γ-Tubulin-containing abnormal centrioles are induced by insufficient Plk4 in human HCT116 colorectal cancer cells

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Summary

Cancer cells frequently induce aberrant centrosomes, which have been implicated in cancer initiation and progression. Human colorectal cancer cells, HCT116, contain aberrant centrioles composed of disorganized cylindrical microtubules and displaced appendages. These cells also express unique centrosome-related structures associated with a subset of centrosomal components, including γ-tubulin, centrin and PCM1. During hydroxyurea treatment, these abnormal structures become more abundant and undergo a change in shape from small dots to elongated fibers. Although γ-tubulin seems to exist as a ring complex, the abnormal structures do not support microtubule nucleation. Several lines of evidence suggest that the fibers correspond to a disorganized form of centriolar microtubules. Plk4, a mammalian homolog of ZYG-1 essential for initiation of centriole biogenesis, is not associated with the γ-tubulin-specific abnormal centrosomes. The amount of Plk4 at each centrosome was less in cells with abnormal centrosomes than cells without γ-tubulin-specific abnormal centrosomes. In addition, the formation of abnormal structures was abolished by expression of exogenous Plk4, but not SAS6 and Cep135/Bld10p, which are downstream regulators required for the organization of nine-triplet microtubules. These results suggest that HCT116 cells fail to organize the ninefold symmetry of centrioles due to insufficient Plk4.

Key words: Centrosomes, Centrioles, Nine-triplet microtubules, Hydroxyurea, γ-tubulin, Plk4, SAS6, Cep135, HCT116 cells

Introduction

The major microtubule-organizing center in animal cells is the centrosome, which is composed of a pair of centrioles and the surrounding amorphous cloud of pericentriolar material. During cell division, one centrosome is located at each spindle pole to organize the mitotic spindle. The establishment of spindle bipolarity is a prerequisite for high fidelity of chromosome segregation, thereby maintaining genomic stability. It is, therefore, important to understand how centrosome replication is controlled in the cell.

The centrioles play a central role in determining the number of microtubule-organizing sites. Indeed, the total number of centrioles is precisely controlled during the cell cycle, as is evident from their characteristic morphological changes in cells at different cell cycle stages (Kuriyama and Borisy, 1981): daughter cells receive a pair of full-length centrioles after cell division. The orthogonal configuration of two centrioles becomes disoriented during G1, and a new centriole appears at a right angle to the proximal side of each parent centriole during late G1 to early S phase. Daughter centrioles slowly elongate to attain almost full length at the onset of M phase, during which one pair of centrioles is positioned at each spindle pole, leading to its separation from another pair after mitosis and cytokinesis.

Centriole duplication in mammalian cells has been shown to be under the direct control of Cdk2-cyclin A (Meraldi et al., 1999). In *C. elegans* embryos, centriogenesis is triggered by a signal mediated by several key molecules, including ZYG-1 (O'Connell et al., 2001), which causes the targeting of SAS5 and SAS6 to a nascent centriole (Delattre et al., 2006; Pelletier et al., 2006). These molecules are required for formation and elongation of a central tube, a structural intermediate of the centriole, onto which nine sets of centriolar microtubules become organized in a SAS4-dependent manner (Pelletier et al., 2006). Despite morphological variations of the centriole, evolutionary and/or functional homologs of most of those molecules have been identified in other species (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Leidel et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007; Yabe et al., 2007). It is thus plausible that the basic mechanism of centriole biogenesis is common among diverse organisms (for reviews, see Bettencourt-Dias and Glover, 2007; Dutcher, 2007). In mammalian cells, additional molecules, such as γ-tubulin, α-tubulin, Cep135, and CP110, appear to be essential for centriole replication (Keylein-Sohn et al., 2007). Among them, Cep135 would be of particular interest as it has recently been identified as a cartwheel component important for establishment of ninefold symmetry of the centriole in collaboration with another cartwheel protein, SAS6 (Hiraki et al., 2007; Nakazawa et al., 2007). γ-Tubulin is a well-known component of the pericentriolar material that is responsible for microtubule nucleation by forming a multiprotein ring complex (γ-TuRC) (Moritz and Agard, 2001). γ-Tubulin is also present in close association with centrioles (Fuller et al., 1995; Moudjou et al., 1996) and basal bodies (Silflow et al., 1999; Klotz et al., 2003). When expression of γ-tubulin and γ-TuRC components is silenced, centriole and basal body assembly becomes severely impaired, suggesting that these proteins are essential for centriogenesis (Ruiz

et al., 1999; Raynaud-Messina et al., 2004; Shang et al., 2002; Dammermann et al., 2004; Haren et al., 2006). γ-Tubulin might be required for new centriole replication by stabilizing centriolar microtubules (Dammermann et al., 2008).

To study the mechanism of centrosome duplication and its regulation, it is useful to analyze cells that produce aberrant centrosomes. Many cancer cells are known to possess centrioles and centrosomes that are abnormal in number and structure, which has been postulated to contribute to their genomic instability in cancer cells (for reviews, see Brinkley and Goepfert, 1998; Krämer et al., 2002; Nigg, 2002). During the screening of human cancer cells, we identified one cell line, human HCT116 colorectal cancer cells, that expressed unique centrosome-related structures associated with only a subset of centrosomal proteins, including γ-tubulin. These cells with abnormal centrosomes contain less Plk4 at each centrosome than normal cells, and ectopic expression of Plk4 suppressed the induction of the abnormal structures, suggesting that insufficient Plk4 results in the formation of aberrant centrioles in HCT116 cells.

Results

HCT116 cells contain abnormal centrosomes

To study the centrosome in cancer cells, we immunostained HCT116 cells with anti-γ-tubulin (Fig. 1A',C') along with Cep135 (Fig. 1A) or pericentrin antibodies (Fig. 1C). Each cell revealed the presence of one or two centrosomal dots at the juxtanuclear position. It was, however, noted that there were extra sites labeled only with γ-tubulin antibodies but not with Cep135 and pericentrin antibodies (arrows). These γ-tubulin-specific sites generally appeared as a small spot in about 5-10% of total cell populations. In some cells, the abnormal structure formed an elongated fiber that reached nearly 10 microns in length. During treatment with the DNA synthesis inhibitor, hydroxyurea, the cells induced more elongated γ-tubuilin-containing abnormal centrosomes (see below; arrowheads in Fig. 1B',D').

Because γ-tubulin, pericentrin and Cep135 are ubiquitous centrosomal components co-localized at the centrosome in all of the mammalian cells tested thus far, their differential distribution urged us to examine additional centrosomal antibodies in HU-treated HCT116 cells. Nek2 (Fig. 1E-E") and ninein (Fig. 1F-F") are core components of the centrosome; neither of these proteins were present in any of the abnormal centrosomal sites revealed by γ-tubulin staining. Negative immunostaining of γ-tubulin-specific sites was also seen in cells labeled with TACC2 (Fig. 1G-G"), CTR453 (Fig. 1H-H"), and SAS6 (Fig. 1I-I") antibodies, in addition to cenexin, Cep170, C-Nap1, Plk4/SAK, SAS4/CPAP, and Aurora-A antibodies (Table 1). Not all components were undetectable: we found two molecules (centrin and PCM1) specifically localized at every site containing γ-tubulin (Fig. 2A-C"). Although centrin showed identical distribution to that of γ-tubulin, PCM1 labeling was much more intense in the abnormal structure than in centrosomes (arrowheads). γ-Tubulin forms a multiprotein ring complex (γ-TuRC), which is targeted to the centrosome for initiation of microtubule assembly (Moritz and Agard, 2001; Harren et al., 2006). We immunostained HU-treated cells with γ-tubulin antibodies along with probes for γ-TuRC-associated components, GCP2 (Fig. 2D'-E'), GCP4 (not shown) and Nedd1 (Fig. 2F'-G'). These proteins were present in the abnormal centrosomes labeled with γ-tubulin (Fig. 2D,F), but not CTR453 (Fig. 2E,G), suggesting that γ-tubulin exists as a multiprotein ring complex in the abnormal structures of the HCT116 cell.

