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Report

The Mammalian SPD-2 Ortholog Cep192 Regulates Centrosome Biogenesis

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Summary

Centrosomes are the major microtubule-organizing centers of mammalian cells. They are composed of a centriole pair and surrounding microtubule-nucleating material termed pericentriolar material (PCM) [1]. Bipolar mitotic spindle assembly relies on two intertwined processes: centriole duplication and centrosome maturation. In the first process, the single interphase centrosome duplicates in a tightly regulated manner so that two centrosomes are present in mitosis [2, 3]. In the second process, the two centrosomes increase in size and microtubule nucleation capacity through PCM recruitment, a process referred to as centrosome maturation [4]. Failure to properly orchestrate centrosome duplication and maturation is inevitably linked to spindle defects, which can result in aneuploidy and promote cancer progression [5]. It has been proposed that centriole assembly during duplication relies on both PCM and centriole proteins, raising the possibility that centriole duplication depends on PCM recruitment [6]. In support of this model, *C. elegans* SPD-2 and mammalian NEDD-1 (GCP-WD) are key regulators of both these processes [7–13]. SPD-2 protein sequence homologs have been identified in flies, mice, and humans, but their roles in centrosome biogenesis until now have remained unclear [10, 14–16]. Here, we show that Cep192, the human homolog of *C. elegans* and *D. melanogaster* SPD-2, is a major regulator of PCM recruitment, centrosome maturation, and centriole duplication in mammalian cells. We propose a model in which Cep192 and Pericentrin are mutually dependent for their localization to mitotic centrosomes during centrosome maturation. Both proteins are then required for NEDD-1 recruitment and the subsequent assembly of γ-TuRCs and other factors into fully functional centrosomes.

Results and Discussion

Cep192 Is a Pericentriolar Protein that Accumulates at Centrosomes during Mitosis

C. elegans SPD-2 is a centrosome protein that plays an upstream role in centriole duplication and centrosome maturation [7, 8, 10, 11, 17–20]. A sequence homolog of *C. elegans* SPD-2, called Cep192, has been identified in humans [10, 14]. Cep192 is present in centrosome preparations, and green fluorescent protein (GFP) chimeras of Cep192 localize to centrosomes [14, 21]. By immunofluorescence microscopy, we found that Cep192 colocalized with γ-tubulin, Centrin, and other centrosomal markers on interphase and mitotic centrosomes (Figures 1A and 1B, data not shown). In interphase cells, Cep192 distribution was similar to that of γ-tubulin, whereas it overlapped partially with Centrin. Analysis by immuno-electron microscopy (immuno-EM) revealed that Cep192 localizes in close proximity to the outer wall of interphase centrioles (Figure 1C). By using fluorescence recovery after photobleaching (FRAP) assays to examine protein dynamics, we found that centriolar Cep192 is in dynamic equilibrium with its cytoplasmic pool, in sharp contrast to Centrin, a stably associated centriolar protein (Figure 1D).

In mitotic cells, Cep192 distribution closely overlapped that of γ-tubulin, although Cep192 appeared to preferentially surround one of the two Centrin-positive structures per pole (Figures 1A and 1B, bottom panels, and data not shown). In fixed cells, Cep192 protein levels at centrosomes increased from interphase to mitosis about 12-fold, more than any other centrosome protein tested, including Pericentrin (4-fold), γ-tubulin (4-fold), and NEDD-1 (7-fold) (Figures 1A and 1B and Figure S1 available online). In order to determine the onset of Cep192 recruitment to centrosomes, we used cell lines stably integrated with a bacterial artificial chromosome (BAC) containing the murine homolog of Cep192 tagged with GFP (mCep192::GFP) at the C terminus, a construct we have shown to be functional [21] (Figure S2). We measured total mCep192::GFP centrosome intensity during the cell cycle by using time-lapse microscopy and observed that mCep192::GFP levels stay relatively constant prior to the G2/M transition, which coincides with the onset of centrosome separation, before rapidly increasing approximately 3-fold, in agreement with published results on γ-tubulin recruitment during mitosis [22] (Figure 1E, Figure S3, and Movies S1–S3). This increase does not reflect an overall increase in total Cep192 protein, which remains constant upon entry into mitosis (Figure S4). We note that the increase observed in Cep192 amounts on mitotic centrosomes is lower than that observed in fixed samples, which might be due to endogenous Cep192 competing with mCep192::GFP or antibody accessibility issues. Taken together, the results

