CCDC57 Cooperates with Microtubules and Microcephaly Protein CEP63 and Regulates Centriole Duplication and Mitotic Progression

Graphical Abstract

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In Brief

Centrosome dysfunction is linked to cancer and neurodevelopmental disorders such as primary microcephaly. Gurkaslar et al. identify CCDC57 as a pleiotropic regulator of centriole duplication, mitosis, and ciliogenesis. They show that it mediates its functions by regulating recruitment of centriole duplication proteins to the centrosome and microtubule nucleation.

Highlights

- CCDC57 localizes to pericentriolar material, microtubules, and satellites
- CCDC57 co-localizes with CEP63 at the proximal end of centrioles
- CCDC57 binds to CEP63 and functions during initiation of centriole duplication
- CCDC57 interacts with microtubules and ensures proper mitotic progression

Article

CCDC57 Cooperates with Microtubules and Microcephaly Protein CEP63 and Regulates Centriole Duplication and Mitotic Progression

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SUMMARY

Centrosomes function in key cellular processes ranging from cell division to cellular signaling. Their dysfunction is linked to cancer and developmental disorders. Here, we identify CCDC57 as a pleiotropic regulator of centriole duplication, mitosis, and ciliogenesis. Combining proximity mapping with superresolution imaging, we show that CCDC57 localizes to the proximal end of centrioles and interacts with the microcephaly protein CEP63, centriolar satellite proteins, and microtubules. Loss of CCDC57 causes defects in centriole duplication and results in a failure to localize CEP63 and CEP152 to the centrosome. Additionally, CCDC57 depletion perturbs mitotic progression both in wild-type and centriole-less cells. Importantly, its centrosome-targeting region is required for its interaction with CEP63 and functions during centriole duplication and cilium assembly, whereas the microtubule-targeting region is required for its mitotic functions. Together, our results identify CCDC57 as a critical interface between centrosome and microtubule-mediated cellular processes that are deregulated in microcephaly.

INTRODUCTION

Centrosomes function as main microtubule-organizing centers of animal cells and mediate various key cellular and developmental processes, including cell shape, polarity, and motility, as well as spindle formation and chromosome segregation (Paz and Lüders, 2018; Wu and Akhmanova, 2017). At the core of centrosomes are two centrioles, which are barrel-shaped, microtubule-based structures characterized by evolutionarily conserved radial 9-fold symmetry (Azimzadeh and Marshall, 2010; Gönczy, 2012; Winey and O'Toole, 2014). Centrioles form the centrosome by recruiting pericentriolar material (PCM), which nucleates and organizes microtubules. Additionally, centrioles nucleate the formation of the cilia and flagella, microtubule-based cellular extensions with key functions in cellular signaling and cell motility (Heydeck et al., 2018; Mirvis et al., 2018). In addition to centrioles and PCM, vertebrate cells have an array of membrane-less 70- to 100-nm granules termed “centriolar satellites,” which localize and move around the centrosomes (Hori and Toda, 2017; Kubo and Tsukita, 2003).

Deregulation of the biogenesis and functions of centrosomes and cilia are associated with various human diseases (Bettencourt-Dias et al., 2011; Nigg and Raff, 2009). Structural and numerical centrosome abnormalities were among the first cell biological defects reported in cancer (Nigg and Holland, 2018). Centrosome amplification promotes aneuploidy and invasive behavior in cultured cells (Ganem et al., 2009; Godinho et al.,

2014; Kushner et al., 2014; Silkworth et al., 2009) and development of spontaneous tumors in multiple tissues in mouse models (Levine et al., 2017; Serçin et al., 2016). Mutations affecting centrosome components also cause various human developmental disorders, including the autosomal primary recessive microcephaly (MCPH) and ciliopathies. MCPH is a neurodevelopmental disorder characterized by small brain size and mental retardation, which is caused by reduced neuronal proliferation during embryonic development. Importantly, the majority of the genes mutated in MCPH encode centrosome proteins with functions ranging from centriole duplication to spindle pole organization (Jayaraman et al., 2018; O'Neill et al., 2018). Eight of these proteins—including PLK4, STIL, CEP63, and CEP152—function in centriole duplication, suggesting a strong link between neurogenesis and centriole number control (Nigg and Holland, 2018). To determine how centrosomal defects contribute to these diseases, it is essential to elucidate the control mechanisms that regulate centrosome biogenesis and function.

Centrioles duplicate exactly only once in most cell types, and this canonical duplication cycle is tightly regulated in a cell-cycle-linked manner (Nigg and Holland, 2018). Centriole duplication begins with the assembly of a procentriole orthogonal to the proximal end of each pre-existing centriole during G1/S transition. Procentrioles elongate throughout S and G2 phases and are then segregated to the daughter cells by the mitotic spindle in mitosis. The mechanisms that determine the location of centriole duplication on the preexisting centrioles and dictate

the orientation and copy number of procentrioles have long been areas of extensive investigation (Firat-Karalar and Stearns, 2014). Genomics, proteomics, and bioinformatics studies and genetic screens identified five conserved components required for initiation of centriole duplication—which are PLK4, CEP192, SAS-6, STIL, and CPAP—in mammalian cells (Dammermann et al., 2004; Delattre et al., 2004; Kirkham et al., 2003; Leidel et al., 2005; Leidel and Gönczy, 2003; O’Connell et al., 2001; Pelletier et al., 2004).

Centriole duplication initiates with the recruitment of the Polo-like kinase family member PLK4 to origin of centriole duplication (Bettencourt-Dias et al., 2005; Habadanck et al., 2005). Timely and efficient centriolar recruitment of PLK4 is regulated at multiple levels. First, the cellular abundance and kinase activity of PLK4 is regulated through phosphorylation and ubiquitination (Cunha-Ferreira et al., 2013; Guderian et al., 2010; Holland et al., 2012; Klebba et al., 2013). Second, its centrosomal targeting requires the cooperative action of multiple protein scaffolds at the proximal end of centrioles in mammalian cells. CEP152 and CEP192 are the components of the first scaffold, which recruit PLK4 to the centrioles through the electrostatic interactions with its negatively charged polo-box domain of PLK4 (Cizmecioglu et al., 2010; Hatch et al., 2010; Kim et al., 2013; Sonnen et al., 2013). The centrosomal recruitment of CEP152 is, in turn, regulated by CEP63, which functions in ensuring efficient centriole duplication (Brown et al., 2013; Sir et al., 2011). Recent *in vitro* studies identified the self-assembly of CEP63 and CEP152 into higher order cylindrical architectures as the underlying mechanism for PLK4 recruitment (Kim et al., 2019).

In addition to conserved components of the centriole duplication pathway, some proteins are unique to vertebrates, where centrosomes have more complex roles during development and differentiation. An example to this diversity is in the composition of the protein scaffolds that recruit PLK4 to the origin of centriole duplication. While PLK4 recruitment depends on Asterless in flies and Spd-2 in nematodes, the homologs of these two distinct proteins, CEP152 and CEP192, cooperate during this process in mammalian cells (Dzhindzhev et al., 2010; Kemp et al., 2004; Kim et al., 2013; Pelletier et al., 2004; Sonnen et al., 2013). Another example is the regulation of centriole duplication by centriolar satellites, which are vertebrate-specific, membrane-less granules that localize and move around the centrosomes in a microtubule- and motor-dependent manner (Prosser and Pelletier, 2020; Odabasi et al., 2020; Hori and Toda, 2017; Kubo et al., 1999). The centriolar satellite proteins CCDC14, KIAA0753, SPAG5, CEP72, and CEP90 were shown to function in centriole duplication by regulating centrosomal targeting of duplication proteins; in particular, the ones mutated in primary microcephaly such as CEP63 (Firat-Karalar et al., 2014; Kodani et al., 2015).

To identify regulators of centrosome biogenesis in mammalian cells, we previously generated proximity interaction maps for proteins that function during initiation of centriolar duplication, including CEP63 and its paralog, Deup1; PLK4; CEP152; CPAP; and CEP192 using the proximity-dependent biotin identification (BioID) approach (Firat-Karalar et al., 2014). CCDC57 was one of the uncharacterized proteins that stood out in these

maps due to its high proximity to centriole duplication proteins. Notably, CCDC57 was recently identified as a component of the centriolar satellite interactome, which corroborates the functional relationship between centriolar satellites and centriole duplication (Gheiratmand et al., 2019). Here, we characterized CCDC57 as a centriole duplication protein required for recruitment of the microcephaly-associated proteins CEP63, CEP152, and PLK4 to the centrosome. Moreover, independent of its centrosomal targeting, CCDC57 localized to and interacted with microtubules and regulated microtubule nucleation, stability, and mitotic progression. Together, our results identify CCDC57 as a pleiotropic regulator of centriole duplication, ciliogenesis, and mitosis.

RESULTS

CCDC57 Localizes to the Centrosome and Centriolar Satellites

To determine where CCDC57 localizes at the mammalian centrosome/cilium complex, we examined its endogenous localization in human osteosarcoma U2OS cells using a CCDC57 polyclonal antibody that recognizes the 508- to 620-amino-acid (aa) fragment of human CCDC57 (Figure 1A). Immunoblotting experiments showed that CCDC57 is ubiquitously expressed in different mammalian cell lines (Figure 1B). CCDC57 localized to the centrosome throughout the cell cycle, as assessed by co-localization with the centriole marker centrin (Figure 1C). Following microtubule depolymerization by nocodazole treatment, the majority of CCDC57 remained associated with the centrosome (nocodazole-treated cells, 78.8% $\pm$ 1.8%; control, 100%; $p < 0.0001$; $n = 100$ in 2 independent experiments) (Figure 1D). While a localization pattern similar to that of the centrosome was observed for transiently expressed Myc-CCDC57, ectopic fusion protein also localized to a subset of centriolar satellites, as assessed by co-localization with the centriolar satellite marker PCM1 (Figure 1E). The satellite pool of CCDC57, identified when it is expressed, ectopically confirms its affinity to the centriolar satellites but does not reveal satellites. Together, these results identified CCDC57 as a bona fide centrosome protein.

