Aneuploid colon cancer cells have a robust spindle checkpoint

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Colon cancer cells frequently display minisatellite instability (MIN) or chromosome instability (CIN). While MIN is caused by mismatch repair defects, the lesions responsible for CIN are unknown. The observation that CIN cells fail to undergo mitotic arrest following spindle damage suggested that mutations in spindle checkpoint genes may account for CIN. However, here we show that CIN cells do undergo mitotic arrest in response to spindle damage. Although the maximum mitotic index achieved by CIN lines is diminished relative to MIN lines, CIN cells clearly have a robust spindle checkpoint. Consistently, mutations in spindle checkpoint genes are rare in human tumours. In contrast, the adenomatous polyposis coli (APC) gene is frequently mutated in CIN cells. Significantly, we show here that expression of an APC mutant in MIN cells reduces the mitotic index following spindle damage to a level observed in CIN cells, suggesting that APC dysfunction may contribute to CIN.

INTRODUCTION

Prior to cell division, the replicated genome is segregated such that the daughter cells receive all the genetic information required for further growth and development. The fate of the daughters is dependent on the accuracy of this process: missegregation dramatically alters gene dosage and can result in cell death or birth defects such as Down's syndrome (Nicklas, 1997). Human tumour cells often have abnormal chromosome complements suggesting that aneuploidy may also cause cancer. However, whether aneuploidy is a contributing factor, or merely a consequence of tumorigenesis has remained controversial (Li et al., 2000).

It is now apparent that the majority of aneuploid colon cancer cells missegregate their chromosomes with high frequency, that is they exhibit chromosome instability (CIN) (Lengauer et al., 1997). In contrast, diploid colon cancer lines missegregate their

chromosomes with much lower frequency, and thus while not CIN they frequently exhibit minisatellite instability (MIN). Although a minority of colon cancers display both CIN and MIN, or neither, these two phenotypes are generally mutually exclusive (Lengauer et al., 1997; Georgiades et al., 1999; Abdel-Rahman et al., 2001), suggesting that one or the other is required for the evolution of the majority of colon cancers (Lengauer et al., 1998; Cahill et al., 1999b).

While it is now widely accepted that MIN is due to mutations in mismatch repair genes (Kinzler and Vogelstein, 1996), the genetic lesions which give rise to CIN remain undefined. However, the very nature of the CIN phenotype suggests that the cause may be mutations in genes required to maintain the fidelity of chromosome segregation (Lengauer et al., 1997). Support for this notion came from the observation that, in contrast to the MIN lines, CIN lines fail to undergo mitotic arrest in response to spindle damage (Cahill et al., 1998). However, we show here that the CIN lines do undergo mitotic arrest in response to spindle damage. Interestingly, the maximum mitotic index achieved by the CIN lines is diminished relative to MIN lines suggesting that although the CIN cells have a robust checkpoint it may well be compromised. Significantly, we also show that expression of an APC mutant can compromise the spindle checkpoint response in MIN cells to a level observed in CIN cells, thus raising the possibility that APC dysfunction may contribute to CIN.

RESULTS

Following observations that aneuploid colon cancer cells exhibit CIN (Lengauer et al., 1997), we analysed the spindle checkpoint status of the MIN lines HCT-116 and DLD-1; the CIN lines HT-29, LoVo, SW480 and SW837; and HeLa cells. When grown in plastic dishes and viewed by phase-contrast microscopy, the

