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Cdc42 and Gsk3 modulate the dynamics of radial glial growth, inter-radial glial interactions and polarity in the developing cerebral cortex

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SUMMARY

Polarized radial glia are crucial to the formation of the cerebral cortex. They serve as neural progenitors and as guides for neuronal placement in the developing cerebral cortex. The maintenance of polarized morphology is essential for radial glial functions, but the extent to which the polarized radial glial scaffold is static or dynamic during corticogenesis remains an open question. The developmental dynamics of radial glial morphology, inter-radial glial interactions during corticogenesis, and the role of the cell polarity complexes in these activities remain undefined. Here, using real-time imaging of cohorts of mouse radial glia cells, we show that the radial glial scaffold, upon which the cortex is constructed, is highly dynamic. Radial glial cells within the scaffold constantly interact with one another. These interactions are mediated by growth cone-like endfeet and filopodia-like protrusions. Polarized expression of the cell polarity regulator Cdc42 in radial glia regulates glial endfeet activities and inter-radial glial interactions. Furthermore, appropriate regulation of Gsk3 activity is required to maintain the overall polarity of the radial glia scaffold. These findings reveal dynamism and interactions among radial glia that appear to be crucial contributors to the formation of the cerebral cortex. Related cell polarity determinants (Cdc42, Gsk3) differentially influence radial glial activities within the evolving radial glia scaffold to coordinate the formation of cerebral cortex.

KEY WORDS: Radial glia, Cerebral cortical development, Cdc42, Gsk3 (GSK-3), Mouse, Schizophrenia, Neurodevelopmental disorders

INTRODUCTION

Polarized radial glial cells provide a template for the generation and migration of neurons that eventually form the different cortical layers (Rakic, 2003). During early stages of cortical development, radial progenitors divide symmetrically to expand the pool of radial glia. Later, asymmetric divisions of radial glia give rise to pairs of neuron, radial glial cell or a subventricular zone (SVZ) intermediate precursor (Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2004; Noctor et al., 2008; Anthony et al., 2004; Malatesta et al., 2000). The daughter neurons may retain the radial fiber and somally translocate or use the radial glial scaffold as a migrational guide (Miyata et al., 2001; Noctor et al., 2001; Ayala et al., 2007; Mason et al., 1988; Marin and Rubenstein, 2003; Rakic, 2003). Additionally, radial glia can modulate the radial migratory patterns of interneurons invading the dorsal cerebral wall from the ganglionic eminence (Yokota et al., 2007; Poluch and Juliano, 2007). Once neurons migrate into the cortical plate, anti-adhesive radial glial surface cues and basal radial glial endfeet-pial membrane interactions are thought to contribute to the final placement of neurons in the cortex (Beggs et al., 2003; Halfter et al., 2002; Haubst et al., 2006; Graus-Porta et al., 2001; Gongidi et al., 2004). As neuronal migration dwindles and neurons settle in their respective laminar positions, the radial glia differentiate into astrocytes and ependymal cells (Schmechel and Rakic, 1979a; Schmechel and Rakic, 1979b; Spasskey et al., 2005; Culican et al., 1990; Voight, 1989).

A defining feature of radial glial cells as they undergo various stages of differentiation in the developing cerebral cortex is their polarity (see Fig. S1 in the supplementary material). Radial glial cell polarity is evidenced by: (1) the soma situated at the ventricular zone (VZ) and an elongated basal process that extends the width of the cortical wall; (2) the selective orientation and expression of microtubules and microtubule-associated proteins, respectively, in their radial processes; (3) the deployment of migration-modulating cell surface molecules (e.g. astrotactin, Sparcl1) to distinct locales along the radial glial processes to facilitate distinct phases of glial-guided neuronal migration; (4) the apical localization of signaling cues such as β-catenin, β-integrin, N-cadherin, Par3, Cdc42 or Numb/Numbl to regulate apical adhesion/proliferation of radial glia; and (5) the targeting of adhesion receptors such as GPR56 to the endfeet to facilitate basal end adhesive interactions with pial basement membrane (Ayala et al., 2007; Rakic, 1972; Rakic, 2003; Bultje et al., 2009; Cappello et al., 2006; Li et al., 2008; Rasin et al., 2007; Gongidi et al., 2004; Zheng et al., 1996; Loulier et al., 2009; Zhang et al., 2010). Apicobasal expression of adenomatous polyposis coli (Apc) also promotes radial glial characteristics (Yokota et al., 2009). The function of radial glia during corticogenesis depends on the generation and dynamic modulation of this morphological, cytoskeletal and molecular polarity (Gaiano et al., 2000; Patten et al., 2003; Schmid et al., 2003; Hunter and Hatten, 1995). In spite of its significance, the dynamics of the polarized radial glial scaffold, the nature of radial glial cell-cell interactions, the molecular signals controlling distinct aspects of radial glial polarity and the contributions of these activities to corticogenesis are poorly defined.

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Here, we demonstrate that radial glia provide a surprisingly dynamic scaffold for the generation and guidance of neurons during cortical development. Live imaging of large cohorts of radial glial cells, using single- and multi-photon laser-scanning microscopy, indicates that radial glial cells within the scaffold actively extend, retract or interact with each other. Radial glia actively probe each other along their entire length using transient, filopodia-like protrusions. Surprisingly, leading edges or endfeet of radial processes, oriented towards the pial surface, are characterized by robust growth cone-like extension or retraction, rather than by stably attached endfeet as previously thought. These radial glial leading edges can be categorized as either club-like or branched and the ratio of these different types of radial glial growth cones changes as the cerebral cortex expands. Distinct members of related cell polarity pathways [i.e. Cdc42 and Gsk3 (GSK-3)] differentially modulate these activities. Live imaging of Cdc42 activity, dominant-negative inhibition of Cdc42 and conditional *Cdc42* gene inactivation indicate that polarized expression of Cdc42 in radial glia regulates glial endfeet activities and inter-radial glial interactions. Furthermore, pharmacological inhibition, shRNA-mediated knockdown, dominant-negative disruption and conditional deletion of *Gsk3a* and *Gsk3b* indicate that appropriate regulation of Gsk3 activity is required to maintain the overall polarity of the radial glia scaffold. Inactivation of both Cdc42 and Gsk3 in radial glia disrupted the migration and final placement of cortical neurons. We propose that dynamic interactions between neighboring radial glial cells may coordinate the neuronal generation and migration that underlie the radial columnar organization of the cerebral cortex.