Spatial localization of proteins at the centrosome informs hypothesis about their function. Thus, we determined the sub-centrosomal localization of CCDC57 by performing three-dimensional structured illumination (3D-SIM) superresolution microscopy in U2OS cells stained for CCDC57 along with markers of the distal lumen proteins (Centrin 3), distal appendage proteins (CEP164), proximal end linker proteins (C-Nap1 and Rootletin), and PCM proteins (CEP63, gamma-tubulin) (Figure 2). CCDC57 localization at the centrosome was proximal to that of centrin and distal to that of C-Nap1 and Rootletin (Figures 2A, 2E, and S1A). Like CEP63 and gamma-tubulin, CCDC57 localized to resolvable rings at the proximal end of centrioles (Figure 2A). To measure ring diameters, we performed plot profile analysis for fluorescence intensities relative to distance and determined the distances between intensity maxima (Figure 2B). Notably, CEP63 and CCDC57 were arranged as rings of similar diameters (CCDC57: 302 $\pm$ 5 nm; CEP63: 307 $\pm$ 6 nm) (Figure 2C). The diameter of the gamma-tubulin ring was in agreement with previous studies (402 $\pm$ 9 nm) and was larger than the diameter

Figure 1. CCDC57 Localizes to the Centrosome and Centriolar Satellites

(A) Schematic of human CCDC57 full-length (FL) construct. Numbers indicate CCDC57 amino acids. CC indicates coiled-coil domains. CCDC57 antibody was raised against the 508- to 620-aa fragment of human CCDC57.

(B) CCDC57 is expressed in various mammalian cell lines. Extracts from IMCD3, RPE1, U2OS, and HEK293T cells were blotted for CCDC57 and actin (loading control).

(C) Localization of endogenous CCDC57 at the indicated cell-cycle stages. Asynchronously growing U2OS cells were fixed and stained for CCDC57, alpha-tubulin, and centrin 3.

(D) Effect of microtubule depolymerization on centrosomal CCDC57 levels. U2OS cells were treated with DMSO or 10 μ M nocodazole for 1 h, fixed, and stained for CCDC57 and alpha-tubulin. Relative quantifications of CCDC57 centrosomal levels are presented as the mean of two independent experiments $\pm$ SEM (50 cells per experiment; ****p < 0.0001).

(E) Myc-CCDC57 localizes to the centrosome and centriolar satellites. U2OS cells transfected with Myc-CCDC57 were fixed and stained for Myc, PCM1, and γ -tubulin. DNA was stained with DAPI.

Scale bars, 10 μ m. All insets show 4 $\times$ enlarged centrosomes.

Figure 2. CCDC57 Localizes to the Proximal End of the Centriole

(A) Representative DeltaVision OMX 3D-SIM SR micrographs were shown for CCDC57 relative to different markers of the centriole and PCM. U2OS cells were fixed and stained for CCDC57 and markers of the centriole, the distal lumen proteins (Centrin 3), distal appendage proteins (CEP164), proximal end linker proteins (Rootletin), and PCM proteins (CEP63 and gamma-tubulin). Scale bar, 3 μ m.

(B) Corresponding intensity plots of the centrosome rings for CCDC57, CEP63, and gamma-tubulin, which were calculated by scanning the fluorescence intensities and determining the distances between intensity maxima.

(C) Plots for the ring diameters of CCDC57, CEP63, and gamma-tubulin, which were calculated by scanning the fluorescence intensities and determining the distances between intensity maxima.

(D and E) Corresponding intensity plots of the rings for the indicated proteins, (D) CCDC57, CEP63, Centrin and (E) CCDC57, Rootletin, Centrin.

(F) Schematic representation of the localization of different PCM and centriole markers at the centrosome and localization of CCDC57 relative to PCM markers from a cross-section view at the PCM.

of the CEP63 and CEP152 rings (Sonnen et al., 2013). Like endogenous CEP63, GFP-CEP63 formed a resolvable ring at the proximal end of centrioles that co-localized with the CCDC57 ring (Figure S1B). These results show that CCDC57 co-localizes with CEP63 at the proximal end of the centriole.

CCDC57 Localizes to Centrosomes and Microtubules through Distinct Regions

CCDC57 has four coiled-coil domains at its N-terminal 1- to 605-aa region (CC1: 92–173; CC2: 214–422; CC3: 456–483; and CC4: 521–548) and no identifiable domains at its C-terminal 606- to 916-aa region (Figure 3A). To determine the regions required for its localization to centrosomes, we generated a series of GFP-fusion deletion constructs and ectopically expressed them in U2OS cells for localization experiments (Figure 3A). The GFP-CCDC57 (1–502) and (1–605) fragments localized to the centrosome during interphase and mitosis (Figures 3B and S2A). Their partial co-localization with two centrin foci in interphase and four centrin foci in mitosis confirm their localization at the centrosome and identify the first 502 aas as the centrosomal targeting domain (Figure 3B).

The C-terminal GFP-CCDC57 (502–916) and (606–916) fragments localized only to the microtubules but not to the centrosome (Figures 3B and S2A). In mitotic cells, GFP-CCDC57 (503–916) and (606–916) localized to spindle microtubules during metaphase (Figures 3B and S2A). Supporting its affinity to microtubules, GFP-CCDC57 (606–916), but not GFP, pelleted with microtubules in co-sedimentation experi-

ments (Figure 3C). In U2OS cells stably expressing mNeonGreen fusions of CCDC57 (1–502) and CCDC57 (503–916) that are resistant to CCDC57 small interfering RNA (siRNA), the fusion proteins localized to centrosomes and cytosol along with slight microtubule localization, respectively (Figure S2B). We used these stable lines for subsequent phenotypic rescue experiments. Together, the localization experiments show that CCDC57 is targeted to centrosomes and microtubules through distinct domains at its N and C termini.

In contrast to its C-terminal region, full-length GFP-CCDC57 did not localize to microtubules (Figure 3B), suggesting that the interaction between its C-terminal domain and microtubules might be inhibited by the N-terminal domain. In U2OS cells stably expressing mNeonGreen-CCDC57, the fusion protein formed large cytoplasmic aggregates (Figure S2B). Due to this aggregation phenotype, we did not use this cell line for the phenotypic rescue experiments. To test whether endogenous CCDC57 interacts with microtubules, we performed *in vitro* microtubule co-sedimentation assays with cell lysates. Endogenous CCDC57 co-pelleted with taxol-stabilized microtubules (Figure 3D). As controls, the microtubule-associated protein (MAP) gamma-tubulin also co-pelleted with microtubules, while the cytoplasmic protein GAPDH remained in the supernatant (Figure 3D). To test whether the microtubule interaction is of centrosomal origin or not, we performed similar experiments in HeLa::SAS-6^{-/-} centriole-less cells and found that a fraction of CCDC57 still pellets with microtubules (Figures S3A and S3B). These results show that endogenous CCDC57, either directly or indirectly, binds to microtubules in cells and suggest that it might function in microtubule-mediated cellular processes.

Figure 3. CCDC57 Localizes to the Centrosome and Microtubules through Distinct Domains

(A) Schematic representation of human CCDC57 full-length (FL) and deletion constructs and summary of their localization to the centrosome and microtubules. Cent, centrosome; MT, microtubules.

(B) CCDC57 (1–605) localizes to the centrosome and CCDC57 (606–916) localizes to the microtubules. Co-localization was determined by analyzing U2OS cells transfected with GFP-CCDC57 constructs and staining fixed cells for GFP and centrin. DNA was stained with DAPI.

(C and D) CCDC57 binds to microtubules *in vitro*. Microtubule pelleting assays were performed using extracts from (C) cells expressing GFP-CCDC57 (606–916) or (D) untransfected cells. Indicated cell extracts were incubated with taxol-stabilized microtubules or buffer, loaded onto a 40% glycerol cushion, and spun down. Input (I), supernatant (S), and pellet (P) fractions were blotted for GFP or CCDC57. Gamma-tubulin and GAPDH were used as controls. Microtubules were detected by immunoblotting for alpha-tubulin.

(E) Overexpression of CCDC57 and its deletion mutants disrupts centriolar satellite distribution and microtubule network organization. U2OS cells were transfected with the indicated GFP-CCDC57 constructs; treated with DMSO or 10 μ M nocodazole for 1 h; fixed; and stained for GFP, alpha-tubulin, and PCM1. DNA was stained with DAPI. Scale bars, 10 μ m; all insets show 4 $\times$ enlarged centrosomes.

Overexpression of CCDC57 and Its Deletion Mutants Disrupts Centriolar Satellite Distribution and Microtubule Network Organization

The pericentrosomal distribution of centriolar satellites depends on an intact microtubule network. We determined the effects of CCDC57 overexpression on the microtubule network and centriolar satellite distribution. In high expressing cells, GFP-CCDC57 formed aggregates that sequestered endogenous PCM1 and resulted in loss of pericentrosomally distributed centriolar satellites (Figure 3E). In contrast, it did not change the organization of the microtubule network (Figure 3E). The size of the aggregates varied between different cells, which likely correlated with the relative expression level of GFP-CCDC57 (Figure S2D). Interestingly, PCM1 was enriched at the periphery of the aggregates, while GFP-CCDC57 was distributed homogeneously. These aggregates also stained positive for centrin, but not for

other centrosome proteins, including CEP63 and CEP290, suggesting that there is specificity in which proteins are sequestered to the aggregates (Figure S2E).

Overexpression of the GFP fusions of CCDC57 (1–502) and CCDC57 (503–916) resulted in formation of filamentous and granular aggregates in cells. GFP-CCDC57 (503–916)-induced bundles, but not GFP-CCDC57 (1–502)-induced ones, co-localized with microtubules (Figure 3E). Microtubule depolymerization upon nocodazole treatment disrupted the GFP-CCDC57 (503–916)-positive microtubule bundles, indicating that they are microtubule-based structures and that expression of this fragment does not stabilize them (Figure 3E). In contrast, GFP-CCDC57 (1–502)-positive bundles and aggregates remained intact in nocodazole-treated cells (Figure 3E). Because this fragment contains three coiled-coil domains, it is likely that these bundles resulted from dimerization of this fragment with itself or the full-length CCDC57. Finally, although GFP-CCDC57 (503–916) overexpression disrupted centriolar satellite distribution, GFP-CCDC57 (1–605) did not, suggesting that the former phenotype is likely due to changes in microtubule organization (Figure 3E).

Figure 4. Localization, Activity, and Proximity Interactors of BioID-CCDC57 Fusion Protein

(A) BioID-CCDC57 stimulates biotinylation at the centrosome and centriolar satellites. U2OS cells were transfected with Myc-BirA*-tagged CCDC57. After 18 h incubation with biotin, cells were fixed and stained for biotinylated proteins with fluorescent streptavidin and centrosome with anti- γ -tubulin antibody. DNA was stained with DAPI. Scale bar, 10 μ m; insets show 4 $\times$ enlarged centrosomes.

(B) HEK293T cells transfected with BioID or BioID-CCDC57 were lysed and biotinylated proteins were precipitated by streptavidin beads. The initial sample (IS) and immunoprecipitated biotinylated proteins (IP) were run on a gel and immunoblotted with fluorescent coupled streptavidin.

