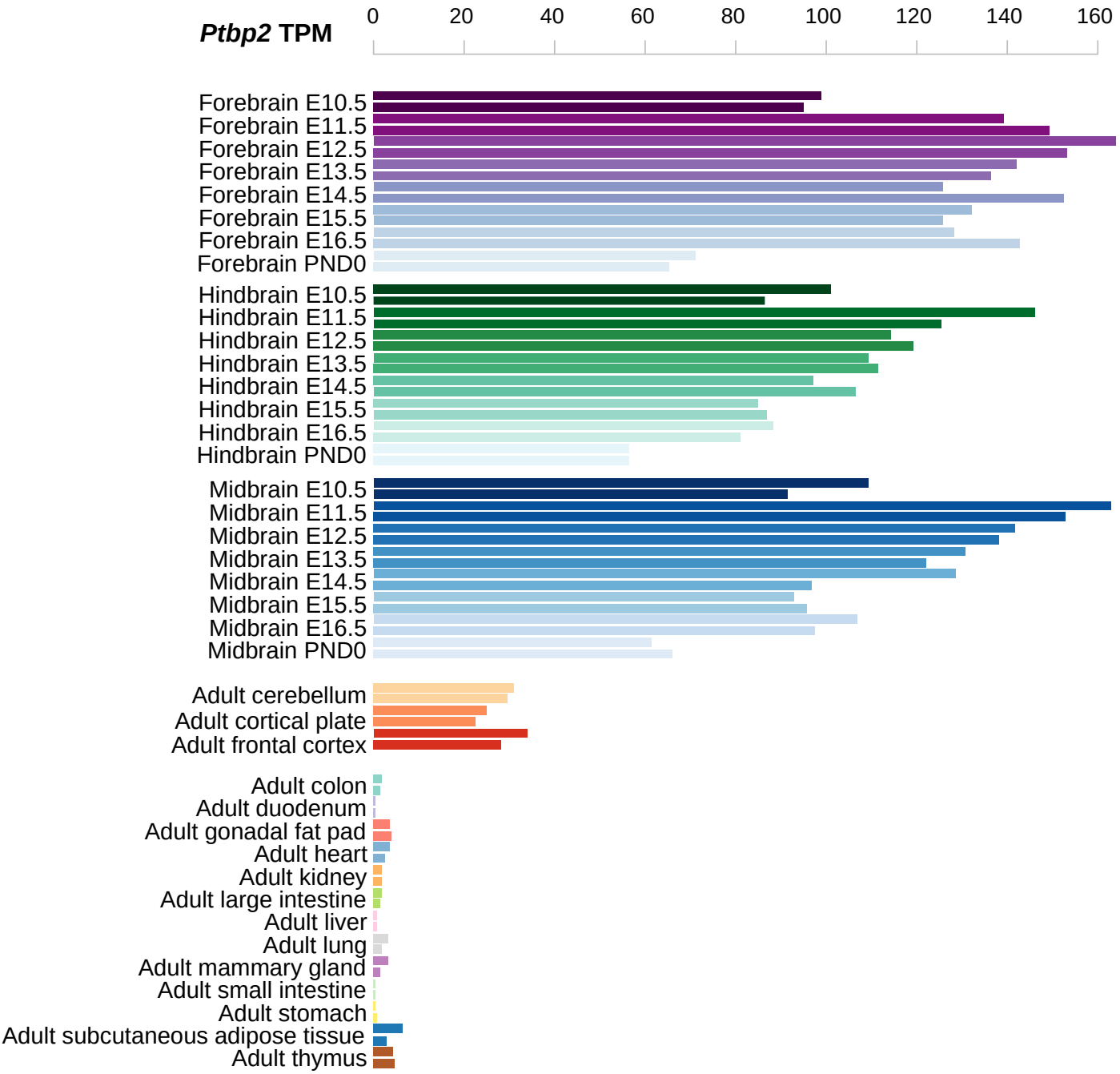

Ptbp2 TPM
0
20
40
60
80
100
120
140
160
Forebrain E10.5
Forebrain E11.5
Forebrain E12.5
Forebrain E13.5
Forebrain E14.5
Forebrain E15.5
Forebrain E16.5
Forebrain PND0
Hindbrain E10.5
Hindbrain E11.5
Hindbrain E12.5
Hindbrain E13.5
Hindbrain E14.5
Hindbrain E15.5
Hindbrain E16.5
Hindbrain PND0
Midbrain E10.5
Midbrain E11.5
Midbrain E12.5
Midbrain E13.5
Midbrain E14.5
Midbrain E15.5
Midbrain E16.5
Midbrain PND0
Adult cerebellum
Adult cortical plate
Adult frontal cortex
Adult colon
Adult duodenum
Adult gonadal fat pad
Adult heart
Adult kidney
Adult large intestine
Adult liver
Adult lung
Adult mammary gland
Adult small intestine
Adult stomach
Adult subcutaneous adipose tissue
Adult thymus

