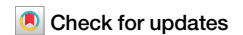


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Versatile gamma-tubulin complexes contribute to the dynamic organization of MTOCs during *Drosophila* spermatogenesis



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The initiation of microtubule formation is facilitated by γ -tubulin and γ -Tubulin Ring Complex (γ -TuRC) in various microtubule-organizing centers (MTOCs). While the heterogeneity of tissue-specific MTOCs and γ -TuRC in *Drosophila* testis has been described, their molecular composition and physiological significance are poorly understood. We investigated the testis-specific distribution and biochemical interaction of the canonical γ -TuRC proteins Grip163 and Grip84. We found that while Grip163 is present on the centrosome and basal body, Grip84 localizes to the centrosome and Golgi in spermatocytes and colocalizes with the testis-specific γ -Tubulin complexes (t- γ -TuC) at the basal body, apical nuclear tip, and near the elongated mitochondria after meiosis. We also showed the apical nuclear tip localization of some γ -TuRC interacting partners and proved their binding to t- γ -TuC proteins. These results highlight and prove the importance of the different γ -TuRCs in organizing the diverse MTOCs present during the extensive rearrangement of cell organelles during the spermatogenesis of *Drosophila*.

The microtubule (MT) network in eukaryotic cells plays a crucial role in cell differentiation and division, as well as in the movement through the cilia and flagella. The spatial re-organization of the MT cytoskeleton is a fast and dynamic process that requires distinct subcellular nucleation sites called microtubule organizing centers (MTOCs) that act as templates for the MT arrays to initiate. The main MTOC in dividing cells is the centrosome, which consists of a pair of centrioles (an older mother and a newer daughter centriole), and the pericentriolar material (PCM). Apart from the centrosome, non-centrosomal microtubular organizational centers (ncMTOCs) are present in different cell types, but it is not known how these structures regulate the organization and dynamics of microtubules. The heterogeneity of ncMTOCs is reflected in the molecular composition and the cellular localization, and their function is unique to each cell type¹.

MTs are polar structures containing plus and minus ends. The highly dynamic plus end is generally where the majority of MT polymerization and depolymerization occurs, while the minus end is often stabilized and capped². The main factor known to specifically associate with microtubule

minus ends is the γ -tubulin ring complex (γ -TuRC)^{3,4}. PCM and ncMTOCs both recruit γ -TuRCs to the microtubule nucleation site^{5,6}. γ -TuRC is a multiprotein complex consisting of γ -tubulin and its associated proteins (γ -tubulin ring proteins (Grips) in *Drosophila*, or γ -tubulin complex proteins (GCPs) in human) that are essential for γ -TuRC integrity and MT nucleation activity. The conserved and essential core of the γ TuRC is the small complex (γ TuSC) that consists of two γ -tubulins, one copy of Grip84 (human GCP2) and Grip91 (human GCP3) in *Drosophila*⁷. The large ring complex comprises multiple γ -TuSCs and additional Grips, including Grip75 (human GCP4), Grip128 (human GCP5), Grip163 (human GCP6), and Grip71 (NEDD1)^{8,9}. An additional component of the microtubule nucleation machinery is Mzt1, which can bind to both γ -TuSC and γ -TuRC in many eukaryotes^{10–12}. Although Mzt1 is essential in most organisms, it exhibits testis-specific expression and binds to Grip128, Grip91 and t-Grip91 in *D. melanogaster*^{13,14}. It was shown that γ -TuRC genes are not essential for viability, but γ TuRC is able to nucleate microtubule organization more efficiently than the small complex alone⁹.

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Similarly to mammals, *Drosophila* spermatogenesis involves a series of stage-dependent morphological changes when stem cells give rise to mature sperm. During this process, round spermatids of 10 μm in diameter elongate to become 1850 μm in length¹⁵, which requires an extensive remodeling of existing cellular organelles, as well as the addition of new structures. After meiosis, each spermatid inherits a centriole that will serve a basal body around a ring-shaped PCM-like macromolecular structure called the centriole adjunct. The growth of the axoneme starts from the basal body and continues to elongate towards the spermatid tail (Fig. 1a)¹⁶. Opposite to the basal body, on one side of the nucleus, the acroblast starts to develop, which is a Golgi-derived organelle. During the later stages of elongation, the acroblast will be converted into the acrosome. Another important event that occurs after meiosis is the fusion of the mitochondria into two giant mitochondrial derivatives, called the nebenkern. These mitochondrial

derivatives elongate with the axoneme during sperm maturation and accumulate paracrystalline material in one of them, which could provide structural support for the growing tail (Fig. 1a)^{15,17}.

Growing evidence suggests that testis-specific MTOC components play important roles in basal body and ncMTOC formation and also in the positioning and shaping of the post-meiotic organelles during *Drosophila* spermatogenesis^{13,14,18}. For instance, testis-specific CnnT expression contributes to the conversion of mitochondria into a ncMTOC, independent of core pericentriolar Cnn¹⁸. In addition to the canonical γ -TuRC genes (Grip84, Grip91, Grip75, Grip128, Grip163, and Grip71), we have recently identified three additional testis-specific paralogs of the γ -TuRC components, t-Grip84, t-Grip91, and t-Grip128^{14,19}. We have shown, that from the round spermatid stage onward, these testis-specific γ -Tubulin complex (hereafter referred to as t- γ -TuC) proteins start to localize to the centriole

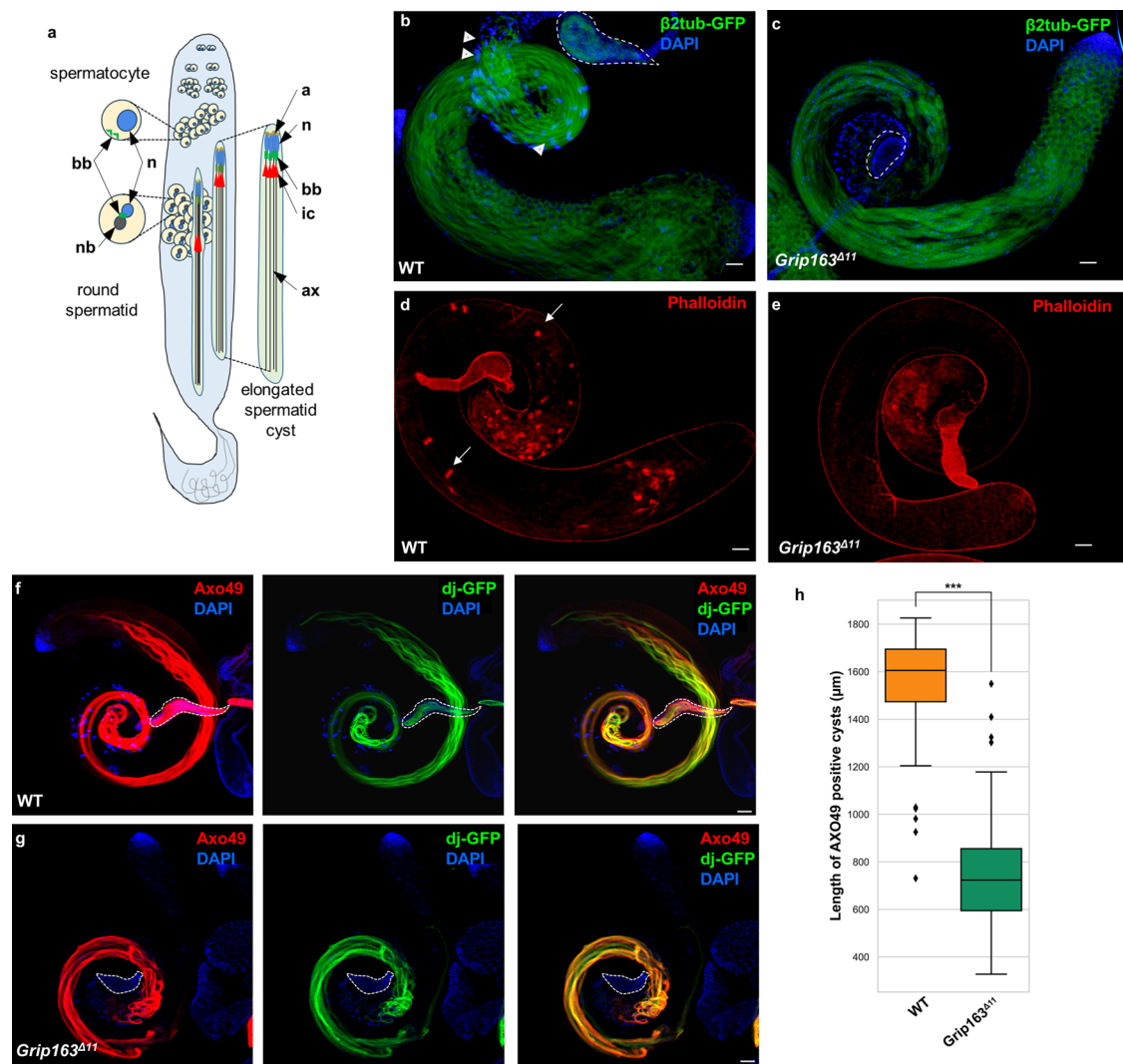


Fig. 1 | Mitochondrial and axonemal abnormalities lead to abnormal spermatid elongation in *Grip163 Δ 11* mutant. **a** Schematic representation of *Drosophila* spermatogenesis stages in the testis, highlighting the major cellular organelles (bb basal body, n nucleus, nb nebenkern, a acrosome, ax axoneme and cytoskeletal structures (ic investment cone) of the secondary spermatocyte, round spermatids and the elongated spermatid cyst. **b, c** Microtubules are visualized with the β 2tub-GFP transgene (green), while the nuclei stained with DAPI (blue) in WT **b** and *Grip163 Δ 11* mutant spermatids (**c**) Elongated cysts are present both in WT and *Grip163 Δ 11*, however, the seminal vesicle (dashed lines) of *Grip163 Δ 11* mutant does not contain β 2tub-GFP positive mature sperms. **d, e** The individualization complexes (IC) were

stained with Phalloidin (red), ICs are formed normally (arrows) in WT, (**c**) and missing in *Grip163 Δ 11* mutant (**e**). **f, g** Fully developed axonemes are visualized by Axo49 tubulin antibody (red) and elongated mitochondria are with DJ-GFP transgene (green) both in the testis and seminal vesicle (dashed line). Elongated polyglycylated axonemes and mitochondria were shorter in *Grip163 Δ 11* mutant and the seminal vesicle was empty (dashed line) (**g**) compared to the WT (**f**). **h** Boxplot shows the length of AXO49 positive cysts in WT and *Grip163 Δ 11* mutant. (WT $n_{\text{testis}} = 12$, *Grip163 Δ 11* $n_{\text{testis}} = 22$, WT $n_{\text{measurement}} = 36$, *Grip163 Δ 11* $n_{\text{measurement}} = 64$) Statistical significance was tested by one-way ANOVA. (scale bars: 50 μm).

