Formation of extra centrosomal structures is dependent on β-catenin

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Summary

β-Catenin has important roles in cell–cell adhesion and in the regulation of gene transcription. Mutations that stabilize β-catenin are common in cancer, but it remains unclear how these mutations contribute to cancer progression. β-Catenin is also a centrosomal component involved in centrosome separation. Centrosomes nucleate interphase microtubules and the bipolar mitotic spindle in normal cells, but their organization and function in human cancers are abnormal. Here, we show that expression of stabilized mutant β-catenin, which mimics mutations found in cancer, results in extra non-microtubule nucleating structures that contain a subset of centrosome proteins including γ-tubulin and centrin, but not polo-like kinase 4 (Plk4), SAS-6 or pericentrin. A transcriptionally inactive form of β-catenin also gives rise to abnormal structures of centrosome proteins. HCT116 human colon cancer cell lines, from which the mutant β-catenin allele has been deleted, have reduced numbers of cells with abnormal centrosome structures and S-phase-arrested, amplified centrosomes. RNAi-mediated depletion of β-catenin from centrosomes inhibits S-phase-arrested amplification of centrosomes. These results indicate that β-catenin is required for centrosome amplification, and mutations in β-catenin might contribute to the formation of abnormal centrosomes observed in cancers.

Key words: β-Catenin, Centrosomes, γ-Tubulin

Introduction

Mutations in β-catenin are found in many cancers and are an early event in colon cancer (Polakis, 1999; Sparks et al., 1998). β-Catenin has essential roles in cell–cell adhesion, and in the Wnt pathway in which a β-catenin–T-cell factor (TCF) complex transcriptionally regulates gene expression (Nelson and Nusse, 2004). β-Catenin levels are tightly controlled by a destruction complex of Adenomatous polyposis coli (APC) and Axin, which facilitates the phosphorylation of β-catenin by casein kinase I (CKI) and glycogen synthase kinase 3β (GSK3β), leading to β-catenin ubiquitylation and degradation by the proteasome (Easwaran et al., 1999; Hart et al., 1999; Kishida et al., 1998; Kitagawa et al., 1999; Nakamura et al., 1998; Rubinfeld et al., 1996). Mutations in the CKI and GSK3β phosphorylation sites of β-catenin (Fig. 1A) are found in human tumors and cancer cell lines and result in stabilization of β-catenin (Polakis, 1999). It is thought that the primary consequence of β-catenin accumulation is unregulated β-catenin-mediated transcription of genes controlling cell proliferation (Morin et al., 1997; Polakis, 1999). However, expression of stabilized mutant β-catenin with increased transcriptional activity in a non-transformed cell line resulted in only a very low, albeit significant, percentage of transformed cells, as measured by anchorage-independent growth in soft agar (Barth et al., 1999). Thus, additional defects caused by stabilization and accumulation of β-catenin might be important in cell transformation.

A newly identified target for β-catenin function is the centrosome. We reported recently that β-catenin is a component of centrosomes and interacts with centrosomal proteins to regulate centrosome separation (Bahmanyar et al., 2008). The number, organization and function of centrosomes is important in chromosome segregation. Human tumors are characterized by numerical and structural chromosomal aberrations that are probably due to abnormal chromosome segregation (Lengauer et al., 1997; Lengauer et al., 1998). Increased centrosome numbers and centrosome defects have been observed in aggressive and low-grade tumors, and precancerous lesions (D'Assoro et al., 2002; Nigg, 2002; Pihan et al., 1998; Pihan et al., 2001; Pihan et al., 2003; Salisbury et al., 2004), and chromosomal instability is common in colon cancers with stabilized β-catenin (Hadjihannas and Behrens, 2006; Hadjihannas et al., 2006).

Centrosomes comprise a pair of centrioles that must be duplicated once per cell cycle (Stearns, 2001; Tsou and Stearns, 2006), so that at the onset of mitosis, a cell has two centrosomes that separate to form the poles of the bipolar mitotic spindle (Doxsey et al., 2005; Stearns, 2001; Tsou and Stearns, 2006). Genetic studies in *C. elegans* and later work in vertebrates showed that SAS-4 (CPAP/CENPJ), SAS-6 and ZYG-1 (Plk4/SAK) are core proteins required for the templated formation of centrioles (Bettencourt-Dias et al., 2005; Dammermann et al., 2008; Habedanck et al., 2005; Kirkham et al., 2003; Leidel et al., 2005; Leidel and Gonczy, 2003). Extra centrosomes can also form de novo, indicating that templated over-duplication is not the only mechanism for formation of extra centrosomes or centrosome-like structures (Khodjakov et al., 2002; La Terra et al., 2005). Here, we show that expression of stabilized β-catenin, which mimics mutations found in cancer, directly induces formation of abnormal centrosome structures; in addition, oncogenic β-catenin contributes to the formation of abnormal centrosome structures found in cancer cells.

Results

β-Catenin stabilization induces extra γ-tubulin-labeled puncta

We examined MDCK cells (a non-transformed cell line with normal centrosomes) that stably express β-catenin mutated in its CK1 and GSK3β phosphorylation sites (Fig. 1A; referred to as β-cat*) (Barth et al., 1999) to determine whether β-catenin stabilization affects centrosome organization. The total amount of β-catenin in the β-cat* stable lines was higher than that of wild-type β-catenin in parental cells (Fig. 1B), as shown previously (Barth et al., 1999). Asynchronous parental cells and cells expressing β-cat* were immunostained for γ-tubulin (Fig. 1C), a centrosome component, and the number of γ-tubulin puncta in the cytoplasm was determined. Very few parental MDCK cells had more than two γ-tubulin-labeled puncta (0.7±0.2% in three experiments; n>300). However, significantly more β-cat*-expressing cells had extra γ-tubulin-labeled puncta (9.9±3.1% in three experiments; n>300); switching off β-cat* expression with doxycycline for 5 days reduced the percentage of cells with extra γ-tubulin puncta from 9.9% to 3.4% (one experiment, number of cells counted 1367). Cells with extra γ-tubulin-labeled structures had mostly only one extra γ-tubulin puncta, and very few cells had more than one extra puncta (Fig. 1C; S.B., unpublished results).

To investigate the time course of formation of additional γ-tubulin puncta after β-catenin accumulation, we took advantage of transient transfections that result in an initial higher expression level of protein. As soon as 18–24 hours after transfection of GFP-β-cat* in MDCK cells, three or more γ-tubulin puncta were induced in ~12% of transfected cells (Fig. 1D). MDCK cells have a cell cycle time of 16–18 hours (Bauer et al., 1998), thus the effect of β-catenin accumulation on formation of these abnormal centrosome structures probably occurred within one cell cycle. Significantly, transient transfection and overexpression of wild-type GFP-β-catenin in MDCK cells (Fig. 1D), U-2OS (S.B., unpublished results) and Ptk2 cells (Ligon et al., 2001) also induced formation of extra γ-tubulin puncta, whereas transfection of GFP did not. Thus, increased levels of either wild-type or mutant β-catenin (β-cat*) results in the induction of extra γ-tubulin structures within one cell cycle, and hence within one centrosome duplication cycle.

Extra γ-tubulin puncta form independently of β-catenin-mediated transcription

The β-catenin armadillo repeat domain (ARM) is necessary and sufficient for localization of β-catenin to centrosomes (Bahmanyar et al., 2008). In cells that transiently express a β-catenin mutant lacking the ARM domain (ΔARM; Fig. 1D), 2.7±1.3% of cells had increased γ-tubulin puncta, which is similar to that in control cells expressing GFP (Fig. 1D).

The ARM domain contains binding sites for the transcription factor TCF (Fig. 1A), raising the possibility that induction of extra γ-tubulin puncta by β-catenin was due to increased β-catenin–TCF-mediated gene transcription. Therefore, we expressed a transcriptionally inactive form of β-catenin in which the β-catenin C-terminal domain was replaced with the transcriptional repressor engrailed (ENG) (Montross et al., 2000). Transient expression of Myc-tagged β-catenin-ENG induced three or more γ-tubulin puncta in 21% of MDCK cells (ENG; Fig. 1D), which was similar to that induced by overexpression of β-cat* (Fig. 1D). However, unlike overexpression of β-cat*, β-catenin-ENG did not increase TCF-mediated gene transcription (Fig. 1E). We conclude that the formation of extra γ-tubulin puncta is independent of β-catenin-mediated transcription, but requires the β-catenin ARM domain, which is both necessary and sufficient for localization of β-catenin to centrosomes (Bahmanyar et al., 2008).

