Lack of Adenomatous Polyposis Coli Protein Correlates with a Decrease in Cell Migration and Overall Changes in Microtubule Stability[□]

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Most sporadic colorectal tumors carry truncation mutations in the adenomatous polyposis coli (APC) gene. The APC protein is involved in many processes that govern gut tissue. In addition to its involvement in the regulation of β -catenin, APC is a cytoskeletal regulator with direct and indirect effects on microtubules. Cancer-related truncation mutations lack direct and indirect binding sites for microtubules in APC, suggesting that loss of this function contributes to defects in APC-mutant cells. In this study, we show that loss of APC results in disappearance of cellular protrusions and decreased cell migration. These changes are accompanied by a decrease in overall microtubule stability and also by a decrease in posttranslationally modified microtubules in the cell periphery particularly the migrating edge. Consistent with the ability of APC to affect cell shape, the overexpression of APC in cells can induce cellular protrusions. These data demonstrate that cell migration and microtubule stability are linked to APC status, thereby revealing a weakness in APC-deficient cells with potential therapeutic implications.

INTRODUCTION

Loss of full-length adenomatous polyposis coli protein (APC) is common to most colorectal cancers due to truncation mutations that delete large regions of the C terminus of APC (Polakis, 1999; Näthke, 2004). Consistent with the highly penetrant phenotype in the gut that accompanies such mutations, APC has emerged as a multifunctional protein that is involved in a number of processes that govern the normal architecture of this tissue (Näthke, 2004). APC is required for canonical Wnt signaling and thus supports normal differentiation via regulation of β -catenin (Polakis, 2000; Fodde, 2002). APC is also involved in organizing the cytoskeleton, in particular, microtubules (Näthke, 2004). Loss of APC correlates not only with defects in chromosome segregation in mitosis but also with defects in the organization of parallel microtubule arrays in highly polarized cells (Fodde *et al.*, 2001; Kaplan *et al.*, 2001; Mogensen *et al.*, 2002; Dikovskaya *et al.*, 2004). The ability of APC to stabilize microtubules in vitro together with its peculiar localization to the end of microtubules that invade cellular protrusions gave rise to the hypothesis that APC is directly involved in supporting cell migration by locally stabilizing microtubules

(Näthke *et al.*, 1996a; Zumbunn *et al.*, 2001). More recently, the localization of APC to early axons while they are still morphologically indistinguishable from dendrites in the same cells has been shown as well as a role for APC in helping to promote axon outgrowth (Votin *et al.*, 2005).

Tissue cells usually possess a population of microtubules that resist depolymerization induced by either nocodazole or low temperature. In migrating cells, these “stabilized” microtubules usually emanate preferentially toward the direction of migration, whereas microtubules that are labile to depolymerizing conditions fill the rest of the cytoplasm. Such stabilized microtubules do not exchange tubulin subunits at their plus ends with the soluble tubulin pool, and they are thought to be “capped” by a mechanism requiring the microtubule plus-end-associated protein EB1 and possibly also APC (Wen *et al.*, 2004). Stabilized microtubules are frequently enriched in tubulin that has undergone posttranslational modifications. One modification is cleavage of the carboxy-terminal tyrosine of α -tubulin by tubulin tyrosine carboxypeptidase to generate detyrosinated or “Glu” microtubules. Another modification is acetylation (Westermann and Weber, 2003). These modifications are independent of the mechanism of microtubule stabilization; however, it seems that microtubules accumulate modifications as they “age” so that these modifications serve as a measure of the time a microtubule has remained assembled (Webster and Borisy, 1989). Although microtubule age is a measure of turnover and thus stability per se (i.e., the longer a microtubule lives, the more stable it must be), a correlation between acetylation and microtubule stability against nocodazole cannot always be established (Westermann and Weber, 2003). Nonetheless, it is likely that microtubule stabilization and/or tubulin modifications play a role in cell migration by creating different cellular domains that contain differentially

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stabilized microtubules. Indeed, inhibiting tubulin deacetylation results in decreased cell migration (Haggarty *et al.*, 2003), whereas overexpression of the enzyme responsible for this reaction correlates with an increase in cell movement (Hubbert *et al.*, 2002). Furthermore, the presence of deacetylases in neuronal cells correlates with protrusive activity where active remodeling of microtubules takes place, suggesting a connection between microtubule stability and protrusive activity (Southwood *et al.*, 2006). Consistent with this idea, inhibiting microtubule dynamics, leads to a loss of cell migration (Liao *et al.*, 1995; Kole *et al.*, 2005). These observations suggest that the balance between dynamic and stabilized microtubules is crucial for normal cell migration.

