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ORIGINAL ARTICLE

Centriole overduplication through the concurrent formation of multiple daughter centrioles at single maternal templates

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Abnormal centrosome numbers are detected in virtually all cancers. The molecular mechanisms that underlie centrosome amplification, however, are poorly characterized. Based on the model that each maternal centriole serves as a template for the formation of one and only one daughter centriole per cell division cycle, the prevailing view is that centriole overduplication arises from successive rounds of centriole reproduction. Here, we provide evidence that a single maternal centriole can concurrently generate multiple daughter centrioles. This mechanism was initially identified in cells treated with the peptide vinyl sulfone proteasome inhibitor Z-L_3VS. We subsequently found that the formation of more than one daughter at maternal centrioles requires cyclin E/cyclin-dependent kinase 2 as well as Polo-like kinase 4 and that overexpression of these proteins mimics this phenotype in the absence of a proteasome inhibitor. Moreover, we show that the human papillomavirus type 16 E7 oncoprotein stimulates aberrant daughter centriole numbers in part through the formation of more than one daughter centriole at single maternal templates. These results help to explain how oncogenic stimuli can rapidly induce abnormal centriole numbers within a single cell-division cycle and provide insights into the regulation of centriole duplication.
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Introduction

Centrosomes are the major microtubule organizing centers in most animal and human cells (Bornens, 2002; Nigg, 2004). The single centrosome duplicates precisely once before mitosis through mechanisms that remain to be understood. In a normal cell cycle, each of the two centrioles that make up a G1 phase centrosome is thought to function as a template for the formation of precisely one newly synthesized daughter centriole (Sluder, 2004). The restriction to a single round of centriole duplication per cell division cycle (Tsou and Stearns, 2006a) contributes to the prevention of aberrant centrosome numbers, multipolar mitoses and chromosomal instability (Salisbury *et al*., 1999; Brinkley, 2001; Nigg, 2002).

In contrast to normal cells, tumor cells frequently contain abnormal centrosome numbers (Lingle *et al*., 1998; Pihan *et al*., 1998). Although various oncogenic stimuli have been found to provoke abnormal centrosome and centriole numbers *in vitro*, relatively little is known about the precise mechanisms of their origin. In principal, aberrant centrosome numbers can arise through cell division failure or a genuine disruption of the centriole duplication cycle itself (Nigg, 2002; Duensing, 2005). Only the second category should be considered as centriole overduplication and it has been proposed that overduplication can be distinguished from centriole accumulation through the presence of excessive numbers of immature daughter centrioles (Guarguaglini *et al*., 2005).

The premise that each maternal centriole serves as a template for the formation of one and only one daughter centriole during each cell division cycle does not readily explain the rapid induction of aberrant centriole numbers detected under certain experimental conditions. The human papillomavirus type 16 (HPV-16) E7 oncoprotein has been found to rapidly stimulate supernumerary centrosomes in primary human cells and tumor cell lines (Duensing *et al*., 2000, 2004, 2006a). HPV-16 E7 binds and degrades the retinoblastoma tumor suppressor protein (pRB), inactivates cyclin-dependent kinase inhibitors such as $p21^{Cip1}$ and causes a deregulated expression of cyclin E (McIntyre *et al*., 1996; Martin *et al*., 1998) thereby creating an aberrant S phase-like state that supports viral replication (Munger *et al*., 2001). How these activities can trigger centriole overduplication within a time period that corresponds to approximately a single cell-division cycle (Duensing *et al*., 2004, 2006a) in currently unknown.

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In the present report, we provide evidence for centriole overduplication through the concurrent formation of multiple daughter centrioles at single maternal centrioles in human cells. This mechanism was first identified using the proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L_3VS) but further analyses showed a requirement for cyclin E/cyclin-dependent kinase 2 (CDK2) and Polo-like kinase 4 (PLK4). Combined overexpression of these proteins in fact mimicked the Z-L_3VS-induced phenotype in both tumor cells and in non-transformed cells, even in the absence of a proteasome inhibitor. Finally, we discovered that HPV-16 E7-induced centriole overduplication also involves the formation of more than one daughter centriole per maternal centriole. These findings help to explain the rapid induction of abnormal centriole numbers by this viral oncogene and provide evidence for a role of proteolysis in the regulation of centriole duplication.

