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The γTuRC components Grip75 and Grip128 have an essential microtubule-anchoring function in the *Drosophila* germline

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The γ-tubulin ring complex (γTuRC) forms an essential template for microtubule nucleation in animal cells. The molecular composition of the γTuRC has been described; however, the functions of the subunits proposed to form the cap structure remain to be characterized in vivo. In *Drosophila*, the core components of the γTuRC are essential for mitosis, whereas the cap component Grip75 is not required for viability but functions in *bicoid* RNA localization during oogenesis. The other cap components have not been analyzed in vivo. We report the functional characterization of the cap components Grip128 and Grip75. Animals with mutations in *Dgrip128* or *Dgrip75* are viable, but both males and females are sterile. Both proteins are required for the formation of distinct sets of microtubules, which facilitate *bicoid* RNA localization during oogenesis, the formation of the central microtubule aster connecting the meiosis II spindles in oocytes and cytokinesis in male meiosis. Grip75 and Grip128 anchor the axoneme at the nucleus during sperm elongation. We propose that Grip75 and Grip128 are required to tether microtubules at specific microtubule-organizing centers, instead of being required for general microtubule nucleation. The γTuRC cap structure may be essential only for non-centrosome-based microtubule functions.

KEY WORDS: γ-Tubulin ring complex, *Drosophila*, *bicoid* RNA localization, Meiosis, Spermatogenesis

INTRODUCTION

γ-Tubulin is essential for microtubule nucleation in vivo (Wiese and Zheng, 1999). Two γ-tubulin containing complexes, the γ-tubulin small complex (γTuSC) and the γ-tubulin ring complex (γTuRC), have been isolated from a variety of sources (Murphy et al., 1998; Oegema et al., 1999; Zheng et al., 1995). The *Drosophila* γTuSC, which contains γ-tubulin, Grip91 and Grip84, displays low microtubule-nucleating activity in vitro (Oegema et al., 1999). The larger γTuRC consists of a lockwasher-like structure and a globular cap that decorates one end of the complex (Moritz et al., 2000). It contains several γTuSCs and Grip71, Grip75, Grip128 and Grip163 (Gunawardane et al., 2000; Gunawardane et al., 2003; Oegema et al., 1999). The γTuSC has been suggested to form the subunits of the lockwasher, whereas the remaining Grip proteins may build the cap (Moritz et al., 2000; Zhang et al., 2000). The γTuRC is associated with microtubule minus ends, possesses high microtubule-nucleating activity in vitro and forms a template for microtubule nucleation in vivo (Moritz et al., 2000; Zheng et al., 1995).

The structural organization of the γTuRC into the lockwasher and the cap may reflect a functional subdivision. The components of the γTuSC appear to be required for microtubule organization. In *Drosophila*, mutations in *Grip91* (*l(1)dd4* – FlyBase) or *Grip84* are lethal and display defects in spindle assembly (Barbosa et al., 2000; Colombie et al., 2006). *Drosophila* has two *γ-tubulin* genes, *γTub23C* and *γTub37C*. Whereas γTub37C expression is restricted to the female germline and the early embryo, γTub23C is almost ubiquitously expressed and crucial for mitosis (Sunkel et al., 1995; Tavosanis et al., 1997). In contrast to the γTuSC components, the function of the cap components is poorly understood. Although in vitro data suggest that only the γTuRC but not the γTuSC provides high microtubule-nucleating activity, null mutations in the γTuRC component *Grip75* are viable (Schnorrer et al., 2002). Furthermore, depletion of cap components by RNAi in S2 cells results in mild mitotic defects (Verollet et al., 2006). These data suggest that either the γTuSC can nucleate microtubules in vivo to an extent that is sufficient for life, or that Grip75 is dispensable for γTuRC function in microtubule nucleation.

γTub37C and Grip75 are essential for the microtubule-dependent localization of *bicoid* (*bcd*) RNA to the anterior cortex of the *Drosophila* oocyte (Schnorrer et al., 2002). In the oocyte, *bcd* RNA initially localizes in a ring at the anterior cortex. At stage 10b, a transition into a disc-like localization pattern occurs. *bcd* RNA remains at the anterior cortex until the egg is laid (St Johnston, 2005). In *Grip75* and *γTub37C* mutant oocytes, relocalization during stage 10b fails and *bcd* RNA diffuses away from the anterior cortex (Schnorrer et al., 2002). Grip75 and γTub37C are concentrated together with *bcd* RNA at the anterior cortex at this stage, and, thus, it has been proposed that a new microtubule-organizing center (MTOC) assembles at the anterior cortex at stage 10b.

Are Grip71, Grip128 and Grip163 required for the same processes as Grip75, or do the individual subunits have different functions from Grip75? As *Grip71*, *Grip128* or *Grip163* mutants were not available, it was unclear if the *Grip75* mutant phenotype resembles a 'cap-null' situation, and why the cap structure of the γTuRC was not essential for the microtubule-nucleating activity of the γTuRC.

We have isolated mutants in *Grip128*, which mislocalize *bcd* RNA during late oogenesis in the same way as *γTub37C* and *Grip75* mutants. *Grip75* and *Grip128* mutants are viable but display

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defects in male and female meiosis, as well as in sperm motility. We provide evidence that a γTuRC forms in *Grip128* and *Grip75* mutants, suggesting that the γTuRC is functional in microtubule nucleation without the full cap structure. However, specific functions of the γTuRC require the additional proteins Grip128 and Grip75. We propose that Grip128 and Grip75 anchor the γTuRC at special MTOCs, rather than being essential for microtubule nucleation.

MATERIALS AND METHODS

Fly strains

The wild-type stocks were Oregon R* or *y w*. We used the following mutant alleles or transgenes: swa^{VA11} (Schnorrer et al., 2000), $Grip75^{175}$ and $\gamma Tub37C^{139}$ (Schnorrer et al., 2002), $Grip128^{326}$ and $Grip128^{352}$ (this study), and *NZ143.2* (Clark et al., 1997). Transgenic flies were generated in a *y w* background according to standard methods. Germline clones were induced by incubating third instar larvae grown in vials once for 1 hour or in bottles twice for 2 hours on 2 consecutive days in a 37°C water bath. Homozygous mutant *Grip128* females were generated by rescuing the sterility of *Grip128* mutant males with the genomic rescue construct and backcrossing to *Grip128* heterozygous females.

Screening procedure

Grip128 mutants were isolated in the course of an F1 screen designed to identify new mutants that disrupt *bcd* RNA localization during oogenesis. Screening was carried out as described (Luschnig et al., 2004; Schnorrer et al., 2002) with the modification that we established lines from females that produced eggs with early arrest phenotypes, but also with other strong developmental defects. Mutations were induced on a *w f hs-Flp122 FRT9-2* chromosome.

Identification of *Grip128* mutants

The mutation *X-326* was mapped between 14,650 kb and 15,591 kb on the physical map (release 3.2) using a combination of conventional and SNP-based meiotic mapping (Berger et al., 2001). Details are available upon request. We PCR-amplified suitable fragments from genomic DNA and sequenced the candidate gene *Grip128* from *X-326*, *X-352* and the parental chromosome.

