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APC is a component of an organizing template for cortical microtubule networks

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Abstract

A microtubule network on the basal cortex of polarized epithelial cells consists of non-centrosomal microtubules of mixed polarity. Here, we investigate the proteins that are involved in organizing this network, and we show that end-binding protein 1 (EB1), adenomatous polyposis coli protein (APC) and p150^{Glued} — although considered to be microtubule plus-end-binding proteins — are localized along the entire length of microtubules within the network, and at T-junctions between microtubules. The network shows microtubule behaviours that arise from physical interactions between microtubules, including microtubule plus-end stabilization on the sides of other microtubules, and sliding of microtubule ends along other microtubules. APC also localizes to the basal cortex. Microtubules grew over and paused at APC puncta; an *in vitro* reconstituted microtubule network overlaid APC puncta; and microtubule network reconstitution was inhibited by function-blocking APC antibodies. Thus, APC is a component of a cortical template that guides microtubule network formation.

Microtubule interactions with the cell cortex are thought to be important in a wide variety of cell functions, including cytokinesis, cell migration, membrane retraction during cell motility, and vesicle and protein delivery to, and retrieval from, the plasma membrane. However, molecular mechanisms that are involved in specifying microtubule attachment to, and organization at, the cell cortex are poorly understood.

Proteins that associate with microtubule plus ends (+Tip proteins) might act as one point of regulation for microtubule–cortex interactions, because the plus ends are localized to the cell periphery in close apposition to the plasma membrane¹. Several +Tip proteins have been identified, including APC, CLIP-170, EB1 and p150^{Glued} (reviewed in refs 2, 3). However, many questions remain unanswered regarding the regulation and function of +Tip proteins.

Although +Tip proteins have been highlighted as regulators of microtubule dynamics and stability^{4–9}, their role in microtubule binding and organization at the cell cortex remains poorly understood. Overexpression studies indicate that +Tip proteins are delivered to the cortex in association with microtubules^{10,11}, and that they colocalize on the membrane cortex, perhaps in interacting complexes^{8,12}. Examination of endogenous EB1 and APC showed that these +Tip proteins generally co-distribute in the same areas of the cell, but that their subcellular localization is not identical¹³.

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To address questions concerning the organization of microtubules and the functions of +Tip proteins at the cell cortex, we isolated intact basal plasma membranes with the associated cytoskeleton (basal patches) from polarized epithelial cells. We show that cortical microtubules are organized into a network through interactions between individual microtubules, and between microtubules and a basal-cortex-associated template. We exploited the accessibility of these membrane patches for high-resolution microscopy and protein reconstitution to identify microtubule-associated proteins that regulate network organization. Our results define roles for APC and other +Tip proteins in organizing microtubules at the basal cortex of polarized epithelial cells.

RESULTS

To investigate microtubule organization at the plasma membrane, we adopted a simple, reproducible technique termed ‘unroofing’ (reviewed in ref. 14) that uses a brief pulse of sonication to remove the tops of cells and leave behind the basal membrane, with associated cytoskeleton attached to the filter substratum. The resulting basal patches comprise a lipid bilayer, actin filaments and microtubules, but no nuclei and few organelles¹⁵. Scanning electron microscopy (A.R., unpublished observation) revealed that some basal patches remained open, which we exploited for reconstitution experiments, whereas others had sealed over to form very flat basal cytoplasts that were suitable for direct analysis of microtubule dynamics.

Microtubule dynamics on the basal cortex

Microtubule dynamics were imaged in basal cytoplasts that had been isolated from polarized MDCK cells expressing green fluorescent protein (GFP)-tagged tubulin. The majority of cytoplasts were bounded by circumferential microtubules that enclosed a flexible network with multiple microtubule–microtubule connections (Fig. 1a; Supplementary Information, Movie 1). Microtubule activities that arose from interactions of microtubules with one another and with the underlying membrane cortex were identified. These activities are described first, and then we describe the distribution of +Tip proteins that were analysed.

Analysis of microtubule dynamics revealed stabilization points on the basal cortex and on microtubules themselves. Growing microtubules often paused for several minutes when encountering another microtubule (Fig. 1b, c; Supplementary Information, Movies 2 and 3), or a specific location on the basal cortex (see Supplementary Information, Movies 3 and 4). When a microtubule depolymerized, it often did not completely disappear, but remained as a stub on the cortex (Fig. 1b, c). In addition, many microtubules remained fixed in place — even in sharply curved shapes — for long periods, while at the same time exhibiting dynamic instability at the plus end (see Supplementary Information, Movies 3 and 4).

Microtubule ends were often observed to slide along the sides of other microtubules (Fig. 1b–e; Supplementary Information, Movies 2, 3, 5 and 6). In most cases, sliding was predominately in one direction along a microtubule, either to the plus or minus end (Fig. 1b–d), but could occur bidirectionally (Fig. 1e). Two sliding events in Fig. 1b are unidirectional over distances of 0.85 μm (white arrow) and 1.1 μm (green arrow) and occur at instantaneous rates of 0.7 and 1.1 $\mu\text{m min}^{-1}$, respectively, with many pauses in between sliding movements (see Supplementary Information, Movie 2). Although both of these events are towards the minus end, the rates are much slower than dynein-driven movement (1–2 $\mu\text{m s}^{-1}$; refs 16–19). The sliding event in Fig. 1d, although strongly biased in one direction, clearly involved small movements in either direction along the microtubule, with rates as high as 2.5 $\mu\text{m min}^{-1}$ towards the direction of bias and 1.9 $\mu\text{m min}^{-1}$ in the other direction (see Supplementary Information, Movie 5). Thus, sliding might arise from

unbinding, diffusion and rebinding of the microtubule end on the side of another microtubule.

Another property of these interacting microtubules is microtubule bending. Bending occurred when one end of a microtubule slid along the side of another microtubule while its other end remained fixed in place (Fig. 1b–e), or when a microtubule pulled (Fig. 1b, e) or pushed (Fig. 1e; Supplementary Information, Movie 6) on the side of another microtubule. Bending also seemed to arise from an indirect linkage to a moving microtubule (Fig. 1b). Microtubule bending is further evidence that microtubules are physically linked together and firmly attached to the basal cortex.