(C) Mass spectrometry analysis of proximity interactors of Myc-BirA*-CCDC57. Proximity interactors are ranked in the order of their average NSAF from three independent experiments, and the first 200 proteins were plotted in the Cytoscape interaction network. Proteins in diamonds indicate those that localize to the centrosome; red lines around the proteins indicate those that localize to the centriolar satellites.

(D) Sub-interaction STRING interaction networks of CCDC57 based on biological process. Green lines indicate the validated interactions in this study.

(E) GO-enrichment analysis of the first 200 proximity interactors of CCDC57 based on their biological process and cellular compartment.

(F) CCDC57 interacts with CEP63 and centriolar satellite proteins CEP131 and PCM1. HEK293T cells were transfected with BioID or BioID-CCDC57 and incubated with biotin for 18 h. Complexes were immunoprecipitated with streptavidin beads, and co-precipitated proteins were detected with anti-Myc, anti-PCM1, anti-CEP63, and anti-CEP131 antibodies.

(G) CEP63 interacts with CCDC57. HEK293T cells were transfected with GFP-CEP63, complexes were immunoprecipitated with GFP-Trap beads, and co-precipitated proteins were detected with anti-GFP and anti-CCDC57 antibodies.

CCDC57 Has Extensive Proximity Interactions with Components of the Centrosome/Cilium Complex

CCDC57 was previously identified as a proximity interactor, Deup1, which functions in deuterosome-mediated centriole duplication in multiciliated epithelia, and OFD1, which functions in cilium assembly (Gupta et al., 2015; Zhao et al., 2013). To gain unbiased insight into cellular functions and mechanisms of CCDC57, we identified the spatial proximity interactome of full-length CCDC57 and its microtubule-targeting 606- to 916-aa region using the BioID approach. N-terminal Myc-BirA* fusion of CCDC57 (BioID-CCDC57) localized to and stimulated biotinylation at the centrosome and centriolar satellites, as assessed by Myc and streptavidin staining (Figures 4A and S4A). BioID-CCDC57 (606–916) localized to the microtubules and stimulated biotinylation both at the microtubules and the centrosome, suggesting that either it transiently localizes to the centrosome, or

its interacting partners at the microtubules exchange with the centrosome (Figure S4A). After lysing cells expressing Myc-BirA* fusions, biotinylated proteins were precipitated by streptavidin beads, as assessed by immunoblotting for Myc and streptavidin (Figure 4B). Following mass spectrometry, the proximity interactors of CCDC57 were ranked based on their normalized spectral abundance factor (NSAF) values (Tables S1 and S2). CCDC57 and CCDC57 (606–916) had both overlapping and distinct interactions (Figure S4E; Table S3). Gene ontology (GO) analysis of their proximity interactome based on their cellular compartment and biological processes revealed significant enrichment of components of centrosomes, satellites, and microtubule cytoskeleton, as well as their associated functions (Figures 4E and S4B; Tables S4 and S5). In agreement with their distinct cellular localization, components of the centriole duplication were enriched in the full-length CCDC57 interactome, and

components of the microtubule organization were enriched in the CCDC57 (606–916) interactome (Figures 4C, 4D, and S4D).

Several of the functional clusters among the proximity interactors of CCDC57 stood out by their association with key centrosome/cilium-mediated cellular processes and their disease links. CEP63 interacts with CEP152 and functions during initiation of centriole duplication, and its mutations were implicated in microcephaly (Brown et al., 2013; Sir et al., 2011). KIAA0753 and OFD1 interact and function in cilium assembly and centriole duplication, and their mutations were linked to the oral-facial-digital syndrome (Chevrier et al., 2016; Firat-Karalar et al., 2014; Stephen et al., 2017). PCM1, CEP72, and CEP131 are core centriolar satellite proteins implicated in cilium assembly and cell-cycle progression (Hall et al., 2013; Odabasi et al., 2019; Stowe et al., 2012; Wang et al., 2016). CEP192 and HAUS complex function during organization of PCM (Paz and Lüders, 2018). To validate these proximity interactions, we performed streptavidin pull-down experiments in HEK293T cells expressing BioID-CCDC57. CEP63, PCM1, and CEP131 pelleted with BioID-CCDC57, but not with BioID itself, confirming the proximity of these proteins to CCDC57 in cells (Figure 4F).

Given the initial identification of CCDC57 as a proximity interactor of centriole duplication proteins that function during the initiation stage (Firat-Karalar et al., 2014), we tested whether this proximity relationship is reflected at the physical level. Immunoprecipitation experiments in cells expressing GFP-CEP63 confirmed pull-down of endogenous CCDC57 (Figure 4G). Of note, GFP-CEP63 still interacted with CCDC57 in HEK::PCM1^{-/-} satellite-less cells, showing that their interaction is not mediated at the satellites (Figures S3C and S3D). In reciprocal immunoprecipitation experiments, we found that Myc-CEP63 co-pelleted with GFP-CCDC57 and GFP-CCDC57 (1–502) but not GFP (Figure S4F). In agreement, cytoplasmic aggregates that formed upon ectopic expression of GFP-CCDC57 (1–502) sequestered Myc-CEP63 (Figure S4G). Together, these results show that CCDC57 interacts with CEP63 through its N-terminal centrosomal targeting region.

CCDC57 Is Required for Efficient Centriole Duplication

Given its proximity and physical interactions with proteins implicated in centriole duplication, we tested whether CCDC57 is required for canonical centriole duplication and centriole amplification using loss-of-function studies. CCDC57 was efficiently depleted from U2OS cells 86 h after transfection with siRNAs against CCDC57, as assessed by immunoblotting, immunofluorescence, and real-time qPCR experiments (Figures S5A–S5C). We determined the centriole numbers in control and CCDC57-depleted cells by centrin labeling and identified a significant increase in cells that had either one centriole or no centrioles (siControl: 3.3% ± 0.5%; siCCDC57: 21.7% ± 3.2%; $p < 0.001$) (Figures 5A and 5B). CCDC57 depletion also compromised centriole reduplication during prolonged S phase arrest in U2OS cells. While 61.0% ± 5.8% of control cells had more than four centrioles, 27.7% ± 5.3% of the CCDC57-depleted cells had centriole amplification, and the majority had four or fewer centrioles ($p < 0.05$) (Figures 5C and 5D). The centriole reduplication phenotype was rescued upon transient expression of the RNAi-resistant GFP-CCDC57, but not GFP, confirming the

specificity of this phenotype (Figures 5C and 5D). Importantly, canonical centriole duplication and centriole amplification phenotypes were rescued by stable expression of mNeonGreen-CCDC57 (1–502), but not mNeonGreen-CCDC57 (503–916) (Figures S5D and S5E). These results show that CCDC57 mediates its functions in centriole duplication through its N-terminal centrosome targeting region.

Because CCDC57 interacts with CEP63, we hypothesized that CCDC57 might function in centriole duplication through regulating centrosomal recruitment of CEP63. To test this, we quantified CEP63 centrosomal levels in control and CCDC57-depleted cells and showed that it decreased significantly upon CCDC57 depletion (siRNA [si]Control: 1 ± 0.03; siCCDC57: 0.5 ± 0.03; $p < 0.0001$) (Figure 5E). Immunoblotting of cell extracts indicated that this decrease was not due to changes in CEP63 cellular abundance upon CCDC57 loss (Figure S5B). Analogously, CEP63 depletion resulted in a significant decrease at the centrosomal CCDC57 levels, identifying a mutual localization dependency (siControl: 1 ± 0.04; siCCDC57: 0.4 ± 0.05; $p < 0.0001$) (Figure 5F). Several satellite proteins such as KIAA0753 and CEP90 were previously characterized as regulators of centrosomal CEP63 abundance (Firat-Karalar et al., 2014; Kodani et al., 2015). To assay the functional relationship of CCDC57 to these proteins, we quantified centrosomal CEP90 and KIAA0753 levels upon CCDC57 depletion and found that their levels were not reduced relative to those of control cells (for KIAA0753: control, 1 ± 0.04; siCCDC57, 1.8 ± 0.06; $p < 0.0001$; for CEP90: control, 1 ± 0.04; siCCDC57, 1.2 ± 0.06; $p < 0.01$) (Figures S5F and S5G). These results confirm that CCDC57 is required for efficient recruitment of CEP63 to the centrosome and that it does not mediate these functions via KIAA0753 and CEP90.

CEP63 interacts and co-localizes with CEP152 at the proximal end of centrioles and is required for its centrosomal recruitment during centriole duplication (Brown et al., 2013; Sir et al., 2011). CCDC57 depletion also resulted in a significant decrease in its centrosomal levels (siControl: 0.9 ± 0.05; siCCDC57: 0.7 ± 0.06; $p < 0.0005$) (Figure 5G), which suggests that CCDC57 functions upstream of the CEP63-CEP152 complex. In addition to CEP63, CEP192 also interacts with CEP152 and is required for its centrosomal localization (Kim et al., 2013; Sonnen et al., 2013). To test whether CCDC57 functions upstream of CEP192, we quantified its centrosomal levels upon CCDC57 loss and showed that it was similar to those of control cells ($p = 0.9$) (Figure 5H). These results together show that CCDC57 is required for centrosomal recruitment of CEP63 and CEP152, but not CEP192, during centriole duplication.

To determine whether CCDC57 functions during initiation of centriole duplication via regulating centriolar recruitment of PLK4, we quantified centrosomal PLK4 levels upon CCDC57 loss. Centrosomal PLK4 levels decreased significantly in CCDC57-depleted cells relative to control cells (control: 1 ± 0.35; siCCDC57: 0.6 ± 0.03; $p < 0.0001$) (Figure 5I). To investigate how these defects are reflected during cartwheel assembly and procentriole formation, we counted the number of SAS-6 foci in cells with nuclear proliferating cell nuclear antigen (PCNA) foci, a marker of DNA replication. While the majority of control cells had two SAS-6 foci, there was a significant increase in the percentage of

Figure 5. CCDC57 Is Required for Initiation of Centriole Duplication and Centrosomal Recruitment of CEP63 and CEP152

(A and B) Effect of CCDC57 depletion on centriole duplication. U2OS cells were fixed 86 h after transfection with control siRNA or CCDC57 siRNA, and centriole number was determined by staining for centrin and gamma-tubulin. $n \geq 200$ cells per experiment. Data represent mean value from four experiments per condition $\pm$ SEM (** $p < 0.001$; *** $p < 0.001$; ns, not significant). (A) Representative fluorescent micrographs for centriole numbers in control and CCDC57-depleted cells. (B) Quantification of A.

