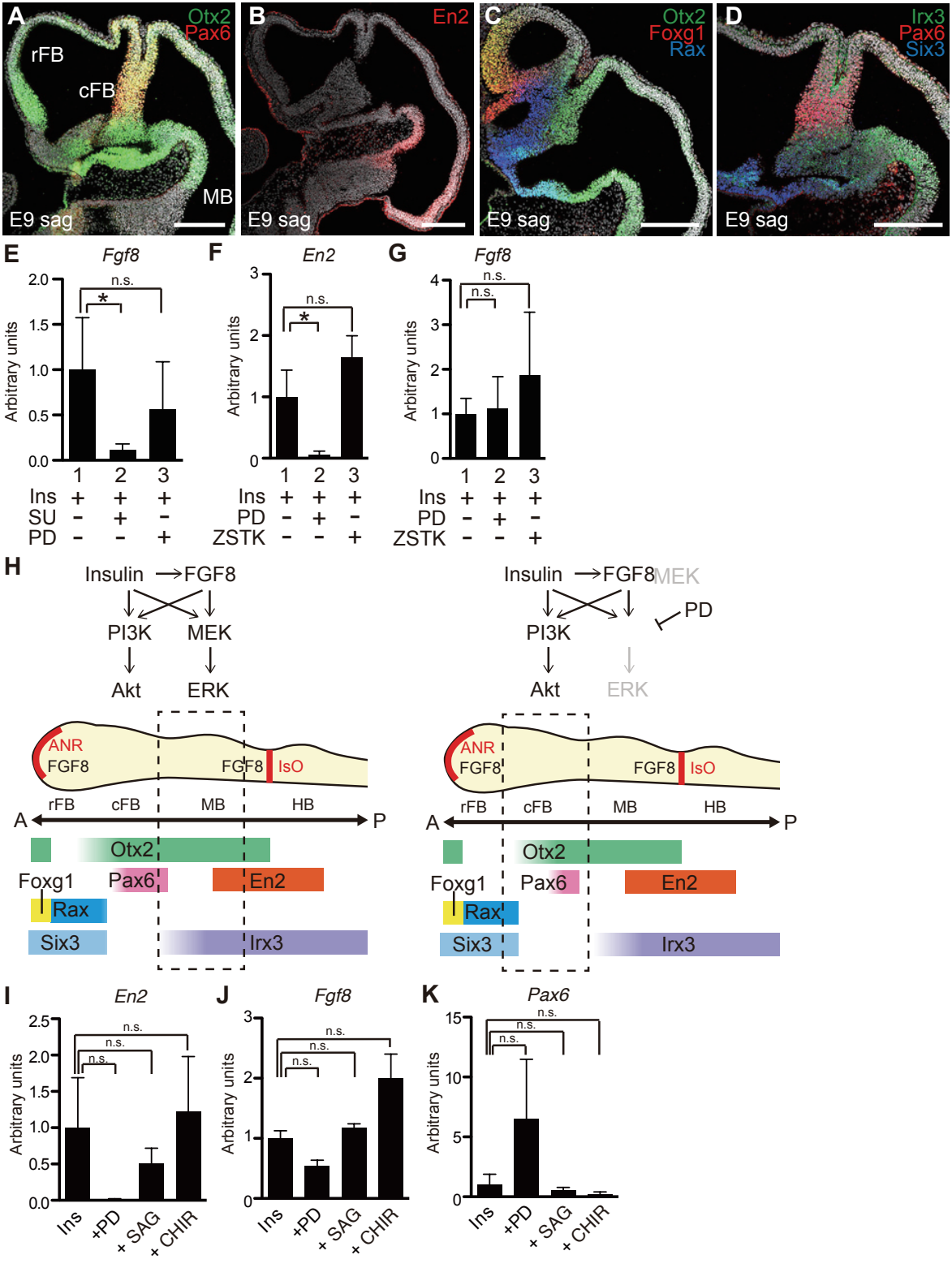

A
Otx2
Pax6
rFB
cFB
MB
E9 sag
B
En2
E9 sag
C
Otx2
Foxg1
Rax
E9 sag
D
Irx3
Pax6
Six3
E9 sag
E
Fgf8
Arbitrary units
n.s.
*
Ins
SU
PD
F
En2
Arbitrary units
n.s.
*
Ins
PD
ZSTK
G
Fgf8
Arbitrary units
n.s.
n.s.
