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Arl13b-regulated activities of primary cilia are essential for the formation of the polarized radial glial scaffold

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Abstract

The construction of cerebral cortex begins with the formation of radial glia. Once formed, polarized radial glial cells divide either symmetrically or asymmetrically to balance appropriate production of progenitor cells and neurons. Upon birth, neurons use the processes of radial glia as scaffolding for oriented migration. Radial glia thus provide an instructive structural matrix to coordinate the generation and placement of distinct groups of cortical neurons in the developing cerebral cortex. Here we show that Arl13b, a cilia-specific small GTPase mutated in Joubert syndrome patients, is critical for the initial formation of the polarized radial progenitor scaffold. Through developmental stage-specific deletion of Arl13b in mouse cortical progenitors, we found that early neuroepithelial deletion of ciliary Arl13b leads to a reversal in the apical-basal polarity of radial progenitors and aberrant neuronal placement. Arl13b modulates ciliary signaling necessary for radial glial polarity. Our findings demonstrate that Arl13b signaling in primary cilia is important for the initial formation of a polarized radial glial scaffold and suggest that disruption of this process may contribute to aberrant neurodevelopment and brain abnormalities in Joubert syndrome-related ciliopathies.

Keywords

Primary cilia; neuroepithelium; cortical progenitors; ciliopathies; cerebral cortex

Introduction

The cerebral cortex emerges as a result of coordinated unfolding of radial progenitor formation, neurogenesis, neuronal migration, post-migratory neuronal differentiation and

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Author Contributions

E.A., H.H., J.G., Y.Y., and T.C. designed the experiments and supervised the project. H.H., J.G., Y.Y., N.L.U., C.S., J.L., and N.V. conducted the experiments and analyzed the data. E.A., H.H., J.G., and T.C. wrote the manuscript.

connectivity. The formation of polarized radial glia is the first step in the construction of cerebral cortex[1]. Once formed, polarized radial glial cells can divide either symmetrically or asymmetrically. Symmetric radial glial divisions occur primarily during the early stages of cortical development to expand the radial glial population. Asymmetric divisions of radial glia result in a daughter neuron and either a radial glial cell or an intermediate precursor. Temporal regulation of the ratio of asymmetric to symmetric divisions of radial glia is critical for balancing appropriate neuronal production with progenitor maintenance[2–4]. Once born, new neurons use radial processes of radial glia as permissive and instructive scaffolding for oriented migration towards their target locations. Polarized radial glia in essence provide an instructive structural matrix to coordinate the generation and placement of distinct groups of cortical neurons in the developing cerebral cortex[1, 5–8].

The initial establishment of apical-basal polarity of radial glial cells is an essential step in the orderly unfolding of cerebral cortical organization. In mice, between embryonic days 9 and 12 (E9 and E12), an undifferentiated sheet of neuroepithelial cells in the telencephalic vesicles transforms into radial progenitors. During this transformation, the psuedostratified neuroepithelial cells stop their interkinetic nuclear migration, which spans their entire apical-basal axis, begin to express specific radial progenitor markers (e.g., BLBP, Glast, and vimentin) and initiate a restricted pattern of basal-apical interkinetic nuclear movement limited to the boundaries of the ventricular zone. Importantly, the cell soma of radial progenitors remain apically positioned within the ventricular zone, with a long basal process extending towards the pial surface, thus enabling the orderly generation and guidance of new neurons in the cerebral cortex. In spite of its importance for the proper unfolding of cortical development, little is known about what determines the initial emergence of apical-basal polarity of the radial glial scaffold in the developing cerebral cortex.

Intriguingly, primary cilia, the microtubule-based, slender projections from cells, are always localized to the apical region of a radial glial cell. The significance of cilia function for cerebral cortical development and function is evident in developmental brain disorders such as Joubert, Meckel-Gruber, orofaciodigital and Bardet-Biedl syndromes (commonly referred to as ciliopathies), where disrupted cilia function and the resulting changes in cortical formation may underlie cognitive deficits and mental retardation[9–16]. Mouse mutations that affect ciliogenesis also lead to hydrocephalus, disruptions in neurogenesis and brain tumor formation[9, 12, 17–24]. Primary cilia, unique in their ability to function as sensors and conveyors of critical signals in a complex environment, may play a guiding role in the establishment of apical-basal polarity of the radial glial scaffold.

To examine this hypothesis, we used a mouse genetic model in which cilia are present, but their function is impaired. Arl13b, a small GTPase of the Arf/Arl family, mutated in Joubert syndrome patients, is specifically localized to cilia and controls the microtubule-based, ciliary axoneme structure[10, 25]. Deletion of Arl13b impairs a cilium's ability to convey critical extracellular signals such as Sonic hedgehog (Shh)[25]. Importantly, this provides us a unique entry point for understanding the role of cilia in radial progenitor development because, in contrast to most cilia null mutants (e.g., intraflagellar transport (IFT) mutants) that ablate cilia formation and all related biological signalling[11–16, 20], Arl13b mutants impair but do not destroy cilia and their downstream pathways. Thus, genetic manipulation

of Arl13b function provides an efficient model to disrupt the signaling functions of cilia and assay the resultant effect. By conditionally deleting Arl13b at distinct stages of cortical progenitor development, we show that primary cilia activity involving Arl13b is necessary for the initial establishment of the apical-basal polarity of the radial glial scaffold. Loss of Arl13b and the resultant loss of cilia function lead to a reversal of the apical-basal polarity of radial progenitors in the developing cerebral wall. As a result, the laminar organization of neurons in cerebral cortex is drastically disrupted. These findings demonstrate that primary cilia activity plays an essential role in the initial establishment of a properly polarized radial glial scaffold, which is necessary for constructing a normal cerebral cortex.

Results

Cilia-specific expression of Arl13b in radial progenitors

To map the expression of Arl13b in neuroepithelial cells and developing radial glia, we immunolabeled E9 telencephalic neuroepithelium (Figure 1A, B) and radial progenitors (Figure 1C–E) from E14 telencephalon with anti-Arl13b antibody. Arl13b expression is localized to the primary cilium of both neuroepithelial and radial progenitor cells (Figure 1 A–F). In radial progenitors, Arl13b^{+} cilia were always found in the apical, cell soma domains of these cells (Figure 1E). Further, intermediate progenitors derived from radial glial cells also display primary cilia (Figure 1D, F).

Effects of Arl13b on radial progenitor organization

Mutations in Arl13b disrupt the ciliary microtubule organization and the ability of cilia to function as conveyors of critical extracellular signals such as Sonic hedgehog (Shh). To examine whether Arl13b is involved in the emergence of apical-basal polarity of the radial glia scaffold, we first examined the radial glial organization in mutant mice carrying a null allele of Arl13b, called *Arl13b*$^{hennin(hnn)}$ [25]. The apical-basal polarity of the developing radial glial scaffold is remarkably perturbed in *Arl13b*$^{hnn/hnn}$ embryos. The soma of radial glia are ectopically located near the pial surface, instead of adjacent to the ventricle (Figure 2A, B), while their neuronal progeny are aberrantly placed near the ventricular surface (Figure 3; SFigure1). Glial endfeet-like structures are evident near the ventricular surface in mutants, instead of adjacent to the pial surface (Figure 4A–D). Although pial membrane is present, it is often discontinuous, and segments were seen on the opposite surface of the cerebral wall (Figure 4E–F). Reelin localization is also reversed in the mutants (Figure 4G–H). Further, the normal apical enrichment of markers such as β-catenin (*CTNNB1)* in radial progenitors is disrupted in mutants; β-catenin is distributed aberrantly near the pial surface (Figure 4I, J). The deletion of Arl13b also disrupts the formation of adherens junctions and the localization of Numb to the apical endfeet (Figure 4M–P). N-Cadherin localizes randomly in the mutants, instead of at apical adherens junctions (Figure 4O–P). Similarly, the normal apical enrichment of Numb is deregulated in Arl13b mutant progenitors (Figure 4M–N). Finally, defined localization of pericentrin, a centrosome marker, in the apical aspects of radial progenitors in the ventricular surface is also lost in Arl13b mutants (Figure 4K–L). These observations suggest that primary cilia dysfunction in Arl13b^{hnn} mutants disrupts the emergence of appropriate apico-basal radial glial polarity.