(C) Effect of CCDC57 depletion on centriole amplification during prolonged S phase. U2OS cells were transfected with control siRNA or CCDC57 siRNA, which was followed by transfection with GFP or GFP-CCDC57. 48 h after hydroxyurea treatment, cells were fixed and stained for GFP and centrin, and the percentage of cells with more than 4 centrioles was determined.

(D) Quantification of rescue experiments in (A). $n \geq 150$ cells per experiment. Data represent mean value from three experiments per condition $\pm$ SEM (* $p < 0.05$ *** $p < 0.001$, ns: not significant).

(E–H) Effect of CCDC57 depletion on (E) CEP63, (G) CEP152, and (H) CEP192 levels at the centrosome. U2OS cells were fixed 86 h after transfection with control siRNA or CCDC57 siRNA and stained for the indicated proteins. Images represent centrosomes in cells from the same coverslip taken with the same camera settings. CEP63, CEP152, and CEP192 fluorescence intensities at the centrosome were measured from SUM projections, and average means of the levels in control cells were normalized to 1. $n = 100$ cells per experiment. Data represent mean value from two experiments per condition $\pm$ SEM (*** $p < 0.001$; **** $p < 0.0001$; n.s., non-significant). (F) Effect of CEP63 depletion on CCDC57 level at the centrosome. U2OS cells were fixed 86 h after transfection with control siRNA or CEP63 siRNA and stained for CEP63. Images were quantified as described for (E), (G), and (H). Data represent mean value from two experiments per condition $\pm$ SEM (**** $p < 0.0001$).

(I) Effect of CCDC57 depletion on PLK4 level at the centrosome. U2OS cells were fixed 86 h after transfection with control siRNA or CCDC57 siRNA

and stained for PLK4. Data represent mean value from two experiments per condition $\pm$ SEM (**** $p < 0.0001$).

(J) CCDC57 depletion compromises SAS-6 recruitment in S-phase cells. SAS-6 foci were counted in U2OS cells with nuclear PCNA foci 86 h after transfection with control siRNA or CCDC57 siRNA. $n > 100$ cells per experiment. Data represent mean value from three experiments per condition $\pm$ SEM (** $p < 0.001$; **** $p < 0.0001$).

Scale bars, 10 μ m; all insets show 4 $\times$ enlarged centrosomes.

cells with 0 or 1 SAS-6 focus upon CCDC57 depletion (Figure 5J), indicating that CCDC57 is required for centrosomal SAS-6 recruitment. Collectively, our results show that CCDC57 regulates centrosomal recruitment of the CEP63-CEP152 complex and functions during initiation of centriole duplication.

CCDC57 Is Required for Efficient Cilium Assembly and Centriolar Satellite Distribution

Centrioles are essential for nucleating the formation of primary cilia, and CCDC57 has proximity interactions with proteins impli-

cated in cilium biogenesis. To investigate whether CCDC57 functions in cilium assembly, we depleted CCDC57 in human RPE1 and mouse IMCD3 cells and quantified the percentage of ciliated cells upon 48-h serum starvation. While control RPE1 cells ciliated 81% $\pm$ 2.6%, CCDC57-depleted cells ciliated 48.7% $\pm$ 4.6% ($p = 0.004$) (Figures 6A and 6B). The cilia that formed in CCDC57-depleted cells were significantly ($p < 0.0001$) shorter in length (Figure 6C). The ciliogenesis defect of CCDC57-depleted RPE1 cells was rescued by stable expression of mNeonGreen-CCDC57 (1–502), but not by mNeonGreen-CCDC57(503–916)

Figure 6. CCDC57 Is Required for Efficient Ciliogenesis and Centriolar Satellite Distribution

(A) Effect of CCDC57 loss on cilium formation. Control and CCDC57-depleted RPE1 cells were serum-starved for 48 h, and percentage of ciliated cells was determined by staining for acetylated-tubulin, glutamylated tubulin, PCM1, and DAPI.

(B) Quantification of ciliogenesis experiments. Results shown are the mean of three independent experiments $\pm$ SEM (>300 cells per experiment; $^{**}p < 0.01$).

(C) Effect of CCDC57 depletion on cilium length. Cilium length was determined by staining for acetylated-tubulin and DAPI. Results shown are the mean of three independent experiments $\pm$ SEM (>100 cells per experiment; $^{****}p < 0.0001$).

(D) mNeonGreen (mNG)-CCDC57 (1–502) stable expression rescues the ciliogenesis defect of CCDC57-depleted cells. U2OS cells stably expressing mNG, mNG-CCDC57 (1–502), or mNG-CCDC57 (503–916) were fixed 86 h after transfection with control siRNA or CCDC57 siRNA and 24 h serum starvation and were stained for cilium markers. Results shown are the mean of two independent experiments $\pm$ SEM (>100 cells per experiment; $^{*}p < 0.05$; n.s., non-significant).

(E) Effect of CCDC57 depletion on centriolar satellite distribution. U2OS cells were fixed 86 h after transfection with control siRNA or CCDC57 siRNA and stained for the indicated proteins. Images represent centrosomes in cells from the same coverslip taken with the same camera settings.

(F) Quantification of (E). PCM1 and CEP131 fluorescence intensities at the centrosome were measured from SUM projections, and average means of the levels in control cells were normalized to 1. Data represent mean values from two experiments $\pm$ SEM (>50 cells per experiment; $^{**}p < 0.01$; $^{****}p < 0.0001$).

(G) Effects of PCM1 depletion on ciliogenesis efficiency of CCDC57-depleted cells. RPE1 cells were transfected with control siRNA, CCDC57 siRNA, or CCDC57/PCM1 siRNAs for 86 h; serum-starved for the last 24 h; fixed; and stained for cilium markers.

(H) Quantification of (G). Results shown are the mean of two independent experiments $\pm$ SEM (>100 cells per experiment; $^{**}p < 0.01$; $^{***}p < 0.001$).

Scale bars, 10 μ m.

Figure 7. CCDC57 Is Required for Timely and Faithful Mitotic Progression

(A and B) Effect of CCDC57 depletion on mitotic phenotypes. Control and CCDC57-depleted U2OS cells stably expressing mCherry-H2B and GFP-centrin2 were imaged every 6 min for 12 h. (A) Representative still frames from time-lapse experiments are shown. (B) Mitotic time was quantified as the time interval from

(legend continued on next page)

(siCCDC57-mNeonGreen: $43.8\% \pm 4\%$; siCCDC57-mNeonGreen (1–502): $82.7\% \pm 4\%$; mNeonGreen (503–916): $66.8\% \pm 11\%$) (Figure 6D). Analogously, CCDC57 depletion in IMCD3 cells resulted in a significant ($p < 0.001$) decrease in the fraction of ciliated cells ($36.8\% \pm 3\%$) as compared with control cells ($63.5\% \pm 3\%$; $n = 300$) (Figures S5H and S5I). These results show that the centrosome-associated pool of CCDC57 is required for efficient ciliogenesis.

We determined the consequences of CCDC57 depletion on satellite distribution by quantifying the pericentrosomal levels of PCM1 and CEP131. CCDC57 depletion resulted in a significant reduction in the amount of pericentrosomal PCM1 and CEP131 levels ($p < 0.0001$ and $p < 0.001$) (Figures 6E and 6F). Given that centriolar satellites have been described as regulators of centrosome and cilium biogenesis, we next investigated whether—and, if so, how—satellite dispersal contributes to the defective ciliogenesis and CEP63/CEP152 centrosomal recruitment phenotypes associated with CCDC57 loss (Hori and Toda, 2017; Prosser and Pelletier, 2020). First, we determined how loss of satellites affected PCM integrity by quantifying centrosomal levels of CEP63 and gamma-tubulin in satellite-less cells. To this end, we generated U2OS::PCM1^{−/−} cells using the CRISPR-Cas9 genome editing approach and validated the lack of PCM1 expression in these cells (Figures S3E and S3F). The centrosomal levels of both CEP63 and gamma-tubulin increased slightly but significantly in U2OS::PCM1^{−/−} cells relative to control cells ($p < 0.0001$) (Figures S3G and S3H). Additionally, to determine whether CCDC57 mediates its functions during ciliogenesis through perturbing satellite distribution, we quantified the fraction of ciliated RPE1 cells depleted for CCDC57 individually or depleted for CCDC57 and PCM1 together. Co-depletion of CCDC57 and PCM1 corroborated the ciliation defect as compared to cells depleted only for CCDC57, suggesting that CCDC57 and PCM1 regulate cilium assembly through different pathways (siControl: $87.94\% \pm 1.05\%$; siCCDC57: $65.98\% \pm 0.05\%$; siCCDC57+siPCM1: $54.65\% \pm 0.3\%$) (Figures 6G and 6H). Collectively, these results suggest that CCDC57 functions during cilium assembly and that centrosomal CEP63 recruitment is not mediated directly by its satellite-associated pool.

CCDC57 Is Required for Mitotic Progression and Microtubule Nucleation

CCDC57 binds to microtubules, localizes to interphase and spindle microtubules, and has proximity interactions with mitotic regulators. Therefore, we hypothesized that CCDC57 regulates microtubule-mediated cellular processes such as mitotic progression. To test this, we performed time-lapse imaging of control and CCDC57-depleted U2OS cells stably expressing the centriolar marker GFP-centrin 2 and the nuclear marker mCherry-H2B over 12 h (Figure 7A). First, we quantified and compared the mitotic time for the group of control and CCDC57-depleted cells that completed mitosis. Mitotic time was determined as the time from nuclear envelope breakdown to anaphase onset by using mCherry-H2B dynamics as a reference. While the average mitotic time was 38.4 ± 0.9 min for control cells ($n = 176$ mitotic cells out of 1,318 cells), it was 51.1 ± 2.4 for CCDC57-depleted cells ($n = 79$ cells out of 1,261 cells; 3 independent experiments; $p < 0.0001$) (Figure 7B). The mitotic delay phenotype was rescued by stable expression of mNeonGreen-fusion of CCDC57 (1–502) and CCDC57 (503–916) (Figure S6A). In addition to mitotic delay, proliferation assays with control and CCDC57-depleted cells over 4 days revealed that CCDC57 loss significantly compromised doubling times around day 3 (Figure 7C). Finally, the percentage of phospho-histone-H3-positive cells was reduced in CCDC57-depleted cells as compared with control cells (siControl: $7.7\% \pm 0.4\%$; siCCDC57: $2.5\% \pm 0.1\%$; 2 independent experiments; $p < 0.05$) (Figure 7D).

