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Cep57, a multidomain protein with unique microtubule and centrosomal localization domains

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Abstract

This study demonstrates different functional domains of a recently described centrosomal protein, Cep57 (Centrosomal protein 57). Endogenous Cep57 protein and ectopic expression of full-length protein or the N-terminal coiled-coil domain localize to the centrosome internal to γ -tubulin suggesting that it is either on both centrioles or a centromatrix component. The N-terminus can also multimerize with the N-terminus of other Cep57 molecules. The C-terminus contains a second coiled-coil domain that directly binds to microtubules. This domain both nucleates and bundles microtubules *in vitro*. This activity was also seen *in vivo*, as overexpression of full-length Cep57 or the C-terminus generates nocodazole resistant microtubule cables in cells. Based on these findings, we propose that Cep57 serves as a link with its N-terminus anchored to the centriole or centromatrix and its C-terminus to microtubules.

Keywords

centrosome; centromatrix; centriole; microtubule; microtubule formation nucleation; Cep57 (centrosomal protein 57)

Introduction

The centrosome is a small organelle that nucleates and regulates microtubules (MTs) of animal cells [1]. It includes a core structure consisting of a pair of centrioles with surrounding accessory proteins referred to as pericentriolar material [2, 3]. In addition, Schnackenberg et al. [4] have proposed a salt or chaotrope insoluble internal substructure called the 'centromatrix.' Centrosomes are the predominant microtubule organization center of animal cells and a central component is the γ -tubulin ring complex, which contains MT nucleation activity. After nucleation the minus ends of some MTs remain anchored at the

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centrosome. In addition to a role as microtubule organization center, recent studies have demonstrated significant participation of centrosomes in signal transduction.

In our study, Cep57, was initially identified as one of the positive candidates through a yeast two-hybrid screen. Cep57 used to be denoted as KIAA0092, the code given by a coding sequence prediction project of the human genome [5] until it appeared in the list of salt insensitive component of purified centrosomes and was denoted as Cep57 (Centrosomal Protein 57) [6]. Bossard et al. [7] showed suppression of endogenous Cep57 resulted in hindrance of translocation of the 18 kDa fibroblast growth factor 2 isoform from the membrane to the nucleus and called the protein Translokin. Kim et al. [8] suggested a role of Cep57 in the postmeiotic phase of sperm cell differentiation based on the observation that Cep57 mRNA is upregulated between Day 21 and Day 25 of postnatal testicular development.

Because of the importance of centrosomes in cell division and the incomplete understanding of their role, the distinct features of Cep57 prompted us to further perform a structure function analysis of Cep57 as a possible key player in the centrosomes function. Recent *in silico* screening suggests two forms of Cep57 exist in *Xenopus*, human and in mouse. We find that Cep57 has distinct functional domains to strictly target it to centrosomes, and to induce nucleation and stabilization of MTs.

Materials and Methods

Cloning of Mouse Cep57 cDNA and plasmid construction

cDNA of mouse Cep57 with flanking restriction sites was cloned from QUICK-Clone cDNA (mouse smooth muscle, CLONTECH) by PCR with a set of primers: 5'-C GCG GAT CCC ATG GCG GCA GCT CCG GTC TCG GCG GCT T-3' and 5'-G CTC TAG AAT TCA GTA ATC CCA ACA CAG ATT ACT GCT CT-3'. The overall sequence of the PCR product was matched with the reported sequence in GeneBank (Accession#: AY225093) except a few mismatches: A9T, G10C, A884G and A887C. The full-length and different segments of Cep57 cDNA were PCR-amplified from the initial PCR product and introduced into various expression vectors; e.g. pGST-Parallel1 [9], pEGFP-C and pDsRed-Express-C (CLONTECH) and pJRed-C (EVROGEN). JRed is a monomeric *Anthomedusae* jellyfish red fluorescent chromoprotein [10].

For low-level expression in mammalian cells, a mammalian expression vector, from which a N-terminally humanized *Renilla reniformis* green fluorescent protein (hrGFP; Stratagene) - fused protein is expressed under the regulation of human ubiquitin C promoter, was constructed. DNA sequence encoding hrGFP was PCR-amplified with flanking restriction sites, 5' *HindIII* and 3' *EcoRI*, and the Kozak translation initiation sequence adjacent to the initiation codon, and recombined between *HindIII* and *EcoRI* sites of pUB/V5-His vector (Invitrogen). DNA sequence encoding either truncated or full-length Cep57 was subsequently introduced to express N-terminally hrGFP-fused protein. Expression test of hrGFP-N-Cep57 promoted by the hUbc promoter showed 5–10 fold less than the one promoted by the CMV promoter (data not shown).