Neuroepithelial cells undergo interkinetic nuclear movement (INM) throughout the apico-basal extent of the neuroepithelial layer as they transform into polarized radial progenitors. Normal patterns of INM result in the apical localization of $PH3^+$, M-phase radial progenitors in the wild-type telencephalon. The significant misplacement of $PH3^+$ progenitors towards the basal surface in Arl13b mutant telencephalon (Figure 2C–F) indicates that disrupted INM may have contributed to the aberrant radial progenitor organization in Arl13b mutants. Arl13b–deficient radial progenitors ectopically divide at or near the pial surface, instead of adjacent to the ventricular surface (Figure 2C–F).

Because radial progenitors actively proliferate and support the migration of their neuronal progeny, we asked how the disrupted polarity in Arl13b mutants might affect the dual function of radial glia as neural precursors and migratory guides of newly generated neurons. As new neurons resulting from radial glial division are guided to distinct laminar positions in the developing cortex, we next examined neuronal migration and positioning in *Arl13b*^{hnn} mice. Analysis of newly generated cortical neurons with different neuron-specific markers indicates profound disruption of neuronal organization (Figure 3, SFigure 1). In control cortices, immunolabeling of different newly generated cortical neurons with Tuj-1, anti-calretinin, anti-reelin or anti-Tbr1 antibodies indicates that they migrate normally and are positioned appropriately within the emerging cortical plate; however, neuronal migration to distinct laminar positions is completely disrupted in the mutants (Figure 3, SFigure 1). Arl13b mutants have enlarged lateral ventricles, but a thin, distinguishable midline is present (SFigure 1). Corresponding to the reversed apical-basal polarity of radial progenitors, mutant neurons are ectopically placed near the ventricular surface. Often these neurons form tuber-like clusters within the developing cerebral wall (Figure 3B–F). The disrupted polarity in Arl13b mutant radial glial scaffold likely affected the subsequent migration and positioning of these cortical neurons. Axial MRI scans through the embryos further illustrate the profound perturbance in normal cerebral cortical structural organization resulting from Arl13b deletion (SFigure 2, Movies 1, 2).

Primary cilia affects the development of radial glia

To further define the role of primary cilia function in the formation and function of the polarized radial glial scaffold, we conditionally inactivated *Arl13b* at three developmental stages: just prior to the formation of radial glia from neuroepithelial cells (at E9, using Foxg1-Cre); when the radial glial population is actively expanding (at E10.5, using Nestin-Cre); and when radial progenitors are actively differentiating to generate and guide new neurons (at E13.5, using hGFAP-Cre [SFigure 3A]). To do this, we crossed the *Arl13b* floxed allele (*Arl13b*$^{lox/lox}$) to *Arl13b*$^{+/hnn}$ mice heterozygous for the respective Cre allele[26–28]. We verified the loss of Arl13b signal in progenitors following Cre-mediated recombination in these lines on a cell-by-cell basis using immunolabeling with an anti-Arl13b antibody (SFigure 3B–C E–F, H–I,). We further confirmed this by immunoblot of whole brain lysates showing a reduction in Arl13b following Cre-mediated recombination of Arl13b floxed alleles (SFigure 3D, G, J, K). In *Arl13b*$^{lox/hnn}$*; Foxg1-Cre* mice, where deletion is initiated at E9 in the neuroepithelium, we found abnormal organization of the radial glial scaffold. Radial progenitors formed, but their apical-basal polarity was lost (Figure 5A–B). As a result, neuronal positioning and layer formation is also perturbed in

these mutants (Figure 5C–H). Further, other brain structures, such as the hippocampus, where radial progenitor polarity is essential for its normal formation, are severely malformed following the disruption of primary cilia function in neuroepithelial cells (Figure 5I–J).

To examine whether Arl13b–regulated primary cilia activity after the formation of the polarized radial glial scaffold is necessary for the ongoing maintenance of radial glial polarity, we used either Nestin-Cre or hGFAP-Cre to inactivate Arl13b. Nestin-Cre initiates deletion at E10.5, soon after the initial establishment of radial progenitors, whereas hGFAP-Cre begins to delete at E13.5, well after the formation of polarized radial glial scaffold. We found that radial progenitor organization is normal in both *Arl13b*$^{lox/hnn}$*; Nestin-Cre* and *Arl13b*$^{lox/hnn}$*; hGFAP Cre* brains (Figure 5K–N), indicating that disruption of primary cilia function, via the deletion of Arl13b, is not necessary to maintain the polarized organization of radial progenitors once it formed.

To further confirm the requirement for Arl13b in the initial formation and organization of radial glial scaffold, we combined a tamoxifen-inducible *Cre* line, *CAGG-Cre*^{ERTM}, with the *Arl13b* floxed allele and injected the pregnant dams with tamoxifen to control the timing of *Arl13b* deletion in the embryos[29]. We deleted Arl13b from E6.5 and analyzed the telencephalic neuroepithelium at E9.5 (SFigure 4C–G). E6.5 injection deletes Arl13b in neuroepithelial cells prior to the formation of radial progenitors. Deletion of Arl13b was confirmed as before with immunohistochemical and immunoblot analysis (SFigure 4E–F, I). Early loss of Arl13b did not affect neuroepithelial organization at E9.5 (SFigure 4). Consistently, telencephalic neuroepithelial organization in E9.5 *Arl13b*$^{hnn/hnn}$ mutants is similar to wild-type (SFigure 4A, B). To examine the effect of Arl13b deletion after the formation of radial glia, we deleted Arl13b from E10.5 and analyzed the radial glial scaffold at E12.5 (SFigure 4G, H, J). As with the observations made earlier with different Nestin and hGFAP Cre lines, deletion of Arl13b after the formation of radial glia did not affect the organization of the radial glial scaffold. Lastly, we also deleted Arl13b from E6.5 and analyzed radial glial scaffold at E14. This early deletion of Arl13b in neuroepithelium, lead to the disruption of polarized radial glial scaffold formation and cortical neuronal organization, similar to *Arl13b*$^{hnn/hnn}$ mutants (SFigure 4M–U). Radial glial polarity is reversed and consequently neuronal laminar formation is disrupted and neurons formed ectopic tuber- like clusters in the cerebral wall (SFigure 4M–R). Together, the neuroepithelial/radial progenitor-specific inactivation of Arl13b at different developmental stages suggests that Arl13b–mediated primary cilia function is critical for the initial establishment of the apical-basal polarity in cortical radial progenitors.

Altered dynamics of cilia in Arl13b mutant progenitors

To assess the cell biological basis of the disrupted apico-basal polarity of radial glial scaffolding in *Arl13b*^{hnn} mutants, we first performed real-time observations of primary cilia activity in radial progenitors in the embryonic cortex. We labeled primary cilia in radial progenitors with 5Htr6-GFP electroporation and live-imaged GFP^{+} cilia activity in radial progenitors. These live-imaging observations demonstrate that primary cilia in wild-type progenitors extend, retract, branch and remodel in the ventricular zone (Figure 6A, C, SFigure 5, Movies 3, 4 and 5). Further, inter-cilia contacts between adjacent progenitors are

also evident (Movie 3). In contrast, primary cilia of Arl13b–deficient radial progenitors are shorter and do not actively remodel (Figure 6B, C; Movie 6). To complement the real-time imaging of radial progenitor primary cilia activity in the ventricular zone, we also measured cilia orientation and length in fixed tissue. In wild-type radial progenitors, the average length of primary cilia is 3.45 ± 0.27 μm (n=30), and at any point in time a significant majority of them were oriented towards the ventricular surface (SFigure 5E–F). In contrast, Arl13b deletion results in significantly shorter primary cilia (average length = 1.51 ± 0.11 μm [n = 30]) with disrupted morphological plasticity (Figure 6; Movie 4). Quantification of changes in cilia length, angle of cilia tip rotation and number of branching events per hour indicates the disrupted dynamism of cilia activity in Arl13 mutants (Changes in cilia length (μm): WT, 2.37±0.49, mutant, 0.3±0.1; Angle (°) of tip rotation: WT, 30.26±7.17, mutant, 12.4±4.65; Branching index: WT, 0.4±0.19, mutant, 0; Number of cilia per group=16; Values are mean ± SEM; $p < 0.05$ [unpaired, two-tailed Student's *t*-test]).

