The transcriptional repressor RP58 is crucial for cell-division patterning and neuronal survival in the developing cortex

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The neocortex and the hippocampus comprise several specific layers containing distinct neurons that originate from progenitors at specific development times, under the control of an adequate cell-division patterning mechanism. Although many molecules are known to regulate this cell-division patterning process, its details are not well understood. Here, we show that, in the developing cerebral cortex, the RP58 transcription regulator protein was expressed both in postmitotic glutamatergic projection neurons and in their progenitor cells, but not in GABAergic interneurons. Targeted deletion of the RP58 gene led to dysplasia of the neocortex and of the hippocampus, reduction of the number of mature cortical neurons, and defects of laminar organization, which reflect abnormal neuronal migration within the cortical plate. We demonstrate an impairment of the cell-division patterning during the late embryonic stage and an enhancement of apoptosis of the postmitotic neurons in the RP58-deficient cortex. These results suggest that RP58 controls cell division of progenitor cells and regulates the survival of postmitotic cortical neurons.

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Introduction

Glutamatergic cortical neurons are generated from progenitor cells in the cortical germinal zone and migrate radially in an inside-to-outside gradient. The earliest neurons form the preplate (together with the Cajal-Retzius cells) and the neurons born subsequently migrate past the earliest-born neurons to intercalate within the preplate, divide it into the marginal zone (MZ; layer 1) and the subplate (layer 6b), and form the lower layers of the cortical plate (CP). Late-born neurons then migrate past the early-born neurons to form the upper layers of the CP, beneath the MZ. In contrast, GABAergic neurons and Cajal-Retzius cells are generated from progenitor cells outside the neocortex, in the ganglion eminence and in the cortical hem, respectively, and migrate tangentially into the neocortex (Bayer and Altman, 1991; Allendoerfer and Shatz, 1994; Molyneaux et al., 2007). The radial glial progenitors (RGPs) in the ventricular zone (VZ) give rise to cortical neurons, while the progenitor cells in the subventricular zone (SVZ) produce a substantial number of upper-layer neurons (Smart and McSherry, 1982, Tarabykin et al., 2001, Sugitani et al., 2002). Some of the SVZ progenitor cells are intermediate progenitors (IMPs), which originate from the VZ and produce neurons by dividing limited times (Noctor et al., 2004; Haubensak et al., 2004; Miyata et al., 2004). In the hippocampus, pyramidal neurons of the Cornu Ammonis (CA) are generated from the VZ and produce neurons by dividing limited times (Noctor et al., 2004; Haubensak et al., 2004; Miyata et al., 2004). In the hippocampus, pyramidal neurons of the Cornu Ammonis (CA) are generated from the VZ and produce neurons by dividing limited times (Noctor et al., 2004; Haubensak et al., 2004; Miyata et al., 2004).
the DG, continue to divide, and undergo further migration to the granule layer of the DG (Forster et al., 2006; Li and Pleasure 2007).

These cortical progenitor cells generate a vast diversity of terminally differentiated neuronal phenotypes. The balance between exit from and reentry into the cell cycle is important for the formation of these cell types at appropriate times; however, the molecular mechanism underlying this regulation is not completely understood (Dehay and Kennedy, 2007).

We have previously described a novel DNA binding protein, RP58 (also known as ZNF238), which shares homology with the POZ domain of a number of zinc finger (ZF) proteins, which are termed POZ-ZF proteins (Aoki et al., 1998). RP58 exhibits a sequence-specific transcriptional repressor activity (Aoki et al., 1998) and probably acts by binding to the DNA methyltransferase Dnmt3a, which associates with histone deacetylase and acts as a corepressor (Fukuda et al., 1997). POZ-ZFs are important for many biological processes, which include B-cell fate determination, DNA damage responses, cell-cycle progression, and a multitude of developmental events (Kelly and Daniel, 2006).

Among the POZ-ZF proteins, the promyelocytic leukemia zinc finger (PLZF) is essential for stem cell self renewal in the murine testis (Buaas et al., 2004; Costoya et al. 2004), Miz1 plays an essential role in the control of the exit from the cell cycle during the hair cycle (Gebhardt et al., 2007), and ZEN0N is involved in the maintenance of panneuronal features and/or in the survival of mature neurons (Kiefer et al., 2005).

We demonstrated that RP58 transcripts are highly expressed in the cerebral cortex in the embryonic mouse brain (Ohtaka-Maruyama et al., 2007). In addition, RP58 is expressed weakly in the VZ and intensely in the SVZ, intermediate zone (IZ), and CP in the embryonic cortex, which suggests that RP58 is important for the early development of cortical neurons. In adult cerebral cortex, the expression of the RP58 transcript is maintained in glutamatergic neurons, but not in GABAergic neurons.

In the present study, we investigated the role of RP58 in the development of the cerebral cortex by generating and analyzing RP58-deficient mice. Our results demonstrate that RP58 deficiency causes enhanced apoptosis and impairs the cell-division patterning in the VZ during late development, which suggests that RP58 is a novel regulator of glutamatergic neuron survival and of progenitor cell division.