Production of GST-C-Cep57 proteins in *E. coli* and purification

E. coli BL21-CodonPlus (Stratagene) was transformed with pGST-Parallel1-C-Cep57, pre-cultured to OD₆₀₀ 2.0 at 37°C and further cultured at 16°C in Terrific Broth containing 100 mg/ml ampicillin and Isopropyl β-D-thiogalactopyranoside (1 mM) for 24 h. Bacteria were harvested by centrifugation, re-suspended in ice-cold PBS containing protease inhibitors (complete protease inhibitor cocktail tablets; Roche) and lysed by French press. Cell lysate was cleared by a two-step centrifugation, first at 20,000 g for 10 min followed by a 65,000 g spin for 1 h (both at 4°C). Resulting supernatant was subjected to glutathione sepharose 4 fast flow beads (Amersham) and rocked overnight at 4°C. After thorough wash by PBS and then 50 mM PIPES buffer (pH 7.0), the beads were packed in a column, and GST-C-Cep57 was eluted by 10 mM reduced glutathione in 50 mM PIPES buffer (pH 7.0).

Production of anti-Cep57 antibodies

The recombinant 6xHis-C-Cep57 protein was used as antigenic peptide for inoculation of rabbit for polyclonal antibody production (BIOSOURCE, Hoplinton, MA). After the epitope region was narrowed to between amino acid residue 332–500 by verifying reactivity of the crude serum to different regions of Cep57, the antibody was affinity purified against the recombinant GST-Cep57_{332–500} protein. To raise monoclonal antibodies in mice, The recombinant GST-Cep57_{332–500} and GST-N-Cep57 proteins were used as antigenic peptides (A&G Pharmaceutical, Columbia, MD).

Analysis of MT stabilization and homo-multimerization by co-localization

NIH 3T3 cells were cultured on glass cover slips in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen-Gibco) supplemented with 10% fetal bovine serum (FBS; Invitrogen-Gibco) at 37°C in 5% CO₂. Plasmids for the ectopic expression of fluorescent-tagged either truncated or full-length Cep57 were transfected into cells using Lipofectamine 2000 (Invitrogen) following manufacture's standard protocol. Twenty-four hours after transfection, cells were either fixed immediately in methanol at –20°C or for MT stabilization analysis, subjected to the medium containing nocodazole (5 μM) for 30 min at 37°C and fixed. The cells were washed with PBS, blocked in 3% BSA in PBS and stained with either a primary antibody or a fluorescent conjugated antibody in the blocking buffer. Fluorescent conjugated antibodies used for epifluorescence confocal microscopy are Cy3-conjugated anti-β-tubulin antibody (SIGMA) and Cy3-conjugated anti-myc antibody (SIGMA) at the dilution of 1:2,000. Combinations of the primary antibody [i.e. anti-γ-tubulin antibody (Abcam; Cambridge, MA) or polyclonal anti-Cep57 antibody at the dilution of 1:2,000 or monoclonal anti-Cep57 antibody (hybridoma supernatant) at the dilution of 1:250] and the secondary antibody [i.e. Alexa Fluor 488 or 594 conjugated anti-rabbit or anti-mouse IgG antibody (Invitrogen-Molecular Probes) at the dilution of 1:2,000] were also used for epifluorescence confocal microscopy. The cells were washed between and after the application of the antibodies with PBS, mounted in Aqua Poly/Mount (Polysciences) and visualized using epifluorescence confocal microscopy on Olympus FV300. When cells were co-stained by the monoclonal anti-Cep57 antibody and Cy3-conjugated anti-β-tubulin antibody (mouse monoclonal), Cy3-conjugated anti-β-tubulin

antibody was applied after the staining process with the monoclonal anti-Cep57 antibody was completed.

RNA interference

Hela cells were cultured in DMEM supplemented with 10% FBS (Invitrogen-Gibco) at 37°C in 5% CO₂. For transfection, Hela cells were prepared in 6-well dishes with 1.7 ml of serum- and antibiotics- free DMEM and transfected by adding OptiMEM (Invitrogen-Gibco) containing 6 µl of DharmaFECT 1 siRNA transfection reagent (Dharmacon, CO) and siGENOME SMARTpool reagent (Dharmacon, CO), a pool of four RNAi oligos targeting segments of human Cep57 sequence: 5'-GAUAAAGCAUGCCGAAAUGUU-3', 5'-GGAAACGCAUGCAAGCUAAUU-3', 5'-CAACAGCAGAGCCAUAUUUUU-3' and 5'-AGUAAGAAGUUGUCAGUAAUU-3'. The final concentration of siGENOME SMARTpool reagent was 50 nM; subconcentration of each RNA oligo was 12.5 nM. After 4 h of transfection in serum-free DMEM, 1 ml of DMEM supplemented with 30% FBS was added. After 24 h of transfection, the transfectant was trypsinized and sparsely re-plated in new 6-well dishes with glass over slips. The cells were fixed, immunostained and subjected to epifluorescence confocal microscopy 4 days after the transfection.