Results

Induction of abnormal centriole duplication by the proteasome inhibitor Z-L_3VS

Components of the ubiquitin–proteasome machinery localize to centrosomes and ubiquitin-mediated proteolysis has been implicated in the regulation of centriole duplication (Freed *et al*., 1999; Gstaiger *et al*., 1999; Wigley *et al*., 1999; Fabunmi *et al*., 2000; Nakayama *et al*., 2000; Wojcik *et al*., 2000). We performed a morphological screen for aberrant centriole duplication events using a panel of proteasome inhibitors in U-2 OS cell populations stably expressing green fluorescent protein (GFP)-tagged centrin (U-2 OS/centrin-GFP) to visualize individual centrioles. Centrin-GFP signals in dimethylsulfoxide (DMSO)-treated cells typically consisted of large centrin-GFP dots (indicating the maternal centriole) with a single smaller centrin-GFP dot (indicating the daughter centriole) in close proximity to the maternal centriole (Figure 1a). Treatment of cells for 48 h with the proteasome inhibitor Z-L_3VS (Bogyo *et al*., 1997) was found to induce multiple centrin-GFP dots in a significant proportion of cells (Figure 1a and b). Centrin-GFP dots were frequently arranged in a 'flower'-like pattern with a large centrin-GFP dot in the center surrounded by multiple smaller centrin-GFP dots (Figure 1a).

Quantification of cells with more than one small centrin-GFP dot adjacent to a large centrin-GFP dot revealed a 55.7-fold increase in populations treated with 1 μM Z-L_3VS for 48 h (39%; $P \leqslant 0.0001$) compared with DMSO-treated controls (0.7%; Figure 1b). This increase was dose-dependent since cell populations treated with lower concentrations of Z-L_3VS contained a reduced fraction of cells with aberrant daughter centriole formation (data not shown). An increase of cells with excessive daughter centriole formation, albeit to a lesser extent, was also detected in cells treated with the proteasome inhibitors MG262 or MG132 but not in cells treated with epoxomicin or lactacystin. The Z-L_3VS-induced aberrant formation of daughter centrioles was significantly less frequently detected in HeLa or T98G cells and was absent in IMR-90 normal human fibroblasts (data not shown).

To determine why U-2 OS cells but not HeLa or T98G cells showed excessive daughter centriole synthesis, we performed a series of additional experiments (see Supplementary Information and Supplementary Figures 1–3 for details). In contrast to HeLa or T98G cells, U-2 OS/centrin-GFP treated with Z-L_3VS showed a marked increase of cyclin E protein expression and an accumulation in G2 phase of the cell division cycle. However, accumulation in G2 phase was not required for excessive daughter centriole formation at maternal templates and several lines of evidence indicate that this process begins before cells reach G2 (see Supplementary Information and Supplementary Figures 1 and 2).

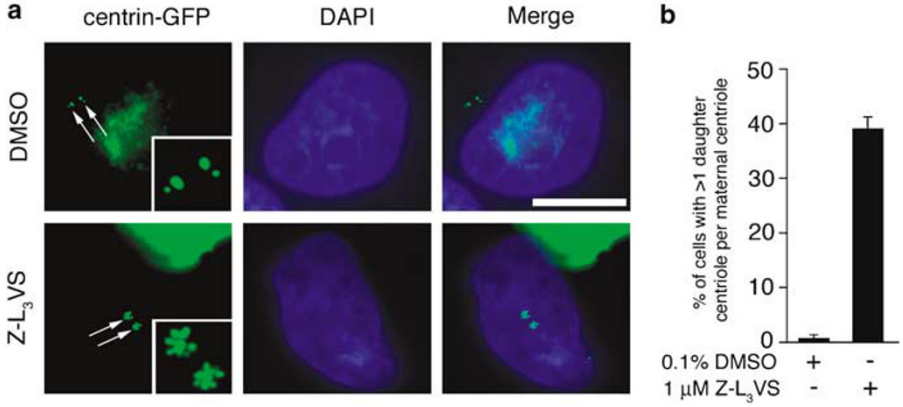

Figure 1 Aberrant centriole configuration following treatment with the proteasome inhibitor Z-L_3VS. (**a**) Fluorescence microscopic analysis of U-2 OS cells stably expressing centrin-GFP to visualize centrioles (arrows; inserts) after either treatment with 0.1% DMSO (top panels) or 1 μM Z-L_3VS (bottom panels) for 48 h. Nuclei stained with DAPI. Scale bar indicates 10 μm. (**b**) Quantification of U-2 OS/centrin-GFP cells with more than one daughter centriole per maternal centriole after treatment with either 0.1% DMSO or 1 μM Z-L_3VS for 48 h. Each bar represents mean + s.e. of at least three independent experiments with a minimum of 100 cells counted per experiment.