Molecular biology

For the genomic rescue construct, *Grip128* was amplified from genomic DNA and cloned into pCaSpeR4 using a *Xba*I compatible *Nhe*I site about 1.4 kb upstream of the start codon and a *Xho*I site (underlined) introduced with primer aactcgagtgaggagttcgagtgaggttttg.

Protein biochemistry

Preparation of ovarian extracts, protein expression analysis and immunoprecipitations were performed as described (Schnorrer et al., 2002). We used extract buffer [50 mM HEPES-KOH pH 7.6, 75 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.05% NP-40, 1 mM DTT, 1 mM PMSF, protease inhibitor mix (Roche)] for all experiments. Sucrose density gradients were prepared as described (Moritz et al., 1998), with the following modifications. Ovarian extract (100 μl) was loaded onto each 5-40% sucrose gradient (50 mM HEPES-KOH pH 7.6, 75 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT), and the gradients were centrifuged at 237,000 ***g*** in a SW60 rotor (Beckman) for 4 hours at 4°C. The ribosomal profile was measured at 260 nm, and the peak of the small ribosomal subunit was used as a 40 S size standard. Homozygous mutant tissue was used for all biochemical assays. For detection, we used the following primary antibodies: rabbit anti-Grip128 (1:5000) and anti-Grip163 (1:5000) (Gunawardane et al., 2000); rabbit anti-Grip91 (1:2000), anti-γ-tubulinC12 (1:5000) and anti-Grip84 (1:2000) (Oegema et al., 1999); rabbit anti-Grip75 affinity-purified (1:2000) (Schnorrer et al., 2002); rabbit anti-Swa serum (1:20000) (Schnorrer et al., 2000); and mouse anti-γ-tubulin GTU88 (1:5000) (Sigma). Both γ-tubulin antibodies show the same specificity on western blots. Primary antibodies were detected with goat anti-mouse-HRP (1:5000) (Dianova), donkey anti-rabbit-HRP (1:10000) (Amersham) or ProteinA-HRP (1:5000) (Amersham) followed by enhanced chemiluminescence.

Cytology

In situ hybridization and analysis of cytoplasmic streaming were performed as described (Schnorrer et al., 2002). Stage 14 oocytes were fixed as described (Tavosanis et al., 1997) and stained with 1 μg/ml DAPI.

For microtubule staining in oocytes, ovaries were dissected and fixed in methanol, rehydrated into PBT (PBS containing 0.1% Tween-20) and blocked in 5% normal goat serum (NGS). Incubation with FITC-conjugated anti-α-tubulin DM1A (Sigma) at 1:100 was overnight at 4°C. Ovaries were then washed in PBT, dehydrated in methanol and embedded in a 2:1 mixture of benzyl benzoate and benzyl alcohol.

For other oocyte staining, ovaries were fixed in 4% paraformaldehyde in PBT, washed in PBTx (PBS containing 0.2% Triton-X-100) and blocked in 5% NGS. Incubation with primary antibody was overnight at 4°C. Ovaries were then washed in PBTx, incubated with the secondary antibody for at least two hours, washed and embedded in Aqua/Polymount (Polysciences).

Zero- to 30-minute-old embryos were fixed in methanol/heptane, washed in methanol, rehydrated into PBT and further processed as above. Embryos were embedded in Aqua/Polymount or benzyl benzoate/benzyl alcohol.

Testes were dissected in testes buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl pH 6.8, 1 mM EDTA) and squashed on SuperFrost slides. The slides were frozen in liquid nitrogen, the coverslip was removed and the testes fixed in cold methanol. Testes were rehydrated into PBT and blocked in 5% NGS. Incubation with primary antibody containing 20 μg/ml RNAse A was for 1 hour at room temperature. Testes were then washed in PBT and incubated with the secondary antibody and 25 μg/ml propidium iodide for one hour. After washing, the testes were embedded in Aqua/Polymount.

We used the following antibodies: mouse anti-β-Gal (1:2000) (Promega), anti-γ-tubulin GTU88 (1:100) and anti-α-tubulin DM1A (1:1000) (Sigma), and goat anti-mouse-Al488 (Molecular Probes) (1:500). Images were collected on a confocal microscope (Zeiss LSM510).

Electron microscopy

Testes were prepared for electron microscopy with the DMSO-trialdehyde fixation method (Kalt and Tandler, 1971). Briefly, testes were incubated in fixative (100 mM sodium cacodylate, 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, 2.5% DMSO) for 30 minutes at room temperature and then kept on ice for further 3 hours. Samples were postfixed on ice with 1% osmium tetroxide in 100 mM phosphate buffer and then embedded in 2% agarose. The testes were contrasted with 1% tannic acid and then 1% uranyl acetate in water, dehydrated with ethanol, embedded in epon and sectioned for transmission electron microscopy. Images were acquired with a Philips CM10 transmission electron microscope at 60 kV.

RESULTS

A genetic screen for factors involved in *bcd* RNA localization

exuperantia, *swallow* (*swa*) and *staufen* are involved in the localization of *bcd* RNA to the anterior pole of the oocyte during oogenesis (St Johnston, 2005). In an F1 screen designed to identify lethal and/or early embryonic arrest mutants with defects in *bcd* RNA localization, mutants in *Grip75* and *γTub37C* were identified (Luschnig et al., 2004; Schnorrer et al., 2002).

To identify additional mutants that disrupt *bcd* RNA localization, we extended the F1 screen to the X chromosome. In brief, males were mutagenized and crossed to females containing a GFP marker on the X chromosome. Using the Flp-FRT system, clones were induced in the germline by mitotic recombination. Eggs derived from homozygous mutant germline clones were identified by the absence of GFP fluorescence. When eggs showed defects in their development, lines were established from sibling eggs derived from heterozygous germline clones. These resulting lines were again tested for their phenotype using the Flp-FRT/DFS system that eliminated all but homozygous mutant clones (Chou and Perrimon, 1992; Chou and Perrimon, 1996), and upon confirmation, ovaries were assayed for *bcd* RNA localization.

Nine and a half thousand females produced non-fluorescent progeny that were scored for developmental defects. 174 lines were established and screened by in situ hybridization. In this paper, we describe two mutant alleles of the same gene, *X-326* and *X-352*, with a specific defect in *bcd* RNA localization at stage 10b. Both *X-326* and *X-352* mutants are viable but male and female sterile. Eggs derived from homozygous mutant germline clones do not undergo nuclear divisions, as judged by DIC microscopy of embryos under oil (data not shown).

Identification of *Grip128* mutants

To identify the gene disrupted in the *X-326* and *X-352* mutants, we mapped *X-326* by meiotic recombination between the visible markers *garnet* and *forked*. To refine our mapping, we used single nucleotide polymorphisms and mapped the mutation between 14,650 kb and 15,591 kb on the physical map (release 3.2). A candidate gene in this interval was *Grip128*. Sequencing this gene revealed nonsense mutations in the *X-326* mutant (Gln^{662}→stop) and in the *X-352* mutant (Gln^{706}→stop) (Fig. 1A).

To support these data, we generated a 5.2 kb genomic rescue construct encompassing 1.4 kb of upstream sequences, the full coding region, the introns and the predicted 3′ untranslated region of the *Grip128* gene. The corresponding transgene fully rescued the male and female sterility of both mutants. Therefore, we conclude that the mutations causing the sterility in the *X-326* and *X-352* mutants are in the *Grip128* gene and we named the two mutants $Grip128^{326}$ and $Grip128^{352}$.