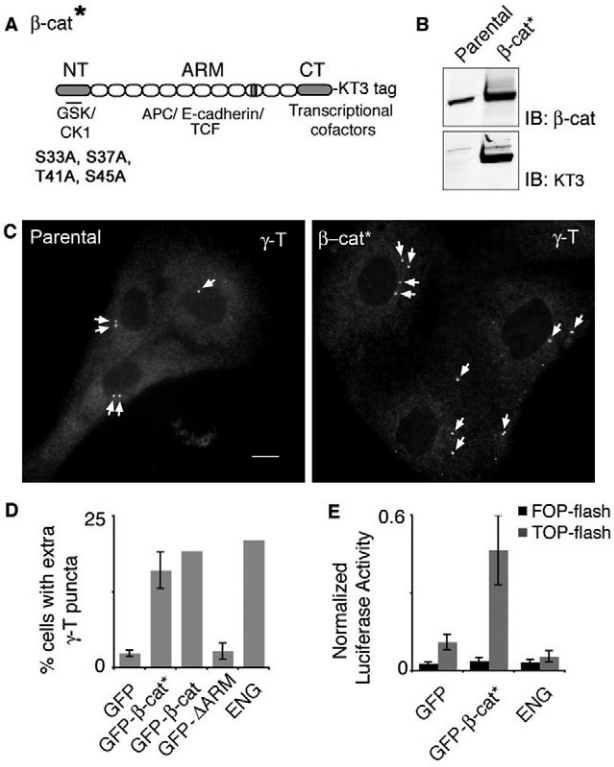

Fig. 1. Increased levels of β-catenin induce formation of extra γ-tubulin puncta. (**A**) Schematic representation of β-catenin protein. N-terminal (NT) and C-terminal (CT) domains and the 12 central armadillo repeats are indicated (ARM). Mutations in the CK1 and GSK3β phosphorylation sites to generate stabilized β-cat* were made: S33A, S37A, T41A and S45A. A stretch of unrepeated amino acids in repeat 10 (gray box) is shown. The CK1 and GSK3β phosphorylation sites (NT), Adenomatous polyposis coli (APC), E-cadherin, and T-cell factor (TCF) binding sites (ARM), transcriptional cofactor binding region (CT) and the KT3 tag used in stable cell lines are shown. (**B**) Immunoblot of whole-cell lysate from parental MDCK and β-cat*-expressing cells. (**C**) Parental and β-cat*-expressing cells immunostained for γ-tubulin (arrows). β-cat*-expressing cells have three or more γ-tubulin puncta. Scale bar: 10 μm. (**D**) Percentage of cells with three or more γ-tubulin puncta in MDCK cells transiently transfected with indicated constructs: pEGFP-C1 (GFP), GFP-S33A/S37A/T41A/S45A-β-cat* in pEGFP-C1 (GFP-b-cat*), GFP-β-catenin in pEGFP-C1, GFP-β-catenin without the ARM domain (GFP-ΔARM; Elul et al., 2003), β-catenin–engrailed chimera (ENG; Montross et al., 2000). Bars show s.e.m. of three experiments. Mean value is shown for experiments performed twice. (**E**) TCF reporter assays (TOP-Flash) and control reporter assays (FOP-Flash) were performed on lysates from MDCK cells transiently transfected with the indicated constructs. Error bars represent s.e.m.

Extra γ-tubulin puncta induced by β-cat* do not nucleate microtubules and contain a subset of centrosomal proteins

We tested whether these extra γ-tubulin puncta were functional in the nucleation of cytoplasmic microtubules (MTs) after nocodazole washout (Fig. 2A-D). At room temperature, 15 minutes after nocodazole washout, anti-α-tubulin antibody-labeled MTs (blue in

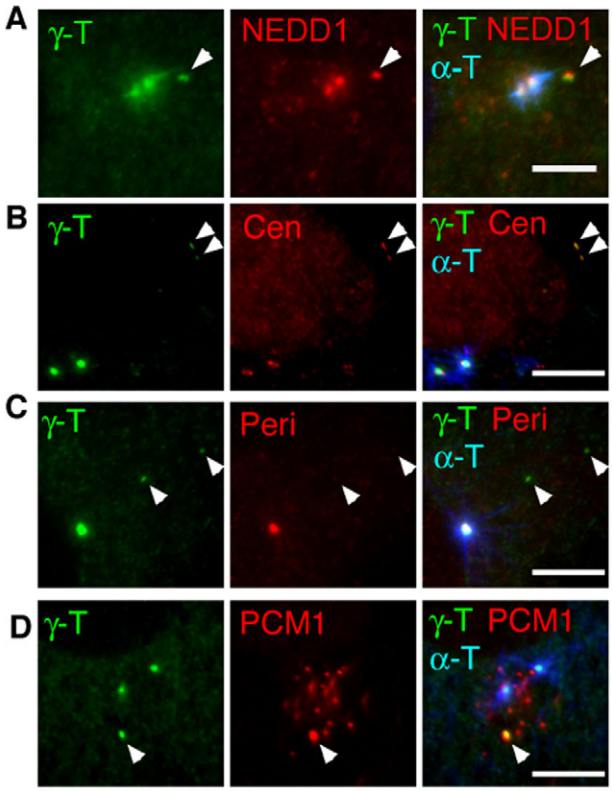

Fig. 2. Non-MT-nucleating γ-tubulin puncta contain NEDD-1, centrin and PCM-1, but not pericentrin. (**A–D**) Centrosomal areas of β-cat*-expressing cells immunostained after nocodazole washout for γ-tubulin (green in A–D) and co-immunostained for NEDD-1 (red in A), centrin (red in B), pericentrin (red in C) or PCM1 (red in D) and α-tubulin (blue in merge A–D). Arrowheads indicate non-MT-nucleating puncta. Scale bars: 5 μm.

Fig. 2A–D) had repolymerized from original centrosomes, but not from extra γ-tubulin puncta (arrowheads in Fig. 2A–D).

We noticed that immunostaining of γ-tubulin at non-MT-nucleating puncta (white arrowheads in Fig. 2A–D) was less bright than that at MT-nucleating centrosomes, raising the possibility that these puncta lack key centrosomal components. Mature centrosomes have a centriole pair containing centrin, pericentriolar material containing pericentrin, MT-nucleating γ-TuRC ring components, and centriolar satellites labeled with PCM-1 (Dammermann and Merdes, 2002; Zimmerman et al., 1999). PCM-1 has a punctate distribution in the vicinity of centrosomes and is thought to be involved in delivery of intermediate centrosomal protein complexes (Dammermann and Merdes, 2002). The non-MT-nucleating puncta contained the outer γ-TuRC ring component NEDD-1/GCP-WD (Haren et al., 2006; Luders et al., 2006) and inner ring component γ-tubulin (Aldaz et al., 2005) (Fig. 2A), centrin (Fig. 2B) and PCM-1 (Fig. 2D). By contrast, pericentrin was absent from these non-MT-nucleating puncta (Fig. 2C). Pericentrin might be required for the proper scaffolding of the γ-TuRC proteins (Gomez-Ferreria et al., 2007; Zhu et al., 2008) and, therefore, in its absence, these structures might not be able to nucleate cytoplasmic MTs.

We observed a small increase in mitotic cells with multipolar spindles (supplementary material Fig. S1) in the cells expressing β-cat* (1.5±0.01% compared with 0.1±0.03% in parental MDCK cells; with two experiments, $n \geq 600$ mitotic cells), indicating that some of the abnormal γ-tubulin spots mature into functional microtubule-nucleating structures. Thus, expression of β-cat* in MDCK cells results mostly in extra centrosome structures that are not functional in nucleating cytoplasmic MTs and contain only a subset of centrosomal proteins; a few of these structures might contribute to abnormal mitoses.

β-Cat*-expressing cells have increased PCM-1 and centrin-containing satellites around centrosomes

Fifty-one percent of cells expressing β-cat* had more than 10 extra centrin-labeled puncta close to centrosomes (Fig. 3). In contrast to increased centrin puncta around bona fide centrosomes, extra γ-tubulin-labeled puncta were present in only 9.9% of cells, and most of these had only one or two extra γ-tubulin and centrin co-labeled puncta (S.B., unpublished results; see also Figs 1 and 2).

Since most of these centrin-containing puncta did not contain γ-tubulin (Fig. 3A) and colocalized with the centriolar satellite protein PCM-1 (Fig. 3B,C), they represent a second subtype of centrosomal protein structure that is specifically enriched in cells expressing β-cat*. We defined them as centrin–PCM-1 satellites and quantified them by measuring the number of centrin–PCM-1 satellites in a 400 μm^2 area around centrosomes that were labeled with RFP-pericentrin (Fig. 3B,C). Cells expressing β-cat* had significantly more centrin–PCM-1 satellites close to centrosomes compared with parental cells (Fig. 3B,C; parental, n=44; β-cat*, n=39; ***P=0.0004). When β-cat* expression was suppressed, the number of satellites containing centrin–PCM-1 was reduced (Fig. 3B,C; β-cat* dox, n=36; *P=0.02).

β-Cat* does not effect centrin turnover at the original centrosome, but increases the mobile pool of γ-tubulin

We analyzed the turnover of centrin and γ-tubulin at original centrosomes in cells expressing β-cat*. MDCK cells stably expressing β-cat* and parental cells were co-transfected with the centrosome-targeted C-terminal domain of pericentrin fused to RFP (Gillingham and Munro, 2000) to label the original centrosomes, and either GFP-centrin-2 (Fig. 4A–D) or GFP-γ-tubulin (Fig. 4E–G). Fluorescence recovery after photobleaching (FRAP) of GFP-centrin-2 showed no difference in turnover ($t_{1/2}$: 7±0.9 minutes for parental and 9±2.2 minutes for β-cat*) or mobile fraction (17±0.7% for parental and 15±1.4% for β-cat*) at the original centrosomes in cells expressing stabilized β-cat* compared with parental MDCK cell (Fig. 4A–D; n=8 for β-cat* and n=6 for parental cells). Maximum recovery of GFP-centrin-2 in MDCK cells (17±0.7%) was lower than that of centrin-1-GFP in CHO cells [~ 30% (Prosser et al., 2009)], but the half-lives were similar (7±0.9 minutes vs. >5 minutes).

There was no significant difference in the turnover of GFP-γ-tubulin at original centrosomes in cells expressing β-cat* (Fig. 4G; $t_{1/2}$=2.0±0.7 minutes for parental; 2.2±0.4 minutes for β-cat* and 1.9±0.5 minutes for β-cat* + dox). However, the mobile fraction of GFP-γ-tubulin was significantly increased from 20±1% maximum recovery in parental cells to 53±2% maximum recovery in cells expressing β-cat* (Fig. 4F,G; n=9 for β-cat* and n=7 for parental cells). This effect could be rescued by doxycycline-induced suppression of β-cat* (Fig. 4F,G; n=5 for β-cat* + dox; maximal recovery 20±1%, measured for the first 15 minutes only). Maximum recovery of γ-tubulin at interphase centrosomes in MDCK cells expressing β-cat* was similar to that for γ-tubulin at an interphase centrosome in a Ptk cells (Khodjakov and Rieder, 1999). However, parental MDCK cells have a much lower mobile pool of γ-tubulin at interphase centrosomes, indicating that there are differences between cell lines.