Microtubules overlay cortical APC puncta

Our analysis has revealed unexpected microtubule properties on these basal membranes. First, microtubules appeared to interact with specific sites on the basal cortex that influenced their dynamics and distribution; second, microtubules interacted physically with other microtubules; and third, together these interactions generated and maintained a crosslinked, dynamic microtubule network on the basal cortex. We next sought to define proteins that could regulate these microtubule properties at the basal cortex.

Scanning electron microscopy of microtubules on basal membrane patches (Fig. 2a) showed the presence of microtubule–microtubule junctions (Fig. 2b). Microtubule ends that formed junctions with the sides of other microtubules often were enlarged, suggesting the presence of proteins or a protein complex at the microtubule end. Along the length of the microtubules were small, and occasionally larger, bumps indicative of bound proteins (Fig. 2b). Below we describe the distributions of +Tip proteins at microtubule junctions and along the length of microtubules, consistent with these images.

APC forms clusters at the tips of cell extensions in association with microtubule plus ends^{11,20–22} and localizes at the base of mammalian polarized epithelial cells^{23,24}. Using an affinity-purified antibody, we detected two localizations of APC on basal membrane patches: one associated with the basal cortex in areas devoid of microtubules, and the other along the entire length of individual microtubules (Fig. 2c–e) and at points of microtubule–microtubule intersection (Fig. 2e, f).

Multiple domains of APC can mediate microtubule binding (Fig. 3a). Carboxy-terminal domains of APC bind directly to microtubules²⁵ and EB1 (ref. 26), and an amino-terminal Armadillo repeat domain binds kinesin-associated protein 3 (KAP3)²⁷, which binds the kinesin KIF3A (ref. 28). We examined basal membrane patches from SW480 and Caco-2 cells, two epithelial cell lines that express a truncated APC protein that lacks the C-terminal microtubule- and EB1-binding domains (Fig. 3a)^{29,30}. APC formed punctate, microtubule-associated arrays and microtubule-independent puncta on the basal membrane of SW40 and Caco-2 cells, similar to those in MDCK cells (compare Fig. 3 with Fig. 2). Thus, APC attachment to the plasma membrane cortex and to microtubules does not require the APC C-terminal microtubule- or EB1-binding domains. These data are consistent with previous results that show that APC that lacks these domains localized with microtubules when overexpressed in cells³¹, and that *Drosophila* APC2, which lacks the C-terminal microtubule-binding domain, localized with microtubules³².

KAP3 and p150^{Glued} localize along microtubules

Because the APC N terminus is sufficient for microtubule binding to the basal cortex, we examined the distribution of KAP3, which binds the N-terminal Armadillo repeats of APC²⁷. KAP3 localized in a punctate pattern on basal membranes from both MDCK and Caco-2 cells. A subset of KAP3 localized to microtubule–microtubule intersections and

microtubule ends (Fig. 4a), and to spots on the cortex that lacked microtubules (Fig. 4a, b). The patterns of KAP3 and APC appeared to be very similar, but because both KAP3 and APC antibodies are made in rabbits, we were unable to test directly for protein colocalization.

We next examined the distribution of p150^{Glued}, a component of the dynactin complex that binds to microtubules³³, KAP3 (ref. 34) and EB1 (ref. 35). p150^{Glued} puncta localized along the length of microtubules on basal patches from both MDCK and Caco-2 cells (Fig. 4c, d). In many cases, p150^{Glued} localized to intersections between microtubules and to microtubule ends (Fig. 4c, d). p150^{Glued} can bind microtubule plus ends independently of dynein and dynactin³⁶, but because it is a component of the dynactin complex, we also examined the distribution of dynein. Dynein, however, was localized to very few spots, scattered over the membrane cortex but not in a pattern like that of either p150^{Glued} or microtubules (data not shown).

EB1 binds microtubules along their length

We extended our analysis to the +Tip protein EB1. EB1 puncta bound the entire length of microtubules and microtubule–microtubule intersections (Fig. 5a), similar to its distribution in intact cells (Fig. 5b). EB1 also bound to growing microtubule plus ends, seen by imaging basal cytoplasts prepared from red fluorescent protein (RFP)-tagged EB1-expressing MDCK cells (A.R., unpublished observation). EB1 was often present at gaps in tubulin antibody staining (Fig. 5a). The gaps in tubulin staining are probably due to the presence of microtubule-associated proteins, including EB1, APC and p150^{Glued}. Note that scanning electron micrographs (Fig. 2) showed many puncta, indicative of bound proteins spanning the length of these microtubules.

EB1 staining was not absolutely coincident with, nor qualitatively similar to, that of APC, even though both proteins were present along the same microtubules (Fig. 5d, f). EB1 staining coincided with microtubules, and there was very little EB1 staining in areas of the basal cortex that are devoid of microtubules, unlike APC, which was also found on microtubule-free areas of the cortex. Thus, despite the fact that EB1 and APC bind to each other *in vitro*³⁷, bind microtubules³⁸, and are localized at the basal membrane of polarized epithelial cells, they were not colocalized on basal microtubules (see also ref. 13). As a further test of the independence of EB1 distribution from APC, we examined EB1 localization in Caco-2 cells in which the truncated APC lacks the EB1-binding domain (Fig. 3a). EB1 was located along the length of microtubules on basal patches from these cells (Fig. 5e, f) and from SW480 cells (A.R., data not shown), similar to its distribution on microtubules on MDCK cell basal patches (Fig. 5a). Therefore, a direct APC–EB1 interaction is not required for either APC or EB1 localization to microtubules at the basal cortex.

APC distribution and microtubule dynamics

APC has characteristics of a protein that could link microtubules to the basal cortex, because it localizes both to microtubules and to the basal cortex, and promotes microtubule stability^{8,31}. To investigate a role for APC in microtubule organization on the basal cortex, we sought to define where APC was in relation to microtubule growth and dynamics. Rather than overexpressing a tagged form of APC, we analysed the distribution of endogenous APC by retrospective staining after imaging microtubule dynamics.

Analysis of individual microtubule dynamics revealed that microtubule plus ends often grew over and paused for long periods at specific points on the basal cortex (Figs 1 and 6; Supplementary Information, Movies 3 and 8). Retrospective staining showed that basal

cortex-associated APC puncta were present at points along the path of microtubule growth, at points where microtubule plus ends paused, and at points where microtubules were rescued (see Fig. 6e for a representative life-history plot). In addition, APC localized in prominent puncta where multiple microtubules converged (Fig. 6a; Supplementary Information, Movie 7), and at points along sharply curving microtubules. Together these results provide evidence that APC puncta on the basal cortex are sites of microtubule attachment and stabilization.

