Current Biology 17, 1960–1966, November 20, 2007 ©2007 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2007.10.019

Report

Human Cep192 Is Required for Mitotic Centrosome and Spindle Assembly

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Summary

As cells enter mitosis, centrosomes dramatically increase in size and ability to nucleate microtubules. This process, termed centrosome maturation, is driven by the accumulation and activation of γ-tubulin and other proteins that form the pericentriolar material on centrosomes during G2/prophase. Here, we show that the human centrosomal protein, Cep192 (centrosomal protein of 192 kDa), is an essential component of the maturation machinery. Specifically, we have found that siRNA depletion of Cep192 results in a complete loss of functional centrosomes in mitotic but not interphase cells. In mitotic cells lacking Cep192, microtubules become organized around chromosomes but rarely acquire stable bipolar configurations. These cells contain normal numbers of centrioles but cannot assemble γ-tubulin, pericentrin, or other pericentriolar proteins into an organized PCM. Alternatively, overexpression of Cep192 results in the formation of multiple, extracentriolar foci of γ-tubulin and pericentrin. Together, our findings support the hypothesis that Cep192 stimulates the formation of the scaffolding upon which γ-tubulin ring complexes and other proteins involved in microtubule nucleation and spindle assembly become functional during mitosis.

Results and Discussion

We used automated microscopy to screen an RNAi library targeting 23,835 human genes for siRNAs inducing a mitotic-arrest phenotype. Control HeLa or U2OS cells have a mitotic index of ~5%, and our screen identified over 200 genes whose silencing elevated the mitotic index in both cell lines to 10%–40% [1]. Among these, two nonoverlapping siRNAs targeting the centrosomal protein Cep192 were found to induce a particularly strong phenotype: Mitotic indices in both cell lines were elevated above 30% 48 hr after transfection with either siRNA. (Cep192 knockdown was confirmed by quantitative RT-PCR [Figure S1 in the Supplemental Data available online] and by immunofluorescence [Figure 1A] with a specific antibody raised against the C-terminal region of Cep192.) Cep192 was originally described in a proteomic analysis of the human centrosome and shown to maintain its association with the organelle after relatively harsh salt treatment [2]. Thus, it is probably a core component of the centrosomal scaffolding.

Followup immunofluorescence indicated a fundamental role for Cep192 in mitotic-spindle assembly; 80% of spindles were aberrant after 72 hr siRNA treatment (Figures 1A and 1B). Spindle defects could be grouped into three phenotypic categories: (1) monopolar, (2) small bipolar, and (3) disorganized spindles. The "disorganized-spindle" phenotype in which microtubules appear randomly scattered around chromosomes was by far the most prevalent (~70% of all spindles observed). Entirely consistent results were obtained with either Cep192 siRNA sequence.

Loss of Cep192 Induces the Formation of Spindles without Centrosomes

To specify Cep192's impact on spindle assembly, we visualized mitosis in living cells. In controls, two obvious microtubule organizing centers (MTOCs) corresponding to centrosomes were always apparent during prophase and subsequently directed the formation of bipolar spindles after nuclear-envelope breakdown at prometaphase. Control spindles typically segregated their chromosomes during anaphase and proceeded through telophase (Figure 1C and Movie S1). In contrast, centrosomes were not apparent in prophase cells lacking Cep192. Instead, nucleation of microtubules occurred randomly after nuclear-envelope breakdown, resulting in the formation of disorganized nonbipolar structures (Figure 1C and Movie S2).

Similar analyses of GFP-centrin-2-expressing cells indicated that centrioles were still present after Cep192 depletion but did not localize to opposite sides of chromosomes to form spindle poles (Figure S2 and Movies S3 and S4). Additionally, further analysis of centriole number indicated that the loss of Cep192 does not impact centriole duplication but induces premature centriole disengagement (Supplemental Data, Figure 3B, and Figure S3).

