DOCK7 interacts with TACC3 to regulate interkinetic nuclear migration and cortical neurogenesis

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Neurogenesis in the developing neocortex relies on the ability of radial glial progenitor cells (RGCs) to switch from proliferative to differentiative neuron-generating divisions, but the molecular mechanisms that control this switch in a correct temporal manner are not well understood. Here, we show that DOCK7, a member of the DOCK180 family of proteins, regulates RGC proliferation versus differentiation. Silencing of DOCK7 in RGCs of developing mouse embryos impedes neuronal differentiation and maintains cells as cycling progenitors. In contrast, DOCK7 overexpression promotes RGC differentiation to basal progenitors and neurons. We further present evidence that DOCK7 influences neurogenesis by controlling apically directed interkinetic nuclear migration of RGCs. DOCK7 exerts its effects by antagonizing the microtubule growth-promoting function of the centrosome-associated protein TACC3. Thus, DOCK7 interaction with TACC3 controls interkinetic nuclear migration and the genesis of neurons from RGCs during cortical development.

The six-layered neocortex is formed by the orderly generation of postmitotic neurons through proliferative and differentiative divisions of neural progenitors during neurogenesis1–3. A principal subtype of progenitor that gives rise to most pyramidal neurons in the developing neocortex is the radial glial cell (RGC). These cells stem from neuroepithelial cells located at the apical-most region of the neuroepithelium, the ventricular zone (VZ)6–11. RGCs are highly polarized cells, extending an apical process into the ventricular lining and a long thin basal process to the pial surface1,2. A hallmark of these cells, responsible for the pseudostratified appearance of the VZ, is interkinetic nuclear migration (INM), whereby nuclei change position along the apical–basal axis during the course of the cell cycle12–14. Nuclei move away from the apical surface during the G1 phase of the cell cycle, undergo S phase at a basal location in the VZ and return to the apical surface during G2 to divide in the vicinity of the centrosomes, located in the apical endfeet of neuroepithelial and RGCs. Because of the apical location of their nuclei undergoing mitosis, these cells are also referred to as apical progenitors.

Before the peak of neurogenesis, RGCs undergo predominantly symmetric proliferative divisions to amplify the progenitor pool. However, during the peak of neurogenesis (embryonic day (E) 13–18 in mice) they largely divide asymmetrically to self-renew while giving rise either to one neuron or to an intermediate (basal) progenitor, which subsequently divides symmetrically in the subventricular zone (SVZ) to produce two neurons7–9,15–17. Unlike apical progenitors, basal progenitors do not display apical–basal polarity or INM9,17,18. While the differentiating progeny progressively migrate away to form the cortical plate, renewing RGCs remain in the VZ for subsequent divisions, ensuring sufficient progenitors to produce later born neurons and glial cells2–4. Self-renewability of RGCs must be tightly balanced with differentiation for proper neocortical development19. Disruption of this balance has been associated with neurological and neuropsychiatric disorders, including microcephaly and schizophrenia20,21.

How apical progenitors precisely control self-renewal versus differentiation is not well understood. Besides cell cycle kinetics and asymmetric inheritance14,22–27, recent evidence suggests that INM can influence the balance between neurogenesis and progenitor pool maintenance by controlling the exposure time of apical progenitor nuclei to neurogenic versus proliferative signals along the basal–apical axis12,13,28–34. A high apical to low basal gradient of Notch activity, known to prevent apical progenitors from differentiating, has been reported in developing chick and zebrafish retina28,30. Furthermore, perturbations of INM in mouse neocortex have been linked to cell fate changes31–34, with impaired basal-to-apical (bl-to-ap) INM being associated with a decrease in apical progenitors and a concomitant increase in basal progenitors and/or neurons31,32,34. However, the consequences of the converse situation, accelerated bl-to-ap INM, for apical progenitor proliferation and neuronal differentiation remain unaddressed.

The cellular machinery that controls INM involves actomyosin- and microtubule-dependent systems12,13, with the former generally linked to the ap-to-bl leg of INM and the latter to the bl-to-ap leg in the developing rodent cortex32,33,35,36 (but see ref. 37). Ample evidence indicates that the centrosome, the main microtubule-organizing center in animal cells, is important in bl-to-ap INM13,31,32. Depletion of centrosomal proteins, such as Cep120 and TACCs, which associate with the microtubule lattice linking the centrosome and nucleus, disrupts this leg of INM and causes increased neuron production at the expense of progenitors32. But although these studies point to the importance of centrosomal proteins in INM and maintenance of the progenitor pool, little is known about their regulation in these events.

Here we have investigated the role of DOCK7, a member of the DOCK180-related protein superfamily, in cortical neurogenesis. The DOCK180 family emerged as a distinct class of Rac and/or Cdc42 GTPase guanine nucleotide exchange factors (GEFs), which have

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Figure 1 Distribution and localization of DOCK7 in the developing mouse cortex. (a) DOCK7 levels in cortical lysates at different developmental stages. γ-tubulin was used as a loading control. Full-length western blot is shown in Supplementary Figure 12. (b) Coronal cryosections of the neocortex at E13.5 immunostained for DOCK7 and neural stem/progenitor marker nestin or neuronal marker class III β-tubulin Tuj1. CP, cortical plate; IZ, intermediate zone. (c) Apical endfeet of VZ progenitors in coronal brain slices of the neocortex at E13.5 immunostained for DOCK7 and centrosomal marker γ-tubulin, with nuclei counterstained with DAPI. (d) Cultured cortical progenitors isolated from E13.5 neocortices immunostained for DOCK7 and centrosomal marker pericentrin, and counterstained with DAPI. Scale bars: 15 μm in b; 10 μm in c; 5 μm in d.

Results

DOCK7 expression and localization in developing neocortex

To assess DOCK7’s role during cortical development, we began by examining its expression patterns in the developing cortex. Western blot
Analysis of cortical lysates showed DOCK7 to be expressed at E11, and expression persisted until at least postnatal day (P) 3, implying that DOCK7 is expressed during active neocortical neurogenesis (Fig. 1a).

