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ARTICLES

Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex

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Projection neurons migrate from the ventricular zone to the neocortical plate during the development of the mouse brain. Their overall movement is radial, but they become multipolar and move nonradially in the intermediate zone. Here we show that Reelin, the Rap1 GTPase and N-cadherin (NCad) are important for multipolar neurons to polarize their migration toward the cortical plate. Inhibition and rescue experiments indicated that Reelin regulates migration through Rap1 and Akt, and that the Rap1-regulated GTPases RalA, RalB, Rac1 and Cdc42 are also involved. We found that Rap1 regulated the plasma membrane localization of NCad and NCad rescued radial polarization when Rap1 was inhibited. However, inhibition of Rap1 or NCad had little effect on glia-dependent locomotion. We propose a multistep mechanism in which Reelin activates Rap1, Rap1 upregulates NCad, and NCad is needed to orient cell migration.

The mammalian neocortex is patterned by the coordinated migration of immature neurons[1]. Most neocortical projection neurons originate by asymmetric division of radial glia progenitors in the ventricular zone. They then move radially to the subventricular zone (SVZ) and lower intermediate zone, where they become multipolar. They dynamically extend and retract multiple long projections and move in apparently random directions[1–3]. Axonogenesis starts as cells approach the middle of the intermediate zone. After the axon emerges, the cells reorient their centrosomes and Golgi upwards, toward the developing cortical plate, and resume radial migration[4]. As they move to the upper part of the intermediate zone, their morphology changes from multipolar to bipolar. Bipolar cells have a thick, radially oriented leading process and a thin, trailing axon, and move by locomotion along the radially oriented processes of radial glia or other neurons[1,5].

The transition from multipolar to radial migration can be inhibited by many mutations and experimental manipulations, suggesting that it is a complex process[1]. Among the requirements are cytoskeletal regulators including dynein-associated proteins and extracellular signals including semaphorin 3A, but little is known about how the signals are interpreted by the cell[1,6,7].

In many cell types, cell orientation and polarity are controlled by local activation and global inhibition of signaling pathways that organize the cytoskeleton and direct vesicle traffic[8]. Positive feedback loops coordinated by small GTPases stabilize cell polarity in response to intracellular and extracellular cues. GTPases are molecular switches that are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs). The GTP state is active and binds to effector molecules. In yeast and leukocytes, the Ras-related GTPase Rap provides a key nexus for activating other small GTPases, which in turn regulate the actin cytoskeleton and membrane traffic[8]. Rap proteins are also important for polarization of nonmotile cells; for example, for specifying the axonal-dendritic polarity of cultured hippocampal neurons[9] and the apical-basolateral polarity of epithelial cells[10]. However, the roles of Rap proteins *in vivo* in mammals have been unclear, perhaps partly because of possible redundancy between the five Rap genes (Rap1A, 1B, 2A, 2B and 2C)[11].

Here we have studied the role of Rap proteins in cortical development and discovered a key role for Rap1 in polarizing radial migration of multipolar cells. Specifically, we found that Reelin, which is known for its role in neuron lamination in the cortical plate[1,12], activates Rap1 in multipolar neurons in the intermediate zone. In turn, Rap1 regulates neural cadherin (NCad or CDH2). NCad is a classical cadherin: a single-pass transmembrane receptor that regulates cell-cell contact by calcium-dependent homophilic binding[13]. We find that NCad is needed to orient the migration of multipolar cells toward the cortical plate. Our results suggest a multistep model for orienting the migration of cortical neurons in the intermediate zone.

RESULTS

Rap1 regulates migration into the upper intermediate zone

We monitored cortical development *in vivo* by electroporation of ventricular zone progenitor cells with green fluorescent protein (GFP)[14]. We then observed the positions of daughter neurons at various times thereafter. Previous studies have shown that most cells in the lower intermediate zone and SVZ are multipolar, whereas most cells in the upper intermediate zone and cortical plate are bipolar, so we name these regions the multipolar migration zone (MMZ) and radial migration zone (RMZ), respectively[1–3]. Control neurons reach the MMZ a day after electroporation (embryonic day (E)15.5) and are still there a day later (E16.5; **Supplementary Fig. 1**). During the third day (E17.5), most of the neurons have passed into the RMZ, and some have reached the top of the cortical plate[1–3].

To test the role of Rap proteins in this process, we expressed dominant-negative Rap1A^{17N} or Rap1GAP, which inhibits all Rap family members but not Ras[14]. Neurons in which Rap was inhibited reached the MMZ normally in the first day, but their movement into the

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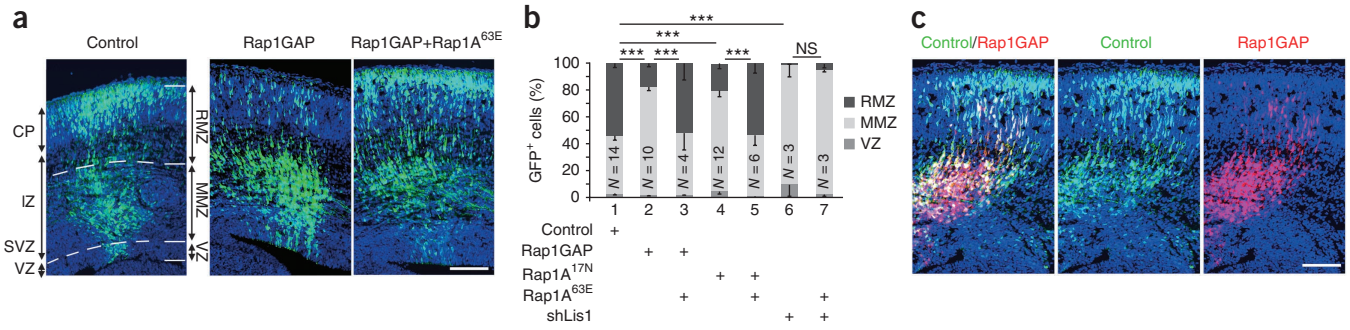

Figure 1 Rap activity is required cell-autonomously for neurons to enter the upper intermediate zone and cortical plate. (**a**–**c**) Fetal brains were electroporated *in utero* at embryonic day 14.5 (E14.5) with plasmids expressing the indicated proteins along with GFP or ChFP. Rap1GAP was expressed in progenitors and postmitotic neurons under the CAG promoter; Rap1A^{17N} and Rap1A^{63E} were expressed in postmitotic neurons using the NeuroD promoter. (**a**) Positions of transfected cells at E17.5. The cerebral wall was subdivided into radial morphology zone (RMZ; cortical plate and upper intermediate zone), multipolar morphology zone (MMZ; middle and lower intermediate zone and subventricular zone (SVZ)) and ventricular zone (VZ). GFP, green; DAPI, blue. (**b**) Percentage of electroporated cells in each region. (**c**) Sequential electroporation with GFP, followed, 10 min later, by Rap1GAP and ChFP, shows that Rap role in migration is cell autonomous. Scale bars, 100 μm. CP, cortical plate; IZ, intermediate zone. Error bars, s.e.m. ***$P < 0.001$; NS, not significant.

RMZ on the third day was delayed (**Fig. 1a** and **Supplementary Fig. 1**). The decrease in the proportion of cells in the RMZ was highly significant (**Fig. 1b**). To test whether the defect was due to the inhibition of Rap activity in neurons or in other cells, we expressed Rap1GAP or dominant-negative Rap1A^{17N} from the NeuroD promoter, which is expressed only in postmitotic neurons (**Supplementary Fig. 2**). Entry to the RMZ was inhibited (**Fig. 1b** and **Supplementary Fig. 3**), indicating that Rap is required in postmitotic neurons. Moreover, inhibition of entry to the RMZ by Rap1GAP or Rap1A^{17N} could be rescued by the co-expression of the active mutant Rap1A^{63E} in postmitotic neurons (**Fig. 1b**) although the active form did not affect cell migration when expressed alone (**Supplementary Fig. 3**). As a further control for specificity, we used short hairpin (sh)RNA to deplete the dynein regulator Lis1. Lis1 is needed for entry into the RMZ[7], but the effects of its depletion were not rescued by Rap1A^{63E} (**Fig. 1b**). Moreover, sequential electroporation of GFP followed by a mixture of cherry fluorescent protein (ChFP) and Rap1GAP showed that Rap-inhibited cells do not affect the migration of control cells (**Fig. 1c**). To confirm our results, we knocked down Rap1A and Rap1B using specific shRNAs. Knockdown of Rap1A or both Rap1A and Rap1B gave a similar phenotype to that caused by Rap1GAP, whereas Rap1B had less effect (**Supplementary Fig. 3a**,**b**). We confirmed the specificity of *Rap1A* shRNA by the rescue of the phenotype when we co-expressed a nontargetable *Rap1A* cDNA (**Supplementary Fig. 3b**). Further controls showed that Rap1 inhibition did not detectably affect Pax6$^+$ apical progenitor cells, Nestin$^+$ radial glia fibers, Tbr2$^+$ basal progenitors in the SVZ, Cux1$^+$ neuronal fate, cell proliferation or apoptosis (**Supplementary Fig. 4**). These results show that Rap1 family GTPases, principally Rap1A, have a cell-autonomous role in regulating the exit of neurons from the MMZ.

