Supplementary information

Methods

In situ hybridization

RT-PCR was performed to obtain *Cyclin D2*, *Cyclin D3* cDNA clones for synthesis of RNA probes. Total RNA was purified from the brains of E10.5 ICR mouse embryos by RNeasy column (Qiagen, Maryland, USA) and cDNA was synthesized using reverse transcriptase and oligo dT primer (SuperScript preamplification system; Gibco, Carlsbad, CA). The Oligonucleotide primers used to amplify cDNAs are as follows: *Cyclin D2* (5'-CGCATTCAGCCAAAGGAAGG-3' and 5'-CCGAATGGCTTCCTCACAGG-3') *Cyclin D3* (5'-CTTCCAGTGCGTGCAAAAGG-3' and 5'-ACACCTTTGGAAGGTACTGG-3'). Amplification was performed for 35 cycles under the following conditions: denaturation, 95°C, 5 min; annealing, 61°C (*Cyclin D2*), 50°C (*Cyclin D3*), 1 min; and extension, 72°C, 1 min. To clone amplified products, these fragments were blunted using T4 DNA polymerase (Invitrogen, Carlsbad, CA) and inserted into the *Eco*RV site of pBluescriptII SK (-) (Stratagene, La Jolla, CA). *mRFP* RNA probe was produced by pRSET (Invitorgrn). Ngn2 probe was kindly obtained from Dr. M. Nakafuku.

Immunohistochemistry and Western blotting

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 h or overnight at 4°C. Embryos were cut into 8-μm or 12-μm sections and incubated

with primary antibodies at 4°C overnight. Primary and secondary antibodies used are listed below. For observation, sections were mounted with VECTASHIELD mounting medium (Vector laboratories, Burlingame, CA) and visualized with a confocal laser-scanning microscope (LSM5 Pascal, Carl Zeiss, Jena, Germany) and an AxioplanII fluorescent microscope equipped with an AxioCam CCD camera (Carl Zeiss, Jena, Germany). Detection of BrdU was as described in a previous paper with minor modifications (Takahashi & Osumi, 2002). 50 mg/ml BrdU/PBS solution was directly injected into the abdominal cavity of pregnant mice. Sections were treated in 2 N HCl for 12 min at 37°C and stained with anti-BrdU and anti-mouse IgG-Cy3 donkey antibodies. Using peptides corresponding to the C-terminal region of mouse Ngn2 (CEKHRYAPHLPLARDI), a rabbit polyclonal anti-Ngn2 antibody was generated. The antibodies used in this study are summarized in Table S1. Western blot analysis was performed essentially as described previously (Sakurai & Osumi, 2008) except with a different sample lysis solution and blocking solution. E12.5 embryo brains were homogenized in T-PER (Pierce, Rockford, IL) supplemented with a protease inhibitor mixture (Roche, Indianapolis, IN), blocking was performed with ODYSSEY blocking buffer (LI-COR). Antibodies were to Cyclin D2 (Santa Cruz, Exalpha). Western blots were probed with goat anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor 680 (LI-COR) and IRdye 800 (LI-COR). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR).

Expression constructs

For the *cis*-acting transport element assay, parts of the *Cyclin* D2 3'UTR sequence were subcloned into *pCAX-EGFP*, downstream of the EGFP sequence. *pCE/CD2/3'* (nucleotides 1106-6274); *pCE/CD2/3'/3023-6274* (nucleotides 3023-6274); *pCE/CD2/3'/4039-6274* (nucleotides 4039-6274); *pCE/CD2/3'/3023-4039* (nucleotides 3023-4039); *pCE/CD2/3'/3731-4015* (nucleotides 3731-4015); *pCE/CD2/3'/3764-4297* (nucleotides 3764-4297); *pCE/CD2/3'/ Δ3764-4293* (nucleotides 1106-3764 and 4297-6274); *pCE/CD2/3'/3764-3863* (nucleotides 3764-3863 x3 tandem repeat); *pCE/CD2/3'/3814-3913* (nucleotides 3814-3913 x3 tandem repeat); *pCE/CD2/3'/3864-3963* (nucleotides 3864-3963 x3 tandem repeat); *pCE/CD2/3'/3914-4015* (nucleotides 3914-4015 x3 tandem repeat); *pCE/CD2/3'/3930-3980* (nucleotides 3930-3980 x3 tandem repeat); *pCE/CD2/3'/3950-4000* (nucleotides 3950-4000 x3 tandem repeat); *pCE/CD2/3'/3965-4015* (nucleotides 3965-4015 x3 tandem repeat). To generate *pCAX-EGFP*, *EGFP* cDNA originating from *pEGFP-C1* (BD–Clontech, CA, USA) was inserted into the *Eco*RV site of *pCAX*. To generate *pCAX-EGFP-NLS*, the nuclear localization signal sequence from *pDsRed2-Nuc* (BD-Clontech, CA, USA) was inserted into *pCAX-EGFP*. For local translation assays, the 3'UTR sequence of *Cyclin D2* mRNA (nucleotides 1496-5457) was subcloned into the *Eco*RV site of *pCAX-EGFP-NLS*, which was located downstream of the *EGFP-NLS* sequence in the forward or reverse direction (*pCEN/CD2/3'/1496-5457; pCEN/CD2/3'/5457-1496*).

Gene transfer into mouse embryos by electroporation

In utero electroporation was performed as described previously with minor modifications (Osumi & Inoue, 2001; Takahashi et al, 2002). Timed pregnant ICR mice at E12.5-13.5 were anesthetized with 97% 2, 2, 2-tribromoethanol (Sigma, St. Louis, MO) in tert-amyl alcohol (Sigma, St. Louis, MO). After the abdomen was cleaned with 70% ethanol, a midline laparotomy of about 3 cm was performed, and the uterus was pulled out. All expression constructs were purified with ultracentrifugation and resuspended in PBS buffer; 1-3 μl of DNA solution was injected into the lateral ventricle with a mouth-controlled glass capillary pipette, and square pulses (38-40 V, 50 ms, 5 pulses at 1 s intervals) were delivered into embryos with an electroporator and tweezer-type electrodes, which consist of a pair of round platinum plates of 1 mm diameter (BEX, Tokyo, Japan).

