RESEARCH ARTICLE

Drosophila sensory cilia lacking MKS proteins exhibit striking defects in development but only subtle defects in adults

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ABSTRACT

Cilia are conserved organelles that have important motility, sensory and signalling roles. The transition zone (TZ) at the base of the cilium is crucial for cilium function, and defects in several TZ proteins are associated with human congenital ciliopathies such as nephronophthisis (NPHP) and Meckel–Gruber syndrome (MKS). In several species, MKS and NPHP proteins form separate complexes that cooperate with Cep290 to assemble the TZ, but flies seem to lack core components of the NPHP module. We show that MKS proteins in flies are spatially separated from Cep290 at the TZ, and that flies mutant for individual MKS genes fail to recruit other MKS proteins to the TZ, whereas Cep290 seems to be recruited normally. Although there are abnormalities in microtubule and membrane organisation in developing MKS mutant cilia, these defects are less apparent in adults, where sensory cilia and sperm flagella seem to function quite normally. Thus, localising MKS proteins to the cilium or flagellum is not essential for viability or fertility in flies.

KEY WORDS: Ciliogenesis, MKS module, Sensory cilia, Transition zone

INTRODUCTION

Cilia and flagella are microtubule (MT)-based extensions of the plasma membrane present in evolution since the last eukaryotic common ancestor (Ishikawa and Marshall, 2011; Nigg and Raff, 2009; Sung and Leroux, 2013). Cilia have diverse roles in cellular sensing, signalling and motility (Basten and Giles, 2013; Berbari et al., 2009; Nigg and Raff, 2009) and are formed when the centriole pair migrates to the plasma membrane (PM). The older, mother, centriole forms a basal body (BB) that docks at the PM and MTs extend from the distal end of the BB to form a membrane-bounded axoneme. Concomitantly, many proteins are recruited to the transition zone (TZ), a complex structure assembled where the BB meets the PM (Garcia-Gonzalo and Reiter, 2012; Ishikawa and Marshall, 2011). The TZ is thought to be essential for cilium function and helps assemble a membrane and cytoplasmic barrier that allows the cilium to form a distinct cellular compartment (Czarnecki and Shah, 2012; Hsiao et al., 2012; Hu and Nelson, 2011; Nachury et al., 2010; Reiter et al., 2012).

Interestingly, many of the genes linked to cilia dysfunction in humans, such as those associated with Meckel–Gruber syndrome (MKS), Joubert syndrome (JBT) and nephronophthisis (NPHP), encode proteins that localise to the TZ (Czarnecki and Shah, 2012; Davis and Katsanis, 2012; Hu and Nelson, 2011; Reiter et al., 2012). Several studies have indicated that the TZ proteins function in broadly three distinct complexes or ’modules’: an MKS module (comprising proteins such as MKS1, Tectonic, B9D1 and B9D2), an NPHP module (comprising proteins such as NPHP1 and NPHP4) and a Cep290 module (comprising proteins such as CEP290) although there is variability and some overlap of module components depending on cell type and species (Garcia-Gonzalo et al., 2011; Chih et al., 2011; Sang et al., 2012; Schouteden et al., 2015; Yee et al., 2015; Williams et al., 2008, 2011; Winkelbauer et al., 2005). Proteins of one module are generally required for the recruitment of other members of the same module, whereas the recruitment of each module can occur at least partially independently of the other modules (Basirli et al., 2014; Bialas et al., 2009; Schouteden et al., 2015; Williams et al., 2008, 2011).

Studies, primarily in worms, indicate that there is a modular hierarchy of TZ assembly with NPHP8 (also known as RGRIP1L and MKSS) (Jensen et al., 2015; Li et al., 2016) initially recruiting Cep290 to form the central cylinder of the TZ (Schouteden et al., 2015) and the MKS and NPHP modules then cooperating to form the Y-links (Williams et al., 2008, 2011). Only when co-mutating or co-depleting components of both MKS and NPHP modules at the same time can a strong effect be observed (Dawe et al., 2007; Tamamachote et al., 2009; Weatherbee et al., 2009; Williams et al., 2008, 2011; Yee et al., 2015). Interestingly, the same complex might perform slightly different functions in different organisms, or even different tissues, as observed in a mouse model of MKS1-associated MKS (Weatherbee et al., 2009).