Dynamism of cilia morphology indicates changes in cilia signalling[30, 31]. A plausible mechanism behind the disrupted dynamics of primary cilia is the abnormal ciliary signaling and distribution of critical signaling receptors needed to sense extrinsic cues vital for radial progenitor development. For example, the localization of apical complex receptors, such as IgfR1, is required for the appropriate sensing of diffusible CSF-borne signals critical for the early development of radial progenitors[32]. We found that apical localization of IgfR1 is significantly disrupted in Arl13b mutants (Figure 7A–B). IgfR1 is localized to progenitor primary cilia, and the activation of IgfR signaling in cilia normally results in the accumulation of several downstream, direct signaling targets of IgfR1 (e.g., phosphorylated-Akt and phosphorylated-insulin receptor substrate 1 [IRS-1][33, 34]) at the base of the cilium (Figure 7C, G). The ciliary localization of these downstream signaling targets is significantly altered in Arl13b mutants, but is rescued by re-expression of Arl13b (Figure 7D–F, H–J). Together, these observations suggest that Arl13b–deficient cortical progenitor cilia are unable to undergo the characteristic remodeling and orientation, as well as the appropriate ciliary signaling needed to form a normal apico-basally polarized radial glial scaffold within the developing cerebral cortex.

Discussion

Formation of radial glia is the first step in the construction of the cerebral cortex. Appropriately polarized radial glia provide an instructive scaffold to coordinate the generation and placement of distinct groups of cortical neurons in the developing cerebral cortex. Our studies show that primary cilia activities regulated by Arl13b are essential for the formation of apical-basal polarized radial progenitor scaffold. Arl13b inactivation leads to cilia that are defective in their ability to respond to critical extracellular signals. Arl13b signaling thus provides a unique avenue to examine the mechanisms that modulate primary cilia function in cortical progenitors and their contribution to the formation of cerebral cortex. To define the role of Arl13b in the formation and differentiation of radial glia, we conditionally inactivated Arl13b at three distinct developmental time points: 1) just prior to the formation of radial glia from neuroepithelial cells (E9), 2) when the radial glial population is actively expanding (E10.5), and 3) when radial progenitors are actively differentiating to generate neurons (E13.5 onwards). Early deletion of Arl13b in

neuroepithelial cells leads to a drastic disruption of normal radial glial scaffold formation. Instead of a radial glial grid with soma localized to the apical end of the developing cerebral wall, adjacent to the ventricles, the apical-basal polarity of Arl13b–deficient radial progenitors is severely altered (Figures 2–5). Progenitors divide on the basal end, adjacent to the pial surface, and neurons migrate aberrantly inwards, leading to bulb-like ectopia and a complete lack of neuronal laminar organization (Figure 3). Conditional inactivation of Arl13b in telencephalic neuroepithelial cells in *Arl13b*$^{lox/hnn}$*; Foxg1-Cre* mice also leads to apical-basal polarity defects. However, conditional deletion of Arl13b activities in primary cilia following the formation of polarized radial progenitors in *Arl13b*$^{lox/hnn}$*; Nestin-Cre* or *Arl13b*$^{lox/hnn}$*; hGFAP-Cre* mice does not affect their organization. Although radial progenitor polarity is perturbed in both *Arl13b*$^{hnn/hnn}$ and *Arl13b*$^{lox/hnn}$*; Foxg1-Cre* mutants, additional differences in radial progenitor disruptions between *hnn/hnn* and *Arl13b*$^{lox/hnn}$*; Foxg1-Cre* mutants (e.g., the formation of convoluted collections of radial progenitors in Foxg1-Cre mutants [Figure 5B]) needs further exploration. Together, these observations suggest that Arl13b–mediated primary cilia activity in neuroepithelial cells is essential for the appropriate formation of radial progenitor scaffold in the developing cerebral cortex. CAG-Cre induced deletion of Arl13b at different stages of telencepahlic development (SFigure 4) further supports this conclusion.

Disrupted radial progenitor development in Arl13b mutants underlies the aberrant formation of cortex and neuronal heterotopias. Using neuron-specific inactivation of Arl13b, we found that neuronal disruption of Arl13b does not lead to heterotopia. Since deletion of Arl13b in newborn, radially migrating projection neurons does not affect their migration or layer formation[35], disrupted Arl13b activities in progenitors in Arl13b mutants exerts a significant effect on the eventual organization of the cerebral cortex.

Live-imaging indicates that primary cilia of cortical progenitors are surprisingly dynamic, reflecting their ability to probe their environment for critical signals. The dynamism of cilia activity in progenitors suggests that cilia are likely to function as sensors of critical signals in the complex telencephalic environment[12–14, 20, 35]. Primary cilia actively relocalize within the apical processes or cell soma (Figure 6; SFigure 5; Movies 3–5). As they relocalize, they also extend, retract or branch, and make contacts with adjacent cilia (Movies 3, 5). Deletion of Arl13b disrupts these activities and suggests that Arl13b's role is in primary cilia. Recent studies have shown that remodeling of cilia morphology is reflective of changes in cilia signalling[30–31, 36]. Accordingly, Arl13b–deficient cilia appear to be compromised in their IgfR1 signaling functions (Figure 7). In addition, imaging of the localization of critical signaling molecules, such as Smo, indicates that following Arl13b deletion, localization of these molecules within the primary cilium is altered[37, 38]. Further, any changes in the cellular positioning of primary cilia may also compromise the progression of progenitor differentiation[39].

As neuroepithelial cells undergo interkinetic nuclear movement throughout the apico-basal extent of the neuroepithelial layer, prior to transformation into radial progenitors, disruption of cilia activities in Arl13b mutants may have perturbed their ability to sense and respond appropriately to the environmental signals necessary for the neuroepithelial-radial progenitor transformation[40]. Thus, in combination, the altered signaling capabilities and the disrupted

plasticity of *Arl13b*^{hnn} mutant primary cilium are likely to have rendered it incapable of processing critical signals essential to establish the apical-basal polarity of radial progenitors. For example, recent studies revealed that diffusible cerebrospinal fluid (CSF) signals presented to the apical surfaces of cortical progenitors play an essential role in organizing the apical domain of cortical progenitors[32]. The primary cilium is likely to be an important sensor of these signals, and disruption of primary cilium in Arl13b mutants may have led to an inability to sense and process such signals, resulting in the loss of apical-basal polarity in Arl13b mutants. Consistently, Igf1R localization in the apical domain of progenitors, necessary to generate and maintain apical progenitor polarity via CSF-borne Igf ligands[32], is lost in Arl13b mutants.

Although the polarity was altered, radial progenitors continued to divide and generate new intermediate precursors and neurons from the apical surface; however, these neurons appear to be unable to migrate and organize themselves into layers. They migrated away from the radial progenitor cell soma region towards the ventricular surface, but they often collected in large undefined tuber-like ectopias (Figure 2). These observations suggest that, even with disrupted polarity, neurogenesis could proceed to some extent, yet the oriented migration and laminar organization of neurons rely critically on the appropriately polarized radial glia scaffold.

Signals derived from meninges are known to be essential for some of the critical initial steps of corticogenesis, such as radial glial endfeet formation and the invasion of layer 1 Cajal-Retzius cells into the cerebral wall[41]. Notably, in spite of malformed, mislocalized pial membrane in Arl13b mutants, radial glial cell endfeet formed, and the migration of reelin^{+} Cajal-Retzius cells into the cerebral wall occurred (albeit aberrantly), suggesting that meninges-independent mechanisms may also influence these developmental landmark events in the cerebral cortex.