Several lines of evidence indicate that binding of microtubules to cortex-associated APC occurred as microtubules grew to, and over, an APC punctum. First, APC puncta were present in the retrospective analysis without an overlying microtubule, but were seen in the movie to be in the path of microtubule growth or at a point of pause (Fig. 6c, d). Second, large puncta or clusters of APC were crossing points for multiple microtubules, and microtubules coming from different directions grew over the same APC punctum (Fig. 6). Third, microtubule depolymerization was rescued at APC puncta (Fig. 6d, e). Although we do not exclude that APC rides along on the plus end of growing microtubules¹¹, our results reveal that basal-cortex-bound APC puncta provide attachment points for growing microtubules and thereby contribute to the organization of the microtubule network on the basal cortex.

Assembly of microtubules on the basal cortex

To test directly whether basal cortex-bound APC puncta are involved in microtubule binding and organization on the basal cortex, we examined the distribution of exogenous tubulin that polymerized into microtubules on open basal membrane patches. We found that pre-polymerized microtubules did not bind to basal patches (A.R., unpublished observation). Therefore, tubulin dimers were added to allow direct polymerization of microtubules over the basal cortex, and their distribution was compared with that of endogenous APC puncta. Strikingly, these newly polymerized microtubules superimposed precisely over APC puncta on the basal cortex (Fig. 7a, b).

We repeated this experiment on basal patches from Caco-2 cells. Again, we found that exogenous tubulin polymerized over APC puncta (Fig. 7c) and formed a pattern similar to that of endogenous microtubules (Fig. 7d). Note that exogenous microtubules could be distinguished from endogenous microtubules owing to discontinuities in the staining of endogenous microtubules (Fig. 7d). Because Caco-2 cells have a C-terminal truncation of APC, the N-terminal domain of APC is sufficient for microtubule capture on the basal cortex, consistent with our earlier findings (see Fig. 3).

To obtain functional evidence that APC directly regulates microtubule organization on the basal cortex, we premixed tubulin dimers with an affinity-purified APC polyclonal antibody (APC2; ref. 25) to determine whether antibody binding to APC would sterically inhibit tubulin polymerization on membrane patches (Fig. 8). Compared with the control (Fig. 8a), microtubules polymerized onto the patches with a lower frequency in the presence of the APC antibody (Fig. 8b). However, many spots of tubulin or very short microtubules were found on membrane patches (Fig. 8b).

Our reconstitution experiments showed that pre-polymerized microtubules do not bind to the basal cortex, but actively polymerizing microtubules bind in association with endogenous basal-cortex-bound APC. APC might be required to attach or stabilize microtubules as they polymerize, or to lower the critical concentration of tubulin required for nucleation. To address these questions, we performed the reconstitution experiment in the presence of 5 μ M taxol and APC antibody. Taxol enhances microtubule polymerization by lowering the critical concentration for microtubule nucleation³⁹, and stabilizes polymerized

microtubules⁴⁰, thus relieving these potential roles from APC and allowing us to focus on microtubule attachment to the basal cortex. In the presence of taxol, but absence of APC antibody, exogenously polymerized microtubules were shorter but more numerous than in the absence of taxol (Fig. 8c), consistent with increased microtubule nucleation on the basal cortex. In the presence of APC antibody and taxol, the number of microtubules on patches was considerably lower (Fig. 8d). Thus, at least one function of APC is to act as binding sites for polymerizing microtubules on the basal cortex. The small spots of tubulin or short microtubules present on the basal cortex in the absence of taxol might indicate that APC is not required for microtubule nucleation, but rather acts as a succession of binding sites on the basal cortex that stabilize polymerizing microtubules. Indeed, the retrospective analysis (Fig. 6) indicated that microtubules paused and were rescued at APC puncta, providing evidence that APC has a role in stabilizing as well as binding microtubules on the cortex.

We repeated the reconstitution experiment on Caco-2 cell basal patches using rhodamine-labelled tubulin in the presence of a control antibody or APC antibody (Fig. 8e). We quantified the results by tracing the area of the basal membrane patches, and by measuring the number and total length of microtubules per area. In the presence of control antibody, the total microtubule length per area was $0.33 \pm 0.14 \mu\text{m} \mu\text{m}^{-2}$ ($n = 21$ patches) compared with $0.09 \pm 0.04 \mu\text{m} \mu\text{m}^{-2}$ in the presence of APC antibody ($n = 24$ patches). The number of microtubules per patch was 0.07 ± 0.04 per μm^2 for the control, compared with 0.02 ± 0.01 per μm^2 in the presence of APC antibody (Fig. 8e). Thus, in the presence of an APC antibody, microtubule growth is inhibited on the basal cortex of cells expressing full-length or truncated APC.

DISCUSSION

Interactions between microtubules and the cell cortex are thought to regulate many important cellular processes. However, it remains unclear how microtubules are organized at the cell cortex by cortical proteins. Using basal membrane patches from polarized epithelial cells to directly analyse microtubule–cortex interactions, we provide evidence that APC and other +Tip proteins regulate microtubule dynamics and organization at the cortex.

We found that microtubules are attached to the basal cortex as a dynamic network with many microtubule–microtubule intersections. Although intersections have been reported between actin filaments (reviewed in ref. 41), this is, to the best of our knowledge, the first report that microtubules can be organized in this way. Several behaviours arose from microtubule–microtubule interactions, including bending of microtubules, stabilization of microtubule ends on the sides of other microtubules, and sliding of microtubule ends along the sides of other microtubules. Our parallel analyses of +Tip protein distribution and function suggest how they might regulate the organization and dynamics of this cortical microtubule network.

Our results show that endogenous EB1, APC and p150^{Glued} localized along the entire length of microtubules, not just at plus ends. Although EB1 is implicated in stabilizing microtubules by localizing at the plus ends of microtubules⁸, EB1 has also been shown to bind the side of microtubules — albeit with a lower affinity than the plus end — in *Xenopus* egg extracts⁴². In addition, inhibition of phosphorylation by protein kinase A caused p150^{Glued} to bind to the sides of microtubules and not just to the plus ends³⁶. We also found that +Tip proteins localized to microtubule–microtubule junctions. In the same way that +Tip proteins may function at the ends of microtubules to promote contact with the cell cortex², they may be responsible for contacts between microtubule ends and the sides of other microtubules.