MATERIALS AND METHODS

Analysis of radial glia in slice preparations of embryonic brains

Lateral ventricles of E14-16 mouse embryos were injected with 2.5 μl of a plasmid mixture containing 3 μg/μl DNA (pBLBP-EGFP, pBLBP-DsRed2, pBLBP-Cdc42-EGFP, pBLBP-DN-Cdc42-GFP, pBLBP-Cdc42A-EGFP or pCdc42-EGFP) diluted 1:1 with Mouse Neuron Nucleofector Solution (Amaxa Biosystems)/0.001% Fast Green, using a Parker Hannifin Picospritzer II. Immediately after injection, heads were subjected to electroporation (BTX/Genetronics) under the following conditions: LV mode, 70 Volts, 100 mseconds pulse length, 100 mseconds pulse interval, eight pulses, unipolar (polarity). Following electroporation, cortices were removed from the embryos, embedded in 3% low-melting-point agarose in complete Hanks Balanced Salt Solution and coronally sectioned (150 μm) in a vibratome (Leica VT 1000S). Sections were mounted on Nucleopore membrane filters, placed in glass-bottom Mat-Tek dishes, and cultured in MEM/10% fetal bovine serum (FBS) at 37°C and 5% CO_2. GFP-labeled radial glia spanning the cerebral wall in the mediodorsal region of the cerebral cortex were repeatedly imaged using a Zeiss inverted microscope (attached to a Pascal confocal laser-scanning system and a live cell incubation chamber) or a Zeiss LSM 510 multi-photon microscope for 1-24 hours. Slices were then fixed with 4% paraformaldehyde and immunolabeled with RC2 and anti-GFP antibodies as described previously (Yokota et al., 2007; Yokota et al., 2009). To some cultures, 10 μM BrdU was added 12 hours prior to fixation and immunolabeling with anti-BrdU and anti-GFP antibodies. Actively proliferating radial progenitors (GFP^+ $BrdU^+$) were counted from these slices.

GFP^+ radial glia endfeet types and radial glial morphology (i.e. full length, spanning the width of cortex and shorter) were measured in electroporated slices. Time-lapse movies were used to measure the rates of extension or retraction of radial glia tips. The number of contacts between 100 μm-long segments of adjacent radial fibers, separated by less than 25 μm in distance, was measured per hour and used as the cell-cell contact index. All quantifications of radial fiber and cellular dynamics were performed using the Zeiss Pascal or LSM510 software.

The background-normalized fluorescence intensities of Cdc42-GFP in a 25 μm^2 area of the apical and basal poles of apically or basally moving progenitors were measured using the Zeiss LSM image browser and Image J and used to obtain the ratio of basal to apical Cdc42-GFP fluorescence intensity.

Antibodies, immunohistochemistry and immunoblot analysis

The following primary antibodies were used for immunolabeling or immunoblotting: RC2 (Developmental Studies Hybridoma Bank, University of Iowa), anti-actin (Sigma), anti-Cdc42 (Santa Cruz Biotechnology; Cytoskeleton), anti-Gsk3β (Transduction Labs) and anti-phospho-Gsk3β (Biosource International; Cell Signaling Technology). Immunoreactivity was detected with Cy2- or Cy3-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch). Immunoprecipitation and immunoblotting of whole-cell extracts of embryonic cortices were performed as described (Yokota et al., 2007; Yokota et al., 2009). Immunoblot films were scanned and densitometric analysis was performed using Image J. Intensity values were normalized to loading control (actin) levels.

Mice

Mice were cared for according to animal protocols approved by the University of North Carolina. Nervous system-specific conditional knockout *Cdc42* mice (*Cdc42*$^{lox/lox}$*;hGFAP-Cre*) were generated by mating mice carrying a *Cdc42* allele flanked by loxP sites (Chen et al., 2006) with *hGFAP-Cre* (Zhuo et al., 2001) mice. Littermate *Cdc42*$^{lox/+}$*;hGFAP-Cre* mice served as controls. In some experiments, E16 embryos were electroporated with pBLBP-GFP and Gsk3β-S9A expression vectors (Hetman et al., 2000) as described above and radial progenitors in the VZ of control and *Cdc42* conditional knockout embryos were analyzed after 36 hours. The generation of *Gsk3b* floxed alleles and of *Gsk3a*$^{-/-}$*;Gsk3b*$^{lox/lox}$*;Nestin-Cre* mice have been described (Kim et al., 2009).

ShRNA-mediated inhibition of Gsk3 and other modulators of Gsk3 activity

The *Gsk3* target sequence oligos, mutated target sequence oligos, and dominant-negative Gsk3 constructs have been described (Kim et al., 2006). *Gsk3a* and *Gsk3b* unique target sequence (5′-GAACCGAGAGCTC-CAGATC-3′) was used to generate shGsk3. The scrambled sequence of shGsk3 was used to generate control shRNA. The target sequence oligos and scrambled target sequence oligos were subcloned into pSuper-Basic vector (OligoEngine). Immunoblotting and immunohistochemical analysis of shRNA-transfected cells was used to demonstrate that shGsk3 drastically knocks down (~90% of control) endogenous Gsk3α and Gsk3β in CAD cells and embryonic brain neurons (Kim et al., 2006). Targeting constructs were co-electroporated into embryonic cerebral cortex (E16) with radial glia-specific pBLBP-GFP cDNA. The effect of disruption of Gsk3 activity on the characteristic polarized morphology of radial glia (GFP^+) was quantified as described previously (Yokota et al., 2009).

RESULTS

Dynamic activities of radial glia in the developing cerebral cortex

To visualize distinct aspects of radial glial development and differentiation during corticogenesis, we developed an assay in which large cohorts of radial glial cells can be monitored over extended periods of time in the developing cerebral cortex. Radial glia-specific BLBP promoter-EGFP cDNA (pBLBP-EGFP) was electroporated into E14-16 mouse cortices to label radial glia with GFP. Radial glial cells in cortical slices from electroporated brains were repeatedly imaged for extended periods using a single- or multi-photon confocal laser-scanning microscope attached to a live cell incubation chamber (Fig. 1; see Movie 1 in the supplementary material). Pial membrane is intact in these slices. The GFP-positive cells span the width of the cerebral wall, display the characteristic polarized morphology of radial glia, and immunolabel with radial

