Bug22 influences cilium morphology and the post-translational modification of ciliary microtubules

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Summary
Cilia and flagella are organelles essential for motility and sensing of environmental stimuli. Depending on the cell type, cilia acquire a defined set of functions and, accordingly, are built with an appropriate length and molecular composition. Several ciliary proteins display a high degree of conservation throughout evolution and mutations in ciliary genes are associated with various diseases such as ciliopathies and infertility. Here, we describe the role of the highly conserved ciliary protein, Bug22, in Drosophila. Previous studies in unicellular organisms have shown that Bug22 is required for proper cilia function, but its exact role in ciliogenesis has not been investigated yet. Null Bug22 mutant flies display cilia-associated phenotypes and nervous system defects. Furthermore, sperm differentiation is blocked at the individualization stage, due to impaired migration of the individualization machinery. Tubulin post-translational modifications (PTMs) such as polyglycylation, polyglutamylation or acetylation, are determinants of microtubule (MT) functions and stability in centrioles, cilia and neurons. We found defects in the timely incorporation of polyglycylation in sperm axonemal MTs of Bug22 mutants. In addition, we found that depletion of human Bug22 in RPE1 cells resulted in the appearance of longer cilia and reduced axonemal polyglycylation. Our work identifies Bug22 as a protein that plays a conserved role in the regulation of PTMs of the ciliary axoneme.

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Key words: Basal bodies, Cilia, Sperm individualization, Spermatogenesis, Tubulin post translation modifications

Introduction
Cilia are specialized cell organelles that have motility and sensory functions. Their axoneme is built through nucleation of MTs from a basal body anchored at the plasma membrane, while assembly of the remaining cilia components normally relies on cargo transportation, in a process known as intraflagellar transport (IFT). Several ciliary proteins display a high degree of conservation, appearing widely present throughout eukaryotes. Mutations that perturb basal body anchoring, transition zone and ciliary assembly, or the transport of specific signaling molecules to the cilium, are associated with a variety of diseases known as ciliopathies. These include several clinical manifestations such as retinal degeneration, polydactyl, kidney cysts, cranial malformations, mental retardation, obesity and sterility.

In the last decade several high throughput studies have contributed to the identification of cilia and centrosome components (Andersen et al., 2003; Avidor-Reiss et al., 2004; Keller et al., 2005; Li et al., 2004; Pazour et al., 2005; Stolc et al., 2005). One of these components is the Basal body up regulated gene 22 (Bug22), initially identified as a basal body component in the green algae Chlamydomonas reinhardii (Keller et al., 2005). Bug22 is a remarkably conserved protein with homologs in all flagellated eukaryotes, but also in non-flagellated eukaryotes, being absent only from unicellular fungi and non-flagellated algae genomes (Broadhead et al., 2006). Bug22 is not exclusively associated with basal bodies, but also with cilia, in Chlamydomonas, Tetrahymena, Paramecia, mouse and human cells (Dawe et al., 2007; Pazour et al., 2005; Laligné et al., 2010; Ostrowski et al., 2002; Smith et al., 2005; Ishikawa et al., 2012).

In Paramecium, Bug22 is localised along the axonemes of motile cilia. Its depletion causes defects in ciliary morphology and motility without affecting overall axoneme structure (Laligné et al., 2010). Because functional analyses of Bug22 have only been performed in unicellular organisms (Hodges et al., 2011; Laligné et al., 2010), we decided to investigate its functions in a multicellular organism: the fruit fly Drosophila melanogaster. Here, we show that Bug22 in Drosophila associates with nucleus, basal bodies, sensory cilia and sperm flagella. Analysis of Bug22 mutants revealed an uncoordinated phenotype, confirming a role for this protein in ciliogenesis. Unexpectedly, we have also found overly long basal bodies and defects in sperm individualization in Bug22 mutants. Axonemal size control seems to be a general function of Bug22, as the depletion of the human homolog in RPE1 cells resulted in the formation of longer primary cilia. Interestingly both fly and human axonemes showed defects in the levels of tubulin post-translational modifications (PTMs) in the absence of Bug22. Our work suggests that Bug22 might play a conserved function in the regulation of axonemal size and functionality through the regulation of tubulin PTMs.
Results

*Drosophila* and human Bug22 are associated with cilia

In *Drosophila*, Bug22 is encoded by the CG5343 gene, which codes for a protein with an estimated mass of ~23 kDa. In order to study its localisation, we raised antibodies and generated constructs to express GFP-tagged versions of full length Bug22. We were unable to obtain any specific immunoreactivity from sera of animals immunised with either human or *Drosophila* Bug22, probably due to its high conservation and thus poor antigenicity. A commercially available antibody (GTL-3, see Materials and Methods) specifically recognised Bug22 on western blots (see below) but not in immunostainings. Hence, analysis of Bug22 in flies was based on transgenic lines that express GFP-Bug22 under a ubiquitous promoter, termed Ubi (Basto et al., 2008; Peel et al., 2007). We found that GFP-Bug22 localised to cilia of chordotonal organs in sensory neurons localised in the fly antenna (Fig. 1A) and was also associated with the sperm flagellum (Fig. 1B). While analyzing the male testis, we also noticed a clear signal of GFP-Bug22 at the tip of the giant centrioles of primary spermatocytes (Fig. 1C). This localization was more distal than that of other known centriole proteins such as Asterless (Asl) (Fig. 1C) or Sas4 and PACT (data not shown). Ultrastructural analysis of these centrioles has shown their distal-most segment actually corresponds to a small primary cilium, composed of a transition zone and a short axoneme (Carvalho-Santos et al., 2012; Riparbelli et al., 2012; Tates, 1971) and so we conclude that GFP-Bug22 is associated with this primary cilium. In sensory neurons and in sperm cells, Bug22 appeared localized to the nucleus (data not shown).

