

## Original Article

# Neurogenin2-d4Venus and Gadd45g-d4Venus transgenic mice: Visualizing mitotic and migratory behaviors of cells committed to the neuronal lineage in the developing mammalian brain

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To achieve highly sensitive and comprehensive assessment of the morphology and dynamics of cells committed to the neuronal lineage in mammalian brain primordia, we generated two transgenic mouse lines expressing a destabilized (d4) Venus controlled by regulatory elements of the *Neurogenin2* (*Neurog2*) or *Gadd45g* gene. In mid-embryonic neocortical walls, expression of *Neurog2-d4Venus* mostly overlapped with that of *Neurog2* protein, with a slightly (1 h) delayed onset. Although *Neurog2-d4Venus* and *Gadd45g-d4Venus* mice exhibited very similar labeling patterns in the ventricular zone (VZ), in *Gadd45g-d4Venus* mice cells could be visualized in more basal areas containing fully differentiated neurons, where *Neurog2-d4Venus* fluorescence was absent. Time-lapse monitoring revealed that most d4Venus<sup>+</sup> cells in the VZ had processes extending to the apical surface; many of these cells eventually retracted their apical process and migrated basally to the subventricular zone, where neurons, as well as the intermediate neurogenic progenitors that undergo terminal neuron-producing division, could be live-monitored by d4Venus fluorescence. Some d4Venus<sup>+</sup> VZ cells instead underwent nuclear migration to the apical surface, where they divided to generate two d4Venus<sup>+</sup> daughter cells, suggesting that the symmetric terminal division that gives rise to neuron pairs at the apical surface can be reliably live-monitored. Similar lineage-committed cells were observed in other developing neural regions including retina, spinal cord, and cerebellum, as well as in regions of the peripheral nervous system such as dorsal root ganglia. These mouse lines will be useful for elucidating the cellular and molecular mechanisms underlying development of the mammalian nervous system.

**Key words:** differentiation, *Gadd45g*, neurogenesis, *Neurogenin2*, ventricular zone.

## Introduction

The vertebrate central nervous system develops through accumulation of neurons in the outer region

of the primordial brain wall, while neural progenitor cells stay in the inner region and maintain a neuroepithelial structure called the ventricular zone (VZ). Recent advances in live-imaging techniques have enabled tracking of morphological changes and principal cell-division modes (i.e., asymmetric or symmetric) in sporadically and fluorescently labeled cells in the VZ of mammalian brains. *Neurogenin2* (*Neurog2*) has been implicated in differentiation into the neuronal lineage in a variety of neurodevelopmental contexts and regions, including the neocortex (reviewed in Wilkinson *et al.* 2013), retina (Ma & Wang 2006; Hufnagel *et al.* 2010), cerebellum (Dastjerdi *et al.* 2012; Florio *et al.* 2012), and spinal cord (reviewed in Alaynick *et al.* 2011). Because anti-*Neurog2* immunostaining visualizes only nuclei in the VZ, previous observation of the full morphology and behavior of individual *Neurog2*<sup>+</sup> cells has only been achieved

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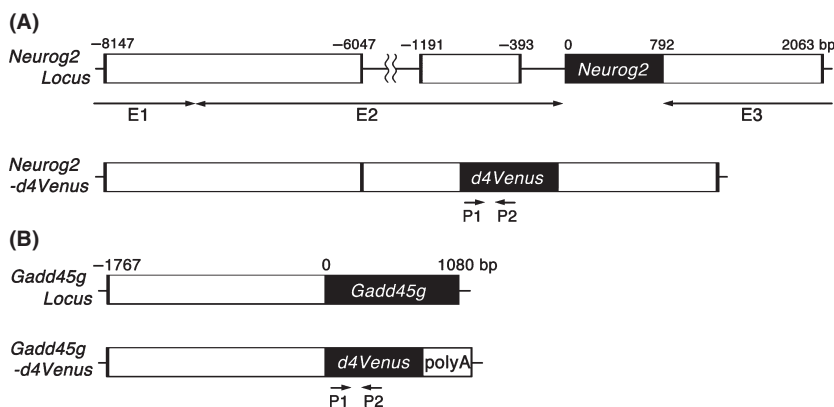
indirectly, by combining 3D information obtained in slice culture (in which sporadic fluorescent labeling was performed with dyes such as Dil) with the results of subsequent immunostaining (Miyata *et al.* 2004; Ochiai *et al.* 2009).

In order to better study the cell–cell interactions (such as one via Delta–Notch signaling) underlying cell-fate determination, as well as the cellular movements that maintain the VZ structure, we wished to directly observe the morphological changes undergone by such lineage-committed (differentiating) cells. To that end, we generated a transgenic mouse line in which *Neurog2*-expressing cells can be sensitively and comprehensively detected within the VZ. Because the expression and activity of *Neurog2* coincide with those of cell-cycle inhibitors (Nguyen *et al.* 2006; Kawaguchi, A. 2008; Shimojo *et al.* 2008; Lacomme *et al.* 2012), we reasoned that another transgenic line with the regulatory elements of *Gadd45g*, a negative cell-cycle regulator (Huang *et al.* 2010; Kaufmann & Niehrs 2011; Ishida *et al.* 2013) expressed in the mammalian VZ (Kawaguchi, A. 2008; Shimojo *et al.* 2008; Arai *et al.* 2011), could also contribute to 4D monitoring of differentiating cells. We carefully characterized *Neurog2*-d4Venus<sup>+</sup> or *Gadd45g*-d4Venus<sup>+</sup> cells in various regions of the developing murine nervous system, especially the neocortex, where lineage-tracking and profiling of progenitors have been performed most extensively (reviewed in Franco & Müller 2013; Greig *et al.* 2013). We found that these two transgenic lines (*Neurog2*-d4Venus and *Gadd45g*-d4Venus) are very useful for studying progenitor heterogeneity and differentiation, as well as morphological dynamics, during development of the nervous system.

## Materials and methods

### *Generation of Neurog2-d4Venus and Gadd45g-d4Venus mice*

To visualize transcriptional activity mediated by the enhancer and promoters of *Neurog2* and *Gadd45g*, we used the fluorescent protein Venus, a derivative of enhanced yellow fluorescent protein (EYFP) characterized by its rapid maturation and increased brightness (Nagai *et al.* 2002). The d4Venus reporter protein includes the mouse ornithine decarboxylase PEST sequence, which acts as a proteolytic signal for intracellular protein degradation (Li *et al.* 1998). The gene for d4Venus was amplified by polymerase chain reaction (PCR) using a plasmid encoding d4Venus (Sunabori *et al.* 2008) as the template and a specific primer set (Table S1). To generate *Neurog2*-d4Venus reporter mice, we identified the *Neurog2* enhancer and promoter from sequences conserved among the human, cattle, mouse, and chicken *Neurog2* loci (Fig. 1A). These regions are partially included in previously reported *cis*-regulatory elements (Scardigli *et al.* 2001; Simmons *et al.* 2001; Fig. 1A). A 2101-bp (–8147 to –6047) fragment of the enhancer region of *Neurog2*, a 799-bp (–1191 to –393) fragment of the promoter region of *Neurog2*, and a 1272-bp (792–2063) fragment of the enhancer and poly-A region of *Neurog2* were amplified by PCR using the C57BL/6N mouse BAC clone (B6Ng01-170F09, purchased from RIKEN BRC) as the template and individual primer sets (Table S1). These amplified fragments were subcloned into the modified pEGFP-N1 plasmid (Clontech), which lacks the cytomegalovirus (CMV) promoter and SV40 poly-A region. The gene for d4Venus was inserted into this plasmid in