Virus production and injection into the brain

For virus vector transfection, HEK293T cells were plated at 5 x 10^6 cells/plate on a 15 cm Tissue Culture Dish (FALCON, NJ, USA) and cultured in 30 ml DMEM containing 10% FCS and 1% PSF at 37 °C in 5% CO_2 for 24 h. Opti-MEM (1 ml, GIBCO, Carlsbad, CA) and Trans IT-293 Transfection Reagent (72 μl, TAKARA, Otsu, Japan) were mixed and incubated for 15 min at RT. *pCS-EF-EGFP* plasmid (8 μg), *pCMV-VSV-G-RSV-Rev* (8 μg), and *pCAG-HIVgp* (8 μg) were added into the mixture and incubated for 15 min. The entire mixture was added dropwise to the culture dish. After 24 h, the medium was replaced with 30 ml of advanced DMEM containing 10% FCS and 1% PSF, and cells were cultured for 72 h. Supernatants were cleared by a pass through a 0.45 μm Millipore

filter and centrifuged at 6000 G at 4°C for 16 h. After removal of the supernatant, pellets of virus were resuspended gently in 100 μl PBS and stored in aliquots at - 80°C until use. Pregnant ICR mice were anesthetized, and the solution containing virus particles was injected into the telencephalic ventricle of embryos at E12.5-13.5 by *in utero* manipulation. For clonal analysis of apical progenitors, low-titer EGFP- lentivirus was injected into the lateral ventricle of E12.5 embryos and harvested at E14.5. In total, 32 of 0.25 μm confocal Z-sectioned images were used for 3D reconstruction using NIS-Elements software (Nikon, Tokyo).

References

Sakurai K, Osumi N (2008) The neurogenesis-controlling factor, Pax6, inhibits proliferation and promotes maturation in murine astrocytes. *J Neurosci* **28**(18)**:** 4604-4612

Table 1 antibodies used in this study

Antibodies	Species	Dilution	Vender
Primary antibodies			
Cyclin D2	Rabbit polyclonal	1:1000	Santa Cruz
Cyclin D2	Mouse monoclonal	1:5000	Exalpha
Class III β-tubulin	Mouse monoclonal	1:2000	Covance
Phospho-histone H3	Rabbit polyclonal	1:600	Upstate
Laminin	Rat monoclonal	1:100	Chemicon
GFP	Chicken polyclonal	1:1000	Abcam
MPM2	Mouse monoclonal	1:200	Upstate
Neurogenin 2	Mouse monoclonal	1:20	From Dr. D. Anderson
Neurogenin 2	Rabibit polyclonal	1:1000	In this study
BrdU	Mouse monoclonal	1:50	BD Bioscience
Phospho vimentin	Mouse monoclonal	1:200	Abcam
SOX2	Goat polyclonal	1:500	R&D
Tbr2	Rabibit polyclonal	1:500	Abcam
Secondary antibodies			
Rabbit IgG-Cy3	Donkey	1:400	Jackson
Mouse IgG-Cy3	Donkey	1:400	Jackson
Rat IgG-Cy3	Donkey	1:400	Jackson
Chicken IgY-Alexa488	Donkey	1:400	Molecular Probes
Goat IgG-Cy5	Donkey	1:400	Jackson

Supplementary figure legends

Figure S1 Specificity of Cyclin D2 antibodies. **(**A-C**)** Double-fluorescence Western blots using E12.5 whole brain lysates reveal a single 34kDa band using DCS-3 (Exalpha) monoclonal antibody (lanes A and C). With M-20 (Santa Cruz) polyclonal antibody (B), a single 34kD band is coincident with DCS-3 (C, white arrowhead), although some extra bands are also present (B, C, black arrowhead). (D, E) M-20 antibodies stain for cyclin D2 in the E12.5 neocortex of wildtype littermate mice (D), but no positive staining is observed in the cortex of Cyclin D2 knockout mice (E).

Figure S2 Expression patterns of D-type *Cyclin* mRNA in the mouse telencephalon at E10.5, E12.5 and E14.5. *Cyclin D1* and *Cyclin D3* mRNAs in the ventricular zone (VZ) of the telencephalon at E10.5. At E12.5 and E14.5, *Cyclin D1* mRNA was strongly expressed in the VZ, whereas expression of *Cyclin D3* was no longer seen in the telencephalon. Scale bars: 100 μm.

Figure S3 Function of the *cis*-acting transport element is conserved in the cortex. (A) GFP reporter construct carrying full 3' UTR elements of *Cyclin D2* was electroporated together with *pCAGGS-mRFP* into E12.5 forebrain vesicles and cultured for 12 h before analysis**.** (B, C) *pCE/CD2/3'/3965-4015* (see Fig. 2) and *pCAGGS-mRFP* were introduced into the E14 mouse cortex by *in utero* electroporation. Embryos were sacrificed 24 h after electroporation, and the mRNA localization pattern was checked by *in situ* hybridization. EGFP transcripts were observed both in the basal endfoot

(arrowheads) and VZ (B), whereas control *mRFP* transcripts were observed only in the VZ (C). Dotted lines indicate the basal lamina, Scale bars: 600 μm.