Fig. 1. *Drosophila* and human Bug22 localise to the nucleus and cilia. (A) GFP-Bug22 (left and in green in the merged panel) localises to the cilia of antennal chordotonal organs. The proximal segments of the two cilia of each scolopale (sensory unit of the chordotonal organs, schematized on the right) can be identified by their position next to the Eys protein (middle panel, shown in red in the merged panel) labeling the scolopale extracellular space. Scale bar: 5 μm. (B) GFP-Bug22 (left and in green in the merged panel) localizes along the entire length of the sperm flagella and the nucleus. α-tubulin (middle panel is shown in red in the merged panel) and DNA is in blue. The inset in the merged panel shows a higher magnification view of the sperm nuclei region with α-tubulin and GFP-Bug22 to illustrate the nuclear localisation of Bug22. Scale bar: 5 μm. (C) Primary spermatocyte expressing GFP-Bug22 (left and in green in the merged panel) and stained for the centriole marker Asterless (Asl) that labels the entire centriole at this stage (middle and shown in red in the merged panel) and for DNA (shown in blue in the merged panel). GFP-Bug22 localises to the distal segment of the giant centrioles, a region that is not labeled by the centriole marker Asl and that is composed of doublets of MTs (Carvalho-Santos et al., 2012; Riparbelli et al., 2013; Riparbelli et al., 2012; Tates, 1971). In addition GFP-Bug22 also localizes to the nucleus and it is enriched in the nucleolus (arrow in the left panel). Scale bar: 10 μm. (D) hTERT-RPE1 cells transfected with GFP-hBug22 (left, in green in the merged panel) and stained for Ac-Tubulin to label the primary cilium (shown in red in the merged panel) and DNA (shown in blue in the merged panel). Scale bar: 3 μm.
shown for sensory neurons, Fig. 1B-inset). In primary spermatocytes, Bug22 in addition to the nucleus appeared strongly enriched at the nucleolus (Fig. 1C, arrow). Importantly, we have never seen Bug22 associated with centrosomes or basal bodies in other cell types (data not shown).

We finally confirmed that, as described previously (Ishikawa et al., 2012), human Bug22 (hBug22) is also ciliary. In hTER-T-RPE1, GFP-hBug22 distributes along the length of primary cilia and the nucleus (Fig. 1D). Thus, Bug22 is a conserved ciliary protein.

Bug22 plays essential roles in ciliated and non-ciliated tissues in Drosophila
To analyse the function of Bug22 in flies we generated a null allele for Bug22 by homologous recombination (supplementary material Fig. S1A). We obtained one single allele (supplementary material Fig. S1B) that will be referred to as Bug22. Importantly, GFP-Bug22 transgenes rescued this mutation (Fig. 2C,D), showing that the phenotypes described below are solely due to the loss of Bug22.

Fig. 2. Characterisation of Bug22 flies. (A) Images from WT and Bug22 flies. From left to right: a WT adult fly at rest; a Bug22 fly, showing an abnormal positioning of its wings; a Bug22 fly, showing an improperly unfolded wing (right); a Bug22 fly, showing severe morphological defects in its wings and legs and so it cannot stand in an upright position. (B) Image from vials containing WT (left) and Bug22 (right) flies. While the majority of WT flies rapidly migrate to the top (arrowhead) of the vial after this has been tapped, Bug22 mutants remain at the bottom of the tube (arrowhead). (C,D) Graphs representing rescue experiments for Bug22 phenotypes of wing posture/inflation and climbing capacity. Percentage of flies belonging to various phenotypic classes is represented. At least 25 flies from each genotype were scored. Chi-square tests were used to assess statistical differences between the mutant and the rescue experiments considering the “Normal” phenotype. Only statistical significances are shown. JO15-Gal4 driver was used for chordotonal organ expression, which contain ciliated neurons. elav-Gal4 and DJ6844-Gal4 for neuronal expression, 24B-Gal4 and Mef2-Gal4 for mesoderm and muscle expression. Ubq promoter drives constitutive transgenic expression.
Bug22 flies were viable and could develop until late pupal stages. However, eclosed adult flies presented reduced lifespan, which could vary from a few hours to up to a few days. Furthermore, and consistently with the suspected functions of Bug22 in cilia, about half of the mutants displayed an uncoordinated phenotype (here referred to as Unc) similar to Drosophila mutants with defects in ciliogenesis (Baker et al., 2004; Martinez-Campos et al., 2004; Basto et al., 2006; Enjolras et al., 2012). Bug22 flies were morphologically normal, but presented defects in the positioning of their wings (Fig. 2A) and, despite being able to walk, did not present the same feeding and foraging behaviour as wild-type (WT) flies. Indeed, Bug22 males only produced immotile sperm. The remaining adult mutant progeny, however, presented more severe phenotypes. These included defects in wing inflation (unfolding), a “slimy” body wall and incapacity to stand in an upright position (Fig. 2A), which altogether resulted in the death of the mutant flies only a few hours after eclosion. Thus, Bug22 flies fall on two distinct phenotypic classes: one that displays an Unc phenotype and a second one with even more severe phenotypes.

To better understand the reasons for these differences, we decided to investigate the nature of the defects found in Bug22 mutants. We used the UAS/Gal4 system to express a UAS-Bug22 construct in different cell types in the Bug22 background. Expression of Bug22 exclusively in the mesoderm (MeF2-Gal4, 24B-Gal4), which has been shown to restore climbing activity and wing posture in mutants presenting mitochondrial defects affecting the skeletal muscle system (Greene et al., 2003), did not rescue any trait of Bug22 flies (Fig. 2C,D). Using a chordotonal-organ-specific Gal4 driver (JO15-Gal4) (Sharma et al., 2002), we obtained only a partial rescue of Bug22 phenotypes as some of these flies still presented defects in locomotion and climbing activity (Fig. 2C,D). Strikingly, an almost complete rescue of all defects, including body morphology (non-inflated wings and “slimy” body wall), locomotion, climbing activity and lifespan was obtained when we used a pan-neuronal (elav-Gal4) (Luo et al., 1994) or adult nervous system (DJ684-Gal4) (Seroude et al., 2002) Gal4 drivers (Fig. 2C,D). These results lead us to conclude that Bug22 functions in both ciliated and non-ciliated neurons. Importantly, ubiquitous expression of Bug22 using a ubiquitous

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![Figure 3](image-url) **Fig. 3. Characterisation of Bug22 testes.** (A) Pictures from adult WT (left) and Bug22 testes (right) showing regular testes morphology. Scale bar: 100 μm. (B) High magnification pictures of WT (left) and Bug22 (right) spermatids showing the presence of thicker sperm tails in the mutant. Scale bar: 10 μm. (C) Phase contrast image showing that Bug22 (right) onion stage spermatids are indistinguishable from WT (left). Every post-meiotic round spermatid contains one nucleus (white circle) adjacent to one nebenkern (black circle), both structures having approximately the same size. Onion stages from at least 20 males were analysed. Scale bar: 10 μm. (D) Images of WT and Bug22 dividing primary spermatocyte centroinies expressing RFP-PACT (top panel and shown in red in the merged panel) and Asl (shown in green in the merged panel). Scale bar: 2 μm. (E) Graphs showing measurements of centriole (left) and basal body (right) lengths in WT and Bug22. Measurements were based on the fluorescence signal of the transgenic protein RFP-PACT (red bars) and Asl (green bars). Mean values of length (∆) represent the standard deviation from more than 50 centroinies from meiosis I or II spermatocytes (left) and elongated spermatids (right). Student’s t-tests were performed to assess statistical differences.
promoter (pUbq) largely rescued the wing, Unc and sperm immotility phenotypes (Fig. 2C,D). Overall, our analysis shows that *Drosophila* Bug22 plays essential functions in ciliated cells: sensory neurons and sperm, and suggests that Bug22 has additional functions in the nervous system.

