Published in final edited form as:

Curr Biol. 2015 October 5; 25(19): 2591–2596. doi:10.1016/j.cub.2015.08.039.

A short CEP135 splice isoform controls centriole duplication

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Summary

Centriole duplication is coordinated such that a single round of duplication occurs during each cell cycle. Disruption of this synchrony causes defects including supernumerary centrosomes in cancer and perturbed ciliary signaling [1–5]. To preserve the normal number of centrioles, the level, localization, and post-translational modification of centriole proteins is regulated so that when centriole protein expression and/or activity is increased, centrioles self-assemble. Assembly is initiated by the formation of the cartwheel structure that comprises the base of centrioles [6–11]. SAS-6 constitutes the cartwheel and SAS-6 levels remain low until centriole assembly is initiated at S-phase onset [3, 12, 13]. Cep135 physically links to SAS-6 near the site of microtubule nucleation and binds to CPAP for triplet microtubule formation [13, 14]. We identify two distinct protein isoforms of Cep135 that antagonize each other to modulate centriole duplication: full length Cep135 (Cep135^{full}) promotes new assembly while a short isoform, Cep135^{mini}, represses it. Cep135^{mini} represses centriole duplication by limiting the centriolar localization of Cep135^{full} binding proteins (SAS-6 and CPAP) and the pericentriolar localization of γ-tubulin. The Cep135 isoforms exhibit distinct and complementary centrosomal localization during the cell cycle. Cep135^{mini} protein decreases from centrosomes upon anaphase onset. We suggest that the decrease in Cep135^{mini} from centrosomes promotes centriole assembly. The repression of centriole duplication by a splice isoform of a protein that normally promotes it serves as a novel mechanism to limit centriole duplication.

Keywords

centriole; microtubule; centrosome; Cep135; alternative splicing

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Supplemental Information

Supplemental information includes four figures and Supplemental Experimental Procedures.

Author Contributions

KDD designed and performed experiments, DGS, BAB, MEP, LRH, and THG performed experiments, DFG designed and produced macros for image analysis, and CGP designed and performed experiments and wrote the manuscript.

Results and Discussion

Cep135 is a microcephaly associated (MCPH8), cartwheel protein that promotes centriole assembly and stability [9, 13, 15–19]. The protein contains a coiled-coil domain and conserved regions near the N-terminus and the C-terminus, respectively (Figure 1A). The Cep135 N-terminus binds to microtubules and CPAP [20, 21]. The Cep135 C-terminus binds to the cartwheel protein SAS-6 and maintains it at the cartwheel in *Chlamydomonas* [22]. Thus, Cep135 links the cartwheel with centriole triplet microtubule assembly, an early event in centriole biogenesis [13]. How Cep135 activity is regulated remains unclear [3].

A short isoform of Cep135 localizes to centrioles and the PCM

We identified an alternative splice isoform of human *Cep135* (Figures 1A and S1A; Cep135^{mini}). Intron 5 is retained in the mRNA of this short isoform causing translation read-through into the intron thereby adding 16 amino acids followed by a stop codon to generate Cep135^{mini}. The 29 kDa Cep135^{mini} isoform contains the Cep135 N-terminus required for microtubule binding but lacks the predicted CPAP and SAS-6 binding domains found in the 134 kDa Cep135^{full} isoform [13, 16]. Both Cep135^{full} and Cep135^{mini} transcripts are detected in U2OS, RPE1, and HeLa cells (Figure S1B; data not shown).

Affinity purified antibodies that specifically recognize the Cep135^{mini} isoform were generated using two peptides containing amino acid sequences specific to Cep135^{mini}'s divergent C-terminus (Figures 1, S1). Cep135^{mini} localizes to centrosomes in RPE1, U2OS, and HeLa cells (Figures 1B–F, S1; data not shown). Similar localization is found in cells expressing fluorescent protein fusions to Cep135^{mini} (Figure S1I). Cep135^{mini}'s localization pattern changes through the cell cycle. During G1-phase of the cell cycle, Cep135^{mini} localizes predominantly to the proximal-end of centrioles but is slightly spread compared to Cep135^{full} (Figures 1B, S4C–F), while in G2 it localizes both to centrioles and to the pericentriolar material (PCM) as judged by co-staining with γ-tubulin (Figures 1B, C and S4D–F). Cep135^{full} localization to the PCM was not observed in either G1 or G2 (Figures 1B, C and S1I) [16, 20, 23–25]. Immuno-EM localization also places Cep135^{mini} at the proximal end of the centriole co-incident with the triplet microtubules and the PCM (Figures 1D–F). Thus, the Cep135 protein isoforms exhibit distinct localization patterns at the centrosome.