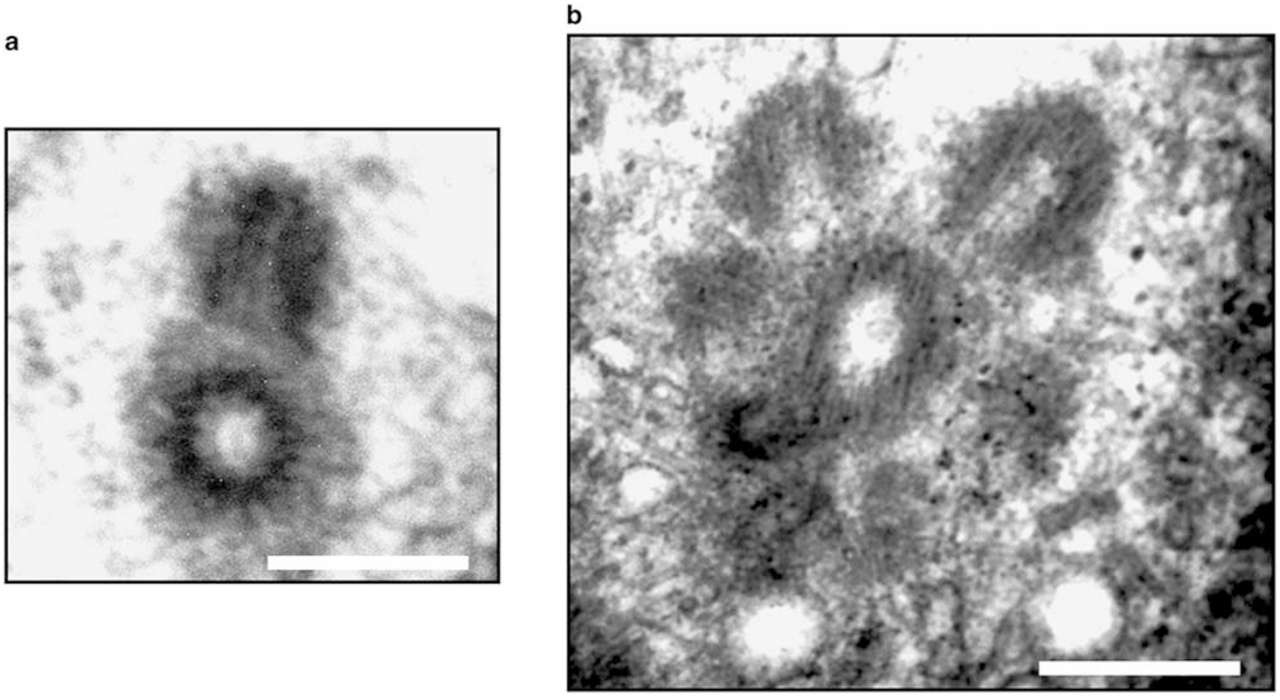

Figure 2 Concurrent formation of multiple daughter centrioles at a single maternal centriole following Z-L_3VS. Electron microscopic analysis of U-2 OS/centrin-GFP cells treated with 0.1% DMSO (**a**) or treated with 1 μM Z-L_3VS for 48 h (**b**). Scale bars indicate 500 nm.

Overexpression of cyclin E in Z-L_3VS-treated HeLa or T98G cells, which showed no significant response to Z-L_3VS alone, could induce aberrant centriole duplication at single maternal templates, albeit not to the extent detected in U-2 OS/centrin-GFP cells (Supplementary Figure 3). These results suggest that cyclin E may not be solely responsible for aberrant excessive daughter centriole formation and that changes in the expression level of additional proteins are likely to be involved.

Ultimately, we sought to determine whether the aberrant centrin-GFP dots were in fact intact daughter centrioles. We examined over 100 Z-L_3VS-treated U-2 OS cells by electron microscopy. A total of 24 centrioles were analysed in serial sections of which three had multiple daughter centrioles. Figure 2 demonstrates multiple daughter centrioles radially arranged around a central maternal centriole in comparison to a normal mother–daughter centriole pair.

Taken together, our results suggest that Z-L_3VS leads to an accumulation of proteins in U-2 OS cells that trigger an excessive daughter centriole formation at single maternal templates.

Aberrant centriole duplication requires cyclin E/CDK2 and PLK4

Previous studies have demonstrated a role of cyclin E, cyclin A, CDK2 and PLK4 in centriole overduplication (Hinchcliffe *et al*., 1999; Lacey *et al*., 1999; Matsumoto *et al*., 1999; Meraldi *et al*., 1999; Bettencourt-Dias *et al*., 2005; Habedanck *et al*., 2005; Duensing *et al*., 2006b). To analyse the role of these proteins in Z-L_3VS-induced abnormal daughter centriole formation in greater detail, small interfering RNA (siRNA) experiments (Elbashir *et al*., 2001) were performed (Figure 3a). As expected, U-2 OS/centrin-GFP cells transfected with control siRNA duplexes and treated with 1 μM Z-L_3VS for 48 h showed excessive daughter centrioles at maternal centrioles in 46.6% of cells in comparison to 1.3% in DMSO-treated controls ($P \leqslant 0.0001$; Figure 3b and c). The proportion of cells showing this configuration was significantly reduced in cells transfected with siRNA duplexes targeting cyclin E1 (21.9%; $P \leqslant 0.0001$), or, to a lesser extent, cyclin E2 (36.3%; $P \leqslant 0.05$). Depletion of CDK2 caused a significant decrease of cells with aberrant centriole formation (29.9%; $P \leqslant 0.005$), whereas knockdown of cyclin A2 had no effect (Figure 3c).