Rap1 orients the migration of multipolar neurons

We reasoned that Rap1 might be needed before the start of radial migration. We first checked the morphology of control and Rap-inhibited cells. Two days after electroporation, most control neurons in the lower part of the RMZ, close to the transition zone, had bipolar morphology (90.0 ± 2%; mean ± s.e.m.) but most Rap-inhibited neurons were multipolar (10.5 ± 0.5% with bipolar morphology; **Fig. 2a**). We then examined the orientation of the Golgi apparatus in multipolar cells in the upper MMZ by staining for the Golgi protein GM130. After control electroporation, most upper MMZ neurons had their Golgi apparatus oriented toward the cortical plate, but the Golgi was disoriented if Rap was inhibited (**Fig. 2b**). We then tracked the movement of multipolar neurons in the upper MMZ using time-lapse video-microscopy (**Supplementary Movie 1**). Most of the multipolar neurons in this region of control cortex moved upwards toward the cortical plate (91.1 ± 1.1%; **Supplementary Movie 1**), and only a minority moved randomly (typical paths shown in **Fig. 2c**). By contrast, most Rap-inhibited neurons moved in random directions (**Supplementary Movie 1**) with only 38.7 ± 1.2% migrating toward the cortical plate. Rap1 inhibition did not affect the speed of multipolar migration (**Fig. 2d**). These results suggest that Rap1 activity is needed for multipolar cells to orient their migration toward the cortical plate. Neurons in which Rap1 was inhibited or knocked down still extended axons (**Supplementary Fig. 5**), suggesting that inhibition of Rap1 does not prevent cortical axonogenesis *in vivo*. This contrasts with the requirement for Rap1B for hippocampal axonogenesis *in vitro*[9]. However, we cannot rule out the possibilities that residual Rap activity might be sufficient for axonogenesis *in vivo* or that the requirement for Rap might differ depending on cell type or *in vivo* versus *in vitro* system.

The requirement of Rap1 for migration was not absolute. Populations of cells that expressed Rap1GAP or Rap1A^{17N} were migrating through the cortical plate with bipolar morphology by 5 d after electroporation (**Supplementary Fig. 6a**). These cells were not normal, as they did not form an apical dendritic tree at the top of the cortical plate (**Supplementary Fig. 6b**). This implies that cells may be capable of locomotion despite low Rap activity. Indeed, time-lapse video-microscopy of the RMZ at E16.5 showed that control and Rap-inhibited cells migrated at similar speeds (**Fig. 2d** and **Supplementary Movie 2**). To further test whether Rap1 is required for neuron migration, we dissociated E15.5 cerebral cortex, electroporated the cells with GFP and Rap1GAP or vector, and measured their migration on lattices comprised of radial glia and neuronal processes, similar to the substrate they encounter *in vivo*[15]. Control and Rap1GAP-expressing cells migrated equally (**Fig. 2d** and **Supplementary Movie 3**). As shown below, Rap1GAP inhibits Rap1 in these cells. Together, these results suggest that Rap1 is transiently involved in the orientation of multipolar migration in the intermediate zone, but is unimportant for radial migration through the cortical plate.

Rap1 regulates N-cadherin localization in cortical neurons

In many types of cell, Rap1 regulates traffic between endosomes and the plasma membrane[16]. We detected epitope-tagged Rap1A on both endosomes and the plasma membrane of electroporated neurons

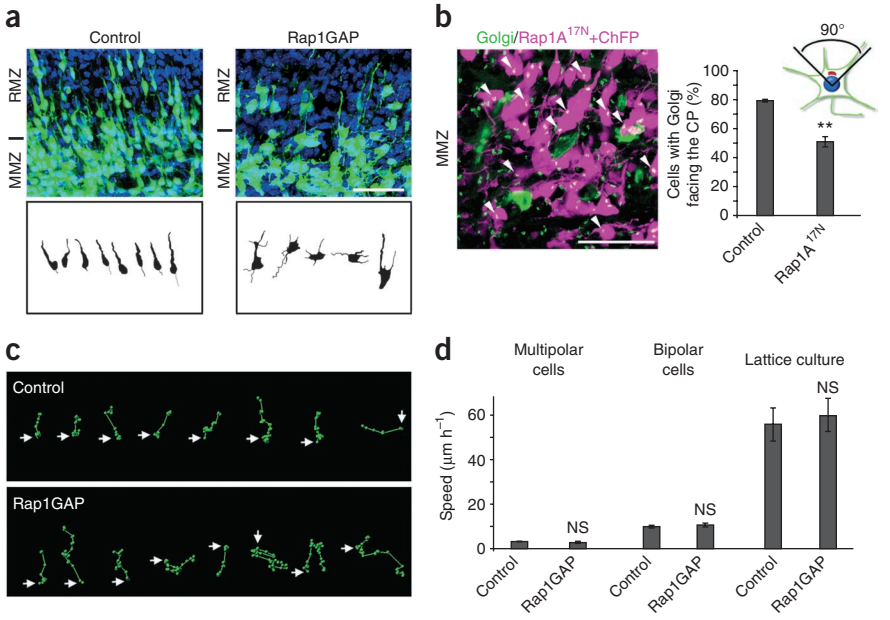

Figure 2 Rap1 is required to orient multipolar cells but not for migration of bipolar neurons. The indicated plasmids were electroporated *in utero* at E14.5 along with plasmids expressing GFP or ChFP. (**a**) Computer-based reconstruction of shapes of GFP$^+$ neurons in E16.5 cortices at the transition between MMZ and RMZ. (**b**) Golgi staining (green, arrowheads) of MMZ neurons (magenta). The percentage of cells with Golgi facing the cortical plate was calculated (mean ± s.e.m.). (**c**) Tracks of migration paths followed by control and Rap-inhibited multipolar cells in the upper part of the MMZ. Positions of cell centroids in successive frames (circles) are linked by lines. The start position is marked by an arrow. (**d**) Migration of control and Rap inhibited GFP$^+$ multipolar (left; control, $N = 28$ cells in 3 movies; Rap1GAP: $N = 21$ in 3 movies) and bipolar (right; control, $N = 121$ cells in 6 movies; Rap1GAP, $N = 51$ in 4 movies) neurons in cortical slices prepared at E16.5 from brains electroporated at E14.5. Right, migration of bipolar GFP$^+$ cortical neurons in lattice culture (control, $N = 22$ cells; Rap1GAP, $N = 14$). Mean ± s.e.m. of average migration speed. Scale bars, 50 μm. $^{**}P < 0.01$; NS, not significant.

in vivo (**Supplementary Fig. 7a**). Therefore we considered the possibility that Rap1 might regulate cell orientation by controlling the cell-surface expression of membrane proteins that sense the environment.

In other cell types, Rap1 regulates membrane proteins of the integrin and classical cadherin families[11,17,18]. The role of integrins in neuronal migration has been studied before and is still unclear[19]. Therefore we tested whether cadherins might be involved in neuron migration. We focused on NCad because it is expressed throughout the developing cortex, including the region in which radial migration starts (see below).

To test whether Rap1 regulates the expression of NCad on cell surfaces *in vivo* we introduced HA-tagged, full-length, wild-type NCad (NCad^{WT}-HA) by *in utero* electroporation together with control vector or Rap1GAP. We detected NCad^{WT}-HA on the plasma membrane and internal compartments under both conditions, but the amount on the membrane was relatively decreased when Rap1GAP was expressed (**Fig. 3a**,**b**). To test whether the localization of endogenous NCad is regulated by Rap1, we electroporated embryonic cortical neurons with GFP and Rap1GAP or vector and examined the cells by immunofluorescence. Although endogenous NCad localized to the plasma membrane of control cells, it was primarily in a perinuclear structure in Rap1GAP-expressing cells (**Fig. 3c**). To test whether Rap1 modulates functional NCad on the cell surface, we measured the homophilic adhesion of electroporated neurons to surfaces coated with the NCad extracellular domain (NCad-Fc). We counted GFP$^+$ adherent cells that expressed the neuron marker MAP2 (**Supplementary Fig. 8**). Although control neurons bound to NCad-Fc or control poly-D-lysine (PDL), Rap1GAP specifically inhibited the binding of neurons to NCad-Fc (**Fig. 3d**). As a control, the active form of Rap1A was able to rescue the binding defect (**Supplementary Fig. 9a**). Rap1GAP also inhibited neurite extension on NCad-Fc but not PDL (**Fig. 3e**). These phenotypes depended on NCad, as overexpression of full-length, wild-type NCad^{WT} rescued binding and neuritogenesis by Rap1GAP-expressing cells on NCad-Fc (**Fig. 3d**,**e**). To inhibit cadherin function we used a mutant NCad^{DN} that lacked most of the extracellular domain. Similar mutants have been used in other studies[20,21], and induce internalization of endogenous cadherins[22]. As expected,

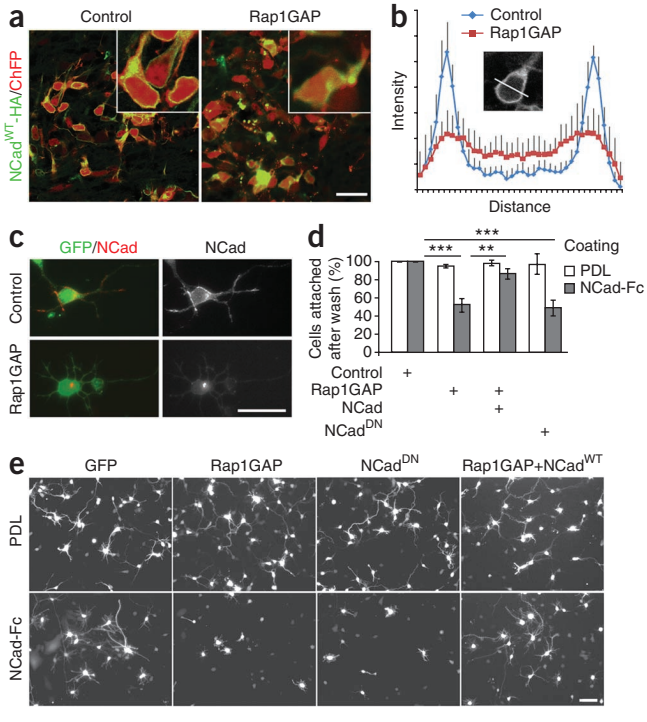

Figure 3 Rap1 regulates adhesion to NCad and membrane levels of NCad. (**a**) HA antibody staining (green) of MMZ neurons that were electroporated *in utero* with HA-tagged NCad^{WT}, ChFP and vector (control) or Rap1GAP. (**b**) Mean ± s.e.m. of HA-NCad fluorescence intensity profiles across the bodies of $N = 9$ control and $N = 9$ Rap1GAP-expressing cells. (**c**) Immunofluorescence of the endogenous NCad in dissociated cortical neurons electroporated with GFP expressing plasmid alone or along with the Rap1GAP-expressing plasmid. (**d**,**e**) E15.5 dissociated cortical neurons were electroporated with the indicated plasmids and incubated overnight on dishes coated with NCad-Fc or PDL. (**d**) GFP-expressing cells were counted before and after washes to determine the percentage of attached cells (mean ± s.e.m.). (**e**) The morphology of GFP-expressing attached cells was observed after a further 1 d of differentiation. Scale bars, 20 μm.