Figure S4 Immunohistochemical detection of Cyclin D2 in daughter cells following cell division. (A) Low-titer lentiviral particles were injected *in utero* into the ventricle of E12.5 embryos and harvested at E14.5. (B) Example of a cortical hemisphere harboring only one cluster of EGFP cells, presumed to be clonal offspring of an infected cell, with descendants distributed in the SVZ (arrow) and also in the VZ (box D). (C-C") Basal processes (box C in B) revealed by EGFP shows Cyclin D2 staining (arrowheads). (D-D") In the VZ, two daughter cells, one basal and the other apical, shows differential staining with Cyclin D2 present at higher level in the basally-position offspring (nucleus is outlined with white line in D"). Scale bars: 100 μm in B, 10 μm in C and D.

Figure S5 The neuronal marker Ngn2 is expressed by the apically-positioned daughter following asymmetric divisions at E12.5. (A-C) At early G1, a pair of daughter cells close to the apical membrane are both devoid of Ngn2 staining. (D-F) At late G1, a pair of EGFP-positive daughter cells are situated away from the apical membrane. Ngn2 is expressed by the apically-positioned daughter cell in 50% of the pairs (9/18). (G) Schematic showing Ngn2 inheritance in the apically-positioned cell at early G1 (top row), while the neuronal marker is absent from late G1 offspring (bottom row). Scale bar: 10 μm.

Figure S6 (A-C) Cyclin D2 protein is symmetrically expressed in two M-phase cells identified by phosphorylated vimentim (pVim). (D-F') *Cyclin D2* mRNA between daughter cells of an EGFP-positive clone (labeled at E11.5) show similar staining levels revealed by *in situ* hybridization. Note high levels of *Cyclin D2* mRNA in the basal endfeet of the basally-positioned daughter. (G-I') In a control experiment, *Ngn2* mRNA is present at higher levels in the apically-positioned daughter cell of an *EGFP*-labeled clone. Scale bar: 10 μm in A-C, 25 μm in D-I.

Figure S7 Perturbation of global Cyclin D2 levels in E13.5 neuroepithelium by *in utero* electroporation of Cyclin D2 over-expressing or siRNA knockdown constructs followed by analysis 24 h later. (A) Schematic diagram depicting introduction of overexpressing *pCAX-Cyclin D2-ORF or* control *pCAX*, and Stealth RNAi for mouse *Cyclin D2* (*si1726*) or control siRNA. Overexpression would increase Cyclin D2 globally in all transfected cells, while RNAi knockdown would downregulate Cyclin D2 in the germinal zones. (B-E) *In situ* hybridyzation for *Cyclin D2* shows increased mRNA levels with Cyclin D2-ORF compared to the control *pCAX* in VZ but not so much in basal endfeet region. In contrast, knockdown with siRNA greatly reduced *Cyclin D2* mRNA from both in the VZ and basal endfeet regions, while this effect was not observed with the control siRNA constructs. (F-I) Immunostaining for Cyclin D2 shows increased protein levels with Cyclin D2-ORF compared to the control plasmid *pCAX*. In contrast, siRNA knockdown virtually removed Cyclin D2 from the neuroepithelium, while this effect was not observable with the control siRNA construct. Insets show the basal side of radial glia. (J)

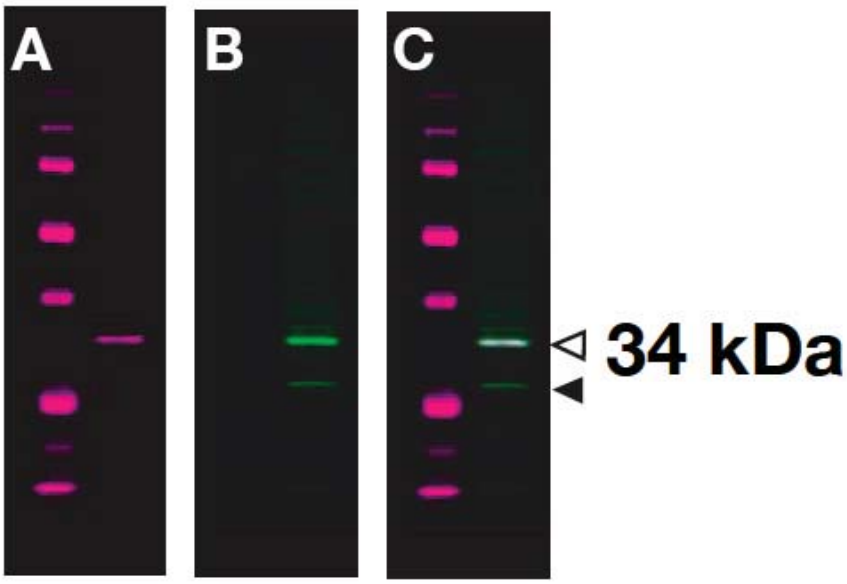
Quantification for Cyclin D2-positive cells shows a statistically significant effect for Cyclin D2 overexpression in EGFP cells compared to the controls. A similar effect was also observed when comparing Cyclin D2 knockdown with the control plasmid. (K-N) Co-electroporation of *pCAX-EGFP-NLS* allows visualization of the cell nuclei for quantification of cellular populations that are apical progenitors (SOX2+/Tbr2-), intermediate progenitors (Tbr2+), or differentiated neurons (SOX2-/Tbr2-). (O) Overexpression of Cyclin D2 leads to increase in the proportion of apical progenitors compared to the control; conversely, Cyclin D2 knockdown leads to a reduction in numbers of apical progenitors. On the other hand, Cyclin D2 overexpression is associated with a reduction in the numbers of intermediate progenitors plus neurons, while Cyclin D2 knockdown was associated with increased numbers of intermediate progenitors plus neurons. Error bars indicate SEM *P < 0.05, **P < 0.001, ***P < 0.0001, Student's *t*-test. Scale bar: 100 μm in B-E, 50 μm in F-N.

