Cep97 Is Required for Centriole Structural Integrity and Cilia Formation in Drosophila

Graphical Abstract

Highlights
- Cep97 stably binds to fully formed centrioles
- Cep97 is retained on mature basal bodies and is required for ciliogenesis
- Cep97 promotes centriole stability
- Cep97 regulates centriolar microtubule acetylation potentially via Sirt2

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In Brief
Centrioles are highly stable structures. Here, Dobbelaere et al. use the fruit fly Drosophila as an experimental model to show that the distal end protein Cep97 is required for centriole stability and function in ciliogenesis. Cep97 function is mediated at least in part by regulating centriolar microtubule acetylation via an interaction with the deacetylase Sirt2.
Cep97 Is Required for Centriole Structural Integrity and Cilia Formation in Drosophila

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https://doi.org/10.1016/j.cub.2020.05.078

SUMMARY

Centrioles are highly elaborate microtubule-based structures responsible for the formation of centrosomes and cilia. Despite considerable variation across species and tissues within any given tissue, their size is essentially constant [1, 2]. While the diameter of the centriole cylinder is set by the dimensions of the inner scaffolding structure of the cartwheel [3], how centriole length is set so precisely and stably maintained over many cell divisions is not well understood. Cep97 and CP110 are conserved proteins that localize to the distal end of centrioles and have been reported to limit centriole elongation in vertebrates [4, 5]. Here, we examine Cep97 function in Drosophila melanogaster. We show that Cep97 is essential for formation of full-length centrioles in multiple tissues of the fly. We further identify the microtubule deacetylase Sirt2 as a Cep97 interactor. Deletion of Sirt2 likewise affects centriole size. Interestingly, so does deletion of the acet- ty lase Atat1, indicating that loss of stabilizing acetyl marks impairs centriole integrity. Cep97 and CP110 were originally identified as inhibitors of cilia formation in vertebrate cultured cells [6], and loss of CP110 is a widely used marker of basal body maturation. In contrast, in Drosophila, Cep97 appears to be only transiently removed from basal bodies and loss of Cep97 strongly impairs ciliogenesis. Collectively, our results support a model whereby Cep97 functions as part of a protective cap that acts together with the microtubule acetylation machinery to maintain centriole stability, essential for proper function in cillum biogenesis.

RESULTS AND DISCUSSION

Centrioles generally assemble adjacent to pre-existing parental centrioles in a series of steps that have been extensively studied in a range of experimental models. First, PLK4/Sak/ZYG-1 is recruited to the mother centriole, where it concentrates into a single focus that marks the site of daughter centriole assembly. PLK4 then recruits and phosphorylates STIL/Ana2/SAS-5 as well as SAS-6 to form the hub-and-spoke structure of the cartwheel. Finally, CPAP/SAS-4 along with γ-tubulin directs assembly of the microtubule-based centriole wall [7]. Overexpression of CPAP as well as its interacting proteins CEP120 and SPICE1 results in over-elongation of centrioles [4, 5, 8–10], and recent studies demonstrate that CPAP imparts slow, processive growth on centriolar microtubules [11, 12]. The distal (plus) ends of centriolar microtubules are bound by a complex of Cep97 and CP110 [6, 13, 14]. In vertebrates, this complex is thought to counteract CPAP activity, as loss of either component results in over-elongated centrioles, while overexpression of CP110 suppresses the effect of excess CPAP [4, 5]. Cep97 and CP110 have been shown to interact with the depolymerizing kinesin KIF24, which specifically acts on centriolar, but not cytoplasmic microtubules to limit centriole size [15]. An interaction between CP110 and another depolymerizing kinesin, Klp10A, was reported in Drosophila, and loss or depletion of Klp10A results in a dramatic extension of centriole size, an effect suppressed by overexpression of CP110 [16, 17]. Surprisingly, however, loss of CP110 in the fly has only minor effects on centriole elongation and, indeed, results in shortening of centrioles in Dmel cells, hinting at context-dependent differences [16, 17]. Further, while Cep97 and CP110 are thought to function together, it has been noted that hypomorphic Cep97 mutants display stronger phenotypes than CP110-null mutants, suggesting a role for Cep97 independent of CP110 [18]. We therefore sought to carry out a comprehensive analysis of Cep97 in the fly, using newly developed tools to monitor protein localization and function.

Cep97 stably caps mature centrioles

Both in vertebrates and Drosophila, Cep97 has so far been exclusively studied by immunofluorescence microscopy in fixed cells or fluorescent fusions expressed under a heterologous promoter. To examine the in vivo dynamics of Cep97 without the potential complications of overexpression, we generated a GFP fusion under the control of endogenous regulatory sequences. This fusion was found to be fully functional in rescuing the phenotypes of Cep97 deletion and expressed at levels similar to the endogenous protein (see below). GFP fluorescence was consequently used to monitor Cep97 localization and dynamics for all experiments in this study. Cep97 was found to localize to...
Figure 1. Cep97 stably localizes to mature centrioles and basal bodies in Drosophila
(A) Schematics of tissues used to determine Cep97 localization and function. Immunofluorescence micrographs show Cep97 colocalizing with centriolar and centrosomal markers (Asl, Sas4, and γ-tubulin) in all tissues examined, including at the basal bodies of chordotonal neurons (bb, basal body; dc, daughter

(legend continued on next page)
centrioles throughout the animal (Figure 1A). A detailed examination of the giant centrioles in spermatocytes by stimulated emission depletion (STED) microscopy found Cep97 concentrated in the distal lumen (Figure S1A), consistent with Drosophila CP110 [17, 18] but considerably narrower than the distribution reported for both proteins in human RPE cells [14]. The reasons for this discrepancy are not immediately clear. However, we could confirm these findings by expansion microscopy on retinal pigment epithelial (RPE) cells in our own hands (Figures S1B and S1E).

We next examined the dynamics of Cep97 in syncytial embryos. New centrioles assemble in each S phase and separate at the end of the subsequent mitosis. While mother and daughter centrioles remain in close proximity and cannot be resolved by confocal microscopy, previous work has shown that the recruitment and turnover of centriolar proteins can be assessed by fluorescence recovery after photobleaching (FRAP) [20]. When applied to Ana2, a component dynamically associated with the cartwheel [13], this assay shows rapid recovery, reaching pre-bleach signal intensities within ~100 s. In contrast, Sas4, a component stably incorporated into the centriolar microtubule wall [21], shows more gradual recovery and reaching only 50% of pre-bleach signal (Figure 1B), reflecting recruitment to newly forming centrioles, Sas4-GFP on the original parental centriole remaining photobleached, and accounting for the missing 50%. Cep97 recovers even later, when Sas4 levels have plateaued and daughter centrioles have reached their mature length [13] (Figure 1B). The failure to reach the original pre-bleach signal intensity further indicates that centriolar Cep97, like Sas4 and Sas6 [13, 21, 22], does not exhibit cytoplasmic exchange and forms a stable cap at the distal end of fully grown centrioles. It should be noted that these results conflict with [13], which found Drosophila Cep97 dynamically associated with the growing end of centrioles, which we believe reflects an excess of Cep97 due to GFP overexpression [23].