adjunct, then to the nuclear tip, and finally surrounding the mitochondria of the elongating cyst. We also presented that they bind to γ -Tubulin and Mzt1 and interact with each other in the post-meiotic stages¹⁴. We and others have shown that Mzt1 binds to the N-termini of Grip/GCP proteins and interacts with the N-terminal regions of Grip91, t-Grip91 and Grip128 in *Drosophila*^{13,14}. Our finding raised the question of whether the canonical γ -TuRC proteins are replaced with their testis-specific counterparts, or whether they are able to cooperate to meet the needs of microtubule nucleation during the late stages of spermatogenesis. We also aimed to understand whether the t- γ -Tu complexes can interact with the canonical γ -TuRC or with any other proteins. In order to address these questions, we created in situ tagged *Grip163* and *Grip84* transgenic lines to elucidate the adaptable localization and identify interacting partners of the γ -TuRC during the development of male germ cells.

Results

Phenotypic characterization of the *Grip163*^{Δ11} mutant line

We have reported previously that t- γ -TuC proteins are present only in the post-meiotic spermatids¹⁴. However, there is a lack of data regarding the relationship between the canonical and the t- γ -TuCs, as well as the presence or localization of ubiquitously expressed γ -TuRC components in the late stages of spermatogenesis.

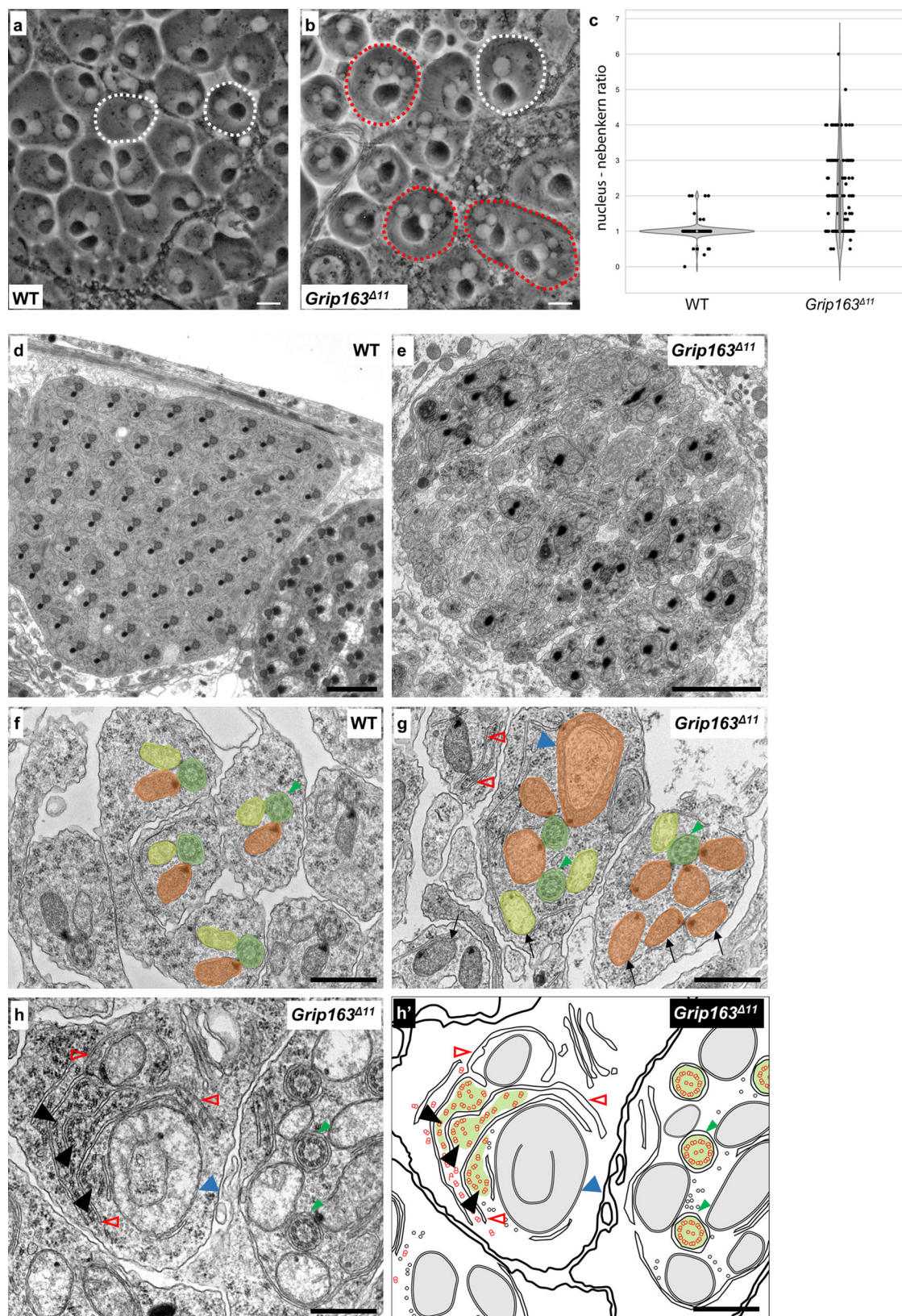
Therefore, we tested the presence and possible function of the γ -TuRC component Grip163, which has no testis-specific paralogs, in sperm development^{8,14,20}. To achieve this, we induced mutation in the *Grip163* gene, and performed an in situ tagging of the *Grip163* gene, using the CRISPR-Cas9 gene editing technology. Using a specific guided RNA designed for *Grip163*, we deleted 11 base pairs from the coding region of the gene (hereafter, *Grip163*^{Δ11}). This deletion caused a frameshift leading to a premature stop codon in the coding region of *Grip163* (Supplementary Fig. 1a). Interestingly, we found that in contrast to the *Grip84* and *Grip91* homozygotes, which are lethal^{21,22}, the homozygous *Grip163*^{Δ11} mutant animals are viable, but male and female sterile (Supplementary Fig. 1c, Supplementary Data). It is known that the γ -TuSC is essential for centrosome formation, mutants of the canonically expressed γ -TuRC components, *Grip128* and *Grip75*, are viable and similar to *Grip163*^{Δ11} male and female sterile^{9,21,23–25}. The previously characterized *Dgrip163*^{GE27087} mutant allele is male fertile and female sterile⁹, therefore we decided to test the spermatogenesis phenotype of *Grip163*^{Δ11}. *Grip163*^{Δ11} is male and female sterile in hemizygous combination with the overlapping *Df(3L)BSC574* deficiency, suggests that *Grip163*^{Δ11} is a strong hypomorph or a null allele (Supplementary Fig. 1c, Supplementary Data). The seminal vesicles of the *Grip163*^{Δ11} males were empty, without mature sperms (Fig. 1b, c). Based on this phenotype we investigated both the early and late stages of spermatogenesis in *Grip163*^{Δ11} flies. We found an abnormal nebenkern-nucleus ratio in the round spermatids, which is a hallmark of abnormal mitotic division (Fig. 2a–c, Supplementary Data). Nevertheless, many of the cysts are able to elongate in the *Grip163*^{Δ11} mutant, visualized by the β 2tub-GFP transgene (Fig. 1b, c). To further investigate the elongation of the cysts, we tested whether the individualization proceeds normally. After the elongation of the cyst, an actin rich cytoskeletal structure, the individualization complex (IC) is formed at the caudal end of the nuclei surrounding the basal-body and axoneme. During individualization the IC moves towards the tip of the tail, expelling and degrading all the cytoplasmic content from the spermatid and tightly cover the axoneme-mitochondria complex with an individual plasma membrane (Fig. 1a)²⁶. To visualize the elongated post-meiotic cysts, we stained the actin cones of the IC in the *Grip163*^{Δ11} testis and found the lack of them in *Grip163*^{Δ11}, providing evidence for the failure of proper cyst elongation, which is the prerequisite of individualization (Fig. 1d, e). For further evidence, we used anti-pan polyglycylated tubulin (Axo49) antibody to visualize mature axonemes of the spermatids, at the onset of individualization²⁷. We observed significantly shorter axonemes in the *Grip163*^{Δ11} mutant compared to the wild-type (WT), indicating abnormal spermatid elongation (Fig. 1f–h, Supplementary Data).

Axoneme elongation depends on the mitochondria, which provide a structural platform for MT reorganization to support the robust elongation taking place at the tail tip of the sperm²⁸. Based on this we tested the presence and appearance of late elongating mitochondria using dj-GFP as a marker. In accordance with the defect observed in axoneme elongation, it was detected that the mitochondria in the *Grip163*^{Δ11} mutants also exhibited incomplete elongation (Fig. 1f, g).