The fruit fly Drosophila melanogaster has proved a powerful experimental system, yet comparatively little research has focused on the TZ in flies. The fly offers several advantages to study TZ function. Perhaps most importantly, flies do not use cilia for hedgehog or wingless signalling, so flies lacking cilia develop largely normally, without the gross morphological perturbations associated with defects in these signalling pathways in vertebrate systems (Basto et al., 2006). Indeed, most adult fly cells do not contain cilia or flagella, which are restricted to certain sensory neurons and sperm lineages (Kernan et al., 1994). Adult flies lacking centrioles and cilia are severely uncoordinated owing to the lack of cilia in their mechanosensory neurons and die shortly after eclosion (Basto et al., 2006). The analysis of TZ assembly and function is also potentially simplified in Drosophila as flies seem to
lack a core NPHP module. Although two putative NPHP genes, niki (CG10951) and Atxn10 (CG4975) were recently identified in flies (Basiri et al., 2014), no orthologues for the key NPHP module proteins, NPHP1, NPHP4 and NPHP8, have been found. In contrast, Drosophila orthologues of Cep290 and several members of the MKS module (including all but one of the ‘core’ MKS module proteins: TMEM67, CC2D2A, B9D1, B9D2, Tectonic and MKS1 – but not AH1), have recently been identified (Barker et al., 2014; Basiri et al., 2014), suggesting that flies might rely only on the MKS20 and MKS modules for TZ assembly.

Moreover, it has recently been shown that Cep290 and Chibby (a conserved TZ protein involved in Wnt signalling in vertebrates, but not in flies) are required for cilia function in flies (Basiri et al., 2014; Enjolras et al., 2012). Cep290 mutants are uncoordinated and although several MKS module proteins (MKS1, B9D1 and B9D2) were still recruited to the growing ciliary cap structure in elongating mutant spermatids, their localisation was abnormally diffuse (Basiri et al., 2014). Cby mutants exhibit reduced mechanosensation and male fertility, and have structural defects in their sensory neuron cilia and in the short primary cilia found in maturing spermatocytes as well as in the axonemes of mature sperm (Enjolras et al., 2012). The function of the conserved MKS module of proteins, however, has not been directly addressed in flies.

Here, we analyse the distribution of several MKS module proteins in flies and generate mutations in Mks1 and B9d1. We show that the MKS-proteins are recruited to an area of the TZ that is spatially distinct from Cep290, and that mutations in Mks1 and B9d1 disrupt the TZ localisation of the other MKS proteins, supporting the idea that these proteins form a functional complex. Despite the lack of detectable MKS proteins at the TZ, Mks1 and B9d1 mutants are viable and fertile and, although MKS1 mutants exhibit structural defects at sensory cilia during development, these defects are largely absent in adults. Thus, even though flies lack an obvious NPHP module, they can still form functional cilia and flagella without an MKS module, given enough developmental time.

RESULTS

TZ proteins in Drosophila melanogaster

We previously performed a bioinformatics analysis of putative TZ proteins across species and identified a core conserved group of proteins that are present in >50% of ciliated organisms: AH1, B9D1, B9D2, CC2D2A, Tectonic, TMEM67 and probably MKS1 (Barker et al., 2014). Drosophila melanogaster has identifiable homologues of all of these proteins except AH1, and also has homologues of the TZ proteins Cep290, TMEM216, TMEM231 and TMEM237; no components of the core NPHP TZ module were identified (Barker et al., 2014). These findings are in broad agreement with another bioinformatics analysis of TZ proteins in Drosophila, although this study identified two putative non-core NPHP genes, niki (CG10951) and Atxn10 (CG4975) (Basiri et al., 2014). In addition, Chibby (Cby) and Dilatory (Dila) have also recently been identified as components of the Drosophila TZ (Enjolras et al., 2012; Ma and Jarman, 2011).

Drosophila TZ proteins occupy distinct regions in spermatocyte cilia

To better understand how TZ proteins are organised in Drosophila cilia we generated fly lines individually expressing GFP fusions to the core MKS module proteins MKS1, B9D1, B9D2, TMEM216, CC2D2A and Tectonic (see Materials and Methods). We also obtained lines expressing the TZ proteins Cep290–GFP (Basiri et al., 2014) and Chibby (Cby)–GFP (Enjolras et al., 2012). We used dual-colour 3D super-resolution structured illumination microscopy (3D-SIM) to image the distribution of each GFP-fusion at the BB–axoneme complex in vivo in fixed mature spermatocytes labelled with anti-Asterless (Asl) antibodies to mark the outer wall of the BB (Fraz et al., 2013; Varmark et al., 2007) (Fig. 1A). These spermatocytes have long BBs that extend a short axoneme (A.D. Tates, PhD thesis, Rijksuniversiteit Leiden, The Netherlands, 1971) (Fig. S1), and several genes encoding TZ proteins are highly expressed in testes. Moreover, mutations in Cep290, Cby and Dila all lead to defects in spermatogenesis (Basiri et al., 2014; Enjolras et al., 2012; Ma and Jarman, 2011).