Cep97 is not universally stripped from the ciliary base
Removal of CP110 from the maturing basal body is a hallmark event in vertebrate cilogenesis, occurring after distal appendage-mediated ciliary vesicle docking and requiring TTBK2 [24, 25]. This removal appears to trigger transition zone assembly and extension of the ciliary axoneme [26]. Although most work has focused on CP110, Cep97 appears to follow the same pattern [5] (Figures S1C and S1D). We first examined Cep97 localization in spermatogenesis (Figure 1A). Spermatogenesis in Drosophila Cep97 can be divided into a mitotic stage, in which male germ cells divide asymmetrically to generate gonialblasts, which in turn divide four more times to generate a cyst of 16 primary spermatocytes; a meiotic stage, in which primary spermatocytes divide to produce 64 haploid spermatids; and finally a differentiation stage, in which spermatids mature into spermatozoa [27]. Centrioles elongate considerably during spermatogenesis, doubling in size between the early spermatocyte stage and meiosis I. They also form a short primary cilium, which is partially resorbed and internalized at the onset of meiosis [28]. Upon meiotic exit, further remodeling results in formation of the sperm flagellum. This process involves separation of the transition zone from the basal body [29]. Additionally, a procentriole-like structure forms adjacent to the maturing basal body [30]. In contrast to CP110, removed at the onset of centriole elongation in early spermatocytes [17], Cep97 persists throughout early spermatogenesis up to and including meiosis (Figure 1A).

Removal of Cep97 is therefore clearly not required for initial basal body elongation or formation of the primary cilium in primary spermatocytes. Cep97 is, however, transiently lost as basal bodies are remodeled to give rise to the sperm flagellum before reappearing on the mature basal body. While this loss coincides with migration of the transition zone, we found no colocalization with Cep290, a reported CP110 interactor in vertebrates [31] during migration of the transition zone (Figure S1F). Late in spermatocyte maturation, Cep97 was, however, observed along the ciliary axoneme ahead of the traveling actin cones that mediate sperm individualization (Figure S1G). Recruitment to the elongating axoneme by end-binding proteins, including the Cep97 interactor CEP104, has been proposed as a means for removal of Cep97/CP110 from basal bodies [32], but this has hitherto not been observed in unperturbed cells. Finally, as in the case of sperm, Cep97 was localized to the mature basal body of mechanosensory primary cilia in chordotonal neurons (Figure 1A). Collectively, these results indicate that Cep97 need not be removed for centriole elongation or (primary) cilogenesis to occur. Although this clearly goes against the dogma in the field, it is not entirely unprecedented since CP110 has been reported to be present at the mature basal bodies of Xenopus multiciliated cells [33]. It is also potentially significant given the requirement of Cep97 for proper cilogenesis (see below).

Cep97 mutant flies are viable but uncoordinated
Previous work on Drosophila Cep97 employed a transposable element insertion, which was shown to behave as a hypomorph [18]. To obtain a true null mutant, we therefore used the ends-out method [34] to replace the entire coding sequence of Cep97 with the White marker gene (Figures S2A–S2D and 2A). As shown to be the case for CP110 mutants [17], Cep97-null flies were viable and fertile, with no obvious morphological defects (Figure S2E). However, mutants displayed behavioral defects characteristic of impaired mechanosensory function. Thus, touch sensation in larvae [35] and negative geotaxis in adult flies [36] was found...
to be strongly reduced (Figures 2B and 2C). Mutants also displayed reduced male fertility (Figure 2I). Defects were rescued by introduction of Cep97-GFP, confirming specificity of the mutant phenotype and functionality of our GFP transgene.

**Cep97 mutants are cilia defective**

Mechanosensory neurons in the fly are ciliated, raising the possibility that behavioral defects stem from defects in ciliary architecture. We therefore examined the sensory organs responsible for proprioception in *Drosophila*, the chordotonal organs. Chordotonal organs are made of multiple scolopidia, each of which contains a pair of ciliated nerve endings ensheathed by a scolopale glia cell and attached with their ciliary tips to the cuticle via a cap cell [37]. Antibody staining for Sas4 and NompC, which localizes to the distal end of mechanosensory cilia [38], in Cep97 mutants showed that basal bodies are present but cilia are frequently missing (Figure 2D). To analyze these defects in more detail, we examined ciliary ultrastructure in Cep97 mutant flies (Figures 2E–2H). Cross-sectional views of wild-type flies always showed a pair of cilia, positioned side by side with their tips embedded in the cap cell. In contrast, one or both cilia were frequently missing in Cep97 mutants (Figures 2F and 2H). In cases where both cilia were still present, they frequently appeared to be laterally displaced with features such as the ciliary dilation found in different sections, suggesting defects in basal body positioning. Longitudinal sections support this idea with what appear to be maloriented basal bodies found in certain sections (Figures 2G and 2F). Close-up views revealed no marked defects of the axoneme, transition zone, or rootlets (Figure 2F). However, basal bodies frequently appeared abnormal, with mother and daughter centrioles not always clearly distinct and instances of rootlets apparently emanating directly from the mother centriole instead of enveloping the daughter as usually the case in *Drosophila* [39]. Other centriole pairs did not appear to be associated with ciliary structures (Figures 2G and 2F). Collectively, these results suggest Cep97 is required for proper formation of cilia, with basal body function impaired in Cep97 mutants.

Since male fertility was also affected, we wondered whether these defects extend to the sperm flagellum. Examining the testes of Cep97 mutant flies by thin-section transmission electron microscopy, we found early spermatid cysts to contain 64 spermatids (12/12 cysts), indicating proper completion of meiosis (Figure 2J, data not shown). However, even at early stages, abnormal spermatids could be observed in which axonemes were incomplete or missing. These defects became more pronounced in mature spermatids, consistent with impaired axoneme elongation and/or stability (Figures 2J and 2K). Thus, Cep97 is also required for ciliogenesis in sperm. However, a sizeable number of structurally normal axonemes was still observed and live microscopy showed the presence of motile sperm in the seminal vesicle (Figure S3F; Video S1), consistent with the residual fertility of Cep97 mutant males.

While both Cep97 and CP110 were originally described as inhibitors of ciliogenesis, it has recently been reported that CP110 also has a positive role in promoting ciliogenesis, with impaired docking of basal bodies following CP110 depletion or mutation in vertebrates [33, 40]. Our results are consistent with this idea and suggest Cep97 is likewise required for proper basal body function.

