Rac-deficient cerebellar granule neurons die before they migrate to the internal granule layer

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Granule neurons are the most common cell type in the cerebellum. They are generated in the external granule layer and migrate inwardly, forming the internal granule layer. Small Rho GTPases play various roles during development of the nervous system and may be involved in generation, differentiation and migration of granule neurons. We deleted Rac1, a member of small Rho GTPases, by GFAP-Cre driver in cerebellar granule neurons and Bergmann glial cells. Rac1lox/lox; Cre mice showed impaired migration and slight reduction in the number of granule neurons in the internal granule layer. Deletion of both Rac1 and Rac3 resulted in almost complete absence of granule neurons. Rac-deficient granule neurons differentiated into p27 and NeuN-expressing post mitotic neurons, but died before migration to the internal granule layer. Deletion of Rac3 has little effect on granule neuron development. Rac1lox/lox; Rac3+/−; Cre mice showed intermediate phenotype between Rac1lox/lox; Cre and Rac1lox/lox; Rac3−/−; Cre mice in both survival and migration of granule neurons. Rac3 itself seems to be unimportant in the development of the cerebellum, but has some roles in Rac1-deleted granule neurons. Conversely, overall morphology of Rac1lox/lox; Rac3−/−; Cre cerebella was normal. One allele of Rac1 is therefore thought to be sufficient to promote development of cerebellar granule neurons.

Small Rho GTPases play a variety of roles during development of the nervous system, including neurogenesis, differentiation, migration, dendritogenesis, axon guidance and synapse formation1,2. Rac subfamily of small Rho GTPases consists of three members: Rac1, Rac2 and Rac3. Physiological functions of Rac1 have been studied using knockout mice. Although Rac2 expression can be found only in hematopoietic cells3, Rac3 is ubiquitously expressed in the nervous system4,5. Rac3 may therefore have some roles in Rac1-deficient neuronal cells, creating difficulty in studying the functions of “Rac” in the nervous system.

Granule neurons are the most common cell type in the cerebellum. Cerebellar granule neuron precursors are generated in the rhombic lip and migrate tangentially along the surface of the cerebellar primordium to form the external granular layer (EGL). Granule neuron precursors continue to proliferate in the EGL and then exit the cell cycle to differentiate into granule neurons. Differentiated granule neurons then migrate inwardly past the Purkinje cells and eventually form the internal granular layer (IGL), where they extend their axons into the molecular layer to form parallel fibers6. Cerebellar granule neurons were thought to be devoid of Rac3 and predominantly express Rac14, and deletion of Rac1 in cerebellar granule neurons resulted in impaired migration and axon formation7. Detailed analysis revealed, however, that cerebellar granule neurons express not only Rac1, but also Rac3 during development, and that deletion of Rac3 in addition to Rac1 resulted in much more severe phenotype than that of Rac1-knockout mice8. Deletion of Rac1 by Atoh1-Cre driver in addition to Rac3 resulted in agenesis of the IGL in the anterior medial part of the cerebellum. Cerebellar granule neurons deficient in both Rac1 and Rac3 can differentiate normally until the expression of NeuN, a marker for postmitotic cerebellar granule neurons. After that, however, they exhibit defects in neuritogenesis and die by apoptosis in the deep layer of the EGL before migrating to the IGL, resulting in agenesis of the IGL.

Small Rho GTPases play a variety of roles during development and they may play different roles within the different developmental stages. For example, deletion of RhoA in the cerebral cortical neurons by FoxG1-Cre driver induced disruption of adherens junctions and hyperproliferation of neural progenitor cells9, whereas that by Emx1-Cre mouse resulted in double cortex formation due to radial glial scaffold disruption10. To elucidate the variety of roles of small Rho GTPases, each of them need to be deleted by different Cre drivers that can induce Cre/loxP recombination within different developmental stages. In the present study, we therefore deleted Rac1...
in cerebellar granule neurons and Bergmann glial cells using GFAP-Cre mice in addition to Rac3 and examined the roles of Rac in the development of the cerebellum.

