Figure S1

A

Cep135full (intron 5 removal)

= Peptide sequence for α-Cep135mini

... D Y S K Q v g f l f t c i v g i e i g m l * I E L R E ...

...GACTATAGCAAGCAGgtaggatttttatttacttgcatagtagggattgagataggtatgctgtga aatattcagATTGAGCTAAGAGAA ...

Exon5 Cep135mini (intron 5 retention) Exon6

= siRNA (Cep135mini #1)

B

Size (bp) 4,000 2,000 1,000 600

CEP135full CEP135mini

CEP135full (rtPCR = 1,954 bp)

Exon2 Exon5 Exon16

CEP135mini (rtPCR = 580 bp)

Exon2 Exon5

C Predicted MW (kDa) α-Cep135mini 82 64 49 37 26

D α-Cep135mini 82 64 49 37 26

E Serum only α–Cep135mini α-Cep135mini + peptide 82 64 49 37 26

F Control Cep135mini knockdown 82 64 49 37 26 1.00 0.31 α-tubulin

G Control Peptide Block Cep135mini Merge

p<0.01

Relative fluorescence 1.2 0.8 0.4 0.0

Control Peptide Block

H Control siRNA Depleted Cep135mini Merge

p<0.01

Relative fluorescence 1.2 0.8 0.4 0.0