Three separate mutations in either the GTP-binding domain or the coiled-coil region of Arl13b have been linked to Joubert syndrome[10]. Joubert syndrome patients often display cognitive impairments indicative of disrupted cortical development. The molar tooth sign of malformed superior cerebellar peduncles and cerebellar malformations are the main MRI-based diagnostic features of Joubert syndrome patients. Other changes, such as periventricular heterotopias, hippocampal malformation, temporal lobe hypoplasia and enlarged ventricles, are also seen in some Joubert syndrome patients[42]. Expression of human Arl13b mutations in a cell type-specific manner in different Arl13b null neural cells will help determine the relevance of Joubert-causing mutations to the variety of patient brain phenotypes observed. While the developmental effects of the mutations identified in human Joubert syndrome patients remain to be fully elucidated, the analysis of Arl13b–related primary cilia function in cortical progenitors suggests that disruptions in the early organization of radial progenitor scaffold in ciliopathies and the resultant changes in the functional organization of neurons may contribute to the brain structure and cognitive deficits seen in these disorders.

Methods

Mice

Mice were cared for according to animal protocols approved by the University of North Carolina and Emory University. Light/dark cycle in the vivarium is 7/7 hours. Animals were housed in groups of 3 adults per cage. Arl13b null mice (*Arl13b*$^{hnn/hnn}$) were generated as described in Caspary et al., 2007[25]. Arl13b was conditionally inactivated in cortical progenitors by crossing *Arl13b*$^{Lox/Lox}$ mice, in which Arl13b exon 2 is flanked by LoxP sites (*Arl13b*^{tm1Tc}, MGI: 4948239, Su et al., 2012[29]), with *Cre*$^{+}$;*Arl13b*$^{+/hnn}$ mice. Four different Cre lines, *Foxg1-Cre*[26], *Nestin-Cre*[27], *hGFAP-Cre*[28] and *CAGG-CreERTM*[29] were used to inactivate Arl13b in neuroepithelial cells and radial progenitors at different developmental time points. Littermate *Arl13b*$^{lox/+}$ *Cre*$^{+}$ mice served as controls. For inducible deletion of Arl13b using the *CAGG-Cre*^{ERTM} line, tamoxifen was injected as previously described[29]. *Arl13b*^{hnn} and *Arl13b*$^{lox/lox}$ lines are maintained on C3H/HeJ background.

Immunohistochemistry

Immunohistochemical labeling of embryonic brain sections or isolated neural cells was performed as described earlier[43–45]. The following primary antibodies were used: Anti-Arl13b (Caspary et al., 2007), RC2 (Iowa Hybridoma), anti-Brn1 (gift from Dr. A. Ryan, McGill University), anti-Zic (gift from Dr. J. Aruga, Riken Brain Science Institute, Japan), anti-BLBP (LV1392131, Chemicon, R&D Systems), anti-Tbr1 (AB31940, Abcam), anti-Cux-1 (SC13024, Santa Cruz Biotechnology), anti-Ctip2 (Abcam, AB18645), anti-phospho-histone (Millipore, 06–570), anti-BrdU (Becton Dickinson, 347580), anti-pIRS-1 (SC17200, Santa Cruz Biotechnology), anti-pAKT (S473, Cell Signaling), anti-N-Cadherin (Zymed, 11268187), anti-Reelin (MAB5366, Millipore), anti-ACIII (SC32113, Santa Cruz Biotechnology), anti-Numb (07–147, Upstate), and anti-β-catenin (C2206, Sigma). Appropriate Cy2, Cy3 or Alexa dye-conjugated secondary antibodies (Jackson ImmunoResearch, Molecular Probes) were used to detect primary antibody binding. DRAQ5 (Alexis) or Nissl (Molecular probes) was used as nuclear counterstain.

Cortical progenitor assays

Cortical progenitors from WT and *hnn/hnn* embryos were established and transfected with Arl13b DNA as described in Yokota et al., 2009[45].

Magnetic Resonance Imaging

Wild type and Arl13b$^{hnn/hnn}$ embryos at e12.5 were imaged by Numira Biosciences (http://www.numirabio.com).

Analysis of primary cilia activity in the developing cerebral cortex

Live-imaging analysis of GFP^{+} primary cilia in WT and Arl13b–deficient embryonic cortices was performed as follows. Lateral ventricles of embryonic day 11–13 wild-type and littermate mutant mouse embryos were injected with 2.5 μl of a plasmid mixture containing 3 μg/μl Arl13b–GFP, 5Htr6-GFP[46], and/or pBLBP-DsRed2 DNA with 0.001% fast green, and were then subjected to electroporation as described previously[8]. Following

electroporation, cortices were removed from the embryos, coronally sectioned (200 μm) in a vibratome (Leica VT 1000S), mounted on nucleopore membrane filters and maintained in DMEM/10% FBS at 37°C/5% CO_2. In some experiments, dissected halves of embryonic cortices were whole-bath electroporated with specific DNA mix prior to slicing as described in Yokota et al. 2007. Within 24 hours, XFP^+ primary cilia in the medio-dorsal region of the cerebral cortex were repeatedly imaged at 5–10 minute intervals using an Olympus inverted microscope attached to a FluoView confocal laser scanning system and a live-cell incubation chamber. Changes in cilia length, angle of tip rotation and number of branching events (branching index) were measured during one hour of observation.

Statistical analysis

Statistical analyses used for each experiment are provided in the figure legends or text. GraphPad or Excel was used for data analysis. Two-tailed Student's *t*-test and One way ANOVA with Tukey-Kramer multiple comparison test were performed using GraphPad. Data distribution was assumed to be normal. Data were collected and processed blindly. No statistical methods were used to predetermine sample size. But sample sizes are similar to those described in previous related publications[36, 38, 43–45].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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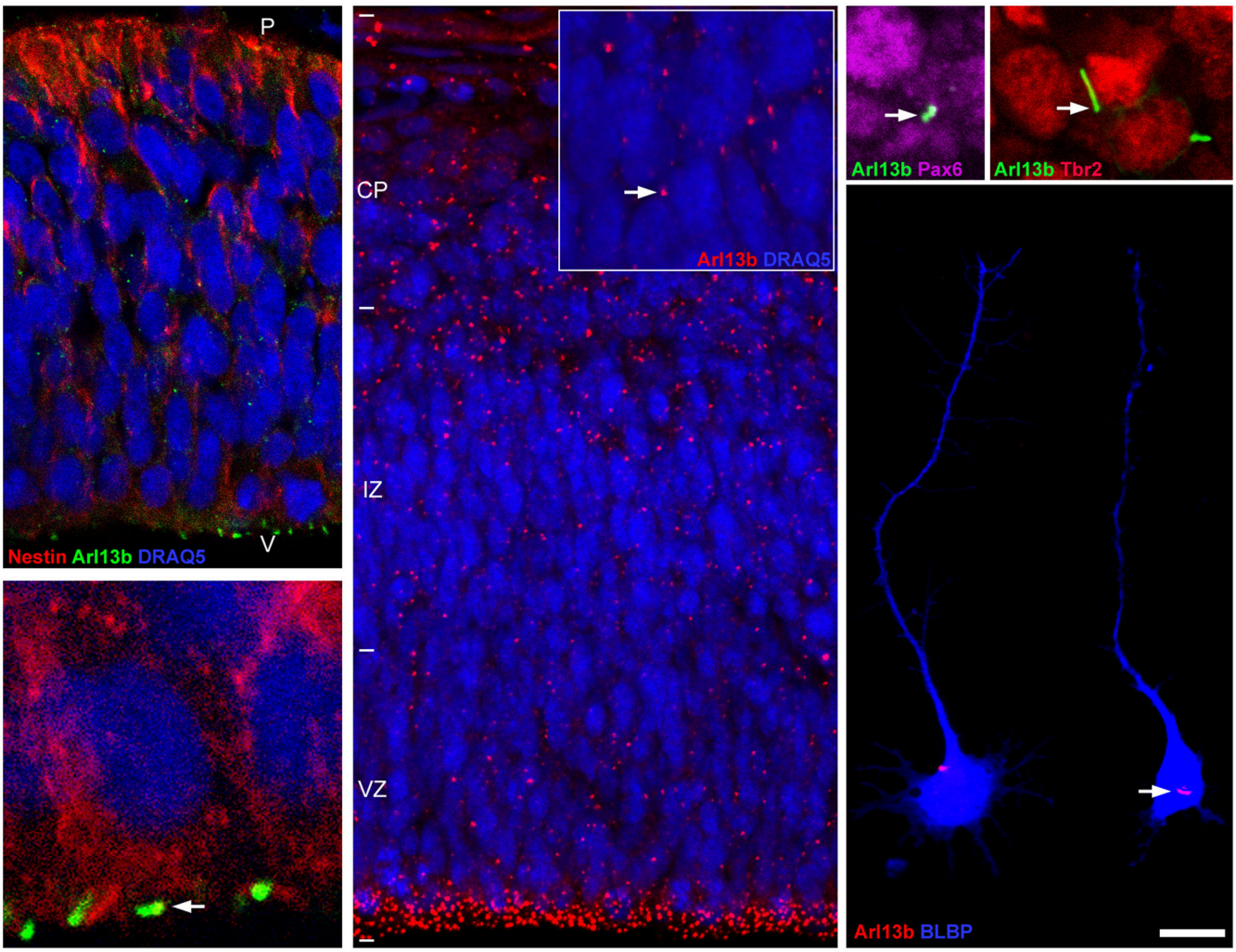

