

Chromosome instability in colorectal tumor cells is associated with defects in microtubule plus-end attachments caused by a dominant mutation in *APC*

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The attachment of microtubule plus ends to kinetochores and to the cell cortex is essential for the fidelity of chromosome segregation. Here, we characterize the causes underlying the high rates of chromosome instability (CIN⁺) observed in colorectal tumor cells. We show that CIN⁺ tumor cells exhibit inefficient microtubule plus-end attachments during mitosis, accompanied by impairment of chromosome alignment in metaphase. The mitotic abnormalities associated with CIN⁺ tumor cells correlated with

status of *adenomatous polyposis coli* (*APC*). Importantly, we have shown that a single truncating mutation in *APC*, similar to mutations found in tumor cells, acts dominantly to interfere with microtubule plus-end attachments and to cause a dramatic increase in mitotic abnormalities. We propose that *APC* functions to modulate microtubule plus-end attachments during mitosis, and that a single mutant *APC* allele predisposes cells to increased mitotic abnormalities, which may contribute to tumor progression.

Introduction

Chromosome segregation during cell division requires proper orientation of the mitotic spindle and successful movement of chromosomes on spindle microtubules. These events involve microtubule plus-end attachments with the cell cortex and kinetochores (for review see McNally, 2001; Carvalho et al., 2003). After attachment, a combination of forces generated by motor proteins and microtubule dynamics are thought to contribute to the movement of chromosomes during mitosis (Scholey et al., 2003). Therefore, failure to properly attach spindle microtubules during mitosis can result in mispositioning of the mitotic spindle or defects in chromosome segregation. Despite the identification of a large number of proteins critical for chromosome segregation, the precise nature of the microtubule attachments and the pathways that regulate attachments are poorly understood (for review see Cheeseman et al., 2002; Biggins and Walczak, 2003).

One approach to studying the mechanisms underlying microtubule attachment and chromosome segregation is to focus on naturally occurring mutations that compromise this process. Colorectal tumor cells are particularly prone to chromosome instability (CIN), and allelic imbalances have

been observed in early colonic adenomas, consistent with a potential role for CIN in tumor progression (Lengauer et al., 1997; Nowak et al., 2002). However, the significance of these changes for the progression of cancer remains controversial (Sieber et al., 2002). In the context of the tumor, it is likely that changes that affect chromosome segregation will be subtle, balancing cell viability with the potential selective advantages derived from changes in ploidy. Despite its prevalence in tumors, little is known concerning the mechanisms that contribute to CIN. Cell fusion experiments have shown that CIN has a dominant genetic quality, and this has led to the proposal that mutation of a so-called “gatekeeper” gene can contribute to CIN (Lengauer et al., 1997). However, the nature of this gatekeeper gene is not clear. It has been suggested that mutations in mitotic checkpoint proteins might induce CIN, although to date, such mutations have only rarely been found in human cancers (Cahill et al., 1998; Gascoyne et al., 2003). The proposed role of CIN in tumor progression and the high percentage of colorectal tumor cells with CIN suggest that the gatekeeper gene is mutated early and frequently in colorectal tumors (Shih et al., 2001; Nowak et al., 2002).

The most frequent mutation found in human colorectal tumors is in *adenomatous polyposis coli* (*APC*), typically resulting in a truncated gene product (Lamlum et al., 1999; Sieber et al., 2002). Significantly, loss of heterozygosity at

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Abbreviations used in this paper: ACA, anti-centromere antibody; *APC*, *adenomatous polyposis coli*; CIN, chromosome instability.

the *APC* locus is observed early in human lesions, and patients who inherit a single mutant allele of *APC* are at increased risk of additional genetic instability (Lamlum et al., 1999; Shih et al., 2001). *APC* has been primarily implicated in the Wnt signal transduction pathway as a regulator of β -catenin, a protein that controls the transcription of developmentally important genes (for review see Polakis, 1997, 2000). Although the requirement of *APC* for β -catenin degradation is clear, its precise biochemical role in protein degradation remains less certain. Complicating the simple assignment of function to *APC* are findings that suggest *APC* is important for a number of cellular pathways, including cytoskeleton regulation (Dikovskaya et al., 2001; Bienz, 2002).

Various types of interactions between *APC* and microtubules have been reported, including its direct interaction with microtubules and its association with EB1, a microtubule-associated protein that is enriched at microtubule plus ends (Su et al., 1995; Deka et al., 1998; Juwana et al., 1999; Zumbunn et al., 2001). The interaction between *APC* and EB1 is particularly intriguing, as EB1 has recently been shown to play a critical role in the proper assembly and positioning of the mitotic spindle in *Drosophila* tissue culture cells (Rogers et al., 2002), a role similar to its yeast homologues, BIM1 and MAL3 (Beinhauer et al., 1997; Schwartz et al., 1997; Tirnauer et al., 1999). In addition, both EB1 and *APC* have been implicated in maintaining proper spindle positioning in the developing nervous system of *Drosophila*, possibly by tethering microtubules to cortical actin elements (Lu et al., 2001; McCartney et al., 2001; Rogers et al., 2002).

Recent analyses of *APC* in tissue culture cells provide evidence that *APC* may also function in mitosis. Murine embryonic stem cells with the *APC*^{min/min} mutation exhibit CIN as well as an increase in the number of torn or lagging chromosomes, raising the possibility that mitosis is compromised in these cells. In addition, *APC* has been observed at the plus ends of kinetochore microtubules and in association with the mitotic checkpoint proteins Bub1p and Bub3p (Fodde et al., 2001; Kaplan et al., 2001). Although intriguing, these analyses neither define the precise role of *APC* in mitosis nor how *APC* mutations compromise the mitotic process. In addition, some controversy surrounds the significance of these findings, possibly because the role of *APC* in mitosis is only one of its many cellular functions.

To examine the cause of CIN and the potential role of *APC* in mitosis, we have carefully analyzed the mitotic apparatus in an array of colorectal tumor cell lines. We demonstrate that both astral and midzone microtubules are compromised in tumor cells with CIN. Multiple lines of evidence suggest that the defect in spindle microtubules is due to inefficient microtubule plus-end attachments. Conditional expression of the amino terminus of *APC* in cells with wild-type *APC* is sufficient to dominantly interfere with microtubule plus-end attachments, recapitulating the phenotype observed in *APC* mutant tumor cells. These results argue that *APC* modulates microtubule plus-end attachments during mitosis, and that *APC* is a gatekeeper gene whose mutation may contribute to the CIN phenotype observed in colorectal tumor cells.

Results

The mitotic spindle is compromised in CIN⁺ colorectal tumor cells

To address the underlying causes of CIN in tumor cells and to evaluate the role of *APC* in mitosis, we analyzed the spindle apparatus in a series of colorectal tumor cell lines. Two categories of colorectal tumor cell lines were chosen: (1) cell lines previously characterized to have high rates of CIN (CIN⁺ cells; HT29, SW480, Caco2, and LoVo); and (2) cell lines previously characterized to have relatively stable genomes (CIN⁻; HCT116, and RKO; Lengauer et al., 1997; Tsushimi et al., 2001). The CIN⁺ colorectal tumor cell lines all contain homozygous mutations in *APC*, resulting in carboxy-terminal truncated gene products ranging from aa 1250 to 1500; the CIN⁻ colorectal tumor cell lines are wild-type for *APC*, but have mutations in other genes, including mutations in *β -catenin* that result in a constitutively active gene product (Ilyas et al., 1997). Mitotic spindles in tumor cells were analyzed using restoration deconvolution microscopy after staining with tubulin mAbs. Metaphase spindles in CIN⁻ tumor cells were robust, exhibiting numerous astral microtubules that extended to the cell cortex and abundant midzone microtubules (Fig. 1 A); the “midzone” is defined as the region between the two spindle poles that includes both kinetochore and interpolar microtubules. In contrast, we observed a reproducible decrease in the level of tubulin staining in spindles of CIN⁺ metaphase cells. Spindles in multiple CIN⁺ cell lines exhibited a reduction in both astral and midzone microtubules, particularly apparent when single optical sections from the middle of the spindle were observed (Fig. 1 A, bottom panels). We took several approaches to quantify the apparent differences in tubulin staining. First, we measured the fluorescence intensities of tubulin at the spindle midzone relative to the maximal tubulin staining in the cell (Fig. 1 B, diagram). The values for each cell line were subjected to a multiple comparison test, and those cell lines that did not vary significantly were plotted together (Fig. 1 B, red and blue brackets). Cell lines clustered into two statistically distinct categories, with CIN⁺ cell lines falling below the mean (Fig. 1 B). The restoration of near-normal tubulin intensities for CIN⁺ cells after taxol treatment confirmed the validity of this approach (Fig. 1 B). It is possible that the reduced tubulin staining in the midzone of CIN⁺ cells is due to hyper-elongated spindles; however, measurements of pole–pole distances show that spindle size does not vary with respect to the CIN phenotype (Table I). Additionally, the ploidy of each cell line could influence the number of microtubules observed in the midzone. Numbers reported from the literature as well as our own independent ploidy measurements argue that tubulin intensity does not vary with respect to chromosome number (Table I and Fig. 1 A). These observations led us to speculate that CIN⁺ tumor cells have decreased tubulin staining in the spindle midzone as a result of fewer kinetochore or interpolar microtubules.

