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CEP215 is involved in the dynein-dependent accumulation of pericentriolar matrix proteins for spindle pole formation

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CEP215 is a human orthologue of Drosophila centrosomin which is a core centrosome component for the pericentriolar matrix protein recruitment. Recent investigations revealed that CEP215 is required for centrosome cohesion, centrosomal attachment of the γ-TuRC, and microtubule dynamics. However, it remains obscure how CEP215 functions for recruitment of the centrosomal proteins during the centrosome cycle. Here, we investigated a role of CEP215 during mitosis. Knockdown of CEP215 resulted in characteristic mitotic phenotypes, including monopolar spindle formation, a decrease in distance between the spindle pole pair, and detachment of the centrosomes from the spindle poles. We noticed that CEP215 is critical for centrosomal localization of dynein throughout the cell cycle. As a consequence, the selective centrosomal proteins were not recruited to the centrosome properly. Finally, the centrosomal localization of CEP215 also depends on the dynein-dynactin complex. Based on the results, we propose that CEP215 regulates a dynein-dependent transport of the pericentriolar matrix proteins during the centrosome maturation.

Introduction

The centrosome consists of a pair of centrioles surrounded by amorphous pericentriolar material (PCM). The PCM is a lattice-like structure consisting of a number of coiled-coil proteins, such as pericentrin and CG-NAP, and provides a docking site for the minus-end of microtubules.[1] Therefore, the size of PCM is correlated with the amount of the centrosomal γ-tubulin ring complex (γ-TuRC), and eventually with the microtubule-organizing activity of the centrosome.[2] The amount of PCM fluctuates during the cell cycle, so that it is minimal after the exit from mitosis and gradually increases during the cell cycle progression. Especially the centrosome at late G_2 phase actively recruits the PCM components to prepare for mitosis in which a robust microtubule-organizing activity is required for the bipolar spindle formation.

A few mechanisms have been proposed for centrosomal recruitment of the PCM components.[3,4] A group of the PCM proteins including PCM-1, NuMA, and pericentrin are recruited to the centrosomes via a microtubule-dependent pathway.[5] Cytoplasmic dynein, a minus end-directed microtubule motor, plays a key role for this transport. Microtubule-independent mechanisms are also proposed, so that γ-tubulin is recruited to the centrosome, in part, by a simple diffusion.[6] It is possible that there exist additional transport mechanisms for the recruitment of specific centrosomal proteins.

The Drosophila centrosomin (Cnn) is a core centrosome component which is involved in the PCM protein recruitment.[7,8] In the *cnn* mutant embryos, a group of selected PCM proteins such as γ-tubulin, D-TACC and Msps are improperly recruited to the centrosome.[9] In consequence, the centrosomes fail to function as microtubule-organizing centers during mitosis, leading to severe mitotic defects in embryos.[8-10] Therefore, Cnn is considered as an essential centrosome maturation factor.[11]

CEP215/CDK5RAP2 is a human orthologue of Cnn.[9] It is interesting that homozygous mutations in *CEP215* cause autosomal recessive primary microcephaly (MCPH), a neurogenetic disease in which the brain is small at birth and thereafter.[12] The underlying cause of MCPH is unknown, but it is linked to defects in mitosis of neuroepithelial cells.[13,14] The centrosomal functions of CEP215 have been investigated recently.[15-17] Knockdown of CEP215 caused premature centrosome separation, suggesting that it is required for centrosome cohesion, possibly through a regulatory pathway independent of rootletin and C-NAP1.[15] CEP215 is also required for centrosomal attachment of the γ-TuRC, playing a role in the microtubule organizing function of the centrosome.[16] In addition, CEP215 interacts directly with EB1 and regulates microtubule dynamics and stability.[17] However, the function of CEP215 in mitosis is still poorly understood.

In this study, we investigated a role of CEP215 during mitosis. Our results revealed that CEP215 is important for proper progression of mitosis, possibly in conjunction with dynein during centrosome maturation and spindle assembly.

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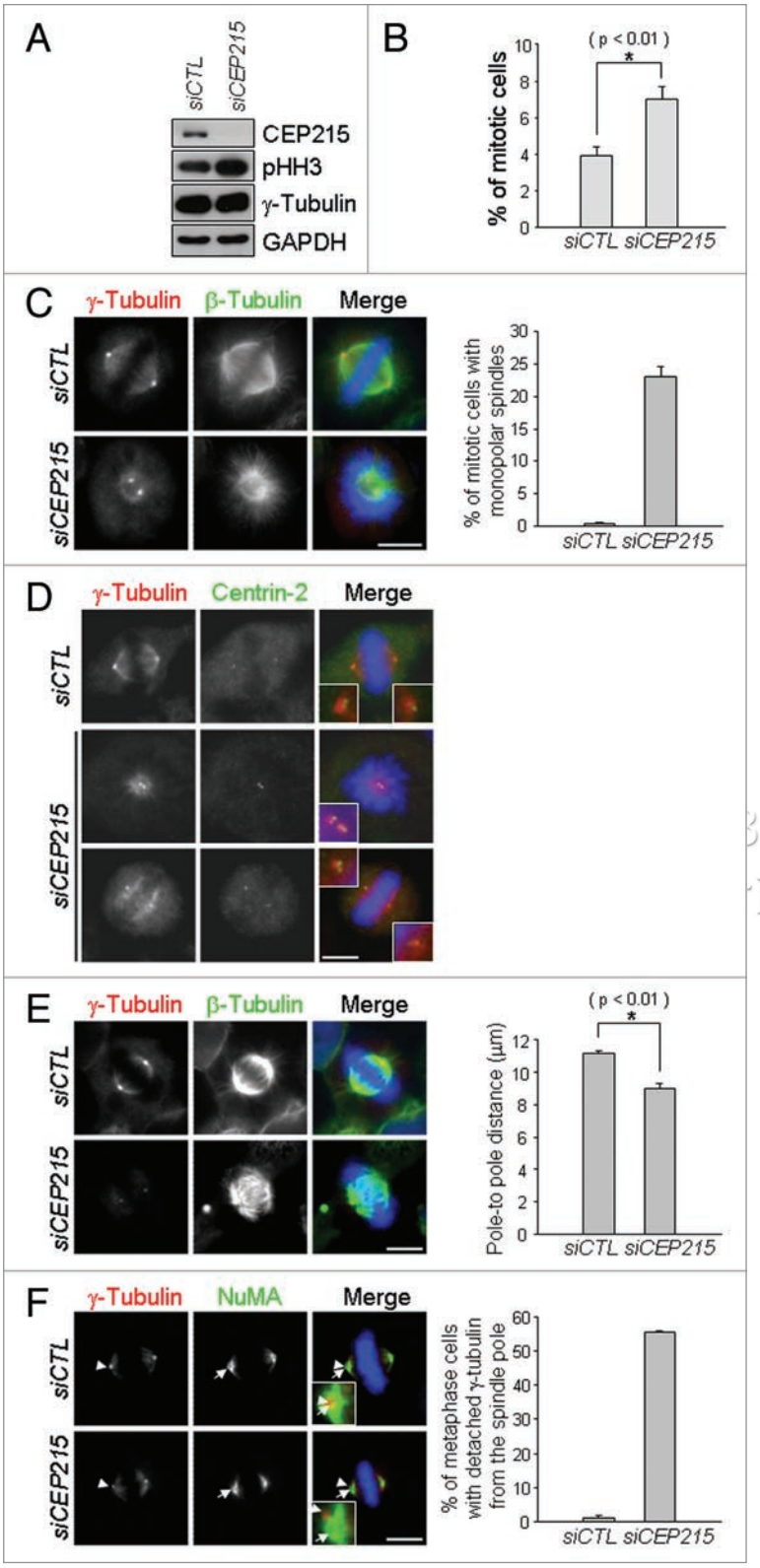