To determine the specific mitotic defects associated with CCDC57 depletion, we quantified the percentage of cells that (1) underwent apoptosis after a long mitotic arrest, (2) had chromosome alignment defects, and (3) had spindle multipolarity and cytokinetic defects associated with concomitant multinucleation. After a prolonged mitotic arrest, $19.8\% \pm 2.2\%$ of CCDC57-depleted cells underwent apoptosis relative to $2\% \pm 2.4\%$ of control cells, as assessed by membrane blebbing and DNA fragmentation ($p < 0.01$; $n = 3$ experiments) (Figures 7E and S6B). In agreement, the levels of cleaved caspase-3 relative to uncleaved caspase-3 increased in CCDC57-depleted cells as compared with control cells (Figure 7F). Additionally, flow

nuclear envelope breakdown (NEBD) to anaphase onset (AO). $n > 40$ mitotic cells per experiment. Data represent mean $\pm$ SEM of four independent experiments (**** $p < 0.0001$).

(C) Effect of CCDC57 depletion on cell proliferation. Proliferation assays for control and CCDC57-depleted cells. Numbers were normalized to day 1. Data represent mean $\pm$ SEM of three independent experiments (**** $p < 0.0001$).

(D) Mitotic index of control and CCDC57-depleted cells were quantified as the percentage of phospho-histone H3 positive cells. Data represent mean $\pm$ SEM of two independent experiments (* $p < 0.05$).

(E) Effect of CCDC57 depletion on mitotic index. Percentage of control and CCDC57-depleted mitotic cells was quantified based on cells that completed bipolar mitosis (Normal), cells that underwent apoptosis (Apoptosis), and cells with cytokinetic defects (Multinucleation). $n > 40$ cells per experiment. Data represent mean $\pm$ SEM of three independent experiments (* $p < 0.05$; ** $p < 0.01$).

(F) Effect of CCDC57 depletion on apoptosis. Cleaved caspase-3 levels were normalized to uncleaved caspase-3 levels to compare the apoptotic control and CCDC57-depleted cells.

(G) Effect of CCDC57 depletion on percentage of cells in different cell-cycle phases. Control and CCDC57-depleted cells were fixed, and their DNA content was assessed by flow cytometry. Data represent mean $\pm$ SEM of two independent experiments (* $p < 0.05$).

(H) Percentage of control and CCDC57-depleted mitotic cells were quantified based on chromosome alignment and segregation phenotypes that included normal, misaligned, and lagging chromosomes. $n > 40$ cells per experiment. Data represent mean $\pm$ SEM of three independent experiments (** $p < 0.01$, n.s., not significant).

(I) Effect of CCDC57 depletion on mitotic phenotypes of HeLa::SAS-6^{−/−} centriole-less cells. Control and HeLa::SAS-6^{−/−} cells were fixed and stained for SAS-6, centrin, and DAPI to confirm the lack of centrioles and SAS-6 expression upon SAS-6 loss. Percentage of control and CCDC57-depleted mitotic centriole-less cells was quantified based on cells that completed mitosis, cells that underwent apoptosis, and cells with cytokinetic defects. $n > 30$ cells per experiment. Data represent mean $\pm$ SEM of three independent experiments (*** $p < 0.001$; **** $p < 0.0001$).

cytometry analysis of the asynchronous control and CCDC57-depleted cells showed an increase in the fraction of apoptotic cells upon CCDC57 depletion (Figure 7G). The increased apoptosis phenotype was rescued by stable expression of mNeonGreen fusions of CCDC57 (1–502) and CCDC57 (503–916) (Figure S6C). Finally, $58.7\% \pm 9.0\%$ of CCDC57-depleted cells had chromosome misalignment defects, relative to the $9.1\% \pm 3.4\%$ of control cells ($p < 0.01$; $n = 3$ experiments) (Figures 7H and S6B), which was rescued by stable expression of mNeonGreen-CCDC57 (1–502). To directly test the contribution of the centrosome-independent pools of CCDC57 to its mitotic functions, we performed similar experiments in HeLa::SAS-6^{-/-} centriole-less cells. CCDC57 loss exacerbated the mitotic defects such as apoptosis and multinucleation in centriole-less cells (Figure 7I). Collectively, these results demonstrate that CCDC57 is required for proper mitotic progression, and its centrosome and microtubule-associated pools cooperate in this function.

The mitotic phenotypes associated with CCDC57 depletion might be due to its microtubule-associated functions. To investigate the nature of these functions, we performed microtubule regrowth experiments to determine whether CCDC57 functions in microtubule nucleation and/or anchoring. Microtubule depolymerization in control and CCDC57-depleted cells was induced by 1-h nocodazole treatment, and cells were then fixed and stained for alpha-tubulin and acetylated-tubulin at different time points after nocodazole washout. Notably, there were higher levels of microtubule regrowth and acetylated microtubules in CCDC57-depleted cells, as evidenced by a significant increase in microtubule aster size (Figures S7A and S7B). Immunoblotting experiments identified a significant increase in the total cellular levels of acetylated tubulin upon CCDC57 loss and no change for alpha-tubulin (Figure S7C). To determine whether altered abundance of PCM proteins underlies the increase in microtubule nucleation in CCDC57-depleted cells, we quantified centrosomal levels of gamma-tubulin and CDK5RAP2. While gamma-tubulin centrosomal levels were unaltered in CCDC57-depleted cells ($p = 0.1$), centrosomal CDK5RAP2 levels were significantly higher relative to those of control cells (CDK5RAP2: control, 1 ± 0.03 ; siCCDC57, 1.2 ± 0.05 ; $p < 0.001$) (Figure S7D). These results together showed that CCDC57 negatively regulates microtubule nucleation and stability.

DISCUSSION

In this study, we characterized CCDC57 as a regulator of centriole duplication, ciliogenesis, and mitosis. CCDC57 interacts with CEP63 through its centrosomal targeting domain and functions during initiation of centriole duplication through regulating CEP63, CEP152, and PLK4 recruitment to the centrosome. Additionally, CCDC57 localizes to interphase and mitotic microtubules and is required for microtubule nucleation and stability, mitotic progression, and chromosome alignment. Collectively, our results provide insight into the mechanisms that underlie the key centrosome- and cilium-mediated processes deregulated in disease.

Characterization of CCDC57 deletion mutants for their cellular localization showed that CCDC57 is targeted to centrosomes

and microtubules through distinct regions at its N and C termini. The N-terminal centrosomal targeting region comprises three coiled-coil domains, which likely mediates its dimerization with other centrosome proteins. In agreement, immunoprecipitation experiments revealed that this region is required for CCDC57 interactions with CEP63. In contrast to the centrosomal targeting domain, the C-terminal microtubule targeting region has no identifiable domains. The stretches of amino acids conserved in CCDC57 orthologs neither were enriched in basic amino acids nor had homology to other microtubule-binding proteins, including centrosomal MAPs such as CEP120, CEP135, CPAP, Mdm1, and CCSAP (Backer et al., 2012; Carvalho-Santos et al., 2012; Hsu et al., 2008; Lin et al., 2013b; Van de Mark et al., 2015). Future *in vitro* microtubule sedimentation, polymerization, and dynamics experiments with purified CCDC57 and its fragments are required to elucidate on the nature of the relationship between CCDC57 and microtubules, and these studies will inform the microtubule-mediated functions of CCDC57 during mitosis and microtubule nucleation.

The separation of centrosome and microtubule localization and interaction affinities within CCDC57 allowed us to dissect the mechanisms by which CCDC57 mediates its pleiotropic functions. Of note, we could not perform the rescue experiment with full-length CCDC57, as its ectopic expression induced the formation of cytoplasmic aggregates and inhibited proper centriolar satellite distribution and ciliogenesis even in low-expressing cells. The defective canonical centriole duplication, centriole amplification, and ciliogenesis phenotypes in CCDC57-depleted cells were rescued by this centrosomal targeting domain, but not the C-terminal microtubule targeting domain. CCDC57 interacts with CEP63 through this domain, and overexpression of this domain recruits CEP63 to cytoplasmic aggregates. Based on these lines of evidence, we propose that interaction of CCDC57 with its binding partners at the centrosome, such as the CEP63-CEP152 microcephaly module, is required for its functions during the initiation of centriole duplication. As for the C-terminal microtubule targeting domain, the enrichment of mitotic regulators and MAPs in its proximity interactome and the absence of centriole duplication proteins such as CEP63 relative to the interactome of the full-length CCDC57 corroborate its regulatory roles in microtubule-mediated processes. Depletion of CCDC57 in centriole-less cells exacerbated the mitotic phenotypes of these cells, which demonstrates that its mitotic functions are not solely regulated by its centrosome-associated pool. Notably, the mitotic phenotypes of CCDC57-depleted cells were rescued by the expression of both its centrosomal and microtubule targeting domains. Collectively, these results show that centrosome and microtubule-associated activities of CCDC57 cooperate during mitosis.

How does CCDC57 regulate microtubules to mediate its functions during mitotic progression and fidelity? Microtubule regrowth experiments identified CCDC57 as a suppressor of microtubule nucleation and acetylation. Increase in microtubule nucleation was not due to an increase in the amount of centrosomal gamma-tubulin levels, which is different than the inhibitory mechanisms reported for other centrosome proteins, such as the daughter centriole protein CEP120 (Betleja et al., 2018). A possible mechanism for this inhibition is that CCDC57

cooperates or competes with other MAPs to regulate microtubule nucleation and stability. The proximity interactome of the CCDC57 microtubule-targeting region identified MAPs, including CAMSAP1 and KIAA0753, as potential candidates for these proteins (Hueschen et al., 2017; Yadav et al., 2014). Supporting this possibility, we identified an increase in the centrosomal levels of KIAA0753 in CCDC57-depleted cells. Despite these compelling models, we would like to highlight that the mitotic defects upon CCDC57 loss are, in part, consequences of defective CEP63 centrosomal recruitment and centriole duplication. In fact, analogous to CCDC57, loss of CEP63 was shown to cause increased apoptosis, mitotic errors, and delays (Marjanović et al., 2015).

Proximity and physical interactions of CCDC57 with CEP63 and its function in centriole duplication together identify CCDC57 as part of the CEP63-CEP152 functional module at the origin of centriole duplication. While our data showed that CCDC57 regulates centrosomal recruitment of CEP63 and CEP152, how this regulation is imparted at the molecular level is not known. Analogous to the relationship between CEP63 and CEP152, it could be a simple targeting mechanism where CCDC57 and CEP63 provide binding sites for each other at the centrosome (Sir et al., 2011). In a complementary mechanism, the satellite pool of CCDC57 might also take part in centrosomal targeting of duplication proteins, as previously reported for CEP63 (Firat-Karalar et al., 2014; Kodani et al., 2015). Future studies that specifically dissect functions of the CCDC57 satellite pool will be essential to correlate its pleiotropic functions and mechanisms.

