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The Adenomatous Polyposis Coli (APC) Protein is an Essential Regulator of Radial Glial Polarity and Construction of the Cerebral Cortex

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Abstract

Radial glia are highly polarized cells that serve as neuronal progenitors and as scaffolds for neuronal migration during construction of the cerebral cortex. How radial glial cells establish and maintain their morphological polarity is unknown. Using conditional gene targeting in mice, we demonstrate that Adenomatous Polyposis Coli (*APC*) serves an essential function in the maintenance of polarized radial glial scaffold during brain development. In the absence of APC, radial glial cells lose their polarity and responsiveness to the extracellular polarity maintenance cues, such as neuregulin-1. Elimination of APC further leads to marked instability of the radial glial microtubule cytoskeleton. The resultant changes in radial glial function and loss of APC in radial glial progeny lead to defective generation and migration of cortical neurons, severely disrupted cortical layer formation, and aberrant axonal tract development. Thus APC is an essential regulator of radial glial polarity and is critical for the construction of cerebral cortex in mammals.

Keywords

Radial glia; Cortical development; APC

Introduction

Among the early developing cells within the mammalian brain are highly polarized neural precursor cells, radial glia, which give rise to most cortical neurons and guide their appropriate migration and placement (reviewed in Ayala et al., 2007; Rakic, 2003). The polarity of radial glial cells is manifested by the positioning of a pear shaped cell soma in the ventricular zone (VZ) and a long, slender process extending from the VZ towards pia (Schmechel and Rakic, 1979). Polarized radial glial cells can divide symmetrically or asymmetrically. Symmetric radial glial divisions give rise to two daughter radial glial cells and occurs 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 a radial glial cell or an intermediate

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precursor (Anthony et al., 2004; Miyata et al., 2001; Malatesta et al., 2000; Noctor et al., 2004; Noctor et al., 2008). The pially-directed radial process provides a permissive and instructive scaffolding for the oriented migration of newly generated neurons (Ayala et al., 2007). As neurogenesis and neuronal migration ends, radial glia transform to give rise to glial progeny (Noctor et al., 2008). Disruptions in polarized radial glial scaffold could therefore affect both neurogenesis and migration, leading to aberrant generation, positioning and connectivity of neurons in cerebral cortex.

Recent studies indicate that selective localization and activity of polarity cues such as Cdc42 or Numb/Numbl within the apical regions of radial glial cell soma and associated ventricular process are required for the appropriate generation of neurons from radial glia (Cappello et al., 2006; Rasin et al., 2007). Dynamic regulation of apical adherence junctions by these proteins appears to be critical for this process. During radial glial proliferation, oriented interkinetic translocation of the radial glial nucleus to the ventricular surface, mediated by the centrosomal protein Cep120 or Cdc42, modulates radial progenitor self renewal and neurogenesis (Cappello et al., 2006; Xie et al., 2007). As neurons are generated, polarized, pially-directed radial glial processes guide neurons from their sites of birth in the ventricular zone to their final destinations in distinct cortical layers. Once neurons reach the top of the developing cortical plate, selective adhesive interactions between glial endfeet and pial basement membrane or between neurons and basal radial glial processes modulate the final neuronal positioning (Haubst et al., 2006: Gongidi et al., 2004). Radial glial endfeet-basement membrane attachment abnormalities mainly affected neuronal placement, rather than radial progenitor proliferation or neurogenesis (Haubst et al., 2006). Although these studies demonstrate the importance of apical and basal adhesive interactions in the radial progenitor proliferation and final neuronal placement, little is known about how the polarized radial glial scaffold is constructed and maintained during corticogenesis. We therefore aimed to identify the molecular regulators and mechanisms involved in this process.

One potential candidate for the regulation of radial glial polarity is the tumor suppressor gene, Adenomatous Polyposis Coli (*APC*), which is highly expressed in the developing brain (Bhat et al., 1994). Since the discovery of germ-line mutations of the APC gene in familial adenomatous polyposis (FAP) (Kinzler et al., 1991), APC has been intensively studied to delineate its functions related to intestinal tumorogenesis (Su et al., 1992). The discovery that APC regulates Wnt/β-catenin signaling has provided at least a partial explanation for its tumor suppressor effects (Nathke, 2006; Korinek et al., 1997; Morin et al., 1997). However, distinct domains of APC are now known to interact with a variety of targets in addition to Wnt pathway components, including microtubules, the cytoskeletal regulators DLG (discs large protein), kinesin-2, mDia, EB1 and IQGAP1, and the Rac guanine-nucleotide-exchange factor (GEF) Asef1 (Aoki and Taketo, 2007). The N- terminus of APC can also modulate self association of APC and clustering (Li et al., 2008). It has been proposed that these interactions mediate a variety of cellular functions related to microtubule stability, microtubule “capture” by cellular cortex and plasma membrane, and interactions between microtubles and polyemerized actin (Aoki and Taketo, 2007; Nathke, 2005). Association of APC with the plus ends of microtubules is thought to be critical to the establishment or maintenance of cell polarity (Barth et al. 2008; Collin et al., 2007; Mogensen et al., 2002; Solecki et al., 2006). These ideas are based for the most part on *in vitro* model systems many of which have relied on components of the nervous system. For example, migration of astrocytes in wounding assays is associated with regulated APC expression at the leading edge, and APC is found at growth cones of hippocampal axons, where disruption of APC interferes with axon specification (Etienne-Manneville and Hall, 2003; Etienne-Manneville et al., 2005; Votin et al., 2005). In spite of these important proposed roles of APC, the *in vivo* significance of these observations remain to be established. Indeed whether APC has any function related to mammalian brain development is unknown.

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We show here that APC is expressed in a polarized manner in radial glia, highly concentrated at the pially directed tip and cell soma. Cre-LoxP- mediated inactivation of APC in radial progenitors of embryonic cerebral cortex leads to progressive dismantling of the radial glial scaffold. Live imaging of radial glial development demonstrate a failure to maintain the proper orientation of the pially directed process and marked microtubule instability. Surprisingly, loss of APC results in a reduction in proliferation of radial precursors even though nuclear localized β-catenin is increased. Finally, loss of APC leads to disrupted neuronal placement and aberrant connectivity. These findings demonstrate that APC is essential to the development and maintenance of a polarized radial glial scaffold and construction of cerebral cortex.

Results

Conditional deletion of APC disrupts radial glia polarity and cortical development

We inactivated APC in early embryonic cerebral cortex using an APC floxed allele line known to yield APC loss of function after Cre mediated recombination (Shibata et al.,1997; Sansom et al., 2004; Gallagher et al., 2002; Hasegawa et al., 2002) and a Nestin-Cre line that drives Cre recombinase in developing radial glia (Tronche et al., 1999; Anton et al., 2004). The Nestin-Cre transgene induces widespread recombination in the CNS neural progenitors from around embryonic day 10 and loss of APC is evident in the embryonic cortices of *APC*$^{lox/lox}$Nestin-Cre [APC cKO] mice (Fig. 1). Deletion of APC significantly disrupts the formation and organization of cerebral cortex. Prominent cellular disorganization of the developing cerebral wall and increased size of the lateral ventricles are readily apparent from E13 (Fig. 1).

APC is highly expressed in the developing cerebral wall. Significantly enriched expression of APC is seen in the sub- pail zone, where APC is localized to the radial glial endfeet (Fig. 2A, B). Similarly, *in vitro*, APC is highly expressed at the tips of radial glial cells (Fig. 2C). This characteristic pattern of APC immunoreactivity is absent in APC deficient cortices (data not shown).

The construction of cerebral cortex depends on radial glia. We therefore analyzed radial glial development at early, middle, and late embryonic stages (E13, 16, and P0, respectively) in the absence of APC. Radial glia are generated in APC deficient mice. However, at E13, although they span the width of the developing cortex, deficits in their radial organization are apparent (Fig. 3A, B). Radial glia end feet are aberrantly arborized in APC mutants and often do not extend perpendicularly towards the pial surface (Fig. 3A, B; Supp. Fig. 1). By E16, it is apparent that the characteristic radial glial scaffolding within the emerging cerebral cortex does not form (Fig. 3C, D). The entire radial glial scaffold is drastically altered with most radial glial cells elaborating short, misoriented radial fibers (Fig. 3C, D). By P0, only a few radial glial cells remain in the APC deficient cerebral cortex. Strikingly, the remaining radial glial cells have lost their pially directed processes (Fig. 3E, F). These observations demonstrate that although radial glia are initially generated in the APC deficient cortex, they are unable to maintain the polarity required for the patterned organization of neocortex. Correspondingly, immunohistochemical labeling indicates that APC protein is heavily concentrated at the tip of the pially directed, polarized radial process (Fig. 2B, C).

To further delineate the effect of APC on radial glial scaffold, we inactivated APC in radial glia using the hGFAP promoter- Cre line. The hGFAP promoter is active in radial glia from E13.5 and hGFAP promoter directed transgene expression initially occurs primarily in radial progenitors of dorsal, but not ventral, cortex (Zhuo et al., 2001; Anthony et al., 2004; Malatesta et al., 2003; L. Pevny, personal communications). At E16, complete disruption of radial glia scaffold is evident in dorsal cortex of *APC*$^{lox/lox}$hGFAP-Cre mice (Fig. 4A–D). In contrast, in ventral cortex of E16 *APC*$^{lox/lox}$hGFAP-Cre mice where Cre mediated inactivation of APC is not expected, radial glia extend long polarized processes as in controls (Fig. 4A, B, E, F; Supp.

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Fig. 2). Lastly, electroporation of Cre plasmids into radial glia of *APC*$^{lox/lox}$ embryonic (E14) cortices also lead to significant disruption of polarity in Cre expressing radial glia (5.83± 0.49 fold increase in radial glia without polarized radial process; Supp. Fig. 3). These observations further support the central role of APC in radial glial polarity and suggest that the effect is cell autonomous.

Evaluation of apical markers of radial progenitors supports the conclusion that APC deletion disrupts radial glial polarity. Normal, apical enrichment of Numb is severely disrupted in APC deficient radial progenitors. Instead Numb is distributed aberrantly in a non- polarized manner throughout these cells (Supp. Fig. 4A, B). Similarly, localization of pericentrin, a centriole marker, in the apical aspects of radial progenitors in the ventricular surface is lost in APC deficient progenitors (Supp. Fig. 4C, D). The normal enrichment of Cdc42 localization in apical membranes of radial progenitors is also perturbed (data not shown). At the basal end, the pial basement membranes is present in the APC deficient cortex, but is often undulated in shape (Supp. Fig. 4E, F).