Figure 1. Abnormalities in the mitotic spindles of CEP215-depleted cells. (A) HeLa cells were transfected with either nonspecific control (*siCTL*) or *CEP215*-specific (*siCEP215*) siRNAs. Forty-eight hours later, the cells were subjected to immunoblot analysis with antibodies specific to CEP215, phospho-histone H3 (pHH3), γ-tubulin and GAPDH. (B) Mitotic indices of the *siCTL*- and *siCEP215*-transfected cells were quantified by immunostaining the cells with the pHH3 antibody. Over 500 cells per each group were counted in three independent experiments. (C) The *siCTL*- or *siCEP215*-transfected cells were coimmunostained with antibodies specific to γ-tubulin (red) and β-tubulin (green). The proportion of the mitotic cells with monopolar spindles was determined. One hundred mitotic cells per each group were analyzed in three independent experiments. (D) The *siCTL*- or *siCEP215*-transfected cells were coimmunostained with antibodies specific to γ-tubulin (red) and centrin-2 (green). Representative bipolar and monopolar mitotic cells are shown. The insets are magnified views of the centrosomes. (E) The *siCTL*- or *siCEP215*-transfected cells were coimmunostained with antibodies specific to γ-tubulin (red) and β-tubulin (green). The distance between two mitotic spindle poles was measured. Thirty metaphase cells per each group were analyzed in three independent experiments. (F) The *siCTL*- or *siCEP215*-transfected cells were coimmunostained with antibodies specific to γ-tubulin (red) and NuMA (green) to discriminate the centrosomes (γ-tubulin, arrowheads) from the mitotic spindle poles (NuMA, arrows). The insets are magnified views of the spindle poles. The number of spindle poles with detached centrosomes was counted. Fifty metaphase cells per each group were analyzed in three independent experiments. The graphs in Figure 1 were expressed as means and standard errors. Asterisk, $p < 0.01$ in a paired t-test. Scale bars, 10 μm.

Results

CEP215 is required for a proper bipolar spindle formation during mitosis. To study the role of CEP215 during mitosis, we examined mitotic cells after CEP215 suppression. Endogenous CEP215 levels were reduced to less than 5% of the control levels in 48 h after transfection of the *CEP215*-specific siRNA in HeLa cells (**Fig. 1A**). At the same time, the phospho-histone H3 levels increased, indicating mitotic accumulation in the CEP215-depleted cells (**Fig. 1A**). In fact, proportion of the mitotic cells in the CEP215-depleted population increased about 2-fold in comparison to the control population (**Fig. 1B**). Abnormalities in the mitotic spindle were frequently accompanied in the CEP215-depleted cells. First, monopolar spindles were observed in 25% of the mitotic population of the CEP215-depleted cells (**Fig. 1C**). It is not resulted from a centriole duplication defect, since the monopoles contained two pairs of centrioles as stained by the γ-tubulin and centrin-2 antibodies (**Fig. 1D**). Even most of the bipolar spindles in the CEP215-depleted population did not look normal. For example, the distance between two poles of the CEP215-depleted cells was reduced by 80% of that of the control cells (**Fig. 1E**). The centrosomal γ-tubulin intensity in the CEP215-depleted cells was reduced significantly, and the astral spindles did not actively sprout from the spindle poles, as reported previously (**Fig. 1E** and reviewed in refs. 16