Whether APC contributes to overall microtubule stability in cells and how such an effect contributes to cell migration is not known. Inactivation of APC specifically in mouse intestinal tissue leads to an abrupt drop in the migration of intestinal epithelial cells *in situ* (Sansom *et al.*, 2004). In this case, differentiation of cells is also altered, making it difficult to discern whether direct effects on cytoskeletal proteins mediate these changes or whether changes in differentiation also play a role. Furthermore, changes in migration and/or cytoskeletal organization in response to loss of APC in cultured cells have mostly been described in cells expressing N-terminal fragments of APC together with full-length APC. Such fragments have effects of their own. Most importantly, they can regulate and can be regulated by interactions with other domains in full length APC (Li and Näthke, 2005). So in this situation, both the absence of functional APC and the presence of N-terminal APC fragments are likely to contribute in separate (although possibly also interactive) ways to phenotypes induced by APC mutations. In the systems described here, we determine the effects mediated by loss of APC itself.

To determine how APC affects cell migration in a situation where differentiation is not a factor, we examined the effect of loss of APC on microtubule posttranslational modification and stability and also on cell migration. To this end, we measured migration of three different types of cells where APC was constitutively or conditionally inactivated. In addition, the distribution pattern of posttranslationally modified tubulin was determined. In all cases, loss of APC led to a measurable loss of cellular protrusions and an overall decrease in cell migration. These changes were accompanied by a relative decrease of acetylated microtubules in the cell periphery, particularly in migrating cells. Our data reveal a complex interplay between the direct effects of APC on microtubules and its overall effect on cell morphology and behavior.

MATERIALS AND METHODS

Cell Culture and Transfections

Cells were maintained in 5% CO₂ atmosphere in DMEM/F-12 (for PTK2) or DMEM media, supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO), 1% Pen/Strep stock solutions (MP Biomedicals, Irvine, CA), 1% L-glutamine (MP Biomedicals) (only PTK2), and nonessential amino acids (only for U2OS, 1:100; Sigma-Aldrich).

Cre-recombinase producing adenovirus (kind gift of Khasahyarsha Khazaie, Dana-Farber Cancer Institute, Boston, MA) was propagated in 293 T-cells. To inactivate APC, cells were incubated with 300 μ l of virus-containing supernatant in 2.5 ml of media per 3.5-cm² dish for 2 days. To eliminate APC using RNA interference (RNAi), human osteosarcoma cells, U2OS, were treated with small interfering RNA (siRNA) directed against APC with Oligofectamine (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, by using 5 nM siRNA targeting human APC (SmartPool reagent; Dharmacon RNA Technologies, Lafayette, CO), or 5 nM nontargeting siRNA. Cells were maintained for 2–3 d after siRNA treatment. The sense sequences in the SmartPool were RNAi duplex 5: GAUGAUAGUCGCGAACUUUU, RNAi duplex 6: GAGAAUACGUCCACACCUUUU, RNAi duplex 7: GAAC-

UAGAUACACCAAUAAUU, and RNAi duplex 8: CCAAUUAUAGUGAACGUUUUU.

For transient transfections, cells were seeded onto ethanolized (fibroblasts; U2OS) or collagen-coated (PTK2) coverslips approximately 14 h before transfection with FuGENE6 (Roche Diagnostics, Indianapolis, IN) for PTK2 or Lipofectamine 2000 (Invitrogen) for cotransfection of siRNA and green fluorescent protein (GFP). Cells were fixed for immunofluorescence 36–48 h after transfection.

Fibroblasts constitutively lacking APC and wild-type control cell lines were kindly provided by R. A. Weinberg (Whitehead Institute, Cambridge, MA); fibroblasts for conditional inactivation of APC were a kind gift from Owen Sansom and Alan Clark (Sansom *et al.*, 2004).

Immunofluorescence

Cells cultured on coverslips were fixed in –20°C methanol for 5 min. Slides were washed once with phosphate-buffered saline (PBS) at room temperature and blocked for 1 h in blocking solution (PBS with 0.1% Triton X [TX] 100, 20% bovine serum albumin, 0.1% Na-azide, and 2% donkey serum (Scottish Antibody Production Unit [SAPU])). All antibodies were diluted in blocking solution. Antibodies were diluted as follows: acetylated tubulin clone 6-11B-1 (T6793; Sigma-Aldrich), 1:500; YL1/2 (Serotec, Oxford, United Kingdom), 1:500; and APC (Näthke *et al.*, 1996b), 1:1000. Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA) (raised in donkey) or from Invitrogen (raised in goat). Cells were stained with 1 μ g/ml 4',6-diamidino-2-phenylindole and mounted in 90% glycerol, 20 mM Tris, pH 8.8, and 0.5% *p*-phenylenediamine.

For the images shown in Figure 3, B and C, cells were fixed in 3.7% paraformaldehyde in PHEM buffer containing 60 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 25 mM HEPES, 10 mM EGTA, 4 mM MgSO₄, and 0.5% TX 100, pH 6.9. F-actin was detected with rhodamine-phalloidin (Invitrogen) diluted 1:200.

