Table S3: Related to Figure 1. Systematic analysis of transcriptomic changes between Layer $5^{wildtype}$ (L5 WT) vs. Layer $6^{wildtype}$ (L6 WT) identified 35 DEX genes, denoted as layer 5/6 DEX genes. Comparing layer 5/6 DEX genes to the DEX genes identified from *Tbr1*^{layer6} homozygous mutant (L6 Null) vs. Layer $6^{wildtype}$ (L6WT) revealed that 60% of genes showed layer 5-like transcriptomic profile, with the remaining 40% resembling layer 6-like expression profile. Upregulated (red) and downregulated (blue) genes are shown. FDR=False Discovery Rate

	L6 Null vs. L6 WT		Layer 5/6 DEX Genes	L5 WT vs. L6 WT	
	Log_2FC	FDR		Log_2FC	FDR
Genes with Layer 5-like Transcriptomic Profile in *Tbr1*^{layer6} Mutant	6.737310931	4.97E-06	*Hist1h2ba*	2.807164923	0.00055482
	1.424969748	3.01E-05	*Nrgn*	1.621444668	5.22E-09
	1.432850639	0.006055173	*Lypd1*	2.472478419	4.30E-08
	1.357252354	0.021315518	*Thrb*	2.184771654	1.17E-08
	0.624247989	0.001197141	*Dio2*	2.220811689	8.94E-09
	0.539736836	0.004363945	*Slc1a2*	2.70091325	1.84E-10
	0.409638798	0.001461498	*Slc1a3*	1.781181721	3.25E-08
	0.357083033	0.003621591	*Rab6b*	1.699729354	7.14E-08
	-0.304630679	0.008111647	*Tmem132a*	-1.892074985	1.33E-09
	-0.370212227	0.002302499	*Cntn2*	-4.037806798	1.84E-10
	-0.384313076	0.008225695	*Neurod6*	-2.364077368	2.24E-09
	-0.39326935	0.018765766	*Igfbpl1*	-4.483958943	1.84E-10
	-0.4374153	0.032619571	*Arrdc3*	-1.56712654	1.20E-08
	-0.788856814	0.004070628	*Nr4a2*	-2.713989807	1.57E-09
	-0.964631245	6.63E-05	*Tbr1*	-1.830409679	6.18E-09
	-1.158695777	3.01E-05	*Tle4*	-1.502403009	2.68E-09
	-1.306658068	0.002083987	*Pappa2*	-1.962391615	4.97E-05
	-1.347941779	3.01E-05	*Cwc22*	-2.029674747	3.26E-09
	-1.456893237	0.005477162	*Gdf10*	-1.756525649	8.63E-05
	-1.492895413	0.001197141	*Nfe2l3*	-2.601836747	3.73E-08
	-1.955887421	0.00111894	*Ngfr*	-2.6711269	8.53E-07
Genes with Layer 6-like Transcriptomic Profile in *Tbr1*^{layer6} Mutant	1.42892783	0.009485133	*Trhr*	-1.898305294	0.021285003
	1.113802644	0.007109418	*Fst*	-3.369416755	2.21E-07
	0.987710061	0.015978998	*Flrt3*	-1.657309888	6.85E-08
	0.637557225	0.016960976	*Dusp4*	-2.97016098	1.06E-08
	0.597053976	0.025684283	*Neurod1*	-2.174024707	7.71E-08
	0.500622213	0.002749805	*Dcc*	-1.792335786	2.73E-09
	0.409419236	0.008251616	*Nrp2*	-2.270825159	2.77E-09
	0.393980357	0.003165434	*Sez6*	-2.172856327	2.68E-10
	0.282732079	0.042277293	*Lrrn3*	-1.900328258	2.24E-09
	0.213373288	0.019803984	*Nrp1*	-2.731092717	8.01E-10
	-0.884539393	0.00102823	*Rorb*	1.614978724	2.77E-08
	-0.571829565	0.001310348	*Prkcb*	2.263609306	1.98E-10
	-0.456131889	0.017122861	*Gnal*	1.994550669	8.70E-10
	-0.408460371	0.000737954	*Adcy1*	1.638115766	1.98E-10

Table S4: Related to Figure 2. Complete list of DNA FASTA sequences that were cloned to generate DIG-labeled RNA probes for ISH experiments.

Recombinant DNA

>*Tbr1*

CACTCGCTCTTTCACTTGACCCTCGATGACCGTCTGCGGGGATAAGTGCAGGTCTCTCACTATGATTTTAAAACTCTTCTTTTTTCTTTCTTTCTTTCTCTCTACA
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>*Nr4a2*

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>*Foxp2*

CATCTGCTCAGCCTTCAGCGCCAGGGCCTCATCTCCATCCCACCCGGCCAAGCAGCCCTTCCTGTCCAGTCGCTGCCTCAAGCTGGCTTAAGTCCTGCTGAGATTC
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>*Tle4*

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>*Wnt7b*

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>*Bcl11a*

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>*Bcl11b*

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CTTCAGCGTCTACAGCACCCTGGAGAAACACATGAAAAAGTGGCACGGTGAACACTTGCTGACTAATGATGTCAAAATCGAGCAGGCTGAGAGGA

>*Fezf2*

GCAAGGTGTTCAATGCTCACTATAACCTCACCCGCCACATGCCTGTCCACACCGGAGCTAGACCGTTTGTGTGCAAAGTCTGTGGCAAAGGCTTCCGCCAGGCCAG
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>*Foxp1*

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>*Sst*

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Table S5: Related to Figure 3. Correlation between downregulated (red), upregulated (green) and unchanged (black) genes in *Tbr1*^{layer6} mutant and TBR1genomic distribution on the promoter and distal regions of those genes. The changes in the transcript levels are derived from FACS purified RNA-Seq layer 6 neurons of *Tbr1*^{layer6} mutant and compared to *Tbr1*^{wildtype} at P5. TBR1 ChIP-Seq from whole wildtype cortex at P2 was used to investigate TBR1 genome-wide binding. TBR1 canonical motif sequence was used to examine whether the presence or absence of a TBR1 motif results in the dysregulation of the target gene. ([a]) indicates the transcript levels that were measured using qPCR. Ambiguous base pairs: W=A/T, H=A/C/T.

