STED nanoscopy of the centrosome linker reveals a CEP68-organized, periodic rootletin network anchored to a C-Nap1 ring at centrioles

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The centrosome linker proteins C-Nap1, rootletin, and CEP68 connect the two centrosomes of a cell during interphase into one microtubule-organizing center. This coupling is important for cell migration, cilia formation, and timing of mitotic spindle formation. Very little is known about the structure of the centrosome linker. Here, we used stimulated emission depletion (STED) microscopy to show that each C-Nap1 ring at the proximal end of the two centrioles organizes a rootletin ring and, in addition, multiple rootletin/CEP68 fibers. Rootletin/CEP68 fibers originating from the two centrosomes form a web-like, interdigitating network, explaining the flexible nature of the centrosome linker. The rootletin/CEP68 filaments are repetitive and highly ordered. Staggered rootletin molecules (N-to-N and C-to-C) within the filaments are ∼75 nm apart. Rootletin binds CEP68 via its C-terminal spectrin repeat-containing region in 75-nm intervals. The N-to-C distance of two rootletin molecules is ∼35 to 40 nm, leading to an estimated minimal rootletin length of ∼110 nm. CEP68 is important in forming rootletin filaments that branch off centrioles and to modulate the thickness of rootletin fibers. Thus, the centrosome linker consists of a vast network of repeating rootletin units with C-Nap1 as ring organizer and CEP68 as filament modulator.

The centrosome is the main microtubule-organizing center of animal cells. It consists of centrioles of nine triplet microtubules that are surrounded by the pericentriolar material (PCM) (1). Centrioles provide the duplication capability of centrosomes and have a scaffold function for the PCM. The PCM carries the microtubule-organization activity of centrosomes (2, 3). Human interphase cells contain two centrosomes that are joined together by the centrosome linker. The centrosome linker is an oligomeric proteinaceous structure that connects the two centrosomes of a cell together into one microtubule-organizing unit (4).

The centrosome linker is composed of the proteins C-Nap1 (encoded by CEP250), rootletin (CROCC), and CEP68 (5–7). Rootletin is a long, filamentous protein that has self-assembly properties (8, 9). It is also a component of the cilia rootlets that dock the basal body of cilia to the nuclear surface by binding to the nuclear envelope protein Nesprin1 (8, 10). Additional proteins, namely LRRC45 and centlein, have been implicated in linker formation; however, their role is less clear (11, 12). The centrosomal linker has to be resolved before or at the beginning of mitosis to allow formation of the mitotic spindle (13). The timing of linker disassembly in the cell cycle determines the requirement of the mitotic motor protein Eg5 in spindle formation and has an impact on the efficiency of mitotic chromosome segregation (14, 15). In addition, in cancer cells, the elevated cyclin B2 levels hyperactivate polo-like kinase (i.e., PLK1), leading to the accelerated dissolution of the centrosome linker and the appearance of lagging chromosomes (16). Furthermore, a defective centrosome linker in interphase cells impairs Golgi organization and cell migration and influences the cellular position of cilia (17, 18). Interestingly, a truncating mutation in CEP250 (C-Nap1) affects centriole cohesion and is associated with Seckel-like syndrome in cattle, implicating a role of the centrosome linker during development (19).

Most studies to date have focused on identifying linker components, yet our understanding of the molecular architecture of the centrosome linker and the function of linker components remains rudimentary. In a simple model, rootletin has been described to connect the two centrosomes of an interphase cell by forming a linear filament between the C-Nap1 anchor at the proximal end of each mother centriole (20). Considering the importance of the centrosome linker for mitosis, cancer development, and cilia organization, it is crucial to understand its architecture and the role of linker proteins in its organization.

Significance

The integrity of the centrosome linker that joints both centrosomes of a cell into one microtubule-organizing unit plays important roles in cell organization, chromosome segregation, and cancer development. However, little is known about the structure of the linker. Here, we show by stimulated emission depletion microscopy that the centrosome linker consists of a vast network of repeating rootletin units with a C-Nap1 ring at centrioles as organizer and CEP68 as filament modulator. Rootletin filaments originating from the two centrosomes form a web-like, interdigitating filamentous network, explaining the flexible nature of the centrosome linker and the ability of the kinesin motor Eg5 to disrupt the linker function by force.