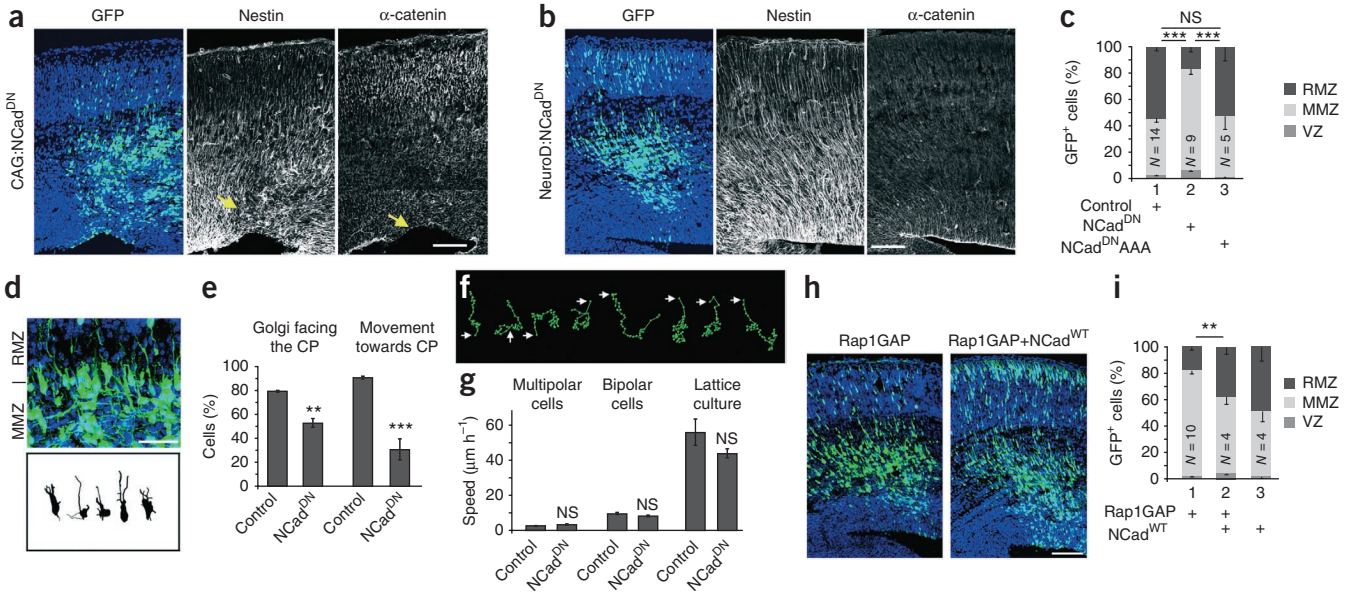

Figure 4 NCad is required to orient multipolar cells, under control of Rap1. (**a**,**b**) Electroporation of dominant-negative NCad^{DN} in progenitor cells (**a**) or postmitotic cells (**b**) at E14.5 and labeled for the indicated proteins 3 d later. (**c**) Percentage of electroporated cells in each region (mean ± s.e.m.). NCad^{DN}AAA, inactive point mutant of NCad^{DN}. (**d**) Computer-based reconstruction of shapes of GFP$^+$ neurons in E16.5 cortices at the transition between MMZ and RMZ. (**e**) Percentage of cells with Golgi facing the cortical plate and fraction of multipolar neurons migrating toward the cortical plate in time-lapse videomicroscopy (mean ± s.e.m.). (**f**) Tracks of migrating NCad^{DN}-expressing multipolar cells in the upper part of the MMZ. Positions of cell centroids in successive frames (circles) are linked by lines. Arrow, start position. (**g**) Migration of GFP$^+$ neurons in cortical slices prepared at E16.5 from brains electroporated at E14.5. Cadherin-inhibited multipolar (left; control, $N = 28$ cells in 3 movies; NCad^{DN}, $N = 27$ in 3 movies) and bipolar (middle; control, $N = 121$ cells in 6 movies; NCad^{DN}, $N = 65$ cells in 4 movies) neurons migrate at a normal speed. Right, migration of GFP$^+$ neurons in lattice culture ($N = 16$ cells). Mean ± s.e.m. of average migration speed. (**h**) Wild-type NCad^{WT}, expressed from the NeuroD promoter, partly rescues the migration defect of Rap1GAP-expressing cells. (**i**) Percentage of electroporated cells in each region (mean ± s.e.m.). Scale bars represent 20 μm (**d**) and 100 μm (**a**,**b**,**h**). $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant.

NCad^{DN} inhibited cortical neuron attachment and neuritogenesis on NCad-Fc (**Fig. 3d**,**e**). These results suggest that Rap1 activity maintains functional cadherins on the cell surface of neurons *in vivo* and *in vitro*. However these data do not show whether the primary effect is on endocytosis, exocytosis or the overall level of NCad.

Several Rap effectors regulate cadherins in other systems. The Rap1 effector Vav2 and its substrate GTPase Cdc42 inhibit endocytosis of ECad[23], whereas the Rap1 effector RalGDS activates RalA, which docks secretory vesicles to the exocyst complex and recycles ECad to epithelial cell-cell junctions[24]. As a preliminary test of which Rap effectors regulate NCad in multipolar neurons, we performed *in utero* electroporation with GTPase binding domains from NWASP, POSH and Sec5, which are semi-specific inhibitors of Cdc42, Rac1 and RalA/B, respectively[25,26]. Each of these proteins partially inhibited entry into the RMZ, suggesting that it requires both Rac1-Cdc42 and RalA/B activity (**Supplementary Fig. 9b**). To further confirm the involvement of Ral enzymes in cortical development, we knocked down RalA and RalB separately using shRNA. Knockdown of either RalA or RalB inhibited RMZ entry. Inhibition of RalA or RalB was rescued by human RalA or RalB, respectively (**Supplementary Fig. 9b**). This suggests that both Ral proteins have an important role in this process. Activated Rap1A^{63E} did not overcome inhibition by the POSH binding domain, suggesting that Rap1 is upstream of or parallel to Rac1 (**Supplementary Fig. 9b**). We then tested whether activated Vav2, RalA, RalB, Cdc42 and Rac1 could rescue entry into the RMZ by Rap-inhibited neurons. Vav2 produced almost complete rescue, whereas each of the small GTPases individually allowed partial rescue without affecting migration when electroporated alone (**Supplementary Fig. 9c**,**d**). Inhibition of Ral enzymes also affected the presence of wild-type NCad-HA at the plasma membrane (**Supplementary Fig. 9e**). Moreover, the active form of Vav2 was able to rescue the NCad-binding defect of neurons in which Rap was inhibited (**Supplementary Fig. 9a**). These results suggest that RalA/B and Rac1-Cdc42 regulate NCad traffic and that Rap1 might regulate localization of NCad to the membrane by several mechanisms.

Cadherins regulate radial orientation of multipolar neurons

We next used *in utero* electroporation of NCad^{DN} to test whether cadherins regulate multipolar neuron migration. Expression of NCad^{DN} in progenitor cells using the ubiquitous CAG promoter disrupted α-catenin$^+$ apical junctions and Nestin$^+$ radial glia (**Fig. 4a**), as expected[27]. However, expression of NCad^{DN} in postmitotic neurons from the NeuroD promoter inhibited the appearance of multipolar neurons in the RMZ (**Fig. 4b**,**c**) without affecting apical junctions or radial glia (**Fig. 4b**). A mutant NCad^{DN} (NCad^{DN}AAA) that binds α-catenin and β-catenin but not p120Ctn (ref. 28) did not inhibit neuron movement, consistent with the idea that p120Ctn regulates cadherin traffic[22], which suggests that α- and β-catenin are not needed, or are not limiting, for NCad function (**Fig. 4c**).

More detailed study revealed further similarities between cells in which Rap was inhibited and cells in which NCad was inhibited. NCad^{DN}-expressing cells in the upper transition zone between the MMZ and the RMZ retained multipolar morphology (only 21.1 ± 1.1% showed bipolar morphology; **Fig. 4d**), Golgi orientation was impaired (**Fig. 4e**), and multipolar neuron migration was disoriented, although the speed was unaffected (**Fig. 4e–g**). Moreover, like Rap inhibitors, NCad^{DN} did not affect the speed of migration of bipolar neurons in the cortical plate or of bipolar neurons migrating in lattice culture (**Fig. 4g**).