Materials and methods

Generation of RP58-deficient mice

Similarly to what is observed for the human RP58 gene, the sequence of the mouse RP58 gene that encodes the functional protein is uninterrupted over its entire 4.2 kb length (Meng et al., 2000). A gene-targeting construct was prepared by deletion of the entire exon (5.4 kb). The resulting RP58 targeting vector (Supplementary Fig. 1A), which was constructed from a mouse strain 129 library (Stratagene) and consisted of a 4.2 kb homology arm derived from the 5' end of the exon, a PGK promoter-neomycin expression cassette, and a 2.7 kb sequence of the mouse RP58 gene that encodes the functional protein, was linearized with XbaI and integrated into the genome of embryonic stem cells (ES cells). The homologous integration experiment was performed by electroporation. Colonies that survived after selection were picked and expanded for DNA analysis. Targeted ES cells were injected into C57BL/6 blastocysts, and chimeric mice, which were then crossed with C57/BL6 females to obtain homozygous mutant animals. These mice were, in turn, interbred to produce homozygous RP58+/− mice at the expected Mendelian frequency.

Southern blot analysis of genomic DNA isolated from the tails of embryonic day (E) 18.5 fetuses confirmed the homologous integration of the target vector (Supplementary Fig. 1B), which resulted in the replacement of the entire RP58 exon (5.4 kb) with the neomycin resistance gene. Northern blot analysis of total RNA extracted from genotyped embryonic brains (Supplementary Fig. 1C) showed that the RP58 transcript was present only in wild-type and heterozygous embryos. In homozygous mutant embryos, no RP58 transcript of any size was observed. Embryonic brain extracts were incubated with anti-RP58-conjugated Sepharose 4B beads. The beads were washed extensively and boiled in SDS sample buffer. After centrifugation, the supernatant was analyzed for the presence of RP58 by immunoblotting, as described previously (Ishida et al., 2002). To confirm the specificity of the interactions between the antigen and the antibody, the peptide (CLPTVRDWTLGEDSSQELWK) used for the generation of the anti-RP58 antibody was added during the immunoprecipitation experiment. Antibodies specific to RP58 detected the protein in brain extracts from wild-type, but not homozygous mutant, embryos (Supplementary Fig. 1D). The day after the mating was designated E0.5.

Immunohistochemistry

Heads of embryos were removed, fixed in Bodian’s fixative (3.7% formaldehyde, 80% ethanol), embedded in paraffin, and sectioned at an 8 μm thickness. A few embryos were perfused with 4% paraformaldehyde and sectioned using a cryostat (10–25 μm thickness). In most cases, the antigens in these sections were reactivated by heating in 10 mM citrate buffer (adjusted to pH 6.0) using a microwave or an autoclave.

We used the following antibodies: rabbit anti-mouse RP58(1:500, Takahashi et al., 2008), mouse anti-reelin(1:200, Chemicon), rabbit anti-MAP2 (1:500, Chemicon), rabbit anti-Tbr1 (1:500, Chemicon), rabbit anti-Prox1 (1:1000, Covance), chicken anti-Tuj1 (1:200, Chemicon), mouse anti-Brdu (1:50, Becton-Dickinson), rat anti-Brdu (1:200, Abcam), mouse anti-α-synuclein, mouse anti-β-synuclein (1:200, BD Transduction Lab), rabbit anti-Pax6 (1:200, Chemicon), mouse anti-P-CNA (1:200, Chemicon), mouse anti-nestin (1:200, Rat-401), mouse anti-NeuN (1:100, Chemicon), mouse anti-ki67 (1:100, Novoceastra), rabbit anti-ki67 (1:500, Novoceastra), goat anti-NeuroD (1:100, Santa Cruz Biotechnology), guinea pig anti-Dlx2 (1:100, gift from Dr. Yoshikawa; Kuwajima et al., 2006), mouse anti-Neurogenin2 (1:5, gift from Dr. Anderson), rabbit anti-phosphohistone H3 (P-H3) (1:200, Upstate), rat anti-neurofilament (1:500, Fukuda et al., 1997), rabbit anti-ssDNA (1:400, DAKO), rabbit anti-active caspase 3 (1:400, R&D), and goat anti-Unc5d (1:200, R&D).

Anti-IgG antibodies conjugated to biotin (Vector, 1:200), Alexa 488, Alexa 546, Alexa 555, Cy3, or Cy5 (1:500) (Molecular Probes or Jackson Laboratories) were used as secondary antibodies and the ABC kit (Vector) or the TSA Fluorescence System (PerkinElmer) were used to detect biotin. After nuclear staining with DAPI and Topro3, the sections were mounted with PermaFluor (Immuron) or dehydrated and mounted with Entellan Neu (Merk). A laser-scanning confocal microscope was used to image fluorescence signals.

To perform RP58/Pax6 and RP58/Tbr2 double labeling using rabbit polyclonal antibodies, we used the TSA or TSA Plus Fluorescence System (PerkinElmer), according to Fricourt et al. (2008). Sections were first incubated with diluted anti-RP58 antibody (1:8000), for the TSA Plus Fluorescence System, and were then incubated with rabbit anti-Pax6 antibody (1:200), anti-Tbr2 antibody (1:200), or no antibody (negative control). For RP58/P-H3 double labeling, sections were first incubated with anti-RP58 antibody (1:500), for the TSA Fluorescence System, and were then incubated with rabbit anti-P-H3 antibody (1:200). For Pax6/Tbr2 double labeling, sections were first incubated with diluted anti-Pax6 antibody (1:30000), for the TSA Plus Fluorescence System, and were then incubated with rabbit anti-Tbr2 antibody (1:200). For Pax6/Tbr2/Unc5d triple labeling, sections were first incubated with diluted anti-Pax6 antibody (1:15000), for the TSA Plus Fluorescence System, and were then incubated with rabbit anti-Tbr2 (1:200) and anti-Unc5d (1:200) antibodies.
RNA in situ hybridization