Figure S8 Effects of Cyclin D2 overexpression on cell proliferation. Control plasmid *pCAX, pCAX-Cyclin D2-ORF,* control Stealth RNAi, or si1726 was electroporated into the E12.5 forebrain. Forty-eight hours after electroporation, BrdU was pulse-labeled for 15 min before sacrifice, and immunostained for BrdU (A-D) and PHH3 (E-H). Tbr2 positive-cells designated the subventricular zone (SVZ) (data not shown), and intermediate zone (IMZ) containing outer subventricular zone (OSVZ) and cortical plate (CP) were distinguished by morphology. (A-D) An increase in BrdU-labeled cells was observed in VZ, SVZ, and IMZ following *Cyclin D2*-overexpression (B) compared with

the control (A). Conversely, Cyclin D2 knockdown using siRNA decreased the number of BrdU-labeled cells in the VZ and SVZ (D) compared with the control (C). (E-H) Similarly, increased PHH3-positive cells were observed in VZ, SVZ and IMZ following *Cyclin D2*-overexpression (F) compared with the control (E), whereas Cyclin D2 knockdown is associated with a decrease in the number of PHH3-positive cells in the VZ and SVZ (H) compared with the control (G). Scale bar: 50 μm.

Movie 1 3D reconstruction of the shape of EGFP-labeled daughter cells at E14.5. Two dividing daughter cells derived from an apical progenitor are visualized by infection of low-titer EGFP-lentiviral particles. The rotated 3D movie clearly shows that the basal process of an apical progenitor is inherited by the basally positioned daughter cell.