Control siRNA Depleted

I G1 Centrin Cep135full Cep135mini

Centrin Cep135full Centrin Cep135mini

G2 Centrin Cep135full Cep135mini

Centrin Cep135full Centrin Cep135mini

Figure S2

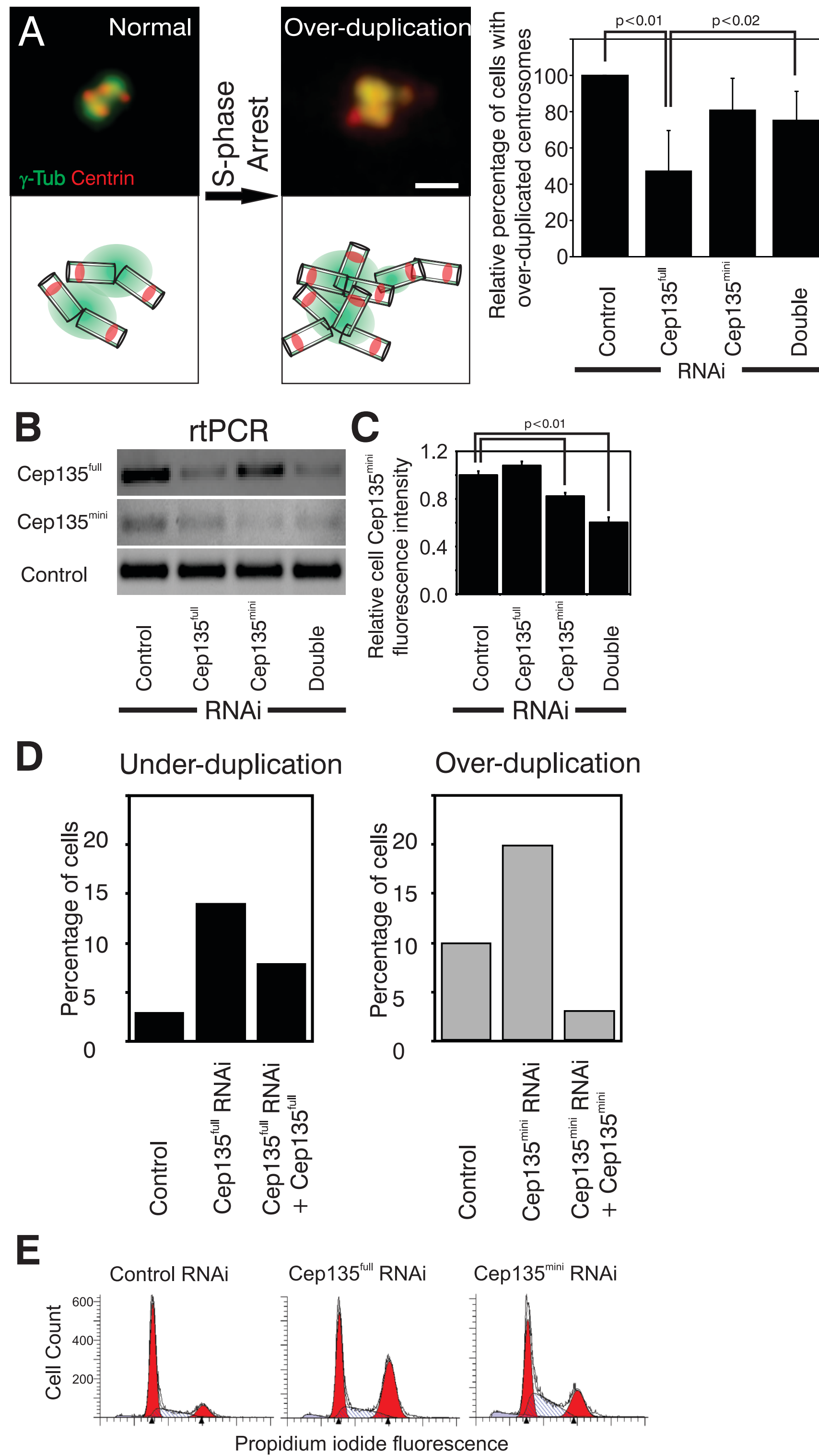


Figure S3

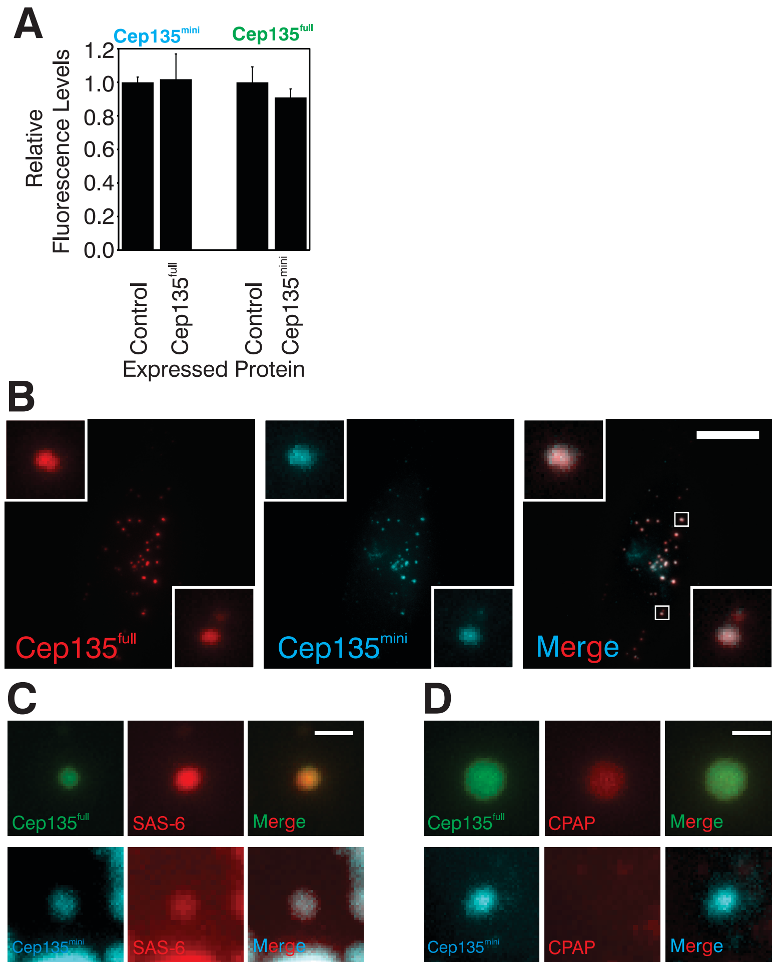
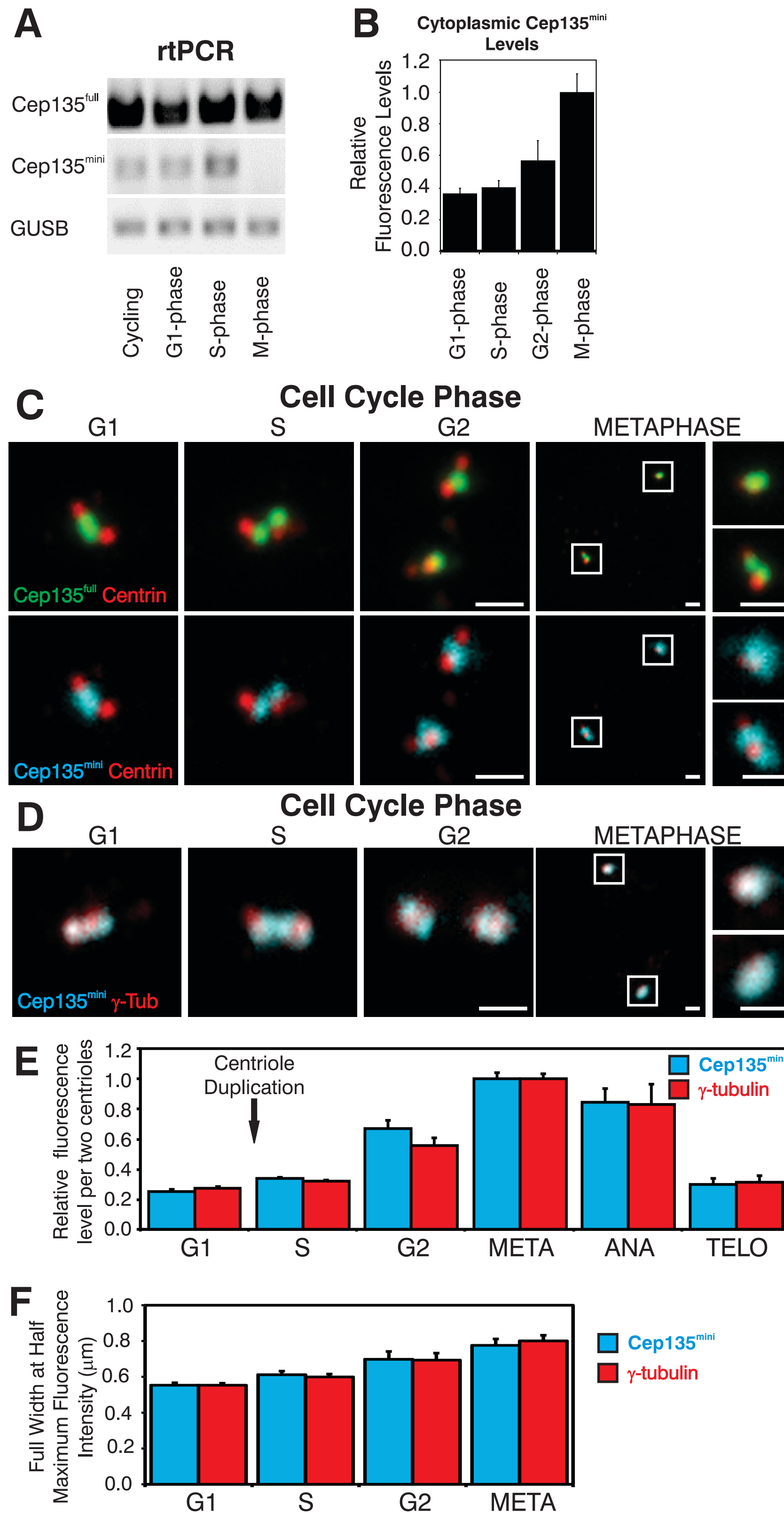


Figure S4



Supplemental Data

Figure S1. Related to Figure 1. Cep135 isoforms exhibit unique localization patterns.