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Here, we have analyzed the centrosome linker proteins C-Nap1, rootletin, and CEP68 by stimulated emission depletion (STED) microscopy (21–23), and direct 3D stochastic optical reconstruction microscopy (STORM) (24–26). Rootletin/CEP68 filaments form an extended, web-like network that spreads up to 1 to 2 μm outward from the C-Nap1 ring at the proximal end of both centrioles. Rootletin filaments coming from opposite centrioles are weaved into each other, which probably is the basis of centrosome linkage. STED-based statistical analysis showed that rootletin forms regular filaments, with a repeat organization of 75 nm (N-to-N or C-to-C). The N-to-C-distance of two rootletin molecules was measured to be ∼35 to 40 nm, which leads to an estimated minimal rootletin length of ∼110 nm. CEP68 binds to rootletin filaments every 75 nm via its C-terminal end that contains a conserved spectrin repeat. CEP68 affects the thickness of rootletin filaments and promotes filament formation from the rootletin ring that encircles C-Nap1 at centrioles. Based on these data, we suggest a model for the centrosome linker formation.

Results

The Centrosome Linker Is a Flexible Entity. Nontransformed human telomerase-immortalized retinal pigmented epithelial (RPE)-1 cells have a robust centrosome linker and are, therefore, ideally suited for the analysis of this structure by microscopy (17). Live-cell imaging analysis of RPE-1 FRT/T-Rex mNeonGreen-CEP68-P2A-mRuby2-PACT cells revealed that the two centrosomes in most cells were kept close together (<2 μm) during interphase (Movies S1–S3). However, in about 5% of the cells, both centrosomes moved several micrometers (>2 μm) apart. In many cases, this centrosome distance was >5 μm, exceeding the length of the centrosome linker (Movies S4–S6). Eventually, the centrosomes joined together and reestablished a functional centrosome linker, as indicated by the closeness of the two centrosomes over at least 20 min (Movies S4–S6). These data indicate that some cells lose centrosome linker function in a reversible manner, suggesting that the centrosome linker is a flexible structure.

CEP68 and Rootletin Form an Extended, Colocalizing Filamentous Network with a Repeat Organization of 75 nm. To understand the architecture of the centrosome linker, we localized the proteins rootletin and CEP68 in the centrosome linker by STED microscopy (5, 6). Analysis of rootletin with regional antibodies directed against the C terminus of the protein (named root-C1) and of CEP68 with a polyclonal antibody (SI Appendix, Fig. S1 A–D) detected both proteins as web-like filamentous networks that originated at each of the two centrioles marked by polyglutamylated tubulin, γ-tubulin, or C-Nap1 (Figs. L4 and 24). Each centriole was associated with several rootletin and CEP68 filaments that radiated outward from the centriole into the cytoplasm.

STED images showed striated patterns for root-C1 and CEP68 fibers, indicating a highly ordered organization of both proteins in the filaments (Figs. 1A and B and 2A and B). Statistical analysis revealed a repeat organization of root-C1 and CEP68 of 75 nm along the filaments (Figs. 1C and 2C and SI Appendix, Fig. S2). The periodicity was independent of the filament length (Figs. 1A and B). The length of the rootletin/CEP68 filaments varied strongly. Most filaments were a few hundred nanometers long, but lengths just over 2 μm were also found (SI Appendix, Fig. S2 E and F). Filaments were often thicker close to centrioles and tapered off toward the tip (Figs. 1D and 2D). In some cases, these tapered filaments appeared to be one fiber; in other cases, multiple fibers came together and aligned their periodicity (red arrows, Bottom).

We used nonsynchronized RPE-1 cells for analysis of the centrosome linker. About >80% of these RPE-1 cells are in G1 and S phases. To exclude that rootletin images were influenced by the dissolution of the linker in G2/M (4), we analyzed the root-C1 pattern in cells that were stained for the cell cycle counts.
Cep68 binds to rootletin filaments in 75-nm intervals. (± | μ STED; www.pnas.org/cgi/doi/10.1073/pnas.1716840115)

3 nm repeat organization [three samples, plus one data point Vlijm et al. analyzed by 3D-STORM. (All and three other cells (red dotted lines Right Bottom B and 2 γ To understand whether the highly organized cen-