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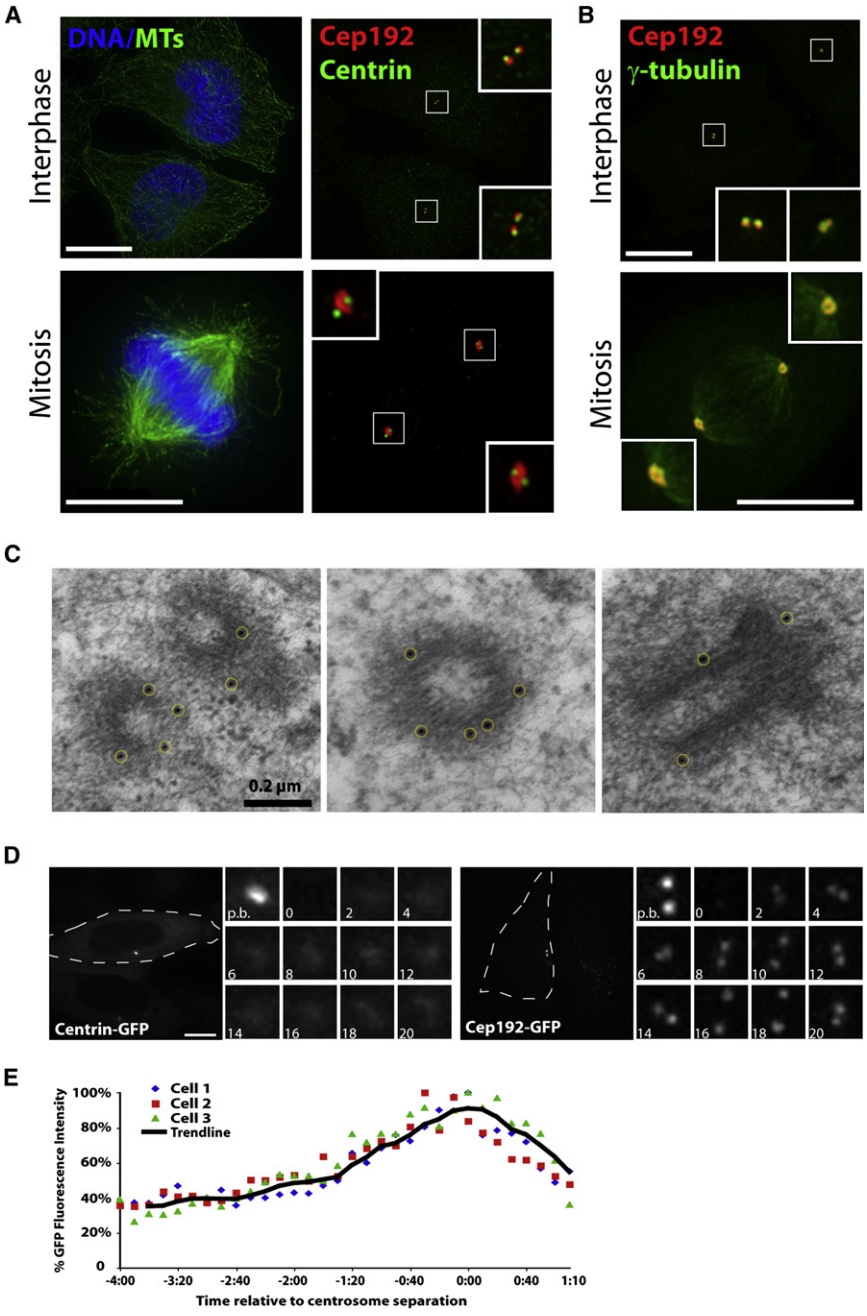

Figure 1. Cep192 Is a Pericentriolar Protein that Accumulates on Centrosomes during Mitosis

(A) Interphase (top panel) and mitotic (bottom panel) HeLa cells were labeled for DNA, microtubules, Cep192, and Centrin as a centriole marker. Insets are 4-fold magnifications of boxed regions. Scale bars represent 10 μm.

