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POSH Localizes Activated Rac1 to Control the Formation of Cytoplasmic Dilation of the Leading Process and Neuronal Migration

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SUMMARY

The formation of proximal cytoplasmic dilation in the leading process (PCDLP) of migratory neocortical neurons is crucial for somal translocation and neuronal migration, processes that require the elaborate coordination of F-actin dynamics, centrosomal movement, and nucleokinesis. However, the underlying molecular mechanisms remain poorly understood. Here, we show that the Rac1-interacting scaffold protein POSH is essential for neuronal migration in vivo. We demonstrate that POSH is concentrated in the PCDLP and that knockdown of POSH impairs PCDLP formation, centrosome translocation, and nucleokinesis. Furthermore, POSH colocalizes with F-actin and the activated form of Rac1. Knockdown of POSH impairs F-actin assembly and delocalizes activated Rac1. Interference of Rac1 activity also disrupts F-actin assembly and PCDLP formation and perturbs neuronal migration. Thus, we have uncovered a mechanism by which POSH regulates the localization of activated Rac1 and F-actin assembly to control PCDLP formation and subsequent somal translocation of migratory neurons.

INTRODUCTION

Radial migration of neurons plays a pivotal role in neocortical lamination (Hatten, 2002; Kriegstein, 2005; Rakic, 1990; Torii et al., 2009). During corticogenesis, pyramidal neurons derived from the ventricular zone (VZ) pass through a multipolar stage to become bipolar and then undergo radial-glia-guided migration to reach their final destination within the cortex. Disturbance of any single step will cause specific neurological syndromes (Ayala et al., 2007; Kriegstein, 2005; Marín and Rubenstein, 2003; Rakic, 2003; Valiente and Marín, 2010).

The migration of bipolar neurons requires a concerted coordination between leading process extension and soma translocation (Ayala et al., 2007; Nadarajah et al., 2001). At the initial phase of neuronal somal migration, the stabilization of the leading process is marked by an increase in volume of the proximal leading process, referred to as the proximal cytoplasmic dilation in the leading process (PCDLP) (Bellion et al., 2005; Schaar and McConnell, 2005). After the formation of the PCDLP, the centrosome moves forward and enters the leading process, and then the nucleus translocates toward the centrosome (Ayala et al., 2007; Tsai and Gleeson, 2005). Although PCDLP formation has been predicated to be required for centrosomal movement and nucleokinesis (Ayala et al., 2007; Bellion et al., 2005; Schaar and McConnell, 2005; Umeshima et al., 2007; Valiente and Marín, 2010), how it is orchestrated is still unclear.

Recent evidence indicates that F-actin assembly in the leading process plays an essential role in centrosomal translocation and nucleokinesis during neuronal migration (Norden et al., 2009; Solecki et al., 2009). F-actin dynamics, centrosome translocation, and nucleokinesis are well coordinated in the PCDLP to ensure proper somal translocation and neuronal migration (He et al., 2010; Schaar and McConnell, 2005; Solecki et al., 2009). However, the underlying molecular mechanism has still not been elucidated.

POSH (plenty of SH3s) was identified initially as a Rac1-interacting protein (Tapon et al., 1998). It interacts with the activated form of Rac1, but not its inactivate form (Tapon et al., 1998; Xu et al., 2003). We have shown previously that POSH interacts with JIPs (c-Jun N-terminal kinase [JNK]-interacting proteins) to serve as scaffold proteins for a JNK pathway protein complex, the POSH-JIP apoptotic complex (PJAC) that includes Rac1 (a member of the GTPase family), the MLK family (subfamily of the MAP kinase kinase kinase), MKK4/MKK7 (the MAP kinase kinase family members), and the JNK family (JNK1–JNK3) (Kukekov et al., 2006; Xu et al., 2003, 2005). POSH plays an important role not only in the organization of PJAC but also in the activation of the JNK pathway (Kukekov et al., 2006; Tapon et al., 1998; Xu et al., 2003, 2005). Studies with conditional knockout mice and in utero electroporation (IUE) of mouse brain indicate that many PJAC components including Rac1, DLK (a member of the MLK family), MKK4 and MKK7, and JNKs are indispensable for radial migration in the developing brain (Chen et al., 2007; Kawauchi et al., 2003; Konno et al., 2005; Westerlund et al., 2011; Yamasaki et al., 2011). An intriguing question remains to be answered is whether and how POSH might regulate radial migration in the developing brain.

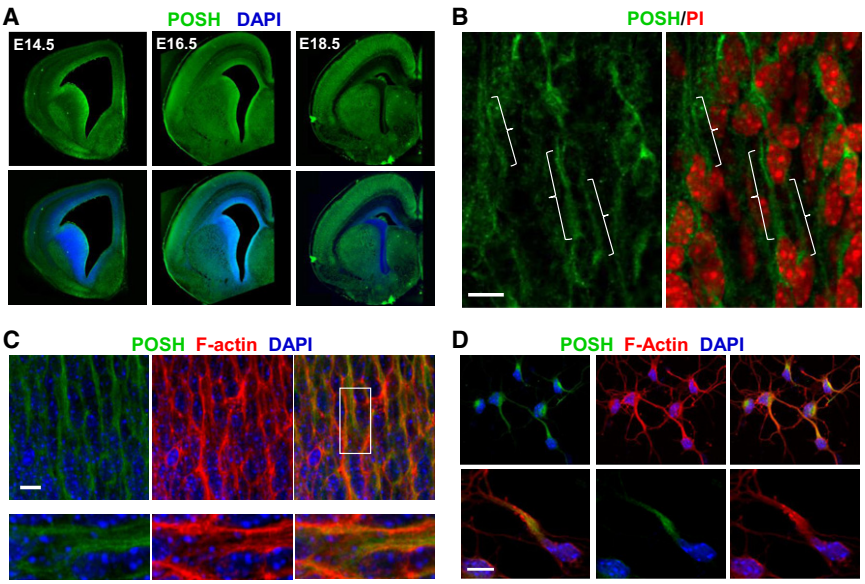

Figure 1. POSH Is Highly Expressed in the Leading Process of Radially Migrating Neurons

(A) Immunohistochemistry of coronal cortical sections at E14.5, E16.5, and E18.5 with POSH antibody (green). Nuclei were labeled with DAPI (blue).

(B) POSH expression (green) in migrating neurons in the IZ of E16.5 neocortex. Nuclei were labeled with PI (red). POSH is concentrated in the leading process (labeled by {).

(C and D) POSH colocalized with F-actin in migrating neurons in the IZ and cultured cortical neurons. E16.5 brain slices (C) and primary cultured cortical neurons (dissected from E14.5 brain and cultured for 24 hr) (D) were fixed and stained with POSH antibody for endogenous POSH (green), and phalloidin for F-actin (red) and DAPI for nuclei (blue). Enlarged view of rectangle in (C) is shown at the bottom.

Scale bars, 10 μm.

See also Figure S1.

Here, we show that POSH is both essential and sufficient for neuronal migration in vivo. Knockdown of POSH expression in cortical neurons by IUE impairs PCDLP formation, centrosome translocation, and nucleokinesis. We further demonstrate that POSH colocalizes with the activated Rac1 and F-actin in the cell cortex, and knockdown of POSH delocalizes activated Rac1 and disrupts F-actin assembly. Finally, we found that knockdown of Rac1 expression in cortical neurons also leads to defects in PCDLP formation and neuronal migration in vivo. Thus, our findings indicate that POSH is a regulator that controls PCDLP formation and proper cortical neuronal migration through localizing Rac1 activity and F-actin assembly.