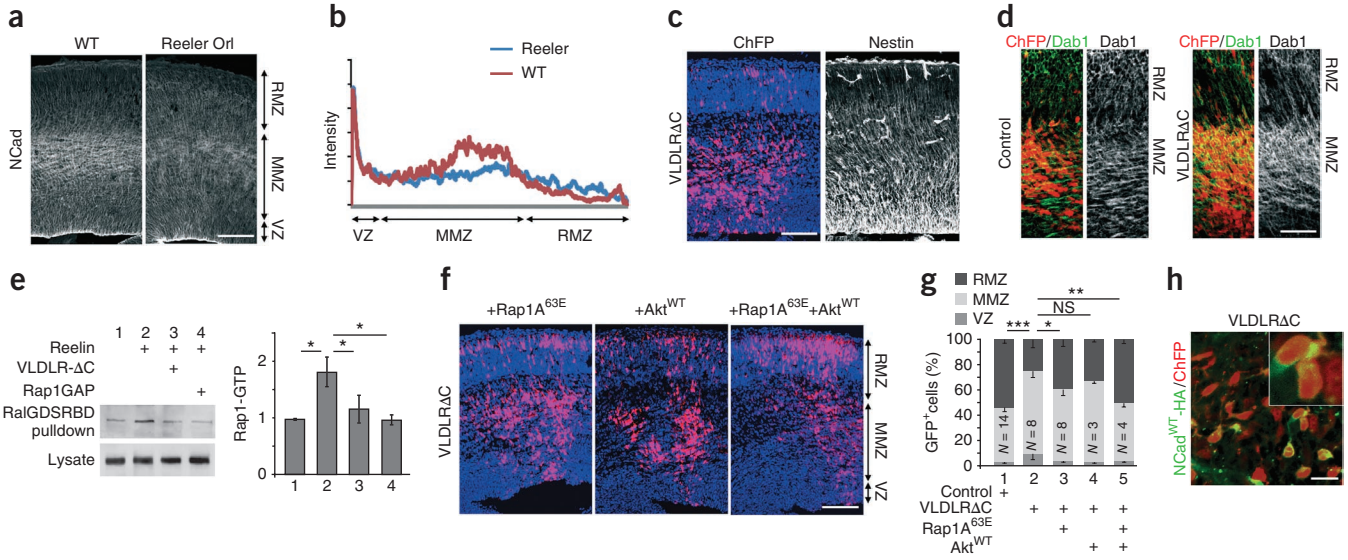

Figure 5 Reelin regulates Rap1 and entry into the RMZ. (**a**) Immunostaining for NCad in the cerebral wall of wild-type and Reeler mutant embryos at E16.5. (**b**) Quantification of the NCad staining for two brains in each genotype. (**c**) Expression of dominant-negative VLDLRΔC interferes with neuronal entry to the RMZ without affecting the morphology of radial glia. Left, red, ChFP; blue, DAPI. Right, Nestin. (**d**) Dominant-negative VLDLRΔC interferes with Reelin-dependent Dab1 degradation in the MMZ. *In utero* electroporation with ChFP and vector or VLDLRΔC plasmids, stained for ChFP (red) and Dab1 protein (green). (**e**) Rap1-GTP loading. Neurons were electroporated with HA-Rap1A^{WT} and GFP-RalGDSRBD along with Rap1GAP or VLDLRΔC. Five days after electroporation, neurons were treated with control (lane 1) or Reelin-containing supernatants (lanes 2–4) for 20 min before lysis. Lysates were analyzed directly or after pull-down with GFP-RalGDS-RBD to purify Rap1-GTP. Samples were immunoblotted with HA antibodies. The graph shows the Rap1-GTP levels from two experiments (mean ± s.e.m.). Similar results were obtained in two additional experiments with stimulation 3 d after electroporation. (**f**) Rap1 and Akt partly rescue inhibition by VLDLRΔC. (**g**) Percentage of electroporated cells in each region (mean ± s.e.m.). (**h**) HA antibody staining (green) of MMZ neurons that were electroporated *in utero* with HA-tagged NCad, ChFP and VLDLRΔC. Scale bars, 100 μm (**a**,**c**,**f**), 50 μm (**d**) and 20 μm (**h**). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant.

Full-length NCad^{WT} partly rescued the migration of Rap-inhibited neurons (**Fig. 4h**,**i**). Together, these results suggest that Rap1 and NCad are on the same signaling pathway, with Rap1 regulating cell-surface localization of NCad. Immunofluorescence microscopy showed that, like HA-Rap1A^{63E} (**Supplementary Fig. 7a**), NCad^{WT}-HA is present on the surface and in a perinuclear compartment (**Supplementary Fig. 7b**). We therefore suggest that Rap1 might regulate the presence of NCad on the cell surface, and NCad might be responsible for orienting the migration of multipolar cells out of the MMZ.

Reelin receptors regulate Rap1 and N-cadherin

The secreted signaling protein Reelin stimulates Rap1 in cultured neurons[29]. Reelin is secreted by Cajal-Retzius neurons above the top of the cortical plate, but Reelin cleavage products diffuse to the bottom of the cortical plate[30]. Furthermore, there is some evidence that Reelin affects neurons in the intermediate zone. First, migrating neurons downregulate their Reelin receptors when they reach the upper intermediate zone[31]. Second, mutations in the Reelin-response pathway inhibit exit from the intermediate zone[32,33]. Moreover, we found that NCad protein levels were lower in the MMZ of Reelin mutants than wild-type controls (**Fig. 5a**,**b**). We therefore tested whether Reelin was required for Rap1 activation at the time when multipolar neurons commence radial migration.

We prepared a dominant-negative mutant of one of the Reelin receptors, very low density lipoprotein receptor (VLDLR), by deleting part of its cytoplasmic domain. We reasoned that this construct—VLDLRΔC—would compete with endogenous Reelin receptors for Reelin but would not relay the intracellular signal. When VLDLRΔC was expressed from the CAG promoter by *in utero* electroporation, there was no detectable effect on Nestin$^+$ radial glia, but neurons expressing VLDLRΔC were delayed in the MMZ (**Fig. 5c**). We tested whether VLDLRΔC was inhibiting the Reelin response by assaying the level of the intracellular Reelin signal mediator Dab1. Reelin stimulates the degradation of Dab1 by the ubiquitin-proteasome system, and Dab1 levels are decreased when Reelin is present[34,35]. Immunofluorescent staining showed that multipolar neurons expressing VLDLRΔC contained more Dab1 protein than corresponding control neurons, consistent with a reduced Reelin response *in vivo* (**Fig. 5d**). We also tested whether VLDLRΔC inhibited Reelin-induced activation of Rap1 by using a pull-down assay in dissociated neurons. VLDLRΔC inhibited Reelin-stimulated activation of Rap1, similarly to Rap1GAP (**Fig. 5e**).

To test the relationship between Reelin, Rap1 and NCad in MMZ neurons, we first tested whether we could rescue MMZ neurons with blocked Reelin receptors by expressing active Rap1A^{63E}. Indeed, Rap1A^{63E} partly rescued radial migration by Reelin-inhibited cells (**Fig. 5f**,**g**). However, we achieved almost complete rescue by co-expressing Rap1A^{63E} with the protein kinase Akt (**Fig. 5f**,**g**), which is also involved in Reelin signaling[36,37]. Akt alone had little effect when used at this level (**Fig. 5f**,**g**) even though overexpression of Akt^{WT} could increase Akt signaling (**Supplementary Fig. 10**). These results suggest that Reelin is important for regulating Rap1 and Akt in MMZ neurons. Second, we tested whether Reelin was involved in upregulation of NCad on the cell surface. Membrane levels of NCad^{WT}-HA were decreased in VLDLRΔC-expressing cells in the multipolar zone (**Fig. 5h**). Reeler mutant neurons form distinctive aggregates in culture[38], and changes in neuron-neuron interactions have been proposed to underlie the altered development of the Reeler mutant neocortex[39]. Our results suggest that altered Rap1 and NCad levels may underlie these properties of Reeler mutant cortex.

DISCUSSION

Our results are consistent with a multi-step model that regulates the radial orientation of multipolar neurons. First, randomly moving multipolar neurons near the middle of the intermediate zone encounter Reelin, and perhaps other signals that activate Rap1 GTPases (step 1; **Supplementary Fig. 11**). The active Rap1 then increases the level of NCad on the surface (step 2). It is not clear exactly how Rap1 regulates NCad, but the cytoskeleton and vesicle traffic may be involved because Rho- and Ral-family GTPases have a role. We suspect that Rap1 may regulate NCad endocytosis, exocytosis or stability on the membrane, although other mechanisms cannot be excluded. Surface NCad then allows cells to sense environmental signals that orient the neuron toward the cortical plate, and the multipolar cells migrate radially into the upper intermediate zone (step 3). The nature of the signaling mediated by cell surface NCad is unclear. Finally, after the cells change to bipolar morphology and begin to travel along radial glia, they become relatively independent of Rap1 and NCad for movement. Other molecules, such as connexin-43, may take over to provide adhesive forces for glial-guided locomotion[40].

When our work was nearly complete, a study implicated NCad and Rab-regulated vesicle trafficking in neuronal migration[41]. Like us, the authors found that NCad was required for neuron migration, but did not test whether NCad could overcome a block in migration caused by inhibiting recycling. Instead, they found that partial knockdown of NCad would rescue neurons in which endocytosis of NCad was blocked by knockdown of Rab5. This suggests that excess surface NCad inhibits migration. We agree, in that high level overexpression of NCad blocks neurons in the multipolar zone. However, we also found that expression of NCad at lower levels, too low to cause a phenotype alone, could rescue Rap1-inhibited neurons. Our results suggest a chain of causality, with Rap1 increasing surface NCad on multipolar cells so that the cells can sense directional signals in the environment. We also identified Reelin as an extracellular signal that regulates NCad surface localization.

We used Rap1GAP, NCad^{DN} and VLDLRΔC to interfere with Rap1, NCad and Reelin receptors, respectively. This allowed us to manipulate signaling in postmitotic neurons specifically and to regulate groups of paralogs simultaneously, but raises concerns about specificity. The following results support the specificity of the reagents. First, for Rap1, we observed the same phenotypes whether we inhibited Rap1 with Rap1GAP or Rap1^{17N}, or knocked down Rap1A and Rap1B with shRNA. Moreover, Rap1A^{63E} rescued the migration block caused by Rap1GAP, Rap1^{17N} or Reelin inhibition but not the block by Lis1 shRNA or Rac1 inhibition. Next, for NCad, another group found that NCad shRNA caused migration arrest in the intermediate zone[41], resembling the arrest we detected using NCad^{DN}. Moreover, wild-type NCad rescued neurons in which Rap1 signaling was inhibited. Finally, for Reelin signaling, recent studies support our finding that neurons are delayed in the intermediate zone when Reelin signaling is absent[32,33].

The proposed role for NCad in orienting multipolar cells is reminiscent of the role of ECad in setting up apical-basolateral polarity during the mesenchymal-epithelial transition[10]. When migrating mesenchymal cells first touch, ECad forms *trans*-dimers and generates signals that recruit the actin cytoskeleton and focus active ECad into the junctional region[10]. *Trans*-interacting ECad, or other cell surface receptors such as nectins, locally activate Rap1 and create a positive feedback loop that routes more ECad to the junctions, stabilizes the junctions and interacts with other receptors and signaling molecules that maintain cell polarity. By analogy, we speculate that NCad on multipolar cells may sense directional cues in the environment by forming *trans*-dimers with NCad or other cadherins expressed on radially oriented processes from other neurons or radial glia. However, it is also possible that NCad may interact with other cell-surface receptors that respond to directional signals from the cortical plate.