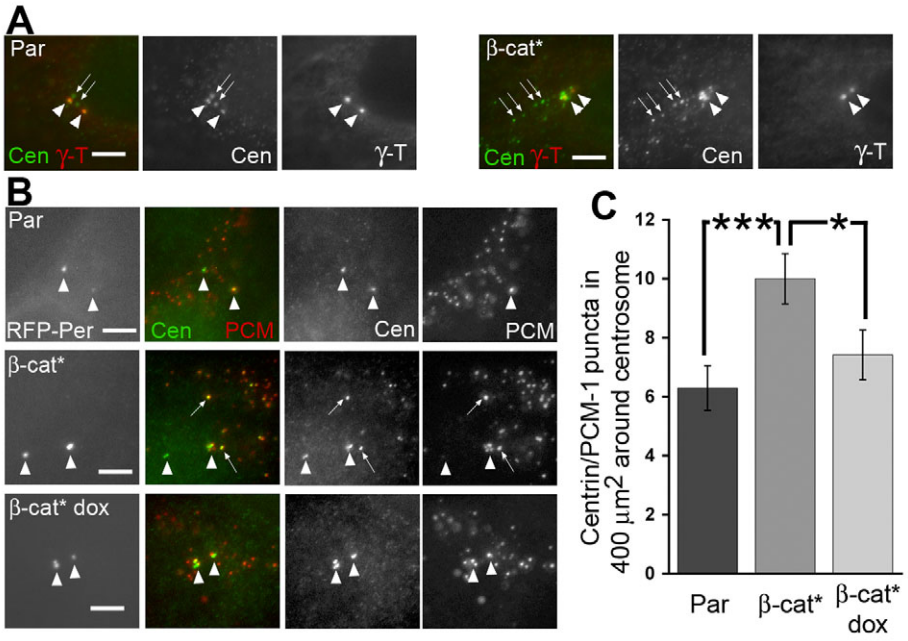

Fig. 3. β-Cat* induces accumulation of satellites containing PCM-1 and centrin around centrosomes. (**A**) 400 μm^2 centrosome areas of parental MDCK cells (Par) or MDCK cells expressing β-cat* magnified to show centrin puncta (some are indicated with arrows) close to centrosomes (arrowheads) stained with γ-tubulin and centrin. Each panel shows, from left to right, the merged image, the single image for centrin and the single image for γ-tubulin. Additional centrin-containing puncta (arrows) around centrosomes (arrowheads) in cells expressing stabilized β-cat* do not colocalize with γ-tubulin. (**B**) MDCK cells were transfected with RFP-pericentrin as a marker for centrosomes and co-immunostained for centrin and PCM-1. Magnified 400 μm^2 centrosome area in parental cell (Par in top row), in a cell expressing stabilized β-cat* (β-cat* in middle row) or in a cell in which expression of β-cat* was turned off with dox (β-cat* dox in bottom row) each expressing RFP-pericentrin (first column) and co-immunostained for centrin and PCM-1 (second column, centrin and PCM-1 merged images; third column, centrin; fourth column, PCM-1). (**C**) Mean number (± s.e.m.) of centrin and PCM-1 puncta in 400 μm^2 around centrosomes labeled with RFP-pericentrin (Parental, n=44; β-cat*, n=39; β-cat* dox, n=36). ***P=0.0004; *P=0.02 in two-tailed Mann-Whitney test. Scale bars: 5 μm.

In summary, there are three distinct effects of β-cat* on centrosomal proteins in MDCK cells: (1) increased mobile pool of γ-tubulin at original centrosomes; (2) increased numbers of centrin–PCM-1 satellites; and (3) non-MT-nucleating, abnormal γ-tubulin structures that do not have pericentrin, but contain PCM-1, centrin and NEDD-1/GCP-WD.

Removal of β-cat* from HCT116 cells reduces abnormal γ-tubulin structures, and amplification of centrioles in S-phase-arrested cells

We tested the role of mutant β-catenin in the formation of extra centrosomal structures in HCT116 cells which have one wild-type (wt) allele of β-catenin and one mutant allele with a deletion of the S45 codon (termed Par β*/wt) (Morin et al., 1997), and cell lines derived from these parental cells in which either the wt allele (termed 18 β*/– and 68 β*/–) or the mutant allele (termed 85 –/wt and 92 –/wt) was deleted by somatic cell gene targeting (Kim et al., 2002). Since S45 is the CK1 priming site required for subsequent GSK3 phosphorylation of the other sites (Liu et al., 2002), this deletion abolishes both CK1 and GSK3β phosphorylation of β-catenin similarly to the quadruple S to A mutations (β-cat*) that we expressed in MDCK cells. Significantly, non-MT-nucleating, abnormal γ-tubulin puncta that have no pericentrin have been described in parental HCT116 cells and correlated with polo-like kinase (Plk) 4 levels (Kuriyama et al., 2009), but the role of mutant β-catenin was not tested.

All five HCT116 cell lines had a low percentage of abnormal γ-tubulin puncta that did not contain pericentrin (Fig. 5A, top panel, 0 D HU). The number of cells with abnormal γ-tubulin puncta was significantly lower in cell lines that had only the wild-type β-catenin allele (Fig. 5B, 0 days HU, 85 –/wt, 92 –/wt; **P<0.009 compared with parental cells). Abnormal γ-tubulin puncta were elongated, and the number of cells containing these structures increased significantly when cells were arrested in S-phase for 3 days with hydroxyurea (Fig. 5A, bottom panel, 3 D HU). However, even after 3 days in HU, the number of cells expressing only the wild-type allele of β-catenin that had abnormal γ-tubulin puncta was significantly lower than in the parental cell line (Fig. 5B, 3 days HU, 85 –/wt, 92 –/wt; **P<0.009). These data show that the presence of mutant β-catenin significantly increased the formation of extra γ-tubulin puncta in asynchronous HCT116 cells, and during S-phase arrest, which increased the length of abnormal γ-tubulin puncta. Significantly, abnormal γ-tubulin puncta were also elongated in S-phase-arrested MDCK cells expressing β-cat* (supplementary material Fig. S2).

To distinguish between abnormal γ-tubulin structures and amplified centrioles, we used the centriole marker SAS-6 (Fig. 6A). There was a small, but significant decrease in the percentage of cells with amplified SAS-6-positive centrioles in HCT116 lines, which have only one wild-type allele of β-catenin, compared with parental HCT116 cells, with a wild-type and mutant allele, and HCT116 lines, with only the mutant allele (Fig. 6B).

β-Cat* does not affect Plk4 levels at original centrosomes in HCT116 cells and localizes to only some of the extra γ-tubulin structures

In order to analyze whether mutant β-catenin expression affected Plk4 localization to centrosomes, we quantified Plk4 fluorescence levels in the five HCT116 cell lines (Fig. 7). Plk4 and γ-tubulin