A. Tighe et al.

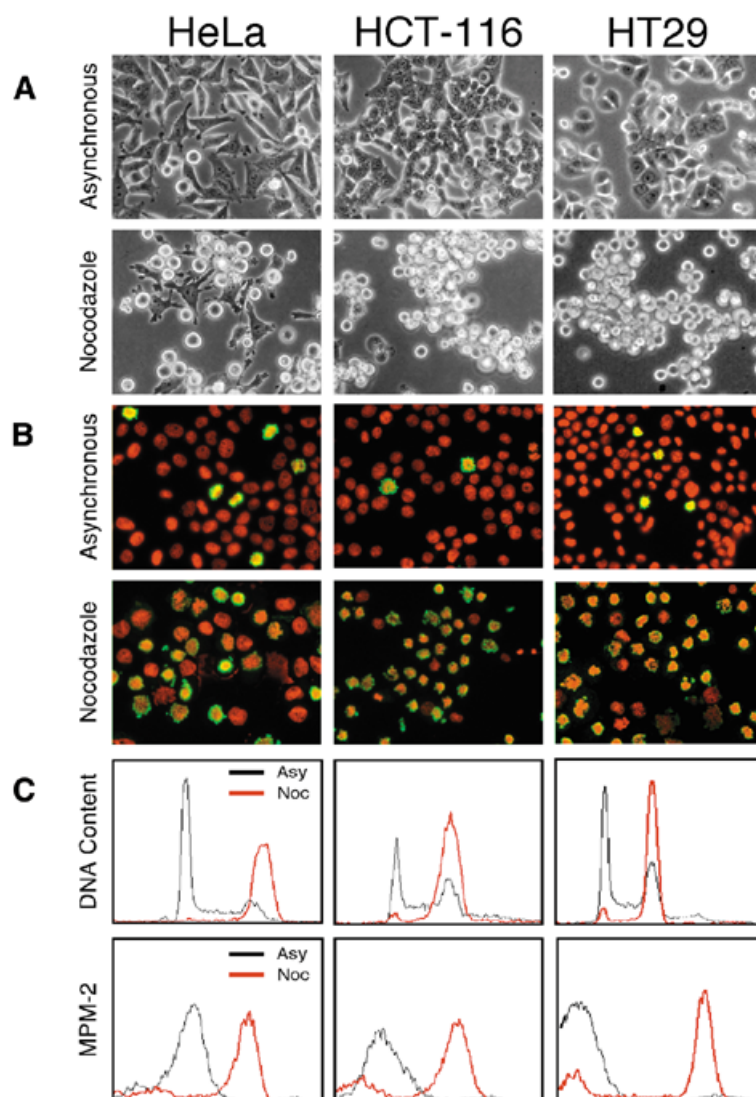**Fig. 1.** CIN cells undergo mitotic arrest following spindle damage. HeLa, HCT-116 (MIN) and HT-29 (CIN) cells were treated without or with 0.2 μg/ml nocodazole for 18 h. (**A**) Phase-contrast microscopy shows the majority of the cells in all lines round up following nocodazole treatment. (**B**) Immunofluorescence microscopy shows that in nocodazole, the majority of cells have condensed chromosomes (red) which are positive for phospho-histone H3 (green). (**C**) Flow cytometry shows that in the absence of nocodazole all three lines exhibit typical DNA content profiles (upper panels) with few MPM-2 positive cells (lower panels). In the presence of nocodazole all the lines exhibit large G_2/M peaks and the majority of the cells are MPM-2 positive indicating that they are mitotic.

vast majority of the cells in each line remained flat and adhered to the dish (Figure 1A). Following treatment with 0.2 μg/ml nocodazole for 18 h, the cells rounded up and detached from the dish (Figure 1A). Immunofluorescence analysis of asynchronous cultures showed that the vast majority of the cells had round nuclei with decondensed chromatin which did not express phospho-histone H3 (Figure 1B). Following nocodazole treatment however, most of the cells in all the lines had condensed chromosomes positive for phospho-histone H3 (Figure 1B). When analysed by flow cytometry, the untreated cells exhibited typical asynchronous DNA content histograms (Figure 1C) and few cells expressed mitotic specific MPM-2 epitopes. DNA

content histograms of nocodazole-treated cells displayed large 4N peaks, consistent with a G_2/M arrest. The majority of cells also expressed MPM-2 epitopes consistent with these cells being in mitosis. Taken together, these observations suggest that all the lines analysed undergo mitotic arrest following spindle damage.