In wild-type (WT) spermatocytes all of the TZ proteins were located distally to the BB (stained by Asl, red in Fig. 1A) and extended into the axoneme to varying extents (Fig. 1A). We measured the average length and width of these axoneme extensions (Fig. 1D,E; note that for CC2D2A–GFP we could not accurately measure the width as its distribution was too irregular). We then created compound images (Fig. 1F) by overlaying the average distribution of each protein on an EM-tomogram of a typical WT BB–axoneme complex, aligning the markers so that the Asl staining terminated at the distal end of the BB (see Materials and Methods). These studies revealed that Cep290 occupied a distinct inner region of the TZ (shown in green in all the compound images) that seemed to overlap with the axonemal MTs. The distribution of Cby (blue) overlapped the outer portion of the Cep290 region, whereas Tectonic (purple) and MKS1 (white) occupied a region between the axonemal MTs and the ciliary membrane that was largely outside of the Cep290 region. TMEM216 (orange), B9D1 and B9D2 (both olive green) all also occupied a similar region between the axoneme and the membrane, but these regions extended distally beyond the other TZ proteins, with B9D1 and B9D2 extending the furthest. These studies demonstrate that individual TZ proteins occupy distinct regions within the TZ, in agreement with recent findings in cultured RPE-1 cells (Yang et al., 2015) as well as predictions based on the domain structure of these proteins (Garcia-Gonzalo et al., 2011). The localisation patterns of individual TZ proteins, including Cep290, MKS1, B9D1, B9D2, Cby observed here are in broad agreement with earlier studies (Basiri et al., 2014; Lee et al., 2014).

MKS1 and B9D1 are required to localise the MKS module to the TZ

To study the function of the Drosophila MKS complex in TZ formation we generated an Mks1 mutation by imprecise P-element excision. This generated a 1.4 kb deletion that removed the N-terminal 474 aa of the MKS1 protein, including the start codon and part of the conserved B9 domain (Fig. 1B), a domain of unknown function often found in cilia- and/or flagella-associated proteins. We hereafter refer to flies homozygous for this mutation as Mks1Δ1 mutants.

We analysed the localisation of GFP fusions of the other TZ proteins by 3D-SIM in an Mks1Δ1 background. The TZ localisation of Cep290–GFP and Cby–GFP was not detectably perturbed, but B9D1–GFP, B9D2–GFP, TMEM216–GFP and Tectonic–GFP were no longer detectable at the TZ in Mks1Δ1 mutants (Fig. 1A). Interestingly, although CC2D2A–GFP was also no longer detectable at the TZ in Mks1Δ1 mutants (Fig. 1A). These findings strongly suggest that the recruitment of the entire MKS module of proteins to the TZ is dependent on MKS1, although a TZ that can recruit Cep290 and Cby is still formed in Mks1Δ1 mutants.
Fig. 1. MKS proteins occupy distinct regions within the TZ and depend on MKS1 for their TZ localisation.

(A) 3D-SIM micrographs of Drosophila spermatocyte cilia showing the localisation of various GFP-tagged TZ proteins (green, as indicated) in relation to the BB protein Asterless (Asl, red) in WT (left panels) and Mks1Δ1 mutants (right panels).

(B,C) Schematic representations of (B) the Mks1Δ1 deletion and (C) the B9d1Δ1 mutation that creates a frameshift at amino acid 41 and a premature stop codon at amino acid 77 (blue asterisk).

(D) Graph quantifies the longitudinal length of Asl and the TZ proteins in 3D-SIM micrographs. Isolated GFP dots, sometimes present at the distal tips of the TZ (arrows in A), were ignored for these measurements.

(E) Graph quantifies the diameter of the ‘barrel’ formed by Asl and TZ proteins in 3D-SIM micrographs (see Materials and Methods).