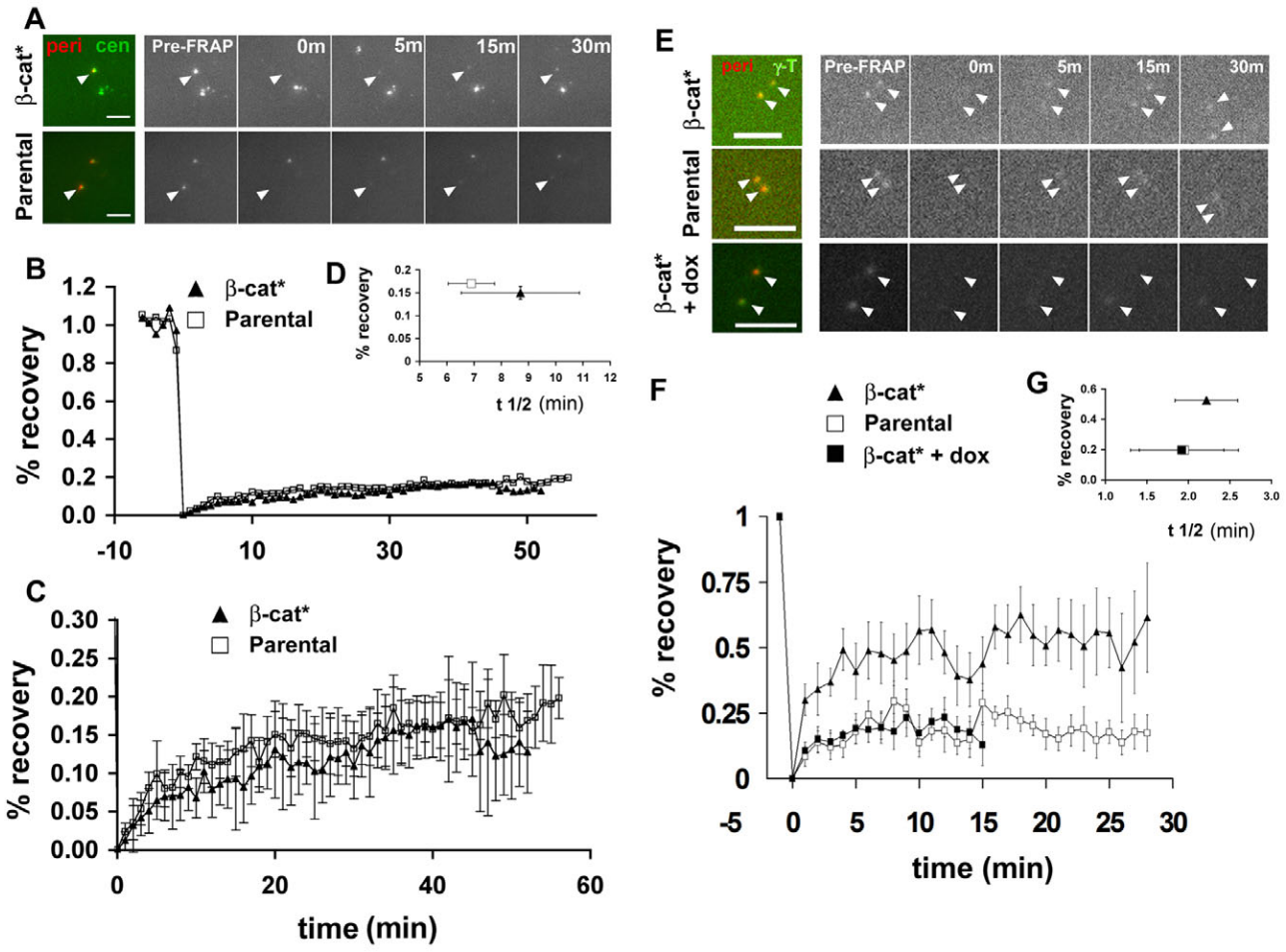

Fig. 4. β-Cat* does not affect centrin turnover, but increases the mobile fraction of γ-tubulin at centrosomes. (**A**) Representative montages of GFP-centrin FRAP experiments in β-cat* and parental MDCK cell lines. These cell lines were transiently transfected with GFP-centrin and RFP-pericentrin. Left panel is a merged image showing colocalization at centrosomes. Right panel shows GFP-centrin fluorescence before (Pre-FRAP), immediately after (0m) and 5, 15 and 30 minutes after photobleaching. Scale bars: 5 μm. (**B**) Quantification of centrin FRAP experiments. GFP-centrin was measured centered on the pericentrin marker (see Materials and Methods) and values were normalized. (**C**) Same quantification as shown in B, focusing on values in the lower part of the *y*-axis. Error bars indicate s.e.m. (n=6 for parental, n=8 for β-cat*). (**D**) Data were fitted to an exponential; maximal recovery is 17±0.7% for parental and 15±1.4% for β-cat*. The half-life ($t_{1/2}$) is 7±0.9 minutes for parental and 9±2.2 minutes for β-cat*. (**E**) Representative montages of GFP-γ-tubulin FRAP experiments in β-cat* and parental MDCK cell lines, and β-cat* MDCK cells in which expression of β-cat* was repressed with doxycycline (+dox). These cell lines were transiently transfected with GFP-γ-tubulin and RFP-pericentrin. Left panel is a merge showing colocalization at centrosomes. Right panel shows GFP-γ-tubulin fluorescence before (Pre-FRAP), immediately after (0m) and 5, 15 and 30 minutes after photobleaching. Scale bars: 10 μm. (**F**) Quantification of γ-tubulin FRAP experiments. GFP-γ-tubulin was measured centered on the pericentrin marker and values were normalized. Error bars indicate s.e.m. (n=7 for parental, n=9 for β-cat.*, n=5 for β-cat.* + dox). (**G**) Data were fitted to an exponential and values for maximal recovery and half-life were calculated (maximal recovery, 20±1% in parental cells and β-cat* + dox; 53±2% in cells expressing stabilized β-cat*; $t_{1/2}$=2.0±0.7 minutes for parental; 2.2±0.4 minutes for β-cat* and 1.9±0.5 minutes for β-cat* + dox). For β-cat* + dox, FRAP was measured for the first 15 minutes only.

colocalized at centrosomes (black arrowheads in Fig. 7A), but elongated γ-tubulin structures did not stain with anti-Plk4 antibodies (white arrowheads in Fig. 7A). Plk4 levels were increased at centrosomes in S-phase-arrested HCT116 cells compared with untreated cells, but there was no significant correlation between the level of Plk4 at centrosomes and different combinations of wt and mutant β-catenin alleles in HCT116 cell lines (Fig. 7B).

Antisera to the C- or N-terminus of β-catenin showed little or no localization of β-catenin to the elongated γ-tubulin puncta in S-phase-arrested β-cat*-expressing MDCK and HCT116 parental cells, although they consistently immunostained centrosomes (shown for the C-terminal β-catenin antiserum in supplementary material Fig. S3A,B). Transiently expressed GFP-β-cat* localized to pericentrin-positive centrosomes, and occasionally to the elongated extra γ-tubulin structures in S-phase-arrested cells (supplementary material Fig. S3C, MDCK cell with extra γ-tubulin structure that does not have GFP-β-cat*; supplementary material Fig. S3D, HCT116 cell with extra γ-tubulin structure that has GFP-β-cat*).

Thus, the presence of extra γ-tubulin structures does not correlate with an increase in Plk4 levels at centrosomes and neither Plk4 nor β-catenin itself localizes strongly at these structures.

β-Cat* does not change total levels of γ-tubulin and centrin in MDCK or HCT116 cells

We examined whether β-cat* expression affected the total levels of centrosomal proteins in cells. We did not detect significant differences in the levels of PCM-1, centrin or γ-tubulin between

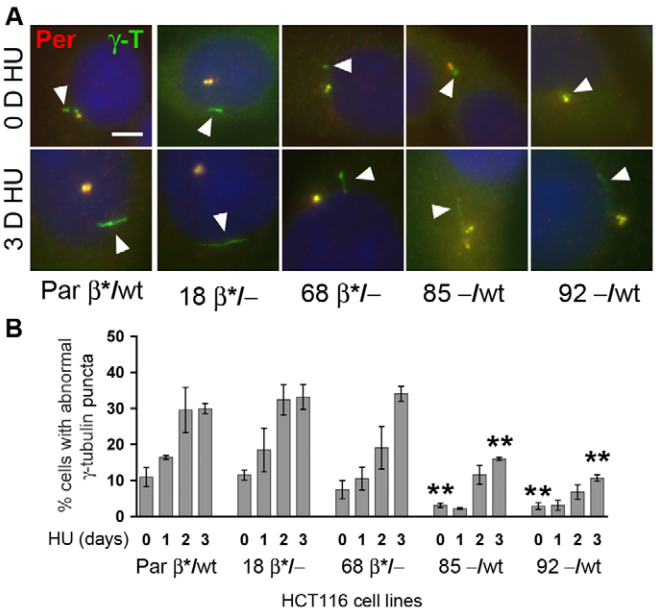

Fig. 5. Removal of the mutant β-cat* allele from colon cancer cell line HCT116 decreases the number of cells forming abnormal γ-tubulin puncta. (**A**) Parental (Par) HCT116 cells that have a mutant (β*) β-catenin allele and a wild-type (wt) β-catenin allele (Par β*/wt) or independent clones of HCT116 cells in which either the wt allele has been removed (18 β*/–; 68 β*/–) or the mutant allele has been removed (85 –/wt; 92 –/wt) have been left untreated (0 D HU) or S-phase-arrested for 3 days with hydroxyurea (3 D HU). Images show magnified 400 μm^2 areas around centrosomes co-stained for pericentrin (red) and γ-tubulin (green). Parental and all clones show extra γ-tubulin-containing puncta near centrosomes that do not contain pericentrin (arrowheads). In S-phase-arrested cells (3 D HU) these extra γ-tubulin puncta are elongated (arrowheads in bottom panel). Scale bar: 5 μm. (**B**) Quantification of cells with abnormal γ-tubulin puncta. Bars represent s.e.m. of five experiments for 0 and 3 days of hydroxyurea (HU) treatment and s.e.m. of three independent experiments for 1 and 2 days of hydroxyurea (HU) treatment. $^{**}P<0.009$ for 0 and 3 days HU compared with Par β*/wt.

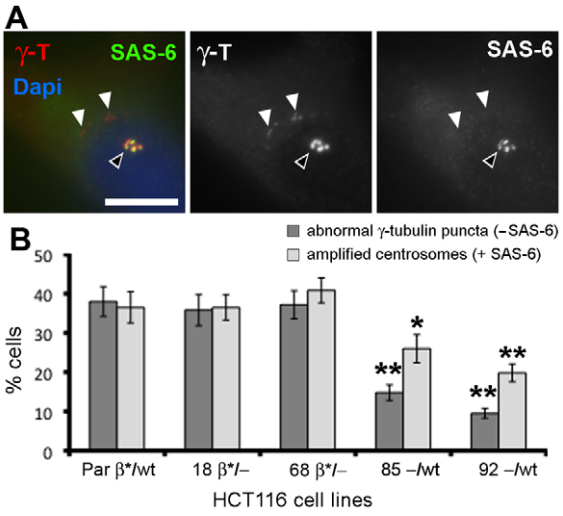

Fig. 6. The centriole marker SAS-6 does not localize to abnormal γ-tubulin puncta in S-phase-arrested HCT116 cells and removal of the mutant β-cat* allele inhibits centriole amplification. (**A**) HCT116 line 18 with one mutant (β*) β-catenin allele (18 β*/–) was S-phase-arrested for 3 days with hydroxyurea. Images show the magnified area around amplified centrosomes co-stained for γ-tubulin (red) and SAS-6 (green). SAS-6 localizes to amplified centrosomes (black arrowheads) but not to extra γ-tubulin puncta (white arrowheads). Scale bar: 10 μm. (**B**) HCT116 cell lines S-phase-arrested for 3 days with hydroxyurea were quantified for the number of cells with abnormal γ-tubulin puncta (dark bars) that are SAS-6-negative or number of cells with amplified centrosomes (light bars) that have more than four centrioles labeled with SAS-6. Bars represent s.e.m. of five to six experiments; $^{*}P<0.05$ and $^{**}P\leq0.008$ compared with parental cells in a one-tailed Mann-Whitney test. Cells with only one wild-type allele of β-catenin have significantly fewer abnormal γ-tubulin puncta and amplified centrosomes.

parental MDCK cells (Par), cells expressing β-cat* (–DOX), or cells in which β-cat* expression was suppressed for 5 days (+DOX) (Fig. 8A). MDCK cell line 4 was used for analysis of extra γ-tubulin puncta and PCM-1–centrin satellites (Figs 1 and 3).

HCT116 cell lines 85 and 92 with only one wild-type allele of β-catenin had 70% and 60%, respectively, of the total β-catenin protein levels observed in parental HCT116 cells that have both wild-type and mutant β-catenin alleles (Fig. 8B). Total levels of centrin and γ-tubulin were not significantly different in the HCT116 cell lines, although a 20–30% decrease of PCM-1 was detected in cell lines 85 and 92 with only one wild-type β-catenin allele compared with parental HCT116 cells (Fig. 8B). After 3 days of S-phase arrest with hydroxyurea (+), the level of β-catenin was slightly increased, whereas the level of PCM-1 decreased in all HCT116 cell lines (Fig. 8B).

Centrosomal β-catenin is required for centrosome amplification in S-phase-arrested U-2 OS cells

We used siRNA depletion of β-catenin as an independent assay to test whether β-catenin is required for centrosome amplification during S-phase arrest. Human osteosarcoma U-2OS cells are commonly used to analyze the role of centrosomal proteins in HU-induced centrosome amplification (Fisk et al., 2003; Leidel et al., 2005; Warnke et al., 2004).