RESULTS

Expression of POSH in Migratory Cortical Neurons

Because many components of the JNK pathway have been demonstrated to play an essential role in cortical neuronal migration (Chen et al., 2007; Kawauchi et al., 2003; Konno et al., 2005; Westerlund et al., 2011; Yamasaki et al., 2011) and POSH is important for the activation of JNK signaling (Tapon et al., 1998; Xu et al., 2003, 2005), we investigated whether POSH is also involved in this process. We first examined POSH expression in embryonic mouse brain (Figures 1A and S1) and found that POSH is expressed strongly in the VZ, subventricular zone (SVZ), and lower intermediate zone (IZ) but weakly in the upper IZ and cortical plate (CP) at embryonic day 14.5 (E14.5). At E16.5, POSH expression increases in the IZ and CP. At E18.5, it increases significantly in the CP but decreases in the IZ, SVZ, and VZ. Interestingly, POSH is localized predominantly in the leading process of migratory bipolar neurons in the IZ (Figure 1B). In addition it partially colocalizes with F-actin in the leading processes of bipolar neurons both in vivo and in vitro (Figures 1C and 1D). These cells are β-III-tubulin positive and migrate along the radial glial fiber as reported previously by Elias et al. (2007), Noctor et al. (2001), Rakic (1978), and Wang et al. (2009) (Figures S1E and S1F).

POSH Is Essential for Cortical Neuronal Migration

The distinct temporal and spatial expression pattern of POSH suggests its role in neuronal behavior during brain development. To test this possibility, we made bicistronic constructs encoding both dsRed and shRNA to knockdown overexpressed as well as endogenous POSH (Figure S2). We employed IUE to deliver POSH shRNA into neural progenitor cells of the VZ of E14.5 mouse. The most striking difference between POSH shRNA and control (Ctrl) shRNA-transfected brains inspected at E18.5 was the distribution of neurons. In Ctrl brains, ~50% of transfected cells were in the CP and ~25% in the IZ (Figure 2A). In contrast only 10% or less (around one-fifth that of the Ctrl) cells reached the CP in POSH shRNA-transfected brains. The majority of cells (~66%) were distributed in the IZ (Figure 2A), indicating that POSH knockdown perturbs neuronal migration. It is noteworthy that many POSH knockdown neurons tended to accumulate in the IZ just below the subplate of the CP (Figures 2A, 3A, and S3). The aforementioned finding indicates that POSH plays an important role in neuronal migration. We therefore concentrated on investigating the role of POSH in neuronal migration.

To validate target specificity of POSH shRNA and to ensure the specificity of the cell-positioning phenotype for POSH shRNA, three different shRNAs targeting different regions of POSH were employed (Figure 2A; data not shown). To further confirm that the migratory defects were caused by POSH shRNA, we coelectroporated POSH shRNA and *Xenopus POSH* into the embryonic mouse brain and found that the migration defect could be partially rescued by coexpression (Figure 2B). This indicates that the phenotype was POSH dependent, and not the result of off-target effects. Other possibilities that might affect cell migration were excluded, including cell death (analyzed by activated caspase-3 staining), aberrant morphology of

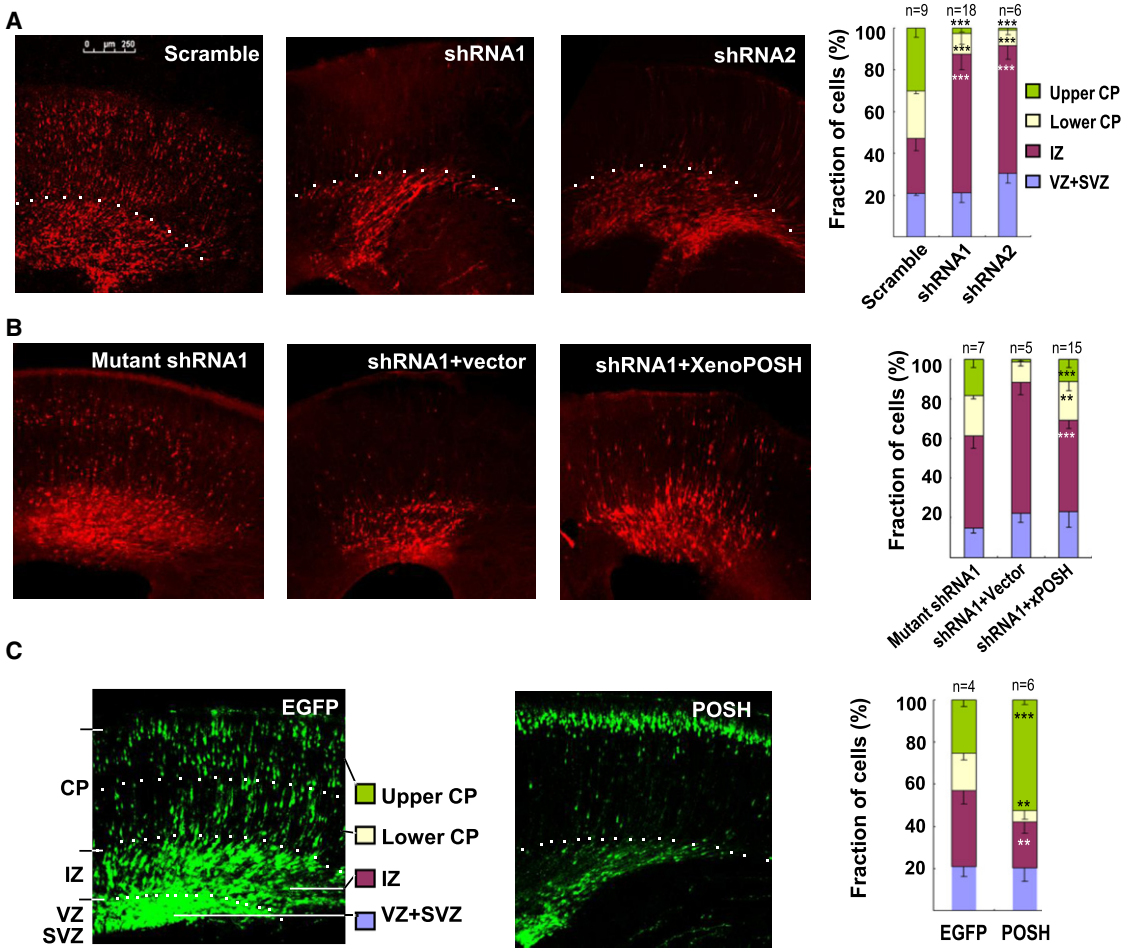

Figure 2. POSH Is Both Essential and Sufficient for Neuronal Migration

Coronal sections from brains electroporated at E14.5 and inspected at E18.5.

(A) POSH shRNAs reduce the fraction of cells in the CP (left panels, subplate was labeled by dashed lines). Quantification of cell distribution (right panel, refer to C for definition of different zones). One-way ANOVA, VZ/SVZ, $p = 0.0015$; IZ, ***$p < 0.0001$; Lower CP, ***$p = 0.0001$; Upper CP, ***$p < 0.0001$.

(B) The migration defect caused by POSH knockdown can be partially rescued by coexpression of Xenopus POSH. One-way ANOVA, VZ/SVZ, $p = 0.036$; IZ, ***$p < 0.0001$; Lower CP, **$p = 0.00067$; Upper CP, ***$p < 0.0001$.

(C) Overexpression of POSH enhances neuronal migration. Coronal section outlining different zones in the neocortex (left panel). One-way ANOVA, VZ/SVZ, $p = 0.9$; IZ, **$p = 0.002$; Lower CP, **$p = 0.00032$; Upper CP, ***$p < 0.0001$.

n, slice numbers from different brains.

See also Figure S2.

radial glia (analyzed by Nestin staining), and general brain structure (Figures S1 and 2A–2C; data not shown).