If mitotic centrosomes are not present in cells lacking Cep192, where do spindle microtubules originate? To address this question, we depolymerized microtubules in mitotic-arrested HeLa cells by cold treatment and repolymerized them by rapid warming, thus allowing the speed and position of microtubule polymerization in control versus Cep192-depleted cells to be determined. In controls, microtubules reappeared rapidly (within 1 min) and were organized in astral arrangements focused at the centrosomes (Figure S4). In contrast, MT growth in the absence of Cep192 occurred much more slowly (4 min) and was not organized at distinct foci. Instead, repolymerized microtubules appeared randomly at sites coincident with chromosomes. Interestingly, this effect was confined to mitosis in light of the fact that microtubule regrowth in Cep192 siRNA-treated interphase cells appeared normal (Figure S5). Thus, Cep192

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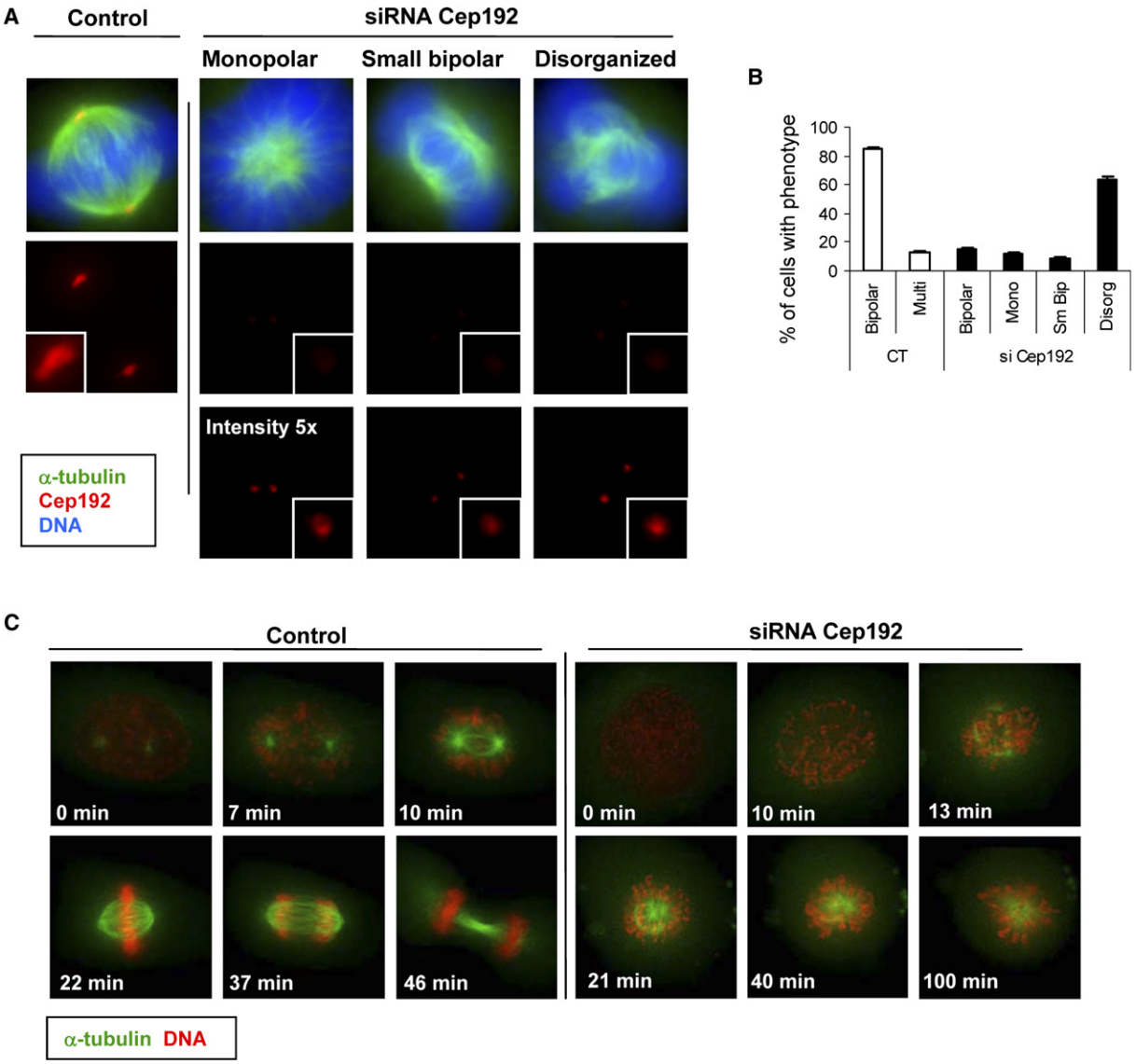