Importantly, PLK4 has recently not only been implicated in centriole overduplication (Bettencourt-Dias *et al*., 2005; Habedanck *et al*., 2005) but has furthermore been shown to trigger a deposition of centriole precursor material in a rosette-like arrangement around maternal centrioles (Habedanck *et al*., 2005). Since the aberrant centriole configuration observed here in Z-L_3VS-treated cells was highly reminiscent of the rosette-like pattern of centriolar precursor material in cells overexpressing PLK4, we tested whether depletion of PLK4 interferes with the induction of this phenotype. We found that siRNA duplexes targeting PLK4 abolished the abnormal centriole formation induced by Z-L_3VS (2%; $P \leqslant 0.0001$; Figure 3c).

Taken together, these results indicate that both cyclin E/CDK2 and PLK4 are required for the formation of multiple daughter centrioles at maternal templates.

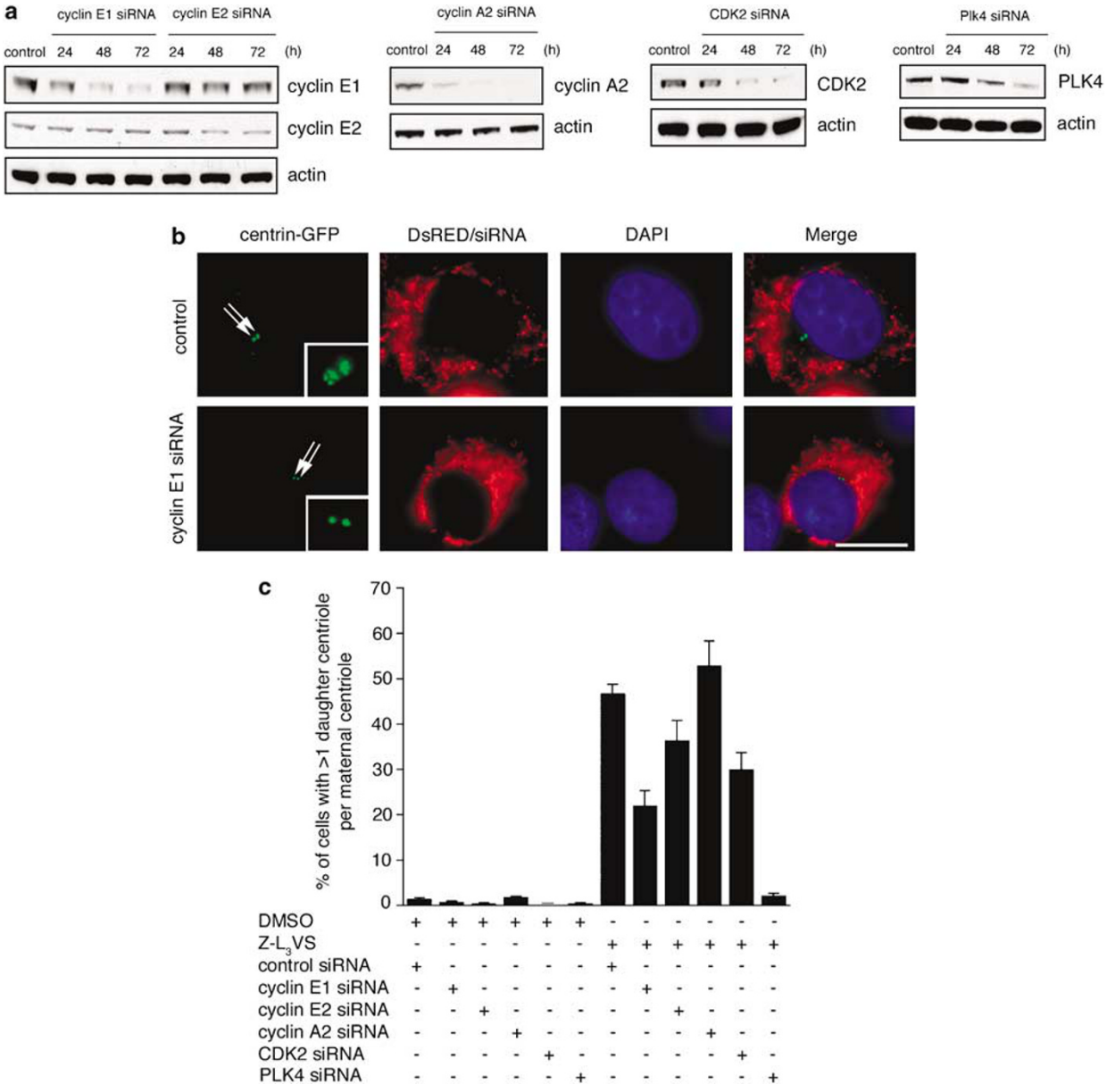

Figure 3 Z-L_3VS-induced formation of excessive daughter centrioles at maternal centrioles requires cyclin E, CDK2 and PLK4. (**a**) Immunoblot analyses of U-2 OS/centrin-GFP cells transfected with either control siRNA duplexes (control) or siRNAs targeting cyclin E1, cyclin E2, cyclin A2, CDK2 or PLK4 for the indicated time intervals. (**b**) Fluorescence microscopic analysis of U-2 OS/centrin-GFP cells transfected with control siRNA duplexes (top panels) or siRNAs targeting cyclin E1 (bottom panels) following treatment with 1 μM Z-L_3VS for 48 h. Cells were co-transfected with DsRED fluorescent protein as transfection marker. Nuclei stained with DAPI. Scale bar indicates 10 μm. (**c**) Quantification of U-2 OS/centrin-GFP cells transfected with the indicated siRNAs followed by either control treatment with 0.1% DMSO or 1 μM Z-L_3VS for 48 h. Each bar represents mean + s.e. of at least three independent experiments with a minimum of 100 cells counted per experiment.

Overexpression of cyclin E/CDK2 and PLK4 stimulates the concurrent formation of multiple daughter centrioles at single maternal centrioles