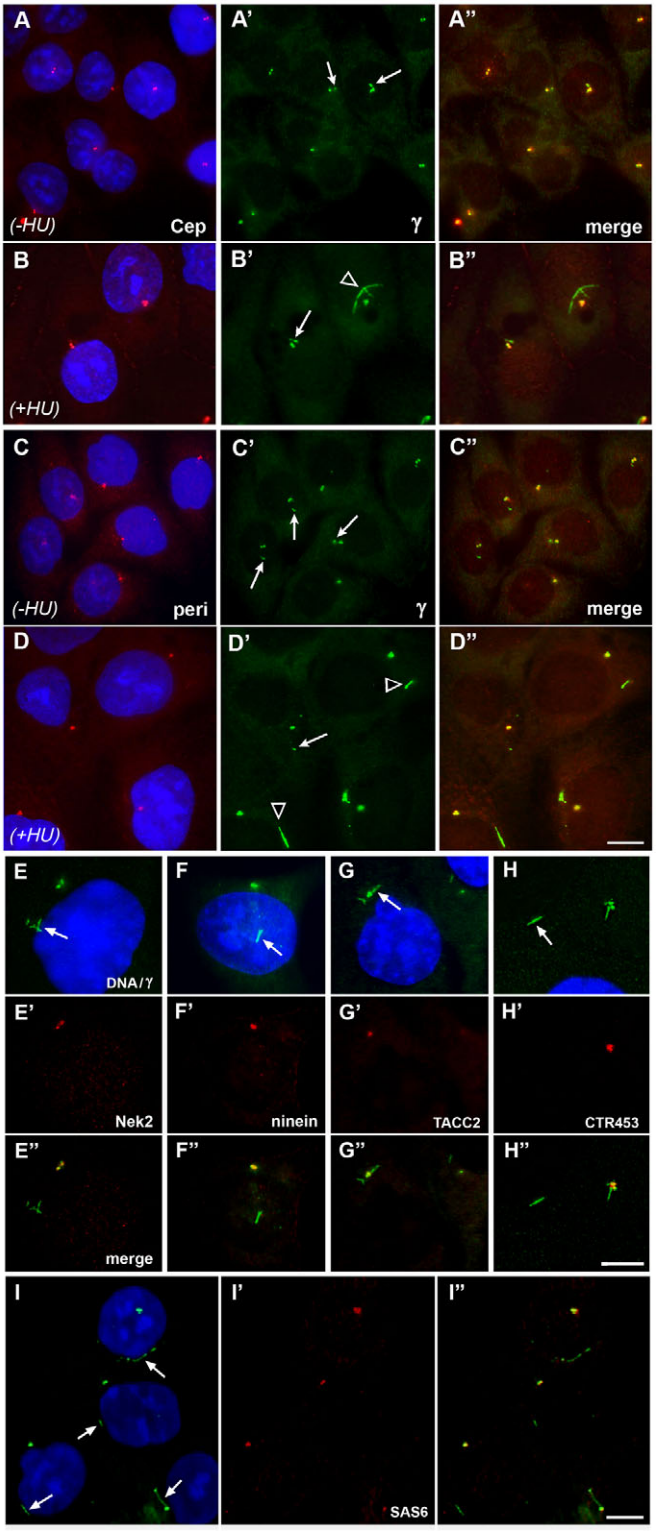

Fig. 1. Identification of γ-tubulin-containing abnormal centrosomes in HCT116 cells. Cells were double immunostained with γ-tubulin (A'-D') and Cep135 (A,B) or pericentrin (C,D) after treatment (B,D) or no treatment (A,C) with 2 mM hydroxyurea (HU) for 3 days. The structures associated with γ-tubulin alone are present (arrows). They appear as small dots, but sometimes form fibrous structures, which become more prominent during HU treatment (arrows). (E-I) HU-arrested cells double-stained with γ-tubulin (E-I) and Nek2 (E'), ninein (F'), TACC2 (G'), CTR453 (H') and SAS6 (I'). Those components are not associated with γ-tubulin-specific abnormal centrosomes (arrows). Merged images are shown in A"-I". Scale bars: 10 μm.

Table 1. Centrosome proteins present or absent in the abnormal centrioles

Present	Absent
γ-Tubulin	Cep135
Centrin	Pericentrin
PCM1	Ninein
GCP2	Cenexin
GCP4	Nek2
Nedd1	C-Nap1
	Plk4/SAK
	CTR453
	Cep170
	Aurora-A
	SAS6
	TACC2
	SAS4/CPAP

Hydroxyurea treatment stimulates the induction of the γ-tubulin-specific structure

As shown in Fig. 1B-B",D-D", abnormal centrosomes changed their shape drastically in cells treated with hydroxyurea (HU), which is known to induce multiple centrosomes in certain cell types by uncoupling the centrosome cycle from the cell cycle (Balczon et al., 1995; Kuriyama et al., 2007). To determine whether not only the shape but also the ability to induce abnormal centrosomes were affected by HU treatment, we counted numbers of normal and abnormal centrosomes by double staining with γ-tubulin and Cep135 antibodies (Fig. 3). Abnormal structures became more abundant as cells were treated with HU for a longer period of time, and we could find them in nearly a half of the total cell population after 3 days of treatment (hatched red bar in Fig. 3). The emergence of multiple centrosomes was also noted (solid red bar); however, they were always less abundant than abnormal centrosomes.

The tumor suppressor protein p53 has been implicated to be involved in the formation of multiple centrosomes in cancer cells (Fukasawa et al., 1996). Therefore, we next compared the abundance of normal and abnormal centrosomes in HCT116 cells lacking p53 (Bunz et al., 1998). Control $p53^{-/-}$ cells probed for γ-tubulin contained a similar level of abnormal structures as $p53^{+/+}$ cells before HU treatment. Like $p53^{+/+}$ cells, the cells lacking p53 induced more abnormal centrosomes (hatched bars in Fig. 3) in response to HU treatment. Unlike $p53^{+/+}$ cells, however, $p53^{-/-}$ cells induced more centrosomes labeled with both γ-tubulin and Cep135 antibodies than abnormal γ-tubulin-specific structures. From these results, we concluded that abnormal centrosomes could be induced in HCT116 cells, regardless of the presence or absence of p53.

HCT116 cells contain disorganized centrioles

Immunofluorescence staining showed that HCT116 cells induced prominent fibrous structures. To examine the ultrastructure of the γ-tubulin-specific abnormal centrosomes, we carried out thin section electron microscopy. $p53^{+/+}$ cells treated with HU for 3 days frequently revealed the presence of microtubule bundles running parallel in the same direction (Fig. 4). The number and length of clustered microtubules were highly variable. Some were similar in appearance to a centriolar cylinder, although they were longer and much thinner than normal centrioles (Fig. 4A). Fig. 4B-H shows loosely packed microtubule bundles, some of which were surrounded by amorphous material with differing degrees of electron density (Fig. 4B-C). These incomplete centrioles and/or microtubule bundles are probably related to the fibers of various lengths detected in HU-arrested HCT116 cells by immunofluorescence staining.