To examine the role of APC in radial glial polarity further, we analyzed the response of APC deficient (*APC*$^{lox/lox}$Nestin-Cre) radial glia to Neuregulin-1 (NRG-1), a molecular signal known to promote radial glial polarity and growth of radial processes (Anton et al., 1997; Rio et al., 1997; Gierdalski et al., 2005). Wild type radial glia (E15) extend their radial processes in response to NRG-1 (Supp. Fig. 5A–D, I). In contrast, APC deficient radial glia have significantly shorter radial processes and do not respond to NRG-1 (Supp. Fig. 5E–I). Similar deficits in radial glia were noticed when radial progenitors from APC$^{lox/lox}$ mice were transfected with Cre plasmids to inactivate APC (Supp. Fig. 6). These Cre transfected APC$^{lox/lox}$ radial glial cells also do not respond to NRG1 (Supp. Fig. 6). These results suggest that in the absence of APC, radial glia in the developing cerebral cortex are unable to respond to signals critical for the maintenance of polarity.

To assess the cell biological basis of misorientation and disrupted maintenance of radial glial scaffolding, we performed real-time observations of radial glial development in the embryonic cortex (Fig. 5, Supp. Movies 1–4). We labeled radial glia in embryonic day 15 cortex with BLBP promoter-GFP electroporation and live imaged their actvity. These long- term live imaging observations demonstrate that radial glial cells in wild type mice extend a polarized leading process towards the pial surface. Over an 8 hour period, active movement at the radial glial tips and cell soma is evident in normal radial glia, but no loss of polarity. In contrast, processes of APC deficient radial glia are nowhere near the pial surface. Radial glia lacking APC extend processes randomly in multiple directions, showing a striking deficiency of polarized extension towards the pial surface. Time-lapse images from control and APC deficient radial glia illustrating this failure of polarized extension are shown in Fig. 5 and the full movies are included as Supp Movies 1–4. Thus, although the processes of APC deficient radial glia are highly motile, they are unable to establish and maintain the characteristic polarized, pially-directed growth needed to form and maintain the radial glial scaffold within the developing cerebral cortex (Fig. 5, Supp. Movies 1–4).

Loss of APC impairs radial glial functions in neurogenesis and migration

We next asked how the disrupted polarity in APC mutants affected the dual function of radial glia as a source of new neurons and migratory guide of newly generated neurons. To measure the effects of APC on radial glial proliferation, mitotically active, M-phase radial glial cells in control and APC deficient brains at E12, 14, and 16 were immunolabeled with anti-phospho-histone H3 (PH3) antibodies. APC deletion significantly reduced the number of PH3^{+}, M-phase radial glial progenitor cells at each of these ages (Fig. 6A–C). Similar reduction in progenitor proliferation was also noticed when actively proliferating radial progenitors in embryonic cortices were pulse labeled by administering the S-phase marker Bromodeoxyuridine (BrdU)

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an hour prior to sacrifice (Fig. 6D–F). Fewer radial glial cells actively divide in the absence of APC. Furthermore, we double labeled $BrdU^+$ cells with Ki67, a marker for cells in S, G2, and M phases. $BrdU^+/Ki67^+$ cells represent the fraction of progenitor cells that are actively cycling and this fraction was reduced significantly (−21± 3%) in APC deficient cortex (Fig. 6F). Analysis of mitotic spindle assembly and organization in APC deficient radial progenitors *in vivo* indicate that in the absence of APC, mitotic spindles are disorganized and lack properly formed spindle poles (Fig. 6G). Reduced precursor proliferation is paralleled by enhanced apoptotic cell death in APC deficient cortex (Fig. 6H–J).

New neurons resulting from radial glial division are guided to distinct laminar positions in the developing cortex. A strong prediction from results presented above is that cortical layer formation would be profoundly disrupted in APC deficient mice. Analysis of newly generated cortical neurons with different cortical layer specific markers indicate that not only are fewer neurons generated in the APC mutants as a result of reduced radial glial proliferation, but their migration to distinct laminar positions is also completely disrupted (Fig. 7). Neuronal misplacement is prominent adjacent to aberrantly polarized radial glia (Supp. Fig. 1). Similar disruptions in neuronal placement was also evident in $APC^{lox/lox}$hGFAP-Cre cortex (Fig. 4). Both the disrupted radial morphology of APC mutant radial glia and the loss of neuronal APC likely affect the migration and positioning of these cortical neurons.

Neuronal connectivity is disrupted in APC- deficient cerebral cortex

The progressive dismantling of radial glial scaffold in the developing cortices of APC mutants may not only affect the generation and migration of cortical neurons, but also the post-migratory axonogenesis and connectivity of these neurons. Labeling of axonal fiber tracts in cortex with anti-L1 antibodies indicate that instead of the characteristic outgrowth of cortical axons towards other areas of cortex and distant CNS regions, APC mutant axons swirl around, are grossly misrouted, and do not orient or fasciculate towards their appropriate targets (Fig. 8A–B). At E14 when the initial axon outgrowth of newly arrived deeper layer cortical neurons starts to occur in normal cortex (asterisk, Fig. 8C), extension of APC deficient axons is disrupted and is characterized by clustering of misrouted axons (arrow, Fig. 8D). By E16, prominent defects are evident in the growth, orientation, and organization of major cortical fiber tracts in the brain, such as the internal capsule, posterior commisure and habenulo-peduncular tract (Fig. 8E–H). In addition, extension of thalamic axons towards cortex is also disrupted. Thalamocortical fibers do not orient or fasciculate properly and are misrouted in the developing cerebral wall (arrowheads, Fig. 8I, J).

Presumably, both the deficiency in radial glial scaffolding and neuron cell autonomous mechanisms may underly these dramatic axonal effects *in vivo*. Interestingly, dissociated cortical neurons from APC deficient mice maintained *in vitro* for varying time periods (48 hours to one week) on a laminin substratum can grow axons. However axons of these APC deficient neurons are abnormal, exhibiting markedly excessive branching of the primary and secondary axonal processes at all time points examined (Fig. 8K–M). Often, the tips of these axonal branches, rather than growing straight, curve back towards the parent axon, a behavior seen in less than 10% of control neurons (Fig. 8K, L, N). Similar excessive branching of axons is also observed when normal neurons from $APC^{lox/lox}$ mice are cultured and then transfected with expression vectors encoding Cre-EGFP (total number of branches: control, 2.87± 0.22; APC $^{lox/lox}$ + Cre-EGFP, 4.56± 0.55). Further, APC deficient neurons do not respond to the neuronal growth factor, BDNF, which normally induces branching at this age. Total number of branches in wild type neurons increase in response to 50 ng/ml BDNF. (WT, 6.6± 0.5%; WT+BDNF, 10.6± 0.8%), whereas BDNF-did not induce increased branching in APC deficient neurons (APC cKO, 10.8± 0.8%; APC cKO+BDNF, 8.6± 0.7%). Together, these *in vivo* and *in vitro* observations indicate that cell autonomous and non- cell autonomous APC signaling

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may influence the polarity and functional connectivity of distinct groups of neurons both within the developing cerebral cortex and other brain regions.

Altered β-catenin expression and microtubule stability in APC- deficient cerebral cortex

How does APC affect the development of radial glial scaffolding and the resultant patterns of neuronal positioning and connectivity in cerebral cortex? We have demonstrated that APC's effects on radial glial cells are two fold: it is necessary for appropriate division and expansion of radial glial population, and it is needed for the maintenance of polarized morphology of radial glia. APC can potentially influence radial glial proliferation/differentiation by influencing gene transcription through the β-catenin/T cell factor [TCF] transcriptional program or through association with microtubule cytoskeleton (Nathke, 2006; Aoki and Taketo, 2007; Bommer et al., 2007; Sansom et al., 2007). We investigated both of these aspects of APC interactions. As expected, both total and dephosphorylated, active β-catenin are significantly upregulated in APC mutant cortices (Fig. 9A). Early changes in the apical distribution of β-catenin are evident in E13 APC deficient cortex. By E16, widespread expression of β-catenin is noticed throughout the APC mutant cortex (Fig. 9B–E). *In vitro*, β-catenin is normally distributed around intercellular adherens junctions of cortical cells (Fig. 8F, arrow). In APC deficient cells, this characteristic pattern of β-catenin expression is lost and a prominent upregulation of β-catenin localized to nucleus is noticed (Fig. 9G–H). To examine if the nuclear localized β-catenin is transcriptionally active in APC deficient cortex, we electroporated a reporter construct that expresses a destabilized GFP variant under the control of a β-catenin responsive promoter (TOPdGFP; Dorsky et al., 2002). It also contains in tandem, constitutive CAG promoter driven mCherry (TOPdGFP-CAG mCherry; gift from Dr. A. Chenn, Northwestern University). Drastic up regulation of GFP expression is evident in ventricular progenitors of APC deficient cortex within 24 hours of electroporation, suggesting that nuclear localized β-catenin in APC deficient cortex is transcriptionally active (Fig. 9I–K). Over expression of β-catenin would be expected to enhance neural precursor proliferation by promoting their reentry into cell cycle after mitosis as is the case in mice that overexpress stabilized β–catenin in the developing central nervous system (Chenn and Walsh, 2002; 2003). Surprisingly, in APC mutants, in spite of enhanced β-catenin expression, radial glia proliferation is down regulated (Fig. 6).