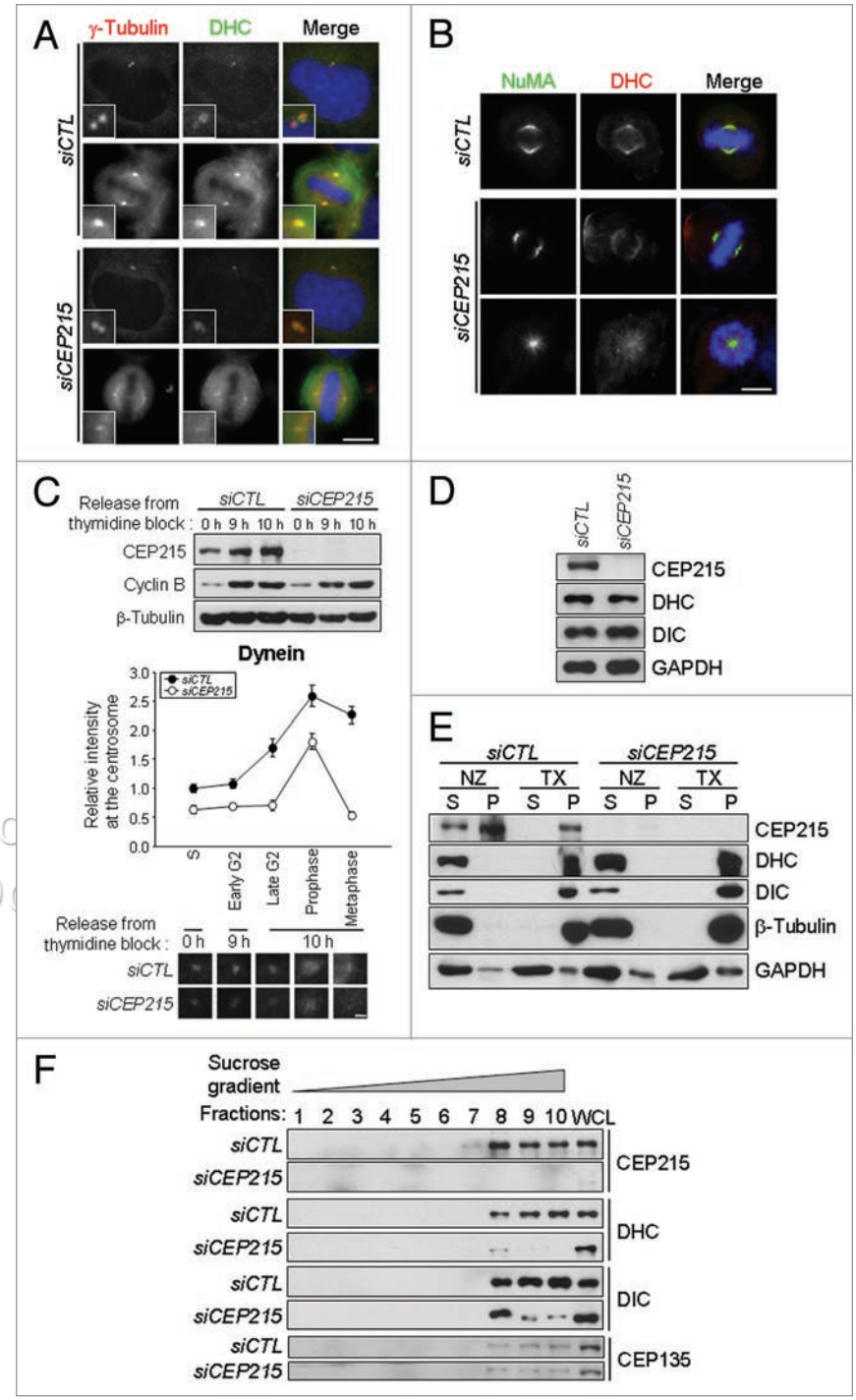

Figure 2. The centrosomal dynein levels were reduced in the CEP215-depleted cells. (A) HeLa cells were transfected with *siCTL* or *siCEP215*. Forty-eight hours later, the cells were coimmunostained with antibodies specific to γ-tubulin (red) and dynein heavy chain (DHC, green). Representative interphase and mitotic cells are shown. The insets are magnified views of the centrosomes. Scale bar, 10 μm. (B) The *siCTL*- or *siCEP215*-transfected cells were coimmunostained with antibodies specific to NuMA (green) and DHC (red). Scale bar, 10 μm. (C) The *siCTL*- or *siCEP215*-transfected cells were treated with 2 mM thymidine for 18 h to arrest the cell cycle at S phase. The cells were then cultured in a fresh medium for 0, 9 or 10 h and subjected to immunoblot analysis with antibodies specific to CEP215, cyclin B and β-tubulin. The same sets of cells were coimmunostained with antibodies specific to cyclin B and DHC. The exact cell cycle stages were determined based on the cell morphology and cyclin B expression. The centrosomal dynein levels were quantified by measuring the fluorescent intensity of DHC at the centrosome and compared with those of the control S phase cells. Over 20 cells per each group were analyzed, and the graphs were expressed as means and standard errors. The representative pictures are magnified views of the centrosomes at each experimental group. Scale bar, 2 μm. (D) Forty-eight hours after the *siCTL* or *siCEP215* transfection, the cells were subjected to immunoblot analysis with antibodies specific to CEP215, DHC, DIC (dynein intermediate chain) and GAPDH. (E) The *siCTL*- or *siCEP215*-transfected cells were treated with nocodazole (NZ) or Taxol (TX), and the cell lysates were fractionated into supernatant (S) and pellet (P) by ultracentrifugation. The samples were subjected to immunoblot analysis with indicated antibodies. (F) The centrosomes from the *siCTL*- or *siCEP215*-transfected cells were enriched using discontinuous sucrose gradient ultracentrifugation. The fractions were subjected to immunoblot analysis with indicated antibodies. The whole cell lysates (WCL) were used as a control.

and 18). We also observed that the centrosome which was immunostained with the γ-tubulin antibody did not position at the center of the spindle pole, but was detached from the pole (**Fig. 1F**). These results suggest that CEP215 is critical for a proper spindle pole formation during mitosis.

CEP215 is critical for centrosomal localization of dynein throughout the cell cycle. The mitotic defects in CEP215-depleted cells are reminiscent of those seen after dynein depletion. First, mitotic cells were accumulated in both the dynein- and CEP215-depleted populations (**Fig. 1B** and reviewed in refs. 19 and 20). Second, the mitotic spindle poles were frequently unfocused (**Fig. 1E** and reviewed in ref. 20). Third, the centrosomes were frequently detached from the spindle poles (**Fig. 1F** and reviewed in refs. 20 and 21). Similarities in the mitotic phenotypes of the CEP215- and dynein-depleted cells allowed us to investigate a functional relationship between them. We initially investigated whether dynein localization is affected by CEP215 depletion or not. Cytoplasmic dynein is known to localize to microtubules, cell cortex, kinetochores and the centrosome.[22-24] In CEP215-depleted cells, the dynein levels in the centrosomes were reduced significantly in both interphase and mitotic cells, while those in the other parts of the cell were not affected (**Fig. 2A and B**). It was reported that dynein binds to the centrosome in a cell cycle-dependent manner.[24] To determine when the centrosomal localization of dynein is disturbed by CEP215 depletion, we arrested the cells at S phase using thymidine block and released them synchronously