The γ-tubulin-specific structures are also associated with centrin, which is a widely used centriole marker (Fig. 2). This finding supports the observations by electron microscopy described above that the abnormal centrosomes are indeed morphologically defective centrioles. To confirm this, we carried out the additional experiments described below.

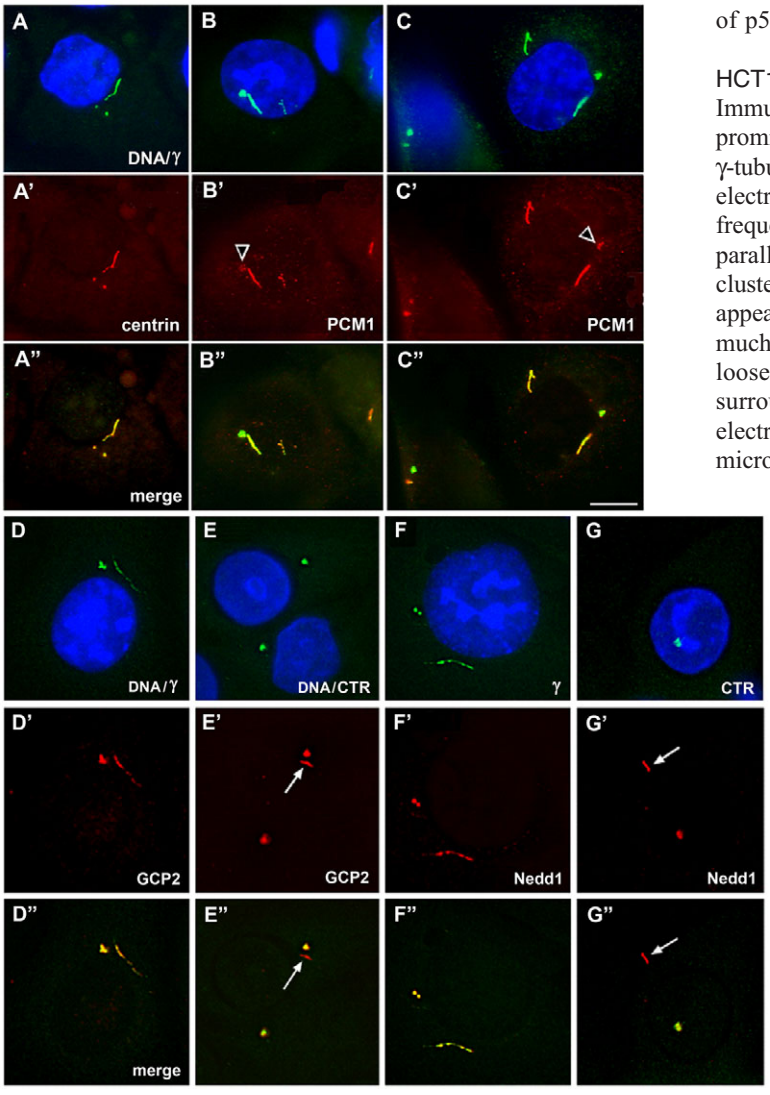

Fig. 2. γ-Tubulin-containing abnormal centrosomes are associated with a subset of centrosomal proteins. HU-arrested HCT116 cells were stained with centrin (A'), PCM1 (B',C'), GCP2 (D',E') and Nedd1 (F',G') antibodies along with either γ-tubulin (A-D,F) or CTR453 (E,G) antibodies. Merged images are shown in A"-G". All structures revealed by γ-tubulin antibodies are labeled with antibodies specific to those components. In contrast to the intensely stained abnormal centrosomes, PCM1 shows relatively weak staining of the centrosome (arrowheads in B' and C'). Arrows indicate the position of abnormal centrosomes revealed by GCP2 and Nedd1, but not CTR453. Scale bars: 10 μm.

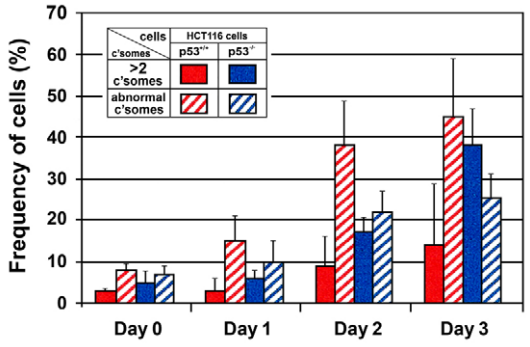

Fig. 3. Frequency of HCT116 cells with abnormal centrosomes (hatched bars) and over two centrosomes (solid bars) in $p53^{+/+}$ (red) and $p53^{-/-}$ (blue) cells. Cells were treated with 2 mM HU and structures staining positively for both γ-tubulin and Cep135 or γ-tubulin alone were counted at different time points in 150-250 cells for each experiment. The same experiment was repeated five to ten times.

Non-tyrosinated α-tubulin is preferentially localized at the abnormal centrosome

The C-terminal tyrosine residue encoded in the α-tubulin subunit (tyrosinated tubulin; Y-tub) is removed by a tubulin-specific carboxypeptidase resulting in non-tyrosinated tubulin with glutamic acid at the C-terminus (detyrosinated tubulin; E-tub). This post-translationally modified tubulin subunit is preferentially localized to stable, long-lived microtubules, including centriolar microtubules (Bulinski and Gundersen, 1991). Fig. 5 shows double immunostaining of HCT116 cells with γ-tubulin (Fig. 5A-C) and α-tubulin antibodies specific to either E-tub (Fig. 5A'-B') or Y-tub (Fig. 5C'). To obtain a clearer view of centriolar microtubules, we pretreated cells with nocodazole to depolymerize in situ microtubules before fixation. Virtually no fluorescence signals were detected in Y-tub immunostained cells (Fig. 5C'), whereas E-tub was specifically recognized at all structures probed by γ-tubulin staining (Fig. 5A-A',B-B'). E-tub antibody further revealed thin fibers extending from γ-tubulin-containing fibers (Fig. 5B', arrowheads), which might represent stable microtubules that had remained intact after depolymerization of cytoplasmic microtubules by nocodazole treatment.

Abnormal centrosomes are unable to polymerize microtubules

Although γ-tubulin appears to exist as a multiprotein ring complex (Fig. 2D-G) capable of microtubule nucleation in vivo and in vitro (Moritz and Agard, 2001), the γ-tubulin-containing abnormal centrioles were, like normal centrioles, unable to nucleate cytoplasmic microtubules. Fig. 5D-E shows HU-arrested cells in which cytoplasmic microtubules were depolymerized by nocodazole treatment. After brief recovery from the microtubule drug, short microtubule asters were formed onto the centrosome (Fig. 5D'-E'). As shown by arrows, the abnormal centrosomes were specifically devoid of microtubule asters, indicating that no cytoplasmic microtubules were polymerized onto the γ-tubulin-containing structure.