Ins
PD
ZSTK
H
Insulin
FGF8
PI3K
MEK
Akt
ERK
ANR
FGF8
IsO
rFB
cFB
MB
HB
A
P
Otx2
Foxg1
Pax6
En2
Rax
Six3
Irx3
PD
I
En2
Arbitrary units
n.s.
Ins
+PD
+ SAG
+ CHIR
J
Fgf8
Arbitrary units
n.s.
K
Pax6
Arbitrary units
n.s.

Fig. S1. Effects of insulin and MEK inhibitor on anterior-posterior axis formation in SFEBq culture. (**A-D**) Expression of regional markers in the mouse E9 sagittal sections. Pax6 and Otx2 were co-expressed in caudal forebrain. (**E-G**) qPCR analysis of SFEBq-cultured mESC aggregates on day 7 for the expression of (E) Fgf8 (n = 40 aggregates from 5 independent experiments), (F) En2 and (G) Fgf8 (n = 96 aggregates in each from 6 independent experiments). Insulin (1 μg/ml) was added to the culture on day 0. FGF inhibitor (SU5402, 10 μM), MEK inhibitor (PD0325901, 1 μM) and PI3K inhibitor (ZSTK474, 250 nM) were added to the culture on day 4. Statistical differences were calculated between insulin-treated (lane 1) and additional treated mESCs (lane 2 and 3). (**H**) Schematic of caudal forebrain induction by the treatment with insulin and MEK inhibitor. (**I-K**) qPCR analysis of SFEBq-cultured mESC aggregates on day 7 for the expression of En2 (I), Fgf8 (J), and Pax6 (K) (n = 24 aggregates from 3 independent experiments). MEK inhibitor (PD0325901, 1 μM), Shh agonist (SAG, 10 nM) or GSK3β inhibitor (CHIR99021, 1 μM) were added to the culture on day 4. rFB, rostral forebrain; cFB, caudal forebrain; MB, midbrain; HB, hindbrain; ANR, anterior neural ridge; IsO, isthmic organizer. Scale bars: 200 μm in A-D. Each bars represents mean ± S.D. Nuclear counter staining (grey in A-D), DAPI.