**Cep97 is required for assembly of proper length centrioles**

Defective basal body function likely stems from defects in the underlying centriole template. Centriole numbers were found to be unchanged in mutant embryos, suggesting centriole duplication is not appreciably affected (not shown). Pericentriolar material recruitment and mitotic progression were also unaffected. Centrioles likewise appeared superficially normal in the early stages of spermatogenesis. However, as centrioles elongate in primary spermatocytes, Cep97 mutant centrioles grew significantly longer than in wild-type controls (Figures 3A and 3B). This marked size difference was maintained for the remainder of spermatogenesis and still evident for mature basal bodies (not shown). Interestingly, centrioles in wing discs, a non-ciliated somatic tissue, behaved very differently. Here, Cep97 mutant centrioles were markedly shorter, a phenotype rescued by expression of Cep97-GFP (Figures 3C–3E and S2G). Interestingly, centrioles displayed prominent extensions of individual microtubules emanating from one end (Figures 3C and 3F), as previously reported for CP110 [17]. However, CP110 mutants otherwise displayed much less severe phenotypes, with centriole length...

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**Figure 2. Cep97 deletion mutants display ciliary defects**

(A) Immunoblotting confirms loss of Cep97 in Cep97Δ animals and expression of Cep97-GFP transgene to levels similar to controls.
(B and C) Behavioral assays in larvae (touch assay) (B) and adult flies (climbing/bang assay) (C) show loss of coordination in Cep97 and CP110 mutants, rescued for Cep97 by expression of Cep97-GFP.
(D) Schematic and immunofluorescence micrographs of scolopidia in chordotonal organ of the fly. Sas4 and NompC were used to visualize basal body and ciliary tip, respectively. Scolopidium outline is based on differential interference contrast (DIC). Each scolopidium contains two cilia with their ends embedded in the cap cell. Cep97 mutants display severe disorganization of ciliary morphology.
(E–G) Cross (F) and longitudinal sections (G) through control and Cep97 mutant scolopidia analyzed by transmission electron microscopy (TEM). Positions are indicated by numbers in schematic in (E). Cross sections reveal missing cilium at all levels, though remaining cilium appears morphologically normal. Longitudinal sections reveal mispositioned basal bodies (arrow) as well as rootlets emanating directly from docked basal body rather than enveloping daughter centriole as in controls (arrowhead). See also Figure S2F.
(H) Quantitation of ciliary defects revealed by ultrastructural analysis in Cep97 mutants and GFP rescue animals as in (F). n = 90 cilia control, 118 Cep97Δ, and 95 Cep97Δ Rescue.
(I) Cep97 and CP110 mutants display reduced male fertility, assessed as number of offspring per single male.
(J and K) TEM analysis of sperm axonemes in control and Cep97 mutant testes. Cross-sectional views (J) reveal missing axonemes (each cyst normally contains 64 sperm developing synchronously) as well as fragmented axonemes in Cep97 mutants. Defects are quantitated in (K). Numbers are % of axonemes defective/misleading per cyst. n = 16 cysts control and 11 Cep97Δ.

Scale bars are 5 μm (J), 1 μm (D), 500 nm (G, panel 7), 200 nm (F, panels 1–6), and 100 nm (G, panel 8, and J, insets). Error bars are SD (B, C, I, and K). t-test; *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S2.
only slightly different from wild type in both spermatocytes and wing discs [17] (Figures 3B and 3D). Further, Cep97;CP110 double mutants displayed a phenotype essentially indistinguishable from Cep97 single mutants. Cep97 is generally thought of as purely a recruitment factor for CP110 [6]. Cep97 indeed recruits CP110 to centrioles also in the fly (Figure 3G). However, our results clearly indicate that Cep97 has functions in centriole length control beyond CP110 recruitment. Further, both Cep97 and CP110 are generally thought to limit centriole elongation by opposing the activity of proteins, such as CPAP [4, 5]. This has also been reported for CP110 in Drosophila [17]. Yet the consequences of Cep97 loss are clearly more differentiated, with both abnormal elongation (spermatocytes) and shrinkage (wing discs) occurring depending on cellular context, with a high degree of variability observed in both cases. As previously proposed [16], we therefore see the role of Cep97 and CP110 not as limiting centriole elongation but rather acting as a protective cap limiting further centriole growth or potentially shrinkage once the proper length has been reached.

Cep97 interacts with the microtubule acetylation machinery to stabilize centrioles

How might Cep97 and CP110 function in capping centrioles? Given the stable association of Cep97 with the distal end of centrioles, a direct effect on centriolar microtubules cannot be excluded. However, an examination of the recruitment dynamics of Sas4, a component of the centriolar wall, revealed no obvious difference compared with controls (Figure 3H). Furthermore, Sas4 continues to be stably incorporated, with no detectable exchange on mature centrioles. This is in contrast to depletions of γ-tubulin or tubulin itself, which result in Sas4 remaining partially or fully dynamic [2]. Therefore, the early stages of centriole assembly up to and including centriolar microtubule wall assembly appear to be unaffected by loss of Cep97.

What then might explain the stabilizing influence of Cep97? A hint came from an effort to identify interactors of the deacetylation enzyme SirT2 by using a combined tandem affinity purification and proximity biotinylation approach [41]. SirT2 is known to shuttle between nucleus and cytoplasm, where it acts alongside Hdac6 to deacetylate cytoplasmic microtubules [42, 43]. Cep97 was found as one of the top interactors of SirT2 in cytoplasmic fractions of human cell extracts [41]. Reciprocal pull-downs of Cep97 from human HEK293T cells reliably recovered SirT2, confirming this interaction (Figure 4A). Acetylation of lysine 40 on α-tubulin by Atat1 is associated with stable microtubules, including those at centrioles and cilia, although the functional significance of this modification remains poorly understood [44, 45]. RNAi-mediated depletion of Atat1 has been reported to delay ciliogenesis in serum-starved vertebrate cells [46], while Hdac6 and SirT2 are thought to function in cilium resorption upon resumption of the cell cycle [47, 48]. To study the role of Atat1 (Tat in Drosophila), SirT2, and Hdac6 in the fly, we obtained putative loss-of-function mutants in their respective homologs. As previously observed for Cep97, we found all three mutants to be viable, with no obvious morphological defects (Figures S3A and S3B). However, all also displayed uncoordinated movement, as well as reduced male fertility and sperm motility (Figures S3C–S3F). Ultrastructural analysis of mutant sperm revealed similar axonemal defects as for Cep97, albeit at lower frequencies (Figures 4B, 4C, and S4C). Double mutants of Cep97 with SirT2 and Hdac6 did not result in any synergistic phenotypes, either at the whole animal behavior or cilium morphology level (Figures 4B, 4C, S3C–S3F, and S4C). However, co-deletion of Cep97 and Atat1 was synthetetic lethal (Figure S3B). Double mutants of Cep97 with a weaker transposon insertion mutant of Atat1 (Atat1<sup>−/−</sup>; see Figures S4A and S4B) were viable but displayed severe uncoordination and fully penetrant male infertility with entirely immotile sperm (Figures S3C–S3F). Consistent with this, examination of Cep97;Atat1 double-mutant sperm revealed almost complete disruption of axonemal architecture (Figures 4B, 4C, and S4C). Thus, Cep97 and components of the microtubule acetylation machinery share similar ciliary phenotypes in the fly, although the synergism between Cep97 and Atat1 also suggest independent functions of each protein.