Nocodazole blocks the formation of γ-tubulin-containing abnormal structures

Microtubule depolymerizing reagents, such as colcemid and nocodazole, block the formation of centrioles by interfering with the polymerization of microtubules forming the wall of the centriole (Kuriyama, 1982). To determine whether the formation of γ-tubulin-containing fibers and/or dots was inhibited by nocodazole, we double-treated cells with HU and nocodazole. As summarized in Fig. 6, the structures labeled by γ-tubulin antibodies, but not Cep135 antibodies, were detected in up to 50% of total cell populations after 3 days of HU treatment. When cells were treated with both HU and nocodazole, virtually no γ-tubulin-containing fibers and/or dots were formed, indicating that abnormal centriole formation was efficiently blocked by interfering with microtubule assembly.

Besides abnormal centrosomes associated with only a subset of centrosome components, we also noted that over 90% of the HU-arrested cells examined by electron microscopy (n>100) contained aberrant centrioles. In a cell shown in Fig. 7A, cytoplasmic microtubules were emanating from the centrosome, and mitochondria were aligned prominently with these microtubules. This centrosome contained three centrioles, two of which were perpendicular to one another, as typically seen in normal centriolar profiles (Fig. 7C). Although the centrioles looked normal, some of the microtubules forming the wall of one centriole appeared to protrude, which might correspond to the extension of the centriolar microtubules in one direction. A more severe abnormality was seen

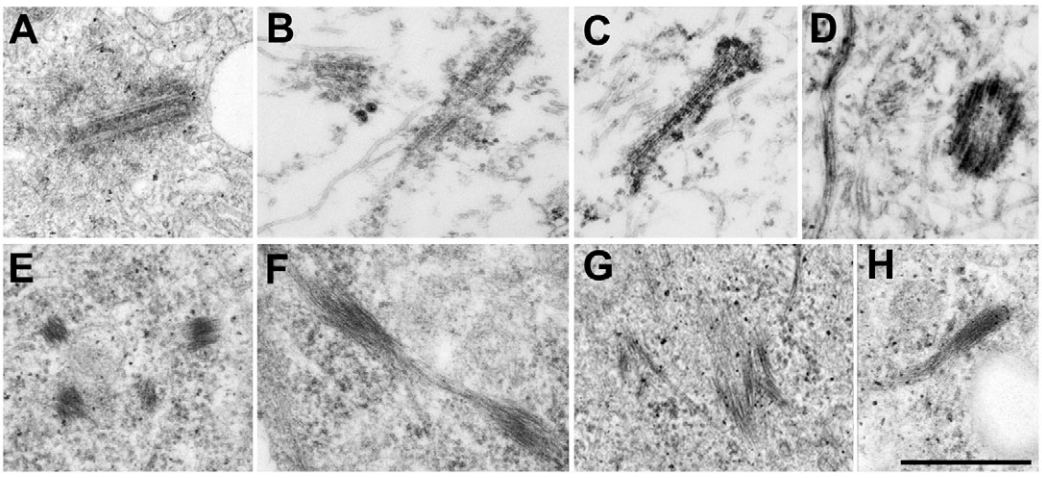

Fig. 4. Fibrous structures composed of microtubule bundles of various lengths and sizes are detected in HU-arrested HCT116 cells by thin-section electron microscopy. Images shown in B-D were obtained from cells extracted with a detergent-containing medium prior to fixation. Some microtubule bundles appear to be forming the centriole barrel (A), whereas others are seen as more loosely connected microtubule bundles (D-H). Scale bar: 0.5 μm.

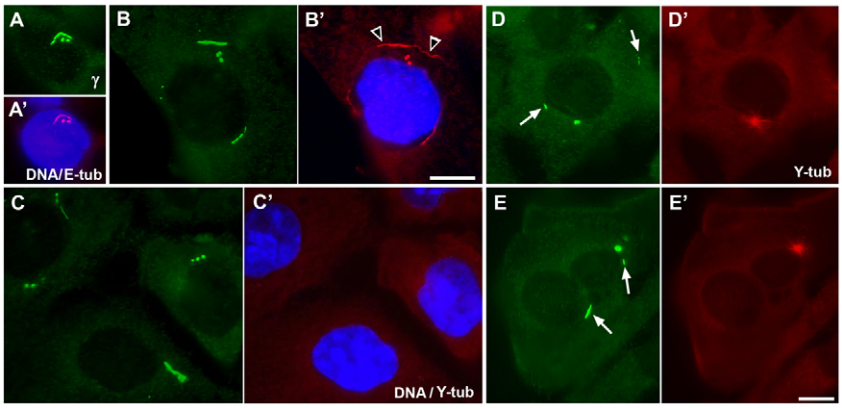

Fig. 5. Abnormal centrosomes are associated with non-tyrosinated tubulin, but devoid of the microtubule-nucleating activity. Cells were treated with 2 mM HU for 3 days and then incubated with 1 μg/ml nocodazole for 1-3 hours to depolymerize in situ microtubules. In A-C', cells were double-stained with γ-tubulin (A,B,C) and antibodies to non-tyrosinated (E-tub: A',B') and tyrosinated (Y-tub: C') α-tubulin. Tyrosinated tubulin is undetectable in nocodazole-treated cells, whereas non-tyrosinated tubulin specifically associates with the abnormal centrosomes revealed by γ-tubulin staining. Arrowheads in B' indicate positions of thin fibers extending from the side of γ-tubulin-containing fibers. In D-E', cells were double-stained with γ-tubulin (D,E) and tyrosinated α-tubulin antibodies (D',E') after a brief recovery from nocodazole treatment. Normal centrosomes are able to form asters with short microtubules, but no cytoplasmic microtubules are polymerized onto the γ-tubulin-containing abnormal centrosomes (arrows). Scale bars: 10 μm.

in the third centriole outlined in Fig. 7A. Here, the wall was thinner than those of the other centrioles and its entire surface was surrounded by electron-dense material. The material sometimes formed a round structure protruding from the centriolar cylinder (Fig. 7A').

Fig. 7E-M shows various degrees of morphological abnormality in centrioles detected in both extracted (Fig. 7E-H) and non-extracted cells (Fig. 7A,I-M) after a 3-day treatment with HU. Some centrioles were composed of doublet (Fig. 7F) or singlet microtubules (not shown) rather than triplet microtubules as seen normally (Fig. 7B). In some cells, we detected bundles of microtubules (Fig. 7E), which might represent a cross-sectional view of the centriole-related fibers shown in Fig. 4D-H. Most frequently, a subset of microtubules was seen as an outgrowth from one end of the centriolar wall (Fig. 7K-M). Some centrioles were shorter (Fig. 7I), whereas others were thinner (Fig. 7J) than controls. Electron-dense structures were attached to the centriolar cylinder (Fig. 7G-H,J,M); these structures resembled the distal and subdistal appendages normally located at the distal end of mother centrioles (Fig. 7D). From these results, we concluded that in the abnormal centrioles of HCT116 cells, the microtubules forming the centriolar wall were disorganized and appendage-like structures were displaced. Unlike the γ-tubulin-specific abnormal centrosomes, the centrosomes that contained abnormal centrioles apparently possessed microtubule-nucleating capacity, because astral microtubules appeared to converge at the dense material surrounding the aberrant centrioles (Fig. 7A,I).