(A) DNA and protein sequence showing the alternative splicing that leads to the formation of Cep135^{full} and Cep135^{mini}. Green line denotes peptide sequence used for antibody production. Blue line denotes the CEP135^{mini} target sequence for siRNA. **(B)** rtPCR detection of CEP135^{full} and CEP135^{mini} transcripts in RPE1 cells. Red arrows denote sites of PCR primer recognition. **(C)** Western blot shows that high levels of recombinant 6XHIS-Cep135^{mini} is detected by affinity purified α-Cep135^{mini}. **(D)** Western blot shows antibody recognition of Cep135^{mini} in an RPE1 cell lysate. Asterisk denotes Cep135^{mini} band. **(E)** Pre-immune serum does not recognize Cep135^{mini} and immunogenic peptide incubation with α-Cep135^{mini} blocks Western detection of Cep135^{mini} in RPE1 cell lysates. **(F)** siRNA knockdown of Cep135^{mini} reduces Cep135^{mini} staining (69% reduction) but not control (DM1a; α-α-tubulin). Asterisk depict Cep135^{mini} band. **(G)** Immunogenic peptide incubation with α-Cep135^{mini} blocks (81% decrease; $p<0.01$) immuno-fluorescence detection of Cep135^{mini} in RPE1 cells. Centrin is used to label centrioles (red). Scale bar, 1 μm. **(H)** siRNA knockdown of Cep135^{mini} in HeLa cells decreases (74% decrease; $p<0.01$) immuno-fluorescence detection of Cep135^{mini}. Cytoplasmic levels of Cep135^{mini} also decreased after Cep135^{mini} knockdown suggesting that the α-Cep135^{mini} antibody is specific (data not shown). **(I)** Expressed GFPLAP-Cep135^{mini} (cyan) localizes in the same pattern as α-Cep135^{mini} antibody staining, respectively. Expressed GFPLAP-Cep135^{mini} is localized relative to Cep135^{full} (green) and Centrin (red). Scale bars, 1 μm.

Figure S2. Related to Figure 2. Cep135^{mini} inhibits centriole duplication.

(A) Centriole over-duplication is induced in 45±24% of U2OS cells arrested in S-phase using aphidicolin treatment for 48 hrs. Centriole and centrosome frequencies are quantified using α-

centrin (red) and α-γ-tubulin (green) staining, respectively. Cep135^{full} knockdown (2.1-fold reduction compared to control; $p<0.01$), but not Cep135^{mini} knockdown (1.2-fold reduction compared to control), causes a significant suppression of centriole over-duplication. Simultaneous knockdown of both isoforms returned the level of over-duplication to near control levels of centriole over-duplication (1.3-fold reduction compared to control; $p<0.02$). Mean±SD represents eight separate experiments for >1100 cells for each condition. Scale bar, 1 μm. **(B)** rtPCR shows that Cep135^{full} and Cep135^{mini} transcript levels are reduced upon siRNA treatment. **(C)** Total α-Cep135^{mini} cellular fluorescence is decreased upon Cep135^{mini} siRNA treatment in U2OS cells (Mean±SEM). **(D)** Rescue of Cep135^{full} and Cep135^{mini} knockdown effects. RNAi impervious versions of mCherry:Cep135^{full} and mCherry:Cep135^{mini} were transfected into cells depleted of Cep135^{full} and Cep135^{mini}, respectively, to rescue the phenotypes. **(E)** FACS analyses in cycling U2OS cells show that Cep135^{full} knockdown causes a 25% increase in G2/M phase cells while Cep135^{mini} knockdown causes a 15% increase in S-phase cells. X-axis denotes DNA content measured by propidium iodide staining. Red area defines the region counted for 2n or 4n DNA content.

Figure S3. Related to Figure 3. Cep135^{mini} expression displaces SAS-6, CPAP, and γ-tubulin from centrioles and centrosomes.

(A) Exogenous expression of mCherry:Cep135^{full} or mCherry:Cep135^{mini} does not affect the endogenous levels of Cep135^{mini} or Cep135^{full}, respectively. Endogenous levels were quantified using antibody staining. Mean±SEM represents four separate transfection experiments. **(B)** Exogenously expressed Cep135^{full} and Cep135^{mini} at high levels form ectopic foci in RPE1 cells. Both isoforms co-localize to these foci, in addition to association with centrin stained centrioles (data not shown). Insets display two representative co-localizing foci. Scale bar, 10 μm. **(C)** Expressed SAS-6 associates with ectopic Cep135^{full} foci in HeLa cells suggesting these

components interact. Expressed Cep135^{mini} weakly and variably associates with ectopic SAS-6 foci. **(D)** Expressed CPAP associated with ectopic Cep135^{full}, but not Cep135^{mini}, foci in HeLa cells. **(C-D)** Scale bar, 1 μm.