Using SAS-6 as a marker, we defined the percentage of S-phase-arrested U-2OS cells with abnormal γ-tubulin puncta (SAS-6-negative) or amplifying γ-tubulin centrosomes (SAS-6-positive) (Fig. 9A,B); note that SAS-6-positive, amplifying centrosomes nucleated microtubules in nocodazole-washout experiments (A.B., unpublished result). Unlike HCT116 cells (see Fig. 6), S-phase-arrested U-2OS cells form very few abnormal SAS-6-negative γ-tubulin puncta (Fig. 9B), and, therefore, are suited for analysis of the role of β-catenin in amplifying functional SAS-6-positive centrosomes.

We depleted β-catenin protein levels by siRNA in U-2OS cells and then treated these cells with HU for 96 hours. RNAi resulted in a 75% reduction in the total level of β-catenin (Fig. 9C), and a 73% decrease in β-catenin fluorescence intensity at centrosomes (arrows in Fig. 9D,E).

In a whole-population analysis, in which the degree of β-catenin depletion in each cell was not measured, HU treatment of control cells resulted in 64±3.7% of cells with three or more centrosomes (n=368). By contrast, 37±2.9% of siRNA-treated cells had three or more centrosomes (n=321; total population, Fig. 9F). BrDU incorporation in cells treated with control siRNA or siRNA to knock down β-catenin showed that 69% (n=95) and 61% (n=88) of cells, respectively, were arrested in S-phase, demonstrating that HU had similar affects on S-phase arrest in cells treated with control and *β-catenin* siRNA.

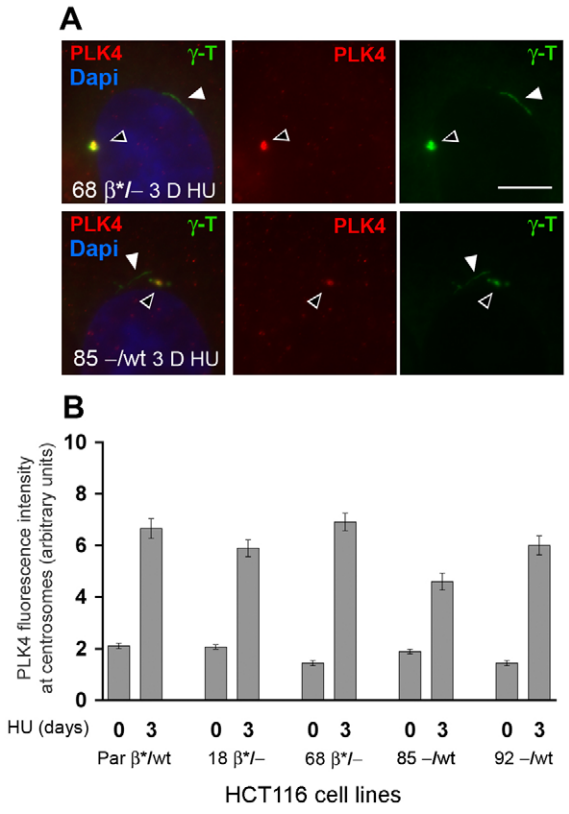

Fig. 7. Mutant β-cat* does not affect Plk4 levels at centrosomes in colon cancer HCT116 cells. (**A**) Plk4 (red), DAPI (blue) and γ-tubulin (green) co-staining of HCT116 cell lines 68 β*/– and 85 –/wt treated for 3 days with hydroxyurea (3 D HU). Plk4 (red) colocalizes with γ-tubulin at centrosomes (black arrowheads), but not at abnormal elongated γ-tubulin structures (white arrowheads). Scale bar: 10 µm. (**B**) Quantification of Plk4 fluorescence intensity at centrosome areas in untreated HCT116 cell lines (0 days HU) or in S-phase-arrested HCT116 cell lines (3 days HU). Bars represent s.e.m. of more than 50 centrosome areas measured for each cell line. Plk4 levels are increased in S-phase-arrested compared with untreated cells because of centrosome amplification. Although there are some variations in Plk4 levels at centrosome areas between different cell lines, cell lines with mutant β-cat* do not show a consistent increase or decrease in Plk4 levels compared with cell lines that have only wt β-catenin.

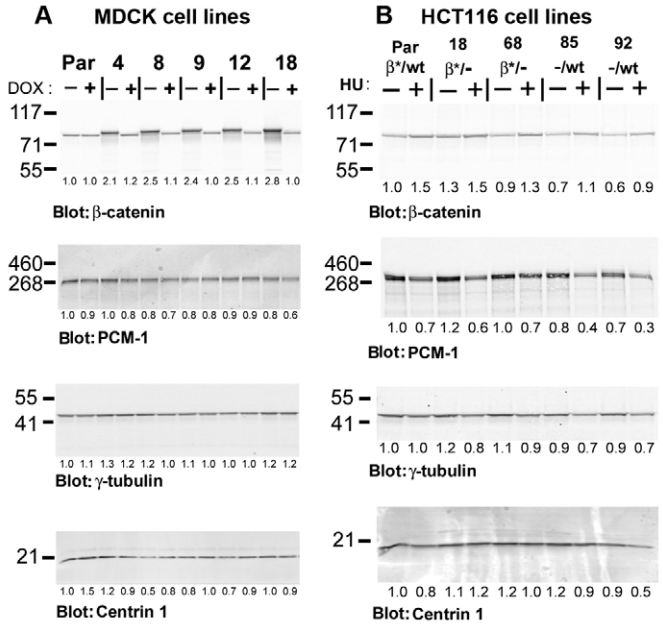

Fig. 8. Expression of mutant β-cat* does not significantly change total cellular levels of PCM-1, γ-tubulin or centrin. (**A**) SDS extracts from MDCK cell lines expressing mutant S33A/S37A/T41A/S45A β-cat* (4, 8, 9 and 12, –DOX) or not expressing mutant β-cat* (Par ±DOX and 4, 8, 9 and 12, +DOX) were immunoblotted for total β-catenin, PCM-1, γ-tubulin or centrin 1. (**B**) SDS extracts from asynchronous (–HU) or 3 days S-phase-arrested (+HU) HCT116 cell lines containing (Par, 18, 68) or not containing (85, 92) the mutant ΔS45 β-catenin allele were immunoblotted for total β-catenin, PCM-1, γ-tubulin or centrin 1. Numbers below blots represent the percentage of signal intensity for each protein compared with signal intensity for parental lines (Par, –DOX for MDCK and Par for HCT116) in each blot.

We directly measured the level of β-catenin and the number of centrosomes in individual cells, because siRNA treatment does not deplete protein equally in all cells. Only 14% (n=42) of cells depleted of β-catenin had extra centrosomes (β-catenin-depleted population, Fig. 9F). The average fluorescence intensity of β-catenin at centrosomes in cells treated with control siRNA was 13.8 a.u. Significantly, in cultures treated with *β-catenin* siRNA, cells with two or fewer centrosomes had an average β-catenin fluorescence intensity of 3.0 a.u. (Fig. 9G, grey diamonds), whereas cells with three or more extra centrosomes had an average β-catenin fluorescence intensity of 7.7 a.u. (Fig. 9G, black squares). Approximately 70% of β-catenin siRNA treated cells that had β-catenin fluorescence intensities of less than 4.5 a.u. at centrosomes did not have extra centrosomes (Fig. 9G). Thus, depletion of β-catenin at centrosomes strongly correlated with inhibition of formation of extra centrosomes in response to HU treatment.

Discussion

β-Catenin is a component of centrosomes (Bahmanyar et al., 2008; Corbit et al., 2008; Huang et al., 2007), forms a complex with the centrosomal proteins Nek2, C-Nap1 and Rootletin, and is involved in mitotic centrosome separation (Bahmanyar et al., 2008; Hadjihannas et al., 2010). Depletion of β-catenin in asynchronous cells results in monopolar spindles with unseparated centrosomes (Bahmanyar et al., 2008; Kaplan et al., 2004), whereas expression of β-cat* causes increased centriole splitting in G1–S (Bahmanyar et al., 2008; Hadjihannas et al., 2010). These studies raise important questions about the affects of β-catenin levels on centrosome organization, duplication and function.

Here, we showed that expression of stabilized mutant forms of β-catenin (β-cat*) induces formation of extra centrosomal structures in normal MDCK epithelial cells and HCT116 cancer cells. Removal of the mutant β-catenin allele from HCT116 cells significantly decreased the number of abnormal γ-tubulin structures in asynchronous and S-phase-arrested cells, and decreased amplification of SAS-6-positive centrioles during S-phase arrest. Depletion of β-catenin also decreased centriole amplification in S-phase-arrested U-2OS cells.

Plk4 is required for templated centrosome duplication (Habedanck et al., 2005; Kuriyama et al., 2009), and insufficient amounts of Plk4 at centrosomes have been implicated in the formation of the abnormal γ-tubulin structures in S-phase-arrested HCT116 cells (Kuriyama et al., 2009). However, we showed directly that Plk4 levels at centrosomes were not significantly