**Bug22 is required for differentiation of the sperm flagellum**

To understand the requirements for Bug22 in male fertility, we analysed the genital tracts of Bug22 males and found defects in spermatogenesis. Indeed, although Bug22 male reproductive system appeared morphologically normal (Fig. 3A), it contained empty seminal vesicles (not shown). Furthermore, under the light microscope various defects at the level of elongated spermatid flagella could be perceived: these were immotile, appeared much less flexible than the WT and finally, the flagella appeared thicker and presented cytoplasmic bulges (Fig. 3B).

We had observed that GFP-Bug22 localised to the distal tip of spermatocyte centrioles. Defects in meiosis are frequently present in centriole or centrosome mutants (Basto et al., 2006; Martinez-Campos et al., 2004; Mottier-Pavie and Megraw, 2009; Rodrigues-Martins et al., 2007). Characterisation of meiotic divisions showed that spindle poles and spindle morphology appeared normal and we never detected chromosome segregation defects (data not shown). Further inspection of the ploidy and number/size of nebenkerns (mitochondrial derivatives) in post-meiotic cells (onion stage spermatids) by phase contrast microscopy confirmed the absence of meiotic defects in Bug22 (Fig. 3C). Strikingly, we noticed that Bug22 primary spermatocyte centrioles were longer than their WT counterparts and assembled centriole pairs with strange bends and/or arrangement (Fig. 3D,E). This difference in length was still observed at later stages of spermatogenesis, when these centrioles behave as basal bodies to nucleate sperm flagella (Fig. 3E). Note that the extent of the signal occupied by Asl decreases in basal bodies while PACT continues to increase. This is in agreement with the characterization of Asl localization to the a proximal centriole-like structure after meiosis (Blachon et al., 2009).

<table>
<thead>
<tr>
<th>No. cysts with open axonemes</th>
<th>No. axonemes/cyst</th>
<th>No. cysts with reduced number of mitochondrial derivatives</th>
</tr>
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<tbody>
<tr>
<td>WT cyst (n=24)</td>
<td>0</td>
<td>63 (11 cysts contained 64 axonemes)</td>
</tr>
<tr>
<td>Bug22 cyst (n=24)</td>
<td>4</td>
<td>62 (7 cysts contained 64 axonemes)</td>
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**Fig. 4. TEM analysis of Bug22 testes.** (A) TEM micrographs of cross sections of WT (left) and Bug22 (right) post-individualized cysts. In the WT cyst a highly ordered arrangement of sperm tails axonemes surrounded by membrane can be seen, while the Bug22 mature cysts at a comparable stage of differentiation present a high level of disorganization with most axonemes appearing un-individualized. Large membrane delimited non-electron dense inclusion bodies can occasionally be seen in non-individualized cysts (arrow). Scale bar: 1 μm. (B) TEM micrographs from cross-sections of sperm flagella from WT and Bug22 spermatid cysts. In both genotypes, axonemes form correctly, displaying a 9+9+2 MT arrangement. However, in a few post-elongation cysts abnormal axonemes (right) were found that appeared either slightly opened or completely disassembled. Scale bar: 0.2 μm. (C) Representative sperm tail from WT (left) and Bug22 (right) testes. In both genotypes a major (MMD) and a minor (mmD) mitochondrial derivative (arrows) can be seen associated with each axoneme. In the mutant spermatid tails, the appearance of two axonemes sharing one major mitochondrial derivative was frequently seen (arrows) in late-elongation or individualizing cysts. Scale bars: 0.2 μm. (D) Quantification of defects in Bug22 spermatid cysts analysed by TEM.
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organelles, dispensable for individual sperm cell function as ICs tails. Individualization results in the reduction of spermatid cell requires the movement of multiprotein complexes, called axoneme structure, rather than defects in the assembly process. While Bug22 sperm tails appeared morphologically normal in most cases, we also observed defects in mitochondria derivatives (Fig. 4C, arrows). Normally, a triad of one axoneme, one major and one minor mitochondrial derivative can be seen. In mutant tails, however, a high portion of cysts (45.6%, n=24) contained a few sperm tails with mitochondrial derivatives in insufficient number, a defect that was not associated with meiotic defects. Since this type of defect was infrequent and never observed in testes taken from pupal stages, we interpret them as resulting from a failure in the maintenance of the axoneme structure, rather than defects in the assembly process.

While Bug22 sperm tails appeared morphologically normal in most cases, we also observed defects in mitochondria derivatives (Fig. 4C, arrows). Normally, a triad of one axoneme, one major and one minor mitochondrial derivative can be seen. In mutant tails, however, a high portion of cysts (45.6%, n=24) contained a few sperm tails with mitochondrial derivatives in insufficient number, a defect that was not associated with meiotic defects. In Drosophila, at the end of spermatogenesis, a process known as sperm individualization generates 64 mature and individualized sperm cells within a cyst. Individualization requires the movement of multiprotein complexes, called individualization complexes (ICs), along the length of sperm tails. Individualization results in the reduction of spermatid cell volume, through the expulsion of large masses of cytoplasm and organelles, dispensable for individual sperm cell function as ICs migrate (Tokuyasu et al., 1972a). We conclude that Bug22 is required for sperm individualization and thus maturation.

**Bug22 is required for the migration of individualization complexes**

Sperm individualization starts with the assembly of ICs around each of the elongated nuclei. ICs are composed, among many other components of F-actin structures, called actin cones, which have been well characterised (Noguchi and Miller, 2003). Subsequently, the ICs begin to move along the sperm tail. Because this migration is accompanied by the continuous accumulation of extruded cell material around the IC, a voluminous structure called the cystic bulge (CB) is created at this stage (Fig. 5A, arrows). Finally, when the complex reaches the end, of the now individualized tails, the CB turns into a waste bag (Fig. 5A, arrowheads), which is eventually degraded. In Bug22 flies, just like in WT, ICs were correctly assembled at the spermatid nuclei (Fig. 5A, insets). Moreover, the ICs moved and migrated away from the nuclei (Fig. 5A, arrows and insets). However, unlike in WT individualizing cysts, the ICs appeared dispersed and somehow lagged along the sperm tails (Fig. 5A, arrows and insets). Asynchronous movement of actin cones is commonly observed in Drosophila mutants with defective sperm individualization (Fabrizio et al., 1998; Riparbelli and Callaini, 2007; Zhou et al., 2011). Accordingly, CBs and waste bags in Bug22 appeared smaller and contained less material than in WT (Fig. 5A, arrowheads). Activation of caspases in a spatially regulated manner is also required during sperm individualization, to promote the formation of the CB (Arama et al., 2003; Kaplan et al., 2010), but we did not observe any differences between WT and Bug22 cysts. We concluded that in the absence of Bug22, ICs can assemble correctly at the sperm nuclei, but then are not able to migrate properly to individualize sperm tails.