Three-dimensional image data sets were acquired using a Delta Vision Spectris Restoration Microscope built around an Olympus IX70 stand, with a 100 $\times$ /1.4 numerical aperture lens or an Olympus 60 $\times$ /1.40, Plan Apo lens (Applied Precision, Issaquah, WA). Optical sections were recorded every 0.2 μ m, and three-dimensional data sets were deconvolved using the constrained iterative algorithm (Swedlow *et al.*, 1997; Wallace *et al.*, 2001).

Generation of APC-GFP Constructs

All sequence residues used in this article refer to human APC sequence. Full-length and C-APC constructs with or without the microtubule binding site were previously generated in pEGFPC1 or pEGFPC3 (Zumbrunn *et al.*, 2001).

For the deletion of the EB1 binding site, the C-terminal 510 base pairs were deleted. The following polymerase chain reaction (PCR) primers were used: for full-length APC Δ EB1 and for full-length APC Δ MT Δ EB1: *tac tga ata tag gac acg tgt aag aa* and *acg gat cct tag gag atc ttc cag atc tag ga*; for C-APC Δ EB1 and full-length APC Δ MT Δ EB1: *tgc aat tct gca gtc gac ggt* and *acg gat cct tag gag atc ttc cag atc tag ga*; and the resulting fragment was cloned via BamH1 and Pml1 (full-length constructs in pEGFPcAPC-C1) and BamH1 and Sal 1 (for C-APC constructs in pEGFPcAPC-C3) sites into the appropriate existing vectors.

Protein Sample Preparation

Cells were placed on ice for 5 min, medium was aspirated, and plates were washed twice with chilled PBS. Then, plates were incubated for 5 min with 500 μ l of MEBC Lysis buffer per 10-cm dish (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM Na-EDTA, 5 mM Na-EGTA, 40 mM β -glycerophosphate, and 0.5% NP-40) containing the following protease inhibitors: leupeptin, pepstatin A, chymostatin (each 10 μ g/ml), 1 mM NaVO₄, and 10 mM NaF. Cells were scraped off and spun at 14,000 rpm for 20 min at 4°C and frozen on dry ice.

Western Blotting

Extracts were applied to 4–12% gradient SDS-gels (Invitrogen), by using 3-(*N*-morpholino)propanesulfonic acid running buffer and transferred to Protran nitrocellulose membrane (0.1- μ m pore size; Whatman Schleicher and Schuell, Keene, NH). For APC detection, crude serum from rabbit immunized with middle portion of APC was used (Näthke *et al.*, 1996b), diluted 1:1000 in blocking solution (Tris-buffered saline: 5% nonfat milk, 1% donkey serum, and 0.02% Triton X-100). Antibody dilutions were as follows: α -tubulin DM1 α (Sigma-Aldrich), 1:500; N-APC polyclonal (Midgley *et al.*, 1997), 1:1000; anti-GFP (Roche Diagnostics), 1:1000; actin (MP Biomedicals), 1:500; op18/stathmin O138 (Sigma-Aldrich), 1:1000; and KAP3 (K55520; BD Biosciences Transduction Laboratories, Lexington, KY), 1:200. The mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich) was diluted 1:2500. Secondary anti-rabbit, anti-mouse, and anti-sheep horseradish peroxidase-labeled antibodies (SAPU) or IRDye800/700-conjugated anti-sheep and anti-mouse secondary antibodies (either Invitrogen or Rockland, Gilbertsville, PA) were diluted 1:5000.

Migration

Cells were grown to confluence under normal tissue culture conditions. Monolayers were scratched with a needle or a razor blade, and cell movement