We used single-stranded digoxigenin (DIG)-UTP-labeled RNA probes generated from the mouse RP58 cDNA (approximately 1.6 kb); mouse ER81 (a gift from Dr. Jessell; Arber et al., 2003); mouse RO8/1 (a gift from Dr. McConnell; Weimann et al., 1999); Svet1 (a gift from Dr. Tarabykin; Tarabykin et al., 2001); NT3 (a gift from Dr. Azawawa; Shinozaki et al., 2004); rat SCIP (a gift from Dr. Lemke), mouse α-crystalline (a gift from Dr. Funatsu; Funatsu et al., 2004), rat KAI (a gift from Dr. Boultier; Better et al., 1990), for mouse Tbr1, mouse mSorLA, mouse CTFG, and mouse Tailless (gifts from Drs. Y Sugitani and T Noda; Sugitani et al., 2002); HESS cDNA (a gift from Dr. Guillemot; Cau et al., 2000). Some probes were hydrolyzed to a length of about 500 bp. RNA in situ hybridization was performed on Bodian’s-fixed paraffin sections, according to the method of Ohtaka-Maruyama et al. (2007), and on 4% paraformaldehyde-fixed frozen sections, according to the method of Sugitani et al. (2002). In some cases, the counterstaining was performed using Nuclear Fast Red (Kernechtrot).

BrdU- and IdU-labeling experiments

Bromodeoxyuridine (BrdU) or iododeoxyuridine (IdU) (50 mg/kg of body weight) were injected intraperitoneally into pregnant mice at various developmental stages. To estimate the rates of cell-cycle exit, randomly selected BrdU-positive cells (about 50 cells) were examined for PCNA or Pax6 immunoreactivity 24 h after the incorporation of BrdU. In particular, the rates of cell-cycle exit were estimated in the lower region (which corresponded to the VZ) and in the upper region (which corresponded to the SVZ and IZ) of E16.5 embryos in which BrdU was incorporated on E15.5. The total number of BrdU-positive cells was counted and examined for Pax6 immunoreactivity (which corresponds to 0.09 mm of the ventricular surface). The SVZ was identified by staining with Unc5d/Svet1.

To estimate the production of progenitor cells, randomly selected Ki67-positive cells were examined for BrdU immunoreactivity 0.5 h after the incorporation of BrdU.

The estimation of cell-cycle kinetics was performed according to Martyngna et al. (2005). Pet, was estimated by counting the total number of cells in the prospective VZ within the sampling area.

TUNEL assay

Apoptosis was detected using a TUNEL assay kit (Dead End Fluorometric TUNEL system, Promega). Deparaffinized sections were treated with proteinase K (20 μg/ml) in 100 mM Tris–Cl and 50 mM EDTA (pH = 8.0) for 15 min at room temperature (RT), followed by treatment with FITC-nucleotide containing TdT or H2O (as a negative control), and counterstaining using propidium iodide.

Results

Targeted disruption of the RP58 gene

To study the role of RP58 in the development of the central nervous system, we disrupted the RP58 gene in embryonic stem cells using the target vector (see Supplementary Fig. 1A and “Materials and methods” section). Heterozygous (RP58+/−) mice were phenotypically indistinguishable from their wild-type littermates, whereas all homozygous (RP58−/−) mice, which were generated from intercrosses of the heterozygotes, died shortly after birth. The cause of the death remains unknown and is currently under investigation.

Hypoplasia of the hippocampus and neocortex in RP58-deficient mice

Because RP58 transcripts are expressed abundantly in the brain of the wild-type mice (RP58+/+; Ohtaka-Maruyama et al., 2007) and RP58−/− mice die shortly after birth, we performed histological analyses of brains isolated from null, heterozygous, and wild-type animals at neonatal and embryonic stages. We observed hypoplasia of the neocortex and hippocampus in RP58−/− mice, whereas the brains of RP58+/− mice appeared to be normal (Fig. 1; Supplementary Figs. 1E–M). Therefore, we compared RP58−/− mice with either wild-type or RP58+/− mice in subsequent experiments. The neocortex of RP58−/− mice displayed a reduced thickness and its layers were disorganized. Furthermore, the VZ appeared to expand radially in the mutant cortex (asterisk in Fig. 1). In the mutant hippocampus, the pyramidal cell layer and the typical V-shaped granule cell layer of the DG were not evident (Fig. 1). Additionally, the cerebellum of RP58−/− mice lacked the typical foliation observed in wild-type and heterozygous animals (see Supplementary Figs. 1K–M). In the present study, we focused our analysis on the neocortex and hippocampus of mutant mice.

Reduced numbers of mature neurons in the mutant neocortex and hippocampus

Double staining of the neocortex with MAP2 and β-III-Tubulin (TuJ1) showed that postmitotic neurons were present in the mutant neocortex; however, the subplate layer was incompletely formed in the medial region of the mutant neocortex (arrowheads in Supplementary Figs. 2A–B’).