Figure 1. Depletion of Cep192 Disrupts Mitotic-Spindle Assembly

(A–C) HeLa cells stably expressing GFP-α-tubulin were transfected with Cep192 siRNA. Cells were fixed and stained with Cep192 and Hoechst 33258 72 hr after siRNA transfection, as shown in (A) and (B). (A) shows representative images of the different observed phenotypes. We obtained images in the middle row with identical acquisition conditions to show the efficiency of Cep192 knockdown (~90%). The bottom row are the same images after we increased the intensity gain 5-fold in order to visualize the two dots of Cep192 still associated with centrosomes in treated cells. (B) shows the frequency analysis of spindle phenotypes: bipolar, multipolar (Multi), monopolar (Mono), small bipolar (Sm Bip), and disorganized (Disorg). Nontreated cells are indicated as CT (control). As shown in (C), 48 hr after siRNA transfection, live cells stained with Hoechst 33258 were imaged in a DeltaVision microscope. Images are individual frames of video recordings (see Movies S1 and S2 in the Supplemental Data). Error bars in (B) show standard deviations (SDs).

is entirely and specifically required for the nucleation of centrosomal microtubules during mitosis.

Cep192 Accumulates on Mitotic PCM

To better understand how Cep192 activates mitotic centrosomes, we examined its localization throughout the cell cycle. Immunofluorescence revealed that Cep192 associates with centrosomes during both interphase and mitosis, closely colocalizes with centrosomal γ-tubulin, and surrounds both mother and daughter centrioles (Figure 2A and Figure S6). Thus, Cep192 is a component of the PCM. It is notable that, although γ-tubulin immunofluorescence is also present on mitotic-spindle microtubules ([3] and Figure 3A), no Cep192 was observable on microtubules or any discrete noncentrosomal structures.

Although Cep192 associates with centrosomes throughout the cell cycle, its levels do not remain constant: They increase 10-fold in prophase, remain high through metaphase, and fall during anaphase and telophase (Figures 2B and 2C). The accumulation of Cep192 on mitotic centrosomes mirrors that of γ-tubulin [4], providing a circumstantial link to centrosome maturation.