Myosin VI (myo VI) is known to stabilize the actin cones during spermatogenesis and the activity of this motor is essential during individualization (Hicks et al., 1999; Noguchi et al., 2006). Since defects in the migration of the actin cones were noticed in Bug22 sperm, we investigated whether myo VI recruitment or localisation was perturbed in mutant sperm tails. Just like in WT, myo VI was correctly recruited to the actin cones at IC assembly and relocalized to the front of the cones during IC migration (Fig. 5A and insets) in Bug22 flagella. Taken together, characterization of Bug22 cysts undergoing spermatid differentiation both by TEM and immunofluorescence showed that Bug22 is essential during this step of sperm maturation.

The incorporation of the post-translational modification, polyglycylation is defective in Bug22 mutant flagella

The function of Bug22 in IC movement could be linked to its localization to sperm tails, likely at the level of the axonemal MTs. A clear evidence for the ability of this protein to bind the MT cytoskeleton has been provided in a proteomic study of C. reinhardtii flagella (Pazour et al., 2005), in which Bug22 ortholog was found enriched mainly in the fraction of proteins that were more strongly associated with the axoneme. Although not much is known about the MT properties that allow the movement of actin cones along the axoneme, tubulin polyglycylation, a post-translational modification (PTM) (Redeker et al., 1994) mostly present in ciliary MTs, was suggested to play a direct role in IC migration and in Drosophila sperm individualization (Rogowski et al., 2009). The same study also showed that reduced levels of glycylation cause the formation of a structurally normal but unstable sperm axoneme, similarly to what we saw in Bug22 flies.

Drosophila sperm tails are known to contain high levels of polymodified MTs, namely of polyglutamylation and polyglycylation (Bressac et al., 1995; Hoyle et al., 2008; Kavlje et al., 2010; Rogowski et al., 2009), particularly the accessory and central pair of MTs in the sperm axoneme (Hoyle et al., 2008). Therefore, we decided to determine whether these modifications were correctly incorporated in Bug22 axonemes. In western blot and immunostaining procedures, using antibodies that recognize long chains of glutamate residues (polyE) to detect polyglycylation, a post-translational modification (PTM) (Redeker et al., 1994) mostly present in ciliary MTs, was suggested to play a direct role in IC migration and in Drosophila sperm individualization (Rogowski et al., 2009). The same study also showed that reduced levels of glycylation cause the formation of a structurally normal but unstable sperm axoneme, similarly to what we saw in Bug22 flies. Drosophila sperm tails are known to contain high levels of polymodified MTs, namely of polyglutamylation and polyglycylation (Bressac et al., 1995; Hoyle et al., 2008; Kavlje et al., 2010; Rogowski et al., 2009), particularly the accessory and central pair of MTs in the sperm axoneme (Hoyle et al., 2008). Therefore, we decided to determine whether these modifications were correctly incorporated in Bug22 axonemes. In western blot and immunostaining procedures, using antibodies that recognize long chains of glutamate residues (polyE) to detect polyglycylation (Eddé et al., 1990; Janke and Bulinski, 2011), we obtained inconclusive results that were highly variable. On the other hand, when we analysed polyglycylation, using polyG antibodies that recognize long glycine chains (3 and more residues) (Eddé et al., 1990; Janke and Bulinski, 2011; Redeker et al., 1994), we found a strong reduction in the mutant (Fig. 5B,C; supplementary material Fig. S2A). Since in extracts that combined testes from different males, a large variation in signal was noticed (data not shown), we analysed four independent experiments in which samples of single testes from WT and Bug22 flies were compared (Fig. 5B,C; see Materials and Methods). This procedure revealed a significant reduction of polyglycylation in the mutant. Importantly, this decrease was never accompanied by equivalent alterations in the total levels of
Fig. 5. Analysis of sperm individualization and tubulin modifications in WT and Bug22 testes. (A) Left – immunostaining of whole mount WT and Bug22 testes stained for F-actin (shown in green), cleaved caspase3 (Cleaved-C3, shown in red) and DNA (shown in blue). In the WT panel individualizing cysts can be observed with migrating ICs (arrows) and waste bags at the extremity (arrowheads). In Bug22, the ICs (arrows) lagged and the only waste bag detected (arrowheads) appeared very abnormal and reduced in size. Right – higher magnifications of WT (top) and Bug22 (bottom) sperm tails undergoing individualization. F-actin is shown in green, MyoVI is shown in red and DNA/Cleaved-C3 in blue. ICs recruitment to the sperm nuclei occurs similarly to WT (top and bottom left), but the IC migration (top and bottom right) was not synchronous. MyoVI localisation did not seem to be perturbed in Bug22 testes. Scale bars: (left) 100 μm, (right) 10 μm. (B) Immunoblots from WT and Bug22 testes extracts probed with polyG antibodies that recognise polyglycylation. The bracket indicates the tubulin region and the arrow points to a higher molecular weight band corresponding to unidentified polyglycylated proteins that are also reduced in the mutant. (C) Quantification of tubulin polyglycylation levels detected by western blot in WT and Bug22 single testes. Values shown are relative to a WT sample set as reference within each of four experiments performed, and had been previously normalized to a loading control. The lines show the mean value ± SD. A two-tailed unpaired t-test was used to assess statistical differences between polyG levels in WT and mutant. (D) Immunoblots from WT and Bug22 testes extracts probed with polyG (that recognise polyglycylation) and α-tubulin antibodies. Both lanes contain equivalent protein amounts determined by Coomassie staining. This result shows that in the mutant, tubulin levels are similar to WT. (E) Definition of the four stages of spermatid differentiation. Nuclei are shown in blue and sperm tails in red. (F) Representative images of WT and Bug22 cysts, stained with polyG antibodies (to reveal polyglycylation, shown in red) and for DNA (shown in blue), during spermatid differentiation. Scale bar: 5 μm. (G) Plots showing polyG fluorescent intensity measurements in WT (gray) and Bug22 mutant cysts (red). The median intensities of all cysts analysed (each dot corresponds to a different cyst) ± SD are shown. A two-tailed unpaired t-test was used to assess statistical differences between WT and mutant at each stage. n.s. stands for no statistical significance. Cysts were classified according to their differentiation stage defined in E. Polyglycylation of WT sperm tails occurs at a defined developmental timing during spermatid differentiation. Such defined program appears altered in Bug22 sperm tails from canoe stages onwards.
tubulin, which appeared similar to WT in Bug22 testes (Fig. 5D).
Interestingly, the difference in PTM levels was not only restricted
to α- and β-tubulins, as the polyglycylation levels of other yet
unidentified proteins of higher molecular weight were also
reduced in the mutant (Fig. 3B, arrow).