To further characterize this abnormality of the neocortex, we examined the expression of various layer markers. The number of E19 subplate neurons positive for the connective tissue growth factor (CTGF), which labels maturing subplate neurons in layer 6b (Friedrichsen et al., 2003; Heuer et al., 2003), was drastically decreased in the mutant neocortex when compared with the wild type (Figs. 2A and B). To detect the subplate neurons at the earlier stage, we examined the staining for β-synuclein, which is an inhibitor

![Fig. 1](image-url)
of the aggregation of α-synuclein (Hashimoto et al. 2001), as a marker for subplate neurons. Since β-synuclein is mostly detected in the deepest region of layer 6, identified with Tbr1 immunoreactivity, β-synuclein-positive cells correspond to the subplate neurons in the wild-type cortex at E16.5 (arrows in Supplementary Figs. 2E–E'). In mutant neocortices, the number of subplate neurons was severely reduced and a part of the surviving subplate neurons was displaced superficially at E16.5 (Supplementary Figs. 2C–F). In addition, in the RP58 mutants, a fraction of the neurofilament-positive thalamocortical fibers (Kawano et al., 1999), which use subplate neurons for their pathfinding, abnormally projected towards the surface of the neocortex (Fig. 3).

Reelin-positive Cajal-Retzius neurons (Ogawa et al., 1995) developed normally in layer 1 in the E18.5 mutant (Figs. 2C and D, green). In the E18.5 mutant cortex, the majority of Tbr1-positive cells was located in the deeper part of cortical plate (Fig. 2C, Supplementary Fig. 2C–F); however, many of these cells were also detected diffusely throughout the CP (Fig. 2D; Supplementary Fig. 3F). ER81, which is a layer 4 cortical neuron (I and J), mSorLA-labeled layer 2/3 cortical neurons, and (K and L) Dlx2-labeled GABAergic neurons. In the mutant neocortex, the subplate neurons were sharply reduced in number (A and B), Cajal-Retzius neurons were normal (C and D), Tbr1-positive cells were shifted more superficially and were more widely scattered when compared with the wild type (C and D), ER81- and mSorLA-positive cells were located diffusely and in reduced numbers (arrows in F and J), the expression level of RoRβ was dramatically reduced (G and H), and Dlx1-positive cells were roughly normal (K and L). Scale bar, 0.1 mm (A–L).

The diffuse distribution of Tbr1-positive and other cortical neurons in the mutant cortex raised the possibility that the RP58 deficiency impaired the inside-out layer formation. To examine this possibility, we performed double labeling by injecting iododeoxyuridine (IdU) at E12.5 and 5-bromo-2-deoxyuridine (BrdU) at E14.5, followed by examination of the brains at E19 (Figs. 2M–N'). Most late-born cortical neurons (Figs. 2M' and N', yellow) crossed over early-born cells (Figs. 2M' and N', red) in the wild-type cortex, while many late-born neurons were abnormally located beneath early-born cells in the mutant cortex. The defects of laminar organization observed in the

Fig. 2. Dysplasia of the neocortex and hippocampus in RP58−/− mice. (A–L) The disorganized laminar structures of the neocortex and hippocampus of the mutant were demonstrated by various layer-specific markers at E19 (A and B) or E18.5 (C–L) in the wild-type (+/+) and RP58-deficient (−/−) neocortex. (A and B) CTGF-labeled subplate neurons. (C and D) reelin-labeled layer 1 Cajal-Retzius neurons and Tbr1-labeled layer 6 cortical neurons. (E and F) ER81-labeled layer 5 cortical neurons. (G and H) RoRβ-labeled layer 4 cortical neurons. (I and J) mSorLA-labeled layer 2/3 cortical neurons, and (K and L) Dlx2-labeled GABAergic neurons. In the mutant neocortex, the subplate neurons were sharply reduced in number (A and B), Cajal-Retzius neurons were normal (C and D), Tbr1-positive cells were shifted more superficially and were more widely scattered when compared with the wild type (C and D), ER81- and mSorLA-positive cells were located diffusely and in reduced numbers (arrows in F and J), the expression level of RoRβ was dramatically reduced (G and H), and Dlx1-positive cells were roughly normal (K and L). Scale bar, 0.1 mm (A–L).
RP58 mutant cortices suggest that RP58 may play a role in neuronal positioning or migration.

The RP58-deficient hippocampus was reduced in size and had no identifiable CA pyramidal layer or DG granular layer in sections stained with Nissl (Fig. 1), NeuN (Supplementary Figs. 2C and H), or MAP2 and TuJ1 double stain (see Supplementary Figs. 2A–B’).

Cajal-Retzius cells play an important role in the normal layer formation of the hippocampus. The Tbr1/reelin double staining revealed that Cajal-Retzius cells (Nakajima et al., 1997), some of which were Tbr1-positive, were present in the mutant (Fig. 2P). The hippocampal fissure, which is characterized by Reelin-positive cells, was poorly developed (asterisk in Figs. 2O and P). In the developing p73^-/- hippocampus, the most striking abnormality is the absence of the hippocampal fissure, which suggests a role for p73 in cortical folding (Meyer et al., 2004). Therefore, p73 and Reelin expression were examined at the cortical hem (see Supplementary Fig. 4), which revealed that the expression of p73 and reelin was both normal in RP58-deficient cortical hem. We next examined the CA and DG. The pan-hippocampal plate marker, α-crystalline (Funatsu et al., 2004), was expressed in a more dorsal cortical region in the mutant than in the wild type (arrows in Figs. 4A and E). Since α-crystalline is also expressed in the neocortex as well as in the hippocampus, we used another hippocampal marker, α-synuclein, together with the DG marker, Prox1. We found that α-synuclein was expressed in the hippocampal region and its staining did not overlap with the Prox1-positive region in the wild-type brain. In contrast, although α-synuclein expression was detected in the more dorsal cortical region in the mutant brain, it did not overlap with the Prox1-positive dentate region (see Supplementary Figs. 5A–B”). These results suggested that the hippocampus was formed in a more dorsal region in the mutant, probably because of an insufficiency in hippocampal folding; however, the basic positional relationship between the CA and DG remained intact.