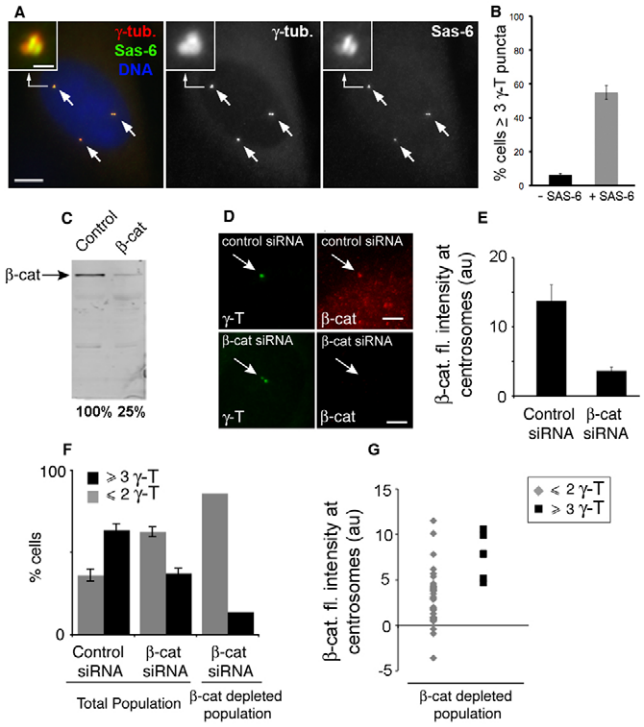

Fig. 9. Depletion of β-catenin with siRNA inhibits amplification of centrosomes in hydroxyurea-treated U-2OS cells. (**A**) U-2OS cultures were S-phase-arrested for 96 hours in HU and co-stained for γ-tubulin (red in A), SAS-6 (green in A) and DNA (blue in A). Scale bar: 10 μm. Inserts in A show magnified area of one of the three γ-tubulin and SAS-6 puncta in the cell. Scale bar: 1 μm. (**B**) Percentage of cells with three or more SAS-6-negative or SAS-6-positive γ-tubulin puncta was determined. Error bars are s.e.m. of three experiments. (**C**) SDS lysates from U-2OS cultures treated with control and *β-catenin* siRNA incubated for 96 hours in HU were loaded equally and immunoblotted for β-catenin. (**D**) Representative examples of centrosome region (arrows) of cells treated with control and *β-catenin* siRNA immunostained for γ-tubulin (green) and β-catenin (red). Scale bars: 2 μm. (**E**) Normalized fluorescence intensity of β-catenin at centrosomes in control (*n*=14) and *β-catenin* siRNA cells (*n*=42). Fluorescence intensity of β-catenin at centrosomes in siRNA-treated cells was significantly reduced. (**F**) Histogram showing in a total population the percentage of HU-treated control (*n*=368) or *β-catenin* siRNA (*n*=321) cells with two or fewer γ-tubulin puncta (gray bars) or three or more γ-tubulin puncta (black bars). Bars represent s.e.m. of four experiments. Portion of graph representing β-catenin-depleted population shows percentage of HU-treated *β-catenin* siRNA cells depleted for β-catenin at centrosomes, with indicated number of γ-tubulin puncta (*n*=42). Percentage of cells with three or fewer γ-tubulin puncta is significantly reduced in cells depleted of β-catenin. (**G**) Fluorescence intensity measurements of β-catenin at centrosomes in knockdown cells from F. Diamonds represent cells with two or fewer γ-tubulin puncta; squares represent cells with three or more γ-tubulin puncta. 71% of cells with two or fewer γ-tubulin puncta in *β-catenin* siRNA cells had fluorescence intensities less than 4.5 a.u.

affected by the presence of the mutant β-catenin allele in either asynchronous or S-phase-arrested HCT116 cells.

The mechanism by which β-cat* expression induces abnormal centrosome structures in cycling cells and promotes centriole amplification in S-phase-arrested cells is not understood. β-Cat*-expressing cells have a normal DNA content determined by FACS analysis (Bahmanyar et al., 2008), and 98% of β-cat*-expressing cells complete cytokinesis (S.B., unpublished results). Thus, β-cat*-induced extra γ-tubulin puncta are not caused by incomplete cytokinesis. As shown in this study, they are also independent of TCF-mediated transcription. One possibility is that increased centriole splitting caused by β-cat* accumulation at centrosomes (Bahmanyar et al., 2008) destabilizes the scaffolding of pericentriolar proteins, which causes dissociation of fragments of pericentriolar material. Indeed β-cat* increases the mobile pool of γ-tubulin at centrosomes, indicating that the dynamics of some components of the pericentriolar material are changed. Abnormal γ-tubulin puncta contain the centriolar marker centrin and probably have centriolar microtubule walls (see below), but little or none of the other core centriolar proteins, such as glutamylated tubulin (supplementary material Fig. S3E,F), SAS-6 and Plk4. Because SAS-6 and Plk4 are required for templated centriole duplication, the absence of these components indicates that these extra γ-tubulin puncta arise by a mechanism that is distinct from that of normal centriole duplication.

Electron microscopy showed that elongated γ-tubulin structures in S-phase-arrested HCT116 cells contain aberrant centrioles composed of disorganized cylindrical microtubules (Kuriyama et al., 2009). The abnormal γ-tubulin puncta that we observed in HCT116 and MDCK cells correspond to the elongated γ-tubulin structures identified by Kuriyama and colleagues by the following criteria: (1) both studies observed small extra γ-tubulin puncta in 5–10% of HCT116 cells; (2) both studies observed that abnormal γ-tubulin puncta become elongated and their numbers per cell increased during S-phase arrest by HU treatment; (3) both studies showed that abnormal γ-tubulin structures contain γ-tubulin, centrin, PCM-1 and Nedd1, but not Pericentrin, SAS-6 and Plk4/SAK, and that they do not nucleate microtubules.

The presence of γ-tubulin and NEDD-1/GCP-WD in these extra γ-tubulin puncta indicates that γ-TuRC rings are present but are not able to nucleate cytoplasmic MTs (Aldaz et al., 2005; Haren et al., 2006; Luders et al., 2006; Stearns and Kirschner, 1994). γ-Tubulin-containing structures can form de novo in the absence of cytoplasmic MTs (Khodjakov et al., 2002). The pathway of de novo centrosome assembly might involve the aggregation of centrin into pre-centrioles that further coalesce to become immature centrosomes (La Terra et al., 2005). Centrin and PCM-1 are components of centriolar satellites, which are involved in centriole biogenesis (Balczon et al., 1994; Baron and Salisbury, 1988; Dammermann and Merdes, 2002; Kubo and Tsukita, 2003). In β-cat*-expressing cells, we detected centrin and PCM-1 in extra γ-tubulin puncta, and additional centrin–PCM-1 satellites that did not have γ-tubulin. PCM-1 might provide sites in which intermediate centrosomal complexes can undergo folding (Dammermann and Merdes, 2002). Thus, centrin–PCM-1 satellites could be precursors that subsequently accumulate components of the γ-TuRC complex and transform into abnormal γ-tubulin-containing puncta.

We suggest that the majority of extra non-functional γ-tubulin puncta in dividing MDCK cells do not mature. However, we observed a small increase in mitotic cells with multipolar spindles in MDCK cells expressing stabilized β-cat*, indicating that some of the abnormal γ-tubulin puncta mature into functional MT-nucleating structures. Alternatively, β-cat* might promote, independently of S-phase arrest, the amplification of SAS-6-positive centrioles at a very low frequency, which subsequently form additional MT-nucleating structures. The origin and maturation of these extra centrosome structures requires further investigation and

could provide insight into a new mechanism for centrosome amplification in cancer cells.

Centrosome amplification has been observed in 20–30% of cells deleted of tumor suppressors or overexpressing oncogenes including p53, BRCA-1, PML3 and HPV-16 E (Duensing et al., 2000; Fukasawa et al., 1996; Xu et al., 1999; Xu et al., 2005). Our study is the first to demonstrate that accumulation of a mutant form of β-catenin found in early stages of many cancers (Sparks et al., 1998) directly induces centrosome aberrations in normal epithelial cells, and is required to maintain abnormal centrosome structures in cancer cells. Centrosome aberrations could contribute to spindle defects and abnormal chromosome segregation that could promote unregulated growth characteristics (Sluder and Nordberg, 2004). Although overall ploidy is not changed in non-cancerous epithelial cells expressing β-cat* (Bahmanyar et al., 2008), a significant, albeit small percentage of these cells shows loss of growth control as measured by anchorage-independent growth in soft agar (Barth et al., 1999). Taken together, these studies suggest a new pathway in which increased levels of β-catenin induce abnormal centrosome organization, which could synergize with other known effects of mutant β-catenin on gene transcription and cell–cell adhesion to induce cell transformation.

Materials and Methods

Cell lines

Growth conditions for Madin–Darby canine kidney (MDCK) type II/G cells and U-2 OS have been described previously (Barth et al., 1997; Chang and Stearns, 2000). The parental MDCK cell line T23 and doxycycline-repressible β-cat* MDCK cells have been described previously (Barth et al., 1999). For transient transfection of cDNA constructs, cells were plated at ~2×10^5 cells per 22×22mm coverslip and transfected the next day using lipofectamine 2000 reagent as described by the manufacturer (Gibco BRL, Gaithersburg, MD). HCT116 parental line and HCT116 cell lines derived from these by somatic cell gene targeting were a gift from Todd Waldman (Georgetown University School of Medicine, Washington, DC) and grown as described (Kim et al., 2002). HCT116 and MDCK cells were plated onto coverslips, cultured for 3 days without treatment, or treated for 1, 2 or 3 days with 2 mM hydroxyurea (Sigma, St Louis, MO) and fixed in cold methanol as described below.

Nocodazole washout

Cells were cultured as above and treated either at 4°C for 30 minutes or with 33 μM nocodazole (Sigma) for 1 hour at 37°C. Cells were cooled to room temperature, and nocodazole was washed out with three changes of fresh medium. Cells were incubated at room temperature with fresh medium for different times and processed for immunofluorescence.

DNA constructs

β-Cat* was generated by mutating the four CK1/GSK3β phosphorylation sites (Ser33, Ser37, Thr41 and Ser45) to alanine and cloning into the tet-off expression vector pUDH10-3, as described previously (Barth et al., 1999). Myc-tagged β-catenin/engrailed was a gift from Pierre McCrea at University of Texas, Houston, TX (Montross et al., 2000) and GFP-tagged ΔARM was a gift from Tamira Elul at Touro University, Vallejo, CA (Elul et al., 2003). For transient transfection experiments of GFP constructs, the *Sac*II-*Bam*HI fragments encoding β-cat and β-cat* were removed from pUDH10-3 (Barth et al., 1997) and cloned into pEGFP-C1 (Clontech). GFP-centrin-2 was a gift from Jeffrey Salisbury at the Mayo Clinic, Rochester, MN. C-terminal RFP-pericentrin domain was a gift from Sean Munro at Cambridge University, Cambridge, UK and GFP-γ-tubulin was a gift from Tim Stearns at Stanford University, Palo Alto, CA.

TCF reporter gene assay

TCF (TOP-Flash) and control (FOP-Flash) reporter assays were done as previously described (Barth et al., 1999). In brief, MDCK cells were transiently co-transfected with indicated GFP or β-catenin expression plasmids, pSV-β-galactosidase to measure galactosidase activity as a control for transfection efficiency and either pTOPFLASH to measure TCF activity or control vector pFOPFLASH which is mutated in the TCF recognition sites (vectors were gifts from Marc van de Wetering and Hans Clevers, Hubrecht Institute and University Medical Center, Utrecht, The Netherlands). Twenty-four hours after transfection, cells were extracted and Dual-Light reporter assays were performed as previously described (Barth et al., 1999).