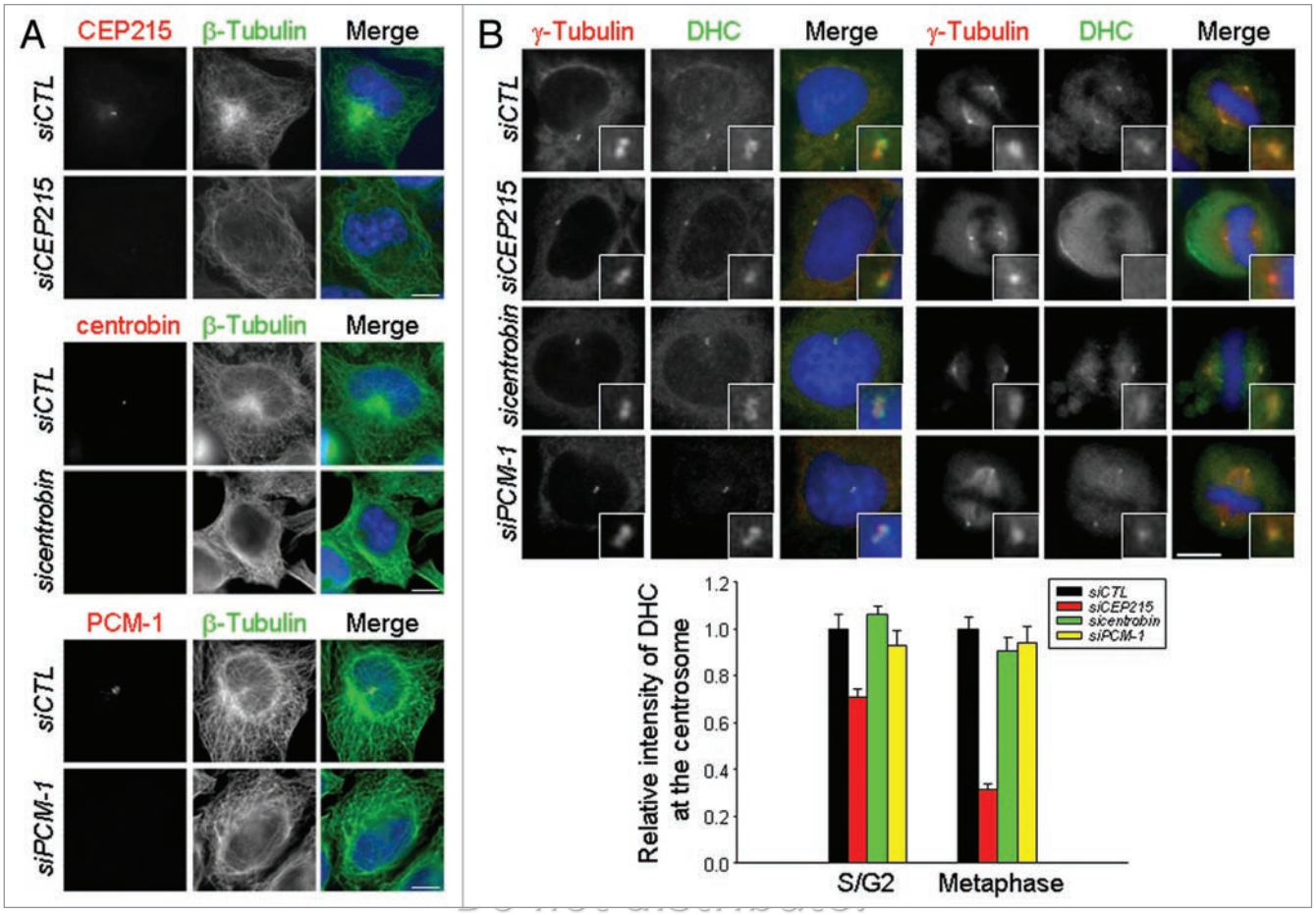

Figure 3. Specific reduction of the centrosomal dynein in the CEP215-depleted cells. (A) HeLa cells were transfected with siRNAs specific to *CEP215*, *centrobin* and *PCM-1*, and then coimmunostained with β-tubulin (green) along with the corresponding antibodies (red). (B) The *siCTL-*, *siCEP215-*, *sicentrobin-* and *siPCM-1*-transfected cells were coimmunostained with antibodies specific to γ-tubulin (red) and DHC (green). The insets are magnified views of the centrosomes. The centrosomal dynein levels were quantified by measuring the fluorescent intensity of DHC at the centrosome and compared with those of their respective controls. Over 20 cells per each group were analyzed in three independent experiments. The graphs were expressed as means and standard errors. Scale bars, 10 μm.

(**Fig. 2C**). At indicated time periods, the cells were immunostained with antibodies specific to dynein heavy chain and cyclin B. The centrosomal dynein intensity increased from S phase to mitotic entry, but appeared significantly reduced in the CEP215-depleted cells throughout the cell cycle (**Fig. 2C**).

Total cellular levels of dynein were quantified in the CEP215-depleted cells. Although depletion of CEP215 reduced the dynein intensity at the centrosome, its total protein levels were not affected (**Fig. 2D**). We assessed the levels of dynein associated with microtubules using microtubule fractionation assay. The result showed that the amount of dynein in association with microtubules was not reduced after CEP215 depletion (**Fig. 2E**). This suggests that depletion of CEP215 did not affect the microtubule binding activity of dynein. Finally, we enriched the centrosomes from the *siCTL-* or *siCEP215-*transfected cells and observed the dynein levels which were determined by immunoblotting with antibodies specific to dynein heavy and intermediate chains. The results showed that the centrosomal dynein levels were reduced significantly in the CEP215-depleted centrosome fractions (**Fig. 2F**). In contrast, the centrosomal CEP135 levels were not reduced in the *siCEP215*-transfected cells (**Fig. 2F**). Based on these results, we conclude that CEP215 is required for dynein localization at the centrosome.

Reduction of the centrosomal dynein levels in CEP215-depleted cells is not a general consequence of the abnormal microtubule formation. As reported previously, depletion of CEP215 disrupted the interphase microtubule network (**Fig. 3A** and reviewed in ref. 16). One might suspect that dynein cannot be concentrated into the centrosome efficiently, because the microtubule network was not focused into the centrosome. To investigate whether reduction of dynein at the centrosome is a general consequence of an abnormal microtubule network formation or a specific phenotype of CEP215 depletion, we examined levels of the centrosomal dynein after suppression of other centrosomal proteins which are known to be critical for microtubule network formation. We selected centrobin and PCM-1 of which the depletion disrupted radial microtubule networks in the interphase cells.[25,26] Depletion of each protein caused an abnormal microtubule network formation similar to CEP215-depleted cells (**Fig. 3A**).