(B) Interphase (top panel) and mitotic (bottom panel) HeLa cells were stained as in (A) except that γ-tubulin was used as a PCM marker. Insets are 2-fold magnifications of boxed regions. Scale bars represent 10 μm.

(C) Immuno-EM images showing that Cep192 is localized in close proximity to the outer wall of interphase centrioles. The scale bar represents 0.2 μm.

(D) FRAP analysis of HeLa cells expressing GFP-tagged murine Centrin-2 or murine Cep192. Images were taken before (p.b.) and immediately after photobleaching (0) and every 2 min afterwards. Insets show 6-fold magnifications of the centrosomal region. The scale bar represents 10 μm.

(E) HeLa cells expressing murine Cep192 (mCep192) tagged at the C terminus with a bacterial artificial chromosome were imaged 4 hr prior to centrosome separation until cytokinesis and total centrosomal fluorescence was quantified. Also shown is a trend-line of a three-point moving average.

thus far indicate that Cep192 is a pericentriolar protein in dynamic equilibrium with its cytoplasmic pool and that Cep192 accumulates on centrosomes during mitosis like other pericentriolar material (PCM) proteins [12, 13, 22, 23].

Cep192 Is Required for Mitotic Spindle Assembly

In order to gain insight into the potential function of Cep192 in centrosome biogenesis, we depleted Cep192 in HeLa cells by using enzymatically prepared small interfering RNAs (esiRNAs) [24–26]. Transfection of Cep192 esiRNA resulted in a marked reduction of Cep192 levels either in whole-cell extracts (Figure S5) or at mitotic centrosomes (Figures 2A and 2B) to less than 5% those of the control. A significant decrease in a high-molecular-weight band corresponding to Cep192, which migrates slower than its predicted molecular weight of 192 kDa, was observed [14]. Cep192 depletion led to a 4-fold to 5-fold increase in the mitotic index relative to the control, consistent with our previous results (Figure 2C) [21]. This increase in mitotic index is explained by a high incidence of aberrant spindles upon Cep192 RNA interference (RNAi) (>80%), most of which (>90%) are monopolar (Figures 2A and 2D). Similar results were obtained with chemically synthesized siRNAs, although higher mitotic indices (15%–20%) were observed (Figure S2). To rule out potential off-target effects [27], we generated stable cell lines by BAC transgenesis expressing an RNAi-resistant mCep192::GFP messenger RNA (mRNA) [21]. In the two clones tested, we observed no increase in mitotic indices or abnormal mitotic spindles after endogenous Cep192 depletion (data not shown). These results showed that mCep192::GFP can functionally complement endogenous Cep192, thereby validating the specificity of our Cep192 RNAi phenotype (Figure S2).

Cep192 Is Required for PCM Recruitment and Centrosome Maturation

The spindle assembly defect observed upon Cep192 RNAi suggests that it might be required for centrosome assembly and/or centrosome maturation. Consistent with this possibility, we observed that the depletion of Cep192 lead to a decrease in γ-tubulin and NEDD-1 on interphase centrosomes, whereas Pericentrin levels were not significantly affected (Figure 3A). Consistent with the known role for NEDD-1 in γ-tubulin ring complex (γ-TuRC) recruitment and microtubule nucleation from interphase centrosomes, we observed a marked defect in microtubule repolymerization after recovery from