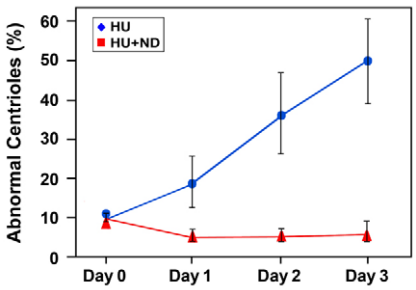

Fig. 6. Abnormal centrosome formation is blocked by nocodazole treatment. HCT116 cells were treated with 2 mM HU in the presence (red) or absence of 1 μg/ml nocodazole (blue). Cells fixed at different time points were double-stained with γ-tubulin and Cep135 antibodies to detect normal and abnormal centrosomes.

Formation of the γ-tubulin-containing structure is dependent on Plk4, but not SAS6 and Cep135

Plk4 has been shown to initiate centriole assembly in diverse organisms (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). To determine whether this kinase is involved in the formation of abnormal centrosomes, we immunostained endogenous Plk4 with antibodies raised against the C-terminal domain of human Plk4 (M.A. and I.H., unpublished results). Fig. 8A-C shows three different fields where two cells were side-by-side: one cell contained γ-tubulin-specific abnormal centrosomes, whereas the other cell did not. Although the intensity of the Plk4 fluorescence signal was generally not as strong as that of γ-tubulin, it was possible to detect Plk4 at the centrosome in both types of cells. It was consistently noted that the Plk4 fluorescence was more intense in cells without abnormal centrosomes (arrowheads) than in cells expressing γ-tubulin-specific structures (arrows). Table 2 summarizes the quantitative analysis of the comparison of Plk4 level between normal and abnormal centrosomes: regardless of the presence or absence of p53. We detected less Plk4 in both HU-treated and untreated HCT116 cells expressing abnormal centrosomes. Thus, cells with the γ-tubulin-specific structure express a lower level of Plk4 fluorescence than cells containing no abnormal structures.

We next prepared a V5-tagged Plk4 construct to express ectopic proteins in HCT116 cells by transient transfection. After HU-treatment for 2-3 days, the cells were fixed and immunostained with monoclonal anti-V5 and polyclonal Cep135 antibodies to screen for cells that were expressing Plk4 at the centrosome. The same cells were labeled further with monoclonal γ-tubulin antibody to determine the presence or absence of abnormal centrioles. As summarized in Fig. 9 (Plk4 (V5) columns), induction of abnormal centrioles were detected in 7.1% of total transfected cell populations (n=453). This number is significantly smaller than that of non-transfected cells counted on the same coverslips (29.8%; n=1,030). Overexpression of ectopic Plk4 also resulted in the formation of multiple centrosomes (>2 centrosomes detected in 35-45% and 3-5% transfected and non-transfected cell populations, respectively), which is consistent with previous observations (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). In contrast to the abnormal centrosomes that are associated with only a subset of centrosomal components, Plk-induced multiple centrosomes were positive in immunostaining with the centrosomal antibodies tested thus far, including Cep135, pericentrin, PCM1, cenexin, and Cep170 (Fig. 10). The inhibitory effect of Plk4 on the formation of abnormal centriole-related structures is not due to a leader sequence as similar

results were obtained by expression of TAP (Flag-His-calmodulin binding site)-conjugated Plk4 [Fig. 9, Plk4 (TAP) columns].

It has been shown that SAS6 and Cep135 are components of the radial spokes of the cartwheel, which is responsible for organization of ninefold symmetry of the centriole and basal bodies (Hiraki et al., 2007; Nakazawa et al., 2007). Unlike Plk4, exogenous GFP-SAS6 and HA-Cep135 could not suppress the abnormal centriole formation; rather, ectopic expression of these molecules caused the induction of more γ-tubulin-specific structures than non-transfected cells (Fig. 9). Because SAS6 and Cep135 are not associated with the abnormal structures (Fig. 1), we concluded that it is the upstream regulator Plk4 that is insufficient, causing the induction of abnormal centrioles in HCT116 cells.

Discussion

HCT116 cells are well-characterized and widely used human cancer cells, and they contain unique centrioles abnormal in morphology and macromolecular composition. This suggests that the mechanism controlling the organization of nine triplets of centriolar microtubules is not functioning correctly in this particular cancer cell line.

Abnormal centrosomes in cancer cells

As abnormal centrosomes are found so frequently in numerous cancers, centrosome aberration has been postulated as one of the leading factors in carcinogenesis (Brinkley and Goepfert, 1998; Krämer et al., 2002; Nigg, 2002). In their pioneering studies, Lingle and coworkers (Lingle et al., 1998; Lingle and Salisbury, 1999) and Pihan and coworkers (Pihan et al., 1998) reported detailed morphological abnormalities in cancer cells, such as supernumerary centrioles and centrosomes, disorganized centriolar structures, excess pericentriolar clouds, elevated levels of microtubule-nucleating activity and unusual phosphorylation properties of the centrosomal components. Overall, these profiles fit the abnormal centrosomes that were detected in HCT116 cells. Particularly prominent are structural alterations in the centriole, and we noted significant disruption of the centriolar barrel structure with disorganized microtubules and displaced appendages, which become more prominent in HU-arrested cells (Fig. 4). The centrosomes in HCT116 cells displayed a further unique feature in being associated with only a limited number of centrosomal proteins: γ-tubulin and γ-TuRC, centrin and PCM1. As far as we are aware, this is the first report of differential distribution of centrosomal components in the centriole. Supernumerary centrosomes have been determined by antibody labeling of various cancer cells, including HCT116 cells (Wong and Stearns, 2003). The fact that centrosomal probes stain centrosomes and centrosome-related structures differently provides a cautious reminder that centrosome numbers quantified by antibody staining might not always be accurate.

Pihan and coworkers reported that nearly all tumors and tumor-derived cell lines expressed high levels of pericentrin, which caused the formation of ectopic fibers capable of microtubule nucleation in vivo (Pihan et al., 1998). In this study, fibrous structures were also detected in HCT116 cells, but neither pericentrin nor microtubule-nucleating activity was associated with them. It is thus probable that the nature of centrosomal aberrations is