We then analysed the distribution of polyglycylation in WT
and Bug22 cysts. Surprisingly, we noticed the occurrence of
highly complex patterns of tubulin polyglycylation in WT testes
reflecting a developmentally regulated process. A detailed
analysis of the precise timely incorporation of this tubulin
modification during Drosophila sperm maturation has never been
reported. Therefore, we decided to characterise polyglycylation
(using polyG antibodies) in semi-squashed preparations (see
Materials and Methods). We focused our analysis in the period
of spermatogenesis that ranged from the initial phases of sperm
differentiation, when sperm tails start to elongate, until the final
stages, where fully mature individual sperm cells coil and
separate from each other, in order to be mobilised into the
semenal vesicle (Fabrizio et al., 1998; Tokuyasu et al., 1972a;
Tokuyasu et al., 1972b) (Fig. 5E).

After having subdivided the spermatogenesis period in four
different stages following to the distinct nuclear morphologies
progressively acquired by the maturing spermatids (see Materials
and Methods for a detailed analysis of the sub phases), analysis of
WT cysts revealed that, from the beginning of elongation until
the formation of individual coiled sperm cells, polyglycylation
appeared according to a defined order. In pre-elongation stages,
polyglycylation was relatively low with a clear signal
surrounding each nuclei (Fig. 5F,G) both in WT and mutant
cysts. In the canoe stage, however, polyglycylation levels were
increased in the mutant, suggesting premature polyglycylation of
Bug22 sperm tails. At later stages, polyG signals never reached
WT levels (Fig. 5F,G). In Drosophila, two enzymes, TTLL3A
and TTLL3B, are able to initiate and elongate glycine chains in
opposition to mammals, where separate enzymes specifically
catalyze each step: initiation (TTLL3/8) or elongation (TTLL10)
(Rogowski et al., 2009). Previous studies suggested that
polyglycylation occurs during IC passage and that the enzymes
responsible for this modification could be transported by these
complexes (Bressac et al., 1995; Rogowski et al., 2009). In light
of this hypothesis, it was possible to conceive that some of the
defects in polyglycylation, observed in Bug22 mutants could
result from defects in IC migration. To investigate this question
we analysed whole-mount preparations of WT testes co-stained
for F-actin and PolyG. In sperm tails that contain ICs that were
just starting to assemble near the sperm nuclei, polyglycylation
was already detected along sperm tails (supplementary material
Fig. S3). Furthermore, when we analysed sperm tails that
contained migrating IC cones positioned away from the sperm
nucleus, a strong and comparable polyG signal was noticed both
in front and at the rear of the IC complex (supplementary material
Fig. S3). Altogether, these results showed that sperm tail
polyglycylation takes place earlier than initially proposed, either
before or during IC recruitment to the sperm head but,
importantly, before IC passage. These observations lead us to
conclude that polyglycylation is incorporated independently of
IC passage. Interestingly, this first description of in vivo PTM
regulation during Drosophila spermatogenesis is in agreement
with in vitro data describing the appearance of tubulin
polyglycylation independently of IC passage (Hoyle et al., 2008).

Our results suggested that Bug22 plays an important function
in maintaining polyglycylation levels in Drosophila sperm tails.
To ascertain if the defects observed in polyglycylation could
be rescued by overexpression of glycytating enzymes, we
overexpressed a GFP fusion of TTLL3B using a ubiquitous
promoter that induces moderate overexpression (Basto et al.,
2008; Peel et al., 2007). Overexpression of TTLL3B-GFP in WT
flies did not have any deleterious effect. Flies eclosed normally,
displayed normal climbing and flying activity and both female
and males were fertile. In contrast, the overexpression of
TTLL3B-GFP in Bug22 had a deleterious effect. These flies
were delayed during development and most flies died at late
pupal stages or just after eclosion. These results suggested that
the overexpression of TTLL3B could not rescue the
individualization phenotype of the mutant. Examination of
TTLL3B-GFP, Bug22 testes showed an aggravation of the
spermatogenesis phenotype when compared to Bug22 testes
(Fig. 6C). IC recruitment and migration were severely perturbed
and nuclei appeared dispersed along the sperm tails. In the few
partially intact cysts in the canoe/needle stages that we were able
to analyse (Fig. 6B), high-intensity polyG aggregates were
noticed accumulating in vesicle-like structures that also
contained TTLL3B-GFP, suggesting ectopic, exaggerated and
premature enzyme activity. Importantly, the later stages of sperm
maturation, such as post-elongation, were only rarely seen in
TTLL3B-GFP, Bug22 testes.

Furthermore, we noticed a dose-dependent effect of TTLL3B-
GFP transgenes when expressed in Bug22 mutant background
(Fig. 6C). When two copies were present, IC recruitment to the
sperm nuclei and migration (analysed by the morphology and
position of the ICs) were severely impaired and we never
observed the formation of waste bags when compared to testes
that only contained one TTLL3B-GFP copy (Fig. 6C). These
results suggest that the overexpression of TTLL3B in the absence
of Bug22 results in premature and (severely increased)
polyglycylation during the canoe stages, which causes extreme
defects in spermatogenesis.

Regulation of ciliary tubulin modifications by Bug22 is
conserved in vertebrate cells
Given the high degree of conservation between the human and fly
Bug22 orthologs (92%) and the ciliary localisation of hBug22 in
hTERt-RPE1 cells (Fig. 1D and Ishikawa et al., 2012), we
decided to test whether primary cilia tubulin PTMs were also
dependent on Bug22. PTMs are known to be essential for normal
cilia function and architecture. Therefore, we hypothesized
that if a function for Bug22 in regulating PTMs was conserved in
vertebrates, cilia defects should be seen in the absence of Bug22.
To start this analysis we characterised PTMs in primary cilia of
RPE1 cells. Using polyG, polyE and acetylated tubulin antibodies
we observed that the last two antibodies recognise centrioles and
primary cilia, suggesting that in RPE1 cells these structures are
polyglutamylated and acetylated but not polyglycylated.
Knockdown of hBug22 using small interfering RNAs (siRNAs)
resulted in a ~80% decrease in Bug22 levels as determined by
RT-PCR (Fig. 7A).