We asked whether ectopic expression of cyclin E/CDK2 and/or PLK4 could aberrant daughter centriole formation in the absence of a proteasome inhibitor. Transient transfection of U-2 OS/centrin-GFP cells with cyclin E/CDK2 did not lead to a significant increase in the percentage of cells with excessive daughter centriole formation at maternal templates (Figure 4a), although in line with previous experiments (Duensing *et al*., 2004), a 2.2-fold increase in cells with more than four centrioles arranged in a random fashion was detected (data not shown). Overexpression of PLK4, however, caused a significant 17.9-fold increase of abnormal centriole formation at maternal centrioles from 1 to 17.9% ($P \leqslant 0.0005$) in agreement with previous results (Habedanck *et al*., 2005). This increase was further enhanced when cyclin E/CDK2 and PLK4 were expressed simultaneously as evidenced by the 27-fold

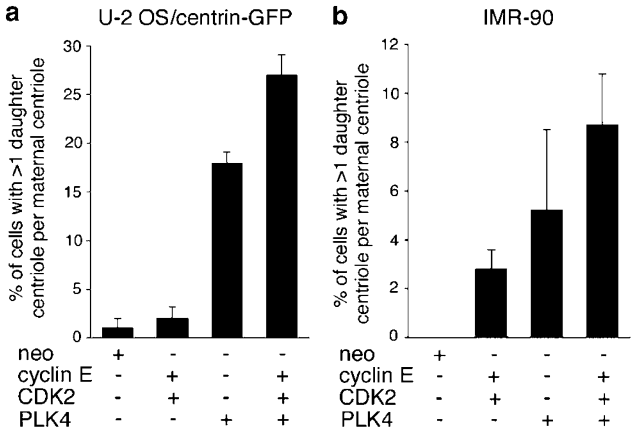

Figure 4 Overexpression of cyclin E/CDK2 and PLK4 stimulates the concurrent formation of multiple daughter centrioles at single maternal centrioles. (**a** and **b**) Quantification of U-2 OS/centrin-GFP or IMR-90 cells with more than one daughter centriole per maternal centriole at 48 h after transfection with empty vector (neo), cyclin E, CDK2 and PLK4 either alone or in combination. Each bar represents mean + s.e. of at least three independent experiments with a minimum of 50 cells counted per experiment.

increase of excessive daughter centriole formation (27%; $P \leqslant 0.0005$).

IMR-90 normal human fibroblasts did not show aberrant daughter centriole formation following treatment with Z-L_3VS. Nonetheless, ectopic expression of PLK4 either alone or in combination with cyclin E/CDK2 caused a strikingly similar phenotype after immunofluorescence staining for centrin with centrioles arranged in a 'flower'-like pattern. Overexpression of cyclin E/CDK2 resulted in an increase of cells with abnormal daughter centriole formation from 0% in controls to 2.8% ($P \leqslant 0.05$; Figure 4b). Cells transfected with PLK4 alone showed abnormal centriole formation in 5.2% of cells, whereas co-expression of cyclin E/CDK2 and PLK4 led to 8.7% of IMR-90 cells displaying more than one daughter centriole per maternal template ($P \leqslant 0.01$).

Taken together, these results show that overexpression of PLK4 can stimulate the formation of multiple daughter centrioles at maternal centrioles (Habedanck *et al*., 2005) and that cyclin E/CDK2 enhances this process. The cooperative effects between cyclin E/CDK2 complexes and PLK4 appear to be particularly important for aberrant daughter centriole formation in non-transformed cells.