The phenotypic similarities with Cep97 prompted us to examine centrioles and basal bodies in acetylation-defective animals. In wing discs, SirT2 mutants displayed severely truncated centrioles to an extent similar to Cep97. Interestingly, so did mutants of Hdac6 and Atat1. Centriole lengths were also more variable, again as with Cep97 (Figures 4D and 4E). One notable difference was the absence of extensions of individual centriolar microtubules found with Cep97 and CP110 (Figure 4F). Combined loss of the acetylation machinery and Cep97 did not result
in further shortening of centrioles. However, the centriolar microtubule extensions found in Cep97 single mutants were largely absent in Cep97;Atat1 double mutants, suggesting they are destabilized (Figures 4D–4F). In spermatogenesis, Atat1, Sirt2, and Hdac6 mutant basal bodies did not display the over-elongation phenotype observed with Cep97, although lengths were more variable than in controls (Figures 4G and 4H). Sir2 mutants did, however, display apparent basal body extensions positive for Ana1 (Figures 4I and 4J).

Thus, the phenotypes of Cep97 and components of the acetylation/deacetylation machinery are remarkably similar, though not identical, suggesting a shared function in regulating centriolar microtubule stability. This prompted us to examine the acetylation of centriolar microtubules in the various conditions. Basal body signal in sperm was found to be dependent on Atat1, the major though not only microtubule acetylase in the fly (Figure 4G). Acetylated tubulin signal was similarly reduced in Cep97 mutants, as well as interestingly in mutants of Sir2 and Hdac6 (Figures 4G and S4D). Thus, the centriolar/ciliary phenotypes in Cep97 mutants might at least in part be explained by disruption of centriolar microtubule acetylation. It might at first seem paradoxical that loss of the acetyltransferase Atat1 yields largely hyp-acetylation [49] and -glutamylation [50], another normally stabilizing post-translational modification, being reported to destabilize microtubules, the implication being that a large imbalance in modification is as destabilizing as complete loss of modification.

A revised model of Cep97 function

In summary, our results indicate that Cep97 forms part of a capping structure that is recruited to fully elongated, mature centrioles. Removal of Cep97 need not occur to allow initiation of the ciliary axoneme. Rather, Cep97 is required for proper basal body function in ciliogenesis. Consistent with its late loading onto centrioles, loss of Cep97 does not affect procentriole assembly up to and including formation of the CPAP/Sas4-containing centriole wall. However, failure to assemble the Cep97 cap structure leaves centriolar microtubules exposed, which can result in abnormal extension or shrinkage, depending on cytoplasmic context. Instead of counteracting the activity of centriole elongation factors, such as CPAP, CEP120, or SPICE1, Cep97 therefore acts to limit microtubule dynamics once the proper length has been reached.

Our work provides novel mechanistic insight into how this might occur. A hallmark feature of centrioles is their remarkably constant size and stability, with no detectable turnover of tubulin subunits after their incorporation [51], enabling individual centrioles to be traced through many cell divisions [52] and indeed the lifetime of an animal [53]. The extensive post-translational modification of centriolar microtubules, including by acetylation, is likely key for this stability, as memorably demonstrated by the dissolution of centrioles after microinjection of antibodies against poly-glutamylated tubulin [54]. Here, we identify the microtubule deacetylase Sir2 as a Cep97 interactor. We further show that loss of Cep97 results in loss of centriolar microtubule acetylation. Remarkably, perturbation of components of the microtubule acetylation machinery (Sir2, Hdac6, and Atat1) largely phenocopies loss of Cep97. All three components have been localized to stable microtubules, including those at centrioles [43, 55, 56]. We could recapitulate this localization by overexpression of GFP transgenes in Drosophila S2 cells (Figure S4E). The simplest explanation then is that Cep97 regulates this machinery at centrioles to stabilize centriolar microtubules and confer on them their remarkable lack of dynamics. Consistent with this, bimolecular fluorescence complementation analysis shows an interaction between Cep97 and Sir2 at centrioles (Figure 4K). Finally, it bears remarking that the effects of Cep97 perturbation are much more severe than for CP110 [17]. Cep97 is also more widely conserved across eukaryotes than CP110, which is found exclusively in metazoans [57]. Cep97 is therefore clearly more than just a CP110 recruitment factor. The work presented...
here should serve as a foundation for studies examining Cep97 function also in other experimental models.

ACKNOWLEDGMENTS

We thank members of the Dammermann and Slade labs for discussions; Jordan Raft and Mustafa Aydogan for sharing data prior to publication; Balazs Czeglédi of the IMBA fly house and members of the Dickson and Knoblich labs for help with the preparation of samples for EM; the Bloomington Drosophila Resource Center (VDRC) for strains and reagents; and Josef Gotzmann and Thomas Peterbauer of the MFPL Bio-Optics facility for technical assistance. This work was supported by grants Y597-B20 from the Austrian Science Fund (FWF) to A.D. and 17-20613Y from the Czech Science Foundation to M.H. as well as a Lisa-Methner Fellowship of the FWF (M1293-B09) and a VIPS post-doctoral fellowship of the University of Vienna to J.D.