**Fig. 1.** Generation of the *Neurog2*-d4Venus and *Gadd45g*-d4Venus mice. (A, B) Structure of the *Neurog2*-d4Venus (A) and *Gadd45g*-d4Venus transgenes (B). Diagrams represent gene loci (upper) and transgene sequences (lower). The translation start site is defined as 0 bp. Black box: coding sequence. Open boxes: enhancers or promoters. In (A), the *Neurog2* enhancer regions E1, E2, and E3 (Scardigli *et al.* 2001) are indicated. P1 and P2 are polymerase chain reaction (PCR) primers for routine genotyping.

place of enhanced green fluorescent protein (EGFP) (Fig. 1A). To generate *Gadd45g-d4Venus* reporter mice, we identified the *Gadd45g* enhancer and promoter in sequences conserved among the human, cattle, mouse, and chicken *Gadd45g* loci (Fig. 1B). A 1768-bp (–1767 to 0) fragment containing the enhancer and promoter region of *Gadd45g* was amplified by PCR using the C57BL/6N mouse BAC clone (B6Ng01-110O13, purchased from RIKEN BRC) as the template and a specific primer set (Table S1). The gene for d4Venus was inserted into the modified pEGFP-N1 plasmid, which lacks the CMV promoter and contains the SV40 poly-A region, in place of EGFP. The enhancer and promoter fragments of *Gadd45g* were subcloned into this plasmid using the In-Fusion HD Cloning kit (TaKaRa) (Fig. 1B). Both purified transgenes (Fig. 1) were individually microinjected into pronuclei of ICR zygotes to generate *Neurog2-d4Venus* (Acc. No. CDB0490T) and *Gadd45g-d4Venus* transgenic mice (Acc. No. CDB0491T: <http://www.cdb.riken.jp/arg/TG%20mutant%20mice%20list.html>). Offspring and embryos of both transgenic mouse lines were routinely genotyped by PCR; primers used to detect both transgenes were as follows: forward P1 (5'-acgtaaacggccacaagtc-3'), reverse P2 (5'-gtcctcctgaagtgcgatgc-3') (Fig. 1). Amplification of genomic DNA using these primers yielded 337-bp product. Details of reporter mouse production will be provided upon request. *Neurog2-d4Venus* (Acc. No. CDB0490T: <http://www.cdb.riken.jp/arg/TG%20mutant%20mice%20list.html>) (Fig. 1A). *Gadd45g-d4Venus* (Acc. No. CDB0491T: <http://www.cdb.riken.jp/arg/TG%20mutant%20mice%20list.html>) (Fig. 1B).

### Plasmids

For overexpression of *Neurog2*, pEF1-*Neurog2* was generated by inserting the open reading frame encoding Neurogenin2 into pEF1. Either mixture of pEF1-*Neurog2* (1.0 µg/L) and pCAGGS-DsRed (Okamoto *et al.* 2013) (0.5 µg/µL) or pCAGGS-DsRed alone (0.5 µg/µL; negative control) was introduced into cells via electroporation.

### Slice culture and imaging

Brain walls and slices were mounted in collagen gel (Miyata *et al.* 2001, 2004), and time-lapse confocal microscopy was performed using an upright CSU-X1 microscope (Yokogawa) equipped with an iXon+ CCD camera (Andor), as described previously (Okamoto *et al.* 2013). Chambers for on-stage culture were filled with 40% O<sub>2</sub>. In some cases, nuclei of all cells were visualized using H2B-mCherry transgenic mice (Abe *et al.* 2011), and cell–cell borders were visualized using

FM4-64 dye (Molecular Probes). Nuclear tracking was performed using the Move-tr software (Library Company, Japan).

### Dissociation cell culture

Neocortical cells were dissociated using trypsin and plated at a density of 1–5 × 10<sup>4</sup>/cm<sup>2</sup> in a small area (0.5 cm<sup>2</sup>) surrounded by a feeder layer of astrocytes (Kawaguchi *et al.* 2004). Imaging was performed manually using an inverted fluorescence microscope (IX70; Olympus), or automatically in on-stage culture using another IX70 microscope; both microscopes were equipped with CCD cameras (Orca ER, Hamamatsu). On-stage imaging was automatically controlled by the METAMORPH 6.3r-1 software (Universal Imaging).

### Immunohistochemistry

Cross-sectional and tangential immunohistochemistry were performed as described previously (Okamoto *et al.* 2013). Brains were fixed with periodate–lysine–paraformaldehyde (PLP) fixative (McLean & Nakane 1974), immersed in 20% sucrose, embedded in OCT compound (Miles), and then frozen and sectioned coronally and tangentially (16 µm). Frozen sections were treated with the following primary antibodies: anti-Pax6 (rabbit, Covance); anti-Ngn2 (mouse, R&D Systems); anti-Tbr2 (EOMES) (rabbit, Abcam); anti-pH3 (rabbit, Millipore); anti-BrdU (rat, Novus Biologicals); anti-p27 (mouse, Transduction Laboratories); anti-βIII-tubulin (mouse, Covance); anti-Lhx1/5 (mouse, Hybridoma Bank); anti-RFP (rabbit, MBL) or anti-GFP (rat, Nacalai Tesque; rabbit, MBL; chicken, Aves Labs). After washes, sections were treated with secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 647 (Molecular Probes, A-11029, A-11006, A-11034, A-11030, A-11035, A-11081, A-21236, A-21245) and subjected to confocal microscopy (Olympus FV1000).