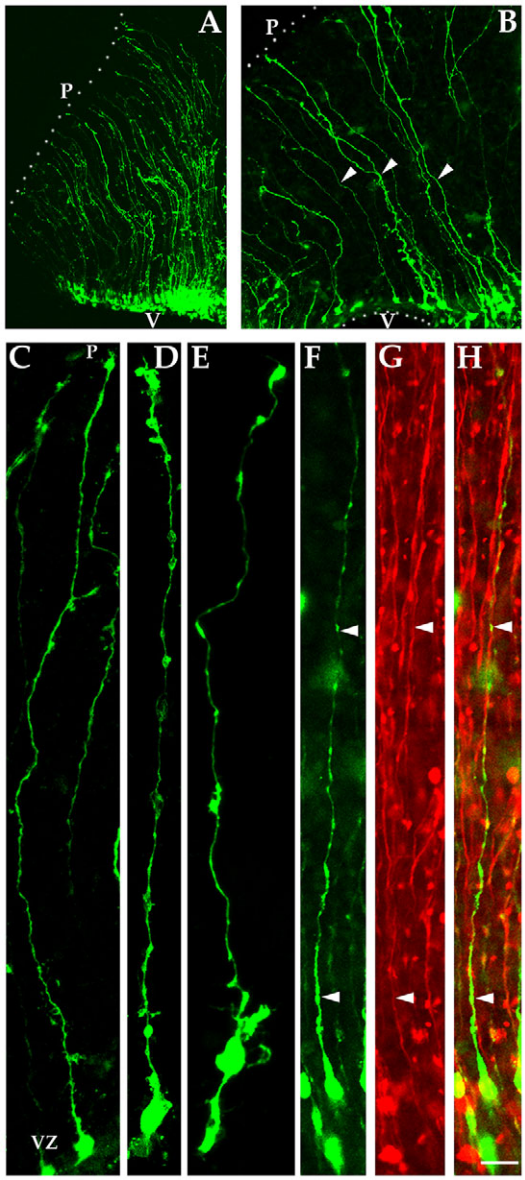

Fig. 1. Assay for radial glial development in mouse embryonic cerebral cortex. (**A**,**B**) Large cohorts of radial glial cells (green) spanning the entire width of the developing embryonic cerebral cortices were labeled with GFP by electroporation with pBLBP promoter-GFP plasmids. Labeling of multiple, definable, adjacent radial glia (arrowheads, B) is essential for the examination of inter-radial glial interactions and developmental potential of these cells. (**C-E**) Higher magnification views of labeled radial glial cells, illustrating the polarized morphology of radial glial cell soma in the ventricular zone (VZ), with a long, polarized basal process oriented towards the pia. (**F-H**) pBLBP-EGFP-labeled radial glial cells (arrowheads) immunolabeled with radial glial-specific RC2 antibody (red). P, pial surface; V, ventricular surface. Scale bar: 90 μm in A; 70 μm in B; 30 μm in C-H.

glia-specific RC2 antibodies (Fig. 1). As noted previously (Miyata et al., 2001; Noctor et al., 2001), these cells can undergo symmetric or asymmetric divisions and transform into multipolar astroglial cells (see Fig. S1 in the supplementary material). Importantly, these long-term live imaging studies of radial glial populations revealed several previously uncharacterized aspects of radial glial development. A prominent feature of the developing radial glia scaffold is the extensive inter-radial glial interactions (see Movie 2 in the supplementary material). Adjacent radial glial cells extended filopodia- or spine-like protrusions all along their processes to contact each other (Fig. 2; see Movies 2-4, 6 and 7 in the supplementary material). The average length of these protrusions was 5.42±0.5 μm. These inter-radial glial interactions were extensive and dynamic and appeared to occur concurrently with radial glial proliferation or guidance of neuronal migration. They also extended longer processes (10-40 μm) that could span the width of several radial glial cells, enabling interactions between non-adjacent radial glia (see Movies 5 and 6 in the supplementary material). Similar interactions between radial glial cell soma in the VZ were also evident (see Movie 8 in the supplementary material).

Another surprising, yet hitherto undefined, feature of developing radial glia was the dynamic nature of their leading edges/endfeet near the pial surface (Fig. 2F-J). They appeared to be similar to axonal growth cones and were highly active (see Fig. S2 and Movies 9 and 10 in the supplementary material). Based on their shape, they could be characterized either as club-like or branched (Fig. 2F-J). The number of radial glia with club-like endfeet increased as the cerebral cortex expanded during development. Equal levels of both types of endfeet were noted during early embryonic stages (E14; branched:club endfeet ratio of 47±9:53±9%), whereas the club-like form was more prevalent at late embryonic stages (E16; branched:club endfeet ratio of 21±5:79±6%). These radial glial leading edges extended and retracted at a rate of 7.5±2.4 μm/hour and 15.3±1.9 μm/hour, respectively. Importantly, the radial glia endfeet did not appear to be stable structures attached to the pial basement membrane, as previously thought. Rather, they are highly motile and constantly remodeling, continuously probing adjacent radial glial endfeet, and may interact with neurons passing through the marginal zone (see Movies 9 and 10 in the supplementary material).

Together, these live imaging observations suggest that extensive inter-radial glial interactions and highly dynamic radial glial growth cone activities are key features of the polarized radial glial scaffold in the developing cortex.

Role of Cdc42 in radial glial polarity

To explore the molecular control of distinct activities of the polarized radial glial scaffold, we analyzed the function of Cdc42, a small GTPase that is thought to be a principal determinant of cell polarity in response to environmentally derived cues (Allen et al., 1998; Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2002; Gotta et al., 2001; Garvalov et al., 2007; Heasman and Ridley, 2008). Cdc42 is expressed throughout the developing cerebral wall. To selectively analyze the dynamics of Cdc42 localization in radial glia, we electroporated Cdc42-EGFP plasmids into radial glia in E15 cerebral cortex. Previous studies have indicated that Cdc42-GFP accurately localizes to the same cellular locales as endogenous Cdc42 (Michaelson et al., 2001). Cdc42-GFP preferentially localized to the leading edge of radial glial fibers near the pial surface and in cell soma (Fig. 3; see Movie 11 in the supplementary material). Cdc42 appeared to associate with the plasma membrane of the leading edge undergoing oriented extension towards the pial surface (Fig. 3; see Fig. S3 in the supplementary material). Dynamic changes in Cdc42 localization were evident in the active radial glial endfeet (see Fig. S3 and Movies 12 and 13 in the supplementary material). De novo Cdc42-GFP expression was also noticeable along radial glia processes where new lateral process/spine growth is initiated (see Movie 14 in the supplementary material).

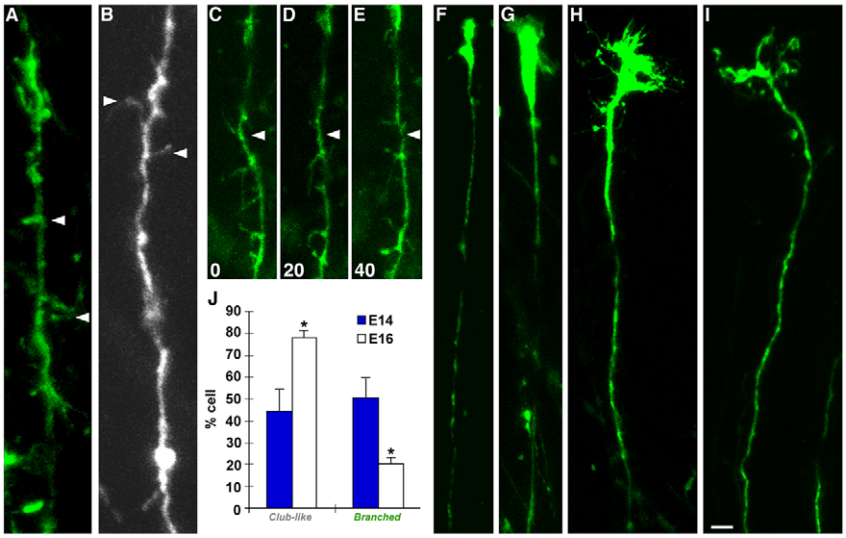

Fig. 2. Dynamics of radial glial spines and growth cones. (**A**,**B**) All along the radial processes, spine-like protrusions are evident (arrowheads). (**C-E**) Time-lapse observations indicate dynamic activity and rearrangements of these protrusions (see changes in protrusions adjacent to arrowhead). Time elapsed is indicated in minutes. (**F-I**) Radial glial endfeet are dynamic and vary in shape from club-like (F,G) to highly branched (H,I). (**J**) As cortical development proceeds, proportionately more radial glia exhibit club-like endfeet. Data shown are mean±s.e.m. **P*<0.001 (Student's *t*-test). Also see Movies 1-4 in the supplementary material. Scale bar: 100 μm in A; 28 μm in B-F; 66 μm in G-I.

