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RP58 Regulates the Multipolar-Bipolar Transition of Newborn Neurons in the Developing Cerebral Cortex

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SUMMARY

Accumulating evidence suggests that many brain diseases are associated with defects in neuronal migration, suggesting that this step of neurogenesis is critical for brain organization. However, the molecular mechanisms underlying neuronal migration remain largely unknown. Here, we identified the zinc-finger transcriptional repressor RP58 as a key regulator of neuronal migration via multipolar-to-bipolar transition. *RP58*$^{-/-}$ neurons exhibited severe defects in the formation of leading processes and never shifted to the locomotion mode. Cre-mediated deletion of RP58 using in utero electroporation in *RP58*$^{flox/flox}$ mice revealed that RP58 functions in cell-autonomous multipolar-to-bipolar transition, independent of cell-cycle exit. Finally, we found that RP58 represses *Ngn2* transcription to regulate the Ngn2-Rnd2 pathway; *Ngn2* knockdown rescued migration defects of the *RP58*$^{-/-}$ neurons. Our findings highlight the critical role of RP58 in multipolar-to-bipolar transition via suppression of the Ngn2-Rnd2 pathway in the developing cerebral cortex.

INTRODUCTION

Proper neuronal migration is critical for brain organization. It is known that defects in neuronal migration during embryogenesis are related not only to brain malformation but also to psychiatric disorder (Kähler et al., 2008; Verrotti et al., 2010; Liu, 2011). However, the genetic and developmental pathways that regulate neuronal migration remain to be elucidated. During glutamatergic neurogenesis, newborn neurons migrate from the ventricular zone (VZ) toward the pial surface. This process comprises several steps: proliferation of progenitor cells in the VZ and their cycle exit and differentiation, multipolar migration in the subventricular zone (SVZ) and intermediate zone, and radial migration in the cortical plate after multipolar-to-bipolar transition. Many genes reportedly participate in each step of neurogenesis (Hevner et al., 2006; Guillemot, 2007; Barnes and Polleux, 2009). In particular, many transcription factors play key roles as molecular switches in regulating the gene expression of downstream effectors to control this process. For example, Pax6 (Georgala et al., 2011) and Sox2 (Hutton and Pevny, 2011) are expressed in neural progenitor cells. Ngn2 plays an important role in the specification of glutamatergic neurodifferentiation (Fode et al., 2000) and regulation of neuronal migration (Hand et al., 2005; Ge et al., 2006; Heng et al., 2008). It activates downstream effector genes *Tbr2(Eomes)* and *NeuroD* in intermediate progenitor cells and postmitotic neurons (Hevner et al., 2006; Seo et al., 2007; Ochiai et al., 2009). Besides Ngn2, Brn1/2 and REST are also involved in neuronal migration (Sugitani et al., 2002; Mandel et al., 2011). Each of these transcription factors is expressed in certain cell populations during neurogenesis. Therefore, in normal cortical development, every step of neurogenesis involves precise temporal regulation of the gene expressions of downstream effectors. However, the molecular mechanisms underlying such regulation are not yet clearly understood.

RP58, a zinc-finger transcriptional repressor belonging to the BTB/POZ-domain family (Aoki et al., 1998), is highly expressed in the developing cerebral cortex (Ohtaka-Maruyama et al., 2007). *RP58* is a downstream target of Ngn2 (Seo et al., 2007) and is prominently activated in multipolar migrating cells in the embryonic cortex. Here, we report that RP58 controls neuronal migration via the regulation of multipolar-to-bipolar conversion in the developing cerebral cortex, in addition to its role in cell-cycle exit of progenitor cells (Okado et al., 2009; Hirai et al., 2012). *RP58*$^{-/-}$ brains show severe defects in neuronal migration through failed repression of *Ngn2* as a downstream effector. Ngn2, a proneural basic-helix-loop-helix (bHLH) transcriptional activator, is known as a master regulatory factor in glutamatergic

neuronal differentiation. It is transiently expressed in committed neuronal progenitor cells (Ochiai et al., 2009) and downregulated in postmitotic neurons after activating many target genes, including *RP58* and *Rnd2* (Seo et al., 2007; Heng et al., 2008; Gohlke et al., 2008). Our study reveals that negative feedback regulation of *Ngn2* transcription by RP58 is important for maintaining the Ngn2-Rnd2 pathway at an appropriate level and plays an essential role in promoting normal neuronal migration in the developing cerebral cortex.

RESULTS

RP58 Controls Neuronal Migration in the Developing Cerebral Cortex

RP58 is strongly expressed in the developing cerebral cortex, especially on E15–16, during which extensive neuronal production, as well as migration of new cortical neurons, occurs (Ohtaka-Maruyama et al., 2007; Figure S1A). RP58 is expressed in a fraction of progenitor cells, and it is specifically expressed in excitatory glutamatergic neurons from the embryonic stage to adulthood (Ohtaka-Maruyama et al., 2007; Okado et al., 2009). Birthdating experiments with bromodeoxyuridine (BrdU) suggest the involvement of RP58 in cell-cycle exit of neural progenitor cells and neuronal migration of late-born neurons (Okado et al., 2009; Figures S1B and S1C). The mechanism underlying cell-cycle exit has been elucidated elsewhere (Hirai et al., 2012), whereas the details of its function in migration remain unknown.

To clarify the role of RP58 in neuronal migration, we first investigated neuronal migration in $RP58^{-/-}$ mouse brains by in utero electroporation. Green-fluorescent-protein (GFP)-positive electroporated cells showed severely impaired migration compared with wild-type (WT) cells (Figure 1A). Few GFP-positive cells had reached the pial surface 3 days after electroporation in $RP58^{-/-}$ brains. However, this defect was completely rescued by coelectroporation of RP58-expression plasmids (Figures 1A and S1F).

To determine whether small-interfering-RNA (siRNA)-mediated knockdown of *RP58* affects neuronal migration in WT brains (Figures S1D and S1E), we chose two small-hairpin RNA (shRNA) constructs (#30 and #36) that effectively inhibited *RP58* expression (Figure S1D), performed in utero electroporation on E14, and harvested the brains on E17 (Figure S1E). *RP58* knockdown resulted in migration defects, suggesting that RP58 has cell-autonomous functions in neuronal migration. GLAST and nestin immunostaining suggested that radial glial fibers of $RP58^{-/-}$ brains remained intact (Figures 1B and S1G). Magnified images revealed that $RP58^{-/-}$ brains coelectroporated with the RP58-expression plasmids had normal cell shape and migration (Figure 1C).

One characteristic of the defects observed in $RP58^{-/-}$ brains is the increased population of Pax6-positive neural and Tbr2-positive immature intermediate progenitor cells (Okado et al., 2009), suggesting that RP58 controls the neural progenitor kinetics. Ki67 and Tbr2 immunostaining revealed that the increased population of Ki67- and Tbr2-positive cells in the VZ of $RP58^{-/-}$ brains was normalized by electroporation of the RP58-expression plasmids (Figures 2A and 2C). This normalization occurred only in the GFP-positive electroporated regions (Figure S2B). To confirm the rescue of the increase in neural-progenitor proliferation, BrdU immunostaining was performed 2 hr before harvesting E17 brains (electroporated on E14). The increased ratio of BrdU-positive cells to GFP-positive cells normally observed in $RP58^{-/-}$ brains normalized in the brains electroporated with the RP58-expression plasmids (Figure S2). These results suggest that extrinsic expression of RP58 rescues the abnormal increase in neural-progenitor proliferation observed in the $RP58^{-/-}$ VZ.

GFP-positive $RP58^{-/-}$ cells located above the VZ were Ki67 negative (Figure 2B), suggesting a functional role for RP58 in neuronal migration. However, the migration defect of $RP58^{-/-}$ neurons may be attributable to impairment of neural-progenitor proliferation, as migration occurs after progenitor proliferation. Therefore, it is difficult to distinguish whether RP58 functions in neuronal migration independently of cell-cycle exit by using conventional RP58 knockout mice. To clarify this issue, we next used conditional knockout mice to generate a situation in which *RP58* is deleted only in migrating cells.

RP58 Has Cell-Autonomous Functions in Multipolar-to-Bipolar Transition

We generated conditional knockout mice ($RP58^{flox/flox}$) and examined the migration defect following complete deletion of *RP58* by in utero electroporation of Cre-expression plasmids. $RP58^{-/-}$ neurons showed dramatic migration defects, with migrating cells exhibiting migration arrest in the middle of the cortex (Figure 3A). Again, this phenotype was completely rescued by electroporation of RP58-expression plasmids (Figure 3A). Immunostaining confirmed that RP58 expression is responsible for this dramatic phenotype (Figures 3B and 3C), indicating that RP58 has cell-autonomous function in neuronal migration.

A few RP58-positive cells entered the cortical plate (CP), probably because of insufficient expression of Cre (Figures 3B and 3C, arrows). These results suggest that the migration of immature neurons is associated with a permissive boundary requiring RP58 functions. MAP2 immunostaining revealed that the subplate layer likely represents this boundary (Figure 3D), because $RP58^{-/-}$ cells failed to cross this layer, thus accumulating under the subplate and failing to enter into the CP. These migration-arrested cells near the subplate layer showed Ki67 negativity and Tbr1 positivity (Figure 3E). Detailed observation of cell morphology revealed that $RP58^{-/-}$ neurons failed to undergo multipolar-to-bipolar transition (Figures 3B, 3C, and 3E), suggesting that they exited the cell cycle but failed to convert to the bipolar morphology before entering the nascent CP because of RP58 deficiency.

