Supplementary Material

Supplementary Experimental Procedures

Molecular Biology and Antibody Generation

Full length CPAP in pEGFP-C1 was kindly provided by T.K.Tang [1]. Two amino acids (R129 and S1333) were reverted by PCR (L129 and L1333) to correspond to the CPAP RefSeq sequence (accession number NM_018451). The full-length cDNA was then cloned into pEGFP-C3 (Clonetech, Mountain View, CA) for transient transfection. For inducible expression of EGFP-CPAP and mCherry-CPAP, the corresponding cDNAs were cloned into pEBTet [2].

The C-terminal region of CPAP (from RZPD clone IRAUp969C1177D, GenBank entry AF139625) corresponding to amino acids 1070 – 1338 of the full length protein was cloned into pGEX-6P-3 (Amersham Pharmacia Biotech, Freiburg, Germany) to express a GST-CPAP fusion. Polyclonal rabbit antibodies were raised against the affinity purified recombinant protein (Eurogentec, Seraing, Belgium). The antigenic CPAP fragment was cleaved from GST using Prescission Protease (Amersham Pharmacia Biotech) and coupled to Affi-Gel-15 (Bio-Rad Laboratories, Hercules, CA) for antiserum affinity purification. CPAP antibodies were eluted in 200 mM glycine (pH 2.2) / 500 mM NaCl, neutralized with 1.5M Tris-HCl (pH 9.2), dialysed in phosphate-buffered saline (PBS) with 10% glycerol and concentrated to 1μg/μl using Centriprep columns (Millipore, 10kD exclusion volume).

Cell Culture and Cell Lines

Human cells were cultured in high-glucose DMEM with GlutaMAX (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) in a humidified 5% CO2 incubator at 37°C. Cells were split every 3-4 days or at confluency. To generate inducible cell lines, U2-OS cells were transiently transfected with pEBTet-EGFP-CPAP or pEBTet-mCherry-CPAP at 80-90% confluency. 24 h after transfection, cells were exposed to selective medium

containing 1 μg/ml puromycin, which led to substantial death of nontransfected cells over 4-5 days. After amplification of the transfected cell population under selective conditions for 1-2 weeks, cells were frozen in 10% DMSO and stored at -80°C. Expression was induced using 1 μg/ml doxycyclin. Quantification of signal intensities in i-GFP-CPAP cells that harbor threads 24 hours after induction with doxycyclin indicated that the protein is overexpressed ~10 fold on average over endogenous levels (N=10 cells of each kind). Aggregates of GFP-CPAP often formed in the cytoplasm when expression levels were very elevated. Live imaging experiments also indicated that very elevated expression levels often ultimately result in cell death.

Primary human cells were prepared and cultured as previously described [3]. Human umbilical vein endothelial cells (HUVECs) and human keratinocytes were obtained from the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne). HUVECS were electroporated and cultured for 72 hours thereafter. Human keratinocytes were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described below and also cultured for 72 hours after transfection.

Cell Cycle Synchronization and FACS Analysis

Cells were synchronized at the G1/S transition by a double thymidine block. Cells were incubated in medium containing 2 mM thymidine for 18 h, released for 6 h and again incubated in thymidine containing medium for 18 h. Cells were then released and samples taken at the indicated timepoints following release, stained with Propidium Iodide and the DNA content measured by flow cytometry (FACScan, BD Biosciences).

RNAi and Transient Plasmid Transfection

For RNAi experiments, ~100.000 cells were seeded on 18 mm sterile glass coverslips in 6-well plates. 6 μl of 20 μM siRNA in 100 μl OptiMEM medium (Invitrogen, Carlsbad, CA) and 3 μl of Oligofectamine (Invitrogen) in 27 μl OptiMEM were incubated in parallel for 5 minutes, mixed for 15 minutes and

then added to 1 ml medium per well. 1 ml medium was added to each well after 24 h, and cells were analyzed after 48 or 72 h. Double stranded siRNA oligonucleotides were synthesized with 3'-UU overhangs (Dharmacon, Lafayette, CO) with the sequences GGACUGACCUUGAAGAGAA (CPAP-siRNA1), AGAAUUAGCUCGAAUAGAA (CPAP-siRNA3), UCUAUAUCAUGGCCGACAA (control-siRNA). siRNA against CP110 and HsSAS-6 were as described [4, 5]. For generating a GFP-CPAP construct resistant to siRNA3, four silent nucleotide changes were introduced in the corresponding region of CPAP (AGAGTTAGCTAGGATCGAA) to render the construct resistant to these siRNAs. The corresponding sequence-verified cDNA (CPAP-R) was cloned into pEBTet as above.