Analysis of hBug22-depleted cells revealed the presence of a
large proportion of cells that grew longer cilia (Fig. 7B,C).
Furthermore, these cilia frequently displayed a curved
morphology that was never seen in control cells (Fig. 7B,C). We did not find differences in the levels of acetylated
tubulin in hBug22-depleted cells (Fig. 7C). In contrast, the levels of polyE were significantly decreased (Fig. 7C) in the longer cilia. Importantly, we were able to rescue ciliary size defects in hBug22-depleted cells by expressing an siRNA-resistant GFP-hBug22 fusion protein (supplementary material Fig. S4), showing the specificity of the cilia size and morphology phenotypes. We conclude that in both Drosophila and human cells, Bug22 plays an essential role in maintaining cilia morphology, which might depend on the correct incorporation of tubulin PTMs.

Discussion

Here we have investigated the role of Bug22 proteins in Drosophila and RPE1 ciliogenesis. In both experimental models, we demonstrated a requirement for Bug22 in maintaining ciliary morphology, as well as defects in the levels of tubulin PTMs. We show that in flies, Bug22 modulates the timely incorporation of polyglycation during spermatogenesis.

Analysis of Bug22 mutants

Bug22 mutants did not show defects during development. At birth, however, Bug22 flies could be subdivided into two main classes. An Unc-type class, where mutant flies, similarly to other cilia mutants, were uncoordinated and presented defects in locomotion, gravitaxis and were unable to feed. These flies died within a few days after eclosion, probably do to dehydration, similarly to other cilia mutants (Baker et al., 2004; Martinez-Campos et al., 2004; Basto et al., 2006). Unexpectedly, the other class of Bug22 mutants presented an even more severe phenotype. They were unable to inflate their wings, presented defects in cuticle deposition and remained paralyzed. They invariably died just a few hours after eclosion. This type of defects has not been reported in centrosome or cilia mutants and we think they result from a yet uncharacterised function of Bug22. In flies, two types of non-visual sensory organs, Type-I (also known as sensilla), that harbor ciliated neurons and Type II, which consist of single, non-ciliated multi-dendritic neurons can be found (Avidor-Reiss et al., 2004; Gogendeau and Basto, 2010; Kernan et al., 1994; Kernan and Zuker, 1995). Since all Bug22 defects were rescued when we specifically overexpress Bug22 with pan-neuronal Gal4 drivers (Fig. 2C), but not with the JO15Gal4 (expressed in the Johnston organ, which contains only ciliated neurons), we propose that this protein probably plays essential functions in both ciliated and non-ciliated neurons.

Bug22 mutants present overly long centrioles and hBug22 depletion causes lengthening of primary cilia

During the course of this study we found that Bug22 centrioles and basal bodies were longer than the equivalent WT organelles.
In addition, during meiotic stages, Bug22 centrioles lose their typical V-shape. Importantly, defects in centriole assembly were not found in any other cell type in Bug22 mutants (data not shown), not even in the mitotic stages that precede the formation of the large primary spermatocyte centrioles. The abnormal lengthening probably reflects the unusual property of these centrioles, which is the nucleation of a primary cilium at their most distal end (Tates, 1971; Varmark et al., 2007; Carvalho-Santos et al., 2012; Riparbelli et al., 2012). Mutations in centriole components are known to perturb meiotic divisions or to cause fragmentation of primary spermatocyte centrioles, which leads to multipolar spindle formation (Bettencourt-Dias et al., 2005; Delgehyr et al., 2012; Martinez-Campos et al., 2004; Riparbelli and Callaini, 2011; Rodrigues-Martins et al., 2007). However, this was not the case in Bug22 mutants, as we have never observed defects in spindle assembly or chromosome segregation (data not shown) consistent with the lack of abnormalities at the onion stage (Fig. 3C).

Depletion of hBug22 in RPE1 cells resulted in the formation of an elongated primary cilium. In control cells, a strong polyglutamylation signal was detected at the proximal end just above the transition zone. In hBug22-depleted cells, a significant decrease in polyE levels was also found in hBug22-depleted cells (unpaired Student’s t-tests, two-tailed, \( P < 0.0001 \)). In contrast, Ac-Tubulin levels were not significantly changed in hBug22-depleted cells (unpaired Student’s t-tests, two-tailed, \( P > 0.1 \)).
Bug22 plays a role in sperm individualization
All Bug22 males produce immotile sperm that contained correctly assembled axonemes most of the time (Fig. 4B–D). Defects in sperm individualization were noticed, which did not result from defects in the initial recruitment of ICs. Instead, defects in IC migration were frequent, and the waste bags that normally form at the end of individualization, were either small or absent (Fig. 5A). Together these results suggest that the initial recruitment of the individualization machinery takes place in Bug22, although the synchronous migration is compromised.

When we analysed tubulin polyglycylation in Bug22 we found two types of defects. First, polyglycylation was deposited prematurely during the canoe stages, while in WT sperm tails this modification was noticed mainly at the needle stages (Fig. 5F,G). Then, at late stages, during the needle and post-elongation periods, polyglycylation levels did not reach high levels in the mutant (Fig. 5F,G). Thus, it is possible that the initial addition of the modification can take place in Bug22 axonemes even if prematurely, but the second polyglycylation wave (or the elongation of glycine chains) during the post-individualization stages fails.

The observations that overexpressing TTLL3B-GFP results in the formation of polyG aggregates along the sperm tail only in the mutant support a role for Bug22 in exerting a buffering effect on the formation of polyglycylation in the Drosophila sperm tail. It is possible that Bug22 functions as a filter that occupies the axoneme surface to work as a steric hindrance that limits the access of active enzyme to MTs, accounting for the defects observed in IC recruitment at the sperm nucleus (Fig. 6B) might depend on these substrates.

Conclusions
Our work shows that Bug22 influences the size of organelles that contain an axoneme such as centrioles and basal bodies (the unusual primary cilium present in fly centrioles and basal bodies), both in the male germline in Drosophila as well as the primary cilium of RPE1 cells. Very likely, Bug22 also contributes to the morphology of other MT structures that are not organised in axonemes, as expression of Bug22 in neurons rescued the severe phenotypes associated with the Bug22 mutation. MTs in neurons are highly modified and stabilized (Janke and Bulinski, 2011) and so, Bug22 likely plays a function in these cells.

Bug22 is remarkably conserved across evolution and it is even present in non-ciliated genomes as seed plant genomes (Hodges et al., 2011; Laligné et al., 2010). It will be interesting in the future to understand whether Bug22 also influences tubulin PTMs in cell types or organisms that do not contain cilia.