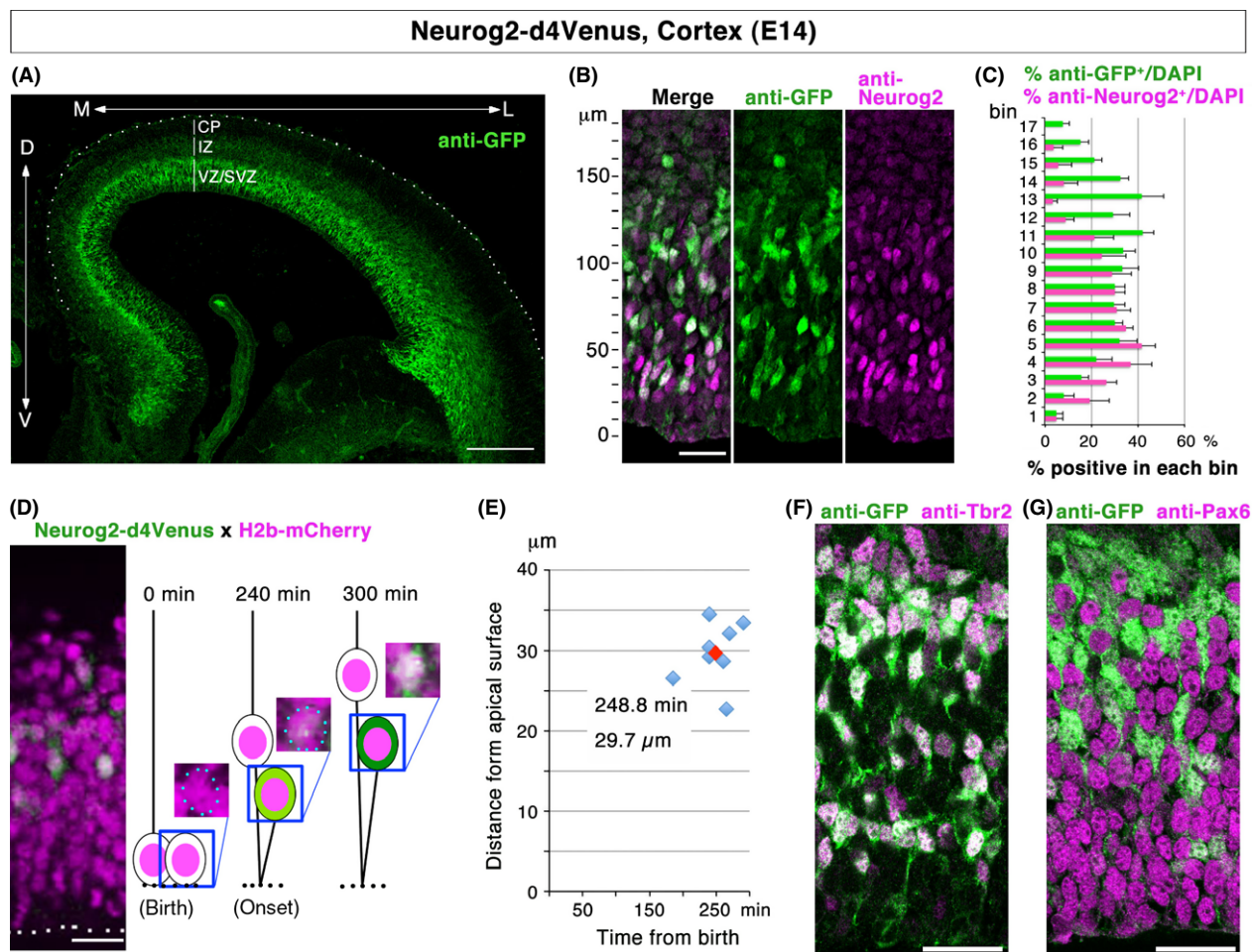
## Results

### *Neurog2-d4Venus*<sup>+</sup> or *Gadd45g-d4Venus*<sup>+</sup> cells in the developing neocortex are under differentiation into the neuronal lineage

To determine whether the *Neurog2-d4Venus* and *Gadd45g-d4Venus* mouse lines allow appropriate visualization of cells differentiating into the neuronal lineage, we first examined developing brain specimens immunohistochemically. In the neocortical wall during the mid-embryonic stage (embryonic day 13–14 [E13–14]), the *Neurog2-d4Venus* signal (anti-GFP immunoreactivity)

was detected from the ventricular zone (VZ) to the sub-ventricular zone (SVZ), mostly overlapping with anti-Neurog2 immunoreactivity (Fig. 2A–C). In the SVZ, however, many anti-GFP<sup>+</sup> cells were negative for Neurog2 protein, whereas in the apical half of the VZ, some Neurog2<sup>+</sup> cells were negative for d4Venus. Together, these observations suggest that expression of d4Venus was slightly delayed (in apical VZ) as well as a bit more persistent (in SVZ) compared to expression of Neurog2

protein. To quantitate the possible delay in detection of differentiating cells, we performed time-lapse observations in mice generated by crossing the *Neurog2-d4Venus* line with an *H2B-mCherry* transgenic line, in which a histone H2B-mCherry fusion protein is ubiquitously expressed under the ROSA26 genomic locus (Abe *et al.* 2011) (Fig. 2D). In this dual-color monitoring system, we could monitor movement of all the nuclei and identify division of progenitors (i.e., the birth of their