Supernumerary daughter centrioles contribute to mitotic spindle pole formation

To determine whether supernumerary daughter centrioles are able to mature and to become functional, we assessed mitotic spindle pole abnormalities in U-2 OS/centrin-GFP cells treated with 1 μM Z-L_3VS in comparison to cells treated with 0.1% DMSO (Figure 5a). Cells were treated for 24 h, washed thoroughly and released into normal media for an additional 24 h. A significant decrease in normal bipolar mitotic spindle formation was detected from 80.5% in DMSO-treated controls to 20% in Z-L_3VS-treated populations ($P \leqslant 0.005$; Figure 5b). At the same time, a significant increase of multipolar mitoses from 5.6% in controls to 30.7% in Z-L_3VS-treated cells was detected ($P \leqslant 0.05$). Even more significant was the increase of pseudobipolar mitoses with multiple centrioles at each spindle pole of an apparently bipolar spindle (Brinkley, 2001). Such cell division abnormalities increased from 13.1% in controls to 48.7% in Z-L_3VS-treated cells ($P \leqslant 0.005$). These findings suggest that supernumerary centrioles that are induced by Z-L_3VS can become functional and contribute to mitotic spindle pole formation.

HPV-16 E7-induced centriole overduplication involves concurrent formation of more than one daughter at maternal centrioles

We next determined whether HPV-16 E7-induced centriole overduplication involves the formation of more than one daughter centriole per maternal centriole.

Immature daughters were occasionally seen to surround mature centrioles in HPV-16 E7-transfected U-2 OS/centrin-GFP cells (Guarguaglini *et al*., 2005). Cells transfected with HPV-16 E7 showed an increase of abnormal daughter centriole formation at maternal centrioles (mostly two) at 24 h after transfection from 1.1% in empty vector controls to 3.2% in HPV-16 E7-expressing cells ($P \leqslant 0.05$; Figure 6a and b). At 48 h after transfection, aberrant daughter centriole formation was 2.2-fold increased in HPV-16 E7-expressing cells (1.7%) when compared with controls (0.3%; $P \leqslant 0.05$). At the same time point, cells with a random arrangement of supernumerary centrioles was 2.2-fold increased from 3.6% in controls to 8% in HPV-16 E7-expressing cells ($P \leqslant 0.0001$).

Besides HPV-16 E7, treatment of cells with the ribonucleotide reductase hydroxyurea has been shown to stimulate a bona fide centriole overduplication (Guarguaglini *et al*., 2005). U-2 OS/centrin-GFP cells with more than one daughter per maternal centriole were detected after 24 h (7.6%), 48 h (9.5%) and 72 h (5.8%) treatment with hydroxyurea (Figure 6c). Such cells were increased when compared with mock-treated controls (0.3, 0 and 1%, respectively; differences at 24 h and 48 h were statistically significant, $P \leqslant 0.05$).

Taken together, these findings show that excessive daughter centriole formation at single maternal templates is involved in centriole overduplication induced by both, HPV-16 E7 or hydroxyurea.

Discussion

Here, we present evidence for a mechanism of centriole overduplication in human cells that involves the concurrent formation of more than one daughter centriole at single maternal centrioles. We first identified this mechanism in cell populations treated with the peptide vinyl sulfone proteasome inhibitor Z-L_3VS (Bogyo *et al*., 1997). In contrast to lactacystin, which did not induce this phenotype, the activities of Z-L_3VS include inhibition of

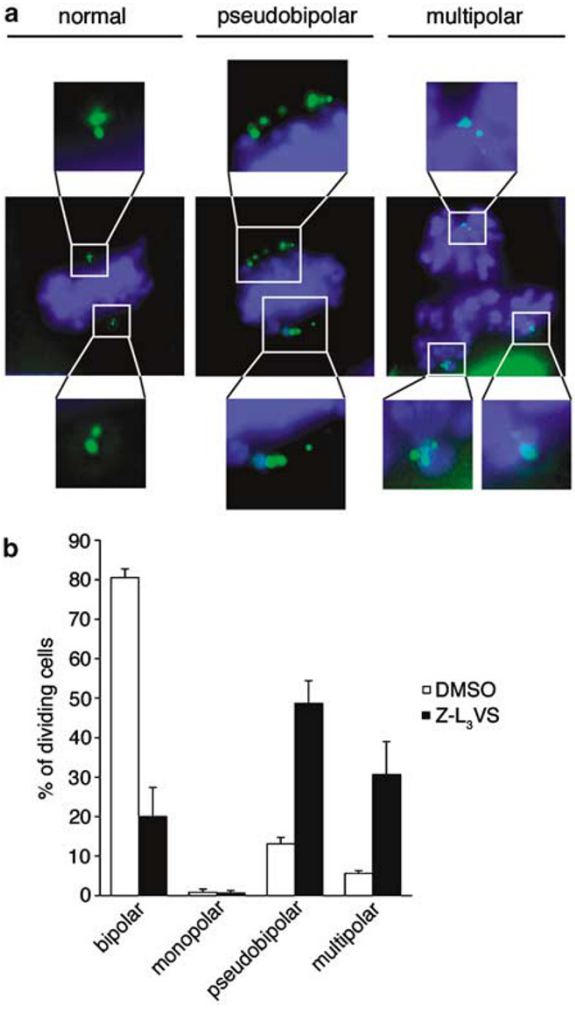

Figure 5 Aberrantly synthesized daughter centrioles can function as mitotic spindle poles. (**a**) Fluorescence microscopic analysis of mitotic U-2 OS/centrin-GFP cells. A normal bipolar metaphase is shown in comparison to a pseudobipolar metaphase and a multipolar (tripolar) anaphase. More than two images are merged for middle and right panels to show all centrioles. Chromosomes stained with DAPI. (**b**) Quantification of mitotic abnormalities in U-2 OS/centrin-GFP populations after treatment with 0.1% DMSO or 1 μM Z-L_3VS for 24 h and incubation in normal media for an additional 24 h. Only dividing cells were evaluated. Each bar represents mean + s.e. of three independent experiments with at least 100 mitotic cells counted per experiment.