To characterize the spindle checkpoint response in more detail, we treated the lines with nocodazole and determined the mitotic index every 6 h (Figure 2A). The mitotic index of each line increased ~10-fold during the first 12 h and in each case there is a clear peak between 18–24 h, which falls significantly by 36–42 h. Thus, in contrast to a previous report (Cahill *et al.*, 1998), we conclude that both the MIN and CIN lines do undergo a
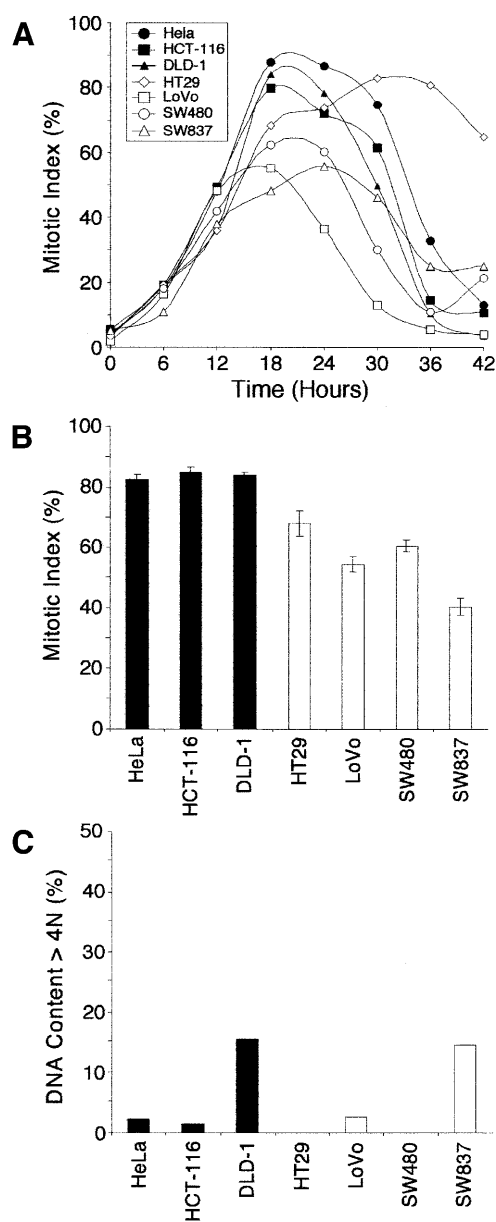
Fig. 2. CIN lines exhibit a robust spindle checkpoint. The lines indicated were plated out and treated with nocodazole to prevent spindle assembly. (**A**) Plot of mitotic index every 6 h. (**B**) Mitotic index at 18 h with each value representing the average and standard error from four independent experiments. (**C**) Percentage of cells with DNA contents >4N after 48 h.

transient mitotic arrest in response to spindle damage. Interestingly, there do appear to be differences in the maximum mitotic index achieved by the various lines. To characterize this further, the mitotic index of each line was determined in four separate experiments following an 18 h nocodazole block (Figure 2B). While the mitotic indices achieved by the HeLa and MIN cells was typically >80%, the CIN cells achieved mitotic indices of only 40 to 68%.

Previously, we showed that cells with an abrogated spindle checkpoint can bypass apoptosis following prolonged mitotic

arrest (Taylor and McKeon, 1997), suggesting that aneuploidy may not be due to elevated chromosome loss in the CIN cells but rather due to the ability of the CIN cells to survive an aberrant mitosis. However, only DLD-1 and SW837 cells showed any significant signs of DNA re-replication in the presence of nocodazole with ~15% of the cells having DNA contents >4N after 48 h (Figure 2C). In contrast, when the spindle checkpoint is abrogated in either HeLa cells by expressing a dominant-negative Bub1 or in HCT-116 cells by mutating the *MAD2* gene, >70% of the cells accumulate DNA contents >4N after 48 h in nocodazole (Taylor and McKeon, 1997; Michel *et al.*, 2001).

Taken together our results show that the CIN cells do indeed have a robust spindle checkpoint. Consistent with this notion, mutations in known spindle checkpoint genes are extremely rare in human tumours (Cahill *et al.*, 1998; Cahill *et al.*, 1999a; Imai *et al.*, 1999; Yamaguchi *et al.*, 1999; Myrie *et al.*, 2000; Sato *et al.*, 2000). However, our data suggest that the spindle checkpoint may be partially compromised in CIN cells, raising the possibility that mutations in genes not directly involved in the spindle checkpoint but rather involved in microtubule dynamics and/or spindle positioning may be mutated in these cells. Interestingly, the APC gene is frequently mutated in CIN cells (Cahill *et al.*, 1999b; Rowan *et al.*, 2000). Furthermore, APC can stabilize microtubule ends (Zumbrunn *et al.*, 2001) and also binds EB1 (Su *et al.*, 1995), a protein involved in spindle positioning (Tirnauer and Bierer, 2000).

To test whether APC dysfunction may play a causal role in CIN, we stably expressed an N-terminal APC mutant in HCT-116 cells which are MIN and have a wild-type APC gene (Lengauer *et al.*, 1997; Rowan *et al.*, 2000). The mutant expressed encodes the first 750 amino acids of APC and is thus typical of APC mutants expressed in colon cancer cells (Fearnhead *et al.*, 2001). Consistently, expression of N-APC was detectable in lines transfected with the N-APC vector but not a control vector (Figure 3A). When treated with nocodazole for 18 h, the mitotic index of the four control cell lines ranged from 67 to 83% (Figure 3B), similar to that of parental HCT-116 cells. In contrast, the mitotic index of the six cell lines expressing N-APC ranged from 31 to 57% (Figure 3B). Thus, expression of an N-terminal APC mutant in a MIN line can reduce the mitotic index following spindle damage to levels observed in CIN cells.