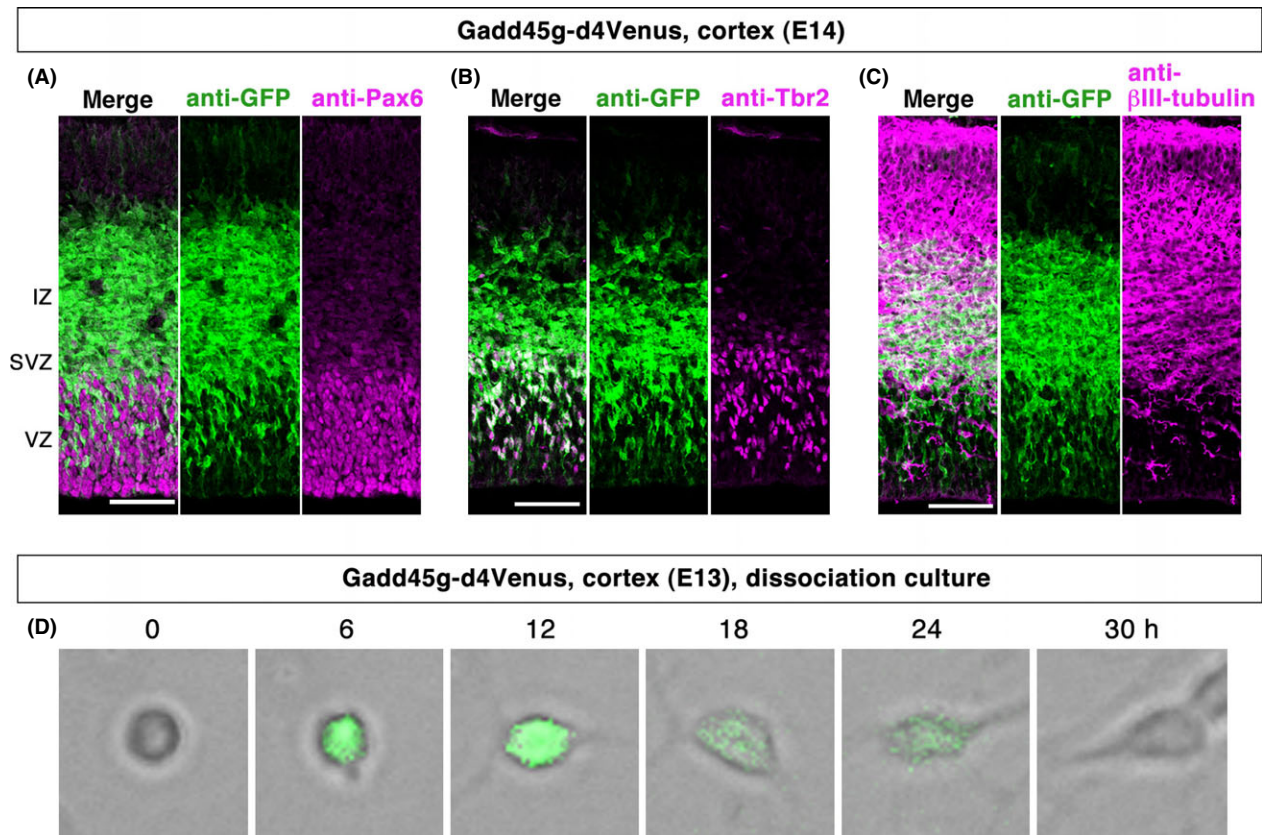


**Fig. 2.** Distribution and emergence of *Neurog2-d4Venus*<sup>+</sup> cells in the developing cerebral wall. (A–C) Anti-GFP immunoreactivity observed in coronal sections of E14 *Neurog2-d4Venus* mice. Intensely labeled cells were observed from the ventricular zone (VZ) to the subventricular zone (SVZ), whereas much weaker signals were observed in the intermediate zone (IZ). The cortical plate (CP) was completely negative. (B–C) Anti-green fluorescent protein (GFP) (green) and anti-Neurog2 (magenta) double immunostaining. (C) Histogram showing the distribution of GFP- or Neurog2-positive cells in the VZ and SVZ. The proportion of total DAPI<sup>+</sup> (4'6'-diamidino-2-phenylindole dihydrochloride) nuclei that were positive for GFP or Neurog2 is indicated in each of the bins (10- $\mu\text{m}$  increments from the ventricular surface). Scaling along the ventricular-pial axis is identical in B and C. (D) Time-lapse observation (every 5 min) on a cerebral wall slice prepared from an E13 *Neurog2-d4Venus* (green)  $\times$  *H2B-mCherry* (magenta) double-transgenic mouse. Schematic diagram and a corresponding image sequence showing that a daughter cell generated from a d4Venus<sup>-</sup> H2B-mCherry<sup>+</sup> progenitor cell at the apical surface began d4Venus expression at 240 min, and that the intensity increased further by 300 min. (E) Graph spatiotemporally depicting the onset of d4Venus expression. Blue dots represent results from eight cells; the average (244.8  $\pm$  11 min, 29.7  $\pm$  1.4  $\mu\text{m}$ ) is shown red. (F) Anti-GFP (green) and anti-Tbr2 (magenta) double immunostaining showing that almost all GFP<sup>+</sup> cells are Tbr2<sup>+</sup>. (G) Anti-GFP (green) and anti-Pax6 (magenta) double immunostaining. Scale bar: 200  $\mu\text{m}$  in A; 20  $\mu\text{m}$  in B, D, F, and G.

daughter cells). It was therefore possible to tell when a given daughter cell generated at the apical surface (from a d4Venus<sup>-</sup> H2B-mCherry<sup>+</sup> cell) became positive for d4Venus (Fig. 2D,E). We found that the expression of Neurog2-d4Venus began approximately 4 h after the birth of a daughter cell at the apical surface, when the nucleus/soma of the positive cell was about 30  $\mu$ m from the apical surface (Fig. 2D,E). The timeline of Neurog2-d4Venus expression was only slightly (approximately 1 h) later than that of Neurog2 protein (Ochiai *et al.* 2009). The Neurog2-d4Venus signal was intense in Tbr2<sup>+</sup> cells (Fig. 2F), but not in fully differentiated neurons occupying the far basal areas (Fig. 2A). These results indicate that *Neurog2-d4Venus* mice are useful for sensitive and specific detection of cells committed to the neuronal lineage in the developing neocortex.

The pattern of Gadd45g-d4Venus expression in the VZ (Fig. 3A) was apparently similar to that of Neurog2-d4Venus in VZ (Fig. 2G). We also characterized this pattern more quantitatively. First, 29% of all VZ cells

(4'-6'-diamidino-2-phenylindole dihydrochloride [DAPI]-labeled) were also Gadd45g-d4Venus<sup>+</sup>, and 30% of all VZ cells were Neurog2-d4Venus<sup>+</sup>. Second, in both lines, 100% of d4Venus<sup>+</sup> cells in the VZ were also Neurog2<sup>+</sup>. Third, the percentage of Neurog2<sup>+</sup> cells that were also d4Venus<sup>+</sup> was similar between the two mouse lines: 84% in *Neurog2-d4Venus* ( $n = 1096$ ) and 82% in *Gadd45g-d4Venus* ( $n = 748$ ). In the SVZ, the Gadd45g-d4Venus signal was more intense than the Neurog2-d4Venus signal. Most Gadd45g-d4Venus<sup>+</sup> cells in the VZ and SVZ were Tbr2<sup>+</sup> (Fig. 3B), and Gadd45g-d4Venus<sup>+</sup> cells in areas from the upper part of the SVZ to the intermediate zone were mostly positive for the neuronal marker  $\beta$ III-tubulin (Fig. 3C). Time-lapse monitoring of single dissociated VZ cells prepared from Gadd45g-d4Venus mice revealed that d4Venus<sup>-</sup> cells became d4Venus<sup>+</sup> and remained fluorescent for up to 24 h; subsequently, as they started to extend neurite-like processes, they converted back to d4Venus<sup>-</sup> (Fig. 3D).



**Fig. 3.** Distribution of Gadd45-d4Venus<sup>+</sup> cells in the developing cerebral wall. (A–C) Double immunostaining with anti-green fluorescent protein (GFP) (green) and either anti-Pax6 (magenta) (A), anti-Tbr2 (magenta) (B), or anti- $\beta$ III-tubulin (magenta) (C), on cortical sections prepared from E14 *Gadd45g-d4Venus* mice. IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bar: 50  $\mu$ m. (D) Time-lapse monitoring of a single VZ cell isolated from an E13 *Gadd45g-d4Venus* mouse. Note that the cell became d4Venus<sup>+</sup> by 6 h, maintained its fluorescence intensely until 12 h, became less intense as it extended neurite-like processes (18–24 h), and finally became d4Venus-negative (30 h).

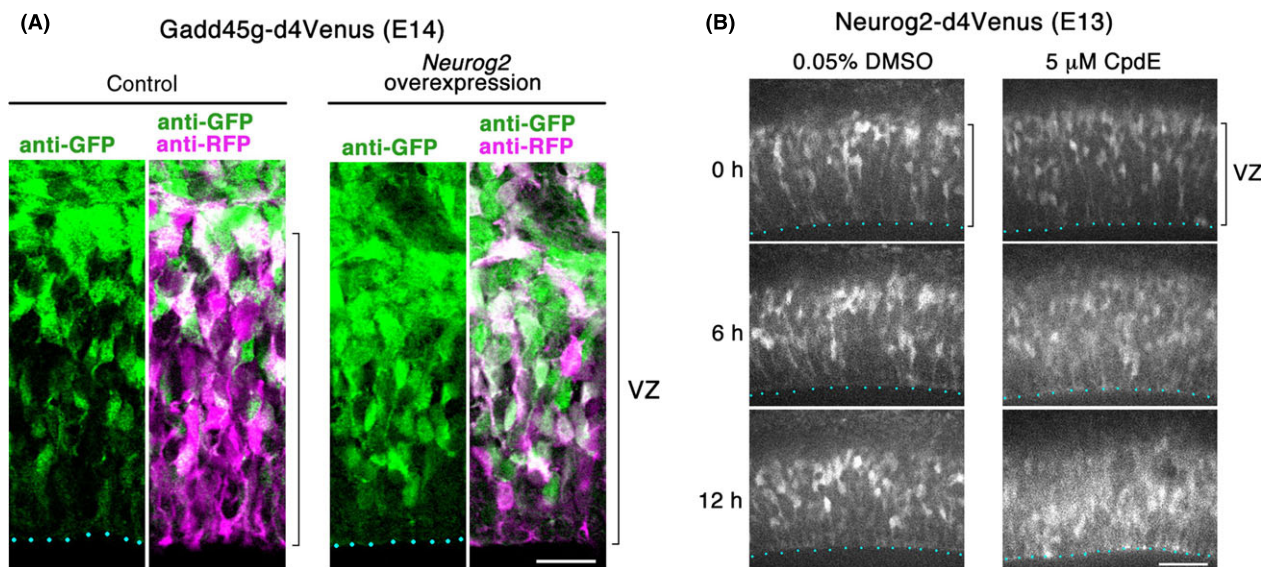
We conducted additional functional evaluations by experimentally inducing differentiation. Artificial expression of *Neurog2* increased the proportion of *Gadd45g-d4Venus*<sup>+</sup> cells in VZ (Fig. 4A). Addition of  $\gamma$ -secretase inhibitors, DAPT (data not shown), or compound E (Fig. 4B) to 3D slice cultures, in order to compromise Notch signaling, dramatically increased *Neurog2-d4Venus* expression. These results suggest that these reporter lines enable faithful visualization of cells that are differentiating into the neocortical neuronal lineage.