To examine cell-cycle exit in these migrating defective neurons, BrdU was injected at E14, just after electroporation of GFP, or GFP together with Cre expressing plasmids, into $RP58^{flox/flox}$ mice. Embryos were harvested 24 hr later and immunostained with anti-BrdU and anti-Ki67 antibodies (Figure 3Fa and 3Fb). As a result, no significant differences were observed between control and Cre-electroporated brains regarding the ratio of cell-cycle exit, which is estimated as the percentage of $Ki67^{-}$ cells/$GFP^{+}BrdU^{+}$ cells (Figure 3Fc). We also performed a BrdU pulse-labeling experiment before harvesting at 24 hr after

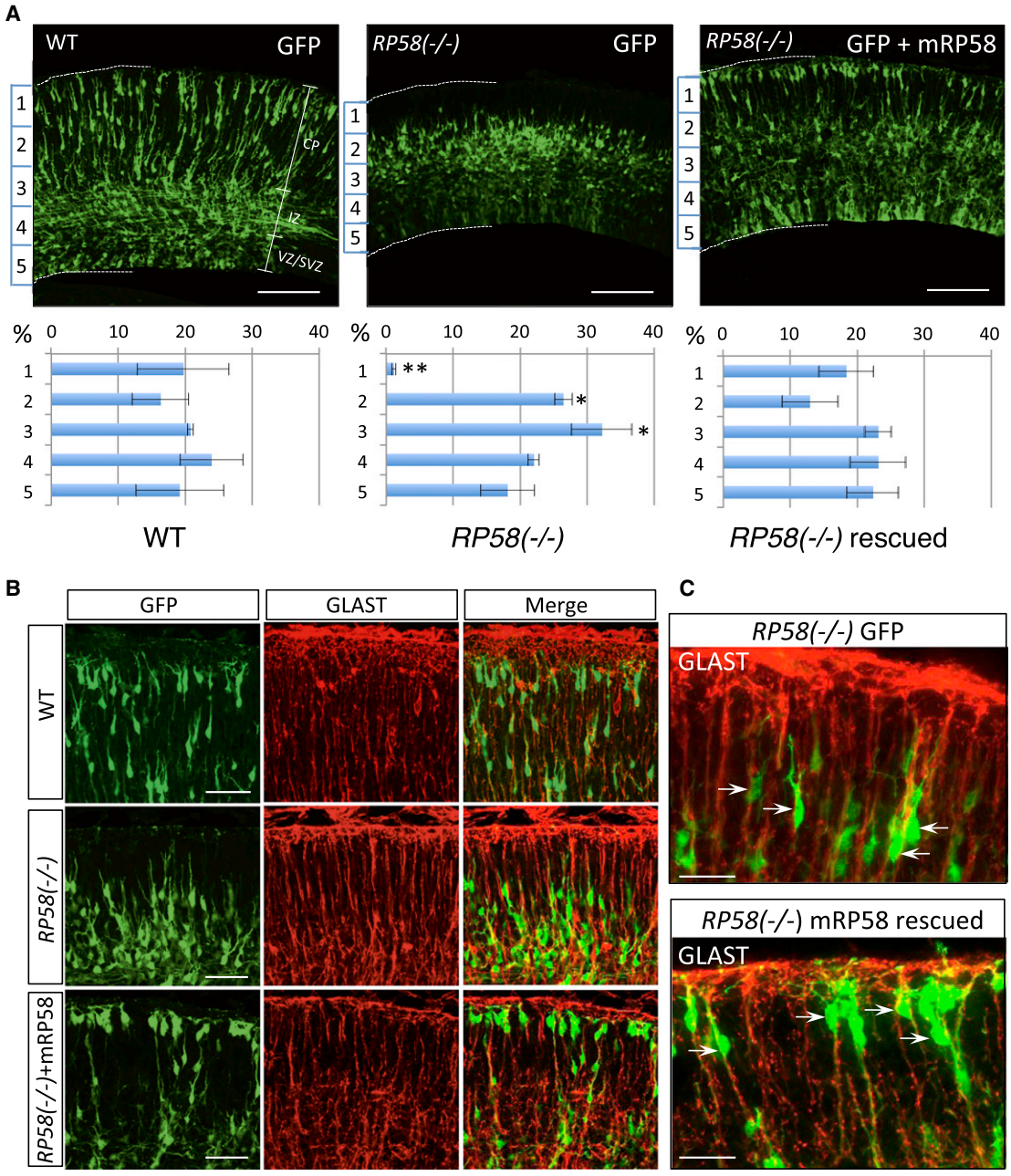

Figure 1. Migration Defects in *RP58*$^{-/-}$ Brains

(A) GFP-positive cells did not reach to the pial surface of *RP58*$^{-/-}$ brains, but the migration defects were completely rescued by electroporation of mouse RP58-expression plasmids (mRP58). Scale bars, 100 μm. Data represent the mean ± SD (n = 6 slices from three mice); *p < 0.05, **p < 0.01 (Student's t test). See also Figures S1B, S1C, and S1E.

(B) GLAST immunostaining revealed that radial glial fibers were not affected in *RP58*$^{-/-}$ brains. Scale bars, 50 μm. See also Figure S1G.

(C) Magnified images of (B) reveal that GFP-positive cells did not reach the pial surface of *RP58*$^{-/-}$ brains, but rescued cells migrated to the pial surface. Scale bars, 25 μm.

See also Figure S1.

electroporation. The ratio of intermediate progenitor (IMP) and BrdU-Tbr2 double positive cells to GFP-positive cells also did not differ between control and Cre-electroporated brains (Figure S3A). This suggests that cell-cycle exit is not impaired in *RP58* $^{flox/flox}$ cells and that the effects of *RP58* deletion by Cre likely emerged after the exit of progenitor cells from the cell cycle in this experimental condition. Immunostaining for RP58 confirmed that the level of RP58 proteins was diminished

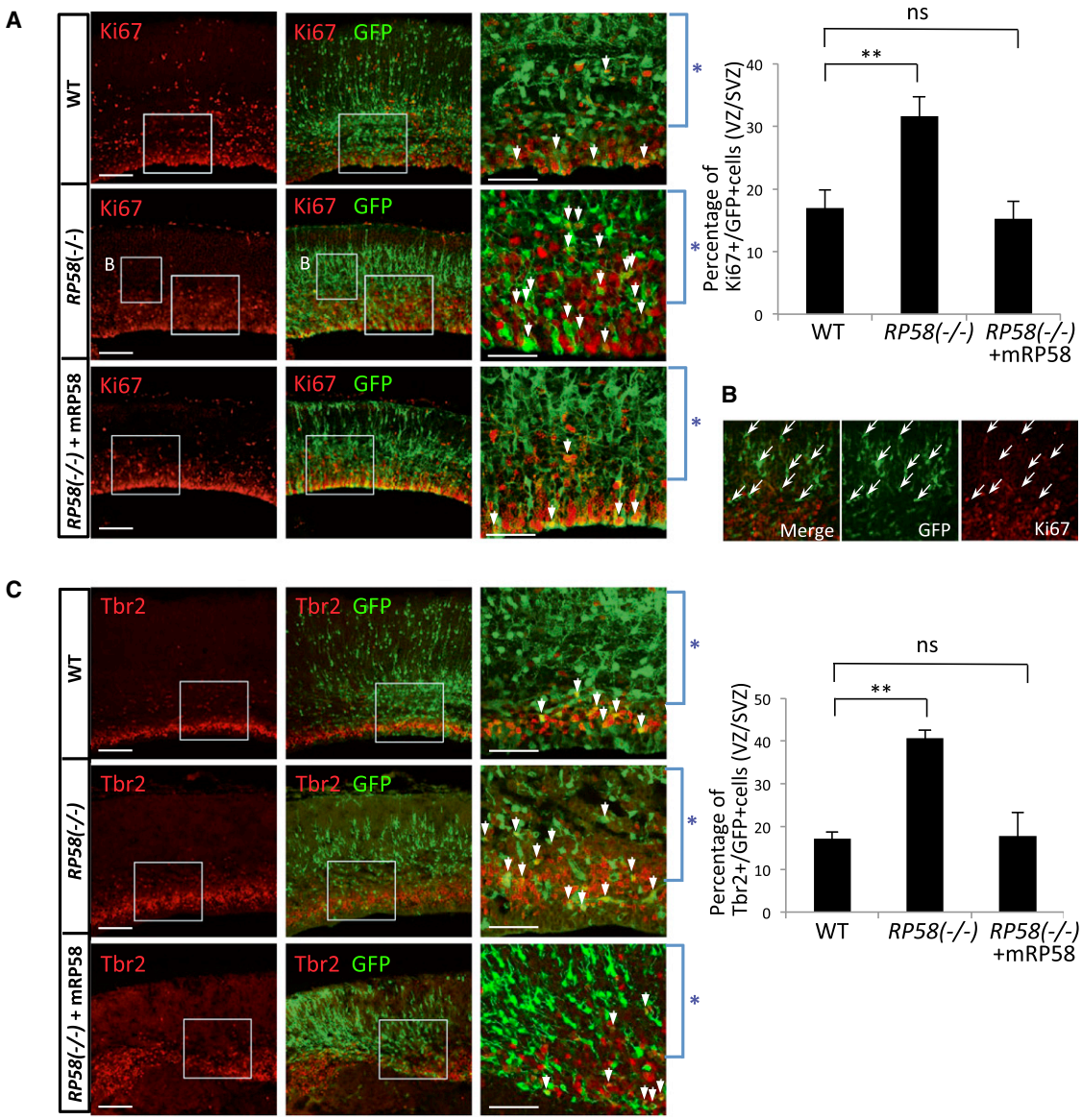

Figure 2. Rescue of the Abnormal Increase in Neural Progenitor Proliferation Observed in *RP58*$^{-/-}$ Brains

(A) The number of Ki67-positive cells (arrows) in rescued *RP58*$^{-/-}$ brains decreased to the same level as that of GFP-positive cells in the upper VZ and SVZ (asterisks) compared with control *RP58*$^{-/-}$ brains. See also Figure S2A.