Figure 1. Primary cilia in neuroepithelial cells and cortical progenitors

(A) Primary cilia (green dots) in a cross section of telencephalic neuroepithelium [E9] were labeled with anti-Arl13b antibodies. Neuroepithelial (red) cells were labeled with anti-nestin antibodies. (B) Higher magnification view of neuroepithelial cells with primary cilia (arrow). (C) Primary cilia (red dots) in a cross section of the developing cerebral wall [E14] were labeled with anti-Arl13b antibodies. Higher magnification inset (C) illustrates primary cilia (arrow) in VZ cells. (D–F) Different types of cortical progenitors were co-labeled with cell type-specific and anti-Arl13b antibodies. Radial progenitors ($Pax6^+$[purple], D; $BLBP^+$[blue], E) and intermediate progenitors ($Tbr2^+$[red], F) possess primary cilia (arrow, D–F). Cells in A–C were nuclear counterstained (blue) with DRAQ5. Ventricular surface is towards the bottom in panels C (inset), D and F. Panels shown are representative examples from analysis of ten embryos per group. P, pial surface; V, ventricular surface; VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bar: A, 5 μm; B, D, F, 14 μm; C, 34 μm; E, 20 μm.

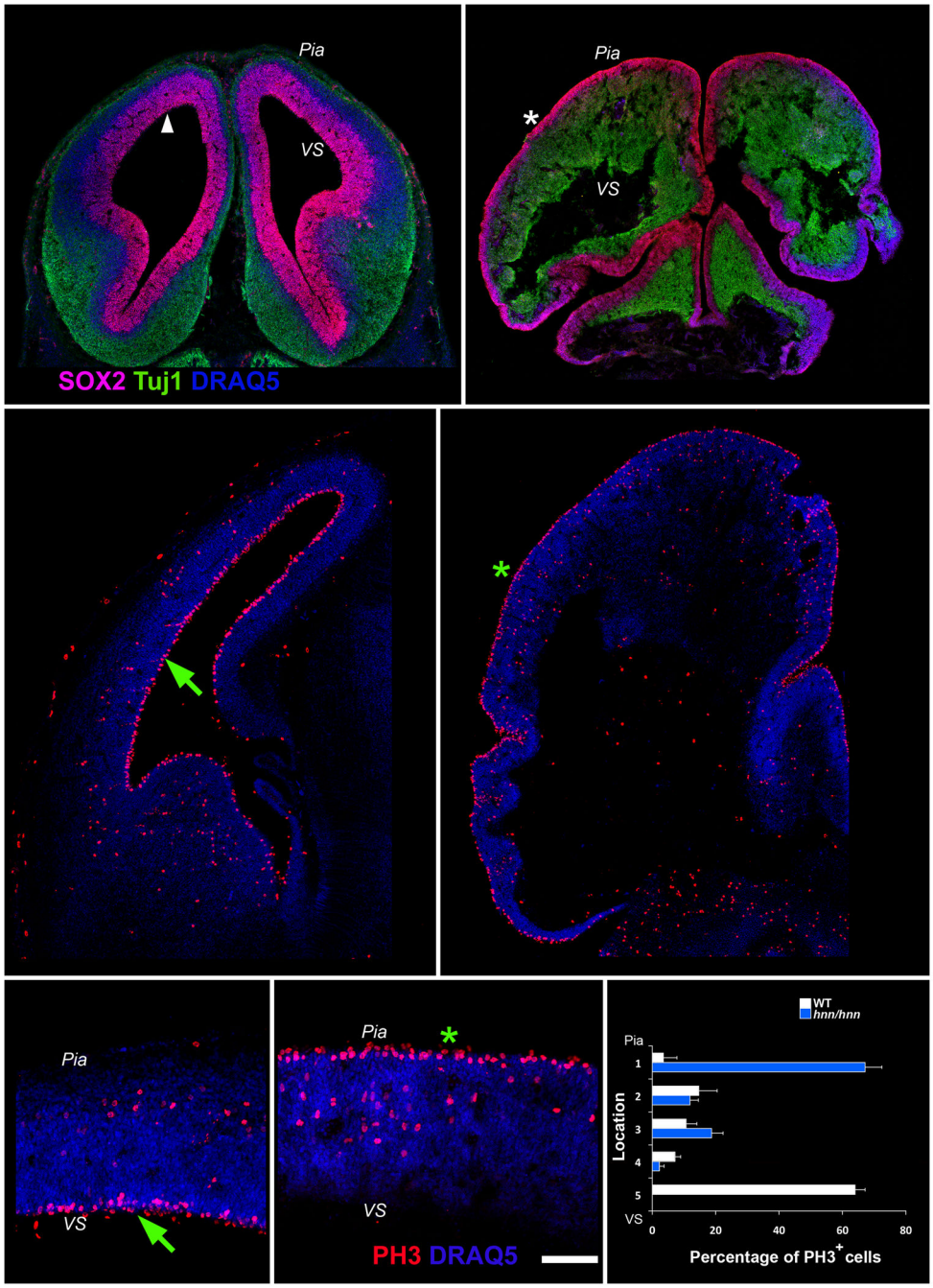

Figure 2. Disrupted formation of polarized radial progenitor scaffold in Arl13b mutants

(A) Loss of Arl13b and the resultant loss of primary cilia function lead to reversal of radial progenitor polarity. In E13 wild-type (WT) cortex, $Sox2^{+}$ radial glial cell soma (purple, arrowhead) are localized near the ventricular surface (VS). In *Arl13b*$^{hnn/hnn}$ cortex, they are localized on the pial surface (asterisk, B). (C–F) Ectopic proliferation of radial progenitors in Arl13b mutants. Immunolabeling with anti-mitotic marker PH3 indicates that, compared to control (C, arrow), progenitors proliferate near the pial surface (D, asterisk) in Arl13b$^{hnn/hnn}$ cortex. Panels E and F are higher magnification views of the cerebral wall

illustrating the proliferation of progenitors near the ventricular surface (arrow, E) and pial surface (asterisk, F) in WT and *Arl13b*$^{hnn/hnn}$ cerebral wall, respectively. (G) Normal interkinetic nuclear movement is required for the progenitors to migrate to the apical end (ventricular surface) to divide. Quantification of the location of proliferating progenitors in WT and *Arl13b*$^{hnn/hnn}$ cerebral wall indicates severe disruption of this process as most of the Arl13b mutant progenitors divide at the opposite end. Data shown are mean ± SEM (n=3 independent experiments; number of brains per group=3). Images shown are representative examples from analysis of 10 control and 10 mutant embryos. VS, ventricular surface; P, pial surface. Scale bar: A–B, 650 μm; C–D, 330 μm; E–F, 100 μm.