Materials and methods

Animals. The following mouse strains were used in this study: Rac1 flox/flox and GFAP-Cre Cre. Mice of both sexes were used. To obtain Rac1 flox/+ ; Cre and Rac1 ′/+ ; Cre mice, Rac1 ′/+ ; Cre males were crossed with Rac1 flox/− ; Cre male mice. To obtain Rac1 ′/+ ; Cre, Rac1 ′/+ ; Cre flox/− ; Cre, Rac1 ′/+ ; Cre flox/− ; Cre, Rac1 ′/+ ; Cre, Rac1 ′/+ ; Cre flox/− ; Cre mice, Rac1 ′/+ ; Cre female mice were crossed with Rac1 flox/− ; Cre male mice. To obtain Rac1 ′/+ ; Cre, Rac1 ′/+ ; Cre flox/− ; Cre, Rac1 ′/+ ; Cre, Rac1 ′/+ ; Cre flox/− ; Cre mice, Rac1 ′/+ ; Cre female mice were crossed with Rac1 ′/+ ; Cre; Rac1 ′/+ ; Cre male mice. Mice were genotyped by polymerase chain reaction (PCR) of genomic DNA extracted from tails using the following primers: 5′-TCAATCTGTCGTCAGCTAC-3′ and 5′-GATGCTTCCTAGGATTGGAAGCAGCC-3′ for Rac1 ′/+ ; Cre, 5′-CATTCTGTCGTCAGCTAC-3′ and 5′-CACGCGCGCGCGCTGTTGTCG-3′ and 5′-TTGTCTGGTCACAGACATC-3′ for Rac3 ′/+ ; Cre, 5′-GCCGTCGTCAGTAAACTATC-3′ and 5′-TGAAACGACATTGGCTGCATT-3′ for GFAP-Cre. All animal experiments were performed in accordance with the relevant guidelines and regulations and approved by the Animal Care and Use Committee of Wakayama Medical University. ARRIVE guidelines were followed in all animal experiments.

Histology. Cerebella were fixed in paraformaldehyde and embedded in paraffin. Sagittal and coronal sections were made with a microtome and subjected to TUNEL staining and immunohistochemistry. The TUNEL method was carried out using Apoptag Fluorescein In Situ Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO). For immunohistochemistry, the following primary antibodies were used in this study: rabbit anti-cleaved Caspase-3 (Cell Signaling Technology, Danvers, MA), rabbit anti-Ki67 (Abcam, Cambridge, UK), rabbit anti-NeuN (Cell Signaling Technology), mouse anti-phospho-histone H3 (Abcam), rabbit anti-GFAP (Protein tech, Rosemont, IL), mouse anti-BrdU (MBL, Tokyo, Japan), mouse anti-p27 (BD Transduction Laboratories, San Jose, CA), mouse anti-Sox2 (Protein tech), and mouse anti-Calbindin-D-28 K (Sigma-Aldrich). Immunopositive signals were detected using Alexa Fluor 488 and 568-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA). Nuclei were visualized with DAPI, and sections were viewed and images were taken using an LSM700 confocal laser-scanning microscope (Zeiss, Oberkochen, Germany). Images of Nissl-stained sections were taken by a BZ9000 microscope (Keyence, Osaka, Japan).

5-Bromo-2′-deoxyuridine (BrdU) labeling. To assess the cell-cycle withdrawal, differentiation and migration of cerebellar granule neurons, mice were injected with 25 mg/kg of BrdU intraperitonially on postnatal day 7 (P7) and sacrificed 24, 48 and 72 h after the injection. Cerebella were fixed in paraformaldehyde and embedded in paraffin. Sagittal sections were subjected to immunohistochemistry mentioned above.

Statistical analysis. For quantitation of the immunohistochemical results, 3 animals (2 sections/animal) were examined in each genotype. Immuno-positive signals were analyzed using ImageJ (https://image.nih.gov/ij/). Statistical analyses were carried out by one-way ANOVA followed by a Tukey’s post hoc test. Jarque–Bera test was used to determine whether sample data were normally distributed. Differences were defined as statistically significant when P < 0.05.