During neurogenesis, radial glial cell soma undergo oscillatory, bidirectional (apical↔basal) interkinetic nuclear movement (INM) in the VZ (Rakic, 2003). Polarized localization of Cdc42 within the tips of cell soma was seen to precede and correlate with the direction of movement. Cdc42 localization changed in parallel to alterations in the direction of radial glial somal movement [Fig. 4E; number of radial glial cells imaged was 16 (*n*=3)]. Measurement of the relative intensity of Cdc42 distribution at the apical and basal somal poles of the progenitors undergoing INM indicated an accumulation of Cdc42 in the direction of cell movement (Fig. 4F). By contrast, control GFP localized uniformly throughout radial glia soma during INM. Together, these observations indicate that the polarized recruitment of Cdc42 within the developing radial glia might play an influential role in promoting radial glial cell polarity and the resultant functions.

To determine the functional significance of Cdc42 in developing radial glia, we first analyzed the effects of dominant-negative Cdc42 (DN-Cdc42) (Etienne-Manneville and Hall, 2001) on radial glial development. We electroporated BLBP promoter-driven DN-Cdc42 plasmids (pBLBP-DN-Cdc42-IRES-EGFP) into embryonic cortex (E15). The BLBP promoter enables selective expression of DN-Cdc42 in radial glia. DN-Cdc42 expression resulted in excessive branching near the pial surface (Fig. 4A-C; see Movie 15 in the supplementary material). Nearly three times as many radial glia displayed this defect following DN-Cdc42 expression (GFP, 21±5%; DN-Cdc42-GFP, 61±4.8%) (Fig. 4C). The majority of DN-Cdc42-expressing radial glia were shorter than controls and did not reach the pial surface (quantified in Fig. 4D). The rates of extension and retraction of the radial glial leading edges were also retarded in DN-Cdc42-expressing glia (extension: GFP, 7.5±2.4 μm/hour; DN-Cdc42-GFP, 1.8±0.12 μm/hour; retraction: GFP, 15.3±1.9 μm/hour; DN-Cdc42-GFP, 3.9±0.5 μm/hour). Live imaging of control GFP- and DN-Cdc42-expressing radial glia indicated that there is a general reduction in inter-radial glial interactions or contacts between adjacent radial glial fibers following Cdc42 inhibition (cell-cell contact index: GFP, 0.7±0.15; DN-Cdc42, 0.29±0.11).

To further evaluate the significance of Cdc42 activity in vivo, we conditionally inactivated Cdc42 in radial progenitors of the developing cerebral cortex. *Cdc42* floxed mice were mated with *hGFAP-Cre* lines (Zhuo et al., 2001). The *hGFAP-Cre* transgene induces widespread recombination of the floxed *Cdc42* allele in the radial progenitors of the telencephalon from E13.5. Cdc42 inactivation resulted in excessive radial glial branching near the pial surface, similar to what was observed in DN-Cdc42-expressing radial glia (Fig. 5A-C; see Fig. S4 in the supplementary material). In addition, as seen previously (Cappello et al., 2006), radial glial cell soma appeared to be less densely packed and were displaced away from the ventricular surface (see Fig. S5 in the supplementary material). The radial

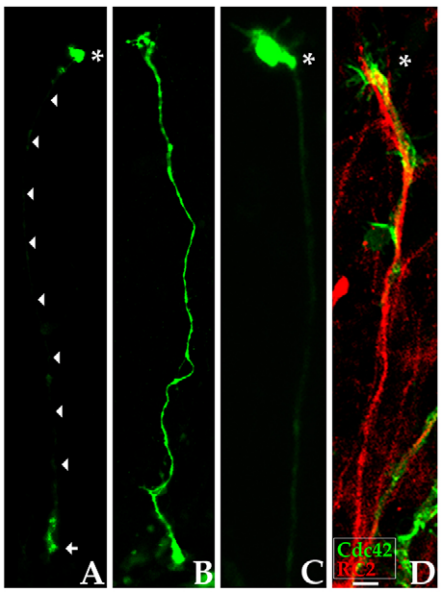

Fig. 3. Expression of Cdc42 in the leading edges of polarized radial glia. Mouse E15 cerebral cortex was electroporated with Cdc42-GFP or GFP plasmids, sliced, and imaged within 8 hours. (**A**) Low-magnification view of an entire radial glial cell indicates preferential localization of Cdc42-GFP at the leading tip (asterisk). Arrow, cell soma; arrowheads, polarized process. By contrast, control GFP is localized uniformly throughout radial glia (**B**). (**C**) Higher magnification view of the radial process shows Cdc42 accumulation at the leading tip (asterisk). (**D**) Double labeling with radial glia-specific RC2 antibody (red) confirms preferential accumulation of Cdc42 (green) in radial process tips. Also see Movies 11-14 in the supplementary material. Scale bar: 35 μm in A,B; 90 μm in C,D.