Figure S4. Related to Figure 4. Cep135^{mini} levels are controlled through the cell cycle. **(A)** mRNA levels of Cep135^{full} and Cep135^{mini} in cycling, G1-phase arrested, S-phase arrested, and M-phase arrested HeLa cells. GUSB is an internal control. mRNA levels represent three separate experiments. **(B)** Relative cytoplasmic levels of Cep135^{mini} through the cell cycle. Mean±SEM represents three separate experiments. **(C)** Localization of α-Cep135^{full} (green; top panel) and α-Cep135^{mini} (cyan; middle panel) relative to Centrin (red) during the cell cycle. Scale bars, 1 μm. **(D)** Localization of α-Cep135^{mini} (cyan; bottom panel) relative to α-γ-tubulin (red) during the cell cycle. Scale bars, 1 μm. **(E)** Quantification of the relative levels of Cep135^{mini} and γ-tubulin through the cell cycle. **(F)** Quantification of the full width at half maximum fluorescence intensity of Cep135^{mini} and γ-tubulin through the cell cycle.

Supplemental Experimental Procedures

Cep135^{mini} identification

The predicted 29 kDa protein (hCG2039594; Cep135^{mini}) was determined to be an alternatively spliced variant of Cep135 (Figure S1). The expression of both isoforms (Cep135^{full} and Cep135^{mini}) were confirmed both by rtPCR and sequencing of PCR products and by Western blot (Figures S1 and S2 and data not shown).

Recombinant expression constructs

Cep135^{full} and Cep135^{mini} were expressed as N-terminal fusions to either GFP-LAP (pIC113), mCherry-LAP (pIC194), pEGFP-C1, or pmCherry-C1 [S1]. Recombinant Cep135^{mini} protein was expressed in *E. coli* as a GST- fusion in pGEX-6p-1 (GE Healthcare) or 6xHIS fusion in pQE30 (Qiagen). All constructs were sequenced to confirm their proper integration and sequence. Plasmids were transiently transfected into RPE1, U2OS, or HeLa cells using Effectene Transfection reagent (Qiagen).

Cell culture and drug treatments

Mammalian tissue culture lines, U2OS, RPE1, and HeLa cells were all grown in 10% FBS in DMEM media at 37°C and 5% CO_2. Cells were arrested in S-phase by treatment for 24 or 48 hrs with 95 μM aphidicholin or 5 mM thymidine and double blocked, depending on the experiment. HeLa cells were arrested in G1 by serum starvation for 48 hr. Cells were arrested in mitosis by release from S-phase block for 4.5 hrs before incubating in 166 nM nocodazole for 12 hrs.

The Plk4 over expressing, centrin-GFP RPE1 cell line was a generous gift from Dr. Bryan Tsou (Memorial Sloan-Kettering Cancer Center). Doxycyclin (0.5 μg/mL) was used to induce Plk4 expression in S-phase arrested cells.

To determine the effect of Cep135^{full} and Cep135^{mini} over expression on SAS-6 and CPAP levels, RPE1 cells were transfected with mCherryLAP, mCherryLAP-Cep135^{full} or

mCherryLAP-Cep135^{mini} for 24 hours prior to S-phase arrest for 24 hours and staining for centrin with either SAS-6 or CPAP. To determine the effect of Cep135^{full} and Cep135^{mini} over expression on γ-tubulin levels, cycling RPE1 cells were transfected with mCherryLAP, mCherryLAP-Cep135^{full} or mCherryLAP-Cep135^{mini} for 48 hours prior to staining for centrin and γ-tubulin. Relative fluorescence intensities were measured by comparing centrosomal fluorescence levels of mCherry transfected control cells to mCherryLAP-Cep135^{full} or mCherryLAP-Cep135^{mini} transfected cells.

To test whether Cep135^{full} and Cep135^{mini} associated with each other, SAS-6 and CPAP, RPE-1 or HeLa cells were co-transfected with the appropriate expression constructs for 6 to 24 hrs, depending on the experiment. Cells expressing high levels of Cep135^{full} or Cep135^{mini} produce ectopic foci in the cytoplasm. We then tested whether Cep135^{full}, Cep135^{mini}, SAS-6 or CPAP fusions to EGFP or mCherry, respectively, co-associated with these foci. Identical results were observed for both RPE-1 and HeLa cell lines.

