

RhoA and Cdc42 are required in pre-migratory progenitors of the medial ganglionic eminence ventricular zone for proper cortical interneuron migration

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SUMMARY

Cortical interneurons arise from the ganglionic eminences in the ventral telencephalon and migrate tangentially to the cortex. Although RhoA and Cdc42, members of the Rho family of small GTPases, have been implicated in regulating neuronal migration, their respective roles in the tangential migration of cortical interneurons remain unknown. Here we show that loss of *RhoA* and *Cdc42* in the ventricular zone (VZ) of the medial ganglionic eminence (MGE) using *Olig2-Cre* mice causes moderate or severe defects in the migration of cortical interneurons, respectively. Furthermore, *RhoA*- or *Cdc42*-deleted MGE cells exhibit impaired migration *in vitro*. To determine whether RhoA and Cdc42 directly regulate the motility of cortical interneurons during migration, we deleted *RhoA* and *Cdc42* in the subventricular zone (SVZ), where more fate-restricted progenitors are located within the ganglionic eminences, using *Dlx5/6-Cre-ires-EGFP* (*Dlx5/6-CIE*) mice. Deletion of either gene within the SVZ does not cause any obvious defects in cortical interneuron migration, indicating that cell motility is not dependent upon RhoA or Cdc42. These findings provide genetic evidence that RhoA and Cdc42 are required in progenitors of the MGE in the VZ, but not the SVZ, for proper cortical interneuron migration.

KEY WORDS: RhoA, Cdc42, Interneuron, Tangential migration, Mouse

INTRODUCTION

Proper neural circuit formation and function in the mammalian cerebral cortex requires the appropriate generation and migration of cortical interneurons, together with the precise development of pyramidal projection neurons. Cortical interneurons are mostly inhibitory, use γ -aminobutyric acid (GABA) as a neurotransmitter and comprise 20–30% of all cortical neurons (Di Cristo, 2007). During development, cortical interneurons originate from the ganglionic eminences in the ventral telencephalon, and migrate tangentially to populate the cortex (Corbin et al., 2001; Marin and Rubenstein, 2001). Although recent studies have revealed that extracellular signaling molecules and transcription factors play important roles in the tangential migration of cortical interneurons (Métin et al., 2006; Wonders and Anderson, 2006; Nakajima, 2007), little is known about the roles played by intracellular signaling molecules in cortical interneuron migration.

Members of the Rho family of small GTPases, including RhoA, Cdc42 and Rac1, are key regulators of cytoskeletal dynamics that have a variety of effects on cellular processes during nervous system development, including neuronal migration, axon guidance and synapse formation (Govek et al., 2005; Govek et al., 2011; Hall and Lalli, 2010). Loss of Rac1 in neural progenitor cells in the developing brain induces defects in both radial and tangential migration, but the defects in tangential migration seem to arise from

a failure to develop migratory competency during progenitor differentiation (Chen et al., 2007; Vidaki et al., 2012). In addition, previous studies mainly using dominant-negative and constitutively active constructs suggest important roles for RhoA and Cdc42 in neuronal migration, yet a recent study using mouse genetics has shown that RhoA is largely dispensable for radial migration in neurons in the developing mouse cortex (Cappello et al., 2012). To date, the roles of RhoA and Cdc42 in tangential migration have not been examined.

In this study, we have explored the roles of RhoA and Cdc42 in the tangential migration of cortical interneurons using conditional gene-targeting approaches.

MATERIALS AND METHODS

Animals

The following mouse strains were used: *RhoA*-floxed (Chauhan et al., 2011; Katayama et al., 2011; Melendez et al., 2011), *Cdc42*-floxed (Yang et al., 2006), *Olig2-Cre* (Dessaud et al., 2007; Sürmeli et al., 2011), *Dlx5/6-CIE* (Stenman et al., 2003) and stop-floxed *EGFP* (Nakamura et al., 2006). *RhoA*^{flox/+}; *Olig2-Cre* or *RhoA*^{flox/+}; *Dlx5/6-CIE* were used as controls for *RhoA* mutants, and *Cdc42*^{flox/+}; *Olig2-Cre* or *Cdc42*^{flox/+}; *Dlx5/6-CIE* mice were used as controls for *Cdc42* mutants. As no differences were observed among these controls, we present data from only one control in the figures.

Histological and western blot analyses

Histological and western blot analyses were performed as described previously (Katayama et al., 2012; Leslie et al., 2012). Bromodeoxyuridine (BrdU) labeling and analysis of cell-cycle exit were performed as described (Chenn and Walsh, 2002). The primary antibodies utilized in this study were: mouse anti-N-cadherin (Invitrogen), mouse anti- β III-tubulin (Covance), mouse anti- β -catenin (BD Transduction Laboratories), mouse anti-BrdU (GE Healthcare), rabbit anti-GFP (Invitrogen), rabbit anti-phospho-histone H3 (Cell Signaling Technology), rabbit anti-parvalbumin (Swant), rabbit anti-Ki67 (Novocastra), rabbit anti-Sox2 (provided by M. Nakafuku, Cincinnati Children's Hospital Medical Center), rabbit anti-RhoA (Cell Signaling Technology) and mouse anti-Cdc42 (BD Transduction Laboratories). F-actin

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and nuclei visualization, as well as apoptotic cell detection (TUNEL assay), were performed as previously described (Katayama et al., 2011).