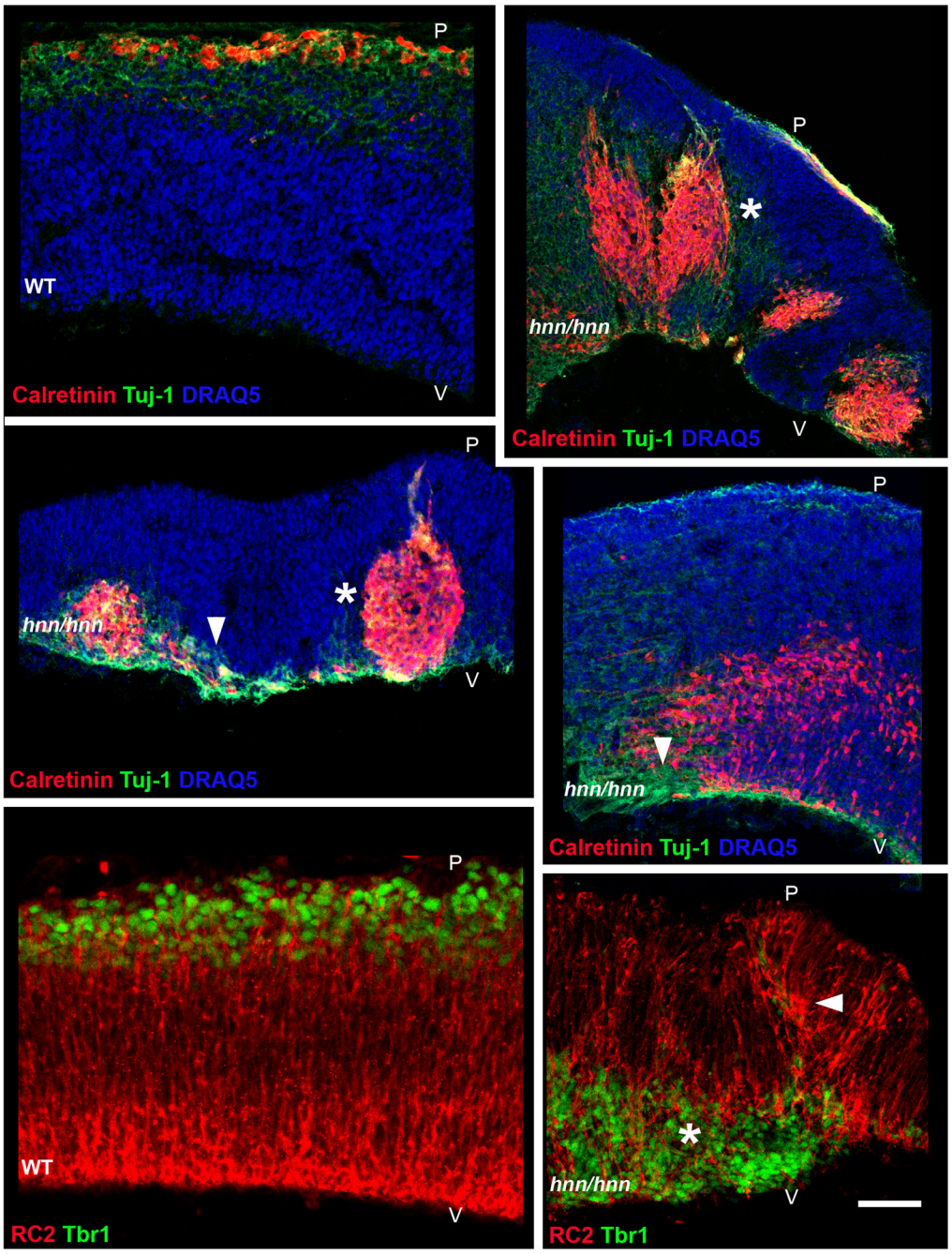

Figure 3. Disrupted cortical layer formation in Arl13b mutants

(A–D) The reversal of radial progenitor polarity affects neuronal migration and layer formation. Instead of the layer-like organization of different types of cortical neurons (red [Calretinin^{+}]; green [Tuj-1^{+}]) in E13 wild-type cortex (A), neurons in Arl13b$^{hnn/hnn}$ cortex (E13) migrate aberrantly and are placed ectopically (B–D). Neurons are frequently found near the ventricular surface (arrowheads, C, D) away from the cortical plate and often form tuber-like clusters (asterisk, B and C). (E–F) Co-immunolabeling of E13 WT (E) and mutant (F) cortices with radial progenitor-specific RC2 antibodies (red) and early generated, deeper

layer neuronal marker Tbr1 (green) further illustrates the disrupted radial progenitor scaffold formation (arrowhead, F) and ectopic neuronal placement (asterisk, F) in Arl13b mutant cortex (F). Sections in A–D were nuclear counterstained (blue) with DRAQ5. Pial (P) and ventricular surface (V) are indicated. Images shown are representative examples from analysis of 10 control and 10 mutant embryos. Scale bar: A–D, 275 μm; E–F, 130 μm.