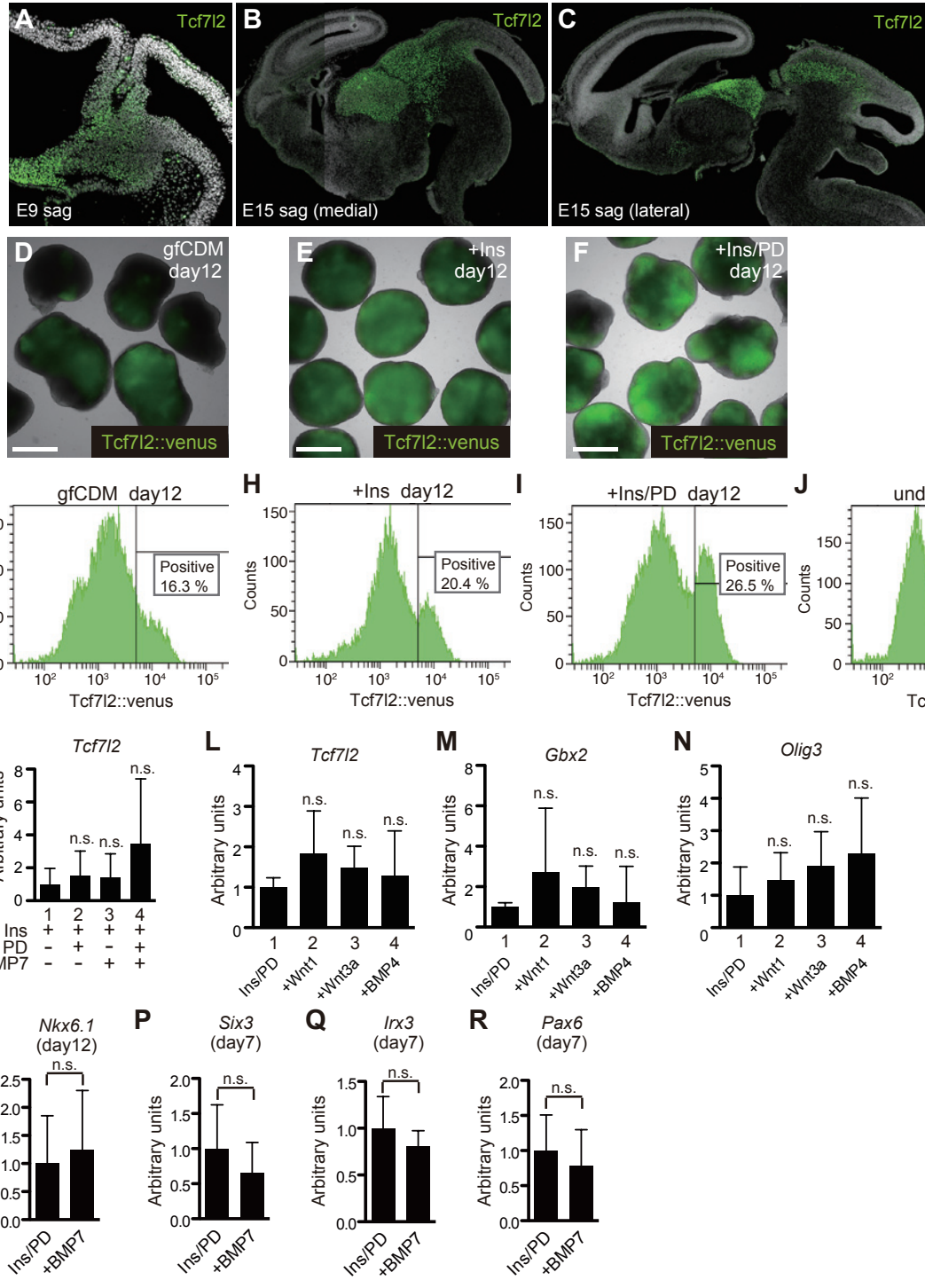


Fig. S2. BMP7 induces thalamic progenitors with little effects on anterior-posterior or dorso-ventral axes. (**A-C**) Expression of Tcf7l2 in the mouse E9 and E15 sagittal sections. (**D-F**) SFEBq-cultured mESC aggregates (Tcf7l2::venus) on day 12. (**G-J**) FACS analysis of Tcf7l2::venus fluorescence. (**K**) qPCR analysis of SFEBq-cultured mESC aggregates on day 12 for the expression of Tcf7l2 (n = 112 aggregates from 7 independent experiments). MEK inhibitor (1 μM) and/or BMP7 (30 ng/ml) were added to the culture on day 4. Statistical differences were calculated between insulin-treated (lane 1) and additional treated mESCs (lane 2-4). (**L-N**) qPCR analysis of SFEBq-cultured mESC aggregates on day 12 for the expression of Tcf7l2 (L), Gbx2 (M), and Olig3 (N) (n = 80 aggregates from 5 independent experiments). Wnt1 (50 ng/ml), Wnt3a (50 ng/ml), BMP4 (30 ng/ml) were added to the culture on day 4. Statistical differences were calculated between insulin/PD-treated (lane 1) and additional treated mESCs (lane 2-4). (**O**) qPCR analysis of SFEBq-cultured ESC aggregates on day 12 for the expression of Nkx6.1 (n = 80 aggregates from 5 independent experiments). BMP7 (30 ng/ml) was added to the culture on day 4. (**P-R**) qPCR analysis of SFEBq-cultured mESC aggregates on day 7 for the expression of Six3 (P), Irx3 (Q), or Pax6 (R) (n = 96 aggregates from 6 independent experiments). BMP7 (30 ng/ml) was added to the culture on day 4. Scale bars, 500 μm in D-F. Each bars represents mean ± S.D.

