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SHORT REPORT

Phosphatidylinositol 4,5-bisphosphate regulates cilium transition zone maturation in *Drosophila melanogaster*

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ABSTRACT

Cilia are cellular antennae that are essential for human development and physiology. A large number of genetic disorders linked to cilium dysfunction are associated with proteins that localize to the ciliary transition zone (TZ), a structure at the base of cilia that regulates trafficking in and out of the cilium. Despite substantial effort to identify TZ proteins and their roles in cilium assembly and function, processes underlying maturation of TZs are not well understood. Here, we report a role for the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) in TZ maturation in the *Drosophila melanogaster* male germline. We show that reduction of cellular PIP_2 levels through ectopic expression of a phosphoinositide phosphatase or mutation of the type I phosphatidylinositol phosphate kinase Skittles induces formation of longer than normal TZs. These hyperelongated TZs exhibit functional defects, including loss of plasma membrane tethering. We also report that the *onion rings* (*onr*) allele of *Drosophila Exo84* decouples TZ hyperelongation from loss of cilium–plasma membrane tethering. Our results reveal a requirement for PIP_2 in supporting ciliogenesis by promoting proper TZ maturation.

KEY WORDS: PtdIns(4,5)P_2, Skittles, Cep290, Cilia, Transition zone, Basal body

INTRODUCTION

Cilia are sensory organelles important for signalling in response to extracellular cues, and for cellular and extracellular fluid motility (Satir and Christensen, 2010; Marshall and Nonaka, 2006; Eley et al., 2005; Brooks and Wallingford, 2014). Consistent with their importance, defects in cilium formation are associated with genetic disorders known as ciliopathies, which can cause neurological, skeletal and fertility defects, in addition to other phenotypes (Waters and Beales, 2011; Valente et al., 2014; Hammarsjö et al., 2017; Inaba and Mizuno, 2016). Many ciliopathies are associated with mutations in proteins that localize to the transition zone (TZ), the proximal-most region of the cilium that functions as a diffusion barrier and regulates the bidirectional transport of protein cargo at the cilium base (Reiter et al., 2012; Szymanska and Johnson, 2012). For example, the conserved TZ protein CEP290 is mutated in at least six different ciliopathies (Rachel et al., 2012), and is important for cilium formation and function in humans (Shimada et al., 2017; Stowe et al., 2012) and *Drosophila* (Basiri et al., 2014). Although the protein composition of TZs has been investigated in various studies (Gonçalves and Pelletier, 2017), the process of TZ maturation, through which it is converted from an immature form into one competent at supporting cilium assembly, is relatively understudied.

Ciliogenesis begins with assembly of a nascent TZ at the tip of the basal body (BB) (Reiter et al., 2012). During TZ maturation, its structure and protein constituents change, allowing for establishment of a compartmentalized space, bounded by the ciliary membrane and the TZ, where assembly of the axoneme, a microtubule-based structure that forms the ciliary core, and signalling can occur. In *Drosophila*, nascent TZs first assemble on BBs during early G2 phase in primary spermatocytes (Riparbelli et al., 2012). This occurs concomitantly with anchoring of cilia to the plasma membrane (PM), microtubule remodelling within the TZ (Riparbelli et al., 2013; Gottardo et al., 2013), and establishment of a ciliary membrane that will persist through meiosis (Riparbelli et al., 2012) (Fig. 1A). TZ maturation has been described in *Paramecium* (Aubusson-Fleury et al., 2015), *Caenorhabditis elegans* (Serwas et al., 2017) and *Drosophila* (Gottardo et al., 2013), and is most readily observed in the *Drosophila* male germline as an increase in TZ length.