*Intermediate neurogenic progenitors dividing basally or apically in the neocortical wall*

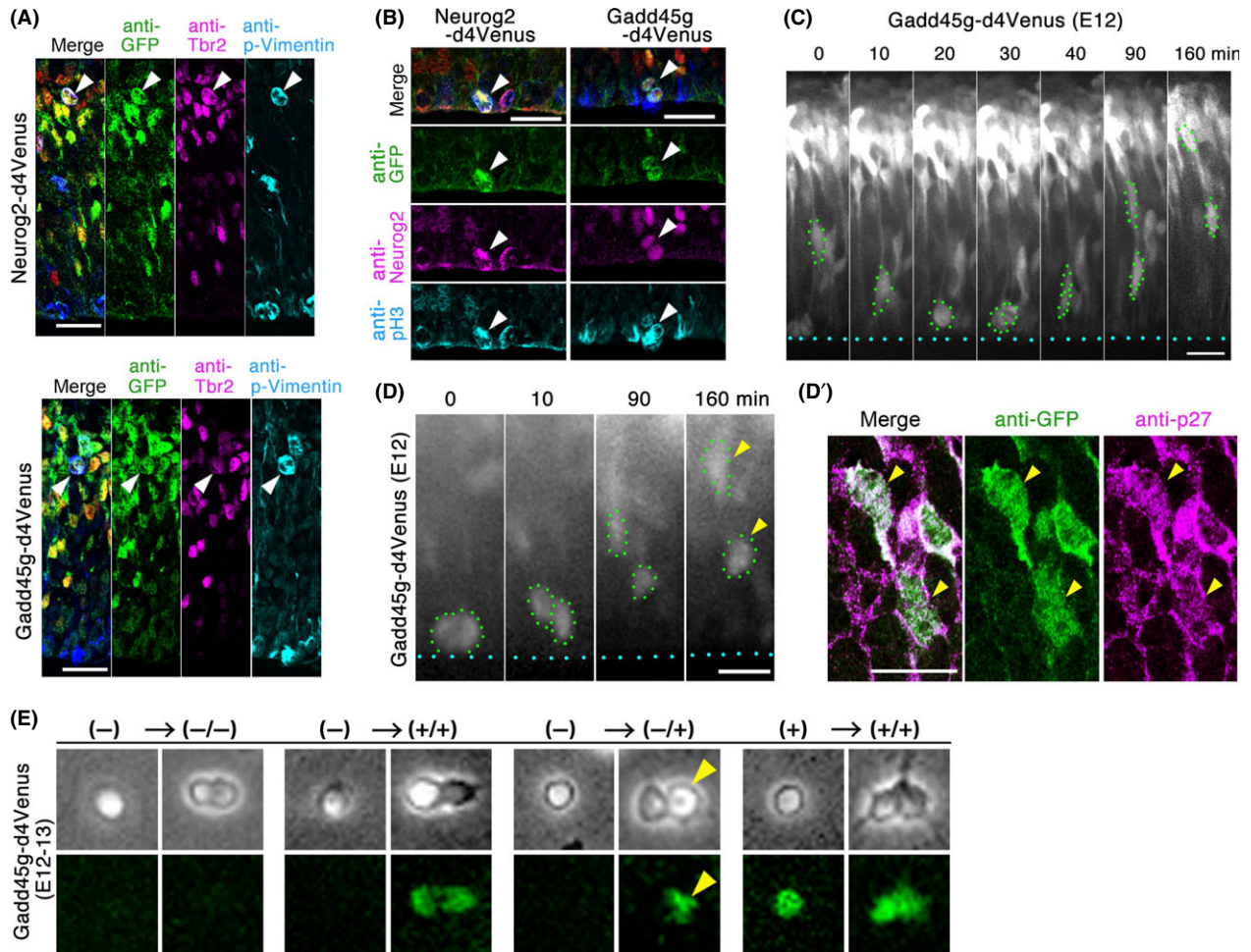
In E13–14 neocortical walls, we observed *Neurog2-d4Venus*<sup>+</sup> or *Gadd45g-d4Venus*<sup>+</sup> cells in S-phase (labeled with BrdU administrated 20 min before fixation) (data not shown) or M phase (pH3<sup>+</sup> or p-Vimentin<sup>+</sup>) (Fig. 5A,B) of the cell cycle. Most of the *Neurog2-d4Venus*<sup>+</sup> or *Gadd45g-d4Venus*<sup>+</sup> M-phase cells were in the SVZ, corresponding to the well-known non-stem-like, lineage-restricted progenitor cells that divide basally to give rise to neuron pairs (Haubensak *et al.* 2004; Miyata *et al.* 2004; Noctor *et al.* 2004) (called “basal progenitors” [BP]). We also observed *Neurog2-d4Venus*<sup>+</sup> or *Gadd45g-d4Venus*<sup>+</sup> cells in M phase at the apical surface (Fig. 5B); we

speculated that these cells ( $4.8 \pm 1.2\%$  of the total M-phase population at the apical surface) may be another type of progenitor committed to the neuronal lineage. The existence of such cells was previously suggested based on expression patterns of *Tbr2-GFP* or *Tis21-GFP*, and they are called the intermediate neurogenic progenitors dividing apically (Kowalczyk *et al.* 2009). They are referred to as  $\text{INP}_{\text{VZ}}$  (in contrast to  $\text{INP}_{\text{SVZ}}$  which corresponds to the BP [Nelson *et al.* 2013;]) and are also known as the short neural precursors (SNPs) (Tyler & Haydar 2013). The behaviors of  $\text{INP}_{\text{VZ}}$  and their daughter cells are not well understood.