Based on this general spermatid elongation defect of *Grip163*^{Δ11} flies, the detailed morphology of post-meiotic cysts was further investigated using transmission electron microscopy (TEM). In wild-type testis, the 64 spermatids develop simultaneously and on the TEM image of elongated cysts we observed the two mitochondrial derivatives attached to the axoneme, which has the classical 9 + 2 arrangement of microtubules in each spermatid (Fig. 2d–f). In *Grip163*^{Δ11} mutants, cysts became disorganized, with fewer spermatids per cyst and with disturbed mitochondrial:axonemal ratio (Fig. 2e). Frequently, multiple mitochondrial derivatives were attached to a single axoneme (Fig. 2g, h), and we also observed disintegrated axoneme remnant with doublet microtubules surrounded by unfurled axial membrane (Fig. 2h, h'), supporting that Grip163 is necessary for normal axoneme formation and/or stability. Since the γ -TuRC was proposed to be essential for centrosome formation in somatic cells¹¹, we examined the centrosome/basal body distribution at different stages of spermatogenesis in the testes of *Grip163*^{Δ11} males. We tested the presence and the distribution of the centrosomal/basal body localized γ -Tubulin and the PACT domain of the *Drosophila* Pericentrin-like Protein (DPlp) fused with GFP (referred to as GFP-PACT)²⁹. In the wild-type animals both proteins localize to the centrosome/basal body during different stages of spermatogenesis (Fig. 3a, b, Supplementary Fig. 2b). In wild-type males, GFP-PACT is located close to the nucleus and the nebenkern, as expected in round spermatids. In contrast, multiple and scattered GFP-PACT signals were observed in the *Grip163*^{Δ11} mutants' testis (Fig. 3c, d, Supplementary Fig. 2b, c). We calculated both the nucleus-nebenkern and the basal body-nebenkern ratio in the round spermatids (Supplementary Fig. 2a, Supplementary Data). Theoretically, these ratios should be close to 1 in WT, as each spermatid is supposed to have one of each organelle per cell. However, we found that the *Grip163*^{Δ11} mutant was significantly different from the expected 1. In the *Grip163*^{Δ11} mutant, the average ratio is higher than one (~2.06, ~2.01) indicating that there are less nebenkerns, but approximately equal amount of basal bodies and nuclei (Supplementary Fig. 2a, Supplementary Data). These abnormalities could be the consequence of cytokinetic defects of the meiotic cysts of *Grip163*^{Δ11} mutant.

We also measured the fluorescence intensity of γ -Tubulin and GFP-PACT in premeiotic and post-meiotic cysts. Both in WT and the *Grip163*^{Δ11} mutant flies we found strong GFP-PACT positive centrosomal and basal body signals (Fig. 3c, d), however, the γ -Tubulin signal was weak in spermatocytes compared to the WT spermatocytes (Supplementary Fig. 2d, e, Supplementary Data).

Surprisingly, after meiosis the γ -Tubulin signal was stronger and accumulated on the basal body both in WT and *Grip163*^{Δ11} mutant spermatids, but those were scattered along the cysts (Fig. 3c, d, Supplementary Fig. 2e, Supplementary Data). It was previously proposed that γ -Tubulin could be recruited to the centrosomes in somatic cells by γ -TuSCs, independently of the γ -TuRCs⁹. Mzt1 is a well-characterized binding partner of the γ -TuSC and we showed previously, that the post-meiotically localizing three t-Grip proteins (t-Grip84, t-Grip91, t-Grip128) bind to γ -Tubulin and t-Grip91 and also to Mzt1^{14,30}. The γ -TuRC interacting Mzt1 is localized to the centrosome, basal body and the surface of the mitochondria from the meiotic stages onward and also to the apical tip of the elongating nuclei (Fig. 9)¹⁴. We found Mzt1 localization on the surface of the mitochondria in *Grip163*^{Δ11} mutant, suggesting that Grip163 and probably γ -TuRC are not essential for Mzt1 mitochondrial anchoring. Similar to γ -Tubulin, Mzt1 is not present on the meiotic basal body, however, in the post-meiotic stages, it



localizes to the dispersed basal body in the *Grip163^{A11}* mutant spermatids (Supplementary Fig. 3). All these data suggest that Grip163 could be essential for the formation of stable γ -TuRC before and at the onset of meiosis, however, the newly organized testis-specific γ -TuRC could contribute to the γ -Tubulin and Mzt1 binding after meiosis (Fig. 9).

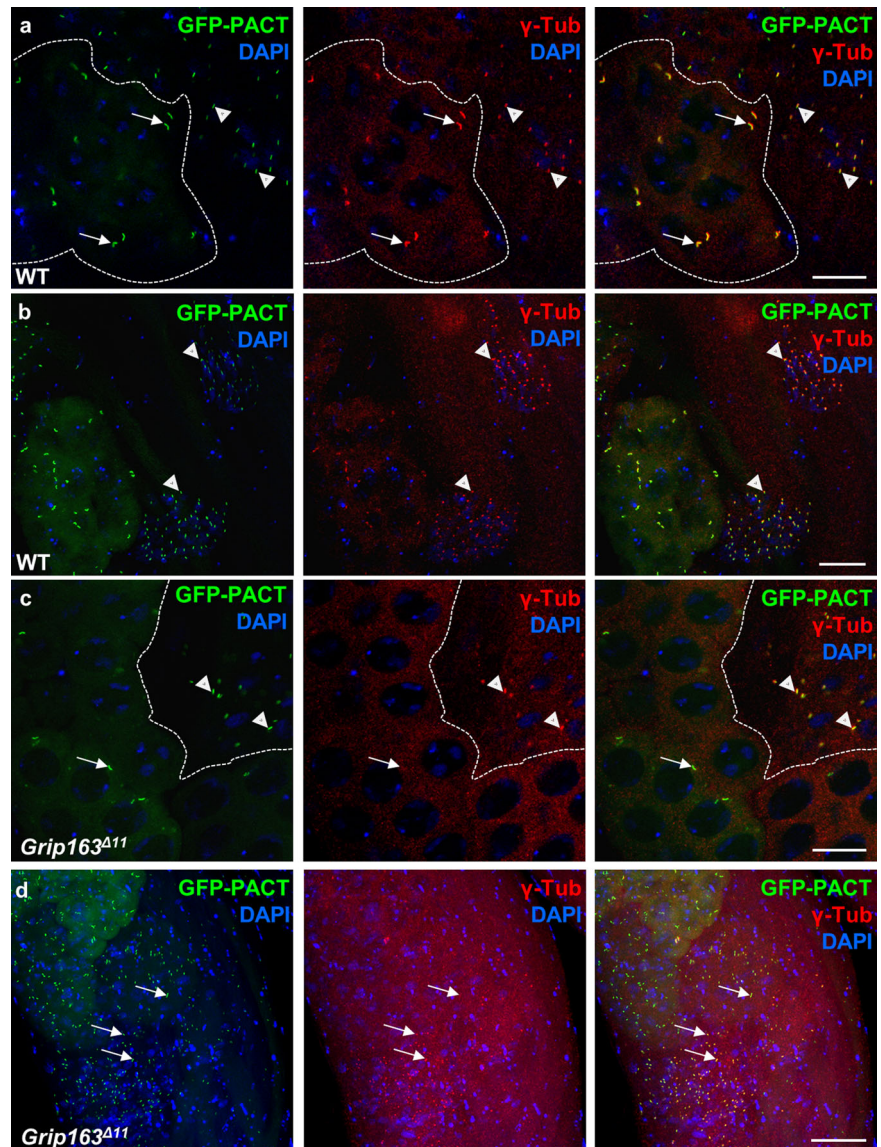
Localization of γ -TuRC proteins during spermatogenesis

To test the subcellular localization of Grip163 at different stages of spermatogenesis, we generated endogenously GFP-tagged Grip163 transgenic lines. We and others have shown that the N- or C-termini of the γ -TuRC proteins are responsible for the binding of their interacting

Fig. 2 | Abnormal mitochondrial and axonemal structures in the post-meiotic cysts of *Grip163^{Δ11}* mutant. **a, b** Phase contrast images show a normal 1:1 (white outline), nucleus: nebenkern ratio in WT (**a**) and an abnormal ratio (red outline) in *Grip163^{Δ11}* mutant (**b**). **c** Violin plot showing the nucleus: nebenkern ratio in WT ($n = 186$) and *Grip163^{Δ11}* mutant round spermatids ($n = 112$). **d, h** Transmission electron micrograms of testis cross-sections, representing elongating cysts of WT (**d, f**) and *Grip163^{Δ11}* mutant (**e, g, h**). Disorganized cysts with axonemal and mitochondrial abnormalities are characteristic in *Grip163^{Δ11}* mutant. **f** WT elongating cyst shows normal axoneme structure with 9 + 2 tubulin dimers (highlighted

in green and green arrowhead) and the attaching two mitochondrial derivatives (yellow and orange), with paracrystalline material formation in one of them (orange). **g, h** In *Grip163^{Δ11}* mutant the mitochondria and the axoneme detached from each other (black arrows), and multiple mitochondrial derivatives (highlighted in orange and yellow) attached to one axoneme (green and green arrowhead). **h, h'** Mitochondria fused, enlarged (blue arrowheads) and detached from the axoneme (green) and doublet microtubules representing the disintegrated axonemes (black arrowheads) and the open axial membranes (red empty arrowheads) are characteristic of *Grip163^{Δ11}* mutant cyst. (scale bars: (a–e) 2 μm, (f–h') 500 nm).

Fig. 3 | Mislocalization of centrosomal markers in *Grip163^{Δ11}* mutant. **a, b** GFP-PACT (green) and γ -Tubulin (red) are colocalizing on the centrosome (arrows) and basal body (arrowheads) in WT spermatocytes and spermatids (**a, b**). γ -tubulin, but not GFP-PACT centrosomal localization is affected in *Grip163^{Δ11}* mutant spermatocytes. In the round spermatids, GFP-PACT and γ -Tubulin are colocalised (**c**), but the post-meiotic elongating cysts are disorganized with scattered basal bodies (**d**). (scale bars: 20 μm).