Fig. 2. Cep68 binds to rootletin filaments in 75-nm intervals. (A) STED images of CEP68 together with C-Nap1 (Top) or γ-tubulin (Bottom). (B) Single CEP68 filaments from the cell in A and three other cells (red dotted lines 75 nm apart are a guide to the eye). Although the majority of the CEP68 spots are separated by 75 nm, a few locations show slightly different distances (red arrows). (C) Histogram of fitted periodicity. CEP68 shows a regular 75 ± 3 nm repeat organization [three samples, plus one data point from a fourth sample; 38 cells; 142 line profiles; total length, 37.54 μm (∼984 periods); maximum single fiber length, 1.34 μm]. (D) CEP68 filaments show thick to thin filaments (white arrow, Top), and even separate filaments synchronize their phase (white arrows, Bottom). (E) Rootletin (red) and CEP68 (green) colocalize along the same fiber [Left, STED; Right, intensity line profile and the filament (no. 1 or 2) it is taken from]. (F) Color-coded 3D CEP68 network of the cell shown in Movie S9 analyzed by 3D-STORM. (All scale bars, 500 nm.) The STED images are Wiener deconvolved; the raw data are shown in SI Appendix, Fig. S11.

marker CENP-F. CENP-F does not stain the nucleus in G1- and S-phase cells; however, it decorates the nuclear envelope and associates with kinetochores starting in G2 (27). Analysis of interphase cells lacking a nuclear CENP-F signal resulted in the same rootletin pattern as what was observed for CENP-F-positi-

The very similar localization pattern of rootletin and CEP68 along centrosome linker filaments suggested that both proteins colocalize on the fibers. Indeed, the root-C1 and CEP68 signals localized dot-like along the same fibers in STED microscopy (Fig. 2E). Line profiling of the signal intensities showed near colocalization of the peak intensities in most cases (Fig. 2F, Right). Thus, CEP68 and the C terminus of rootletin localize close together on the filaments.

Lack of antibody accessibility to some epitopes could cause the striated C-rootletin staining pattern. To exclude this possibility, we measured localization of the N terminus of rootletin with a regional antibody root-N. Also, the root-N antibody resulted in a 75-nm periodic signal along the rootletin fiber (Fig. 3 A–C). We next analyzed localization of N- and C-terminal regions of root-

Rootletin Filaments Have a Similar Periodicity in Human Primary and Cancer Cells. To understand whether the highly organized centrosome linker of RPE-1 cells is a common feature in other cell types, we imaged rootletin and CEP68 localization by STED microscopy in RPE-1, primary human umbilical vein endothelial cells (HUVECs), and HCT116 colon cancer cells in relationship to the centrosomal marker γ-tubulin. The 75-nm repeat unit organization of rootletin and CEP68 was observed in all cell types (SI Appendix, Fig. S5). However, in HCT116 cells, the rootletin/CEP68 fibers were, in general, shorter and denser around the centrosomes and therefore more difficult to be re-

C-Nap1 is the anchor of the centrosome linker at the proximal end of centrioles (5). It
C-Nap1 forms a ring at centrioles and functions as rootletin fila-

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A

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E

Answer has been suggested that rootletin and CEP68 localization with
centrioles depends on C-Nap1 (6, 7). We confirmed these data by using a CEP250 (C-Nap1) knockout (KO) cell line and siRNA
depletion of C-Nap1 (SI Appendix, Fig. S6A and B) (17). Consistent with published data (6), siRNA depletion of C-Nap1 can result in
centrioles carrying only one or very few elongated rootletin fibers
(SI Appendix, Fig. S6F), probably because of a minor residual pool
of C-Nap1 at centrosomes despite efficient depletion (SI Appendix,
Fig. S1E and F). Interestingly, ~10 to 20% of CEP250 (C-Nap1) KO cells contained rootletin filaments in the cytoplasm, with no connection to the γ-tubulin–marked centrosome (SI Appendix, Fig.
S6C and D). A similar frequency of free-floating rootletin filaments
was observed in RPE-1 wild-type cells (SI Appendix, Fig. S6D).

C-Nap1 forms a ring at centrioles and functions as rootletin fila-
ments organizer. (A) Three examples of dual-color STED analysis of C-Nap1
[monoclonal anti-C-Nap1 mouse antibody (34)] labeled with kk114L and of
CEP68 (polyclonal anti-rabbit antibody) labeled with star600 (Top) and
star580 (Middle and Bottom). (Top) For optimal imaging of the C-Nap1 ring,
C-Nap1 was imaged first, followed by CEP68, in each scanned line. (Middle
and Bottom) The imaging order in Top was reversed to increase the reso-
lution of the CEP68. The C-Nap1 ring is illustrated in the Middle and Bottom
Insets. (B) Dual-color STED analysis of CEP68 and rootletin shows ringlike
colocalization of both proteins. (Scale bars, 500 nm.) The STED images are
Wiener deconvolved; the raw data are shown in SI Appendix, Fig. S11.