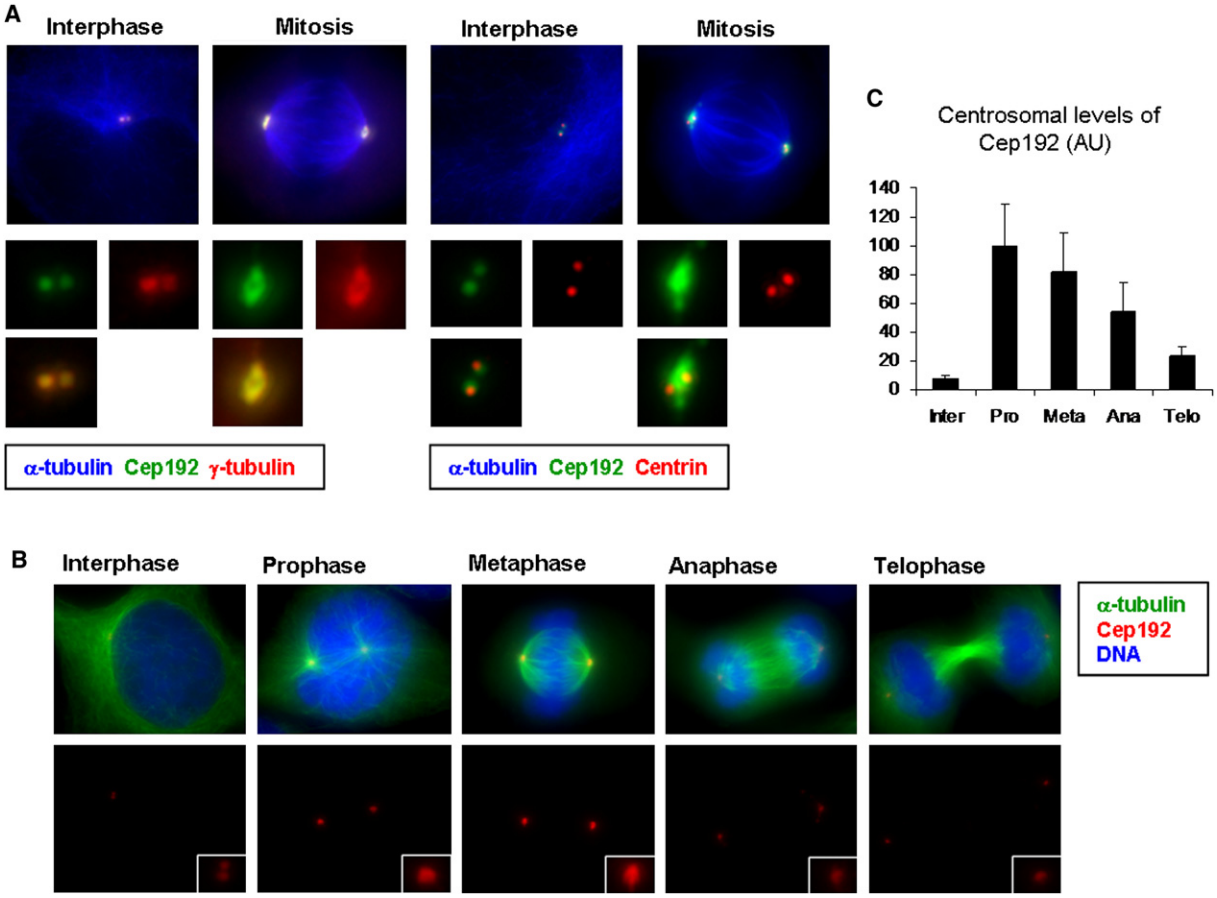


Figure 2. Cep192 Is a Pericentriolar Protein that Is Recruited to Mitotic Centrosomes

HeLa cells stably expressing GFP-α-tubulin were fixed and immunostained with the indicated antibodies and Hoechst 33258. (A) shows immunostaining of Cep192 and either γ-tubulin or centrin in interphase and mitotic cells. (B) shows cells in the different stages of mitosis. In the bottom row, only Cep192 immunostaining is shown. (C) shows the quantification of centrosomal Cep192 immunofluorescence levels throughout the cell cycle. We quantified at least 15 cells in each stage in three different experiments (AU, arbitrary units). Error bars in (C) show +SDs.

Cep192 Recruits Key PCM Components to Mitotic Centrosomes

Because Cep192 associates with the PCM and is required for the activation of mitotic centrosomes, we examined how its depletion impacted the assembly of other key PCM components. Strikingly, depletion of Cep192 was found to completely abolish γ-tubulin's association with centrosomes in most mitotic cells (Figure 3A). Moreover, in the few mitotic cells in which γ-tubulin immunostaining remained visible as small discrete dots (probably centrosomes), the fluorescence intensity of these dots was even lower than of interphase centrosomes. Thus, Cep192 depletion results in an effective loss of γ-tubulin from centrosomes when cells enter mitosis, rather than simply preventing the accumulation of additional γ-tubulin as centrosomes mature. Note that γ-tubulin retains its ability to associate with spindle microtubules under these conditions (Figure 3A). Although Cep192 depletion also caused some loss of γ-tubulin from interphase centrosomes (30% reduction, Figure 3A and Figure S7A), the effect was marginal relative to the mitotic phenotype. Consistent with this observation, microtubules were well organized during interphase, and as mentioned above, no significant deficiency in microtubule regrowth after cold treatment was observed in Cep192-depleted cells (Figure S5). Thus, Cep192 is absolutely required for the localization of γ-tubulin to the centrosome during mitosis, but only partially so during interphase.