A positive role for NCad in migration is unexpected. In general, cadherins are downregulated before cell migration[13,42]. Indeed, NCad is downregulated when neurons leave the ventricular zone[43]. However, there are a few examples in which cadherins are required for cell migration. In some cases, cadherins may provide traction forces between moving and stationary cells, and in other cases they connect groups of cells undergoing collective migration[20,21,44]. During the group migration of *Drosophila* border cells, DECad is required on the border cells as well as the cells they migrate through, and DECad is needed for the first cell of the group to extend a leading process along a growth factor gradient[45]. Exactly how this happens is unclear. However, it is possible that surface NCad on multipolar neurons allows them to sense a chemoattractant. It will be interesting to dissect downstream signaling mechanisms from NCad that orient multipolar cell migration in the mammalian neocortex.

While this paper was under review, Reelin, Rap1 and cadherins were reported to regulate the somal translocation of early-born cortical neurons[46]. Apparently, cadherins stabilize the leading process in the marginal zone. With our results, this indicates that Reelin, Rap1 and cadherins are likely to mediate two key events during cortical development: glia-independent somal translocation of early-born neurons and radial migration of multipolar late-born neurons.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.J. performed the experiments. Y.J. and J.A.C. conceived the experiments and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Experiments were approved by the Hutchinson Center Institutional Animal Care and Use Committee and performed using CD1 mice from Charles-River Laboratories or Reeler (*Reln*$^{rl-Orl}$) mice that had been backcrossed into the Balb/c genetic background.

Vector construction. Plasmids containing the coding sequences for Rap1GAP, Rap1A^{17N}, Rap1A^{63E}, VAV2DPC, Rac1^{61L}, Cdc42^{61L}, NWASP-RBD-GFP, POSH-RBD-GFP (W.T. Arthur, Fred Hutchinson Cancer Research Center); RalA^{72L}, RalB^{72L} (Addgene); Sec5-RBD-GFP (M. White, University of Texas Southwestern Medical Center); and NCad (V. Vasioukhin, Fred Hutchinson Cancer Research Center) were used as templates to insert the sequences into pCAGIG (K. Sanada, Harvard Medical School) or pNeuroD (F. Polleux, Scripps Research Institute). shRNA for Lis1 (L.-H. Tsai, Massachusetts Institute of Technology) was expressed from the H1 promoter in pCAGGFP. shRNA sequences targeting Rap1A[47] and Rap1B[9] sequences were introduced into the pSUPERretro vector (Oligoengine). The RNAi construct for Rap1B pSHAG-Rap1B was provided by A. Puschel (University of Münster)[9]. RalA and RalB shRNA expression vectors were purchased from Open Biosystems. pCAGGFP (Addgene) and pCAGCherryFP were used for co-electroporation. NCadDN contains the transmembrane and intracellular domains (deletion of residues 99–708). A soluble form containing the intracellular domain alone gave similar results (residues 747–906). The AAA mutation was inserted by PCR replacing the GGTGGAGGA sequence at residues 777–779 with GCGGCCGCA. HA tags were at the C terminus of NCad and the N terminus of Rap1A and Rap1B. Wild-type pCAG-Akt1 was provided by Y. Gotoh (University of Tokyo). Mouse VLDLRΔC, lacking part of the cytoplasmic domain including the Dab1 binding site by deletion of residues 828–873, was cloned by PCR into pCAG-IRES-ChFP.

Antibodies. For immunofluorescence, we used rabbit anti-Cux1 (Santa Cruz Biotechnology), rabbit anti-Tbr2 (R. Hevner, Seattle Children's Hospital Research Institute), mouse anti-Nestin (Millipore), cleaved caspase-3 (Cell Signaling), Pax6 (Developmental Studies Hybridoma Bank), mouse anti-HA (Covance), Ki67 (Novacastra), GM130 (BD Biosciences), αN-catenin (Developmental Studies Hybridoma Bank), rabbit anti-MAP2 (Chemicon), mouse anti NCad (Invitrogen) and donkey secondary antibodies labeled with Alexa 488, 568, and 647 (Invitrogen).

***In utero* microinjection and electroporation.** *In utero* microinjection and electroporation was performed at E14.5 essentially as described[48], using timed pregnant CD-1 mice (Charles River Laboratories). Needles for injection were pulled from Wiretrol II glass capillaries (Drummond Scientific) and calibrated for 1-μl injections. DNA solutions were mixed in 10 mM Tris, pH 8.0, with 0.01% Fast Green. Forceps-type electrodes (Nepagene) with 5 mm pads were used for electroporation (five 50-ms pulses of 25 V at E12.5 or five 50-ms pulses of 40 V at E14.5).

Histology and cytology. Embryos were collected at E17.5 or E19.5. Brains were dissected and successful electroporations identified by epifluorescence microscopy. Positive brains were fixed in a 3.7% paraformaldehyde (wt/vol) in phosphate-buffered saline (PBS) and cryoprotected in a 30% sucrose PBS solution (wt/vol) overnight at 4 °C. Brains were frozen in optimal cutting temperature compound (OCT) before 14-μm-thick brain cross-sections were obtained with a cryostat and placed on slides. For selected antibodies, sections were antigen-retrieved by immersion of the slides in 0.01 M sodium citrate buffer, pH 6.0 at 95 °C for 20 min. Dissociated neurons were fixed for 20 min in 3.7% paraformaldehyde PBS solution. Sections and fixed neurons were permeabilized for 30 min in PBS 0.4% Triton X-100 (wt/vol), then blocked for 2 h with 5% normal goat serum (vol/vol) and 0.4% Triton X-100 in PBS at 25 °C. Primary antibodies were incubated overnight at 4 °C. Slides were washed four times for 10 min with 0.4% Triton X-100 in PBS. Secondary antibodies were added for 2 h at 25 °C. Slides were washed as before and coverslipped with 0.1 M Tris pH 8.5, 25% glycerol (vol/vol), 10% Mowiol 4-88 (vol/vol, Calbiochem) including 0.1% diazobicyclo-octane (wt/vol, Sigma) as anti-fade reagent. Most images were obtained with a 20× objective and were captured using Zeiss LSM 510META confocal microscope with Zeiss LSM Image Browser. Images were assembled in Adobe Photoshop. Brightness and contrast were adjusted equally on figures.

Organotypic slice culture and time-lapse confocal microscopy. Embryonic brains were electroporated at E14.5 and 300-μm embryonic brain slices were prepared at E16.5 using a vibratome (World Precision Instruments) as described[3,49]. Time-lapse confocal microscopy was performed using an Achroplan × 20/0.50 with a Zeiss LSM 5 Pascal confocal on an Axioskop2 upright microscope. Slices were embedded in a drop of 3% agarose and cultured in a chamber on a heated stage (Warner Instruments) in DMEM-F12 (Invitrogen) supplemented with B27 (Invitrogen) and 10% serum. The medium was preheated at 37 °C and equilibrated with 95% O_2 and 5% CO_2. The medium was flowed into the chamber at ~5 ml h^{-1}. Repetitive acquisitions were performed every 30 min for up to 20 h in laterodorsal regions of the cortex in which 25 successive *z* optical planes spanning 120 μm were acquired. *Z*-stacks were selected and combined in Zeiss LSM Image Browser. Slight drifts of the slices were corrected using the ImageJ registration tool Turboreg (P. Thévenaz, Biomedical Imaging Group, Swiss Federal Institute of Technology, Lausanne, Switzerland). Average velocity of migrating cells was obtained using Imaris (Bitplane). Histograms were compiled using Microsoft Excel.

Lattice culture. Dorsal neocortices were dissected and cortical cultures were prepared at embryonic day 14.5 (E14.5) using Accutase (Sigma) dissociation. After electroporation (Amaxa) cells were plated in DMEM-F12 medium with 2% B27 (vol/vol), 1× Glutamax, 1× penicillin-streptomycin and 30 mM glucose (all reagents from Invitrogen) on 96-well plates coated with 25 μg ml^{-1} poly-D-lysine (Sigma). Cultures were maintained in a 37 °C, 5% CO_2 incubator. After 1 day *in vitro*, lattice cultures were induced with Invitrogen 293 SFM (20%, vol/vol) as described[15]. Movies were acquired on a Cellomics ArrayScan with 5 min time interval between frames.

Cortical neuron culture, *in vitro* electroporation and binding assay. Binding of neurons to N-cadherin extracellular domain-Fc (NCadECD-Fc) fusion p rotein (human) (R&D Systems #1388-NC) was done by a procedure adapted from reference 50.

N-CadECD-Fc was diluted in HBSS (Hank's balanced salt solution) + 1 mM $CaCl_2$ and aliquots of 50 μg ml^{-1} were stored at −80 °C in tubes blocked with 3% BSA (wt/vol). 96-well ELISA high binding plates (Corning #9018) were coated with 12.5 μg ml^{-1} NCadECD-Fc or 20 μg ml^{-1} poly-D-lysine, overnight at 4 °C. Wells were then blocked with 3% BSA for 2 h at 25 °C, then washed 3 times with HBSS + 1.2 mM $CaCl_2$. Neuron suspensions were prepared from embryonic day 15.5 (E15.5) mouse embryo telencephalons and electroporated using Amaxa electroporator as described by the manufacturer. Equal cell numbers (10^5 cells) from each electroporation were plated in each well of the prepared plates. Cells were cultured overnight in DMEM-F12 medium with 2% B27 and 1 × penicillin-streptomycin, counted, vigorously washed then counted again. Cells remaining on the plate were incubated for 1 more day to assess neurite outgrowth.

Statistical analysis. Statistical analysis made use of Student's *t*-test across *N* samples, where *N* is the number of embryos or cells as defined in the figure legends.

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Supplementary Information to:

Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex

Yves Jossin and Jonathan A. Cooper

This Supplement contains three Supplementary movies and eleven Supplementary figures.