Cep135^{full} and Cep135^{mini} have opposing functions in centriole duplication

The unique localization patterns of the two Cep135 isoforms suggest that Cep135^{full} and Cep135^{mini} have distinct functions. To determine how Cep135^{full} and Cep135^{mini} expression impacts centriole duplication, we knocked down or over expressed these isoforms in U2OS cells and quantified the frequency of centriole over- and under-duplication [26]. Cep135^{full} knockdown significantly increases the number of cells with under-duplicated centrioles (Figures 2A, B) while Cep135^{full} over expression increases the number of cells with over-duplicated centrioles (Figure 2C). These results confirm prior studies showing that Cep135^{full} promotes centriole assembly and that its levels must be controlled to limit centriole over-duplication [9, 13]. Conversely, Cep135^{mini} knockdown increases the number of cells with over-duplicated centrioles (Figure 2A, B) while over expression of Cep135^{mini} causes an increase in cells with under-duplicated centrioles (Figure 2C). These results suggest that Cep135^{mini} represses centriole assembly. Cep135^{mini} knockdown was confirmed by measuring transcript and protein levels (Figure S1F,H and S2B,C). All phenotypes were rescued by exogenous expression of siRNA impervious mutants (Figure S2D). Finally, the Cep135^{full} and Cep135^{mini} knockdown had a minimal impact on the cell cycle (Figure S2E); suggesting, the phenotypes are not due to changes in the cell cycle or in aberrant cell divisions. We did observe a low level of multipolar mitoses upon Cep135^{mini} knockdown as is expected with centrosome amplification (data not shown). Together, the knockdown and over expression experiments support a model in which Cep135^{mini}, in contrast to Cep135^{full}, negatively regulates centriole and centrosome duplication.

To determine whether Cep135^{full} and Cep135^{mini} perturbations affect centriole duplication independent of the cell cycle, we next examined whether these isoforms modulate aberrant Plk4-induced centriole amplification in S-phase arrested RPE1 cells [8–11]. 61% of control cells that over express Plk4 over-duplicate their centrioles (Figures 2D, E). Cep135^{full} knockdown blocks Plk4-induced centriole over-duplication (Figures 2E) while Cep135^{full} over expression moderately augments centriole over-duplication (Figure 2G, [9, 13]). In contrast, Cep135^{mini} knockdown moderately increases over-duplication and also increases the total number of centrioles per cell (Figure 2F), while Cep135^{mini} over expression dramatically reduces the number of cells with over-duplicated centrioles (Figure 2G). Concurrent knockdown of both isoforms partially rescues the loss in centriole over-duplication that is seen in Cep135^{full} only knockdown (Figure 2E). A nearly identical trend was observed in S-phase arrested U2OS cells that normally over-duplicate their centrioles (Figure S2A). Collectively, these results suggest that the balance between the Cep135 isoforms is important for the normal homeostasis of centriole numbers and that Cep135^{mini} counteracts Cep135^{full} function in promoting centriole duplication.

Cep135^{mini} expression limits the centriolar levels of essential assembly factors

The negative effect of Cep135^{mini} on centriole duplication led us to ask whether Cep135^{mini} disrupts the ability of Cep135^{full} to promote centriole duplication. A simple explanation is that Cep135^{mini} functions as a dominant negative molecule to remove Cep135^{full} from the centriole. Over expression of either Cep135 isoform does not disrupt the localization of the other (Figure S3A) suggesting that Cep135^{full} is able to localize to centrioles even when Cep135^{mini} levels are high.