To confirm the predicted truncations, we analyzed Grip128 expression in wild-type and mutant ovarian and male extracts. An antibody specific to Grip128 recognized a protein at the expected size of ~130 kDa in wild-type ovaries and males (Fig. 1B) (Gunawardane et al., 2000). However, we could not detect any protein of the wild-type or predicted truncated sizes of 76 kDa and 81 kDa in extracts of $Grip128^{326}$ and $Grip128^{352}$ mutant ovaries or males, even though the available antibody was generated against the first 200 amino acids of Grip128 (Gunawardane et al., 2000). The additional bands detected by the antibody are not specific, as an antibody we raised against amino acids 192-479 shows a different background pattern (data not shown). $Grip128^{326}$ over the deficiency In(1)AC2[L]AB[R], which uncovers *Grip128*, is viable and shows the same *bcd* mislocalization phenotype as $Grip128^{326}$ or $Grip128^{352}$ homozygotes (data not shown). We therefore conclude that both alleles are protein-null alleles and also behave genetically as null alleles.

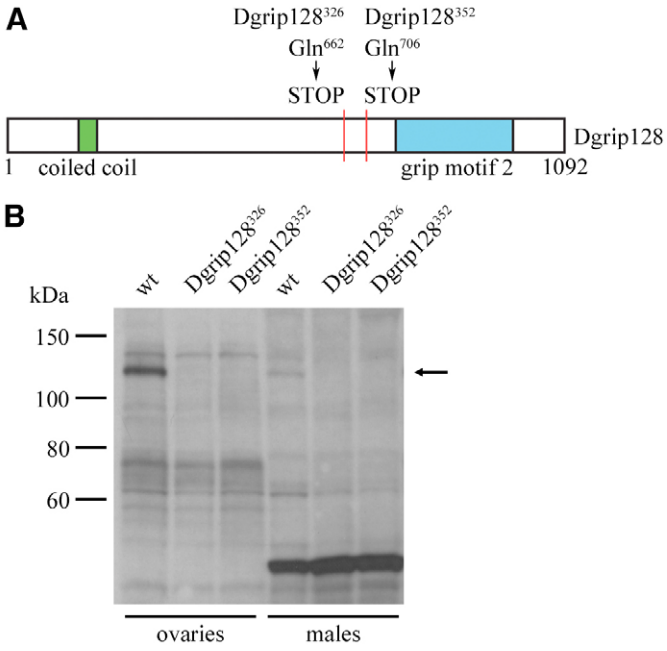

Fig. 1. Molecular characterization of *Grip128* mutant alleles. (**A**) Schematic overview of the Grip128 protein and the mutant alleles. Motifs are indicated (Gunawardane et al., 2000). C to T changes result in STOP codons replacing Gln^{662} in $Grip128^{326}$ and Gln^{706} in $Grip128^{352}$. (**B**) Wild-type and homozygous mutant ovarian and male extracts were analyzed by western blotting with a Grip128 antibody (Gunawardane et al., 2000). Grip128 is indicated by an arrow. Additional bands are non-specific.

bcd RNA localization requires Grip128

Grip75 and γTub37C are required for *bcd* RNA relocalization in stage 10b (Schnorrer et al., 2002). To determine whether Grip128 has a similar function, we analyzed *bcd* RNA distribution in *Grip128* mutant oocytes and eggs. In wild-type and *Grip128* mutant oocytes, *bcd* RNA is localized in a ring at the anterior cortex prior to stage 10b (Fig. 2A,D). However, the transition into the disc-like pattern only partially occurs and *bcd* RNA then diffuses away from the anterior cortex in *Grip128* mutants (Fig. 2E). *bcd* RNA is completely unlocalized in mutant stage 12-13 oocytes (data not shown), whereas in *Grip128* mutant eggs, *bcd* RNA is distributed in a graded manner (Fig. 2F), which is probably due to the *bcd* RNA destabilizing activity of the posterior system. Hence, the defect in *bcd* RNA localization in *Grip128* mutants is identical to the defects of *Grip75* and *γTub37C* mutants, suggesting a similar role in the *bcd* RNA localization machinery. Moreover, the *bcd* mislocalization phenotype of $Grip128^{352}$;$Grip75^{175}$ double mutant oocytes is the same as in the single mutants (data not shown), without obvious differences in the strength of the phenotype.

Grip75 and γTub37C are enriched at the anterior cortex of stage 10b and 11 oocytes, and participate in a new MTOC, which directs the relocalization of *bcd* RNA (Schnorrer et al., 2002). To demonstrate a functional requirement of Grip128 in this MTOC, we analyzed Nod:βgal and microtubule distribution in wild-type and *Grip128* mutant oocytes. Nod:βgal is a marker for microtubule minus-ends, which recapitulates *bcd* RNA localization in wild-type oocytes (Clark et al., 1997; Schnorrer et al., 2002). In *Grip128* mutant oocytes, however, the Nod fusion is not enriched at the anterior margin in stage 11 (Fig. 2K), whereas the earlier ring-like localization pattern is indistinguishable from the wild-type pattern (data not shown). In wild-type stage 11 oocytes, microtubules extend from the center of the anterior cortex towards the lateral margin, whereas these microtubules are strongly reduced in *Grip128* mutant oocytes (Fig. 2G,H). By contrast, the subcortical microtubule array, which has been proposed to mediate cytoplasmic streaming (Theurkauf and Hawley, 1992), and cytoplasmic streaming itself appear to be normal in *Grip128* mutant stage 11 oocytes (compare Movies 1 and 2 in the supplementary material). Furthermore, nuclear migration and the organization of the microtubule cytoskeleton in oocytes prior to stage 10b are normal in *Grip128* mutant oocytes (data not shown). We conclude that Grip128, Grip75 and γTub37C establish a set of microtubules, which are presumably nucleated from the anterior pole and are essential for *bcd* RNA localization at stage 10b to 11.

To determine whether the defect in RNA localization is specific to *bcd* RNA, we analyzed the distribution of other localized transcripts such as *osk*, *grk* and *orb* RNAs. Both *osk* and *grk* RNA localization is unaffected in *Grip128* mutants throughout oogenesis