(B) Magnified images of a part of the nonrescued intermediate zone. Ki67 immunostaining revealed that the migration-impaired GFP-positive cells in nonrescued *RP58*$^{-/-}$ brains were Ki67 negative (arrows).

(C) The number of Tbr2-positive cells decreased in rescued *RP58*$^{-/-}$ brains. The increased number of Tbr2-positive cells in the VZ of *RP58*$^{-/-}$ brains normalized in brains electroporated with RP58-expression plasmids. All electroporation experiments were performed in utero on E14 and tissues were harvested on E17. Data represent the mean ± SD (n = 6 slices from two to three mice); **p < 0.01 (Student's t test). Scale bars, 100 μm.

See also Figure S2.

between 2 and 3 days after electroporation. Many GFP-positive E16 Cre-electroporated cells still expressed the RP58 proteins (Figure S3B). This suggests that there is a time lag between Cre expression and complete loss of expression of RP58 proteins, and that Cre-electroporated cells may exit the cell cycle normally together with endogenous RP58 proteins. However, by E17, cells exhibited a complete loss of expression of the RP58 protein, showed a severe defect in multipolar-bipolar conversion, and were not able to cross the subplate layer (Figure S3B). Fixation of the Cre-electroporated *RP58* $^{flox/flox}$ mouse brain 5 days after electroporation at E19 revealed that RP58-deficient cells still had not entered into the cortical plate, suggesting that the migration defect is not simply due to their migrating speed (Figure S3C).

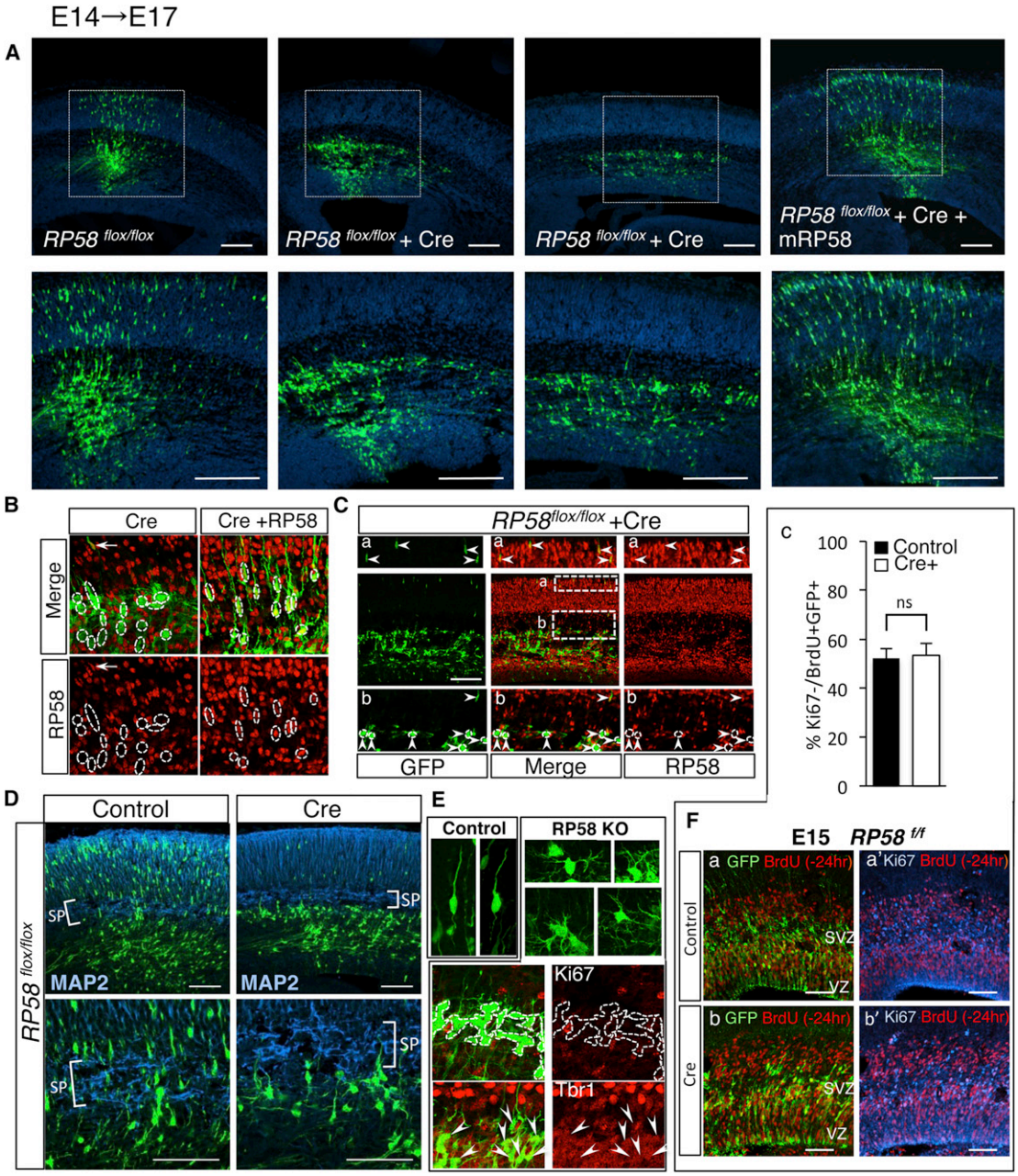

Figure 3. Striking Migration Defects by Acute Deletion of *RP58*

(A) Coelectroporation of Cre-expression plasmids with GFP in E14 mouse brains homozygous for a conditional null mutant allele of *RP58* (*RP58*$^{flox/flox}$) resulted in acute deletion of *RP58* and prevented neurons from entering the CP. Coelectroporation of RP58-expression plasmids completely rescued these migration defects.

(B and C) RP58 immunostaining confirmed that all the stagnating cells were RP58 negative. Migrating cells that entered the CP (arrows) and RP58-rescued cells expressed RP58 (B, right panels). Scale bars, 100 μm.

(D) MAP2 immunostaining revealed that the permissive boundary was the subplate layer. Magnified images show that *RP58*$^{-/-}$ cells failed to enter the CP and stagnated immediately under the subplate layer. SP, subplate.

(E) *RP58*$^{-/-}$ cells failed to undergo multipolar-to-bipolar transition and possessed multiple thin neurites. These cells were Ki67 negative and Tbr1 positive.

(F) The exit of progenitor cells from the cell cycle was not impaired in Cre-electroporated GFP-positive cells. GFP plasmids were coelectroporated with or without Cre-expression plasmids into brains of *RP58*$^{flox/flox}$ mice at E14, and BrdU was injected just after electroporation. Embryos were fixed 24 hr later and the brain sections were immunostained with Ki67 and BrdU antibodies. There was no significant difference in the ratios of Ki67 negative cells to BrdU and GFP-double-positive electroporated cells between control and Cre-introduced cells in the VZ. Data represent the mean ± SD (n = 6 slices from two individuals); ns > 0.05 (Student's t test).

See also Figures S3A and S3B.