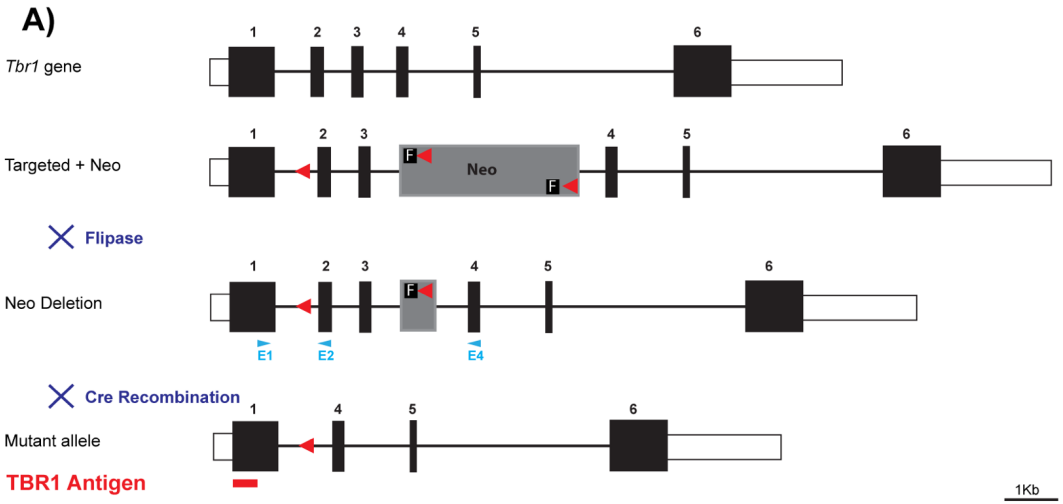
Gene	Change in Transcript Levels	# of TBR1 Peaks		# of TBR1 motifs		TBR1 Canonical Motif 
		Promoter	Distal	Promoter	Distal	
Tbr1	0.512	1	2	1	10	WTTTCACACHTH
Nr4a2	0.579	1	3	2	5	WTTTCACACHTH
Tle4	0.448	1	19	-	8	WTTTCACACHTH
Wnt7b	0.410	1	12	1	10	WTTTCACACHTH
Drd1	0.104	1	7	-	2	WTTTCACACHTH
Mc4r	0.124	1	12	-	5	WTTTCACACHTH
Hs3st5	0.239	1	5	-	3	WTTTCACACHTH
Bcl6	0.249	1	3	1	-	WTTTCACACHTH
Ngfr	0.258	1	4	-	2	WTTTCACACHTH
Nfe2l3	0.355	1	2	-	3	WTTTCACACHTH
Bcl11a	0.454	1	19	2	3	WTTTCACACHTH
Foxp1	1.412	1	21	4	7	WTTTCACACHTH
Fezf2	1.705	1	3	-	3	WTTTCACACHTH
Bcl11b	1.42[a]	1	10	-	4	WTTTCACACHTH
Nefl	3.094	1	2	-	1	WTTTCACACHTH
Ntng1	2.910	1	6	2	2	WTTTCACACHTH
Vwc2l	2.775	1	7	1	2	WTTTCACACHTH
Nrgn	2.685	1	2	-	-	WTTTCACACHTH
Thrb	2.562	1	10	-	5	WTTTCACACHTH
Zfp521	2.167	1	12	-	7	WTTTCACACHTH
Nefm	2.362	1	3	-	1	WTTTCACACHTH
Fgf9	2.554	1	9	-	4	WTTTCACACHTH
Dlx5	No Change	1	-	-	-	-
Sst	No Change	-	4	-	-	-
Sox2	No Change	1	-	-	-	-
Otx1	No Change	1	3	-	-	-
Gnb1	No Change	1	2	-	-	-
Gpd1l	No Change	1	2	-	-	-
Szt2	No Change	1	1	-	-	-
Chmp1a	No Change	1	1	-	-	-
Col1a1	No Change	1	4	-	-	-
Slc35a4	No Change	1	-	-	-	-

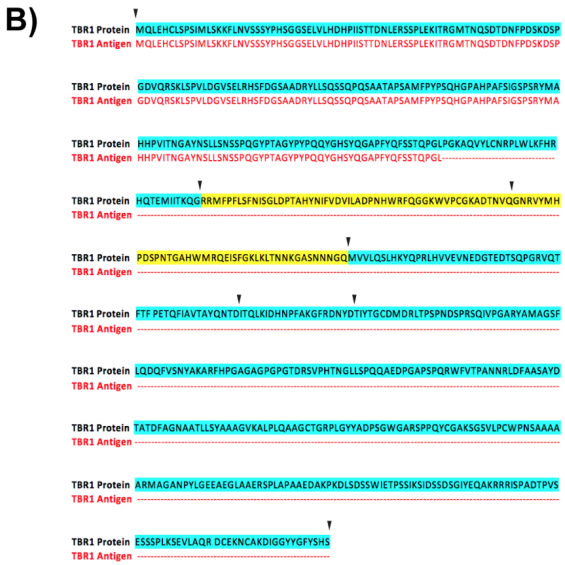
Table S6: Related to Figure 3. List and genomic location of putative regulatory elements (REs) that were utilized in luciferase transcription assay. REs were selected from downregulated (red), upregulated (green) and unchanged (black) genes. Transcript levels were deduced from RNA-Seq and qPCR (a) at α=0.05. Changes in transcript levels are shown in Log_{10}FC. Each RE had at least one degenerate form of TBR1 canonical motif. The motif sequence and location of the motif is shown.