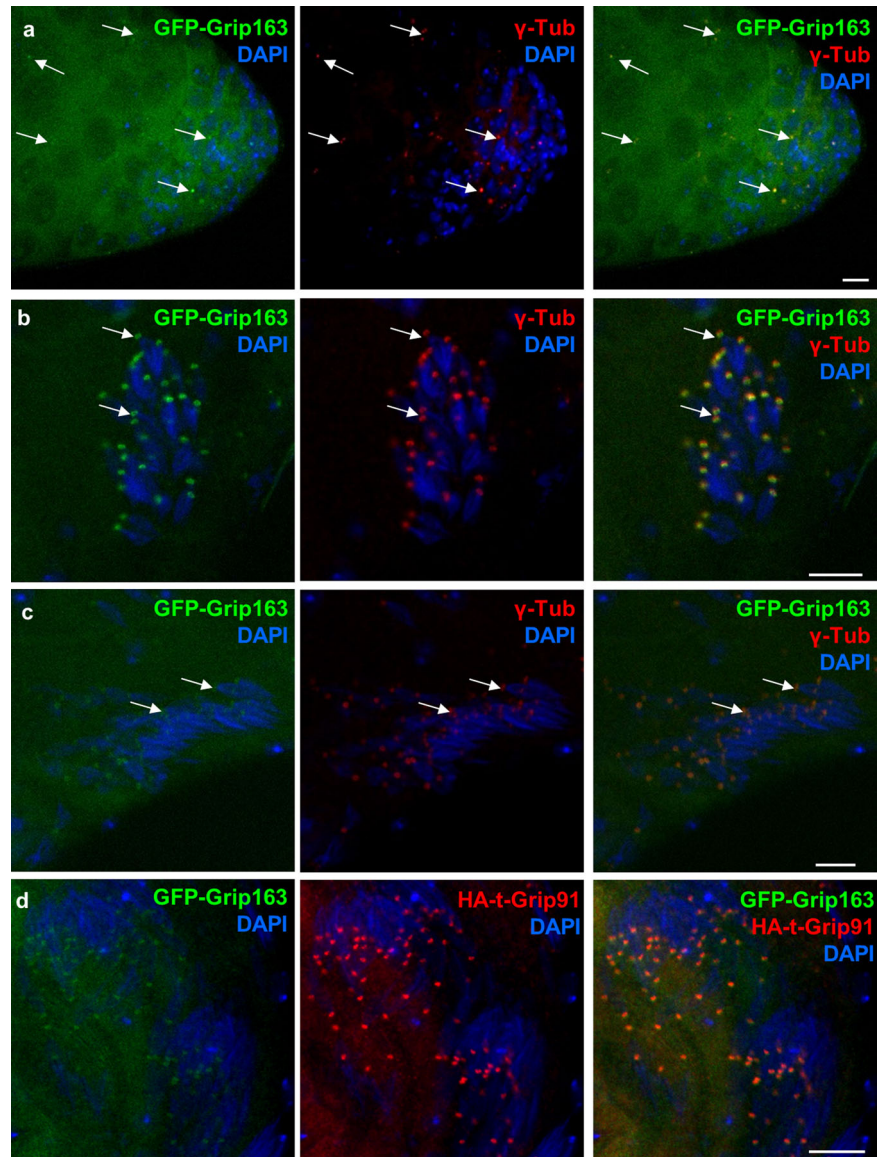
partners^{13,14}, therefore we tagged the Grip163 close the N-terminus (aa150), without interfering with the functional GCP domains of the protein (Supplementary Fig. 1a). The fertility of the GFP-Grip163 males is comparable to the wild-type animals, indicating that the inserted fluorescent tag does not alter the function of the protein (Supplementary Fig. 1c, Supplementary Data). GFP-Grip163 signal is present and colocalised with γ -Tubulin on the centrosomes and basal body on the developing spermatids (Fig. 4a, b).

We also showed the colocalization of GFP-Grip163 with γ -Tubulin and also with HA-t-Grip91 on the post-meiotic basal body/centriole adjunct (Fig. 4c, d). These results strongly suggest that the testis-specific γ -TuRC

does not replace the canonical γ -TuRC, but both are present on the basal body/centriole adjunct of the developing spermatids (Fig. 9).

The localization of GFP-Grip163 to the post-meiotic centriole adjunct has prompted an inquiry into the potential presence of other canonical γ -TuSC components in the subsequent developmental stages. To investigate this, we applied CRISPR technology to in situ tag Grip84 with GFP. We designed the incorporation in a way that all 5 potential isoforms of Grip84 will have the N-terminally inserted GFP tag, outside of the GCP domain (Supplementary Fig. 1b). It was previously reported that the classical mutant of *Grip84* is lethal, therefore the normal viability and fertility of the in situ GFP-tagged Grip84 serves as evidence that the GFP tag does not alter the

Fig. 4 | Localization of GFP-Grip163 during spermatogenesis. a–c The endogenously tagged GFP-Grip163 colocalizes with γ -Tubulin at the (a) centrosome (arrows) and the (b, c) basal body (arrows) throughout spermatogenesis. d GFP-Grip163 has an overlapping localization with the t- γ -TuC represented by HA-t-Grip91 on the basal body/centriole adjunct in the elongating spermatids. (scale bars: 10 μ m).



normal function of Grip84 (Supplementary Fig. 1c, Supplementary Data)³¹. We found that GFP-Grip84 is present on the centrosomes of spermatocytes, similarly to the GFP-Grip163 protein (Fig. 5a–e). This localization of GFP-Grip84 was also verified by its colocalization with the centrosomal component Asterless (Asl) (Fig. 6a, Supplementary Fig. 4a, b, Fig. 9)³².

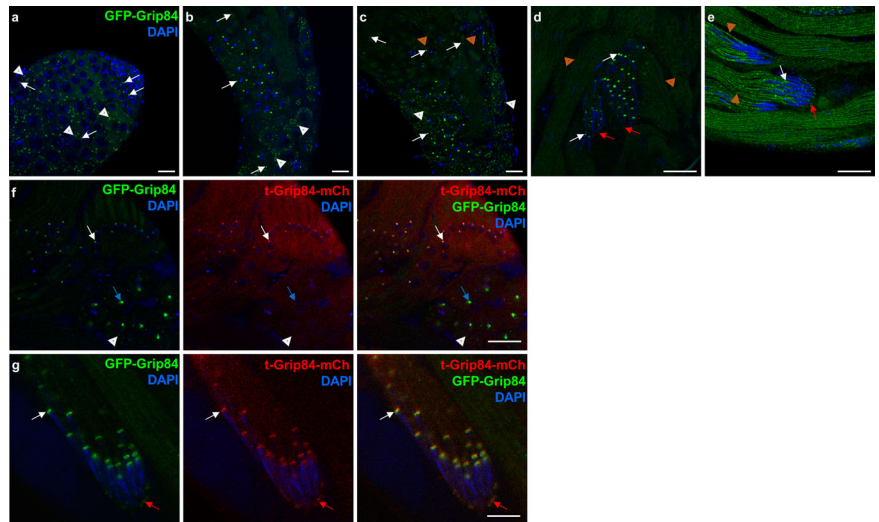
Following meiosis, GFP-Grip84 is present on the centriole adjunct and then appears at the apical nuclear tip and exhibits a strong overlap with the elongated mitochondria, akin to the GFP-Grip163. We wanted to know whether the post-meiotic localization of GFP-Grip84 overlaps with its testis-specific paralogue, t-Grip84. To do this we co-expressed GFP-Grip84 and t-Grip84-mCherry together and found complete colocalization after the meiotic stages (Fig. 5f, g)¹⁴. These results provide additional evidence that both the ubiquitously expressed γ -TuRC and the testis-specific γ -TuRC may collectively contribute to the formation of the diverse microtubule organizing centers during the post-meiotic stages of spermatids.

Apart from the centrosomal localization of GFP-Grip84, we also noticed strong bright punctae in the cytoplasm of spermatocytes, whose distribution is reminiscent of tER-Golgi units. It is known that centrosomal proteins are associated with the Golgi apparatus in mammalian cells³³. Golgi organization is special in *Drosophila*, as the scattered Golgi stacks are found in close proximity to the ER exit sites (tER-Golgi)³⁴. To test the tER-Golgi

localization of GFP-Grip84, we used GM130 as a *cis*-Golgi, as well as GCC88, as a *trans*-Golgi-specific antibody for co-immunostaining (Fig. 6b–e, Supplementary Fig. 4c, d, Fig. 9)³⁵. We observed strong colocalization of GFP-Grip84 with the *cis*-Golgi, suggesting that GFP-Grip84 is connected to the tER-Golgi units of spermatocytes.

In a comprehensive analysis of the effector proteins of Rab GTPases, Grip84, Grip91 and γ -Tubulin were identified as interacting partners of the Golgi-localizing Rab2 and Rab4³⁶. Rab2A and Rab2B proteins were shown to be essential for acrosome formation and male fertility in mice³⁷, therefore it seemed reasonable to check whether the Golgi-localizing Rab2 might also be present at the apical end where the acrosome is located during nuclear elongation in *Drosophila* spermatids. We tested the germline-specific BamGal4 driven pUASP-Rab2-YFP localization in spermatocytes and spermatids. We detected Rab2-YFP on the t-ER-Golgi units adjacent to the *trans*-Golgi-specific antibody GCC88 in spermatocytes and on the acrosome containing apical nuclear tip of the elongated spermatids (Fig. 6d, e). Localization of GFP-Grip84 to the Golgi apparatus led us to hypothesize that *Drosophila* γ -TuRC, similarly to the mammalian γ -TuRC might be necessary to the Golgi organization and also could have a possible function of Rab2 in acrosome formation (Fig. 9). Additional experiments can address these highly intriguing prospects.