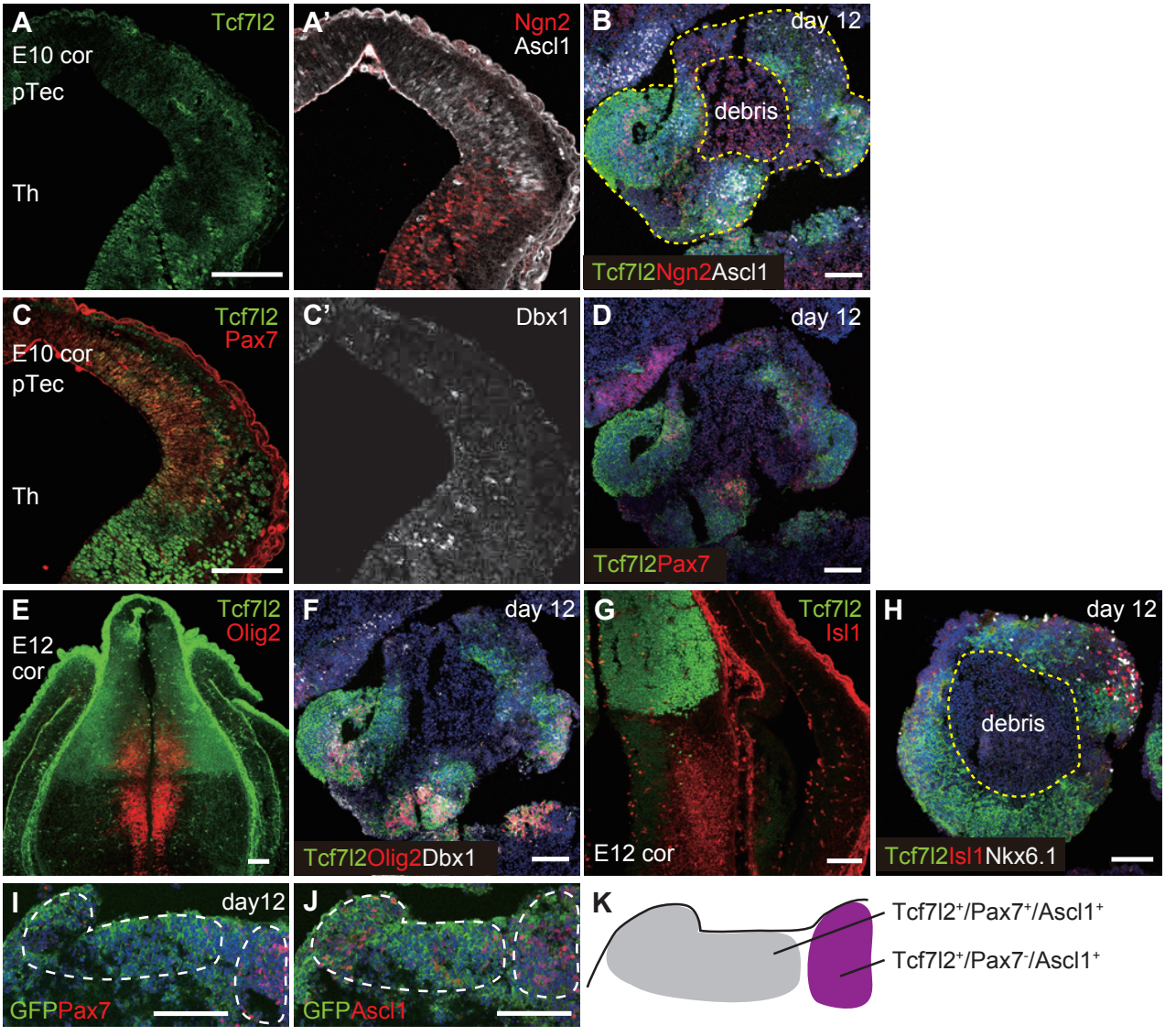


Fig. S3. Expression of specific markers for caudal forebrain in mESC-aggregates.

(**A,C,E,G**) Expression pattern of E12 caudal forebrain markers. (**B,D,F,H-J**) Cryosections of SFEBq-cultured mESC aggregate on day 12. (**K**) Schema for the expression pattern of thalamic markers in the mESC aggregate (I,J). Th, thalamus; pTec, pretectum. Yellow broken line demarcates the outline of the aggregate in (B, H). White broken line in (I, J) demarcates each structure in (K). Scale bars, 100 μm in A-J.