Supplementary movies:

Movie 1: Movement of multipolar cells in the MMZ of control, Rap1GAP and NCad^{DN} electroporated cortex.
Cortices were electroporated at E14.5 and slices were prepared at E16.5.
One frame per 30 min. Playback speed 7 frames/s. Compressed z-stacks spanning around 90 μm of cortical depth. Height of image is approximately 100 μm.

Movie 2: **Movement of bipolar cells in the RMZ of control, Rap1GAP and NCad^{DN} electroporated cortex.**
Cortices were electroporated at E14.5 and slices were prepared at E16.5.
One frame per 30 min. Playback speed 7 frames/s. Compressed z-stacks spanning around 90 μm of cortical depth. Height of image is approximately 100 μm.

Movie 3: **Movement of control, Rap1GAP and NCad^{DN} neurons migrating in vitro in lattice culture.**
One frame per 5 min. Playback speed 7 frames/s. Superimposed phase contrast and GFP epifluorescence images.

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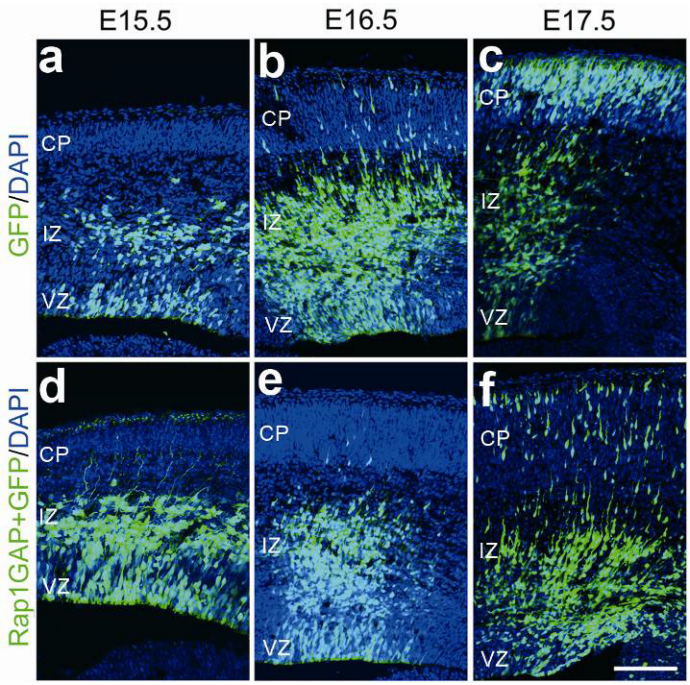

Figure S1. Rap inhibition has no obvious effect on early stages of migration.
(**a-f**) In utero electroporation at embryonic day 14.5 (E14.5) with GFP (**a-c**) or Rap1GAP and GFP (**d-f**). Positions of GFP-positive cells were observed at E15.5 (**a,d**), E16.5 (**b,e**) and E17.5 (**c,f**). CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. Blue: DAPI. Scale bar, 100 μm.

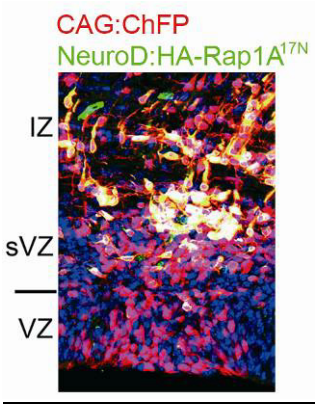

Figure S2. **NeuroD promoter is active in SVZ/IZ neurons but inactive in VZ progenitors.** Co-electroporation of CAG-ChFP with NeuroD-HA-Rap1A^{17N}. The NeuroD promoter is expressed in sVZ and IZ neurons but not VZ progenitors (green), while the CAG promoter is expressed throughout (red). IZ, intermediate zone; sVZ, sub-ventricular zone; VZ, ventricular zone. Blue: DAPI.

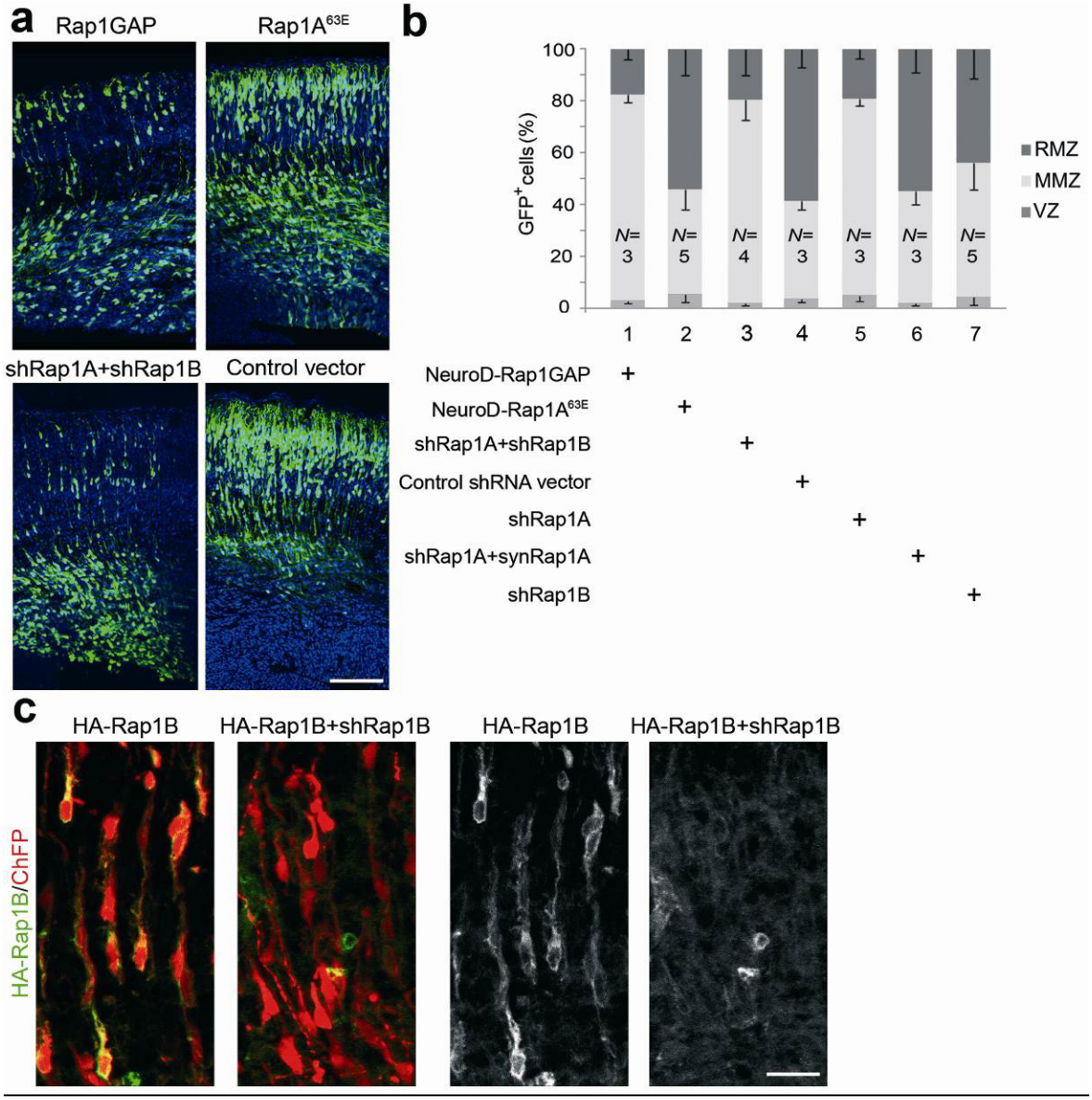

Figure S3. Effects of Rap1 inhibition in post-mitotic neurons and of shRNAs targeting Rap1A and Rap1B.

(a) In utero electroporation at E14.5 of the indicated genes under the NeuroD promoter and of shRNAs for Rap1A and Rap1B. Brains were analysed 3 days after electroporation. **(b)** Percentage of electroporated cells in each region. N is the number of individual electroporated embryo brains analyzed in a minimum of two independent electroporations. Rescue experiments for shRap1A were performed by co-electroporating a modified cDNA sequence of the Rap1A gene (synRap1A) that is not targeted by the shRNA. **(c)** Efficiency of Rap1B knock down. HA tagged Rap1B (green) was co-electroporated with CherryFP (red) and a control vector or Rap1B shRNA. Blue: DAPI. Scale bars, 100 μm in (a) and 30 μm in (c).