A possible explanation for the lack of hyperproliferation in the setting of excess β-catenin is that APC interaction with microtubule cytoskeletal machinery is needed for efficient cell division. APC- microtubule interactions may also be expected to influence the polarized morphology of radial glia. APC's localization at sites of microtubule attachment and stabilization suggests that it plays an essential role in microtubule dynamics and stability (Mimori-Kiyosue et al., 2000; Mogensen et al., 2002; Reilein and Nelson, 2005). In order to assess this idea, we labeled microtubules in radial glial progenitors in APC mutant cortex with the microtubule binding domain of ensconsin fused to GFP [EMTB-3GFP] (Lechler and Fuchs, 2007; Faire et al., 1999) and live imaged microtubule dynamics in these cells (Fig. 10, Supp. Movie 5, 6). Comparison of microtubule cytoskeleton from adjacent time points of observation can be used as an indicator of the extent of MT rearrangement and stability. Since dynamically unstable microtubules continuously remodel their organization, the level of overlap of microtubule cytoskeleton between adjacent time points of observation indicates the extent of stability of microtubule network and the overall dynamic changes in microtubule cytoskeleton (Reilein et al., 2005). In control radial glial cells, extensive overlap of the microtubule cytoskeleton was noticed between images obtained at 6 minute intervals (Fig. 10A–C). In contrast to control cells, the less stable microtubule cytoskeleton in APC deficient cells is rearranged rapidly, as indicated by the non-overlapping alignment of the microtubule cytoskeleton between adjacent time points of observation (Fig. 10A–C, Supp. Movie 5, 6). These long term imaging of microtubule dynamics in APC deficient cortex suggests that in the

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absence of APC microtubule networks do not achieve stability. Consistent with the hypothesis that APC deficiency leads to unstable microtubules, the level of acetylated tubulin (aged, stable form of tubulin) (Westermann and Weber, 2003) is reduced in APC deficient cortices (Fig. 10D). Moreover, when control and APC deficient radial progenitors were treated with nocodazole to depolymerize unstable microtubules, we noticed that lack of APC resulted in marked changes in stable microtubules (Fig. 10E, F). Compared to the extensive network of stable microtubules in control radial progenitors, APC deficient radial glia contained fewer nocodazole- resistant, stable microtubules (Fig. 10E, F; % of radial glia with developed network of stable microtubules: Control, 86±10%; APC cKO, 14.7±2.4% [number of cells: Control, 233; APC cKO, 261])

To further investigate the microtubule dynamics, we labeled microtubule plus ends in radial progenitors with EB1-GFP and analyzed changes in microtubule plus end growth rate and orientation. 100 microtubule plus ends from 10 cells were analyzed for each group. $EB1^+$ microtubule plus end velocity in APC deficient cells (7.96±0.42 μm/min.) is not markedly different from that in wild type cells (7.12±0.26 μm/min.; also see Supp. Movie 7, 8). However, EB1 tipped microtubules often extended in directions away from cell cortex in APC deficient cells (Fig. 10G). Compared to controls, 16 fold more EB1 comets in APC deficient cells moved in orientations away from the leading edge (n=145 EB1 comets from 10 control or APC cKO cells). Furthermore, the average length of microtubule plus end extension was significantly reduced in APC deficient cells (control, 3.72±0.27 μm [n=50]; APC cKO, 2.31±0.16 μm [n=50]). On average microtubules extended for shorter periods in APC deficient cells (control, 29.12±2 sec.[n=50]: APC cKO, 19.4±1.26 sec.[n=50]), further suggesting disrupted microtubule stability in the absence of APC. To further characterize the dynamics of microtubule instability in APC deficient cells, we also measured the frequency of microtubule plus end catastrophe in EMTB-3GFP labeled radial glia as described in Reilein et al (2005). Compared to wild type cells, the frequency of catastrophe was increased APC deficient cells (control: 0.309±.02 min^{-}; APC cKO: 0.384±.02 min^{-}; also see Supp. Fig. 7). Together, these observations on microtubule growth dynamics indicate that in the absence of APC microtubules in radial progenitors are unstable and do not extend or orient appropriately.

Stabilizing microtubules in APC deficient radial glia with taxol (5μM) partially rescued the defects in radial progenitor proliferation (% of proliferating radial glia: control, 13.25±.0.9; control+ taxol: 5.92±0.68; APC cKO: 8.7±1, APC cKO+ taxol: 11.21±1.3). Similar rescue was also noticed in the ability of APC deficient radial glia to extend radial processes (radial process length: control, 85.3±.4.7μm; control+ taxol: 76.47±4.48μm; APC cKO: 56.3±4.9μm, APC cKO+ taxol: 78.4±6.2μm). These observations support the idea that microtubule instability in radial glia contributes to the defects in their function in APC deficient cerebral cortex.

APC deficient neurons also demonstrated evidence of microtubule instability. High power confocal imaging of neurons stained to visualize tubulin demonstrated that microtubule organization was frequently disrupted at or near the point of branching (Fig. 10H, I). Instead of bundling tightly along the core of the axonal processes at branch points, microtubule filaments are unbundled and extend diffusely at these points in APC deficient neurons, indicating microtubule instability (Bielas et al., 2007; Lewcock et al., 2007).

Discussion

The results described here demonstrate a novel and essential role for APC in the construction of cerebral cortex in mammals. APC is required to maintain and extend the radial glial scaffolding during the formation of the cortex. Further, APC activity in radial glia progenitors is essential for the expansion of the radial glial population and for the generation of neurons

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in normal numbers. Deletion of APC disrupts the appropriate positioning and connectivity of cortical neurons.

Despite the critical importance of APC in regulating proliferation and its role in Wnt signaling, the only prior *in vivo* work on APC in central nervous system development has been done in Drosophila where it has been shown that APC proteins (Drosophila APC 1 and 2) regulate neural progenitor differentiation, neuronal adhesion and growth, and photo receptor fate (Ahmed et al., 1998; Akong et al., 2002; Benchabane et al., 2008; Hayden et al., 2007). These abnormalities in Drosophila have been ascribed mainly to the regulation of transcription via the β-catenin/TCF pathway, but recent work in Drosophila also provided evidence for APC regulation of epithelial mitotic spindles downstream of Akt (Buttrick et al. 2008; Rusan et al., 2008). Although there is another APC homologue in mammals, APC-L (APC2) that is heavily expressed in brain, its functions are apparently non-redundant as APC-L deficient mice survive into maturity (Yamanaka et al., 2002; vanEs et al., 1999; Snider, Van Es, and Clevers, unpublished observations).

APC is required for the development and maintenance of the radial glial scaffold

We have shown here that APC deletion comprehensively disrupts radial glial development and corticogenesis. Radial glial cells normally provide the matrix for the construction of cerebral cortex. Previous studies on apical polarity of cortical progenitors and radial glia- pial membrane attachments have demonstrated that adhesive interactions at the apical and basal ends of radial glia are necessary to facilitate appropriate progenitor proliferation and neuronal placement, respectively (Cappello et al., 2006; Haubst et al., 2006; Rasin et al., 2007). However, relatively little is known about how the polarized radial glial scaffold is established, maintained, and expanded in the developing cerebral cortex. We found that in the absence of APC polarized radial glia scaffolding is gradually dismantled (Fig. 3, 4). The apical polarity of these cells as defined by the expression of markers such as Cdc42, Numb and pericentrin is lost. Live cell imaging of radial glia in the developing cortex definitively shows that the radial process can neither polarize properly nor orient and extend towards the pial surface (Fig. 5; Supp. Movies 1–4). Instead these radial processes wander around the developing cerebral wall, leading ultimately to profound deficits in both progenitor proliferation and guidance of neuronal placement (Fig. 5–8). Without APC, the ability of these cells to respond appropriately to secreted or extracellular substrate bound cues in the developing cerebral wall appears to be perturbed. This effect of APC on radial glial development is cell autonomous. In $APC^{lox/lox}$ hGFAP-Cre cortex, APC deleted radial glia in dorsal cortex collapse, whereas in adjacent ventral cortical domains where APC was not affected, radial glia remains intact (Fig. 4, Supp. Fig. 2). Electroporation of Cre into $APC^{Lox/Lox}$ cortex and the resultant loss of radial glia polarity further supports the cell autonomous role for APC.

APC is highly expressed at the tips of polarized radial glial cells like in other highly polarized cells (Fig. 2). In reactive astrocytes, APC is required for them to orient and migrate towards the edge in wounding assays (Etienne-Manneville et al., 2003). Similarly, abundant APC is found at growth cones of PC12 cells, DRG neurons and hippocampal neurons. *In vitro* loss of function assays in these systems suggest that APC contributes to efficient axon elongation (Shi et al., 2004;Zhou et al., 2004;Votin et al., 2005;Koester et al., 2007;Purro et al., 2008). Based on our results presented here, we suggest that polarized APC expression at the tips of radial glia may provide them with the necessary competence to orient and extend towards the pial surface as the cerebral wall expands during embryogenesis. Similar mechanisms may also influence polarized process extension in APC deficient migratory and post migratory cortical neurons. However, dismantling of the radial glial scaffold in the developing cortex appear to have a profound indirect effect on the placement and wiring of cortical neurons.

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APC and radial glial cell proliferation

As embryonic cortex expands, radial glial cells divide symmetrically, to enlarge the radial progenitor population, or asymmetrically, to generate neurons, radial glia, and/or intermediate precursors (Haydar et al., 2003; Mizutani et al., 2007; Noctor et al., 2004; Noctor et al., 2008; Yoon et al., 2008). A major surprise from our results is that radial progenitor proliferation is reduced in the absence of APC. Analysis of radial progenitor proliferation in APC deficient cortex at different embryonic stages suggests that, both symmetric and asymmetric divisions are affected throughout cortical development.

APC was identified originally as a tumor suppressor gene and in most tumor models APC deficiency results in uncontrolled proliferation. APC's effects on cell proliferation are thought to depend critically on β-catenin activity downstream of Wnt signaling (Nathke, 2006; Bienz, 2002; Aoki and Taketo, 2007). APC is an integral component of the destruction complex that normally promotes β-catenin phosphorylation and proteosomal degradation. Inhibition of this destruction complex, through the activation of Wnt signaling pathway, enables β-catenin to translocate to nucleus, bind to the TCF family of DNA binding proteins, and regulate gene transcription (reviewed in Willert and Jones, 2006). Importantly, transgenic expression of constitutively active β-catenin in the developing cortex promotes radial progenitor expansion dramatically with obvious infolding of the cortical plate to accommodate the increase in precursors and their neuronal progeny (Chenn and Walsh, 2002, 2003; Wrobel et al., 2007). Thus the level of β-catenin is a critical regulator of neural precursor proliferation in the developing cortex.

We demonstrate here that APC appears to function in the cerebral cortex to regulate β-catenin much as it does in other tissues. Indeed we found dramatic upregulation of β-catenin in APC deficient brain. However, in APC mutants, in spite of drastically enhanced β-catenin expression and activation of TCF/LEF signaling, radial glia proliferation is down regulated. There is no infolding or expansion of the cortical plate or persistence of radial glial cells as seen in β-catenin over expressing mice (Fig. 1, 3, 4, 7). Instead, labeling with multiple indicators of cell proliferation indicates progressive reduction of radial progenitor proliferation in APC deficient cortex (Fig. 6). As a consequence, the numbers of radial glia and post mitotic neurons are noticeably reduced in the absence of APC.