Because knockdown of POSH impairs neuronal migration, we went on to investigate whether expression of POSH can promote the migration of neurons. We overexpressed POSH in the E14.5 brain. Four days later, ~60% POSH-expressing cells reached the upper CP, more than double the number of Ctrl cells (~25%) (Figure 2C). On the other hand, POSH-expressing cells in the IZ and lower CP dropped substantially to ~27% compared to ~53% for the Ctrl. The aforementioned results indicate that POSH plays a crucial role in neuronal migration.

In order to confirm the role of POSH in migration in real time, we performed live-cell imaging analysis of bipolar neurons in the IZ of organotypic cortical slice cultures prepared from electroporated mouse embryos. We found that the motility of POSH shRNA-transfected cells was dramatically slower than that of Ctrls (Figure 3A; Table S1; Movies S1 and S2). Most of them failed to move during our observation period (4–6 hr; Movie S2). Even for those that migrated, their waiting time (time between somal translocations) was doubled (>214 versus ~107.5 min for Ctrls). In addition they took much longer to migrate (~123.8 versus ~89.7 min for Ctrls) and, consequently, migrated more slowly (~0.54 versus ~0.93 μm/min for Ctrls). In contrast, cells expressing POSH had much higher motility and moved faster (Figure 3B; Table S1; Movies S3 and S4). Compared with the Ctrls, the average number of migration (nucleokinesis or somal translocation) during the period of observation for POSH knockdown neurons dropped significantly with only

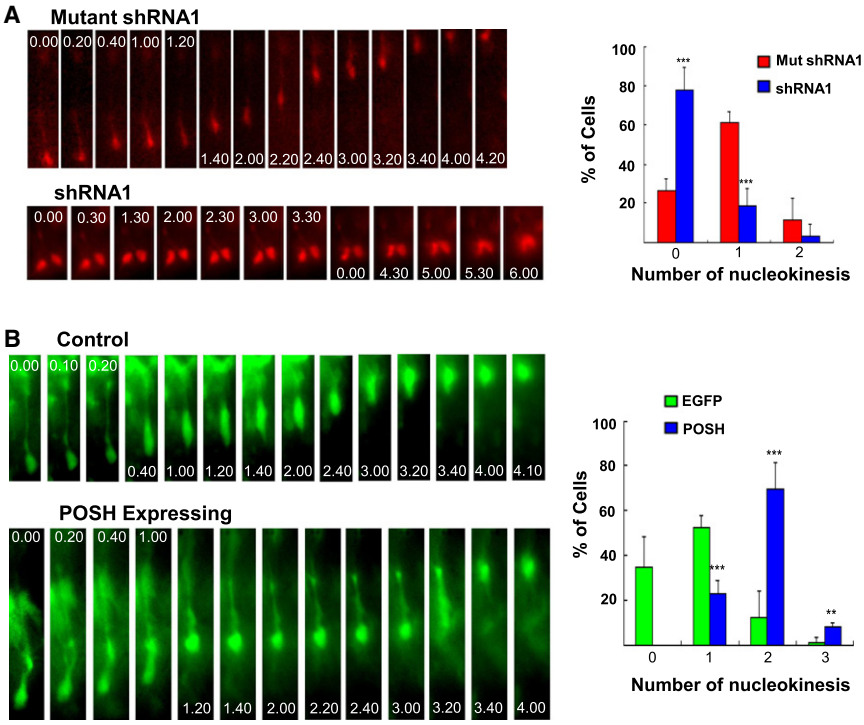

Figure 3. POSH Controls the Motility of Migratory Neurons

Brains were electroporated with POSH shRNA1 (A), POSH (B), or Ctrl constructs at E14.5. Three days later, brains were sectioned and cultured. Live-cell imaging of slices was performed on cells below the subplate in the IZ.

(A) Live images are of Ctrl bipolar cell or POSH knockdown cells (left panels). The number of nucleokinesis in 4 hr is analyzed in the right panel. Mutant shRNA1, n = 4. POSH shRNA1, n = 4. Zero times, ***$p < 0.0001$; once, ***$p < 0.0001$; twice, $p = 0.09$, t test.

(B) Images of Ctrl bipolar cell and POSH-expressing cell. The number of nucleokinesis in 4 hr was analyzed in the right panel. EGFP, n = 4. POSH, n = 4. Zero times, $p = 0.0008$; once, ***$p < 0.0001$; twice, ***$p < 0.0001$; three times, **$p = 0.0047$, t test.

n, slice numbers from different brains.

See also Movies S1, S2, S3, and S4.

~22% moving once or twice compared with ~76% for the Ctrls (Figure 3A). In contrast, migration times increased substantially for POSH-expressing neurons (Figure 3B); about 77% of POSH-expressing neurons moved twice or more within 4 hr compared with ~13% for the Ctrls. Therefore, POSH controls the motility of migratory neurons.

Knockdown of POSH Impairs PCDLP Formation and Centrosome Translocation in Bipolar Neurons

To decipher the underlying mechanism by which POSH controls radial migration, we analyzed the morphology of migratory neurons in the IZ in detail. We noticed that POSH knockdown neurons formed very thin and elongated leading processes with a large portion of cells (~83%, compared to ~32% in the Ctrl) lacking the characteristic proximal cytoplasmic dilation of migratory neurons presented in most of Ctrl neurons (Figures 4A–4C). They looked similar to neurons that had just finished somal movement (Tsai et al., 2005), except that their leading processes were longer. In contrast the percentage of cells without apparent PCDLP in POSH-overexpressing neurons dropped to ~16% compared to ~36% in Ctrls (Figure 4C). We measured the average width of PCDLP and found that it was significantly narrower in POSH knockdown neurons, whereas it was wider in POSH-overexpressing neurons (Figure 4D). This indicates that POSH plays an essential role in PCDLP formation. PCDLP is crucial for soma translocation during neuronal migration (Ayala et al., 2007; Bellion et al., 2005; Schaar and McConnell, 2005). Thus, our data suggest that POSH is likely to control the formation of PCDLP to regulate neuronal migration.

The centrosome has previously been demonstrated to move ahead of nuclear translocation (Tsai et al., 2007; Tsai and Gleeson, 2005; Zhang et al., 2009). As shown in Figures 4E and 4F, ~61.6% of centrosomes in Ctrl migratory neurons were located in the PCDLP. However, in POSH knockdown neurons the percentage dropped by more than half with only ~20% being located in the leading processes. Interestingly, centrosomes in POSH knockdown neurons were located much closer to or even beside the nuclei, with only 8% of centrosomes in POSH knockdown neurons being located more than 10 μm away from nuclei compared with 38% in Ctrl neurons (Figures 4E and 4G). These results suggest that defective PCDLP formation in POSH knockdown neurons may interrupt the entrance of the centrosome into the leading process.

Knockdown of POSH Leads to Changes in the Assembly/Distribution of F-Actin and Cell Morphology

F-actin has been shown to be enriched in the PCDLP, and the actomyosin complex in this region is essential for the coordinated movement of the centrosome and soma during neuronal migration (Norden et al., 2009; Solecki et al., 2009). We found recently that POSH is likely to regulate the formation of the F-actin network in epidermal cells and its migration during dorsal closure in *Drosophila* (Zhang et al., 2010). Given the evidence that POSH is enriched in the leading processes and colocalizes with F-actin (Figure 1), we examined whether POSH is engaged in F-actin assembly to regulate radial migration. Our results showed that F-actin filaments were concentrated in the PCDLP in ~89% of the migratory neurons in the Ctrl (Figures S4A and S4B) compared to in only ~26% of the POSH knockdown neurons. In addition the amount of actin filaments in soma appeared to decrease in POSH knockdown neurons, although not as apparently as in the PCDLP. This suggests that POSH controls F-actin assembly in migrating neurons, especially in the PCDLP.