Plk4 antibody generation

A full-length cDNA clone of *Xenopus laevis* Plk4 (Plx4) was obtained from OpenBiosystems (MGC68791; Huntsville, AL). Anti-Plk4 antibody was obtained from rabbits immunized with the C-terminal 85 amino acids of the *Xenopus laevis* homolog of Plk4 fused to GST (Cocalico Biologicals, Reamstown, PA). Plk4-specific antibodies were purified by acid elution after binding to GST-C-Plk4 immobilized on Ultralink Biosupport resin (Pierce, Rockford, IL). GST-reactive antibodies were removed by initially passing the serum over an Ultralink Biosupport column with immobilized GST. For antibody-blocking experiment, 0.25 μg anti-Plk4 antibody was diluted in buffer and incubated with 3.6 μg GST-C-Plk4 before incubation on cells. HEK 293T cells were fixed and co-immunostained with untreated or epitope-blocked anti-Plk4 antibody diluted 1:200 and anti-γ-tubulin antibody GTU-88 and secondary antibodies coupled to Alexa Fluor 488 and Alexa Fluor 594 and imaged as described above (supplementary material Fig. S4).

Fluorescence microscopy of fixed cells

Cells were fixed in –20°C methanol for 5 minutes and stained as described elsewhere (Louie et al., 2004). The following antibodies were used: rabbit polyclonal β-catenin, 1:200 (Nathke et al., 1994); γ-tubulin clone GTU88, 1:1000 (Sigma); rabbit polyclonal pericentrin, 1:200 (Covance/BabCO); rabbit polyclonal centrin was provided by Timothy Mitchison (Harvard Medical School, Cambridge, MA), 1:1000; mouse monoclonal centrin, 1:1000, was provided by Jeffrey Salisbury; mouse monoclonal GT335 to glutamylated tubulin was provided by Carsten Janke (Centre National de la Recherche Scientifique, Montpellier, France), rabbit polyclonal SAS-6 was provided by Alexander Dammermann and Karen Oegema (Ludwig Institute for Cancer Research, La Jolla, CA) and rabbit polyclonal PCM-1 antiserum 1:3000 was provided by Andreas Merdes (Centre National de la Recherche Scientifique, Toulouse, France); secondary antibodies against mouse, rat, rabbit, or chicken IgG with minimal species cross-reactivity coupled to FITC, Rhodamine or Cy5 were used at 1:200 for FITC and Rhodamine and 1:100 for Cy5 (Jackson ImmunoResearch). Epifluorescence was analyzed using a Zeiss Axioplan microscope (Carl Zeiss Meditec, Dublin, CA), as described elsewhere (Louie et al., 2004). Images were taken using identical exposure times for measurements of Plk4 fluorescence intensity and analyzed using ImageJ. Mean Plk4 fluorescence intensity was measured at centrosomes marked by co-staining of γ-tubulin and corrected for background Plk4 fluorescence intensity of an equivalent area next to each centrosome.

Fluorescence recovery after photobleaching

GFP-centrin and GFP-γ-tubulin turnover were measured using the Marianas System FRAP module (Yamada et al., 2005). Two protocols were used with γ-tubulin: (a) images were captured in a single focal plane, GFP-γ-tubulin fluorescence was measured using ImageJ in a 10-pixel-diameter circle centered on the pericentrin marker, and background fluorescence in the immediately surrounding region was subtracted (n=4 parental, n=6 β-cat*); (b) using the Slidebook software package, z-stacks (8 per minute, 2 μm steps) were acquired, maximum projected, and GFP-γ-tubulin fluorescence was measured in a mask defined by the pericentrin marker (n=3 parental; n=3 β-cat*; n=5 β-cat.* + dox). Both protocols produced similar results when analyzed separately; the data were combined for subsequent analysis. To measure FRAP of GFP-centrin, z-stacks were acquired and analyzed as described above (n=6 parental; n=8 β-cat*).

Immunoblotting for β-catenin and centrosome proteins

MDCK cell lines were cultured for 5 days in the absence of (–DOX) or with 40 ng/ml doxycycline (+DOX) to turn off expression of β-cat*. HCT116 cell lines were cultured for 3 days with or without 2 mM hydroxyurea (HU). MDCK and HCT116 cell lines were SDS-extracted and equal amounts of total extracted protein were loaded into each lane for SDS-PAGE and immunoblotting, as described before (Barth et al., 1999; Bahmanyar et al., 2008). Immunoblotting was done with β-catenin mouse monoclonal antibody at 1:500 (BD Biosciences Pharmingen, San Diego, CA), PCM-1 and γ-tubulin antibodies as described, for fluorescence, and centrin-1 rabbit antiserum at 1:1000 (Abcam, Cambridge, MA); secondary goat anti-mouse antibody IRDye 800 (1:15,000) was from Li-COR Biotechnology (Lincoln, NE), anti-rabbit Alexa Fluor 650 (1:30,000) was from Molecular Probes (Eugene, OR). Membranes were scanned at 680 and 800 nm and antibody signals were quantified using an Odyssey infrared imaging system (Li-COR Biotechnology, Lincoln, NE).

HU arrest of β-catenin-depleted cells

Small interfering RNAs to knock down human β-catenin (Dharmacon smart pool) or GFP, as a control, were transiently transfected into U-2OS cells with Oligofectamine (Invitrogen, Carlsbad, CA) as described by the manufacturer (Dharmacon Research, Lafayette, CO). Hydroxyurea (Sigma) was added at 2 mM 64 hours after transfection and for a further 96 hours. BrDU (Roche, Indianapolis, IN) was added to cells 30 minutes before fixation using the manufacturer's methods (Roche, Indianapolis, IN). Levels of β-catenin in cultures were analyzed by loading equal amounts of total protein for immunoblotting and quantification of bands was carried out as described in the previous section. Levels of β-catenin at centrosomes were analyzed as described for Plk4.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/18/3125/DC1

References

Aldaz, H., Rice, L. M., Stearns, T. and Agard, D. A. (2005). Insights into microtubule nucleation from the crystal structure of human gamma-tubulin. *Nature* **435**, 523-527.

Bahmanyar, S., Kaplan, D. D., Deluca, J. G., Giddings, T. H., Jr, O'Toole, E. T., Winey, M., Salmon, E. D., Casey, P. J., Nelson, W. J. and Barth, A. I. (2008). b-Catenin is a Nek2 substrate involved in centrosome separation. *Genes Dev.* **22**, 91-105.

Balczon, R., Bao, L. and Zimmer, W. E. (1994). PCM-1, A 228-kD centrosome autoantigen with a distinct cell cycle distribution. *J. Cell Biol.* **124**, 783-793.

Baron, A. T. and Salisbury, J. L. (1988). Identification and localization of a novel, cytoskeletal, centrosome-associated protein in PtK2 cells. *J. Cell Biol.* **107**, 2669-2678.

Barth, A. I., Pollack, A. L., Altschuler, Y., Mostov, K. E. and Nelson, W. J. (1997). NH2-terminal deletion of beta-catenin results in stable colocalization of mutant beta-catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J. Cell Biol.* **136**, 693-706.

Barth, A. I., Stewart, D. B. and Nelson, W. J. (1999). T cell factor-activated transcription is not sufficient to induce anchorage-independent growth of epithelial cells expressing mutant beta-catenin. *Proc. Natl. Acad. Sci. USA* **96**, 4947-4952.

Bauer, A., Lickert, H., Kemler, R. and Stappert, J. (1998). Modification of the E-cadherin-catenin complex in mitotic Madin-Darby canine kidney epithelial cells. *J. Biol. Chem.* **273**, 28314-28321.

Bettencourt-Dias, M., Rodrigues-Martins, A., Carpenter, L., Riparbelli, M., Lehmann, L., Gatt, M. K., Carmo, N., Balloux, F., Callaini, G. and Glover, D. M. (2005). SAK/PLK4 is required for centriole duplication and flagella development. *Curr. Biol.* **15**, 2199-2207.

Chang, P. and Stearns, T. (2000). Delta-tubulin and epsilon-tubulin: two new human centrosomal tubulins reveal new aspects of centrosome structure and function. *Nat. Cell Biol.* **2**, 30-35.

Corbit, K. C., Shyer, A. E., Dowdle, W. E., Gaulden, J., Singla, V. and Reiter, J. F. (2008). Kif3a constrains beta-catenin-dependent Wnt signalling through dual ciliary and non-ciliary mechanisms. *Nat. Cell Biol.* **10**, 70-76.

D'Assoro, A. B., Lingle, W. L. and Salisbury, J. L. (2002). Centrosome amplification and the development of cancer. *Oncogene* **21**, 6146-6153.

Dammermann, A. and Merdes, A. (2002). Assembly of centrosomal proteins and microtubule organization depends on PCM-1. *J. Cell Biol.* **159**, 255-266.

Dammermann, A., Maddox, P. S., Desai, A. and Oegema, K. (2008). SAS-4 is recruited to a dynamic structure in newly forming centrioles that is stabilized by the gamma-tubulin-mediated addition of centriolar microtubules. *J. Cell Biol.* **180**, 771-785.

Doxsey, S., Zimmerman, W. and Mikule, K. (2005). Centrosome control of the cell cycle. *Trends Cell. Biol.* **15**, 303-311.

Duensing, S., Lee, L. Y., Duensing, A., Basile, J., Piboonniyom, S., Gonzalez, S., Crum, C. P. and Munger, K. (2000). The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc. Natl. Acad. Sci. USA* **97**, 10002-10007.

Easwaran, V., Song, V., Polakis, P. and Byers, S. (1999). The ubiquitin-proteasome pathway and serine kinase activity modulate adenomatous polyposis coli protein-mediated regulation of beta-catenin-lymphocyte enhancer-binding factor signaling. *J. Biol. Chem.* **274**, 16641-16645.