We hypothesize that multiple other functions of APC, in addition to its role in regulating β-catenin levels, are required for appropriate proliferation of cortical precursors. First, APC can modulate the export of nuclear β-catenin to cytoplasm for association with specialized plasma membrane compartments such as adherens junction membranes (Henderson, 2000), which are essential for normal proliferation (Cappello et al., 2006; Rasin et al., 2007). The disruption of such intracellular β-catenin targeting may have negatively influenced radial progenitor division in APC cKO mice (Fig. 6, 8F, G). Furthermore, residual APC function appears to be required for optimal transduction of Wnt signaling and β-catenin driven cell proliferation (Takacs et al., 2008). This study in Drosophila demonstrated that APC has dual functions: it facilitates destruction complex dependent β-catenin degradation, but it also can negatively regulate axin to promote Wnt-β catenin signaling (Tackacs et al., 2008). Increased β– catenin may require residual APC to promote proliferation. Thus opposing activities of APC to repress as well as activate Wnt regulated β-catenin signaling may be an essential toggle in the appropriate extent and type of radial progenitor proliferation. Finally, APC interactions with the mitotic spindle may be essential for the appropriate radial progenitor proliferation. During cell division, APC is known to associate with microtubule based spindle apparatus and the kinetochores and regulate the spindle orientation and appropriate chromosomal segregation, respectively (Fodde et al., 2001; Kaplan et al., 2001; Buttrick et al., 2008; Dikovskaya et al., 2007). APC mediated tethering of spindle microtubules to adherens junctions and the resultant spindle orientation is crucial in determining the symmetric or asymmetric nature of cell divisions (Lu et al., 2001;

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Haydar et al., 2003). Improper chromosome segregation, resulting in chromosome instability, can lead to defective cell cycle progression and apoptosis (Kaplan et al., 2001; Fodde et al., 2001). Indeed, we have demonstrated here striking disruption of mitotic spindles in APC deficient radial progenitors (Fig. 6G). Progenitors with disrupted polarity may also not be able to respond appropriately to maintenance or proliferation cues. Thus, in APC deficient cortex, disrupted mitosis and chromosomal instability of progeny may lead to both the progressive depletion of radial glial progenitors and eventual radial glial cell death.

APC-microtubule interactions in radial glia

The ability to maintain and expand the polarized radial glial scaffolding within the developing cerebral cortex is essential for the orderly emergence of cerebral cortical organization. Dynamic regulation of the radial glial cytoskeleton underlies this ability. We therefore live imaged microtubule dynamics in radial glia (Fig. 10; Supp. Fig. 7; Supp. Movie 5–8). We found that in the absence of APC, microtubules in radial glial cells constantly remodel without generating stable microtubule networks (Fig. 10; Supp. Movie 5, 6), a characteristic of dynamically unstable microtubules (Reilein et al., 2005). Microtubule stabilization lead to rescue of radial progenitor deficits. Nocodazole treatment of control and APC deficient radial glia demonstrated that APC deletion results in fewer nocodazole resistant, stable microtubules (Fig. 10E, F). In addition, the orientation and extension of plus end microtubule growth towards the leading edge is retarded (Fig. 10G; Supp. Movie 7, 8). The frequency of microtubule catastrophe is increased as well (Supp. Fig. 7). This loss of system wide stability within the microtubule network in highly polarized cells such as radial glia would presumably affect their ability to extend or maintain the polarized radial morphology in the expanding embryonic cerebral cortex.

Several earlier observations about APC functions related to microtubules might explain this microtubule instability in radial glial cells. APC is present along the paths of microtubule growth, points where microtubule plus ends pause, where multiple microtubules converge, and where microtubules are rescued (Mogenesen et al., 2002; Reilein et al., 2005; Koester et al., 2007; Kroboth et al., 2007; Mimori- Kiyosue et al., 2000). This pattern of APC localization among the interacting networks of microtubules is thought to guide microtubule network formation (Reilein and Nelson, 2005). Moreover, microtubules preferentially stabilize near the leading edge of polarized cells (Gundersen et al., 1988; Palazzo et al., 2004). APC has also been localized to microtubule plus ends and to the plasma membrane of polarized cells where it is thought to function to direct or stabilize new microtubules on or towards the membrane during oriented process extension (Etienne-Manneville and Hall., 2003; Reilein and Nelson, 2005; Koester et al., 2007; Mimori- Kiyosue et al., 2000; Purro et al., 2008). Such APC regulated capture and anchoring of microtubules to radial glial cell cortex may facilitate the maintenance and expansion of polarized radial glia scaffold. Finally, APC binding to actin at actin-rich leading edges or intercellular junctions to enable coordinated modulation of actin and microtubule cytoskeleton has also been proposed (Moseley et al., 2007). A very recent study also suggests that APC is also required for anchoring of mRNAs at the plus ends of stable microtubules in cellular protrusions (Mili et al., 2008), where local translation may promote cell polarity related functions. Disruption of these diverse APC regulated microtubule-microtubule or microtubule-cell cortex interactions may underlie the microtubule instability and the resultant morphological deficits we observed in APC-deficient radial glia.

APC- microtubule interactions could also indirectly influence radial glial polarity. For example, APC mediated tethering of microtubules to polarized cell-cell contacts, be it adherens junctions in the ventricular surface or glial endfeet-basement membrane adhesion sites in the basal end, may facilitate microtubule-based transport and delivery of polarity cues such as Cdc42 to the appropriate regions of cell where they are needed. Consistent with this idea, recent

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studies indicate that targeted delivery of membrane proteins such as connexin 43 to adherens junctions depends on microtubule capture at the cortex (Shaw et al., 2007). Thus, altered microtubule tethering and the resultant disruptions in targeted delivery of crucial receptors and signaling proteins to appropriate cell domains may also have indirectly contributed to altered polarity, adhesion, and function of APC deficient radial glia.

While our observations highlight the significance of APC-microtubule interactions in radial glial development, the relative contributions of APC-β catenin and APC-MT interactions to corticogenesis remains to be fully characterized. Similarly, the differential regulation of membrane, microtubule, and β-catenin targeting complex associated pools of APC in the developing neocortex needs to be delineated (Penman et al., 2005; Bienz, 2002; Mimori-Kiyosue and Tsukita, 2001; Rusan et al., 2008). The currently available APC mutant models with truncations in APC domains are of limited use since, the deletions often span multiple functional domains that control APC's interactions with β-catenin, microtubules, axin, DLG and EB1 (Fodde et al., 2001). Generation and analysis of mutant models in which distinct APC domains are deleted will be valuable as will be post mortem brain analysis of FAP patients with specific APC deletions. In addition, silencing of APC mediated effects on β-catenin function, by inhibiting the upregulated β-catenin signaling (Korinek et al., 1997; Tago et al., 2000; Rusan et al., 2008) in APC null background would also be informative. Such studies will help clarify whether specific or synergistic interactions of APC with microtubule and β-catenin pathways play a determinant role in corticogenesis.

Conclusions

Collectively, our observations indicate a central cell autonomous role for APC in radial glial scaffold maintenance and radial glial function. APC activity enables radial glia to maintain appropriate molecular and morphological polarity in support of appropriate types of cell divisions and oriented guidance of neurons. The multidomain structure of APC is ideally suited to control these diverse functions of radial glia during corticogenesis.

Materials and Methods

Mice

Mice were cared for according to animal protocols approved by the University of North Carolina. Nervous system–specific conditional knockout *APC* mice ($APC^{lox/lox}$Nestin-Cre or $APC^{lox/lox}$hGFAP-Cre) were generated by mating mice carrying an *APC* allele flanked by *lox*P sites (Shibata et al., 1997) with either Nestin-Cre (Tronche et al., 1999) or hGFAP-Cre (Zhuo et al., 2001) mice. Littermate $APC^{lox/+}$ Nestin-Cre^{-} or $APC^{lox/+}$hGFAP-Cre^{-} mice served as controls.

Immunohistochemistry and Immunoblot Analysis

See Supplemental Methods for details.

In vitro assays with neuregulin 1

See Supplemental Methods for details.

Analysis of radial glial cell activity in the developing cerebral cortex

Long- term live imaging analysis of BLBP-GFP^{+} radial glial cells in APC deficient embryonic cortices were performed as described in Yokota et al. (2007). See Supplemental Methods for details.

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Analysis of cortical neuron axon growth

See Supplemental Methods for details.

β-catenin activity assay

See Supplemental Methods for details..

Analysis of microtubule dynamics

To visualize microtubule dynamics in radial glia, EMTB-3GFP (gift from Dr. Bulinski, Columbia University) and BLBP promoter-DsRed2 plasmids were electroporated into E15 control and $APC^{lox/-}$ Nestin-Cre cortex as described earlier (Yokota et al., 2007b). Cortices were then vibratome sliced into 200μm thick sections and maintained in MEM/10% BGS. Twenty-four hours later, slices were placed in a live cell incubation chamber attached to a Zeiss Pascal confocal microscope, and $EGFP^+$ cells in the ventricular zone area were repeatedly imaged at 5–10-minute intervals for up to two hours. Changes in EMTB-GFP^+ microtubule organization within the cell soma was quantified using Zeiss LSM image browser. Images from adjacent time points of observation were superimposed, a 25μm length line scan (Zeiss LSM image browser) spanning the length of the cell soma was used to quantify the number of spots of microtubule co-localization at three different locations within the cell soma and used as microtubule stability index. Measurements obtained for control cells (normalized to one) were compared to APC deficient cell measurements. To measure catastrophe and rescue frequencies, EMTB-GFP^+ microtubules in these cells were imaged every minute. The number of transitions from growth or pause to shortening was measured for active, individual microtubules and divided by the total time observed to obtain catastrophe frequency. The number of transitions from shortening to pause or growth was divided by total time to ascertain rescue frequency (Reilein et al., 2005).

To measure microtubule plus end dynamics, we electroporated EB1-GFP plasmids (Mimoti-Kiyosue et al., 2000) into E15 control and $APC^{lox/-}$ Nestin-Cre cortex. Electroporated radial progenitors from ventricular zone were microdissected, dissociated, and maintained *in vitro* for 24 hours (Yokota et al., 2007). EB1-GFP^+ microtubule tips were imaged every 10 seconds for 2–5 minutes. The average velocity of EB1-GFP^+ microtubule ends was measured using Zeiss LSM image browser.

To assay for stable microtubules, radial glia from E15 cerebral cortex were prepared and maintained for 24 hours as described in Schmid et al., 2003. Cells were then placed into 4°C for 30 minutes, followed by incubation at 37° C for 60 minutes with media containing 10μg/ml nocodazole. Cells were immunolabeled with anti-BLBP and anti-acetylated microtubule antibodies. BLBP is a radial glial marker and nocodazole resistant, stable microtubules in these cells are detected with anti-acetylated microtubule antibodies. Stable microtubules in control and APC deficient radial progenitors were imaged and percentage of radial glia with network of stable microtubules were quantified for each group.

To measure the effects of microtubule stabilization, radial glia from E15 cerebral cortex were prepared and maintained for 24 hours as described earlier. 5μM taxol was then added to radial glial cells for 15 hours. Cells were also pulsed with 10μm BrdU for the same time. Cells were then fixed in 4% paraformaldehyde and co- immunolabelled with anti- BrdU and anti- BLBP anitobodies. Radial glial process length and percentage of $BrdU^+$ radial glial cells were quantified for control and APC deficient groups.