To unequivocally delineate the role of POSH in F-actin assembly, we cotransfected GFP-fused G-actin together with POSH shRNA and found that POSH knockdown affected F-actin

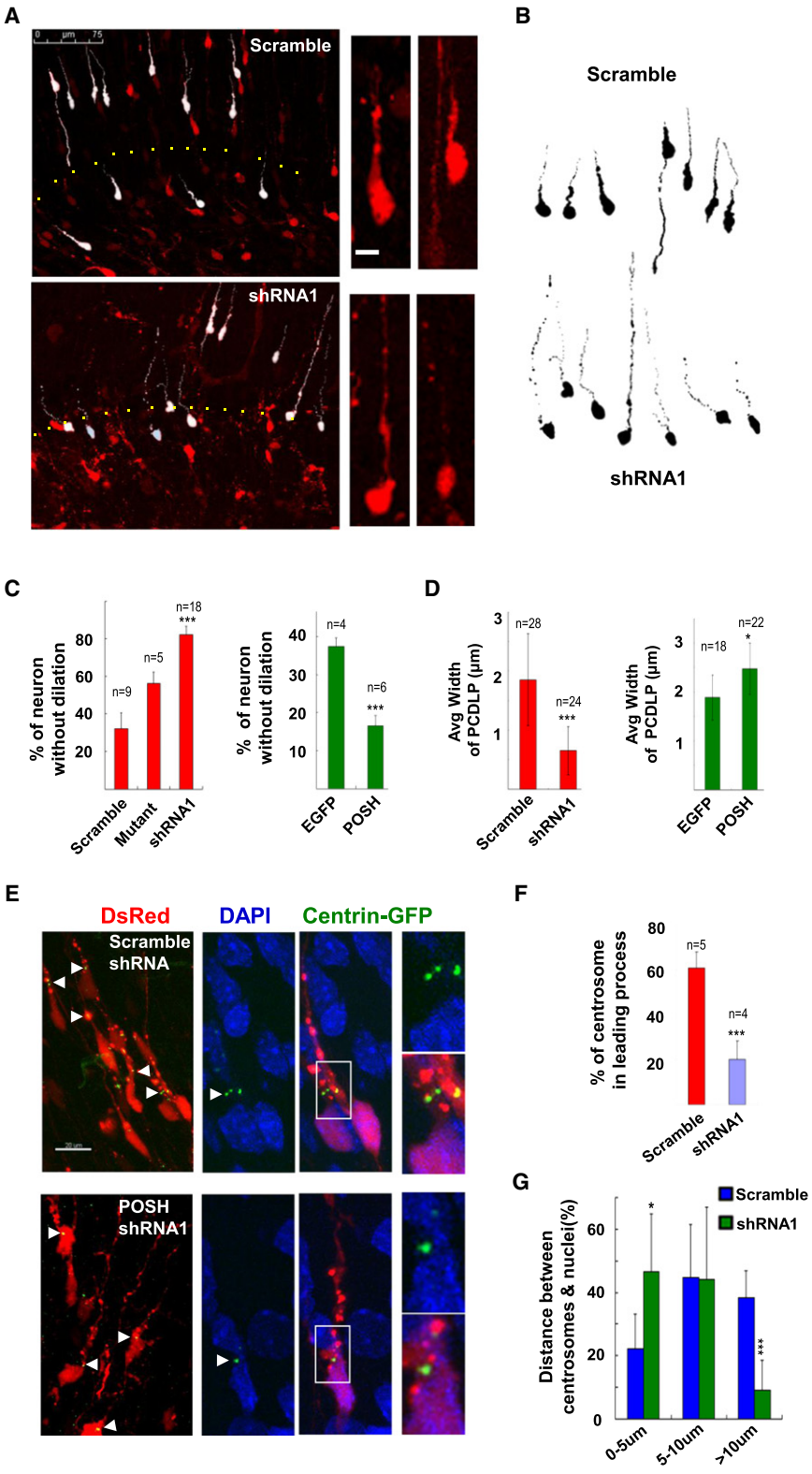

Figure 4. POSH Knockdown Inhibits PCDLP Formation and Interrupts Centrosomal Translocation in Bipolar Neurons

(A) Coronal sections around the subplate from brains electroporated at E14.5. POSH shRNA1 inhibits the formation of PCDLP at E18.5. Scale bar, 10 μm.

(B) Tracings of representative transfected neurons from (A).

(C) Quantification of neurons without dilation. Left panel: ***$p < 0.0001$, t test. Right panel: ***$p < 0.0001$, t test.

(D) The average width of PCDLP. Left panel: ***$p < 0.0001$, t test. Right panel: *$p = 0.0162$, t test.

(E) The localization of the centrosomes (labeled by arrowheads) in migrating neurons in the IZ (centrosome, Centrin-GFP; nucleus, DAPI; migrating neurons, dsRed). Scrambled shRNA: Upper panels. POSH shRNA1: Lower panels. Scale bar, 20 μm.

(F) The percentage of neurons with a centrosome in the leading process. ***$p < 0.0001$, t test.

(G) Distance between centrosome and nucleus. Only those centrosomes localized in front of nuclei were counted. $\leq$5 μm, *$p = 0.012$; 5–10 μm, $p = 0.747$; $\geq$10 μm, ***$p = 0.00021$, t test.

n, number of slices from different brains except in (D), which is neurons from different brains.

See also Figure S3.

assembly and distribution in bipolar neurons in vivo (Figure 5A). We further examined the role of POSH in cultured cells. First, we electroporated brains with POSH shRNA at E14.5 and cultured the dissected neurons 12 hr later. Surprisingly, most POSH knockdown cells could not attach to the plate and generate typical axon or dendrites. As a result, we analyzed only those neurons cultured for less than 12 hr that could attach to the plate and showed no signs of cell death as evaluated by nuclear staining and morphology. As shown in Figure 5B, well-organized F-actin was detected in the lamellipodia of Ctrls. In POSH knockdown cells, however, very few typical lamellipodia were found, indicating a defect in the actin mesh network. Although processes with multiple branches could be detected in some cells, no well-organized actin filaments could be identified in them (Figure 5B). To further confirm the effect of POSH knockdown on F-actin assembly, we knocked down POSH in HeLa cells and noticed the emergence of process-like structures (Figure S4C). Interestingly, most F-actin bundles in these POSH knockdown cells were concentrated at the front of the process-like structures, whereas cortical circumferential F-actin and stress fibers mostly disappeared. All these results indicate that POSH plays an essential role in F-actin assembly.

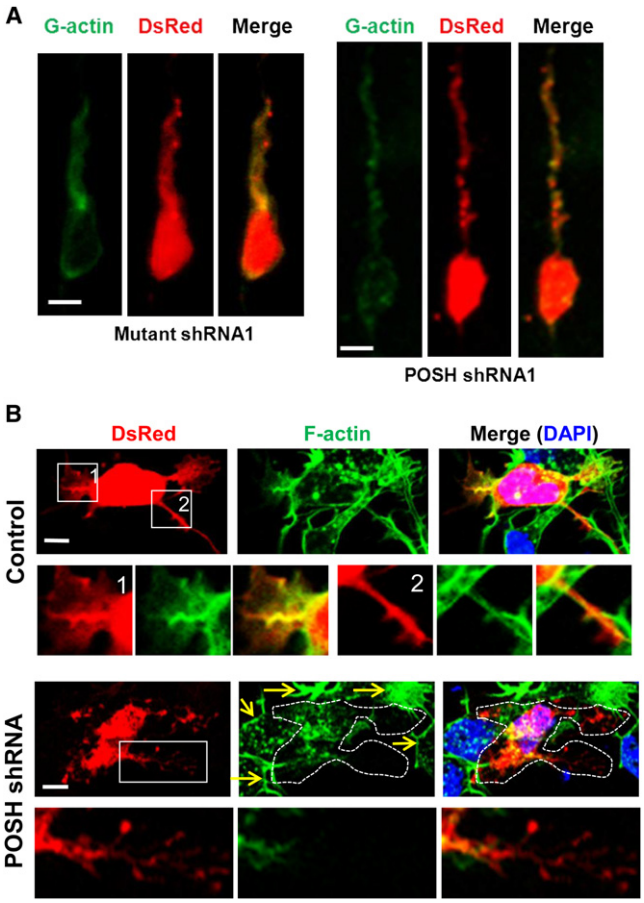

Figure 5. POSH Knockdown Interrupts the Normal Assembly of F-Actin and Cell Morphology

Coronal sections in the IZ from brains electroporated at E14.5 and analyzed at E18.5.