Explant culture of the MGE

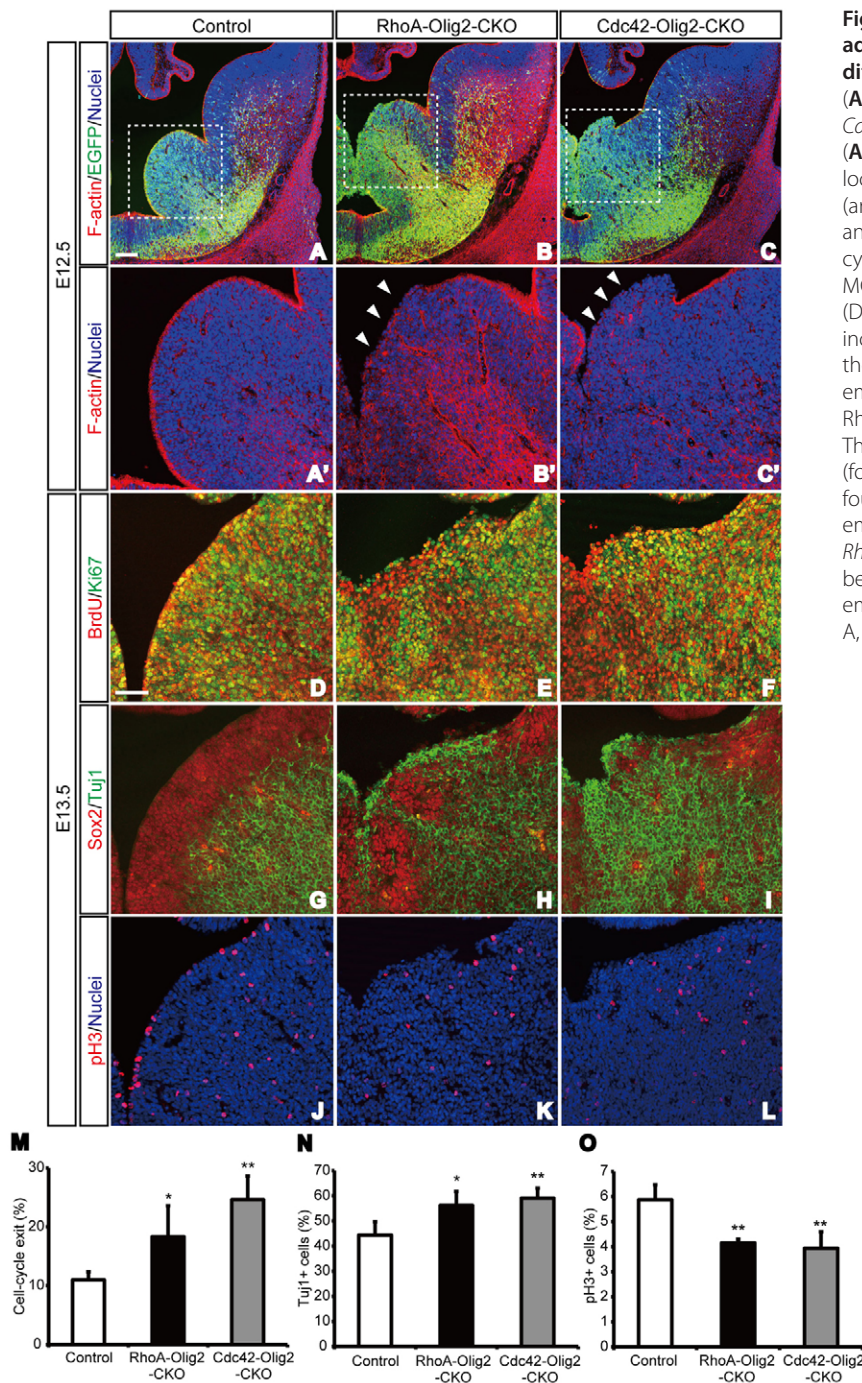
Explant cultures of MGE were performed as previously described (Shinohara et al., 2012).

RESULTS AND DISCUSSION

Defects in adherens junctions in the MGE in *RhoA-Olig2-CKO* and *Cdc42-Olig2-CKO* embryos

As *Olig2* has been shown to be expressed in neural progenitor cells within the VZ of the MGE (Miyoshi et al., 2007; Ono et al.,

2008), we used *Olig2-Cre* mice (Dessaud et al., 2007; Sürmeli et al., 2011) to delete *RhoA* and *Cdc42* in those progenitors to investigate their roles in the development of cortical interneurons. We first examined the formation of adherens junctions in the MGE as both *RhoA* and *Cdc42* have been shown to mediate the formation of adherens junctions in the developing nervous system (Cappello et al., 2006; Cappello et al., 2012; Chen et al., 2006; Herzog et al., 2011; Katayama et al., 2011; Katayama et al., 2012). In *RhoA^{flox/flox}; Olig2-Cre* (hereafter referred to as *RhoA-Olig2-CKO*) and *Cdc42^{flox/flox}; Olig2-Cre* (hereafter referred to as *Cdc42-Olig2-CKO*) embryos, apical disorganization of the MGE appeared at around embryonic day (E)12.5 (Fig. 1A-C). We



crossed mutant mice with stop-floxed *EGFP* reporter mice (Nakamura et al., 2006) and found that the dysplastic region corresponded well with the *EGFP*⁺ (i.e. Cre/loxP recombined) region (Fig. 1A-C). We then determined the localization of the adherens junction components F-actin, N-cadherin and β -catenin, and found that apical staining of these molecules was lost in the dysplastic region in both mutants (Fig. 1A-C; supplementary material Fig. S1). These results indicate that the loss of RhoA and Cdc42 disrupts adherens junctions in neural progenitor cells of the MGE, similar to the deletion effects reported for other regions of the central nervous system (Cappello et al., 2006; Cappello et al., 2012; Chen et al., 2006; Herzog et al., 2011; Katayama et al., 2011; Katayama et al., 2012).