To determine if kinetochore microtubules are specifically affected in CIN⁺ tumor cells, we measured kineto-

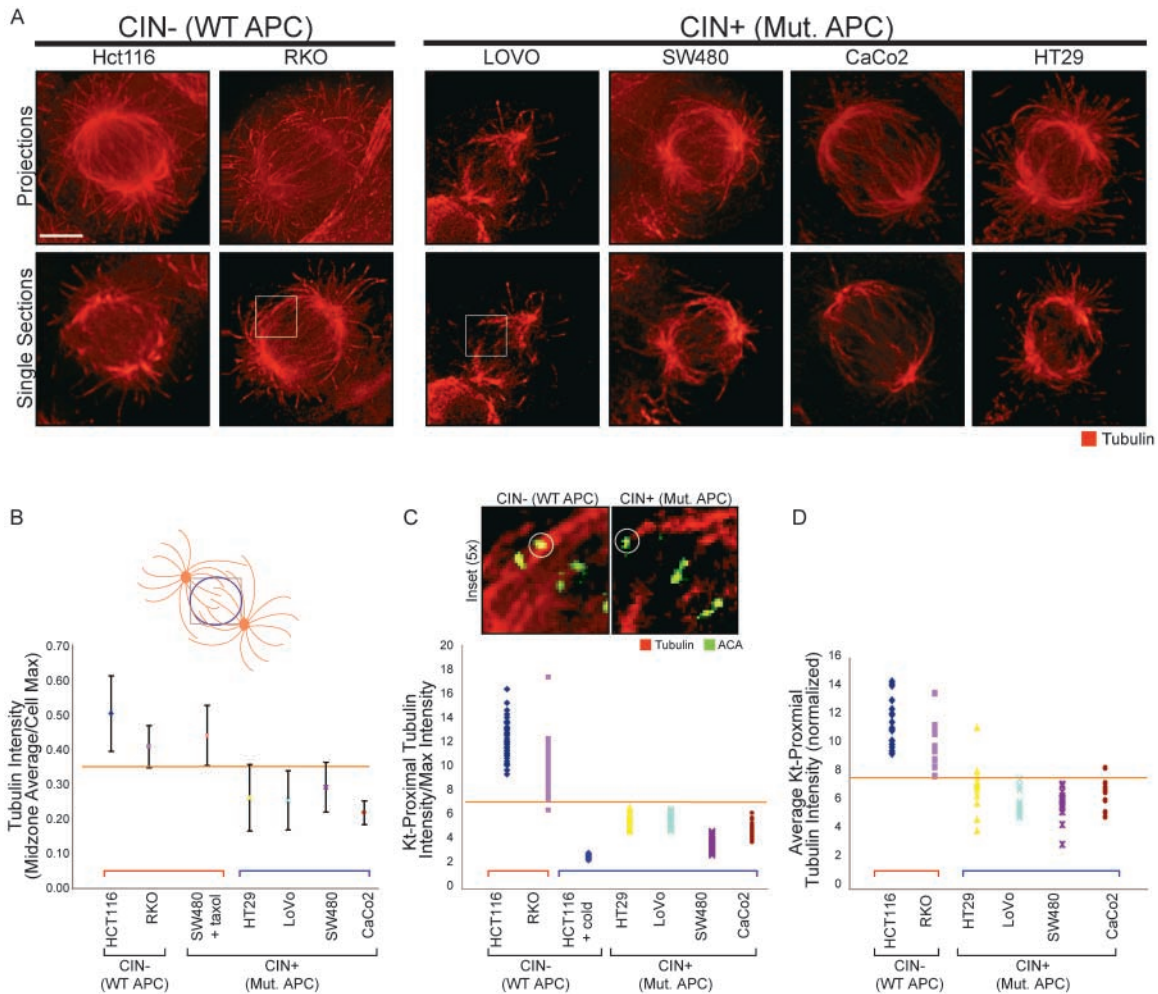


Figure 1. Mitotic spindles are compromised in CIN⁺ tumor cells. (A) CIN⁻ tumor cells (HCT116 and RKO) and CIN⁺ tumor cells (LoVo, SW480, CaCo2, and HT29) were fixed and stained with antibodies against tubulin. Both projections of z-stacks (top) and single z-sections are shown (bottom). Bar, 5 μ m. (B) The data representing midzone tubulin intensities (see schematic) of multiple cells ($n_{\text{avg}} = 15$) for each tumor cell line were analyzed by Tukey's multiple comparison analysis and statistically similar data were plotted together, indicated by red and blue brackets. (C) Images are 5 \times the regions outlined by boxes in A. Circles indicate the typical area surrounding kinetochores used to determine kinetochore-proximal tubulin intensity. Each data point represents the tubulin intensity proximal to a single kinetochore for each cell line indicated. Data were analyzed and statistically similar groups were plotted together as above. (D) All kinetochore-proximal tubulin intensities were averaged for individual cells and each data point represents a single cell. Data were analyzed and statistically similar groups were plotted together as above. Orange lines represent the grand means in B–D.

chore-proximal tubulin intensities, normalizing these values to the maximal tubulin intensity observed in the cell (Fig. 1 C). When these values were determined for individual metaphase cells from each line, we observed a three- to fourfold reduction in CIN⁺ compared with CIN⁻ cells, consistent with a decrease in kinetochore microtubules in CIN⁺ cells (Fig. 1 C). To assure that our measurements were specific to microtubules, we showed that kinetochore-proximal tubulin values fell to background levels after cold-induced depolymerization (Fig. 1 C). We applied this quantification method to a large number of metaphase cells from each tumor cell line and again observed two statistically distinct populations of cells (Fig. 1 D, brackets), with CIN⁺ cells falling below the mean, arguing that the reduction of midzone microtubules specifically reflects a loss of kinetochore microtubules.

CIN⁺ tumor cells have reduced kinetochore–microtubule attachments

To assess the extent of kinetochore–microtubule attachments more directly, CIN⁺ and CIN⁻ tumor cells were cold treated or treated with calcium-containing buffer. Both treatments preferentially depolymerize unattached microtubules while leaving behind kinetochore microtubules that have made stable attachments (Mitchison et al., 1986; Nakamura et al., 1998, 2001; Kapoor et al., 2000). In the following experiments, we present data from HCT116 (CIN⁻) and SW480 (CIN⁺) cells, but other CIN⁻ and CIN⁺ tumor cell lines showed similar properties. After 10 min of cold-treatment, cells were fixed and stained for tubulin. CIN⁻ metaphase cells showed a loss of astral microtubules, but retained robust kinetochore microtubule staining. In contrast, CIN⁺ metaphase cells rapidly lost both astral and kineto-

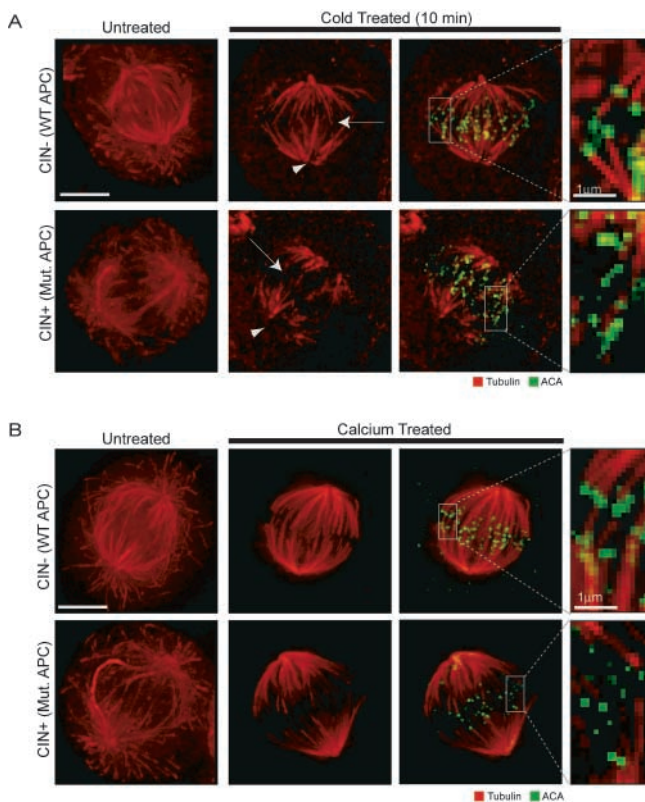


Figure 2. Kinetochores–microtubule attachments are less stable in CIN⁺ tumor cells. (A) CIN[−] (HCT116) and CIN⁺ (SW480) metaphase cells were fixed and stained with antibodies against tubulin (red) and kinetochores (ACA, green) before (untreated) or after cold treatment (at 4°C for 10 min). Projections of z-sections containing the spindle are presented; insets represent a 2.5-fold magnification of the area defined by the box. Arrows indicate the spindle midzone and arrowheads indicate the position of astral microtubules. (B) CIN[−] (HCT116) or CIN⁺ (SW480) tumor cells were incubated with calcium-containing buffer (calcium treated) or fixed normally (untreated), and were stained to visualize tubulin (red) and kinetochores (ACA, green). Projections of z-sections containing the spindle are presented; insets are as above. Bars, 5 μm; inset, 1 μm.