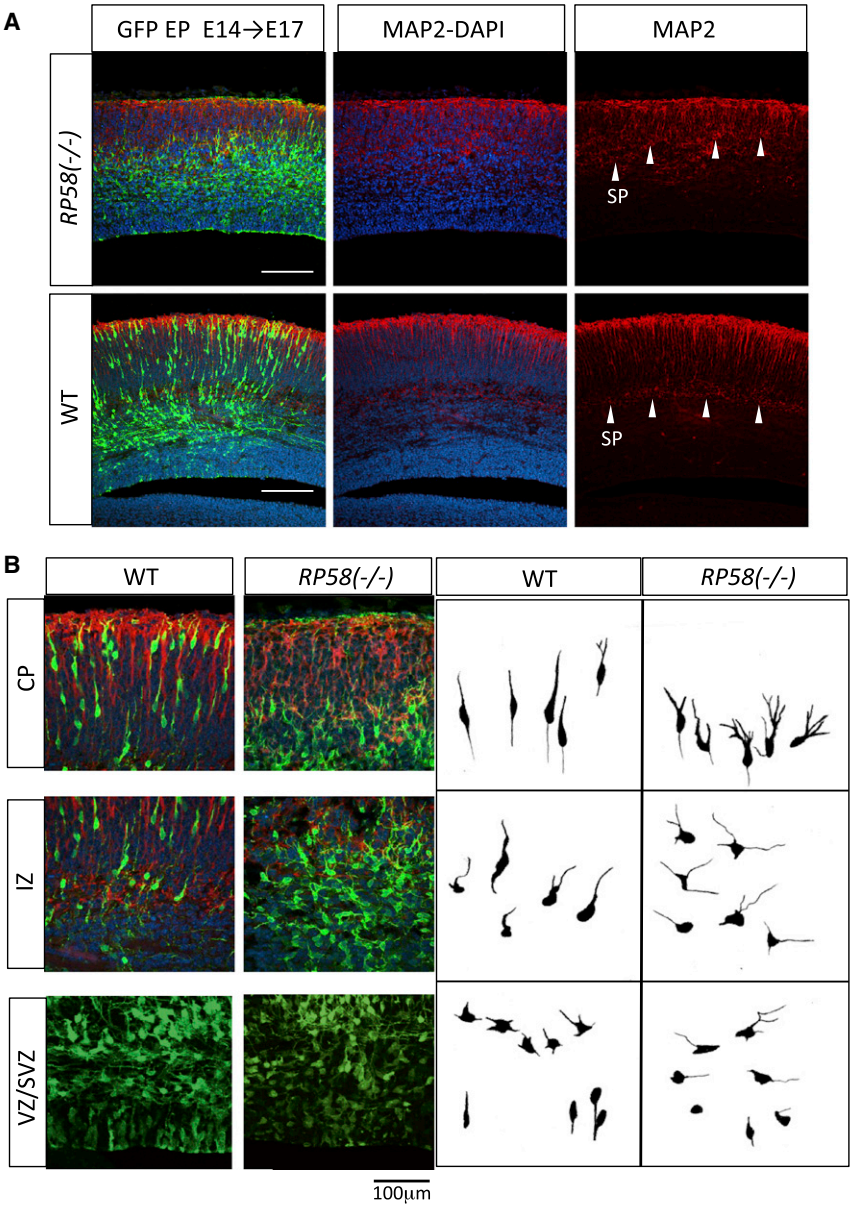

Figure 4. Failure of *RP58*$^{-/-}$ Cells to Cross the Subplate Layer

(A) MAP2 immunostaining revealed that the subplate layer (arrowheads) of *RP58*$^{-/-}$ brains was disorganized compared with that of WT brains. GFP-positive migration-defective cells failed to enter the CP by crossing the disorganized subplate layer. Scale bars, 100 μm. See also Figure S4A.

(B) Magnified images of each area and tracings of GFP-positive electroporated cells revealed abnormal morphology of *RP58*$^{-/-}$ neurons. Scale bar, 100 μm.

RP58 Regulates Neuronal Cell Morphology

The results of the conditional knockout experiment prompted us to reexamine the migration defect of *RP58*$^{-/-}$ neurons from this standpoint. MAP2 immunostaining revealed that the subplate layer of *RP58*$^{-/-}$ brains was disorganized and that GFP-positive electroporated cells could not enter into the CP, similar to *RP58*$^{flox/flox}$ cells (Figure 4A). BrdU labeling at E11 and BrdU and Neurocan immunostaining at E15 supported the observation that the subplate layer of *RP58*$^{-/-}$ brains is disorganized (Figure S4A). *RP58*$^{-/-}$ neurons also failed to convert from the multipolar morphology to the bipolar morphology, as in the case of *RP58*$^{flox/flox}$ cells (Figure 4B). Furthermore, multipolar cells located in the SVZ and intermediate zone did not have the same morphology as WT cells: they harbored thinner and longer neurites than did icontrol cells (Figure 4B). In a previous study, immunostaining of Tuj1, which is immunoreactive in neurites, was found to be decreased in *RP58*$^{-/-}$ cortices (Okado et al., 2009; Figure S2), suggesting the involvement of RP58 in the regulation of axonal and dendritic development.

To examine the development of neurites of *RP58*$^{-/-}$ neurons, we prepared dissociated primary cultured neurons from E14 WT and *RP58*$^{-/-}$ hippocampal regions. Tau-1 immunostaining of these neurons on DIV7 revealed that *RP58*$^{-/-}$ cells extended shorter and thinner axons than did WT cells (Figure S4B). Next, we examined the development of dendrites of primary cultured neurons prepared from WT and *RP58*$^{-/-}$ cerebral cortical cells by MAP2 immunostaining on DIV3, DIV5, and DIV7. In comparison with WT cells, which extended thick and long dendrites in reticular formation on DIV7, *RP58*$^{-/-}$ neurons extended poor and thin dendrites at the same stage (Figure S4C). It is worth noting that although equal cell numbers were plated on DIV0, fewer neurons were observed in the *RP58*$^{-/-}$ dishes (Figure S4C, arrows), supporting previous observations that apoptosis is enhanced in the *RP58*$^{-/-}$ cerebral cortex (Okado et al., 2009; Figure 6). Taken together, these results support the notion that RP58 functions in axonal and dendritic growth, as well as in neuronal survival.

RP58 Represses Ngn2 Transcription by Directly Binding to Its Transcriptional Regulatory Regions

Ngn2 is a master transcriptional activator for glutamatergic neurogenesis in the cerebral cortex (Fode et al., 2000; Guillemot, 2007); it is also involved in regulating neuronal migration (Hand et al., 2005; Ge et al., 2006; Heng et al., 2008). *RP58* is a downstream target gene of Ngn2 (Seo et al., 2007; Gohlke et al., 2008; Yokoyama et al., 2009). We also confirmed by luciferase reporter assay that Ngn2 activates *RP58* promoter activity in Neuro2A cells (Figure 5A). Moreover, we confirmed the induction of *RP58* expression in dissociated cerebral cortical cells

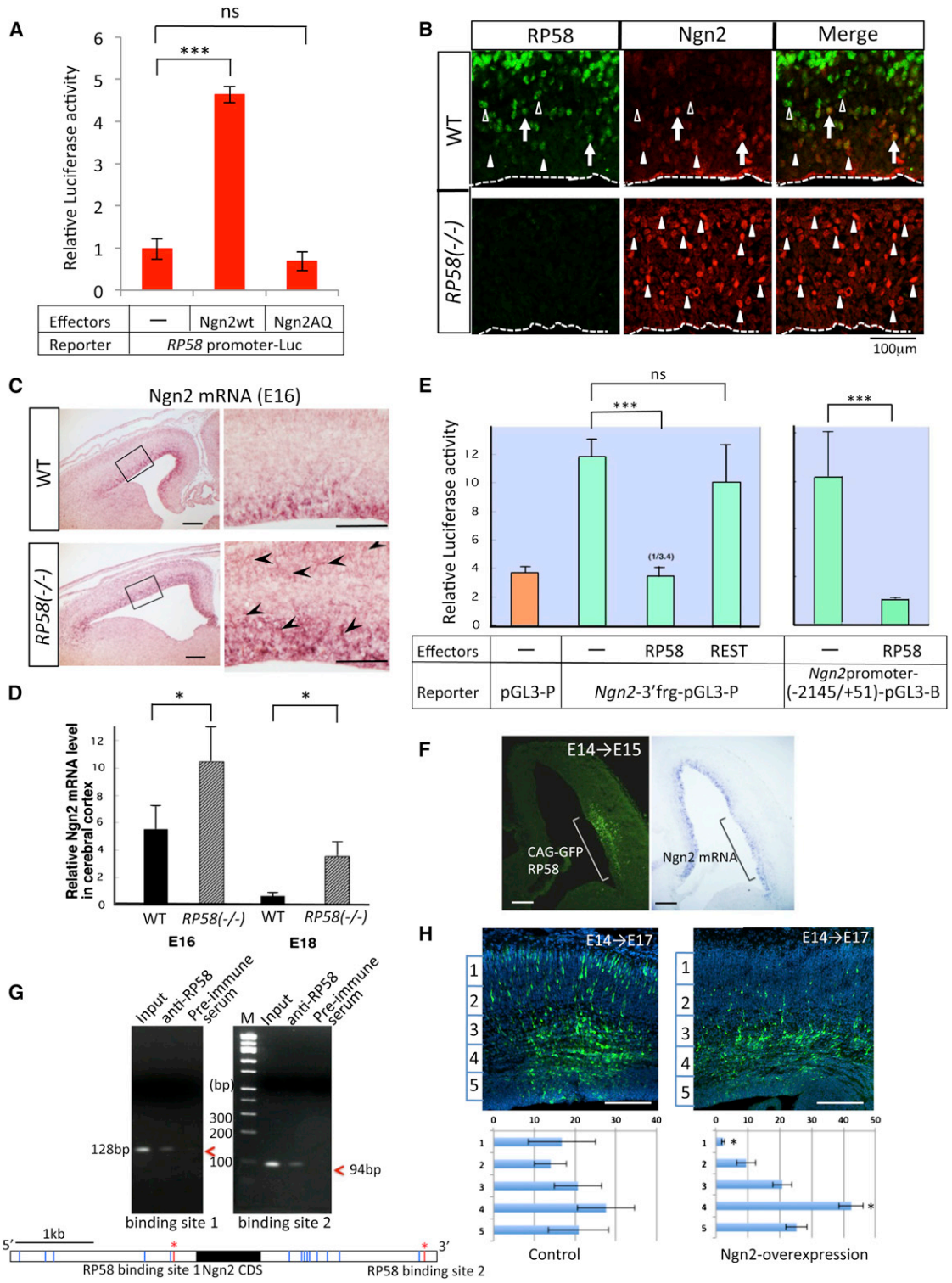

Figure 5. RP58 Binding to the Ngn2 Promoter and 3′-Downstream Enhancer Region

(A) Ngn2-mediated RP58 promoter activation was detected by luciferase reporter assay. The promoter was activated by the Ngn2 WT but not the Ngn2-AQ mutant plasmid, which lacks transactivation activity. Data represent the mean ± SD (n = 6 transfections); ***p < 0.001 (Student's t test).