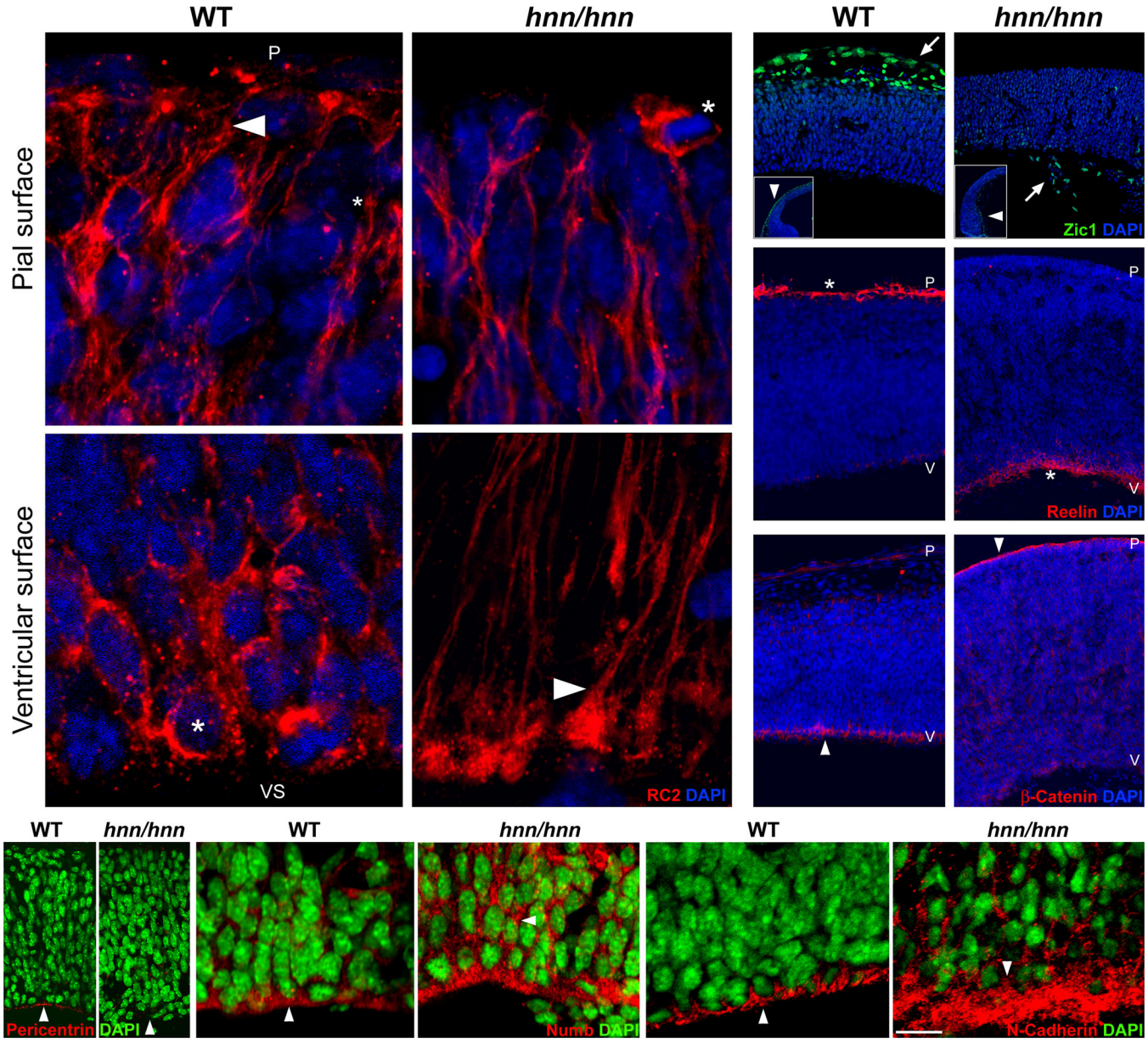

Figure 4. Cortical developmental disruptions in Arl13b mutants

(A–D) Aberrant localization of RC2^{+} radial glial endfeet near the ventricular surface. Consistent with the reversed polarity of radial progenitors, the normal localization of radial progenitor endfeet near the pial surface (arrowhead, A) and cell soma near the ventricular surface (asterisk, C) are reversed in E13 Arl13b mutants (B, D). (E–F) Disrupted formation of Zic1^{+} pial membrane in mutants. Compared to the WT pia (E, arrow), mutant (F, arrow) pia is discontinuous and misplaced. Inset panel (E) indicates normal cortical covering by pial membrane (arrowhead), whereas mutant pia (arrowhead, F [inset]) is discontinuous and often found on the opposite side of the cerebral wall. (G–P) Altered apical-basal organization of the cerebral cortex in mutants. (G–H) Reelin^{+} Cajal-Retzius neurons are normally found at the top of the cortex in layer 1 (asterisk, G). In contrast, they were localized to the apical surface (asterisk, H), near the ventricles in mutants. (I–J) Anti-β-

catenin prominently labels the apical surface of progenitors (arrowhead, I), adjacent to the ventricles in WT cortex. In contrast, β-catenin labeling is evident in the opposite, basal surface (arrowhead, J) in mutants. Further, apical localizations of pericentrin (K), Numb (M) and N-Cadherin (O) are disrupted in mutants (L, N, P; compare arrowheads). P, pial surface; V, ventricular surface. Images are representative examples from analysis of 7 control and 7 mutant embryos. Scale bar: A–D, 17 μm; E–F, 110 μm; G, I, 190 μm; H, J, 300 μm; K–L, 60 μm; M–P, 30 μm.

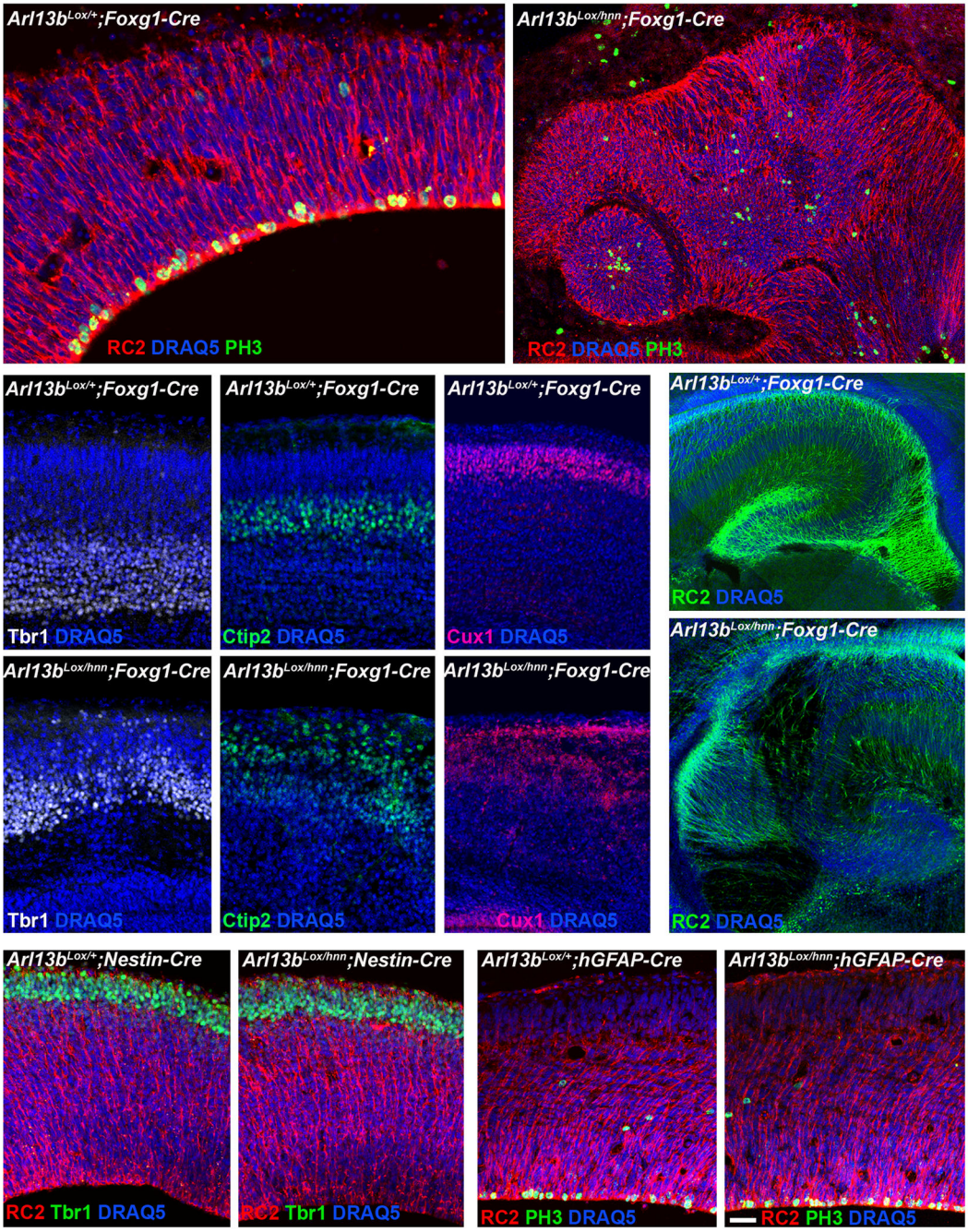

Figure 5. Arl13b deletion in neuroepithelial cells disrupts radial progenitor scaffold organization and laminar organization of neurons in cerebral cortex

(A–B) Normal radial progenitor scaffold (RC2^{+}) and progenitor proliferation (PH3^{+}) in E13 control cerebral wall. (B) Loss of polarized radial progenitor organization and ectopic proliferation in E13 *Arl13b$^{lox/hnn}$; Foxg1-Cre* cortex following deletion of Arl13b in neuroepithelial cells. (C–H) Deeper and upper-layer neurons in E16 cortices from control and *Arl13b$^{lox/hnn}$; Foxg1-Cre* mice were labeled with anti-Tbr1, -Ctip2 (*Bcl11b*) and -Cux1 antibodies. Characteristic laminar organization of different classes of neurons was evident in

control cortices (C–E), but was disrupted in *Arl13b*$^{lox/hnn}$*; Foxg1-Cre* cortex (F–H). (I–J) Hippocampus, which also contains polarized radial progenitors (I, green), does not form properly in *Arl13b*$^{lox/hnn}$*; Foxg1-Cre* mice (J). (K–N) Deletion of Arl13b following the formation of polarized radial progenitor scaffold does not affect radial progenitors. Nestin-Cre and hGFAP-Cre lines were used to ablate Arl13b from E10.5 and E13.5 radial progenitors, respectively. (K–L) In *Arl13b*$^{lox/hnn}$*; Nestin-Cre* cortex (L), RC2^{+} radial progenitors form normally and neuronal migration and placement resembles control cortex (K). (M–N) Similarly, radial progenitor scaffold is not disrupted in *Arl13b*$^{lox/hnn}$*; hGFAP-Cre* cortex (N), and progenitor proliferation is identical to that of control cortex (M). Early-born neurons (K–L) and mitotic progenitors (M–N) were labeled with anti-Tbr1 and anti-PH3 antibodies, respectively. Nuclei were counterstained with DAPI. Images are representative examples from analysis of 10 control and 10 mutant mice per group. Scale bar: A, 40 μm; B, 90 μm; C–H, 75 μm; I–J, 300 μm; K–N, 725 μm.