To gain insight into the mechanism by which APC dysfunction might compromise spindle checkpoint function, we over-expressed N-APC mutants in BHK and HeLa cells. Significantly, the N-terminal 750 amino acids of APC localized to the cytoplasm and to a structure which resembled the centrosome (Figure 4A and B). The N-terminal 1314 amino acids exhibited a similar pattern (data not shown). In contrast, full length APC localized only in the cytoplasm (Figure 4C). Co-staining with antibodies against cenexin (Lange and Gull, 1995) confirmed that N-APC does indeed localize to the centrosome (Figure 4D). Furthermore, the endogenous N-APC mutant expressed in DLD-1 cells, corresponding to amino acids 1 to 1416 (Homfray *et al.*, 1998), localizes to the centrosome (Figure 4E).

DISCUSSION

We show here that CIN colon cancer cells undergo transient mitotic arrest in response to spindle damage. However, the
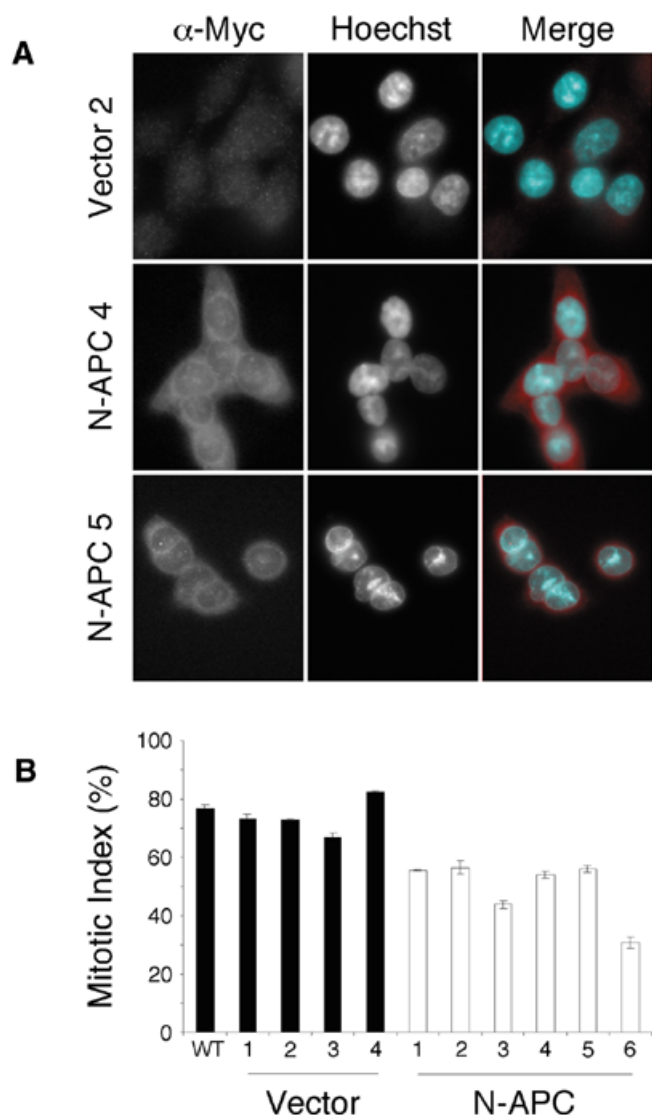
A. Tighe et al.

Fig. 3. Expression of N-APC compromises spindle checkpoint function. (**A**) HCT-116 cells stably transfected with control or N-APC vectors stained with anti-Myc antibodies and Hoechst dye. Only background staining is observed in the control line whereas extensive cytoplasmic staining is visible in the N-APC lines. (**B**) Stably transfected HCT-116 lines were treated with nocodazole to prevent spindle assembly. After 18 h the mitotic index was determined. Each value represents the average and standard error from three independent experiments. The mitotic index of lines expressing N-APC is reduced compared with the controls.