Antibodies

A rabbit polyclonal antibody that is specific to the Cep135^{mini} isoform was generated using a small peptide that includes the unique C-terminal 16 amino acids of Cep135^{mini} (Ac-YSKQVGFLFTC-amide; Figure S1A). Antibodies were purified on a peptide conjugated affinity column using the antigens. Two distinct peptides and purifications generated similar centrosomal localization (data not shown). Further purification using a GST-Cep135^{mini} affinity column also produced a α-Cep135^{mini} antibody with the same centriolar and centrosomal localization. Peptides and antibodies were produced by 21st Century Biochemicals (Marlboro, MA). Purified antibodies were then confirmed by Western (1:1,000) to recognize an approximately 29 kDa protein, among additional bands (Figure S1C-F). Specificity of the antibody was confirmed by peptide blocking of Western blot and immunofluorescence signal, by recognition of a recombinant form of Cep135^{mini} and by decrease in total cellular α-Cep135^{mini}

fluorescence signal upon siRNA knockdown in HeLa and RPE1 cells. α-Cep135^{mini} was used at 1:50,000 for immunofluorescence. Because antibodies to both isoforms of Cep135 were generated in rabbit, we directly labeled α-Cep135^{mini} with Alexa488 dye (Molecular Probes), used at a dilution of 1:1,000 to 1:2,000, and pseudo-colored the resulting images cyan. α-centrin (1:2,000; 20H5; Abcam [S2]), α-Cep135^{full} (1:5,000 generous gift from Dr. T.K. Tang ;[S3]) α-SAS-6 (1:2,000, Bethyl A301-802A), α-CPAP (1:350, Proteintech CENPJ 11517-1-AP) and α-γ-tubulin (1:500; Sigma DQ-19). Alexa-fluor secondary antibodies were used at 1:1,000 for all experiments (Molecular Probes).

Immunofluorescence

Cells were fixed with 100% methanol at −20°C, washed with PBS/Mg (1x PBS, 1mM $MgCl_2$), and then blocked with Knudsen Buffer (1x PBS, 0.5%BSA, 0.5% NP-40, 1mM $MgCl_2$, 1mM NaN_3). DNA was visualized using Hoechst 33342 (Sigma).

RNAi knockdown

Knockdown of Cep135^{full} and Cep135^{mini} was performed by transfecting RPE1, U2OS or HeLa cells with single siRNA oligos using Oligofectamine Transfection Reagent (Invitrogen) or RNAiMAX Reagent (Invitrogen). Knockdown was confirmed by rtPCR and by immunofluorescence quantification of total cellular protein levels. Two separate siRNA oligos were used for each Cep135^{full} and Cep135^{mini} and both produced similar phenotypes. The siRNA oligo with the stronger phenotype was used for experiments (Cep135^{full}#2 – AAUGAGAGACAUACAGUUAAA[dT][dT] and Cep135^{mini}#1 – UAGUAGGGAUUGAGAUAGGUA[dT][dT]). Non-specific scrambled siRNAs were used as controls. The knockdown phenotypes were rescued by exogenous expression of siRNA impervious mCherry-Cep135^{full} and mCherry-Cep135^{mini} constructs (Figure S2D).

Microscopy

The fluorescence imaging system was previously described [S4]. Briefly, images were acquired using a Nikon Ti (Nikon Instruments, Inc.) inverted microscope stand equipped with a 100X PlanApo DIC, NA 1.4 objective. Images were captured using an Andor iXon EMCCD 888E camera or an Andor Xyla 4.2 CMOS camera (Andor Technologies). Localization experiments described in Figure 4B were performed using a Swept Field Confocal system (Prairie Technologies / Nikon Instruments) on a Nikon Ti inverted microscope stand equipped with a 100X Plan Apo λ, NA 1.45 objective. Images were captured with an Andor Clara CCD camera (Andor Technologies). Nikon NIS Elements imaging software was used for image acquisition and ImageJ and NIS Elements were used for data analysis. Image acquisition times were kept constant within a given experiment and ranged from 50 to 400 msec depending on the experiment. All images were acquired at room temperature.