For plasmid transfections, cells were seeded at 80-90% confluency. 1 μg of plasmid DNA in 50 μl OptiMEM and 2 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 50 μl were incubated in parallel for 10 minutes, mixed for 10 minutes and added to each well. The medium was changed after 12-24 h to reduce toxicity of the reaction mix.

Multi-mode time-lapse microscopy

For time-lapse microscopy, cells grown on coverslips were mounted in Rose chambers. Full Z-series through the cell (0.7 μm steps) were recorded at 15 min intervals using either 60x1.4 or 100x1.4 PlanApo lens. Images were captured on Andor iXon back-illuminated EM CCD. The system was driven by IPLab software. During the experiment, cells were maintained at 37^{o}C, and the light exposure was minimized by using shutters directly synchronized with the camera. We deemed a spindle multipolar when more than one cleavage furrow was initiated, indicative of multipolar spindle assembly, even if some of these furrows regressed subsequently.

Immunoblotting and Immunofluorescence Microscopy

To determine total CPAP levels, cells from different timepoints after G1/S-phase release were lysed in buffer containing 50 mM HEPES (pH 7.4), 250 mM

NaCl, 5 mM EDTA, 1% NP-40 and protease inhibitors (P8340, Sigma-Aldrich) by three freeze-thaw cycles in liquid N2 and centrifugation at 13.000 rpm for 10 minutes to remove cell debris. Protein concentration was determined by Bradford analysis. 80 μg of lysate were resolved by SDS-PAGE on a 8-13% gradient gel and immunoblotted on Immobilon-P transfer membrane (Millipore Corporation, Bedford, MA). Primary antibodies were 1:500 rabbit anti-CPAP (this study), 1:1000 mouse anti-γ-tubulin (GTU88, Sigma-Aldrich) and 1:500 rabbit anti-HsSAS-6 [5] diluted in 3% non-fat dry milk in PBS. Secondary antibodies were 1:5000 HRP-conjugated anti rabbit or mouse IgG (Promega, Madison, WI). Washes were performed in tris-buffered saline (TBS) containing 0.05% Triton X-100 (TBST).

For immunofluorescence analysis, cells were fixed in -20°C methanol for 10 minutes and washed in PBS containing 0.05% Triton X-100 (PBST). After blocking in 1% bovine serum albumin (BSA) in PBS for 1 hour, cells were incubated with primary antibodies over night at 4°C. Following three washes in PBST for 5 minutes, cells were incubated with secondary antibodies and 1 μg/ml Hoechst 33258 for 1 hour at room temperature, washed four times for 5 minutes in PBST and mounted. Primary antibodies were 1:1000 rabbit anti-CPAP (this study), 1:200 mouse anti-tubulin (DM1A, Sigma-Aldrich), 1:2000 mouse anti-γ-tubulin (GTU88, Sigma-Aldrich), 1:25 goat anti-pericentrin (E17, Santa-Cruz), 1:1000 rabbit anti-NEDD1 [6], 1:500 rabbit anti-Cep192 [7], 1:1000 mouse anti-acetylated tubulin (6-11B-1, Abcam, UK), 1:1000 mouse anti-polyglutamylated tubulin (gift from B. Eddé), 1:1000 mouse ’20H5’ anti-Centrin-2 [8] (gift from J.L. Salisbury), 1:2000 rabbit anti-Centrin-3 [9] (gift from M. Bornens), 1:1000 rabbit anti-Cep135 (gift from R. Kuriyama), 1:200 rabbit anti-CP110 [10], 1:200 rabbit anti-Odf2 (gift from H. Ishikawa) [11, 12], 1:20’000 rabbit anti-Ninein (gift from M. Bornens) [13], 1:2000 rabbit anti-C-Nap1 (gift from E. Nigg) [14], 1:200 rabbit anti-HsSAS-6 [5] and 1:200 rabbit anti-GFP (gift from V. Simanis). Secondary antibodies were 1:1000 Alexa488-coupled anti-mouse, Alexa568-coupled anti-rabbit, Alexa488-coupled anti-goat (Molecular Probes, Eugene, OR), Cy3-coupled anti-rabbit (Dianova) and 1:400 Cy5-

coupled anti-mouse antibodies (Dianova). Confocal images were taken on the Leica TCS SP2 inverted microscope using a 63x oil immersion objective (Zeiss, Germany). Confocal sections of relevant structures were taken with 0.2 μm – 0.4 μm intervals and maximum intensity projected using the Leica LCS Lite software (Leica Microsystems, Germany). Images were processed in Adobe Photoshop. Thread complexity was defined as the number of free ends per thread determined by collecting through confocal z-series and marking the end of each elongation with a ~300 nm radius. Non-overlapping radii were counted as one free end, whereas multiple ends located in a single radius were counted as one (branched) end.