chore microtubules (Fig. 2 A). Similarly, treatment of permeabilized cells with calcium-containing buffer left kinetochores microtubules intact in CIN[−], but not in CIN⁺ metaphase cells, arguing that stable kinetochores–microtubule attachments are less abundant in CIN⁺ tumor cells. Quantification revealed that very few kinetochores have adjacent microtubules in CIN⁺ cells (3%) compared with CIN[−] cells (56%; Fig. 2 B, inset). However, by anaphase both CIN[−] and CIN⁺ cells exhibited similar levels of kinetochores–microtubule attachments (unpublished data), suggesting that attachments can occur in both cell types, albeit less efficiently in CIN⁺ cells.

To test if microtubule nucleation or growth is compromised in CIN⁺ cells, we measured the ability of microtubules to regrow after cold-induced depolymerization. After the return of cold-treated cells to warm media, cells were fixed and visualized with antibodies against tubulin. After 30 s, newly formed microtubule asters were observed at the majority of spindle poles in CIN[−] and CIN⁺ mitotic cells. By 60 s, similar numbers of microtubule projections were ob-

served to have grown to average lengths >4 μm in both CIN[−] and CIN⁺ tumor cells (Fig. 3, A and B; unpublished data). We interpret these data to suggest that early events, associated with microtubule nucleation and growth, are relatively similar between CIN[−] and CIN⁺ cells.

Although initial microtubule nucleation and growth appear normal, we observed a significant decrease in the number of microtubule projections in CIN⁺ cells compared with CIN[−] cells after 90 s of recovery (Fig. 3, A and C). The decrease in the number of microtubule projections at later time points of recovery is consistent with a defect in forming microtubule plus-end attachments. The status of APC in CIN⁺ cells and previous reports that place APC in a position to affect plus end attachments led us to examine APC localization during microtubule regrowth (Mimori-Kiyosue et al., 2000; Fodde et al., 2001; Kaplan et al., 2001). Both wild-type and mutant APC similarly localize along the length and at the plus ends of growing microtubules, suggesting they can influence plus-end binding (Fig. 3 D). In summary, we propose that defects in plus-end attachments lead to fewer kinetochores and astral microtubules in CIN⁺ cells, and that both wild-type and mutant APC proteins are positioned to affect plus-end attachments.

CIN⁺ tumor cells have defects in chromosome congression

To determine if the subtle defect in microtubule plus-end attachments affected chromosome congression in CIN⁺ cells, we measured the ratio of the width to the height of the chromosomal mass (congression index) in CIN[−] and CIN⁺ cells that had achieved a metaphase type alignment of chromosomes. Analysis of a representative sample of cells showed that, on average, CIN⁺ tumor cells fail to congress their chromosomes to the same extent as CIN[−] tumor cells (Table I, see Fig. 4a for example). Although congression could, in theory, be influenced by ploidy and/or spindle length, no correlation was observed (Table I). CIN⁺ cells also exhibited a dramatic reduction (3–5 fold) in metaphase cells and a corresponding increase in cells where chromosomes were not maximally aligned (i.e., prometaphase cells; Fig. 4 A, right panel and Fig. 4 B). Together, these data argue that defects in proper microtubule plus end attachment results in a less efficient movement of chromosomes to the metaphase plate.

Proper bivalent attachment of chromosomes to the mitotic spindle is also required for diminution or loss of mitotic checkpoint proteins (such as BubR1, Bub1, and Mad2) bound to kinetochores. Therefore, we examined BubR1 levels in cells with maximally congressed chromosomes, as determined by their congression index. BubR1 staining was reduced in CIN[−] metaphase cells relative to unattached kinetochores, whereas CIN⁺ cells with maximally aligned chromosomes failed to down-regulate BubR1 from the kinetochores, resulting in a 2–5 fold increase in BubR1 intensities (Fig. 4 C and Table I). The retention of BubR1 at the kinetochores is not due to a general failure in the down-regulation of checkpoint proteins, as anaphase CIN⁺ cells down-regulate BubR1 as expected (Fig. 4 D). The retention of checkpoint proteins was not limited to BubR1, as Mad2 and Bub1 also persisted in CIN⁺ metaphase cells (unpublished

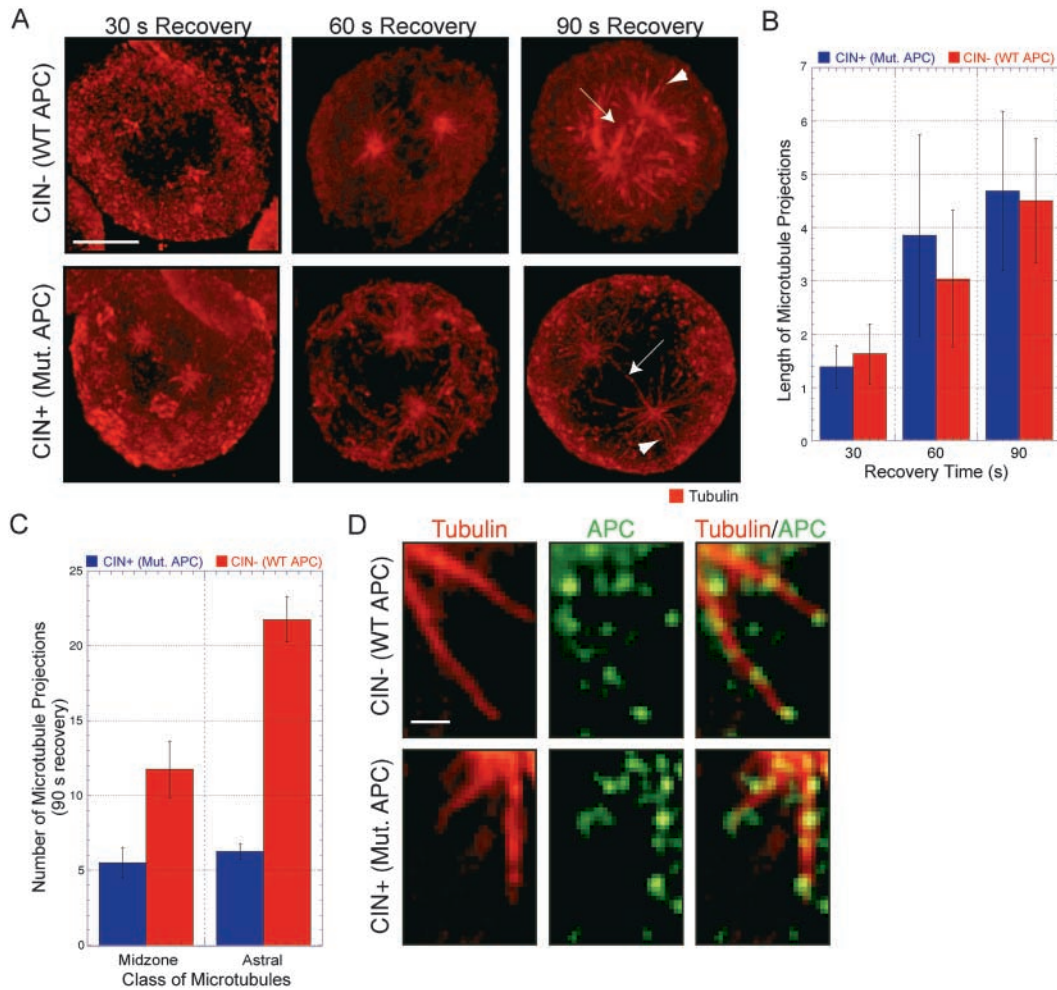


Figure 3. Microtubule growth and nucleation are normal in CIN⁺ tumor cells. (A) CIN⁻ (HCT116) and CIN⁺ (SW480) cells were stained for tubulin after cold treatment and recovery at 37°C for 30, 60, or 90 s. Projections of z-sections containing the spindle are presented. Bar, 5 μ m. (B) The length of microtubules was measured from each pole for both CIN⁻ (red bars) and CIN⁺ (blue bars) tumor cells. Error bars represent the SD of microtubule length. (C) The numbers of microtubule projections emanating from each pole toward the cortex (astral, arrowhead) and toward the chromosomes (kinetochore, arrow) were counted in CIN⁻ (red bars) and CIN⁺ (blue bars) tumor cells. Error bars represent the SD of microtubule numbers. (D) CIN⁺ and CIN⁻ cells were methanol fixed and stained for tubulin (red) and APC (green) after cold treatment and recovery. Single z-sections are presented. Bar, 1 μ m.