AUTHOR CONTRIBUTIONS

J.D. conception and design; acquisition, analysis, and interpretation of data; and drafting or revising the article. M.S.C. and M.H. contributed unpublished essential data or reagents (RPE cell expansion microscopy). D.S. contributed unpublished essential data or reagents (Sirt2/Cep97 interaction in human cells). A.D. conception and design, analysis and interpretation of data, and drafting or revising the article.
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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Alpaca GFP-Booster Atto 488-conjugated nanobody | ChromoTek | Cat#gba488-100; RRID: AB_2631386 |
| Alpaca GFP-Booster Atto 594-conjugated nanobody | ChromoTek | Cat#gba594-10; RRID: AB_2631387 |
| Anti-FLAG M2 magnetic beads | Sigma-Aldrich | Cat#M8823; RRID: AB_2637089 |
| Mouse monoclonal anti-acetylated Tubulin antibody | Sigma-Aldrich | Cat#T7451; RRID: AB_609894 |
| Mouse monoclonal anti-acetylated Tubulin antibody | [58] | N/A |
| Mouse monoclonal anti-alpha Tubulin antibody | Sigma-Aldrich | Cat#T9026; RRID: AB_477593 |
| Mouse monoclonal anti-FLAG HRP-conjugated antibody | Sigma-Aldrich | Cat#A8592; RRID: AB_439702 |
| Mouse monoclonal anti-gamma Tubulin antibody | Sigma-Aldrich | Cat#T6557; RRID: AB_477584 |
| Mouse monoclonal anti-HA antibody | Covance | Cat#MMS-101R-200; RRID: AB_291263 |
| Rabbit polyclonal anti-Ana1 antibody | [60] | N/A |
| Rabbit polyclonal anti-Asl antibody | [60] | N/A |
| Rabbit polyclonal anti-Cep97-C-ter antibody | This study | N/A |
| Rabbit polyclonal anti-Cep97-N-ter antibody | This study | N/A |
| Rabbit polyclonal anti-CP110 antibody | [17] | N/A |
| Rabbit polyclonal anti-Sas4 antibody | [61] | N/A |
| Rabbit polyclonal anti-hsCep97 antibody | Proteintech | Cat#22050-1-AP; RRID: AB_11182378 |
| Rabbit polyclonal anti-hsCP110 antibody | Proteintech | Cat#12780-1-AP; RRID: AB_10638480 |
| Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488-conjugated antibody | Thermo Fisher | Cat#A-21206; RRID: AB_2535792 |
| Goat anti-Mouse IgG (H+L) Alexa Fluor 488-conjugated antibody | Thermo Fisher | Cat#A-11001; RRID: AB_2534069 |
| Goat anti-Mouse IgG (H+L) Alexa Fluor 568-conjugated antibody | Thermo Fisher | Cat#A-11004; RRID: AB_2534072 |
| Goat anti-Mouse IgG (H+L) Alexa Fluor 647-conjugated antibody | Thermo Fisher | Cat#A-21236; RRID: AB_2535805 |
| Goat anti-Rabbit IgG (H+L) Alexa Fluor 555-conjugated antibody | Thermo Fisher | Cat#A-21428; RRID: AB_2535849 |
| Goat anti-Rabbit IgG (H+L) Alexa Fluor 568-conjugated antibody | Thermo Fisher | Cat#A-11011; RRID: AB_143157 |
| Goat anti-Rabbit IgG STAR RED-conjugated antibody | Abberior | Cat#2-0012-011-9; RRID: AB_2620152 |
| Goat anti-Rabbit IgG HRP-conjugated antibody | Cell Signaling Technology | Cat#7074; RRID: AB_2099233 |
| Horse anti-Mouse IgG HRP-conjugated antibody | Cell Signaling Technology | Cat#7076; RRID: AB_330924 |
| **Biological Samples** |        |            |
| BAC containing Cep97 in DH10B E. coli | BACPAC Resources | RP96-06H02 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Hoechst 33258 | Thermo Fisher | Cat#H1398; CAS: 23491-45-4 |
| Phalloidin Alexa Fluor 568 | Thermo Fisher | Cat#A12380 |
| 3X FLAG Peptide | Sigma-Aldrich | Cat#F4799 |
| TransIT Insect Transfection reagent | Mirus Bio | Cat#MIR 6104 |
| **Critical Commercial Assays** |        |            |
| RNasey Mini Kit | QIAGEN | Cat#74134 |
| QuantiTect Multiplex RT-PCR Kit | QIAGEN | Cat#205311 |

(Continued on next page)
| REAGENT or RESOURCE SOURCE | IDENTIFIER |
|----------------------------|------------|
| Human: hTERT RPE-1 cells   | ATCC       |
| Human: HEK293T cells       | ATCC       |
| D. melanogaster: S2 cells  | Life Technologies |
|                            | Cat#R69007; RRID: CVCL_Z232 |

**Experimental Models: Organisms/Strains**

| D. melanogaster: Wild-type/w[1118] | BDSC | 6326; FlyBase ID: FBst0006326 |
| D. melanogaster: Sas4-GFP/Sas-4UbiLP.GFP | [62] | FlyBase ID: FBal0240464 |
| D. melanogaster: Cep97Δ/Cep97Δ | This study | N/A |
| D. melanogaster: Cep97ΔRescue/Cep97Δ; Cep97GFP | This study | N/A |
| D. melanogaster: y[1] w[1118]; Df(3L)BSC113/TM6B, Tb [1] | BDSC | 8970; Flybase ID: FBst0008970 |
| D. melanogaster: Hdac6Δ/HDAC6KO | BDSC | 51182; Flybase ID: FBst0051182 |
| D. melanogaster: Sirt2Δ/Sirt2BB-2-35 | BDSC | 8839; Flybase ID: FBst0008839 |

**Oligonucleotides**

See Table S1

**Recombinant DNA**

| pU6-BbsI-chiRNA | Addgene Plasmid # 45946 |
|----------------|-------------------------|
| pU6-BbsI-chiRNA-Cep290 (target sequence: GAGACCGTATCCCTGCGCA) | This study | pJD78 |
| pBluescript II KS(+) | Stratagen | Cat#212207 |
| GFP-Cep290 Donor template in pBluescript | This study | pJD151 |
| pMAL-c2X | NEB | Cat#N8076S |
| pGEX-6P-1 | GE Healthcare | Cat#28954648 |
| pMAL-Cep97-NT | This study | pJD93 |
| pMAL-Cep97-CT | This study | pJD90 |
| PGEX-6P-1-Cep97-NT | This study | pAD632 |
| PGEX-6P-1-Cep97-CT | This study | pAD641 |
| pGX-attP | N/A | |
| pGX-attP-Cep97 | This study | pJD39 |
| pCM43 | Barry Dickson | N/A |
| pCM43-Cep97-GFP | This study | pJD72 |
| p3xFLAG-CMV-10 | Sigma-Aldrich | Cat#E4401 |
| p3xFLAG-CMV-10-hsCep97 | This study | N/A |
| pDONR221-hsSirt2 | Joanna Loizou | N/A |
| pDEST 2xHA-hsSirt2 | This study | N/A |
RESOURCES AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alexander Dammermann (alex.dammermann@univie.ac.at).

Materials Availability
Fly strains, plasmids, antibodies and cell lines generated in this study are available upon request from the Lead Contact.