Almost all proteins mutated in primary microcephaly are centrosome proteins that function in centriole duplication and spindle assembly, which highlights the crucial roles imparted by centrosomes during neurodevelopment as well as the importance of centriole number control. The results of our study place CCDC57 in the context of the CEP63-CEP152 functional module associated with microcephaly, which ensures efficient centriole duplication and faithful mitotic progression (Brown et al., 2013; Marjanović et al., 2015; Sir et al., 2011). Although no microcephaly patient mutations have been reported for CCDC57 to date, our data suggest that CCDC57 might be a candidate microcephaly gene, and targeted searches for CCDC57 mutations in microcephaly patients by whole-exome sequencing are required to address this possibility. Loss-of-function studies in animal models and brain organoids will also be essential to determine whether—and, if so, how—CCDC57 contributes to neurodevelopment.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.107630>.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Acetylated-K40 tubulin (6-11-B)	Sigma-Aldrich	Cat#T6793; RRID: AB_477585
Mouse monoclonal anti-Polyglutamylation (GT335)	Adipogen	Cat#AG-20B-0020; RRID: AB_2490210
Mouse monoclonal anti-gamma-tubulin clone (GTU-88)	Sigma	Cat#T6557; RRID: AB_477584
Mouse monoclonal anti-alpha-tubulin (DM1A)	Sigma	Cat#T6199; RRID: AB_477583
Rabbit polyclonal anti-CCDC57	Sigma	Cat# HPA023344; RRID: AB_477584
Rabbit polyclonal anti-KIAA0753	Sigma	Cat# HPA023494; RRID:AB_1852339
Rabbit polyclonal anti-CEP131	Proteintech	Cat#25757-1-AP; RRID:AB_2784548
Rabbit polyclonal anti-PIBF1 (CEP90)	Proteintech	Cat# 14413-1-AP; RRID:AB_10858916
Rabbit polyclonal anti-CEP192	Proteintech	Cat# 18832-1-AP; RRID:N/A
Rabbit polyclonal anti-PCM1	Proteintech	Cat#19856-1-AP; RRID:N/A
Mouse monoclonal anti-Caspase 3/P17/P19	Proteintech	Cat#66470-2-Ig; RRID:N/A
Mouse monoclonal p-Histone H3 (C-2)	Santa Cruz Biotechnology	Cat# sc-374669; RRID:AB_11150094
Mouse monoclonal anti-SAS-6	Santa Cruz Biotechnology	Cat# sc-81431; RRID:AB_1128357
Mouse monoclonal anti-PCNA (PC10)	Santa Cruz Biotechnology	Cat# sc-56; RRID:AB_628110
Mouse monoclonal anti-Rootletin (C-2)	Santa Cruz Biotechnology	Cat# sc-374056; RRID:AB_10918081
Mouse monoclonal anti-C-Nap1 (F-7)	Santa Cruz Biotechnology	Cat# sc-390540; RRID: AB_2810862
Mouse monoclonal anti-c-Myc (9E10)	Thermo Fisher	Cat# 13-2500; RRID:AB_2533008
Rabbit polyclonal anti-CEP290	Abcam	Cat#ab85728; RRID: AB_1859783
Rabbit polyclonal anti-PCM1	Conkar et al., 2017	N/A
Rabbit polyclonal anti-GFP	Conkar et al., 2017	N/A
Rabbit polyclonal anti-PLK4	Hatch et al., 2010	N/A
Rabbit polyclonal anti-CEP152	Bethyl Laboratories	Cat#A302-480A; RRID:AB_1966084
Mouse monoclonal anti-CETN3 (M01, 3E6)	Abnova	Cat# H00001070-M01; RRID: AB_464016
Rabbit polyclonal anti-β-Actin	Cell Signaling Technology	Cat#4967; RRID:AB_330288
IRDye 680RD goat anti-mouse IgG (H+L)	LI-COR	Cat#926-68070; RRID: AB_10956588
IRDye 800CW goat anti-rabbit IgG (H+L)	LI-COR	Cat#926-32211; RRID: AB_621843
Goat anti-rabbit Alexa Fluor 488	Thermo Fisher	Cat#R37116; RRID: AB_2556544
Goat anti-rabbit Alexa Fluor 568	Thermo Fisher	Cat#A-11011; RRID: AB_143157
Donkey anti-mouse Alexa Fluor 568	Thermo Fisher	Cat#A-11031; RRID: AB_144696
Donkey anti-mouse Alexa Fluor 488	Thermo Fisher	Cat#A28175; RRID: AB_2536161
Goat anti-rabbit Alexa Fluor 647	Thermo Fisher	Cat#A-27040; RRID:AB_2536101
Goat anti-mouse Alexa Fluor 647	Thermo Fisher	Cat#A-28181; RRID:AB_2536165
Goat anti-mouse IgG2a Alexa Fluor 568	Thermo Fisher	Cat#A-21134; RRID:AB_2535773
Goat anti-mouse IgG1 Alexa Fluor 568	Thermo Fisher	Cat#A-21124; RRID:AB_141611
Goat anti-mouse IgG2b Alexa Fluor 568	Thermo Fisher	Cat#A-21144; RRID:AB_2535780
Streptavidin, Alexa Fluor 488 conjugate	Thermo Fisher	Cat# S11223; RRID:AB_2315383
Bacterial and Virus Strains		
Stb13	Thermo Fisher	Cat#C737303
Chemicals, Peptides, and Recombinant Proteins		
Prolong Gold anti-fade Mounting Media	Life Technologies	Cat#P36935
Lipofectamine RNAimax Transfection Reagent	Life Technologies	Cat#13778150
Lipofectamine LTX with Plus Reagent	Thermo Fisher	Cat#15338100

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Paraformaldehyde 16% Solution	Thermo Fisher	Cat#28908
Gateway BP Clonase II Enzyme mix	Thermo Fisher	Cat#11789020
Gateway LR Clonase II Enzyme mix	Thermo Fisher	Cat#11791100
Gotaq qpcr master mix	Promega	Cat#A6001
Propidium Iodide solution	Sigma-Aldrich	Cat#P4864-10ML
Nocodazole	Sigma-Aldrich	Cat#M1404
Hydroxyurea	Sigma-Aldrich	Cat#H8627
Streptavidin Agarose	Thermo Fisher	Cat#20353
GFP-Trap Agarose	Chromotek	Gtma-20
MycoAlert Mycoplasma Detection Kit	Lonza	Cat# LT07-118
Polyethylenimine	Polysciences	Cat#23966-1
Protease-inhibitor cocktail tablets	Roche	Cat#37378900
Puromycine	InvivoGen	Cat#ant-pr-1
DAPI	Life Technologies	Cat#D1306
Experimental Models: Cell Lines		
hTERT RPE-1	ATCC	Cat#CRL-4000
U2OS	ATCC	Cat# HTB-96
HEK293T	ATCC	Cat#CRL-3216
HeLa	ATCC	Cat#CCL2
mIMCD-3 Flip-In	Max Nachury, University of California, San Francisco	N/A
HeLa PCM1 ^{-/-}	This paper	N/A
U2OS PCM1 ^{-/-}	This paper	N/A
HeLa Sas-6 ^{-/-}	This paper	N/A
U2OS::mNeonGreen	This paper	N/A
U2OS::mNeonGreen-CCDC57 (1-502 a.a.)	This paper	N/A
U2OS::mNeonGreen-CCDC57 (503-916 a.a.)	This paper	N/A
U2OS::mCherry-H2B	This paper	N/A
U2OS::mCherry-H2B/GFP-Centrin 2	This paper	N/A
Oligonucleotides		
CCDC57 siRNA: AGCAGGACAUUGAAAGGUA	Thermo Fisher	Cat#4392420
PCM1 siRNA: GGGCUCUAAACGUGCCUCCUU	Dharmacon	N/A
CEP63 siRNA: AGUCCAGCGAUUUCUGACGUU	Dharmacon	N/A
Control siRNA	Dharmacon	Cat#D-001210-02-05
mCCDC57 shRNA CCAACAGGACATAGACAGGTA	This paper	N/A
PCM1 gRNA	Odabasi et al., 2019	N/A
hSAS-6 gRNA	Wang et al., 2015	N/A
Primer CCDC57 (1-916)_gatewayFp: GGGGACAACCTTTG TACAAAAAAGTTGATATGCTGCCACTGGGCTCAGAG	Macrogen	N/A
Primer CCDC57 (1-916)_gatewayRp: GGGGACAACCTTTG TACAAGAAAGTTGTTAACTACAACATTATGGACTGA	Macrogen	N/A
Primer CCDC57(1-502)_gatewayRp: GGGGACAACCTTTG TACAAGAAAGTTGTCTGCCTGAGAAGCATCTGTGC	Macrogen	N/A
Primer CCDC57(503-916)_gatewayFp: GGGGACAAC TTTGTACAAAAAAGTTGATGGCACAGATGCTTCTCA GGCAG	Macrogen	N/A
Primer CCDC57(1-605)_gatewayRp: GGGGACAACCTTTG TACAAGAAAGTTGTCTGCCTGAGAAGCATCTGTGC	Macrogen	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer CCDC57(606-916)_gatewayFp: GGGGACAAC TTTGTACAAAAAGTTGATGGCACAGATGCTTCTCA GGCAG	Macrogen	N/A
CCDC57_qPCR_fp: GAA ACA AGA CCT CAC CAG GG	Macrogen	N/A
CCDC57_qPCR_rp: TGGAAGTCATGCTCTCTTCTCT	Macrogen	N/A
Recombinant DNA		
pcDNA-DEST47	Thermo Fisher	Cat#12281010
pDEST-myc-BirA*	Christian Hoerner, Stanford University	N/A
pLVPT2-GFP-N1	Christian Hoerner, Stanford University	N/A
pEGFP-C1-CEP63	Firat-Karalar et al., 2014	N/A
pCDH-EF1-mNeonGreen-T2A-Puro	This paper	N/A
pCS2+6xMyc-CEP63	This paper	N/A
pCDH-EF1-mNeonGreen-CCDC57	This paper	N/A
pCDH-EF1-mNeonGreen-CCDC57(1-502) siRNA resistant	This paper	N/A
pCDH-EF1-mNeonGreen-CCDC57(503-916)	This paper	N/A
pCDH-EF1-mNeonGreen-mCCDC57	This paper	N/A
pcDNA-DEST47-CCDC57	This paper	N/A
pcDNA-DEST47-CCDC57 (1-502)	This paper	N/A
pcDNA-DEST47-CCDC57 (1-605)	This paper	N/A
pcDNA-DEST47-CCDC57 (503-916)	This paper	N/A
pcDNA-DEST47-CCDC57 (606-916)	This paper	N/A
pDEST-myc-BirA* CCDC57	This paper	N/A
pDEST-myc-BirA* CCDC57 (606-916)	This paper	N/A
pLVPT2-GFP-Centrin 2	This paper	N/A
pLVPT2-mCherry-H2B	Odabasi et al., 2019	N/A
pLKO.1-shCCDC57	This paper	N/A
pLKO.1-shControl	Sigma Aldrich	Cat# SHC002
Software and Algorithms		
Leica Las X	Leica Microsystems	N/A
Fiji/ImageJ	Fiji/ImageJ	https://imagej.net/Fiji/
Prism	Graphpad software	N/A
Adobe Illustrator CC 2017	Adobe	N/A
Huygens for image deconvolution	Scientific Volume Imaging	N/A
softWoRx v. 7.0.0 software	GE Healthcare	N/A

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elif Nur Firat-Karalar (ekaralar@ku.edu.tr).