Gene	Transcript Levels in Mutant	TBR1 Peak Location	TBR1 Canonical Motif 	TBR1 Motif Location
Tbr1	-1.952	chr2: 61494203-61494886	TCACAGAT	chr2:61,494,590-61,494,604
Foxp2	-1.422	chr6: 15097236-15098146	TCACAGCCAT	chr6: 15097865-15097875
Grin2b	-0.28[a]	chr6: 135813640-135814770	TCACAGAT	chr6: 135814230-135814238
Bcl11a	-2.2[a]	chr11: 24270818-24271924	TAACACCT	chr11: 24271476-24271484
Foxp1	1.021	chr6: 99325484-99327361	TAACACTT	chr6: 99325950-99325958
Fezf2	1.705	chr14: 13170235-13171693	TGACAGTT	chr14: 13170709-13170717
Hcn1	2.2[a]	chr13: 118669041-118670541	TCACAGTA	chr13: 118670417-118670424
Dlx5	Not expressed in layer 6 neurons	chr6: 6819420-6819819	Not found	N/A

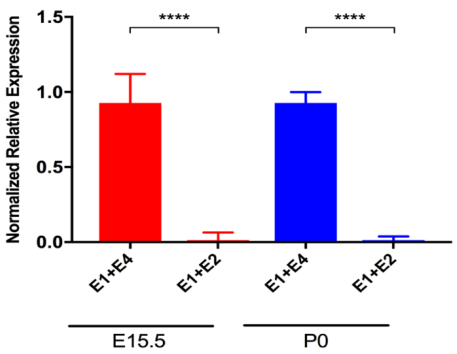
[a] Transcript levels were measured using qPCR

Figure S1. Related to Figure 1. Organization of the *Tbr1* wildtype and conditional mutant (*Tbr1^{f}*) alleles.
(A) Schematic representations of the *Tbr1* wildtype allele, *Tbr1* targeting vector, and *Tbr1* conditional mutant allele (*Tbr1^{f}*). The wildtype *Tbr1* allele has six known exons (numbered black boxes, 1-6); the initiation codon is in exon 1, and the termination codon is in exon 6. The white boxes indicate the 5′ and 3′ UTRs. Red arrowheads correspond to the location of LoxP sites; the black boxes with an F inside are Frt sites. Flipase removes the Neomycin expression cassette (grey box with Neo inside). Upon *Cre* recombination, exons 2 and 3 are deleted to generate *Tbr1* mutant allele. The positions of the qPCR primers used for genotyping are indicated with blue arrowheads under exons 1, 2 and 4. The location of TBR1 antigen that was detected with antibodies for western blotting (panel E) and for immunohistochemistry (panel F) is indicated by the red line under part of exon 1. **(B)** Amino acid alignment of full-length TBR1 protein (black) with TBR1 antigen (red). Black arrowheads represent exonic boundaries. Region highlighted in yellow indicates the amino acid sequence corresponding to the deleted region in the *Tbr1* mutant allele. **(C)** RT-qPCR results corresponding to relative expression levels of *Tbr1* exons 1 and 2 (E1+E2) as well as exons 1 and 4 (E1+E4) transcripts in the cortex of *Tbr1* constitutive null and wildtype littermates at E15.5 and P0. Gene expression levels were analyzed using two biological replicates, each assayed in experimental triplicates. The error bars represent the standard error of the mean of all replicates; gene expression is normalized relative to a housekeeping gene (*Ef1α*). Relative expression levels in wildtype mice were defined as 1.0 (*P <0.05) (**P < 0.01) (***P<0.001). **(D)** *In situ* hybridization (ISH) on coronal sections of P0 brain from wildtype and *Tbr1* constitutive null. *Tbr1* full-length (FL) probe and *Tbr1* probe corresponding to exons 2 and 3 (E2-3) were used during the hybridization step. Layer 6 is labeled as VI. Scale bar = 100 μm. **(E)** Western blot (WB) to detect TBR1 protein isolated from E15.5 and P0 cortex of a *Tbr1* constitutive null and wildtype littermate. Black arrowhead indicates the TBR1 protein (~75 kDa) detected in wildtype at E15.5 and P0. **(F)** Immunohistochemistry (IHC) against TBR1 on coronal sections of P0 brain from *Tbr1^{wildtype}* and *Tbr1^{layer6}* homozygous mutant. TBR1 protein levels were reduced ~90% in *Tbr1^{layer6}* homozygous mutant neurons. Scale bar = 50 μm.