To determine the spatial expression pattern(s) of DOCK7 in the developing neocortex, we performed immunohistochemistry on cryosections of mouse brains around the onset of neocortical neurogenesis (E9.5, E10.5) and at E13.5. DOCK7 immunoreactivity was undetectable at E9.5 and weak at E10.5 but relatively high at E13.5 in (E9.5, E10.5) and at E13.5. DOCK7 immunofluorescence in the VZ of E13.5 cortices overlapping staining of DOCK7 with the centrosomal marker γ-tubulin, suggesting the presence of DOCK7 at the centrosome (Fig. 1c). To corroborate this, we examined the subcellular localization of DOCK7 in cultured cortical progenitors derived from E13.5 mouse cortices. Although DOCK7 fluorescence was detectable throughout the cytoplasm, an intense signal was noticeable at the centrosome, where DOCK7 colocalized with the centrosomal marker pericentrin (Fig. 1d). These data confirm the presence of DOCK7 at the centrosome in cortical progenitors.

DOCK7 depletion enhances VZ progenitor proliferation in utero

Next, we asked whether DOCK7 affects progenitor proliferation in the embryonic mouse neocortex. To approach this, we generated a vector that expresses a short hairpin RNA (shRNA Dock7#2) targeting the 3′ untranslated region (UTR) of mouse Dock7 mRNA. DOCK7#2 shRNA substantially reduced DOCK7 levels in cortical progenitors, whereas control shRNA (scr#1) had no effect (Supplementary Fig. 2). DOCK7 knockdown did not affect DOCK6 levels, and DOCK8 expression was not detectable in cortical progenitors; these proteins are most closely related to DOCK7 (Supplementary Fig. 3).

We introduced the Dock7#2 or scr#1 shRNA construct, together with an EGFP-NLS plasmid (expressing enhanced GFP fused to a nuclear localization signal), into E13.5 mouse brains by in utero electroporation, administered BrdU at E15.5 and analyzed brains 2 h later (Fig. 2). The percentage of BrdU-positive progenitors in the VZ was considerably higher in the Dock7#2 shRNA group than in the scr#1 shRNA group (Fig. 2a,b). Rescue experiments using a Dock7 cDNA that lacks the 3′ UTR and is therefore resistant to Dock7#2 shRNA-mediated RNA interference (Supplementary Fig. 2) demonstrated that the Dock7 RNAi effect was specific (Fig. 2a,b).

We further performed immunostainings for phosphorylated histone H3 (PH3), a mitotic marker, and determined the mitotic index (the percentage of transfected cells PH3-positive) in the VZ. This index was considerably higher in the Dock7#2 shRNA group than in control and rescue groups (Fig. 2c,d). Thus, DOCK7 knockdown increased the percentage of proliferating VZ progenitors in the embryonic neocortex. When examining the impact of ectopic expression of Flag-tagged DOCK7, we observed the opposite effects. The percentage of BrdU-positive transfected cells, as well as the mitotic index, was significantly lower in the Flag-DOCK7–expressing group than in the control vector.
DOCK7 controls bidirectional INM of RGCs. (a–d) Mouse embryos were electroporated at E13.5 with a plasmid expressing EGFP-NLS together with one of the indicated plasmids, pulse-labeled with BrdU at E15.5 and killed 15 min, 2 h, 4 h or 6 h after BrdU injection. Brain slices were immunostained for EGFP and BrdU (a, b) and EGFP, BrdU and PH3 (c, d, left). (a, b) Left: confocal images showing position of BrdU+ and EGFP-NLS+ nuclei in neocortices transfected with plasmids expressing scr#1 shRNA (scr#1) or Dock7#2 shRNA (Dock7#2) (a) or control vector (vector) or Flag-DOCK7 (DOCK7) (b), at indicated times after BrdU injection. Scale bars, 20 µm. Arrowheads indicate BrdU+ nuclei of transfected cells. Right: distribution of BrdU+ transfected cells quantified as percentage of all transfected cells per 20-µm-high bin across VZ and part of SVZ, which were divided into six such bins. Data are mean ± s.e.m.; n = 383–601 cells for each condition. (c, d) Quantification of BrdU+ and PH3+ transfected cells at apical (<20 µm) or more basal (>20 µm) locations relative to ventricular surface in VZ at 2 and 6 h after BrdU injection. Data are mean ± s.e.m.; n = 754–1,009 cells for each condition. *** P < 0.001; NS, P > 0.05 compared with scr#1 (c) or vector (d); Student’s t-test. Right: representative images of RGCs expressing EGFP and Dock7#2 shRNA (c) or Flag-DOCK7 (d) immunostained for EGFP, PH3 and centrosomal marker γ-tubulin. Scale bars, 10 µm. For details on quantifications, see Supplementary Data.

To gain further insight into how altered DOCK7 expression could affect the progenitor pool size, we analyzed the proliferative status of VZ progenitors in more detail by performing BrdU pulse-labeling, to visualize S-phase cells, combined with Ki67 staining to reveal cells in the cell cycle. First we electroporated embryos at E13.5, 2 d later we administered a 24-h pulse of BrdU, and then we quantified the fractions of transfected cells that entered the cell cycle (BrdU*Ki67+) or withdrew from the cell cycle (BrdU*Ki67−) (Fig. 2i–n). The percentage of BrdU and Ki67 double-positive cells was elevated in the Dock7#2 shRNA group (Fig. 2j) and diminished in the Flag-DOCK7 group (Fig. 2i). Conversely, the percentage of BrdU-positive and Ki67-negative cells was diminished in the Dock7#2 shRNA group (Fig. 2k) and elevated in the Flag-DOCK7 group (Fig. 2l). These data indicate that DOCK7 knockdown favors maintenance of cells as cycling progenitors, whereas ectopic DOCK7 expression promotes cell cycle exit. In a second set of experiments, we delivered a short BrdU pulse (30 min) and measured the VZ ratio of cycling Ki67-positive progenitors in S phase (BrdU*) to Ki67-positive progenitors. This ratio was not significantly different between Dock7#2 shRNA, Flag-DOCK7 and control transfected groups (Supplementary Fig. 5a, b), implying that altering DOCK7 levels does not appreciably affect cell cycle duration. In concurrence, DOCK7 knockdown in the mouse neuroblastoma Neuro-2A cell line did not alter cell cycle distribution, as revealed by flow cytometric analysis (Supplementary Fig. 5c). Thus, DOCK7 likely affects progenitor pool size by influencing cell cycle exit or reentry, but not cell cycle duration of VZ progenitors.