Materials Availability

All unique/stable reagents and cell lines generated in this study are available from the Lead Contact without restriction.

Data and Code Availability

This study did not generate any unique code. All data generated in this study are available from the Lead Contact without restriction. Original/source mass spectrometry data for [Figure 4/Figure S4](#) in the paper is available in [Table S1](#) and [Table S2](#).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human hTERT RPE-1, U2OS, HEK293T, HeLa were purchased from the American Type Culture Collection. Mouse IMCD3 Flip-In cells were shared by the laboratory of Max Nachury at University of California San Francisco. hTERT RPE-1 and IMCD3 Flip-In cells were cultured in Dulbecco's modified Eagle's F12 (DMEM:F12, Pan Biotech) supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies) and Penicillin/Streptomycin (GIBCO). HeLa, HEK293T and U2OS cells were cultured in Dulbecco's modified Eagle's (DMEM, Pan Biotech) supplemented with 10% FBS and Penicillin/Streptomycin. HeLa Sas-6^{-/-}, U2OS PCM1^{-/-} and HeLa PCM1^{-/-} were generated in this study using CRISPR/Cas9 genome editing approached and maintained in DMEM supplement with 10%FBS and Penicillin/Streptomycin. U2OS-mNeonGreen, U2OS::mNeonGreen-CCDC57 (1-502 aa), U2OS::mNeonGreen-CCDC57 (503-916 aa), U2OS::mCherry-H2B and U2OS::mCherry-H2B/GFP-Centrin 2 stable cell lines were generated in this study by lentiviral transduction and maintained in DMEM supplement with 10%FBS and Penicillin/Streptomycin.

METHOD DETAILS

Cell culture and transfection

Human telomerase immortalized retinal pigment epithelium cells (hTERT-RPE1, ATCC, CRL-4000) and mouse kidney inner medullary collecting duct (IMCD3 Flip-In) were cultured with Dulbecco's modified Eagle's Medium DMEM/F12 50/50 medium (Pan Biotech, Cat. # P04-41250) supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies, Ref. # 10270-106, Lot # 42Q5283K) and 1% penicillin-streptomycin (GIBCO, Cat. # 1540-122). Human embryonic kidney (HEK293T, ATCC, CRL-3216) and osteosarcoma epithelial (U2OS, ATCC, HTB-96) cells were cultured with DMEM medium (Pan Biotech, Cat. # P04-03590) supplemented with 10% FBS and 1% penicillin-streptomycin. All cell lines were authenticated by Multiplex Cell Line Authentication (MCA) and were tested for mycoplasma by MycoAlert Mycoplasma Detection Kit (Lonza). RPE1 and U2OS cells were transfected with the plasmids using Lipofectamine 2000 according to the manufacturer's instructions (Thermo Fisher Scientific). HEK293T cells were transfected with the plasmids using 1 µg/µl polyethylenimine, MW 25 kDa (PEI). For serum starvation experiments, RPE1 cells were washed twice with PBS and incubated with DMEM/F12 50/50 supplemented with 0.5% FBS and 1% penicillin-streptomycin for the indicated times. For microtubule depolymerization experiments, cells were treated with 10 µg/ml nocodazole (Sigma-Aldrich, Cat. #M1404) or vehicle (dimethyl sulfoxide) for one hour at 37°C. For microtubule regrowth experiments, cells were washed 3X with PBS after microtubule depolymerization, incubated in warm media for the indicated times, fixed and stained. For proliferation assays, 1*10⁴ cells were plated to 24 well plates, transfected with siRNAs for two consecutive days. 40 h post first siRNA transfection, and cells were counted at 24, 48, 72 and 96 h. At each count, cells were splitted at 1:2.

Lentivirus production, cell transduction and genome editing

Lentivirus were generated using pLKO.1-shScrambled, pLKO.1-shCCDC57, pcDH-mNG-CCDC57 full-length and truncations, and pLVPT2-mCherry-H2B plasmids as transfer vectors. For U2OS::PCM1^{-/-} and HEK293T::PCM1^{-/-} cell generation, we used the gRNA plasmids and sequences as previously described (Odabasi et al., 2019). For HeLa::SAS-6^{-/-} cell generation, we used the gRNA plasmids and sequences as previously described (Wang et al., 2015). For infection, 1 × 10⁵ U2OS or RPE1 cells were seeded on 6-well tissue culture plates the day before infection, which were infected with 1 mL of viral supernatant the following day. 48 hour post-infection, cells were split and selected in the presence of 6 µg/ml puromycin for RPE1s and 4 µg/ml puromycin for U2OS cells for four to six days until all the control cells died. PCM1 and SAS-6 knockout efficiency was determined in the heterogeneous pool using immunofluorescence experiments with specific antibodies. Following serial dilutions of the heterogeneous pool and colony growth, cells were trypsinized and expanded for screening for knockouts by immunofluorescence and/or western blotting. U2OS and RPE1 cells stably expressing mCherry-H2B and mNeonGreen fusions of full-length CCDC57 and its truncations were generated by infection of cells with lentivirus expressing the fusions.

Plasmids

Full-length cDNAs of *Homo sapiens* CCDC57 (GenBank accession number 284001) and *Mus musculus* CCDC57 (GenBank accession number 71276) were obtained from Open Biosystems. The ORF of CCDC57 was amplified by PCR and cloned into pDONR221 using the Invitrogen Gateway system. Subsequent Gateway recombination reactions using pCS2+6xMyc DEST, pcDNA-DEST47, pDEST-myc-BirA* and pLVPT2-GFP-N1 were used to generate GFP-CCDC57, LAP-CCDC57, BioID-CCDC57 and pLVPT2-GFP-CCDC57. Deletions constructs of CCDC57 were made by PCR amplification of the indicated regions. PCR products were cloned into pDONR221 and gateway recombination reactions using pcDNA-DEST47 and pLVPT2-GFP-N1 were used to produce GFP-tagged truncations. pEGFP-C1-CEP63 (GenBank/EMBL/DBJ accession number NM_001042383.1) was used for CEP63 expression.

pLVPT2-GFP-N1 was used to generate GFP-CCDC57 lentiviral expression vector. Deletions constructs of CCDC57 were made by PCR amplification of the indicated regions. PCR products were cloned into pDONR221 and gateway recombination reactions using pcDNA-DEST47 and pLVPT2-GFP-N1 were used to produce GFP and Lenti-GFP deletion constructs. pcDH-EF1-mNeonGreen-T2A-Puro plasmid was used to generate mNG-CCDC57, mNG-CCDC57(1-502)^{RR} and mNG-CCDC57 (503-916)^{RR}. The siRNA-resistant CCDC57(1-502)^{RR} clone was generated by making five consecutive synonymous base pair changes at the center of the

siRNA-targeted region (mutated base pairs in bold: AGCAGG**ACATTGAAAGGTA**) by using overlapping PCR. Mouse CCDC57 shRNA oligonucleotides (nucleotides 1194-1214, 5'-CCAACAGGACATAGACAGGTA-3') were cloned into pLKO.1 (Invitrogen).

RNAi and rescue experiments

CCDC57 was depleted using an siRNA with the sequence 5'-AGCAGGACAUUGAAAGGUA-3' (nucleotides 1196-1213) ordered from Thermo Fisher Scientific. PCM1 was depleted using an siRNA (Dharmacon) with the sequence 5'-GGGCUCUAAACGUGCCUCUUU-3' as previously described (Conkar et al., 2019). CEP63 was depleted using an siRNA (Dharmacon) with the sequence 5'-AGUCAGCGAUUUCUGACGUU-3' as previously described (Brown et al., 2013). Control siRNA#1 (Dharmacon, Cat. # D-001210-02-05) was used as control. Cells were seeded onto coverslips at 70% confluency and transfected with 50 nM of siRNA in two sequential transfections using Lipofectamine RNAiMAX (Life Technologies, Ref. # 13778-150, Lot # 2009103) in OPTI-MEM (Life Technologies, Cat. # 31985062) according to the manufacturer's instructions. Depletion of proteins was confirmed 48 h, 72 h and 86 h after transfection by immunofluorescence and immunoblotting. For rescue experiments, cells were transfected with control and CCDC57 siRNAs using Lipofectamine RNAiMax followed by transfection with GFP or GFP-mCCDC57 using Lipofectamine 2000. 86 h post transfection with the siRNAs, cells were fixed and stained.