Figure 1. Loss of APC reduces cell migration in three different cell systems. Fibroblasts constitutively lacking APC and wild-type controls, or fibroblasts with wild-type (wt) or floxed APC (fl) were treated with Cre-recombinase (Cre) to inactivate APC in the floxed cells and grown to confluence. Similarly, U2OS cells were treated with siRNA to target APC or control siRNA and grown to confluence. A free margin was introduced in all three different cell systems, and cells were allowed to migrate. (A) Examples of cells immediately ($t = 0$) and 20 h (APC mutant and wt) or 24 (U2OS) h after the gap was introduced, to illustrate that cells lacking APC filled free space less efficiently than control cells. The grid marks on the coverslips used for these experiments are separated by 600 μm . (B) Cells were imaged at $t = 0$, and either $t = 8$ h (APC mutant versus WT), $t = 24$ h (U2OS), or $t = 3.5$ h (Cre/lox fibroblast), and Openlab software (Improvision) was used to determine the area free of cells at the beginning and end of the recording to then calculate the area covered by cells over time. A specific width was set using gridlines that were marked on the coverslips (i.e., 1800 $\mu\text{m} = 3$ boxes). The distance covered by the migrating margin per hour was calculated and is summarized in a box-plot (Tukey, 1977) that was prepared using SigmaStat for Windows (Systat Software, Point Richmond, CA). The bar in the middle of each box shows the mean, the boxes above and below show the upper and lower quartile of the data, respectively, and the “whiskers” indicate the highest and lowest data point. The n-value for each case is indicated on the x-axis below the box. APC-depleted conditions are shown in red. Inactivation of APC led to a decrease in cell movement over the time course, whereas treatment of control cells did not produce this effect. For all three different cell systems, a highly statistical difference in migration compared with controls was detected ($p < 0.001$ for APC mutant versus wt and floxed $\pm$ Cre by t test and $p < 0.001$ for Kruskal-Wallis analysis of variance on ranks with 1 degree of freedom for siRNA-treated cells) (<http://dorakmt.tripod.com/mt/d/glosstat.html>). Please note that cell growth as measured by BrdU incorporation of cells in the migrating margin and by monitoring cell number over 24 h was not different between cell pairs with or without APC (Supplemental Figure 1). (C) Protein lysates from the same cells used in A and B were probed with antibodies against N-terminal regions in APC and GAPDH as indicated. Full-length APC was only detected in cells with normal, wild-type, or untreated mutant cell lines (APC-wt, cont siRNA; fl-ctrl, wt-cre, wt-ctrl). (For quantitation, see Supplemental Figure 1G.)

into the gap was imaged by recording one image per minute by using an Axiovert 40J M with a Cool View camera from Photonic Science (Robertsbridge, East Sussex, United Kingdom) by using IonBiosion III 1-58 capturing software. Quantitation of the covered area over time was carried out using Openlab image software (Improvision, Lexington, MA). The area was measured by tracing the area free of cells by using gridlines that were marked on the coverslips to define a specific width (for example, for U2OS cells, we used 1800 μm , which corresponds to three grid boxes on the slide; Bellco Biotechnology, Vineland, NJ) at the beginning and end of the recording. These values were subtracted from each other to yield the area filled by cells over the time of the recording (see Figure 1A for schematic). The migration rate was then calculated by dividing this area by the width of the margin and the time of the recording. For the U2OS cells, the time interval was 24 h, for the Cre/lox fibroblasts, it was 225 min, and for the constitutive fibroblasts it was 8 h.

A similar approach was used to measure the length of the migrating margin (Figure 3A). Instead of the area, only the margin itself was traced between two set points (600 μm apart) immediately (t_0) and 24 h after making the scratch (t_{24}). The difference between the two measurements was calculated and is plotted in Figure 3A.

The migration of single cells in the margin of a migrating monolayer was monitored in U2OS cells that were transfected with GFP and treated with control or APC targeting siRNA at the same time. A scratch was made 36–48 h after transfection, and cells were imaged every 10 min for 12 h in an environmental chamber in CO_2 independent media (Invitrogen) using a Delta Vision Spectris restoration microscope built around an Olympus IX70 stand, with a 40 $\times$ lens (Applied Precision). Maximum intensity projections were

analyzed using Volocity (Improvision) by tracing the outline of GFP-positive cells in the margin of the migrating front for every 10th time point. The centroid of each cell was tracked using these tracings to yield the length, velocity, and meandering factor for each cell (Figure 2). The meandering factor is the ratio between the displacement (i.e., the straight line between start and end point) and the actual path length, so that movement along a straight line yields a meandering factor of 1.

RESULTS

Loss of APC Induces a Decrease in Cell Migration

To investigate whether loss of APC resulted in changes in cell migration in cultured cells, scratch assays were performed using three different cell systems: 1) fibroblasts isolated from mice carrying a mutation in exon 8 of APC, which renders the APC mRNA unstable so that these cells contain significantly reduced levels of APC (APC-mutant), were compared with matched wild-type control cells (APC-WT) (kind gift from Robert Weinberg and Amin Fazeli, Whitehead Institute); 2) fibroblasts containing APC flanked by LoxP sites so that they lose APC when exposed to Cre-recombinase, were compared before (fl Ctrl) and after (fl

Figure 2. The migration path of APC-negative cells is not altered. (A) U2OS cells were treated with control or APC-targeting siRNA and also transfected with GFP. A scratch was made into a confluent monolayer, and cells were monitored for 12 h. The migration of individual GFP-positive cells in the migrating margin was recorded by tracing the outline of individual cells at specific time points. Volocity (Improvision Ltd., Coventry, United Kingdom) was then used to identify the centroid of each cell and to calculate the length of its migration path over the entire time course, the average speed of the migration, and the meandering factor. Panels show examples of cells with the migrating path shown by a red line. The edge of the migrating margin is outlined by a white line. (B) Length of migration, velocity, and meandering factor are shown using box-plots. The overall length of the displacement and the speed of APC-negative cells were significantly less (similar to what was detected in the overall migration shown in Figure 1). However, their path did not seem to be more convoluted as indicated by similar meandering factors.