Results

Deletion of Rac in cerebellar granule neurons results in loss of granule neurons. In this study, Rac1 was deleted using GFAP-Cre mice, which induces Cre/loxP recombination in cerebellar granule neuron precursors from embryonic day 14.5 and also in Bergmann glial cells (Supplementary Fig. 1). At birth, cerebella of Rac1 flox/+ ; Cre mice were slightly smaller than those of Rac1 ′/+ ; Cre mice (Fig. 1A). The difference in cerebellum size became larger as the mice grew up, and cerebella of Rac1 ′/+ ; Cre mice were markedly smaller than those of Rac1 ′/+ ; Cre mice on P14. Rac3, a close homolog of Rac1, is expressed in the nervous system4. Although Rac3-knockout mice showed no obvious histological abnormalities in the brain1, Rac3 may play some roles in the Rac1-deleted brain, Rac1/Rac3 compound knockout (Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre) mice were therefore generated. The cerebella of Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice were much smaller than those of Rac1 ′/+ ; Cre mice on P14 (Fig. 1A). Sagittal and coronal sections revealed that not only anterior medial parts, but also caudal lateral parts of the cerebellum were affected in Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice. Immunohistochemistry showed NeuN-positive cerebellar granule neurons were almost completely absent in Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice (Fig. 1B). The phenotype of Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre, Rac1 ′/+ ; Cre mice was milder than that of Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice (Fig. 1A, B). Presence of at least one allele of Rac1 appeared sufficient to maintain cerebellum structures because overall morphology of the cerebellum of Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice was normal (Fig. 1A, B). Rac is suggested by these results to be essential for the development of granule neurons in the entire cerebellum. Loss of Rac3 itself has little effect on development of cerebellar granule neurons, but it can play some roles in Rac1-null situation. Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice were small and showed very severe ataxic gait on P14, so no further examinations were performed.

Enhanced apoptotic cell death in the EGL of Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice. As granule neurons were lost in Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice, we next examined cell death and proliferation of granule neurons on P3 and P7. The proportion of TUNEL-positive apoptotic cells was markedly increased in the EGL of Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre and Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice on both P3 and P7 (Fig. 2A, B). TUNEL-positive cells in the cerebellum of Rac1 ′/+ ; Cre; Rac3 ′/+ ; Cre mice were observed in the inner most part of the EGL (Fig. 2A, B). Cell proliferation was assessed using immunohistochemistry for phospho-histone...
H3 (p-HH3) that labels M-phase cells, and the fraction of p-HH3-positive mitotic cells in the EGL was slightly increased in Rac1flox/flox; Rac3+/−; Cre mice and Rac1flox/flox; Rac3−/−; Cre mice that retain only one allele of Rac1 showed morphologically normal development of the cerebellum. (B) Immunohistochemistry for NeuN and Calbindin around preculminate fissure of the sagittal section. NeuN-positive granule neurons were almost completely absent in Rac1flox/flox; Rac3−/−; Cre mice on P14. n = 3 animals/genotype. Scale bars, 100 μm.

**Figure 1.** Deletion of Rac in cerebellar granule neurons results in loss of granule neurons. (A) Nissl-stained sections of the cerebellum on P0, P7 and P14. Development of the cerebellum was severely impaired in Rac1flox/flox; Rac3+/−; Cre mice. The cerebellum of Rac1flox/flox; Cre mice was slightly hypoplastic on P0, but it showed severe aplasia of the IGL in the entire cerebellum on P14. Rac1flox/flox; Rac3−/−; Cre mice showed intermediate phenotype between Rac1flox/flox; Cre and Rac1flox/flox, Rac3−/−; Cre mice. Rac1+/−; Rac3+/−; Cre mice that retain only one allele of Rac1 showed morphologically normal development of the cerebellum. (B) Immunohistochemistry for NeuN and Calbindin around preculminate fissure of the sagittal section. NeuN-positive granule neurons were almost completely absent in Rac1flox/flox; Rac3−/−; Cre mice on P14. n = 3 animals/genotype. Scale bars, 100 μm.