Fig. S1 Tsunekawa



Cyclin D2 +/+

Cyclin D2 -/-

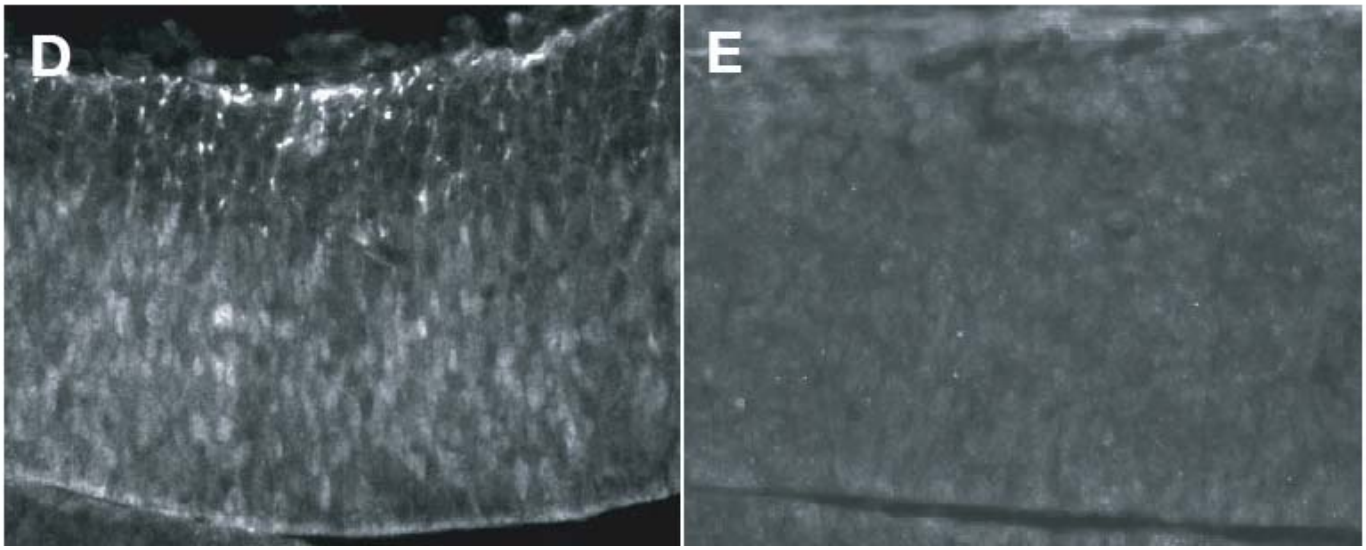


Fig. S2 Tsunekawa

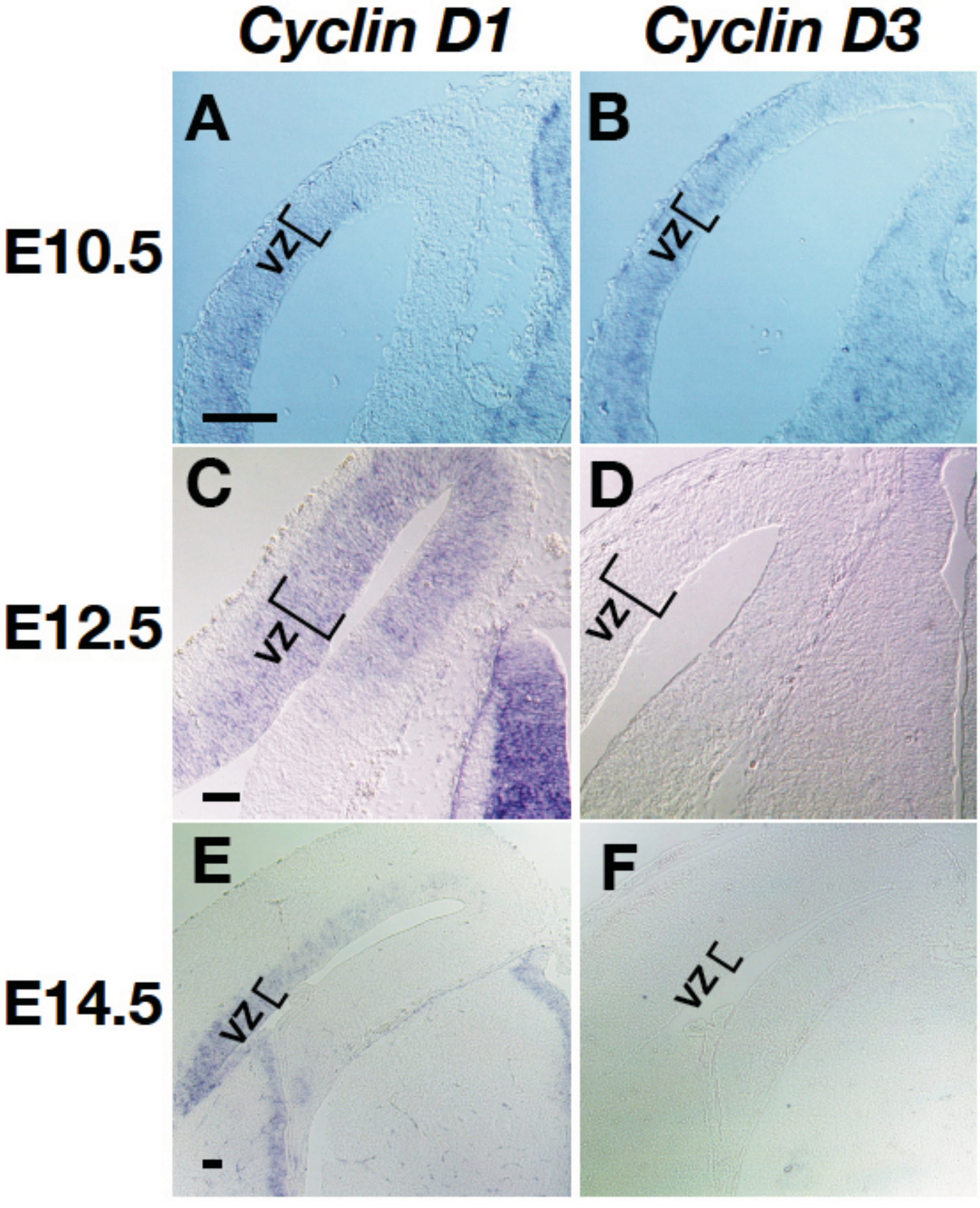
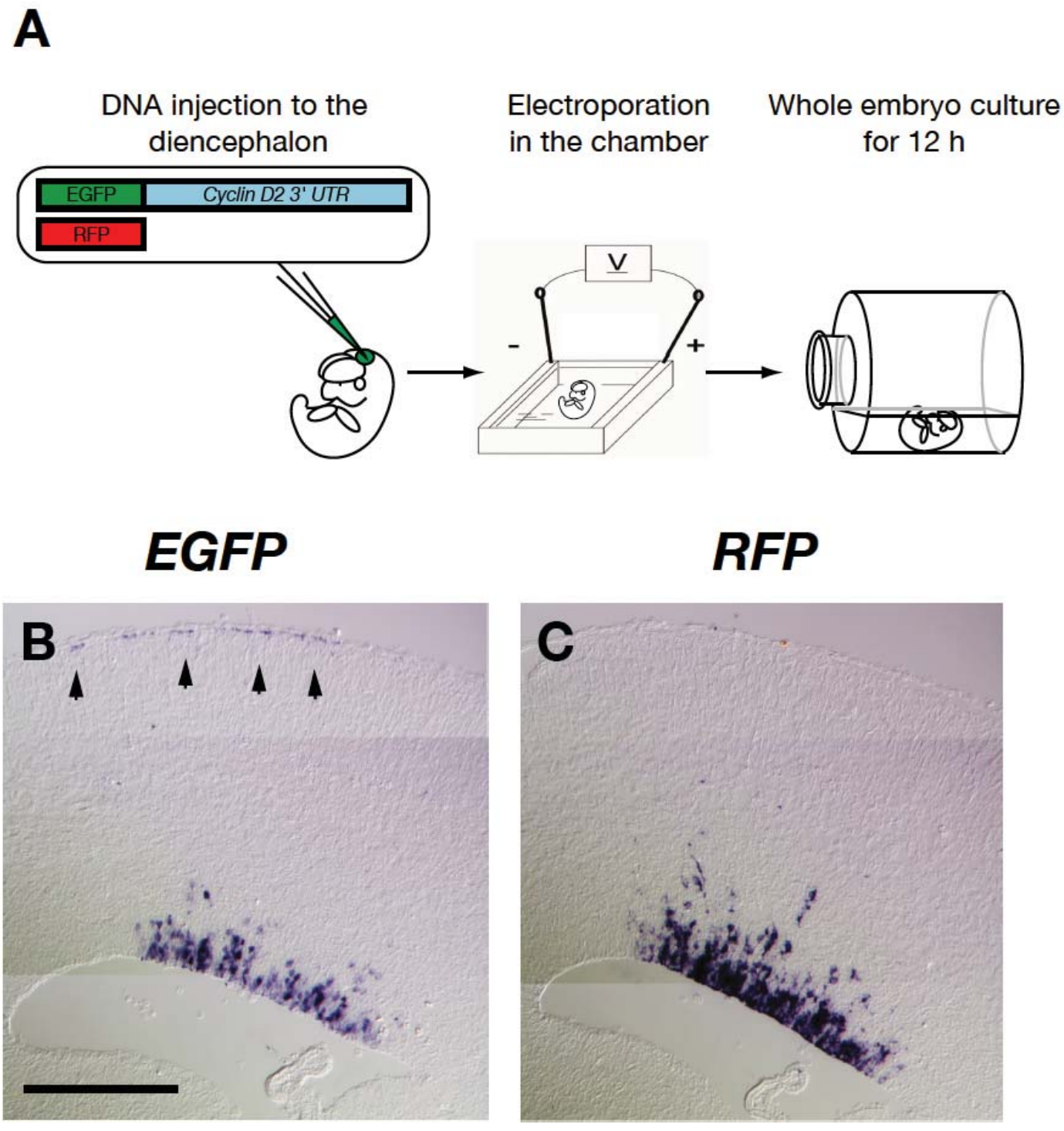