data). Together these data argue that inefficient plus-end microtubule attachments in CIN⁺ cells compromises chromosome congression and bivalent attachment of chromosomes to the mitotic spindle.

Mutant APC (1-1450) acts dominantly to disrupt kinetochore-microtubule attachments

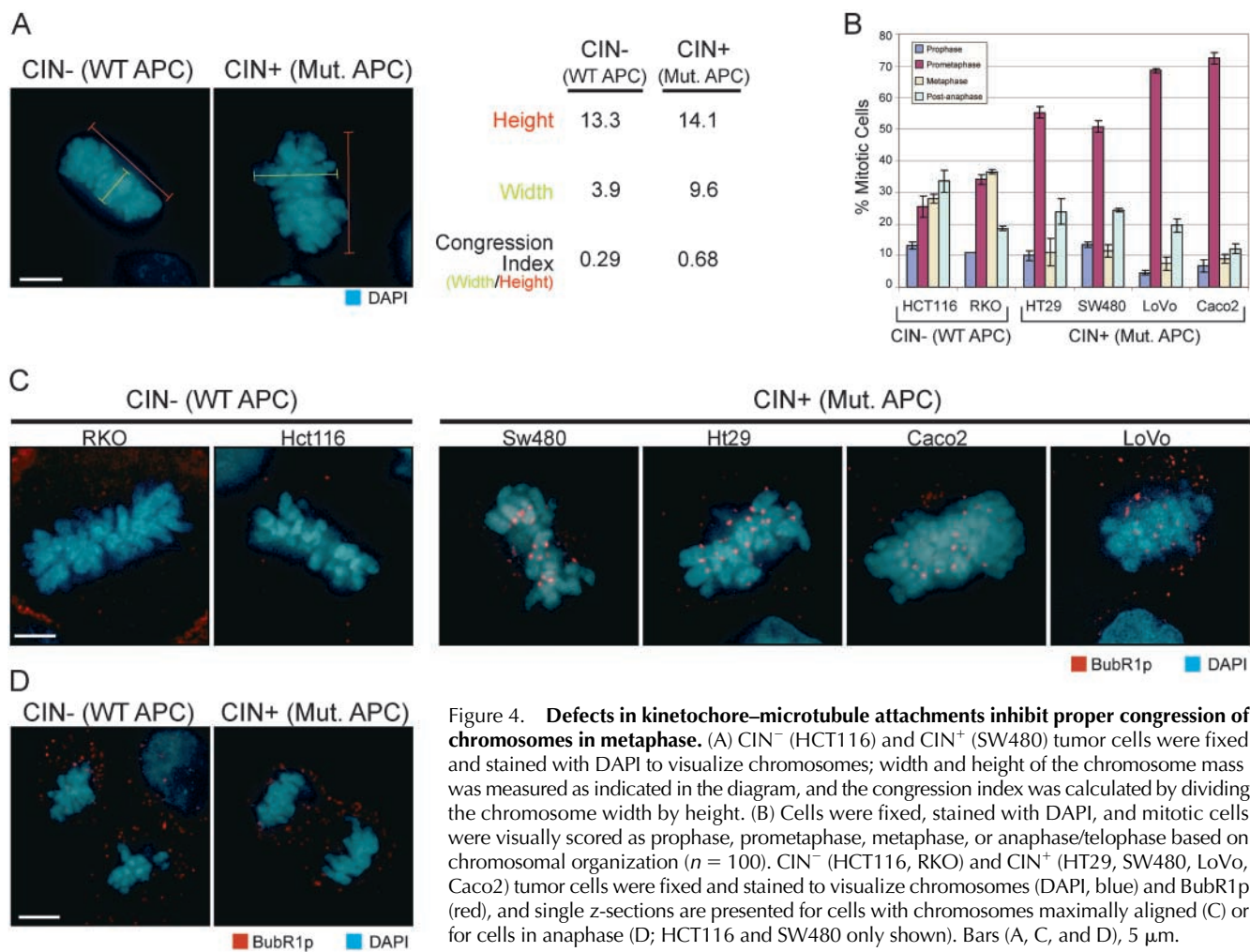
The defect in microtubule attachment observed in CIN⁺ mitotic cells may be caused by any one of the multiple ge-

Table I. Mitotic characteristics of colorectal tumor cell lines

Cell line	CIN ⁻		CIN ⁺			
	HCT116	RKO	SW480	HT29	Caco	LoVo
Chromosomal ploidy ^a	Near diploid (65)	ND (140)	Hypotriploid (111)	Hypertriploid (90)	Modal = 96 (92.5)	Hyperdiploid (69)
Pole-pole distance ^b (μ m; $n_{\text{avg}} = 15$)	8.46 (1.30)	11.03 (2.63)	8.97 (1.33)	8.65 (1.92)	13.73 (1.79)	8.95 (1.45)
Congression index ^b ($n_{\text{avg}} = 15$)	0.30 (0.06)	0.33 (0.06)	0.58 (0.09)	0.53 (0.12)	0.56 (0.10)	0.49 (0.10)
BubR1:ACA intensity ^b ($n = 10$)	2.29 (0.60)	2.80 (0.84)	4.82 (0.95)	7.48 (1.34)	10.69 (2.10)	5.02 (0.77)

^aPloidy status as reported by American Type Culture Collection. Values in parentheses are our ploidy estimates from cell lines used (based on ACA counts).

^bValues in parentheses represent SD.



netic lesions associated with these tumor cells. However, all CIN⁺ cells examined have been reported to contain truncating mutations in *APC*, raising the possibility that microtubule attachment defects are linked to mutations in *APC*. Furthermore, the reported dominant nature of the CIN phenotype and reports that *APC* mutations could act dominantly led us to ask whether a single truncating mutation in *APC* is sufficient to cause the microtubule plus-end attachment defect observed in CIN⁺ tumor cells (Lengauer et al., 1997; Tighe et al., 2001; Ditchfield et al., 2003). To test this possibility, we introduced an archetypal truncation of the carboxy-terminus of *APC*, typically found in human tumors, into human 293 cells that are wild type for *APC* (Fig. 5 A, top). To avoid toxicity issues associated with APC expression in cultured cells, we used an ecdysone regulated expression vector in which the sequences encoding a 13myc epitope tag were fused to the cDNAs encoding the following APC proteins: (i) the full length APC (mycAPC-wt), (ii) an amino terminal fragment that mimics mutations typically found in human tumors (mycAPC1-1450) and (iii), a carboxy-terminal fragment containing the EB1 binding domain of APC (mycAPC2560-2843; Fig. 5 A). Multiple clones were isolated after the cotransfection of 293 cells with the *APC* constructs and the ecdysone nuclear hormone receptor plasmid. Stable 293 clones containing the expression

vector but not the hormone receptor were generated in parallel to control for the effects of unregulated expression of the APC fragments. The expression of APC fragments was dependent on the hormone receptor and elevated levels of protein were observed as hormone concentration was increased (Fig. 5 B). Importantly, the levels of induced mycAPC1-1450 were equal to or lower than the endogenous levels of full length APC when immunoblotted with the same polyclonal APC antibody, arguing that mycAPC1-1450 is not overexpressed in these cells (Fig. 5 B; compare “anti-APC” lanes). Similar results were obtained for mycAPC2560-2843 (Fig. 5 C). Despite screening numerous clones, we were unable to obtain stable cells expressing mycAPC-wt, consistent with previous observations that full length APC expression is extremely toxic to cells (Morin et al., 1996; Rosin-Arbesfeld et al., 2001). Therefore, we used transient cotransfections in 293 cells to assess the effects of mycAPC-wt on mitotic spindles.