An alternative possibility is that over expressed Cep135^{mini} disrupts centriole duplication by binding to Cep135^{full} and precluding its association with its binding partners (SAS-6 and CPAP). To explore the potential interaction between the Cep135 isoforms, we took advantage of the ectopic cytoplasmic foci that Cep135 forms upon over expression [21, 27]. Such foci are presumably the result of oligomerization of Cep135 protein. When both isoforms are expressed in RPE1 cells, ectopic foci that are not associated with centrosomes contain both Cep135^{full} and Cep135^{mini}. This suggests that Cep135^{mini} is competent to associate with Cep135^{full} (Figure S3B).

We next asked whether Cep135^{mini} affects the localization of Cep135^{full} binding proteins that function in centriole duplication (SAS-6 and CPAP). SAS-6 dependent cartwheel assembly initiates centriole biogenesis [12, 28–30]. Over expression of Cep135^{full} and Cep135^{mini} decreases SAS-6 levels at centrioles by 48% and 44%, respectively (Figure 3A). The reduced SAS-6 levels are consistent with the loss of SAS-6 from centrioles in *Chlamydomonas bld10* (Cep135) mutants and suggests that the interplay between SAS-6 and Cep135^{full} is important for new centriole biogenesis [21, 22]. Overexpressed Cep135^{full} protein binds SAS-6 and might sequester SAS-6 from the centriole (Figure S3C). In contrast, we speculate that Cep135^{mini}, which only weakly and variably interacts with SAS-6, promotes a cartwheel conformation that disrupts SAS-6 localization to the centriole (Figure S3C). We next assessed whether the centriolar localization of the Cep135^{full} binding protein and centriole duplication factor, CPAP, is affected by Cep135 isoform levels [9, 21, 31]. Cep135^{full} over expression modestly reduces centriolar CPAP localization (Figure 3B), which we predict is because expressed Cep135^{full} binds to and sequesters CPAP to the cytoplasm (Figure S3D). Conversely, Cep135^{mini}, which does not interact with CPAP, exhibits a potent inhibitory effect on CPAP localization (Figure S3D). This may allow Cep135^{mini} to selectively inhibit centriole duplication. Collectively, our data suggest that Cep135^{mini} prevents centriole duplication by limiting Cep135^{full}-interacting, centriole assembly factors from associating with the centriole.

The PCM network of proteins surrounding centrioles is organized into distinct functional domains [32–35]. The PCM is required for centriole biogenesis and CPAP is important for PCM organization [28, 36–38]. The PCM establishes a nucleation domain from which nascent centrioles are built, beginning with the cartwheel [32–35]. Over expression of Cep135^{mini}, but not Cep135^{full}, causes a 56% decrease in γ-tubulin from the PCM (Figure 3C). Thus, Cep135^{mini} over expression causes loss of both centriolar (SAS-6 and CPAP) and PCM (γ-tubulin) components suggesting that it regulates multiple facets of centriole and centrosome biogenesis.

Cep135 isoforms localize in a cell cycle dependent manner

The antagonistic functions of the Cep135 isoforms suggests that their centriolar levels and localization are modulated through the cell cycle to promote centriole assembly only during G1/S-phase of the cell cycle. To test this, we staged cycling cells and examined the centriolar level of the Cep135 isoforms. Cep135^{full} levels are high during centriole duplication at the G1/S-phase boundary (Figure 4A). Effectively one half of the total Cep135^{full} protein is predicted to be associated with each G1 centriole or new centriole pair following S-phase. These levels increase by 15% through metaphase of mitosis (Figure 4A) and then increase further (11%) by telophase (Figure 4C). Consistent with a role for Cep135^{mini} in repressing centriole assembly, Cep135^{mini} protein levels are lowest during G1/S when centrioles duplicate. After centriole duplication, the centriolar levels of Cep135^{mini} increase and peak at metaphase of mitosis. Because Cep135^{mini} over expression decreases γ-tubulin levels at the centrosome (Figure 3C), the high mitotic Cep135^{mini} levels are inconsistent with the high γ-tubulin levels found at mitotic centrosomes [39]. Perhaps the Cep135^{mini} over expression results represents non-physiological Cep135^{mini} activity. Alternatively, Cep135^{mini} may normally control γ-tubulin recruitment to the centrosome. In summary, at G1/S, Cep135^{full} levels are high and Cep135^{mini} levels are low to promote new centriole biogenesis while Cep135^{mini} increases through the remainder of the cell cycle until metaphase, perhaps to repress promiscuous centriole duplication and PCM expansion.