Supplementary Material

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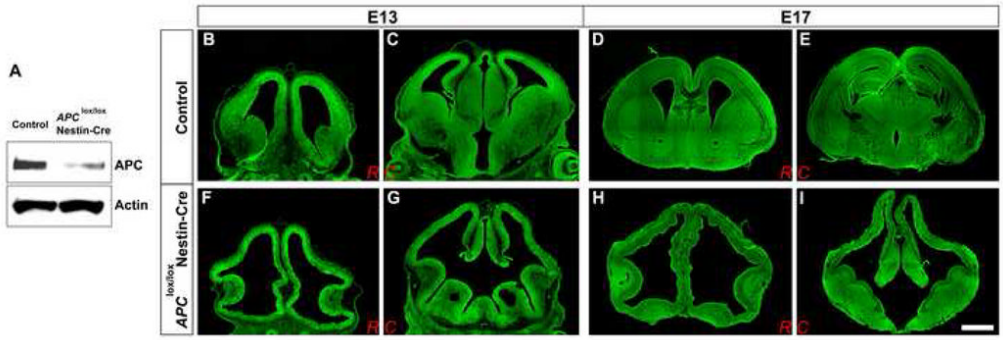

Figure 1. Disrupted corticogenesis in conditional mutants of APC

(A–H) Nestin-Cre mediated recombination was used to inactivate APC expression in the developing brain. (A) Immunoblot analysis of whole cell extracts of E16 cerebral cortex indicates significant reduction in APC following Nestin-Cre mediated recombination of APC *floxed* alleles. Nissl labeled cortical sections from embryonic day (E) 13 [B, C, F, G] and 17 [D, E, H, I] indicate widespread cellular disorganization of cortical structures throughout the rostro-caudal extent of developing brain in mice deficient in APC ($APC^{lox/lox}$Nestin-Cre; F–I). R-rostral, C- caudal. Scale bar: 230 μm (B, C, F, G), 1100μm (D, E, H, I).

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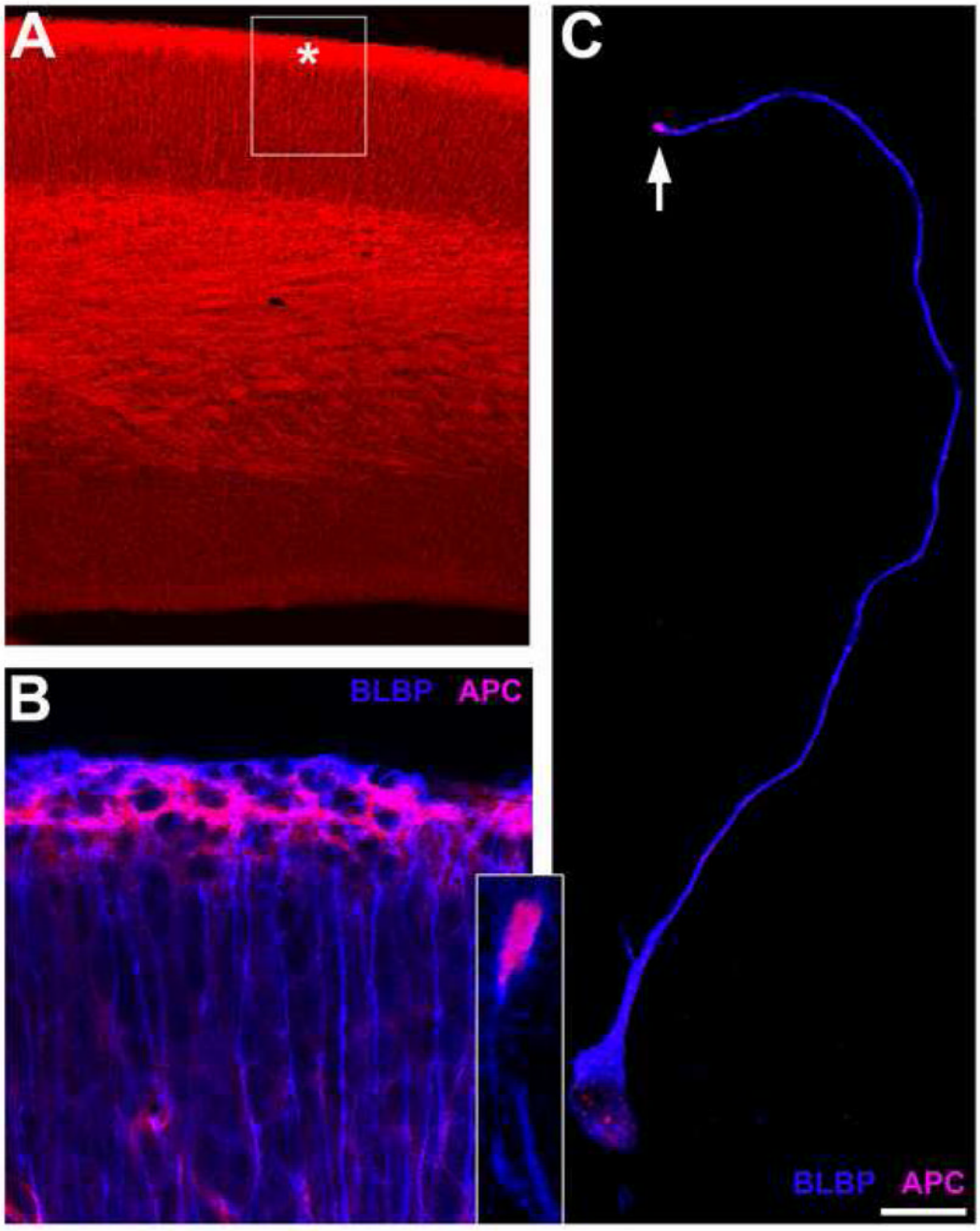

Figure 2. APC expression in developing cerebral cortex

(A) Immunohistochemical localization of APC at E16 indicates prominent APC expression throughout the developing cerebral wall. Enriched level of expression is evident in the subpial region (asterisk). Panel B is a higher magnification image of an area outlined region in panel A. Here, co-localization with radial glial specific anti- BLBP antibodies demonstrates preferential localization of APC in radial glial endfeet (B). Inset (B) shows an isolated radial glial endfeet *in vivo* with enriched APC localization. *In vitro,* APC is expressed in a polarized manner (highly at the tips of the elongated process[arrow] and in the soma) in radial glia labeled with antibodies to radial glial specific marker, BLBP (C). Scale bar: A, 42 μm; B, 27μm; C, 20 μm.

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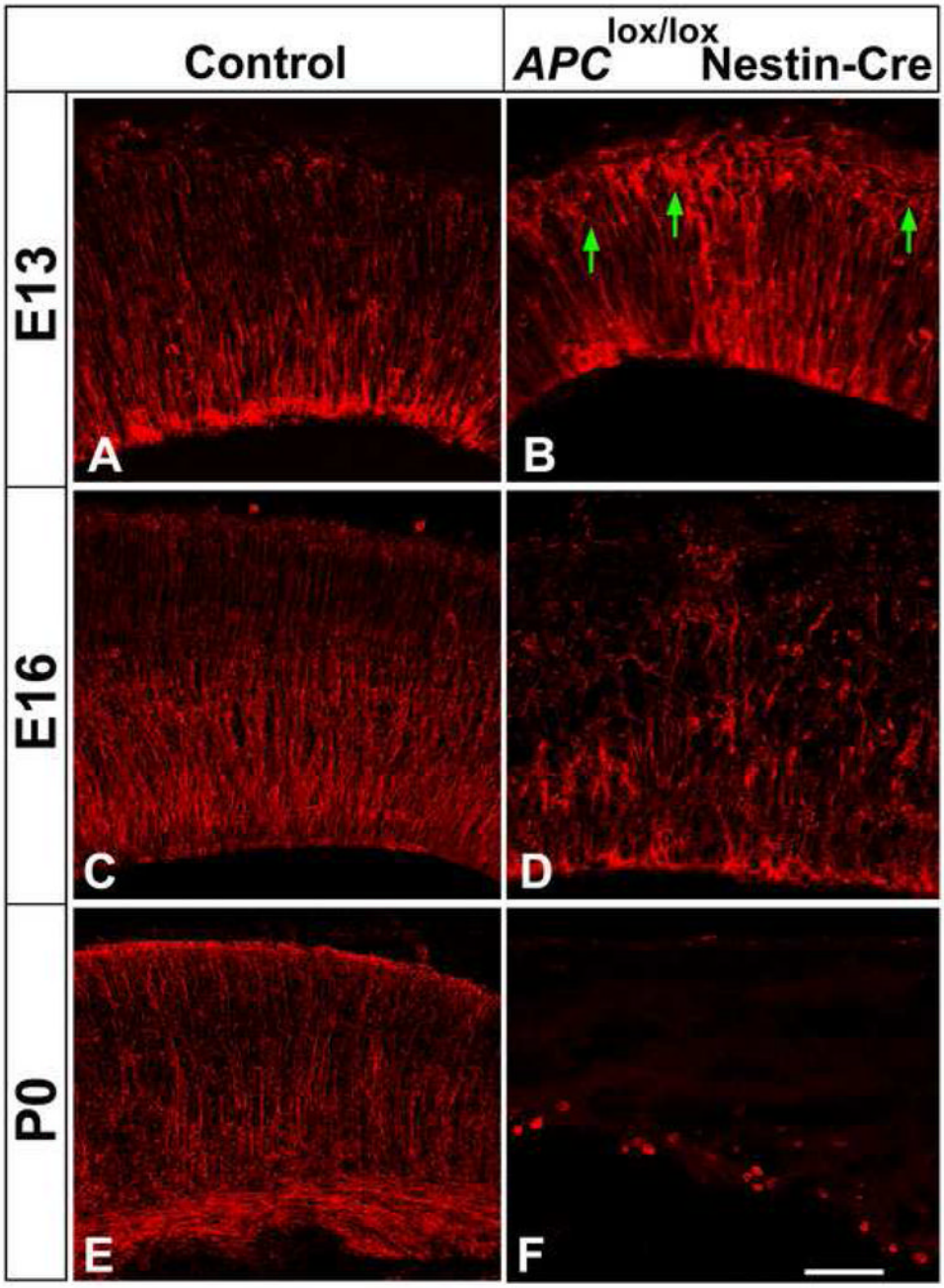

Figure 3. Loss of radial glia polarity following APC deletion

Radial glia in E13, 16, and P0 cortices were labeled with radial glial specific RC2 antibodies. In control cortex, polarized radial glia span the width of the cerebral wall (A, C, E). In contrast, in APC deficient cortex, this characteristic polarized organization of radial glia is drastically disrupted (B, D, F). Radial glial cells are clearly generated in APC deficient mice as evident by their presence at E13(B). However, their elongated processes near the pial surface often points in directions away from the pial surface and are aberrantly branched (arrowheads, B). As cortex develops, they are unable to maintain their polarized morphology-by E16, APC deficient radial glial cells lack the elongated processes spanning the entire width of cortex, instead radial glial processes are shorter and misoriented away from the pial surface (C, D).