We previously showed that the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is essential for proper axoneme structure in the *Drosophila* male germline (Wei et al., 2008; Fabian et al., 2010). PIP_2, which is one of seven different phosphoinositides (PIPs) present in eukaryotes, localizes primarily to the PM, where it is required for vesicle trafficking, among other processes (Balla, 2013). PIP_2 has recently been linked to cilium function. Although the ciliary membrane contains very little PIP2 due to action of the cilium-resident PIP phosphatase INPP5E, the cilium base is enriched in PIP_2 (Nakatsu, 2015). Inactivation of INPP5E causes a build-up of intraciliary PIP_2, which disrupts transport of Hedgehog signalling proteins in vertebrates (Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Conduit et al., 2017) and ion channels involved in mechanotransduction in *Drosophila* (Park et al., 2015). In light of current understanding of PIP_2 as a modulator of cilium function, we sought to investigate the cause of defects we had observed in axoneme assembly in *Drosophila* male germ cells with reduced levels of PIP_2 (Wei et al., 2008; Fabian et al., 2010).

RESULTS AND DISCUSSION

PIP_2 is essential for transition zone maturation

To investigate how reduction of cellular PIP_2 affects ciliogenesis in the *Drosophila* male germline, we used transgenic flies expressing the *Salmonella* PIP phosphatase SigD under control of the spermatocyte-specific β_2-tubulin promoter (hereafter β_2t-SigD) (Wong et al., 2005). To examine whether axoneme defects in β_2t-SigD were caused by aberrant TZ function, we investigated localization of fluorescently tagged versions of the core centriolar/BB protein Ana1 (CEP295) (Goshima et al., 2007; Blachon et al., 2009) and the conserved TZ protein Cep290 (Basiri et al., 2014) during early steps of cilium assembly. Cep290 distribution appeared

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Received 6 April 2018; Accepted 11 July 2018