The separate localization of APC on the cell cortex indicates an additional function in microtubule organization. APC can link microtubules to the cell cortex^{32,43}, stabilize microtubules^{8,31} and stimulate their polymerization^{25,44}, which could mean that it lowers the critical concentration for microtubule nucleation, or stabilizes polymerized microtubules. Our results indicate that APC is involved in both microtubule stabilization and microtubule attachment to the membrane cortex, and thereby has a role in organizing microtubule networks on the basal cortex. Retrospective staining of APC after imaging microtubule dynamics showed that points where microtubule plus ends paused and were rescued were coincident with puncta of APC; these behaviours could indicate stabilization of microtubules by APC. Retrospective staining also showed that microtubules grew over paths where individual APC puncta were located on the basal cortex, indicating that APC is involved in attaching microtubules to the membrane cortex. Mechanisms involved in microtubule stabilization on the cell cortex by APC may include stabilization of microtubule plus ends at the cortex, and APC-mediated attachment along the length of microtubules to stabilize the polymer. In a second approach, exogenous tubulin dimers were added to open patches and were found to polymerize in association with APC puncta. Fewer microtubules polymerized on patches when tubulin dimers were premixed with an APC antibody, even when microtubule polymerization and stabilization was promoted by taxol, again indicating a role for APC in microtubule attachment to the cortex. APC may be retained at the cortex after being transported to the plasma membrane at the tips of microtubules¹¹, where, as shown here, it would then be available for additional interactions that direct and stabilize new microtubules that polymerize on or towards the membrane⁴⁵.

METHODS

Cell lines

The MDCK tubulin-GFP cell line (constructed by A. Barth and E. de Hostos) was made by inserting human α -tubulin, which was cloned from a cDNA library (Genbank accession number K00558), into vector pEGFP-C1 from Clontech (Palo Alto, CA), transfecting this vector into MDCK II cells, and selecting clones of cells in G418. MDCK type II cells and SW480 cells were grown in DMEM that was supplemented with 10% fetal bovine serum. The Caco-2 'brush-border-expressing' human colon adenocarcinoma cell line C2BBE1 is from The American Type Culture Collection (Manassas, VA) and was grown in DMEM without phenol red and supplemented with 4.5 g l⁻¹ glucose, 1.5 g l⁻¹ sodium bicarbonate, 0.01 mg ml⁻¹ human transferrin and 10% fetal bovine serum.

Basal membrane isolation

Cells were plated at confluent density on 12-mm Transwell polycarbonate filter membranes with 0.4 μ m pore size (Corning, Corning, NY), and grown for 3–5 days (MDCK cells) or 2 weeks (Caco-2 cells) to allow for cell polarization. Cells were rinsed and incubated for 10 min in hypotonic buffer (15 mM HEPES, pH 7.3; 15 mM KCl; 1 mM MgCl₂; and 1 mM EGTA), and sonicated at 4 °C with a brief (less than 1 s) pulse using a Branson sonifier 250 (Branson Ultrasonics, Danbury, CT), set at duty cycle 20 and output 19–22%, with a one-eighth inch microprobe held approximately 5–7 mm above the surface of the cells (for a detailed protocol, see ref. 15). Membrane patches were rinsed briefly in buffer before fixation or reconstitution of microtubules.

Fixation and antibodies

Fixation of microtubules on isolated membranes was with 0.3% glutaraldehyde or cold methanol for 10 min. Glutaraldehyde fixation was followed by quenching with 1 mg ml⁻¹ NaBH₄. Microtubules were stained with DM1 α mouse monoclonal α -tubulin antibody (Sigma, St Louis, MO) diluted 1:200. For co-staining of microtubules with p150^{Glued} or

EB1, rat monoclonal α -tubulin antibody YL1/2 was used at 1:200 (Accurate Chemical & Scientific Corporation, Westbury, NY) using the protocol from Jackson Labs for co-staining with mouse and rat monoclonal antibodies (Jackson ImmunoResearch, West Grove, PA). For examination of APC and EB1 in intact cells, fixation was with cold methanol. For visualization of APC and EB1 on isolated membranes, cold methanol or 0.3% glutaraldehyde gave the same pattern of staining. APC was stained with an affinity-purified polyclonal antibody to a central APC domain²⁰. EB1 was stained in MDCK cells with a monoclonal antibody (discontinued from Oncogene Research Products, San Diego, CA) and in Caco-2 cells with monoclonal antibody from BD Biosciences Pharmingen (San Diego, CA). Polyclonal antibodies to KAP3 were a kind gift from N. Hirokawa²⁸ and Y. Takai⁴⁶. For detection of p150^{Glued}, isolated membranes were fixed in cold methanol and probed with a monoclonal antibody to p150^{Glued} from BD Transduction Labs. Secondary antibodies, conjugated to FITC or rhodamine, were from Jackson Labs.

Fluorescence microscopy and image processing of fixed basal membrane patches

Image z-stacks were collected in 0.20- μ m steps on an Olympus IX-70 inverted microscope with a $\times 100$ 1.35 N.A. oil-immersion objective (Olympus America, Melville, NY), and captured with a cooled CCD camera (Photometrics, Tuscon, AZ). Images were collected and processed using DeltaVision deconvolution software (Applied Precision, Issaquah, WA) on a Silicon Graphics workstation (Silicon Graphics, Mountain View, CA).

Scanning electron microscopy

Basal membranes were prepared from MDCK cells that were polarized on Transwell filters, and fixed for 30 min in 2% glutaraldehyde in BRB80 buffer (80 mM PIPES, pH 6.9; 1 mM MgCl₂; and 1 mM EGTA). For gold-labelling of microtubules, unreacted aldehydes were quenched with NaBH₄, and microtubules were stained with the DM1 α monoclonal antibody diluted 1:50, followed by 15-nm gold-labelled secondary antibody. Samples were fixed again after immunolabelling in 2% glutaraldehyde. Samples were processed according to previously described methods⁴⁷. Briefly, samples were changed from glutaraldehyde to 0.1% tannic acid for 20 min, 0.1% uranyl acetate for 20 min, dehydrated through an ethanol series, and critical-point dried in ethanol. Filters were cut from the plastic holders only after critical-point drying. Samples were rotary shadowed with platinum at a 45° angle. Samples were imaged with an FEI Company XL30 Sirion scanning electron microscope at 5 kV, with spot size 3, in ultra-high resolution mode.