Pericentrin, which has been proposed to serve as the primary scaffolding that binds and anchors γ-tubulin to centrosomes [5], was also affected by the loss of Cep192. Centrosome-associated pericentrin decreased by ~80% in mitotic Cep192 siRNA-treated cells (Figure 3B). The remaining pericentrin staining in these cells always appeared as two dots that were associated with one of the two members of the centriole pair (Figure 3B, bottom-right panel). This staining most probably corresponds to kendrin [6] because these structures cannot recruit γ-tubulin or function as MTOCs (Figure 3C).

Finally, we found that two additional components of the mitotic PCM, Aurora A kinase [7] and katanin p60 [8], were also mislocalized after Cep192 depletion (Figure S7B). Cep192 siRNA did not impact the overall expression levels of any of the PCM components analyzed, consistent with the hypothesis that Cep192 regulates localization but not stability of these proteins (Figure S7C).

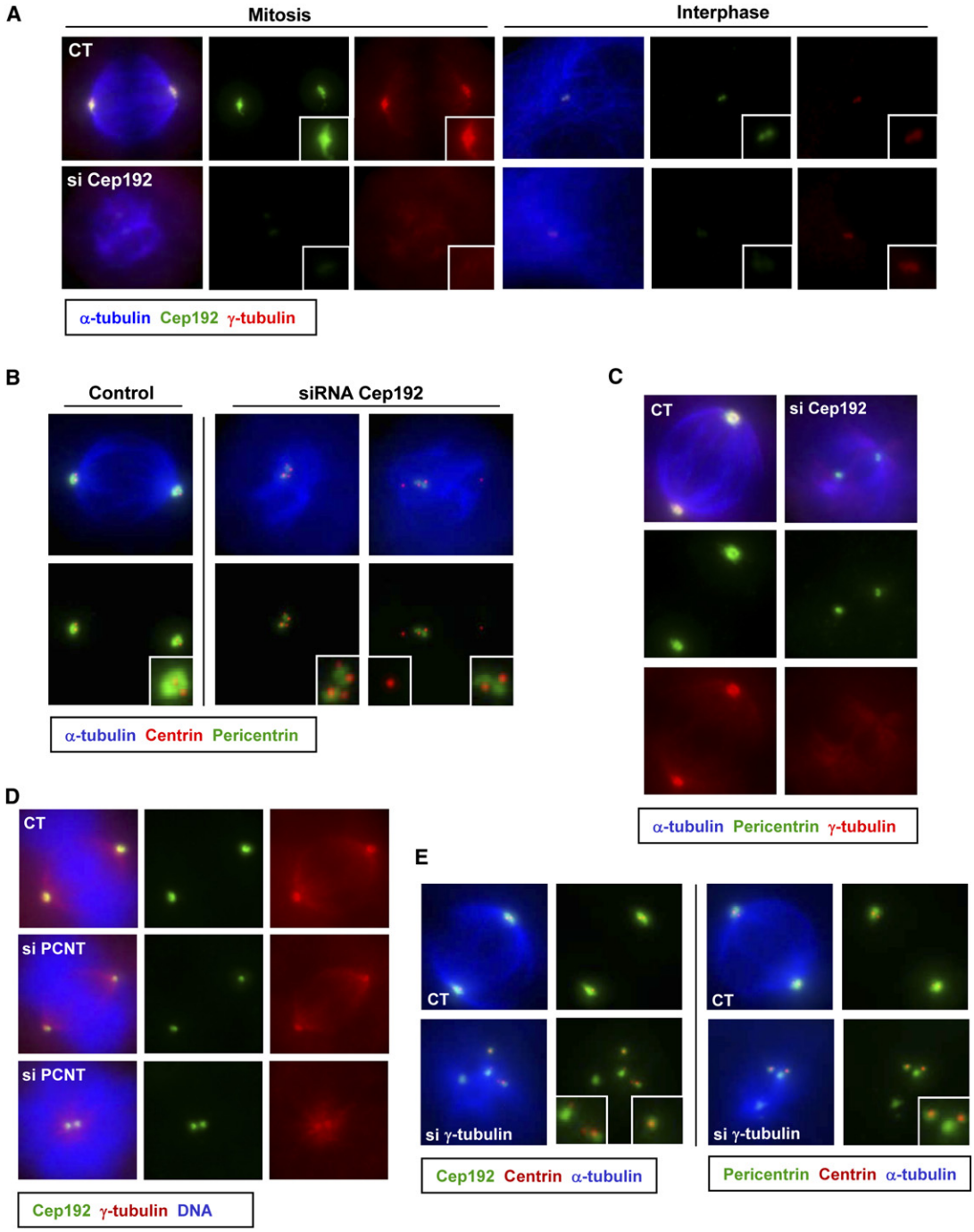
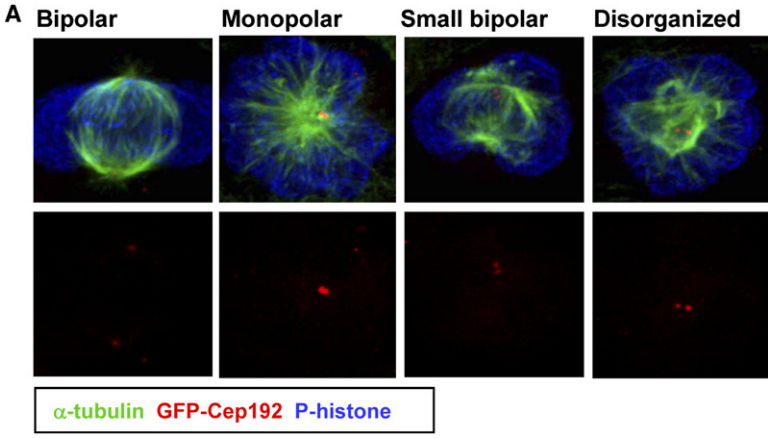