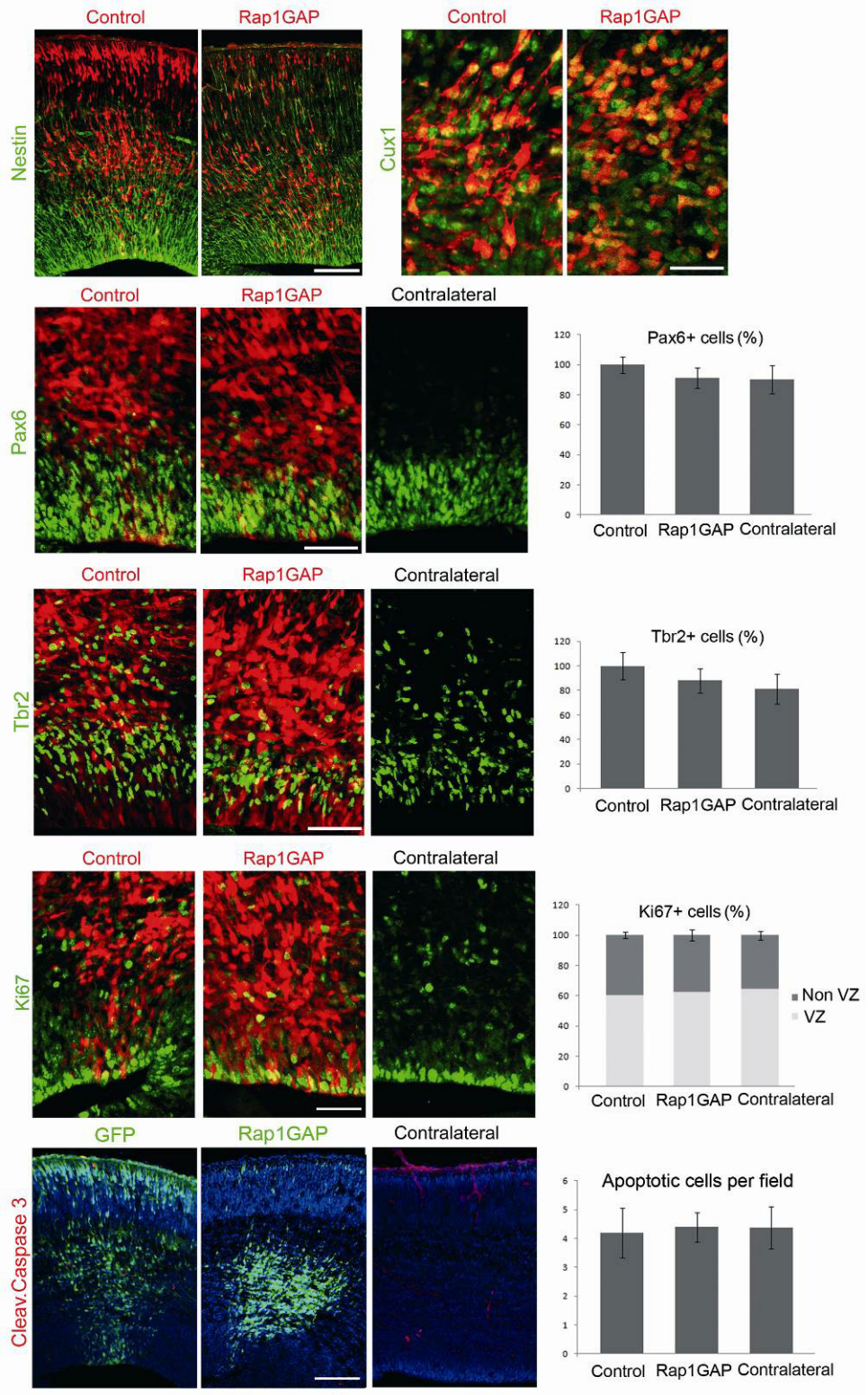

Figure S4. **Inhibition of Rap1 by in utero electroporation did not affect radial glia organization (Nestin), neuronal commitment (Cux1), apical (Pax6) or basal (Tbr2) progenitor cells, cell division (Ki67) or survival (cleaved Caspase-3).**

In utero electroporation at E14.5 with Rap1GAP and ChFP or GFP as indicated. Rap1GAP was expressed in progenitors and post-mitotic neurons under the CAG promoter. The results were quantified by counting the number of stained cells in a constant area of each section, and averaged across sections from at least 3 different embryos for each antibody. Immunostaining at E17.5 for the indicated markers Scale bars, 100 μm for Nestin and cleaved Caspase 3, 50 μm for Cux1, Pax6, Tbr2 and Ki67.

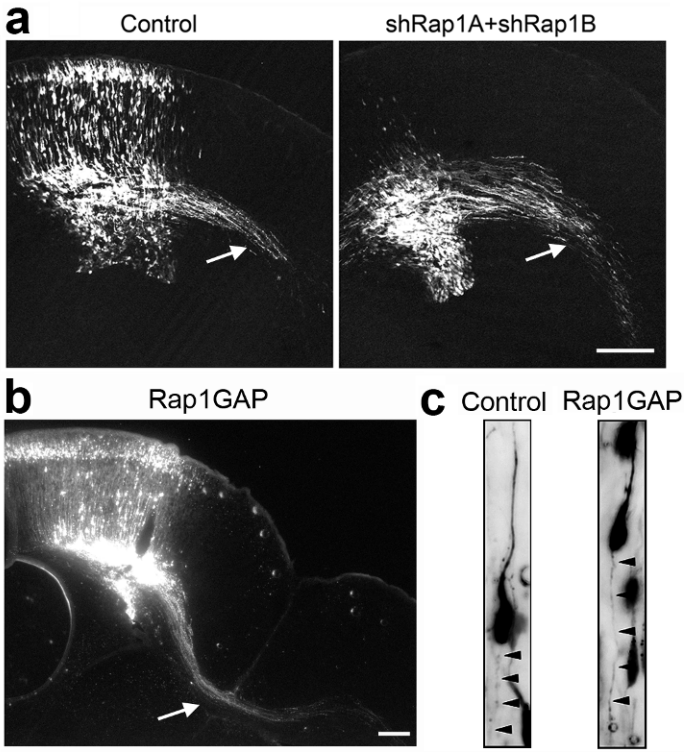

Figure S5. **Axonal growth is not inhibited when Rap is inhibited.**
Brains were electroporated at E14.5 with the indicated plasmids and collected 3 days later at E17.5 **(a)** or 5 days later at E19.5 **(b-c)**.
(a-b) Axons elongate and cross the midline. **(c)** Axons behind migrating bipolar neurons in the CP. Scale bars, 100 μm.

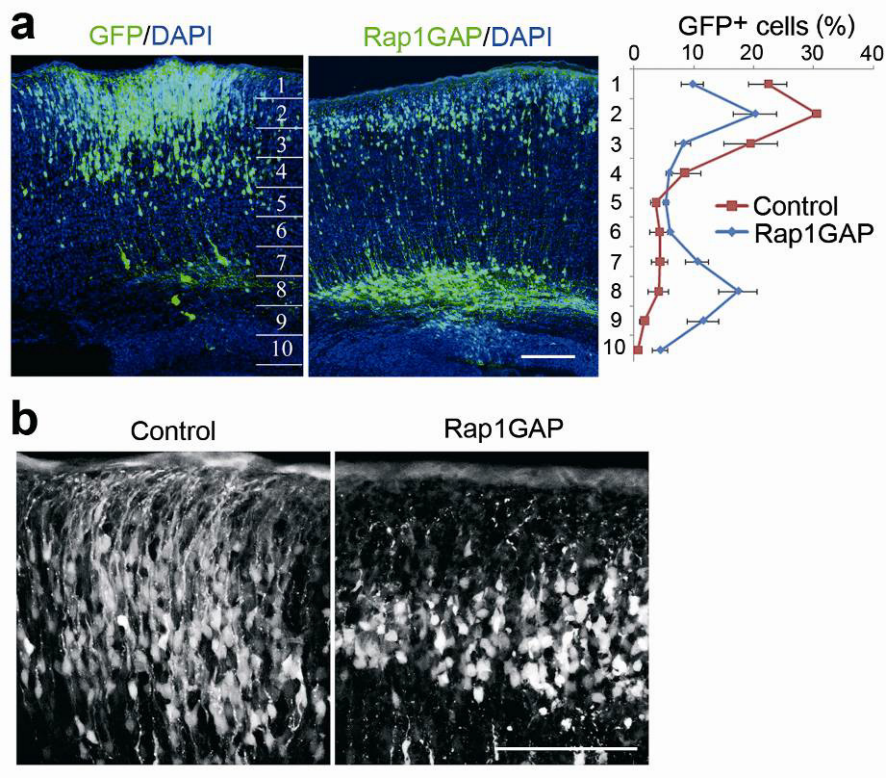

Figure S6. **Rap-inhibited cells that reach the top of the cortical plate exhibit defective apical dendrites**

(a) A population of Rap-inhibited cells reaches the top of the cortical plate at E19.5. The indicated plasmids were electroporated in utero at E14.5, and the distribution of GFP-positive cells examined at E19.5. The cerebral wall was divided into 10 equally sized bins. Percentage of electroporated cells in each bin (mean ± s.e.m., $N > 3$ brains).

(b) Rap1GAP inhibits apical dendrite formation. In utero electroporation at E14.5 with vector or Rap1GAP and GFP. GFP-expressing cells were observed at E19.5. Arrows indicate apical dendrites in the marginal zone of control but not Rap-inhibited cortex.

Scale bar, 100 μm in (a) and 50 μm in (b).

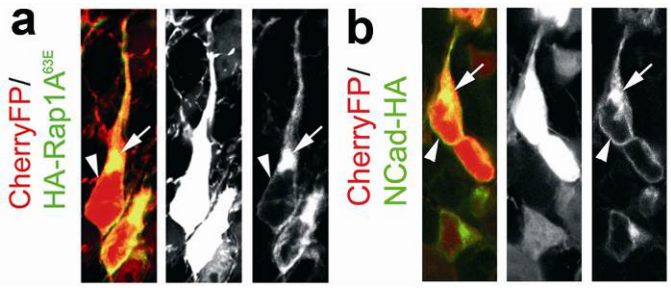

Figure S7. **Localization of Rap1A and NCad in bipolar neurons**.
In utero electroporation of ChFP and HA-Rap1A^{63E} **(a)** or HA-NCad **(b)**, followed by immunofluorescence with anti-HA antibodies (green) and visualization of ChFP (red). Note that both HA-Rap1A^{63E} and NCad-HA are present at the plasma membrane (arrowheads) and in front of the nucleus (arrows) of bipolar neurons in the lower RMZ.