Furthermore, we examined whether specific hippocampal sub-regions were generated in the RP58-deficient mice. The expression of the CA3-species marker KA1 (Bettler et al., 1990) was almost undetectable (an arrow in Figs. 4B and F). The CA1-specific marker SCIP (Pou3f1) (Frantz et al., 1994; arrows in Fig. 3C) was not detected (Fig. 4G). NT3, which is expressed in the cingulate neopallium (Friedman et al., 1991; Lee et al., 2000; an arrow in Fig. 4D), was also not detected in the mutant (Fig. 4H). To examine the DG, we used Prox1 and NeuroD (Figs. 2Q–T), which are markers of immature dentate granule cells (Measure et al., 2000; Galichet et al., 2008). In the wild type, Prox1- and NeuroD-positive cells formed a V-shaped structure, which is typical of the DG, whereas in the mutant they formed an inverted V-shaped structure (arrowhead in Figs. 2Q–T). The DG region that was positive for Prox1 appeared to extend throughout the RP58 mutant hippocampus (Supplementary Fig. 6), suggesting that loss of RP58 function may result in an increase in the number of Prox1-positive dentate granule cells. It is reported that Thr1 is expressed after onset of NeuroD expression (Hevner et al., 2006). Thr1 was expressed in many NeuroD-positive dentate granule cells in the wild type, whereas its expression was severely reduced in the mutant (Figs. 2S and T), suggesting that the production of mature neurons is impaired in the mutant dentate granule cells. These results suggest that, although major areas of the hippocampus were probably retained in the mutant, the CA1, CA3 fields, the cingulate cortex, and DG were not, indicating that the hippocampal neurons had maturation defects like those seen in the neocortex.

Expression pattern of RP58 protein

The abnormality of neurons generated in the mutant cortex indicates that RP58 functions during the development of the neocortex.
and of the hippocampus. To further understand the function of RP58, we examined the expression patterns of the RP58 protein using an RP58-specific antibody (Takahashi et al., 2008). The immunostaining pattern obtained was almost identical to that of the RP58 mRNA in situ hybridization pattern (Figs. 5A and B). The specificity of the RP58 antibody was confirmed by immunostaining of an RP58−/− brain (Fig. 5C). Double staining using the nuclear marker TOPR3 showed that RP58 was localized in the nucleus and that it was absent from the cytoplasm (Supplementary Figs. 7B and B'). At E12.5, RP58 was detected in preplate neurons and in some cells in the VZ (arrows in Fig. 5D). At E16.5, RP58 was present in the CP, IZ, SVZ, and in some cells in the VZ (arrows in Figs. 5E and E').

Double staining with β-synuclein or Reelin indicated that RP58 was expressed in subplate neurons (arrows in Fig. 5F), but not in cells of the MZ (Fig. 5E). RP58 was not detected in Dlx2-positive cells (arrows in Fig. 5G), which correspond to GABAergic neurons. In the E16.5 hippocampus, RP58 was detected in most developing neurons and in some progenitor cells in the VZ (Fig. 5J). At E18.5, RP58 was detected in migrating neurons, pyramidal layer cells of the CA, and dentate granule cells (Fig. 5K), which were identified by immunoreactivity for NeuroD (Figs. 5L and L'). RP58 was not detected in reelin-positive Cajal-Retzius cells in the hippocampal fissure (asterisk in Fig. 5K). Therefore, RP58 is expressed in migrating and postmigratory glutamatergic neurons, which are impaired in the mutant, whereas RP58 is not expressed in the Cajal-Retzius cells and GABAergic neurons, which are not impaired in RP58-deficient animals, as shown in Fig. 2. Interestingly, RP58 is also expressed in the progenitor cells in the VZ. Interestingly, some cells in the VZ expressed the RP58 protein at a high level (arrows in Fig. 5H; Supplementary Figs. 7A–B''), and other cells expressed this protein at a low level (arrowheads in Supplementary Figs. 7A–B''). As all of these cells were positive for Ki67, a nuclear protein expressed only in cycling cells, this result suggests that RP58 is expressed by neural progenitors.

To examine whether the VZ cells that express RP58 are RGPs and/or IMPs, we performed double labeling of RP58 with Pax6 (which is an RGP marker) and Tbr2 (which is a pan-IMP marker). Most of the RP58-positive cells in the VZ were Tbr2-positive (arrows in Supplementary Figs. 9C–C''), whereas some RP58-positive cells were Pax6-positive (arrows in Supplementary Figs. 9A–B'') and the others were Pax6-negative (arrowhead in Supplementary Figs. 9A–B''). RP58 was expressed in P-H3-positive cells in the basal regions of the VZ, but not in the apical region of the VZ (Supplementary Fig. 10). RP58 was also detected in some of Ngn2-positive cells (Supplementary Fig. 11). These results suggest that the onset of RP58 expression happens during the transition from Pax6-positive cells to Tbr2-positive cells, or, in other words, at the initial stage of IMPs.