Journal of Cell Science (2018) 131, jcs218297. doi:10.1242/jcs.218297

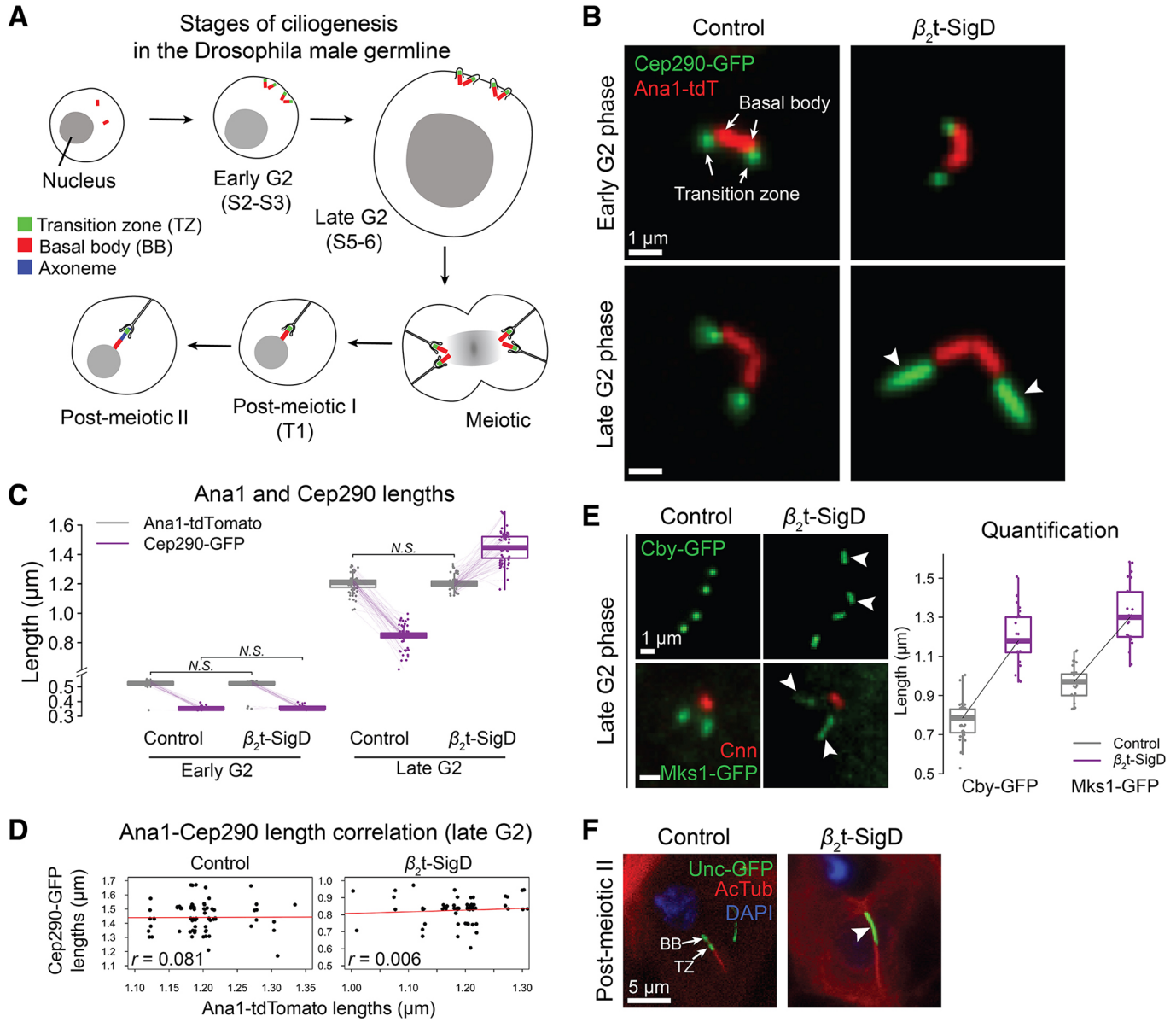

Fig. 1. SigD expression induces TZ hyperelongation. (A) Schematic diagram of ciliogenesis in the *Drosophila* male germline. Stages in parentheses correspond to those in Cenci et al. (1994). (B) β_2t-SigD expression induces hyperelongation in cilia at late G2 phase (arrowheads) as revealed by examination of Cep290–GFP. (C) Quantification of paired Ana1–Cep290 lengths in early and late G2 spermatocytes (n>30 and >65, respectively). (D) Lengths of Ana1–tdTomato versus Cep290–GFP in control and β_2t-SigD cells at late G2 from (C) showing negligible correlation. Regression lines (red) and Pearson correlation coefficients (r) are shown. (E) β_2t-SigD expression induces hyperelongation of the area occupied by the TZ proteins Chibby (Cby) and Mks1 in late G2 (arrowheads). Box plots (right) show quantifications of lengths (n>25). (F) TZ hyperelongation in β_2t-SigD persists through meiosis (arrowhead) but does not prevent axoneme outgrowth. Acetylated tubulin (AcTub) labels the axoneme.

similar in control and β_2t-SigD cells in early G2 phase, when TZs are still immature. In contrast, Cep290-labelled TZs were significantly longer in β_2t-SigD compared to controls by late G2, following TZ maturation (Fig. 1B,C). Unlike *Drosophila cep290* mutants, which contain longer than normal BBs (Basiri et al., 2014), Ana1 length was not affected in β_2t-SigD, and we did not observe a strong correlation between Cep290 and Ana1 lengths (Fig. 1D). Consistent with this result, the ultrastructure of BBs in β_2t-SigD is normal, and localization of the centriolar marker GFP–PACT (Basto et al., 2006) is similar in control and β_2t-SigD cells (Wei et al., 2008). In contrast, the TZ proteins Chibby (Cby) (Enjolras et al., 2012) and Mks1 (Vieillard et al., 2016; Pratt et al., 2016) exhibited hyperelongation in β_2t-SigD cells (Fig. 1E), indicating that this phenotype is not unique to Cep290. TZ hyperelongation was highly penetrant (>70%, n>200) and showed a high correlation (>0.95) within syncytial germ cell cysts, suggesting a dosage-based response to a shared cellular factor, presumably SigD. Despite persistence of hyperelongated TZs through meiosis, axonemes were able to elongate in post-meiotic cells (Fig. 1F). Nonetheless, the ultrastructure of these axonemes is frequently aberrant, either lacking nine-fold symmetry or containing triplet microtubules in addition to the usual doublets (Wei et al., 2008).