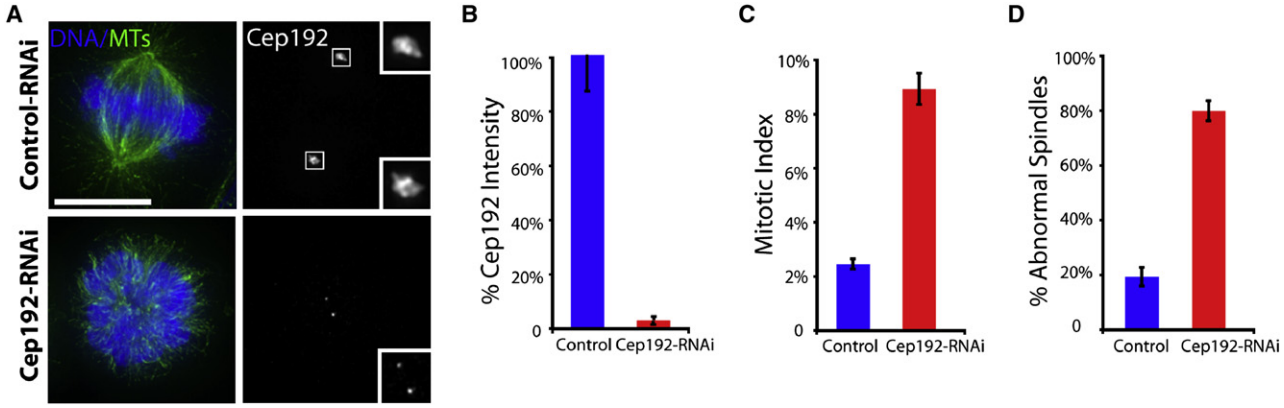

Figure 2. Cep192 Is Required for Mitotic Spindle Assembly

(A) Control and Cep192-esiRNA-transfected cells were stained for DNA, microtubules, and Cep192. The scale bar represents 10 μm, and insets show 2-fold magnifications of boxed regions.

(B) Cep192 fluorescence intensity at centrosomes in control and Cep192-esiRNA-transfected cells.

(C) Mitotic index of control and Cep192-esiRNA-transfected cells.

(D) Percentage of abnormal spindles in control and Cep192-esiRNA-treated cells.

In (B)–(D), results are shown as mean ± standard error of the mean (SEM) of 300–400 total cells (mitotic index) or 100 mitotic spindles (% abnormal spindle) and seven mitotic cells (Cep192 fluorescence intensity).

cold treatment in Cep192-RNAi-treated cells (Figure 3B) [12]. Together, these results suggest that Cep192 depletion impairs NEDD-1 recruitment to interphase centrosomes, which in turn causes a reduction in γ-TuRC recruitment, which then causes the observed defects in microtubule nucleation [12, 13].

The high degree of colocalization between Cep192 and γ-tubulin (Figure 1B), its role in microtubule nucleation, and its accumulation during mitosis prompted us to investigate whether Cep192 was required for centrosome maturation. We looked at the effects of Cep192 depletion on the accumulation of γ-tubulin, Pericentrin, and NEDD-1 on centrosomes at the onset of mitosis (Figure S1) [12, 13, 22, 23]. A marked decrease in the recruitment of NEDD-1, Pericentrin, and γ-tubulin to mitotic centrosomes was observed after Cep192 RNAi, as compared to the control transfection conditions (Figure 3C and Figure S1). These results suggest that Cep192 is required for centrosome maturation and are particularly interesting in light of recent results showing that NEDD-1 plays an upstream role in the recruitment of γ-TuRC components to mitotic centrosomes [12, 13].