To test if the mycAPC1-1450 fragment dominantly interferes with microtubule plus-end attachments, we applied the identical analyses performed for tumor cells to assess the integrity of microtubules in mycAPC1-1450 clones in the presence (induced) or absence (mock induced) of hormone and in the presence or absence of the plasmid encoding the nuclear hormone receptor (no receptor). Although the mi-

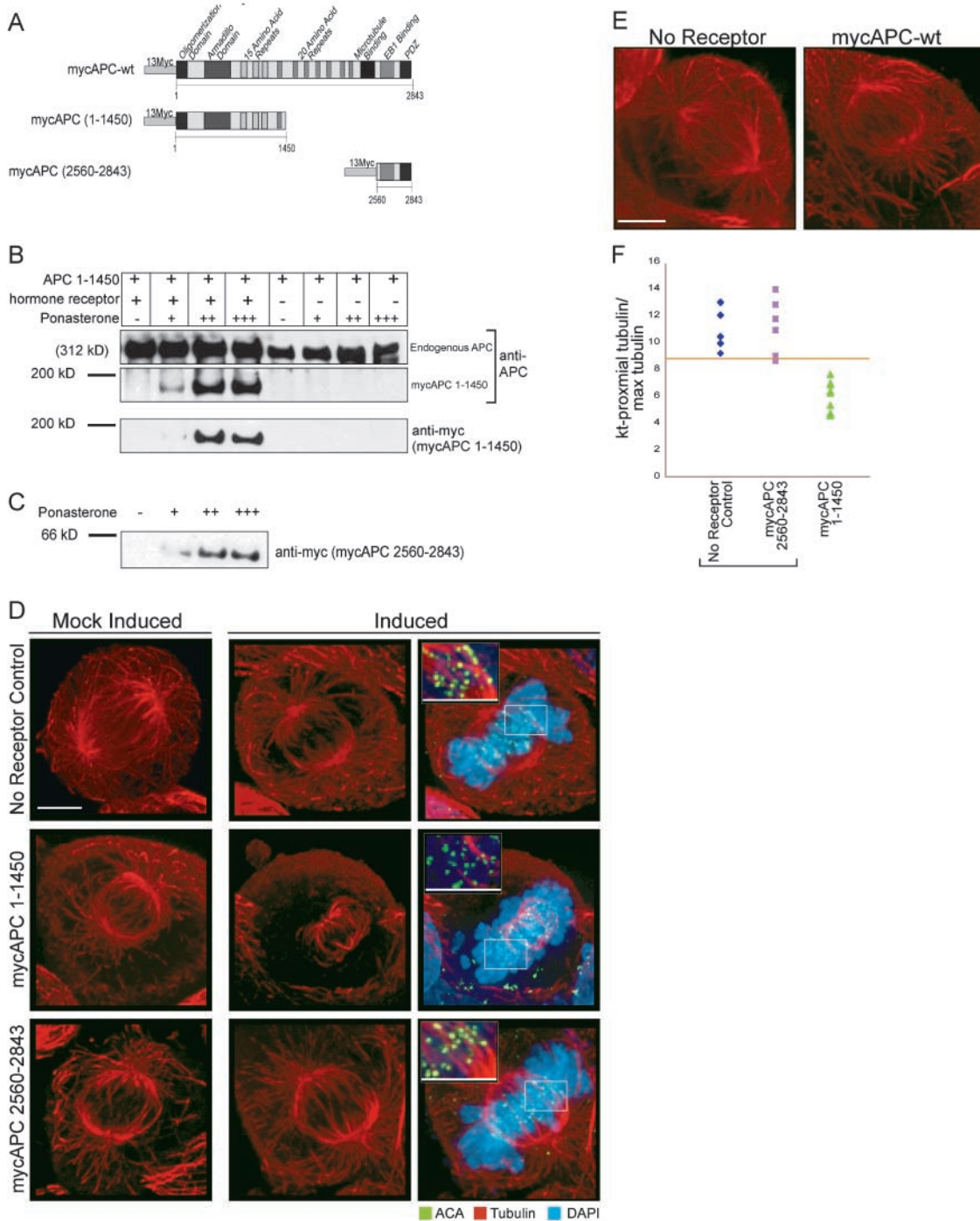


Figure 5. **Mutant APC (1-1450) acts dominantly to disrupt spindle microtubules.** (A) Diagram shows a schematic of the APC fragments expressed in human 293 cells. Stable cell lines containing the nuclear hormone receptor and mycAPC1-1450 (B) or mycAPC2560-2843 (C), with or without the hormone receptor, were grown in the presence of indicated amounts of ponasterone, lysed, and immunoblots were performed with antibodies against the myc epitope or against human APC. The top and middle panels in B were taken from the same film, and are directly comparable (brackets). Position of the nearest mol wt marker is indicated. (D) The indicated stable cell lines were grown in the absence (mock induced) or presence (induced) of ponasterone (2 μ M) for 72 h, fixed, and stained to visualize tubulin (red), chromosomes (blue), and kinetochores (ACA, green). Projections of z-sections containing the spindle are presented. Inset represents a 2.5-fold magnification of the region indicated by the box. (E) Human 293 cells were transiently transfected with mycAPC-wt with and without the nuclear hormone receptor (no receptor control), fixed, and stained to visualize tubulin (as above). (F) Quantification of the average kinetochore-proximal tubulin staining for metaphase cells for each stable cell line (each point represents the average kinetochore-proximal tubulin intensity value for one cell). Statistically similar data were plotted together (indicated by the bracket). Orange line represents the grand mean. Bars (D and E), 5 μ m.

totip spindles in the no receptor control in cells expressing mycAPC2560-2843 and in transient transfections with mycAPC-wt were robust, induction of mycAPC1-1450 for

72 h resulted in the complete disruption of astral microtubules and a dramatic reduction in the staining intensity of midzone microtubules (Fig. 5, D and E; Fig. S2, available at

Table II. Mitotic characteristics of 293 cells expressing APC fragments

Construct induced in 293 clones	mycAPC1-1450 (no receptor)	mycAPC1-1450	mycAPC2560-2843
Percentage of collapsed spindles ($n_{\text{avg}} = 100$)	4%	21%	4%
Pole–pole distance ^a (μm ; $n_{\text{avg}} = 28$)	8.78 (1.86)	6.83 (1.83)	8.13 (2.21)
Congression index ^a ($n_{\text{avg}} = 10$)	0.26 (0.04)	0.43 (0.16)	0.28 (0.04)

^aValues in parentheses represent SD.

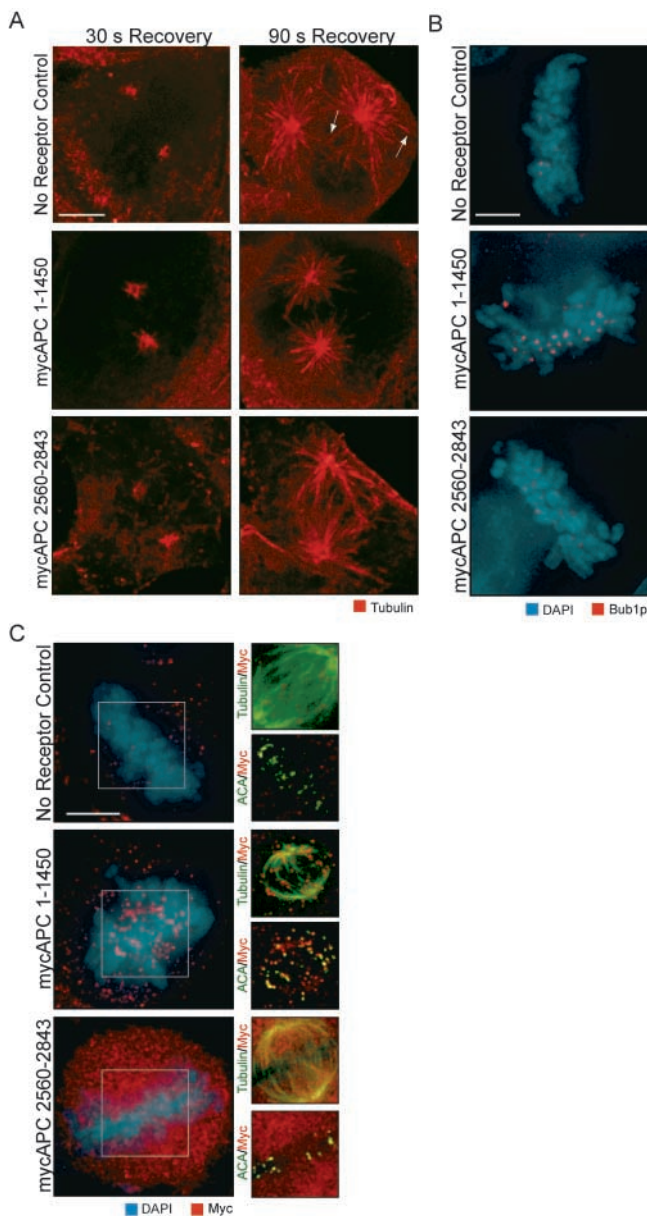


Figure 6. **mycAPC1-1450 interferes with microtubule plus-end attachments and localizes to the tips of kinetochore microtubules.**