The type I PIP kinase Skittles regulates TZ length

Although PIP_2 is its major substrate in eukaryotic cells *in vivo* (Terebiznik et al., 2002; Zhou et al., 2001; Sengupta et al.,

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2013), SigD can dephosphorylate multiple PIPs *in vitro* (Norris et al., 1998). To address whether TZ hyperelongation observed in β_2t-SigD represented a physiologically relevant phenotype due to decreased PIP_2, we attempted to rescue this phenotype by co-expressing β_2t-SigD with fluorescently tagged Skittles (Sktl) under control of the β_2-tubulin promoter. Sktl expression was able to suppress TZ hyperelongation to various degrees in a cilium-autonomous manner (Fig. 2A,B). Furthermore, the BB/TZ protein Unc–GFP (Baker et al., 2004; Wei et al., 2008) revealed TZ hyperelongation at a low penetrance in *sktl*$^{2.3}$ mutant clones (Fig. 2C), indicating that Sktl is important for TZ maturation.

Vertebrate type I PIP kinase PIPKIγ is important for cilium formation in cultured cells (Xu et al., 2016). The *Drosophila* PIPKIs, Sktl and PIP5K59B, arose from recent duplication of the ancestral PIPKI gene, and are not orthologous to specific vertebrate PIPKI isoforms (Fig. 2D). Sktl has diverged more than its paralogue PIP5K59B and seems, based on our data, to be functionally related to PIPKIγ and the *C. elegans* PPK-1 in having roles at cilia (Xu et al., 2014). However, unlike human PIPKIγ, which licenses TZ assembly by promoting CP110 removal from BBs (Xu et al., 2016), our results suggest that Sktl functions in regulating TZ length but not TZ assembly. Consistent with this, neither inactivation nor overexpression of *cp110* affects cilium formation in *Drosophila*,