Cre) inactivation of APC to wild-type (wt Ctrl and wt Cre) fibroblasts derived from mice with identical genetic background but wild-type APC (Sansom *et al.*, 2004); and U2OS cells that lacked APC after treatment with the appropriate siRNA reagent (APC siRNA) were compared with U2OS cells treated with control siRNA (ctrl siRNA).

Cells were grown to confluence on a coverslip marked with grids, and a free margin was introduced with a razor blade. Movement of cells was determined by subtracting the area free of cells over a set width (which was established with the aid of the grids on the coverslips) at the start from that at the end (see schematic in Figure 1A). Figure 1A shows representative examples of control and APC-deficient cells before and after various times to reveal the decreased migration of cells lacking APC. Measuring the area covered by cells over time and using this number to calculate the average distance the cell margin migrated revealed a highly significant decrease in the ability of cells lacking APC to migrate compared with the appropriate control cells (Figure 1B).

To ensure that the detected decrease in migration reflects the situation in individual cells, individual cells in a migrating margin were also monitored (Figure 2). To facilitate the ability to follow single cells in a migrating cell layer, cells were transiently transfected with GFP and the entire cell outline was traced at various points during the migration over 12 h. This allowed the centroid of each cell to be established, which was then used to calculate the length of migration, speed, and the meandering factor (Figure 2). The latter provides a means to estimate directionality of movement (with a factor of 1 indicating movement along a straight line [see *Materials and Methods* for explanation]). The data, summarized in Figure 2, show that cells lacking APC simply did not migrate as quickly as control cells but that there was no difference in the overall directionality of APC-

negative cells as indicated by similar meandering factors for cells with and without APC (Figure 2B).

The difference in the relative speed of migration between cells shown in Figures 1B and 2B is likely due to their exposure to a more toxic transfection reagent that was used for the cells in Figure 2B, which was required to introduce GFP in addition to the siRNA (Oligofectamine for Figure 1 versus Lipofectamine 2000 for Figure 2). The repeated exposure of the cells in Figure 2 to UV light (18 sections were collected every 10 min over 12 h) may also have produced an adverse effect.

Importantly, differences in the migration of APC negative cells could not be attributed to differences in growth rates of the APC-deficient cells in any of the systems used (as determined by monitoring cell number over time and 5-bromo-2'-deoxyuridine (BrdU) incorporation into cells in migrating margins (Supplemental Figure 1). Together, these data show that loss of APC renders cells less able to migrate efficiently.

The decreased expression levels of APC protein were confirmed by immunoblotting (Figure 1C) and revealed a reduction by 80% for floxed Cre-treated cells, 90% for mutant compared with controls, and 96% for siRNA-treated cells (Supplemental Figure 1G).

Lack of APC Correlates with Less Protrusive Activity

To examine how the decreased migration in APC-deficient cells correlated with cell morphology during migration, we compared the overall length of the migrating edge in control and APC-deficient cells by tracing along the leading edge of migrating cells and then determining the length of this line between two fixed points on a grid (see Figure 3A for schematic that illustrates how these measurements were made). The data displayed in Figure 3A show the difference in the length of this line measured at 24 h and the length immediately after the scratch was made. Cells lacking APC

Figure 3. Loss of APC reduces protrusion formation. (A) APC was inhibited in U2OS cells by RNAi, and cells were grown to confluence. A free edge was introduced with a razor blade, and cells were allowed to migrate for 24 h. Images were taken at $t = 0$ and $t = 24$ h. The difference in the formation of protrusions was measured by tracing along the leading edge of migrating cells between two points that were at a fixed distance (600 μm , by using grid marks on the coverslips we used) at $t = 0$ and $t = 24$ h. The difference between the margin length at $t = 0$ (l_0) and $t = 24$ (l_{24}) hours is shown and reveals a significant reduction in the formation of protrusions in APC-depleted U2OS cells. Fibroblasts with wild-type (WT) or floxed APC (FI) were treated with Cre-recombinase (Cre) (B) or U2OS cells treated with either nontargeting siRNA (ctrl siRNA) or APC interfering siRNA (APC siRNA) (C) were grown to confluence. A gap in the cell monolayer was introduced by scratching with a needle, and cells were allowed to migrate into the gap for 2 h. They were fixed and stained with antibodies against APC (green), tubulin (red), and actin (blue). Images of cells at the margin facing the gap reveal endogenous APC protein accumulated in small clusters in protrusions that point toward the gap. After inactivation of APC, cell margins were mostly devoid of protrusions and APC clusters were not present. Bar, 15 μm (B) and 10 μm (C). The approximate position of the wound edge is indicated by the white line. White boxes outline regions that are shown at twice the magnification to the right of each image.