(F) Using the mean lengths and diameters obtained in D,E we created composite images (top panels) superimposing the localisation of Asl (red) and the TZ proteins (various colours, as indicated) on a typical Drosophila spermatocyte EM micrograph. A schematic of the transverse profile of the BB–axoneme complex (lower panel) shows the various protein localisations in relation to the ninefold symmetry MT blades (grey triplets) and the axoneme membrane (black circle). Error bars represent the s.e.m. Scale bar: 2 µm.
We also generated a mutation in the B9d1 gene using CRISPR–Cas9 technology. This mutation generated a frame shift leading to the introduction of a premature stop codon and so presumably to the effective deletion of the C-terminal 164 aa of the B9d1 protein (Fig. 1C); we hereafter refer to flies homozygous for this mutation as B9d1Δ1 mutants. The TZ localisation of MKS1–GFP and B9D2–GFP were no longer detectable in B9d1Δ1 mutants (Fig. S2). As MKS1 is required for the localisation to the TZ of all the other MKS module proteins we have examined, the absence of MKS1 from the TZ of B9d1Δ1 mutants means that the other MKS module proteins are also probably absent from the TZ in B9d1Δ1 mutants. Thus, in agreement with previous reports (Bialas et al., 2009; Williams et al., 2008), MKS module proteins seem to be interdependent for their localisation to the TZ, and are not detectable at the TZ in Mks1Δ1 and B9d1Δ1 mutants.

**Mks1Δ1** and **B9d1Δ1** mutants are viable, fertile and do not exhibit dramatic defects in sensory cilia function

To our surprise, Mks1Δ1 and B9d1Δ1 mutants were not noticeably uncoordinated. We have maintained homozygous stocks of these mutants in the laboratory for more than two years, indicating that they are both male and female fertile. This is in contrast to previously described mutations in the TZ protein encoding genes Cep290 (Basari et al., 2014), Cby (Enjolras et al., 2012) and dila (Ma and Jarman, 2011) that are uncoordinated and exhibit reduced male fertility. In quantitative fertility tests Mks1Δ1 mutant males were not significantly less fertile than WT controls, indicating that mutant sperm flagella were largely functional (Fig. 2A).

We next performed a series of assays to test sensory cilia function. We first tested the flies’ geotaxis response (Hirsch and Tryon, 1956). When WT flies are knocked down to the bottom of a tube they quickly climb to the top, whereas flies with defective sensory cilia function are uncoordinated and cannot climb efficiently. There was no significant difference in the number of flies counted at the top of the vial 10 s after knock down between WT and Mks1Δ1 mutants (Fig. 2B), but we noticed that Mks1Δ1 flies seemed to climb faster than WT. We therefore repeated the assay, but measured the average distance climbed per vial after 7 s of knockdown. This confirmed that the Mks1Δ1 mutants climb slightly faster than WT (Fig. 2C). We next compared the dust grooming response of WT and Mks1Δ1 mutant flies, which is driven by the mechanical stimulation of external bristles by dust (Phillis et al., 1993). This revealed that Mks1Δ1 flies had a significantly higher cleaned area on their heads but no difference was observed in the notum (Fig. 2D). Taken together, these observations confirm that sensory cilia function in Mks1Δ1 mutants is not dramatically perturbed and that, if anything, mutant flies exhibit a slightly increased response to certain stimuli (see below), although such a subtle difference could simply result from genetic background differences.

**Mechanoreceptor potentials of Mks1Δ1 and B9d1Δ1 mutant sensory cilia are not dramatically perturbed**

To more directly examine sensory cilia function in Mks1Δ1 mutants we measured the electrophysiological response of mechanosensory bristles in the notum. Each of these bristles is innervated by a peripheral sensory neuron that extends a cilium at the base of the bristle. The supporting cells that encapsulate the cilium generate a trans-epithelial electron potential (TEP) that can be detected by placing one electrode over the cut end of a bristle and inserting a reference electrode into the thorax (Kernan et al., 1994). Deflecting the bristle with a mechanical stimulus causes ion channels in the cilium membrane to open, allowing ions to flow from the support cells and generate a mechanical response potential (MRP). MRPs in Mks1Δ1 mutants and in B9d1Δ1 mutants did not differ dramatically from controls; in fact both mutants exhibited a slight, but statistically significant, increase in MRP amplitude (Fig. 2E). As a negative control we made similar recordings in Pericentrin-like-protein (Plp) mutants that have previously been shown to have severely reduced sensory cilia function (Martinez-Campos et al., 2004) (Fig. 2E). We also analysed a line expressing MKS1–GFP in the Mks1Δ1 mutant background and observed that
this strain also had a slight increase in the MRP response (Fig. 2E), suggesting that the slight increase in sensitivity is either a result of genetic background differences between the WT and mutant strains, or is not efficiently rescued by the MKS1–GFP transgene. In either case, the electrophysiological response of the Mks1Δ1 and B9d1Δ1 mutant sensory cilia clearly falls within the normal range (Dubruille et al., 2002).