Centrin localizes to the distal end of centrioles, allowing us to determine the sub-centrosomal localization of the Cep135 isoforms during the cell cycle (Figures 4B and S4C; [40]). As expected, Cep135^{full} localizes at the proximal end of the two centrioles during G1 and at the mother centrioles during S-phase. Consistent with previous reports, Cep135^{full} is not detectable at daughter centrioles until G2 [35]. Maturation of the daughter centriole is accompanied by a slow accumulation of Cep135^{full} so that protein levels are once again high by the G1/S-phase boundary of the following cell cycle. Additionally, we propose that Cep135^{full} at the mother centriole is important for nucleation of the daughter centriole as is shown for SAS-6 [41, 42].

Unlike Cep135^{full}, Cep135^{mini} localizes to the proximal end of the centriole during G1 and, upon progression into the cell cycle, becomes increasingly associated with the PCM (Figures 4B and S4D–F). This redistribution of Cep135^{mini} to the PCM may position Cep135^{mini} to suppress Cep135^{full}-dependent centriole biogenesis by limiting Cep135^{full}'s association with its binding partners. However, the function of the unique localization between the two Cep135 isoforms remains to be discovered. Upon transit from metaphase into the subsequent G1, Cep135^{mini} levels drop. Consistent with the Cep135^{mini} protein decrease, Cep135^{mini} transcript levels also decrease during mitosis (Figure S4A). We suggest this decrease permits centriole assembly.

Conclusions

We discovered a Cep135 splice isoform, Cep135^{mini}, which limits centriole duplication (Figure 4D). Cep135^{mini} is a centrosome localized protein whose levels are regulated through the cell cycle and play an inhibitory role in centriole assembly. Following centriole duplication, Cep135^{mini} levels accumulate to restrict centriole duplication. By surrounding the Cep135^{full}-containing mother centriole, Cep135^{mini} might block the assembly of new centrioles by limiting the ability of SAS-6, CPAP, and the PCM (γ-tubulin) to promote centriole biogenesis. The negative regulation of centriole duplication by a splice isoform of a protein that normally promotes it is a potent and novel mechanism to limit centriole duplication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Christina Clarissa and Courtney Ozzello for immuno-EM imaging expertise, Dr. Iain Cheeseman for vectors, Dr. Brian Tsou for the Plk4 overexpressing cells and discussions, Dr. Tang K. Tang for α-Cep135^{full} antibodies and GFP constructs and Dr. Pierre Gönczy for GFP constructs. Thank you to Drs. Lee Niswander, Alex Stemm-Wolf, Jennifer Deluca, Brian Mitchell, Richard Davis, Mark Winey, and Debbie Klos-Derhing for comments and helpful discussions on the manuscript. CGP is supported by NIGMS (GM099820), the Pew Biomedical Scholars Program, and the Boettcher Foundation.

Abbreviations

IEM	immuno-electron microscopy
PCM	pericentriolar material
TEM	transmission electron microscopy