Analysis of proliferation, differentiation and cell death in the MGE in RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos

Because adherens junctions are important for the proliferation and differentiation of neural progenitor cells (Zhang et al., 2010; Rouso et al., 2012), we examined these processes in neural progenitor cells in the MGE. To determine whether the loss of RhoA or Cdc42 in the MGE causes changes in cell-cycle withdrawal, we performed a BrdU pulse-chase experiment using E13.5 embryonic brains and then stained for the proliferative cell marker Ki67 (Mki67 – Mouse Genome Informatics), and counted the proportion of cells that exited the cell cycle in 24 hours (represented as the ratio of BrdU⁺/Ki67⁻ cells among all BrdU⁺ cells). The cell-cycle exit indices in the

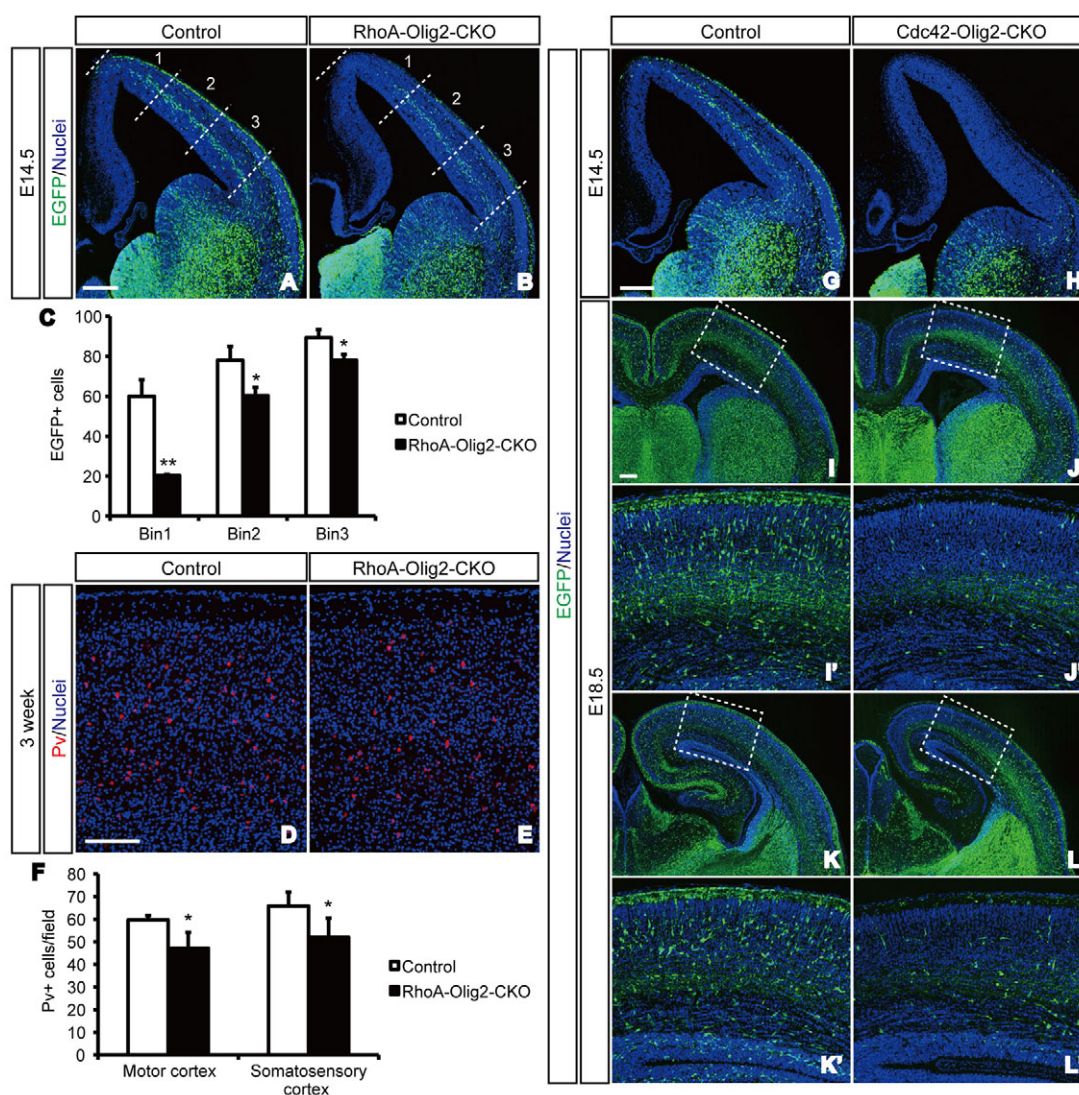


Fig. 2. Cortical interneuron migration was compromised in RhoA-Olig2-CKO and Cdc42-Olig2-CKO mice. (A-C) Impaired migration of cortical interneurons in RhoA-Olig2-CKO embryos at E14.5. For quantification, the cerebral cortex was divided into three equally spaced bins as indicated by dashed lines and numbers. Decrement in the number of EGFP⁺ cells was most prominent in Bin 1. The bar graph represents the mean + s.d. of three embryos. **P*<0.05, ***P*<0.01, Student's *t*-test. (D-F) The number of cortical interneurons was decreased in RhoA-Olig2-CKO mice at 3 weeks of age. Immunohistochemistry for Pv of the motor cortex (D,E). The number of Pv⁺ cells was significantly decreased in the motor cortex as well as in the somatosensory cortex in RhoA-Olig2-CKO embryos compared with control embryos (F). The graph depicts the mean + s.d. of four mice. **P*<0.05, Student's *t*-test. (G-L') Severely impaired migration of cortical interneurons in Cdc42-Olig2-CKO embryos. Only a few EGFP⁺ cells were observed in the cerebral cortex of Cdc42-Olig2-CKO embryos at E14.5 (G,H). The number of EGFP⁺ cells in the cerebral cortex was markedly decreased in Cdc42-Olig2-CKO embryos especially in the dorsal region in both rostral (I-J') and caudal (K-L') areas at E18.5. Boxed areas in I-L are shown at higher magnification in I'-L', respectively. Scale bars: 200 μ m.

MGEs of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos were significantly higher than those of control embryos (Fig. 1D-F,M).