Materials and Methods
Fly stocks
All flies were maintained and handled according to standard Drosophila culture techniques. Stocks used in this study: P(Ubq::RF-P:PACT)CyO (Martinez-Campos et al., 2004), P(Ubq::GFP-Bug22), P(UAST::GFP-Bug22), P(Ubq::TTLL3B-GFP), P(Bug22W9/+/TM3,Sh; Bug22CyO)O. Bug22CyO/P(Ubq::GFP.S65T)PA1D1 (this study), y*w* hs-hid, P[wPFLP23 P701-Sccl/A]ATM Sh hs-hid (Huang et al., 2008), w*, PinCyO.P[Gal4::UAS-GFP]1-5w*, w*, P[Gal4-1-5w*, w*]; P[G21.17-Gal4]JO15/TS.Sb, w9-15, P[Gal4]; P[Gal4]/CyO, w*, w*; [P[Gal4-Mef2.R]J, w*; [P[Gal8B]how24B], from the Bloomington Stock Center. w1118 flies were used as WT controls.

Generation of Bug22 mutant by homologous recombination
For generation of the Bug22 knockout allele, we used the strategy described in Huang et al. (Huang et al., 2008). Briefly, a transgenic fly harboring a “donor DNA” construct to be used for gene targeting was made. To this end, a fragment of 3155 bp of genomic sequence with its 3’s 5’ and 3’ ends was amplified by PCR using primers 1+2 (5’-GGGGACAAGTTTGTACAAAAAAGCAGTGGGTCCTAGG-3’ and 5’-ATACGGTACCAAGATCTCGAAACTCGTACAAACTACG-3’). This fragment was inserted into a Gateway Wt vector. A second insert was cloned into the 3’s flanking the 5’ limit of the Bug22 ATG starting codon (position Ch2L: 10429989) was PCR-amplified from w1118 genomic DNA with primers 5’-ATACCGTACCCGGGATCGATGTCGCGACACATTTAC-3’ and 5’-ATACCTGCATATGTCGACCAAGAATCTGCGCATCCACTCATT-3’ using Plasmid DNA Polymerase (no. F-530, Themoscientific), subsequently sequenced and cloned into the 5’s multiple cloning site of the pRk2 plasmid (Huang et al., 2008), using SacII and NdeI sites. A second insert was cloned into the 3’s cloning site of the same vector using BglII and PstI sites. This 3035 bp fragment, contains the 5’s limit flanking the Bug22 ATG starting codon (position Ch2L: 10430187), was amplified with primers 5’-GAGAAATTCGCTGGAGCTGAAAGGTTGGGTGCTATGAGTTGAGAT-3’ and 5’-ATACGGTACCAAGATCTCGAAACTCGTACAAACTACG-3’. This fragment was cut with BglII and PstI and subcloned in the 3’s cloning site of the same vector using BglII and PstI. The final construct was used for microinjection of a transgenic line expressing TTLL3B-GFP transformed vector, was used for transgenesis by BestGene Inc., USA. A transgenic line carrying the transgene on chromosome III was used for homologous recombination. Putative Bug22 mutant lines, homozygous for the “donor”-DNA construct at chromosome II, were screened for genomic DNA PCR using primers 1+2 (5’-GGGACAAGTTTGTACAAAAAAGCAGTGGGTCCTAGG-3’ and 5’-ATACGGTACCAAGATCTCGAAACTCGTACAAACTACG-3’). After sequencing, the PCR fragments were cloned into Gateway vectors PUbq-GFPNT Gateway vector (Basto et al., 2006) for generating pUbq-GFP-Bug22 lines and pTWG (DGRC) for generating pUbq-GFP-Bug22 lines. Transgenic lines expressing TTLL3B-GFP were made from a construct carrying artificially synthesized TTLL3B cdNA (Genscript USA Inc.) that was cloned into the pUbq-GFP NT Gateway vector (Pee et al., 2007). The final constructs were sent for transgenesis to BestGene Inc., USA.

Fertility and Unc-phenotype tests
At least 15 vials containing single males of a given genotype were mate to two/three w1118 females. Vials were kept at 25°C. In the case of female
fertility tests, the reciprocal cross was made with w^1118 males. Hatching of embryos as first instar larvae was followed for 72 h (normal hatching time at 25°C is 24 h). The phenotype classes were defined in the following way: wings that were closed like WT ones were considered “normal”, wings that appeared normally open were considered “upfold” and when these were folded, they were termed “non-inflated”. As for the climbing activity, phenotypic classification, flies were termed “climbing normal” when they show a “normal climbing capacity” when flies stayed permanently at the bottom of the culture vials, “lack of equilibrium when standing” or “slowness” if they displayed each of these intermediate movement coordination phenotypes.

Electron microscopy
Tests from male pupae (at about 80 h of pupariation) and from adult males (2 days after eclosion) were dissected in PBS and were fixed in chilled glutaraldehyde (2% in 0.1 M phosphate buffer, pH 7.4) overnight. After a 30 min wash, samples were post-fixed in 1% OsO4, dehydrated in graded concentrations of ethanol and subsequently embedded in Epon 812 resin (no. T024, TAAB). Polymerisation was done at 60°C for 48 h followed. Ultrathin sections of the specimens were collected on copper grids, and stained with uranyl acetate and lead citrate. Sample analysis was done in a Philips CM120 electron microscope (FEI, Eindhoven, Netherlands). Image acquisition with a KeenView camera (SIS, Munich, Germany) and measurements were made with the iTEM software (Olympus France SA, Rungis, France).

Immunoblotting
Tests extracts were prepared either by dissecting 16 testes into 50 μl of cold PBS (with 1 mM PMSF and protease inhibitor cocktail from Sigma), to which 50 μl of 2% in 0.1 M phosphate buffer, pH 7.4) overnight. After secondary antibody washes, testes were incubated with Hoechst/PBS or 40 μl for 40 μl. wings that were completely closed and oriented perpendicular to the substrate were termed “lack of climbing capacity” when they stayed permanently at the bottom of the culture vials, “slowness” if they displayed each of these intermediate movement coordination phenotypes.

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Biology Open (0.5%) DMEM/F-12 medium. Cells were incubated for 72 h and then processed for immunofluorescence and gene knockdown analysis by RT-PCR.

Cotransfection of plasmid DNA and siRNAs was done as for plasmid DNA alone. After 24 h of treatment, the culture medium was replaced by DMEM/F-12 medium supplemented with 0.5% FCS, and cells were kept for additional 48 h until being processed for immunofluorescence and gene knockdown analysis.