(A) The indicated stable cell lines were grown for 72 h in the presence of ponasterone ($2 \mu\text{M}$) and stained for tubulin after cold treatment and recovery at 37°C for 30 or 90 s. Projections of z-sections containing the spindle are presented. Arrows indicate sites of microtubule plus-end attachment. (B) The indicated stable cell lines were grown as above, fixed, and stained to visualize chromosomes (DAPI, blue) and Bub1p (red). Single z-sections in the middle of the

<http://www.jcb.org/cgi/content/full/jcb.200307070/DC1>), yielding a remarkably similar phenotype to that observed in CIN^+ tumor cells (Fig. 1 A; Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200307070/DC1>). The effects on the mitotic spindle were more severe in 293 clones expressing mycAPC1-1450. We observed that 21% of the spindles were collapsed (i.e., mitotic spindles where pole–pole distances fell below $5 \mu\text{m}$), a phenotype not observed in the CIN^+ tumor cells. We note that even in the absence of hormone, cells carrying the receptor exhibited subtle defects in spindle microtubules, suggesting that even very low levels of mycAPC1-1450 compromise the mitotic apparatus (Fig. 5 D; compare mycAPC1-1450 mock-induced and induced panels). This phenotype was not limited to 293 cells, as we also observed loss of astral and kinetochore microtubules when mycAPC1-1450 was expressed in the CIN^- RKO colorectal tumor cell line (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200307070/DC1>). Although we have concentrated our analyses on the defects in kinetochore–microtubule attachment, it is striking that astral microtubules are compromised in both CIN^+ tumor cells and in 293 cells expressing mycAPC1-1450.

To assess whether the expression of mycAPC1-1450 alters the ability of 293 cells to form kinetochore–microtubule attachments, we carefully analyzed kinetochore-proximal tubulin staining as described in Fig. 1. Data were collected from numerous stable clones at metaphase, excluding cells with collapsed spindles. Statistical analysis revealed two distinct groups, with cells expressing mycAPC1-1450 falling below the mean (compare Fig. 5 F with CIN^+ cells in Fig. 1 D). The decrease in kinetochore-proximal tubulin staining was not due to increases in spindle pole–pole lengths, as cells expressing mycAPC1-1450 had a similar or reduced pole–pole distance compared with control cells (Table II). Thus, the introduction of the amino-terminal 1450 amino acids of APC dominantly interferes with proper microtubule function during mitosis.

To more closely link the microtubule phenotype observed in 293 cells with that observed in CIN^+ cells, we measured

spindle are presented. (C) Stable cell lines were grown as above and then stained with antibodies to tubulin, ACA, and myc and with DAPI to visualize chromosomes. Chromosomes (blue) and myc (red) staining are presented in the large panels, and tubulin (green) and myc (red) staining or ACA (green) and myc (red) staining of the regions indicated by the boxes are presented as indicated on the right. Projections of 10 z-sections chosen including the middle of the spindle are presented. Bars (A–C), 5 μm .

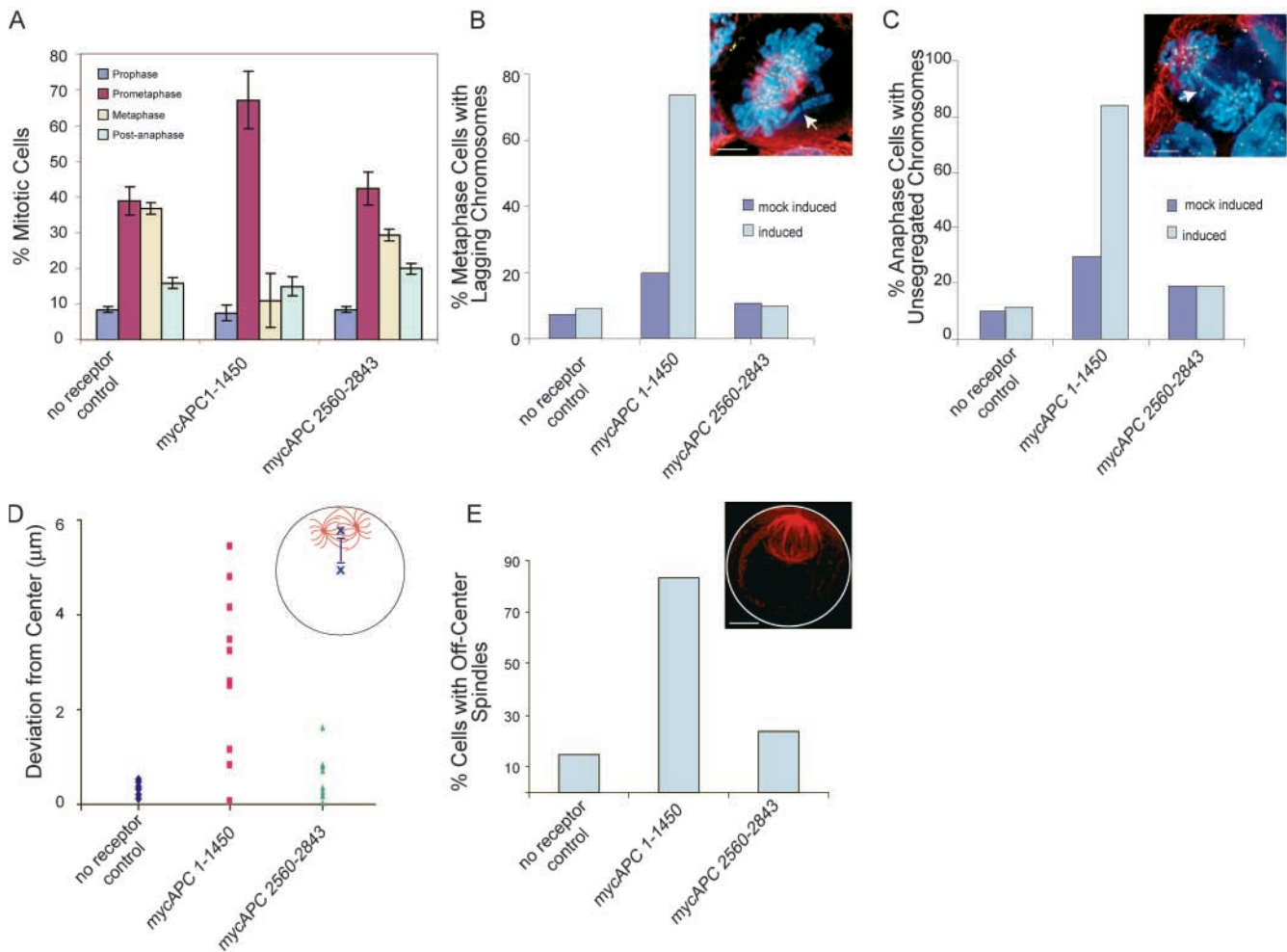


Figure 7. Mutant APC (1-1450) dominantly induces abnormal chromosome segregation. (A) The indicated stable cell lines were grown in ponasterone (2 μM) for 72 h, fixed, stained with DAPI, and the mitotic cells were visually scored as described in Fig. 4 B ($n = 100$). Cells were grown as above and then fixed and stained to visualize tubulin (red), chromosomes (blue), and kinetochores (green; ACA). The percentage of metaphase cells with lagging chromosomes (B; see inset for example, $n = 50$), the percentage of anaphase cells with unsegregated chromosomes (C; see inset for example, $n = 50$), the range of distances of the spindle relative to the center of the cell (D; see inset for diagram of measurement, $n = 10$), and the percentage of cells with the spindle position $> 1 \mu\text{m}$ from the center (E; see inset for example, $n = 50$) was determined for the indicated cell lines. Arrows in B and C indicate mispositioned chromosomes.

the ability of microtubules to regrow after cold-induced depolymerization. As predicted, both control cells and cells expressing mycAPC1-1450 showed similar microtubule nucleation and growth at early time points (Fig. 6 A; 30-s recovery). By 90 s of recovery, cells expressing mycAPC1-1450 show a significant reduction in the number of microtubule projections; very few microtubules reach either the cortex or kinetochores (Fig. 6 A, 90-s recovery; arrows). Consistent with a defect in kinetochore–microtubule attachments, we observed that Bub1p was enriched at kinetochores specifically in metaphase cells expressing mycAPC1-1450 (Fig. 6 B). We conclude from these data that microtubule plus-end attachments are compromised in 293 cells expressing mycAPC1-1450, and this defect is remarkably similar to the defect observed in CIN⁺ tumor cells (Fig. 3).