Fig. S3 Tsunekawa



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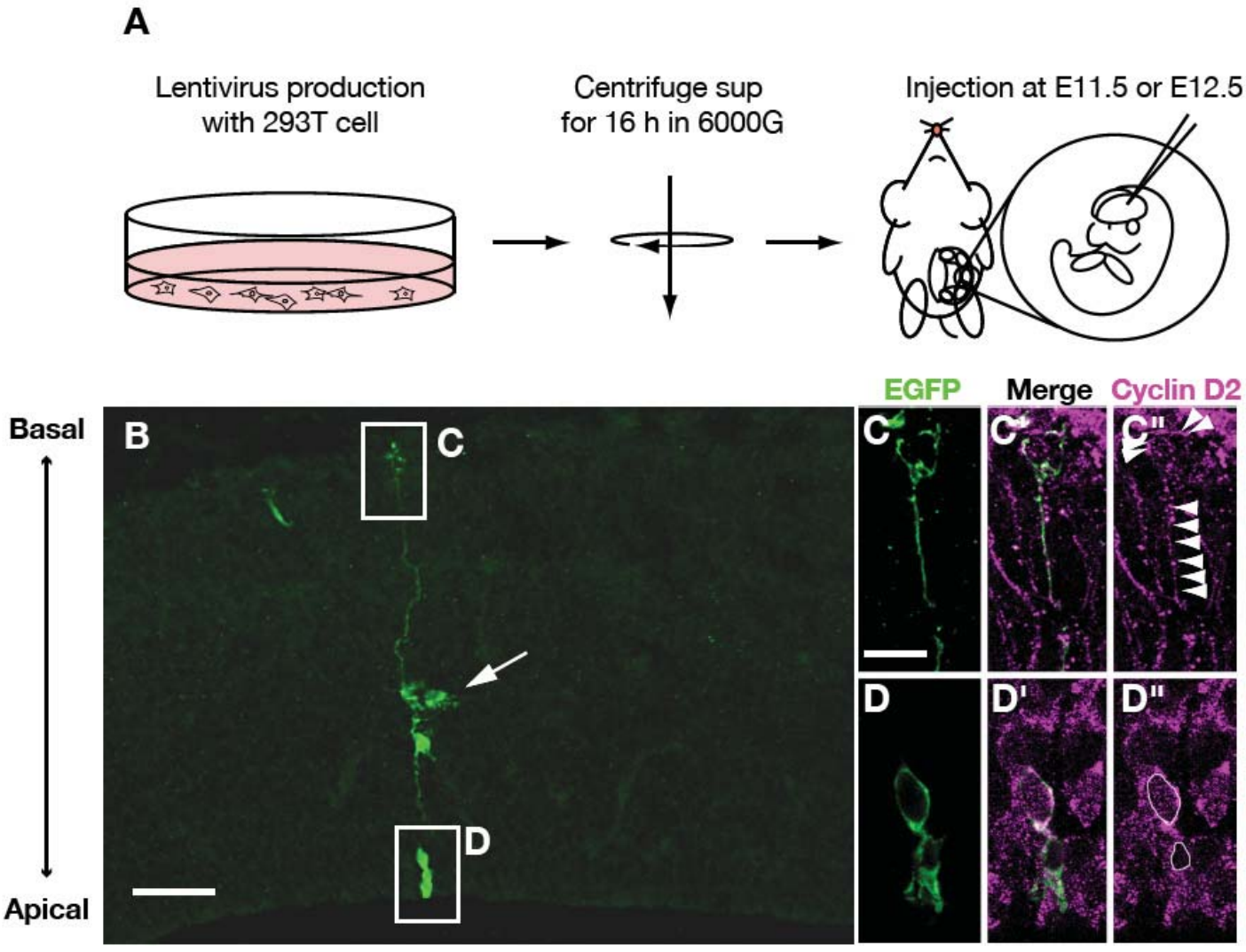