**Mks1**\(^{Δ1}\) mutant spermatocyte axonemes are shortened, but mutant testes exhibit no dramatic defects

To better understand why the lack of MKS module proteins seems to have only a very mild effect on cilia and flagella function, we examined cilia and flagella structure by EM tomography. We first examined testes, as MKS module proteins are most highly expressed in this tissue and mutations in *Cep290*, *Cby* and *dila* all exhibit severe defects in testes (Basiri et al., 2014; Enjolras et al., 2012; Ma and Jarman, 2011). An initial TEM examination of Mks1Δ1 mutant flagella in cross section revealed no obvious structural defects, consistent with our observations that mutant flies are male fertile (Fig. S3). However, we noticed that the short axonemes that are normally assembled in mature spermatocytes (A.D. Bates, PhD thesis, Rijksuniversiteit Leiden, The Netherlands, 1971) were dramatically shortened in mutant cells (Fig. 3A,C,D; Movies 1 and 2), whereas the length of the very long BBs at the base of these axonemes was not dramatically affected (Fig. 3B). Importantly, this short axoneme phenotype was rescued by the expression of the MKS1–GFP transgene (Fig. 3A,C,D; Movie 3). These findings strongly suggest that the proper assembly of the short spermatocyte axoneme is dependent on MKS module proteins.

**The localisation of the transmembrane proteins NompA and NompC is not detectably perturbed in Mks1**\(^{Δ1}\) mutant sensory cilia

The slight increase in signalling response in the bristle hairs suggested a possible defect in protein composition in the ciliary membranes of Mks1Δ1 mutants. We therefore examined the distribution of the transmembrane proteins NompA and NompC. NompA is localised to the dendritic cap at the distal tip of the cilium and is essential for cilia function (Chung et al., 2001; Kernan et al., 1994), whereas NompC forms ion channels in the ciliary membrane that are enriched towards the distal tip of the cilium (Lee et al., 2008). The slight increase in signalling response in the bristle hairs of Mks1Δ1 mutants at 48 h APF (Fig. 5). At 48 h APF WT cilia (n=3) form relatively straight cilia that extend towards the PM of the hair cell (Fig. 5A,B; Movie 4). At their base, these cilia have a BB (BB MTs shown in dark green) that extends MT doublets into the TZ (blue) and then into the ciliary membrane (light green). The TZ is discernible as a straightening (black bar, Fig. 5C) and electron-dense thickening (blue bar, Fig. 5C) of the membrane, similar to that visualised in spermatocyte cilia (Fig. 3A). The ciliary MTs doublets extend for ~2/3 of the cilia length and then continue as singlets of the A MTs; most of these A MTs terminate close to the tip of the cilium. Some MTs that were not connected to the BB were also found in the axoneme in close apposition to the ciliary membrane (purple) and an electron-dense crystalline structure surrounded the top ~1/3 of the axoneme, apparently linking the outer ciliary membrane with the adjacent sheath cell membrane (arrows, Fig. 5D). At the base of the cilia, a daughter centriole was positioned just below the BB and was surrounded by ciliary rootlets (Chen et al., 2015; Styczynska-Soczka and Jarman, 2015) emanating from the distal end of the BB (Fig. 5D) where vesicles could also be observed (blue asterisks, Fig. 5E).

**Intraflagellar transport seems to be subtly perturbed in Mks1**\(^{Δ1}\) mutant cilia

Previous studies have suggested that MKS proteins interact with intraflagellar transport (IFT) proteins in zebrafish cilia (Zhang et al., 2013). To test this possibility more directly in flies, we examined the localisation of NompB–GFP – the IFT88 homolog responsible for anterograde transport (Han et al., 2003) – and of NompA–YFP – the IFT140 homolog that is part of the retrograde transport system (Lee et al., 2008) – at the base of the notum bristle. In WT cells, NompB–GFP localised as a line at the base of the cilium that was enriched at the proximal end of the axoneme, and this was also the case in Mks1Δ1 mutants, although the NompB–GFP signal was slightly, but significantly, extended (Fig. 4D).
disrupted in Mks1Δ1 mutant sensory neurons. Importantly, this is not owing to the Mks1Δ1 mutant developing more slowly than WT, as the time from pupation to eclosion was similar in WT and mutant [4.6±0.1 days (mean±s.d.) at 25°C, P>0.999].