Electron Microscopy

Correlative LM/EM analyses were conducted as described [15]. Cells were fixed by perfusion of 2.5% glutaraldehyde. Spinning-disk confocal Z-series of selected cells were collected immediately before and shortly after fixation. After standard embedment [16], full series of 100-nm sections were obtained for each cell. The sections were examined in Zeiss 910 electron microscope at 80 kV. Images were recorded on film and subsequently scanned.

Electron tomography was carried out on serial sections of select sections as described [17]. Briefly, dual-axis tilt series were collected using a tilt-angle increment that varied according to the cosine of the tilt angle, with a 1.5° increment at the untilted image and a total angular range of ±72°. Digital tilt series images were collected automatically, with a pixel size of 1.6 nm, on an FEI Tecnai F20 electron microscope with a Gatan 2K X 2K camera using the Serial_EM software package [18]. Tilt series images were aligned using 10 nm colloidal gold particles as fiducial markers. Image alignment and three-dimensional reconstruction were computed using IMOD [19].

Supplementary Figure legends

Figure S1

CPAP is required for procentriole formation after HsSAS-6 recruitment in proliferating cells

(A-D) U2-OS (A, B) or HeLa (C, D) cells transfected with the indicated siRNAs for 48 or 72 h were classified in categories based on the number of Centrin-foci present during mitosis (n>100 for each time-point). The quantification of Centrin-foci in CPAP siRNA3 treated U2-OS cells at 72 h (A) is representative of three independent experiments.

(E) Western blot analysis of U2-OS cells transfected with the indicated siRNAs and stained with antibodies against CPAP. Arrowhead indicates CPAP, stars unspecific species that also serve as loading control.

(F) Cells expressing i-GFP-CPAP-R were treated with CPAP-siRNA3 in uninduced and induced conditions for 72 hours. Cells stained with Centrin-3 and α-tubulin were scored for the presence of bipolar or monopolar spindles (N>125 for each). Note that the percentage of monopolar spindles is reduced in cells harboring CPAP-R even in the uninduced condition - presumably due to leaky expression - but reduced to background levels upon doxycycline-mediated induction of the RNAi resistant transgene.

Figure S2

(A-B) U2-OS cells transfected with CPAP siRNAs for 72 hours and stained for γ-tubulin (green) and Centrin-3 (red); DNA is viewed in blue. Note normal bipolar spindle in (A) and asymmetric spindle in (B) with one spindle pole having less Centrin-3 and γ-tubulin.

(C-E) U2-OS cells were treated with CPAP siRNAs for 65 h and synchronized by a thymidine block for the last 18 h. Thereafter, cells were released for the indicated times and stained with antibodies against Centrin-2 (green) and HsSAS-6 (red). At each time-point, transfected cells were scored for the presence of HsSAS-6 signals near single Centrin-foci (N>100 for each). This

experiment demonstrates that HsSAS-6 is not only recruited but also maintained until the onset of mitosis.

(F-G) U2-OS cells transfected with the indicated siRNAs for 72 h and stained for Centrin-2 (green), CPAP (red); DNA is viewed in blue. Note that the CPAP signal does not extend away from the parent centriole as apparent in the cell depleted of HsSAS-6 (G).

Figure S3

Threads are present throughout the cell cycle, in several cell lines and in primary cells

(A-C) U2-OS cells transiently transfected with GFP-CPAP for 72 h and stained with antibodies against GFP (red); DNA is viewed in blue. Prophase (A), anaphase (B) and late telophase (C).

(D-O) U2-OS p53+ (D, E), U2-OS p53- (F, G), Sa-OS (H, I), HCT p53+ (J, K), HCT p53- (L, M) or 293T (N, O) cells transiently transfected with GFP-CPAP for 72 h and stained with antibodies against γ-tubulin (green) and GFP (red); DNA is viewed in blue. The percentages of cells without (left) or with (right) threads is indicated in each case in panels (D-S).