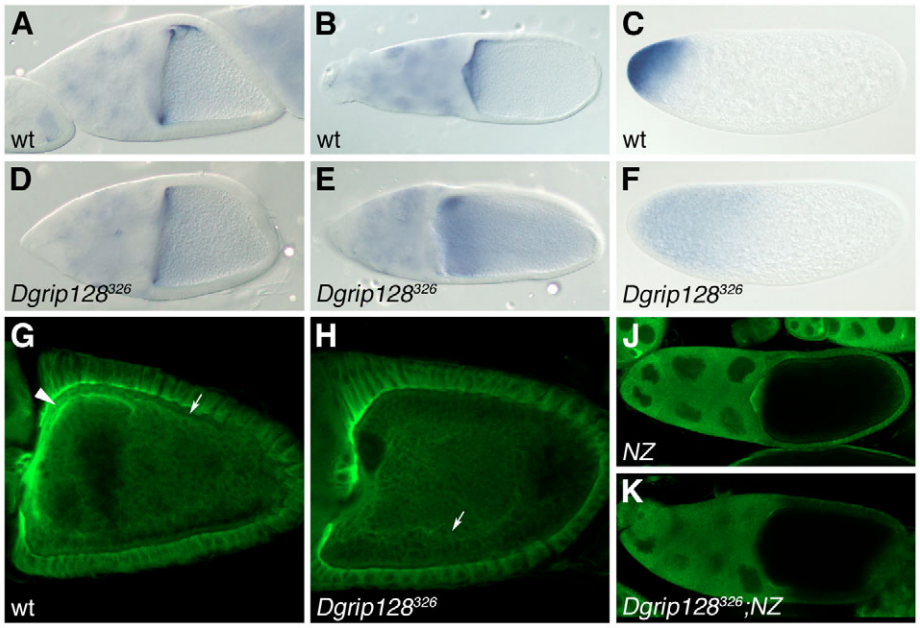

Fig. 2. *bcd* RNA localization requires *Grip128*. (**A-F**) Wild-type (A-C) and *Grip128*^{326} mutant (D-F) oocytes at stages 9 (A,D) and 11 (B,E), and 0- to 30-minute-old eggs (C,F) were stained for *bcd* RNA. (**G**,**H**) Wild-type (G) and *Grip128*^{326} mutant (H) oocytes at stage 11 were stained for microtubules. The anterior microtubule subset is indicated by an arrowhead and the subcortical microtubules by arrows. (**J**,**K**) Stage-11 wild-type (J) and *Grip128*^{326} mutant (K) oocytes carrying the *NZ* transgene were stained for the β-galactosidase reporter.

(see Fig. S1A-D in the supplementary material). Similarly, the localization of *orb* RNA is not disturbed in *Grip128* mutant oocytes, as the RNA initially localizes in a *bcd*-like pattern and is then lost from the anterior cortex during stage 10 in wild-type and mutant oocytes (see Fig. S1E,F in the supplementary material). Taken together, the observed microtubule defect does not result in a general defect in RNA localization.

Composition of the γTuRC during oogenesis

During oogenesis, the composition of γ-tubulin containing complexes has not been analyzed in detail. More importantly, it is unclear whether a γTuRC can form in *Grip75* or *Grip128* mutants.

As a first step towards the characterization of ovarian γTuRCs, we analyzed the expression of the Grips 163, 128, 75, 91 and 84, as well as γTub37C (Fig. 3A). Additionally, we analyzed Swa expression as Swa and the γTuRC have been shown to interact (Schnorrer et al., 2002). Both in wild-type and *swa*^{VA11} ovaries, all γTuRC components we tested were present, suggesting that early embryonic γTuRCs are similar in composition to ovarian γTuRCs. In *Grip128*^{326} and *Grip75*^{175} ovaries, the γTuSC components were present in equal amounts as in wild-type ovaries, whereas the levels of Grip163 were reduced (Fig. 3A). In *Grip75*^{175} ovaries, Grip128 levels were lower, and less Grip75 was present in *Grip128*^{326} ovaries compared with wild type (Fig. 3A). A reduction in the levels of Grip163 and Grip128 has also been observed in S2 cells depleted for Grip75 (Verollet et al., 2006). All of the γTuRC subunits were stable in *γTub37C*^{139} mutants (Fig. 3A), which is a null allele (N.V., I.K. and C.N.-V., unpublished). These data suggest that the cap subunits of the γTuRC depend on each other for their stability in ovaries.

Next, we immunoprecipitated γTub37C-containing complexes from ovarian extracts using an antibody specific to γTub37C. From wild-type extract, γTub37C co-immunoprecipitated with Grip91, Grip84, Grip163, Grip128 and Grip75 (Fig. 3B). In addition, Grip91 and Grip84 co-immunoprecipitated with γTub37C from *Grip75*^{175} or *Grip128*^{326} ovarian extracts, thus the γTuSC forms normally in these mutants.

We wondered whether the γTuSC might still provide microtubule-nucleating activity in vivo or whether a ring complex assembles with an incomplete cap structure. The latter possibility was already supported by co-immunoprecipitation, which showed that some Grip128 was associated with the γTuSC components in the *Grip75* mutant. As immunoprecipitation experiments do not reveal the sizes of γ-tubulin containing complexes, we performed sucrose density gradient centrifugation of wild-type, *Grip75*^{175} and *Grip128*^{326} ovarian extracts (Fig. 3C-E).

γTub37C and Grip128 were present in high molecular weight fractions in wild-type ovaries (Fig. 3C, fractions 9-14), as has been described for embryonic γTub37C and Grip128 (Gunawardane et al., 2000; Moritz et al., 1998; Oegema et al., 1999). Grip128 and γ-tubulin are part of at least two differently sized complexes of ~40 S and ~60 S. The embryonic γTuRC has been shown to have a size of ~37 S (Moritz et al., 1998), thus the 40 S complex in ovarian extract could correspond to the γTuRC. The nature of the larger complex is unknown, but it is possible that it consists of the γTuRC in association with attached MTOC material. Further experiments are necessary to resolve these issues. Both complexes are sensitive to high salt concentrations (e.g. 500 mM KCl; data not shown). Grip128 and γ-tubulin are also present as low molecular weight entities, which presumably correspond to the γTuSC and monomeric Grip128. We also detected a 40 S complex in extracts of *Grip75*^{175} or *Grip128*^{326} ovaries (Fig. 3D,E, fractions 9-10), whereas the larger complex is only present in very small quantities. High salt concentrations lead to the disassembly of these complexes (data not shown). In S2 cells depleted for cap components, severely reduced levels of γTuRC have been observed (Verollet et al., 2006). We also see a reduction in the amount of the large complexes, albeit less severe, which is presumably due to the less stringent salt concentration we use for our experiments, as we noted that the mutant complexes are more labile than the wild-type γTuRC. Although we cannot prove with certainty that the 40 S complexes in *Grip75* and *Grip128* mutants are indeed incomplete γTuRCs, this is a likely possibility because they are similar in size to the wild-type γTuRC and because of the presence of the γTuSC component γ-tubulin and the γTuRC component Grip128 in *Grip75* mutant complexes. The mutant complexes may still be capable of nucleating microtubules, providing an explanation for the observed viability of *Grip75* and *Grip128* mutants.