DOCK7 is required for the genesis of neurons

On the basis of our findings that DOCK7 knockdown expands the VZ progenitor pool and ectopic DOCK7 expression reduces it, we reasoned that DOCK7 could influence the switch of RGCs from proliferation to differentiation and neurogenesis. To assess this, we electroporated E13.5 embryos with a vector expressing Dock7#2 shRNA or Flag-DOCK7, together with an EGFP-NLS or EGFP expression plasmid, and 2 d later determined the number of transfected RGCs, basal progenitors and neurons. To identify RGCs and basal progenitors, we immunostained brain slices for the markers Pax6 and Tbr2, respectively. DOCK7 knockdown resulted in more Pax6-positive cells but fewer Tbr2-positive cells as compared to numbers in control scr#1 shRNA–electroporated cortices (Fig. 3a–d). Conversely,
ectopic DOCK7 expression caused a decrease in Pax6-positive cells but an increase in Tbr2-positive cells, as compared to those in the control vector condition (Fig. 3c–h). Thus, DOCK7 depletion promotes amplification of the RGC pool, whereas DOCK7 overexpression diminishes this pool and promotes the transition from RGCs to basal progenitors.

We further found that the number of EGFP-positive neurons was decreased in cortices of embryos electroporated with Dock7#2 shRNA–expressing vector. This was first revealed by altered cell distribution across the neocortical layers. Newly generated neurons at E15.5 typically migrate toward the cortical plate through the intermediate zone, whereas self-renewing RGCs remain in the VZ. As compared to the control group, Dock7#2 shRNA–transfected cells were more abundant in the VZ and less abundant in the cortical plate and intermediate zone (Fig. 3i,j). We also observed a decrease in the percentage of transfected cells in the SVZ, where basal progenitors reside. Furthermore, the percentage of Tuj1-positive transfected cells was significantly lower in the Dock7#2 shRNA group (Fig. 3k). When E13.5 or E15.5 cortices were electroporated and analyzed 8 or 9 d later, at P1.5 and P4.5, respectively, these perturbations persisted. Whereas in the control groups most transfected cells were located in the cortical mantle at P1.5 and P4.5, with only a small fraction (<10%) residing in the proliferative VZ or SVZ, in the Dock7#2 shRNA groups ≥40% of the transfected cells still resided in the VZ or SVZ, and the percentage of transfected cells in the cortical mantle was correspondingly reduced (Supplementary Fig. 6a,d,g)). Consistently, the percentage of Tuj1-positive transfected cells was considerably lower in the Dock7#2 shRNA and scr#1 shRNA groups (Supplementary Fig. 6b,c,h,k). We did not detect a significant difference in the percentage of GFAP-positive transfected cells between the Dock7#2 shRNA and scr#1 shRNA groups (Supplementary Fig. 6c,f,i,l), implying that DOCK7 knockdown does not induce premature astrocyte differentiation. These data indicate that silencing DOCK7 impairs the genesis of neurons.

When examining the effects of DOCK7 overexpression, we observed a decrease in the percentage of transfected cells in the VZ, an increase in the SVZ and intermediate zone, but, notably, a decrease in the cortical plate (Fig. 3l,m). The number of Tuj1-positive transfected cells was significantly increased upon DOCK7 expression (Fig. 3n); however, most of these cells resided in the intermediate zone, and relatively few were detectable in the cortical plate (Fig. 3l,m). Of note, most of the cells located in the intermediate zone displayed a multipolar phenotype. These data suggest that ectopic DOCK7 expression promotes the genesis of neurons, which are able to reach the intermediate zone, but are defective or delayed in migrating toward the cortical plate, possibly owing to a defect in the transition from multipolar to bipolar phase. Thus, in addition to controlling the genesis of neurons, DOCK7 may also influence the polarization and/or migration of intermediate zone neurons.

**DOCK7 is important for interkinetic nuclear migration**

We next asked how DOCK7 affects neurogenesis and controls RGC proliferation versus differentiation. Cellular parameters that influence RGC progenitor output include cell cycle duration, cell polarity and asymmetric inheritance, and, as shown more recently, INM12–14,19,23–26. Altering DOCK7 levels did not affect cell cycle duration (Supplementary Fig. 5). Also, DOCK7 did not appear to directly affect RGC polarity and adhesion, revealed by RGC coupling by adherens junctions to the apical side of the neuroepithelium (Supplementary Fig. 7). However, a hint of a DOCK7 function in INM came from our BrdU labeling experiments, where we noted a difference in the location of BrdU-labeled nuclei of cells expressing Dock7#2 shRNA and Flag-DOCK7 (Fig. 2a,e). As compared to the control group, the distribution of the BrdU-positive nuclei was shifted toward the apical surface in the Dock7#2 shRNA group, whereas in the Flag-DOCK7 group the nuclei were positioned more basally in the upper VZ. To further establish whether and how altered
DOCK7 expression affects INM, we examined in more detail the migration of RGC nuclei concurrent with cell progression from S to M phase (Supplementary Fig. 8). To this end, we electroporated embryos at E13.5, administered BrdU 2 d later and determined the positions of BrdU-labeled nuclei of transfected cells 15 min, 2 h, 4 h, and 6 h after BrdU injection (Fig. 4).