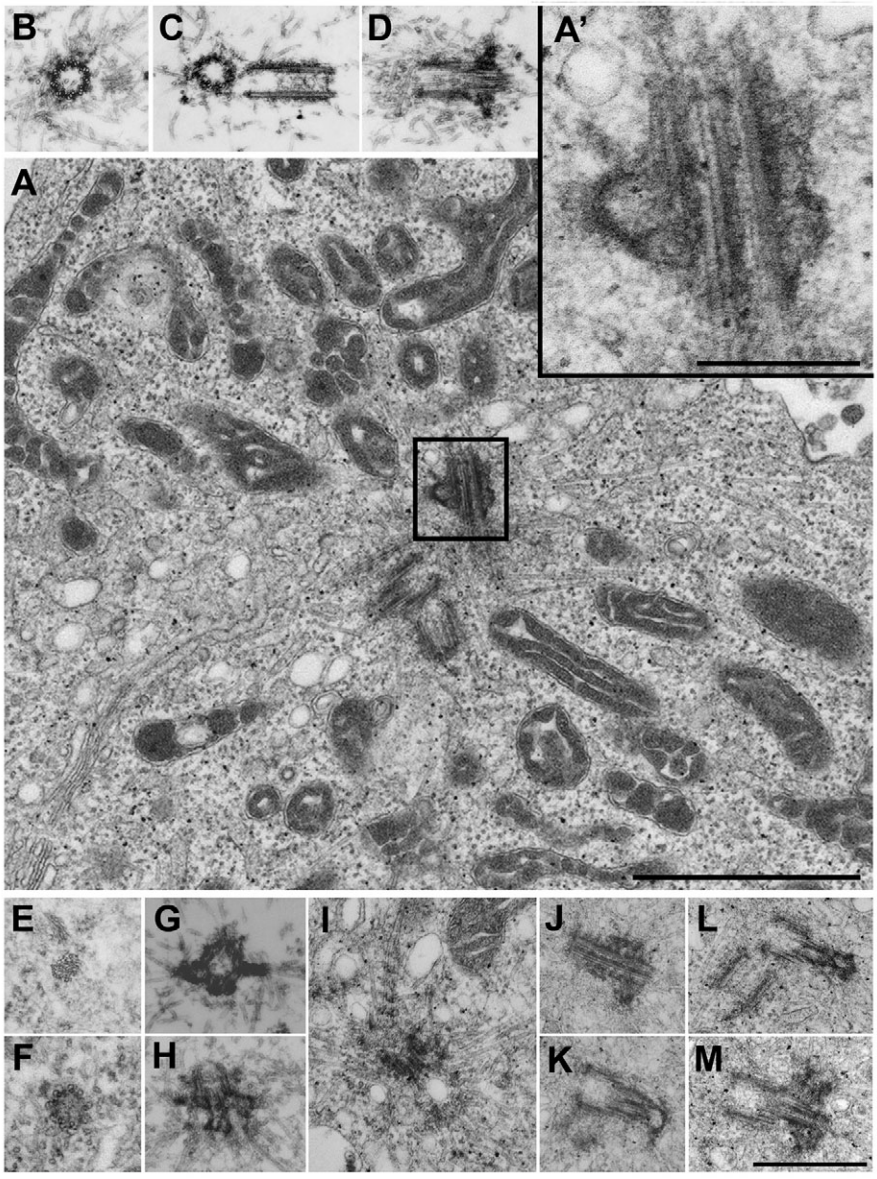

Fig. 7. Abnormal centrosomes detected by electron microscopy in HU-treated HCT116 cells. In A, the centrosome contains three centrioles with various degrees of morphological abnormality. A' shows the area outlined in A at a high magnification. Note the presence of cytoplasmic microtubules emanating from the abnormal centrosome and of mitochondria aligning conspicuously with the microtubules. (B-D) Normal profiles of centrioles with nine-triplet microtubules (B), orthogonal configuration of two centrioles (C), and electron dense appendages at the distal end (D). (E-M) Centrioles with disorganized microtubules and displaced appendages. Images shown in B to I were obtained from cells extracted with detergent. Scale bars: 1 μm (A) and 0.2 μm (A',B-M).

diverse among cancers and cancer cells with different origins. In fact, we have not yet identified the γ-tubulin-specific abnormal structures outside of HCT116 cells. Because cells become transformed through different pathways, it is probable that a wide range of modified gene expression might cause various centrosomal and centriolar alterations unique to each cell type.

In cycling HCT116 cells, aberrant centrioles are detected in 5-10% of the total cell population. The majority of cells are apparently normal in controlling the total number of centrosomes serving as a functional microtubule-organizing center during cell cycle. Thus the question remains as to the origin and continuity of the abnormal centriole. It has been reported that HCT116 cells show relatively normal karyotypes (Masramon et al., 2000; Roschke et al., 2002). We have also confirmed stable karyotypes of the HCT116 cell lines used in the current study (our unpublished results), suggesting that the formation of γ-tubulin-specific structures in 5-10% of the total cell population might not be due to karyotype diversity. p53 has been implicated to be involved in regulation of centriole and centrosome replication (Fukasawa et al., 1996). The number of both centrosomes and abnormal centrioles increases in $p53^{+/+}$ and in $p53^{-/-}$ cells during HU treatment. It was noted that abnormal centrioles are more abundant in $p53^{+/+}$ than $p53^{-/-}$ cells, where centrosomes labeled by both γ-tubulin and Cep135 are numerous (Fig. 3). Because p53 represses Plk4 expression (Li et al., 2005), it is probable that the elevated level of Plk4 proteins results in the formation of more normal centrioles and centrosomes in $p53^{-/-}$ cells than $p53^{+/+}$ cells.

γ-Tubulin in the centriole and centriogenesis

Several lines of evidence have indicated that the γ-tubulin-containing structures identified in HCT116 cells correspond to aberrant centrioles: (1) the fibers resemble centriolar microtubules by thin section electron microscopy; (2) centrin, a widely-used centriolar marker, is present in the structures; (3) the structures are also associated with non-tyrosinated α-tubulin, which is known to be preferentially localized at the centriole; (4) like centriolar microtubules, tubules forming the γ-tubulin-specific structures are stable by nocodazole treatment; (5) nocodazole treatment results in inhibition of the growth of not only centrioles but also γ-tubulin-containing fibers; and (6) as with centrioles, the abnormal structure does not support the formation of cytoplasmic microtubule assembly.

Table 2. Fluorescence intensity of Plk4 at the centrosome in HCT116 cells

HCT116 cells	HU treatment[1]	Counted cells[2]	Fluorescence intensity[3] Abnormal centrosomes	
			–	+
$p53^{+/+}$	+	9	318.5	313.7
$p53^{+/+}$	+	82	496.5	470.0
$p53^{+/+}$	–	13	543.9	467.6
$p53^{-/-}$	+	21	669.0	644.0

[1]Cells were treated with or without hydroxyurea for 3 days.
[2]Numbers of paired cells (+/– γ-tubulin-specific abnormal centrosomes) counted.
[3]Mean intensity (arbitrary units).

One of the unique features of the abnormal centrioles is that they are associated with only a subset of centrosomal components. One of these is γ-tubulin, which is a well-known PCM component responsible for initiation of microtubule polymerization onto the centrosome (Moritz and Agard, 2001). Because molecules required for construction of new centrosomes are transported along cytoplasmic microtubules (Kubo et al., 1999; Young et al., 2000; Dammermann and Merdes, 2002; Hames et al., 2005), γ-tubulin and γ-TuRC might facilitate centrosome assembly by organizing microtubule tracts along which building blocks are carried to the center where new centrioles and centrosomes are formed. We are puzzled by the fact that, despite existing as the γ-TuRC (Fig. 2), γ-tubulin in the abnormal centriole is incapable of microtubule polymerization (Fig. 5). Perhaps, γ-tubulin and γ-TuRC does not acquire the proper microtubule-nucleating capability by attaching to the disorganized centriolar structures. Alternatively, the microtubule-nucleating activity of γ-tubulin and γ-TuRC at the centriole might be restricted to assembling only microtubules of the centriolar wall. Indeed centriole and basal body formation was blocked and/or severely damaged by suppression of γ-tubulin in diverse organisms (Ruiz et al., 1999; Raynaud-Messina et al., 2004; Shang et al., 2002; Dammermann et al., 2004; Haren et al., 2006).