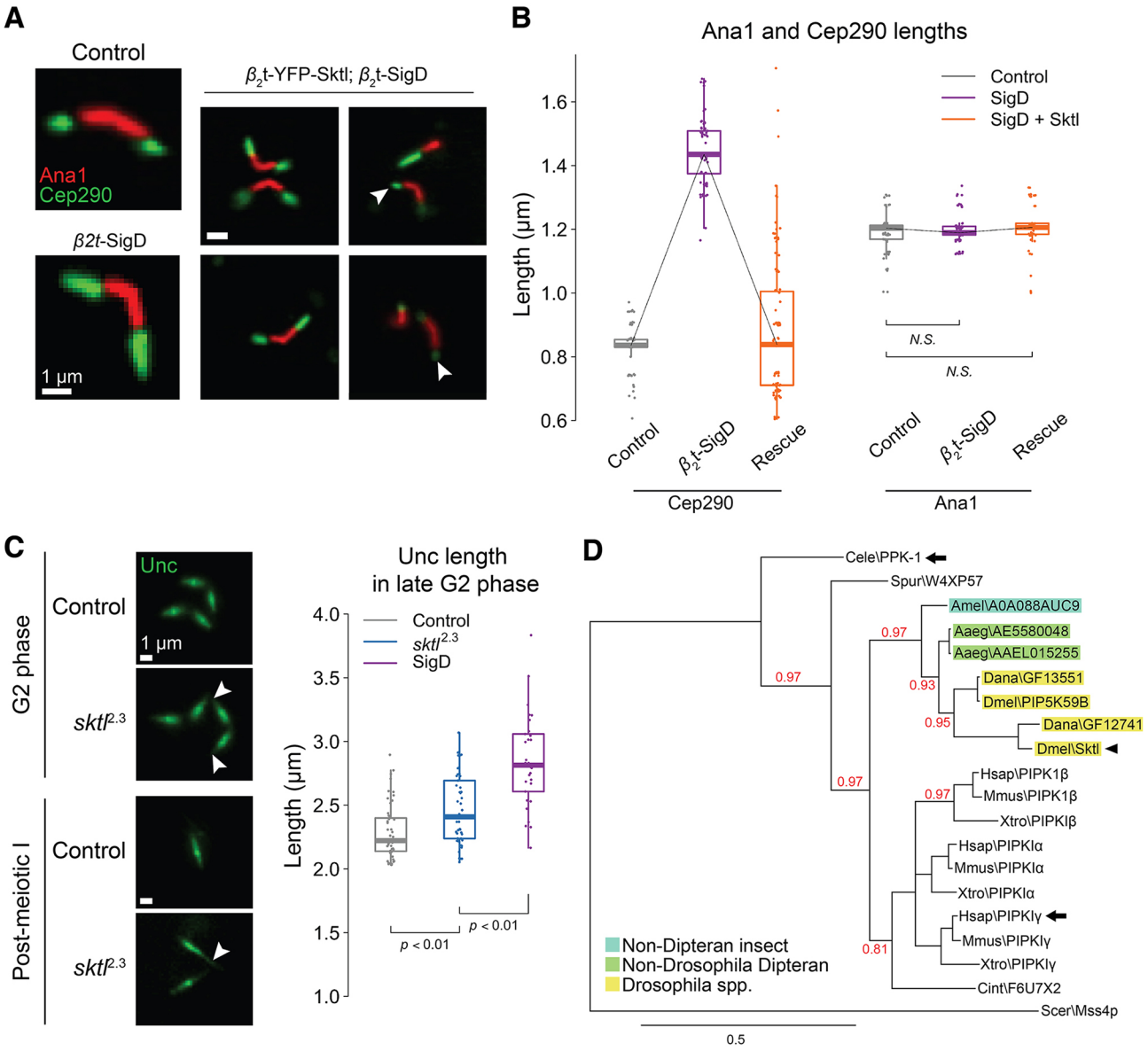

Fig. 2. Sktl is important for TZ maturation. (A) Expression of Sktl suppresses β_2t-SigD-induced TZ hyperelongation in a cilium-autonomous manner. Images demonstrate the varying levels of rescue of the Cep290–GFP-marked length in β_2t-YFP-Sktl; β_2t-SigD cells. Arrowheads mark a fully rescued Cep290 distribution. (B) Quantification of Cep290 and Ana1 lengths from control, β_2t-SigD and β_2t-YFP-Sktl; β_2t-SigD cells from A (n=100). (C) Cilia in *sktl*$^{2.3}$ clones exhibit TZ hyperelongation (arrowheads) marked by Unc–GFP (left). Quantification of Unc–GFP-marked lengths in control (n=53), *sktl*$^{2.3}$ (n=31) and β_2t-SigD (n=51) spermatocytes at late G2 (right). (D) Phylogenetic tree of PIPKIs showing evolutionary conservation of cilium-associated functions. The scale bar (bottom) represents expected amino acid substitutions per site. Branch support values are in red (a value of 1 indicates maximum support). Black arrows, previous evidence of involvement in cilium-associated functions (from Xu et al., 2016). Black arrowhead, Sktl. Cele, *Caenorhabditis elegans*; Spur, *Strongylocentrotus purpuratus*; Amel, *Apis mellifera*; Aaeg, *Aedes aegypti*; Dana, *Drosophila ananassae*; Dmel, *Drosophila melanogaster*; Hsap, *Homo sapiens*; Mmus, *Mus musculus*; Xtro, *Xenopus tropicalis*; Cint, *Ciona intestinalis*; Scer, *Saccharomyces cerevisiae*.

and Cp110 is removed from BBs in early primary spermatocytes (Franz et al., 2013).

Hyperelongated TZs exhibit functional defects

We next sought to examine whether TZ hyperelongation due to SigD expression affected TZ function. Following meiosis in the *Drosophila* male germline, TZs detach from BBs and migrate along growing axonemes, maintaining a ciliary compartment at the distal-most ∼2 µm, where tubulin is incorporated into the axoneme (Basiri et al., 2014; Fabian and Brill, 2012). As revealed by Unc and Cep290 localization, TZs in β_2t-SigD were frequently incapable of detaching from BBs and migrating along axonemes, despite axoneme and cell elongation (Figs 1F, 3A,B). Indeed, the previously reported 'comet-shaped' Unc-GFP localization in β_2t-SigD cells (Wei et al., 2008) persists during cell elongation after meiosis (Fig. 3A, lower row) despite elongation of the axoneme (Fig. 1F).