Figure S6

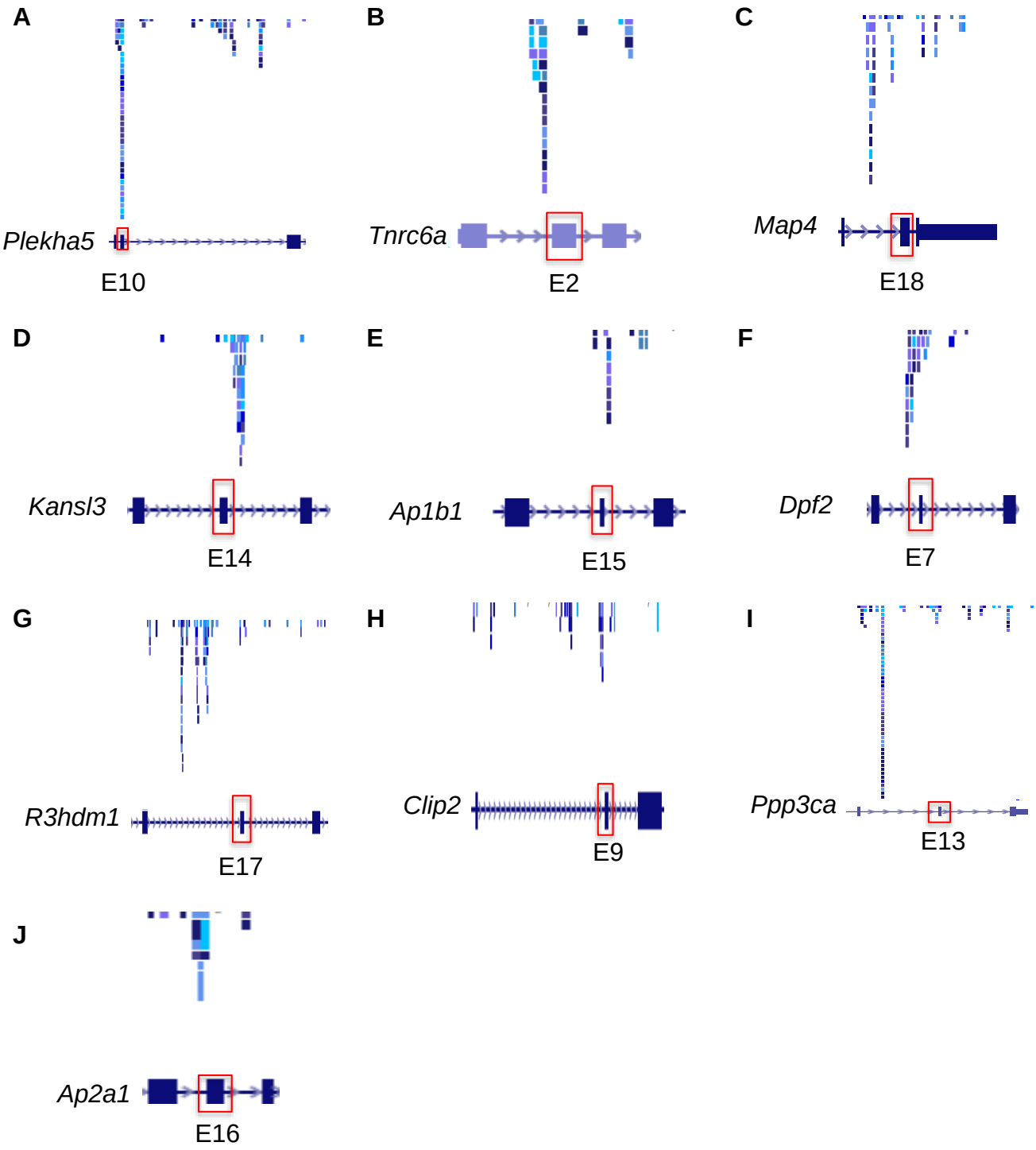
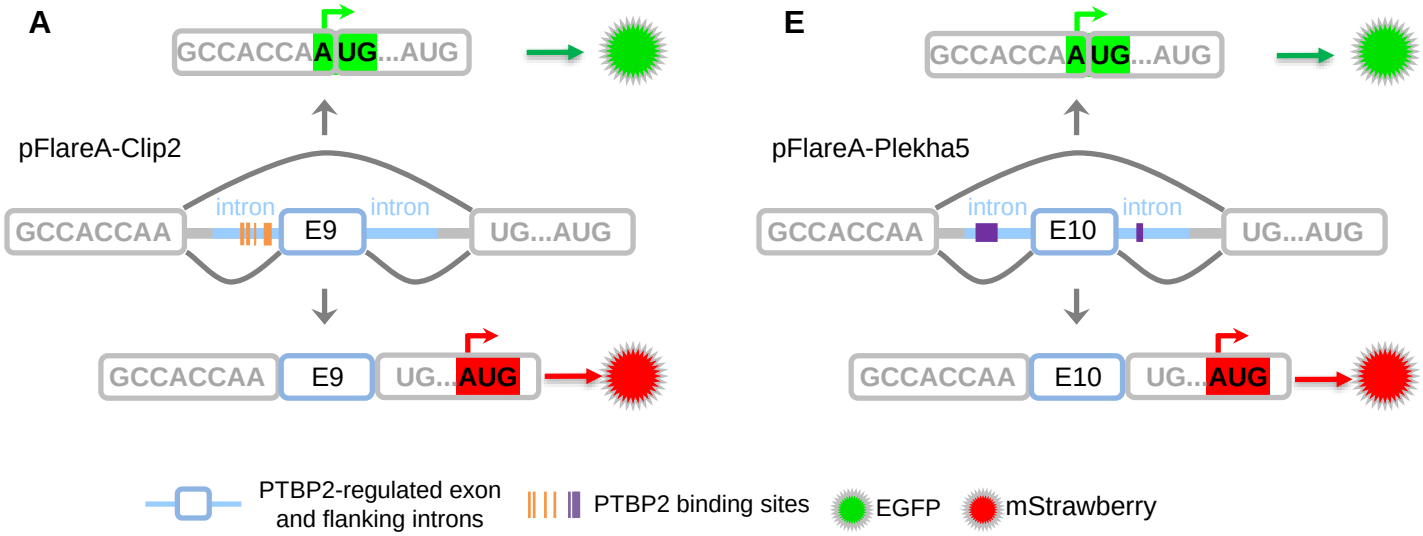
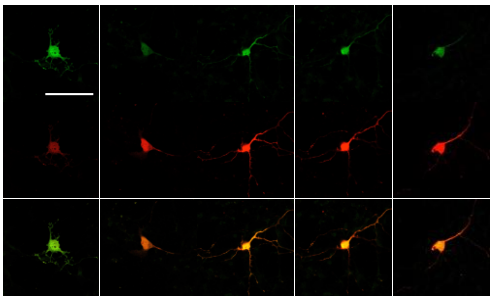

A
Plekha5
E10
B
Tnrc6a
E2
C
Map4
E18
D
Kansl3
E14
E
Ap1b1
E15
F
Dpf2
E7
G
R3hdm1
E17
H
Clip2
E9
I
Ppp3ca
E13
J
Ap2a1
E16