Fig. 5 | Localization of GFP-Grip84 during spermatogenesis. a–e In vivo tagged GFP-Grip84 localizes both to the centrosome (a) and the basal body (b, c) (white arrows) and in the post-meiotic elongating cyst GFP-Grip84 localizes also to the apical tip of the elongating nuclei (d, e) (red arrows). Additionally, it is localized surrounding the elongating mitochondria (c–e) (orange arrowheads). Golgi-like localization of GFP-Grip84 was observed in spermatocytes (c) (white arrowheads). f, g GFP-Grip84 localizes to the centrosome before meiosis (blue arrows) and shows overlapping localization with t-Grip84-mCh at the basal body (white arrows) and the apical tip of the elongating nuclei (red arrows) in the post-meiotic spermatids (Scale bars: 20 μ m).



Post-meiotic ncMTOCs contain centrosomal components

The versatile localization pattern of GFP-Grip84 prompted us to investigate whether any of the already known binding partners of the γ -TuRC could be present on the newly identified localization foci. It was shown previously that γ -Tubulin is localized in the apical region of the spermatid nuclei, specifically at the distal end of the MTs present within the dense complex^{14,38}. We have previously demonstrated the presence of t- γ -TuC and Mtz1 at the apical tip of the nucleus, suggesting the presence of a ncMTOC organized opposite to the basal body during nuclear elongation¹⁴. Therefore, we carefully examined the precise localization of the centriole component, Sas4, a known interacting partner of γ -TuRC proteins by immunostaining using anti-Sas4 antibody^{39,40}. We detected Sas4 on the centrosome of mitotic spermatocytes and the basal body of the spermatids after meiosis (Fig. 7a–c). Intriguingly, we detected endogenous Sas4 on the apical tip of the elongating spermatid nuclei as well (Fig. 7c). Apart from the γ -TuRC proteins, Sas4 has multiple centriolar binding partners, including Sas6, Asl, Ana1, and Dplp^{32,40–43}. Therefore, we wondered whether they are also present on the site, which has been suggested as a ncMTOC at the apical tip of the spermatid nuclei. To achieve this we performed immunostainings using gene-specific antibodies^{32,39,44}.

We detected Sas6 and Asl at the centriole adjunct and also at the apical tip of the nuclei (Supplementary Fig. 5a, b), however, Ana1 and GFP-PACT colocalized with t-Grip91 exclusively at the centriole adjunct region of the elongating leaf and canoe stage nuclei, in line with the reported localization pattern of these proteins (Fig. 7d, Supplementary Fig. 5c)³⁸. These results indicate that the site which has been suggested as a ncMTOC at the apical nuclear tip contains several well-defined binding partners of the γ -TuRC (Fig. 9).

Composition of t- γ -TuC

We next examined whether the partially overlapping localization pattern might result from a physical interaction between the canonical γ -TuRC and the t- γ -TuC and/or between the t- γ -TuC proteins and the γ -TuRC interacting protein Sas4. To address this, we performed a yeast two-hybrid (Y2H) analysis, where we tested t- γ -TuC proteins with the canonical γ -TuRC protein Grip91, Grip84, Grip163, Grip75 and Sas4. Grip proteins contain GCP-N and GCP-C domains based on Pfam database prediction. GCP-N is predicted to be necessary for the incorporation of the proteins into the helix structure of the complex and GCP-C domain mediates longitudinal interactions with γ -Tubulin⁴⁵. Despite the low percentage of similarity between the canonical Grip proteins and the lack of predicted GCP-N domain in Grip128 and also the lack of GCP-C domain in Grip128 and t-Grip91 the core structures are similar between human GCP protein and the *Drosophila* Grip and t-Grip proteins (Supplementary Fig. 6). Due to the large size of

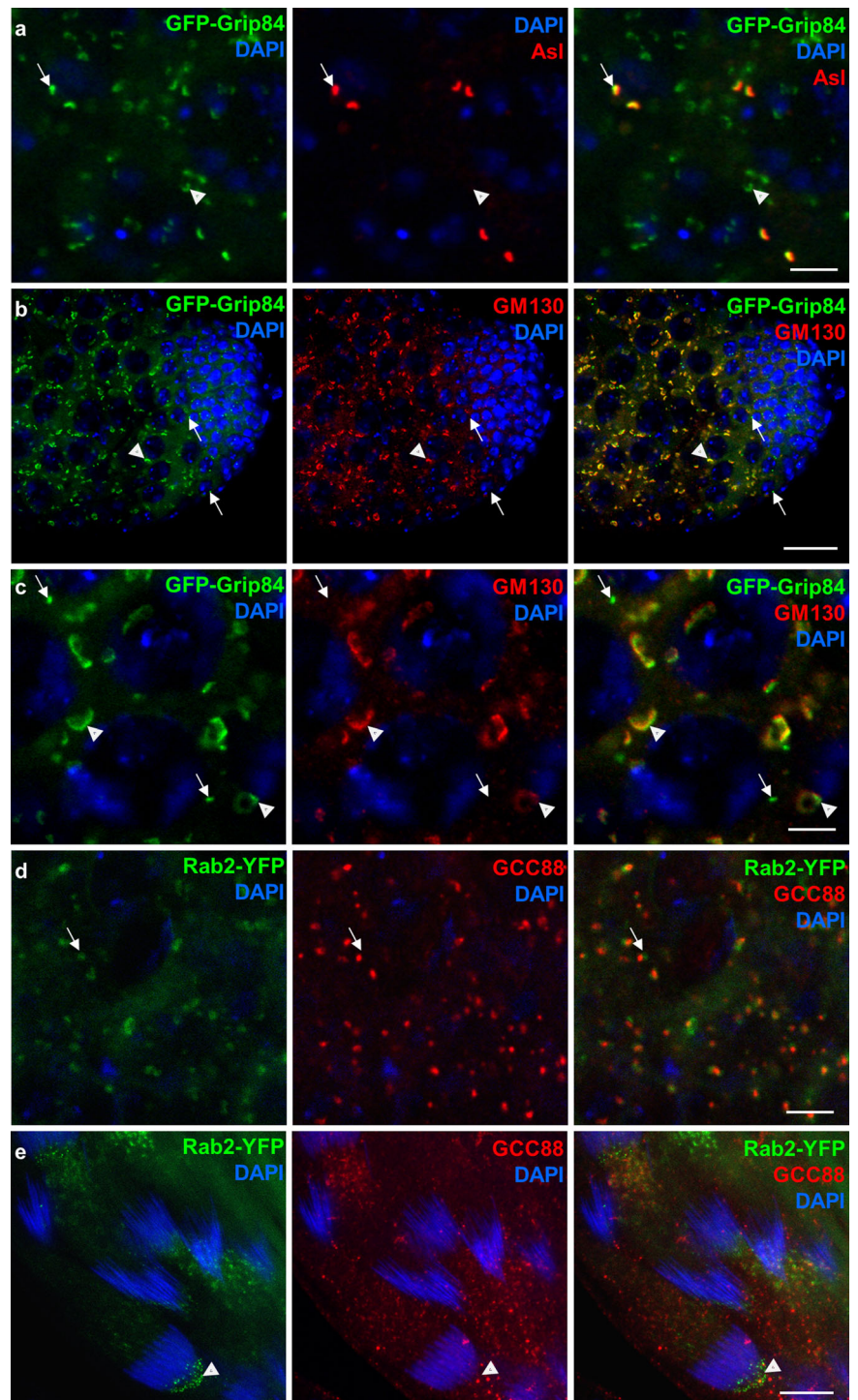
these proteins, we split all three t- γ -TuC proteins as well as Grip91, Grip163 and Grip128 into N-terminal (NT) and C-terminal (CT) parts, while we kept the predicted GCP domains intact. Grip84, Grip75 and Sas4 were used as full-length proteins (Supplementary Fig. 7a).

A specific interaction was found between t-Grip84-CT and Grip91-NT, which suggests the possibility of the presence of a testis-specific and non-testis-specific mixed γ -TuRC (Fig. 8a, Supplementary Fig. 7–8). We also detected interaction between t-Grip84-CT and Grip84, between t-Grip91-NT and Grip84, Grip91-CT, Grip75, Grip163-NT, Grip163-CT and finally between t-Grip91-CT and Grip84, Grip91-CT, Grip75, Grip163-NT, Grip163-CT. Sas4 was able to bind to and t-Grip91-NT, t-Grip91-C, t-Grip128-N and t-Grip128-C, however, we could not find an interaction between Sas4 and t-Grip84-N, t-Grip84-C (Fig. 8a, Supplementary Fig. 7–8).

To support Y2H results and prove the direct interaction between proteins, we performed an in vitro binding experiment and tested several combinations of proteins that showed interaction in the Y2H assay. We used immobilized GST-tagged recombinant proteins (N- or C-terminal parts of the t- γ -TuC proteins, as indicated) as bait, which were mixed with ³⁵S-methionine-labeled Sas4 or Grip91, respectively, produced by a coupled in vitro transcription-translation (IVTT) reaction followed by autoradiography (Fig. 8b–e, Supplementary Fig. 9). The in vitro binding experiments confirmed the results of the Y2H assay (Fig. 8a–e).