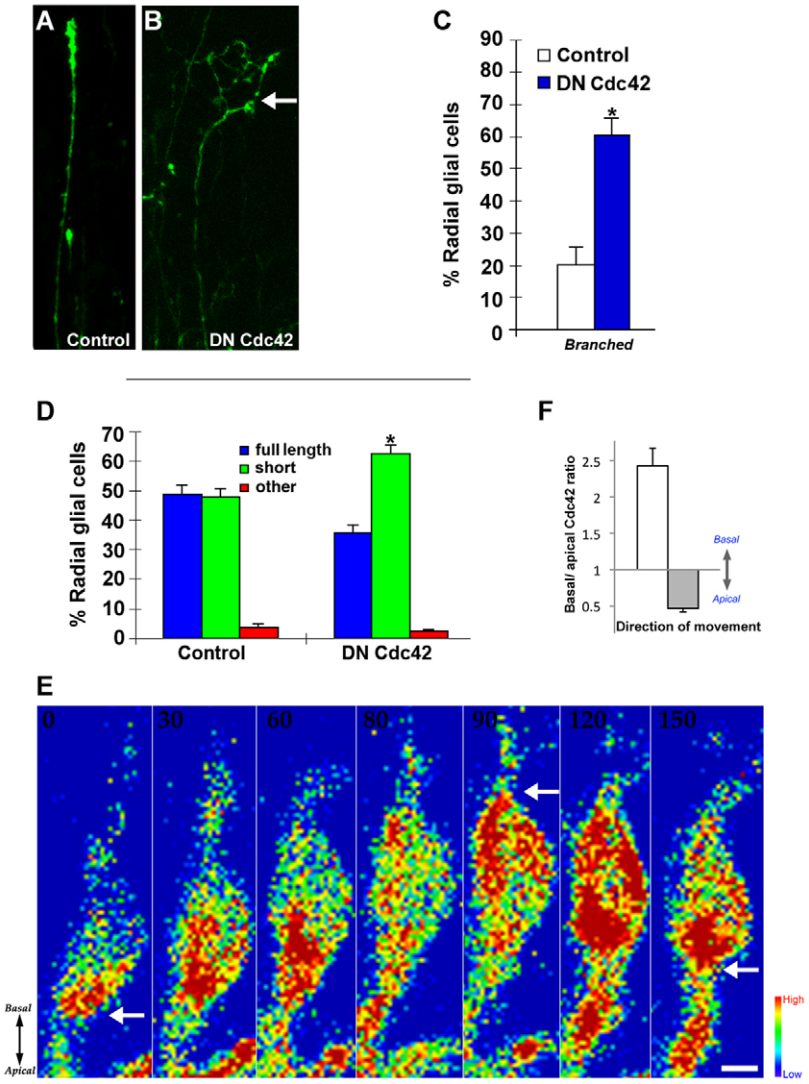

Fig. 4. Cdc42 activity regulates distinct aspects of radial glial morphology. (**A**,**B**) Radial glia in mouse E15 cerebral cortices were electroporated with BLBP promoter-driven dominant-negative Cdc42-GFP or GFP (pBLBP-DN Cdc42-GFP or pBLBP-GFP) plasmids and imaged after 24 hours. (A) Individual radial glia cells expressing control GFP display the characteristic elongated, polarized basal process oriented towards pia. (B) By contrast, DN-Cdc42-expressing radial glia show highly branched basal process ends (arrow). (**C**) Quantification of radial glial endfeet types indicates a significant increase in branched endfeet following DN-Cdc42 expression. (**D**) Quantification of the effect of DN-Cdc42 on radial glial morphology. Radial glia spanning the full length of the cerebral wall or with shorter processes were counted. DN-Cdc42 expression leads to an increase in the number of radial glia with shorter processes. GFP$^+$ cells without processes and multipolar cells were included in the 'other' group. (**E**) Live imaging of Cdc42-GFP expression in radial progenitors in VZ indicates that Cdc42 preferentially accumulates at the apical or basal poles of radial glial cell soma (arrows), corresponding to the direction of interkinetic cell movement in the VZ. Cdc42-GFP images were pseudocolored (red, high Cdc42-GFP; blue, low Cdc42-GFP). Time elapsed is indicated in minutes. (**F**) Measurement of relative fluorescence intensities of Cdc42-GFP in defined areas within the apical or basal poles of progenitors undergoing cell movement confirms the differential distribution of Cdc42-GFP towards the direction of cell movement. A basal/apical Cdc42 fluorescence intensity ratio above 1 is indicative of higher localization at the basal pole, whereas values below 1 indicate accumulation at the apical pole. Arrows indicate the direction of cell movement. Data shown are mean±s.e.m. *, P<0.001 versus controls (Student's t-test). VZ, ventricular zone. Scale bar: 100 μm in A,B; 10 μm in E.

processes of Cdc42-deficient radial glia did not fasciculate as tightly as in controls, suggesting potential deficits in inter-radial glia interactions (Fig. 5A,B; see Fig. S6 in the supplementary material). Labeling with anti-Tbr1 antibodies, a marker for deeper layer neurons, indicated a diffuse distribution and placement of these neurons in the cortical plate of Cdc42-deficient cortex. In caudal regions of embryonic cerebral cortex, pockets of Tbr1$^+$ neuronal ectopias in the VZ were often observed (Fig. 5; see Fig. S7 in the supplementary material). These disruptions in cortical neuronal development suggest potential deficits in the ability of Cdc42-deficient radial glia to function as neural precursors and migratory guides. Together, these observations strongly suggest that Cdc42 plays a pivotal role in the generation of appropriate morphological polarity of radial glia and in their resultant functions in the developing neocortex.

Consistent with disrupted radial glial development during embryogenesis, the laminar organization of neurons and their connectivity were disrupted in *Cdc42* mutants postnatally. *Cdc42$^{lox/lox}$;hGFAP-Cre* mice survived up to 2-4 weeks after birth. Brains of these mutants were hydrocephalic. Labeling with antibodies to specific neuronal layer markers (e.g. Tbr1, Cux1) indicated malformation of neuronal layers and the presence of ectopias in *Cdc42* mutants (see Fig. S8 in the supplementary material). The postmigratory axonogenesis and connectivity of these neurons were also disrupted. Labeling of major axonal fiber tracts in cortex (e.g. corpus callosum) with anti-L1 (L1cam – Mouse Genome Informatics) antibodies indicated that instead of the characteristic outgrowth and organization of cortical axons towards other areas of cortex, *Cdc42* mutant axons are grossly misrouted and appear not to orient or fasciculate towards their appropriate targets (see Fig. S9 in the supplementary material).

Function of Gsk3β in radial glial cell development

Since downstream, indirect regulation of Gsk3β by Cdc42 is thought to play a role in the generation of cell polarity (Etienne-Manneville and Hall, 2003; Heasman and Ridley, 2008; Wu et al., 2006), we next determined whether Gsk3 activity regulates radial glial polarity. Cdc42 and Gsk3β are co-expressed in radial glia (data not shown). The two Gsk3 isoforms, α and β, may functionally compensate for each other. We therefore used a recently characterized Gsk3 inhibitor, 6-bromoindirubin, and a cell-permeable myristylated form of Gsk3 peptide inhibitor to eliminate Gsk3 activity in radial glia (Meijer et al., 2003; Meijer et al., 2004; Kim et al., 2006). 6-bromoindirubin functions by obstructing the ATP-binding pocket of Gsk3. The competitive peptide inhibitor is a substrate-specific inhibitor and is selectively recognized by