(A) POSH regulates the organization of F-actin in migrating neurons. Bipolar neurons (dsRed) in the IZ from brains electroporated with G-actin-GFP together with Ctrl or POSH shRNA at E14.5 were inspected at E18.5 for the distribution of G-actin (green).

(B) Cortex NPCs transfected with either Ctrl or POSH shRNA at E14.5 were dissected 12 hr after transfection and inspected <12 hr after culture. Well-organized F-actin (green) was detected in Ctrl neurons (upper panels), but not in POSH knockdown neurons (lower panels; red, outlined). F-actin was also present in those untransfected cells (labeled by arrows) around the POSH knockdown cell.

Scale bars, 10 μm.

See also Figure S4.

POSH Is Required for the Proper Localization of Activated Rac1

It has been shown previously that Rac1 plays an important role in F-actin assembly and lamellipodia formation (Albertinazzi et al., 1999; Etienne-Manneville and Hall, 2002; Fukata et al., 2003). The phenotype of POSH knockdown cells described above implicates the deregulation of Rac1 activity. In addition POSH is known to interact directly with Rac1 (Tapon et al., 1998; Xu et al., 2003), suggesting that POSH may regulate F-actin assembly via Rac1. To validate this, we investigated the relationship between POSH and Rac1 in cortical neuronal migration. We found that endogenous POSH, Rac1, and F-actin partially colocalized with each other in the neocortex, especially in the leading process of migratory neurons (Figures S5A–S5C). In HeLa cells, endogenous POSH colocalized with F-actin and the transfected activated form of Rac1 (CA Rac1) at the leading edge of lamellipodia (Figure 6A). In POSH knockdown cells, however, we hardly found the typical lamellipodia induced by CA Rac1 and noticed instead that CA Rac1 failed to localize at the cell margins (Figure 6B). We also examined the localization of Rac1 in bipolar neurons in the neocortex. In Ctrl cells, transfected wild-type (WT) Rac1 tended to localize along the plasma membrane of the PCDLP in contrast to the diffused expression pattern in POSH knockdown bipolar neurons (Figure 6C). More Ctrl or POSH knockdown bipolar neurons were shown in Figure S6, including one of the few POSH knockdown neurons that had the dilation of the leading process. This indicates that POSH regulates the localization of Rac1.

We further inspected whether POSH regulates the location of endogenous Rac1 activity. Ctrl shRNA or POSH shRNA was transfected into HeLa cells together with a fluorescence resonance energy transfer (FRET)-based indicator of Rac1 (pRaichu1011×) (Itoh et al., 2002). FRET analysis confirmed previous reports that most Rac1 activity is at the leading edge of lamellipodia in Ctrl cells (Figure 6D) (Machacek et al., 2009). In POSH knockdown cells, most of Rac1 activity was observed inside the cell instead of at the cell membrane (Figure 6D). Taken together, these results indicate that POSH plays an essential role in regulating the localization of the activated Rac1 to cell membrane to Ctrl cell morphology.

Interference with Rac1 Activity Disrupts PCDLP Formation and Perturbs Neuronal Migration

Our results described above show that POSH regulates the localization of activated Rac1 (Figure 6) and that Rac1 likely plays a role in the onset or the speed of cortical neuronal migration (Chen et al., 2007; Govek et al., 2011). Thus, we investigated whether Rac1 plays a similar role to POSH during neuronal migration. In agreement with previous reports, we found that expression of either dominant-negative Rac1 (DN Rac1) or CA Rac1 inhibited neuronal migration (Figures 7A and S7C) (Kawauchi et al., 2003; Konno et al., 2005). The inhibition of neuronal migration induced by DN Rac1 could be rescued by overexpression of POSH (Figure S7A). However, when the expression of endogenous Rac1 was knocked down at the same time, the defect could not be rescued (Figure S7B). This suggests that POSH does not interact with the DN Rac1, but it can interact and promote the localization/activity of endogenous Rac1 to rescue DN Rac1-incurred migration defect. Interestingly, coexpression of POSH with CA Rac1 led to the arrest of most cells even further in the VZ/SVZ (Figure S7C).

Because overexpression of dominant mutants could potentially block the activity of the Rho-GTPase family in general, it may not reveal the specific or physiological functions of Rac1. In addition, expression of either DN Rac1 or CA Rac1 also interrupts the formation of leading processes in many newborn neurons (Chen et al., 2007; Govek et al., 2011). To address this concern, we adopted shRNAs to knockdown Rac1 and expressed WT Rac1 instead of dominant mutants in E14.5 mice.