At the 15-min time point, most BrdU-labeled nuclei of control, Dock7#2 shRNA, and Flag-DOCK7 expressing VZ cells were located in the upper half of the VZ, consistent with the known location of RGC nuclei in S phase (Fig. 4a,b). BrdU-labeled control nuclei began to migrate toward the ventricular surface within 2 h, and continued to do so for about another 4 h (Fig. 4a,b), in concurrence with an 5 phase duration of ~4 h and G2 phase duration of ~2 h at this developmental stage33. After reaching the ventricular surface, they entered apical mitosis (<20 μm from ventricular surface), as judged by their PH3 immunoreactivity (Fig. 4c,d). DOCK7 knockdown accelerated the bl-to-ap INM (Fig. 4a). Whereas only a few control BrdU-labeled nuclei reached the apical region of the VZ after 2 h, a much larger fraction of BrdU-labeled nuclei of transfected cells from the Dock#7#2 shRNA group had already reached this location (Fig. 4a), where most of them stayed for another ~4 h, only to then enter mitosis at the apical surface (Fig. 4c). Indeed, we observed more BrdU+PH3+ VZ cells in the Dock7#2 shRNA group than in the control group at 6 h, but not 2 h, after BrdU injection (Fig. 4c). Conversely, when examining the nuclei position of Flag-DOCK7 expressing VZ cells, we noted that the bl-to-ap migration of BrdU-labeled nuclei was delayed, with many of the nuclei remaining in the upper half of the VZ 6 h after BrdU injection (Fig. 4b). This extended stay at basal locations was associated with an increased number of mitoses away from the apical surface, as judged by the higher percentage of BrdU+PH3+ cells at basal positions (>20 μm from ventricular surface) in the DOCK7 group than in the control vector group (Fig. 4d).

To corroborate and extend the above findings, we carried out time-lapse imaging on acute cortical slices 2 d after in utero electroporation of plasmids expressing empty control vector, Flag-DOCK7, scr#1 shRNA or Dock7#2 shRNA (Fig. 5). We also included plasmids expressing EGFP, to fluorescently label the cytoplasm of the RGCs, and monomeric Kusabira Orange 2, which contains a Cys-Ala-Ala-Xaa farnesylation motif (mKO2-F), to fluorescently label the plasma membrane. Slices were imaged for 8 to 10 h. We found that nucleus-surrounding cell bodies of most control vector–expressing cells migrated steadily toward the ventricle and cells divided at the apical surface (Fig. 5a,e and Supplementary Video 1). In contrast, cell bodies of a substantial fraction of DOCK7-overexpressing cells remained at basal locations and the cells divided away from the ventricular surface (Fig. 5b,e and Supplementary Video 2). We observed some cell bodies of DOCK7-overexpressing cells to move slowly toward the ventricle. However, the distance traveled was short, and those cells divided away from the ventricular surface (Supplementary Video 3). These findings support the idea that DOCK7 overexpression impedes bl-to-ap INM, leading to extended residence time of RGC nuclei at basal locations and ectopic mitoses. Conversely, cell bodies of most Dock7#2 shRNA–expressing cells migrated considerably faster to the ventricular surface than those of control scr#1 shRNA–expressing cells, where, notably, they then resided for several hours before undergoing apical mitoses (Fig. 5c–e and Supplementary Videos 4–6). These findings corroborate the idea that DOCK7 knockdown accelerates bl-to-ap INM, leading to extended residence of RGC nuclei at apical locations and apical mitoses. Taking these findings together, we conclude that DOCK7 critically regulates the bl-to-ap INM process.

DOCK7 interacts with TACC3

To gain insight into the molecular mechanism(s) by which DOCK7 controls INM and neurogenesis, we first explored whether DOCK7’s...
GEF activity is involved. DOCK7, like other DOCK180 members, contains a conserved DHR2 domain that catalyzes the exchange of GDP for GTP on Rac and/or Cdc42 (refs. 38, 44, 45). This domain is required for DOCK7’s role in axon formation44. To determine whether it is important for DOCK7’s role in INM and genesis of neuronal, we tested whether a DOCK7 DHR2 mutant44 could rescue DOCK7 RNAi-evoked effects on INM, RGC expansion and neuron production. Notably, DOCK7ΔDHR2 rescued all phenotypes to a similar degree as that seen with DOCK7 wild type (see below), implying that DOCK7’s DHR2 domain and GEF activity toward Rac and/or Cdc42 are dispensable for its role in INM and cortical overgrowth.

To identify other potentially relevant DOCK7 functions, we decided to search for DOCK7-interacting proteins other than small GTPases. To this end, we used the yeast two-hybrid approach to screen an embryonic mouse brain cDNA library with three different DOCK7 fragments as bait (Fig. 6a). One of these fragments, DOCK7 amino acids 506–1164 (DOCK7-R2), yielded two positive clones containing identical cDNAs matching the TACC domain–encoding sequence of TACC3 and another containing a TACC3 cDNA encoding full-length TACC3. TACC3 is a member of the TACC family, centrosome- and microtubule-associated proteins implicated in centrosome-directed microtubule growth, nuclear migration and, notably, maintenance of the neural progenitor pool during mouse neocortical development12, 46, 47.