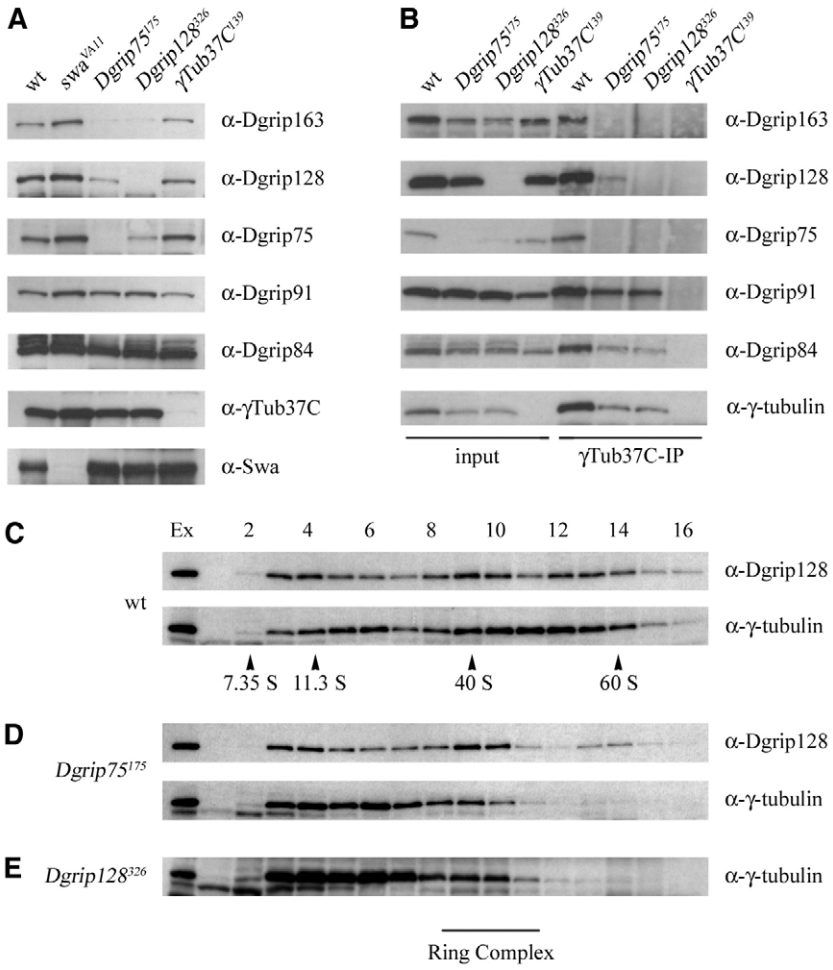

Fig. 3. Analysis of the ovarian γTuRC.
(**A**) Expression analysis of γTuRC components and Swa in ovarian extracts of the indicated genotypes. (**B**) Anti-γTub37C-immunoprecipitation from ovarian extracts of the indicated genotypes. (**C-E**) Wild-type (C), *Grip75*^{175} (D) and *Grip128*^{326} (E) homozygous mutant ovarian extracts were fractionated by 5-40% sucrose density gradient centrifugation. The first lane in each panel corresponds to the input, whereas subsequent lanes contain fractions obtained from top to bottom of the gradient.

Grip128 and Grip75 are required for meiosis in females

To better understand the function of *Grip75* and *Grip128*, we analyzed processes other than *bcd* RNA localization that depend on these genes. In *Grip75* or *Grip128* mutant eggs, we did not detect any nuclear divisions by DIC microscopy or DAPI staining (data not shown), which could be due to either meiotic or mitotic defects. To determine whether meiosis I was impaired in *Grip75* and *Grip128* mutants, we stained stage 14 oocytes with DAPI and analyzed the chromosome arrangement. In oocytes, meiosis is arrested at metaphase I until the egg is laid (King, 1970). In wild-type, *Grip75*^{175} and *Grip128*^{326} oocytes, chromosomes were arranged in a variable but symmetric fashion (Fig. 4A-C) (Theurkauf and Hawley, 1992). Thus, spindle formation in meiosis I appears normal in *Grip75* and *Grip128* mutants.

After passage through the oviduct, the first meiotic division is completed and meiosis II begins with the formation of two spindles in a tandem array, which are connected by a radial array of microtubules (Riparbelli and Callaini, 1996). In *Grip75*^{175} and *Grip128*^{326} eggs, the first meiotic spindle is anastral as in wild type and appears to function properly (Fig. 4D-F). By contrast, meiosis II is severely disrupted. The central array of microtubules is absent, and instead either two anastral spindles formed, which were not properly aligned to each other and to the cortex, or the spindles were strongly disorganized (Fig. 4H,J; data not shown). In older oocytes, the chromosomes dispersed and were associated with small, often misshaped, anastral spindles. Our results suggest that either the central MTOC is not present or that it does not organize microtubules in *Grip75* and *Grip128* mutant eggs.

Grip128 and Grip75 in male meiosis

Both *Grip128* and *Grip75* mutants are not only female sterile but also male sterile. We analyzed spermatogenesis by phase contrast microscopy in wild-type, *Grip128*, *Grip75* and double mutant spermatocytes (Fuller, 1993). At the onion stage, *Grip75*^{175}, *Grip128*^{352} and the double mutant spermatids often displayed a Nebenkern twice the size of a regular Nebenkern, which was associated with two nuclei (Fig. 5B,C; see Table S1 in the supplementary material). Occasionally, we observed some nuclei that were smaller than normal. The mitotic divisions prior to meiosis were not severely affected in single or double mutant males, as we did not observe pre-meiotic cysts with fewer than 16 cells (data not shown).

These observations are further supported by ultrastructural analysis. In ultra-thin sections of wild-type testes, each flagellum contains the axoneme and the associated mitochondrial derivative (Fig. 5D). However, we often observed flagella with two axonemes in *Grip75*^{175} and *Grip128*^{352} spermatids (Fig. 5E,F). Our data suggest that Grip75 and Grip128 might not be essential for mitotic divisions in the male germline, but that both proteins are crucial in male meiosis. More specifically, cytokinesis is impaired in male meiosis and, occasionally, chromosome segregation defects occur. However, we have not vigorously

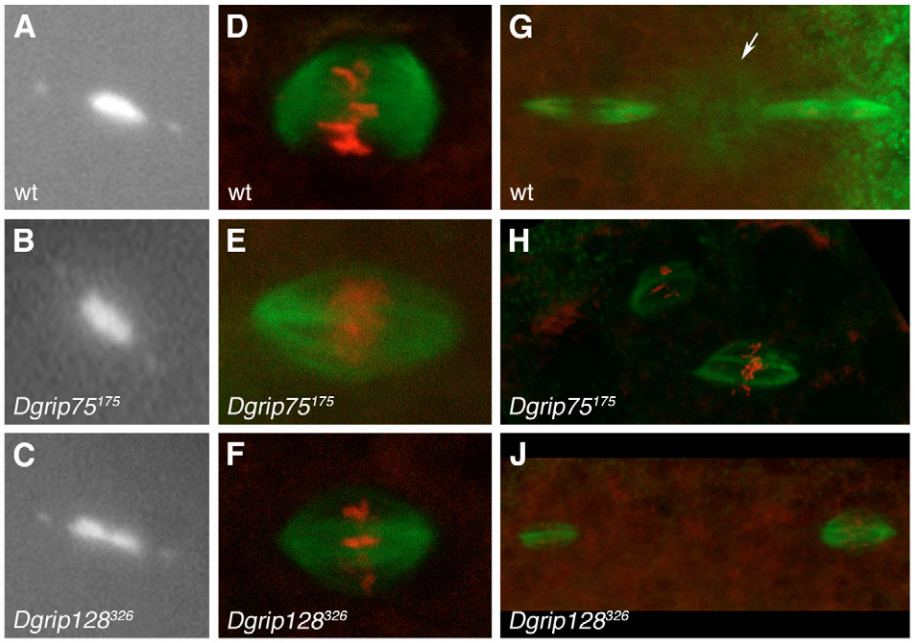

Fig. 4. Meiotic defects in *Grip75* and *Grip128* mutant eggs. (**A-C**) DAPI staining of wild-type (A), *Grip75*^{175} (B) and *Grip128*^{326} mutant (C) stage 14 oocytes. The chromosome arrangement is shown during metaphase arrest. (**D-F**) Meiosis I spindles in wild-type (D), *Grip75*^{175} (E) and *Grip128*^{326} mutant (F) eggs. α-Tubulin is shown in green and DNA in red. (**G-J**) Meiosis II spindles in wild-type (E), *Grip75*^{175} (H) and *Grip128*^{326} mutant (J) eggs, stained as in D-F. The arrow indicates the central MTOC in wild type. Confocal stacks were merged into single images.

excluded functions of Grip75 and Grip128 in pre-meiotic spermatocytes, therefore the observed meiotic phenotypes could also be due to unnoticed mitotic defects.