In addition, we found that the ratios of β III-tubulin (Tuj1; Tubb3 – Mouse Genome Informatics)⁺ post-mitotic neurons were increased whereas the ratios of phospho-histone H3 (pH3)⁺ mitotic cells were decreased in the MGEs of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos (Fig. 1G-L,N,O), which further indicate that more neural progenitor cells have exited the cell cycle in the mutants compared with the controls. In the MGE of control embryos, Sox2⁺ progenitor cells and Tuj1⁺ post-mitotic neurons showed clear restriction to apical and basal compartments, respectively (Fig. 1G). However, in both mutants, these two cell populations were extensively intermingled and Tuj1⁺ neurons were found at the apical surface where VZ progenitors normally divide (Fig. 1H,I).

Finally, we found that the numbers of TUNEL⁺ apoptotic cells were increased in RhoA-Olig2-CKO and Cdc42-Olig2-CKO

embryos compared with control embryos (supplementary material Fig. S2). Collectively, these results indicate that the loss of RhoA or Cdc42 resulted in precocious neuronal differentiation and increased death of MGE cells.

Defects in migration of cortical interneurons in RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos

We examined next the migration of cortical interneurons using the RhoA-Olig2-CKO and Cdc42-Olig2-CKO mice that had been crossed with stop-floxed *EGFP* reporter mice (Nakamura et al., 2006). In RhoA-Olig2-CKO embryos, the numbers of EGFP⁺ cells in the cerebral cortex were decreased compared with control embryos at E14.5 and the decrement was most prominent in the dorsal-most region (Fig. 2A-C). Postnatal 3-week-old RhoA-Olig2-CKO mice showed decreased numbers of parvalbumin (Pv; Pvalb – Mouse Genome Informatics)⁺ and somatostatin (Sst)⁺

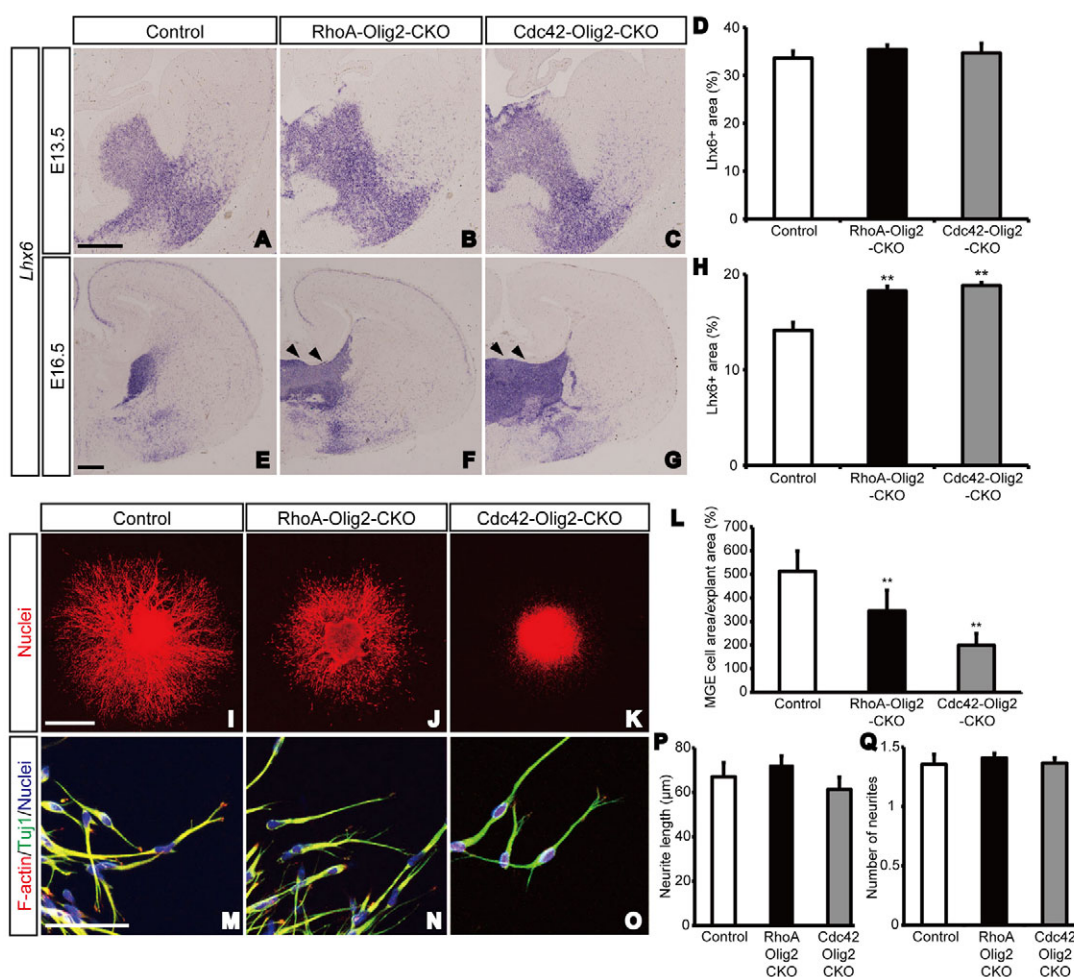


Fig. 3. Cortical interneuron migration was compromised in RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos. (A-H) Specification of *Lhx6*⁺ cells was not altered in the MGE in RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos. At E13.5, *Lhx6* transcripts were expressed in the MGEs of RhoA-Olig2-CKO, Cdc42-Olig2-CKO and control embryos to similar extents (A-D). At E16.5, the *Lhx6*⁺ cells were dispersed throughout the cerebral cortex in control embryos, whereas a significant number of *Lhx6*⁺ cells remained in the MGE in RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos (E-H; arrowheads). The graphs represent the mean + s.d. of six control (three *RhoA*^{fllox/+}; *Olig2*-Cre and three *Cdc42*^{fllox/+}; *Olig2*-Cre), three to four RhoA-Olig2-CKO and four Cdc42-Olig2-CKO embryos. (I-L) Loss of RhoA and Cdc42 in the VZ causes impaired migration of MGE cells from explants *in vitro*. MGE explants from E13.5 embryos were cultured for 48 hours. The graph represents the mean + s.d. of 14 control (eight *RhoA*^{fllox/+}; *Olig2*-Cre and six *Cdc42*^{fllox/+}; *Olig2*-Cre), five RhoA-Olig2-CKO and eight Cdc42-Olig2-CKO embryos. (M-Q) Morphologies of migrating MGE cells in RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos were similar to controls. The graphs represent the mean + s.d. of eight control (three *RhoA*^{fllox/+}; *Olig2*-Cre and five *Cdc42*^{fllox/+}; *Olig2*-Cre), three RhoA-Olig2-CKO and four Cdc42-Olig2-CKO embryos. Statistical analyses were carried out between *RhoA*^{fllox/+}; *Olig2*-Cre and RhoA-Olig2-CKO embryos, and between *Cdc42*^{fllox/+}; *Olig2*-Cre and Cdc42-Olig2-CKO embryos. ***P* < 0.01, Student's *t*-test. Scale bars: in A,E, 200 μm; in I, 400 μm; in M, 50 μm.