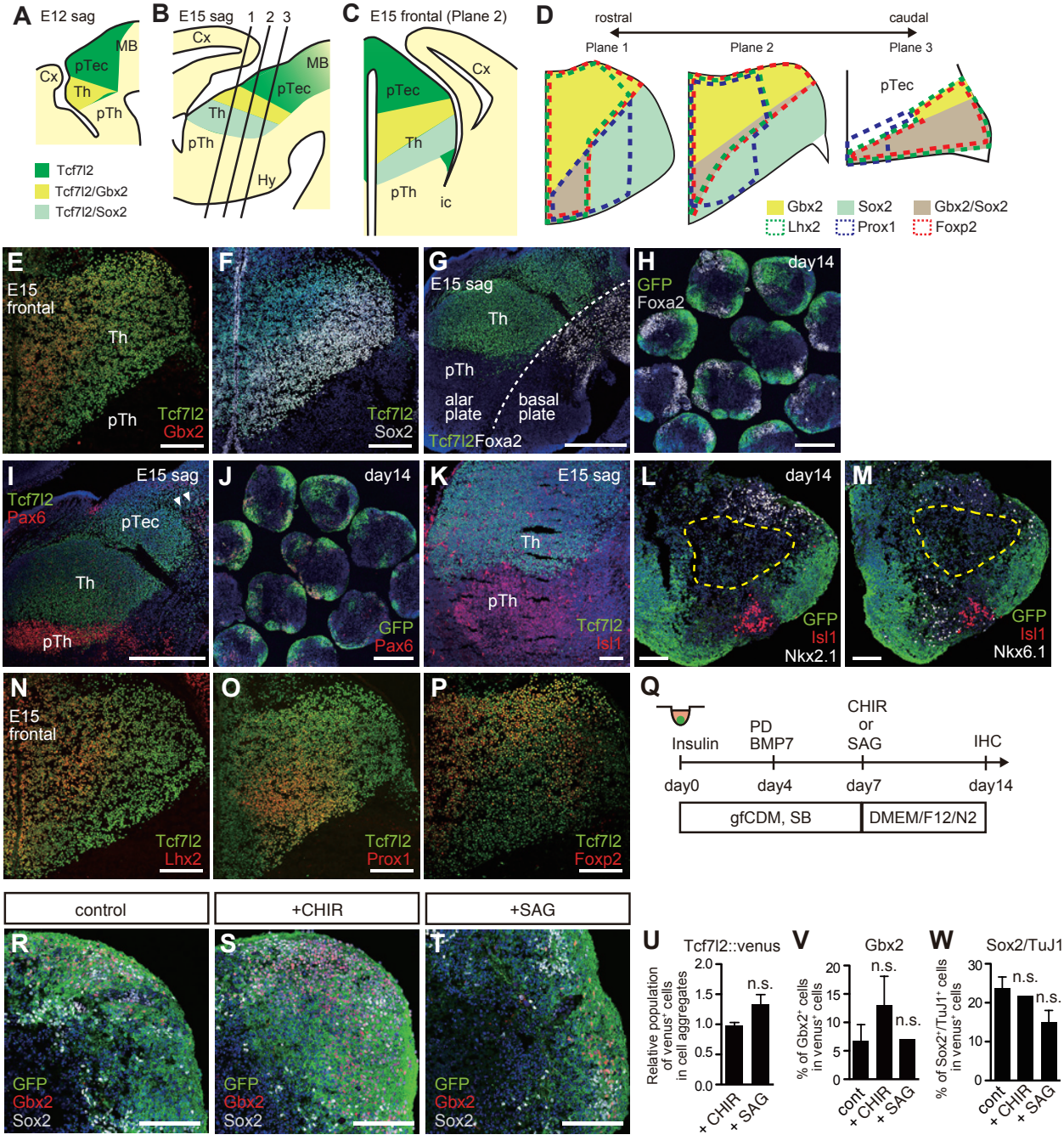


Fig. S4. Thalamic and non-thalamic populations in SFEBq-cultured mESC aggregates. (**A-D**) Schematic diagrams showing the expression patterns of thalamic markers in E12 and E15 mouse caudal forebrain. The lengthwise line represents the plane of frontal section. (**E,F**) Expression of thalamic neuronal markers in the E15 mouse frontal sections immunostained for Tcf7l2, Gbx2, and Sox2. (**G,H**) Expression of Tcf7l2 and basal plate marker Foxa2 in E15 mouse sagittal section (G) and mESC-aggregates on day14 (H). (**I**) Expression of Pax6 in prethalamus. Arrowheads indicate expression in pretectum. (**J**) Cryosection of aggregates on day 14, immunostained for GFP and Pax6. (**K**) E15 mouse sagittal section immunostained for Tcf7l2 and Isl1. (**L,M**) Cryosections of aggregate on day 14 immunostained with GFP, Isl1, Nkx2.1 and Nkx6.1. (**N-P**) Expression of thalamic neuronal markers in the E15 mouse frontal sections immunostained for Lhx2 (N), Prox1 (O), and Foxp2 (P) with Tcf7l2. (**Q**) Procedure for the differentiation of thalamic cells from ESC culture. CHIR99021 (1 μM) or SAG (10 nM) was added to the culture on day 7. (**R-T**) Sections of day 14 ESC aggregates cultured in SFEBq/IPB (R), SFEBq/IPB containing CHIR99021 (S), and SFEBq/IPB containing SAG (T). (**U**) Relative population of Tcf7l2::venus^{+} cells in DAPI^{+} cell aggregates on day 14 to those in the SFEBq/IPB culture (n = 48 aggregates from 3 independent experiments). (**V,W**) Percentage of cells expressing Gbx2^{+} (V) or Sox2^{+} (W) cells in Tcf7l2::venus-positive cells in aggregates on day 14 (n = 48 aggregates from 3 independent experiments). Cx, cortex; pTh, prethalamus; Th, thalamus; pTec, pretectum; MB, midbrain; Hy, hypothalamus; ic, internal capsule. Scale bars, 500 μm in G-J; 200 μm in E,F,N-P; 100 μm in K-M,R-T. Nuclear counter staining (blue), DAPI.