the peptidylglutamyl-peptide hydrolysing (PGPH) activity of the 20S proteasome, and it is possible that such differences in the activity spectrum contribute to the distinct properties of the inhibitors tested.

U-2 OS cells treated with Z-L_3VS contained markedly higher protein levels of cyclin E and accumulated in G2 phase of the cell division cycle when compared to other tumor cell lines. Overexpression of cyclin E and simultaneous treatment with Z-L_3VS could induce the formation of more than one daughter at maternal centrioles in cells that did not respond to Z-L_3VS, albeit not to the level detected in Z-L_3VS-treated U-2 OS cells. These results suggest that changes in the expression of additional proteins may be important for the induction of aberrant daughter centriole formation. Importantly, overexpression of cyclin E/CDK2 together with PLK4 mimicked the centriole overduplication phenotype in the absence of Z-L_3VS and even in cell types in which this inhibitor has no effect. It is therefore possible that subtle changes in PLK4 protein levels and/or changes in its subcellular localization contribute to the observed phenotype.

It has recently been reported that centrioles undergo disengagement at the end of mitosis or early in G1 phase in a process that involves separase. This mechanism restricts centriole duplication to once per cell cycle (Tsou and Stearns, 2006b). Our finding that the formation of 'centriole flowers' occurred predominantly in cells that contained two large centrin-GFP dots suggests that maternal centrioles were disengaged and therefore in a duplication-competent state. However, we provide evidence that maternal centrioles are not limited to nucleate only one daughter centriole. Moreover, a simultaneous growth of two or more progeny is not only found in Z-L_3VS-treated cells but also in fact involved in centriole overduplication triggered by an oncogenic stimulus, the HPV-16 E7 oncoprotein.

The surprisingly rapid induction of aberrant centriole numbers by the HPV-16 E7 oncoprotein has been difficult to reconcile with the idea that centriole overduplication arises from successive rounds of daughter centriole reproduction. By showing that HPV-16 E7 can trigger the concurrent formation of more than one daughter centriole at maternal centrioles, we provide an explanation of how multiple daughter centrioles can be generated within a single S phase. The latter notion represents an important difference to a previous study showing the formation of two daughter centrioles adjacent to a single maternal centriole in *Drosophila* wing disc cells undergoing repeated rounds of S phase (Vidwans *et al*., 2003). Additional support for the notion that mammalian cells have the potential to generate multiple centrioles adjacent to preexisting centrioles stems from early studies on basal body formation in ciliated epithelia (Hagiwara *et al*., 2004). In such cells, most basal bodies form through acentriolar pathways, but flower-like structures similar to those described here have also been observed (Anderson and Brenner, 1971; Dirksen, 1971).

In conclusion, results shown here provide evidence for a concurrent formation of more than one daughter centriole at single maternal centrioles. These aberrantly synthesized daughter centrioles are functional and can contribute to mitotic spindle pole formation. Our results imply that the normal limitation to one and only one daughter per mother centriole and cell division cycle is not owing to inherent structural constraints or limited initiation sites but rather reflects regulation by cyclin E/CDK2 complexes and PLK4. Our results help to explain the rapid centriole overduplication induced by the HPV-16 E7 oncoprotein and, most likely, also other oncogenic insults. Further experiments to elucidate how cyclin E/CDK2 and PLK4 cooperate will provide important insights into the regulation of the centriole duplication cycle.