(P-S) Human umbilical vein endothelial cells (HUVECs) transiently transfected with GFP-CPAP for 72 h and stained with antibodies against Centrin-2 (P-Q) or poly-glytamylated tubulin (R-S) (both viewed in green) and GFP (red); DNA is viewed in blue. Note that no threads were apparent following transient transfection of primary human keratinocytes (data not shown). Why threads in primary cells are either present at lower frequencies than in transformed cells or altogether absent remains to be investigated.

Figure S4

Serial-section EM analysis of cells overexpressing CPAP or depleted of CP110

(A) CPAP-GFP threads in an i-GFP-CPAP cell 72 hours after induction with doxycycline. Maximal-intensity projection of a through-focus series.

(B-K) Complete series of 100-nm sections through the centrosome in the same cell. Three centrioles and four procentrioles were present in this cell. Centrioles "1" and "3" were morphologically normal, while centriole "2" was significantly longer and distorted. Detailed electron-tomography analysis of this centriole is presented in Fig. 2 (see text for details). Procentrioles (1', 2', and 3') resided near the proximal end of each centriole. A second, ectopic procentriole (2'') was found near the distal end of the long centriole.

(L) Contour traces of centriolar cylinders overlaid on the CPAP-GFP distribution. Notice that morphologically-normal centriole "1" resides within a depression in CPAP-GFP signal. Also notice that the distribution of CPAP-GFP along centriole "2" is not uniform.

(M-P) Formation of elongated centrioles and microtubule doublets in U2-OS cells depleted of CP110 using siRNAs. Maximal projections of centrin-GFP (M and O) and corresponding 100-nm EM sections (N and P, selected from full series) illustrating the structure of elongated centrioles (M-N), and microtubule doublets that are not connected to centrioles (O-P).

Figure S5

CPAP levels are highest in G2

(A-B) HeLa cells released from a double thymidine block and analyzed at the indicated time points by Western blot using antibodies against CPAP, HsSAS-6 and γ-tubulin (A), as well as by FACS for DNA content (B).

Figure S6

Cytokinesis failure in subset of cells overexpressing CPAP

(A-B) Selected DIC (single planes, A) and Fluorescence (maximal-intensity projections, B) frames from time-lapse recordings of an i-mCherry-CPAP cell. Times in hours:minutes from the beginning of time-lapse recording. The mother cell assembled an apparently bipolar mitotic spindle (not shown) and two mononuclear cells initially form after mitosis. However, the terminal stages of cytokinesis fail and the two daughter cells fuse back. Note that a role for

centrioles in the terminal stages of cytokinesis has been reported previously [20]. See also Movie S2.