Centriole assembly has been shown to be divided into two steps: formation of the precursor template and organization of nine-triplet microtubules of the centriolar barrel (Pelletier et al., 2006). In *C. elegans*, a signal mediated by a kinase ZYG-1 causes the recruitment of several key molecules, including SAS6, for construction of an intermediate structure. Thereafter, centriolar microtubules are organized onto the hollow precursor called a central tube, in a SAS4-dependent manner. This order of ZYG-1/SAS6/SAS4 in the regulatory hierarchy of centriole biogenesis seems conserved in mammalian cells (Kleylein-Sohn et al., 2007). This was further supported by our observation that expression of Plk4, but not the downstream regulator SAS6, can efficiently reduce the number of aberrant centrioles in HCT116 cells (Fig. 9). It is, therefore, highly plausible that

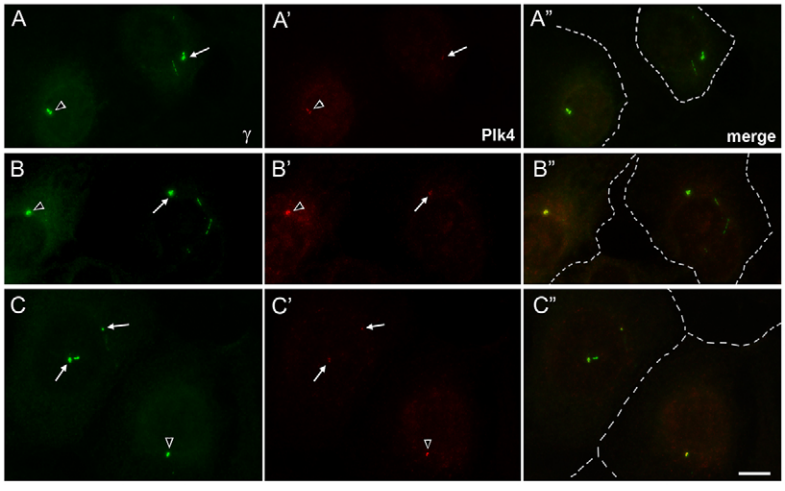

Fig. 8. Quantitative analysis of endogenous Plk4 at the centrosome in cells with and without γ-tubulin-containing abnormal structures. Cells were double-stained with anti-γ-tubulin (A-C) and Plk4 antibodies (A'-C'), and merged images are shown in A"-C". Dotted lines indicate cell borders. Centrosomal positions are indicated by arrows in cells expressing abnormal centrosomes and by arrowheads in cells without γ-tubulin-specific structures. Scale bar: 10 μm.

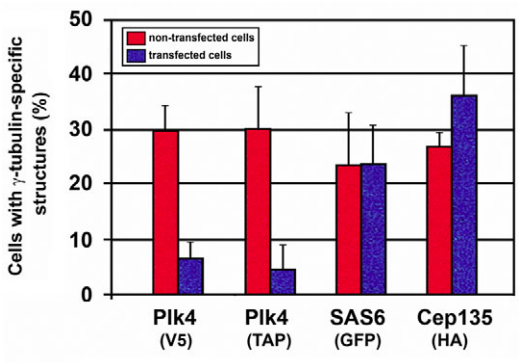

Fig. 9. Frequency histograms of cells containing abnormal centrosomes revealed by γ-tubulin staining. $p53^{+/+}$ HCT116 cells were transfected with constructs encoding V5-Plk4, TAP-Plk4, GFP-SAS6 and HA-Cep135, and further incubated for 2-3 days in the presence of 2 mM HU. Non-transfected (red columns) and transfected cells (blue columns) were counted on the same coverslips. Plk4-expressing cells were first immunostained with monoclonal anti-tag and polyclonal Cep135 antibodies. After recording their staining pattern, the same cells were further probed with monoclonal γ-tubulin antibodies to determine the presence or absence of abnormal centrioles in a total of 500-1000 cells. Centrosomes and γ-tubulin-specific structures in cells expressing SAS6 and Cep135 were calculated by staining with anti-tag and anti-γ-tubulin antibodies, followed by re-staining with anti-Cep135 antibodies. Formation of abnormal centrioles is blocked by expression of Plk4, but not SAS6 and Cep135.

insufficient Plk4 results in the formation of aberrant centrioles in these cells. Nakazawa and coworkers have demonstrated that SAS6 is a component of the central part of the cartwheel located at the proximal end of the *Chlamydomonas* basal body (Nakazawa et al., 2007). In collaboration with Bld10p, which is present in the spoke head to connect the cartwheel to the A-tubule of nine-triplet microtubules (Matsuura et al., 2004), these two proteins play an important role in establishment of the ninefold symmetry in the centriole and basal body (Hiraki et al., 2007; Nakazawa et al., 2007). A mammalian homolog of Bld10p is Cep135 that has already been shown to be essential for centriole and centrosome formation (Ohta et al., 2002; Kleylein-Sohn et al., 2007). Like SAS6, Cep135 is missing in the abnormal centriole in HCT116 cells, and ectopic expression of these molecules causes stimulation of the abnormal centriole formation, rather than suppression (Fig. 9). This is in good agreement with previous observations that overexpression of SAS6 results in the induction of abnormal tube-like structures in *Drosophila* embryos (Rodrigues-Martins et al., 2007). It is interesting to know whether targeting of Cep135 to nascent centrioles is controlled by an upstream regulator of Plk4, as in the case of SAS6.

Because centriolar microtubules are able to be formed in Bld10p- and SAS6-depleted cells (Nakazawa et al., 2007; Rodrigues-Martins et al., 2007), the aberrant centrioles lacking the ninefold symmetrical configuration in HCT116 cells probably represent centriolar microtubules assembled independently of the precursor template. γ-Tubulin in the aberrant centrioles might be important for nucleation, elongation and stabilization of cylindrical microtubules (Dammermann et al., 2008). Independence of microtubule assembly from the precursor formation further suggests the possibility that γ-tubulin and γ-TuRC is targeted to a nascent centriole through a pathway different from Plk4-SAS6-SAS4. It is thus interesting to note that the aberrant centriole is associated with centrin and PCM1, in addition to γ-tubulin. These two components are known to form centriolar satellites that are highly motile and move along cytoplasmic microtubules (Baron and Salisbury, 1988; Baron et al., 1994; Kubo et al., 1999; Kuriyama et al., 2007). PCM1 has also been implicated to be involved in the trafficking of other centrosomal proteins (including centrin, Nek2, pericentrin, ninein and C-Nap1) to the centrosome in a microtubule-dependent manner (Dammermann and Merdes, 2002; Hames et al., 2005). It is, therefore, plausible that γ-tubulin might be recruited to the initial site of centriole assembly through its interaction with PCM1 or centrin-containing structures.

In summary, human cancer cells induce centrioles and centrosomes that are abnormal in their structure and molecular composition. Further analysis of these aberrant centrioles will allow us to gain deeper insight into the mechanism and regulation of centriole biogenesis.