Elul, T. M., Kimes, N. E., Kohwi, M. and Reichardt, L. F. (2003). N- and C-terminal domains of beta-catenin, respectively, are required to initiate and shape axon arbors of retinal ganglion cells in vivo. *J. Neurosci.* **23**, 6567-6575.

Fisk, H. A., Mattison, C. P. and Winey, M. (2003). Human Mps1 protein kinase is required for centrosome duplication and normal mitotic progression. *Proc. Natl. Acad. Sci. USA* **100**, 14875-14880.

Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S. and Vande Woude, G. F. (1996). Abnormal centrosome amplification in the absence of p53. *Science* **271**, 1744-1747.

Gillingham, A. K. and Munro, S. (2000). The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. *EMBO Rep.* **1**, 524-529.

Gomez-Ferreria, M. A., Rath, U., Buster, D. W., Chanda, S. K., Caldwell, J. S., Rines, D. R. and Sharp, D. J. (2007). Human Cep192 is required for mitotic centrosome and spindle assembly. *Curr. Biol.* **17**, 1960-1966.

Habedanck, R., Stierhof, Y. D., Wilkinson, C. J. and Nigg, E. A. (2005). The Polo kinase Plk4 functions in centriole duplication. *Nat. Cell Biol.* **7**, 1140-1146.

Hadjihannas, M. V. and Behrens, J. (2006). CIN By WNT: growth pathways, mitotic control and chromosomal instability in cancer. *Cell Cycle* **5**, 2077-2081.

Hadjihannas, M. V., Bruckner, M., Jerchow, B., Birchmeier, W., Dietmaier, W. and Behrens, J. (2006). Aberrant Wnt/beta-catenin signaling can induce chromosomal instability in colon cancer. *Proc. Natl. Acad. Sci. USA* **103**, 10747-10752.

Hadjihannas, M. V., Bruckner and Behrens, J. (2010). Conductin/axin2 and wnt signalling regulates centrosome cohesion. *EMBO Rep.* **11**, 317-324.

Haren, L., Remy, M. H., Bazin, I., Callebaut, I., Wright, M. and Merdes, A. (2006). NEDD1-dependent recruitment of the gamma-tubulin ring complex to the centrosome is necessary for centriole duplication and spindle assembly. *J. Cell Biol.* **172**, 505-515.

Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R. et al. (1999). The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. *Curr. Biol.* **9**, 207-210.

Huang, P., Senga, T. and Hamaguchi, M. (2007). A novel role of phospho-beta-catenin in microtubule regrowth at centrosome. *Oncogene* **26**, 4357-4371.

Kaplan, D. D., Meigs, T. E., Kelly, P. and Casey, P. J. (2004). Identification of a role for beta-catenin in the establishment of a bipolar mitotic spindle. *J. Biol. Chem.* **279**, 10829-10832.

Khodjakov, A. and Rieder, C. L. (1999). The sudden recruitment of gamma-tubulin to the centrosome at the onset of mitosis and its dynamic exchange throughout the cell cycle, do not require microtubules. *J. Cell Biol.* **146**, 585-596.

Khodjakov, A., Rieder, C. L., Sluder, G., Cassels, G., Sibon, O. and Wang, C. L. (2002). De novo formation of centrosomes in vertebrate cells arrested during S phase. *J. Cell Biol.* **158**, 1171-1181.

Kim, J. S., Crooks, H., Foxworth, A. and Waldman, T. (2002). Proof-of-principle: oncogenic beta-catenin is a valid molecular target for the development of pharmacological inhibitors. *Mol. Cancer Ther.* **1**, 1355-1359.

Kirkham, M., Muller-Reichert, T., Oegema, K., Grill, S. and Hyman, A. A. (2003). SAS-4 is a C. elegans centriolar protein that controls centrosome size. *Cell* **112**, 575-587.

Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S. and Kikuchi, A. (1998). Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. *J. Biol. Chem.* **273**, 10823-10826.

Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K. and Nakayama, K. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J.* **18**, 2401-2410.

Kubo, A. and Tsukita, S. (2003). Non-membranous granular organelle consisting of PCM-1: subcellular distribution and cell-cycle-dependent assembly/disassembly. *J. Cell Sci.* **116**, 919-928.

Kuriyama, R., Bettencourt-Dias, M., Hoffmann, I., Arnold, M. and Sandvig, L. (2009). Gamma-tubulin-containing abnormal centrioles are induced by insufficient Plk4 in human HCT116 colorectal cancer cells. *J. Cell Sci.* **122**, 2014-2023.

La Terra, S., English, C. N., Hergert, P., McEwen, B. F., Sluder, G. and Khodjakov, A. (2005). The de novo centriole assembly pathway in HeLa cells: cell cycle progression and centriole assembly/maturation. *J. Cell Biol.* **168**, 713-722.

Leidel, S. and Gonczy, P. (2003). SAS-4 is essential for centrosome duplication in C elegans and is recruited to daughter centrioles once per cell cycle. *Dev. Cell* **4**, 431-439.

Leidel, S., Delattre, M., Cerutti, L., Baumer, K. and Gonczy, P. (2005). SAS-6 defines a protein family required for centrosome duplication in C. elegans and in human cells. *Nat. Cell Biol.* **7**, 115-125.

Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1997). Genetic instability in colorectal cancers. *Nature* **386**, 623-627.

Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature* **396**, 643-649.

Ligon, L. A., Karki, S., Tokito, M. and Holzbaur, E. L. (2001). Dynein binds to beta-catenin and may tether microtubules at adherens junctions. *Nat. Cell Biol.* **3**, 913-917.

Liu, C., Li, Y., Semenov, M., Han, C. and Baeg, G. H. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechansim. *Cell* **108**, 837-847.

Louie, R. K., Bahmanyar, S., Siemers, K. A., Votin, V., Chang, P., Stearns, T., Nelson, W. J. and Barth, A. I. (2004). Adenomatous polyposis coli and EB1 localize in close proximity of the mother centriole and EB1 is a functional component of centrosomes. *J. Cell Sci.* **117**, 1117-1128.

Luders, J., Patel, U. K. and Stearns, T. (2006). GCP-WD is a gamma-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. *Nat. Cell Biol.* **8**, 137-147.

Montross, W. T., Ji, H. and McCrea, P. D. (2000). A beta-catenin/engrailed chimera selectively suppresses Wnt signaling. *J. Cell Sci.* **113**, 1759-1770.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-1790.

Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, K. and Akiyama, T. (1998). Axin, an inhibitor of the Wnt signalling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level. *Genes Cells* **3**, 395-403.

Nathke, I. S., Hinck, L., Swedlow, J. R., Papkoff, J. and Nelson, W. J. (1994). Defining interactions and distributions of cadherin and catenin complexes in polarized epithelial cells. *J. Cell Biol.* **125**, 1341-1352.

Nelson, W. J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483-1487.

Nigg, E. A. (2002). Centrosome aberrations: cause or consequence of cancer progression? *Nat. Rev. Cancer* **2**, 815-825.

Pihan, G. A., Purohit, A., Wallace, J., Knecht, H., Woda, B., Quesenberry, P. and Doxsey, S. J. (1998). Centrosome defects and genetic instability in malignant tumors. *Cancer Res.* **58**, 3974-3985.

Pihan, G. A., Purohit, A., Wallace, J., Malhotra, R., Liotta, L. and Doxsey, S. J. (2001). Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res.* **61**, 2212-2219.

Pihan, G. A., Wallace, J., Zhou, Y. and Doxsey, S. J. (2003). Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res.* **63**, 1398-1404.

Polakis, P. (1999). The oncogenic activation of beta-catenin. *Curr. Opin. Genet. Dev.* **9**, 15-21.

Prosser, S. L., Straatman, K. R. and Fry, A. M. (2009). Molecular dissection of the centrosome overduplication pathway in S-phase-arrested cells. *Mol. Cell. Biol.* **29**, 1760-1773.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.

Salisbury, J. L., D'Assoro, A. B. and Lingle, W. L. (2004). Centrosome amplification and the origin of chromosomal instability in breast cancer. *J. Mammary Gland Biol. Neoplasia* **9**, 275-283.

Sluder, G. and Nordberg, J. J. (2004). The good, the bad and the ugly: the practical consequences of centrosome amplification. *Curr. Opin. Cell Biol.* **16**, 49-54.

Sparks, A. B., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res.* **58**, 1130-1134.

Stearns, T. (2001). Centrosome duplication. a centriolar pas de deux. *Cell* **105**, 417-420.

Stearns, T. and Kirschner, M. (1994). In vitro reconstitution of centrosome assembly and function: the central role of gamma-tubulin. *Cell* **76**, 623-637.

Tsou, M. F. and Stearns, T. (2006). Controlling centrosome number: licenses and blocks. *Curr. Opin. Cell Biol.* **18**, 74-78.

Warnke, S., Kemmler, S., Hames, R. S., Tsai, H. L., Hoffmann-Rohrer, U., Fry, A. M. and Hoffmann, I. (2004). Polo-like kinase-2 is required for centriole duplication in mammalian cells. *Curr. Biol.* **14**, 1200-1207.

Xu, X., Weaver, Z., Linke, S. P., Li, C., Gotay, J., Wang, X. W., Harris, C. C., Ried, T. and Deng, C. X. (1999). Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. *Mol. Cell* **3**, 389-395.

Xu, Z. X., Zou, W. X., Lin, P. and Chang, K. S. (2005). A role for PML3 in centrosome duplication and genome stability. *Mol. Cell* **17**, 721-732.

Yamada, S., Pokutta, S., Drees, F., Weis, W. I. and Nelson, W. J. (2005). Deconstructing the cadherin-catenin-actin complex. *Cell* **123**, 889-901.

Zhu, F., Lawo, S., Bird, A., Pinchev, D., Ralph, A., Richter, C., Muller-Reichert, T., Kittler, R., Hyman, A. A. and Pelletier, L. (2008). The mammalian SPD-2 ortholog Cep192 regulates centrosome biogenesis. *Curr. Biol.* **18**, 136-141.

Zimmerman, W., Sparks, C. A. and Doxsey, S. J. (1999). Amorphous no longer: the centrosome comes into focus. *Curr. Opin. Cell Biol.* **11**, 122-128.

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