Increased thickness of the EGL in Rac1flox/flox; Cre, Rac1flox/flox; Rac3+/−; Cre and Rac1flox/flox, Rac3−/−; Cre mice is caused by accumulation of p27 and NeuN-positive granule neurons. In spite of enhanced cell death, the EGL of Rac1flox/flox; Cre, Rac1flox/flox, Rac3−/−; Cre and Rac1flox/flox, Rac3+/−; Cre mice appeared thicker than that of the other genotypes on P7 (Fig. 2B), so cellular components of the EGL were examined next. The EGL is divided into an outer layer (oEGL) of proliferating progenitors and inner layer (iEGL) of postmitotic neurons. We examined arrangement of oEGL and iEGL by immunohistochemistry for Ki67 (oEGL cell marker) and p27 (iEGL cell marker). The iEGL area occupied by p27-positive cells was significantly increased in Rac1flox/flox; Cre, Rac1flox/flox, Rac3+/−; Cre and Rac1flox/flox, Rac3−/−; Cre mice, whereas the oEGL area occupied by Ki67-positive cells was not different among genotypes (Fig. 3A, A′, A″). In Rac1+/−; Cre, Rac1flox/flox, Rac3−/− and Rac1+/−, Rac3+/−; Cre mice, Ki67-positive cells and p27-positive cells were clearly separated into outer and inner compartments, respectively. However, these two cell populations were intermingled in Rac1flox/flox; Cre, Rac1flox/flox, Rac3+/−; Cre and Rac1flox/flox, Rac3−/−; Cre mice (Fig. 3A). To examine whether accumulation of p27-positive cells is caused by the accelerated differentiation of progenitor cells, cell-cycle withdrawal of progenitor cells was evaluated. BrdU was injected on P7 and the proportion of cells that exit the cell cycle after 24 h (P8) was counted, which was calculated as the ratio of BrdU+/Ki67− cells among all BrdU-labeled cells. Cell-cycle exit and differentiation into postmitotic neurons of precursors was not altered in Rac1flox/flox; Cre, Rac1flox/flox, Rac3+/−; Cre and Rac1flox/flox, Rac3−/−; Cre mice (Fig. 3B, B′). Accumulation of p27-positive cells in the
EGL is suggested by these results not to be caused by the enhanced differentiation of precursor cells to postmitotic neurons. The innermost part of the EGL, p27-positive cells begin to express NeuN and migrate into the molecular layer to form the IGL. The accumulation of p27-positive cells in the EGL may be caused by impaired migration of NeuN-positive cells, so NeuN expression in the EGL was examined next. The NeuN-positive area in the EGL was significantly increased in Rac1flox/flox; Rac3+/−; Cre and Rac1flox/flox; Rac3−/−; Cre mice (Fig. 3C, C′). Rac-depleted/deleted granule neurons are suggested to differentiate normally as far as they express NeuN but exhibit impaired migration into the molecular layer, resulting in accumulation of p27 and NeuN-positive cells in the EGL. Immunopositive signal of NeuN was strong in granule neurons in the IGL but relatively weak in those in the EGL in Rac1flox/flox; Cre, Rac1flox/flox; Rac3+/− and Rac1flox/flox; Rac3−/−; Cre mice. Conversely, some

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Enhanced apoptotic cell death in the EGL of Rac1flox/flox; Rac3+/−; Cre and Rac1flox/flox; Rac3−/−; Cre mice. TUNEL-staining and immunohistochemistry for phospho-histone H3 (p–HH3) of the sagittal section of the lobule IV/V on P3 (A) and P7 (B). The proportion of TUNEL-positive apoptotic cells was markedly increased in Rac1flox/flox; Rac3+/−; Cre and Rac1flox/flox; Rac3−/−; Cre mice, whereas that of p–HH3-positive mitotic cells was largely unaffected. Graphs depict the fraction of TUNEL-positive cells and p–HH3-positive cells in the EGL. Each bar represents mean ± SD (n = 3). *P < 0.05, **P < 0.01 (significantly different from Rac1+/−/flox; Cre mice), one-way ANOVA followed by a Tukey's post hoc test. Scale bars, 100 μm.
Figure 3. Thickness of the iEGL was increased in $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{+/+}\text{Cre}$ and $\text{Rac}^{1+/+}\text{Cre}$; Cre mice due to impaired migration of NeuN-positive cells. (A) Immunohistochemistry for Ki67 and p27 of the sagittal section of the anterior part of the lobule IV on P7, (B) Immunohistochemistry for BrdU and Ki67 of the sagittal section of the anterior part of the lobule IV. Mice were injected with BrdU on P7 and sacrificed 24 h after the injection (P8). (C) Immunohistochemistry for NeuN of the sagittal section of the anterior part of the lobule IV on P7. Immunopositive signal of NeuN was strong in granule neurons in the IGL but weak in those in the EGL in $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{+/+}\text{Cre}$ and $\text{Rac}^{1+/+}\text{Cre}$; Cre mice. Some NeuN-positive cells in the EGL showed strong immunoreactivity in $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{+/+}\text{Cre}$ and $\text{Rac}^{1+/+}\text{Cre}$; Cre mice (arrowheads). (A’ and A”) The oEGL area occupied by Ki67-positive cells was not different among genotypes, whereas the iEGL area occupied by p27-positive cells was significantly increased in $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{+/+}\text{Cre}$ and $\text{Rac}^{1+/+}\text{Cre}$; Cre mice. (B’) Cell-cycle exit was not affected by Rac depletion/deletion. The proportion of cells that exit the cell cycle (BrdU+/Ki67−) among all BrdU+ cells was not changed among genotypes. (C’) The area occupied by NeuN-positive cells was significantly increased in $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{1+/+}\text{Cre}$, $\text{Rac}^{1+/+}\text{Cre}$; Cre and $\text{Rac}^{1+/+}\text{Cre}$; Cre mice. Each bar represents mean ± SD (n = 3). *$P<0.05$, **$P<0.01$ (significantly different from $\text{Rac}^{1+/+}\text{Cre}$ mice), one-way ANOVA followed by a Tukey’s post hoc test. Scale bars, 50 μm.
NeuN-positive cells in the EGL showed strong immunoreactivity in Rac1<sup>fl/fl</sup>; Cre, Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre and Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice (Fig. 3C, arrowheads). NeuN-positive cells that cannot migrate into the molecular layer may therefore differentiate into cells similar to granule neurons in the IGL in the EGL.