By P0, vast majority of APC deficient radial glia do not have a radial process (E, F). Scale bar: A–B, 30 μm; C–D, 56μm; E–F, 145 μm.

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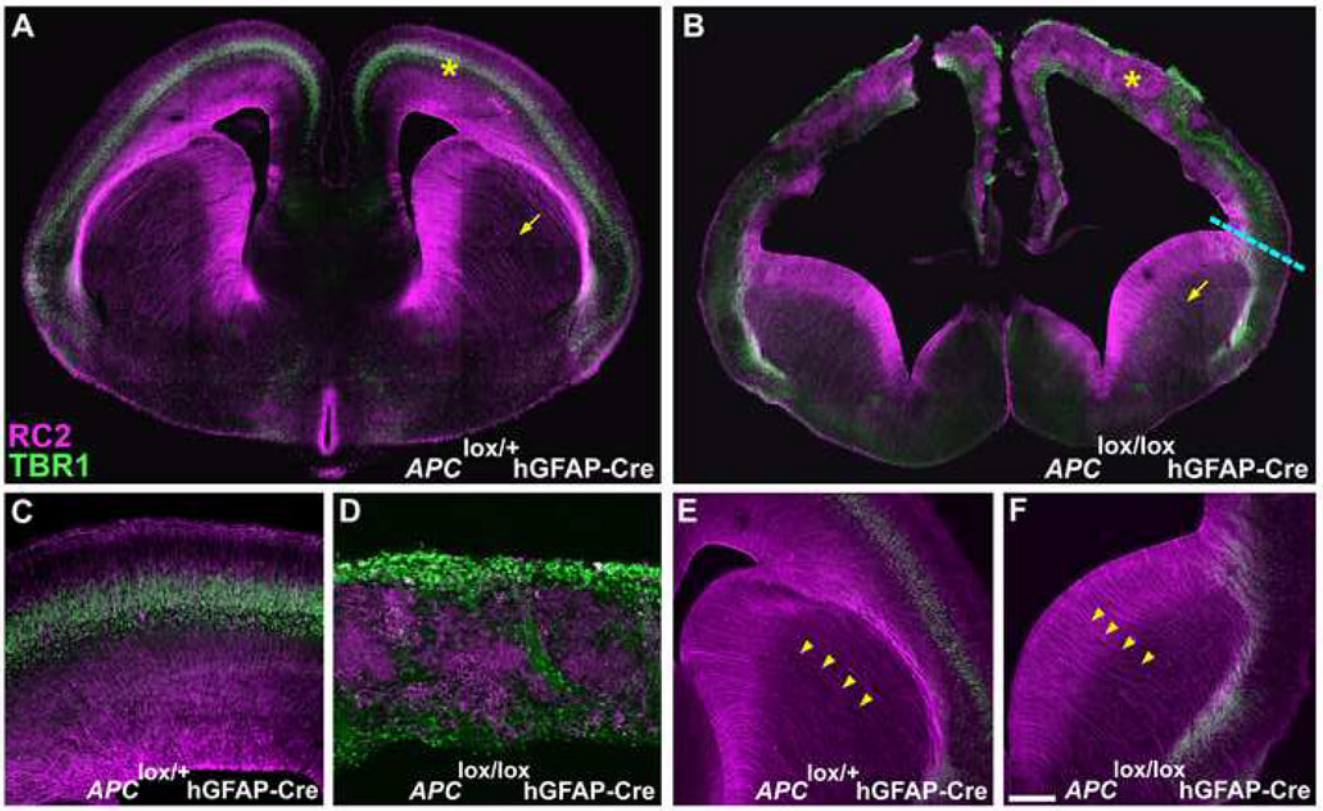

Figure 4. Disrupted Radial glial development in *APC*$^{lox/lox}$hGFAP-Cre cerebral cortex

hGFAP- Cre mediated recombination was used to inactivate APC in radial glia. At E16, hGFAP promoter- Cre mediated recombination occurs widely throughout the dorsal, but not ventral, cortex. (A) Radial glial scaffolding (purple) is evident throughout the normal developing cerebral cortex. In contrast, radial glia development is completely disrupted, specifically in the dorsal regions of cortex in *APC*$^{lox/lox}$hGFAP-Cre mice (B). Dorsal and ventral regions of cortex are indicated by asterisk and arrow, respectively (A, B). Dotted lines in panel B indicates the border between dorsal and ventral cortex. (C, D) Higher magnification images of dorsal cortex from control (C) and *APC*$^{lox/lox}$hGFAP-Cre (D) mice illustrates the lack of radial glial scaffolding in dorsal cortex of APC deficient mice. In contrast, in the ganglionic eminence region of the ventral cortex, radial glial scaffolding appears similar in control (E) and *APC*$^{lox/lox}$hGFAP-Cre mice (F). Arrowheads in panels E and F points to polarized radial processes. Labeling with anti- Tbr1 antibodies (green) indicate normal placement of deeper layer neurons in control (A, C), but not in APC deficient cortex (B, D). Scale bar: A–B, 165 μm; C–D, 60μm; E-F, 90μm.

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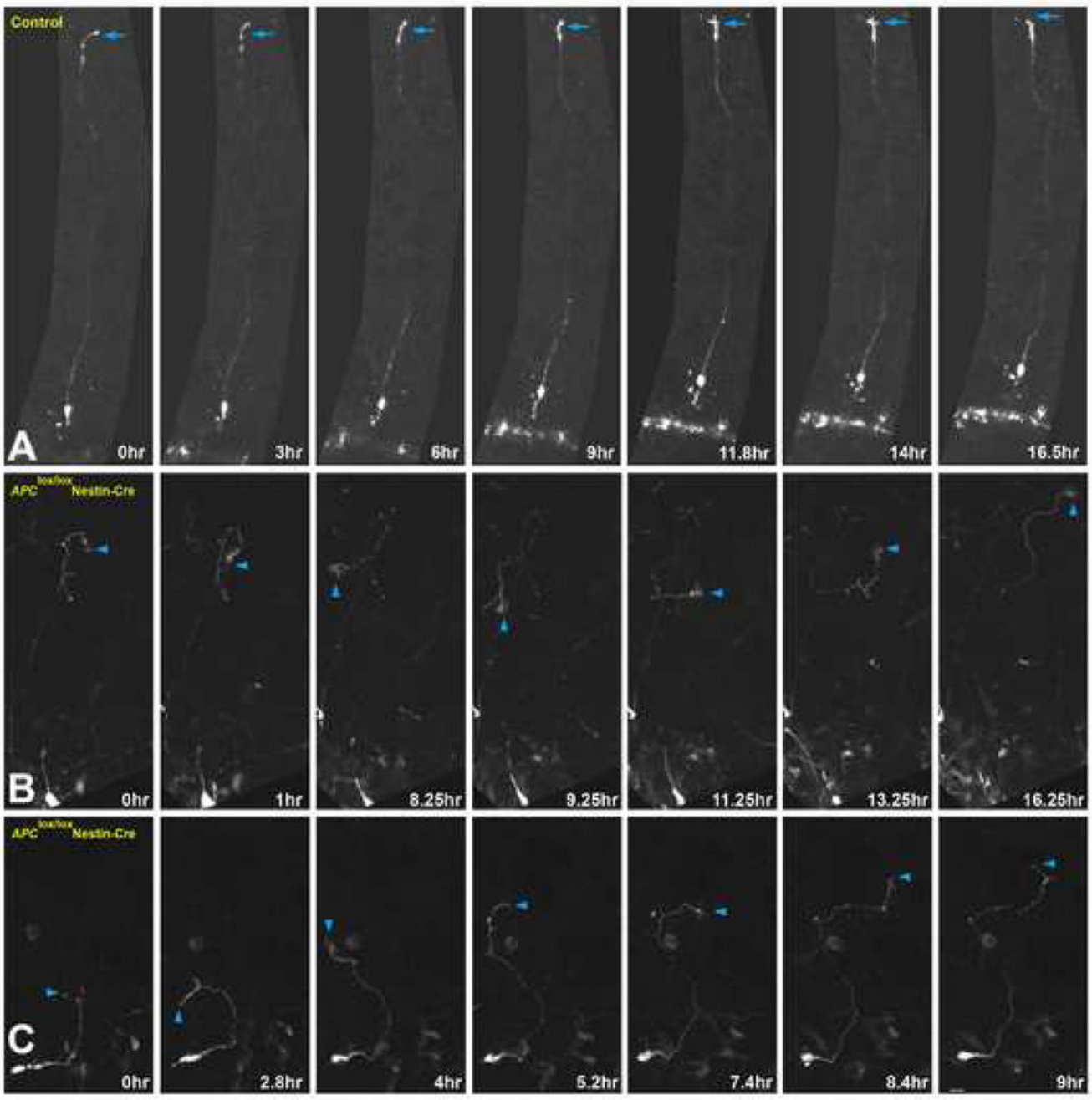

Figure 5. APC is critical for polarized extension of radial glia

Radial glia in E16 control and APC deficient cortices were electroporated with BLBP-GFP DNA. GFP^+ radial glia in control and APC deficient mice were then repeatedly imaged at 5–10 minute intervals for several hours. (A) Control radial glia are polarized and extend their basal process (arrow) towards pial surface. In contrast, APC deficient radial glia appear unable to radially orient and extend towards the pial surface (B, C). Instead, they extend or branch randomly throughout the developing cerebral wall (arrowhead, B, C). Time elapsed between observations are indicated in hours. These images were compiled as AVI movie files to illustrate the differences in radial polarity between control (Supplemental movie 1) and APC deficient (Supplemental movies 2, 3, and 4) radial glia. Time length = 16.5 hrs.(Supplemental

movie 1), 19.5 hrs. (Supplemental movie 2), 10.8 hrs. (Supplemental movie 3), 10.8 hrs. (Supplemental movie 4). Scale bar: 22 μm.