Co-immunoprecipitation

HEK 293 cells were cultured in Minimum Essential Medium (Invitrogen-Gibco) supplemented with 1% nonessential amino acid, 1% Na⁺ Pyruvate and 10% horse serum at 37°C at 5% CO₂ and transiently co-transfected with 1) FLAG-C-Cep57 with myc-N-Cep57 or 2) FLAG-N-Cep57 with myc-N-Cep57 or 3) with myc-Cep57₅₈₋₂₆₉ or 4) with myc-Cep57₁₋₂₃₉ expression vectors by a calcium phosphate method. Following incubation for 36 hours, the transfectants were lysed in buffer [1% Triton X-100 (SIGMA); 150 mM NaCl; 50 mM Tris pH 7.5; 2% protease inhibitor cocktail (SIGMA)] and the lysate was cleared by centrifugation. The supernatant was diluted with an equal volume of lysis buffer without Triton X-100 and protease inhibitor cocktail, hence, the buffer condition for immunoprecipitation was 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.5 and 1% protease inhibitor cocktail. The diluted supernatant was subjected to 15 µl of EZview Red ANTI-FLAG M2 Affinity Gel (SIGMA). Following overnight incubation at 4°C, the gel was washed three times with the immunoprecipitation buffer. Proteins were solubilized in sample buffer (1% SDS, 15% Glycerol, 15 mM dithiothreitol, 62.5 mM Tris pH 6.8, 0.008% Bromophenol blue) and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody or anti-myc antibody. The antibody dilutions were 1:20,000 and 1:10,000, respectively.

Electron microscopy

EM analysis of cells over-expressing Cep57: NIH 3T3 cells over-expressing EGFP-Full-Cep57 were pelleted and trapped in a collagen matrix. Following fixation in glutaraldehyde, tannic acid and osmium tetroxide and en-block staining with 4% uranyl acetate, the cells were embedded in Spurr's resin, and ultra thin sections at 80 nm were prepared. EM of samples used for *in vitro* tubulin-Cep57 polymerization assays: A drop of sample solution containing MTs and/or free tubulin and/or recombinant GST-C-Cep57 protein was applied

on a carbon coated copper grid, proteins were allowed to settle and the grid was negatively stained with 4% uranyl acetate for 1 min. Excess solution was drained by lens paper. EM analyses were performed by an electron microscope (Philips CM12) at 80 KeV.

***In vitro* tubulin polymerization assay**

Tubulin polymerization was monitored at 35°C by light absorbance at 350 nm (OD₃₅₀) using a spectrophotometer (Beckman DU7400). Formation of MTs was confirmed by EM. In all the experiments, the concentration of tubulin was maintained below the critical concentration where spontaneous polymerization occurs. Purified bovine brain tubulin (Cytoskeleton, CO) was suspended in PME buffer (80 mM PIPES, 1 mM MgCl₂, 2 mM EGTA, pH 6.9; 60 µl) with 1 mM GTP, pre-equilibrated in a sample cuvette, and either taxol and/or recombinant GST-C-Cep57 protein was added. Taxol stock solution (2 mM) was prepared in DMSO, and the final concentration of DMSO in the reaction solution did not exceed 5%. Calibration plots (OD₃₅₀ vs [tubulin]) were constructed for tubulin polymerized with 10 µM Taxol and was shown to be linear up to 12 µM of tubulin. All the experiments using GST-C-Cep57 were carried out in parallel with control experiments with GST only.

Assessment of mitotic index and cell cycle analysis using flow cytometry by FACS

NIH 3T3 cells transiently expressing either EGFP-N-Cep57 or myc-N-Cep57 were fixed, immunostained for β-tubulin and/or myc-epitope-tag respectively. Chromosomes were visualized by staining with ToPro3 (Invitrogen-Molecular Probes). Mitotic cells were identified by the typical mitotic appearance of MTs and chromosomes and counted. The cells expressing N-Cep57 were identified either EGFP fluorescence or positive stain of the myc-epitope-tag. Six independent preparations transfected by expression plasmid for EGFP-N-Cep57 and 3 independent preparations for myc-N-Cep57 were assessed. The p-value was calculated by two-tail t-test.

NIH 3T3 cells transfected by myc-N-Cep57 expression plasmid were fixed in cold 2% paraformaldehyde in PBS followed by -20°C 70% ethanol, blocked in 3% BSA in PBS and immuno- and DNA- labeled by FITC-conjugated anti-myc antibody (1:1,000 dilution; SIGMA) and propidium iodide buffer [0.1% Triton X-100 (SIGMA) respectively, 100 µg DNase free RNase (SIGMA) and 10 µg propidium iodide (SIGMA) in PBS]. The FACS analysis was performed by the BD FACSCalibur system (BD Bioscience). The gate for the non-transfected control cells was defined as below the 'gap' in signal intensity from immuno-labeled myc-epitope-tag. The myc-N-Cep57 positive cells were defined as 'all the above' population.