Fig. 4. N-APC localizes to the centrosome. (A–D) Transfected cells stained with anti-Myc antibodies and Hoechst as indicated. (**A**) HeLa and (**B**) BHK cell showing localization of N-APC to the cytoplasm and centrosome. (**C**) BHK cell showing localization of full length APC to the cytoplasm. (**D**) BHK cell showing co-localization of N-APC and cenexin. (**E**) DLD-1 cells stained with antibodies against APC and aurora a (Bischoff *et al.*, 1998) showing co-localization to centrosomes. White arrows identify the centrosomes.

maximum mitotic indices achieved by the CIN cells is lower than that of the MIN cells. Thus, we argue that the aneuploid colon cancer cells examined here have a robust spindle checkpoint. Consistently, it now appears that mutations in known spindle checkpoint genes are extremely rare in human tumours (Cahill *et al.*, 1998, 1999a; Imai *et al.*, 1999; Yamaguchi *et al.*, 1999; Myrie *et al.*, 2000; Sato *et al.*, 2000). The reason for the apparent lack of such mutations may be that they are lethal. Although spindle checkpoint genes are non-essential in yeast
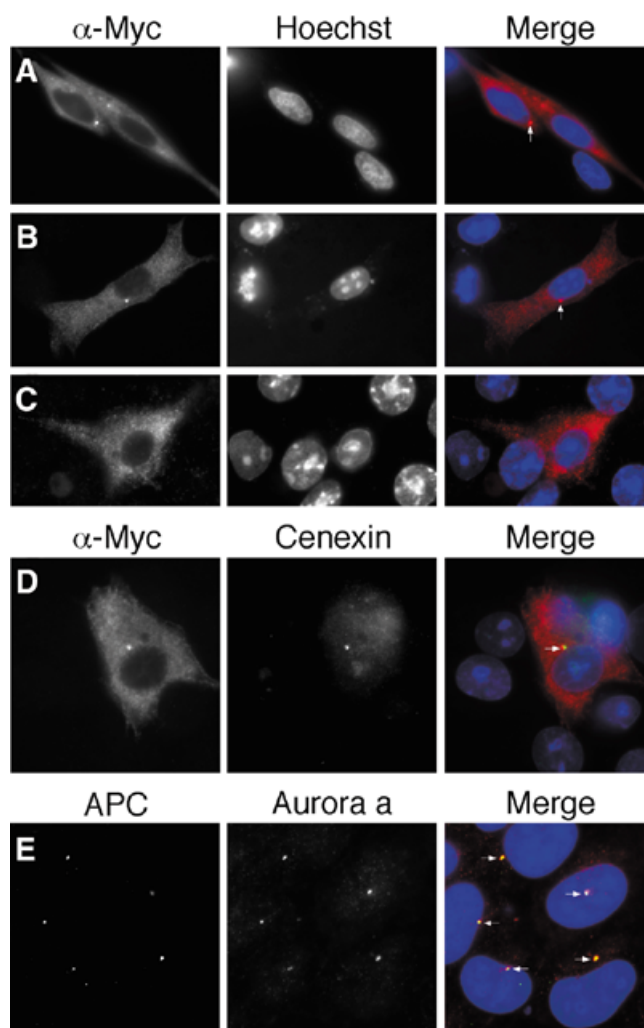
(reviewed in Taylor, 1999) mutations in *MAD2* and *BUB3* result in embryonic lethality in mice (Dobles *et al.*, 2000; Kalitsis *et al.*, 2000). Furthermore, disrupting Bub1 and Mad2 function accelerates mitotic exit in human cells suggesting that spindle checkpoint function may be required to complete normal somatic cell divisions (Taylor and McKeon, 1997; Gorbsky *et al.*, 1998; Michel *et al.*, 2001).

Thus, it appears unlikely that mutations in spindle checkpoint genes underlie the aneuploidies associated with human tumours. In contrast, the APC gene is frequently mutated in aneuploid colon cancer cells. APC is integral to the Wnt signalling pathway where it downregulates β-catenin dependent transcription of genes which promote cellular proliferation (reviewed in Fearnhead *et al.*, 2001; van Es *et al.*, 2001) and

significantly, mutations in either APC or β-catenin are frequently associated with colon cancer. Although not all colon cancer cells have mutations in APC or β-catenin (Salahshor *et al.*, 1999), there is evidence that β-catenin mutations are often an alternative to APC mutations (Lamlum *et al.*, 2000). Furthermore, whereas MIN cells generally have wild-type APC (Salahshor *et al.*, 1999), CIN cells frequently have truncating mutations which result in expression of N-terminal fragments (Cahill *et al.*, 1999b; Rowan *et al.*, 2000). Taken together these observations provide interesting but circumstantial evidence that mutations in APC may play a causal role in CIN.