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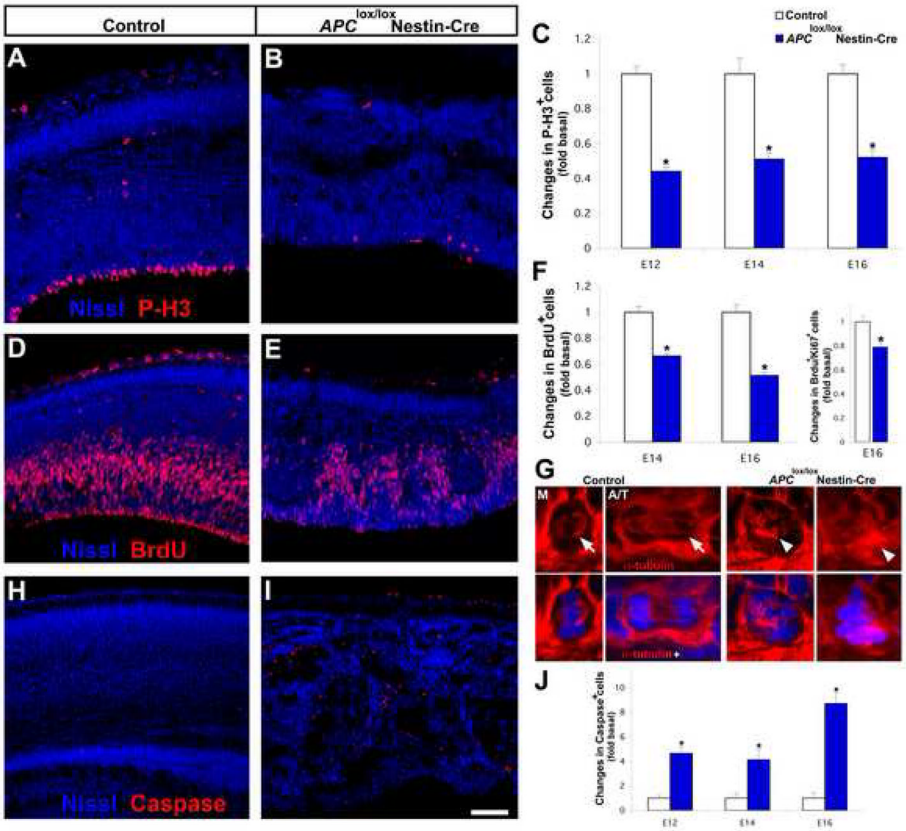

Figure 6. Reduced proliferation of radial glial progenitors in APC deficient cerebral cortex

(A–C) Proliferating radial progenitors in embryonic cortex were labeled with anti- phospho histone antibodies. APC deficiency leads to significant reduction in progenitor proliferation. (D–F) Similar reduction in cortical precursor proliferation was also noticed with BrdU pulse labeling. E14 and 16 mice were given BrdU pulse injections for an hour and BrdU labeled cells were quantified in control and APC deficient cortex (F). Analysis of BrdU and Ki67 double-labeled cells indicates that the fraction of actively cycling $BrdU^+/Ki67^+$ cells was reduced significantly in APC deficient cortex (far right panel, F). (G) Labeling of proliferating radial progenitors in the ventricular zone with anti-α tubulin antibodies and nuclear DNA marker, bis benzimide, indicates the normal formation mitotic spindles (arrows) in control radial progenitors at different stages of cell cycle. In contrast, APC deficient progenitors lack properly organized mitotic spindles (arrowheads). They are often unaligned and unattached. (H–J). Reduction in precursor proliferation is paralleled by enhanced apoptosis in APC deficient cortex. Apoptotic cells were labeled with anti- caspase 3 antibodies. Panels shown in A–B, D–G, and H–I are from E14, 16, and P0, respectively. Sections were Nissl counterstained [blue]. Data shown are mean ±SEM (n=3); asterisk, significant when compared with controls at $p<0.01$ (Student's t test). M-M phase, A/T-anaphase/telophase. Scale bar: A–B, 20 μm; D–E, 60μm; G, 6μm; H–I, 114μm.

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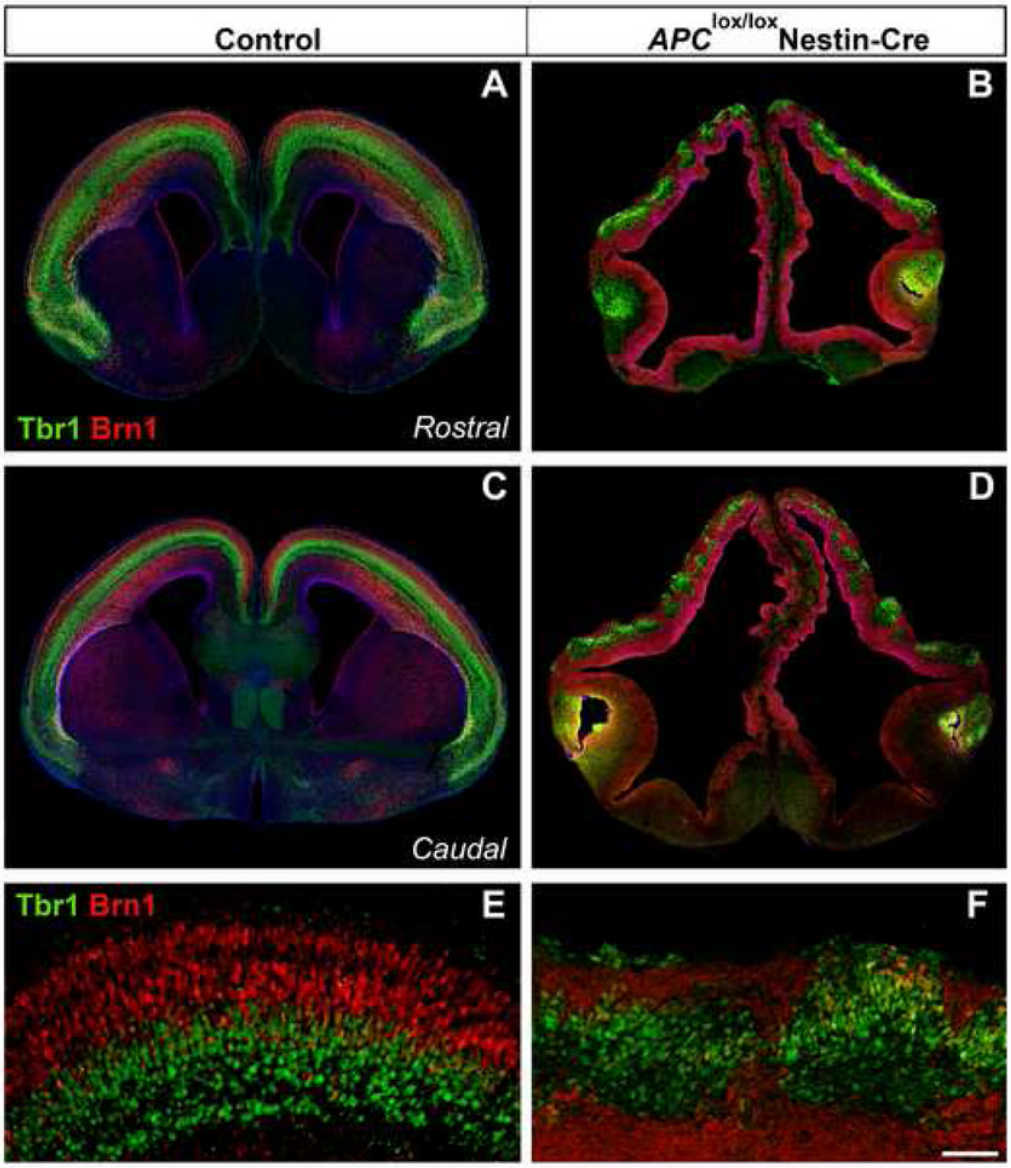

Figure 7. APC deletion disrupts laminar organization of neurons in cerebral cortex

Upper and deeper layer neurons in E16.5 cortices from control and APC$^{lox/lox}$Nestin-Cre mice were labeled with anti- Brn-1 and Tbr1 antibodies, respectively. Characteristic laminar organization was evident throughout the rostro-caudal extent in control cortices (A, C), but was totally disrupted in APC deficient cortex (B, D). APC deficient neurons are distributed as ectopias throughout the cortex. (E, F) Higher magnification images of control (E) and APC$^{lox/lox}$Nestin-Cre cortical plate (F) illustrate the ectopic positioning of neurons destined to distinct layers in APC mutant mice. Scale bar: 900 μm (A–D), 110μm (E–F).

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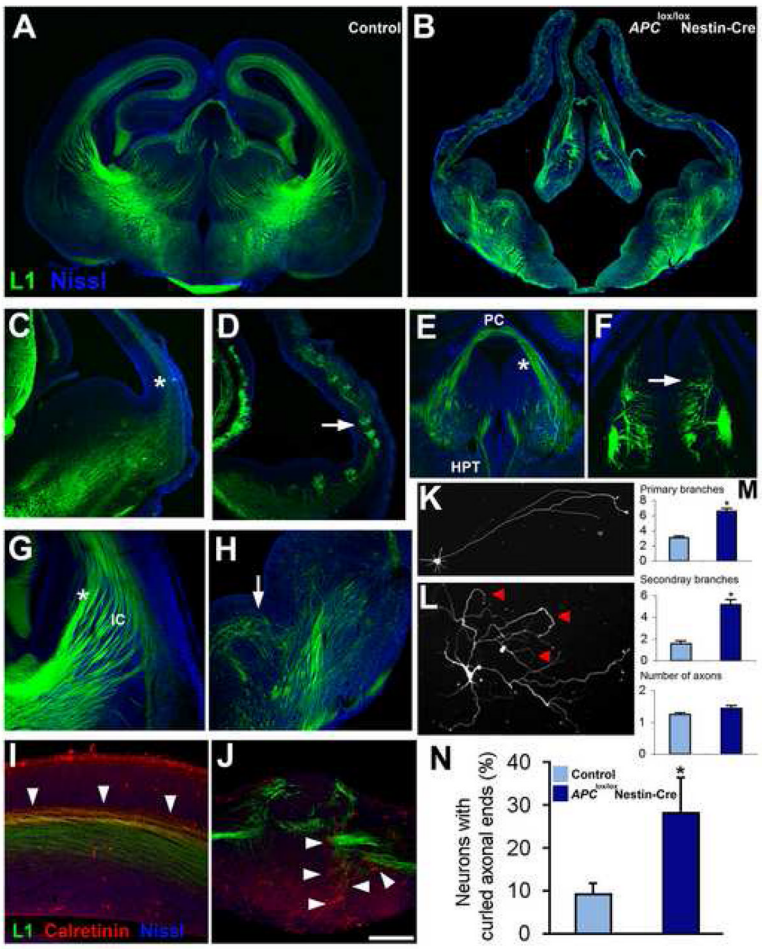

Figure 8. APC regulates patterns of neuronal connectivity in the developing cerebral cortex

(A–B) Labeling of axons entering and exiting the developing cerebral wall (E16) with anti-L1 antibodies indicates that the characteristic patterns of growth of these axonal fiber tracts are disrupted in APC deficient mice. (C, D) At E14 when newly arrived neurons in the cortical plate begin to extend axons (asterisk, C), APC deficiency leads to disrupted axonal extension characterized by clustered swirling of these axons (arrow, D). By E16, the patterned growth of major cortical fiber tracts is altered in APC deficient cortex. Higher magnification images of midbrain regions (E, F) and cortico- striatal boundary (G, H) demonstrate the deficits in the extension and orientation of axons traversing in posterior commisure ([PC]; E, F), habenulo-peduncular tracts ([HPT]; E, F) and internal capsule ([IC]; G, H). Compare the areas indicated by asterisk and arrow (E–H). Labeling of thalamocortical axons with anti- calretinin antibodies (Bloom et al., 2007) indicates the drastic disruption of their extension into the cortex (I, J). Instead of fasciculating adjacent to $L1^{+}$axonal tracts (arrowheads, I), they extend in a totally disorganized manner within the cerebral wall (arrowheads, J). *In vitro,* APC deficient neurons can extend axons, but they are exuberantly branched with increased number of primary and secondary branches (K–M). Compared to controls, the growth tips of these axons consistently curl around (arrowheads, L; N). IC- internal capsule, PC- posterior commisure, HPT- habenulo-peduncular tract. Data shown are mean ± SEM; asterisk indicates significance compared with controls at $p<0.01$ (Student's t-test). Scale bar: 325 μm (A–B), 275μm (C–D), 250μm (E–H), 180μm (K–L).