**Fig. 5.** RP58 expression patterns in the wild-type cerebral cortex. (A) RNA in situ hybridization analysis shows that RP58 transcripts were strongly expressed in cortical cells in the CP, IZ, SVZ, and weakly in the VZ of E15.5 wild-type mice. (B and C) RP58 protein was detected at high levels in the CP, IZ, and SVZ, and weakly in the VZ of E15.5 wild-type mice (B). No signal was detected in RP58−/− brain (C). (D, E, and E') RP58 protein was intensely expressed in developing neurons in the preplate (ppl) at E12.5 (D), in the CP, IZ, and SVZ at E16.5 (E), and in progenitor cells in the VZ at E12.5 (arrows in D) and E16.5 (arrows in E and E'). (F–I) RP58 was detected in β-synuclein-positive subplate neurons at E13.5 (F) and was not detected in the reelin-positive Cajal-Retzius cells at E16.5 (G). Ki67, which is a cell cycling marker, was detected in RP58-positive cells in the VZ at E15.5 (H). A higher magnification view of the region marked by an arrow with an asterisk (*) indicates that RP58 protein was expressed in Ki67-positive progenitor cells. RP58 was not detected in Dlx2-positive GABAergic neurons in the E18.5 neocortex (I). (J) RP58 was expressed in progenitor cells in the VZ (arrows in J) and in the developing neurons of the E16.5 hippocampus. (K–L') RP58 was not detected in reelin-positive Cajal-Retzius cells in the hippocampal fissure (asterisk, K) at E18.5. RP58 was detected in NeuroD-positive DG granule cells (L, L') at E18.5. Scale bars, 1 mm (A–C); 0.1 mm (D and E'); (E), and (J–L'); and 0.05 mm (F, G and I). (H).
Enhanced apoptosis in the RP58-deficient cortex

Next, we examined whether enhanced cell death or reduced production of cortical neurons in the mutant cortex were responsible for the fewer numbers of mature subplate and specified CP neurons observed in the mutant cortex. A larger number of TUNEL-positive cells were found in the postmitotic zone of the mutant neocortex at E15.5 and E18.5 when compared with the wild type, but no differences were observed in the proliferative zone (Figs. 6A, B, E, and F; Supplementary Fig. 12). The mutant hippocampus displayed a significant increase in the number of TUNEL-positive cells at E18.5 when compared with the wild type (Figs. 6M and N). Active-caspase3 immunoreactivity was also enhanced at E16.5 (Figs. 6C, D, I, and J) and E19 (Figs. 6G, H, O, and P) in both the neocortex and the hippocampus of the mutant mice, which suggests that caspase-dependent apoptosis is enhanced in the mutant. Apoptosis was detected in the anterior and posterior neocortex to the same degree (data not shown). Furthermore, single-strand DNA (ssDNA) staining using an anti-ssDNA antibody documented the presence of fragmented DNA (Figs. 6K and L), which confirmed the results of the TUNEL analysis. These results suggest that RP58 deficiency enhances caspase-dependent apoptosis in the cerebral cortex, which may reduce the number of mature cortical neurons.

Expansion of the VZ/SVZ in the RP58-deficient cortex

In addition to enhanced apoptosis, we found that the VZ was likely to be expanded in the postnatal day (P) 0 mutant cortex (asterisk in Fig. 1B). We therefore examined the expression of several markers of the VZ, which included Pax6 (Englund et al., 2005). Pax6 expression expanded radially in the mutant cortex at E19 when compared with the distribution of this protein in the wild-type cortex at E19, as did PCNA immunoreactivity (Figs. 7A–B’). Furthermore, the expression of HES5, which is a basic helix–loop–helix transcription repressor expressed in the VZ (Ohtsuka et al., 2006), and of Tailless, which is an orphan nuclear receptor restricted to the VZ (Monaghan et al., 1995), was also enhanced in the mutant neocortex (Figs. 7C–F). The VZ was expanded in the hippocampus as well as in the neocortex, as determined by double staining of Pax6 with PCNA or Ki67 at E18.5 (Supplementary Fig. 13).

Next, we examined whether IMPs were increased in the mutant. Tbr2-positive cells, which are detected in IMPs and postmitotic immature neurons (Englund et al., 2005), were increased in the E18.5 mutant (Supplementary Figs. 14A and B). The phosphohistone H3 (P-H3)-positive mitotic cells in the SVZ, which correspond to mitotic cells of IMPs, were also increased, together with PCNA-positive cells (see Supplementary Figs. 14C–D’). These results suggest that IMPs were...
increased in the mutant. To examine the developmental stage of IMPS, we performed double staining of Tbr2 and Pax6 (Fig. 8), as Pax6+/Thb2+ cells and Pax6−/Thb2+ cells are early-stage IMPS and late-stage IMPS, respectively (Sasaki et al., 2008). The double staining revealed that, in the E18.5 mutant, Tbr2-positive cells and Pax6-positive cells were increased in number, that both Pax6+/Thb2− cells and Pax6+/Thb2+ cells were increased, whereas Pax6−/Thb2− cells were not (Fig. 8). This result suggests that RGP and early-stage IMPS were increased in the mutant, whereas late-stage IMPS were not. To examine the identity of the SVZ, we performed Svet1 in situ hybridization near the section of the Pax6/Tbr2 double staining from E15.5 to E18.5, which revealed that impairment of the mutant VZ/SVZ progressed from E15.5 to E18.5 (Supplementary Fig. 15). In particular, a tripartite appearance of inner Pax6-dominant/intermediate Tbr2-dominant/outer Pax6-dominant zones was observed in the mutant in later developmental stages (Supplementary Fig. 15). To directly associate these zones with Svet1 expression, we performed a triple staining of Pax6, Tbr2, and Unc5d that corresponds to Svet1 (Sasaki et al., 2008) (Supplementary Fig. 16). Unc5d/Svet1 staining was detected in the upper region of the Tbr2-positive zone in the wild type, while it was also detected, albeit weakly and diffusely, in the upper region of the intermediate Tbr2-positive zone and contained the outer Pax6-dominant zones in the E16.5 mutant mice (Supplementary Figs. 16 A–B′), which suggests that the outer Pax6-dominant zone was located in the SVZ. In the E18.5 mutant, the expression of Unc5d/Svet1 was more diffusely detected in the outer Pax6-dominant/intermediate Tbr2-dominant zone, which suggests that the mutant VZ/SVZ was severely impaired in the late development stages of the mutant (Supplementary Figs. 16 C–D′).