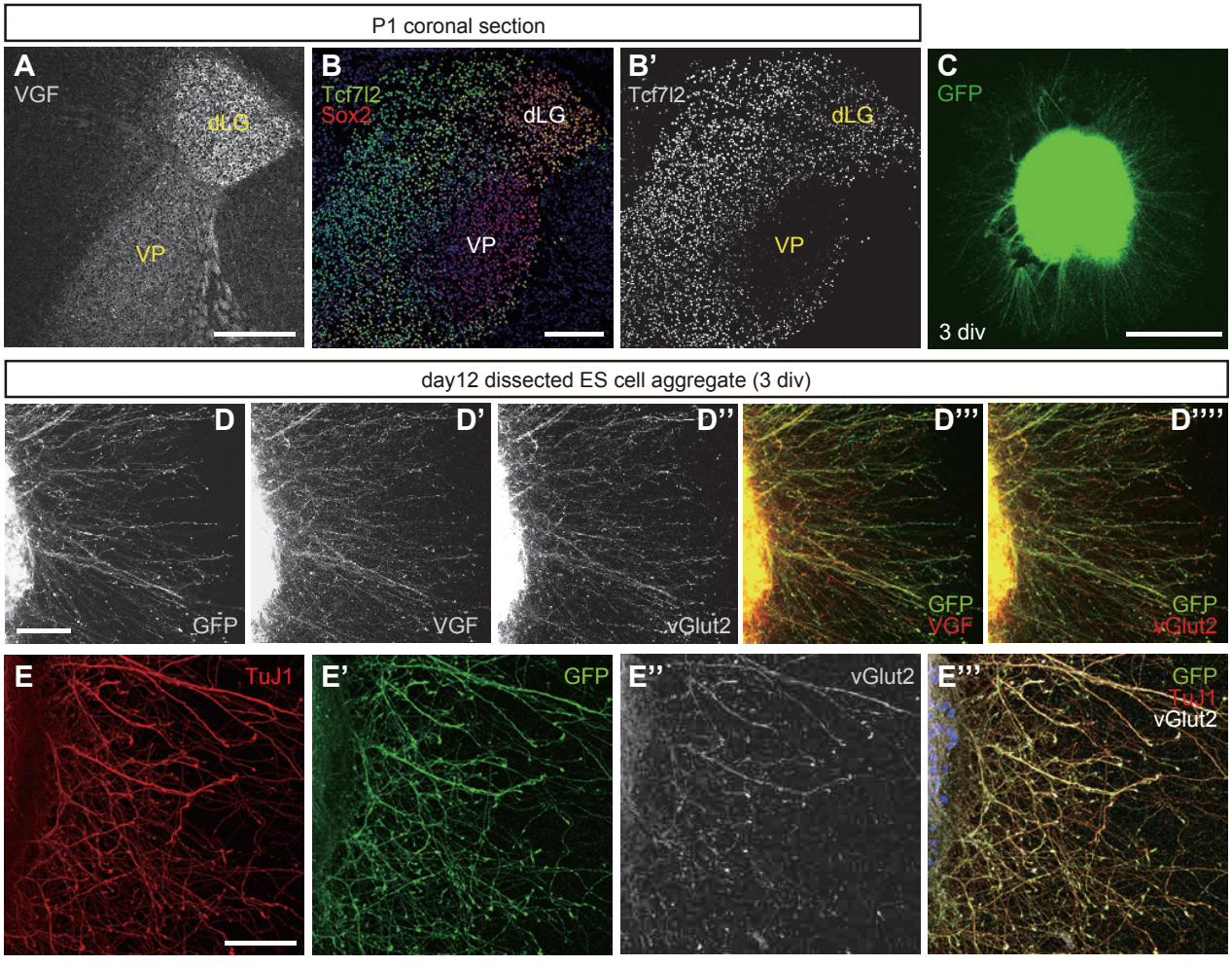
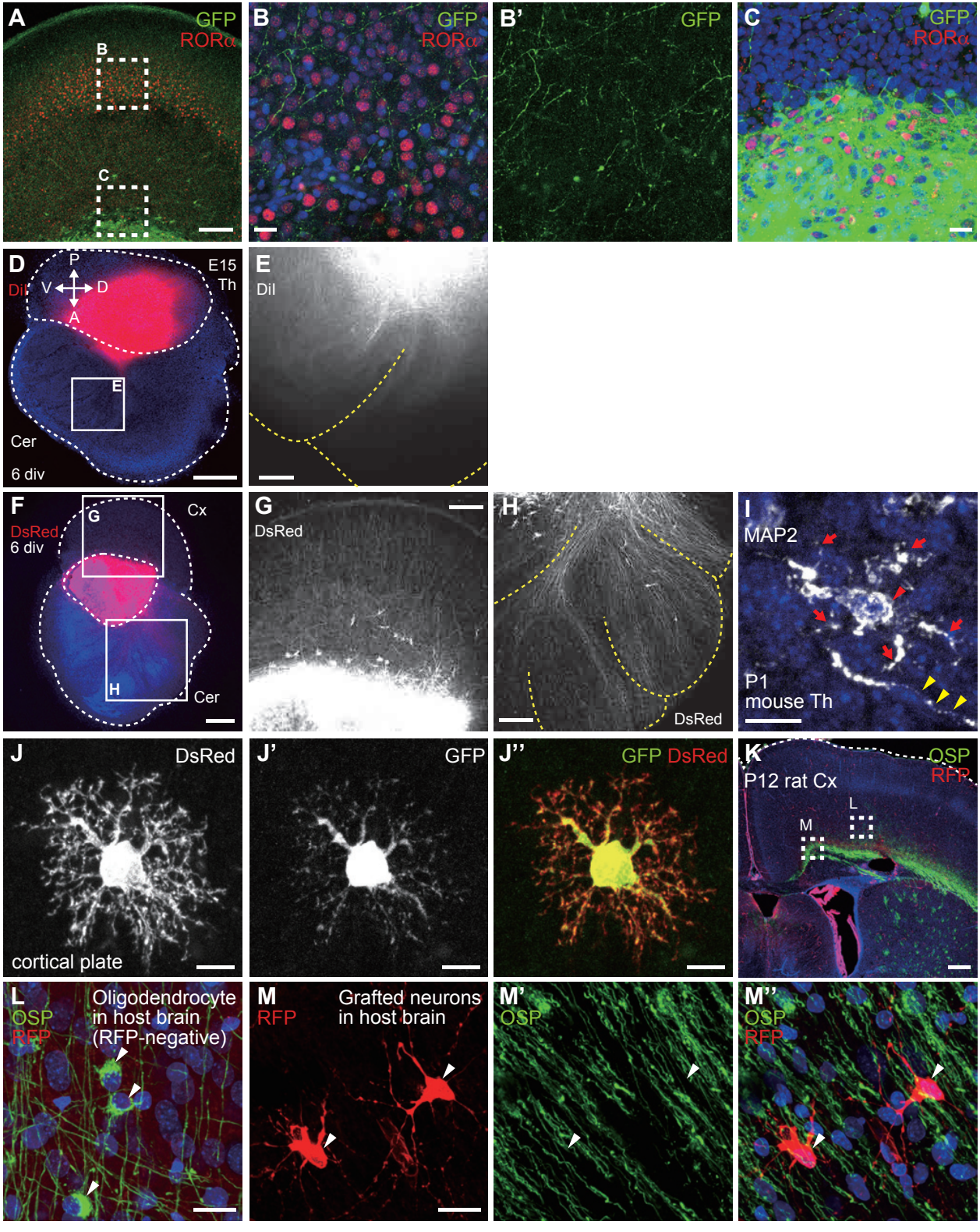


Fig. S5. Expression of Tcf7l2 in a part of thalamus diminishes by postnatal day 1. (**A-B'**) Expression pattern of VGF (A), Tcf7l2 and Sox2 (B) in P1 mouse coronal section. Of VGF^{+} sensory thalamic nuclei, ventroposterior nucleus decreased expression of Tcf7l2 by P1. (**C**) Tcf7l2::venus-positive potion dissected from ESC aggregate on day 12 at 3 days after collagen gel culture. The cells were immunostained with antibodies to GFP. (**D-D''''**) Neurites of dissected mESC-aggregate on day 15, immunostained with GFP, VGF, and vGlut2. (**E-E'''**) Neurites of dissected aggregate on day 15, immunostained with TuJ1, GFP and vGlut2. Scale bars, 200 μm in A,B; 500 μm in C; 100 μm in D,E. Nuclear counter staining (blue), DAPI.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C
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B'
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DsRed
cortical plate
J'
GFP
J''
GFP DsRed
K
OSP
RFP
P12 rat Cx
L
M
L
Oligodendrocyte
in host brain