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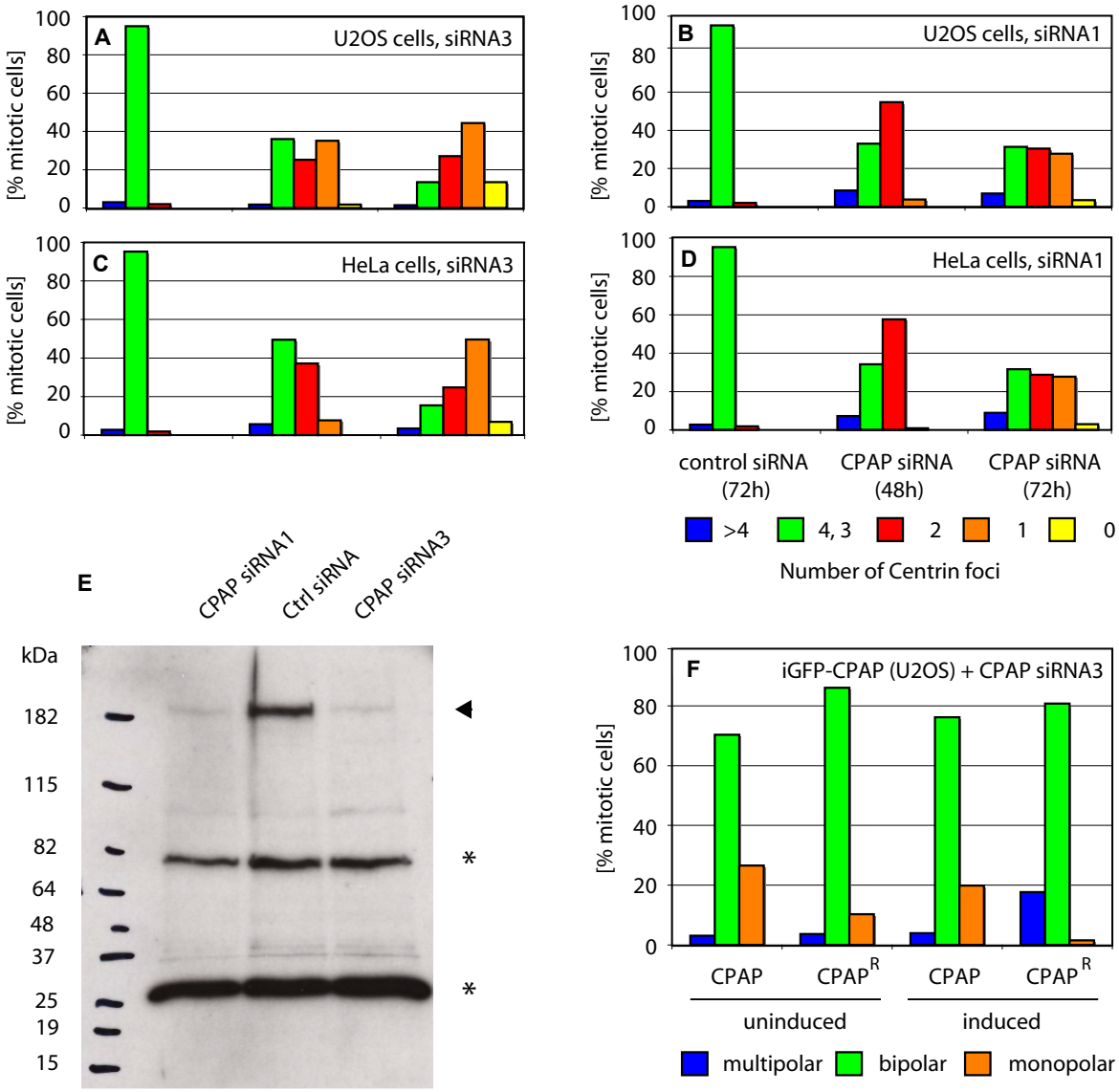
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U2OS cells, siRNA3
B
U2OS cells, siRNA1
C
HeLa cells, siRNA3
D
HeLa cells, siRNA1
[% mitotic cells]
100
80
60
40
20
0
control siRNA (72h)
CPAP siRNA (48h)
CPAP siRNA (72h)
>4
4, 3
2
1
0
Number of Centrin foci
E
CPAP siRNA1
Ctrl siRNA
CPAP siRNA3
kDa
182
115
82
64
48
37
25
19
15
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F
iGFP-CPAP (U2OS) + CPAP siRNA3
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uninduced
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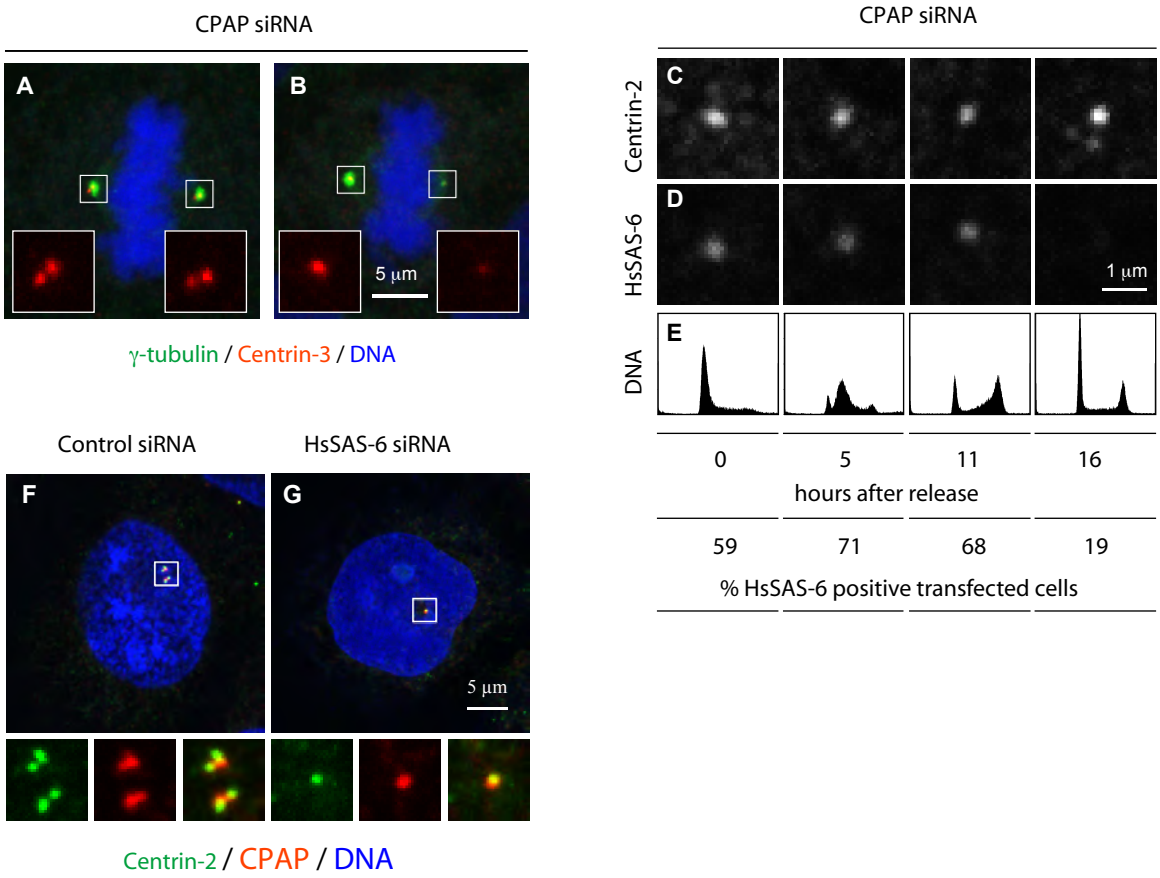
monopolar