Data and Code Availability
The published article includes all datasets generated or analyzed during this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila melanogaster stocks and husbandry
All strains used are listed in the Key Resource Table. w1118 flies were used as wild-type controls. Sas-4-GFP [62], CP110D, and CP110-GFP [17] strains have been described previously. Mutants in Atat1, Hdac6 and Sirt2 were obtained from the Bloomington Drosophila Stock Center (BDSC), except for Atat1KO [63], which was obtained from Jay Parrish, University of Washington. The Cep97 null mutant was created by ends-out or replacement gene targeting [34] using the pGX-attP vector [65]. Briefly, 5kb fragments upstream of the start codon and downstream of the stop codon of the Cep97 gene were cloned on either side of the white gene in the pGX-attP plasmid. This plasmid was then injected into early embryos and flies selected for red eyes. I-SceI endonuclease was then activated by crossing to SceI carrying flies and offspring with red eyes selected. Cep97 deletion was confirmed by Southern, PCR and sequencing. Endogenous promoter Cep97-GFP was created by recombineering. A GFP tag was inserted at the C terminus of Cep97 into BAC RP98-06H02 via bacterial Red/ET recombination (Gene Bridges). Cep97-GFP together with 3.5kb of upstream and 4.2kb of downstream regulatory sequences was transferred to the injection vector pCM43 (gift of Barry Dickson, Janelia Research Campus), again by homologous recombination, and inserted into the landing site attP2 as previously described [66]. All Cep97 localization studies were performed in a Cep97-deletion mutant background. GFP-Cep290 was constructed by CRISPR-mediated genome editing. A guide-RNA to the N terminus of Cep290 and rescue construct with 1kb flanking arms was injected into flies and positive clones identified by PCR and sequencing.

Cell culture
Drosophila S2 cells were cultured in Schneider’s medium containing 10% FCS, penicillin (50 units/ml), and streptomycin (50μg/ml). Cells were grown at 28°C at atmospheric CO2 and passed every 3-4 days. Full-length Sirt2, Hdac6 and Atat1 were amplified from Drosophila cDNA and cloned into the Gateway entry vector pZEO-Entry, then combined with a C-terminal GFP in the expression vector pwUbq [23], placing the gene under the control of a ubiquitin promoter. For BiFC, full-length Cep97, CP110 and Sirt2 were
combined with N-terminal (VN) or C-terminal (VC) fragments of Venus [67] by Gibson cloning in the inducible expression vector pMT (Invitrogen). Stable cell lines were generated by co-transfection with pCoBLAST (Invitrogen) using Trans-IT (Mirus Bio) transfection reagent and selection with blasticidin. For BiFC experiments, moderate expression of split YFP constructs was induced by the addition of 50 μM of CuSO4 for 24h before imaging.

Immortalized human retinal pigment epithelial cells stably expressing telomerase reverse transcriptase (hTERT-RPE1/RPE cells) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS, penicillin (100 units/ml), and streptomycin (0.1mg/ml). Cells were grown at 37°C in 5% CO2 in air and passaged every 2-3 days. For both immunofluorescence staining and expansion microscopy cells were seeded on glass coverslips. To induce ciliation cells were serum starved for 24h prior to fixation. HEK293T cells were cultured as above. Full length Cep97 was amplified from HEK293T cDNA and cloned into the vector p3xFLAG-CMV-10 (Sigma). SIRT2 was transferred from the Gateway donor vector pDONR221 [41] into the destination vector pDEST 2xHA (gift of Joanna Loizou). Transient transfections of HEK293T cells were performed with polyethyleneimine (Polysciences).

METHOD DETAILS

**Drosophila behavioral assays**

**Larval touch assay**

This assay was performed as previously described [35]. In brief, third-instar larvae moving forward were touched with the tip of an eyelash across one side of the thoracic segments and the response measured on a scale of 0–4 (0 no response, 1 hesitation, 2 turn, 3 reversal, 4 complete reversal). This was repeated 4 times for each animal and the total used to calculate a reactive score from 0-16. Experiment was repeated 3 times with > 10 larvae per condition.

**Climbing assay**

To examine fly coordination, 12 two-day-old adult males were collected in 16cm graduated, flat bottom tubes. Flies were allowed to recover from the anesthetic and left in the tube for 15min to acclimatize, then banged to the bottom of the tube and filmed climbing back upward. Videos were analyzed to establish the time at which 10 flies had crossed the half-way (8cm) mark. Banging was repeated four times and the average time recorded. Experiment was repeated 3 times with 12 males per condition.

**Male fertility assay**

To test the fertility of male flies, single males were crossed with four virgin control females in standard culture vials. Flies were mated for 1h. Males were then removed and females left in the vial for 24h. Numbers of offspring were counted prior to hatching. Experiment was repeated 6 times with > 10 males per condition.

**Antibodies for immunofluorescence and immunoblotting**

Rabbit polyclonal antibodies against the N terminus (amino acids 1-328) and C terminus (631-806) of *Drosophila* Cep97 were raised using an MBP fusion as an antigen and purified over a GST fusion as described [68]. The following primary antibodies were used for immunofluorescence in *Drosophila*: GFP-booster-Atto488 (ChromoTek) and mouse anti-acetylated tubulin (6-11B-1; Sigma-Aldrich) at 1:200, rabbit anti-Cep97-N-ter and anti-Cep97-C-ter, rabbit anti-Asl and rabbit anti-Ana1 [60], rabbit anti-Sas4 [61] and mouse anti-NompC [59], all at 1:500; mouse anti-γ-tubulin (GTU88; Sigma-Aldrich) and mouse anti-α-tubulin (DM1a; Sigma-Aldrich), both 1:1000. The following secondary antibodies were used: Alexa Fluor 488, 568 and 647 (Invitrogen), all at 1:1000. To stain actin Phalloidin (Alexa 568) was used at 1:500. DNA was labeled with Hoechst 33342 (Invitrogen). For STED microscopy we used GFP-booster-Atto594 at 1:200 and STAR RED-conjugated secondary antibodies at 1:500. For immunofluorescence in RPE cells the following antibodies were used: mouse anti-acetylated tubulin (C3B9 [58]; gift of Vladimir Varga) at 1:100, rabbit anti-hsCep97 (Proteintech) at 1:150 and rabbit anti-hsCP110 (Proteintech) at 1:500. Anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 secondary antibodies (Invitrogen) were used at 1:1000. For immunoblotting, rabbit anti-Cep97-C-ter antibodies (this study) were used at 1:500, mouse anti-α-HA at 1:1000, mouse anti–α-tubulin (DM1a; Sigma-Aldrich) at 1:1500 and mouse anti-FLAG-HRP at 1:10000. HRP-coupled secondary antibodies were used at 1:7500.

**Immunoprecipitation**

HEK293T cells were co-transfected with 4μg of HA-SIRT2 and 4μg of either FLAG empty vector or FLAG-CEP97. Cells were harvested 48h after transfection and lysed in lysis buffer (50mM Tris-Cl pH 8, 150mM NaCl, 1% Triton, 1x Roche Complete Mini Protease Inhibitor Cocktail, 1mM PMSF, 5 μM trichostatin A, 20mM nicotinamide, 50 units/mL benzonase and 1mM DTT) for 1h at 4°C. 10% of the cleared lysate was kept as input and the rest incubated for 2h on a rotating wheel at 4°C with anti-FLAG M2 magnetic beads (Sigma). Beads were subsequently washed three times with lysis buffer and immunoprecipitated proteins eluted with FLAG peptide.