**The impairment of cell-cycle exit in the RPS8-deficient VZ/SVZ during late development**

We next examined whether the expansion of the VZ/SVZ of the mutant cortex was due to enhanced proliferation and/or impairment of cell-cycle exit. To examine cell proliferation, we counted the number of BrdU-labeled cells in a random selection of 50 Ki67− (which is a proliferating cell marker)-positive cells (which are considered to be progenitor cells) after a 30 min pulse of BrdU. The percentage of progenitor cells labeled with BrdU was not altered in the mutant cortex at E15.5, which suggests that proliferation was not altered in the mutant cortex (Figs. 9A–C). To examine the possibility that the division pattern of progenitor cells was impaired in the mutant cortex, we counted the number of PCNA-negative and Pax6-negative cells in a random selection of 50 BrdU-labeled cells, after a 24 h pulse of BrdU; this corresponds to the fraction of cells exiting the cell cycle. At E16.5, we found that the PCNA−/BrdU+ and Pax6−/BrdU+ ratios were about halved in RPS8 mutant progenitor cells when compared with their normal counterparts, which suggests that cell-cycle exit is inhibited in the mutant VZ progenitor cells in both the medial and lateral neocortices (Figs. 9D–I). This was confirmed by examining the total number of BrdU-positive cells in an area of 0.25 mm², which showed an increase in the number of PCNA− or Pax6+ cells; this suggests that reentry into the cell cycle is enhanced in the mutants (Supplementary Fig. 17). Furthermore, as the characteristic outer Pax6-dominant zone was observed in the mutant cortex (Supplementary Fig. 16B), we examined whether the outer Pax6-dominant zone was involved in the reduction of cell-cycle exit. The Pax6−/BrdU+ ratio was dominantly reduced in the upper region (IZ/SVZ), which contained the abnormal outer Pax6-dominant, when compared with the lower region (VZ) (Supplementary Fig. 18). Therefore, it is possible that the abnormal outer Pax6-dominant zone observed in the mutant reflects the reduction of cell-cycle exit. In contrast, neither proliferation at E12.5 (Supplementary Figs. 19A–C) nor cell-cycle exit at E13.5 (Supplementary Figs. 19D–I) was impaired. These results suggest that cell-cycle exit is reduced in the mutant cortex at late neocortogenesis. The reduction of the cell-cycle exit causes an increase in VZ progenitor cells and thereby leads to the expansion of the VZ. Therefore, it is likely that the reduction of cell-cycle exit, in addition to the enhanced apoptosis, decreases the number of differentiated late-born neurons in the mutant CP.

As cell-cycle kinetics may affect cell-cycle exit, we estimated the duration of the S-phase (Ts) and of the cell-cycle time (Tc) using a BrdU/IdU double labeling paradigm (Martynoga et al., 2005), which revealed no obvious differences in Ts, Tc, and Ts/Tc between wild-type and mutant cortices (Supplementary Fig. 20); however, because this
estimation rested on the assumption that all cells in the VZ are proliferating and that the precursor cells consist of a single proliferating population with the same cycling kinetics (Martynoga et al., 2005), further analyses may be necessary to assess the possibility that RP58 is involved in cell-cycle kinetics.

Discussion

In the present study, we characterized mice carrying disrupted alleles for the POZ/zinc finger transcriptional repressor gene, RP58. We found that homozygous mutants display severe hypoplasia of the cerebral cortex and of the hippocampus, in association with enhanced apoptosis and expansion of the VZ/SVZ. We showed that RP58 is specifically required for the maturation and survival of the excitatory neurons of the cerebral cortex. Furthermore, the present study demonstrated that RP58 is a novel factor that controls the balance of cell division of neuronal progenitors, which remains poorly understood to date.

In the RP58 null mutant, the VZ was expanded and the dorsal cortex appeared like a wild-type younger brain. Therefore, the possibility of developmental delay cannot be excluded. We examined the expression of Tbr1 in the mutant cortex at E13.5, E15.5, and E18.5, which suggested that there is no clear time lag in the Tbr1 expression pattern (Supplementary Fig. 3). In addition, the Tc may cause a developmental delay. The Tc was not altered in the RP58-deficient cortex. In the early embryonic stage, RP58 deficiency did not impair cell-cycle exit, although apoptosis was enhanced in the mutant neocortex at E15. Therefore, the decreased number of mature subplate neurons produced at early embryonic stages could be caused by enhanced apoptosis. On the other hand, the VZ was expanded at later embryonic stages in the mutant, the cell-cycle exit was impaired in RGP, and the level of apoptosis remained high, which suggest that enhanced apoptosis and/or defective cell-cycle control reduce the production of mature cortical neurons at later development stages.