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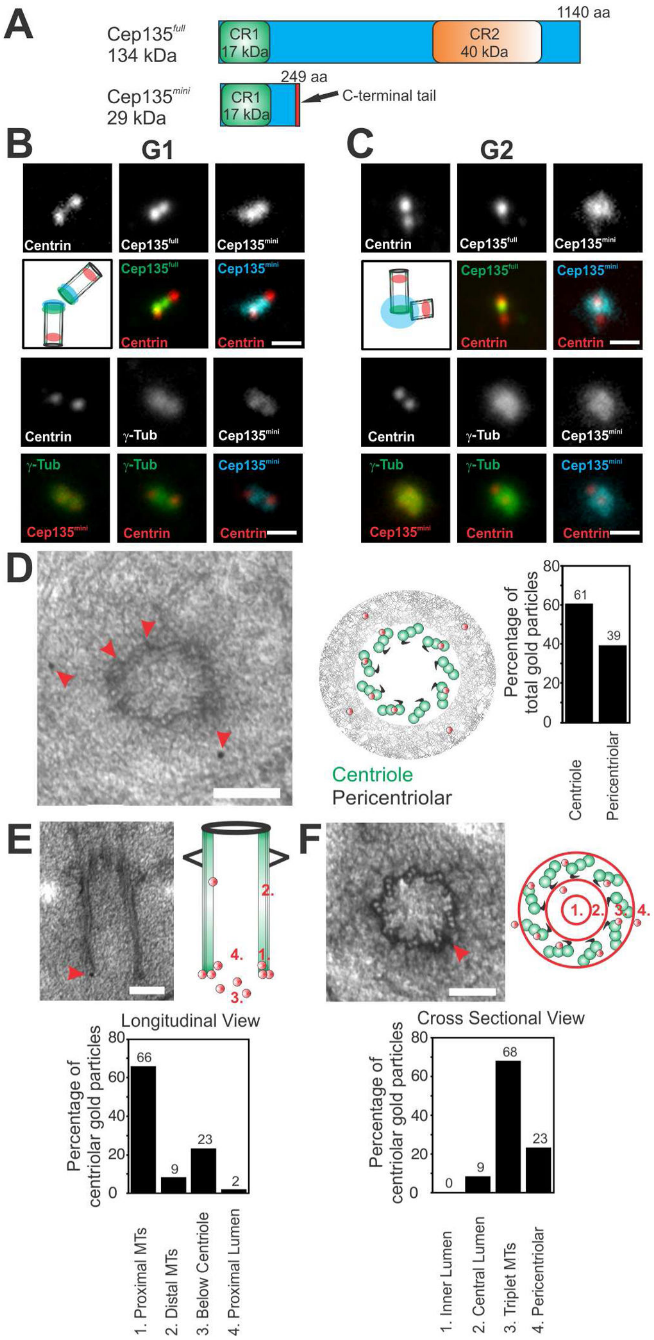

Figure 1. Cep135 isoforms exhibit unique localization patterns

(A) Cep135 protein isoforms. CR, conserved region. Red, C-terminal tail is divergent from Cep135^{full}. **(B)** Localization of Centrin (α-Centrin, red), γ-tubulin (α-γ-Tub; green), Cep135^{full} (α-Cep135^{full}, green), and Cep135^{mini} (Alexa488-α-Cep135^{mini}, cyan and red) during G1. Cep135^{full} and Cep135^{mini} localize to the proximal end of G1 centrioles. Scale bar, 1.0 μm. **(C)** During G2, Cep135^{mini} localizes to centrioles and PCM. Scale bar, 1.0 μm. **(D)** Immuno-EM localization of Cep135^{mini} to the centriolar microtubules and the PCM (n=152 gold particles for 33 centrioles). **(E and F)** Cep135^{mini} localizes to the proximal centriole triplet microtubules. (n=47 gold particles). **(D–F)** The relative distribution of gold particles was quantified for localization to the centrioles and the PCM, centriolar longitudinal sections, and centriolar cross sections, respectively. Red arrowheads denote gold localization. Scale bar, 100 nm.