**Southern and western blotting**

**Southern**

To identify deletion mutants, 20μg of genomic DNA were digested using Smal for 4 hours. Bands were separated on a 0.7% polyacrylamide gel and transferred to nylon membrane, incubated with a P32-labeled probe and washed. Finally, a phosphor-storage screen was exposed to the membrane and developed using a phosphorimager.
Western
To examine Cep97 protein levels in Cep97 mutants, dechorionated embryos were ground with a pestle and mortar and boiled for 10 min in loading buffer. Embryo lysates were separated on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were probed with anti-Cep97 and anti-tubulin antibodies overnight at 4°C, followed by 2 h incubation with HRP-conjugated secondary antibodies and bands visualized by ECL on a Bio-Rad ChemiDoc Touch Imaging System. For western blot analysis of FLAG-hsCep97 immunoprecipitates, 5% of the input and 30% of the eluate were loaded for each sample on a 10% gel and transferred onto nitrocellulose membrane. Blots were probed with anti-FLAG-HRP and anti-HA antibodies overnight at 4°C, followed by 1 h incubation with anti-mouse-HRP secondary antibody in the case of anti-HA antibody. Bands were visualized by ECL.

PCR and RT-PCR
PCR was performed on fly lysates according to standard protocols. For RT-PCR, mRNA was prepared from 10 dissected brains or 10 testes using the RNeasy kit (QIAGEN). cDNA was then generated using the QuantiTect Reverse Transcription kit (QIAGEN).

Immunofluorescence staining of Drosophila cells and tissues
Cep97-GFP, Atat1-GFP, Hdac6-GFP and Sirt2-GFP were detected with anti-GFP. Reconstituted Venus fluorescence signal in the BiFC assay was detected directly. All antibodies were used at dilutions detailed above.

S2 cells
S2 cells were formaldehyde fixed and stained largely as previously described [23]. Briefly, cells seeded on coverslips coated with Concanavalin A were fixed with 4% formaldehyde in PBS for 12 min at room temperature, then briefly washed with PBS. Fixed cells were permeabilized with PBS 0.5% Triton X-100 for 12 min, washed with PBS, then incubated with primary antibodies diluted in PBS 5% BSA overnight in a humid dark chamber. Coverslips were then washed with PBS before incubation with secondary antibodies diluted in PBS 5% BSA for 1 h at room temperature. Finally, coverslips were washed with PBS for 5 min and DNA stained with Hoechst for 10 min. After air-drying, coverslips were mounted in mounting medium (2% w/v n-propyl gallate in 90% Glycerol, 10% Tris–HCl pH 8.0).

Embryos
Embryos were dechorionated and formaldehyde fixed largely as previously described [60]. Briefly, bleach–dechorionated embryos were fixed in a heptane/4% formaldehyde in PBS mixture for 2 min. Heptane was removed and replaced with 1 volume of methanol, which was left on for 1 min. Methanol was replaced once more before rehydrating embryos in PBS 0.1% Triton X-100 3 x 10 min, followed by blocking in PBS 5% BSA for 20 min. Primary antibodies diluted in PBS 5% BSA were then added and embryos incubated overnight at 4°C. The next day embryos were washed 2 x with PBST and incubated with secondary antibodies in PBS 5% BSA for 2 h. After washing twice with PBS, Hoechst was added for 10 min. All liquid was then removed and embryos mounted on slides with mounting medium.

Larval brains
Brains were dissected from 3rd instar larvae and fixed largely as previously described [61]. After dissection in PBS, whole brains were fixed for 20 min in 4% formaldehyde. Primary antibodies diluted in PBS 5% BSA were added and samples incubated overnight at 4°C. The next day samples were washed twice with PBST, incubated with secondary antibodies in PBS for 2 h at room temperature. After washing twice with PBST, Hoechst was added for 10 min. All liquid was then removed and brains mounted on slides with mounting medium.

Wing discs
Wing discs were dissected from 3rd instar larvae and fixed in 4% formaldehyde in PBS 0.1% Triton X-100 (PBST) for 20 min. Samples were then incubated for 15 min in PBST, washed twice with PBS, blocked in 5% BSA in PBST for 30 min before incubating with primary antibodies as for larval brains.

Leg chordotonal organs and testes
Legs chordotonal organs were dissected from 36 h old male pupae and testes from 72-h-old male pupae. Fixation and staining were done essentially as previously described for testes [69]. Briefly, legs and testes were dissected in PBS. Testes were transferred to a microscope slide and cut with a tungsten needle before placing on a coverslip, while leg chordotonal organs were placed between slide and coverslip and pushed out of the cuticle by applying gentle pressure. Slides were then snap-frozen in liquid nitrogen. After recovering slides and removing the coverslip, samples were incubated in ice-cold methanol for 5 min followed by ice-cold acetone for 2 min. Samples were then washed twice with PBS 0.5% Triton X-100, followed by blocking in PBS 0.1% Triton X-100 1% BSA for 30 min. Primary antibodies diluted in blocking solution were then added and samples incubated overnight at 4°C. The next day samples were washed twice with PBS before addition of secondary antibody in PBS for 2 h at room temperature. After washing once with PBS, Hoechst was added for 10 min. Samples were air-dried and mounted with mounting medium. For actin staining, testes were fixed for 25 min in 4% formaldehyde in PBS 0.1% Triton X-100. Samples were then incubated for 10 min in PBST, washed twice with PBS, blocked in 5% BSA in PBST for 30 min before incubating with phalloidin and primary antibodies as above.
Immunofluorescence staining of RPE1 cells

Immunofluorescence staining of non-expanded cells

Coverslips were fixed with 4% formaldehyde in PBS for 10 min at room temperature and briefly washed with PBS. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min and then washed 3x in PBS. Cells were stained with primary antibodies diluted in 2% BSA in PBS for 1 h in a humid dark chamber. After 3x washes with PBS, coverslips were stained with secondary antibodies diluted in 2% BSA in PBS for 1 h. Finally, coverslips were washed in PBS 2x for 5 min and 1x with ddH2O. After air-drying, coverslips were mounted in DAPI-containing ProLong Gold anti-fade mounting medium (Invitrogen).

Expansion microscopy

Protocol is based on [70]. Coverslips with cells were fixed with 4% formaldehyde/4% acrylamide in PBS overnight and then washed 2x with PBS. The gelation was performed by incubating coverslips face down with 45 mM acrylate, 10% (wt/wt) acrylamide, 0.1% (wt/wt) N,N'-methylenebisacrylamide in PBS supplemented with 0.5% TEMED and 0.5% APS, prepared as described in [70] in a pre-cooled humid chamber. After 1 min on ice, chamber was incubated at 37°C in the dark for 30 min. Samples in gel were denatured in denaturation buffer (200 mM SDS, 200 mM NaCl, 50 mM Tris in ultrapure water) at 95°C for 4 h. Gels were expanded in ddH2O for 1 h until they reached full expansion factor (4.2x) and then cut into 1x1 cm pieces. Pieces of gel were incubated with primary antibodies diluted in 2% BSA in PBS overnight at RT. After staining, shrunk pieces of gel were incubated in ddH2O for 1 h, during which time they re-expanded. After reaching their original expanded factor, pieces of gel were incubated with secondary antibodies diluted in 2% BSA in PBS for 3 h at RT. Last expansion in ddH2O with exchange every 20 min was for 1 h until pieces of gel reached full size. Samples were imaged in 35 mm glass bottom dishes (CellVis, USA) precoated with poly-L-lysine. During imaging, gels were covered with ddH2O to prevent shrinking.