Transgenic mice expressing β-catenin precursors also show reduced cell-cycle exit and develop enlarged brains with reduced cortical thickness (Chenn and Walsh, 2002). In contrast, RP58−/− mice showed no enlargement of the brain, although the thickness of the neocortex was reduced. This discrepancy may be due to the reduction...
of cell-cycle exit only at late embryonic stages and/or the presence of high levels of apoptosis in the $RP58^{-/-}$ cortex. We consider RP58 a candidate molecule for the control of the number of mature cortical neurons, as $RP58$ deficiency decreased the number of mature neurons because of enhanced apoptosis and of defects in cell-cycle exit.

A delicate balance in cell proliferation and subsequent cell-cycle withdrawal and differentiation into specific neurons is essential for corticogenesis. The present study indicates the possibility that RP58 regulates this balance, at least at the late embryonic stage. In the wild-type E15.5 VZ, some $RP58$-positive cells showed weak Pax6 immunoreactivity, and almost all $RP58$-positive cells exhibited Tbr2 immunoreactivity (Supplementary Fig. 9), which suggests that the onset of $RP58$ expression happens in IMPs, at the initial stage, when Pax6 and Tbr2 may be coexpressed (Englund et al., 2005).

Pax6+ cells were increased in the $RP58$ null mutant, as were both Pax6+/Tbr2− and Pax6+/Tbr2+ cells. The increase in the number of Pax6+/Tbr2− cells in the mutant is explained by the reduction of cell-cycle exit of VZ progenitors. It is likely that there are extrinsic actions that allow RP58 to activate the expression of extrinsic factors that control cell-cycle exit, because RP58 is not detected in most Pax6+ cells. In fact, it is reported that the generation of projection neurons from cortical progenitors appears to be governed by both cell-intrinsic and environmental cues (Mizutani and Gaiano, 2006); however, we cannot exclude the possibility that a few Pax6+ cells abnormally proliferated in the mutant, as RP58 was detected in some Pax6+ cells in the wild type.

Pax6+/Tbr2− and Pax6+/Tbr2+ cells were increased in the mutant VZ/SVZ, whereas Pax6−/Tbr2+ cells were not increased. Therefore, it is possible that Pax6 is ectopically expressed in Tbr2+ IMPs in the mutant, and that the transition from Pax6+/Tbr2+ cells to Pax6−/Tbr2− cells was inhibited in the mutant, which raises the possibility that RP58 may be an important molecule for the maturation of IMPs. It was reported that (1) Svet1 is a spliced intronic sequence from Unc5d (Sasaki et al., 2008) and (2) Svet1/Unc5d staining is a specific marker of late-stage IMPs. It is likely that Svet1/Unc5d expression was reduced in the mutant, which supports the possibility that RP58 is most important for maturation process from early-stage IMPs to late-stage IMPs; however, as the expression of Svet1/Unc5d is also observed in young neurons (Kawaguchi et al., 2008), the possibility that the reduction of Svet1/Unc5d signal in the mutant reflects the reduction of the number of generated neurons cannot be excluded.
The expression of Pax6, Tbr2, and Svet1/Unc5d reveals that the formation of VZ/SVZ was impaired in the mutant (Supplementary Figs. 15 and 16). The outer Pax6-dominant zone in the E16.5 mutant may be caused by the reduced cell-cycle exit (Supplementary Fig. 18). The outer Pax6-dominant zone in E16.5 was not distinct when compared with that of the E18.5 animals. Therefore, the outer Pax6-dominant zone observed in the E18.5 mutant may partially explain the reduced cell-cycle exit.

Tbr2-positive cells were increased in the mutant VZ/SVZ, as were Pax6+/Tbr2+ cells, but not Pax6+/−/Tbr2+ cells. Tbr2+ IMPs are originated from Pax6+ RGPs. Therefore, the increase in Pax6+/−/Tbr2+ cells in the mutant may be explained by the increase in Pax6+/Tbr2− cells.

We showed that RP58 was expressed in some Ngn2-positive cells (Supplementary Fig. 11). RP58 acts downstream of Ngn2 (Seo et al., 1998). The overexpression of RP58 in the VZ in a sustained manner. As RP58 functions probably (Supplementary Fig. 11). RP58 acts downstream of Ngn2 (Seo et al., 1998). The outer Pax6-dominant zone in the E16.5 mutant may be explained by the increase in Pax6+/Tbr2− cells.

On the other hand, the overexpression of some genes, which may include Pax6, Tbr2, and Ngn2, may explain the abnormalities observed in the RP58-deficient brain. The overexpression of Pax6 affects the proliferation of neuronal progenitors and causes failure of neuronal differentiation (Bel-Vialar et al., 2007) and Tbr2 misexpression inhibits cell-cycle exit (Sessa et al., 2008). We are now analyzing whether the phenotype of the RP58 mutant brain can be explained by the enhanced expression of those genes.

In conclusion, we found that RP58 deficiency reduces the number of mature cortical neurons via strongly enhanced apoptosis and impaired cell-cycle exit, which suggests that RP58 plays a key role in the survival of cortical neurons and in the development of neuronal progenitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2009.04.030.

References