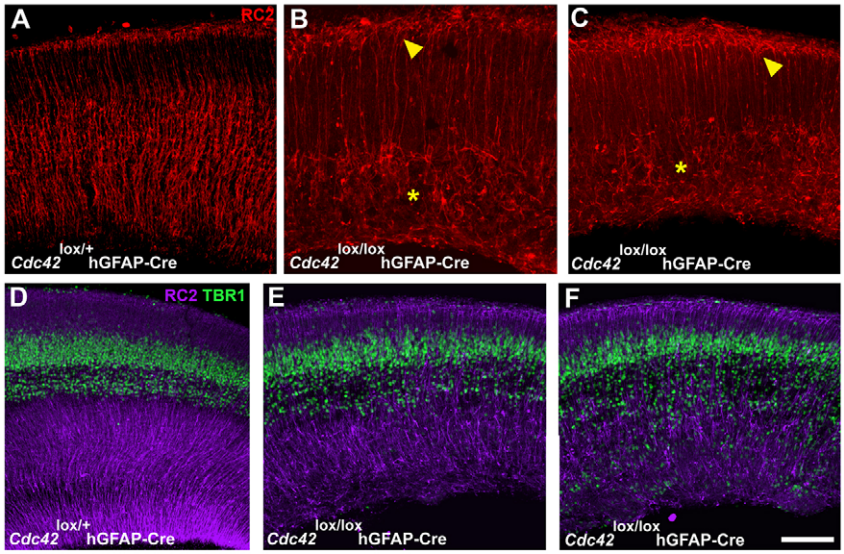

Fig. 5. Disrupted radial glial development in *Cdc42*$^{lox/lox}$*;hGFAP-Cre* cerebral cortex. hGFAP-Cre-mediated recombination was used to inactivate Cdc42 in radial glia. (**A-C**) Conditional deletion of *Cdc42* in radial progenitors leads to branching defects in radial glial endfeet. Although the radial glia scaffold formed in Cdc42-deficient cortex, radial glial endfeet appear to be more branched when compared with controls. Arrowheads point to aberrantly branched endfeet of Cdc42-deficient radial glia (B,C). See Fig. S4 in the supplementary material for a higher magnification illustration. In addition, radial glial processes are less fasciculated (see Fig. S6 in the supplementary material) and their soma appear to be misplaced and less densely packed in Cdc42-deficient cortex (asterisk, B,C). See Fig. S5 in the supplementary material for higher magnification illustration. (**D-F**) Labeling with anti-Tbr1 antibodies (green) indicates normal placement of deeper layer neurons in control (D), but not in Cdc42-deficient (E,F), cortex. Moderate to severe misplacement of neurons was noted within the disrupted radial glia scaffolding of *Cdc42* conditional null mice. Scale bar: 225 μm.

Gsk3α/β. E15.5 embryonic cortices were electroporated with pBLBP-IRES-EGFP plasmids, sliced, and maintained in media supplemented with the respective Gsk3 inhibitors at 300 nM. This concentration is known to produce complete inhibition of Gsk3 activity (Kim et al., 2006). Treatment with Gsk3 inhibitors disrupted the radial organization and pially oriented growth of radial glia (Fig. 6D-F). The radial processes aberrantly extended multiple branches and the glial endfeet were often oriented away from the pial surface (Fig. 6D,E). These results indicate that Gsk3 activity can significantly regulate the polarized growth and organization of radial glial cells.

To further confirm the role of Gsk3 in radial glial development, we tested the effect of shRNA-mediated knockdown of endogenous Gsk3α and β (Kim et al., 2006). Expression of shGsk3, which is known to knockdown endogenous Gsk3α and β (Kim et al., 2006), disrupted the radial glial scaffold significantly (Fig. 6A-C,F; see Movies 16 and 17 in the supplementary material). Instead of displaying the characteristic elongated morphology, the radial glial cells became replete with multiple bends, misoriented endfeet and several branches along the shaft of the radial processes (Fig. 6B; see Movies 16 and 17 in the supplementary material). By contrast, expression of control shRNA induced no changes in radial glial cells. Importantly, expression of a modified, yet functionally active, Gsk3β resistant to shRNA-induced degradation (Kim et al., 2006) rescued shGsk3-mediated disruption of radial glial cells (Fig. 6C,F). Furthermore, two dominant-negative Gsk3 constructs known to inhibit all Gsk3 kinase activity (Gsk3KM) or the activity of Gsk3 towards previously primed (i.e. phosphorylated) substrates (Gsk3 R96A) strongly disrupted radial glial processes (Fig. 6F). These Gsk3-inhibited radial glial processes were wavy and often branched, similar to what was noted when Gsk3 was inhibited with pharmacological inhibitors or shRNA. Together, these results suggest that inhibition of endogenous Gsk3 activity disrupts normal radial glial cell organization.

It is likely that the endogenous Gsk3 isoforms, α and β, exhibit redundancy in their functions in radial glia (Doble et al., 2007). Consistent with this, we noted normal radial glial and cortical development in *Gsk3a* null mice (see Fig. S10 in the supplementary material). To further characterize the significance of Gsk3 signaling in radial glial polarity and to explore the compensatory roles of Gsk3α and β in vivo, we conditionally inactivated both Gsk3α and β in radial progenitors. *Nestin-Cre*, which is active in radial progenitors of the developing telencephalon from ~E10, was crossed to *Gsk3a* null and *Gsk3b* floxed allele lines to generate *Gsk3a*$^{-/-}$*;Gsk3b*$^{lox/lox}$*;Nestin-Cre* mice (Kim et al., 2009). Inactivation of both Gsk3α and β in radial progenitors disrupted their polarized growth spanning the cerebral wall. The basal processes extending towards the pial surface were often wavy, shortened and had frequent bead-like thickenings (Fig. 7). Correspondingly, the migration and placement of cortical neurons were aberrant (Fig. 7A,B). In vitro, Gsk3-deficient radial glia had thicker, shorter and highly branched processes, as compared with the control (see Fig. S11 in the supplementary material). Together, these studies based on pharmacological inhibition, shRNA-mediated knockdown, dominant-negative disruption and conditional deletion of *Gsk3a* and *Gsk3b* confirm the essential nature of Gsk3 signaling for the proper establishment and organization of the polarized radial glial scaffold during corticogenesis.

In astrocytes and keratinocytes in vitro, loss of Cdc42 results in reduced activation of PKCζ (atypical protein kinase C) and in decreased phosphorylation of Gsk3 (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003; Wu et al., 2006). To