We successfully observed apical divisions of  $\text{INP}_{\text{VZ}}$  (Fig. 5C–D'). We found no difference in the intensity of *Gadd45g-d4Venus* between sister cells that were generated from single  $\text{INP}_{\text{VZ}}$  cells (14 of 14 apically generated pairs). We also did not observe mother–daughter differences in *d4Venus* intensity (i.e., the intensity was comparable between approximately 5-h-old daughter cells and their apically dividing *d4Venus*<sup>+</sup> mothers) (14/14). Daughter cells pair-generated from  $\text{INP}_{\text{VZ}}$  were positive for p27 (Fig. 5D,D'), a cell-cycle inhibitor. These results strongly suggest that apically dividing *d4Venus*<sup>+</sup> cells are indeed non-stem-like and committed to the neuronal lineage, and that these cells give rise to daughter cells that adopt differentiated fates. Interestingly, we observed  $\text{INP}_{\text{VZ}}$  division



**Fig. 4.** Functional evaluation of *Gadd45g-d4Venus* (A) and *Neurog2-d4Venus* (B) mice by experimental manipulations that induced differentiation. (A) Anti-green fluorescent protein (GFP) (to detect *d4Venus*, green) and anti-RFP (to detect DsRed, magenta) immunostaining of E14 *Gadd45g-d4Venus* cerebral walls that had been electroporated at E13 with control (DsRed only) or *Neurog2* expression plasmids. Note that % *d4Venus*<sup>+</sup>/DsRed<sup>+</sup> in VZ was much greater in cells overexpressing *Neurog2* (95%) than in control cells (46%). (B) Live observation of cerebral wall slices prepared from E13 *Neurog2-d4Venus* mice. Treatment with compound E, a  $\gamma$ -secretase inhibitor, resulted in a slight increase in the proportion of *d4Venus*<sup>+</sup> cells by 6 h, and more diffuse expression in the VZ by 12 h, whereas the number of *d4Venus*<sup>+</sup> cells was almost constant in the control (DMSO [dimethylsulfoxide]-treated) slice. Apical surface, cyan dashed line. Scale bar: 20  $\mu\text{m}$  in A; 50  $\mu\text{m}$  in B.



**Fig. 5.** Division of neocortical *Neurog2-d4Venus*<sup>+</sup> and *Gadd45g-d4Venus*<sup>+</sup> cells. (A) Triple immunostaining with anti-green fluorescent protein (GFP) (green), anti-Tbr2 (magenta), and anti-p-Vimentin (cyan) (A) on cortical sections prepared from E14 *Neurog2-d4Venus* (upper) or *Gadd45g-d4Venus* (lower) mice. (B) Triple immunostaining with anti-GFP (green), anti-Neurog2 (magenta), and anti-pH3 (cyan) on cortical sections prepared from E14 *Neurog2-d4Venus* (left) or *Gadd45g-d4Venus* (right) mice. Note that both basally dividing (arrowhead in A) and apically dividing cells (arrowhead in B) expressing differentiation markers (Tbr2 or Neurog2) were d4Venus<sup>+</sup>, corresponding to INP<sub>SVZ</sub> (BP) and INP<sub>VZ</sub>, respectively. (C) Time-lapse observation exemplifying division of INP<sub>VZ</sub> in a cerebral wall slice prepared from an E12 *Gadd45g-d4Venus* mouse (Movie S1). (D, D') Another INP<sub>VZ</sub> division in an E12 *Gadd45g-d4Venus* cerebral wall in which both daughter cells (arrowhead, d4Venus<sup>+</sup>) were subsequently (at 160 min) found to be p27<sup>+</sup>, indicating that the original d4Venus<sup>+</sup> cell divided symmetrically, giving rise to two neurons. Cyan dashed line: apical surface. (E) Low-density (clonal) culture of dissociated cells harvested from E12 or E13 *Gadd45g-d4Venus* cerebral walls. Phase-contrast images (upper panels) show division of the original cell and formation of two-cell clones. Both symmetric (- → -/-, - → +/+) and asymmetric (+ → -/+, arrowhead) division patterns were inferred based on d4Venus expression patterns. Scale bar: 20 μm in A, B; 10 μm in C-D'.

cases in which the original *Gadd45g-d4Venus*<sup>+</sup> progenitor was radially elongated spanning from the apical surface to the basal surface and its basal process was subsequently inherited by one of the apically-generated d4Venus<sup>+</sup> daughter cells (Movie S1, Fig. 5C). Basalward nuclear movement of the process-inheriting d4Venus<sup>+</sup> daughter was quicker than that of its sister d4Venus<sup>+</sup> cell (Fig. 5C), which is similar to the pattern observed in clones generated api-

cally from undifferentiated progenitors (Okamoto *et al.* 2013, 2014).

In clonal-density culture, dissociated *Gadd45g-d4Venus*-negative or d4Venus-positive E12 or E13 neocortical cells divided and formed two-cell clones (Fig. 5E). Symmetric (- → -/- or - → +/+) and asymmetric (- → -/+) clones were formed by d4Venus-negative cells. Symmetric division of d4Venus<sup>+</sup> cells (+ → +/+) was also observed. These

results suggest that these transgenic mouse lines are useful for dissecting progenitor heterogeneity, focusing on the place and mode of division.

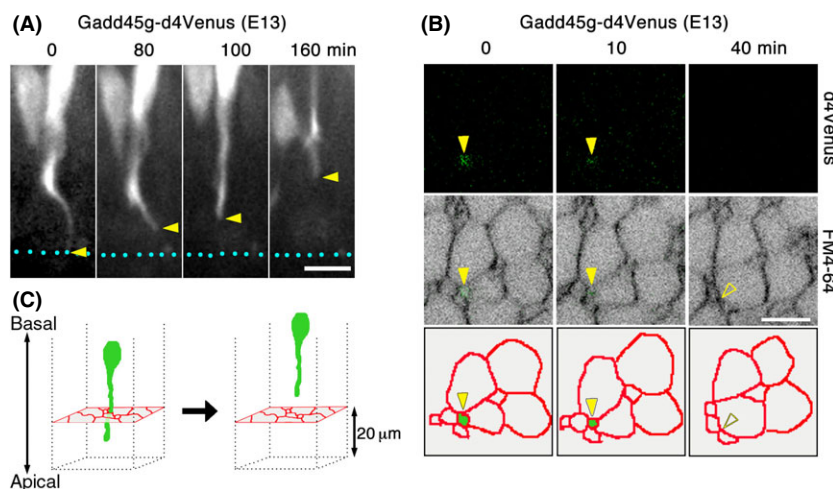
#### Monitoring of delamination from the apical surface

Previous studies suggested that translocation of neocortical cells committed to the neuronal lineage from VZ to SVZ occurs mostly as follows (Miyata *et al.* 2001, 2004; Noctor *et al.* 2004; Ochiai *et al.* 2007; Konno *et al.* 2008; Ochiai *et al.* 2009; Shitamukai *et al.* 2011; Itoh *et al.* 2013). Daughter cells generated from apically dividing progenitor cells are initially attached to the apical surface, with their apical processes integrated into the meshwork comprised by the endfeet of all of the VZ cells. Upon lineage commitment, some of these apically connected daughter cells retract their apical processes and migrate basally to exit the VZ. This developmental scenario has only been inferred indirectly by combining live imaging data and subsequent immunostaining, or by making live observations much longer than the total cell-cycle length, so that particular cells can be identified as non-mitotic (i.e., neurons). *Neurog2-d4Venus* and *Gadd45g-d4Venus* mice have now enabled us to directly monitor how cells committed to the neuronal lineage detach themselves (i.e., delaminate) from the apical surface. Although both lines permit visualization of apically connected processes (Figs 2–5), the *Gadd45g-d4Venus* line was more suitable for high-

resolution live imaging (Fig. 6A). Tangential sectional live observation of *Gadd45g-d4Venus* cerebral tissues labeled with FM4-64 (which visualizes cell–cell borders) demonstrated that *d4Venus*<sup>+</sup> apical processes shrank and disappeared (Fig. 6B).