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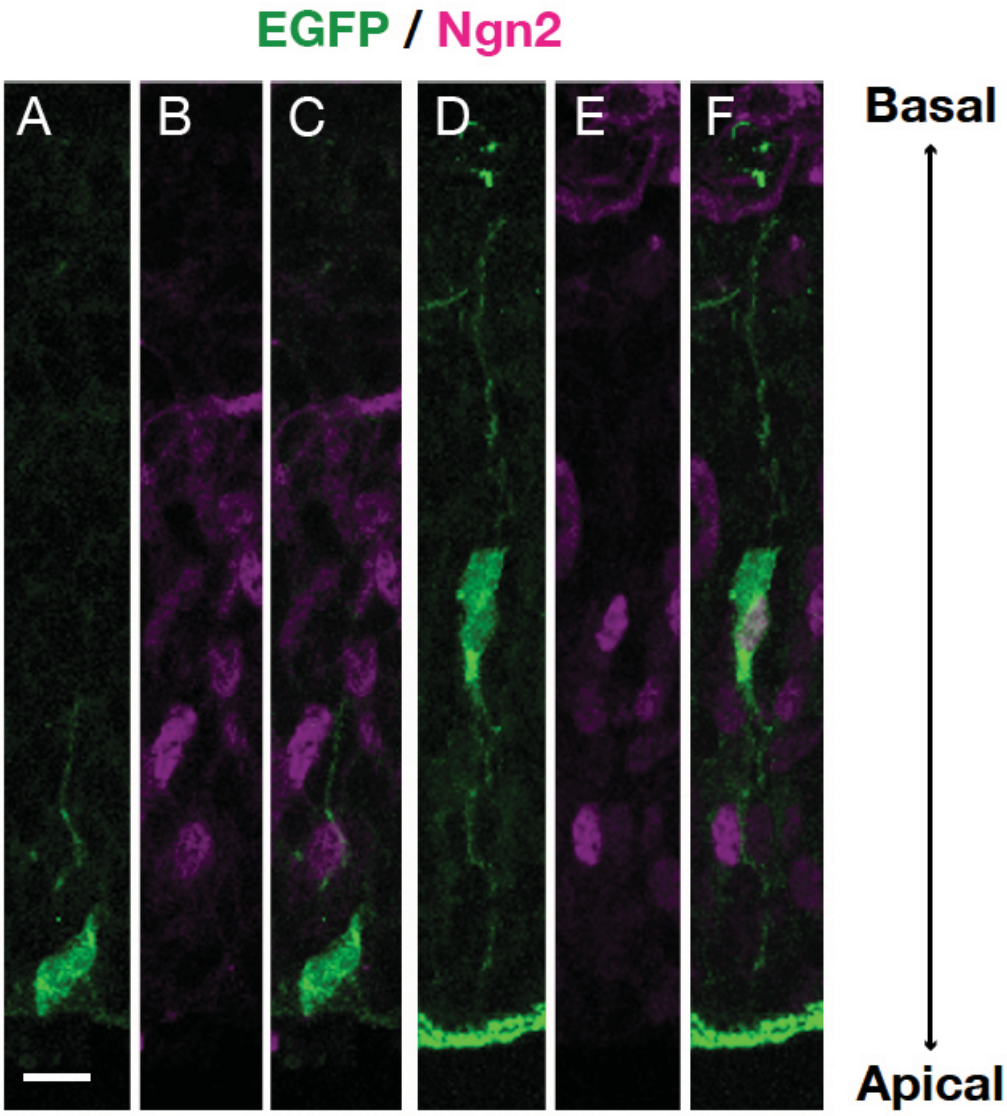
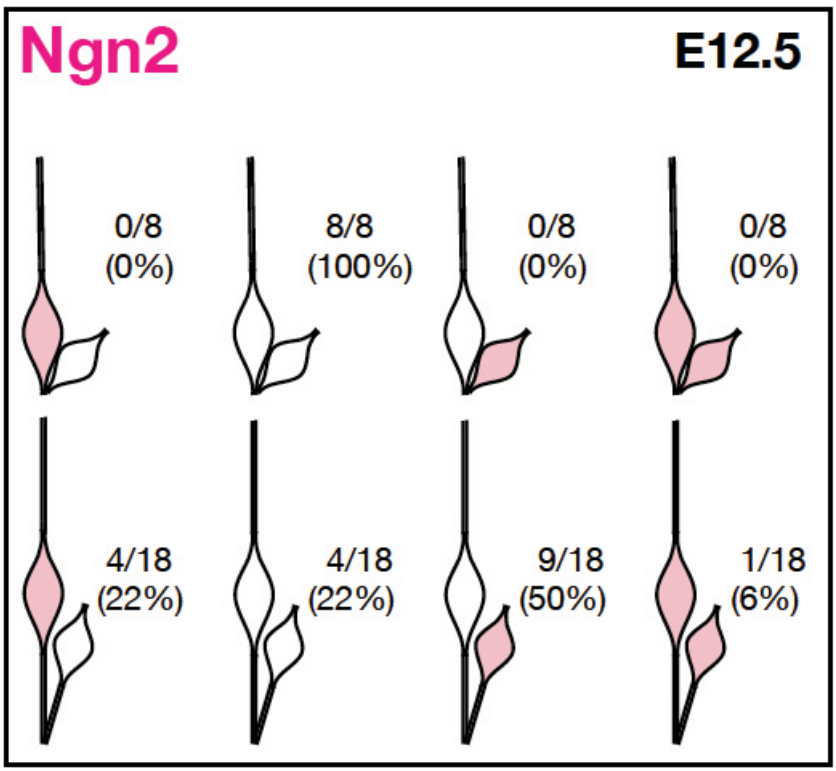