Materials and Methods

Cell culture and drug treatment

Human colorectal cancer cells, HCT116, were obtained from ATCC (Manassas, VA). HCT116 cells with knockout p53 ($p53^{-/-}$) and their parent cell line ($p53^{+/+}$) were a kind gift of Bert Vogelstein (Johns Hopkins University, Baltimore, MD) (Bunz et al.,

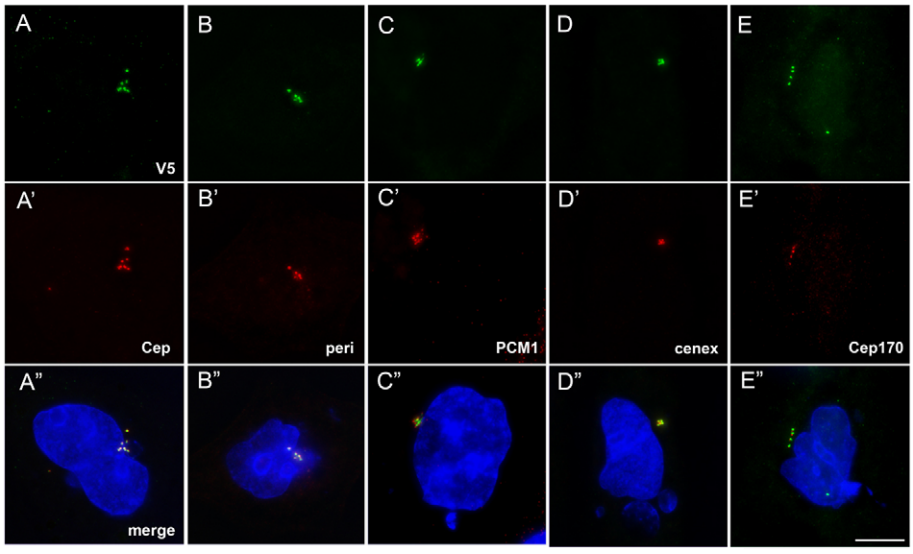

Fig. 10. Centrosomal proteins are targeted to the multiple centrosomes induced by overexpression of ectopic Plk4 in HCT116 cells. After transient transfection with V5-Plk4, cells were treated with HU for 3 days, and then double immunostained with monoclonal anti-V5 antibodies (A-E) and polyclonal anti-Cep135 (A'), pericentrin (B'), PCM1 (C'), cenexin (D'), and Cep170 (E') antibodies. Merged images are shown in A" to E". Unlike abnormal centrosomes labeled with a subset of anti-centrosomal antibodies, Plk4-induced centrosomes recruit other centrosome molecules. Scale bar: 10 μm.

1998). Cells were grown as monolayers in McCoy 5A medium containing 10% fetal calf serum. Drug treatment was done by incubation of cells with hydroxyurea (HU) and nocodazole at final concentrations of 2 mM and 1 μg/ml, respectively.

Immunofluorescence staining and quantification of Plk4 fluorescence

Anti-centrosomal antibodies used in this study include γ-tubulin (Sigma-Aldrich), Cep135 (Ohta et al., 2002), pericentrin (Covance), centrin2/3 from Jeffrey Salisbury (Mayo Clinic, Rochester, MN), PCM1 (Bethyl Laboratories, and a gift of Ron Balczon, University of Alabama, Mobile, AB), Cep170 (Bethyl Laboratories), C-Nap1 (BD Biosciences), TACC2 from Jordan Raff (Gurdon Institute, Cambridge, UK), cenexin (Soung et al., 2006), SAS6 and SAS4/CPAP from Pierre Gönczy (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland), GCP2, GCP4, and Nedd1 from Andreas Meredes (University of Toulouse, Toulouse, France), Nek2 (AbCam), ninein from Jerome B. Rattner (University of Calgary, Calgary, Canada), CTR453 from Michel Bornens (Curie Institute, Paris, France), and Aurora-A from Masashi Kimura (Gifu University, Gifu, Japan). Plk4 antibodies were raised against a C-terminal domain of human Plk4 (M.A. and I.H., unpublished results). Polyclonal antibodies to tyrosinated and non-tyrosinated α-tubulin were a gift of Gregg Gundersen (Colombia University, New York, NY).

For immunostaining, methanol fixed cells were rehydrated with 0.05% Tween-20-containing PBS and incubated with primary antibodies for 30 minutes at 37°C. After washing away excess antibodies, cells were treated further with secondary antibodies (fluorescein-conjugated anti-mouse IgG plus IgM, and Texas red-conjugated anti-rabbit IgG antibodies, Jackson ImmunoResearch). Microscopic observation was made using a Nikon eclipse microscope using ImagePro and SlideBook softwares. For statistic analysis, 150-250 centrosomes were examined in each sample and experiments were repeated five to ten times.

Endogenous Plk4 at the centrosome was quantified by digital analysis of immunofluorescence intensity using the SlideBook 4.1 program. HU-treated and untreated HCT116 cells were double stained with polyclonal anti-Plk4 and monoclonal anti-γ-tubulin antibodies. After mounting on a glass slide, the samples were kept in dark for several days to ensure stabilization and equilibration of the mounting medium. Images of an area where two cells with and without abnormal centrosomes were side-by-side were captured under identical conditions (100 millisecond exposure with constant bin numbers). In some cases, images were acquired by merging five to seven slices of 0.1-0.15 μm sections. Centrosomal spots were designated by creating identical masks and the mean intensity of each spot was measured to compare the Plk4 intensity between the two types of cells. Experiments were repeated four times by preparing immunostaining samples independently.

Expression of Plk4, SAS6, and Cep135

cDNA encoding full-coding sequences of human Plk4 was obtained from Invitrogen (image clone ID 5273226), and SAS6 was cloned from HeLa cDNA. These were cloned into pDONR221 (Invitrogen) and then transferred to pcDNA3.1/nV5-DEST and pcDNA-DEST53 (GFP N-terminus fusion; Gateway system, Invitrogen). Plk4 was also cloned into the TAP expression vector with a leader sequence of Flag-His-calmodulin-binding site at the N-terminus (a gift of Y. Terada, Waseda University, Tokyo, Japan). HA-tagged Cep135 cloned into the pCMV vector were prepared as described previously (Ohta et al., 2002). To introduce exogenous proteins, HCT116 cells cultured on an etched coverslip (Bellco Biotechnology) were transiently transfected using either the nucleotransfection kit (Amaxa) or Lipofectamine 2000 (Invitrogen) according to manufacture's protocols. Right after transfection, cells were treated with 2 mM HU and further cultured for 2-3 days before fixation. For detection of tagged Plk4, cells were immunostained with monoclonal anti-V5 (Clontech) or Flag antibodies (Sigma-Aldrich) along with polyclonal Cep135 antibodies. After recording individual transfected cells, the coverslips were soaked in PBS-Tw20 and carefully removed from a slide glass for re-probing with monoclonal anti-γ-tubulin antibodies to determine the presence or absence of abnormal centrioles in those cells. The localization of Plk4 at abnormal centrioles was also directly analyzed by treatment of cells with monoclonal anti-V5 and polyclonal γ-tubulin antibodies.

Localization of GFP-SAS6 and HA-Cep135 was analyzed by staining with anti-γ-tubulin and polyclonal anti-HA (AbCam)/monoclonal γ-tubulin antibodies, respectively. The same coverslips were re-stained as above with polyclonal anti-Cep135 to score the number of cells with abnormal centrioles. Frequency histograms were constructed by counting 100-200 cells in each experiment, which was repeated three to four times.

Electron microscopy

HCT116 cells treated with or without HU for 3 days were fixed with 2.5% glutaraldehyde in 100PEM (100 mM Pipes at pH 7.0, 1 mM EGTA, 1 mM $MgCl_2$) for 30 minutes at room temperature. In some case, we extracted cells briefly before fixation in a medium containing 0.1% Triton X-100. Cells were washed with 100PEM, postfixed in 1% OsO_4 for 30 minutes, and then block-stained in 2% uranyl acetate for 1 hour. After dehydration through a series of ethanol solutions, samples were infiltrated with and then embedded in Embed 812 plastic (Electron Microscopy Sciences). Specimens of thin sections were examined using a Philips CM12 transmission electron microscope.

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