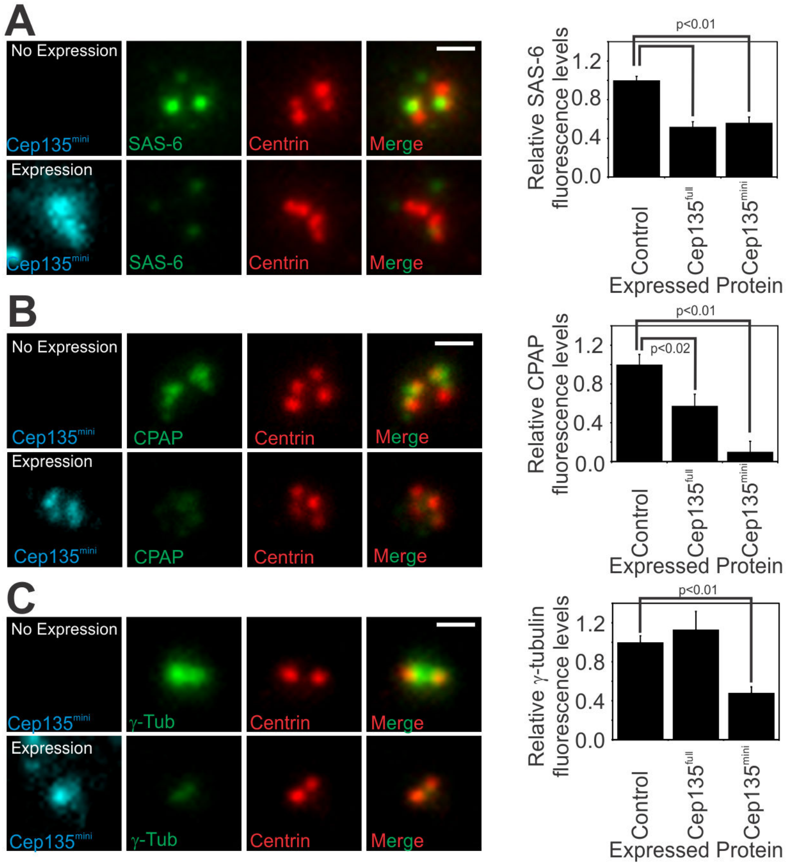

Figure 2. Cep135$^{\text{mini}}$ inhibits centriole duplication

(A) Centrioles (Centrin, red) are visualized relative to PCM (γ-tubulin, green) to quantify centriole under- and over-duplication in cycling U2OS cells. Normal centriole number is characterized as having either two centrioles per PCM focus or closely positioned foci during G1 or two centrioles per PCM focus during S-, G2-, and M-phase of the cell cycle. Scale bar, 1 μm. **(B)** Cep135$^{\text{full}}$ and Cep135$^{\text{mini}}$ depletion inhibits and promotes centriole duplication, respectively. Cep135$^{\text{full}}$ knockdown causes an increase in cells with under-duplicated centrioles (1±1% versus 16±6%). Cep135$^{\text{mini}}$ knockdown causes an increase in centriole over-duplication (7±2% versus 16±5%). Mean±SD represent five separate experiments for >500 cells for each condition. **(C)** Exogenous Cep135$^{\text{full}}$ and Cep135$^{\text{mini}}$ over expression induces an increase in centriole over-duplication (9±5% versus 41±16%) and an increase in centriole under-duplication (2±2% versus 43±19%), respectively. Mean ±SD represents three separate experiments. **(D)** Plk4 over expression causes centriole amplification (Centrin-GFP, green) in 61±6% of S-phase arrested RPE1 cells. Scale bar, 1 μm. **(E)** Cep135$^{\text{full}}$ knockdown reduces the number of cells with amplified centrioles (61±6% versus 22±5%) while Cep135$^{\text{mini}}$ knockdown marginally increases the number of cells with amplified centrioles (61±6% versus 72±4%). Knockdown of both isoforms returned the cells to near control levels of centriole over-duplication (45±6%). **(F)** Cep135$^{\text{mini}}$ depletion increases the number of centrioles per cell (8±2 versus 11±4 centrioles per cell). **(G)** Over expression of Cep135$^{\text{full}}$ causes a moderate increase in the number of cells with amplified centrioles (64±5% versus 80±7%) while Cep135$^{\text{mini}}$ over expression represses centriole amplification (64±5% versus 6±6%). Mean±SD represents three separate experiments.