Immuno-EM localization of $Cep135^{mini}$ was performed as previously described [S5]. $Cep135^{mini}$ was detected using the α-$Cep135^{mini}$ antibody at 1:20 dilution. All images were acquired on an FEI Technai G2 electron microscope equipped with a Gatan Ultrascan digital camera.

Centriole frequency counts

Centriole over- and under-duplication were quantified in cycling U2OS cells that were stained for α-centrin and α-γ-tubulin. A centriole over-duplication event was scored if more than four centrin foci were observed to co-localize with γ-tubulin labeled foci (Figures 2A and S2A). Over-duplication typically led to more than two centrosome foci stained with γ-tubulin (Figures 2A and S2A). Centriole under-duplication was defined as events where a single γ-tubulin foci was associated with only a single centrin foci (Figure 2A). G1 centrosomes were defined as those with a single γ-tubulin focus or two foci separated by less than one μm and contained only

one centrin foci (centriole). Alternatively, G2 centrosomes were separated by greater than one μm and each contained only one centrin foci.

Over-duplication in the Plk4 over expressing RPE1 cell line was visualized by quantifying the total number of GFP-centrin foci (centrioles) per cell. Greater than four GFP-centrin foci was defined as a centriole over-duplication event.

The over-duplication of centrioles was quantified in U2OS cells that were arrested in S-phase with aphidicholin for 48 hrs. 45% of control cells exhibited centriole and centrosome over-duplication. RNAi treatment was performed 24 hrs before S-phase arrest. Over-duplication was measured by staining cells for α-centrin and α-γ-tubulin. An over-duplication event was scored only if more four centrin foci were observed to co-localize with γ-tubulin labeled centrosomes.

Fluorescence quantification

The fluorescence intensity signal of both centrioles and total cell levels was quantified by placing a region of interest around the centrioles or around the cell, respectively, and determining the total integrated intensity for the region of interest. Where appropriate fluorescence was normalized to the peak fluorescence intensity. Background fluorescence was subtracted from these values prior to analysis using Microsoft Excel.

The width of $Cep135^{mini}$ and γ-tubulin localization were measured by using linescan analyses. Linescans were acquired by measuring the maximum fluorescence across a line drawn on cross sectional views of the centriole/centrosome. The full width at half maximum was calculated in ImageJ by measuring the distance across the fluorescence distribution at the half maximum fluorescence intensity.

Cell cycle definition based on centriole morphology

The cell cycle stage for both centriole frequency counts and fluorescence quantification were based on the following criterion. Centrioles were defined based on centrin staining and PCM based on γ-tubulin staining. G1 cells were defined as those with two centrioles and a

single PCM focus or two closely positioned (<1.0 μm) PCM foci. S-phase cells were defined as those with two centriole pairs, each with an associated PCM focus, that were closely positioned (<1.0 μm). G2-phase cells were defined as those with two centriole pairs, each with an associated PCM focus, that were separated (>1.0 μm). Pre-anaphase, mitotic cells were based on having condensed chromosomes. Anaphase cells had separated DNA masses. Telophase cells had separated DNA masses at opposite ends of a divided cell.

Supplemental References.

[S1] Cheeseman, I.M., and Desai, A. (2005). A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. Sci STKE *2005*, pl1.

[S2] Manandhar, G., Simerly, C., Salisbury, J.L., and Schatten, G. (1999). Centriole and centrin degeneration during mouse spermiogenesis. Cell motility and the cytoskeleton *43*, 137-144.

[S3] Lin, Y.C., Chang, C.W., Hsu, W.B., Tang, C.J., Lin, Y.N., Chou, E.J., Wu, C.T., and Tang, T.K. (2013). Human microcephaly protein CEP135 binds to hSAS-6 and CPAP, and is required for centriole assembly. The EMBO journal *32*, 1141-1154.

[S4] Bayless, B.A., Giddings, T.H., Jr., Winey, M., and Pearson, C.G. (2012). Bld10/Cep135 stabilizes basal bodies to resist cilia-generated forces. Molecular biology of the cell *23*, 4820-4832.

[S5] Pearson, C.G., Osborn, D.P., Giddings, T.H., Jr., Beales, P.L., and Winey, M. (2009). Basal body stability and ciliogenesis requires the conserved component Poc1. The Journal of cell biology *187*, 905-920.