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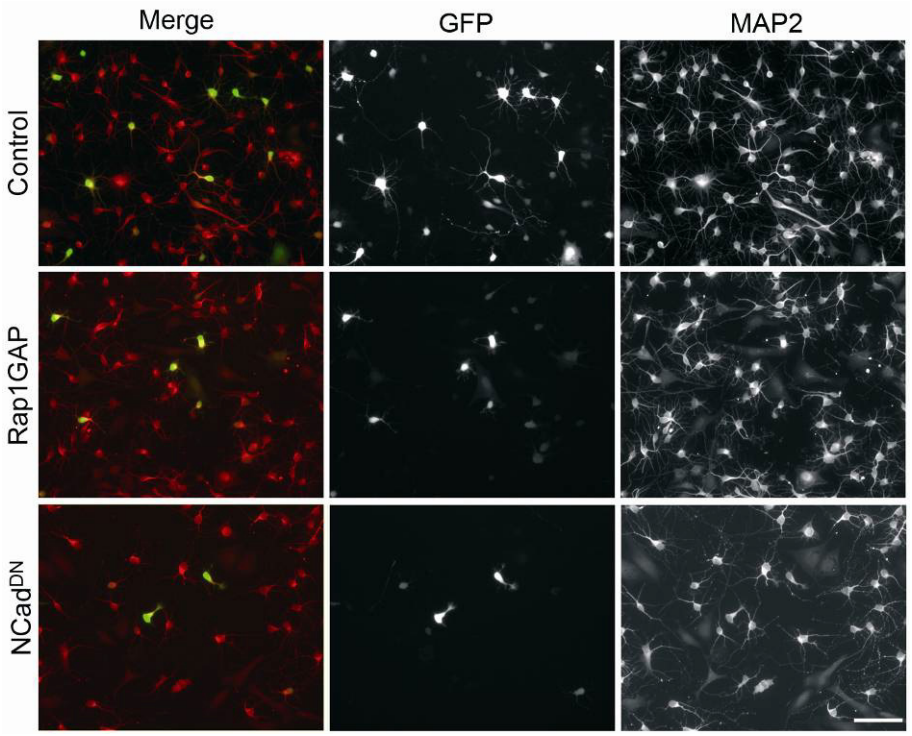

Figure S8. **GFP-expressing cells that bind to NCad-Fc in vitro are MAP2-positive neurons.** Dissociated E15.5 cortical neurons were electroporated with GFP and vector, Rap1GAP or $NCad^{DN}$ and incubated on dishes coated with NCad-Fc. After washing, the attached cells were immunolabeled for MAP2. Note co-expression of GFP and MAP2. Scale bar, 30 μm.

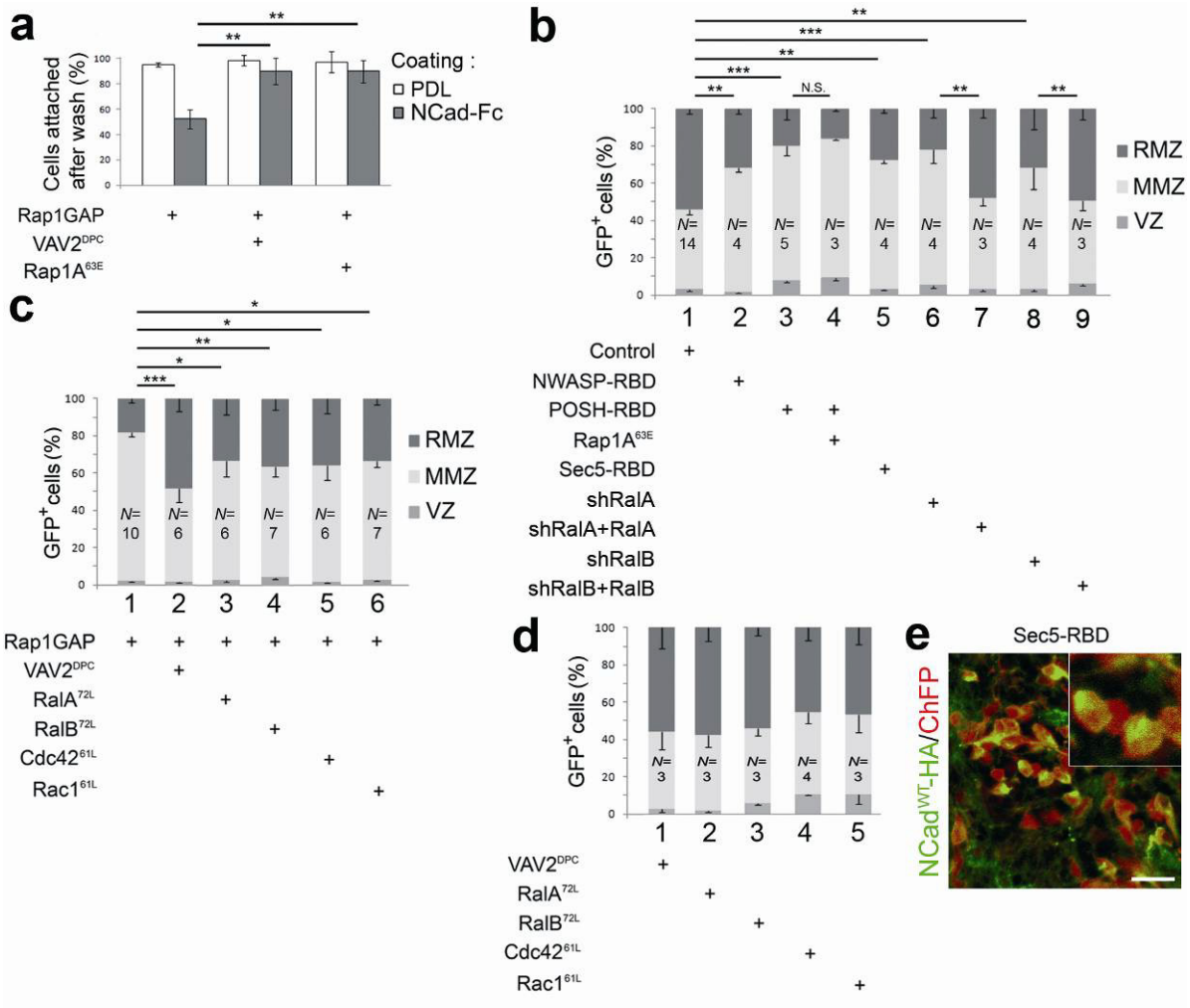

Figure S9. RalA/B, Rac1 and Cdc42 mediate Rap functions in neuron migration.
(a) E15.5 dissociated cortical neurons were electroporated with the indicated plasmids and incubated overnight on dishes coated with NCad-Fc or poly-D-lysine (PDL). GFP-expressing cells were counted before and after washes to determine the percentage of attached cells. **(b-d)** Indicated plasmids were electroporated in utero at E14.5 and the percentage of electroporated cells in each region scored at E17.5 (mean ± s.e.m.). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. N is the number of individual electroporated embryo brains analyzed in a minimum of two independent electroporations **(e)** HA antibody staining (green) of MMZ neurons that had been electroporated in utero with HA-tagged NCad, ChFP and Sec5RBD. Scale bar, 20 μm.

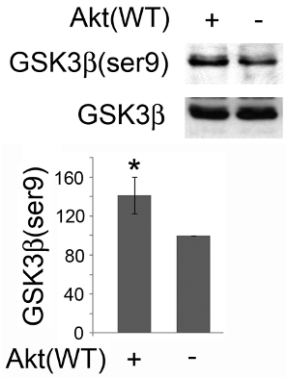

Figure S10. **Over-expression of Akt^{WT} increases Akt signalling.**
293T cells were transfected with a plasmid expressing Akt^{WT} under the CAG promoter, as used for in utero electroporations. Cells lysates were processed for Western blotting using antibodies for phosphorylated GSK3β(Ser 9) and GSK3β. The graph shows that over-expression of Akt^{WT} significantly increases the phosphorylation of GSK3β (mean $\pm$ s.e.m.). *, $P < 0.05$.

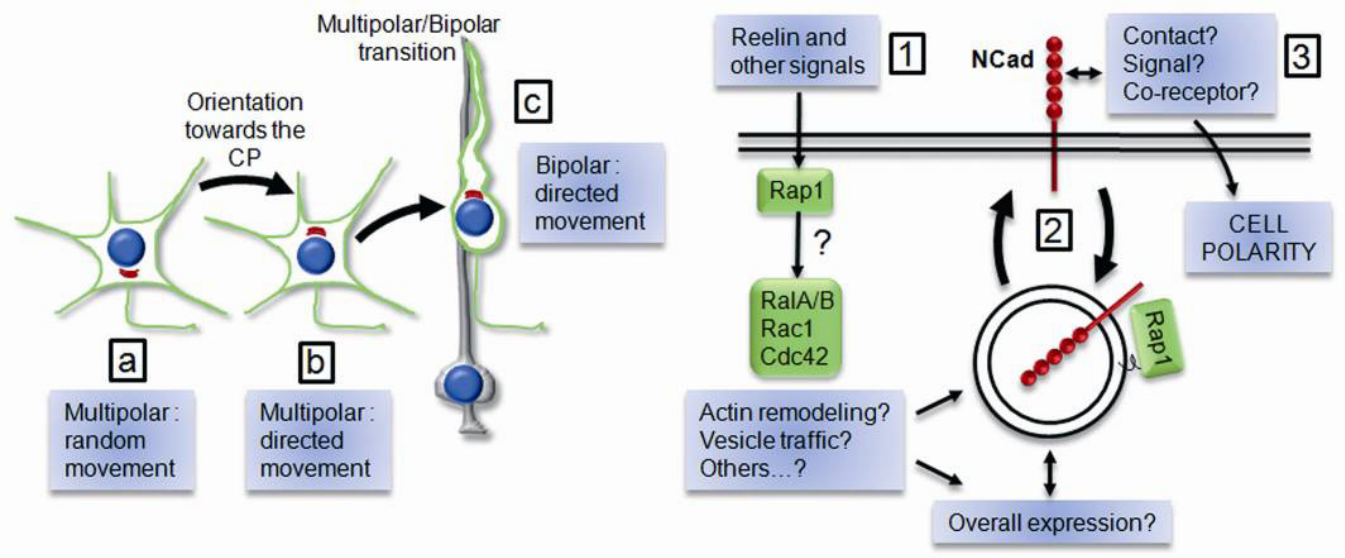

Figure S11. Model for orienting multipolar neurons.

(Left) Cellular events. (a) Most of the multipolar neurons in the MMZ (sVZ and lower IZ) move randomly. **(b)** When they encounter Reelin (and possibly other signals), they orient their Golgi apparatus and their direction of migration towards the cortical plate. **(c)** Later, they transition to bipolar morphology and commence locomotion along radial glia.
(Right) Molecular events. (1) Reelin (and possibly other signals) activate Rap1 and its target GTPases RalA/B, Rac1 and Cdc42. (2) NCad levels increase on the surface and decrease internally, likely due to changes in membrane traffic. (3) NCad on the cell surface helps sense directional cues in the environment that orient the Golgi apparatus and the direction of migration towards the cortical plate. Subsequent locomotion along radial glia appears to be largely independent of Rap and NCad. See text for discussion.