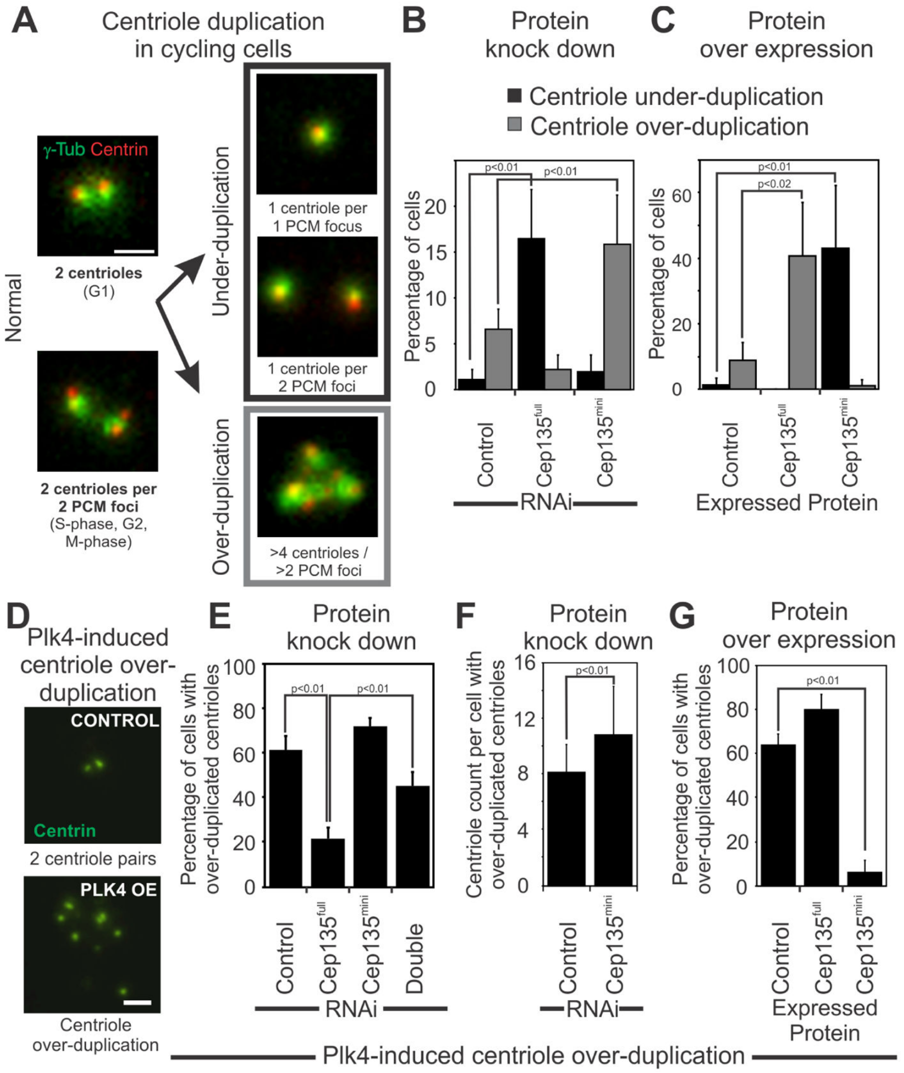

Figure 3. Cep135^{mini} expression displaces SAS-6, CPAP, and γ-tubulin from centrioles and centrosomes

(A) Exogenous Cep135^{full} and Cep135^{mini} expression in S-phase arrested RPE1 cells decreases SAS-6 localization to centrioles. Exogenous Cep135^{full} and Cep135^{mini} expression causes a 48% and 44% decrease in SAS-6 levels, respectively. Upper panels depict SAS-6 (green) in non-transfected cells and lower panels show SAS-6 in Cep135^{mini} transfected cells. Centrin (red) levels were not affected by expression of either protein. **(B)** Cep135^{mini} expression in S-phase arrested RPE1 cells decreases CPAP localization to centrioles. Cep135^{mini} expression causes a 90% decrease in CPAP levels. Cep135^{full} expression causes an intermediate 43% decrease in CPAP levels. Upper panels depict CPAP (green) in non-transfected cells and lower panels show CPAP in Cep135^{mini} transfected cells. **(C)** Exogenous Cep135^{mini}, but not Cep135^{full}, expression in cycling RPE1 cells causes a 52% decrease in γ-tubulin levels. Upper panels depict γ-tubulin (green) in non-transfected cells and lower panels show γ-tubulin in Cep135^{mini} transfected cells. **(A–C)** Mean±SEM represents at least three separate experiments. Scale bar, 1 μm.