We validated the interaction between DOCK7 and TACC3 by several approaches. First, we confirmed the yeast two-hybrid interaction (Fig. 6b) by coimmunoprecipitation experiments, using lysates from a human embryonic kidney cell line (HEK293) expressing Flag-DOCK7 and EGFP-TACC3 (Fig. 6c). Second, we demonstrated the association between DOCK7 and TACC3 by pull-down experiments using glutathione S-transferase (GST)-TACC3 fusion protein. Beads loaded with GST-TACC3 efficiently pulled-down DOCK7 from lysates of E13.5 mouse cortices (Fig. 6d), implying that DOCK7 and TACC3 interact, with the R2 fragment of DOCK7 sufficient for binding. To further delineate the TACC3-binding domain in DOCK7, we generated mutants containing deletions in the R2 fragment (Fig. 6a) and
Figure 8 DOCK7 antagonizes microtubule growth–promoting or microtubule-stabilizing function of TACC3.  
(a,b) DOCK7 antagonizes TACC3’s ability to increase microtubule aster size in COS7 cells. (a) Immunostaining for α-tubulin (tub) and counterstaining with DAPI of COS7 cells expressing Flag-DOCK7 (DOCK7) and EGFP-TACC3 (TACC3), alone or in combination. (b) Measurement of microtubule (MT) aster size. The average aster size of control vector transfected cells was set to 1. \( n = 69–115 \) cells for each condition. 
(c,d) Concurrent silencing of TACC3 prevents enlargement of microtubule fork-like structure caused by DOCK7 knockdown in cultured neocortical cells. E12.5 mouse neocortices were transfected with plasmids expressing EGFP and scr#1 or Dock7#2 shRNA, RFP and Tacc3#1 shRNA, or both Tacc3#1 and Dock7#2 shRNAs together with EGFP and RFP, and were dissociated at E13.5. Cells were cultured for 2 d and immunostained for α-tubulin and counterstained with DAPI. (c) Representative images showing microtubule fork-like structure in transfected neocortical cells. (d) Measurement of perinuclear microtubule bundle size of fork-like structure. The average bundle size of scr#1 shRNA transfected cells was set to 1. \( n = 77–103 \) cells for each condition. (e,f) Silencing TACC3 rescues decrease in nucleus–centrosome distance caused by DOCK7 knockdown in RGCs in vivo. E13.5 embryos were electroporated as in c and examined at E15.5. (e) Brain slices with transfected RGCs, immunostained for γ-tubulin and for EGFP and/or RFP and counterstained with DAPI. Arrowheads indicate centrosomes of transfected cells. (f) Quantification of nucleus–centrosome distance, \( n = 215–371 \) cells for each condition. All data are mean ± s.e.m.; *** \( P < 0.001 \); NS, \( P > 0.05 \) compared with vector (b) or scr#1 (d,f); one-way ANOVA. For details on quantifications, see Supplementary Data. Scale bars, 10 µm (a) and 5 µm (c,e).

DOCK7 controls INM and neurogenesis by antagonizing TACC3

We next investigated the relevance of the DOCK7-TACC3 interaction in INM and neurogenesis. A previous study showed that knockdown of TACC3 halts bl-to-ap INM, decreases the number of proliferating progenitors and increases neuron production32, all opposite to the effects of DOCK7 knockdown. Because the previous study used a mixture of short interfering RNAs targeting all three TACC family members, we first investigated whether knockdown of TACC3 alone is sufficient to produce the above phenotypes. This was indeed the case: knockdown of TACC3 impaired bl-to-ap INM, decreased BrdU labeling and the mitotic index, and increased the percentage of Tuj1+ cells (Fig. 7a–e). These data indicated that DOCK7 and TACC3 have opposing functions during cortical neurogenesis. Therefore, we asked whether DOCK7 exerts its effect on INM and neurogenesis by antagonizing TACC3 function.

If this is the case, simultaneous knockdown of TACC3 and DOCK7 should counteract the phenotypes resulting from DOCK7 deficiency, whereas a DOCK7 mutant defective in TACC3 binding should fail to rescue them. To test the former, we electroporated E13.5 cortices with plasmids coexpressing Dock7#2 and scr#1 shRNA and EGFP-NLS (or EGFP), together with plasmids coexpressing Tacc3#1 shRNA or scr#2 shRNA and RFP-NLS (or RFP), and examined brains 2 d later. As expected, co-electroporation of vectors expressing Dock7#2 and scr#2 shRNA resulted in a higher percentage of cells dividing at apical positions, an increased number of proliferating VZ progenitors and a decrease in neuron production. However, simultaneous TACC3 knockdown completely rescued these phenotypes (Fig. 7f–j). To test whether a DOCK7 mutant defective in TACC3 binding could rescue the DOCK7 RNAi–evoked phenotypes, we co-electroporated plasmids expressing Dock7#2 shRNA and DOCK7ΔTB. In contrast to DOCK7 wild type and DOCK7ΔDH2, DOCK7ΔTB was unable to rescue the INM defect, the increase in dividing VZ progenitors and decrease in neuron production caused by DOCK7 RNAi (Fig. 7k–o). Together, these data indicate that DOCK7 controls INM and cortical neurogenesis by antagonizing TACC3 function.

DOCK7 antagonizes microtubule-associated function of TACC3

The TACC proteins were reported to control INM of cortical neural progenitors by regulating the growth and integrity of centrosome-associated microtubules coupling the centrosome and nucleus32. Hence, we assessed whether DOCK7 antagonizes the microtubule growth–promoting function of TACC3. We first examined this in an African green monkey kidney fibroblast-like cell line (COS7), as TACC3 expression in these cells has been shown to increase the size of the microtubule aster emanating from the centrosome32. In contrast to TACC3 expression, DOCK7 expression decreased the size of the microtubule aster, and, notably, expression of DOCK7 with TACC3 restored the microtubule aster size to that seen in control vector–transfected cells (Fig. 8a,b). To determine whether these manipulations affected microtubule nucleation, we treated COS7 cells with nocodazole (which depolymerizes microtubules) and allowed them to recover after drug washout. After a 5-min recovery period, most of the transfected cells in all groups showed a clear microtubule aster, indicating that microtubule nucleation was not affected. However, after 20 min of recovery, cells expressing DOCK7 and TACC3 showed, respectively, smaller and larger microtubule asters, like those in Figure 8a, as compared to control cells and cells coexpressing DOCK7 and TACC3 (Supplementary Fig. 9).
We then extended these studies to cortical neural progenitors. These cells display a microtubule ‘fork’-like structure consisting of two or more prominent microtubule bundles that couple the centrosome and the nucleus. Whereas knockdown of TACC3 in cultured cortical progenitors decreased the size of the microtubule fork-like structure, knockdown of DOCK7 increased the overall size (Fig. 8c,d). Notably, concurrent knockdown of TACC3 with DOCK7 restored the size of the microtubule fork-like structure to that seen in control cells (Fig. 8c,d).