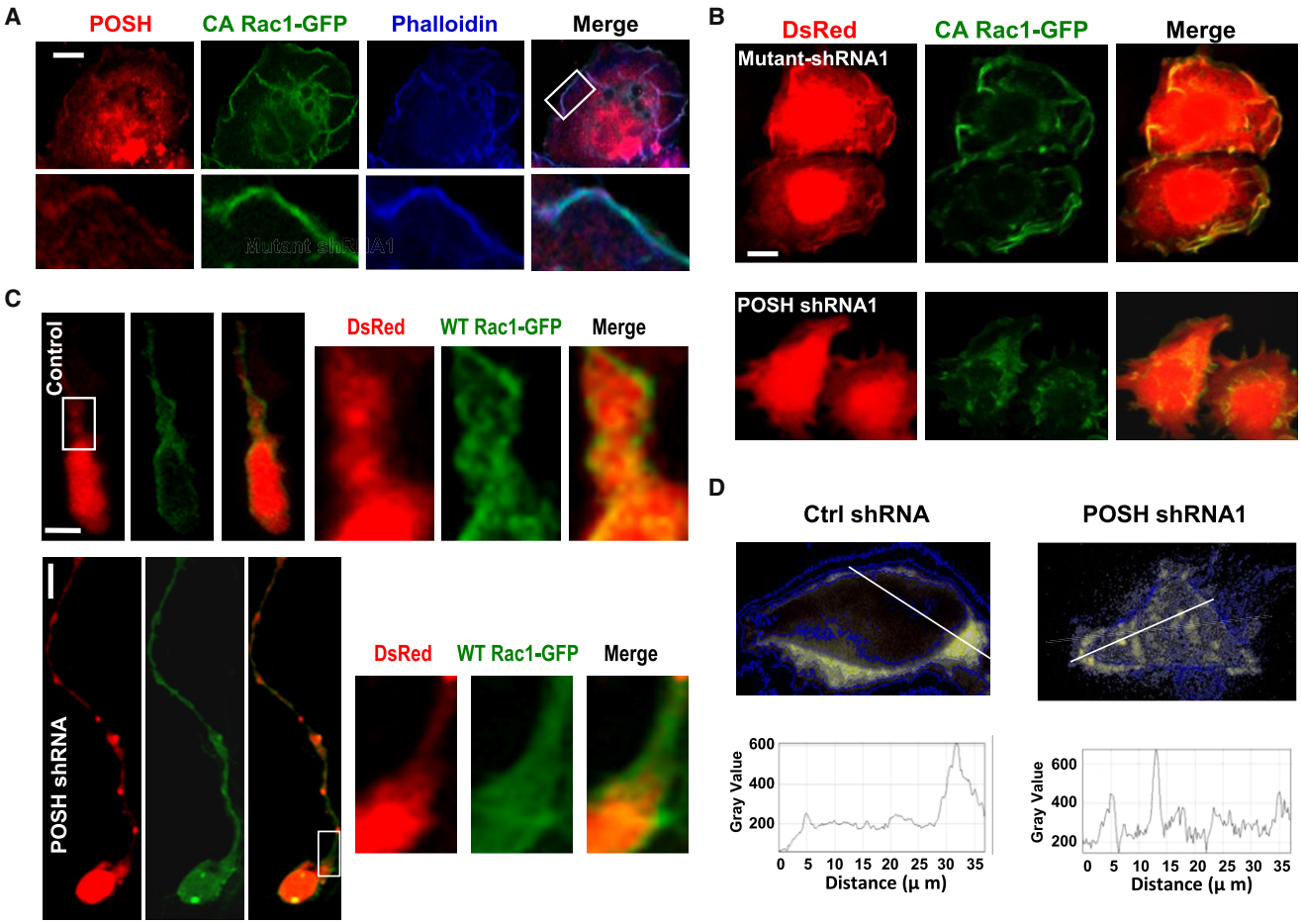

Figure 6. POSH Regulates the Localization of the Activated Form of Rac1

(A) POSH colocalized with F-actin and CA-Rac1 at cell edge. CA Rac1-GFP was transfected into HeLa cells. Twenty-four hours later, cells were stained with POSH antibody for endogenous POSH, phalloidin-FITC for F-actin. Enlarged view of rectangle is shown in the lower panels.

(B) Cell shape and localization of CA Rac1-GFP were affected by POSH knockdown. Ctrl shRNA or POSH shRNA was transfected into HeLa cells together with CA Rac1-GFP. The cell shape (dsRed) and location of Rac1 (green) were examined.

(C) POSH knockdown affects localization of Rac1 in migrating neurons. Bipolar neurons (dsRed) are from brains electroporated with Ctrl or POSH shRNA together with WT Rac1-GFP at E14.5 and inspected at E18.5 for the distribution of Rac1. Enlarged views of rectangles are shown in the right panels.

(D) POSH regulates the location of Rac1 activity. Ctrl shRNA or POSH shRNA was transfected into HeLa cells together with pRaichu1011×. The location of Rac1 activity was analyzed by FRET. FRET efficiency (E%) images are presented in quantitative pseudocolor (upper panels). The indicated linear intensity scans of FRET efficiency for the Ctrl and POSH knockdown cells were analyzed with ImageJ (lower panels).

Scale bars, 10 μm.

See also Figures S5 and S6.

Cell distribution was inspected at E18.5. Interestingly, overexpression of WT Rac1 promoted neuronal migration with ~56% of Rac1-expressing cells reaching the upper CP as opposed to ~26% for Ctrl cells (Figure 7A). Meanwhile, knockdown of Rac1 by RNAi suppressed migration (~3.6% and ~24% for Rac1 knockdown neurons and Ctrls that reached the upper CP, respectively) (Figure 7B). Because we have shown above that POSH controls the localization of Rac1 activity, we tried next to determine whether Rac1 expression-induced neuronal migration requires POSH or not by cotransfecting WT Rac1 with POSH shRNA or Ctrl shRNA. As shown in Figure 7C, cotransfection of WT Rac1 and Ctrl shRNA enhanced neuronal migration to a similar extent as expressing WT Rac1 alone. However, in brains cotransfected with WT Rac1 and POSH shRNA, we noticed that some cells expressing WT Rac1 alone (green) reached the CP, whereas cells expressing mainly POSH shRNA (red) or both POSH shRNA and WT Rac1 (yellow) failed to do so (Figure 7C). To further confirm the relationship between POSH and Rac1, cell lysates from E18 brains were subjected to immunoprecipitation with Rac1 antibody and western blotting with POSH and Rac1 antisera. Analysis of the immunoprecipitates revealed clear interactions between the endogenous proteins (Figure 7D). These results indicate that Rac1-mediated facilitation of radial migration requires POSH.

Finally, we investigated whether Rac1 plays a similar role to POSH in the formation of PCDLP. Our data showed that

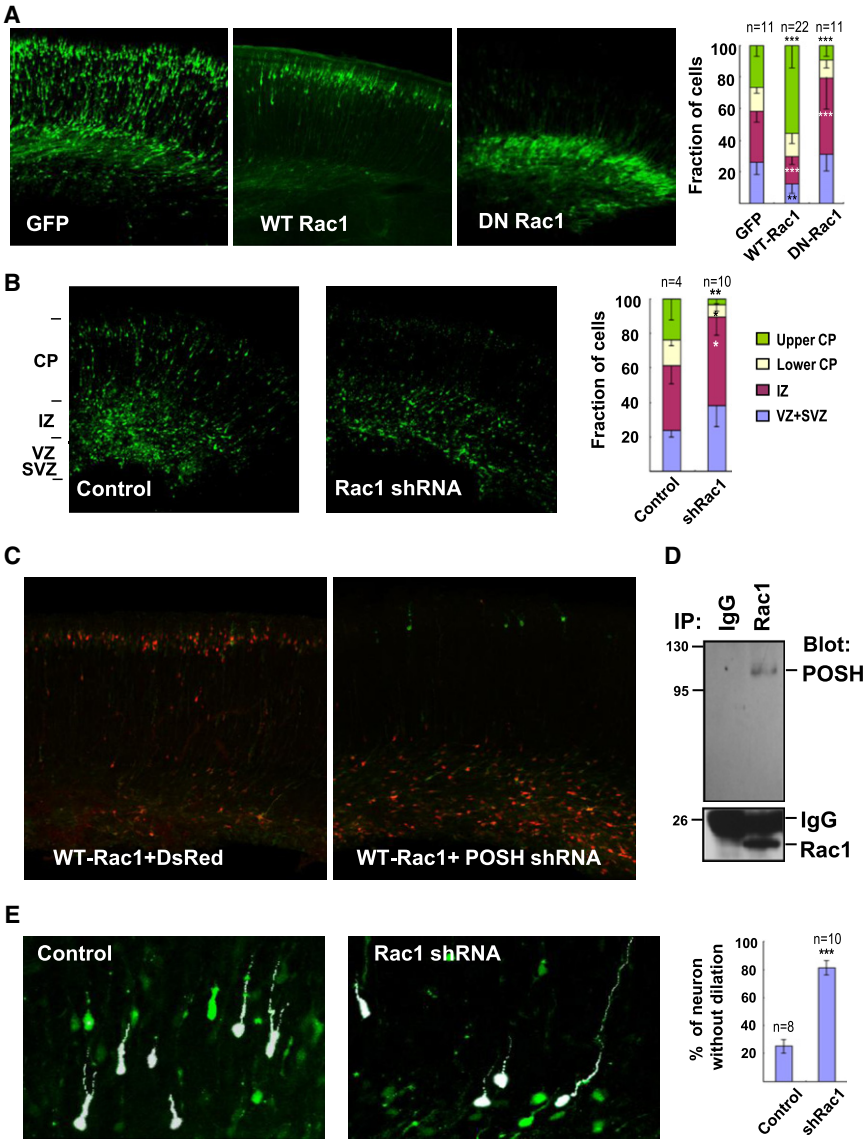

Figure 7. Rac1 Controls Neuronal Migration and PCDLP Formation

Coronal sections are from brains electroporated at E14.5 and inspected at E18.5.