Figure S1


CPAP siRNA
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B
5 μm
γ-tubulin / Centrin-3 / DNA
Control siRNA
HsSAS-6 siRNA
F
G
5 μm
Centrin-2 / CPAP / DNA
CPAP siRNA
Centrin-2
HsSAS-6
DNA
C
D
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1 μm
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16
hours after release
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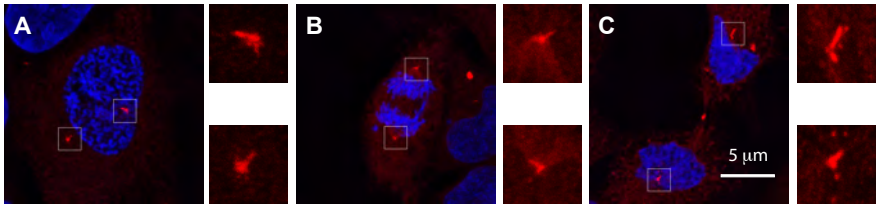
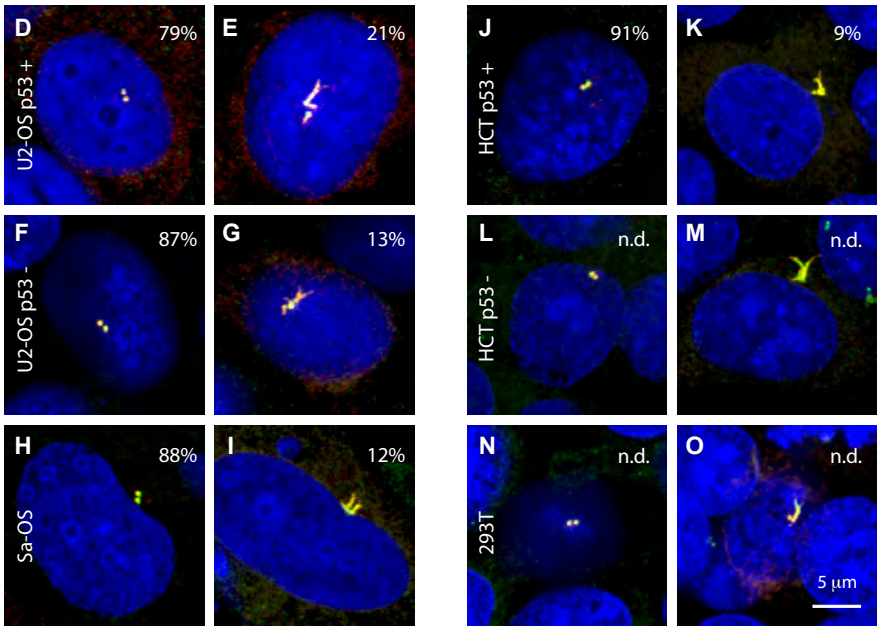
68
19
% HsSAS-6 positive transfected cells