Imaging of microtubule dynamics and retrospective staining of APC

Tubulin-GFP-expressing MDCK cells were plated at confluent density on Transwell filters and allowed to polarize for 3–5 days. After sonication (as described above) filters were cut from plastic supports with a razor edge, a process that created nicks that served as fiduciary marks for orienting the filter after retrospective staining. Filters were rinsed several times in BRB80 buffer, mounted on a glass slide between two strips of double-sided sticky tape, and covered with BRB80 buffer. A 22-mm glass coverslip was placed on top and sealed in place with silicone vacuum grease. Imaging was performed with a Zeiss 200M Axiovert (Carl Zeiss, Thornwood, NY) run by Slidebook software (Intelligent Imaging Innovation, Denver, CO) using a $\times 100$ 1.3 N.A. objective lens heated to 37 °C. Images were captured for 5 s each with no interval between frames. Some patches had microtubules that were not dynamic; we presumed these patches were open or unsealed. Patches that had microtubule dynamics were presumed to be sealed-over cytoplasts. Inclusion of 2 mM ATP and 1 mM GTP in the sonication and imaging buffers resulted in similar parameters of microtubule dynamics to those that occurred without addition of these nucleotides, indicating that cytoplasts sealed over very quickly after sonication. Images were converted to greyscale and sharpened in Image J with the Fourier transform bandpass filter to remove high and low

spatial frequency signals using the limits of 0.192–1.6 μm . Life-history plots were made by registering the coordinates of individual microtubule plus ends in each movie frame using Image J, and graphing the data using Microsoft Excel. For retrospective staining of APC, the location of the imaged cytoplasm was circled on the coverslip, related to the nicks on the filter, and recorded in a laboratory notebook. Filters were fixed in 0.3% glutaraldehyde for 15 min; unreacted aldehyde groups were reduced with three rinses of 1 mg ml⁻¹ NaBH₄; and filters were stained with an affinity-purified antibody to the middle ‘APC2’ domain of APC²⁵. The filter was mounted on a glass slide in the same orientation as during imaging to facilitate location of the imaged cytoplasm. The image of retrospective staining was overlaid on the movie of microtubule dynamics using Adobe Premiere (Adobe Systems, San Jose, CA).

Microtubule reconstitution

Isolated membrane patches were rinsed after sonication in BRB80 buffer and incubated with 1 mg ml⁻¹ phosphocellulose-purified bovine brain tubulin (generously provided by the laboratory of R. Vale) in BRB80, 1 mM GTP, at 37 °C for 20 min. Patches were rinsed free of unbound tubulin with two rinses of warm BRB80 and fixed in 0.3% glutaraldehyde. For antibody inhibition experiments, 5 μl affinity-purified 0.46 mg ml⁻¹ antibody in 50 mM Tris (pH 7.5), raised to the middle ‘APC2’ domain of APC²⁵, was premixed with 70 μl 1 mg ml⁻¹ tubulin in BRB80 buffer on ice (for a final concentration of 30 μg ml⁻¹ antibody). As the control condition, 5 μl 50 mM Tris, pH 7.5, was mixed with 70 μl tubulin. Tubulin, with or without antibody, was added to patches and incubated for 20 min with 1 mM GTP at 37 °C. Antibody inhibition in the presence of taxol was performed as above, except 1 mg ml⁻¹ tubulin (with or without antibody) was premixed with 5 μM taxol before transferring to patches. For quantification, the experiment was performed with rhodamine-labelled tubulin to be sure that only exogenously polymerized microtubules were counted. Rhodamine-tubulin (Cytoskeleton, Colorado Springs, CO) was diluted into 1 mg ml⁻¹ unlabelled tubulin at a ratio of 1:100, and added to patches in the presence of control or APC antibody for 20 min with 1 mM GTP at 37 °C. The control antibody was Biotin-SP affinity-purified goat anti-mouse IgG (H+L) from Jackson Labs; 1 mg ml⁻¹ in 15 mg ml⁻¹ BSA diluted to 30 μg ml⁻¹ antibody. Patches were rinsed with warm BRB80 three times and fixed in 0.3% glutaraldehyde. Image J was used to trace the area of the patch and to measure microtubule length. The number of microtubules was counted per area of patch by eye.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mimori-Kiyosue Y, Tsukita S. “Search-and-capture” of microtubules through plus-end-binding proteins (+TIPs). *J Biochem (Tokyo)*. 2003; 134:321–326. [PubMed: 14561716]
2. Vaughan KT. Surfing, regulating and capturing: are all microtubule-tip-tracking proteins created equal? *Trends Cell Biol*. 2004; 14:491–496. [PubMed: 15350977]
3. Gundersen GG, Gomes ER, Wen Y. Cortical control of microtubule stability and polarization. *Curr Opin Cell Biol*. 2004; 16:106–112. [PubMed: 15037313]