We also observed that PCM proteins such as Pericentrin and γ-tubulin fail to accumulate on mitotic centrosomes upon NEDD-1 RNAi (data not shown) [12, 13]. Upon NEDD-1 depletion, Cep192 fragments were observed in the vicinity of centrioles. Although Cep192 distribution was altered, the total amount of Cep192 associated with these fragments was similar to the amount of Cep192 on control centrosomes (Figure 3D). Similar results were obtained upon RNAi of γ-TuRC subunits (Figure S6), suggesting that efficient clustering of Cep192 around centrosomes requires NEDD-1 mediated γ-TuRC recruitment [12, 13]. Pericentrin depletion induced a 5-fold decrease in Cep192 centrosomal amounts, a more significant decrease than initially reported (Figure 3D) [16]. These results, combined with our observation that Cep192 is required for NEDD-1 recruitment to centrosomes, suggest that Pericentrin and Cep192 are mutually required for their localization to mitotic centrosomes and together are necessary for the efficient recruitment of NEDD-1 and the subsequent assembly of γ-TuRCs during centrosome maturation.

Human Cep192 Is Required for Centriole Duplication

Although a role for NEDD-1 in centriole duplication in mammalian cells has been described, a parallel study did not report such a phenotype [12, 13]. To further scrutinize our observation that Cep192 is required for the recruitment of NEDD-1 to centrosomes, we sought to determine whether Cep192 is required for centriole duplication. First, we counted the centrioles present in control or Cep192-depleted mitotic cells by using antibodies against Centrin (Figure 4A) [28]. We found that the large majority (~80%) of control mitotic cells contained four centrioles, two at each pole of the mitotic spindle (Figures 4A and 4B). In contrast, over 80% of Cep192-depleted mitotic cells contained only two discernable Centrin structures, suggesting either centriole-duplication or -disengagement defects (Figures 4A and 4B). To discriminate between these two possibilities, we employed a centriole-overduplication assay in U2OS cells, in which prolonged S phase arrest induces multiple rounds of centriole duplication [29]. In this assay, inhibition of proteins required for centriole duplication leads to a decrease in the number of cells with more than four centrioles [12, 29–31]. Consistent with our observation in cycling mitotic Cep192-RNAi-treated cells, we observed a 2-fold to 3-fold decrease in the number of cells with more than four centrioles in the centriole-overduplication assay (Figures 4C and 4D). Similar results were obtained upon depletion of SAS-6, a protein known to be required for centriole duplication (data not shown) [6, 14, 21, 31, 32]. This latter result further supports a role for Cep192 in centriole duplication. Lastly, we examined serial-thin sections of Cep192-depleted cells by electron microscopy (Figure 4E). Although two centrioles per pole were observed in control transfected cells (n = 3) [33, 34], all Cep192-RNAi-treated cells analyzed contained less than four centrioles, consistent with a defect in centriole duplication (three centrioles n = 2, two centrioles n = 1, one centriole n = 2). Taken together, our light and electron-microscopy results show that Cep192 is required for centriole duplication in human cells.