To assess the possibility that mycAPC1-1450 may act directly at the site of microtubule plus-end attachments, we examined the localization of APC fragments in 293 cells using antibodies to the myc epitope. The no receptor con-

trol cell line exhibited only background levels of staining as expected, whereas both the mycAPC1-1450 and mycAPC2560-2843 cell lines exhibited strong signals, demonstrating the specificity of the myc antibody in 293 cells. MycAPC1-1450 was observed in puncta along the spindle and juxtaposed to anti-centromere antibody (ACA)–stained kinetochores (Fig. 6 C, insets). In contrast, mycAPC2560-2843 staining filled the entire cell, consistent with it being in a soluble pool and neither along spindle microtubules nor at kinetochores. These results strongly argue that mycAPC1-1450 acts directly on the mitotic spindle and kinetochores to compromise spindle function.

Mutant APC (1-1450) acts dominantly to interfere with chromosome segregation events

The mycAPC1-1450–induced changes to the mitotic spindle led us to examine the consequences of these changes for chromosome alignment and segregation. A visual inspection of cells expressing mycAPC1-1450 revealed a dramatic de-

crease in the number of metaphase cells and a corresponding increase in prometaphase cells (Fig. 7 A), reminiscent of the CIN⁺ tumor cells (Fig. 4 B). To assess whether the chromosome congression is compromised, we measured the congression index as described for the tumor cells (Table I). We observed that cells expressing mycAPC1-1450 have nearly a twofold increase in the congression index, consistent with their failure to fully align chromosomes on the metaphase plate (Table II). These data are consistent with a microtubule plus-end attachment defect that prevents efficient chromosome organization before metaphase.

Unlike CIN⁺ tumor cells, chromosomes in 293 cells are relatively stable and show little segregation abnormalities. If the perturbation in the mitotic spindle contributes to CIN, then we predict that the expression of mycAPC1-1450 will cause an increase in cells that fail to normally segregate their chromosomes. We tested this prediction by monitoring the chromosomal pattern in mitotic cells cultured in the presence of low levels of inducing hormone (2 μ M). The persistent expression of mycAPC1-1450 resulted in two classes of chromosome segregation defects: cells with abnormally positioned chromosomes and cells with mispositioned spindles. Two types of abnormal chromosome positioning were observed: 75% of metaphase cells exhibited chromosomes that fail to position properly between the spindle poles (Fig. 7 B; lagging chromosomes), and 85% of anaphase cells exhibited chromosomes that remain at the midzone after the majority of chromosomes were segregated (Fig. 7 C). In contrast, <10% of no receptor control cells exhibited these defects. Interestingly, a significant increase in lagging chromosomes was observed in mock-induced metaphase cells, a twofold increase over the no receptor control, highlighting how sensitive cells are to even very low levels of mycAPC1-1450 (Fig. 7 B; compare mock-induced mycAPC1-1450 with mock-induced no receptor).

We also observed an increase in the frequency of mispositioned spindles in cells expressing mycAPC1-1450, presumably a manifestation of the loss of astral microtubules in these cells. To categorize spindle position, we determined the center of the mitotic cell by measuring its diameter from cortex to cortex; we calculated how far each spindle varied from the center of the cell and plotted this distance for a number of metaphase cells. In control cells, spindle variation from the center of the cell was minimal (<1 μ m; Fig. 7 D, no receptor and mycAPC2560-2843). In contrast, spindles in cells expressing mycAPC1-1450 were scattered over a wide range of distances from the center of the cell, consistent with the lack of astral microtubules causing free “floating” spindles (Fig. 7 D). Nearly 90% of cells expressing mycAPC1-1450 had mispositioned spindles, a six- or four-fold increase compared with control cells with no receptor or cells expressing mycAPC2560-2843, respectively (Fig. 7 E). Together, these data argue that wild-type 293 cells expressing mycAPC1-1450 are compromised in their ability to properly segregate chromosomes and orient spindles. We conclude that APC functions to regulate microtubule plus-end attachments during mitosis, and naturally occurring mutations in *APC* may contribute directly to mitotic abnormalities and to the CIN phenotype observed in human colorectal tumors.

Discussion

The successful formation of microtubule plus-end attachments is critical in organizing and segregating chromosomes during mitosis. However, little is known about the mechanisms that mediate and regulate microtubule attachment. By analyzing tumor cells that have high rates of CIN, we have identified a role for APC in the regulation of microtubule plus-end attachments during mitosis, and have provided insight into this critical but poorly understood process. Our data also confirm, by completely independent means, previous results that have suggested APC plays a role in chromosome segregation. In addition, our results have important implications for the genesis of colorectal cancers, suggesting that a single mutated allele of *APC* may confer on cells a predisposition to undergo abnormal mitoses. Abnormal mitosis may contribute directly to the genesis of cancer by increasing CIN, or may contribute indirectly by altering the normal division pattern of cells in the colon.

APC mutations induce defects in microtubule plus-end attachments

The cumulative data from CIN⁺ tumor cells carrying mutations in *APC* and from 293 clones expressing mycAPC1-1450 argue that mutant *APC* compromises microtubule plus-end attachments during mitosis. Both astral and kinetochore microtubules were disrupted, resulting in fewer microtubules at the cell cortex and kinetochores, a defect in chromosome congression in metaphase, and a series of spindle-related defects that directly contribute to failures in chromosome segregation. The unstable nature of kinetochore–microtubule attachments in cells with *APC* mutations, coupled with the normal growth properties of microtubules, argues that expression of mutant *APC* specifically affects microtubule plus-end attachments. We speculate that inefficient microtubule capture might result in a shorter microtubule half-life, and thus reduced tubulin staining.

APC mutants may affect the ability of microtubules to locate their binding site, or may affect the integrity of the binding site itself. Our data do not rule out either possibility. The effect of *APC* mutants on both astral and kinetochore microtubules is more consistent with a role for *APC* in guiding microtubules to their binding site, as the cortex and kinetochores are likely to be composed of different proteins. It is possible that mutations in *APC* alter the dynamicity of microtubules, especially after the initial growth phase when plus ends are in close proximity to their binding sites, resulting in plus-end attachment defects. This scenario is consistent with previous observations describing the behavior of newborn interphase microtubules and with our observations that microtubules in cells expressing *APC* 1-1450 grow past cortical and kinetochore attachment sites, as if unable to locate their binding site (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200307070/DC1>; Komarova et al., 2002). Resolution of this question will require careful measurements of microtubule dynamicity in live cells expressing *APC* mutants.

The role of APC in mitosis

Previous works provide correlative data to suggest that APC plays a role in mitosis, primarily based on its localization to kinetochore microtubules, its interaction with the mitotic checkpoint kinase Bub1p, and the increase in aneuploidy observed in embryonic stem cells derived from *APC^{min/min}* mice (Fodde et al., 2001; Kaplan et al., 2001). In this paper, we provide genetic evidence that *APC* mutations dominantly interfere with mitosis by demonstrating that expression of mycAPC1-1450 is sufficient to transfer the mitotic defects found in CIN⁺ tumor cells to 293 cells. The dominant nature of *APC* mutations and the association of both wild-type and mycAPC1-1450 with microtubule plus ends suggests that APC normally acts to regulate microtubule attachments. Therefore, we propose that mycAPC1-1450 acts as a dominant-negative mutation to perturb microtubule plus-end binding. Independent reports support the notion that *APC* mutations may have dominant effects on β -catenin-mediated transcription, interphase microtubule growth, and the mitotic checkpoint (Dihlmann et al., 1999; Tighe et al., 2001; Husoy et al., 2003).