interneurons, which derive from the MGE, in both the motor and somatosensory cortices (Fig. 2D-F; supplementary material Fig. S3).

The Cdc42-Olig2-CKO embryonic phenotype was even more severe, with only a few EGFP⁺ cells observed in the developing cerebral cortex at E14.5 (Fig. 2G,H). Furthermore, the numbers of EGFP⁺ cells in the cerebral cortex were markedly decreased at E18.5, especially in the dorsal region (Fig. 2I-L). Unfortunately, we could not examine postnatal Cdc42-Olig2-CKO mice owing to their perinatal lethality.

To determine whether reduced numbers of MGE-derived interneurons in the cortices of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos are caused by a reduction in the generation of cortical interneurons, we examined the expression of *Lhx6*, a marker for MGE-derived neurons, by *in situ* hybridization. The total areas of *Lhx6* *in situ* hybridization signal were similar in the MGEs of E13.5 RhoA-Olig2-CKO, Cdc42-Olig2-CKO and control embryos (Fig. 3A-D). This suggests that the specification of *Lhx6*⁺ cortical interneurons was not compromised in either mutant at E13.5; however, we observed ectopic localizations of *Lhx6* transcripts in the apical surface in both conditional mutants (Fig. 3B,C) that corresponded with the locations of TuJ1⁺ neurons (Fig. 1H,I). At E16.5, the *Lhx6*⁺ cells were dispersed throughout the cerebral cortex and the striatum in control embryos, whereas

many *Lhx6*⁺ cells remained in the MGEs of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos (Fig. 3E-G). Although the area of *Lhx6* expression was increased in the MGEs of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos at E16.5 (Fig. 3E-H), the numbers of Ki67⁺ cells in the same regions were decreased (supplementary material Fig. S4). Taken together, the decreased numbers of MGE-derived interneurons in the cortices of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos do not seem to be caused by defects in specification of cortical interneurons, but rather result from defects in interneuron migration, although we could not exclude the possibility that the decreased number of progenitors in the MGE affected the number of interneurons in postnatal RhoA-Olig2-CKO mice.

To evaluate directly the migratory competence of the interneurons, we conducted an *in vitro* experiment using MGE explant cultures. MGE cells of control embryos migrated extensively from the explants, whereas those of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos showed defects in migration with impairment being more severe in the *Cdc42* mutant embryos (Fig. 3I-L). We also examined the morphologies of individual neurons in the cultures. There were no obvious defects in length or numbers of neurites in the mutant embryos (Fig. 3M-Q), suggesting that deletion of *RhoA* or *Cdc42* does not affect interneuron morphology.