(APC siRNA) had a less irregular shape, and the cellular front was almost 50% shorter than in control cells (ctrl siRNA; Figure 3A), suggesting that removal of APC compromised the ability of these cells to make protrusions. We chose cells before and after APC inhibition by RNAi to make these measurements because these epithelial cells most closely resemble cells in the relevant tissue and because in this system APC had been inactivated for a short time by using mild conditions.

However, changes in cellular morphology were also visible in fibroblasts after APC was eliminated by treatment with a Cre-recombinase delivering virus. Using these Cre/LoxP fibroblasts, we found that lack of protrusions in cells correlated with a lack of APC clusters near the free edge of migrating cells (Figure 3B). After APC inactivation, little or no APC could be detected in the cytoplasm, and, unlike control cells, these cells did not contain clusters of accumulated APC at the ends of cellular protrusions (Figure 3B). Costaining of these cells with antibodies against actin and tubulin also showed that cells lacking APC formed a more even cell margin and generally lacked long cellular protrusions (Figure 3B). Similar observations were made in epithelial cells after inhibiting APC by using RNAi (Figure 3C). Please note that as reported previously, staining with this particular APC antibody in the nucleus was not altered when APC was inactivated (Brocardo *et al.*, 2005).

The Migrating Margins of in APC-deficient Cells Lack Acetylated Microtubules

The presence of APC protein at microtubule ends correlates with an increase in the lifetime and overall stability of these microtubules (Kita *et al.*, 2006). Our observations that loss of APC led to a decrease in cell migration and protrusions suggested that microtubule stability in cells lacking APC was compromised. To determine whether differences in cell migration in APC-deficient cells correlated with differences in microtubule stability, we compared the distribution of posttranslationally modified microtubules, in particular acetylated microtubules, in migrating cells before and after depletion of APC (Figure 4). Migrating cells lacking APC contained fewer acetylated microtubules and did not position their acetylated microtubules to face the migrating edge as control cells did (Figure 4). Instead, in all migrating APC-deficient cells examined, acetylated microtubules were sometimes dispersed symmetrically throughout cells, but usually they were concentrated in the perinuclear area (Figure 4) similar to the situation in nonmigrating cells (data not shown).

To exclude the possibility that differences in the amount of time spent in mitosis (Gundersen and Bulinski, 1986) contributed to any of the differences we observed, we measured cell DNA profiles of cells using fluorescence-activated cell sorting (FACS), which did not reveal any differences in the

Figure 4. Acetylated microtubules do not invade the migrating margin of APC-deficient cells. Fibroblasts wild-type controls or those constitutively lacking APC (A) and fibroblasts with wild-type (wt) or floxed APC (fl) (B) were grown to confluence and treated with Cre-recombinase (Cre) to inactivate APC in the floxed cells, or U2OS cells were treated with siRNA to target APC or control siRNA and grown to confluence (C). A space devoid of cells was introduced in all three different cell systems with a razor blade, and cells were allowed to migrate for 24 h. Cells were fixed and stained with antibodies against acetylated and unmodified tubulin. Only cells expressing APC showed strong accumulation of acetylated microtubules in the front half of the cells facing the migrating edge, indicated by a line. Bars are shown in micrometers.

relative number of cells in mitosis between APC-positive and -negative cells (data not shown).

We concluded that loss of APC induces an overall decrease in microtubule stability, particularly in the cell periphery, which could explain the reduced protrusive activity of these cells (Figure 3). Indeed, when we examined microtubules after treatment with nocodazole or vinorelbine in cells before or after APC inactivation, cells lacking APC retained fewer polymerized microtubules after treatment with either of these drugs (Supplemental Figure 2). In addition, in fibroblasts the microtubules that remained after nocodazole-induced depolymerization tended to be acetylated (Supplemental Figure 2), confirming that acetylation correlates with increased stability in these cells.

To test whether changes in the expression of other cytoskeletal proteins that have been linked to APC contributed to the effects we observed, we compared the amount of Kap3, a kinesin-associated protein that can bind to APC (Jimbo *et al.*, 2002) and stathmin/OP18, a regulator of microtubule dynamics that has been detected in some tissues as a downstream target of Wnt signaling (Rubin and Atweh, 2004), before and after APC inactivation. No difference in the expression of these proteins was detected in cells lacking APC (Supplemental Figure 3).