Finally, we measured the nucleus–centrosome distance in RGCs expressing Dock7#2 shRNA, Tacc3#1 shRNA or both, as this distance is affected by alterations in microtubule growth and integrity. Brain slices prepared from electroporated embryos were stained with γ-tubulin (centrosomal marker) and counterstained with DAPI (nuclear marker). In contrast to Tacc3#1 shRNA expressing RGCs, where the nucleus–centrosome distance was increased, this distance was considerably decreased in Dock7#2 shRNA–expressing RGCs and could be restored to that seen for scr#1 expressing RGCs by coexpression of Tacc3#1 shRNA (Fig. 8e,f). Combined with the above data, these findings indicate that DOCK7 antagonizes the microtubule growth–promoting or microtubule-stabilizing function of TACC3. Notably, this effect of DOCK7 is independent of its DHR2 domain, as DOCK7ΔDHR2 was able to rescue the Dock7 RNAi–evoked decrease in nucleus–centrosome distance (Supplementary Fig. 10).

DISCUSSION

Fundamental to proper development of the mammalian neocortex is the ability of RGCs to balance self-renewal with neuronal differentiation in a correct temporal manner. Key determinants reported to influence RGC function during neurogenesis include cell polarity, cell migration in a correct temporal manner. Key determinants reported to influence RGC self-renewal with neuronal differentiation and is required for proper genesis of neurons from RGCs. With the onset of neurogenesis, RGCs start switching from proliferative to differentiative neuron generating divisions. Our data implicate DOCK7 in the regulation of this switch. Knockdown of DOCK7 expanded the radial glial progenitor pool by favoring maintenance of RGCs as cycling progenitors at the apical side of the VZ. Notably, with RGC expansion, generation of basal progenitors and neurons was reduced, implying that neuronal differentiation was impaired at the RGC stage. Ectopic DOCK7 expression, by contrast, increased basal progenitor and neuron production and concomitantly reduced cycling RGCs, indicating their accelerated differentiation. These data indicate that DOCK7 regulates RGC proliferation versus differentiation and is required for proper genesis of neurons from RGCs. Our findings further imply that other DOCK180 family members do not compensate for this function of DOCK7, as knockdown of DOCK7 alone was sufficient to cause defects in neurogenesis. This is not surprising, as overall similarity among the DOCK180 members is largely restricted to the DHR2 and DHR1 domains, neither of which appears to be required for DOCK7 function in neurogenesis (Fig. 7 and Supplementary Fig. 11). Also, nonredundant functions for several DOCK180 family members have been previously reported. Although mice carrying a nonsense mutation in the Dock7 locus, originating from a chemical mutagenesis screen, were not reported to exhibit gross neurological or behavioral abnormalities in tests probing innate and/or anxiety- or stress-related behavior, it will be interesting to assess them for specific cognitive or other impairments linked to cortical dysfunction.

Our data support a model in which DOCK7 influences the mode of RGC division and genesis of neurons through its regulatory effects on bl-to-ap INM. Ectopic DOCK7 expression impeded bl-to-ap INM of RGCs, without affecting cell cycle progression. This led to expanded residence of RGC nuclei at basal locations and mitoses at ectopic sites away from the ventricular surface, producing daughter cells that likely differentiated into basal progenitors and/or neurons, as the numbers of both were increased upon DOCK7 overexpression, while RGCs were decreased. In line with this, increased neurogenesis has been reported when INM is impaired upon Cep120 or Hook3 knockdown. Conversely, DOCK7 knockdown accelerated the movement of RGC nuclei from bl-to-ap positions, resulting in extended apical residency of RGC nuclei and apical mitoses. This was associated with an increase in RGCs and reduction in basal progenitors and neurons, implying that extended nuclear residence at the apical side favors symmetrical division of RGCs, with two RGCs as progeny. In concurrence with our model are reports indicating enrichment of proliferative signals at the apical versus the basal side of the neuroepithelium. Thus, we posit that DOCK7 controls the bl-to-ap INM step and thereby influences RGC behavior and neurogenesis. Of note, our studies revealed that progenitors underwent a phase at their normal basal position regardless of the levels of DOCK7 expression, consistent with normal ap-to-bl basal migration of RGC nuclei during the G1 phase.

Although Rho GTPases regulate INM, we found that DOCK7’s effects on INM and neurogenesis did not require its catalytic DHR2 domain and hence are not mediated by DOCK7’s GEF activity toward Rac or Cdc42. Instead, evidence from our studies indicates that DOCK7 exerts its effects by antagonizing the centrosome-associated microtubule growth–promoting or microtubule-stabilizing function of TACC3. Specifically, the two proteins exerted opposing effects on centrosomal microtubule growth, and on INM and neurogenesis. Furthermore, the phenotypes associated with DOCK7 knockdown, including microtubule growth, INM and genesis of neurons, were rescued by reducing TACC3 levels. Finally, a DOCK7 mutant defective in TACC3 binding was unable to rescue the Dock7 RNAi–evoked phenotypes. We posit that fine-tuned regulation of the microtubule–associated function of TACC3 by DOCK7 controls the growth and dynamics of the microtubules coupling the centrosome and nucleus. As such, gain or suppression of TACC3 function facilitates or impedes, respectively, the movement of the nucleus toward the centrosome. Translocation of the nucleus along the microtubules likely involves the minus end–directed motor dynein and associated proteins, as previously reported. Future studies will be required to determine the precise mechanism(s) by which DOCK7 antagonizes TACC3’s microtubule-associated function, how DOCK7 is regulated and whether DOCK7 acts on other microtubule-controlled cellular processes.