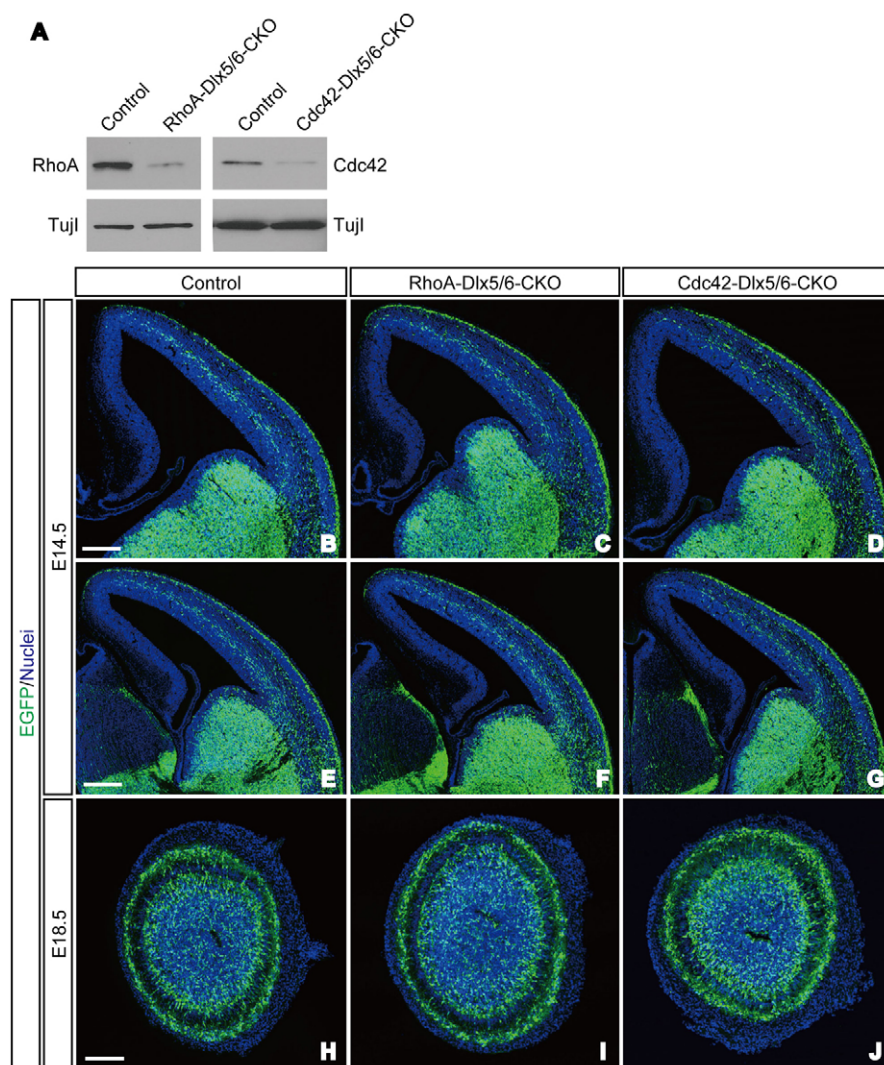


Fig. 4. Interneuron migration was unaffected in RhoA-Dlx5/6-CKO and Cdc42-Dlx5/6-CKO embryos. (A) The protein levels of RhoA and Cdc42 in the SVZ of the ganglionic eminences were markedly decreased in RhoA-Dlx5/6-CKO and Cdc42-Dlx5/6-CKO embryos at E13.5. (B-G) There were no obvious differences in the number or distribution of EGFP⁺ cells in the cerebral cortex between control and RhoA-Dlx5/6-CKO or Cdc42-Dlx5/6-CKO embryos in both rostral (B-D) and caudal (E-G) regions at E14.5. (H-J) No obvious differences were observed in the number and distribution of EGFP⁺ cells in the olfactory bulb between control and RhoA-Dlx5/6-CKO or Cdc42-Dlx5/6-CKO embryos at E18.5. Scale bars: 200 μm.

These *in vitro* and *in vivo* data strongly argue that both RhoA and Cdc42 in VZ progenitors within the MGE are required for proper migration of cortical interneurons. The moderate phenotype exhibited by RhoA-Olig2-CKO mice may indicate a compensatory role played by other Rho family members, such as RhoC, in the absence of RhoA (Leslie et al., 2012).

Indirect roles of RhoA and Cdc42 in cortical interneuron migration

To determine whether RhoA and Cdc42 have direct roles in the migration of cortical interneurons, we deleted *RhoA* and *Cdc42* using *Dlx5/6-CIE* mice in which Cre recombination is induced in the SVZ but not in the VZ of the ganglionic eminences (Stenman et al., 2003). In *RhoA^{flox/flox}; Dlx5/6-CIE* (hereafter referred to as RhoA-Dlx5/6-CKO) and *Cdc42^{flox/flox}; Dlx5/6-CIE* (hereafter referred to as Cdc42-Dlx5/6-CKO) mice, the protein levels of RhoA and Cdc42 were significantly decreased in the SVZ (Fig. 4A). In both mutant embryos, there were no obvious defects in apical organization of the ganglionic eminences compared with control embryos at E12.5 (supplementary material Fig. S5). In contrast to RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos, there were no obvious defects in interneuron migration in E14.5 RhoA-Dlx5/6-CKO and Cdc42-Dlx5/6-CKO embryos (Fig. 4B-D). Because Cre is also expressed in the caudal ganglionic eminence in *Dlx5/6-CIE* mice, the migration of cortical interneurons in the caudal telencephalic region was examined in both mutant embryos. However, no obvious differences between control and RhoA-Dlx5/6-CKO or Cdc42-Dlx5/6-CKO embryos were detected at E14.5 (Fig. 4E-G). We also examined the numbers and distribution of interneurons in the olfactory bulb as Cre is expressed in the lateral ganglionic eminence, a major source of interneurons for the olfactory bulb, in *Dlx5/6-CIE* mice (Wichterle et al., 2001; Stenman et al., 2003). No obvious differences in interneuron migration were observed between control and RhoA-Dlx5/6-CKO or Cdc42-Dlx5/6-CKO embryos at E18.5 in the olfactory bulb (Fig. 4H-J). These findings indicate that RhoA and Cdc42 are not required in SVZ progenitors or in the migrating interneurons, suggesting that RhoA and Cdc42 do not directly regulate cell motility or other general aspects of the migration process.

In conclusion, our genetic findings demonstrate that RhoA and Cdc42 do not directly regulate interneuron migration; however, they are required for progenitors in the VZ to differentiate properly into interneurons with sufficient migratory competency to navigate into the developing cortex.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

K.K., F.I. and Y.Y. performed the research. K.K. and F.I. designed the research. K.K. and F.I. analyzed the data. K.C., R.A.L. and Y.Z. contributed unpublished reagents/analytic tools. K.K., K.C., Y.Z. and Y.Y. wrote the paper.

Supplementary material

Supplementary material available online at
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Fig. S1