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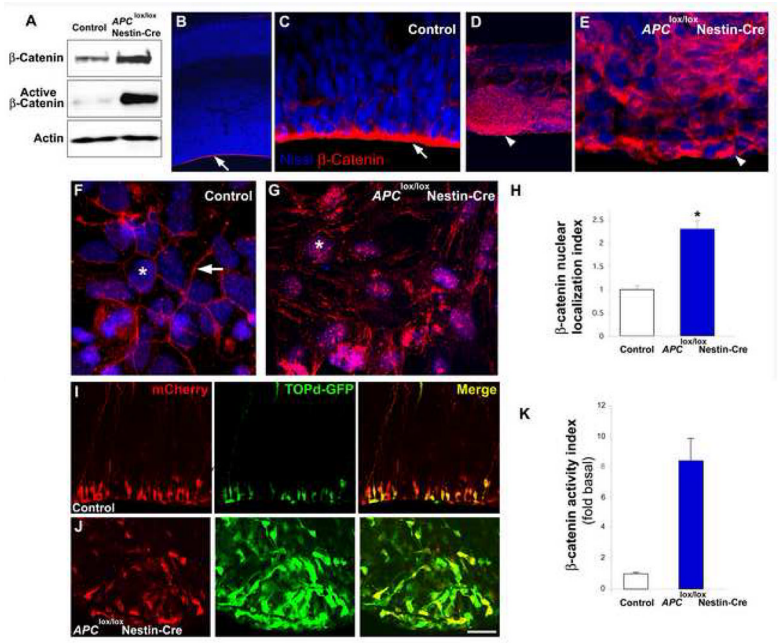

Figure 9. APC deletion dramatically up regulates β-catenin expression in the developing cerebral cortex

(A) Immunoblot analysis of E16 cerebral cortex indicates up regulation of total β-catenin and dephosphorylated (active) β-catenin in APC deficient cortex. (B–E) Immunohistochemical analysis indicates that, instead of the prominent apical expression of β-catenin noticed in the normal cerebral wall (arrows, B, C), significant up regulation of β-catenin is evident throughout the APC deficient cortex (arrowheads, D, E). Panels B and D show low magnification images of the entire cerebral wall, whereas panels C and E are higher magnification images of the ventricular zone region. (F–H) In dissociated cortical cells, prominent β-catenin localization is noticed at sites of cell- cell contacts (arrow, F). In contrast, APC deletion leads to loss of this characteristic pattern of expression at the sites of cell- cell contacts and upregulation of nuclear localized β-catenin (compare asterisks, F, G; H). (I–K) To determine if the up-regulated β-catenin is transcriptionally active, control and APC deficient cortices were electroporated with a β-catenin activity reporter construct, TOPdGFP. mCherry expression serves as control. APC deficiency leads to significant up regulation of active β-catenin as indicated by enhanced GFP expression. Quantification of β-catenin activation (K). Scale bar: 310 μm (B, D), 35μm (C, E), 12.5μm (F, G), 110μm (I), 100μm (J).

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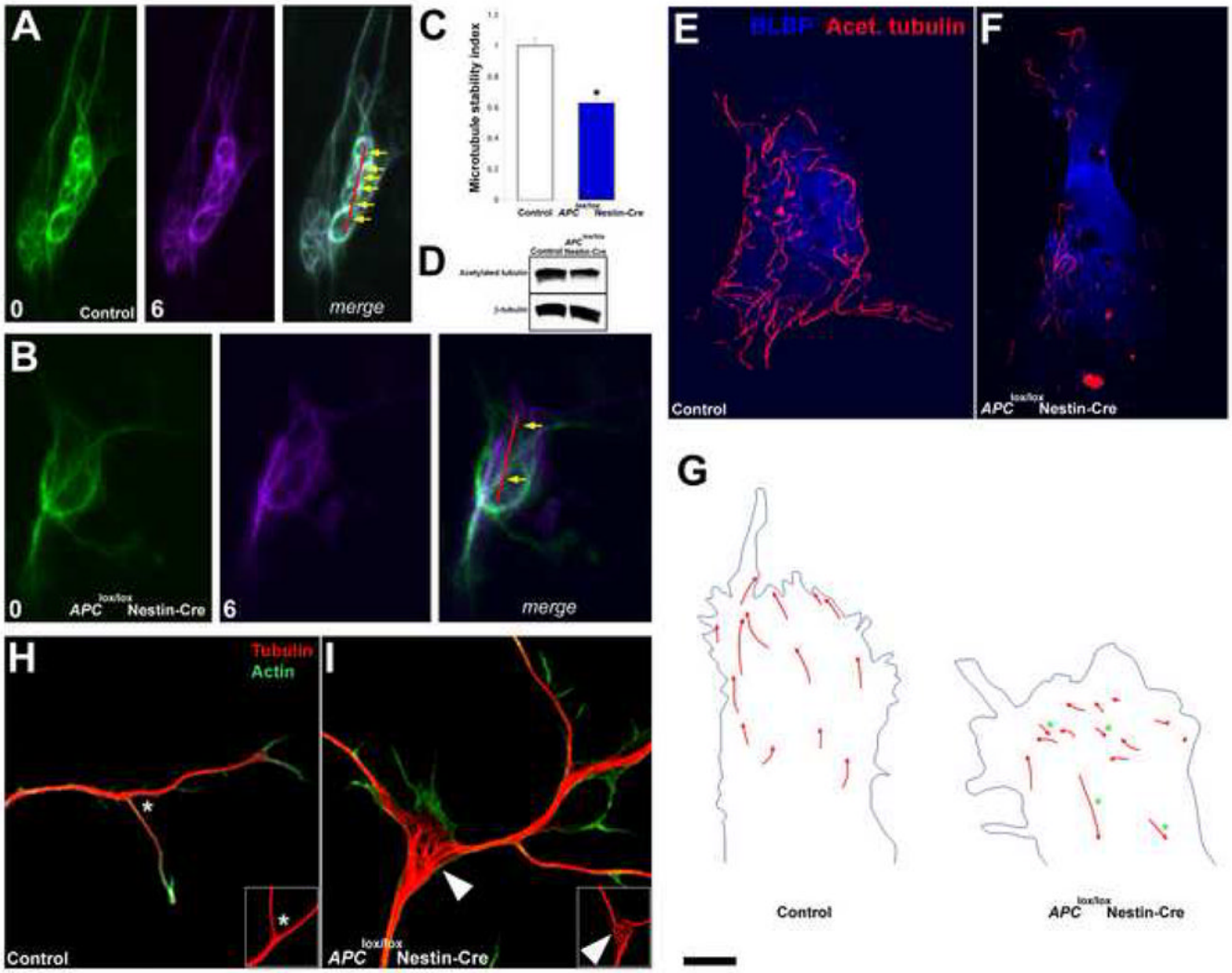

Figure 10. Altered microtubule dynamics in APC deficient cortex

(A–B) Microtubules of radial progenitors in the embryonic cerebral cortex (E15) were labeled with EMTB-3GFP. Labeled cells were imaged at 6-minute intervals and cell soma images from adjacent time intervals were superimposed (merge, A) to evaluate overall changes in microtubule cytoskeleton between different time points of observation. The more overlap of microtubule cytoskeleton between adjacent time points, the more stability of microtubules and less the overall dynamic changes in microtubule cytoskeleton. Compared to control (A), microtubules in APC deficient cells are less stable and rapidly rearranged, thus leading to less overlap (B). Line scans (red line, A–B) spanning the width of the cell soma were used to quantify the spots (yellow arrows, A–B [merge]) of microtubule cytoskeletal overlap between observations. APC deficiency leads to fewer overlaps and thus indicating reduced stability (C). The time-lapse images were also compiled as AVI movie files to illustrate the differences in microtubule dynamics between control (Supplemental movie 5) and APC deficient (Supplemental movie 6) radial progenitors. (D) Immunoblot analysis of acetylated tubulin in control and APC deficient cortex indicates that APC deletion reduced the level of stable, acetylated microtubules. (E–F) Following nocodazole treatment, extensive network of stable, acetylated microtubules is evident in control radial progenitors, whereas fewer stable microtubules were noticed in APC deficient cells. (G) Altered orientation of microtubule plus

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end growth in APC cKO. Patterns of movement of 14 randomly selected EB1-positive microtubule plus ends at the leading edges were traced to indicate the general trajectory and orientation of movement during a period of 60 seconds. Compared to controls (A), many $EB1^{+}$ microtubule tips moved in directions away from cell cortex in APC deficient cells (see asterisks, B). Also see supplemental see movie files #7 (control) and 8 ($APC^{lox/lox}$Nestin-Cre). (H–I) Evidence of microtubule instability is also noticed in APC deficient cortical neurons. Tubulin labeling indicates the presence of diffuse, unfacsiculated microtubule filaments at axonal branch points (arrowheads, I) in APC deficient neurons. In contrast, microtubule filaments in control neurons are tightly facsciculated at branch points (asterisk, H). Number of cells/group= 30 (C). Time elapsed between observations are indicated in minutes (A–B). Data shown are mean ± SEM; asterisk, significant when compared with controls at $p<0.001$ (Student's t test). Also see movie files #5 (Control) and 6 ($APC^{lox/lox}$Nestin-Cre). Time length = 8.3 hrs.(Supplemental movie 5), 6.5 hrs. (Supplemental movie 6), 110 seconds (Supplemental movies 7 and 8). Scale bar: 15 μm (A, B), 10μm (E, F), 2μm (G), 7μm (H, I).