(B) WT and *RP58*$^{-/-}$ brains were double-stained with RP58 and Ngn2 antibodies to determine the number of Ngn2-positive cells. Ngn2-positive (solid arrowheads), Ngn2-and-RP58-double-positive (arrows), and RP58-positive cells were observed in the WT brains. However, in the *RP58*$^{-/-}$ brains, an increased number of Ngn2-positive cells (solid arrowheads) were observed in the upper part. Scale bar: 100 μm.

(legend continued on next page)

transfected with Ngn2-expression plasmids (Figure S5A). These results suggest that Ngn2 activates *RP58* expression in primary cultured neurons.

To examine whether induced RP58 functions as a transcriptional repressor, BS10-Luc plasmid (10 × RP58-binding sites) (Aoki et al., 1998) was cotransfected into these cells. The repressor function of induced RP58 was confirmed by measuring the decreased luciferase activity (Figure S5A). We also confirmed colocalization of Ngn2 and RP58 in some VZ cells by immunostaining (Figure 5B). From the immunostaining pattern, we noticed that Ngn2 expression is transient: first, Ngn2 was expressed in neuronal committed progenitor cells (Figure 5B, solid arrows); then, induced RP58 colocalized in a subpopulation of VZ cells (Figure 5B, arrows), and thereafter, Ngn2 expression was not detected in the RP58-expressing cells (Figure 5B, open arrows). Moreover, *RP58*$^{-/-}$ brains immunostained for Ngn2 showed an increase in the population of Ngn2-expressing cells, which was located ectopically above the VZ (Figure 5B). This unexpected result suggests that Ngn2 induces the expression of RP58, which then represses Ngn2 expression.

To examine this possibility, we first performed in situ hybridization (ISH) and quantitative PCR. *Ngn2* mRNA levels were increased in both E16 and E18 *RP58*$^{-/-}$ cerebral cortices (Figures 5C and 5D, and S5B), and *Ngn2* expression was ectopically upregulated in the upper regions of the VZ (Figures 5C and S5B, arrows). By luciferase reporter and chromatin immunoprecipitation (ChIP) assays, we next examined whether RP58 represses Ngn2 transcription by directly binding to regulatory elements of the *Ngn2* genomic region. When we searched the RP58-binding type E box in the 5′- and 3′-flanking regions of *Ngn2*, we identified two RP58-binding sites: one located in the 5′-flanking region (the previously identified E2 region [Scardigli et al., 2001]) and the other located in the 3′-flanking region (the previously identified E3 region). We constructed reporter plasmids with two genomic fragments including these binding sites (pNgn2 promoter [−2145/+51]-pGL3B and pNgn2-3′frg-pGL3-P) and tested their functions in the presence or absence of *RP58*. The luciferase activities of both reporter plasmids were repressed by RP58 in cotransfected cultured cells compared with empty vector controls (Figure 5E).

Next, we examined the effects of *RP58* overexpression on endogenous *Ngn2* expression in vivo. ISH of specimens fixed at 20 hr after electroporation of RP58-expression plasmids revealed that ectopic *RP58* overexpression disturbed endogenous Ngn2 transcription (Figure 5F). Moreover, ChIP assay of E16 brain extracts with RP58 antibody confirmed direct RP58 binding to the RP58-binding type E boxes in the *Ngn2* genomic region (Figure 5G). Therefore, we concluded that RP58 directly represses *Ngn2* transcription in vivo.

If upregulation of *Ngn2* is a major cause of the migration defects of *RP58*$^{-/-}$ neurons, *Ngn2* overexpression in WT brains should also yield a similar phenotype. To examine this, we performed electroporation of Ngn2-expression plasmids into WT brains. When we used Ngn2-expression plasmids with the β-actin promoter, migration defects were observed (Figure 5H).

Ngn2 Knockdown Rescues Migration Defects in *RP58*$^{-/-}$ Brains

Given that Ngn2-induced RP58 represses *Ngn2* transcription, RP58 may be normally responsible for the transient expression of Ngn2 in vivo. Therefore, the ectopic expression of *Ngn2* in postmitotic neurons of *RP58*$^{-/-}$ brains may disturb their migration. We tested this possibility by knockdown of ectopic *Ngn2* expression with RNAi in *RP58*$^{-/-}$ brains. We used shRNA for Ngn2 (Figure S6A) in the conditional knockdown driven by RP58 promoter (RP58-Cre) (Ohtaka-Maruyama et al., 2012) and observed that *Ngn2* knockdown rescued the defects in migrating distance and cell morphology (Figure 6). Because *RP58*$^{-/-}$ cells cannot enter the CP by crossing the subplate layer, we evaluated the extent of rescue of migrating distance from the subplate layer by dividing the CP into three parts (i.e., upper, middle, and lower). As shown in Figure 6A, shNgn2 electroporation in *RP58*$^{-/-}$ brains partially rescued migration above the subplate layer. Magnified images revealed that migration-rescued cells also had rescued morphology (Figure 6B). In comparison with WT CP neurons, *RP58*$^{-/-}$ neurons possessed mostly thin multipolar neurites (Figure 6B, arrows). However, shNgn2-rescued *RP58*$^{-/-}$ neurons possessed leading processes similar to WT neurons (Figure 6B, lower panel).

To quantitate the extent of rescue, we measured the width of the cell body and thickness of the leading process or leading-process-like neurite measured from the basal region of the process. Figure 6B shows the tendency of shNgn2-rescued *RP58*$^{-/-}$ neurons to have thicker leading processes. Fode et al. (2000) reported that loss of *Ngn2* results in misspecification of projection neurons into interneurons. To exclude the possibility of respecification of GFP-positive rescued cells by shNgn2 electroporation into cortical interneurons, we performed Dlx2 immunostaining to identify interneurons: shNgn2-electroporated GFP-positive cells were negative for Dlx2 expression (Figure S6B).

(C and D) The mRNA level of *Ngn2* was upregulated in *RP58*$^{-/-}$ brains (C) and was confirmed by quantitative PCR at the E16 and E18 stages (D). Scale bars, 100 μm. Data represent the mean ± SD (n = 3); *p < 0.05 (Student's t test). See also Figure S5B.

(E) RP58 mediated transcriptional repression of luciferase linked to the mouse *Ngn2* promoter region and 3′-enhancer region. Luciferase activities were measured in COS-7 cells cotransfected with *Ngn2* reporters and RP58-expression vectors. REST, used as the negative control, did not repress *Ngn2* enhancer activity. Data represent the mean ± SD (n = 6 transfections); ***p < 0.001 (Student's t test).

(F) Ectopic expression of *RP58* caused derangement of the endogenous *Ngn2* expression pattern in the embryonic cortex. RP58 expression vectors and GFP plasmids were electroporated into E14 cortices and fixed on E15. *Ngn2* mRNA localization was detected by ISH. Scale bars, 100 μm.

(G) ChIP assay of lysates prepared from E14 cerebral cortices by using RP58 antibody confirmed the direct binding of RP58 to RP58-binding sequences in the *Ngn2* enhancer and promoter regions.

(H) Ngn2-expressing plasmids driven by the β-actin promoter were electroporated on E14 and fixed on E17. *Ngn2* overexpressing cells exhibited impaired migration. Data represent the means ± SD (n = 6 slices from two individuals); *p < 0.05 (Student's t test). Scale bar, 100 μm.

See also Figure S5.

