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 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 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.
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. 1 A and 2). 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. 1 A and B and 2 A and B). Statistical analysis revealed a repeat organization of root-C1 and CEP68 of 75 nm along the filaments (Figs. 1 C and 2 C and SI Appendix, Fig. S2). The periodicity was independent of the filament length (Figs. 1 D and 2 D and 2 E); however, it was disrupted in some regions. 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.

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.
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.

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 resolved than in RPE-1 and HUVECs. Thus, the centrosome linker is a well-organized repeat structure, a feature that is common to human primary cells, telomerase-immortalized RPE-1 cells, and cancer cells.

C-Nap1 Forms a Ring at the Proximal End of Centrioles That Organizes CEP68 and Rootletin into Rings and Filaments. C-Nap1 is the anchor of the centrosome linker at the proximal end of centrioles (5). It
has been suggested that rootletin and CEP68 localization with centrioles depends on C-Nap1 (6, 7). We confirmed these data by deconvolved images are shown in Fig. S1 1. (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. S1 E 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. S6 C and D). A similar frequency of free-floating rootletin filaments was observed in RPE-1 wild-type cells (SI Appendix, Fig. S6D). siRNA depletion of C-Nap1 did not significantly affect the number of free rootletin filaments of RPE-1 cells (SI Appendix, Fig. S6d). These data indicate that C-Nap1 is not essential for rootletin filament assembly. However, in wild-type cells, C-Nap1 organizes the centriole-associated rootletin network.

To understand the function of C-Nap1 as organizer of rootletin/CEP68 filaments, we determined the structure of C-Nap1 with a regional antibody (SI Appendix, Fig. S1E) directed against the C terminus relative to CEP68 by dual-color STED microscopy. The C-Nap1 signal was resolved as a ringlike structure with an average 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 organizer 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/CEP68 rings that are organized by the C-Nap1 ring and to understand the function of the individual proteins in this organization, we tested how modulation of CEP68, rootletin, and C-Nap1 affected rootletin/CEP68 rings and filaments. Depletion of CEP68 (SI Appendix, Fig. S1C) removed most of the rootletin signal from centrioles in comparison with the nonspecific control (NSC) (SI Appendix, Fig. S7 A and B) (7, 12). Analysis of the
Dox. siRNA depletion of CEP68 in CROCC exposes a strong centriol e rootletin/CEP68 ring. C-Nap1 is essential for shown as comparison. (trosomes in response to siRNA CEP68 depletion. The NSC siRNA control is a single filament of rootletin and residual CEP68 at PCNT-marked cen-

SI Appendix (rootletin) due to the leakiness of the TetON promoter [Fig. S8B, Bottom, Top]. These data suggest that CEP68 plays a role in bundling thin rootletin filaments into thicker filaments. Consis-
tent 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).

The C-Terminal Globular Domain of CEP68 Containing the Spectrin Repeat Interacts with Centrosomes. The regular interaction pattern of CEP68 with rootletin fibers indicates a defined binding site in CEP68 that interacts with a specific region in rootletin. Recently, Nesprin1 was described as a rootletin binding partner (10). We modulated the rootletin/CEP68 system by 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. 6D, 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. 6C 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 was associated with PCNT at centrosomes. However, 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. Consis-
tent 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-depleted cells by STED microscopy revealed rings of rootletin and residual CEP68 at pericentriolar (PCNT)-marked centrosomes (Fig. 5A, 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. 5A 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. 5A, 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 de-

CELIS ET AL.
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Vlijm et al.
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 in 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. See text for details.

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. 5B). 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 radial orientation 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 self-assemble 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 interwoven into a web-like structure (Fig. 5B). 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 interwoven 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 STED data, except Fig. 3 A and B; Fig. 4A, Middle and Bottom; Fig. 5B, csiCEP68; SI Appendix, Figs. S3 and S4 A–C; and the corresponding SI Appendix, Fig. S11 (here we used an Abberior Instruments 775 STED), we used a home-built STED microscope similar to the one published in ref. 23 with a 594- and 650-nm excitation laser combined with a 775-nm STED laser (SI Appendix, Fig. S12). The STED 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 Ethical Commission of the Medical Faculty Mannheim, Heidelberg University (S-175/2015).

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