Besides controlling the genesis of neurons, our data suggest that DOCK7 influences the polarization and/or migration of intermediate zone neurons. This was shown by an increased number of TuJ1-positive neurons in the intermediate zone, but not in the cortical plate, upon ectopic DOCK7 expression. As DOCK7’s GEF activity toward Rac and/or Cdc42 is required for axon formation of hippocampal neurons and migration of Schwann cells, we envisage DOCK7’s GEF activity also to be important for the polarization and/or migration of intermediate zone neurons. Combined, these findings imply that DOCK7 executes several distinct functions in the developing brain by engaging in distinct protein-protein interactions. Here, we show that DOCK7 controls bl-to-ap INM and the genesis of cortical neurons from RGCs, by means of interaction with the centrosome and microtubule-associated protein TACC3. As such, our study not only offers insight into DOCK7 function but also sheds light on the nature neuroscience
INM process as it pertains to the regulation of RGC proliferation versus differentiation in the developing neocortex.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.-T.Y., C.-L.W. and L.V.A. conceived and designed the project. Y.-T.Y. and C.-L.W. performed all the experiments and prepared the figures. L.V.A. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Next, whole embryos and brains were either cryoprotected in 30% sucrose in PBS at room temperature (RT; whole embryos) or overnight at 4 °C (brains of embryos). In 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 h at 37 °C. Animals were sacrificed at indicated time points following BrdU injection. Electroporation reagent Fugene 6 (Roche), as outlined by the manufacturer. Neuro2A cells were electroporated at E13.5 and BrdU (150 mg per kg of body weight) was injected at E13.5 cortices and HEK293 cells expressing Flag-DOCK7, -DOCK7∆-actin (Tacc3#1)32 were cloned into pSUPER (Oligoengine), a modified version of pSUPER in which an EGFP, EGFP-NLS (nuclear localization signal) or a Dox-induced promoter was inserted into the XhoI site of pSUPER, and in the case of Dock7#2 shRNA also into the pTRIPzα-EGFP lentiviral vector21.

Yeast two-hybrid screening. One × 10^6 clones of a mouse fetal brain cDNA library in the pPC6 vector were screened using DOCK7-R1, -R2 or -R3 fragments cloned into pC97 as baits in the pJ69a yeast reporter strain.

Cell culture and transfection/infection. HEK293, COS7, Neuro2A, N1E-115, and SK-N-BE cells were cultured in DMEM containing 10% FBS (HyClone), 4 mM t-glutamine (Gibco BRL), 100 IU/ml penicillin (Gibco BRL) and 100 µg/ml streptomycin (Gibco BRL). Ba2 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and 10% WEHI-3-conditioned medium as a source of interleukin-3 (IL-3). Dissociated cortical progenitors were prepared from E12.5 or E13.5 cortices of CD1 mice and cultured in Neurobasal medium (Gibco BRL) containing 20 ng/ml PFG2 (Promega), 10 ng/ml EGF, 2% B27 (v/v), Gibco BRL), 4 mM t-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin as described52. HEK293 cells were transfected using the calcium phosphate coprecipitation method. COS7 cells were transfected using the transfection reagent Fugene 6 (Roche), as outlined by the manufacturer. Neuro2A cells were infected with lentiviruses, which were generated as previously described11. Dissociated cortical progenitors were transfected using the Amazone Nucleofection system (Amazone, Lonza). We used 3–5 µg total of the indicated plasmids per electroporation.

In utero electroporation and BrdU injections. In utero electroporation was performed largely as described24. In brief, a timed-pregnant CD1 mouse (Charles River) at 13.5 d of gestation was anesthetized, the uterine horns were exposed and approximately 1 to 2 µl of plasmid DNA was injected manually into the lateral ventricles of the embryos using a beveled and calibrated micropipette. Fifty 0–50 µl of 40 V with a 500–900 ms interval were delivered across the uterus with two 5-mm electrode paddles positioned on either side of the head (BTX, ECM830). After electroporation, the uterus was placed back in the abdominal cavity and the wound was sutured. For BrdU-labeling experiments, mouse embryos were electroporated at E13.5 and BrdU (150 mg per kg of body weight) was injected at E15.5. Animals were sacrificed at indicated time points following BrdU injection. All animal care protocols were approved by Cold Spring Harbor Laboratory.

Immunohistochemistry and immunocytochemistry. For immunostaining of tissue sections, whole embryos (E9.5 and E10.5) or brains of embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 1 h at room temperature (RT; whole embryos) or overnight at 4 °C (brains of embryos). Next, whole embryos and brains were either cryoprotected in 30% sucrose in PBS and cut, after embedding in OCT compound, into 16-µm-thick coronal sections on a Leica CM3050S cryostat (whole embryos and brains), or were sectioned into 40-µm-thick coronal sections using a Vibratome (Leica VT1000S; brains). For immunostaining of COS7, SK-N-BE, and dissociated neocortical cells, the cells were fixed for 4 min with ice-cold 100% methanol at −20 °C. Brain sections and cells were blocked and permeabilized with 10% normal goat serum (NGS) and 0.3% Triton X-100 (or 0.1% for cells) in PBS for 1 h at RT, followed by incubation with primary antibodies diluted in 3% NGS and 0.3% Triton X-100 in PBS at concentrations indicated below at 4 °C overnight. We used the following primary antibodies: anti-DOCK7 (ref. 44, rabbit, 1:500), anti-nestin (mouse, 1:200, Chemicon MAB353), anti-Tuj1 (mouse 1:1,500, Covance MMS-435P), anti-γ-tubulin (mouse, 1:2,000, Sigma T6557), anti-EGF (chicken, 1:500, Aves Labs GFP-1020), anti-BrdU (rat, 1:500, AbD Serotec MCA2060), anti-PH3 (rabbit, 1:1,000, Millipore 05-809), anti-Ki67 (rabbit, 1:250, Vector Laboratories VP-K451), anti-Pax6 (rabbit, 1:500, Covance PRB-278P), anti-Tbr2 (rabbit, 1:300, Abcam ab23345), anti-NEF (rabbit, 1:500, Rockland 600-401-379), anti-pericentrin (mouse, 1:1,000, BD Biosciences 611814), anti-TACC3 (mouse, 1:300, Santa Cruz sc-48368), anti-œ-tubulin (mouse, 1:2,000, Sigma T9002), anti-ZO-1 (mouse, 1:100, Invitrogen 339100) and anti-GFAP (chicken, 1:500, Aves Labs GFAP). For BrdU staining, brain sections were incubated in 2 N HCl for 30 min at 37 °C to unmask the antigen, followed by a neutralization step with 0.1 M sodium tetaborate and three washes in PBS. The secondary antibodies used were Alexa Fluor 488, 594 or 647 goat anti-mouse ( Molecular Probes, respectively A-11011, A-11005, A-21235), rabbit ( Molecular Probes, A-11008, A-11012, A-21244), rat ( Molecular Probes, A-11006, A-11007, A-21247) or chicken ( Molecular Probes, A-11039, A-11042, A-21449) (1:500 for immunohistochemistry and 1:1,000 for immunocytochemistry). Nuclei were counterstained with 0.3 µg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) in PBS for 10 min. All brain slices were counterstained with DAPI; images depicting DAPI staining were not always included for reasons of clarity. Images of brain sections and cells were acquired using a spinning disk confocal microscope equipped with an environmental chamber (5% CO2, 37 °C). Pictures were taken at 10-min intervals with 20–25 z-sections (4-µm intervals). The time-lapse data were assembled and analyzed by ImageJ (NIH) and Volocity software (Improvement).