**Rac-deficient granule neurons die before they migrate to the IGL.** To confirm whether Rac-deficient granule neurons differentiate as far as NeuN expression and die in the innermost part of the EGL, mice were injected with BrdU on P7 and differentiation and migration of BrdU-incorporated granule neurons were examined. A large volume of the BrdU-incorporated granule neurons differentiated to NeuN-positive cells in 48 h in all the genotypes (Fig. 4A). However, significantly fewer BrdU-incorporated cells migrated to the IGL (past the Purkinje cell layer) in Rac1<sup>fl/fl</sup>; Cre, Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre and Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice compared with other genotypes at 48 h (Fig. 4A) and 72 h (Fig. 4B). Only a few BrdU-incorporated cells survived in Rac1<sup>fl/fl</sup>; Cre, Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice at 72 h (Fig. 4B). In addition, some BrdU-positive cells in the EGL of Rac1<sup>fl/fl</sup>; Cre, Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice were also positive for cleaved Caspase-3 (CC3), an apoptotic cell marker, at 48 h after the BrdU injection (Fig. 4C, arrowheads). Neuronal apoptosis in granule neurons delayed their migration<sup>17,22</sup>. In Bergman glia, deletion of Cdc42, but not Rac1, impaired migration of granule neurons both in cell-autonomously and non-cell-autonomously. For example, deletion of RhoA or Cdc42 impaired migration of granule neurons, but compound deletion of both Cdc42 and Rac1 much more severely impaired the migration of granule neurons than that of Cdc42-deletion alone<sup>21</sup>. Furthermore, in granule neurons, deletion of β-chimaerin, a GTPase activating protein (GAP) of Rac, or Trio, a guanine nucleotide exchanging factor (GEF) of RhoA, RhoG and Rac, caused defective migration<sup>23,24</sup>. Disruption of Abr and Bcr, RacGAPs, caused misalignment of Bergmann processes and granule cell migration defects<sup>25</sup>. These findings together with ours suggest that small Rho GTPases not only in granule neurons themselves but also in Bergman glial cells play very important roles in the development of cerebellar granule neurons, especially in their migration. The activity of each small Rho GTPase must be tightly regulated.