In *Drosophila* and humans, BBs consist of microtubule triplets (Jana et al., 2016; Lattao et al., 2017), whereas axonemes contain microtubule doublets due to termination of C-tubules at the TZ (Gottardo et al., 2013). Consistent with a defect in this transition and the presence of microtubule triplets in axonemes in β_2t-SigD (Wei et al., 2008), a subset of cilia (<5%) in β_2t-SigD contained Ana1 puncta at the distal tips of TZs (Fig. 3C). Treatment of germ cells with the microtubule-stabilizing drug Taxol increased penetrance of this phenotype from <5% in untreated cells to >25% in cells treated with 4 µM Taxol (arrowheads in Fig. 3D) without significantly affecting Cep290 length (Fig. 3E). Taxol-treated controls did not exhibit TZ-distal Ana1 puncta (P<0.01 at 5% penetrance). Fluorescently tagged Asterless (CEP152), a pericentriolar protein (Blachon et al., 2008; Dzhindzhev et al., 2010), did not localize to TZ-distal puncta in β_2t-SigD cells (P<0.01) suggesting these TZ-distal sites are not fully centriolar in protein composition. Taxol has been hypothesized to disrupt TZ maturation by inhibiting microtubule remodelling in the *Drosophila* male germline (Riparbelli et al., 2013). Indeed, similar to β_2t-SigD, Taxol-treated male germ cells assemble long axonemes that contain triplet microtubules (Riparbelli et al., 2013), further supporting a functional relationship between PIP_2 and microtubule reorganization in TZ maturation.

The *onion rings* mutant decouples defects found in cells with reduced levels of PIP_2

Male flies homozygous for the *onion rings* (*onr*) allele of *Drosophila Exo84* are sterile and exhibit defects in cell elongation and polarity that are similar to those in β_2t-SigD (Fabian et al., 2010). Exo84 is a component of the octameric exocyst complex, which binds PIP_2 at the PM (He et al., 2007). To investigate whether defects in TZ hyperelongation could be explained by defective Exo84 function, we examined TZs in *onr* mutants. Unlike β_2t-SigD, *onr* cells did not display hyperelongated TZs (Fig. 4A), suggesting that Exo84 is dispensable for TZ maturation.

Owing to the involvement of the exocyst in membrane trafficking, we examined whether cilium-associated membranes were affected in β_2t-SigD or *onr* mutants in a manner similar to what is seen in *dilatory; cby* mutants (Vieillard et al., 2016). Dilatory (Dila), a conserved TZ protein, cooperates with Cby to assemble TZs in the *Drosophila* male germline (Vieillard et al., 2016). TZs in β_2t-SigD and *onr* cells were able to dock at the PM initially (Fig. 4B, C,E), but were unable to maintain membrane connections, and were rendered cytoplasmic upon internalization (Fig. 4B,C), similar to TZs in *dila; cby* mutants. In addition, fluorescently tagged Exo70, a PIP_2-binding exocyst subunit, localized to BBs (Fig. 4D). Our results suggest that the exocyst, and Exo84 in particular, regulates cilium–PM association in a similar manner to PIP_2, and that TZ hyperelongation and loss of cilium–PM association are genetically separable phenotypes.

Maturation of a TZ from a nascent to a fully functional state, leading ultimately to axoneme assembly and ciliary signalling, requires orchestration of various proteins and cellular pathways (Reiter et al., 2012; Gonçalves and Pelletier, 2017). Our results indicate that normal execution of this process requires PIP_2, and that depletion of PIP_2 induces TZs to grow longer than normal (Fig. 4F). Similar to β_2t-SigD, *Drosophila dila; cby* and *cby* mutants display hyperelongated TZs (Enjolras et al., 2012; Vieillard et al., 2016). In contrast, *mks1* mutants have shorter TZs (Pratt et al., 2016). Because both Cby and Mks1 are hyperelongated in β_2t-SigD cells, PIP_2 regulates TZ length independently of an effect on Cby or Mks1 recruitment.