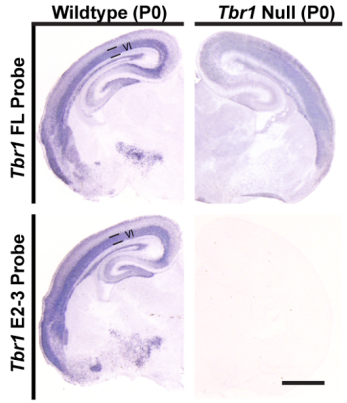




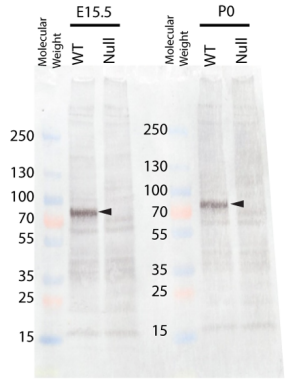
C) qPCR



D) ISH



E) WB



F) IHC

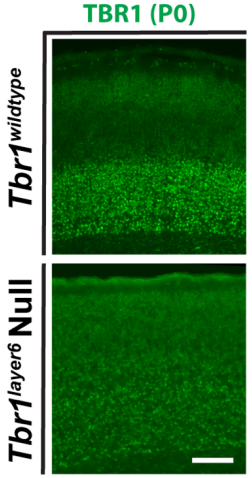
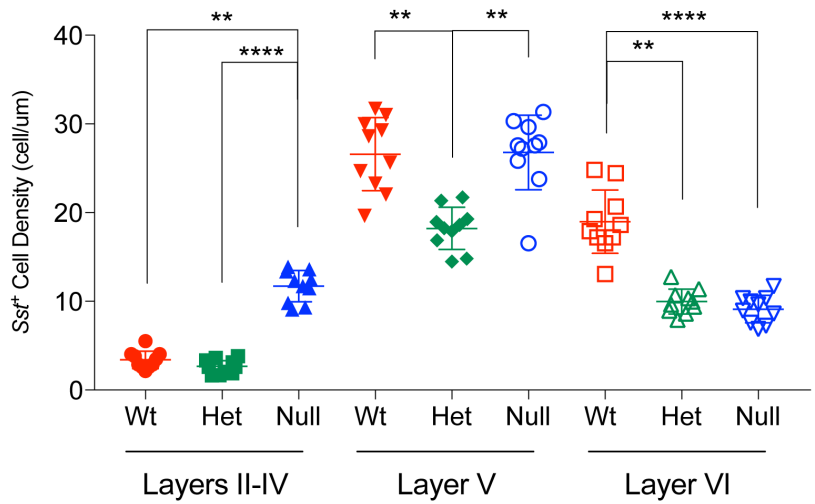


Figure S2: Related to Figure 2. *Tbr1*^{layer6} mutants have altered cortical interneuron number and lamination.
ImageJ software was used to count cell density of **(A)** *Sst*$^{+}$ and **(B)** *PV*$^{+}$ CINs from SSCx of *Tbr1*^{wildtype} (red), *Tbr1*^{layer6} heterozygous (green) and *Tbr1*^{layer6} homozygous mutants (red). *Sst*$^{+}$ CINs were labeled via *in situ* hybridization using a DIG-labeled RNA probe against *Sst* at P3. *PV*$^{+}$ CINs were labeled via immunohistochemistry using anti-parvalbumin antibody at P21. Two-tailed T-test with Tukey correction was used for pairwise comparisons. (*p<0.05) (**p< 0.01) (***p<0.001) (****p<0.0001). Cortical layers are labeled. II/IV = layers 2-4, V = layer 5, and VI = layer 6.

A) *Sst*⁺ CINs



B) *PV*⁺ CINs

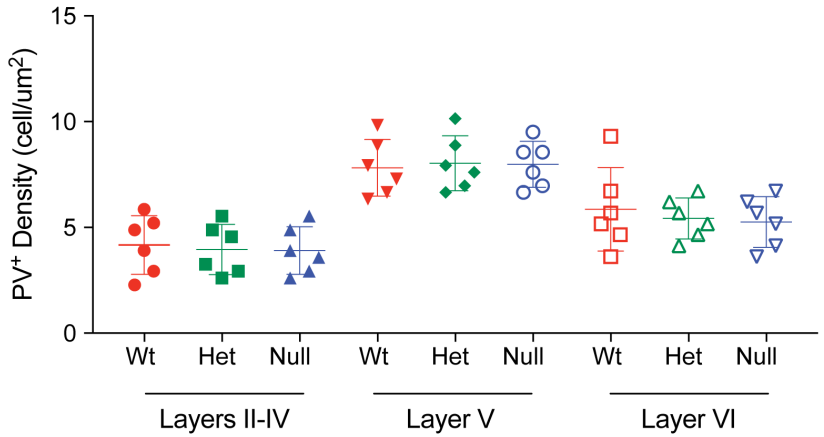
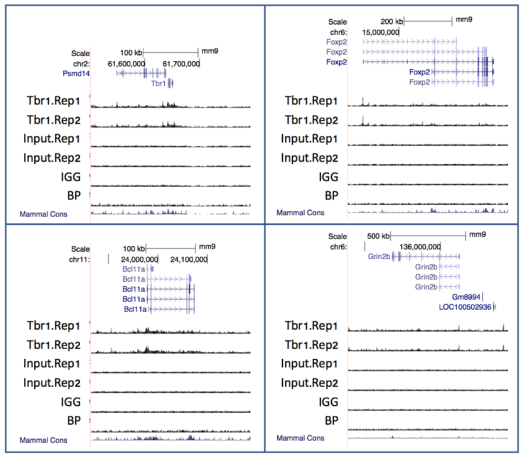
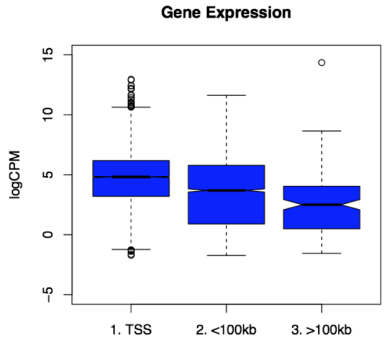