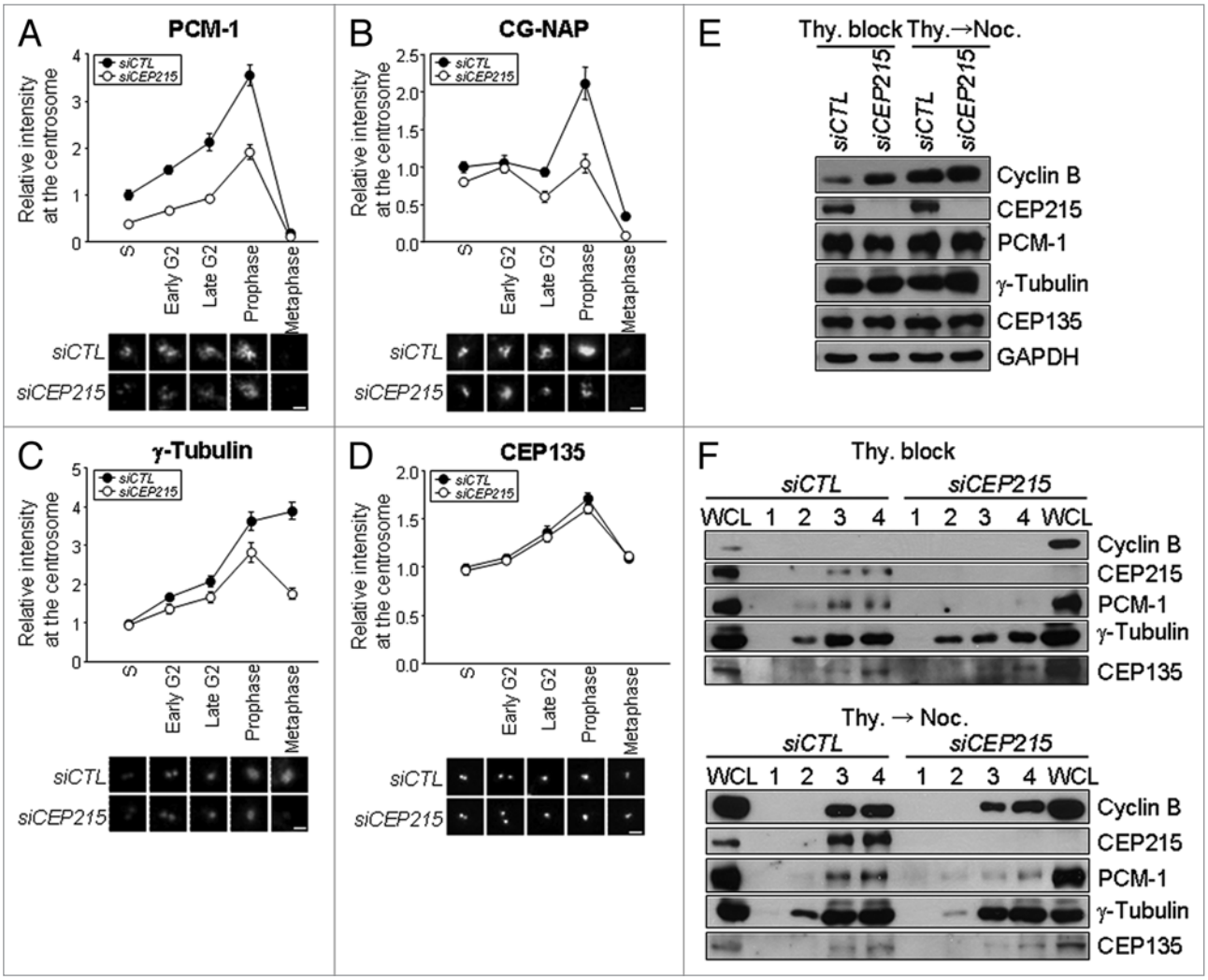

Figure 4. Impaired recruitment of the centrosomal proteins in CEP215-depleted cells. (A–D) HeLa cells were transfected with *siCTL* or *siCEP215*. Thirty hours later, the cells were treated with 2 mM thymidine for 18 h to arrest the cell cycle at S phase, transferred to a fresh medium, and cultured for 0, 9 or 10 additional hours. The cells were then coimmunostained with indicated antibodies (A, PCM-1; B, CG-NAP; C, γ-tubulin; D, CEP135) along with the cyclin B antibody. The exact cell cycle stages were determined based on the cell morphology and cyclin B expression. Over 20 cells per each group were analyzed, and the relative centrosomal intensities were compared with the control S phase cells. The graphs were expressed as means and standard errors. The representative pictures are magnified views of the centrosomes at each experimental group. Scale bars, 2 μm. (E) The *siCTL*- or *siCEP215*-transfected cells were treated with 2 mM thymidine for S phase arrest or sequentially with thymidine and nocodazole for M phase arrest. The cells were then subjected to immunoblot analysis with antibodies specific to cyclin B, CEP215, PCM-1, γ-tubulin, CEP135 and GAPDH. (F) Centrosomes were isolated from the *siCTL*- or *siCEP215*-transfected cells using discontinuous sucrose gradient ultracentrifugation. Only one-third from the bottom fractions were collected and subjected to immunoblot analysis with antibodies specific to cyclin B, CEP215, PCM-1, γ-tubulin, CEP135 and GAPDH. The whole cell lysates (WCL) were used as a control.

Next, we compared dynein intensity at the centrosome in CEP215-, centrobin- and PCM-1-depleted cells (**Fig. 3B**). The results showed that the dynein levels at the centrosome were reduced in the CEP215-depleted cells, but not in the centrobin- or PCM-1-depleted cells (**Fig. 3B**). Such reduction was more obvious in the M phase cells (**Fig. 3B**). These results suggest that reduction of the centrosomal dynein levels in CEP215-depleted cells is not a general consequence of the abnormal microtubule network formation, but a specific effect of CEP215 depletion.

CEP215 is required for recruitment of the selective centrosomal proteins. Many of the centrosomal proteins fluctuate in accord to the cell cycle.[2,4] It is believed that dynein plays a critical role in recruiting centrosomal proteins, including PCM-1, NuMA, pericentrin and CG-NAP.[5] If dynein were disturbed to localize at the centrosome as seen in the CEP215-depleted cells, it could not transport a group of specific cargoes properly. Therefore, we examined recruitment of the centrosomal proteins in the CEP215-depleted cells. The results showed that the centrosomal levels of PCM-1 were reduced in both interphase and mitotic cells, while those of CG-NAP and γ-tubulin were reduced only in mitotic cells (**Fig. 4A–C**). CEP135, a centriolar protein, was not affected by CEP215 depletion (**Fig. 4D**).

We carried out immunoblot analysis to confirm immunocytochemical results shown in **Figure 4A–D**. Sequential treatment