Both the localization and the interaction studies suggest the presence of different γ -TuRC/t- γ -TuC coexisting. To further investigate these complexes, we conducted biochemical isolation and analysis of the subunit composition and migration pattern of various γ -tubulin-containing complexes in testis samples. This involved fractionating protein complexes from testes extracts using Superose 6 size-exclusion chromatography (SEC)⁴⁶. As a control, we fractionated canonical γ -Tubulin complex components from embryo extract (0–5 h embryos contain somatic centrosomal MTOCs with canonical γ -TuRC, but without t- γ -TuRC proteins) of the γ -TuSC component GFP-Grip84 and γ -TuRC component GFP-Grip163, and probed them with anti-GFP, anti- γ -Tubulin and anti-p54 antibodies (Supplementary Fig. 10a–c, 11). To estimate the relative molecular weights of the analysed protein complexes, we compare them to the SEC profile of p54/Rpn10, the ubiquitin receptor subunit of the 2.5 MDa 26S Proteasome⁴⁷. The same extraction and fractionation method was used to analyse testes extracts by SEC, and to investigate the presence of different t- γ -TuC in each fraction. We analysed GFP-Grip163 and GFP-Grip84, γ -Tubulin and members of the testis-specific complexes, HA-t-Grip91 and t-Grip84-mCh. Their SEC profiles suggest that they are part of protein complexes, based on that they co-migrate both in the high molecular weight fractions and partially co-migrate (their peaks partially overlap) in the lower molecular weight

Fig. 6 | γ -TuSC protein GFP-Grip84 is localizing to the Golgi in spermatocytes. a GFP-Grip84 colocalizes with centrosomal protein Asl (anti-Asl, white arrow) and also to the Golgi apparatus (white arrowhead). **b, c** GFP-Grip84 colocalizes with antibody-stained *cis*-Golgi marker GM130 in the spermatocytes (arrowheads). **d, e** Rab2-YFP localizes to the Golgi (arrow) in spermatocytes visualized by the *trans*-Golgi marker GCC88 and also to the apical end of the elongating nuclei (arrowhead). (Scale bars: a, c, d 10 μ m, B, E20 μ m).



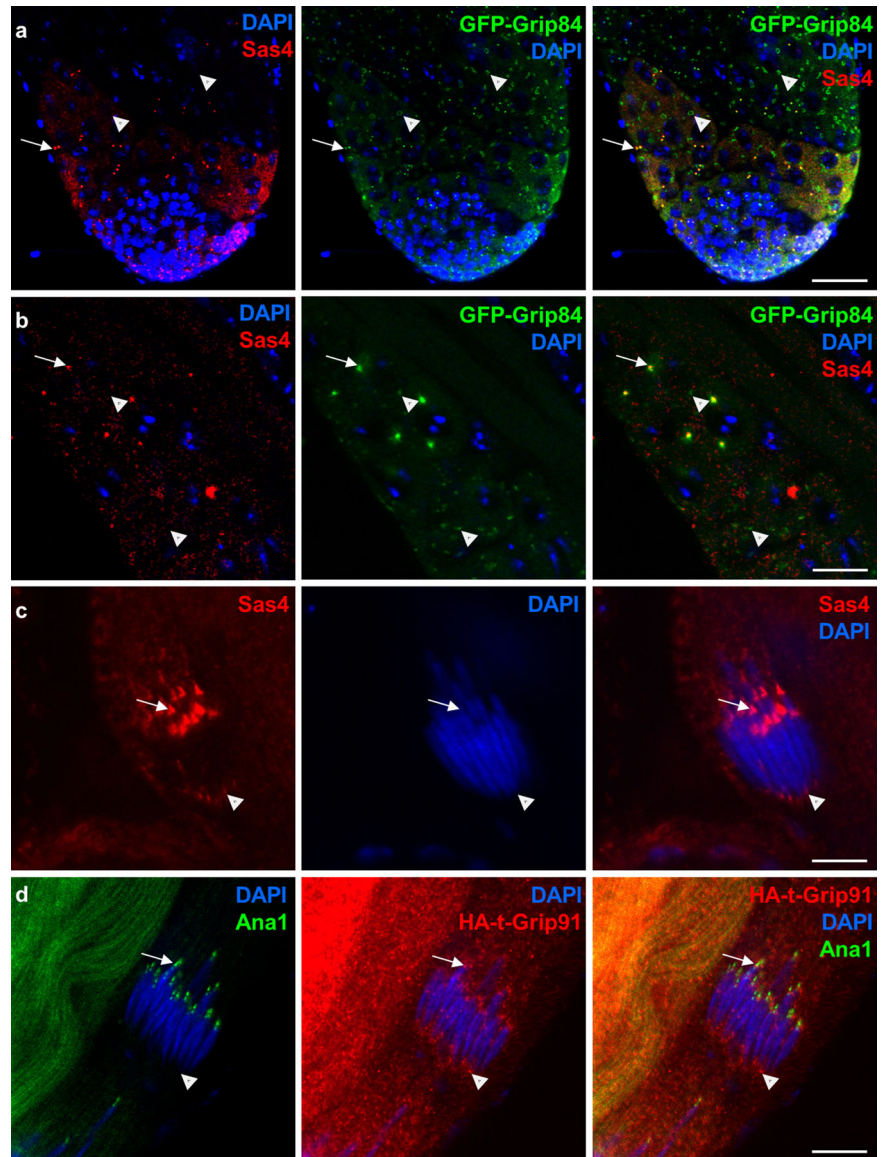
fractions. Although these fractions do not entirely overlap with those of canonical Grip proteins, they are distinctly separated from the free, non γ -TuRC associated gamma-tubulin fractions (fraction 36-39), where we expect to see individual proteins, suggesting that t-Grip proteins are part of a testis-specific proteins complexes (Supplementary Fig. 10a–c, 11).

Discussion

Drosophila spermiogenesis and spermatogenesis represent an ideal model for studying mitotic and meiotic cell division, centrosome and basal body formation and dynamic organelle reorganization after meiosis. It was proven to be a good model for understanding centrosomal and non-

centrosomal MT nucleation due to the intensive morphological changes and organelle movement in the post-meiotic spermatids^{13,14,18}. MT nucleation was reported, among others, at the surface of the Golgi⁴⁸, mitochondria¹⁸, nuclear envelope⁴⁹ and plasma membrane⁵⁰ in mammalian cells. We previously identified and described the function and versatile localization of three testis-specific γ -TuC proteins in the post-meiotic spermatids¹⁴ (Fig. 9). The described centriole adjunct, nuclear tip and mitochondrial surface localization of the t- γ -TuC proteins and their interaction partner Mzt1 raised the question of whether the ubiquitously expressed γ -TuRC is replaced after meiosis for the testis-specific one or both are present due to the multiple suggested ncMTOC formation during

Fig. 7 | Sas4 is a component of the site, which has been suggested as a ncMTOC at the apical tip of the elongating nuclei. a, b Antibody-stained Sas4 (red) colocalizes with the endogenous tag GFP-Grip84 at the centrosome (arrows) but not on the Golgi (arrowheads) in the spermatocytes. **c** Sas4 (red) is localizing to the basal body of the elongating spermatids (arrows) and additionally to the apical tip of the nuclei (arrowheads). **d** Antibody-stained Ana1 localizes only to the basal body (arrows) of the elongating spermatids, while the t- γ -TuC component HA-t-Grip91 localizes to the basal body (arrow) and the apical tip (arrowheads) of the elongating spermatids. (Scale bars: 20 μ m).



spermatid elongation and acrosome formation. γ -Tubulin was already reported to localize to both tips of the elongating nuclei and to the surface of the giant mitochondria during elongation^{14,18,38}. The testis-specific splice variant of Centrosomin (Cnn), CnnT, was also reported to promote non-centrosomal MT growth on the surface of the mitochondrial derivative of spermatids, and the N-terminal region of Cnn-T was shown to bind efficiently to γ -TuRCs, but there was no data about the canonical γ -TuRC localization after the meiotic stages of spermatogenesis^{18,51}. Mutants for the small complex components Grip91 and Grip84 are lethal and have defects in spindle assembly^{21,22}, which indicates their essentiality for the whole fly development. It has been demonstrated that the small complex is the minimal unit needed for MT nucleation⁷. However, RNAi depletion of the ubiquitously expressed Grip163 results in loss of Grip84 recruitment to the ncMTOC in tracheal cells⁹.

Our experiments showed that, in contrast to the essentiality of the γ -TuSC, the γ -TuRC member Grip163 is only essential for fertility, similar to the already characterized γ -TuRC members Grip75 and Grip128²³. From the elongation and basal body stability defects of *Grip163* mutant spermatids, we inferred that the presence of Grip163 protein is essential after meiosis. This was supported by the localization of GFP-Grip163, as we detected its presence in the localization foci of t- γ -TuC members after

meiosis. These results raised the possibility that t- γ -TuC and the canonical γ -TuRC may be present simultaneously, even as a mixed complex, in the aforementioned possible ncMTOCs (Fig. 9). This hypothesis was further supported by the fact that Grip84, a member of γ -TuSC, also exhibits an overlapping localization pattern in the late stages of spermatogenesis and the physical interaction between t-Grip84 and the ubiquitously expressed Grip91. Our results strongly suggest that both testis-specific and canonical γ -Tubulin binding complexes are involved in the formation of post-meiotic ncMTOCs. Although we observed very similar enrichment patterns of the different complex members in testis samples, they were not identical to those of the embryo samples in SEC, suggesting different complexes in somatic cells and testis. Both t-Grip84 and t-Grip91 proteins are expressed exclusively in the testis furthermore they enrich in the high molecular weight fractions in SEC. Additionally, the interactions between the testis-specific and canonical complex members in Y2H and GST-IVTT pull-down experiments suggest the existence of testis-specific complexes. Despite these findings, the precise composition of either mixed or pure t- γ -TuSC and t- γ -TuC in the post-meiotic stages of spermatogenesis is not determined and is an exciting and rather challenging question for the future. Considering both the *in vivo* localization patterns and the *in vitro* interaction findings, we suggest a versatile composition of gamma-tubulin complex formation