Results

To characterize mammalian Cep57, we cloned the mouse Cep57 from a smooth muscle cDNA library. Mouse and human Cep57 are composed of 500 amino acid residues (accession number: Q8CEE0 and Q86XR8 respectively). The secondary structure prediction of Cep57 was performed using COILS (http://www.ch.embnet.org/software/COILS_form.html) [11] as well as the PredictProtein Server (<http://cubic.bioc.columbia.edu/predictprotein/>) [12]. The secondary structure prediction indicated that Cep57 is composed

of two α -helical coiled-coil segments connected by a flexible linker region and this structural information was used to determine where to truncate Cep57, and we generated a set of deletion mutants of Cep57 to study its function and define domains (Figure 1A). Unless otherwise stated, truncations were made at the double prolines (residue 267 and 268) or their proximate residues in the middle flexible linker region. We denote the N-terminal-half truncates as N-Cep57, the C-terminal-half truncates as C-Cep57 and full-length Cep57 as Full-Cep57. Of note, the capability of producing the recombinant N-terminal-half and the C-terminal-half Cep57 proteins in *E. coli*. separately helped to increase production efficiency and retain solubility of the products.

Rabbit polyclonal and mouse monoclonal anti-Cep57 antibodies were produced using C-Cep57 recombinant protein as antigen. Following affinity purification against recombinant C-Cep57, specificity of the polyclonal antibody was verified by 1) specific reactivity to C-Cep57 recombinant protein and over-expressed C-Cep57 in mammalian cells (data not shown), 2) size agreement between endogenous Cep57 and over-expressed Full-Cep57 (Figure 1B) and 3) blockage of reactivity to endogenous Cep57 in the presence of recombinant C-Cep57 (Figure 1B'). Specificity of the monoclonal antibody was verified by the similar manner as for the polyclonal antibody. A tissue screen using the polyclonal antibody shows ubiquitous expression of Cep57 in the tissues tested (Figure 1C). NIH 3T3 cells were immunostained with the polyclonal or monoclonal anti-Cep57 antibody and showed one or two spots adjacent to the nucleus in each cell (Figure 2A). These spots were also stained by anti- γ -tubulin antibody, a marker for the centrosomes. The immunostain by monoclonal antibody in HeLa cells disappeared when the cells were transfected with RNA oligos for RNA interference (RNAi) targeting Cep57 mRNA (Figure 1D). Taken together, we concluded that both the polyclonal and monoclonal antibodies specifically recognize Cep57. Although kinetochore staining is also observed on purified chromosomes from *Xenopus* cells, we did not see it in either *Xenopus*, human or mouse cells [13]. These data suggest that Cep57 is primarily localized to the centrosome in somatic cells.

The cells showing no signal from Cep57 following the transfection for RNAi had reduced pole-to-pole distance of the mitotic spindles. The average pole-to-pole distance of the treated cells ($n=47$) was $8.70\ \mu\text{m}$ as compared to $9.87\ \mu\text{m}$ in the untreated cells ($n=47$; $P<0.00000000002$). This suggests that Cep57 plays a role in mitotic spindle formation in mammalian cells.

The first α -helical, coiled-coil segment of Cep57 contains a centrosome targeting domain

Cells that express low-levels of ectopic expression of hrGFP-Full-Cep57 in NIH 3T3 cells showed localization at the centrosomes (Figure 2B). Both ectopically expressed Cep57 and endogenous Cep57 appeared to be at a cylindrical internal structure in the centrosomes indicated by immunostain with anti- γ -tubulin antibody (Figure 2). This suggests that Cep57 is indeed localized at the centromatrix and/or centrioles and is consistent with its biochemical identification in proteomic analysis of a salt-resistant centrosomal fraction [6].

The centrosome-targeting domain is localized in the N-terminus of Cep57. hrGFP-N-Cep57 expressed in cells localized to the centrosome (Figure 2B). When highly overexpressed this protein could also be found in the cytoplasm (Figure 3B and 4A panel m). This centrosomal

localization was recapitulated by hrGFP-Cep57_{58–239}, whereas hrGFP-C-Cep57 did not show apparent localization to the centrosomes. This strongly suggests that the amino acid residues between 58–239 are responsible for centrosome localization of Cep57 (Figure 1 and 2B). Thus, we define this amino acid stretch as the centrosome localization domain (CLD) of Cep57.

Ectopic expression of the CLD resulted in reduction in the mitotic index and G1 arrest. Among the cells showing ectopic expression of EGFP-N-Cep57, the mitotic index was 0.51%; n=2,744; in contrast to that of the control cells 3.82%; n=12,971, $P<0.000006$. This result is consistent where Myc-N-Cep57 was ectopically expressed resulting in the mitotic index of 0.91% (n=1,694) as compared to 4.21% (n=6,240) in control cells ($P<0.004$). To determine how this cell cycle hindrance occurs, we further analyzed changes in cell cycle by flow cytometry using Fluorescence-Activated Cell Sorting (FACS). The control cells showed a typical distribution among different phases in the cell cycle, whereas the cells ectopically expressing Myc-N-Cep57 were almost exclusively in G1 phase (data not shown): G1 arrest.