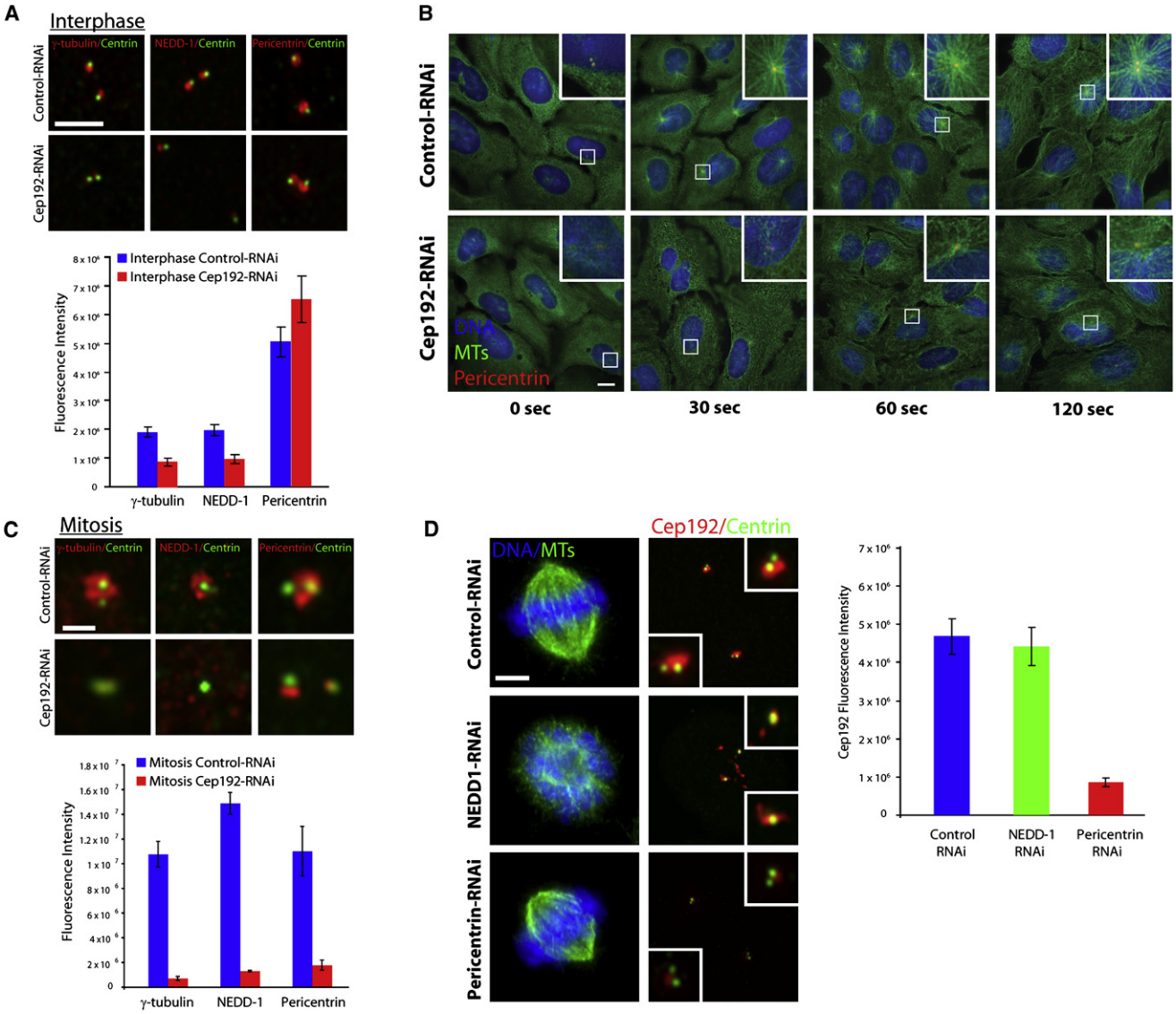

Figure 3. Cep192 Is Required for PCM Recruitment and Centrosome Maturation

(A) Control (top panel) and Cep192-esiRNA-transfected interphase HeLa (bottom panel) cells were labeled for γ-tubulin, NEDD-1, Pericentrin, and Centrin. Only centrosomal regions are shown. The total fluorescence intensity of PCM proteins per cell was quantified. The scale bar represents 2.5 μm.

(B) Repolymerization assay of control (top panel) and Cep192-siRNA-transfected U2OS cells (bottom panel). Cells were fixed and stained for DNA, microtubules, and Pericentrin 0, 30, 60, and 120 s after recovery from cold treatment. The scale bar represents 10 μm, and insets show 4-fold magnifications of boxed regions.

(C) Mitotic HeLa cells transfected with control (top panel) or Cep192 esiRNA (bottom panel) were stained and quantified as in (A).

In (A) and (C), results are shown as mean ± SEM of a minimum of ten cells for each condition. Two-tailed t tests assuming unequal variance were performed on the datasets so that it could be determined whether the differences in fluorescence intensity were significant. The slight increase in Pericentrin intensity after Cep192 RNAi in interphase cells is not statistically significant ($p > 0.05$). However, the decrease in γ-tubulin and NEDD-1 levels in interphase (A), as well as the decrease in γ-tubulin, NEDD-1, and Pericentrin levels in mitotic cells (C), is highly statistically significant ($p < 0.01$).