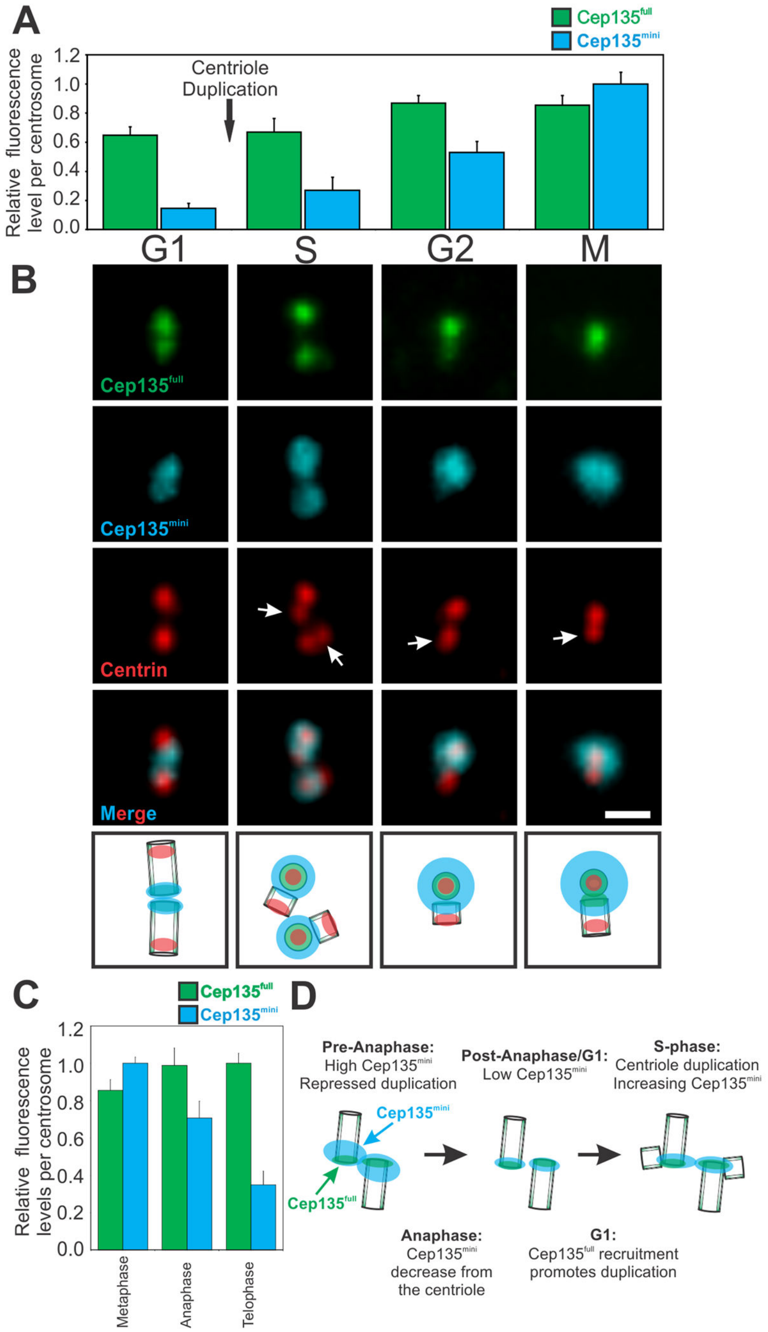

Figure 4. Cep135$^{\text{mini}}$ levels are controlled through the cell cycle

(A) Cep135$^{\text{full}}$ (green) and Cep135$^{\text{mini}}$ (cyan) exhibit differential protein levels at centrioles and centrosomes through the cell cycle. Normalized fluorescence levels were quantified per centrosome (single G1 centriole or S phase, G2, or M centriole pair. The fluorescence of the two G1 centrioles and four S-phase centrioles (two centriole pairs) was normalized by halving the total fluorescence. Mean±SEM represents three separate experiments of >20 cells per condition. **(B)** Cep135$^{\text{full}}$ (α-Cep135$^{\text{full}}$, green) and Cep135$^{\text{mini}}$ (Alexa488 labeled α-Cep135$^{\text{mini}}$, cyan) exhibit distinct and dynamic localization relative to centrioles (Centrin, red). Scale bar, 1 μm. **(C)** Cep135^{full} levels slightly increase during anaphase and telophase while Cep135^{mini} levels drop sharply at anaphase and telophase. Mean±SEM of three separate experiments of >20 cells per condition. **(D)** Model of Cep135 isoform regulation of centriole duplication.