Expression of APC Alters Cell Shape

The decrease in cell protrusions in cells lacking APC prompted us to test whether excess APC in cells could enhance or induce cellular protrusions. To this end, we expressed a number of APC proteins (see Figure 5, A and B, for summary) in PTK2 epithelial cells that endogenously express full-length APC. Expression of proteins of the correct size and antibody reactivity was confirmed by Western blotting with antibodies against GFP and a C-terminal APC domain (Supplemental Figure 4A). Similar expression levels and transfection efficiencies were obtained with these con-

structs as determined by FACS analysis (Supplemental Figure 4B).

In this case, the effect on cell shape was determined measuring the length and width of cells transfected with different APC constructs. The ratio between these two parameters was calculated to obtain a quantitative measure of cellular asymmetry that is independent of cell size. We chose this approach rather than measuring the overall protrusion length in migrating cells (as in Figure 2) because transient transfections did not produce sufficient numbers of mutant cells in the migrating margin to permit statistically significant measurements to be made in this region.

The box-plots in Figure 5C show that only expression of full-length APC induced a significant shift in the asymmetry of cells ($p < 0.001$, Kruskal-Wallis analysis of variance of ranks; Sheskin, 2004), whereas expression of middle, N-, or C-terminal fragments of APC did not produce any statistically significant changes in cell shape. These data suggest that a combination of cytoskeletal interactions of the APC molecule is required for the ability of APC to alter cell shape (median values were APC, 4.0; N-APC, 3.4; M-APC, 2.6; C-APC, 2.4; C-APC Δ MT, 3.1; C-APC Δ EB1, 2.7; GFP, 2.5; and mock transfected, 2.5).

We found that all full-length constructs of APC induced longer protrusions (median values for ratios of asymmetry were APC, 4.0; APC Δ MT, 4.9; APC Δ EB1, 3.9; APC Δ MT Δ EB1, 4.8; compared with GFP, 2.5 and mock transfected, 2.5). These data show that overexpression of neither C-terminal fragments, which bind to and stabilize microtubules efficiently (Zumbrunn *et al.*, 2001), nor N-terminal fragments, which indirectly could affect the actin cytoskeleton by binding to Asef, led to an increase in cellular extensions. On the contrary, the ability to form large clusters at microtubule ends near the plasma membrane was most important for this effect. However, the ability of APC to bind Kap3 was not sufficient to cluster at microtubule ends or induce protrusions, because

Figure 5. Exogenous APC can induce cell shape changes independently of its direct interaction with microtubules. Schematic summary of functional domains of the APC sequence (A) and list of constructs used in this study (B). All constructs were tagged with GFP at their N termini. Their ability to colocalize with microtubules, to accumulate in peripheral clusters, to protect microtubules against depolymerization induced by nocodazole, and to induce protrusions is summarized on the right. (C) Cellular asymmetry was measured in PTK2 cells expressing the indicated APC constructs. The ratio of length versus width was determined in APC-GFP-expressing cells, and the relative abundance of the indicated ratios is shown in a box-plot (Tukey, 1977). (See Figure 2 for explanation of plot.) Typical images of a normal and a highly asymmetrical cell expressing GFP-C-APC and GFP-APC, respectively, are shown above the plot, indicating the axes that were used to calculate the ratio. All full-length APC constructs, but not the N-, middle-, or C-terminal thirds on their own produced a statistically significant increase in cellular asymmetry and thus in the relative length of cellular protrusions regardless of their association with microtubules (Kruskal–Wallis one-way analysis of variance, which is appropriate for this type of nonnormally distributed data; <http://doramkmt.tripod.com/mt/d/glosstat.html>). A histogram summarizing this data in a different format is shown in Supplemental Figure 5.

N-terminal fragments of APC that bind Kap3 did not accumulate in clusters in these cells (Zumbrunn *et al.*, 2001).

Importantly, as expected from our observation that loss of APC led to a decrease in cellular protrusions, increasing APC could enhance cellular asymmetry by inducing longer cellular protrusions.

DISCUSSION

Loss of APC is an early event in almost all sporadic colon cancers, and in most of these cases, the remaining, mutated APC protein is unable to interact with the microtubule cytoskeleton normally. Here, we show that one consequence of such APC loss is a decrease in the overall stability of microtubules and cell migration. Migration of cells is crucial for the normal maintenance of gut epithelium because constant proliferation, coupled with active, directed migration is required to replenish cells that are constantly exfoliated (Näthke, 2004). A decrease in efficient cell migration induced by loss of APC may lead to the accumulation of cells in the toxic environment of the gut, allowing additional mutations to accumulate. This might provide an effective mechanism for the transcriptional changes that accompany APC loss to persist and propagate to produce malignancies.

A role for APC in cell migration has previously been suggested by the observation that inactivating APC in mouse tissue leads to an abrupt decrease in cell migration of enterocytes *in situ* (Sansom *et al.*, 2004). However, in this situation the inactivation of APC is also accompanied by changes in differentiation, making it difficult to dissect the main cause for the decrease in migration. Using our cell

systems, we were able to show directly for the first time that loss of APC immediately results in defects in cell migration. This was detected in cells that constitutively lack APC but also in those where APC loss was induced conditionally using a Cre–Flox system or by RNAi. Migration of whole populations of cells in a monolayer (Figure 1) but also movements of single cells (Figure 2) was reduced by APC depletion.