How does mycAPC1-1450 act dominantly to interfere with microtubule plus-end attachments? One intriguing possibility is that APC influences microtubules through its interaction with EB1, a plus end microtubule-binding protein. EB1 interacts with carboxy-terminal sequences in APC, and this interaction has been suggested to be important for the EB1-induced polymerization of microtubules (Nakamura et al., 2001). EB1 has been implicated in controlling microtubule dynamics, contributing to the dynamicity of microtubules, and therefore, to their ability to make plus-end attachments (Beinhauer et al., 1997; Tirnauer et al., 1999; Rogers et al., 2002). It is tempting to speculate that expression of mycAPC1-1450 inhibits EB1 function and microtubule dynamicity, and therefore makes microtubule plus-end attachments less efficient. However, any model incorporating EB1 would need to rationalize the dominant behavior of APC with the loss of the EB1-binding domain in mycAPC1-1450. One possible explanation may come from the observation that APC forms dimers via amino-terminal sequences; formation of a mixed dimer of wild type and mycAPC1-1450 may result in the nonproductive binding of EB1, and thus act as a dominant negative (Joslyn et al., 1993; Su et al., 1993). Future analysis using recombinant proteins may help to shed light on the types of APC mutant complexes that can assemble.

Although the interaction between EB1 and APC provides a compelling link between APC and microtubule function, there are other APC interactions that may be significant. APC regulates β -catenin levels by forming a complex with β -catenin, Axin, and GSK3 β that leads to the phosphorylation of β -catenin and its degradation through ubiquitin-mediated proteolysis (Polakis, 1999, 2000). It is possible that APC may control mitotic events through its association with Axin and GSK3 β . Interestingly, GSK3 β has been localized to spindle microtubules, and inhibitors of GSK3 β result in defects in microtubule length and chromosomal alignment (Wakefield et al., 2003). It is conceivable that APC links GSK3 β to microtubules, where it may phosphorylate plus-end binding proteins, a modification believed to regulate their association with microtubules (for review see Carvalho

et al., 2003). It will be important to begin to characterize the effect of APC mutations on the localization and biochemical properties of these proteins during mitosis.

Implications for the genesis of colorectal cancer

The genomic instability associated with the CIN phenotype in tumor cells is often cited as contributing to both the proliferative and metastatic potential of tumors. Although there is little dispute that CIN occurs, it is less clear if this genetic instability is a driving force in the onset of cancer. A simple prediction of this model is that CIN should occur early in the genesis of colorectal tumors if it is important for tumor progression. Our data provide a compelling link between APC mutations and mitotic abnormalities, raising the possibility that mitosis may be compromised very early in the development of colonic lesions.

The dominant behavior of APC truncations clearly raises new issues with regard to how colorectal cancers develop. One significant conclusion from our work is that a single genetic lesion can potentially result in a number of critical changes in basic cellular processes. Because these processes involve essential cellular functions, it is not surprising that the effects are subtle, balancing the survival needs of cells with the slow accumulation of aneuploid genomes. We speculate that a single mutant *APC* gene changes the behavior of cells in the intestinal crypt, compromising cell migration, cell cycle advance, symmetry of cell division, and chromosome segregation. It is the combination of these cellular defects that allow abnormal cells to propagate. A major challenge will be to understand how cellular changes in mutant APC cells affect their behavior in their normal cellular environment.

Materials and methods

Plasmids and antibodies

Regions encoding indicated fragments of human *APC* and a 13myc epitope tag were amplified by PCR (the sequence of PCR primers is available upon request) from a previously constructed plasmid (a gift from I. Nathke, University of Dundee, Scotland, UK), and were cloned into the pIND/Hygro ecdysone-inducible mammalian expression vector (Invitrogen). The coding region of each plasmid was confirmed by DNA sequence analysis (Davis Sequencing Facility, Davis, CA). The APC antibody was a gift from I. Nathke. Antibodies against Bub1p (14H5; CHEMICON International), BubR1p (8G1; CHEMICON International), c-Myc (9E10, Santa Cruz Biotechnology, Inc.), tubulin (Tub2.1 or FITC-conjugated Tub2.1, Sigma-Aldrich), and human centromere antigens (ACA; Antibodies, Inc.) were used at the manufacturer's recommendation.

Cell culture

Cells were cultured in Cellgro[®] DMEM high glucose medium (Mediatech, Inc.) supplemented with 10% FCS, 2 mg/ml L-glutamine, 1 mM sodium pyruvate, and 50 U/ml penicillin and streptomycin, with the exception of LoVo cells, which were cultured in F12K medium (Kaughn's modification) with identical supplements. Cells were maintained at 37°C and 5% CO₂. After DNA transfection using the FuGENE[™] 6 reagent (Roche), stable clones were selected and maintained in DMEM as above, supplemented with 200 μ g/ml hygromycin (Sigma-Aldrich). Cells were treated as indicated with ponasterone (1–10 μ M; Invitrogen) or ethanol.

Immunofluorescence

Cells were grown on coverslips coated with 0.5 mg/ml poly-L-lysine (Sigma-Aldrich), fixed in 3.7% PFA (Sigma-Aldrich) for 20 min at 37°C, permeabilized with 0.1% Triton X-100/PBS for 7 min, or methanol fixed for 6 min and acetone permeabilized for 30 s. Samples were blocked with 0.2% gelatin (Sigma-Aldrich) in PBS/0.02% Na₂S₂O₃ for 20 min. In some

cases, cells were treated for 30–60 s with calcium permeabilization buffer as described previously (Mitchison et al., 1986), and were chilled on ice for 1 h (unless noted otherwise) before fixation. Microtubules were allowed to regrow in warm (37°C) media (supplemented with 10 mM Hepes) for indicated times. Fixed cells were incubated with indicated primary and secondary antisera as recommended by the manufacturer and mounted as described previously (Martinez-Exposito et al., 1999). Fluorescence was collected using an inverted microscope (model IX70; Olympus) fitted to a DeltaVision® Restoration System (DV3.0; Applied Precision, Inc.). Images were recorded using a 60× oil immersion lens, NA 1.4 (Olympus) and a Photometrics CCD camera (model CH350; Roper Scientific). Deconvolution and image analysis was performed using tools provided in the softWoRx™ (v2.5) software package (Applied Precision, Inc.).

Quantification, measurements, and statistical analysis

Fluorescence intensity and distances were measured in deconvolved images using tools provided in the softWoRx™ image analysis software (Applied Precision, Inc.). Phenotypic differences in mitotic spindles were assessed by quantifying tubulin intensity using two methods. First, the average midzone tubulin intensity was determined within an inscribed circle fitted between the two spindle poles (Fig. 1 B). To normalize for changes in staining conditions between multiple cells and experiments, a “spindle ratio” was generated by dividing the average pixel intensity value in the circle by the maximum pixel value for the measured cell. In the second method, kinetochore-proximal tubulin intensity was measured within a 0.4-μm circle centered around ACA-positive kinetochores. For each cell, the total kinetochore-proximal tubulin staining was determined for each kinetochore and normalized to the maximal tubulin intensity (single-pixel value) for the measured cell. We also used this analysis to estimate ploidy by calculating the number of ACA-positive signals/cell; the ploidy estimates represent an average >6 mitotic cells. Tukey’s multiple comparison test ($P < 0.001$) was used to analyze differences in spindle ratios and in kinetochore-proximal tubulin intensity for tumor cell lines and stable 293 clones (Prism v3.0; GraphPad Software, Inc.). Measurements of the maximal intensity values for BubR1 and ACA were performed as above and ratios were calculated for each cell line. Measurements of microtubule projection length and calculation of the congression index were performed as described in the text.

Western blot analysis

Cells were lysed in buffer (10 mM Hepes, pH 7.2, 1% Triton X-100, 150 mM NaCl, 0.25 mM EDTA, 50 mM NaF, 50 mM β-glycerol-phosphate, 10% glycerol, and a protease inhibitor cocktail [1 mM TPCK, 10 mM PMSF, and 10 μg/ml leupeptin, pepstatin, and chymostatin]). Proteins were resolved on 3–8% gradient gels using recommended conditions (Invitrogen), transferred to nitrocellulose, and incubated with antibodies as described previously (Nathke et al., 1996). Bound antibodies were detected using the ECL detection system (Amersham Biosciences). Blots were exposed to film for multiple exposure times to ensure the linearity of signal.

Online supplemental material

Figure S1 presents multiple images that detail the range of defects observed in the mitotic spindle for each tumor cell line (HCT116, RKO, HT29, LoVo, SW480, and Caco2). Figure S2 shows several stable 293 clones expressing mycAPC1-1450 or mycAPC2560-2843 to detail the range of defects observed in the mitotic spindles. Figure S3 shows RKO tumor cells (CIN2) transiently expressing mycAPC1-1450 or mycAPC2560-2843 to demonstrate that mycAPC1-1450 dominantly disrupts mitotic spindles in CIN2 tumor cells. Online supplemental material available at <http://www.jcb.org/cgi/content/full/jcb.200307070/DC1>.

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