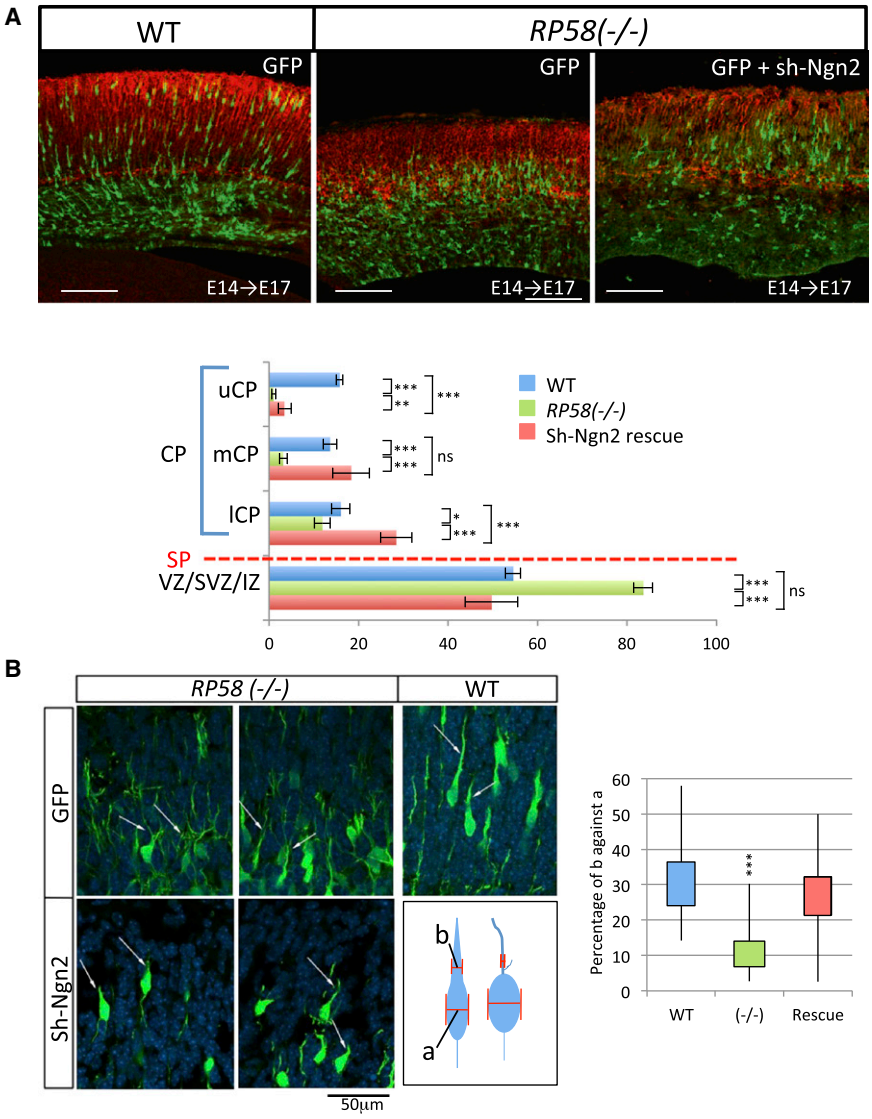

Figure 6. Rescue of Migration Defects in *RP58*$^{-/-}$ Brains by Sh-Ngn2

(A) Knockdown of *Ngn2* by using sh-*loxP*-STOP-*loxP*-Ngn2 expressed by RP58 promoter-driven Cre plasmid rescued the migration defect of *RP58*$^{-/-}$ neurons. The extent of rescue was appreciable by MAP2 immunostaining. In comparison with *RP58*$^{-/-}$ cells, sh-Ngn2-rescued cells migrated farther in the CP after crossing the subplate layer. Data represent the mean ± SD (n = 6 slices from three individuals); *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t test). Scale bars, 100 μm. See also Figure S6.

(B) *Ngn2* knockdown in *RP58*$^{-/-}$ brains rescued the morphology of the migrating neurons. Magnified images reveal that the migration-defective cells did not possess typical leading processes with a single thick basal part (arrows in WT image). Instead, multiple thin neurites were observed (arrows in GFP *RP58*$^{-/-}$ images). Sh-Ngn2-electroporated neurons showed rescue of the formation of leading processes (arrows in sh-Ngn2 *RP58*$^{-/-}$ images). The thickness of the leading processes or thickest neurite of *RP58*$^{-/-}$ neurons was measured at one third of the entire length and recorded as b. The ratio of (b) to the width of the cell body (a) was calculated and is represented graphically. The rescued cells tended to have thicker leading-process-like neurites than nonrescued *RP58*$^{-/-}$ neurons. Over 150 cells were counted in three brains per condition. ***p < 0.01(Student's t test). Scale bar, 50 μm. See also Figure S6B.

Ectopic Ngn2 Expression Induces Migration Defects Mimicking those of RP58 Deficiency

To examine whether the effects of *Ngn2* overexpression depend on its DNA-binding properties, we used an Ngn2-AQ mutant plasmid (pCAG-Ngn2-AQ), which abolishes DNA binding to direct target promoter sequences (Sun et al., 2001; Lee and Pfaff, 2003). When CAG-Ngn2 plasmids were electroporated on E14 and fixed on E17, GFP-positive neurons stagnated below the subplate layer, similar to the case for *RP58*$^{-/-}$ neurons (Figure 7A). Ectopic Ngn2 expression was confirmed by Ngn2 immunostaining (Figure S7). Magnified images revealed that the cells with migration defects possessed thin neurites similar to *RP58*$^{-/-}$ or *RP58*$^{flox/flox}$ neurons (Figures 1B, 1C, 3E, and 7B). However, electroporation of the Ngn2-AQ mutant plasmid did not affect neuronal migration (Figure 7A), suggesting that the effects of *Ngn2* overexpression on neuronal migration depend on its DNA-binding activities.

Ngn2 overexpression reportedly increases the proportion of cells recruited to the SVZ and neurons initiating radial migration and accumulating in the CP (Hand et al., 2005; Ge et al., 2006). We obtained similar results, because GFP-positive cells quickly migrated to the SVZ, but their speed decreased in the CP following *Ngn2* overexpression (Figure 7A). Therefore, ectopic *Ngn2* expression induces migration defects mimicking those of *RP58*$^{-/-}$ cells (Figure 7E).

Ngn2 and its direct downstream effector Rnd2 play significant roles in neuronal migration (Heng et al., 2008). Therefore, RP58 may control the Ngn2-Rnd2 pathway as a repressor to ensure proper neuronal migration, including formation of thick leading processes and entry into the CP for locomotion. Further, RP58 represses *Rnd2* expression by directly binding to its 3′-regulatory region (J.I.H., Z. Qu, C.O.-M., H.O., M.K., D. Castro, F. Guillemot, and S.S. Tan, unpublished data). Therefore, *Rnd2* expression is likely regulated by both Ngn2 and RP58. We confirmed *Rnd2* upregulation by ISH in the region above the VZ in E16 *RP58*$^{-/-}$ brains (Figure 7C). To confirm that *Rnd2* upregulation is responsible for the migration defects following *Ngn2* overexpression, shRnd2 plasmids were coelectroporated with Ngn2-expression plasmids to determine whether the migration defects could be rescued. Electroporation of the conditional shRnd2

(Heng et al., 2008) vector with RP58-promoter-driven Cre plasmid led to partial rescue of the migration defects (Figure 7A). This confirmed that RP58 controls neuronal migration through negative feedback regulation of *Ngn2* transcription and Rnd2 expression (Figure 7D). Rnd2 is a major effector of Ngn2 in neuronal migration and may regulate the cytoskeleton (Heng et al., 2008; Figure 7D). Therefore, RP58 plays an essential role in multipolar-to-bipolar transition and the following locomotion step by regulating the Ngn2-Rnd2 pathway (Figure 7E).

DISCUSSION

Here, we have demonstrated that RP58 is indispensable to neuronal migration during neurogenesis. *RP58*$^{-/-}$ neurons neither convert to the normal bipolar morphology nor enter into the CP. These defects are completely rescued by restoring RP58, suggesting that its functions in neuronal migration are cell autonomous.

RP58 shows a highly specific expression pattern in embryonic brains: weak expression in the VZ and very strong expression in the SVZ and migrating neurons (Ohtaka-Maruyama et al., 2007). This suggests that RP58 has distinct functions in each region. As previously reported, RP58 controls the division of progenitor cells (Okado et al., 2009). This function is likely attributable to its expression in progenitor cells in the VZ, whereas the regulation of neuronal migration may be ascribable to its strong expression in migrating postmitotic neurons in the dorsal pallium.

In the in utero electroporation experiments, Cre-expressing *RP58*$^{flox/flox}$ cells showed unambiguous phenotype compared with *RP58*$^{-/-}$ cells, and this phenotype was completely dependent on *RP58* expression. MAP2 immunostaining revealed that RP58 is important for neuronal entry into the CP. As the development of *RP58*$^{-/-}$ brains is impaired in many aspects, the subplate layer is also disorganized. Our results clearly show that RP58 is indispensable for transition from the multipolar migrating mode to the locomotion mode by altering the cell morphology. In fact, RP58 is strongly expressed in the SVZ, in which many multipolar cells are located at both the mRNA and protein levels (Ohtaka-Maruyama et al., 2007, 2012; Okado et al., 2009). This result suggests that RP58 plays an important role in multipolar migrating cells, in which dynamic cytoskeletal reorganization occurs for conversion to the bipolar morphology. A target gene for such transition is *Ngn2*. Recently, we found that 5.3 kb RP58 promoter activity is highly responsive to Ngn2: the promoter activity was detected in a small population of VZ cells with pin-like morphology and was prominently activated in multipolar migrating cells located in the multipolar cell-accumulation zone (Tabata et al., 2009), which is the lower part of the SVZ (Ohtaka-Maruyama et al., 2012). This suggests that the high expression of RP58 in multipolar migrating cells is attributable to regulation of *RP58* promoter activity.