The N-terminus of Cep57 contains a multimerization domain

Dimerization of Cep57 was suggested by [7]. A portion of the N-terminal-half of Cep57 is homologous to a central region of Epidermal growth factor receptor substrate 15 protein and Epidermal growth factor receptor substrate 15 related protein. The central region of these proteins contain a coiled-coil that is involved in homo- or hetero-dimerization [14, 15]. To test the hypothesis that the N-terminal-half is a dimerization domain in Cep57, co-immunoprecipitation and co-localization assays were performed. Myc-N-Cep57 was co-expressed in HEK293 cells either with FLAG-N-Cep57 or FLAG-C-Cep57 and the whole cell lysate of each treatment was subjected to anti-FLAG antibody-conjugated agarose. As a result, Myc-N-Cep57 was co-immunoprecipitated with FLAG-N-Cep57 but not with FLAG-C-Cep57 (Figure 3A, lanes 1 and 2). Co-localization of ectopically co-expressed various Cep57 truncations also suggested homo-multimerization of Cep57 *via* its N-terminal-half. As discussed later, high-level expression of Full-Cep57 induces a massive fibrous 'basket-like' structure around the nucleus, and this feature is retained in C-Cep57, which is shown by EGFP-fused protein (Figure 4A). As expected, DsRed-C-Cep57 also induced the 'basket-like' structure (Figure 3B). EGFP-N-Cep57 co-expressed with DsRed-Full-Cep57 co-localized to the 'basket-like' structure but the one co-expressed with DsRed-C-Cep57 did not. The results of immunoprecipitation and indirect fluorescence microscopy together suggest homo-multimerization of Cep57 through its N-terminal-half. The crucial amino acid residues involved in multimerization were narrowed by co-immunoprecipitation of further truncated N-terminal-segments. When co-expressed with FLAG-N-Cep57, both Myc-Cep57_{58–259} and Myc-Cep57_{1–239} were co-immunoprecipitated by anti-FLAG antibody-conjugated agarose (Figure 3A lanes 3 and 4). Therefore, the N-terminal amino acid residues between 58–239, the coiled-coil and α -helical segment, namely CLD of Cep57, are also crucial in multimerization of Cep57.

The C-terminus of Cep57 binds, nucleates and bundles MTs

As mentioned above, high-level expression of EGFP-fused Cep57 in mammalian cultured cell lines, such as NIH 3T3 cells, generated massive fibrous 'basket-like' structure around the nucleus. Immunostain for MTs revealed that the 'basket-like' structure was composed not only of EGFP-fused Cep57 but also of MTs and electron micrographs (EM) revealed convoluted thick-cable structures with deposition of dense material on the periphery of longitudinally or transversely sectioned MTs (Figure 5). Formation of the 'basket-like' structure is also in agreement with a similar observation reported by [7]. This feature, the formation of the 'basket-like,' was retained in the C-terminal-half of Cep57. Therefore, to study how C-Cep57 can induce the massive MT network, *in vitro* tubulin polymerization assays were carried out in the presence and absence of the recombinant protein.

A typical time course of tubulin (0.5 mg/ml) polymerization induced by 10 μ M Taxol, monitored by light absorption at 350 nm, is shown in Figure 4B. Critical concentration (minimum [tubulin] capable of initiating tubulin polymerization) was estimated as $<1 \mu$ M and MT formation was confirmed by EM (Figure 4A). Two distinct phases were observed during Cep57 induced MT formation (Figure 4B). Addition of GST-C-Cep57 to tubulin initiated a prompt increase in OD₃₅₀ implying nucleation of MT formation although it takes approximately six times longer to reach the theoretical MT-origin maximum OD₃₅₀ (the first phase) as compared with Taxol induced polymerization. The theoretical MT-origin maximum OD₃₅₀ is the optical density achieved when all tubulin molecules in the sample participate in MT structure. Surprisingly, a second phase occurred with a much higher rate surpassing the MT-origin maximum OD₃₅₀. The increase eventually leveled off at substantially higher OD₃₅₀ than the MT-origin maximum OD₃₅₀; i.e. 1.25 vs 0.4 at 0.25 mg/ml of tubulin.

At high [tubulin]/[Cep57] only the first phase was observed (data not shown). Subsequent addition of GST-C-Cep57 triggered the second phase, which far exceeded the MT-origin maximum OD₃₅₀. This is also observed when GST-C-Cep57 was added to MTs pre-assembled with Taxol. The source of light absorbance in the second phase was explored by EM. In the sample with GST-C-Cep57, we observed side-to-side aggregation of MTs that was not observed among MTs promoted by Taxol (Figure 4A). Among 50 GST-C-Cep57 induced MTs, 48 MTs appeared to participate in side-to-side aggregation, whereas, among 50 taxol induced MTs, no formation of side-to-side aggregation was observed. Taken together, we concluded that GST-C-Cep57 weakly nucleates MT formation and also introduces strong side-to-side aggregation of MTs. These findings were consistent with the side-to-side aggregation of MTs observed by EM in NIH 3T3 cells where Cep57 was over-expressed (Figure 5B). The MTs formed in the presence of GST-C-Cep57 were stable; tolerating prolonged storage at room temperature. Although formation of the 'basket-like' structure is physiologically irrelevant, localization to and stabilization of MTs by over-expression of Cep57 may reflect an important functional feature of Cep57.