Chromosome segregation and cytokinesis depend on an intact meiotic spindle. In *Grip75*^{175} and *Grip128*^{352} spermatocytes, metaphase spindles are formed normally (Fig. 5H,J), although we noticed that sometimes the poles of meiotic spindles were not as focused as in wild type and that chromosomes were occasionally improperly segregated to the spindle poles (data not shown).

Grip128 and Grip75 in sperm morphogenesis

Grip75 and *Grip128* mutant males are completely sterile, even though the cytokinesis defects in these mutants are not fully penetrant. We therefore analyzed sperm morphogenesis to identify further functions of Grip75 or Grip128.

After elongation, sperm are transferred from the testes into the seminal vesicle, where they are stored until mating occurs (Fuller, 1993). In *Grip128* and *Grip75* mutant males, we did not observe sperm in the seminal vesicle. Elongated sperm were present in mutant testes, but they were not motile (data not shown). We

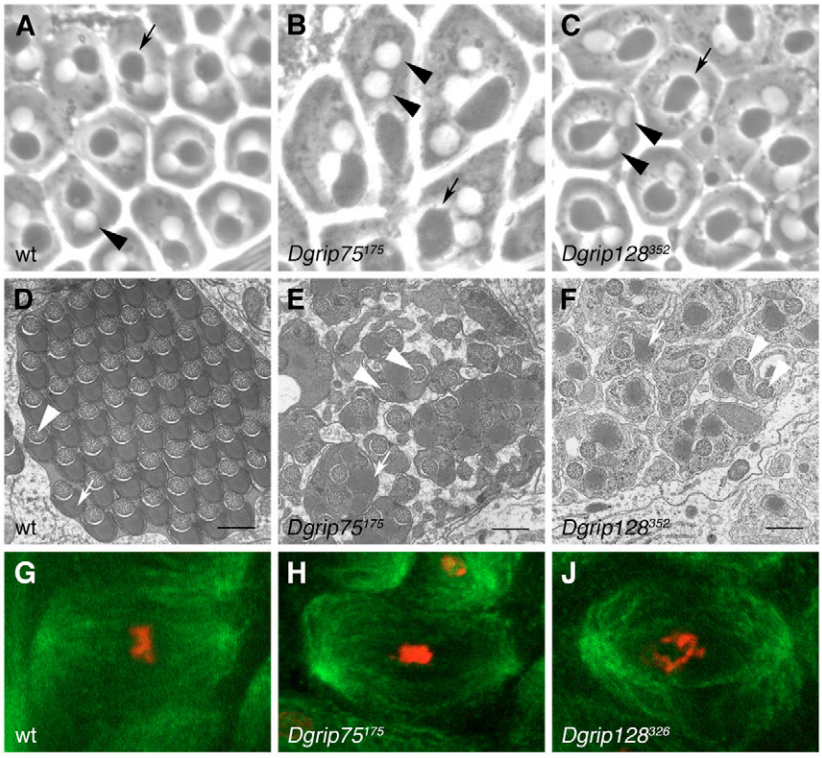

Fig. 5. Meiosis is perturbed in *Grip75* and *Grip128* mutant spermatocytes. (**A-C**) Wild-type (A), *Grip75*^{175} (B) and *Grip128*^{352} (C) mutant spermatids at the onion stage. Nuclei appear bright in phase-contrast microscopy (arrowheads), whereas mitochondria are dark (arrows). (**D-F**) EM cross-sections of wild-type (D), *Grip75*^{175} (E) and *Grip128*^{352} (F) mutant sperm flagella. Scale bars: 500 nm. The arrowheads indicate axonemes, the arrows indicate mitochondria. (**G-J**) Meiosis I spindles at metaphase in wild-type (G), *Grip75*^{175} (H) and *Grip128*^{352} (J) mutant spermatocytes. Microtubules are shown in green, DNA is in red.

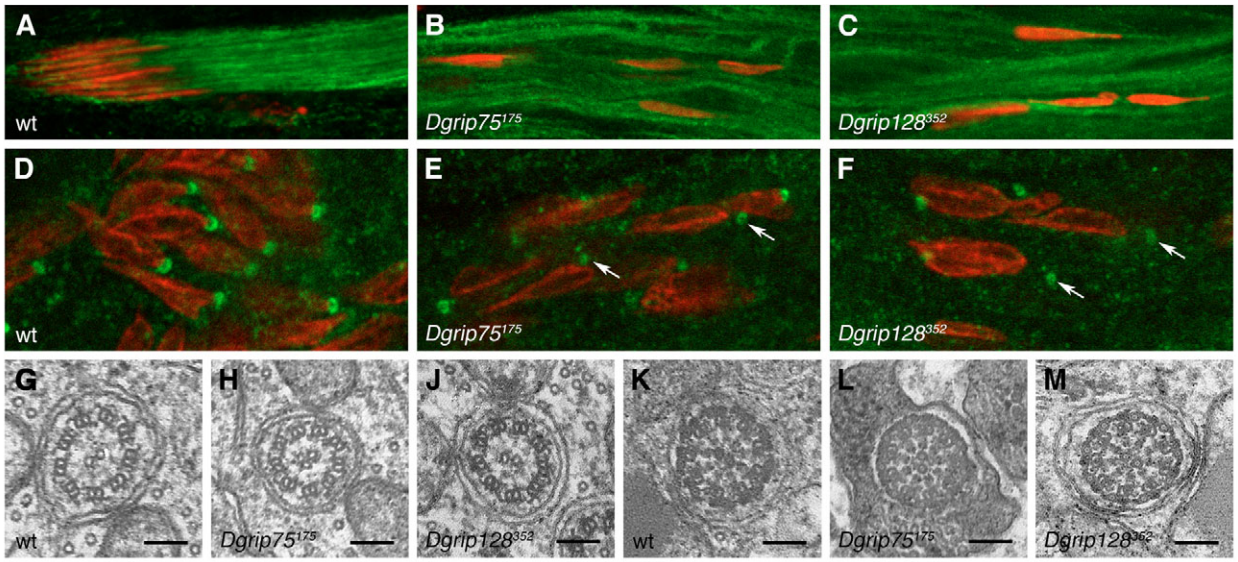

Fig. 6. Grip75 and Grip128 anchor microtubules at the nucleus. (**A-C**) Wild-type (A), *Grip75*^{175} (B) and *Grip128*^{352} (C) mutant sperm were stained for α-tubulin (green) and DNA (red). Nuclei are located at one end of a sperm bundle in wild-type testes but are dispersed along the bundle in mutant testes. (**D-F**) Wild-type (D), *Grip75*^{175} (E) and *Grip128*^{352} (F) mutant elongating spermatids were stained for γ-tubulin (green) and DNA (red). In wild-type spermatids, γ-tubulin is associated with the nucleus, whereas this association is frequently lost in mutant spermatids (arrows). (**G-M**) EM cross-sections of wild-type (G,K), *Grip75*^{175} (H,L) and *Grip128*^{352} (J,M) mutant axonemes in early (G-J) or late (K-M) stages of axoneme assembly. Scale bars: 100 nm.