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Fig. 4.

(C-Nap1) knockout (KO) cell line and siRNA
depletion of C-Nap1 (SI Appendix, Fig. S1C) removed most of the rootletin
signal from centrioles in comparison with the nonspecific control
(NSC) (SI Appendix, Fig. S7A and B) (7, 12). Analysis of the
diameter of 265 ± 35 nm (n = 55, from 34 individual cells) that was
surrounded by a wider CEP68 ring (Fig. 4A; note that in Top, C-
Nap1 was imaged first; in Middle and Bottom, CEP68 was imaged first).

Dual-color STED microscopy showed CEP68 and rootletin rings with attached filaments (Fig. 4B). Each filament originated from
a thicker anchoring point at the rootletin/CEP68 ring. The outcome
of this analysis suggests that C-Nap1 at centrioles functions as organ-
izer of rootletin and CEP68 rings. These rings are then the anchoring
point of rootletin/CEP68 filaments that radiate into the cytoplasm.

The Rootletin Ring on Top of C-Nap1 Is Less Sensitive to CEP68
Depletion than Filaments. To find further support for rootletin/
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signal from centrioles in comparison with the nonspecific control
(NSC) (SI Appendix, Fig. S7A and B) (7, 12). Analysis of the
CEP68-depleted cells by STED microscopy revealed rings of rootletin and residual CEP68 at pericentriolar (PCNT)-marked centrosomes (Fig. S5A, Bottom; see SI Appendix, Fig. S8A for more examples; and see SI Appendix, Fig. S11 for “raw” images). CEP68 depletion resulted in a lower number of rootletin filaments per centrosome without strongly affecting the rootletin ring surrounding the centriole (compare NSC with siCEP68 in Fig. S5A and SI Appendix, Fig. S8A). In about half of the cells, one or two rootletin filaments were visible that were anchored to the centriolar ring by intense, colocalizing CEP68 and rootletin dots (Fig. S5A, Bottom). Thus, rings and filaments of rootletin have different CEP68 requirements.

We modulated the rootletin/CEP68 system by overexpression of the CROCC gene coding for rootletin with or without depletion of CEP68 or C-Nap1. Mild overexpression of CROCC (rootletin) due to the leakiness of the TetON promoter [SI Appendix, Fig. S11, absence of doxycycline (−Dox)] enhanced the rootletin/CEP68 filament network at centrosomes (Fig. S5B, NSC; Fig. S6C, Top). In contrast to the NSC cells that showed thick rootletin filaments in the low image (Fig. S6C, Top), the only detectable rootletin signal of CEP68 cells in the low image was associated with PCNT at centrosomes. However, the high image of siCEP68 cells detected a network of rootletin fibers at the nucleus (Fig. S6C).

The nuclear association possibly reflects the affinity of the nuclear envelope protein Nesprin1 for rootletin (10). STED analysis resolved these rootletin fibers of siCEP68 cells as thin rootletin bundles in TetON-CROCC (rootletin) cells (Fig. 6 C and D). This was particularly obvious in the low brightness and contrast (“low”) image of siCEP68-depleted cells. In contrast to the NSC cells that showed thick rootletin filaments in the low image (Fig. 6C, Top), the only detectable rootletin signal of siCEP68 cells in the low image was associated with PCNT at centrosomes. The high image of siCEP68 cells detected a network of rootletin fibers at the nucleus (Fig. 6C).

The nuclear association possibly reflects the affinity of the nuclear envelope protein Nesprin1 for rootletin (10). STED analysis resolved these rootletin fibers of siCEP68 cells as thin rootletin filaments that lacked a CEP68 signal (SI Appendix, Fig. S9B, Bottom). These data suggest that CEP68 plays a role in bundling thin rootletin filaments into thicker filaments. Consistent with this notion is the finding that co-overexpression of both CEP68 and CROCC (rootletin) promoted the assembly of an extended, dense rootletin/CEP68 network that hardly allowed resolving individual rootletin filaments (SI Appendix, Fig. S8D).