Figure 3. Cep192 Is Necessary for the Organization of the PCM during Mitosis

(A–C) HeLa cells stably expressing GFP-α-tubulin were fixed and immunostained 72 hr after transfection with Cep192 siRNA. (A) shows mitotic and interphase cells immunostained for Cep192 and γ-tubulin. (B) shows centrin and pericentrin immunostaining of mitotic cells. Note that a normal number of centrioles remains after Cep192 knockdown, but they often are disengaged (Discussion in Supplemental Data and Figure S3). (C) shows the staining of γ-tubulin and pericentrin.

(D) Saos-2 cells were fixed and immunostained for Cep192 and γ-tubulin 72 hr after completion of siRNA transfection to knockdown pericentrin (siPCNT) (Loss of pericentrin in HeLa or U2OS cells induces cell death and senescence, so these analyses were performed in Saos-2 cells that have been reported to enter mitosis with low levels of pericentrin [5].).

(E) After γ-tubulin siRNA transfection, HeLa cells stably expressing GFP-α-tubulin were fixed and immunostained for centrin and either Cep192 or pericentrin. Note that the γ-tubulin siRNA-transfected cell on the left has four centrioles, but one of the pairs has disengaged (see Supplemental Data for Discussion and Figure S3B). The treated cell on the right has only two centrioles because of defects in centriole replication after γ-tubulin knockdown, as previously reported [20, 21].