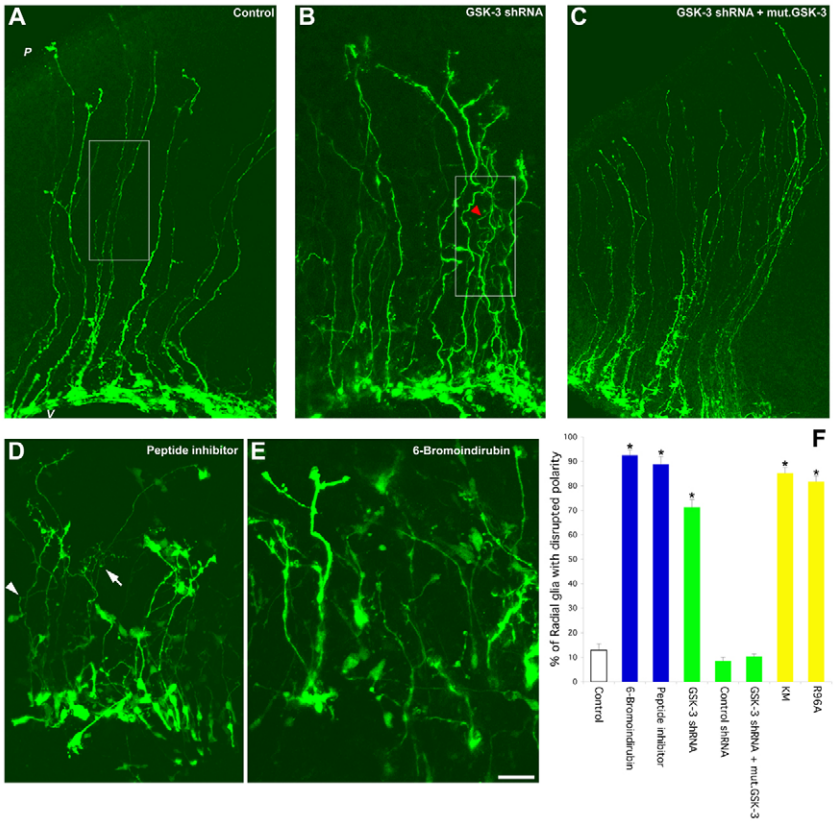

Fig. 6. Gsk3 inhibition disrupts radial glial development and organization. (**A-C**) Cerebral cortices of E15.5 mouse embryos were electroporated with control or Gsk3 shRNA and pBLBP-IRES-EGFP plasmids and radial glia were analyzed 2 days later. (A) Radial glia expressing control shRNA displayed the characteristic polarized morphology. (B) By contrast, Gsk3 shRNA expression resulted in significant disruption to the radial glia scaffold, with many radial glia displaying wavy radial processes (arrowhead, B); compare the radial processes within the boxed areas of control (A) and Gsk3-deficient (B) cortex. (C) This deficit was rescued by co-electroporation of mutant Gsk3β resistant to shRNA-mediated degradation. (**D**,**E**) Inhibition of Gsk3 with a cell-permeable myristylated form of Gsk3 peptide inhibitor (D) or 6-bromoindirubin (E) also resulted in strong defects in the polarized morphology of radial glia. Arrow and arrowhead in D indicate an aberrantly branched and misoriented radial glia cell, respectively. (**F**) Quantification of disrupted radial glial polarity following the different methods of Gsk3 inhibition. Two dominant-negative Gsk3 constructs, Gsk3KM and Gsk3 R96A, which are known to inhibit all Gsk3 kinase activity and the activity of Gsk3 towards previously primed substrates, respectively, also disrupted radial glial polarity. Data shown are mean±s.e.m. *, $P<0.001$ versus controls (Student's *t*-test). P, pial surface; V, ventricular surface. Scale bar: 130 μm in A-C; 120 μm in D,E.

evaluate Cdc42-related changes in Gsk3β in developing cortex, we first examined Gsk3β levels and phosphorylation in control and *Cdc42*$^{lox/lox}$*;hGFAP-Cre* cortex. No significant differences, either in the level or phosphorylation of Gsk3β, were evident in Cdc42-deficient embryonic cortex (see Fig. S12A,B in the supplementary material). To examine whether Gsk3 activity could modulate any of the Cdc42-deficient radial glial phenotypes, we electroporated constitutively active Gsk3β (Gsk3β-S9A) (Stambolic and Woodgett, 1994; Hetman et al., 2000) into control and Cdc42-deficient (*Cdc42*$^{lox/lox}$*;hGFAP-Cre*) radial progenitors in embryonic cortex. Active Gsk3 expression did not rescue the Cdc42-deficient radial progenitor phenotype (see Fig. 12C-G in the supplementary material), suggesting that Gsk3 and Cdc42 exert distinct effects on radial glial development.

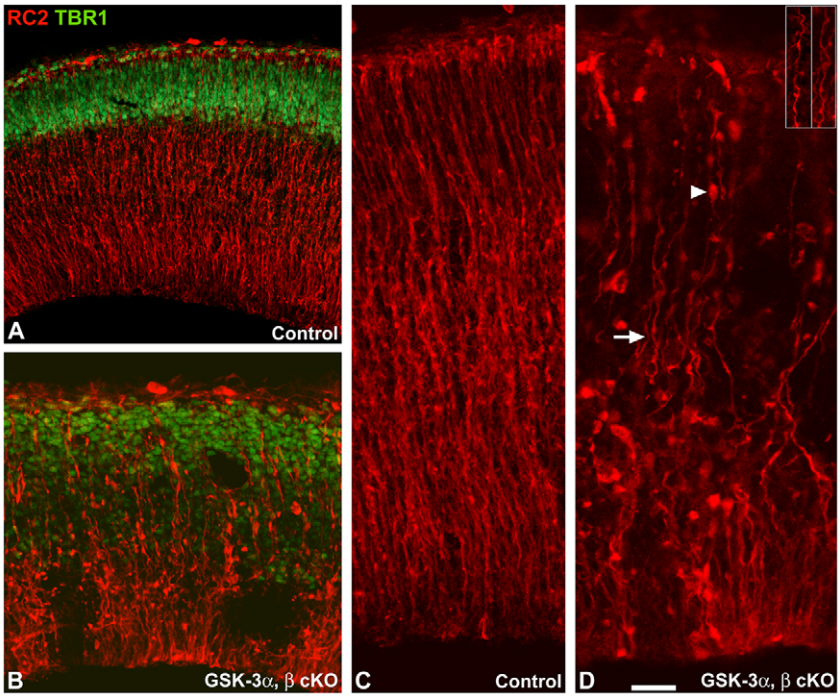

Fig. 7. Disrupted radial glial development in *Gsk3a*$^{-/-}$*;Gsk3b*$^{lox/lox}$*;Nestin-Cre* cerebral cortex. (**A-D**) Inactivation of both Gsk3α and β in radial glial progenitors disrupts radial glial scaffolding. E16 cerebral cortices from control (A,C) and Gsk3-deficient (B,D) mice were labeled with radial glia-specific RC2 antibodies and deeper layer neuron-specific anti-Tbr1 antibodies. Instead of the polarized radial organization of the normally developing cerebral cortex (A,C), Gsk3α- and β-deficient radial glia are wavy (arrow and inset, D), shorter, and often have 'bead'-like thickenings (D, arrowhead). Labeling with Tbr1 antibodies indicates a diffuse placement of neurons within the Gsk3α- and β-deficient cortex. Scale bar: 200 μm in A,B; 100 μm in C,D.

DISCUSSION

We found that inter-radial glial interactions and radial glial growth cone activities are key features of the dynamically active radial glial scaffold in the embryonic neocortex. Critical determinants of cell polarity, Cdc42 and Gsk3, modulate distinct aspects of polarized radial glial scaffold organization and activities during corticogenesis.