(A) Rac1 controls neuronal migration. Coronal sections from brains electroporated with GFP, WT Rac1, and DN Rac1 (left three panels). Quantification of cell distribution in the VZ/SVZ, IZ, lower CP, and upper CP (right panel; refer to Figure 2B for definition of different zones). One-way ANOVA, VZ/SVZ, ***$p < 0.0001$; IZ, ***$p < 0.0001$; Lower CP, $p = 0.208$; Upper CP, ***$p < 0.0001$.

(B) Rac1 is essential for neuronal migration. Coronal sections are from brains electroporated with Ctrl or Rac1 shRNA (left panels). Quantification of cell distribution (right panel). One-way ANOVA, VZ/SVZ, $p = 0.042$; IZ, $p = 0.042$; Lower CP, *$p = 0.002$; Upper CP, **$p = 0.00018$.

(C) Rac1 expression-induced neuronal migration requires the presence of POSH. Representative coronal sections from brains transfected with WT Rac1 together with Ctrl shRNA (left panel) or POSH shRNA (right panel).

(D) Endogenous POSH interacts with Rac1 in the embryonic brain. Cell lysate from the E18 brain was subjected to immunoprecipitation (IP) with anti-Rac1 or IgG antibodies as indicated. Immunocomplexes were probed separately for POSH and Rac1.

(E) Knockdown of Rac1 affects the formation of PCDLP. Coronal sections around the subplate from brains electroporated with Ctrl or Rac1 shRNA (left two panels). Quantification of neurons without dilation (right panel): ***$p < 0.0001$, t test.

n, slice numbers from different brains.

See also Figure S7.

knockdown of Rac1 expression resulted in defect in PCDLP formation of migrating neurons similar to that caused by knockdown of POSH (Figure 7E). It should also be noted that the number of cells shown in Rac1 shRNA-transfected brains (Figure 7E) is apparently less than that in the Ctrl due to the effect of Rac1 RNAi on migration. Thus, our results demonstrate that POSH-dependent cell cortical localization of activated Rac1 is required for PCDLP formation and subsequent neuronal migration.

DISCUSSION

In this study we have characterized a new player in neuronal migration, POSH, which is the scaffold protein for PJAC. We found that POSH is predominantly expressed in the PCDLP of migratory neurons. POSH knockdown by RNAi impairs formation of the PCDLP, and subsequent centrosome translocation, nucleokinesis, and neuronal migration. Detailed analysis revealed that POSH knockdown delocalizes activated Rac1 and disrupts F-actin assembly. Therefore, POSH controls neuronal migration by localizing activated Rac1 and F-actin assembly for formation of the PCDLP.

POSH Plays a Crucial Role in Cortical Neuronal Migration

Neuronal migration requires a sophisticated interplay of complex molecular machines in its multiple steps (Ayala et al., 2007; Govek et al., 2011; Kawauchi and Hoshino, 2008; Kriegstein, 2005; Marín and Rubenstein, 2003; Rakic, 2003; Valiente and Marín, 2010). POSH is expressed in the IZ where it is concentrated in the leading process of migrating neurons and colocalizes with F-actin. This implies that POSH may play a role in radial migration. Our results show that knockdown of POSH expression by RNAi dramatically reduces the percentage of neurons distributed to the CP by around 80%, whereas overexpression of POSH increases the percentage of cells reaching the upper CP

by 2.4-fold (Figure 2). The role of POSH in neuronal migration is further supported by live-cell imaging analysis of bipolar neurons in the IZ, which indicates that POSH controls the motility of migratory neurons. It is interesting to note that many POSH knockdown neurons accumulate in the IZ just below the subplate of the CP. Together with the evidence that POSH expression increases sharply at the subplate, this indicates that POSH may play an important role in neuronal migration, especially from the IZ into the CP.

POSH Controls the PCDLP Formation and Centrosome/Nuclei Translocation

Recent studies have demonstrated that the formation of a cytoplasmic dilation in the proximal leading process precedes centrosome and nuclear translocation, and that nuclei translocate into these dilations in saltatory movements. PCDLP formation has been presumed to be essential for nucleokinesis and neuronal migration (Bellion et al., 2005; Schaar and McConnell, 2005). However, the factors that control the formation of PCDLP remain unknown. We found that POSH knockdown bipolar neurons show curved, thinner, and longer leading processes (Figures 4A, 4B, 5A, 6C, and S4A) as previously reported for LIS1 knockdown cells and, more recently, by our group for CRMP2 knockdown cells (Sun et al., 2010; Tsai et al., 2005). However, the most striking abnormal morphology is the failure of a large portion of neurons accumulated in the IZ below the subplate of the CP to form PCDLP. Therefore, it seems that POSH plays a crucial role in the establishment and perhaps maintenance of the proximal dilation in the leading process. We postulate that the narrowed vessel in the proximal part of the leading process caused by POSH knockdown may physically block the translocation of nuclei and centrosomes into the leading process, consequently blocking neuronal migration. Another possibility for the blockage of the translocation of nuclei and centrosomes is that it is a secondary effect of POSH knockdown on the localization of activated Rac1 and F-actin assembly as discussed below.

Interestingly, knockdown of POSH expression in primary cultured cortical neurons has been reported recently to induce axon outgrowth (Dickson et al., 2010). This scenario is similar to our finding that POSH knockdown leads to longer leading processes in the migratory neuron in vivo even though it happens on the other side of the bipolar neuron. It will be interesting in the future to study whether POSH regulates axon specification or outgrowth in vivo and whether Rac1 and F-actin assembly are involved.

POSH Regulates the Distribution/Assembly of F-Actin

Actomyosin and F-actin dynamics function as the main drivers of the coordinated movement of the centrosome and soma during neuronal migration. Enrichment of F-actin in PCDLP has been assumed to play an essential role in neuronal migration (Norden et al., 2009; Solecki et al., 2009). We found that POSH knockdown leads to the decrease or even loss of F-actin bundles in bipolar neurons, especially in proximal leading processes. In addition, well-organized F-actin structure and cell morphology are disturbed or affected in cultured POSH knockdown cortical neurons and HeLa cells. These results suggest that the decreased F-actin levels in migrating POSH knockdown neurons may account for their defects in radial migration.

Other genes regulating F-actin levels in migratory neurons have been reported recently. LIS1 plays an important role in radial migration, and its haploinsufficiency results in a reduced F-actin content in cultured migrating cerebellar granule cells (Tsai et al., 2005; Kholmanskikh et al., 2006). Knockdown of lamellipodin (Lpd) also results in a reduction in F-actin levels in bipolar pyramidal neurons (Pinheiro et al., 2011). Depletion of POSH and LIS1 reduces F-actin levels locally in different parts of the leading process of migrating neurons. POSH knockdown affects F-actin assembly mainly in PCDLP, whereas LIS1 knockdown does so mainly at the leading edge of migratory neurons (Kholmanskikh et al., 2006). Interestingly, it is the knockdown of POSH but not LIS1 that affects PCDLP formation, although both of them disturb radial migration. Knockdown of Lpd leads to decreased F-actin levels throughout bipolar neurons and causes some bipolar pyramidal neurons to adopt a tangential, rather than a radial-glial, migration mode (Pinheiro et al., 2011). It remains to be seen whether the influence of F-actin assembly in different parts of migratory neurons causes different morphological changes and affects their migration pattern differently.

We have previously shown that POSH is required, in addition to cortical F-actin, for adherens junction formation in epidermal cells during dorsal closure in *Drosophila* (Zhang et al., 2010). Thus, it will be intriguing to investigate in the future whether POSH plays an additional role in neuronal migration by regulating the interaction between migratory neurons and radial glial fibers.