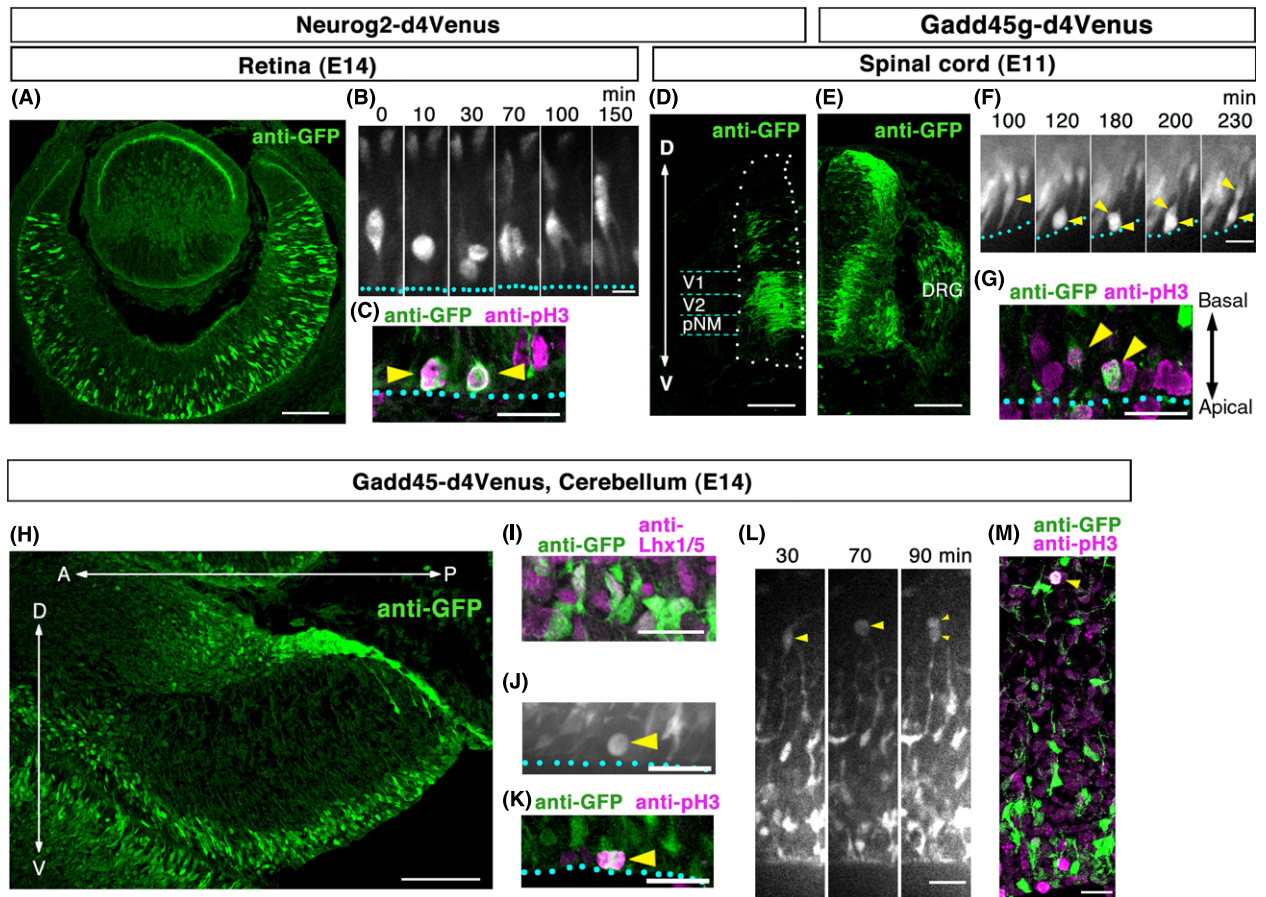
#### *Neurog2-d4Venus*<sup>+</sup> or *Gadd45g-d4Venus*<sup>+</sup> cells in other regions

Because *Neurog2* or *Gadd45g* are also implicated in development of other regions including the retina (Ma & Wang 2006; Hufnagel *et al.* 2010), spinal cord (reviewed in Alaynick *et al.* 2011), dorsal root ganglia (Kaufmann *et al.* 2011), and cerebellum (Dastjerdi *et al.* 2012; Florio *et al.* 2012), we analyzed these tissues. In the developing retina (E14), *Neurog2-d4Venus* mice permitted better visualization of differentiating cells (Movie S2, Fig. 7A) than *Gadd45g-d4Venus* mice, and apically dividing *d4Venus*<sup>+</sup> cells (21.2% of the total apically located M-phase cells) (Fig. 7B,C), as well as occasional non-surface-dividing *d4Venus*<sup>+</sup> cells (Movie S2), could be clearly observed. In the spinal cord (E11) (Fig. 7E–G) and cerebellum (E14) (Fig. 7H–M), *Gadd45g-d4Venus* mice enabled very intense visualization of differentiating cells, including motor neurons, whereas *Neurog2-d4Venus* mice exhibited weaker fluorescence. Apically dividing *d4Venus*<sup>+</sup> cells were more frequently found in the spinal cord (11.1%) than in the neocortex (4.8%) and cerebellum (4.0%). The *Neurog2-d4Venus* signal in the spinal cord was most



**Fig. 6.** Retraction of apical process of basalward-migrating *Gadd45g-d4Venus*<sup>+</sup> cells. (A) Time-lapse sequence showing retraction of the apical process (arrowhead) of a single *d4Venus*<sup>+</sup> cell in an E13 *Gadd45g-d4Venus* mouse cerebral wall slice. Concomitantly with the basalward movement of the soma (disappearing from the field by 160 min), the apical process (arrowhead) was retracted, displaying typical delamination behavior of differentiating VZ cells (Miyata *et al.* 2004; Ochiai *et al.* 2007, 2009). Cyan dashed line: apical surface. (B) Dual-color time-lapse monitoring using an E13 *Gadd45g-d4Venus* mouse and FM4-64 dye to visualize cell–cell borders. Tangential-sectional images obtained at 20 μm from the apical surface are shown. Note the basalward retraction of a *d4Venus*<sup>+</sup> apical process (arrowhead), which is schematically illustrated in (C). Scale bar: 5 μm.





**Fig. 7.** Distribution and division of d4Venus<sup>+</sup> cells in other neurodevelopmental regions. (A–C) Anti-green fluorescent protein (GFP) (A, C) and live (B) observations showing Neurog2-d4Venus<sup>+</sup> cells in the retina (E14). The basalmost zone in the central retina was negative for d4Venus (A). About 21% of apically dividing cells were Neurog2-d4Venus<sup>+</sup> (B, C). (D–G) Anti-GFP (D, E, G) and live (F) observations showing d4Venus<sup>+</sup> cells in the spinal cord of an E11 Neurog2-d4Venus mouse (D) or an E11 Gadd45g-d4Venus mouse (E–G). About 11% of apical M-phase cells were Gadd45g-d4Venus<sup>+</sup>. Although Neurog2-d4Venus expression (D) and Gadd45g-d4Venus expression (E) overlapped in the VZ, Neurog2-d4Venus exhibited a dorsoventrally narrower distribution than Gadd45g-d4Venus; Neurog2-d4Venus signal was absent in the basal (outer) spinal cord regions containing fully differentiated neurons, as well as in the dorsal root ganglion (DRG). (H–M) Anti-GFP (H, I, K, M) and live (J, L) observations in the cerebellum (sagittally sliced) of E14 Gadd45g-d4Venus mice. Many of the d4Venus<sup>+</sup> cells were positive for Lhx1/5, a differentiation marker (Miyata *et al.* 2010). Live images (F, L) are snapshots taken from Supporting Information Movies S3 and S4. Scale bar: 200  $\mu$ m in A, D, E, H; 10  $\mu$ m in B; 20  $\mu$ m in C, F, G, I–M.

intense in a ventral region including the V1, V2, and pMN domains, probably reflecting the activity of the E1 enhancer (Scardigli *et al.* 2001), whereas a dorsal region corresponding to the E3 enhancer-regulated domain also contained fewer clearly labeled cells (Fig. 7D). Moreover, Gadd45g-d4Venus fluorescence was intense in the dorsal root ganglia (Fig. 7E) and the sympathetic trunk (data not shown), both of which are derivatives of the neural crest. In the cerebellum, division of Gadd45g-d4Venus<sup>+</sup> cells away from the apical surface was observed. Time-lapse monitoring showed that these non-surface divisions occurred following somal translocation of Gadd45g-d4Venus<sup>+</sup> cells (Movie S4, Fig. 7L).