Figure S3: Related to Figure 3. TBR1 Genomic Binding. **(A)** ChIP-seq signal for both TBR1 antibody IP replicates, input controls, and negative controls (blocking peptide (BP) and IGG). **(B)** Relationship between TSS and distal binding and target gene expression (logCounts Per Million (logCPM)), stratified for genes with TSS binding, nearest distal binding within 100kb, and nearest distal binding greater than 100kb. **(C)** Relationship between TSS and distal binding and differential gene expression (Log_2Fold change in WT vs KO (Log_2FC)), stratified for genes with TSS binding, nearest distal binding within 100kb, and nearest distal binding greater than 100kb. **(D)** Proportion of TBR1-bound ATAC-seq peaks from fetal human cortex were compared to a control human cardiac mesoderm cell line ATAC-seq dataset. Y-axis shows fold enrichment over control. X-axis shows ATAC-seq peaks separated by TSS and distal and by sub-class for human fetal cerebral cortex reginal specificity. **(E)** Example loci that show concordant TBR1 binding and human fetal cortex ATAC-seq peaks. TSS=Transcriptional Start Site, GZ=Germinal Zone, CP=Cortical Plate. Statistical comparisons of the number of TBR1 bound peaks compared using Fisher's Exact Test ($p < 0.001$).

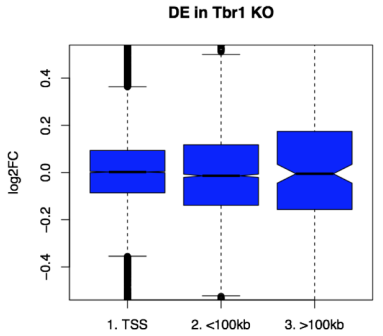
A) TBR1 ChIP-Seq



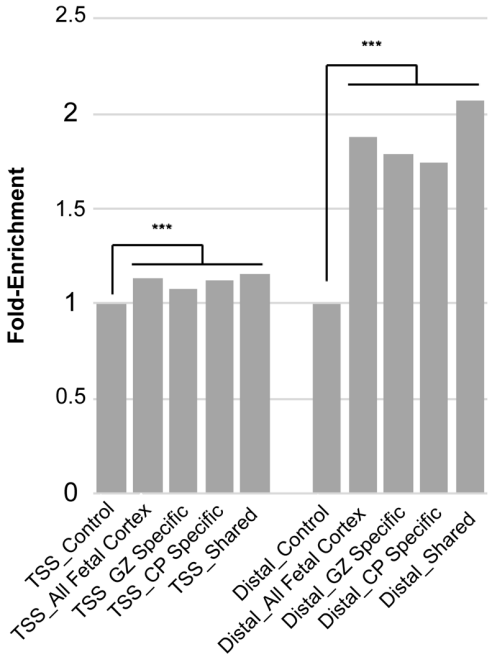
B) TBR1 Genomic Distribution



C) TBR1 ChIP-Seq and RNA-Seq Correlation



D) Enrichment of TBR1 Binding Sites for Orthologous Human Fetal ATAC-seq Peaks



E) Concordant TBR1 Peak with Human Fetal ATAC-seq

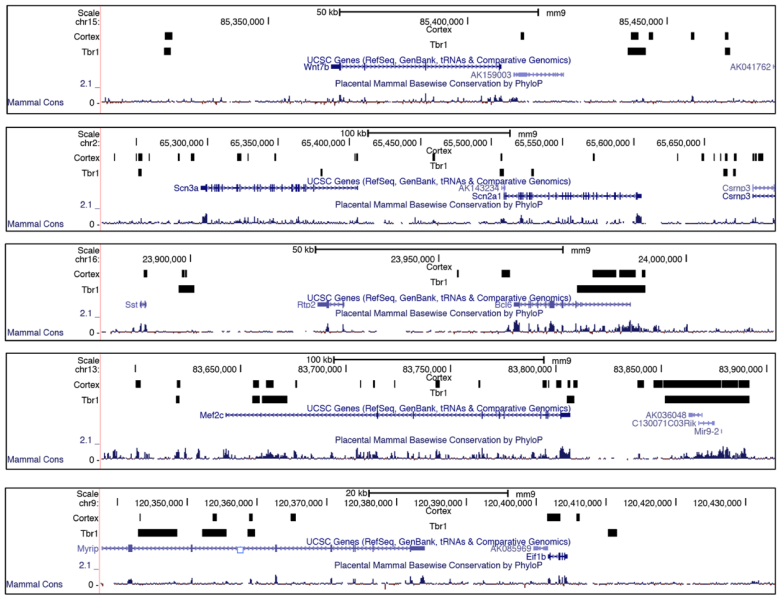


Figure S4: Related to Figure 4. tdTomato expression in layer 6 neurons and axons shows reduced corticothalamic innervation in *Tbr1*^{layer6} mutants at P3 and P21.

(A, B) Rostro-caudal coronal section series shows tdTomato in *Tbr1*$^{\text{wildtype}}$ and *Tbr1*^{layer6} mutants at P3 **(A)** and P21 **(B)**. These lines had the *Ntsr1-cre::tdTomato*$^{f/+}$ alleles to label the layer 6 neurons and their axons by tdTomato's endogenous fluorescence (red). The overlap between DAPI (blue) and tdTomato is shown. White arrowheads in panel 5 (at P3 and P21) correspond to the medial structures in the *Tbr1*^{layer6} mutant thalamus that show reduced corticothalamic projections. **(C)** Schema demonstrating the five different regions (Regions 1-5) that were chosen to quantify the changes in pixel density between *Tbr1*^{wildtype} and *Tbr1*^{layer6} mutants; corresponding to changes in corticothalamic axonal projections between the two genotypes **(D)** Quantification of the tdTomato pixel intensity in thalamic regions 1-5 of *Tbr1*^{wildtype} (red) and *Tbr1*^{layer6} homozygous mutant (blue) at P21. Two-tailed T-test with tukey correction was used for pairwise comparisons. (**p< 0.01) (***p<0.001) (****p<0.0001). Scale: 100 μm in **(A)** and 50 μm in **(B)**.