analyzed chromosome and microtubule distribution in *Grip128*^{352} and *Grip75*^{175} spermatids. Whereas nuclei in wild-type spermatids are packed at one end of the sperm bundle, the nuclei are dispersed along the entire sperm bundle in *Grip128*^{352} and *Grip75*^{175} testes (Fig. 6A-C). In wild-type spermatids, γ-tubulin is localized at the junction between the nucleus and the elongating flagellum (Fig. 6D) (Wilson et al., 1997). Strikingly, in *Grip75*^{175} and *Grip128*^{352} spermatids, the association of γ-tubulin with the nucleus was frequently lost (Fig. 6E,F), suggesting that the axoneme is not tightly attached to the nucleus in these mutants.

To determine whether detachment of the axoneme disrupts axoneme organization, we analyzed the axonemal structure using electron microscopy. Immature axonemes consist of a ring of nine doublet microtubules with a central pair of single microtubules. These microtubule arrangements are completely normal in axonemes of mutant sperm, suggesting that formation of the axoneme does not require Grip128 and Grip75 (Fig. 6G-J). In more mature axonemes, the central pair of microtubules and one microtubule each of the nine doublet microtubules fills with an electron-dense material: the central filament. In addition, nine singlet microtubules appear in an outer ring, and these microtubules also harbor a central filament. All of these features were normal in *Grip128* or *Grip75* mutant sperm (Fig. 6K-M).

In conclusion, Grip128 and Grip75 mediate the attachment of γ-tubulin to the nucleus, which is necessary for alignment of the nuclei at one end of the sperm bundle. However, they are not necessary to build the complex axoneme structures nor are they required for axoneme maturation.

DISCUSSION

The role of the γTuRC in microtubule nucleation has been studied extensively by biochemical assays and electron microscopy. However, for many of the γTuRC components, an understanding of their function in the context of an organism has not yet emerged. We show that components of the γTuRC, which were thought to be required for microtubule nucleation, can have restricted and distinct functions. Our analysis of *Grip128* and *Grip75* mutants suggests that the γTuRC cap structure influences the function of microtubules involved in *bcd* RNA localization during oogenesis, meiosis in males and females, as well as sperm morphogenesis. In *Grip128* and *Grip75* mutants, a γTuRC seems to assemble and to provide basic γTuRC functions, which are sufficient for the viability of adult flies and thus for all the essential processes in somatic cells. Our data support the view that Grip128 and Grip75 anchor the γTuRC at specialized MTOCs, allowing microtubules that are required for a few distinct processes to tightly associate with specific MTOCs.

Grip128 and *Grip75* are not essential for viability

γTuRC function in microtubule nucleation is crucial for viability, as mutations in *Grip91/l(1)dd4*, *Grip84* and *γTub23C* are lethal (Barbosa et al., 2000; Colombie et al., 2006; Sunkel et al., 1995). By contrast, *Grip75*, *Grip128* and the double mutants are viable, showing that both gene products are not essential for the microtubule-nucleating properties of the γTuRC and that the γTuRC formed in these mutants is sufficient for microtubule function in somatic cell types of the fly. However, depletion of cap components such as Grip75, Grip128 or Grip163 by RNAi leads to a higher mitotic index in S2 cells (Verollet et al., 2006), but the cap components are not absolutely essential for mitotic progression. This is not surprising as even mutants with centrosomal defects can survive (Martinez-Campos et al., 2004). Furthermore, γ-tubulin is recruited to centrosomes in *Grip75* or *Grip128* mutant spermatocytes, *Grip75* mutant neuroblasts and in S2 cells depleted for cap components (N.V. and C.N.-V., unpublished) (Verollet et al., 2006), showing that γ-tubulin targeting to the centrosome does not depend on cap components. It has been proposed that γ-tubulin can be recruited to centrosomes as part of the γTuSC, as the amount of large γ-tubulin-containing complexes is severely reduced in cells

depleted for cap components (Verollet et al., 2006). Using buffers with lower salt concentrations, we observe large γ-tubulin containing complexes in *Grip75* and *Grip128* mutants, albeit in reduced amounts compared with wild type. It is likely that these complexes are indeed γTuRCs that lack parts of the cap structure, as they are similar in size to the γTuRC; in addition, Grip128 is present in *Grip75* mutant complexes. The mutant γTuRCs might still be capable of nucleating microtubules.

Whether γ-tubulin forms γTuSCs or incomplete γTuRCs, the cap subunits are dispensable for microtubule nucleation and γ-tubulin recruitment to centrosomes (this study) (Verollet et al., 2006). Moreover, a γTuRC has not been described in *Saccharomyces cerevisiae* and homologs of the cap components have not been identified in yeast, further supporting the notion that microtubule nucleation can occur in the absence of the cap structure.

Overlapping functions of individual γTuRC-specific subunits

In *Drosophila*, it is not known whether individual γTuRC complexes vary in their subunit composition and whether the γTuRC-specific subunits have similar functions. The human γTuRC has been shown to contain all of the described subunits (Murphy et al., 2001). We and others show that the cap components Grip75, Grip128 and Grip163 depend on each other for their stability (Verollet et al., 2006). Furthermore, individual depletion of Grip75, Grip128 or Grip163 results in a similar increase of the mitotic index in treated cells (Verollet et al., 2006). Moreover, *Grip128;Grip75* double mutants show the same phenotypes as the single mutants in the *Drosophila* germline. Taken together, the data support the view that Grip163, Grip128 and Grip75 function in the same processes and are part of the same complexes.

By contrast, Grip71 appears to have a distinct function. On the one hand, depletion by RNAi does not impair protein levels of the other γTuRC-specific proteins or their recruitment to centrosomes; on the other hand, the mitotic phenotypes are much stronger in *Grip71* mutants when compared with *Grip75* mutants (Verollet et al., 2006).

A microtubule-anchoring function of the γTuRC cap structure

Genetic and cell biological data suggest that an intact cap structure is not necessary for microtubule nucleation (this study) (Verollet et al., 2006); thus, the function of the cap is still in question. It could be required for efficient assembly of the γTuRC, for a higher microtubule nucleation rate or for tethering the complex to MTOCs. The former two possibilities predict that all microtubules would be affected to a similar degree, and therefore the most sensitive microtubule-dependent processes would be disrupted in *Grip75* and *Grip128* mutants. The latter possibility predicts that phenotypes would arise when redundant anchoring mechanisms were not available.