G



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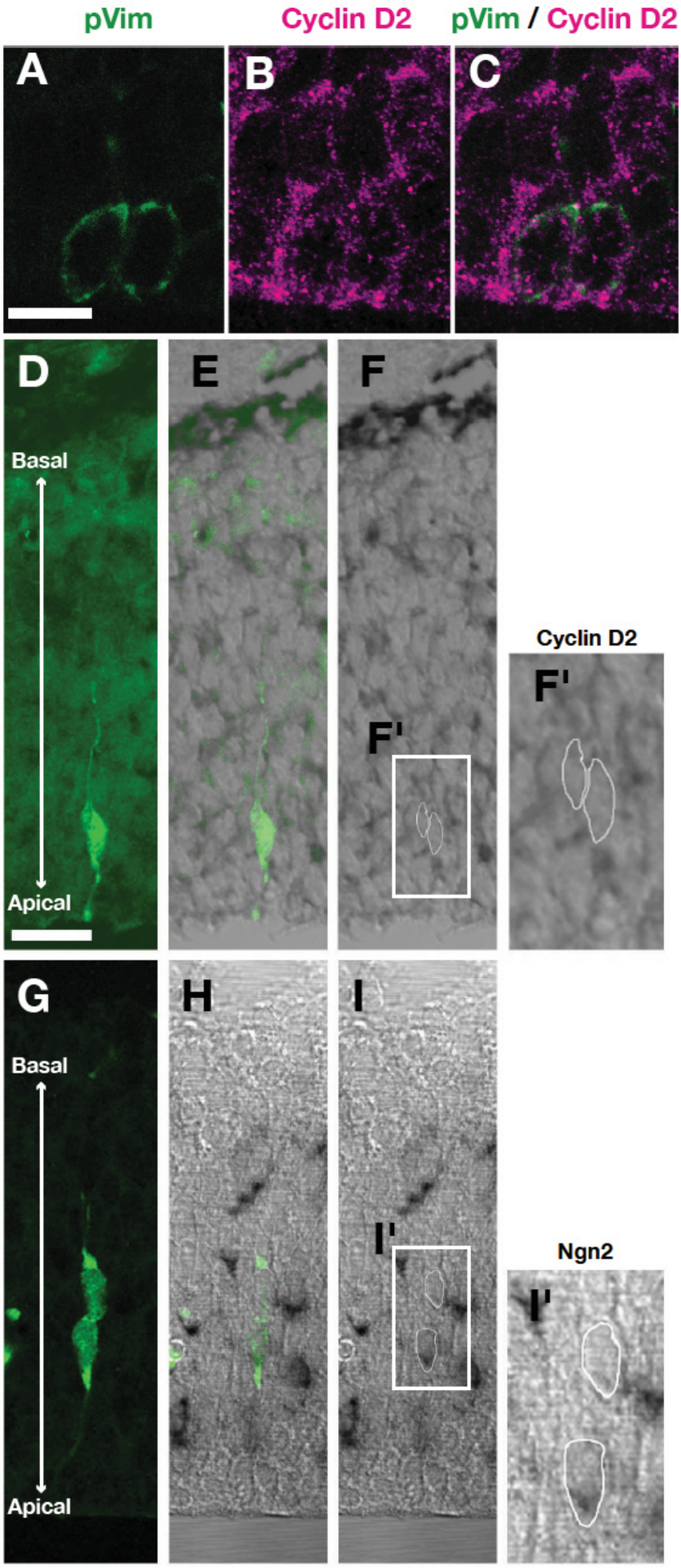

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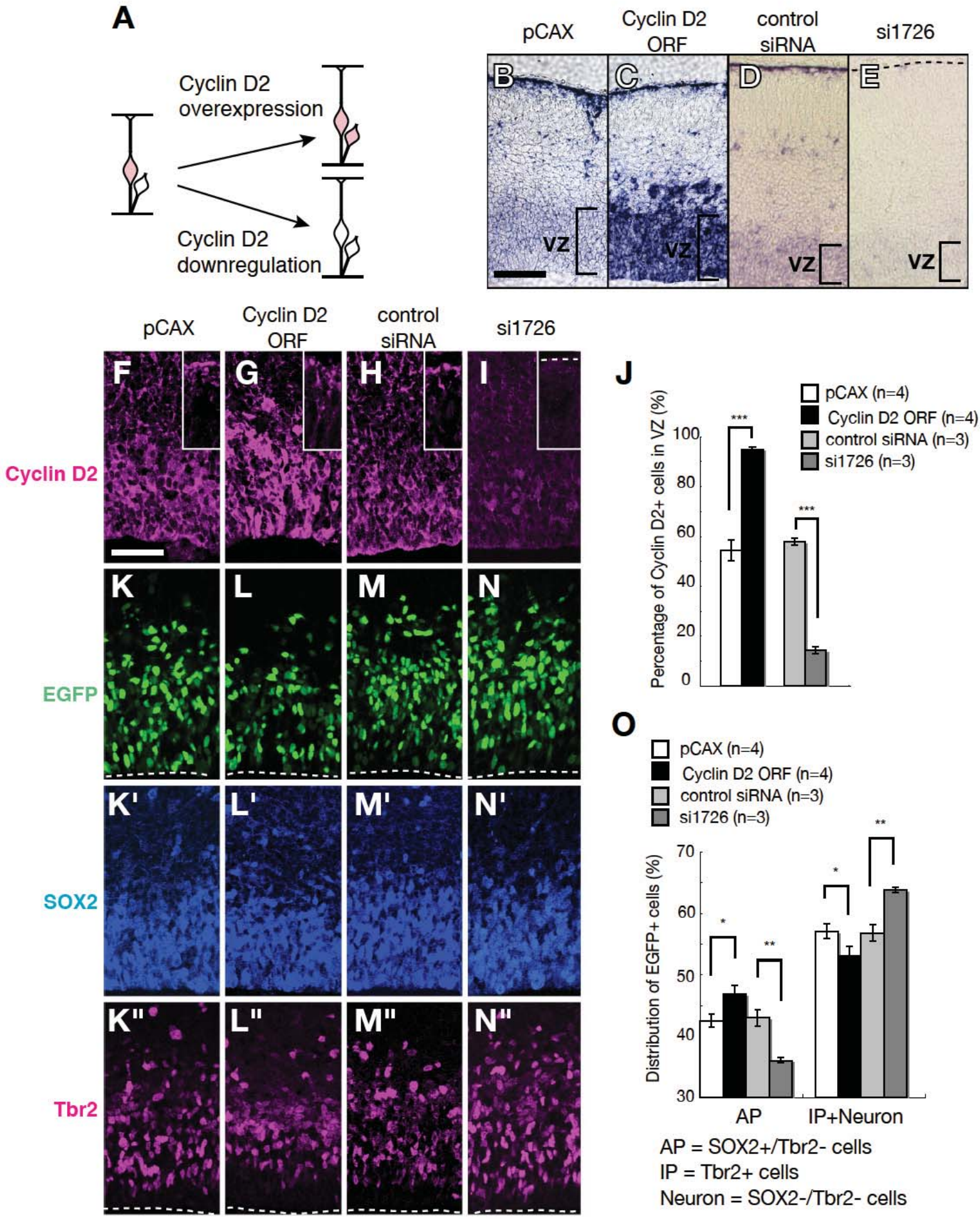

Fig. S8 Tsunekawa