As RP58 is a transcriptional repressor, it represses some downstream effectors at the transcriptional level. Therefore, the phenotypes seen in *RP58*$^{-/-}$ or Cre-electroporated *RP58*$^{flox/flox}$ mice should be the result of upregulation of the downstream genes. In our search for candidate downstream genes, we identified genes *Id1–Id4*. RP58 controls the progenitor-cell kinetics by repressing all *Id* genes, leading to p57 upregulation (Hirai et al., 2012). This would be a candidate for the first function of RP58 in progenitor cells, in which weakly expressed RP58 promotes cell-cycle exit. Oscillatory expressions of *Hes1* and *Ngn2* in neural progenitor cells are important for maintaining their proliferative state (Shimojo et al., 2008). As Ngn2 activates *RP58* transcription, RP58 may accumulate in response to the varying *Ngn2* expression to determine the timing of cell-cycle exit.

The second function of RP58 during cortical development begins in postmitotic migrating neurons. Massive rearrangement of the cytoskeleton and other gene expressions for dynamic morphological conversion should occur in this stage. Many factors are reportedly involved in this step, including Rho and GSK3 signaling-related factors (Barnes and Polleux, 2009; Heng et al., 2010). Surprisingly, *RP58*$^{-/-}$ neurons do not have typical leading processes at all; instead, they possess thin and long neurites extending from the cell bodies. Besides the complete rescue of this phenotype by electroporation of the RP58 expression construct, we found that this phenotype is also rescued by knockdown of *Ngn2* ectopically expressed in *RP58*$^{-/-}$ cells. Moreover, *Ngn2* overexpression in WT cells resulted in similar morphological abnormalities. Several downstream genes directly activated by Ngn2 have been identified, including *RP58* (Seo et al., 2007; Yokoyama et al., 2009), *NeuroD* (Seo et al., 2007; Yokoyama et al., 2009), *Rnd2* (Heng et al., 2008), and *Tbr2* (Ochiai et al., 2009). Rnd2 is a small GTP-binding protein that controls the actin cytoskeleton. Therefore, either overexpression or silencing of *Rnd2* leads to this morphological change. This suggests the importance of maintaining normal *Rnd2* expression in WT neurons. RP58 plays a significant role in this regulation by repressing *Ngn2* expression. Although it has recently been reported that RP58 represses *Ngn2* transcription by its direct binding to the promoter region (Xiang et al., 2012), the functional significance of this feedback regulatory circuit during corticogenesis was not clarified in that study. Our current study reveals that the repression of *Ngn2* by RP58 plays a significant biological role.

Ngn2 is a neuronal determinant that specifies the neuronal lineage (Fode et al., 2000). After this specification, *Ngn2* expression decreases in postmitotic neurons (Miyata et al., 2004; Kawaguchi et al., 2008), indicating that *Ngn2* expression is transient and may be important in advancing immature cortical neurons to the next steps in their development, including their migration within the CP. By this study, we demonstrated that *Ngn2* is downregulated by RP58 for proper neuronal migration, and that failure of this step leads to migration defects. Therefore, in cortical neurogenesis, Ngn2 is a master transcriptional activator for specifying neuronal lineages of progenitor cells, whereas RP58 is a master transcriptional repressor of *Id* genes for the cell-cycle exit of progenitor cells (Hirai et al., 2012) and multipolar-to-bipolar transition by negative feedback regulation of the Ngn2-Rnd2 pathway.

EXPERIMENTAL PROCEDURES

All experimental protocols were approved by the Animal Care and Use Committees of the Tokyo Metropolitan Institute for Neuroscience and the Tokyo Metropolitan Institute of Medical Science.

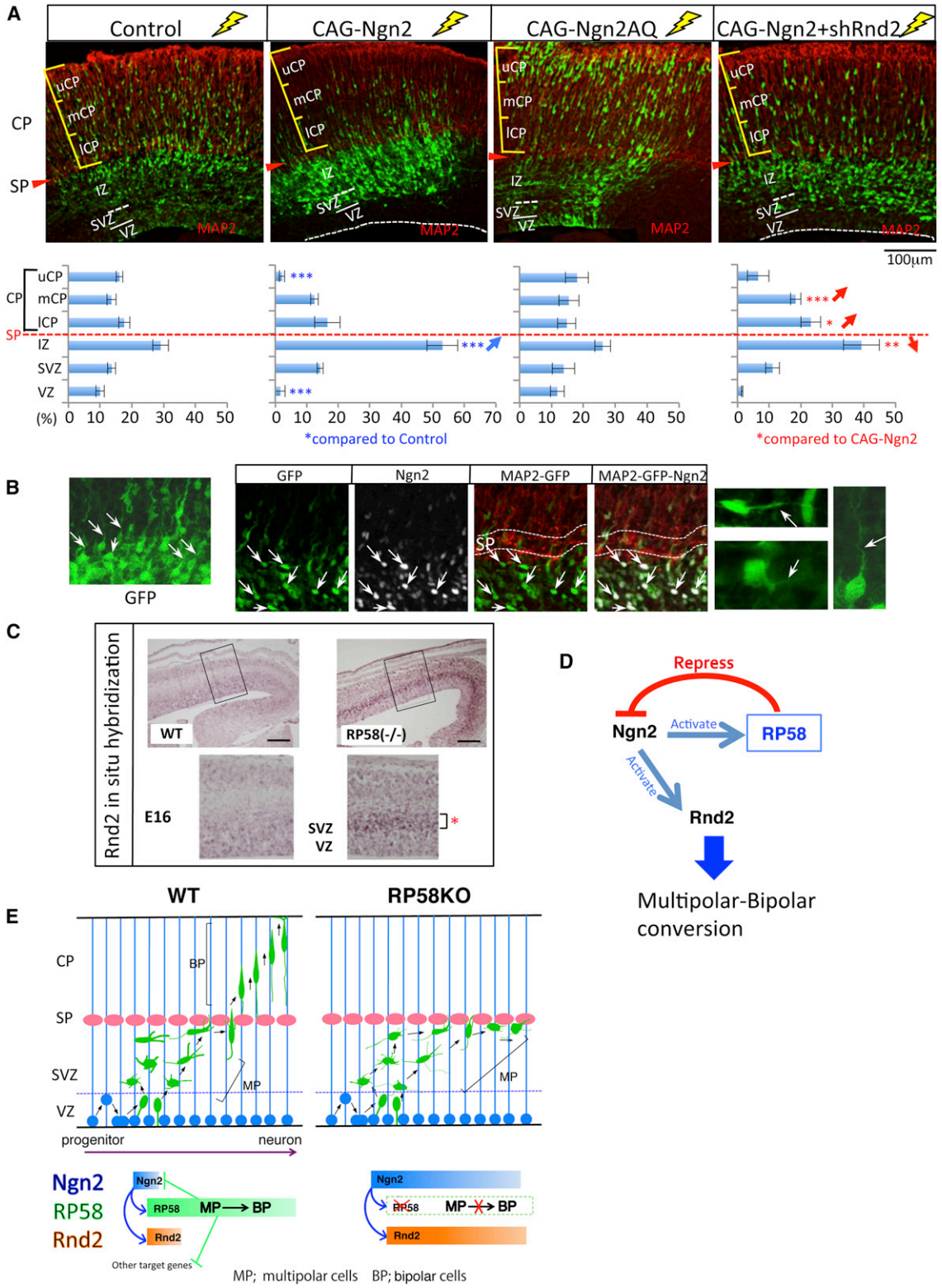

Figure 7. Impaired Neuronal Migration by *Ngn2* Overexpression

(A) The impairment of neuronal migration by *Ngn2* overexpression depended on its transactivation activity. When the pCAG-Ngn2 plasmid was electroporated on E14 and the embryos were harvested on E17, migrating neurons were stagnated beneath the subplate layer, mimicking *RP58*$^{-/-}$ neurons (see Figure 3D). See also Figure S7. Electroporation of the pCAG-Ngn2-AQ plasmid did not affect their migration. Coelectroporation of shRnd2 with pCAG-Ngn2 partially rescued their

(legend continued on next page)

Plasmids

Cre-expression plasmid (pXCANCre; Kanegae et al., 1995) was gifted by Dr. Izumu Saito (University of Tokyo). *RP58* promoter-driven luciferase (5.3 kb, RP58-Luc) and Cre (RP58-Cre) plasmids were constructed as described before (Ohtaka-Maruyama et al., 2012). Reporter plasmids for *Ngn2* regulatory sequences [pNgn2-3′frg-pGL3-P and pNgn2 promoter (−2145/+51)-pGL3B] were constructed as follows. The *Ngn2* promoter fragment was amplified by KOD polymerase and cloned into the NheI-NcoI sites of pGL3B-promoterless luciferase vector (Promega). The 3′-fragment (1935 bp) was amplified and cloned into the SacI–XhoI sites of pGL3P luciferase vector (Promega). BS10-Luc plasmid was constructed as described before (Aoki et al., 1998).