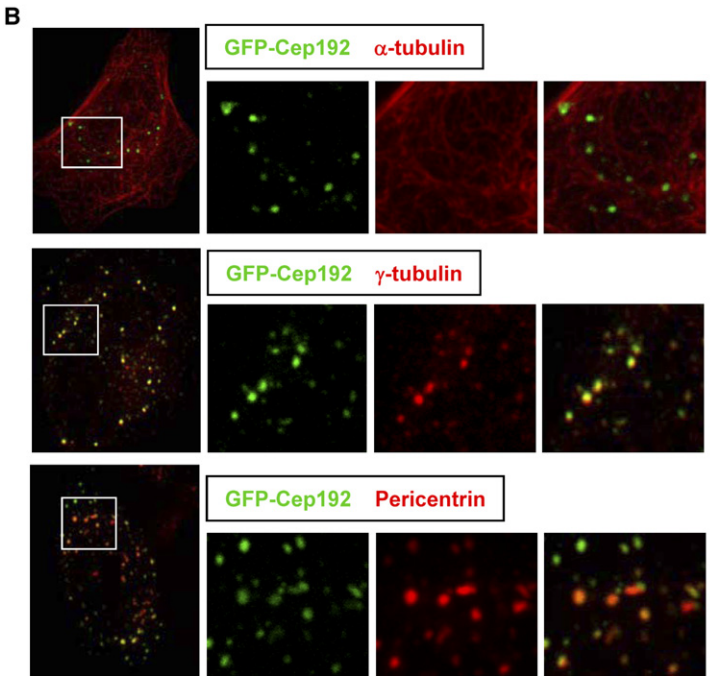


Figure 4. Overexpression of Cep192 Generates Abnormal Spindles

HeLa cells were transfected with a plasmid encoding GFP-Cep192. Cells were fixed and immunostained for α-tubulin, P-Ser10 Histone 3, γ-tubulin, or pericentrin as indicated 24 hr later. In (A), mitotic cells show the different observed phenotypes. (B) shows interphase cells expressing high levels of GFP-Cep192 and immunostained for α-tubulin, γ-tubulin, and pericentrin.

The Targeting of Cep192 Does Not Require Pericentrin, γ-Tubulin, or Aurora A Activation

Next, we sought to determine whether Cep192's centrosomal targeting was conversely dependent on pericentrin or γ-tubulin. Depletion of pericentrin did decrease the levels of mitotic-centrosome-associated Cep192, but the effect was relatively small (~60% reduction) (Figure 3D and Figure S8A). Thus, the targeting of Cep192 is only partially dependent on pericentrin. Depletion of γ-tubulin also had very little impact on the recruitment of Cep192 or pericentrin to discrete cellular foci. However, these foci did not constitute MTOCs and frequently appeared scattered (Figure 3E), suggesting that γ-tubulin might contribute to the overall cohesion of the PCM.

Finally, we examined whether Cep192's accumulation on mitotic centrosomes was stimulated by the Aurora A kinase signaling pathway because Aurora A is required for centrosome maturation in a variety of cell types [9, 10]. Somewhat surprisingly, inhibition of Aurora A with the cell-permeable inhibitor MLN8054 [11–13] had no impact on the centrosomal targeting of Cep192 (Figure S8B). However, we did observe severe defects in centrosomal and spindle morphology consistent with earlier results [12, 13], indicating that Aurora A had been inhibited. These results strongly support the hypothesis that Cep192 lies near the hierarchical base of the centrosome maturation "machinery."

Cep192 Impacts Centrosome Maturation Independently of Pericentrin

The phenotype found in Cep192 siRNA-treated cells is somewhat similar to that reported after pericentrin inhibition (i.e., decreased γ-tubulin on mitotic centrosomes [5]). Thus, it seemed possible that Cep192 influences mitosis solely by targeting pericentrin. However, direct comparative analyses of centrosomal and spindle phenotypes in Cep192 and pericentrin siRNA-treated cells suggest that this is not the case (Figures S9A and S9B). In particular, although Cep192 siRNA completely

abolished mitotic-centrosome-associated γ-tubulin, depletion of pericentrin resulted in a noticeably milder effect—centrosomal γ-tubulin levels decrease to ~60% (Figure S9C). In addition, relative to cells lacking pericentrin, Cep192 siRNA-treated cells displayed a significantly higher frequency of nonbipolar spindles (80% as compared to 15% in pericentrin-depleted cells and 5% in controls). Indeed, the impact of Cep192 siRNA matches more closely, both qualitatively and quantitatively, the effects of depleting γ-tubulin—70% of all spindles in γ-tubulin-depleted cells were disorganized and had morphologies identical to spindles in cells lacking Cep192 (Figure 3E). These data are consistent with the hypothesis that Cep192 influences centrosome maturation and spindle assembly via its relationship with γ-tubulin and, to some extent, does so independently of pericentrin.