We show that hyperelongated TZs are dysfunctional. Similar to *dila*; *cby* (Vieillard et al., 2016) and *cep290* (Basiri et al., 2014) mutants, axonemes can assemble in β_2t-SigD cells despite lack of functional TZs or membrane association, although they show an aberrant ultrastructure (Wei et al., 2008). The presence of TZ-distal Ana1 puncta in β_2t-SigD cells, without the increase in BB length seen in *cep290* mutants lacking a functional TZ barrier, suggests that β_2t-SigD selectively disrupts the ability of TZs to restrict C-tubules and Ana1 without abolishing the TZ barrier entirely. CEP295, the human Ana1 orthologue, regulates post-translational modification of centriolar microtubules (Chang et al., 2016), which might explain the presence of TZ-distal Ana1 along with supernumerary microtubules in β_2t-SigD cells. Asterless (Asl), a pericentriolar protein important for centrosome formation and centriole duplication (Blachon et al., 2008; Dzhindzhev et al., 2010), did not exhibit this TZ-distal localization, possibly due to differences in dynamics of Ana1 and Asl loading onto centrioles (Fu et al., 2016; Saurya et al., 2016) or the more peripheral nature of Asl distribution within the centriole (Blachon et al., 2008).

The majority of PIP_2 at the PM is produced by PIPKIs (Balla, 2013; Hammond et al., 2012). Mutation of the PIPKI Sktl induced hyperelongated TZs, and expression of Sktl could suppress TZ hyperelongation in β_2t-SigD cells, suggesting that Sktl might function *in situ* to regulate TZ length. In humans, *PIPKIC* is linked to lethal congenital contractural syndrome type 3 (LCCS3), which has been suggested to represent a ciliopathy (Xu et al., 2016). The recent discovery of a role for another LCCS-associated protein in cilium function (Jao et al., 2017) corroborates this hypothesis. Our data support the idea that PIPKIs might represent ciliopathy-associated genes or genetic modifiers of ciliary disease.

Members of the exocyst complex are important for cilium formation in cultured cell lines and zebrafish (Zuo et al., 2009; Lobo et al., 2017; Seixas et al., 2016), but their precise roles in ciliogenesis are not well understood. The subunits Sec3 and Exo70 regulate exocyst targeting to the PM through a direct interaction with PIP_2 (He et al., 2007; Zhang et al., 2008). We previously showed that the *onr* allele of *Drosophila exo84* phenocopies defects in male germ cell polarity and elongation observed in β_2t-SigD cells (Fabian et al., 2010). Here, we show that the *onr* mutation phenocopies loss of cilium–membrane contacts in β_2t-SigD cells but not TZ hyperelongation. Thus, TZ hyperelongation is not a prerequisite for failure of cilium–PM association in male germ cells, and Exo84 uniquely regulates the latter process, potentially by supplying membrane required to maintain cilium–PM tethering (Fig. 4F). That the TZ is dispensable for this function is supported by the *Drosophila cep290* mutant, which lacks a functional TZ but