For RP58-expression plasmid construction, the full-length coding sequence of *RP58* was cloned by RT-PCR using RNA isolated from the cerebral cortex of E14 mice as the template and inserted into pAXCAwtit (pAXCA-mRP58) (Niwa et al., 1991). Most of the adenoviral genomic region was deleted by NruI or SalI, and a self-ligated plasmid was obtained as pCA-mRP58. For Ngn2-expression plasmid construction, the full-length coding sequence of mouse *Ngn2* was cloned by RT-PCR using RNA isolated from the cerebral cortex of E14 mice as the template and cloned into the EcoRI site of the CAG vector (pCAG-Ngn2) or bA-GFP vector (pbA-Ngn2) after deletion of the enhanced GFP (EGFP) fragment.

pCAG-EGFP and pbA-EGFP plasmids were donated by Dr. Kawaguchi (Nagoya University) and Dr. Okabe (University of Tokyo), respectively. pCAG-Ngn2-AQ mutant plasmids was constructed by using a KOD-plus mutagenesis kit (Toyobo) in the inverse-PCR method. pREST was donated by Dr. Gail Mandel (Oregon Health and Science University). shRNAmir for Ngn2 (Open Biosystems) was used in this study: the clone (RMM4431-98725508) from the GIPZ Lentiviral shRNAmir library was obtained along with a negative-control shNC clone. To insert a *loxP*-STOP-*loxP* fragment for conversion to a conditional expression vector, the CMV promoter-tGFP fragment of pGIPZshNgn2mir was replaced with the bA-*loxP*-STOP-*loxP*-EGFP fragment of pbA-*loxP*-STOP-*loxP*-EGFP plasmid, donated by Dr. Okabe (University of Tokyo).

For sh-*loxP*-STOP-*loxP*-Rnd2 #1, the original shRnd2 plasmid (Heng et al., 2008) was modified by inserting a *loxP*-STOP-*loxP* sequence between the U6 promoter and the shRnd2 sequence (J.I.H., Z. Qu, C.O.-M., H.O., M.K., D. Castro, F. Guillemot, and S.S. Tan, unpublished data).

In Utero Electroporation

Pregnant C57/BL6 WT or *RP58*$^{-/-}$ mice were deeply anesthetized with pentobarbital, and their embryos were surgically manipulated. For the RP58 shRNA and *RP58* or *Ngn2* overexpression experiments (Figures S1E, S2B, 5F, 5H, 7A, and 7B), pregnant imprinting control region (ICR) mice (Japan SLC) were used. The effector plasmids (shRNA and mouse RP58 or Ngn2-expression plasmids) and 1/3 volume of EGFP (Clontech) expression vector with a modified chicken β-actin promoter containing the CAG promoter (Niwa et al., 1991) were mixed and introduced into the VZ of E14 embryos by in utero electroporation as described previously (Tabata and Nakajima, 2001). For rescue experiments (Figure 6), bA-*loxP*-STOP-*loxP*-shNgn2-IRES-EGFP and RP58 Cre plasmids (Ohtaka-Maruyama et al., 2012) were coelectroporated. In *RP58*$^{flox/flox}$ mice, GFP-only or GFP with CAG Cre plasmid (pXCANCre) was used during electroporation. Embryos were sacrificed on E17, and the brains were dissected, fixed overnight in 4% PFA at 4°C, and placed in 30% sucrose/1 × PBS for cryoprotection. They were then frozen in OCT (Tissue-Tek) and sectioned coronally at 20 μm thickness. To visualize the fluorescent signals more clearly, GFP antibody was used for immunostaining.

Immunostaining

The primary antibodies used in this study were rabbit RP58 (Takahashi et al., 2008) (1:500), GLAST (1:200, Chemicon), rabbit Ki67 (1:500, Novocastra), rat BrdU (1:500, Abcam), rabbit Tbr2(1:500, Abcam), rabbit Tbr1 (1:500, Chemicon), rabbit MAP2 (1:500, Chemicon), mouse Ngn2(1:10, donated by Dr. David Anderson), and chicken GFP (1:500, Abcam). IgG antibodies conjugated to biotin (1:200, Vector Laboratories), HRP (1:200), and Alexa Fluor 488, Cy3, or Cy5 (1:500, Molecular Probes or Jackson Laboratories) were used as secondary antibodies. Images were acquired using a confocal microscope (FV500, Olympus) and a fluorescence microscope (BIOREVO, Keyence).

Generation of *RP58*$^{flox/flox}$ Mice

A genomic fragment containing *RP58* was obtained from mouse strain 129. The coding exon of *RP58* was targeted by homologous recombination in embryonic stem (ES) cells using a targeting vector containing a single *loxP* site and an FRT/*loxP*-neomycin-resistant gene construct (*neo*^{r}-FRT/*loxP*) in the upstream and downstream regions, respectively, of the coding exon. After transfection of the targeting vector into 129 mouse ES cells, the cells were microinjected into ICR mouse blastocysts. The chimeric mice were crossed with B6 mice expressing flippase to generate *RP58*$^{flox/+}$ mice lacking *neo*^{r}. Then, *RP58*$^{flox/flox}$ mice were generated by intercrossing *RP58*$^{flox/+}$ mice. To delete the *RP58* coding exon in the developing neural cells of the cerebral cortex, we performed in utero electroporation with the Cre-expression plasmid.

In Situ Hybridization

In situ hybridization was carried out using digoxigenin-labeled riboprobes as described previously (Ohtaka-Maruyama et al., 2007). Coronal sections of WT and *RP58*$^{-/-}$ brains (E12–E18) were used for this experiment.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described (Ohtaka-Maruyama et al., 2007) using total RNA isolated from the cerebral cortices of E16 or E18 WT and *RP58*$^{-/-}$ mice. The specific primers were 5′-GACAC ATCTGGAGCCGCGTA-3′ (forward) and 5′-CGAGCCCAGCAGCATCAGTA-3′ (reverse) for *Ngn2* and 5′-AAATGGTGAAGGTCGGTGTG-3′ (forward) and 5′-TGAAGGGGTCGTTGATGG-3′ (reverse) for *GAPDH*.

Transfection and Luciferase Reporter Assay

Luciferase reporter assay was performed as described (Ohtaka-Maruyama et al., 2012) using Neuro2A cells (Figure 5A) and COS7 cells (Figure 5E). Transient transfection was performed with Lipofectamine LTX and PLUS reagent (Invitrogen).

migration defect. Data represent the mean ± SD (n = 6 slices from three individuals); *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t tests were performed to compare the control condition with the pCAG-Ngn2, pCAG-Ngn2-AQ, and pCAG-Ngn2 + shRnd2 conditions). Scale bar, 100 μm.

(B) Magnified images of Ngn2-electroporated cells near the subplate layer reveal that Ngn2-expressing cells harbored thin neurites and stagnated immediately under the subplate (arrows).

(C) *Rnd2* expression was upregulated in the SVZ of E16 *RP58*$^{-/-}$ brains. Scale bars, 100 μm.

(D) The Ngn2-Rnd2-RP58 pathway. Ngn2 activates RP58 and Rnd2, and RP58 represses Ngn2 and Rnd2. This negative feedback regulation is important for proper neuronal migration.

(E) Schematic illustrating the *RP58*$^{-/-}$ phenotype. Radial glial progenitor cells proliferate in the VZ (below the dashed line) by symmetrical division. Then, *Ngn2* expression is initiated asymmetrically in one of the daughter cells (pin-like green cell). In the WT, Ngn2 activates RP58 and Rnd2. Conversely, RP58 represses Ngn2, Rnd2, and other target genes. This repression contributes to the transient expression of downstream target genes and multipolar-to-bipolar transition occurs followed by locomotion in the CP. In *RP58*$^{-/-}$ brains, Ngn2 induces Rnd2 but fails to induce RP58. Consequently, Ngn2 and Rnd2 expressions do not decrease, resulting in failure of the multipolar-to-bipolar transition and hence no crossing of the subplate layer (pink) for locomotion in the CP. Probably because the regulation of Rho signaling is disorganized, axonal and dendritic formation is impaired and multiple thin neurites are observed in *RP58*$^{-/-}$ brains compared with WT brains.

ChIP Assay

ChIP assay was performed as previously described (Ochiai et al., 2009) using E14 cerebral wall cells. Immunoprecipittion was carried out using anti-RP58 antibody (Takahashi et al., 2008). The primers used to recognize RP58-binding sites in *Ngn2* 5′- and 3′-flanking regions were as follows: 5′-RP58-binding site (expected PCR product, 128 bp), 5′-ACACCGTGCTCGGTTCCGGG-3′ (forward) and 5′-ACACTGCCTGCGAAGTGGAG-3′ (reverse); 3′-RP58-binding site (expected PCR product, 94 bp), 5′-CAGGTGAATCTCTATGCTAGC-3′ (forward) and 5′-CAATTAACAGATATGCAACA-3′ (reverse).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.01.012.

LICENSING INFORMATION

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