At 72 h APF the overall organisation of the WT cilium (Fig. 5E,F; Movie 6) was similar to that seen at 48 h APF, although the volume of the cilium had increased and the cilium contained many more MTs that ran along the main axis of the cilium but that were not directly connected to the BB. Most of these MTs were in close contact with the ciliary membrane and small links between the MTs and the membranes were visible (black arrows, Fig. 5H), similar to the membrane–microtubule connectors previously observed in chordotonal cilia (Young, 1973). These MTs had a radial separation of ∼16 nm (Fig. 6C) and were on average ∼51 nm (median of ∼41 nm) from the ciliary membrane (Fig. 6D). In addition, the amount of internal membrane present within the 72 h APF cilium also increased (gold, Fig. 5F,G; Fig. 6E), and this extra membrane seemed to originate from invaginations of the cell membrane into the lumen of the cilium (arrow, Fig. S4). The inner volume of this membrane was therefore in contact with the extracellular cytoplasm. The membrane at these regions was very electron-dense, suggesting the presence of high levels of protein material and/or saturated lipids (Fig. 5E).

Interestingly, the 72 h APF Mks1Δ1 mutant cilium had a MT organization that was much more similar to WT than seen at 48 h APF, and many of the BB MTs now extended to the tip of the cilium and large numbers of non-ciliary MTs were also present aligned along the long axis of the cilium (Fig. 5F,G; Movie 7). We noticed,
however, that there was a larger volume of inner membrane within Mks1\(^{Δ1}\) cilia compared with WT cilia (gold, Fig. 5F, G; Fig. 6E), and the ratio of cilium volume to inner membrane volume seemed significantly increased in Mks1\(^{Δ1}\) mutants (Fig. 6E).

**DISCUSSION**

The TZ is essential for proper cilia function (Czarnecki and Shah, 2012; Reiter et al., 2012), and recent studies have suggested that an MKS and an NPHP module work partly redundantly with a Cep290 module to establish the TZ (Basiri et al., 2014; Schouteden et al., 2015; Williams et al., 2008, 2011). The NPHP module seems to have arisen later in evolution and to be largely absent in Drosophila, but six potential members of the MKS module have been identified in flies (Barker et al., 2014; Basiri et al., 2014). We show here that mutations that disrupt the function of either of two members of the MKS module, MKS1 or B9D1, strongly disrupt the TZ-localisation of the other MKS module components, but do not detectably disrupt the TZ localisation of Cep290 or the TZ component Cby, strongly
supporting the idea that the MKS module proteins form a functional unit in flies. Despite the apparent lack of a functional MKS module in \textit{Mks1}\(_\Delta 1\) or \textit{B9d1}\(_\Delta 1\) mutants, these flies exhibit only subtle cilia and flagella defects, and mutant flies are viable and fertile.

Although cilia function is only mildly perturbed in \textit{Mks1}\(_\Delta 1\) and \textit{B9d1}\(_\Delta 1\) mutants, the defects we observe are potentially informative. The MKS module proteins are all highly expressed in testes and two types of cilia are formed in this tissue. Initially, short axonemes extend from the long centrioles found in mature spermatocytes, and these centrioles will later form the BB of the sperm flagella. In \textit{Mks1}\(_\Delta 1\) mutants the short spermatocyte axonemes are dramatically reduced in length, although Cep290 and Cby are both localised normally at the base of the axoneme. The function of these short spermatocyte cilia is unknown, but our findings demonstrate that the axonemes of these cilia can be dramatically shortened without any obvious effect on the fly. In particular, the subsequent formation of the much longer sperm flagella is not detectably perturbed and mutant males seem to exhibit normal levels of fertility. This is in contrast to \textit{Cep290} mutants that also have very short spermatocyte cilia (that seem to lack axonemal MTs), but subsequently exhibit dramatic defects in the formation of the sperm flagella axoneme (Basiri et al., 2014). It therefore remains unclear why MKS module proteins are so highly expressed in testes. We suspect that these proteins must contribute to cilia and flagella.
function in the testes, but that this function is not apparent in the assays we have used here. Nevertheless, it is clear that Cep290 can organise a sufficiently functional TZ in fly testes in the apparent absence of the MKS module, but MKS module proteins cannot do the same in the absence of Cep290 (Basiri et al., 2014).