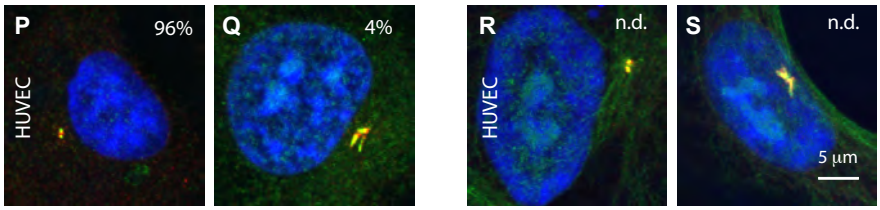
Figure S2



GFP / DNA



γ-tubulin / GFP / DNA



Centrin-2 / GFP / DNA

pg.tubulin / GFP / DNA

Figure S3

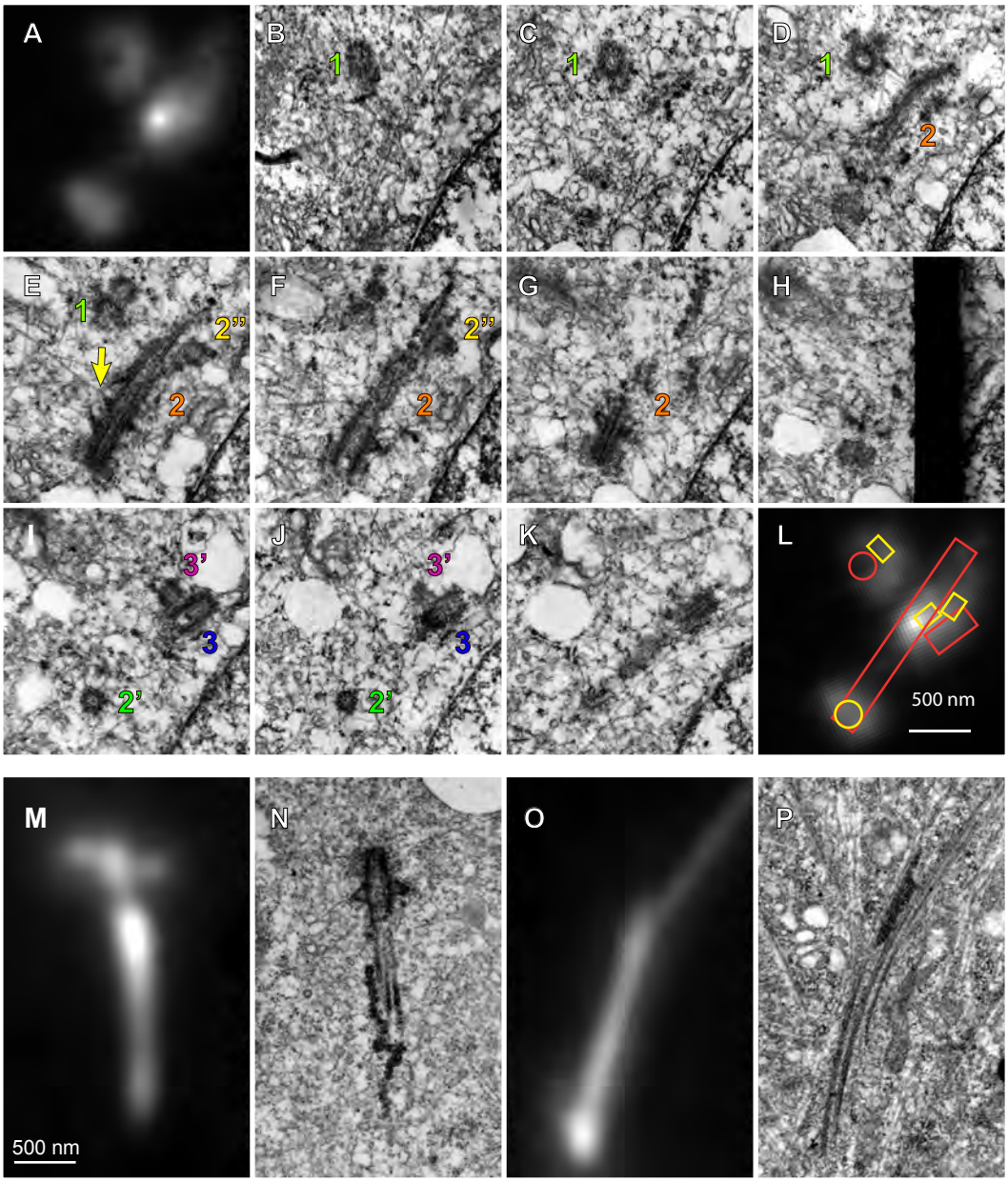


Figure S4

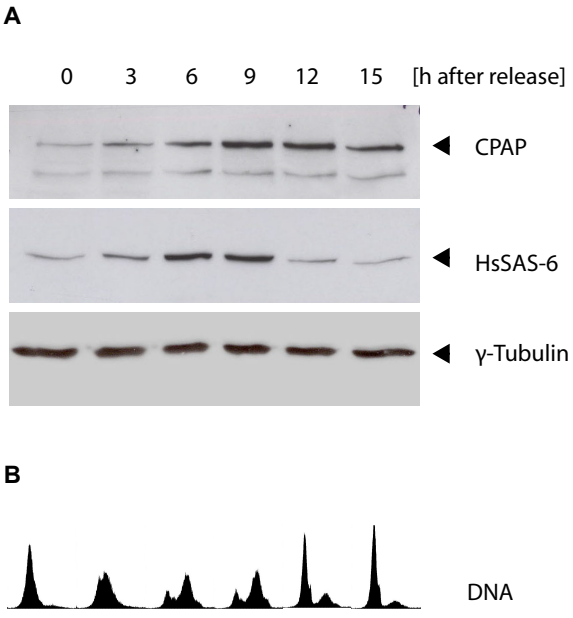


Figure S5

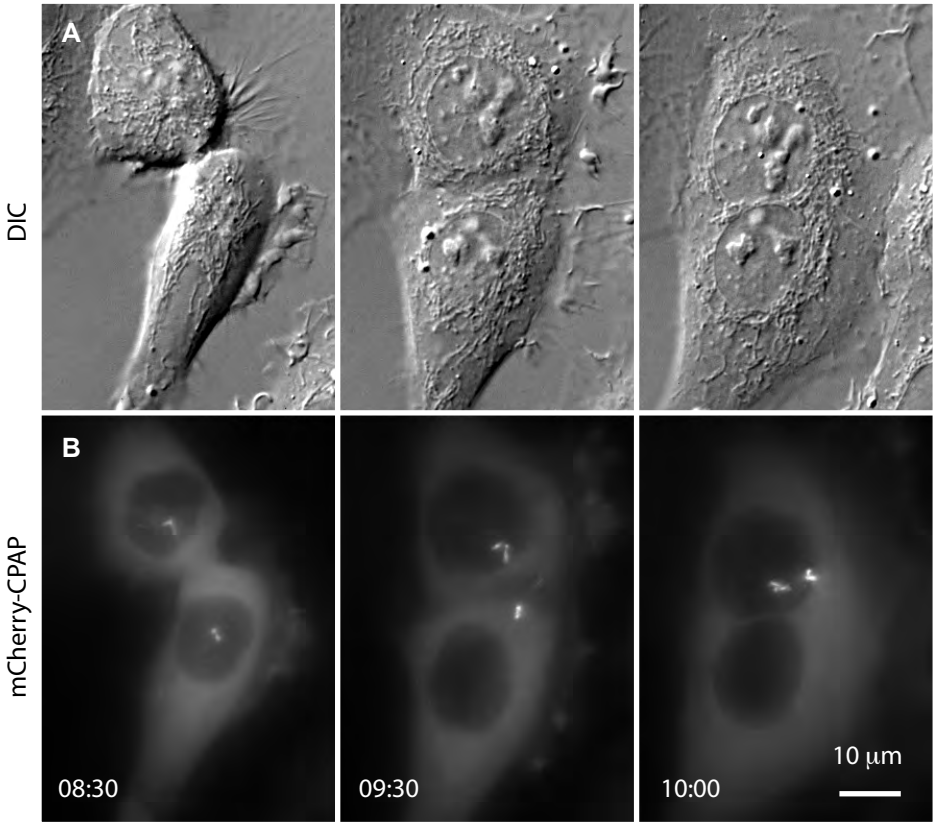


Figure S6