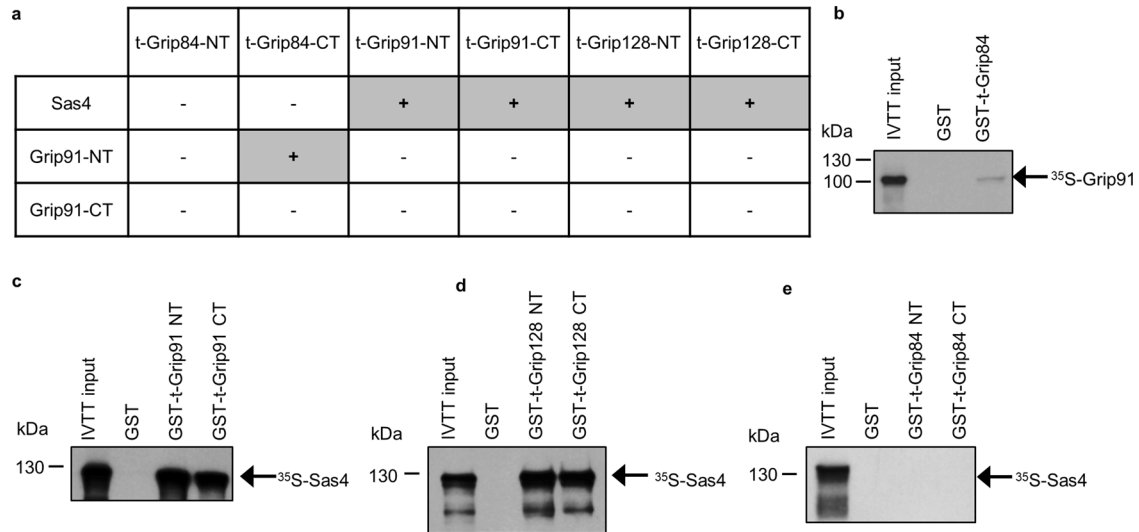
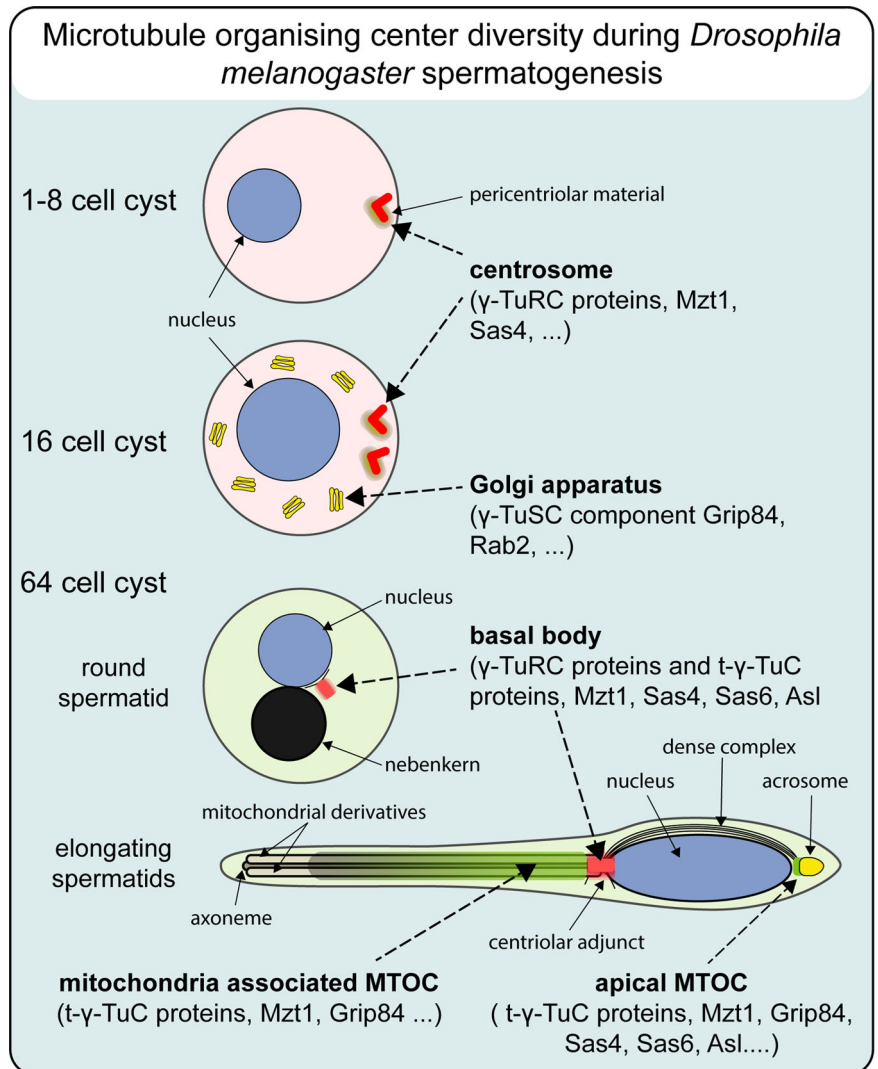


Fig. 8 | Interactions of t- γ -TuC proteins with Sas4 and the canonical Grip91.
a Summary of Y2H analysis of the N- or C-terminal parts of t- γ -TuC proteins (as indicated in the table) with Sas4, and the N- or C-terminal parts of Grip91, respectively. **b–e** Autoradiographic images show the presence or absence of

interaction between purified and immobilized GST-t- γ -TuC protein fragments and ^{35}S -labeled Sas4 or Grip91, synthesized by in vitro transcription-translation (IVTT) reaction.

Fig. 9 | Summary of localization of γ -TuRC and t- γ -TuC (t-Grip84, t-Grip91, t-Grip128) containing MTOCs during different stages of spermatogenesis. γ -TuRC and interactor proteins localize to the centrosome. γ -TuSC member Grip84 localizes to the Golgi apparatus of spermatocytes. Both γ -TuRC and t- γ -TuC present on the basal body and t- γ -TuC, Grip84, and Mzt1 also localize near the elongating spermatids' mitochondria. t- γ -TuC, Grip84, Mzt1, Sas4, Sas6, and Asl enrich at the apical tip of the post-meiotic elongating nuclei.



occurring in distinct MTOCs, like at the basal body, on the mitochondrial surface, and at the apical tip of the nucleus in *Drosophila* spermatids. Nevertheless, the biochemical isolation, identification of additional testis-specific interacting proteins, and the functional diversification of these MTOCs present a significant challenge for upcoming research.

Considering the localization and mutant phenotype of canonical Grip163 and testis-specific t-Grips, it can be assumed that the larger amount and diverse composition of gamma-tubulin complex is required for the cellular reorganization of the elongating spermatids. Based on the mutant phenotypes of the individual complex members, γ -TCs could be essential to the stable attachment of the basal body/centriole adjunct to the sperm nucleus and for the nucleation and stabilization of Tubulins contributing to the formation of the nearly 1,8 mm long axoneme and also to the nucleation of the axoneme independent microtubules around the elongating mitochondrial derivatives^{13,14,28}.

In vivo tagging of Grip84 allowed us to describe the Golgi localization of the γ -TuSC component Grip84 in primary spermatocytes, in addition to its extensively studied centrosomal localization²². Until now γ -Tubulin containing Golgi outpost-associated MT nucleation sites was only described to regulate distal dendritic branching in *Drosophila* neurons⁵². Golgi non-centrosomal MT assembly is mediated by the γ -Tubulin-interacting AKAP450 protein, which is anchored by the cis-Golgi-associated GM130 protein. This provides evidence that the Golgi apparatus may become a major site of MT nucleation by acting as a γ -TuRC docking site in mammalian cells by a mechanism similar to that in the centrosome⁵³. Our results suggest that a similar mechanism could be present in the primary spermatocytes. Further experiments are needed to determine exactly which isoform of the Grip84 protein is associated with the Golgi (Fig. 9).

All three proteins of the γ -TuSC (Grip84, Grip91 and γ -Tubulin) interact with the constitutively active, GTP-bound Rab2 protein³⁶, and Rab2 and Grip84 both localize to both the Golgi and the apical end of the nucleus, suggesting a role for γ -TuSC and Rab2 in the formation of the Golgi and lysosome-associated acrosome. Rab2A and Rab2B proteins were shown to be components of the proacrosomal membrane and essential for acrosome formation and male fertility in mice³⁷.

Drosophila Rab2 is an essential gene and recruitment of Rab2 takes place on the membranes of late endosomes, where it controls the fusion of LAMP-containing biosynthetic carriers and lysosomes to late endosomes in somatic cells⁵⁴. t-ER-Golgi units contribute to the formation of the acroblast, which forms the acrosome by fusing lysosomal structures during nuclear elongation in *Drosophila*⁵⁵. The molecular determinants of acrosome formation are hardly known in *Drosophila*, however, several retrograde tethering complexes were described in the formation of a functional Golgi ribbon in the acroblast (Fári et al. ⁵⁵). Acrosomal proteins such as Sneaky, Lamp1 and Rab2, as well as the ubiquitous and testis-specific γ -TuRC, its partner proteins (Mzt1, Sas4) and other centrosomal proteins (Asl, Sas6), are all localized to the apical tip of the nucleus. Further biochemical studies are needed to clarify the precise interactions between the aforementioned proteins. Nevertheless, these results raise the possibility of a link between the ncMTOC and acrosome organization, which is present at the dense complex forming perinuclear MTs at the apical end of the nuclei (Fig. 9).

Materials and methods

Fly stocks, mutants, and fertility test

Flies were maintained on cornmeal agar medium at 25 °C in standard laboratory conditions. Fly stocks used in this study were obtained from Bloomington *Drosophila* Stock Center, except stated otherwise: *w*¹¹¹⁸ used as wild-type control (3605, BDSC), *w*[1118];*Df*(3 L)BSC574 (25125 BDSC), *P*{UASp-YFP.Rab2.Q65L}02 (9760, BDSC), *P*{DJ-GFP.S}AS1/CyO (5417, BDSC). *bam*-Gal4 testis specific driver⁵⁶ was used (kindly provided by Helen White-Cooper) to express Rab2 in the germline. The β 2-tubulin-GFP line was provided by David M. Glover. GFP-PACT line was obtained from Jordan Raff's²⁹. *Mzt1*-GFP, HA-t-Grip91 and t-Grip84-mCh were described before¹⁴.

To establish the mutant allele *Grip163*^{Δ11} the pCFD5-Grip163-gRNA line was crossed with *vas*-Cas9 source line to generate deletion in *Grip163*. The deletions were identified by *Grip163* specific PCR and further confirmed by direct sequencing. *Grip163*^{Δ11} line was established and used for further analysis.