Cep290 mutant flies are also severely uncoordinated because of defects in their sensory cilia (Basiri et al., 2014), whereas Mks1Δ1 mutants are not noticeably uncoordinated and perform at least as well as WT flies in various assays that assess cilia function. Indeed, mutant flies seem to climb slightly faster than WT flies, and the mechanosensory cilia that attach to the bristles on the notum are, if anything, slightly more sensitive than WT. Although sensory cilia structure was only moderately perturbed in Mks1Δ1 mutant pupae at 72 h APF, cilia structure was more profoundly disrupted earlier in development at 48 h APF. Most strikingly, the axoneme in these developing pupae had severely disrupted MTs, with very few of the BB MTs extending into the axoneme. Supporting this idea, this intriguing possibility that IFT particles can assemble on the inner lumen of the BB wall in these cilia. Clearly, more work will be required to determine the identity and composition of these structures, but these observations raise the intriguing possibility that IFT particles can assemble on the inner lumen of the BB wall in these cilia. Supporting this idea, this accumulation is in agreement with our results showing that NompB–GFP localisation is extended in Mks1Δ1 mutant pupae at 72 h APF, and with previous work that also show an accumulation of NompB in RempA mutants in the chordotonal organ cilia (Lee et al., 2008). Taken together, these results suggest that Mks1 initially affects the localisation of RempA, which leads to the abnormal accumulation of NompB.

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be established and maintained in the absence of these proteins. More work will be necessary to explain the mechanistic basis of these early defects in cilia structure, but as an IFT-B component can regulate ciliary MT growth by negatively regulating the microtubule stabilising protein MAP4 (Bizet et al., 2015), and Mks1ΔI seems to lead to an accumulation of the fly IFT-B component NompB, a similar process could be occurring in flies.

Our results, together with previous studies (Basiri et al., 2014; Enjolras et al., 2012; Ma and Jarman, 2011) suggest a model of sequential assembly of TZ assembly in flies. BB docking to the PM triggers the recruitment of the TZ components, which rely on Cep290 for correct localisation. Cep290 is essential for proper TZ function in flies and for the correct migration of the TZ along the axoneme in growing spermatids (Basiri et al., 2014). Cby plays an important role in these processes, but might not be essential as Cby mutants are uncoordinated, show reduced mechanosensation, and reduced, but not abolished, male fertility – indicating the existence of at least a semi-functional cilium and/or flagellum. In contrast, although the MKS complex is required for the assembly of the short axoneme in spermatocytes, cilia and flagella function in adult flies seems only mildly perturbed in the absence of the complex. Previous studies in mice and worms have also indicated that MKS mutants exhibit relatively mild cilia defects (Dawe et al., 2007; Sluats et al., 2015; Tammachote et al., 2009; Weatherbee et al., 2009; Williams et al., 2008, 2011), but this is often attributed to the MKS module acting redundantly with the NPHP module (Williams et al., 2008, 2011; Yee et al., 2015), something that seems unlikely in flies as they lack most of the core NPHP module proteins (Barker et al., 2014; Basiri et al., 2014).

An intriguing possibility is that the MKS module in flies acts partially redundantly with Cby, and Cby mutants exhibit a similar NompB accumulation at the TZ as MKS mutants (Enjolras et al., 2012). Alternatively, the MKS module could also act partially redundantly with proteins of the Bardet–Beied syndrome complex (BBSome) that is required for cilium function, vesicle trafficking and IFT in several systems (Jin et al., 2010; Wei et al., 2012; Williams et al., 2014). Perturbing BBS function modifies the phenotype of Cep290, MKS or NPHP mutations in other systems (Barbelanne et al., 2015; Yee et al., 2015; Zhang et al., 2014), but the BBS complex remains uncharacterised in flies.

MATERIALS AND METHODS

Fly stocks

w1118 was used as a control in all experiments. Mks1ΔI (this study) was generated by imprecise excision of a p-element [P{Epo}2]CG2556EY2042 (Bloomington); Rubin and Spradling, 1982) 581 bp upstream of Drosophila Mks1. B9d1ΔI (this study) was generated by CRISPR–Cas9. Three guide RNAs (gRNAs: GAAGAGTGCCGAGACTATTGCG, CATCGTGGG-GCAAAATAGTCTCGG and CATCAGTCTCCCGGGCAACGAGG) were cloned into pCFD3-dU6:3gRNA plasmid (Port et al., 2014) and injected into Drosophila embryos expressing the Cas9 gene under the control of the Nanos promoter (Port et al., 2014). P[wy5c] was published previously (Martinez-Campos et al., 2004). Mks1–GFP, B9D1–GFP, B9D2–GFP, TMEM216–GFP, Tectonic–GFP (all this study) are expressed from the Ubiquitin promoter, which drives moderate expression in all tissues (Lee et al., 1988). CC2D2A–GFP (this study) is driven by the endogenous promoter by including a ~2 kb upstream region in the transgene. Transgenes were cloned into Gateway (Invitrogen) vectors as described before (Basto et al., 2006) labelled with w+. The following transgenic lines were published previously and were kind gifts: Cby–GFP (B. Durand; Enjolras et al., 2012), Cep290–GFP (T. Avidor-Reiss; Basiri et al., 2014), NompA–GFP (R. Stanewsky; Chung et al., 2001), NompB–GFP (Maurice Kerman; Han et al., 2003), NompC–GFP (Y. Nung; Yan et al., 2013), NompC-Gal4 (Y. Nung; Yan et al., 2013), Rempa–YFP (A. Jarman; Lee et al., 2008).