Significantly, we show here that expression of an N-terminal APC mutant in a MIN line reduces the maximum mitotic index achieved following spindle damage to a level typical of the CIN lines, providing direct evidence that APC dysfunction may interfere with accurate chromosome segregation. Furthermore, we show that N-APC mutants localize to the centrosome, a key component of the chromosome segregation machinery. These observations suggest that, perhaps as a consequence of an inability to interact with microtubules and/or EB1, N-terminal APC mutants accumulate at the centrosome thus compromising but not abrogating the mechanisms involved in ensuring accurate chromosome segregation. While some segregation errors are likely to be lethal, others may provide a growth advantage. In this model, the original truncating mutation in APC acts as a 'double whammy', destabilizing the genome and setting the stage for dysregulated cellular proliferation upon loss of the second APC allele. In contrast, mutations in spindle checkpoint genes may have such disastrous effects on mitosis that the daughter cells are not viable, explaining why even highly aneuploid colon cancer cells have a robust spindle checkpoint.

METHODS

Cell Culture. HCT-116, DLD-1, HT-29, SW480, SW837, LoVo and BHK cell lines were obtained from the ATCC. TA-HeLa cells were as described (Taylor and McKeon, 1997). All lines were cultured in DMEM plus 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, all from Gibco, at 37°C in a humidified 5% CO_2 incubator. Nocodazole (Sigma) was dissolved in DMSO at 5 mg/ml then used at a final concentration of 0.2 μg/ml. To determine checkpoint status, cells were seeded into plastic dishes and 24 h later nocodazole added. At the time points indicated, loosely attached mitotic cells were isolated and adherent interphase cells harvested by trypsinization. The mitotic and interphase cells were pooled, washed with PBS then processed to determine mitotic index (see below).

Immunofluorescence. For immunofluorescence, cells were centrifuged onto microscope slides then fixed in 3% formaldehyde in PBS (Figure 2B) or grown on coverslips then fixed in methanol (Figures 3 and 4B). Cells were washed in PBS plus 0.1% Triton X-100 (PBST), blocked in 5% non-fat dried milk then incubated at room temperature for 30 min with primary antibodies diluted in PBST. Primary antibodies used were: rabbit anti-phospho-histone H3 (1:200; Upstate Biotech); rabbit anti-cenexin (1:1000; a gift from Keith Gull); rabbit anti-aurora a (1:2000; a gift from Nick Keen); rabbit anti-APC (1:1000; a gift from Inke Nathke); 9E10 mouse anti-Myc tag (1:500); mouse

anti-N-APC (1:20; Oncogene). Following washes with PBST, cells were then stained with Cy2 or Cy3 conjugated donkey anti-rabbit and/or anti-mouse secondary antibodies (Jackson Immunoresearch) diluted 1:500 in PBST for 30 min at room temperature. Following washes, cells were stained with Hoechst at 1 μg/ml in PBST then mounted in 90% glycerol plus 20 mM Tris–HCl pH 8.0. Fluorescence microscopy was performed using a Leica DMR equipped with epifluorescence and a Photometrics CCD camera driven by IP Lab software. Greyscale images were pseudocoloured using PhotoShop 5.0 (Adobe) before printing. To determine the mitotic index at each time point, at least 1000 cells were counted and the state of chromosome condensation used to score the cell as either mitotic or interphase.

Flow cytometry. For FACS analysis, cells were fixed in ethanol, rehydrated in PBS then incubated on ice for 30 min with an FSE conjugated MPM-2 antibody (Upstate Biotech) diluted 1:65 in PBS. Following washing with PBS, the cells were treated with 40 μg/ml propidium iodide and 10 μg/ml RNase for 30 min at room temperature then analysed on a FACSCAN (Becton Dickinson).

Expression of N-APC. N-terminal fragments of APC encoding amino acids 2–750 and 2–1314 were amplified with Pfu polymerase (Stratagene), cloned into pcDNA-3 Myc-tagged expression vectors (Taylor and McKeon, 1997) and sequenced. Plasmids were purified using ion exchange chromatography (Qiagen) then transfected into BHK and TA-HeLa cells using the ProTransfection calcium phosphate kit (Promega) or into HCT-116 cells by electroporation. To generate stable lines, HCT-116 cells were selected in 800 μg/ml geneticin, single colonies were picked and expanded.

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A. Tighe et al.

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