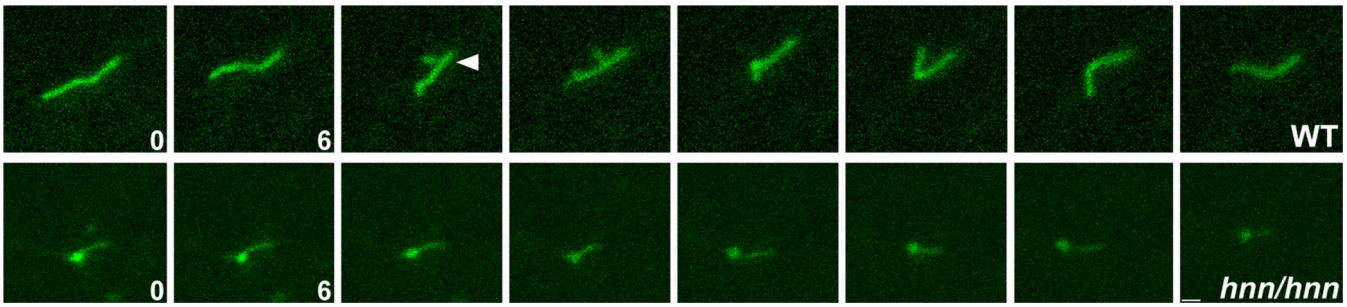

Figure 6. Altered primary cilia dynamics in Arl13b mutants

(A, B). Time-lapse imaging of 5-Htr6-labeled cilia illustrates that in WT progenitors (A), primary cilium changes its length, shape, orientation, and sometimes forms branches (arrowhead, A). In contrast, such dynamism is absent in Arl13b mutant cilium (B). Time interval between each panel is 6 minutes. Scale bar: 1 μm.

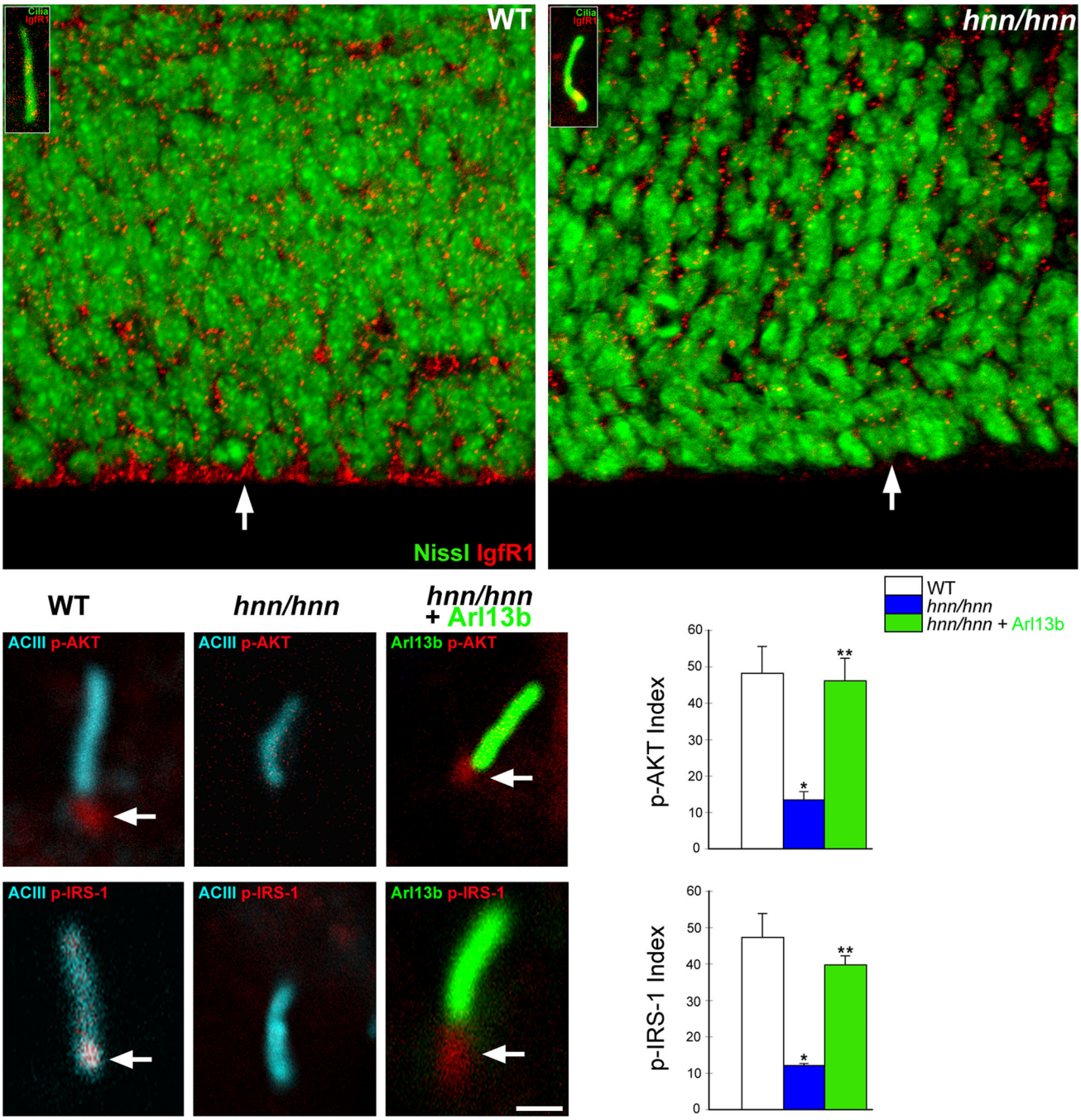

Figure 7. Disrupted localization and signalling of apical complex receptors Igf1R in*Arl13b*$^{hnn/hnn}$ cortex

Immunolabeling of WT and mutant cortices with anti-Igf1R antibodies indicate disrupted localization of Igf1R in apical domains of the radial progenitors following Arl13b deletion (compare arrows in panels A and B). Insets (A, B) show ciliary localization of Igf1R. Ciliary Igf1R signalling leads to phosphorylation of Akt (C, arrow) and accumulation of phosphor-IRS (G, arrow) at the base of the cortical progenitor cilium. Arl13b deletion disrupts these characteristic signs of Igf1R signalling (D, F, H, J). Re-expression of Arl13b in Arl13b mutant primary cilia rescues these deficits (E [arrow], F, I [arrow], J). P-Akt, P-IRS-1 index

indicates percentage of cilia with p-Akt or P-IRS-1 accumulation at the base of the cilium. Data shown are mean ± SEM. Number of cilia analyzed: p-AKT group-WT (77), hnn/hnn (93), hnn/hnn+Arl13b (69); p-IRS-1 group- WT (93), hnn/hnn (96), hnn/hnn+Arl13b (88). * and **, significant when compared with WT or *hnn/hnn*, respectively, at $p < 0.05$ (One way ANOVA with Tukey-Kramer test). Cells were obtained from four different litters. Cilia (A, B, C, D, G, H) were labeled with anti-ACIII antibodies. Scale bar: A–B, 18 μm; C–I, 1 μm.