## Discussion

Several reporter mouse lines designed to allow visualization of cells committed to the neuronal lineage have previously been generated. These include the *Tis21-nucGFP* knock-in (Haubensak *et al.* 2004), the *E1-Ngn2-Cre;Z/AP* double transgenic (Berger *et al.* 2004), the *Eomes::GFP* transgenic (Kwon & Hadjantonakis 2007), and the *Tubb3-mGFP (beta-III-tubulin-GAP43-GFP)* transgenic (Attardo *et al.* 2008). Our *Neurog2-d4Venus* and *Gadd45g-d4Venus* transgenic lines, which confer advantages in sensitivity and morphological discrimination, expand this repertoire. These novel lines will be very helpful in studying

how cell-fate determination (or acquisition) occurs in the VZ.

In the mid-embryonic neocortical VZ/SVZ, overall cytotogenesis is binary, that is, the entire progenitor population gives rise to both undifferentiated (stem-like) and differentiating daughter cells at similar rates. Although progenitors in the SVZ are largely committed to the neuronal lineage (INP<sub>SVZ</sub> or BP generating symmetric neuron pairs), the fate choices adopted by daughter cells generated at the apical surface are more complicated and mysterious (Lui *et al.* 2011; Peyre & Morin 2012; Shitamukai & Matsuzaki 2012). One important mechanism underlying this population-wide asymmetric daughter-cell fate choice with clonal variation is the Delta–Notch interaction within the VZ. Cells committed to the neuronal lineage (represented by INP) express Delta (Kawaguchi, A. 2008; Kawaguchi, D. 2008; Shimojo *et al.* 2008; Yoon *et al.* 2008). These Delta-expressing (i.e., differentiating) cells may dynamically interact with the surrounding VZ cells (Nelson *et al.* 2013) to activate Notch, thereby allowing these Notch-activated cells to remain undifferentiated.

Our previous analysis in slice culture demonstrated formation of three different types of two-cell clones by apically dividing progenitors: (i) Neurog2<sup>+</sup>/Neurog2<sup>+</sup>; (ii) Neurog2<sup>+</sup>/Neurog2<sup>-</sup>; and (iii) Neurog2<sup>-</sup>/Neurog2<sup>-</sup> (Ochiai *et al.* 2009). Although there are several models to explain the aforementioned population-wide asymmetric (binary) fate choice (Peyre & Morin 2012; Shitamukai & Matsuzaki 2012), it is unclear how each of these clones, which differ in cellular composition, arises. Our previous timeline study demonstrated that substantial Neurog2 protein expression in nascent apically generated daughter cells starts around 3 h after their birth (Ochiai *et al.* 2009), suggesting that daughter cells may be neutral in fate (or at least not fully committed) until that age (i.e., 3 h). Therefore, it will be important to investigate the possible association between whether and how such presumably neutral daughter cells (<3 h) encounter the pre-existing Delta-presenting neighbors, and which fate(s) these daughters subsequently choose (i.e., whether they become d4Venus<sup>+</sup> or remain d4Venus<sup>-</sup> at 4 h and later). Such analysis of the relationship between a daughter cell's "encounter histories" and its subsequent fate choice can be performed using the new transgenic mouse lines we describe here. Delta-expressing neighbors can probably be represented by Neurog2-d4Venus<sup>+</sup> or Gadd45g-d4Venus<sup>+</sup> cells, whereas commitment of the youngest (neutral) daughter cells to the neuronal lineage can also be detected sensitively by Neurog2-d4Venus or Gadd45g-d4Venus fluorescence. Our preliminary results suggest that the use of these mice in combination with FM4-64-based visualization of the

borders of all VZ cells will enable us to generate 4D reconstructions (Kawaue, T., Okamoto, M., Shinoda, T., Kawaguchi, A. & Miyata, T unpubl. data, 2014).

Visualization of whole cellular morphologies, including cellular processes, contributes not only to detailed analysis of cell–cell associations/neighborship, as mentioned above, but will also contribute to future studies of the molecular mechanisms by which INPs choose between delamination from and connection to the apical surface. One relevant question to be addressed using these transgenic mice is why most neocortical INP delaminate from the apical surface, and only rarely divide there, whereas other regions (e.g., retina and spinal cord) contain much more abundant apically dividing INPs. It would also be interesting to study whether the somal translocation that progenitors committed to the neuronal lineage exhibit just before their non-surface mitosis (Miyata *et al.* 2004) (Movie S4, Fig. 7L) and that of the outer suventricular zone radial glia-like (oRG) cells, which is termed "mitotic somal translocation" and implicated in the evolution of the neocortex (Lui *et al.* 2011; Gertz *et al.* 2014), are regulated differently or similarly.

## Author contributions

T.K. performed all characterizations of the transgenic mice and wrote the manuscript. K.S. carried out the initial screening of the *Neurog2* and *Gadd45g* gene-regulatory regions and generated the transgene constructs. H.K. generated the transgenic mice. K.O., M.O., and T.S. contributed to live imaging. A.K. and T.M. designed the experiments and wrote the manuscript.

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## Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

**Movie S1.** Time-lapse observation of division of INP<sub>VZ</sub> in a cerebral wall slice prepared from an E12 *Gadd45g-d4Venus* mouse. Note that the apically-dividing *Gadd45g-d4Venus*<sup>+</sup> cell (green arrow) had a process extended to the basal region. The basal process (magenta arrow) was inherited by one of the two daughter cells (arrowhead).

**Movie S2.** Time-lapse observation of division of *d4Venus*<sup>+</sup> cells in a retinal slice prepared from an E14 *Neurog2-d4Venus* mouse. Most divisions occurred at the apical surface (green arrow), while some *d4Venus*<sup>+</sup> cells divided away from the apical surface (magenta arrow).

**Movie S3.** Time-lapse observation in a spinal cord slice prepared from an E11 *Gadd45g-d4Venus* mouse. Apically-dividing *d4Venus*<sup>+</sup> cells (arrow) were observed.

**Movie S4.** Time-lapse observation in a cerebellar slice prepared from an E14 *Gadd45g-d4Venus* mouse. Note that many *d4Venus*<sup>+</sup> cells exhibited somal translocation using processes extended mainly towards the basal (pial) side, and that one of them (cell 1) subsequently divided at a non-surface position.

**Table S1.** Primers for generation of *Neurog2-d4Venus* and *Gadd45g-d4Venus*.