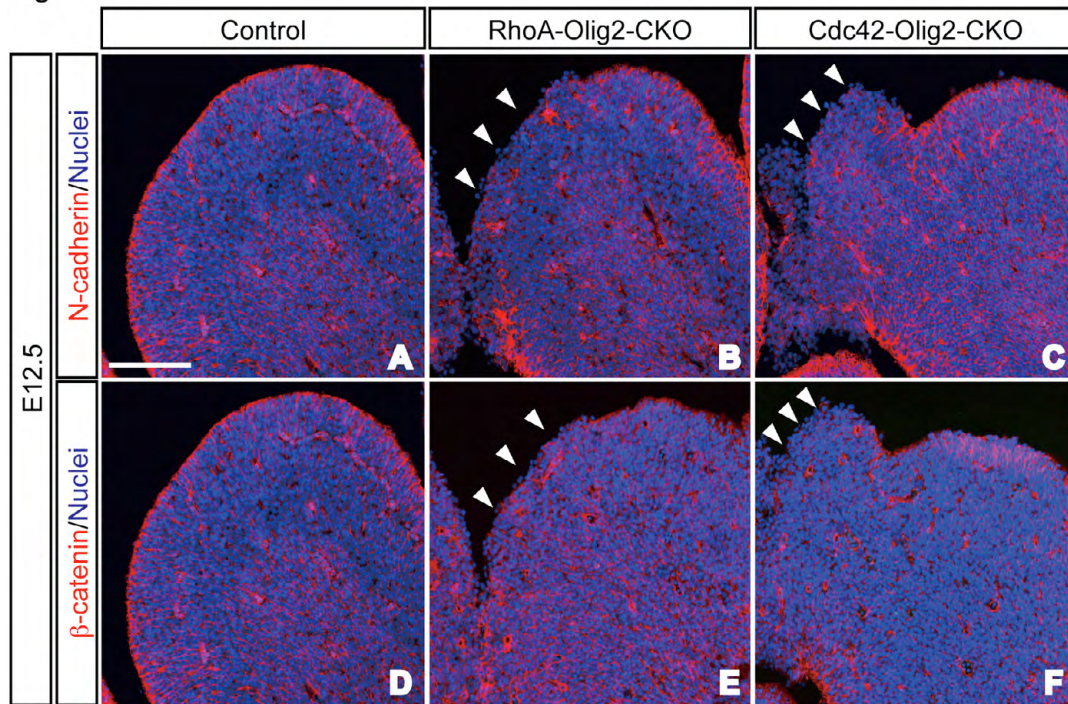


Fig. S1. Disruption of adherens junctions in *RhoA*- and *Cdc42*-deleted neural progenitor cells. (A-F) Apical localization of N-cadherin (A-C) and β -catenin (D-F) was lost in the dysplastic region of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos at E12.5 (arrowheads). Scale bar: 100 μ m.

Fig. S2

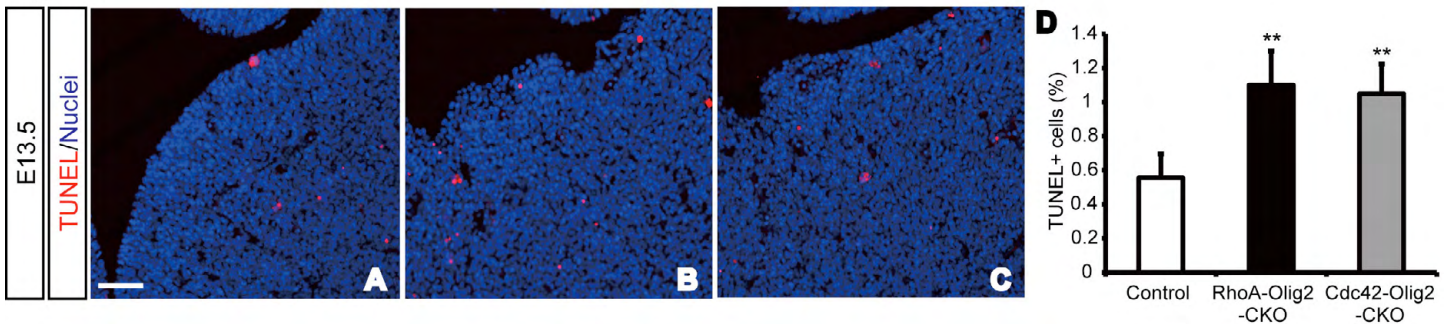


Fig. S2. Loss of *RhoA* and *Cdc42* induced increased apoptosis in MGE cells. (A-D) TUNEL staining revealed increased apoptosis in RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos. The graph represents the mean + s.d. of eight control (four *RhoA*^{fllox/+}; *Olig2*-Cre and four *Cdc42*^{fllox/+}; *Olig2*-Cre), three RhoA-Olig2-CKO and four Cdc42-Olig2-CKO embryos. Statistical analyses were carried out between *RhoA*^{fllox/+}; *Olig2*-Cre and RhoA-Olig2-CKO embryos, and between *Cdc42*^{fllox/+}; *Olig2*-Cre and Cdc42-Olig2-CKO embryos. ***P* < 0.01, Student's *t*-test. Scale bar: 50 μ m.

Fig. S3

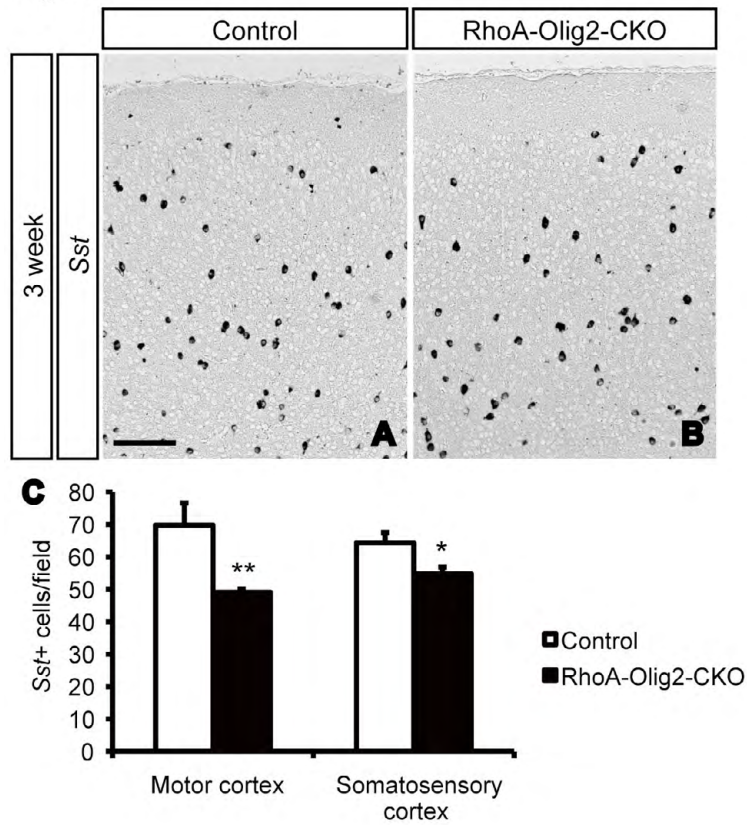


Fig. S3. The number of *Sst*⁺ cortical interneurons was decreased in RhoA-Olig2-CKO mice. (A,B) *In situ* hybridization for *Sst* of the motor cortex at 3 weeks of age. (C) The number of *Sst*⁺ cells was significantly decreased in the motor cortex as well as in the somatosensory cortex in RhoA-Olig2-CKO embryos compared with control embryos. *Sst*⁺ cells in a 10× optical view were counted. The graph depicts the mean + s.d. of three mice. **P*<0.05, ***P*<0.01, Student's *t*-test. Scale bar: 100 μm.