**CEP68 Bundles Rootletin Filaments.** Mild overexpression of HA-tagged CROCC (rootletin-HA) (SI Appendix, Fig. S11, −Dox, long exposure) extended the rootletin/CEP68 network at centrosomes (for an example, see SI Appendix, Fig. S9A). However, it also increased the number of cells with cytoplasmic rootletin filaments that were not associated with centrosomes from ~10 to 30% to nearly 100% [compare SI Appendix, Fig. S8D, i.e., endogenous, with Fig. S6A, i.e., mild CROCC (rootletin) overexpression]. Mild CROCC (rootletin)-overexpressing cells contained thin and thick, noncentrosome-associated rootletin filaments (Fig. 6A). Thick rootletin filaments (Fig. 6A, arrowheads) were associated with CEP68, while thin filaments, which became visible in the high brightness and contrast (“high”) image, were mostly devoid of CEP68 (Fig. 6A, asterisks). STED analysis confirmed that thicker rootletin filaments as seen by confocal microscopy were decorated by CEP68 (Fig. 6B, Bottom). To thin rootletin fibers, CEP68 was either not, or only sparsely, associated (Fig. 6B, Right and SI Appendix, Fig. S9A, Bottom). This suggests that thin rootletin filaments can assemble without the aid of CEP68.

Importantly, siRNA depletion of CEP68 reduced the number of thick rootletin bundles in TetON-CROCC (rootletin) cells (Fig. 6 C and D). This was particularly obvious in the low brightness and contrast (“low”) image of siCEP68-depleted cells. In contrast to the NSC cells that showed thick rootletin filaments in the low image (Fig. 6C, Top), the only detectable rootletin signal of siCEP68 cells in the low image was associated with PCNT at centrosomes. However, the high image of siCEP68 cells detected a network of rootletin fibers at the nucleus (Fig. 6C).

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Fig. S1 J and K). The C-terminal fragment containing the spectrin repeat bound to centrioles, as was the case for full-length CEP68 (Fig. 7B). In contrast, the N-terminal CEP68 fragment was diffusely distributed in cells (SI Appendix, Fig. S10A). Because CEP68 binding to centrosomes requires rootletin (7, 12), these data suggest that the C terminus of CEP68 with the spectrin repeat interacts with centrosomes via rootletin.

To identify rootletin fragments that interact with CEP68, we used a published assay based on the overexpression of rootletin subfragments that form cytoplasmic filaments or larger protein assemblies (SI Appendix, Fig. S1G–I) (7, 9). We asked whether fragments of rootletin recruit endogenous CEP68 as an indication for interaction. Interestingly, the overexpressed rootletin R3 subfragment (amino acids 1,079 to 1,825) recruited endogenous CEP68 (Fig. 7C, enlargement on right and Fig. 7D). Fragment R2 was expressed in RPE-1 cells (SI Appendix, Fig. S1G); however, it did not show formation of dense assemblies (SI Appendix, Fig. S10B). R1 and R4 formed cytoplasmic assemblies or filaments that did not recruit CEP68 (Fig. 7C and D). The larger R123 and R234 rootletin fragments containing R3 recruited CEP68 (Fig. 7C and D). CEP68 recruitment by R3 suggests interaction of CEP68 with this region of rootletin.

Discussion

The centrosome linker has essential functions in connecting both centrosomes of an interphase cell into one microtubule-organizing unit. Failure of this organization has profound consequences for cell organization, cell migration, cilia formation, and chromosome segregation in mitosis (16–18). Despite these important functions, relatively little is known about the structure of the centrosome linker. Rootletin, a linker protein, is also a component of the cilia rootlets. Rootletin fibers in cilia rootlets
have a repeat organization of 60 to 70 nm as seen by electron microscopy (8, 9). Puzzlingly, however, ordered filaments have not been observed in between the two centrosomes by electron microscopy (6), raising the possibility that rootletin in the centrosome linker has a fundamentally different organization than in cilia rootlets.

To solve this riddle, we turned to STED microscopy and 3D-STORM. Superresolution microscopy has paved the way before centrosome biology by showing that the interphase PCM is an ordered array of proteins that surround centrioles (3, 28, 29). A striking feature of the rootletin filaments as resolved by STED microscopy is the 75-nm periodic organization of the N terminus and C terminus of rootletin. This repeat organization suggests that rootletin also assembles in an ordered manner in the centrosome linker and is most easily explained by an N- and C-terminal overlap of two elongated rootletin molecules (Fig. 8A). The N-to-C distance of two rootletin molecules along the filament is in the range of ~35 to 40 nm as determined by dual-color STED microscopy with N- and C-specific rootletin antibodies (root-N and root-C2, respectively) (Fig. 3 and SI Appendix, Fig. S4). Repeating this interaction will result in a protofilament with defined spacing of rootletin N and C termini. Based on our measurements, rootletin has a minimal length of ~110 nm (Fig. 3). However, it may be an even more extended molecule (up to 160 nm), depending on the precise position of the antibody binding sites (SI Appendix, Fig. S4D).