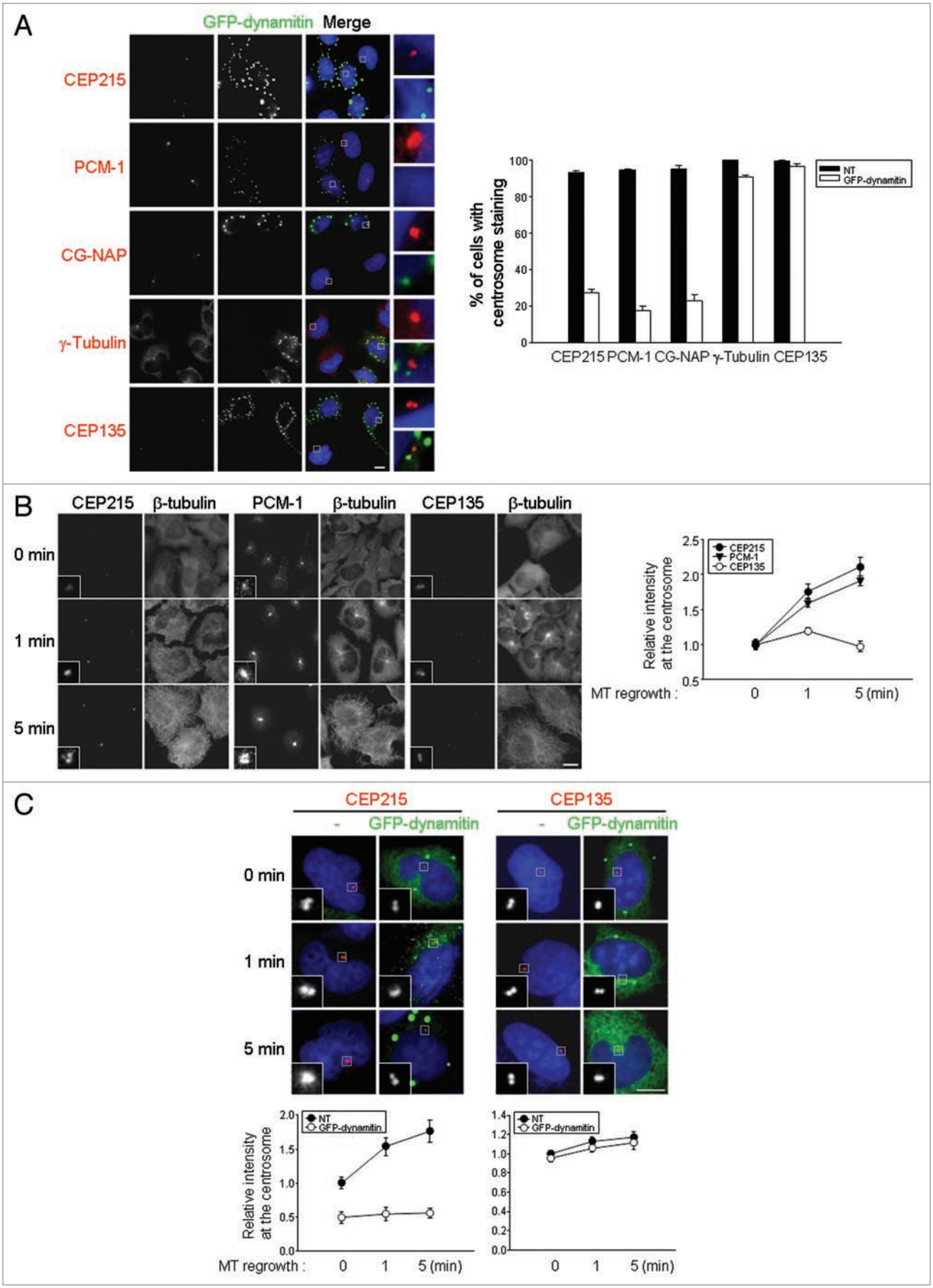
A
GFP-dynamitin
Merge
CEP215
PCM-1
CG-NAP
γ-Tubulin
CEP135
% of cells with centrosome staining
NT
GFP-dynamitin
CEP215 PCM-1 CG-NAP γ-Tubulin CEP135
B
CEP215
β-tubulin
PCM-1
β-tubulin
CEP135
β-tubulin
0 min
1 min
5 min
Relative intensity at the centrosome
MT regrowth :
0
1
5 (min)
C
CEP215
CEP135
-
GFP-dynamitin
0 min
1 min
5 min
NT
GFP-dynamitin
Relative intensity at the centrosome
MT regrowth :
0
1
5 (min)

Figure 5 (See previous page). Centrosomal localization of CEP215 depends on the microtubules and dynein-dynactin complex. (A) HeLa cells were transfected with *GFP-dynamitin*. Twenty-four hours later, the cells were coimmunostained with the GFP antibody (green) along with antibodies specific to CEP215, PCM-1, CG-NAP, γ-tubulin and CEP135 (red). The small panels on the right are magnified views of the centrosomes in the untransfected (upper) and *GFP-dynamitin*-transfected (lower) cells. Over 50 cells per each group were analyzed in three independent experiments. The graphs were expressed as means and standard errors. (B) HeLa cells were incubated on ice for 1 h to disassemble microtubule network, and then incubated in a warm medium for indicated time periods. The cells were coimmunostained with β-tubulin and antibodies specific to CEP215, PCM-1 and CEP135. The insets are magnified views of the centrosomes. The immunofluorescence intensity of each protein at the centrosome was measured at 0, 1 or 5 min after incubating in a warm medium. Relative centrosomal intensities in comparison to the 0-min group were determined. Twenty cells were analyzed per each group and the graphs were expressed as means and standard errors. (C) HeLa cells were transfected with *GFP-dynamitin*. Twenty-four hours later, the cells were incubated on ice for 1 h, and then incubated in a warm medium for indicated time periods. The cells were coimmunostained antibodies specific to CEP215 or CEP135 (red) along with the GFP antibody (green). Scale bars, 10 μm.

of thymidine and nocodazole increased the total protein levels of cyclin B, which indicates that the cells were accumulated in G_2/M phase (**Fig. 4E**). The cyclin B levels in the CEP215-depleted cells increased, probably due to the M phase-arrested population in the previous cell cycle. The total protein levels of PCM-1, γ-tubulin and CEP135 remained constant irrespective of the cell cycle and CEP215 expression (**Fig. 4E**). We obtained centrosome fractions from these cells by discontinuous sucrose gradient ultracentrifugation. Depletion of CEP215 resulted in a remarkable decrease of the PCM-1 levels at the centrosome fraction in both S and M phase cells (**Fig. 4F**). In contrast, the γ-tubulin levels were slightly reduced only at M phase cells. The centrosomal CEP135 levels were not affected at all by CEP215 depletion in both S and M phase cells. These results confirm that CEP215 is important for centrosomal accumulation of the selected proteins.

Centrosomal localization of CEP215 depends on the dynein-dynactin complex. We examined centrosomal accumulation of the selected proteins in dynein-inactivated cells. It was reported that overexpression of dynamitin subunit disrupts the dynein-dynactin complex and impairs dynein-mediated functions.[24] Our results revealed that overexpression of dynamitin caused a significant reduction in the centrosomal levels of PCM-1 and CG-NAP, in comparison to the untransfected cells (**Fig. 5A** and reviewed in refs. 25 and 27). On the other hand, the centrosomal levels of γ-tubulin and CEP135 were not affected at all by dynamitin overexpression (**Fig. 5A** and reviewed in ref. 25). It is interesting that the centrosomal CEP215 levels were also reduced in the dynamitin-overexpressing cells (**Fig. 5A**). These results suggest that the centrosomal localization of CEP215 requires the dynein-dynactin complex.