Overexpression of C-terminus of Cep57 reorganizes microtubules into nocodazole resistant 'basket-like' microtubule structures

To further explore the function of C-Cep57 *in vivo*, EGFP-C-Cep57 was ectopically expressed in NIH 3T3 cells. As a result, the same phenotypic 'basket-like' structure as in the cells over-expressing EGFP-Full-Cep57 at high-level was observed (Figure 5A panel g). Therefore, we concluded that the C-terminal-half is involved in association with MTs *in vivo*. The MT association domain of Cep57 was further narrowed by fusing different tags; i.e. Cep57₂₇₈₋₄₉₁ localized to MTs when it is fused to JRed (Figure 1).

Following NIH 3T3 cells over-expressing EGFP-Full-Cep57 for an extended period of time, we found that the cells eventually died but the 'basket-like' structures remained intact. Furthermore, the 'basket-like' structure consisting of EGFP-Full-Cep57 or EGFP-C-Cep57 and MTs remained intact after the exposure to 5 μ M nocodazole for 30 min, whereas the MTs not co-localized with EGFP-Full-Cep57 or EGFP-C-Cep57 were disrupted by this MT depolymerizing drug (Figure 5A). EGFP alone does not lead to MT stabilization because MTs were completely disrupted when cells over-expressing EGFP alone were subjected to nocodazole (data not shown). Therefore, we concluded that exogenous Cep57 over-expressed in NIH 3T3 cells associates with MTs through its C-terminal-half and promotes a nocodazole resistant MT network. Inhibition of MT formation in culture media supplemented with nocodazole prior to plasmid transfection for ectopic expression of EGFP-Full-Cep57 resulted in no 'basket-like' structures but random aggregation in the cytoplasm (Figure 5C). The different outcomes with pre-and post- treatment of nocodazole in the presence of the 'basket-like' structure implies that over-expression of Cep57 hinders MT dynamics through inhibition of depolymerization.

Discussion

This study focuses on the domains of a recently described centrosomal protein, Cep57 and provides insights into their distinct roles and functions. The N-terminal coiled-coil domain localizes to the centrosome, internal to γ -tubulin demonstrating that it is associated with both centrioles or centromatrix component. Cep57 also directly interacts with MTs through its C-terminal-half, which based on the lack of similarity to known MT binding domains, represents a novel MT binding domain. The ability of this domain to both nucleate and bundle MTs *in vitro* and *in vivo* suggests a role for organizing centrosomal MTs. Thus, the two domains of Cep57 have different functional roles, one to target the protein to the centrosome and the other perhaps for anchoring and organizing MTs at the centrosomes of mammalian cells.

Through the generation of highly specific antibodies to Cep57 as well as expression of hrGFP-Full-Cep57 we have confirmed that Cep57 is indeed a centrosomal protein. Its localization to the core of the centrosome plus its ability to induce side-to-side aggregation of MTs suggest its importance in the structural organization of centrosomal MTs. We did not see kinetochore staining of Cep57 in either *Xenopus*, human or mouse cells, although it is clearly visible on purified chromosomes from *Xenopus* cells [13]. New sequence data has been deposited in the *Xenopus* databases that identified a protein more closely related to Cep57. We propose to call the protein characterized in *Xenopus* the Cep57-related protein

(Cep57R). A Cep57R family member was also found in mouse and human cells. This recent finding suggests a more complex picture of Cep57 or the Cep57 family members and likely accounts for the differences in localization and phenotypes observed in mammalian and *Xenopus* cells. For example, we are currently testing whether *Xenopus* Cep57R reported by Emanuele and Stukenberg [13] is another member of Cep57 protein family that functions at both the kinetochore and the centrosome.

Homology searches were unable to detect similarity to known MT binding domains, thus the C-terminal-half of Cep57 represents a novel MT binding domain. The CLD of Cep57 may also contain a novel centrosomal targeting motif. The CLD has no recognizable similarity to previously known centrosomal targeting signals; i.e. the PACT (pericentrin/AKAP450 centrosomal targeting) domain and cyclin E centrosomal localization signal motif [16, 17]. On the other hand, a homology search using CLD implied its homology to a segment of either Centrosomin (CNN; *Drosophila*) or Spindle-defective protein 5 (SPD-5; *C. elegans*): both centrosomal proteins. Conserved amino acid sequences among Cep57, CNN and SPD-5 are segmental and therefore, it is unlikely that these proteins are homologues as a whole protein. Nonetheless, the fact that the CLD amino acid sequence is conserved among different species implies a common centrosome localization motif and more importantly, functional importance. CNN and SPD-5 both are proposed to recruit γ -tubulin to the centrosomes and to an alternative 'centrosome-like' structure and thus participate in nucleation of tubulin polymerization [18, 19].