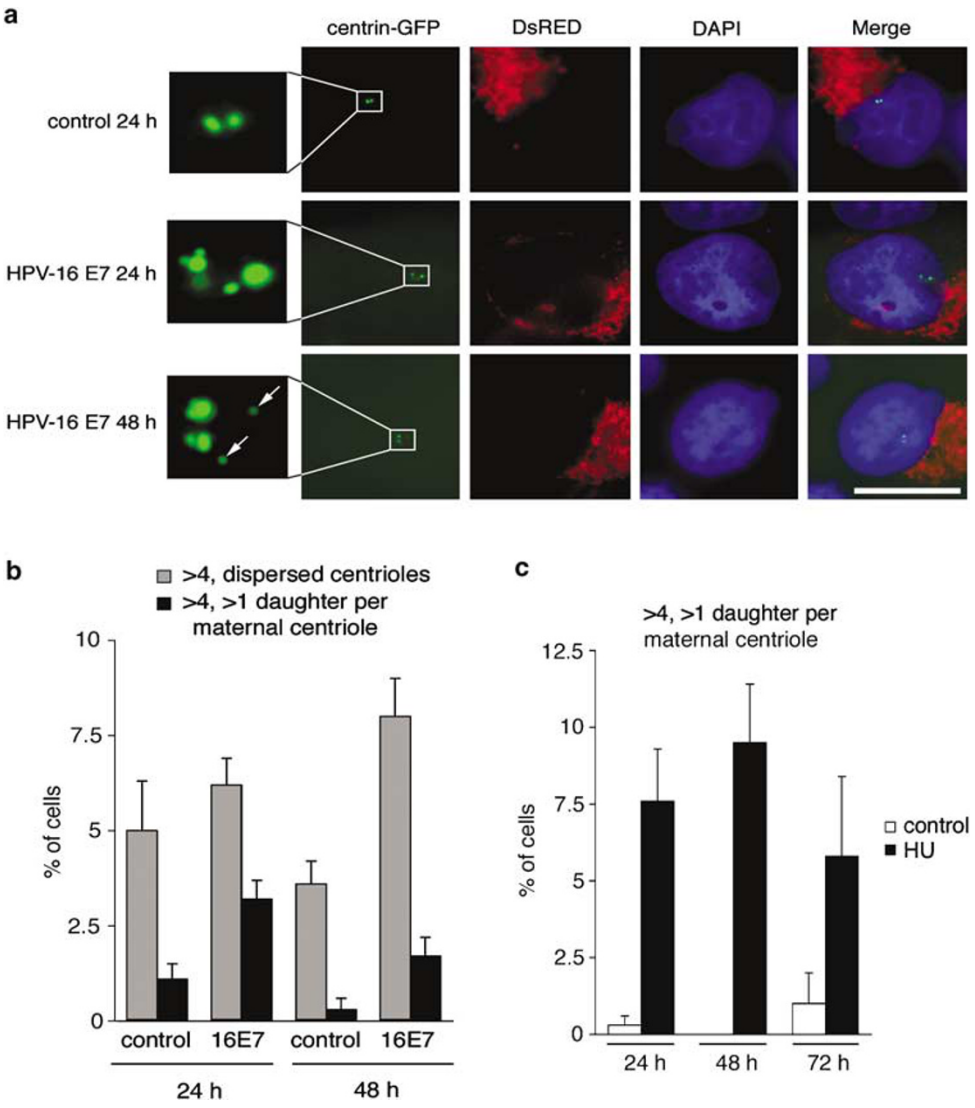

Figure 6 HPV-16 E7 rapidly stimulates the concurrent formation of more than one daughter centriole at maternal templates. (**a**) Fluorescence microscopic analysis of U-2 OS/centrin-GFP cells transiently transfected with either empty vector (control) or HPV-16 E7 after 24 or 48 h. Note the formation of two daughters at a single maternal centriole after 24 h in a HPV-16 E7-transfected cell (middle panels, insert). After 48 h, the majority of cells with abnormal centriole numbers showed a more dispersed arrangement (bottom panels, insert, arrows). Cells were co-transfected with DsRED fluorescent protein as transfection marker. Nuclei stained with DAPI. Scale bar indicates 10 μm. (**b**) Quantification of U-2 OS/centrin-GFP cells with more than four centrioles in a random arrangement (gray bars) in comparison to cells with more than four centrioles and a concurrent formation of more than one daughter per maternal centriole (black bars). Cells were transfected with empty vector (control) or HPV-16 E7 for 24 or 48 h. (**c**) Quantification of U-2 OS/centrin-GFP cells with more than four centrioles and concurrent formation of more than one daughter centriole at a maternal centriole after treatment with 1 mM hydroxyurea for the indicated time intervals. dH_2O-treated cells are shown as controls. Each bar in (**b**) or (**c**) represents mean + s.e. of at least three independent experiments with a minimum of 100 cells counted per experiment.

Materials and methods

Cell culture and transfections
See Supplementary Material for details.

Inhibitors
Proteasome inhibitors Z-L_3VS (Biomol, Plymouth Meeting, PA, USA), MG132, MG262, lactacystin (all Boston Biochem, Cambridge, MA, USA) and epoxomicin (Calbiochem, San Diego, CA, USA) were dissolved in DMSO and used at the indicated concentrations at which cell viability was at least 50% after 48 h treatment. In all experiments, solvent controls were included using 0.1% DMSO. Hydroxyurea (Calbiochem) was dissolved in dH_2O and used at a 1 mM concentration; control cells were treated with dH_2O only.

Immunological methods and siRNA
See Supplementary Material for details.

Electron microscopy
See Supplementary Material for details.

Statistical analysis
Statistical significance was assessed using the two-tailed Student's *t*-test for independent samples. $P \leqslant 0.05$ were considered statistically significant.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).