Fixed and live cell imaging

Standard confocal microscopy

Immunofluorescence samples were analyzed on a Zeiss LSM710 scanning confocal microscope equipped with an Airyscan unit. Stacks of 0.75 m m slices were acquired with a 63x 1.4NA Plan Apochromat lens using single channel mode to avoid cross-illumination. For centriole length measurements in testes, 4x electronic magnification was used. Airyscan images were acquired for selected centrioles using 0.25 m m slices and 10x electronic magnification. Maximum intensity projections of Z stacks prepared in ImageJ were used for image analysis and panel preparation.

STED microscopy

STED images were acquired on an Abberior STEDYCON unit on a Zeiss Axio Imager A2 microscope. Image stacks of 0.1 m m slices were acquired with a 100x 1.46NA alpha Plan-Apochromat lens and using 561/775 nm and 640/775 nm excitation/depletion combinations. Maximum intensity projections of Z stacks prepared in ImageJ were used for image analysis and panel preparation.

Live cell imaging and FRAP analysis

Dechorionated embryos were covered with Voltalef and examined on a Yokogawa CSU X1 spinning disk confocal mounted on a Zeiss Axio Observer Z1 inverted microscope equipped with a 63x 1.4NA Plan-Apochromat lens, 120 mW 405 nm and 100 mW 488 nm solid-state lasers, 2D-VisiFRAP Galvo FRAP module and Photometrics CoolSNAP-HQ2 cooled CCD camera and controlled by VisiView software (Visitron Systems). Z stacks of 0.75 m m were acquired every 15 s. Photobleaching was performed using the galvanometer point scanner to target a region encompassing multiple centrosomes with the 405 nm laser at 120 mW power. Image stacks were imported into ImageJ for post-acquisition processing.

Sperm motility assay

Testes and seminal vesicles were dissected in PBS from 2-day-old males and transferred to 10 m 3045–3056.e1–e7, August 3, 2020

SEM acquisition for RPE cells

Expanded cells were imaged by confocal microscopy on a Leica TCS SP8 scanning confocal microscope using a 63x 1.4NA oil objective with closed pinhole to 0.5 AU. Cilia were acquired in Z stacks at 0.1 m m stack size with pixel size 36 nm. Images were computationally deconvolved using Huygens Professional software (Scientific Volume Imaging) prior to image analysis and panel preparation.

Transmission electron microscopy

Chordotonal organs

Legs from 36 h old pupae were cut off with microscissors and fixed using a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 m l/I sodium phosphate buffer, pH 7.2 for 2 h in a desiccator at room temperature and then overnight on a rotator at 4°C. Legs were then rinsed with sodium phosphate buffer, post-fixed in 2% osmium tetroxide in buffer on ice, dehydrated in a graded series of acetone on ice and embedded in Agar 100 resin. 70 m sections were cut and post-stained with 2% uranyl acetate and Reynolds lead citrate. Sections were examined with a Morgagni 268D microscope (FEI, Eindhoven, the Netherlands) operated at 80 kV. Images were acquired using an 11 megapixel Morada CCD camera (Olympus-SIS).
**Testes/Wing discs**

Late pupal testes and wing discs from third instar larvae were dissected in PBS and fixed using 2.5% glutaraldehyde in 0.1 mol/l sodium phosphate buffer, pH 7.2 for 1h at room temperature. Samples were then rinsed with sodium phosphate buffer, post-fixed in 2% osmium tetroxide in dH2O on ice, dehydrated in a graded series of acetone and embedded in Agar 100 resin. 70nm sections were then cut and processed as above.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Image analysis**

**Centriole length**

Centriole length was measured on maximum-intensity z-projections of testes stained with Ana1 or Asl. At the spermatocyte stage, perfectly oriented centriole pairs (V-shape clearly visible) were selected and only the mother centriole measured in ImageJ, with beginning and end of the centriole defined as where the signal reached 25% of the maximum intensity. > 25 centrioles measured per condition.

**Photobleaching experiments**

Maximum projected stacks were opened and bleach-corrected in ImageJ (simple ratio matrix). Bleached and unbleached centrioles were tracked manually. A circle with a diameter of 7 pixels was used to encompass the centriole and average intensities measured over time for each centriole. Background subtraction was done by measuring the average signal in a 20 pixel circle of nearby cytoplasm. Two bleached centrioles and four unbleached control centrioles were measured per embryo and the signal averaged for each condition. Plots are of 3-point moving average to smooth out signal intensity variation due to centriole movement in z. Time of initial recovery was defined as the time at which centriolar signal initially became detectable again after photobleaching. Time of completion of recruitment was defined as the time at which plateau levels (~50% of original signal) were first reached for three successive frames.

**Centriolar signal quantitation**

To measure the lateral distribution of Cep97 relative to other centriolar markers in *Drosophila* spermatocytes and expanded RPE cells, linescans were performed in ImageJ on maximum-intensity z-projections of perfectly oriented centrioles, that is ones captured lying in or perpendicular to the plane of imaging. Where proteins were located peripherally near the wall of the centriole barrel, the distance between the two peaks of fluorescence intensity was measured for each centriole. For proteins located toward the central lumen of the centriole, distribution was measured as the full width at half maximum. For quantitations in RPE cells, values were divided by the expansion factor of 4.2 to obtain original non-expanded dimensions.

**Quantification of centriole and axonemal defects in electron micrographs**

**Wing discs**

Centrioles of three wing disc were analyzed per condition. Images were acquired at 36000x for > 15 centrioles per wing disc and length and diameter measured using ImageJ.

**Leg chordotonal organs**

Leg chordotonal organs from three different animals were analyzed per condition. Cross sections or longitudinal sections of leg chordotonal cilia were analyzed and scored for presence or absence or other defects at 72000x and 36000x magnification.

**Testes**

Three testes were analyzed per condition. To detect gross defects in cell division, cross sections of whole cysts were analyzed at low magnification (4000x-8000x). Sperm axoneme abnormalities were analyzed at 36000x. Missing or defective axonemes per cyst (normal 64 axonemes) were scored for > 20 cysts per condition.

**Statistical Analysis**

All error bars are standard deviation. To compare samples in a specific experiment, we conducted unpaired t-tests using GraphPad Prism. *, **, *** represent p values of < 0.05, 0.01 and 0.001, respectively. Tests are comparing indicated condition to control unless otherwise specified.