4. Brunner D, Nurse P. CLIP170-like tip1p spatially organizes microtubular dynamics in fission yeast. *Cell*. 2000; 102:695–704. [PubMed: 11007487]
5. Rogers SL, Rogers GC, Sharp DJ, Vale RD. *Drosophila* EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. *J Cell Biol*. 2002; 158:873–884. [PubMed: 12213835]
6. Komarova YA, Akhmanova AS, Kojima S, Galjart N, Borisy GG. Cytoplasmic linker proteins promote microtubule rescue *in vivo*. *J Cell Biol*. 2002; 159:589–599. [PubMed: 12446741]
7. Fukata M, et al. Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. *Cell*. 2002; 109:873–885. [PubMed: 12110184]
8. Wen Y, et al. EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. *Nature Cell Biol*. 2004; 6:820–830. [PubMed: 15311282]
9. Mimori-Kiyosue Y, et al. CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. *J Cell Biol*. 2005; 168:141–153. [PubMed: 15631994]
10. Mimori-Kiyosue Y, Shiina N, Tsukita S. The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules. *Curr Biol*. 2000; 10:865–868. [PubMed: 10899006]
11. Mimori-Kiyosue Y, Shiina N, Tsukita S. Adenomatous polyposis coli (APC) protein moves along microtubules and concentrates at their growing ends in epithelial cells. *J Cell Biol*. 2000; 148:505–518. [PubMed: 10662776]
12. Watanabe T, et al. Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. *Dev Cell*. 2004; 7:871–883. [PubMed: 15572129]
13. Barth AI, Siemers KA, Nelson WJ. Dissecting interactions between EB1, microtubules and APC in cortical clusters at the plasma membrane. *J Cell Sci*. 2002; 115:1583–1590. [PubMed: 11950877]
14. Heuser J. The production of ‘cell cortices’ for light and electron microscopy. *Traffic*. 2000; 1:545–552. [PubMed: 11208142]
15. Drees F, Reilein A, Nelson WJ. Cell-adhesion assays: fabrication of an E-cadherin substratum and isolation of lateral and basal membrane patches. *Methods Mol Biol*. 2004; 294:303–320. [PubMed: 15576920]
16. Allan V. Protein phosphatase 1 regulates the cytoplasmic dynein-driven formation of endoplasmic reticulum networks *in vitro*. *J Cell Biol*. 1995; 128:879–891. [PubMed: 7876311]
17. Paschal BM, Shpetner HS, Vallee RB. MAP 1C is a microtubule-activated ATPase which translocates microtubules *in vitro* and has dynein-like properties. *J Cell Biol*. 1987; 105:1273–1282. [PubMed: 2958482]
18. Schnapp BJ, Reese TS. Dynein is the motor for retrograde axonal transport of organelles. *Proc Natl Acad Sci USA*. 1989; 86:1548–1552. [PubMed: 2466291]
19. Schroer TA, Steuer ER, Sheetz MP. Cytoplasmic dynein is a minus end-directed motor for membranous organelles. *Cell*. 1989; 56:937–946. [PubMed: 2522353]
20. Nathke IS, Adams CL, Polakis P, Sellin JH, Nelson WJ. The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. *J Cell Biol*. 1996; 134:165–179. [PubMed: 8698812]
21. Etienne-Manneville S, Hall A. Cdc42 regulates GSK-3 β and adenomatous polyposis coli to control cell polarity. *Nature*. 2003; 421:753–756. [PubMed: 12610628]
22. Zhou FQ, Zhou J, Dedhar S, Wu YH, Snider WD. NGF-induced axon growth is mediated by localized inactivation of GSK-3 β and functions of the microtubule plus end binding protein APC. *Neuron*. 2004; 42:897–912. [PubMed: 15207235]
23. Rosin-Arbesfeld R, Ihrke G, Bienz M. Actin-dependent membrane association of the APC tumour suppressor in polarized mammalian epithelial cells. *EMBO J*. 2001; 20:5929–5939. [PubMed: 11689433]
24. Mogensen MM, Tucker JB, Mackie JB, Prescott AR, Nathke IS. The adenomatous polyposis coli protein unambiguously localizes to microtubule plus ends and is involved in establishing parallel arrays of microtubule bundles in highly polarized epithelial cells. *J Cell Biol*. 2002; 157:1041–1048. [PubMed: 12058019]
25. Munemitsu S, et al. The APC gene product associates with microtubules *in vivo* and promotes their assembly *in vitro*. *Cancer Res*. 1994; 54:3676–3681. [PubMed: 8033083]

26. Askham JM, Moncur P, Markham AF, Morrison EE. Regulation and function of the interaction between the APC tumour suppressor protein and EB1. *Oncogene*. 2000; 19:1950–1958. [PubMed: 10773885]
27. Jimbo T, et al. Identification of a link between the tumour suppressor APC and the kinesin superfamily. *Nature Cell Biol*. 2002; 4:323–327. [PubMed: 11912492]
28. Yamazaki H, Nakata T, Okada Y, Hirokawa N. Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B. *Proc Natl Acad Sci USA*. 1996; 93:8443–8448. [PubMed: 8710890]
29. Rubinfeld B, et al. Association of the APC gene product with β -catenin. *Science*. 1993; 262:1731–1734. [PubMed: 8259518]
30. Mariadason JM, et al. Down-regulation of β -catenin TCF signaling is linked to colonic epithelial cell differentiation. *Cancer Res*. 2001; 61:3465–3471. [PubMed: 11309309]
31. Zumbunn J, Kinoshita K, Hyman AA, Nathke IS. Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 β phosphorylation. *Curr Biol*. 2001; 11:44–49. [PubMed: 11166179]
32. McCartney BM, et al. *Drosophila* APC2 and Armadillo participate in tethering mitotic spindles to cortical actin. *Nature Cell Biol*. 2001; 3:933–938. [PubMed: 11584277]
33. Waterman-Storer CM, Karki S, Holzbaur EL. The p150^{Glued} component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1). *Proc Natl Acad Sci USA*. 1995; 92:1634–1638. [PubMed: 7878030]
34. Deacon SW, et al. Dynactin is required for bidirectional organelle transport. *J Cell Biol*. 2003; 160:297–301. [PubMed: 12551954]
35. Askham JM, Vaughan KT, Goodson HV, Morrison EE. Evidence that an interaction between EB1 and p150^{Glued} is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. *Mol Biol Cell*. 2002; 13:3627–3645. [PubMed: 12388762]
36. Vaughan PS, Miura P, Henderson M, Byrne B, Vaughan KT. A role for regulated binding of p150^{Glued} to microtubule plus ends in organelle transport. *J Cell Biol*. 2002; 158:305–319. [PubMed: 12119357]
37. Su LK, et al. APC binds to the novel protein EB1. *Cancer Res*. 1995; 55:2972–2977. [PubMed: 7606712]
38. Morrison EE, Wardleworth BN, Askham JM, Markham AF, Meredith DM. EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. *Oncogene*. 1998; 17:3471–3477. [PubMed: 10030671]
39. Howard WD, Timasheff SN. Linkages between the effects of taxol, colchicine, and GTP on tubulin polymerization. *J Biol Chem*. 1988; 263:1342–1346. [PubMed: 2891710]
40. Amos LA, Lowe J. How taxol stabilises microtubule structure. *Chem Biol*. 1999; 6:R65–R69. [PubMed: 10074470]
41. Higgs HN, Pollard TD. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu Rev Biochem*. 2001; 70:649–676. [PubMed: 11395419]
42. Tirnauer JS, Grego S, Salmon ED, Mitchison TJ. EB1-microtubule interactions in *Xenopus* egg extracts: role of EB1 in microtubule stabilization and mechanisms of targeting to microtubules. *Mol Biol Cell*. 2002; 13:3614–3626. [PubMed: 12388761]
43. Yamashita YM, Jones DL, Fuller MT. Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. *Science*. 2003; 301:1547–1550. [PubMed: 12970569]
44. Nakamura M, Zhou XZ, Lu KP. Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. *Curr Biol*. 2001; 11:1062–1067. [PubMed: 11470413]
45. Krylyshkina O, et al. Nanometer targeting of microtubules to focal adhesions. *J Cell Biol*. 2003; 161:853–859. [PubMed: 12782685]
46. Shimizu K, et al. SMAP, an Smg GDS-associating protein having arm repeats and phosphorylated by Src tyrosine kinase. *J Biol Chem*. 1996; 271:27013–27017. [PubMed: 8900189]
47. Svitkina TM, Verkhovsky AB, Borisy GG. Improved procedures for electron microscopic visualization of the cytoskeleton of cultured cells. *J Struct Biol*. 1995; 115:290–303. [PubMed: 8573471]

Figure 1.