CEP68 follows the C-rootletin order because it binds to the R3 region of rootletin. R3 neighbors the C terminus of rootletin (Figs. 7C and 8A). This closeness is consistent with the near colocalization of the CEP68 and root-C2 signals along fibers as shown by STED microscopy. CEP68 binding to rootletin increases the thickness of rootletin fibers. Presently, it is unclear how CEP68 interconnects thinner rootletin filaments.

Interestingly, the C-terminal fragment of CEP68 that interacts with rootletin contains a spectrin repeat. Also, Nesprin1 that interacts with rootletin in rootlets harbors spectrin repeats (10). Spectrin repeats are platforms for cytoskeletal protein assemblies (30). We propose that certain spectrin repeats have the ability to interact with rootletin filaments. Additional experiments are needed to test this model further.

C-Nap1 forms a ringlike assembly at the proximal end of both centrioles (Fig. 8B). C-Nap1 organizes two types of rootletin/CEP68
structures: first, a rootletin ring that surrounds the centriole; and second, filaments as described earlier that originate from the rootletin ring (Fig. SB). The precise structure of the rootletin ring surrounding centrioles is still unclear. It could be composed of short rootletin filaments, or rootletin molecules may have a radially oriented geometry around centrioles similar to what has been observed for PCNT, of which the C terminus is close to centrioles and the N terminus more distant (3). CEP68 depletion mainly affected the outward-radiating rootletin filaments but also affected, to a lower degree, the rootletin pool close to C-Nap1, indicating different CEP68 requirements. Rootletin directly binds to C-Nap1 (20), explaining why the structural integrity of the C-Nap1 pool of rootletin was less dependent on CEP68. Rootletin/CEP68 dots on the centriole ring likely function as nucleation seeds or anchoring points for the assembly of filaments. However, similar to tubulin and G-actin that use nucleators in cells but can also scaffold into polymers when the concentration is high enough (31, 32), rootletin assembles into cytoplasmic filaments without the aid of C-Nap1 and CEP68 when overexpressed (this study and refs. 8 and 9).

How do rootletin/CEP68 filaments connect the two interphase centrosomes of a cell? The STED microscopy and 3D-STORM data suggest that rootletin/CEP68 filaments coming from different centrosomes are intertwined into a web-like structure (Fig. SB). Moreover, live-cell imaging analysis has shown that the centrosome linker is a flexible structure that can lose and regain linker function without the disassembly of centrosome linker filaments (Movies S4–S6). In addition, centrosome linkage can be overcome by microtubule motor forces provided by the kinesin-5 motor Eg5 (33). These data together are most consistent with a model of flexible interweaved rootletin/CEP68 filaments that form regional contacts. However, presently, we cannot exclude the possibility that a single centrosome-to-centrosome fiber can also provide linkage function. The extended web-like centrosome linker potentially has the properties of forming a landing platform for signaling molecules. In addition, it may function as contact sites for cytoskeletal elements with yet unappreciated functions.

Materials and Methods
For all STEM data, except Fig. 3 A and B; Fig. 4 A, Middle and Bottom; Fig. 5B, ciCEP68; SI Appendix, Figs. S3 and S4 A–C; and the corresponding SI Appendix, Fig. 511 (here we used an Abbeirer Instruments 775 STEM), we used a home-built STEM microscope similar to the one published in ref. 23 with a 594- and 650-nm excitation laser combined with a 775-nm STEM laser (SI Appendix, Fig. S12). The STEM images were, unless specified differently, corrected for background (image) background subtraction with a rolling ball radius of 30 to 50 pixels), followed by resampling to half the pixel size (10 nm) and Wiener deconvolution with point-spread functions of 27 to 40 nm and 190 to 250 nm. Due to the large spread in intensities, to show all features, linear background subtraction and intensity scaling was applied. All raw data are shown in SI Appendix, Fig. S11, and an overview of the measured cell is given in SI Appendix, Table S1. Details are in SI Appendix, Details of Materials and Methods, including experimental procedures and reagents.

No animal or clinical experiments were performed. HUVECs were obtained from A. Fischer, German Cancer Research Center, Heidelberg, with the approval of the Medical Ethics Committee of the Faculty Mannheim, Heidelberg University (S-175/2015).

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