Semi quantitative RT-PCR

Total RNA from RPE1 cells was extracted with the RNeasy Minikit (Qiagen), according to the manufacturer’s instructions. Cells from 3 wells of 24-well plates were used for each RNA sample preparation. RNA was resuspended in 30 ml of nuclease-free water and its concentration measured in NanoVue Plus Spectrophotometer (GE Healthcare). cDNA synthesis was done using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to manufacturer’s instructions, except for the fact that oligo(dT)20 primers (Sigma) were used instead of the random primers provided. cDNA was kept at –20°C or used for PCR with GoTaq DNA polymerase (Promega) in 25 ml mixtures. Preliminary PCR amplification was carried out, using a fixed amount of cDNA template and a different number of PCR cycles, in order to determine the linear range of amplification of each product. For the transcription of the cDNA template, we used the following primers CTGCACCACCAACTGCTTAG and AGGTCCACCACTGACAC

Quantification of the intensity of bands on gels was done using the “Gel analysis” tool from ImageJ, according to the instructions present in the following website: http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j.

Quantifications of centriole/cilia lengths

Measurements of centriole and cilia lengths were made in ImageJ software, using the macro 3D-Distance Tool. After setting the voxel dimensions of each Z-series acquisition, the limits of fluorescence signal of the centriole marker considered for the measurement were chosen manually. We used RFP-PACT and Asterless to label centrosomes and basal bodies in both meiotic spermatocytes or early spermatids and acetylated tubulin to label primary cilia in RPE1 cells.

Fluorescence intensity measurements

Mean fluorescence intensities for stained PTM tubulins were measured using the measure tool of the ImageJ software. For measurement in spermatozoids tails, a representative area covering tails from one cyst was selected. The threshold for the subarea corresponding to tubulin signal (filamentous) and to the background signal were then defined manually using the threshold dialog window and the final mean fluorescence intensity determined by subtracting from the thereby assigned mean signal intensity the corresponding mean background intensity. This procedure was partially automatized using a macro created by P. Gilloteaux (Institut Curie). Each dot in Fig. 5G corresponds to the mean intensity of a given cyst at a particular stage of development. For characterization of PTMs in the sperm testes, cysts were classified in four consecutive sub phases of spermatogenesis, identifiable according to their nuclear shape (Lindsley, 1980; Rathke et al., 2010). We counted the number of cysts containing correctly assembled ICs (normal) or improperly assembled ICs (abnormal). In the category ‘cone migration’, we counted the number of cysts containing correctly migrating cones (normal), lagging (as shown in Fig. 5A-inset Bug22) or chaotic (as shown in Fig. 6A-Bug22, arrow).

Quantification of TTLL3B-GFP overexpression in testes

TTLL3B-GFP overexpression in testes was measured by using the measure tool of the ImageJ software. For measurement in spermatozoids tails, a representative area covering tails from one cyst was selected. The threshold for the subarea corresponding to tubulin signal (filamentous) and to the background signal were then defined manually using the threshold dialog window and the final mean fluorescence intensity determined by subtracting from the thereby assigned mean signal intensity the corresponding mean background intensity. This procedure was partially automatized using a macro created by P. Gilloteaux (Institut Curie). Each dot in Fig. 5G corresponds to the mean intensity of a given cyst at a particular stage of development. For characterization of PTMs in the sperm testes, cysts were classified in four consecutive sub phases of spermatogenesis, identifiable according to their nuclear shape (Lindsley, 1980; Rathke et al., 2010). We counted the number of cysts containing correctly assembled ICs (normal) or improperly assembled ICs (abnormal). In the category ‘cone migration’, we counted the number of cysts containing correctly migrating cones (normal), lagging (as shown in Fig. 5A-inset Bug22) or chaotic (as shown in Fig. 6A-Bug22, arrow).

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Author Contributions

T.M.M. and R.B. conceived the project, analysed the data and wrote the manuscript. T.M.M. did the great majority of the experimental procedures. D.G. performed the EM preparation and acquisition and, together with T.M.M., analysed the EM data. C.P. generated tools. C.J. contributed with unpublished tools and advice. R.B. supervised the project.

Competing Interests

The authors have no competing interests to declare.

References


**Supplementary Material**

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**Fig. S1. Generation of a Bug22 null allele.**

(A) Strategy followed to knockout Bug22. A targeting construct was created (P(Bug22") that consisted of an hsp70::white (w") transformation marker, flanked by two loxP sites and surrounded by two arms of homology: "5' arm" and "3' arm", respectively, corresponding to the genomic sequences located immediately upstream and downstream of the Bug22 locus in the chromosome 2L. Following a strategy described by Huang et al. (Huang et al., 2008), homologous recombination event(s) in the germline of females carrying the P(Bug22") construct were induced, in order to delete the CG5343 gene. Recombination events, and thus loss of the junction between Bug22 and its adjacent genes, were tested by PCR using primers 1+2 and 3+4 (see Materials and Methods). (B) Bug22 is detected in WT but not in Bug22 protein extracts by western blot probed with anti-Bug22 antibodies (GTL3, see Materials and Methods). Anti α-tubulin antibody was used as a loading control.
Fig. S2. Quantification of polyglycylation levels in testes. (A) Immunoblots from WT and Bug22 testes extracts probed with antibodies that recognise polyglycylation (polyG). Samples from four WT and Bug22 individuals are shown in each immunoblot. (B) Coomassie staining for analysis of total protein levels is shown. The graphs show the quantification of each lane from immunoblots (C) and from the corresponding protein loadings (D).
**Fig. S3. Sperm polyglycylation takes place before IC migration.** Immunostaining of whole mount WT testis stained for PolyG antibodies (shown in red) and F-actin (shown in green) showing that ICs assemble near the sperm nucleus during the needle stages (top inset) and that during IC migration (bottom inset) tubulin polyglycylation appears already evenly distributed along the front (arrowhead) and rear (arrow). Scale bars: 50 μm (low magnification) 20 μm (high magnification).

**Fig. S4. Control experiments showing specificity of hBug22 depletion.** (A) Estimation of Bug22 depletion by RT-PCR analysis of human Bug22 transcript levels in RPE1 cells co-transfected with negative control (Ctrl) or hBug22 siRNAs + no plasmid/empty pEGFP/pEGP-hBug22RR (“RR” standing for RNAi resistant). GAPDH was used as loading control. The graph bars show average ± SEM from three independent experiments. (B) Representative images of hBug22 siRNA treated RPE1 cells, co-transfected with pEGFP-hBug22RR, stained with antibodies that recognise Ac-Tubulin (shown in red) and DNA (shown in blue). The cell positive for pEGFP-hBug22RR (expressing GFP shown in green) presents normal cilia size, demonstrating that cilia length can be rescued to normal size specifically in cells expressing GFP-hBug22 RNAi resistant transgene. Scale bar: 5 μm. (C) Analysis of cillum length under different transfection conditions. Bars show average ± SEM values from three independent experiments (n>20 per experiment).