Microtubules interact with one another to cause bending, sliding and pausing. Movie frames of MDCK cell basal cytoplasts expressing tubulin-GFP were chosen according to events of interest and thus time intervals (minutes: seconds) are not evenly spaced. Microtubules are identified by arrow colour. Solid arrows designate initial position; open arrows show displacement of a microtubule end. **(a)** Examples of basal patches circumscribed by microtubules enclosing a flexible microtubule network. Arrows indicate end-to-side intersections between microtubules. Scale bars, 5 μm . **(b)** A microtubule network shows microtubule-microtubule and microtubule-cortex interactions. Microtubule ends marked by plus signs grow or exhibit dynamic instability. The white and red microtubules pause while contacting the sides of other microtubules before they continue to grow. The white and green microtubule ends slide towards minus ends of contacting microtubules, and bend in the process. Before contacting the white microtubule, the pink microtubule bends (pink

arrowhead) as the white microtubule slides and bends, although there is no visible linkage. The pink microtubule pulls on a contacting microtubule, causing it to bend (blue arrowhead). The orange microtubule shrinks to a stub but does not completely depolymerize. The microtubules indicated by the blue and yellow plus signs remain in place despite other microtubules sliding and pulling on them. Scale bar, 5 μm . (c) Another example of a network that exhibits microtubule sliding and bending while maintaining attachment to the cortex. Plus and minus ends were identified by dynamic instability of plus ends, and immobility of minus ends. The red microtubule pauses on, and slides along, the side of another microtubule towards its plus end, bending in the process; it continues to grow after 15 min. The pink and yellow microtubules interact at their plus ends and sides. As the green microtubule grows and shrinks, it pauses on the side of the blue microtubule. The blue and white microtubules pause at specific points on the membrane cortex. Scale bar, 5 μm . (d) A microtubule end slides bidirectionally along an immobilized microtubule with a bias in one direction. Scale bar, 2.5 μm . (e) A microtubule end slides bidirectionally along the side of another microtubule, and thus pushes and pulls this microtubule, causing it to bend. For reference, the box remains at a fixed location. Scale bar, 5 μm .

Figure 2.

APC associates both with microtubules and the basal cortex. Scanning electron micrographs of (a) gold-labelled microtubules on an MDCK cell basal patch (scale bar, 500 nm), and (b) two examples of microtubules without labelling on basal patches. Microtubules have small bumps along their length (white arrowheads), as well as larger bumps (white arrows), indicating coating of microtubules by proteins. Microtubule ends terminate on the sides of other microtubules (red arrowheads) and appear to have protein complexes at these junctions. (c) Indirect immunofluorescence of APC (red) and microtubules (green) on basal membrane patches from MDCK cells show microtubule-dependent and -independent localizations of APC. Scale bar, 10 μm . (d) Detailed view of APC (left) and tubulin co-staining (right) on an isolated basal patch. Scale bar, 2.5 μm . (e) APC (left) forms puncta that align with microtubules (middle) and are also present in areas of the membrane patch independent of microtubules (arrow). Right panel shows a merged image. Scale bar, 5 μm . (f) Another example to show that APC (left) binds microtubules (middle) along their length, and also localizes at many sites of microtubule–microtubule intersection (arrows). Right panel shows a merged image. Scale bar, 2.5 μm .

Figure 3.

APC aligns with microtubules on the basal cortex in cell lines that express APC without the C-terminal microtubule-binding domain. (a) Schematic representation of APC protein domains. The location of the truncation in APC expressed in SW480 and Caco-2 cells is marked with an arrow. Grey, dimerization domain; black, highly conserved APC domain; red, Armadillo repeats that bind KAP3; light blue and dark blue, three repeats of 15 amino acids (aa) and seven repeats of 20 amino acids, respectively, which bind β -catenin; purple, three repeats of the amino-acid sequence SAMP that binds axin; yellow, microtubule (MT)-binding region; olive green, EB1-binding domain; pink, PDZ-binding domain. (b) Immunofluorescence of intact SW480 cell extensions stained for APC (red) and microtubules (green) shows alignment of microtubules with a subset of APC punctate arrays (left), and overlap of microtubules with a small APC cluster (right; arrow). The inset shows APC staining in the cluster. Scale bar, 5 μ m. (c) Immunofluorescence of isolated basal patches from SW480 cells shows that microtubules (green) align (arrows) with APC puncta (red). APC is also present in areas of the membrane patch devoid of microtubule staining. Scale bar, 2.5 μ m. (d) Immunofluorescence of isolated basal patches from Caco-2 cells shows binding of APC puncta (red) along the length of microtubules (green). Inset shows a second example. Scale bar, 5 μ m. Left, APC; right, APC and microtubules overlay. (e) APC staining on a Caco-2 cell basal patch from which all microtubules have been removed by sonication, showing that APC remains bound to the membrane cortex in the absence of microtubules. Scale bar, 5 μ m.

Figure 4.

Distributions of KAP3, p150^{Glued} and microtubules on MDCK and Caco-2 cell basal patches. (a, b) Immunofluorescence of microtubules (green) and KAP3 (red) on basal patches from MDCK (a) and Caco-2 (b) cells showing alignment of punctate KAP3 along microtubules, at microtubule–microtubule intersections and at microtubule ends (arrows), and on the membrane cortex where microtubules are absent (arrowheads). Scale bar, 5 μm. (c, d) Immunofluorescence of microtubules (green) and p150^{Glued} (red) on isolated basal patches from MDCK (c) and Caco-2 (d) cells; p150^{Glued} is often localized to microtubule–microtubule intersections and microtubule ends (arrows). Scale bar, 5 μm.

Figure 5.

