SUPPLEMENTAL MATERIAL

Material and Methods

Cell culture and transfections

Human U-2 OS, T98G and HeLa tumor cells lines as well as IMR-90 normal human fibroblasts were obtained from ATCC and maintained in Dulbecco's modified Eagle medium (DMEM; Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA), 50 units/ml penicillin, and 50 μg/ml streptomycin (Cambrex). U-2 OS were engineered to stably expressing a centrin-GPF-encoding construct (kindly provided by Michel Bornens, Institut Curie, Paris). For transient transfections, pCMV- or pcDNA3-based plasmids encoding HPV-16 E7 (kindly provided by Karl Münger, Channing Laboratory, Harvard Medical School, Boston, MA), cyclin E, CDK2 (kindly provided by Philip W. Hinds, Tufts University, Boston, MA), PLK4 (Habedanck et al., 2005) or empty vector were used and transferred by lipofection (Fugene 6; Roche). Cells were co-transfected with a red fluorescent protein-encoding plasmid targeted to mitochondria (DsRED; BD Biosciences Clontech, Palo Alto, CA) as transfection marker.

Inhibitors

Proteasome inhibitors Z-L_3VS (Biomol, Plymouth Meeting, PA), MG132, MG262, lactacystin (all Boston Biochem, Cambridge, MA) and epoxomicin (Calbiochem, San Diego, CA) 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.

HU (Calbiochem, San Diego, CA) was dissolved in dH_2O and used at a 1 mM concentration, control cells were treated with dH_2O only.

Immunological methods

Whole cell lysates were prepared as previously described (Duensing et al., 2006a). Primary antibodies used for immunoblotting were directed against cyclin E (C-19, Santa Cruz, Santa Cruz, CA or Ab-1, Lab Vision, Fremont, CA), cyclin A (6E6, Novocastra, Newcastle upon Tyne, UK), CDK2 (M-2, Santa Cruz), PLK4 (Habedanck et al., 2005), or actin (AC-40, Sigma, St. Louis, MO).

For flow cytometric analysis of DNA content, cells were stained with propidium iodide and analyzed using a CyAn flow cytometer (Dako).

Immunofluorescence staining for centrin (antibody kindly provided by Jeffrey L. Salisbury, Mayo Clinic, Rochester, MN), cyclin B1 (Neomarkers, Fremont, CA) or CEP170 (Guarguaglini et al., 2005) was performed as described previously (Duensing et al., 2006a; Guarguaglini et al., 2005).

Small interfering RNA (siRNA)

Synthetic RNA duplexes were used to reduce protein expression (Elbashir et al., 2001). Oligonucleotides targeting cyclin E1, cyclin E2, cyclin A2, CDK2 and PLK4 were obtained commercially (siGENOME SMARTpool; Dharmacon, Lafayette, CO) and cells were transfected as described previously (Duensing et al., 2006a).

Electron microscopy

For transmission electron microscopy, the samples were fixed for at least 1 hour in a 2% glutaraldehyde solution buffered with PBS. After washing in 3 changes of PBS, the samples were placed in a 1% osmium tetroxide solution buffered with PBS for one hour, followed by a series of rinses with ethanol solutions of increasing concentration (50%, 70%, 95%, and 100%). The samples were then placed in a 1:1 mixture of Epon Araldite resin and propylene oxide, and held overnight in a desiccator. The following day, the Epon Araldite and propylene oxide mixture was removed and replaced with 100% Epon Araldite resin. The samples were infiltrated with the resin for an additional 8 h, placed in embedding molds and polymerized for 48 h at 60°C. Serial thin sections were cut using a Reichert-Jung Ultracut E ultramicrotome and a DDK Diamond knife. Thin sections were picked up on copper grids and stained with 1% uranyl acetate and Reynold's lead citrate. The sections were viewed on a Hitachi H-7100 TEM transmission electron microscope (Hitachi High Technologies America, Pleasanton, CA). Digital images were obtained using an AMT Advantage 10

CCD Camera System (Advanced Microscopy Techniques Corporation, Danvers, MA) and NIH Image software.

Statistical Analysis

Statistical significance was assessed using the two-tailed Student's t test for independent samples. P values ≤0.05 were considered statistically significant.

Legends to Supplementary Figures

Supplementary Figure 1. Z-L_3VS-treated U2OS cells have increased cyclin E protein expression and accumulate in G2 phase of the cell division cycle.

(A) Immunoblot analysis of U-2 OS/centrin-GFP cells treated with 0.1% DMSO or 1 μM Z-L_3VS (48 h) for cyclin E, cyclin A, CDK2 and PLK4. Immunoblot for actin is shown to demonstrate protein loading.

(B) Flow cytometric analysis of U-2 OS/centrin-GFP, HeLa or T98G cells treated with 0.1% DMSO or 1 μM Z-L_3VS for 48 h.

(C) Immunofluorescence analysis of U-2 OS/centrin-GFP cells for cyclin B expression following 48 h treatment with 1 μM Z-L_3VS. Note the absence of cyclin B in cells that contain more than one daughter centriole per maternal centriole (top panels). Arrows indicate centrioles shown in the inserts. Nuclei stained with DAPI. Scale bar indicates 10 μm.

Supplementary Figure 2. Excessive daughter centrioles can form before maternal centrioles fully mature.

Immunofluorescence analysis of U-2 OS/centrin-GFP cells for CEP170, a marker for mature centrioles (Guarguaglini et al., 2005). Arrows indicate centrioles shown in inserts. Note that supernumerary daughters can form at single maternal centrioles that have not fully matured (arrow in merged bottom panel). Nuclei stained with DAPI. Scale bar indicates 10 μm.

Supplementary Figure 3. Overexpression of cyclin E in HeLa or T98G cells treated with Z-L_3VS stimulates aberrant daughter centriole formation.

Quantification of Z-L_3VS-treated HeLa or T98G cells with concurrent formation of more than one daughter centriole per maternal centriole after transfection with empty vector (control) or cyclin E. Cells were treated with 1 μM Z-L_3VS at 24 h after transfection for an additional 48 h. Each bar represents mean and standard error of triple quantification of at least 50 cells of a representative experiment.