(RFP-negative)
OSP
RFP
M
Grafted neurons
in host brain
RFP
M'
OSP
M''
OSP
RFP

Fig. S6. Ectopic projection or integration of thalamic neurons in co-culture or transplantation assay. (**A**) Co-culture of mESC-aggregate with postnatal day 1 (P1) rat cortical slice on culture day 6. (**B-C**) High-magnification views of insets in (A). (**D**) Co-culture of embryonic day 15 (E15) mouse thalamic explant and P1 rat cerebellar slice on culture day 6. DiI was injected in thalamic explant. (**E**) High-magnification view of the inset in (D). Note that thalamic neurons did not extend axons radially but preferentially along the white matter with fasciculation. (**F**) Triple culture of mESC aggregate, P1 rat cortical and cerebellar slices on culture day 6. (**G,H**) High-magnification view of the insets in (F). Yellow broken line in (H) demarcates the cerebellar explant. (**I**) Thalamic neurons in P1 mouse brain section. Red arrows and arrowhead indicate dendrites and soma, respectively. Yellow arrowheads indicate a neurite of another neuron. (**J**) A transplanted mESC-derived neuron in host rat cortex. (**K**) Host rat cortex at 1 week after transplantation. (**L-M"**) High-magnification view of the insets in (K). Cx, cortical slice; Cer, cerebellar slice. Scale bars, 200 μm in A,E,G,H; 20 μm in B,C,L,M; 10 μm in I,J; 500 μm in D,F,K. Nuclear counter staining (blue), DAPI.

Table S1. List of the genes and their primer sequences for qPCR analysis. GAPDH primers were used as an internal control for each specific gene amplification.

	Forward	Reverse
Six3	CCGGAAGAGTTGTCCATGTTC	CGACTCGTGTTTGTTGATGGC
Rax	TTCGAGAAGTCCCACTACCC	TTCATGGACGACACTTCCAG
Irx3	CAACGAGCACCGCAAGAA	TGGTGATGATGGCCAACATG
Fgf8	GTCCTGCCTAAAGTCACACAG	CTTCCAAAAGTATCGGTCTCCAC
En2	ATGGGACATTGGACACTTCTTC	CCCACAGACCAAATAGGAGCTA
Pax6	CAGCTTGGTGGTGTCTTTGT	GCAGAATTCGGGAAATGTCG
Otx2	CGTTCTGGAAGCTCTGTTTG	TTTTCAGTGCCACCTCTTCC
Tcf7l2	GGTGGCCGAATGCACATTGAAAGA	TTTGCCTGTTCT TCCCTGGACA
Gbx2	GCAAGGGAAAGACGAGTCAAA	GGCAAATTGTCATCTGAGCTGTA
Olig3	CAGGAGAGTCGTCTGAACTCG	GTTCGCGTCCGTTGATCTT
Nkx6.1	CTGCACAGTATGGCCGAGATG	CCGGGTTATGTGAGCCCAA
GAPDH	TGACCACAGTCCATGCCATC	GACGGACACATTGGGGGTAG