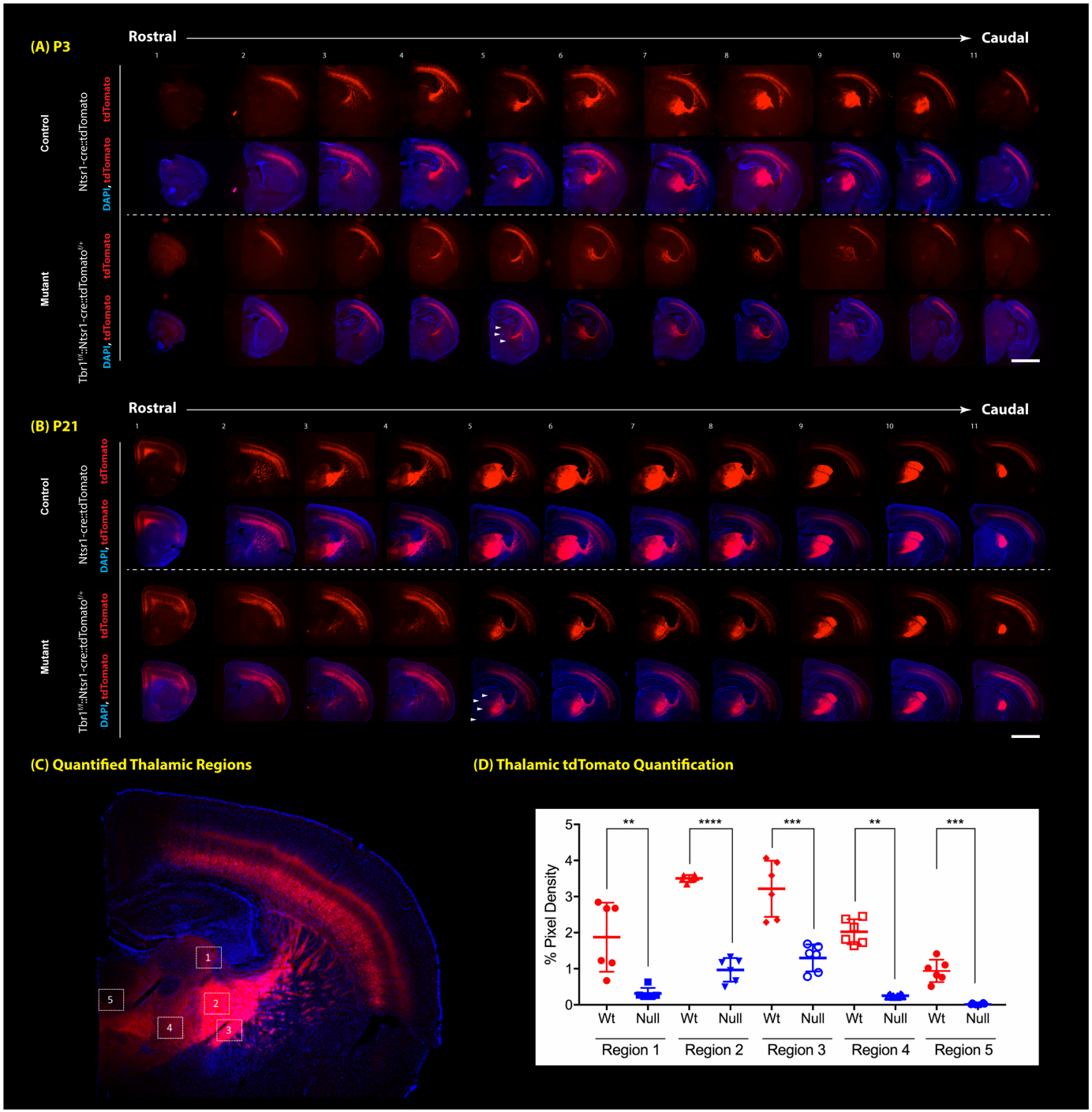

(A) P3
Rostral
Caudal
1
2
3
4
5
6
7
8
9
10
11
Control
Ntsr1-cre::tdTomato
tdTomato
DAPI, tdTomato
Mutant
Tbr1fl/+::Ntsr1-cre::tdTomatofl/+
tdTomato
DAPI, tdTomato
(B) P21
Rostral
Caudal
Control
Ntsr1-cre::tdTomato
tdTomato
DAPI, tdTomato
Mutant
Tbr1fl/+::Ntsr1-cre::tdTomatofl/+
tdTomato
DAPI, tdTomato
(C) Quantified Thalamic Regions
1
2
3
4
5
(D) Thalamic tdTomato Quantification
% Pixel Density
0
1
2
3
4
5
**
****
***
**
***
Wt
Null
Region 1
Region 2
Region 3
Region 4
Region 5

Figure S5: Related to Figure 5. Loss of *Tbr1* in layer 6 reduces excitatory (i) and inhibitory (ii) synaptic input onto the layer 6 pyramidal neurons in the somatosensory cortex at P21.

(A) Schematic representation of somatosensory cortex (SSCx, blue boxes). The blue box represents the region of SSCx utilized for imaging and whole-cell patch clamp experiments. **(B, C)** Schema of layer 6 projection neuron (red) in SSCx of *Tbr1* ^{wildtype} **(B)** and *Tbr1*^{layer6} mutant **(C)**. The rectangles indicate the zone within layer 5 where synapse numbers were analyzed at P21. Pipette tip indicates that the soma was patched during the electrophysiology recordings **(B, C)**.