**Loss of Rac caused disorganization of processes of Bergmann Glia.** The GFAP-Cre mouse line used in this study induces Cre/loxP recombination not only in granule neurons, but also in Bergmann glial cells<sup>26,27</sup>. Several findings suggest that Bergmann glia works as a scaffold for migrating granule neurons<sup>28</sup>. The arrangement of processes of Bergmann glia was therefore examined by immunostaining for GFAP-GFP-positive processes of Bergmann glial cells in Rac1<sup>fl/fl</sup>; Cre, Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre and Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice appeared disorganized probably due to hypoplasia/aplasia of the IGL (Fig. 5). We also immunostained Sox2, which labels nuclei of Bergmann glial cells<sup>19,20</sup>, and counted the number of Sox2-positive cells. The number of Sox2-positive cells in 100 μm width was significantly increased in Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre and Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice. Unlike granule neurons, loss of Rac barely affected the survival of Bergman glial cells. The density of Bergmann glial cell processes was increased, probably due to the hypoplasia of the cerebellar lobules in Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre and Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice. Loss of Rac1 in Bergmann glia reportedly had subtle effect on the organization of glial fibers and migration of granule neurons<sup>21</sup>. Furthermore, Rac3 is absent in glial lineage cells including Bergmann glia<sup>21</sup>; it is unlikely that loss of Rac in Bergmann glia primarily contributes to the impaired migration and loss of granule neurons in Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice. However, the possibility that misalignment of Bergmann glial fibers caused by hypoplasia/aplasia of the IGL secondarily contributes to the impaired migration and loss of granule neurons cannot be ruled out.

**Discussion**

Deletion of both Rac1 and Rac3 was shown to be almost completely absent of granule neurons in the cerebellum. Rac-deficient granule neurons differentiate as far as NeuN expression but die in the innermost part of the EGL and cannot migrate to the IGL. Loss of Rac3 itself has little effect on the development of cerebellar granule neurons, but Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice showed much more severe phenotype than that of Rac1<sup>fl/fl</sup>, Cre mice. In addition, Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice showed intermediate phenotype between Rac1<sup>fl/fl</sup>, Cre and Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice. Rac is suggested by our results to be required for both survival and migration of granule neurons and that Rac3 likely plays roles in Rac1-deleted neurons. Meanwhile, overall morphology of the cerebellum of Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice was normal. Presence of at least one allele of Rac1 is therefore sufficient to promote development of cerebellar granule neurons. In the inner-most part of the EGL, granule neurons begin to express NeuN and migrate into the molecular layer to form the IGL. The immunoreactivity of NeuN in the EGL is weaker than those in the IGL. However, some NeuN-positive granule neurons in the EGL of Rac1<sup>fl/fl</sup>, Rac3<sup>−/−</sup>; Cre mice showed strong immunoreactivity, similar to those in the IGL in other genotypes. Rac-deficient granule neurons that cannot migrate into the molecular layer can differentiate into mature cells in terms of marker expression. However, it is difficult to determine whether granule neurons die because they cannot make complete differentiation or because they cannot migrate to the IGL and are mis-localized. The precise cause of the cell death of Rac-deficient granule neurons remains unknown.