Figure S7



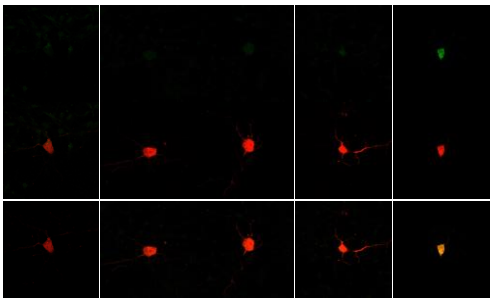
B

Clip2 minigene in WT neurons



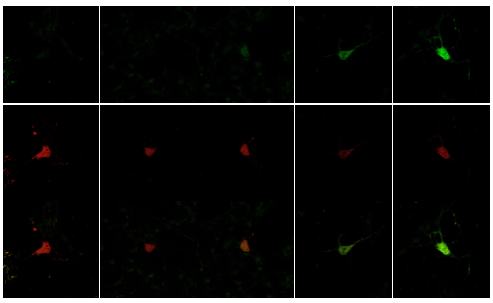
C

Clip2 minigene in *Ptbp2*$^{-/-}$ neurons



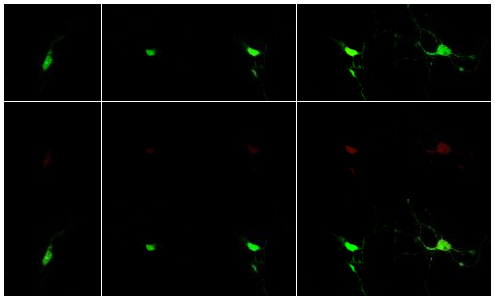
D

Clip2 mutant minigene in WT neurons



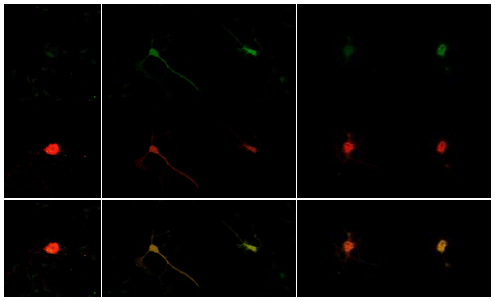
F

Plekha5 minigene in WT neurons



G

Plekha5 minigene in *Ptbp2*$^{-/-}$ neurons



H

Plekha5 mutant minigene in WT neurons

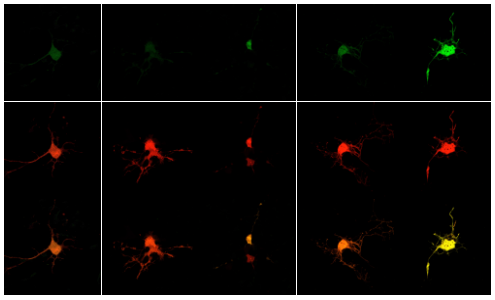


Figure S8

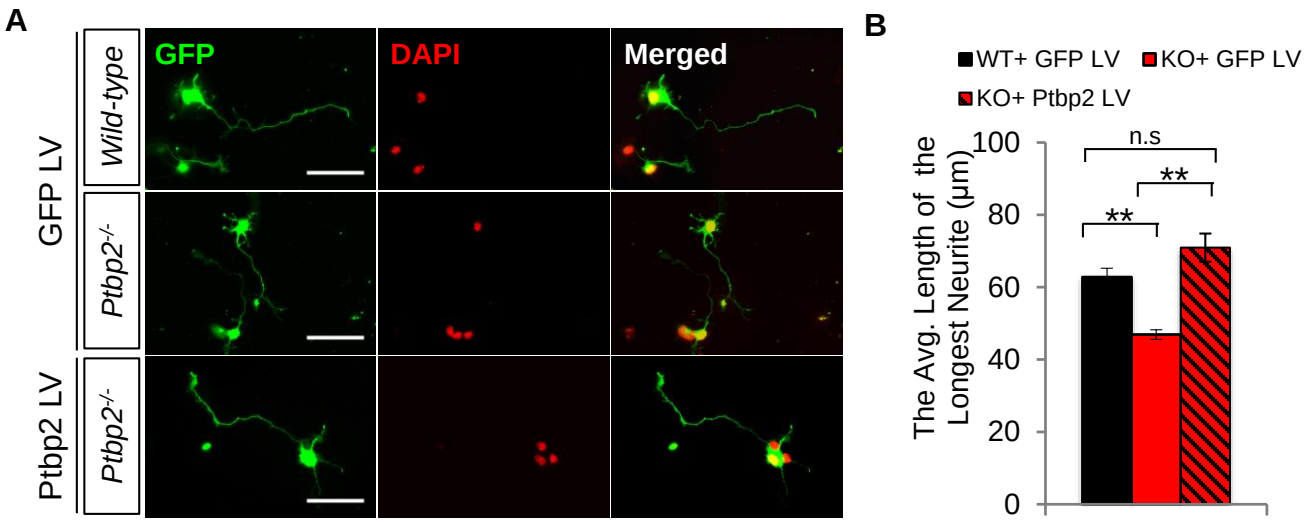

A
GFP LV
Ptbp2 LV
Wild-type
Ptbp2-/-
Ptbp2-/-
GFP
DAPI
Merged
B
WT+ GFP LV
KO+ GFP LV
KO+ Ptbp2 LV
The Avg. Length of the Longest Neurite (μm)
100
80
60
40
20
0
n.s
**
**