(i) Excitatory synapses were analyzed via synaptic bouton staining onto apical dendrites of layer 6 neurons (n=2) and spontaneous EPSC (sEPSC) recordings from the soma of **(D)** *Tbr1* ^{wildtype}, **(E)** *Tbr1*^{layer6} heterozygous, and **(F)** *Tbr1*^{layer6} homozygous mutants at P21 **(D-F)**. *Ntsr1-cre::tdTomato*$^{f/+}$ allele was used to label the layer 6 neurons (red). ImageJ software was used to process confocal images for quantification. **(G)** Quantification of excitatory synaptic density at P21. **(H)** Sample traces of sEPSC recordings at -70mV in SSCx slices from 4 weeks old *Tbr1* ^{wildtype} **(**red**)**, *Tbr1*^{layer6} heterozygous (green), and *Tbr1*^{layer6} homozygous mutants (blue) at P21. **(I)** Quantification of the sEPSC frequency in layer 6 neurons at P21.

(ii) Inhibitory synapses were examined by synaptic bouton staining onto apical dendrites of layer 6 neurons **(J – L)** and spontaneous IPSC (sIPSC) recordings from the soma of the layer 6 neurons of **(J)** *Tbr1* ^{wildtype}, **(K)** *Tbr1*^{layer6} heterozygous, and **(L)** *Tbr1*^{layer6} homozygous mutants at P21. *Ntsr1-cre::tdTomato*$^{f/+}$ alleles was used to label the layer 6 neurons (red). ImageJ software was used to process confocal images for quantification. **(M)** Quantification of inhibitory synaptic density at P21. **(N)** Sample traces of sIPSC recordings in voltage clamp at +10mV in SSCx slices from *Tbr1* ^{wildtype} **(**red**)**, *Tbr1*^{layer6} heterozygous (green), and *Tbr1*^{layer6} homozygous mutants (blue) at P21. **(O)** Quantification of the sIPSC frequency in layer 6 neurons at P21. Two-way ANOVA was used for the statistical analysis of the control, heterozygous and null. Two-tailed T-test with Tukey correction was used for pairwise comparisons. (*p< 0.05) (**p< 0.01) (***p<0.001) (****p< 0.0001).

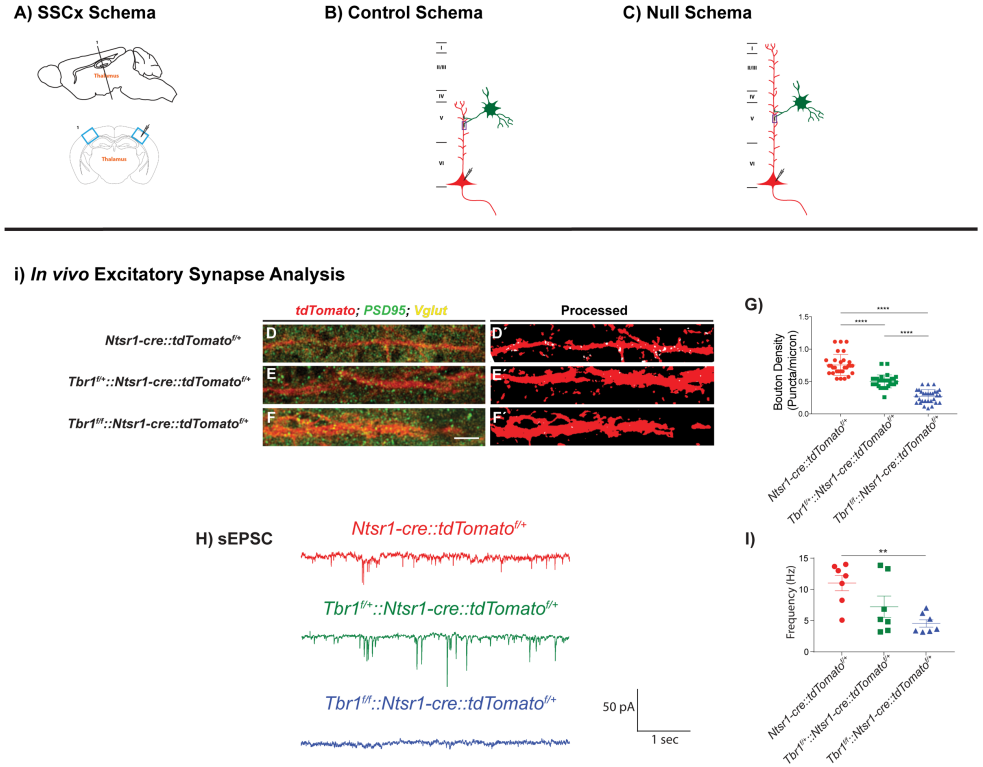
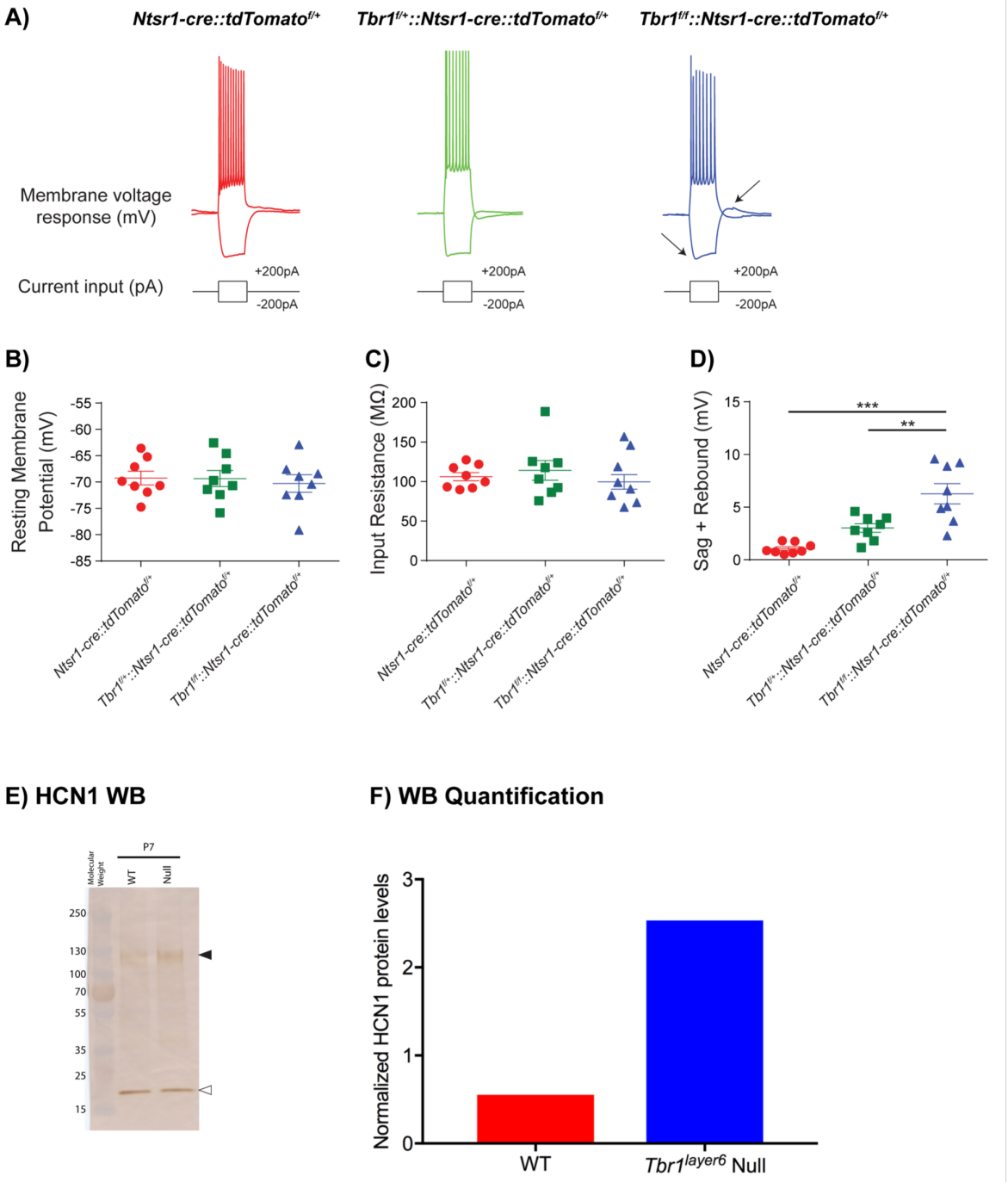