Immunofluorescence, antibodies and microscopy

Cells were grown on coverslips, washed twice with PBS and fixed in either ice cold methanol at -20°C for 10 minutes or 4% PFA in PBS for indirect immunofluorescence. After rehydration in PBS, cells were blocked with 3% BSA (Capricorn Scientific, Cat. # BSA-1T) in PBS + 0.1% Triton X-100 followed by incubation with primary antibodies in blocking solution for 1 hour at room temperature. Cells were washed three times with PBS and incubated with secondary antibodies and DAPI (Thermo Scientific, cat#D1306) at 1:2000 for 45 minutes at room temperature. Following three washes with PBS, cells were mounted using Mowiol mounting medium containing N-propyl gallate (Sigma-Aldrich). For double labeling with rabbit anti-CEP63 and rabbit anti-CCDC57, a monovalent goat anti-rabbit Fab fragment was used as a blocking agent at 20 $\mu\text{g}/\text{ml}$. For double labeling with rabbit anti-CEP63 and rabbit anti-CCDC57, a monovalent Nanotag Camelid sdAb Atto488 anti-Rabbit IgG and Camelid sdAb Atto565 anti-rabbit IgG Fab fragments were used as a blocking agent at 20 $\mu\text{g}/\text{ml}$. Nanobodies were incubated with primary antibodies for 1 hour at room temperature. Following coupling, coverslips were incubated with the nanobody-coupled antibodies overnight at 4°C . Primary antibodies used for immunofluorescence were mouse anti-acetylated tubulin (clone 6-11B, 32270, Thermo Fischer) at 1:10000, mouse anti-glutamylated-tubulin (GT335, Genetex) at 1:500, mouse anti gamma-tubulin (Sigma, clone GTU-88, T5326) at 1:1000, mouse anti GFP (3E6) at 1:750, mouse anti alpha-tubulin (Sigma, DM1A) at 1:1000, rabbit anti CEP152 (Bethyl, A302-480A) at 1:500, rabbit anti-KIAA0753 (Sigma, HPA023494) at 1:1000, rabbit anti-CEP131 (Proteintech, 25757-1-AP) at 1:1000, rabbit anti-CCDC57 (Sigma, HPA023344) at 1:1000, rabbit anti-CEP90 (Proteintech) at 1:1000, rabbit anti-CEP192 (Proteintech) at 1:1000, rabbit anti-phospho-Histone H3 at 1:1000, mouse anti-SAS-6 (Santa Cruz Biotechnology) at 1:500, mouse anti-PCNA (Santa Cruz Biotechnology) at 1:1000 and mouse anti-centrin 3 (Abnova, clone 3E6) at 1:1000, mouse anti-Rootletin (Santa Cruz Biotechnology) at 1:1000 and anti-C-Nap1 (Santa Cruz Biotechnology) at 1:1000. Rabbit anti-PCM1, anti-GFP and anti-PLK4 antibodies were generated and used for immunofluorescence as previously described (Firat-Karalar et al., 2014; Hatch et al., 2010). Secondary antibodies used for immunofluorescence experiments were AlexaFluor 488-, 568- or 633-coupled (Life Technologies) and they were used at 1:2000. Biotinylated proteins were detected with streptavidin coupled to Alexa Fluor 594 (1:2000; Life Technologies).

Time lapse live imaging was performed with Leica DMI8 inverted fluorescence microscope equipped with an incubation chamber. For cell cycle experiments, asynchronous cells were imaged at 37°C with 5% CO_2 with a frequency of 6 minutes per frame with 1.5 μm step size and 12 μm stack size in 1024x1024 pixel format at a specific position using HC PL APO CS2 40x 1.3 NA oil objective. For centrosomal protein level quantifications, images were acquired with Leica DMI8 inverted fluorescent microscope with a stack size of 8 μm and step size of 0.5 μm in 1024x1024 format using HC PL APO CS2 63x 1.4 NA oil objective. Higher resolution images were taken by using HC PL APO CS2 63x 1.4 NA oil objective with Leica SP8 confocal microscope. For superresolution imaging of subcentrosomal CCDC57 localization, images were acquired using DeltaVision OMX 3D-SIM SR (GE Healthcare). All data were captured using an Olympus 60x 1.42 NA oil objective, 640nm/568nm/488 nm laser illumination and standard excitation and emission filter sets. Deltavision immersion oils with RI 1.514 or 1.516 were applied to obtain the best resolution. Sections were acquired at 0.125 μm z steps, and raw three-phase images were reconstructed in three-dimensional and re-aligned by softWoRx v. 7.0.0 software (GE Healthcare).

Quantitative immunofluorescence for CCDC57, CEP63, CEP152, CEP131/AZI1, KIAA0753, CEP90, PLK4, CEP192 and PCM1 was performed by acquiring a z stack of control and depleted cells using identical gain and exposure settings. The SUM slice projections were assembled from z stacks using ImageJ. The centrosome region for each cell were defined by staining for a centrosomal marker including gamma-tubulin and centrin. The region of interest that encompassed the centrosome was defined as a circle 3.5- μm^2 area centered on the centrosome in each cell. Total pixel intensity of fluorescence within the region of interest was measured using ImageJ (National Institutes of Health, Bethesda, MD) (Schneider et al., 2012). Background subtraction was performed by quantifying fluorescence intensity of a region of equal dimensions in the area proximate to the centrosome. Statistical analysis was done by normalizing these values to their mean.

Primary cilium formation was assessed by counting the total number of cells, and the number of cells with primary cilia, as detected by DAPI and acetylated tubulin staining, respectively. Ciliary length was measured using acetylated tubulin as the ciliary length

marker. All values were normalized relative to the mean of the control cells (= 1). For quantification of microtubule regrowth phenotypes, microtubule aster size was determined by drawing a circular region from the centrosome to the distal end of the longest microtubule tip in each cell.

Cell lysis and immunoblotting

Cells were lysed in 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100 and protease inhibitors for 30 min at 4°C followed by centrifugation at 15,000 g for 15 min. The protein concentration of the resulting supernatants was determined with the Bradford solution (Bio-Rad Laboratories, CA, USA). For immunoblotting, equal quantities of cell extracts were resolved on SDS-PAGE gels, transferred onto nitrocellulose membranes, blocked with TBST in 5% milk for 1 hour at room temperature. Blots were incubated with primary antibodies diluted in 5% BSA in TBST overnight at 4°C, washed with TBST three times for 5 minutes and blotted with secondary antibodies for 1 hour at room temperature. After washing blots with TBST three times for 5 minutes, they were visualized with the LI-COR Odyssey® Infrared Imaging System and software at 169 μ m (LI-COR Biosciences). Primary antibodies used for immunoblotting were rabbit anti-PCM1 (Proteintech, 19856-1-AP) at 1:500, rabbit anti-beta-actin (Cell Signaling Technology) at 1:10,000, mouse anti-alpha-tubulin (Sigma, DM1A) at 1:5000, anti-CCDC57 (Sigma HPA023344) at 1:1000, anti-c-Myc (clone 9E10) at 1:500, rabbit anti-capsase-3 (Proteintech) at 1:500 and mouse anti-acetylated tubulin (clone 6-11B, 32270, Thermo Fischer) at 1:5000. anti-PCM1 and anti-GFP antibodies were generated and used for immunoblotting as previously described (Firat-Karalar et al., 2014). Secondary antibodies used for western blotting experiments were IRDye680- and IRDye 800-coupled and were used at 1:15,000 (LI-COR Biosciences).

Immunoprecipitation

HEK293T cells were transfected with the indicated constructs. 24 h after transfection, cells were washed with PBS, lysed in IP buffer (50 mM Tris-HCl pH 7.4, 260 mM NaCl, 2.27 mM KCl, 1.25 mM KH_2PO_4 , 6.8 mM Na_2HPO_4 , 1% NP-40) supplemented with protease inhibitors (10 μ g/ml Leupeptin, Pepstatin and Chymostatin, 1 mM PMSF, 10 μ g/ml Aprotinin), tumbled at 4°C for 45 min, and centrifuged at 13,000 R.P.M. for 15 minutes. Insoluble material was pelleted, and supernatant was incubated with GFP-Trap agarose beads (Chromotek, GTA#10) at 4°C for 45 min. Beads were washed in lysis buffer, eluted in sample buffer, and ran on SDS-PAGE gels.

In vitro microtubule pelleting assay

Untransfected HEK293T cells or HEK293T cells expressing the indicated GFP-fusion proteins were lysed in BRB80 buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM MgCl_2 , 0.1% NP-40, protease inhibitors) at 4°C for 30 min and centrifuged at 13,000 R.P.M. for 15 minutes. Lysates were further cleared by centrifugation at 90,000 R.P.M. for 5 minutes at 4°C with TLA100 rotor (Beckman). 2.5 μ g cell lysate was brought to a final volume of 250 μ l BRB80 supplemented with 1 mM GTP. Lysate was incubated with 25 μ M taxol stepwise at 30°C and with or without 0.5 mM AMP-PNP at 30°C for another 30 minutes. Lysate was centrifuged at 55,000 R.P.M. for 10 minutes at 30°C in a TLA100 rotor (Beckman) through a 125 μ l 40% glycerol cushion prepared from BRB80 buffer supplemented with 1 mM GTP. Pellets were resuspended in the same final volume as the input and equivalent volumes of pellet and supernatant were ran on SDS-PAGE gels and processed by immunoblotting.

Biotin-streptavidin affinity purification

For the BioID experiments, HEK293T cells were transfected with BirA*-tagged proteins. 24 h post-transfection, transfected cells were supplemented with 50 μ M biotin and incubated a further 18 h. After transfection with BirA*-tagged proteins and biotin treatment, cells were directly lysed in lysis buffer. An equal volume of 4°C 50 mM Tris (pH 7.4) was added to the extracts and insoluble material was pelleted. Soluble materials from whole cell lysates and centrosome enriched extracts were incubated with Streptavidin agarose beads (Thermo Scientific) overnight. Beads were collected and washed twice in wash buffer 1 (2% SDS in dH₂O), once with wash buffer 2 (0.2% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, and 50 mM HEPES, pH 7.5), once with wash buffer 3 (250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA and 10 mM Tris, pH 8.1) and twice with wash buffer 4 (50 mM Tris, pH 7.4, and 50 mM NaCl). 10% of the sample was reserved for western blot analysis and 90% of the sample to be analyzed by mass spectrometry was washed twice in 50 mM NH_4HCO_3 .

Mass spectrometry and data analysis

Mass spectrometry analysis was done as described previously with the following change (Firat-Karalar et al., 2014): The MS/MS spectra were searched with the ProLuCID algorithm against the Uniprot human database (downloaded on 03/20/2014). For each BioID experiment data were derived from two biological replicates. Control data were derived from mock-transfected and BirA*-transfected HEK293T cells. To compare data across different runs and assess the abundance of each proximity partner, we applied the normalization of spectral counts (Zybailov et al., 2006). Individual spectral count values were normalized against the sum of all spectral counts for a particular run, resulting in normalized spectral abundance factor (NSAF). To distinguish the nonspecific interactors, we calculated the ratio of the NSAF of each identified protein to the NSAF of the corresponding proteins in the control datasets. A protein was considered a contaminant if the ratio was smaller than 2.5. For analysis of the data from two independent BioID experiments, we only accounted for the proteins that were identified in both replicates. Interaction maps were drawn using

Cytoscape. GO-enrichment analysis was done using DAVID Functional Annotation Bioinformatics Microarray Analysis. For generating sub-interaction maps for specific functional clusters, we performed STRING analysis for the selected set of proteins.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed and plotted using GraphPad Prism 7 (GraphPad, La Jolla, CA). Results are shown as mean $\pm$ standard errors of the mean. Number of biological replicates are indicated in the figure legends. Two-tailed unpaired t tests and one-way analysis of variance (ANOVA) were applied to compare the statistical significance of the measurements. Error bars reflect SD. Following key is followed for asterisk placeholders for *p*-values in the figures: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, **** *p* < 0.0001