Small Rho GTPases including Rac are known to have important roles in the development of cerebellar granule neurons both in cell autonomously and non-cell autonomously. For example, deletion of RhoA or Cdc42 in granule neurons delayed their migration<sup>17,22</sup>. In Bergman glia, deletion of Cdc42, but not Rac1, impaired migration of granule neurons, but compound deletion of both Cdc42 and Rac1 much more severely impaired the migration of granule neurons than that of Cdc42-deletion alone<sup>21</sup>. Furthermore, in granule neurons, deletion of β-chimaerin, a GTPase activating protein (GAP) of Rac, or Trio, a guanine nucleotide exchanging factor (GEF) of RhoA, RhoG and Rac, caused defective migration<sup>23,24</sup>. Disruption of Abr and Bcr, RacGAPs, caused misalignment of Bergmann processes and granule cell migration defects<sup>25</sup>. These findings together with ours suggest that small Rho GTPases not only in granule neurons themselves but also in Bergman glial cells play very important roles in the development of cerebellar granule neurons, especially in their migration. The activity of each small Rho GTPase must be tightly regulated.
Figure 4. Newly generated granule neurons can differentiate into NeuN-positive cells but hardly reach to the IGL in Rac1<sup>flox/flox</sup>, Rac3<sup>−/−</sup>; Cre mice. Immunohistochemistry for BrdU and NeuN of the sagittal section of the anterior part of the lobule IV (A, B). Mice were injected with BrdU on P7 and sacrificed 48 h (A) or 72 h (B) after the injection. Most of the BrdU-incorporated cells differentiate into NeuN-positive cells in 48 h in each genotype. Significantly fewer BrdU-incorporated cells migrate to the IGL in Rac1<sup>flox/flox</sup>; Cre, Rac1<sup>flox/flox</sup>; Rac3<sup>+/−</sup>; Cre and Rac1<sup>flox/flox</sup>; Rac3<sup>−/−</sup>; Cre mice at 48 h and 72 h. Immunohistochemistry for BrdU and cleaved Caspase-3 (CC3) of the sagittal section of the anterior part of the lobule IV (C). Mice were injected with BrdU on P7 and sacrificed 48 h after the injection. The proportion of CC3+/BrdU<sup>+</sup> cells (arrowheads) among all BrdU<sup>+</sup> cells was significantly increased in Rac1<sup>flox/flox</sup>; Cre, Rac1<sup>flox/flox</sup>; Rac3<sup>+/−</sup>; Cre and Rac1<sup>flox/flox</sup>; Rac3<sup>−/−</sup>; Cre mice. Each bar represents mean ± SD (n = 3). *P < 0.05, **P < 0.01 (significantly different from Rac1<sup>+/flox</sup>; Cre mice), one-way ANOVA followed by a Tukey’s post hoc test. Scale bars, 50 μm.
Deletion of both Rac1 and Rac3 in the cerebellum was also previously shown to cause severely impaired development of granule neurons. The biggest difference between current and previous studies is the distribution of hypoplasia/aplasia in the cerebellum. In Atoh1-Cre; Rac1flox/flox; Rac3−/− Cre mice, agenesis of the IGL was observed only in the anterior medial part of the cerebellum, but in our Rac1flox/flox; Rac3−/−; GFAP-Cre mice, it was observed in the entire cerebellum. Furthermore, degeneration of cerebellar granule neurons was much more severe in Rac1flox/flox; Rac3−/−; GFAP-Cre mice than in Atoh1-Cre; Rac1flox/flox; Rac3−/− Cre mice. Deletion of receptor for activated C kinase (Rack1) by GFAP-Cre driver resulted in much more severe phenotype than that by Atoh1-Cre driver. Rack1 deletion by GFAP-Cre mice resulted in agenesis of lobules throughout the rostral to caudal parts of the medial vermis. On the other hand, loss of Rack1 by Atoh1-Cre mice affected only the rostral part of the cerebellum. This difference may be caused by the difference in Cre recombinase activity in GFAP-Cre and Atoh1-Cre mice. Rac was shown in our study to play crucial roles in the development of cerebellar granule neurons not only in the anterior medial part but in the entire cerebellum. Nakamura et al. identified Mid1 as a downstream target of Rac to promote cerebellar development. The human ortholog is a responsible gene for Opitz G/BBB syndrome characterized by malformation of midline structures. Other downstream targets of Rac may play an important role in the caudal lateral part of the cerebellum.

Deletion of Rac1 in telencephalic neuroepithelium resulted in accelerated cell cycle exit of progenitors. Conversely, deletion of Rac1 or Rac1/Rac3 in the medial ganglionic eminence reduced cell cycle exit of interneuron progenitors. Nakamura et al. and our group both showed no effect of deletion of both Rac1 and Rac3 in the cerebellum on cell cycle exit of granule neuron progenitors. Rac may play different roles in cell cycle exit of progenitors depending on cell types.

Rac plays a variety of roles during development of the nervous system, including neurogenesis, differentiation, migration, dendritogenesis, axon guidance and synapse formation, but there is poor current understanding of downstream signaling pathways being involved in each process. In the central nervous system, Rac3, a close homolog of Rac1, is ubiquitously expressed, and it may have roles in Rac1-deficient neurons, making it difficult to recognize the functions of “Rac” and its downstream targets. The present study showed that deletion of both Rac1 and Rac3 in cerebellar granule neurons resulted in much more severe impairment in survival and migration than that of Rac1-deletion alone. Our experimental system can therefore be considered to be an effective tool for identification of downstream targets of Rac in neuronal survival and migration.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 15 March 2022; Accepted: 26 August 2022
Published online: 01 September 2022
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Acknowledgements
This study was supported by KAKENHI (JP16K07000) and Takeda Science Foundation. We acknowledge proof-reading and editing by Benjamin Philiss at the Clinical Study Support Center at Wakayama Medical University.

Author contributions
K.K. and Y.Z. designed research. K.K. performed research and analyzed data. Y.Z. and N.I. contributed unpublished reagents/analytic tools. K.K. and N.I. wrote the paper. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-19252-y.