Importance of the microtubules in accumulation of the centrosomal proteins was examined by the microtubule regrowth assay. As microtubules grew, the levels of CEP215 and PCM-1 increased at the centrosome (**Fig. 5B**). On the other hand, CEP135 which is known to localize to the centrosome in a microtubule-independent manner was not accumulated into the centrosome during this period (**Fig. 5B** and reviewed in ref. 28). Importance of dynein in centrosomal accumulation of CEP215 was reexamined by the microtubule regrowth assay in the dynamitin-overexpressing cells. It is known that overexpression of dynamitin does not disturb microtubule organization, but inhibits dynein-cargo binding in HeLa cells.[29] The centrosomal levels of CEP215 appeared reduced in the dynamitin-overexpressing cells of which the microtubule network was disrupted by cold treatment (**Fig. 5C**). Furthermore, the centrosomal CEP215 levels in the dynamitin-overexpressing cells were not recovered even if the microtubules grew in a warm medium (**Fig. 5C**). These results confirmed that the dynein-dynactin complex is required for centrosomal recruitment of CEP215.

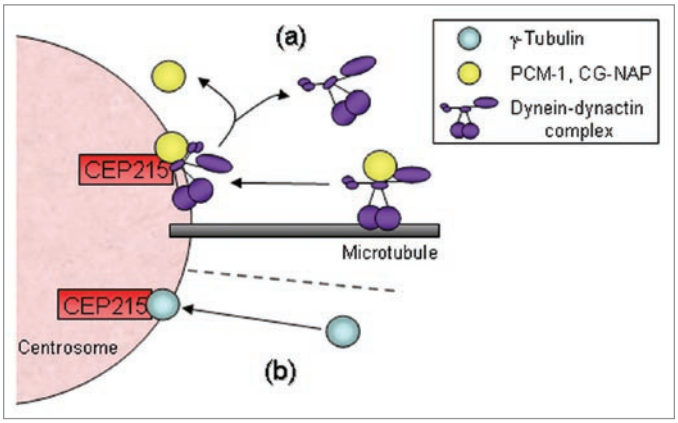

Figure 6. Model. CEP215 is involved in recruitment of the centrosomal proteins through a dynein-dependent and a dynein-independent pathway. (a) The dynein-dynactin complex transports selected centrosomal proteins along the microtubules. After its arrival at the centrosome, dynein leaves the cargoes in the centrosome. CEP215 may function to anchor the dynein-dynactin complex to the centrosome, thereby accumulating the cargo proteins into the centrosome. After the cargo delivery, the dynein-dynactin complex is released from the centrosome. (b) CEP215 may mediate the recruitment of γ-tubulin by direct interaction.[16]

Discussion

In the present study, we observed mitotic phenotypes of the CEP215-depleted cells. Our results revealed that CEP215 is required for proper progression of mitosis. We propose that CEP215 functions as a centrosomal adaptor for dynein, based on the observations that the centrosomal dynein levels are reduced in the CEP215-depleted cells and that the mitotic phenotypes in the CEP215-depleted cells are similar to those in the dynein-depleted cells (**Fig. 6**). Reduction in the PCM size of CEP215-depleted cells may be resulted from a decrease in the dynein-dependent recruitment of the centrosomal proteins (**Fig. 6a**). It was previously known that CEP215 is also involved in centrosomal protein transport in a dynein-independent manner (**Fig. 6b**). A similar mechanism has been proposed for NUDEL which was identified as a regulator of cytoplasmic dynein to localize the centrosome

and kinetochores.[30-33] This work suggests that CEP215 is another centrosomal protein that regulates localization of cytoplasmic dynein at the centrosome. It remains to be investigated whether CEP215 and NUDEL share a functional linkage as dynein adaptors for specific sets of the centrosome proteins or not.

One of the mitotic phenotypes in CEP215-depleted cells is a decrease in the distance between two mitotic spindle poles (**Fig. 1E**). We interpret that this phenotype is resulted from a reduced number of the microtubules from the centrosome. Reduction in the PCM protein levels in the CEP215-depleted cells was reported in this work as well as others (**Fig. 4** and reviewed in refs. 16 and 34). Since the CEP215-depleted cells failed to accumulate PCM proteins in mitosis, they could not produce robust spindles from the centrosomes.[2] In consistent with the observation, reduction in the astral spindles was observed previously in the CEP215-depleted cells.[16] Since the spindle density in the CEP215-depleted cells is not sufficient enough to fix the spindle poles, the distance between two poles of the CEP215-depleted cells may be reduced.

An increase in the number of cells with monopolar spindles was also observed in the CEP215-depleted cells (**Fig. 1C**). Two possible ways have been proposed in the monopolar spindle formation. First, it may be resulted from spindle collapse in which the forces from the plus- and minus-end directed motors such as kinesins and dynein are not balanced for bipolar spindle formation.[35,36] Alternatively, reduction in the microtubule-organizing activity of the centrosome also results in monopolar spindle formation. For example, the CEP192-depleted cells form monopolar spindles and unstable bipolar spindles owing to defects in PCM protein accumulation.[37] We suspect that monopolar spindle formation in the CEP215-depleted cells is attributed to the both reasons: The dynein-dynactin complex does not function properly in the centrosome of the CEP215-depleted cells and, as a consequence, the centrosomal proteins were not accumulated sufficiently during the centrosome maturation process.

Another mitotic phenotype in the CEP215-depleted cells is detachment of the centrosome from the spindle pole (**Fig. 1F**). This may be due to the reduction of the centrosomal dynein levels in the CEP215-depleted cells. Dynein is known to be critical not only for the recruitment of the centrosomal proteins but also for the centrosomal linkage to the spindle pole.[38] It is known that the dynein-dynactin complex functions in tethering spindle microtubules to the centrosome.[38] In fact, the dynein-depleted centrosomes are detached from the spindle poles.[20,21] This is due to a failure in concentration of spindle fibers into the spindle pole. A similar phenotype in the CEP215-depleted cells might be originated from elimination of dynein at the centrosome (**Fig. 1F**).