Activities of polarized radial glia: proliferation, extension, retraction and inter-radial glial interactions

Using long-term time-lapse observations of cohorts of radial glia, we have shown several previously undescribed activities of polarized radial glia (Figs 1 and 2; see Movies 1-10 in the supplementary material). First, the radial glial endfeet are not static, stable structures. Instead, they actively interact with adjacent endfeet and constantly remodel and are highly dynamic in their ability to extend or retract branches. These dynamic behaviors of radial glial endfeet can modulate the behavior of migrating neurons in the marginal zone and may also facilitate meninges-radial glial interactions (Yokota et al., 2007; Siegenthaler et al., 2009). Consistent with the potential importance of the dynamic activity of endfeet, mouse mutants with glial endfeet defects display neuronal placement deficits (Halfter et al., 2002; Haubst et al., 2006; Graus-Porta et al., 2001; Yokota et al., 2007).

The shape of active radial glial endfeet, from club-like to highly branched, also changes as cortical development unfolds. The club-like endfeet, as compared with the highly branched endfeet, tend to advance or retract at a much higher rate. Similar differences have also been noted in axonal growth cones, where growth cones with club-like endings are characterized by a rapid rate of advancement towards targets, whereas branched growth cones tend to be characterized by a slower rate of movement and used when pathway choice decisions are made. Similarly, as the cortex expands, club-like radial glial growth cones may characterize new radial glia, which have to rapidly extend basal processes towards the pial surface following their generation in the VZ. Club-like growth cones were also evident in a subpopulation of radial glia that were undergoing rapid retraction of their leading processes. Radial glia that are about to reorient, transform, divide or undergo apoptosis might rapidly retract their long leading process just prior to these events.

Radial glia also extend multiple, lateral, fine filopodia-like extensions along their basal processes to interact with adjacent radial glial cells (Fig. 2; see Movies 2-8 in the supplementary material) (Schmechel and Rakic, 1979b; Mason et al., 1988; Takahashi et al., 1990). Spontaneous calcium waves propagating through linked clusters of radial glia can modulate the proliferation of cohorts of radial glia, potentially leading to the production of isochronic neurons that are destined to occupy the same cortical layer or column (Weissman et al., 2004). Thus, the dynamic inter-radial glial interactions might help promote and refine the emergence of the ‘radial unit’-like organization of the cerebral cortex (Rakic, 1988).

Cdc42 expression and function in radial glia

Cdc42 localizes to dynamically active regions of radial glia in the developing cerebral cortex (Fig. 3; see Movies 11-14 in the supplementary material). Disruption of Cdc42 activity resulted in altered radial glial endfeet dynamics, inter-radial glial contacts and positioning of radial glial cell soma (Figs 4 and 5; see Figs S4-S7 in the supplementary material). Live imaging of Cdc42 localization dynamics indicates that Cdc42 preferentially accumulates at the front of the nucleus in the direction of migration during INM of radial progenitors (Fig. 4). Disrupted INM following *Cdc42* deletion may thus lead to a displacement of radial glial cell soma within the proliferative niche (Fig. 5) and to the aberrant generation of neurogenic intermediate precursors (Cappello et al., 2006; Xie et al., 2007). This combination of defective radial progenitor proliferation and morphology could be the underlying basis for the aberrant number and placement of neurons noted in Cdc42-deficient cortex (Cappello et al., 2006) (Fig. 5).

Gsk3 functions in radial glia

When the activities of both Gsk3α and β were eliminated using multiple pharmacological inhibitors or shRNAs, polarized radial glial organization was drastically affected (Fig. 6; see Movies 16 and 17 in the supplementary material). Consistently, conditional inactivation of both Gsk3α and β in radial progenitors disrupted their polarized growth spanning the cerebral wall and the resultant migration and placement of neurons (Fig. 7). Gsk3 interactions with pre-phosphorylated, primed substrates such as β-catenin, Apc or CRMP2 (Dpysl2 – Mouse Genome Informatics) and unprimed substrates such as MAP1B (Mtap1b – Mouse Genome Informatics) or CLASP can profoundly modulate the morphology of developing neurons or astrocytes via microtubule cytoskeletal dynamics (Etienne-Manneville and Hall, 2003; Zhou and Snider, 2005; Kim et al., 2006; Hur and Zhou, 2010; Shi et al., 2004; Jiang et al., 2005). The polarized morphology of radial glia is critically dependent on microtubules, but not on actin microfilaments (Li et al., 2003). Inactivation of Gsk3 and the resultant changes in a multitude of substrates capable of modulating microtubule dynamics may have led to the wavy, shortened and thickened radial processes of Gsk3-deficient radial progenitors (Figs 6 and 7).

Local recruitment of Cdc42 is thought to activate several protein complexes, including Par6-PKCζ (Etienne-Manneville, 2003; Heasman and Ridley, 2008). Cdc42 can modulate the stability of β-catenin by controlling the PKCζ-mediated phosphorylation of Gsk3 (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003). Loss of Cdc42 results in reduced activation of PKCζ and decreased phosphorylation (i.e. activation) of Gsk3 (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003; Wu et al., 2006). Although we did not observe changes in serine phosphorylation of Gsk3 in whole cortical extracts of *Cdc42*$^{lox/lox}$*;hGFAP-Cre* embryos, an examination of Gsk3 changes (i.e. serine and tyrosine phosphorylation) specifically in embryonic dorsal cortex, where hGFAP-Cre is predominantly active, and an evaluation of the effect of Gsk3 inactivation in *Cdc42* mutants, will help to further characterize the role of Cdc42-Gsk3 interactions in radial progenitor development.

Manifestation of radial glial polarity and its implications for corticogenesis

The generation and maintenance of polarized morphology and the resultant functions of radial glia guarantee the emergence of a laminar organization of neurons in the cerebral cortex. Inactivation of Cdc42, Gsk3 or Apc in radial progenitors leads to distinct radial glial and cortical phenotypes. Each of these proteins appears to differentially influence the polarized morphology and resultant functions of radial glia. Loss of Cdc42 leads to disrupted radial glial endfeet, inter-radial glia interactions, misplaced radial glial cell soma and the aberrant generation of neurons (Figs 4 and 5) (Cappello et al., 2006). Gsk3 deletion disrupts the symmetric proliferation and the overall organization of elongated, polarized

radial glial cells (Figs 6 and 7; see Movies 16 and 17 in the supplementary material) (Kim et al., 2009), whereas loss of Apc leads to a dismantling of the entire radial glial scaffold (Yokota et al., 2009). Although indirectly related, these distinct members of the cell polarity pathway appear to exert a non-overlapping, hierarchical influence on the structural and molecular polarity of radial glia. A further understanding of these context-dependent activities of cell polarity pathways in the manifestation of radial glial polarity will be essential to define how polarity is translated into the specialized functions of radial glia that underlie the formation of the cerebral cortex. Moreover, the aberrant regulation of Cdc42 and Gsk3 in psychiatric disorders (Mao et al., 2009; Ide and Lewis, 2010) suggests that disruptions in the molecular control of radial glial polarity and the resultant changes in the formation of neural circuitry in the cerebral cortex might contribute to the emergence of neurodevelopmental disorders such as schizophrenia.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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