POSH Regulates the Localization of Rac1 Activity and Cell Morphology in Both Neuronal and Non-Neuronal Cells

The role of Rac1 in F-actin assembly, lamellipodia formation, and cell migration has been extensively studied by Albertinazzi et al. (1999), Etienne-Manneville and Hall (2002), and Fukata et al. (2003). We noted here that most primary cultured POSH knockdown neurons fail to form well-organized F-actin and lamellipodia. In addition to deregulated F-actin assembly, the morphology of POSH knockdown HeLa cells is also reminiscent of abnormal Rac1 activity. We therefore investigated the relationship between POSH and Rac1 in more detail and provide several pieces of evidence indicating that POSH regulates the localization of Rac1 and its activity. First, POSH interacts with Rac1 and colocalizes with Rac1 and F-actin. In the embryonic brain, endogenous POSH interacts with Rac1, and POSH, Rac1, and F-actin partially colocalize with each other. Endogenous POSH colocalizes with the activated form of Rac1 and F-actin at the leading edge of lamellipodia in cultured cells. Second, POSH controls the localization of Rac1. In POSH knockdown HeLa cells, CA Rac1 does not localize at the cell margin and fails to induce F-action assembly and lamellipodia formation as it does in Ctrl cells. In bipolar neurons in the IZ, WT Rac1 tends to have a diffused expression pattern in the leading process in POSH knockdown cells in contrast to localizing along the plasma membrane of the PCDLP in the Ctrl. Third, POSH influences the localization of Rac1 activity. Rac1 activity was detected at the leading edge of Ctrl cells as determined by FRET analysis. In

contrast, most Rac1 activity in POSH knockdown cells was located inside the cell.

Together with previous biochemical studies showing that POSH interacts directly only with the activated form of Rac1 (Tapon et al., 1998; Xu et al., 2003), our results indicate that POSH is likely to play a role in anchoring or possibly recruiting activated Rac1 to the cell cortex of cultured cells and the plasma membrane of the PCDLP in bipolar neurons. The activated Rac1 regulates F-actin assembly and the formation of lamellipodia in cultured cells and the PCDLP formation in bipolar neurons.

Rac1 Is Required for PCDLP Formation and Neuronal Migration

Deletion of *Rac1* in the VZ using *Foxg1-Cre* mice disturbs radial migration (Chen et al., 2007). Expression of DN Rac1 and CA Rac1 by IUE also affects the distribution of neurons (Kawauchi et al., 2003). We confirmed their results and found that the inhibition of neuronal migration induced by DN Rac1 but not CA Rac1 can be rescued by overexpression of POSH. It is possible that POSH does not interact with the DN Rac1. However, it can interact and promote the localization/activity of endogenous Rac1 to rescue neuronal migration. On the other hand, POSH interacts with CA Rac1 and inhibits the migration furthermore, leading to the arrest of most cells in the VZ/SVZ.

We also adopted different strategies to study the specific physiological functions of Rac1. Interestingly, when we expressed WT Rac1, it promoted neuronal migration. On the other hand, knockdown of Rac1 by RNAi suppressed migration. This indicates that Rac1, like POSH, is both essential and sufficient for radial migration. Together with the evidence that expression of either DN Rac1 or CA Rac1 inhibits migration, we have proved the hypothesis that cycles of Rac1 activation and inactivation are important for neuronal migration (Giannone et al., 2004). More interestingly, Rac1 knockdown led to defective PCDLP formation in migrating neurons in a similar manner to POSH knockdown. Furthermore, the enhanced radial migration induced by WT Rac1 depended on the presence of POSH. Together with our findings on the role of POSH in the regulation of F-actin assembly and PCDLP formation, these results support the notion that POSH-dependent cell cortical localization of activated Rac1 is required for F-actin assembly and PCDLP formation and subsequently for neuronal migration.

Rac1 is known to play important roles in the leading edge of the axon and in the leading process of non-neuronal cells in the formation of lamellipodia (Fukata et al., 2003; Govek et al., 2011), and we have shown that POSH plays an important role in lamellipodia formation in cultured cortical cells and non-neuronal cells. Whether lamellipodia formation is involved in the formation of PCDLP in bipolar neurons remains to be determined. If that is the case, it would be intriguing to investigate whether POSH-dependent localization of Rac1 activity is involved. In addition, both POSH and Rac1 have been shown to be important for JNK activation. Therefore, the role of the JNKs in the formation of the PCDLP and in F-actin assembly in PCDLPs should be studied in the future.

In summary, we have uncovered two genes that are essential for PCDLP formation. In addition, we reveal a molecular mechanism for neuron migration in which POSH recruits activated Rac1 to the plasma membrane to ensure actin remodeling and controls the dilation of the leading processes of migratory neurons, which are essential for centrosomal movement and somal translocation and, subsequently, for neuronal migration.

EXPERIMENTAL PROCEDURES

Materials

Antibodies used were mouse monoclonal anti-Nestin (Chemicon), rabbit anti-POSH (MBL), anti-caspase-3-cleaved form (Cell Signaling), anti-Tuj1 (Abcam), rabbit anti-Myc (MBL), mouse anti-GAPDH (Cell Signaling), mouse anti-Rac1 (Cytoskeleton), and mouse anti-GFP (Santa Cruz Biotechnology). F-actin was stained with FITC-phalloidin or TITRC-phalloidin (Sigma-Aldrich). Nuclei were stained with Propidium iodide (PI) or DAPI (Invitrogen).

Plasmids

POSH shRNA oligonucleotides were inserted into pSIREN-RetroQ-DsRed (Clontech). The following target POSH sequences were used: 5′-GGTCA GACTTCTGGATGGCAT-3′ (POSH shRNA1), 5′-TGCTGTGAGGACAGTTGCA GC-3′ (POSH shRNA2), and 5′-CCGCCACTCCATGGAGATCAG-3′ (POSH shRNA3). Rac1 shRNA oligonucleotides were inserted into pSIREN-RetroQ-ZsGreen (Clontech). The target Rac1 sequence was described previously by Weiss-Haljiti et al. (2004). pCMS.EGFP.Myc-POSH was described previously (Xu et al., 2003). pCS2 (+)-*Xenopus*.POSH-EGFP was provided by Dr. Kim (Kim et al., 2005). pcDNA3-WT Rac1-EGFP, pcDNA3-DN Rac1(T17N)-EGFP, and pcDNA3-CA Rac1(Q61L)-EGFP were provided by Dr. Xiaobing Yuan.

Cell Culture, Transfection, and Western Blotting

Cell culture, transfection, and western blotting were performed as previously described (Xu et al., 2003).

Animals and IUE

Mice were provided by the animal center of the Institute of Genetics and Developmental Biology (IGDB), Chinese Academy of Sciences. All experimental procedures involved were performed according to protocol approved by the Institutional Animal Care and Use Committee at IGDB. IUE was performed as described previously (Sun et al., 2010; Tsai et al., 2005).

Brain Sectioning, Organotypic Slice, Time-Lapse or Confocal Imaging

Embryonic brain sectioning and immunohistochemistry were performed using a standard protocol as described before (Sun et al., 2010; Tsai et al., 2005). Sections were imaged on an LSM 700 (Carl Zeiss) or Leica SP1 confocal microscope. Excitation/emission wavelengths were 488/515, 543/572, 568/590, and 633/690 nm. Organotypic slice preparation and time-lapse imaging were performed as described previously (Sun et al., 2010; Tsai et al., 2005).

Quantification of Cell Distribution and the Average Width of PCDLP

Cell counts were performed as described previously (Sun et al., 2010). The average width of PCDLP was analyzed using Imaris software. For the purpose of standardization, the average width of PCDLP between nucleus and 20 μm upward to the leading process was measured. All values represent mean ± SEM, statistics for dual comparisons were generated using the Student's t test, and statistics for triple comparisons were generated using one-way ANOVA: *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

FRET

A method based on FRET that quantifies the location of Rac1 activation in living cells was used. See Extended Experimental Procedures for details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, one table, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.007.

LICENSING INFORMATION

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