(D) Mitotic HeLa cells transfected with control (top panel), NEDD-1 (middle panel), and Pericentrin (bottom panel) esiRNA were labeled for DNA, microtubules, Centrin, and Cep192. Insets show 3-fold magnifications of boxed regions. The scale bar represents 5 μm. The total centrosome-associated Cep192 fluorescence was quantified. Results are shown as mean ± SEM of a minimum of ten cells for each condition.

Conclusions

We have shown that Cep192 is a pericentriolar protein that accumulates at centrosomes during mitosis and is required for PCM recruitment, centriole duplication, microtubule nucleation, and centrosome maturation. At the molecular level, how does Cep192 carry out these functions? A clue might reside in our observation that NEDD-1, a protein required for all of these processes, is dependent on Cep192 to accumulate at centrosomes. Therefore, an important function of Cep192 might be to participate in the centrosomal recruitment of NEDD-1 [12, 13]. Interestingly, depletion of NEDD-1 or γ-TuRC subunits leads to the accumulation of multiple Cep192 structures in proximity of centrioles in mitotic cells, the total amount of Cep192 associated with these structures similar to the total amount of Cep192 present on control mitotic centrosomes. By contrast, Pericentrin depletion impairs Cep192 accumulation at mitotic centrosomes, suggesting that Pericentrin