Endogenous EB1 binds along the length of microtubules on basal patches of MDCK and Caco-2 cells. All scale bars, 5 μm . **(a)** Examples of immunofluorescence staining of EB1 (red) and microtubules (green) shows EB1 puncta along the length of microtubules on basal patches of MDCK cells. EB1 is present at intersection points between microtubules (arrowheads). EB1 often fills in discontinuities in microtubule staining (arrows). **(b)** Immunofluorescence of EB1 staining at the base of an intact MDCK cell shows the same distribution as on isolated basal membrane patches (compare with **a**). The boxed region in the right panel is enlarged in the left panel. **(c)** Co-staining of APC (green) and EB1 (red) on basal patches from MDCK cells shows the difference in protein distributions. APC puncta are localized to the membrane cortex in areas devoid of microtubules, and along the length of microtubules, but not in a pattern overlapping with EB1 puncta. **(d)** Another example of APC and EB1 co-staining on an MDCK basal patch, showing that APC is in a punctate distribution on microtubules and the membrane cortex, whereas EB1 is confined to microtubules in a smoother distribution. **(e)** Staining of EB1 on a Caco-2 cell basal patch shows that EB1 puncta are localized along the length of microtubules. **(f)** Two examples of APC (green) and EB1 (red) staining on Caco-2 cell basal patches show that these proteins do not co-align.

Figure 6.

Retrospective staining of APC after imaging microtubule dynamics. **(a)** Left: movie frame of microtubules in a basal cytoplasm. Scale bar, 5 μm . Centre: tubulin-GFP and APC (red) after fixation and staining. Right: overlay of movie frame with retrospective staining. Microtubules align with APC puncta (yellow arrows) and curve over spots of APC (blue arrows). Larger APC clusters are present where multiple microtubules converge (arrowheads). **(b)** Movie frames from beginning (left) and end (middle) of imaging overlain onto retrospective APC staining. APC retrospective staining with tubulin-GFP (right). Time courses of boxed areas are shown in **c** and **d**. The movie was stopped for refocusing at 3:30 and 11:30 (min: s). Scale bar, 5 μm . **(c)** A microtubule crossed an APC cluster (arrow), and shrank after 11 min 30 s. The cluster remained after the microtubule shrank. **(d)** A microtubule crossed an APC punctum, shown by the orange arrow, and paused at this point for a few minutes; the punctum remained after the microtubule shrank. A microtubule crossed the APC punctum indicated by the blue arrow, was rescued at this location at 2 min, and shrank by 3 min; several minutes later a microtubule growing from a different direction was captured and paused at the same location. This microtubule veered from its original course (7 min 51 s) and bent slightly while crossing over the APC punctum (10 min 25 s).

Two APC puncta in the boxed area are crossed by the microtubule indicated by the white arrow. The microtubule paused at the location of the APC puncta (see life-history plot in **e**). The minus end of the microtubule shrank at 16 min, but did not shrink beyond the first APC punctum. (**e**) Life-history plot of the microtubule plus end indicated by the white arrow in **d**. Yellow shaded areas indicate the locations of the APC puncta. The microtubule plus end paused at the location of the second APC punctum. It grew past but did not shrink beyond this spot for several minutes, and was rescued at this location multiple times. The plus end shrank to the first APC punctum at 17 min 40 s, but did not shrink beyond this spot. The break indicates when the movie was stopped for refocusing.

Figure 7.

Reconstitution of the microtubule network on isolated membrane patches. Exogenous tubulin dimers were added to open basal patches, and the distribution of exogenous microtubules was compared with the distribution of endogenous APC by immunofluorescence. (a) Basal patches from MDCK cells incubated with bovine brain tubulin show exogenous microtubules (green) superimposed over APC puncta (red). The microtubules can be identified as exogenous microtubules because they are longer and straighter than endogenous microtubules and do not have discontinuities in antibody staining. Scale bar, 5 μm . (b) A second example of MDCK cell basal patches incubated with tubulin shows that microtubules (green) formed networks overlying APC puncta (arrows). Left, tubulin and APC staining; right, APC only. There are about three basal patches in the panel. The dimensions of an MDCK cell basal patch are 5–10 μm in either direction. Scale bar, 5 μm . (c) Exogenous microtubules assembled over APC puncta (arrows) on a Caco-2 cell basal patch. Scale bar, 5 μm . (d) Endogenous microtubules on a Caco-2 cell basal patch, which have discontinuities and do not lie as flat as reconstituted microtubules. It was often difficult to capture the length of endogenous microtubules in one image plane on Caco-2 cell patches (inset). Scale bar, 5 μm .

Figure 8.

An APC antibody inhibits microtubule polymerization on the basal cortex. **(a–e)** Exogenous tubulin dimers were added to open basal patches from MDCK **(b–d)** and Caco-2 cells **(e)** in the presence or absence of an affinity-purified APC antibody or taxol, and the distribution of exogenously polymerized microtubules was compared with the distribution of endogenous APC. Left panels in **a–d** show tubulin (green) and APC (red) co-staining; right panels show tubulin staining alone. Scale bars, 5 μ m. **(a)** Control polymerization of tubulin on basal patches results in microtubules overlying APC puncta. Inset shows detail of boxed area. Tubulin polymerized only on areas where basal membrane patches are present. **(b)** Pre-mixing of tubulin with an affinity-purified APC antibody resulted in reduced tubulin polymerization on patches. Few microtubules polymerized; instead short microtubules or spots of tubulin bound to membrane patches (inset, arrows). APC staining is brighter owing to the presence of added APC antibody. **(c)** Control polymerization of tubulin in the presence of taxol resulted in an increased number of shorter microtubules overlying APC puncta. **(d)** Tubulin pre-mixed with APC antibody and added in the presence of taxol resulted in more microtubules bound to patches compared with **b**, but fewer than the control with taxol **(c)**. **(e)** Microtubule reconstitution on basal patches prepared from Caco-2 cells. Rhodamine-labelled tubulin was added to patches in the presence of control or APC antibody. Examples of the distribution of added microtubules are shown on the left. Scale bars, 5 μ m. Exogenous microtubule number and total microtubule length were quantified on membrane patches. The data are represented as a box plot: pink, control ($n = 21$ patches); blue, APC antibody ($n = 24$ patches). The horizontal line through the box shows the median, the whiskers show the range of the data, and circles represent outliers. Total microtubule length per area of patch was reduced 3.8-fold, and the number of microtubules per area was reduced 3.2-fold in the presence of APC antibody.