A) SSCx Schema
B) Control Schema
C) Null Schema
i) In vivo Excitatory Synapse Analysis
tdTomato; PSD95; Vglut
Processed
Ntsr1-cre::tdTomato f/+
Tbr1 f/+::Ntsr1-cre::tdTomato f/+
Tbr1 f/f::Ntsr1-cre::tdTomato f/+
G)
Bouton Density (Puncta/micron)
H) sEPSC
50 pA
1 sec
I)
Frequency (Hz)
ii) In vivo Inhibitory Synapse Analysis
tdTomato; Gephyrin; VGat
M)
N) sIPSC
O)

Figure S6: Related to Figure 6. Intrinsic properties of the pyramidal neurons of *Tbr1*^{layer6} mutants revealed an increase in HCN channels at P21.

(A) The layer 6 pyramidal neurons were patched at P21 and intrinsic properties were collected from whole-cell patch clamp recordings from layer 6 somatosensory cortex. Recordings from *Tbr1*^{wildtype} (red), *Tbr1*^{layer6} heterozygous mutant (green) and *Tbr1*^{layer6} homozygous mutant (blue) show that many intrinsic electrophysiological properties were unaffected by loss of *Tbr1*, including resting membrane potential **(B)**, input resistance **(C)**, and action potential halfwidth (data not shown). We estimated Ih by measuring the membrane potential sag and rebound elicited by a –200 pA current step. **(D)** sag and rebound is increased in *Tbr1*^{layer6} mutants. (**p< 0.01) (***p<0.001). **(E)** Western blot (WB) to detect HCN1 protein isolated from P7 cortex of a *Tbr1*^{wildtype} and *Tbr1*^{layer6} mutant. Black arrowhead indicates the HCN1 protein (~120 kDa, SCBT) and the white arrowhead indicates the *Cyclophilin B* loading control (~20 kDa, Abcam). **(F)** HCN1 protein levels were quantified and normalized to the loading control and corrected for background. WT= *Tbr1*^{wildtype}, Null= *Tbr1*^{layer6} homozygous mutant.


A)
Ntsr1-cre::tdTomato^f/+
Tbr1^f/+::Ntsr1-cre::tdTomato^f/+
Tbr1^f/f::Ntsr1-cre::tdTomato^f/+
Membrane voltage response (mV)
Current input (pA)
+200pA
-200pA
B)
Resting Membrane Potential (mV)
C)
Input Resistance (MΩ)
D)
Sag + Rebound (mV)
***
**
E) HCN1 WB
P7
Molecular Weight
WT
Null
250
130
100
70
55
35
25
15
F) WB Quantification
Normalized HCN1 protein levels
WT
Tbr1layer6 Null