To establish the genomic tagged lines of *Grip84* and *Grip163* pCFD5-Grip84-gRNA and pCFD5-Grip163-gRNA lines were crossed with the *vas*-Cas9 source lines, *w*[1118]; *P*Bac{y[+mDint2] GFP[E.3xP3]=*vas*-Cas9}VK00027 (BL51324), *y*[1] *M*{RFP[3xP3.PB] GFP[E.3xP3]=*vas*-Cas9}ZH-2A *w*[1118]/FM7c (BL51323) respectively, progeny of this cross were injected with the homology arm carrying pJET1.2 plasmid (Thermo Fisher Scientific). Transgenic candidates were identified by gene specific PCR and confirmed by sequencing of the insertion sites. For fertility tests, individual males were crossed with three *w*¹¹¹⁸ virgin females. After 3 days the parents were removed and 14 days after crossing, the hatched progeny was counted in every tube. Experiments were repeated 4 times with >10 males per condition.

Protein alignments

Protein sequences of human GCP proteins were acquired from NCBI. *Drosophila* Grip protein's sequences were retrieved from FlyBase. Multiple sequence alignment with ClustalW and determination of sequence similarity was conducted in Unipro UGENE 1.31.1.^{57,58}

Predicted 3D structures of *Drosophila* Grip proteins (Q9VTS3, Q9VXU8, Q8IRA1, Q4LDP4, Q9XYP8, Q9VKU7, Q9VS91, Q9XYP7) were downloaded from AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk/>). Human 6V6S Ring complex model was downloaded from Protein Data Bank (<https://www.rcsb.org/>). Model alignment and visualization were conducted using UCSF ChimeraX 1.6⁵⁹.

DNA constructs

To make the genomic GFP-Grip84, and GFP-Grip163 line, annealed oligos of the sgRNAs (ATCCTTGAGCGGCTACCGCT), (GTGCTTCTCCGGTCCAGGCT) for Grip84 and Grip163 respectively were ligated into pCFD5 vector (Addgene, 112645) at Bbs1 restriction site. To establish the homology arms 1534 bp upstream, 1529 bp downstream, 1515 bp upstream and 1738 bp downstream of the intended GFP insertion site for Grip84 and Grip163 respectively in addition to GFP were amplified using High fidelity DNA polymerase. The purified PCR products were cloned into pJET1.2 plasmid (Thermo Fisher Scientific) using HiFi DNA Assembly Master Mix (E2621S, NEB)⁶⁰. GFP sequence was introduced 187 bp upstream for Grip84, and 229 bp upstream of the Grip163 sgRNA. Stable transgenic lines were established as described in Port et al.^{61,62}.

For Y2H analysis t-Grip84-NT (1-399 aa) t-Grip84-CT (400-728 aa), t-Grip128-NT (1-550 aa), t-Grip128-CT (551-975 aa) and t-Grip91-NT (1-966 aa), t-Grip91-CT (967-1932 aa) were cloned into pGAD424¹⁴. Full-length of Sas4 (1-901 aa), Grip84 (1-833 aa (Grip84-PD)), Grip75 (1-650 aa) and Grip91-NT (1-545 aa), Grip91-CT (546-917 aa), Grip163-NT (1-685 aa) Grip163-CT (686-1351 aa), Grip128-NT (1-585 aa) Grip128-CT (586-1092 aa) were cloned into pGBT9, using HiFi DNA Assembly Master Mix (E2621S, NEB). To generate GST-tagged bait proteins for the in vitro pull-down assay, full-length Grip84, N- and C-terminal parts of t-Grip84, t-Grip91 and t-Grip128 were inserted into the pDEST15 (11802014, Thermo Fisher Scientific) vector by Gateway cloning (11791-020, Thermo Fisher Scientific). Sas4 and Grip91 cDNAs were obtained from the *Drosophila* Gold collection (DGRC) and inserted into the pDEST17 plasmid (11803012, Thermo Fisher Scientific) by Gateway cloning. Oligonucleotide primers used in cloning are listed in (Supplementary Table 2).

Staining and microscopy

Preparation and staining of testes with formaldehyde or methanol:acetone fixation were performed as described by White-Cooper et al.⁶³. Briefly, testes were dissected and fixed in 4% formaldehyde for 25 min or semi-squashed using a coverslip on poly-L-lysine coated slides, then frozen with liquid

nitrogen and fixed for 5 min in ice-cold methanol followed by 5 min in acetone. After fixation samples were blocked and antibodies were added, the antibodies used in the study are listed in (Supplementary Table 1). Stained samples were mounted in SlowFade Gold antifade reagent (S36967, Life Technologies). Images were taken using Olympus BX51 fluorescent microscope or Olympus Fluoview Fv10i confocal microscope.

Testes for transmission electron microscopy were dissected and fixed overnight at 4 °C in 2% paraformaldehyde and 2% glutaraldehyde. After rinsing in phosphate-buffered saline (PBS), the testes were further fixed for 1 h in 2% OsO₄, followed by dehydration in increasing ethanol concentrations and rinsing in uranyl acetate and acetone. The samples were embedded in Embed812 (Electron Microscopy Sciences, Hatfield, PA, USA) then ultrathin (70 nm) sections were cut on an Ultracut S ultra-microtome (Leica, Wetzlar, Germany), which were mounted on copper grids. The grids were counterstained with uranyl acetate (Merck, Germany) and lead citrate (Merck, Germany) and were examined and photographed with a JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan). The images were processed with the TEM Center software (JEOL, Tokyo, Japan).

Yeast two-hybrid assay

Y2H assays were carried out using the Matchmaker two-hybrid system (K1605, Clontech). One of the baits pGBT9 was cotransformed with one of the preys pGAD424 into PJ69 yeast cells⁶⁴. Individual colonies were streaked out on yeast synthetic double drop-out plates that lacked tryptophan and leucine (Y0750, Merck), after growing, they were inoculated on double and triple dropout plates lacking (tryptophan, leucine, histidine, and adenine (Y2021, Merck) contains 5–10 mM 3-amino-1, 2, 4-aminotriazole (3-AT) (A8056, Sigma).

Recombinant protein production

Recombinant GST-tagged bait proteins were expressed, purified and immobilized onto glutathione sepharose beads as described by Karman et al.⁶⁵.

Coupled in vitro transcription-translation (IVTT) reaction and in vitro binding assay

³⁵S-methionine-labeled prey proteins (Grip91, Sas4) were produced in vitro using the TnT T7 Quick Coupled IVTT kit (L1170, Promega). The detailed protocol of the GST-IVTT binding assay is described in Réthi-Nagy et al.⁶⁶.

Gel-filtration chromatography and western blotting

Separation of γ -Tubulin-containing complexes were carried out by size-exclusion chromatography (SEC) based on the protocol published earlier⁶⁶ with slight modifications. All steps were carried out at 4 °C or on ice unless otherwise stated. Briefly, 25 mg of 0–5 h old dechorionized embryos (expressing GFP-Grip84 or GFP-Grip163) or 500 dissected testes (expressing HA-t-Grip91, t-Grip84-mCh, GFP-Grip84, or GFP-Grip163, respectively) were homogenized in 350 μ l HB buffer (50 mM K-HEPES pH 7.6, 75 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and EDTA-free protease inhibitor cocktail (PIC, Roche, #11873580001)) using a Dounce homogenizer. Ten strokes with a loose pestle followed by ten strokes with a tight pestle were applied, followed by further homogenization by a plastic pestle in microfuge tubes for 5 min. The samples were centrifuged at 15,000 \times g for 30 min at 4 °C. The supernatants were collected and centrifuged again at 21,000 \times g for 10 min at 4 °C. Three hundred microliters of clarified lysates were subjected to SEC using a Superose 6 HR 10/30 FPLC column (Cytiva) pre-equilibrated with HB buffer (without DTT, PMSF, and PIC). Forty-one fractions (0.3 ml each) were collected after 7 ml. Ice-cold acetone (2.1 ml) was added to each fraction, the samples were vortexed, and precipitation was carried out at –20 °C for 24 h followed by centrifugation at 17,000 \times g for 30 min at 4 °C. The precipitated proteins were resolved in Laemmli sample buffer, boiled for 5 min, and loaded onto SDS-PAGE, followed by western blot analysis using the indicated antibodies: anti-GFP mouse mAb (1:1000, Roche, #11814460001), anti-mCherry rabbit mAb

(1:1000, Abcam, #ab213511), anti- γ -tubulin mouse mAb (1:5000, Sigma, clone GTU-88, #T6557), anti-HA mouse mAb (1:40, Roche, clone 12CA5, #11583816001), and anti-p54/Rpn10 mouse mAb (1:400,⁴⁷). The following secondary antibodies were used in western blot: goat anti-mouse IgG conjugated to horseradish peroxidase (1:10,000, Dako, #P044701-2) and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000, Dako, #P044801-2).

Statistics and reproducibility

The exact numbers of repetitions, flies and tests tested for each experiment are indicated in the Source Data file and Figures and the Figure legends. All experiments were performed as two or more independent experiments. The same conclusions were obtained from each experiment. Nuclei and nebenkern were counted based on images taken by Olympus BX51. GFP-PACT signals and nebenkern were counted on living samples. GFP-PACT and γ -Tub intensity were measured by FV10-ASW Viewer software (Ver.4.2) on images taken with the same settings, fluorescence intensity was measured in the axis of the PACT-GFP signal. Fluorescence intensity was adjusted based on background.

Data analysis and graph production was done using Origin 9.0 (OriginLab Corporation, Northampton, MA, USA). One-way ANOVA followed by Tukey's HSD test was used to comparisons between WT and mutants. Additional visualization was made using Python 3.0 seaborn library.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available in the article and its supplementary information. The raw datasets for all graphs and the uncropped images of the immunoblots presented in this study are provided as a Supplementary Fig. or Source Data file.

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Competing interests

The authors declare no competing interests.

Additional information

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