Behaviour assays

To quantify the pupal development, Mks1ΔI or w1118 white pupae were marked with date and time and kept at 25°C. Hatched pupae were counted every morning and evening. Data is presented as the average of three technical repeats of at least five pupae per repeat. For male fertility, single Mks1ΔI or w1118 flies were crossed to three w1118 females. Hatched embryos were counted every morning and evening for 6 days. Each of the 25 biological replicates is presented as the average number of hatched embryos per day. The standard climbing assay was adapted from Ma and Jarman (2011). Briefly, 15 adult flies at 1–3 days old were knocked to the bottom of a cylinder and the number of flies above 12 cm counted after 10 s. Data are the averages of three technical replicates. To measure the distance climbed by individual flies, five technical replicates of 10 adult flies at 1–3 days old were filmed being knocked to the bottom and crawling up the sides of the cylinder. The experimental time was reduced to 7 s to prevent flies reaching the top of the cylinder. The distance climbed per fly was measured in Fiji (Schindelin et al., 2012). For the grooming assay, the average of 10 flies at 1–3 days old per time point were covered in Reactive Yellow 86 dust (Organic Dyestuffs Corporation) (Seeds et al., 2014). The flies were then left to groom themselves for 30 or 90 min before imaging the head and notum on a dissecting microscope equipped with a Digital Sight camera (Nikon). A polygon ROI of the head or notum was used to measure their area. Next, the colour threshold was set to the Reactive Yellow 86 dust to select the areas covered in the dust. The area of these parts within the original ROI was also measured and the ratio was determined.

Electrophysiology

Bristle recordings were collected from individual males at 2–4 days old as described in (Kerman et al., 1994). Humeral and notopleural bristles were cut along their midpoint and a tungsten wire reference electrode was inserted into the thorax. A glass capillary electrode containing 121 mM K+, 9 mM Na+, 0.5 mM Ca2+, 4 mM Mg2+, 35 mM glucose, and 5 mM HEPES, pH 7.1, was placed over the cut bristle to record MRPs. MRPs were evoked by 30 μm deflections of the electrode by software-controlled movement of a PatchStar micromanipulator (Scientifica). Analogue signals were acquired through a MultiClamp-700B amplifier and digitised with an Axon Digidata 1550A A/D board. Data were collected in Clampfit 10.5 (Molecular Devices) and MRP amplitudes were measured offline. Mean amplitudes from each genotype were then compared using a one-way ANOVA with Tukey’s multiple comparisons test in Prism 6 (GraphPad).
NompB-GFP signal was measured in IMOD (Kremer et al., 1996) using open contours.

Electron tomography
Testsis samples were prepared as previously reported (Roque et al., 2012). This protocol was modified to prepare pupal samples for ET. Pupae were removed from their case at 48 or 72 h APF and placed into 2.5–4% glutaraldehyde–paraformaldehyde in 0.2 M PIPES buffer (fixative solution) and heptane for 1–2 h at room temperature. The pupae were transfer to 0.2 M PIPES and dissected with a pair of tungsten needles. The abdomen, head and cuticle were removed. The dissected samples were placed in fixative solution at 4°C overnight. Samples were washed three times for 15 m each. Secondary fixation was performed in 1% uranyl acetate solution for 3742

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.B.P. and H.R. designed and performed the majority of experiments and helped to write the manuscript. I.D. and J.T. designed and J.T performed the electrophysiology experiments. A.R.B. and H.R.D. devised and performed the bioinformatics analysis that identified the genes studied here. J.W.R. helped design experiments and write the manuscript.

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Data availability
Movies are available at https://dx.doi.org/10.6084/m9.figshare.3468638.v1.

Supplementary information
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