GST pull-down assays, communoprecipitation and western blot analysis. For GST pull-down assays, GST-TACC3 fusion protein and GST alone were immobilized onto glutathione-Sepharose beads (GE Healthcare BioSciences). Mouse E13.5 cortices and HEK293 cells expressing Flag-DOCK7, -DOCK7A513–812, -DOCK7∆812–931 or -DOCK7A933–1164 fusion proteins, or empty control vector, were homogenized using micropestles (Eppendorf) in lysis buffer (50 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 5% glycerol, 5 mM NaF, 1 mM Na2VO4 and protease inhibitors). Equal amounts of total lysates were incubated with GST-TACC3 fusion protein or GST. Immunoblots were probed with anti-DOCK7 (ref. 44, rabbit, 1:1,000) or anti-Flag (rabbit, 1:1,000, Sigma F4725) polyclonal antibodies. For communoprecipitations, HEK293 cells expressing EGFP-TACC3 and Flag-DOCK7, or empty control vector, were homogenized in lysis buffer. Total lysates were incubated with anti-Flag M2-agarose beads (Sigma A2220) overnight at 4 °C. Beads were washed six times with lysis buffer. Immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with anti-EGF (rabbit, 1:1,000, Invitrogen A6455) and anti-Flag (rabbit, 1:1,000, Sigma F4725) polyclonal antibodies. For western blots in Figure 1a, mouse cortices (E11–P3) were homogenized using micropestles (Eppendorf) in 75 mM Tris–HCl (pH 6.8), 3.8% SDS, 4 M urea, and 20% glycerol and subjected...
to western blot analysis with anti-DOCK7 antibody (ref. 44, rabbit, 1:1,000), and anti-γ-tubulin (mouse, 1:10,000, Sigma T6557) as a loading control. Western blot analyses depicted in Supplementary Figure 3 were carried out using anti-DOCK7 (ref. 44, rabbit, 1:1,000), anti-DOCK6 (rabbit, 1:1,000, MBL PD016) or anti-DOCK8 (rabbit, 1:1,000, provided by P. Aspenström, Ludwig Institute for Cancer Research, Uppsala, Sweden) antibody and anti-γ-tubulin (mouse, 1:10,000, Sigma T6557) as a loading control.

Analysis of microtubules. COS7 cells transfected with indicated plasmids were immunostained with antibodies against EGFP (chicken, 1:500, Aves Labs GFP-1020) and Flag (mouse, 1:1,000, Sigma F3165, to identify transfected cells) and α-tubulin (mouse, 1:2,000, Sigma T9026, to visualize microtubules), and counterstained with DAPI. Images were acquired using a PerkinElmer spinning disk confocal microscope. The area of the microtubule aster emanating from the centrosome was measured using ImageJ software. Dissociated neocortical cells were prepared from E13.5 neocortices 1 d after electroporation with indicated plasmids as described. Cells were plated on coverslips precoated with poly-d-lysine and laminin, and cultured for 2 d before fixation, immunostaining with antibodies against EGFP (chicken, 1:500, Aves Labs GFP-1020), RFP (rabbit, 1:500, Rockland 600-401-379) and α-tubulin (mouse, 1:2,000, Sigma T9026), and counterstaining with DAPI. Confocal images were acquired and the area occupied by microtubule bundles around the nucleus was measured by ImageJ. Several z-series confocal images of transfected cells were merged to show the entire microtubule fork-like structure.

Quantitative analysis of electroporated neocortices. All the quantification studies were carried out on transfected cells localized within the dorsolateral cortex. To score images, the GFP or RFP channel was first judged independently, followed by judgments of the other markers. A total of 3–6 brain sections were analyzed per animal by taking up to three 10–20-µm-thick sections parallel to the ventricle surface. The centrosome to nucleus distance in transfected RGCs was determined by measuring the distance from the centrosome to the lower edge of the nucleus. To measure the velocity of bl-to-ap nuclear migration, the change in the distance traveled by a transfected RGC (µm) was divided by the time (h) traveled, using Velocity software.

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Statistical analysis. Data were presented as mean ± s.e.m. from at least three independent experiments. Direct comparisons were made using Student’s t-test and multiple group comparisons were made using one-way analysis of variance (ANOVA). Statistical significance was defined as *P < 0.05, 0.01 or 0.001 (indicated as *, ** or ***, respectively). P values ≥0.05 were considered not significant.

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