Figure S9

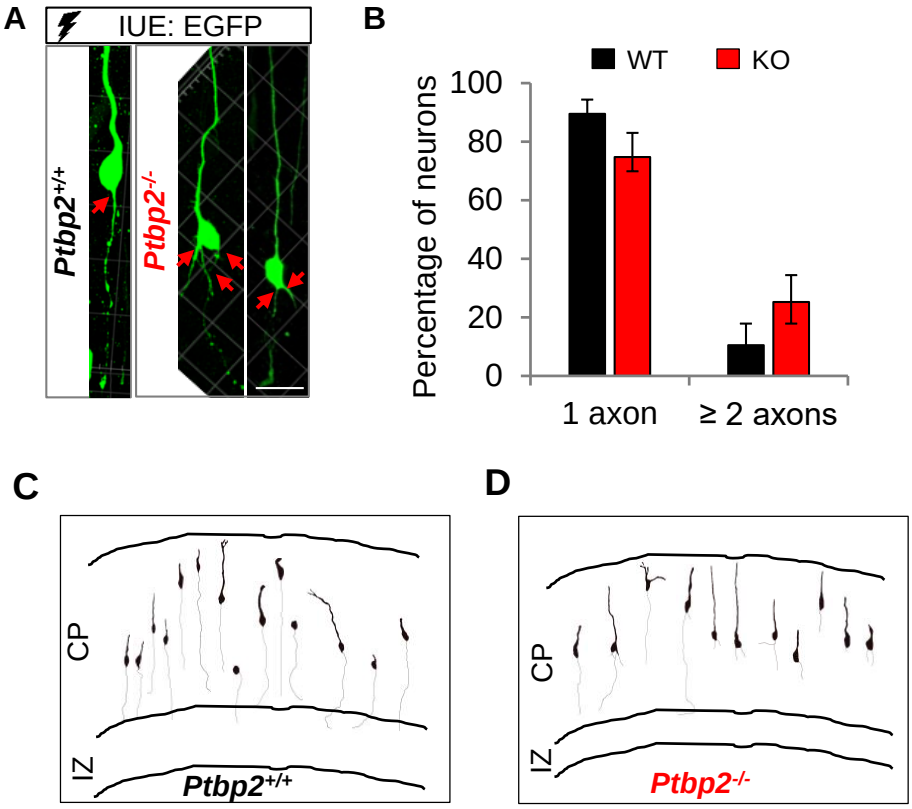
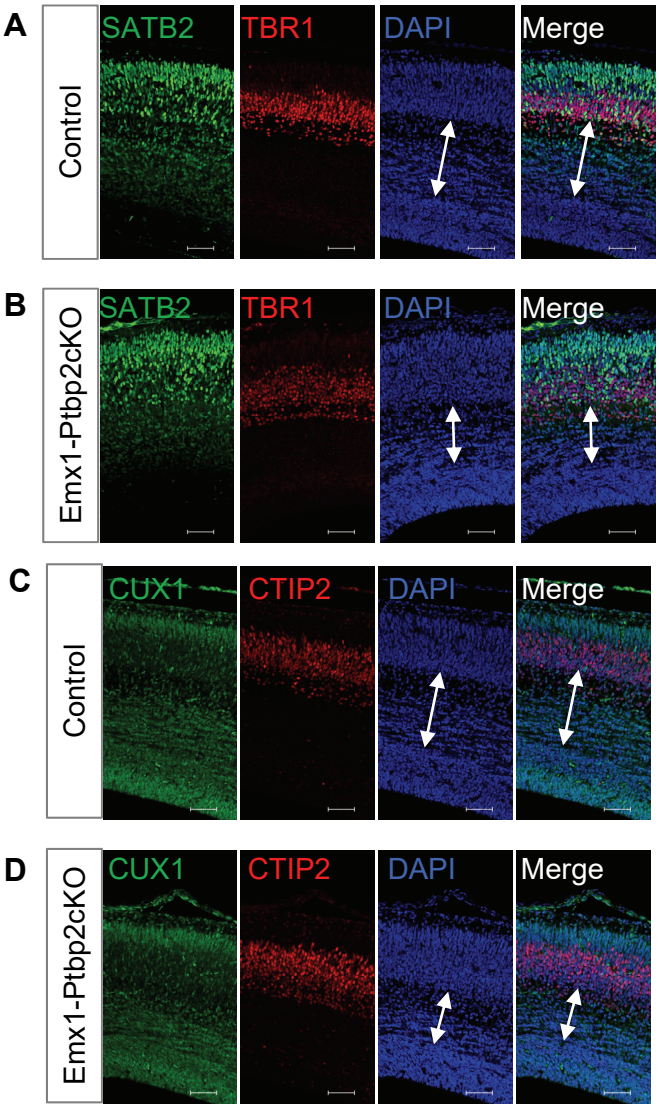
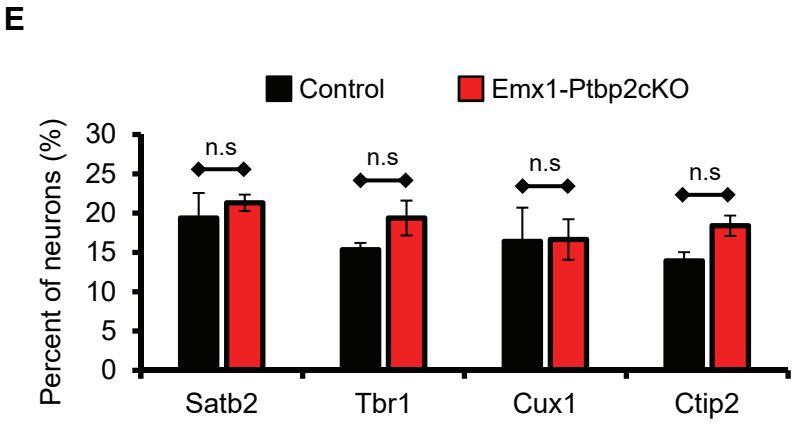

A
IUE: EGFP
Ptbp2+/+
Ptbp2-/-
B
WT
KO
Percentage of neurons
100
80
60
40
20
0
1 axon
≥ 2 axons
C
CP
IZ
Ptbp2+/+
D
CP
IZ
Ptbp2-/-

Figure S10


A
Control
SATB2
TBR1
DAPI
Merge
B
Emx1-Ptbp2cKO
SATB2
TBR1
DAPI
Merge
C
Control
CUX1
CTIP2
DAPI
Merge
D
Emx1-Ptbp2cKO
CUX1
CTIP2
DAPI
Merge


E
Control
Emx1-Ptbp2cKO
Percent of neurons (%)
30
25
20
15
10
5
0
n.s
n.s
n.s
n.s
Satb2
Tbr1
Cux1
Ctip2