Fig. S4

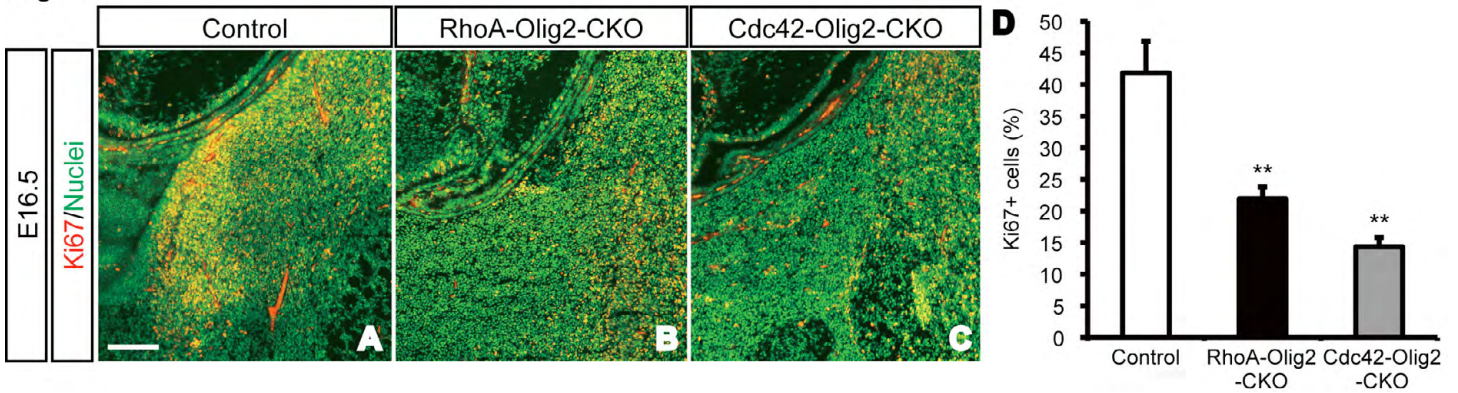


Fig. S4. Reduced proliferating cells in the MGE in RhoA-Olig2-CKO and Cdc42-Olig2-CKO. (A-C) The numbers of Ki67⁺ proliferating cells were significantly decreased in the MGE of RhoA-Olig2-CKO and Cdc42-Olig2-CKO embryos at E16.5. (D) The graph represents the mean + s.d. of seven control (four *RhoA*^{flax/+}; *Olig2*-Cre and three *Cdc42*^{flax/+}; *Olig2*-Cre), four RhoA-Olig2-CKO and four Cdc42-Olig2-CKO embryos. Statistical analyses were carried out between *RhoA*^{flax/+}; *Olig2*-Cre and RhoA-Olig2-CKO embryos, and *Cdc42*^{flax/+}; *Olig2*-Cre and Cdc42-Olig2-CKO embryos. ***P*<0.01, Student's *t*-test. Scale bar: 100 μm.

Fig. S5

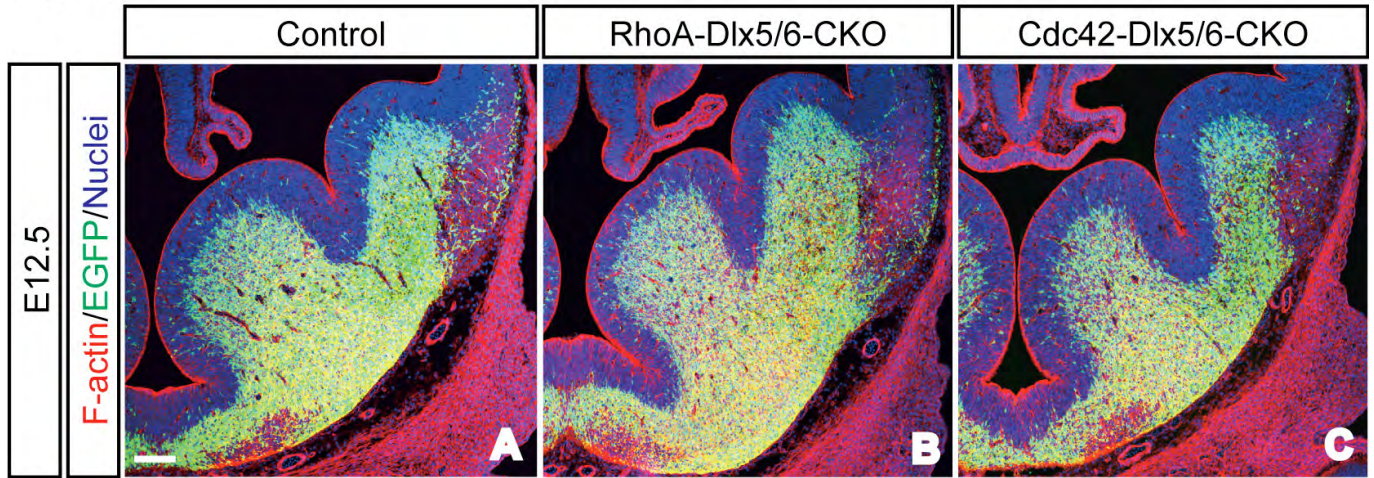


Fig. S5. Apical organization was not disrupted in RhoA-Dlx5/6-CKO and Cdc42-Dlx5/6-CKO embryos. (A-C) Apical localization of F-actin was present in the ganglionic eminences of RhoA-Dlx5/6-CKO and Cdc42-Dlx5/6-CKO embryos at E12.5. Scale bar: 100 μ m.