Cep192 Overexpression Impacts Spindle Morphology

Loss of Cep192 severely perturbs mitotic spindles and centrosomes. To determine whether cells are also sensitive to the overexpression of Cep192, we transfected HeLa cells with a plasmid encoding the fusion protein GFP-Cep192. As expected, GFP-Cep192 localized exclusively to centrosomes. However, 24 hr after transfection with this construct, 40% of the cells showed abnormal mitotic spindles, the majority of which could be categorized as "disorganized" (Figure 4A). Additionally, GFP-Cep192 formed puncta throughout the cytoplasm of both mitotic and interphase cells that had the capacity to recruit γ-tubulin, several γ-tubulin-associated proteins, and pericentrin (Figure 4B and Figure S10). Surprisingly, however, GFP-Cep192 puncta do not form supernumerary MTOCs. These findings contrast earlier work in which the *Drosophila* centrosomal organizer centrosomin was found to form aggregates capable of recruiting γ-tubulin and generating functional MTOCs when expressed in mammalian cells. However, similarly to our findings, overexpression of γ-tubulin also forms cytosolic aggregates that lack microtubule-nucleating activity [14] (see Supplemental Data for further discussion).

Conservation of Cep192

Given the fundamental role of Cep192 in centrosome maturation, one would expect the protein to be highly conserved across phyla. A clear Cep192 homolog is predicted within the mouse genome (76% amino acid sequence similarity with human Cep192), and more distantly related proteins showing similarity to the C terminus of Cep192 are present in *X. laevis* and *D. renio* (Figure S11A). The *C. elegans* protein Spd-2 shows some sequence similarity to Cep192 within a small region of the molecule (Spd-2 domain), leading to the proposal that these proteins are functional homologs [15]. Intriguingly, both Spd-2 and Cep192 are recruited to the centrosome in mitosis and are necessary for the recruitment of PCM [15, 16]. However, unlike Cep192 in HeLa cells, Spd-2 is also required for centriole duplication in *C. elegans* embryos. A Spd-2 homolog in *Drosophila* (D-Spd-2) was recently shown to be partially necessary for γ-tubulin localization to the mitotic-spindle poles in S2 cells [17], although D-Spd-2 RNAi-treated cells do not seem to have defects in spindle morphology. Thus, the function of these three proteins, Spd-2, D-Spd-2, and Cep192, is not entirely conserved.

Cep192 also contains a fragmented PapD-like domain (Ensembl database, http://www.ensembl.org/) consisting of two immunoglobulin-like folds [18] (Figure S11C). Similar domains are found in the nematode MSP (major sperm protein) and mediate its assembly into large macromolecular fibers [19]. By analogy, Cep192 might have the capacity to form a structural lattice or scaffolding. We note that Pap-D domains are not present in Spd-2, which instead is predicted to form coiled coils.

In conclusion, our data identify Cep192 as an essential and specific contributor to mitotic-centrosome function and spindle assembly in human cells. Indeed, our data place this protein at the hierarchical base of the centrosome-maturation pathway. When considered in their entirety, we believe that our findings are consistent with a model in which Cep192 forms or stimulates the formation of a scaffolding on which proteins involved in mitotic microtubule nucleation accumulate and, perhaps become active (an extended discussion is provided in Supplemental Data). Further analysis will be required for determinating the specific mechanisms by which Cep192 performs this function.

Supplemental Data

Additional Discussion, eleven figures, and four movies are available at http://www.current-biology.com/cgi/content/full/17/22/1960/DC1/.

Acknowledgments

We thank J.L. Salisbury (Mayo Clinic Foundation) for the centrin antibody and the HeLa cells expressing GFP-centrin-2 and F.J. McNally (University of California) for the katanin p60 antibody. We are also grateful to L. Haren and A. Merdes (CNRS, Toulouse, France) for the antibodies against NEDD1, GCP-2, and GCP-4. This work was supported by grants from National Institutes of Health to D.J.S., and in part by Philip Morris USA, Inc. D.J.S. is a Scholar of the Leukemia and Lymphoma Society. M.A.G.F. is supported by a fellowship from the Spanish Ministry of Education and Science.

Received: May 16, 2007
Revised: October 2, 2007
Accepted: October 4, 2007
Published online: November 1, 2007

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