Ectopic expression of the CLD resulted in reduction in the mitotic index and G1 arrest. In addition, we found that the CLD of Cep57 also plays a role in multimerization of Cep57 and therefore, the reduction in the mitotic index and the G1 arrest could reflect disrupted multimerization of endogenous Cep57 and perturbed proper structural organization of the centrosomes. Reduction in the mitotic index and G1 arrest induced by ectopic expression of the CLD of Cep57 are reminiscent of the cell cycle hindrance commonly observed with a molecular disturbance of other centrosomal proteins, such as Centriolin and AKAP450 [20, 21] and in physical ablation of the centrosomes [22, 23]. Therefore, reduction in the mitotic index and G1 arrest due to molecular disturbance of Cep57 strongly implicate participation of Cep57 in the complex of centrosomal proteins needed for the normal function of the centrosomes in the cell cycle.

Transfection of RNAi to reduce Cep57 resulted in reduced pole-to-pole distance of the mitotic spindles in cells in which no Cep57 fluorescence was detectable by immunolabeling. This suggests that Cep57 plays a role in mitotic spindle formation in tissue culture cells as has been implicated in *Xenopus* early embryonic cycles [13]. These phenotypes are less dramatic phenotypes than seen after depletion or addition of antibodies in *Xenopus* extracts. This may be a result of poor knockdown or the presence of redundant activities in somatic cells that are not present in embryos.

Little is known about how spindle control size is determined, so it is interesting that spindles assembled in the absence of Cep57 are smaller. Perhaps centrosomes depolymerize MTs at a higher rate in the absence of Cep57. Alternatively, there may be misregulation of astral MTs that anchor the centrosomes and MTOC to the distal ends of the cells and thereby

longitudinally 'stretch' the mitotic spindles in mitosis. Our study clearly shows stringent centrosomal localization of Cep57 and its ability to bind and stabilize MTs. We also obtained data suggesting involvement of Cep57 in anchoring to the centrosomes [13]. Taken together, we prefer a model that the decrease in length of mitotic spindles in Cep57 knockdown cells may reflect astral MTs having a reduced anchoring ability resulting in loss of 'stretch' in the mitotic spindles.

Thus, our analyses have uncovered a number of important activities in Cep57. For example, our observation provides insight into the mechanism of its possible function as a MT anchor at the centrosome. This role as a MT anchor also agrees with the observation in *Xenopus* egg extracts where MTs dislodged from the centrosome after depletion or addition of loss-of-function antibodies [13]. Cep57 directly interacts with MTs. The ability to induce side-to-side aggregation of MTs and the localization of Cep57 to the core of the centrosome implies a role in organizing centrosomal MTs. Finally, structurally Cep57 has separate domains to target to centrosomes and bundle MTs, suggesting a mechanism for anchoring MTs. While the N-terminal domain resides in the core of the centrosomes on the centrioles or centromatrix, the C-terminal domain directly anchors MTs to the centrosomes. Both domains on Cep57 contain numerous Aurora kinase consensus sites so it will be interesting to determine how these activities are regulated. In view of the importance of Taxol in the treatment of cancer, an understanding at the structural level of Cep57 and its targets could provide insights for future drug design.

Abbreviations used

Cep57	centrosomal protein 57
CLD	centrosome localization domain (of Cep57)
CMV	cytomegalovirus
CNN	centrosomin
DsRed	<i>Discosoma corallimorpharian</i> red fluorescent protein
GFP	enhanced green fluorescent protein
GST	glutathione-S-transferases
hUbC	human ubiquitin C
MT	microtubule
OD	optical density
RNAi	RNA-mediated interference
SPD-5	spindle-defective protein 5

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Figure 1. Structural prediction of Cep57, its domain map based on empirical data and characterization of anti- Cep57 antibodies

(A) A schematic diagram of computationally predicted structure of Cep57 and its empirically defined functional domains. Mouse and Human Cep57 include 500 amino acid residues and the residue numbers in this diagram are based on Mouse Cep57. The N-terminal-half and C-terminal-half are separated at the double prolines (residue 267 and 268) and thus each domain was independently characterized. The α -helices in the cartoon represent consensus of different protein structure prediction algorithms. N-terminal-half:

Representative truncates used to define the centrosomal localization and multimerization domain. Not all truncates were used interchangeably in different experiments; therefore, some items are marked as N/A where the exact truncate was not used for the specific assay. C-terminal-half: Representative truncates used to define the MT localization and stabilization domain. **(B)** Polyclonal antibody reacting with a protein (approx. 60 kDa) in NIH 3T3 whole cell lysate (top panel; left lane) at the same size as over-expressed FLAG-Full-Cep57 in HEK293 cells (top panel; middle lane). The over-expressed FLAG-Full-Cep57 is shown to react with both the polyclonal anti-Cep57 and anti-FLAG antibodies (top and bottom panels; middle lane). The polyclonal antibody does not react with HEK293 whole cell lysate (top panel; right lane). **(B')** Polyclonal antibody reacting with a protein (approx. 60 kDa) in NIH 3T3 whole cell lysate (left panel) and the reaction being blocked by addition of recombinant GST-C-Cep57 protein (right panel). **(C)** Endogenous Cep57 expression profile among different tissues in mouse and rat in Western blot analysis using polyclonal anti-Cep57 antibody. **(D)** Representative confocal combined Z-stack images of mitotic Hela cells with and without a treatment for RNAi targeting Cep57. The cells were immunostained for Cep57 and β -tubulin. The signals for Cep57 in the untreated cell (arrows) are absent in the treated cell. The pole-to-pole distance of mitotic spindles (lines) is indicated.