Cnn, a Drosophila homologue of CEP215, is known to be critical for proper spindle formation during mitosis. The mother centrosome of the male germline stem cells in the wild type fly is preferentially located near hub cells, but such preference was disturbed in the *cnn* mutant fly.[39,40] This is probably due to the reduction in the centrosomal microtubules, and, as a result, a spindle pole was not fixed within the cell polarity.[40] Actually, it is supposed that CEP215 is a pericentriolar scaffold component that may play an important role in protein assembly onto the centrosome.[16] A role of dynein in centrosome positioning was also emphasized in mammalian neuronal progenitors.[41] During mammalian neurogenesis, the mother centrosome localizes near the ventricular surface through anchoring to adherens junctions, resulting in asymmetric division. Since dynein associates with β-catenin at the adherens junction, it is thought that the centrosome might be targeted to adherens junctions through the microtubule-minus end-directed motor activity of dynein.[42] We suspect that a similar phenotype may be observed in the CEP215 knockout neuronal progenitor cells, and this may reduce the neuronal cell number, leading to microcephaly.[12]

It is interesting that the centrosomal accumulation of CEP215 and dynein is interdependent. This is in contrast to NUDEL of which the centrosomal localization is dynein-independent.[31] CEP215 may be recruited to the centrosome by a NUDEL-mediated pathway or other unknown transport proteins. It remains to be investigated how CEP215 is linked to the dynein complex in the centrosome.

Materials and Methods

Antibodies. Anti-CEP215 and centrin-2 rabbit polyclonal antibodies were generated and affinity purified against GST-CEP215$^{991-1893}$ and GST-centrin-2, respectively. Antibodies against CEP135 and centrobin were described previously.[26,43] Antibodies against PCM-1 and CG-NAP were kindly gifted from A. Merdes and Y. Ono, respectively. Antibodies against dynein heavy chain, dynein intermediate chain, cyclin B, γ-tubulin (Goat) and GFP were purchased from Santa Cruz. We also purchased antibodies against β-tubulin (Sigma), γ-tubulin (mouse, Sigma), phospho-histone H3 (Upstate), NuMA (Calbiochem) and GAPDH (Ambion).

Cell culture, synchronization, transfection and RNAi experiments. HeLa cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2. HeLa cells were treated with 2 mM thymidine for 18 h to arrest the cell cycle at S phase, and then cultured in a fresh medium for 9 or 10 h to accumulate G_2 or mitotic population, respectively. Transfection into HeLa cells was performed by using Lipofectamine Plus (Invitrogen) for plasmid DNA, and Oligofectamine (Invitrogen) for siRNA as manufacturer's instructions. For RNAi experiments, we used *siCEP215* (5'-GUG GAA GAU CUC CUA ACU AAA-3'), *siPCM-1* (5'-UCA GCU UCG UGA UUC UCA G-3'), *sicentrobin* (5'-GGA UGG UUC UAA GCA UAU C-3') and *siCTL* (control) (5'-AAG TAG CCG AGC TTC GAT TGC-3').[15,25,26]

Immunoblot analysis. HeLa cells were denatured in 1X SDS-PAGE sample buffer for immunoblot analysis. Protein samples were resolved on SDS-PAGE gels and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 0.3% TBST (TBS with 0.3% triton X-100) plus 5% nonfat dried milk for 30 min, incubated with primary antibodies diluted in blocking solution for 4 h, washed four times with 0.3% TBST, incubated with HRP-conjugated secondary antibodies (Sigma) for 30 min, and washed four times with 0.3% TBST.

Immunocytochemistry and image processing. HeLa cells were seeded on a 12 mm cover slip and transfected 18 h later.

At proper time after transfection, the cells were fixed with cold methanol for 10 min. The fixed cells were permeabilized and blocked with 0.1% PBST (phosphate-buffered saline with 0.1% triton X-100) plus 3% BSA for 15 min. Then, the cells were incubated with primary antibodies diluted in 0.1% PBST plus 3% BSA for 1 h, washed with 0.1% PBST three times, incubated with either FITC- or TRITC-conjugated secondary antibody (Jackson Immunoresearch) for 30 min and washed with 0.1% PBST three times. For DNA staining, DAPI solution was used at the final step. The cells were mounted on a slide glass and observed using a fluorescence microscope (Olympus IX51) equipped with a CCD (Qicam fast 1394, Qimaging) camera. The images were analyzed using ImagePro 5.0 (Media Cybernetics, Inc.). The intensity of a specific protein was determined by the sum fluorescent intensity subtracted by background intensity. All statistical data were analyzed with SigmaPlot (Systat Software, Inc.).

Microtubule fractionation assay. Microtubule fractionation was performed as described previously.[44] For microtubule stabilization, HeLa cells were incubated with 20 μM of Taxol for 2 h. The cells were washed twice with PEM buffer (100 mM PIPES, pH 6.6, 1 mM EGTA and 1 mM $MgSO_4$), then lysed with PEM buffer containing 1% NP-40, 20 μM Taxol, 1 mM GTP and 1 mM PMSF. The lysates were centrifugated at 15,000 g for 5 min at room temperature. The supernatant was clarified by centrifugation at 100,000 g for 1 h at 4°C. For microtubule depolymerization, HeLa cells were incubated with 33 μM of nocodazole for 1 h. The cells were then washed twice with PBS, and lysed with TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA and 1 mM PMSF) for 3 min at 4°C. The lysates were centrifugated at 15,000 g for 5 min at 4°C.

Centrosome fractionation assay. Centrosome isolation was performed described previously with some modifications.[45] To depolymerize actin and microtubule filaments, HeLa cells (~2 x 10^7) were incubated with CytochalasinD (1 ug/ml) and nocodazole (10 ug/ml) for 1 h at 37°C. Cells were washed with 1X PBS, 8% sucrose in 1 mM Hepes (pH 7.2) and 1 mM Hepes (pH 7.2). Cells were then lysed in a solution of 1 mM Hepes (pH 7.2), 0.5% NP-40, 0.5 mM $MgCl_2$, 0.1% β-Mercaptoetanol, 50 mM NaF, 1 mM Na_3VO_4 with proteinase inhibitor cocktail for 10 min. To remove chromatin aggregates, the lysates were centrifugated at 2,500 g for 10 min, and filtered through a 50-μm nylon mesh. Hepes was adjusted to 10 mM and DNase I was added (2 units/ml), and the mixture was incubated for 30 min on ice. The lysates were underlaid with 0.5 ml of 60% sucrose solution (60% wt/wt sucrose in 10 mM Pipes (pH 7.2), 0.1% Triton-X100, 0.1% β-mercaptoethanol), then centrifugated at 10,000 g for 30 min. This crude centrosome preparation was purified further by discontinuous sucrose gradient centrifugation at 120,000 g for 1 h. Fractions were collected from the top to bottom, 300 μl per fraction. Each fraction was diluted in 1 ml of 10 mM Pipes buffer (pH 7.2). Centrosomes were recovered by centrifugation at 13,200 rpm for 15 min and denatured in SDS sample buffer.

Microtubule regrowth assay. HeLa cells were placed on ice for 1 h to disassemble microtubule network. After incubation on ice, the cells were incubated with a warm medium for indicated time periods for microtubule regrowth.

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