Mutants with global defects in microtubule function such as hypomorphic *αtub84B* mutants show a wide range of phenotypes such as polyphasic lethality, cuticle defects, short life span and sterility (Matthews and Kaufman, 1987). Similarly, hypomorphic *Grip91/l(1)dd4* mutants are lethal and display both mitotic and meiotic defects in spermatogenesis (Barbosa et al., 2003; Barbosa et al., 2000). As *Grip75* and *Grip128* mutants show very specific phenotypes, a function for the γTuRC cap structure in microtubule anchoring at MTOCs is more conceivable. This is supported by the observed detachment of axonemes from their respective nuclei without any aberrations in axoneme architecture and the undisturbed *orb* RNA localization in *Grip128* mutants. Microtubule recruitment to or anchoring at centrosomes has been shown to depend on a number of factors, such as pericentrin or motor proteins. Redundant mechanisms might act to focus microtubules at conventional MTOCs in somatic cells, but this might not be the case at non-conventional MTOCs in the *Drosophila* germline.

The *Drosophila* pericentrin-like protein D-PLP recruits or anchors γ-tubulin to centrosomes, possibly by direct interaction with γTuSC components (Kawaguchi and Zheng, 2004; Martinez-Campos et al., 2004). Interestingly, D-PLP is only required for efficient anchoring of γ-tubulin to the centrosome in early phases of mitosis, suggesting that a D-PLP independent pathway can recruit and anchor centrosomal components (Martinez-Campos et al., 2004). Maybe D-PLP and the γTuRC cap structure act redundantly in anchoring γ-tubulin at the pericentriolar material during mitosis.

Additionally, microtubule motors focus microtubules at the mitotic centrosome. Inhibition of the dynein-dynactin complex results in disorganized spindles that lack well-focused poles (Gaglio et al., 1997), while analysis of *Dhc64C* mutations in *Drosophila* suggests that dynein is required for the attachment of spindle poles at centrosomes (Robinson et al., 1999). The kinesin-related Ncd is a minus-end directed microtubule motor that also functions in spindle assembly during mitosis (Endow et al., 1994). Depletion of Ncd by RNAi in S2 cells results in frequent release of microtubules from the spindle pole (Goshima and Vale, 2003).

Although the roles of D-PLP and the above mentioned microtubule motors are fairly well established in centrosomes, their contributions to other MTOCs, such as the Grip75- and Grip128-dependent ones, are not as well studied. D-PLP has been shown to maintain the structural integrity of centrioles in male meiosis I (Martinez-Campos et al., 2004); however, a possible function in γ-tubulin anchoring in male meiosis II is difficult to address because of the centriolar defects. Ncd organizes the female meiosis I spindle (Matthies et al., 1996) and also localizes to the meiosis II spindle. *ncd* mutants do not form a structured central aster in meiosis II (Endow and Komma, 1998), but this could be a consequence of defects in meiosis I. We propose that redundant mechanisms focus or anchor microtubules at conventional centrosomes during mitosis. However, some MTOCs in the germline crucially depend on the anchoring function of the γTuRC cap subunits Grip128 and Grip75. Hence, these proteins allow the organization of distinct microtubule populations at particular positions in complex cells, independently of centrosomes.

Grip128 and Grip75 at specialized MTOCs

Interestingly, mutations in *Grip75* or *Grip128* fully disrupt the function of only certain MTOCs. As *Grip128* and *Grip75* mutants are viable, most microtubule-dependent processes in somatic cells function at least to an extent that allows survival of the organism, even though mitosis is delayed (Verollet et al., 2006). These processes are directed by microtubules associated with classical centrosomes, suggesting that somatic centrosomes are less sensitive to the lack of *Grip128* and *Grip75* function than the specialized MTOCs in the male and female germline.

Grip128, Grip75 and γTub37C participate in the formation of a new MTOC at stage 10b, which directs the relocalization of *bcd* RNA during stage 10b (this study) (Schnorrer et al., 2002). They are specifically involved in *bcd* RNA localization, as other microtubule-dependent processes in the oocyte such as oocyte specification, nuclear migration, cytoplasmic streaming, and *orb*, *grk* and *osk* RNA transport are normal in the respective mutants. It has been proposed that different subsets of microtubules could perform this

variety of functions (Schnorrer et al., 2002). Alternatively, loss of *Grip128* or *Grip75* function could lead to a reduction in microtubule number or function, thus impairing only the most sensitive microtubule-dependent processes. Three lines of evidence support a selective function of Grip128 in the organization of the anteriorly originating microtubules during stage 10b and 11. The subcortical microtubule network appears to be normal in mutant oocytes, whereas the anterior set of microtubules is not present. Cytoplasmic streaming is undisturbed in *Grip128* mutants. *orb* RNA localization has been demonstrated to be more sensitive to microtubule-depolymerizing drugs than *bcd* RNA localization (Pokrywka and Stephenson, 1995); however, *orb* RNA is correctly localized in *Grip128* mutant oocytes. These data argue against a general microtubule impairment in *Grip128* mutants.

Female meiosis requires the activities of Grip128 and Grip75 during the second meiotic division. Spindle formation in female meiosis is atypical, with the anastral and acentrosomal first meiotic spindle forming in a chromatin-driven fashion (Matthies et al., 1996). The second meiotic division is characterized by two tandemly arranged spindles, which are connected by a central microtubule aster. This central aster has been proposed to be necessary for correct spacing and alignment of the meiosis II spindles (Riparbelli and Callaini, 2005). It contains γ-tubulin, whereas the distal poles are devoid of γ-tubulin (Endow and Komma, 1998; Matthies et al., 1996). The absence of the central microtubule aster in *Grip75* and *Grip128* mutants could be due either to reduced microtubule nucleation from the MTOC or to a failure in MTOC assembly. We favor the latter hypothesis, as the inner half spindles are formed in the mutants, and the absence of a robust central microtubule aster is also observed in *cnn* and *polo* mutants (Riparbelli and Callaini, 2005; Riparbelli et al., 2000).

As in females, meiosis in males displays special features, such as the reductional segregation of centrioles in the second meiotic division. Thus, the second meiotic spindle is built from centrosomes, which contain a single centriole each, thereby giving rise to unicentriolar cells (Gonzalez et al., 1998). Centrioles in spermatocytes are large and associated with very little pericentriolar material when compared with mitotic centrioles (Fuller, 1993; Riparbelli et al., 2002). These meiotic centrosomes might depend on Grip75 and Grip128 for correct microtubule organization. Alternatively, the central spindle, which is essential for cytokinesis, has been postulated to use transient microtubule organizing centers present between the two daughter nuclei. Grip75 and Grip128 could function in these transient MTOCs to organize the central spindle.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/20/3963/DC1

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Table S1. Quantification of early spermatids

	0:1	1:1	2:1	3:1	4:1	Total
OrR*	1.8%	98.2%	–	–	–	282
Grip75^{175}	–	65.2%	28.6%	4.5%	1.7%	290
Grip128^{352}	2.5%	53.4%	29.5%	12.9%	1.7%	363

Loose spermatids containing identifiable nuclei and Nebenkerns were scored. The percentage of cells with the indicated ratio of nuclei per Nebenkern is given.