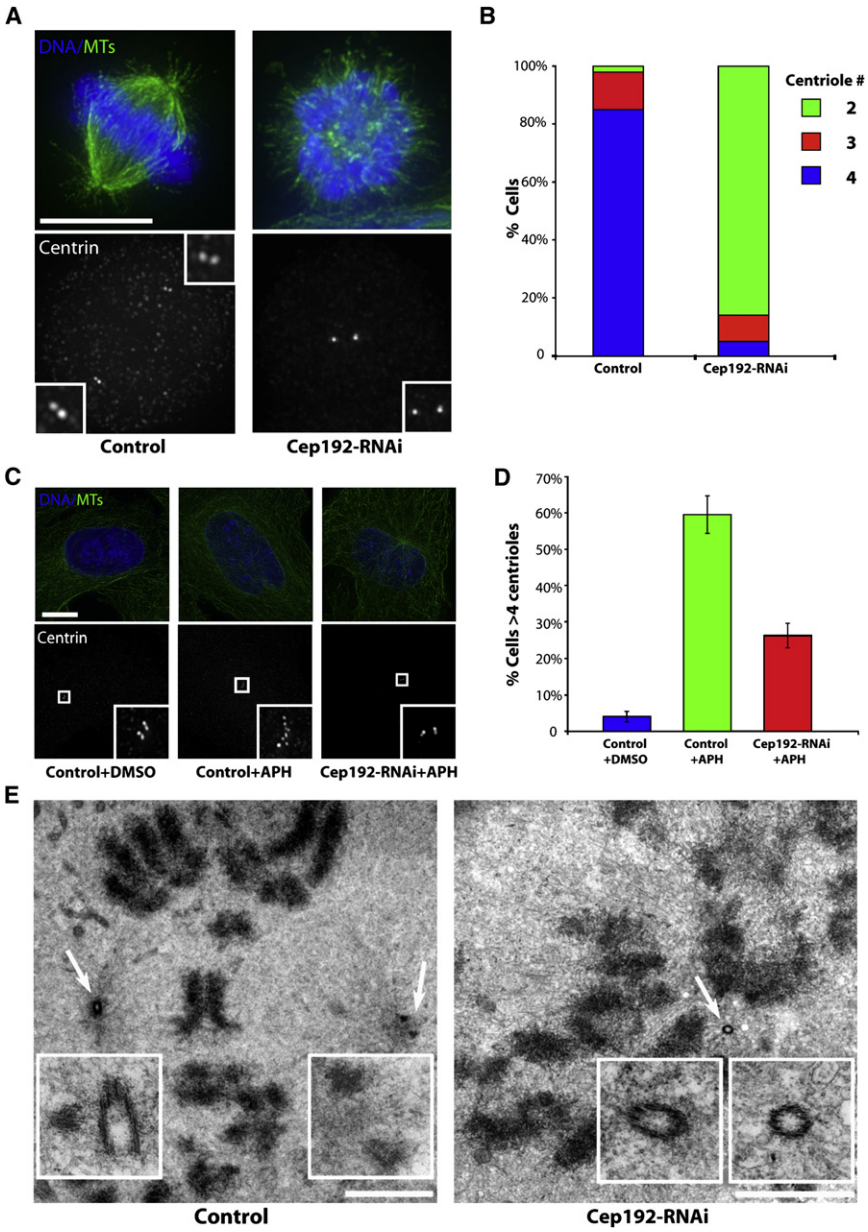

Figure 4. Cep192 Is Required for Centriole Duplication

(A) Control and Cep192-esiRNA-transfected HeLa cells were stained for DNA, microtubules, and Centrin as a marker of centriole number. The scale bar represents 10 μm, and insets show 2.5-fold magnifications of boxed regions.

(B) The percentage of mitotic cells, as judged by the presence of condensed chromatin, with two, three, or four centrioles was quantified (n = 100).

(C) Control and Cep192-siRNA-transfected U2OS cells were treated with aphidicolin (APH) for 60 hr prior to fixation for the induction of S phase arrest and centriole overduplication. Cells were stained for DNA, microtubules, and Centrin as a marker of centriole number. The scale bar represents 5 μm, and insets show 5-fold magnifications of boxed region.

(D) The percentage of cells with more than four centrioles in control transfected cells mock treated with DMSO or aphidicolin (APH) and Cep192 RNAi cells treated with APH was quantified. Results are shown as mean ± SEM (150 cells were quantified for each condition).

(E) Thin-section electron micrographs of control (left panel) or Cep192- (right panel) esiRNA-transfected cells. Arrows indicate the position of centrioles. Insets show magnified views of centrioles. In the Cep192-RNAi-treated cell, one of the centrioles shown in the insets was present in a different section. Scale bars represent 2.5 μm.

might participate in the formation of Cep192 structures. On the basis of the available data, we therefore propose a model whereby Pericentrin and Cep192 are mutually dependent on each other for their localization to mitotic centrosomes, which in turn is required for NEDD-1 recruitment and subsequent assembly of γ-TuRCs and other factors to build robust and functional centrosomes. At present, it remains unclear whether Cep192's role in centriole duplication is direct or indirect. On the one hand, defects in microtubule nucleation are known to impair centriole duplication, and thus the role of Cep192 in centriole duplication might be an indirect consequence of defects in microtubule nucleation or centrosome assembly [35]. On the other hand, work from Kleylein-Sohn and colleagues has shown that only a small subset of centrosome proteins are required for centriole duplication, whereas the vast majority, including the pericentriolar proteins PCM-1 and Pericentrin, appear to be dispensable [23, 36, 37]. In contrast, these results suggest that the Cep192 defect in centriole-duplication we observed might be direct. Surprisingly, Gomez-Ferreria and colleagues, in a recent paper, did not observe centriole-duplication defects in Cep192-depleted cells [16]. Because we observe centriole-duplication defects upon Cep192 RNAi in three independent assays, the nature of this discrepancy is puzzling. However, we contend that the most likely explanation for the difference in centriole-duplication phenotypes relates to differences in silencing efficiency, because we appear to deplete Cep192 more efficiently, pointing perhaps to dosage-specific phenotypes. Altogether, this work is consistent with our previous work in *C. elegans* [6] and supports the idea that the processes of centriole duplication and centrosome maturation are intimately intertwined and that Cep192 links these two processes.

Supplemental Data

Experimental Procedures, six figures, and three movies are available at http://www.current-biology.com/cgi/content/full/18/2/136/DC1/.

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