Figure 2. Centrosome localization of endogenous Cep57 and exogenous Full- and N-Cep57
(A) Confocal images of NIH 3T3 cells immunostained by the polyclonal and monoclonal anti-Cep57 antibodies; top and bottom rows respectively. The centrosomes are located by γ -tubulin except the low magnification image of the cells immunostained by the monoclonal anti-Cep57 antibody, which is co-stained with anti- β -tubulin antibody and thus, MT network is shown. Insets are the mitotic cells corresponding to each treatment. The centrosomes in high magnification shown at the bottom row are the ones in different cells shown in low magnification. **(B)** Confocal images showing centrosome localization of hrGFP-Full-Cep57,

hrGFP-N-Cep57 and hrGFP-Cep57₅₈₋₂₃₉ expressed at low-level in NIH 3T3 cells; top, middle and bottom rows respectively. The centrosomes are located by γ -tubulin stain (middle column). Insets are the mitotic cells corresponding to each sample except the one for hrGFP-Cep57₅₈₋₂₃₉ showing the centrosomes at high magnification; the centrosomes shown in the insets are in different cells from the ones shown at low magnification.

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Figure 3. Co-immunoprecipitation and co-localization of ectopically expressed Cep57 through its proposed multimerization domain

(A) FLAG-C-Cep57 and Myc-N-Cep57 (lane 1), and FLAG-N-Cep57 and Myc-N-Cep57 (lane 2) or Myc-Cep57₅₈₋₂₆₉ (lane 3) or Myc-Cep57₁₋₂₃₉ (lane 4) co-expressed in HEK293 cells, immunoprecipitated by anti-FLAG antibody and blotted by anti-FLAG and anti-Myc antibodies, showing co-immunoprecipitation of Myc-N-Cep57 (lane 2), Myc-Cep57₅₈₋₂₆₉ (lane 3) and Myc-Cep57₁₋₂₃₉ (lane 4) with FLAG-N-Cep57 but not with FLAG-C-Cep57 (lane 1). (B) EGFP-N-Cep57 is co-expressed either with DsRed-C-Cep57 (a and b) or DsRed-Full-Cep57 (c and d) in NIH 3T3 cells, showing co-localization of EGFP-N-Cep57 with DsRed-Full-Cep57 but not with DsRed-C-Cep57.

Figure 4. GST-C-Cep57 induces MT polymerization and MT bundles *in vitro*

(A) Electron micrographs of each samples at maximal OD₃₅₀: taxol induced MTs (a) MTs with recombinant C-Cep57 protein; MT bundles are indicated by arrows (b), and the mixture of tubulin and GST treated in the equivalent condition as the sample with recombinant C-Cep57 protein (c). (B) Transition of OD₃₅₀, reflecting quantity of MTs present in each sample: taxol induced MTs, Cep57 induced MTs and tubulin+10% glycerol.

Figure 5. Ectopic expression of Full-Cep57 and C-Cep57 induces a stable MT 'basket-like' structure and N-Cep57 does not

(A) High-level expression of EGFP-Full-Cep57, EGFP-C-Cep57 and EGFP-N-Cep57 and appearance of MTs in NIH 3T3 cells. Confocal images of EGFP-Full-Cep57 (a and d), EGFP-C-Cep57 (g and j) and EGFP-N-Cep57 (m and p) vigorously expressed in NIH 3T3 cells with corresponding β -tubulin immunostains (b, e, h, k, n and q) and merged images (c, f, i, l, o and r). Cells treated with nocodazole (d-f, j-l and p-r). The cells expressing either EGFP-Full-Cep57 or EGFP-C-Cep57 showing the 'basket-like' phenotype (a, d, g and j) and its co-localization with MTs (c, f, i and l), which is absent in cells expressing EGFP-N-

Cep57 (m, o, p and r). Stabilized nocodazole resistant MTs (e and k) as a result of over-expression of either EGFP-Full-Cep57 or EGFP-C-Cep57. Increased signal of EGFP is observed at the centrosomes along with cytosolic diffusion when EGFP-N-Cep57 was over-expressed at high-level (m and o; arrows). **(B)** Electron micrographs from ultra-thin sections (80 nm) of NIH 3T3 cells over-expressing EGFP-Full-Cep57, showing the convoluted cable-like MT structure; low magnification image (a) and high magnification image of the boxed area in a (b). **(C)** A confocal image of the basket-like structure in cultured cell overexpressing EGFP-Full-Cep57 (a) and nocodazole pre-treated cell over-expressing EGFP-Full-Cep57 (b).