The relative difference between control and APC-deficient cells was highest for cells constitutively lacking APC (Figure 1B) and lowest for epithelial cells immediately after APC loss (Figure 1B). This suggests that long-term changes resulting from APC loss, like alterations in the transcriptional profile, may further contribute to a decrease in migration.

We detected two differences in APC-deficient cells that related to changes in microtubules. First, we found that the ability of cells to induce protrusions was strongly reduced in all three APC-deficient cell systems (Figure 3A). This change correlated with the loss of APC-clusters near the periphery of extending cell margins (Figure 3, B and C). Because cellular protrusions are also dependent on F-actin dynamics and their coordination with microtubules, this observation raises the possibility that loss of APC not only affected microtubules but also produced changes in F-actin. Indeed, N-terminal regions of APC can interact with regulatory proteins for F-actin, including Asef, a Rac-specific guanine nucleotide exchange factor that is stimulated by APC and thus stimulates F-actin (Kawasaki *et al.*, 2000; Kawasaki *et al.*, 2003), and IQGAP to form a ternary complex with Rac1/cdc42 (Watanabe *et al.*, 2004). Furthermore, the link that has been established among mDia, cdc42, and APC may also

contribute to changes in F-actin (Wen *et al.*, 2004; Yamana *et al.*, 2006).

Importantly, we found that the isolated N-terminal domain of APC that contains the full complement of binding sites for IQGAP, Asef, and Kap3 is unable to cluster in cellular protrusions (Zumbrunn *et al.*, 2001) and does not induce protrusions when overexpressed (Figure 5B), suggesting that these protein interactions on their own are not responsible for this effect of APC.

A second change that was common to all APC-deficient cells was a change in the pattern of posttranslationally modified microtubules. Acetylation on lysine residue 78 of α -tubulin is a common modification that occurs in microtubules as they persist, so it is often used as an indicator of microtubule lifetime (age) (Westermann and Weber, 2003). Cells lacking APC contained fewer acetylated microtubules (Figure 4; conversely, microtubules decorated with exogenous GFP-APC were usually acetylated (data not shown). In migrating control cells, acetylated microtubules were always localized to the leading front of cells, whereas in APC-deficient cells, acetylated microtubules were more randomly distributed and often clustered around the nucleus. This suggested that in the short term, loss of APC led to a decrease in stable microtubules specifically in the cellular periphery. Indeed, the presence of endogenous APC protein at microtubule plus ends in the periphery correlates with their longevity (Kita *et al.*, 2006). This suggests that overall APC can contribute to microtubule stability and helps to orient more stable microtubules toward the direction of migration.

We further confirmed that loss of APC rendered microtubules less stable in cells by comparing the sensitivity of microtubules to depolymerizing agents in cells before and after APC inactivation (Supplemental Figure 2). In these experiments, we found that in APC-deficient cells, microtubules depolymerized more readily than in control cells. In addition, we found that the resistant microtubules were usually acetylated, confirming their increased stability. This observation is in contrast to previously published data that showed no correlation between acetylation and increased stability against microtubule-depolymerizing agents (Palazzo *et al.*, 2003). It is possible that this effect is cell type specific or that only specific concentration ranges of microtubule drugs reveal such differences. In contrast, our data are consistent with previously published report that showed a decrease in microtubule stability in cells expressing a dominant fragment of EB1 that can sequester APC away from microtubules (Wen *et al.*, 2004).

Consistent with the ability of APC to affect cell shape by modulating microtubule stability, we found that expressing full-length APC induced the formation of cellular asymmetry by creating longer cellular protrusions (Figure 5). Only full-length APC but not individual fragments had this effect. Most important for this effect was the ability of the exogenous protein to cluster at the end of membrane protrusions, suggesting that locally induced changes were involved in enhancing cellular asymmetry.

In conclusion, we found that loss of wild-type APC, the situation faced in cells at early stages of tumorigenesis, compromised overall microtubule stability and cell migration in monolayers as well as in single cells. This effect was an immediate consequence of APC depletion. These findings could have important implications for therapeutic considerations in APC-deficient tumor cells. The possible weakness created by a decrease in microtubule stability in tumor cells lacking APC, i.e., the majority of colorectal cancers, should prompt the reconsideration of microtubule poisons for treat-

ment of colorectal cancers, particularly recently developed microtubule poisons that are less sensitive to multiple drug resistance (Bacher *et al.*, 2001; Goodin *et al.*, 2004), which minimized the usefulness of this type of agent for treating colorectal cancer in the past.

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