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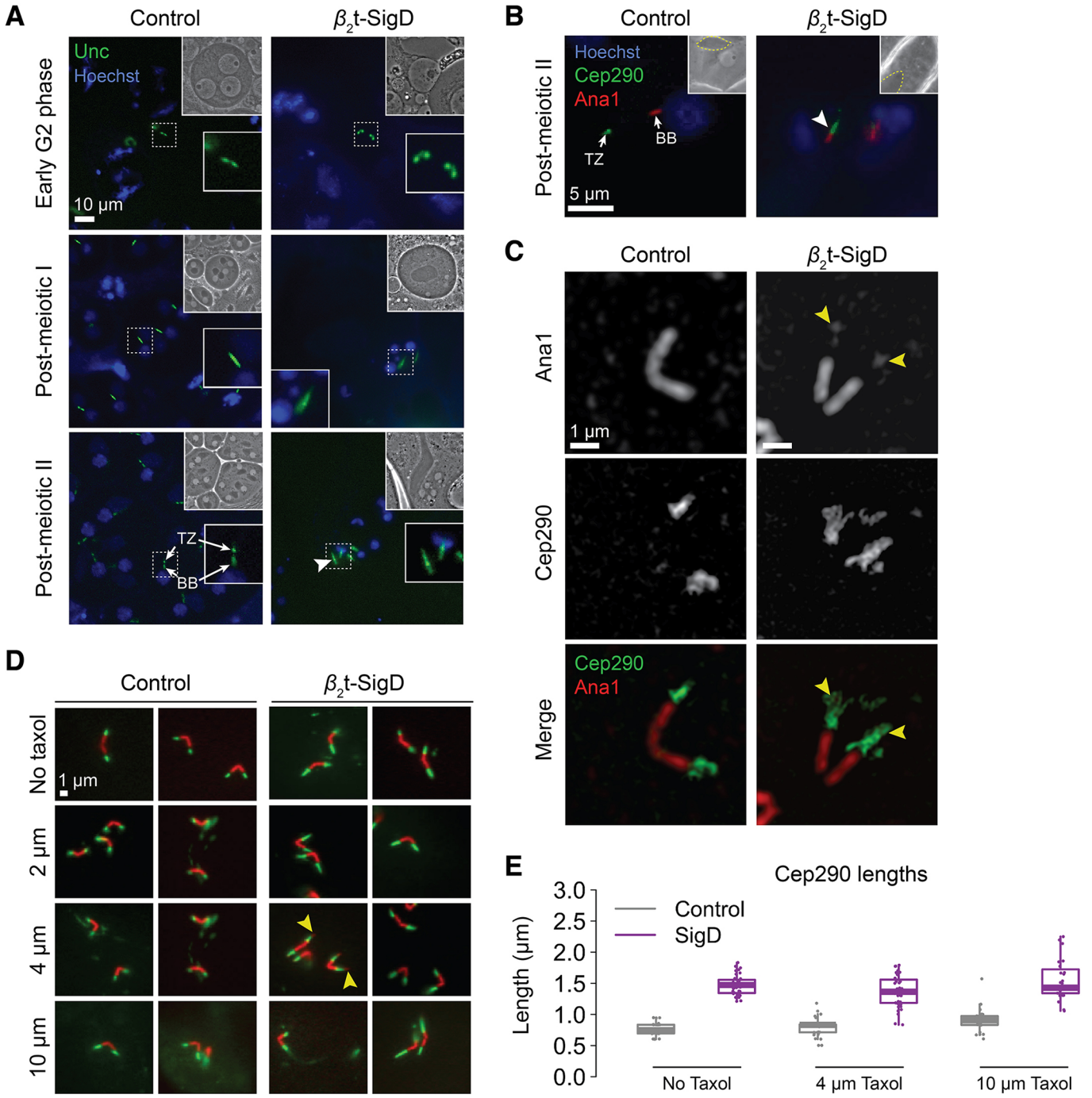

Fig. 3. Hyperelongated TZs display functional defects. (A) The area marked by Unc–GFP is unable to split in spermatids expressing β_2t-SigD (arrowhead). Insets (top, grayscale): phase-contrast images corresponding to regions shown in fluorescence images. Insets (bottom): magnified cilia corresponding to those in areas delimited by dashed white lines. Spermatid cell elongation is concomitant with elongation of mitochondrial derivatives (dark organelles in phase-contrast images). Failure of the Unc–GFP signal to split in β_2t-SigD was highly penetrant (>90%, *n*=63). (B) The area marked by Cep290 is unable to detach and migrate from the basal body at onset of axoneme assembly in β_2t-SigD spermatids (arrowhead). Insets are phase-contrast images corresponding to the regions shown in fluorescence images, with elongating mitochondrial derivatives delineated by yellow dashed lines. (C) Structured illumination micrographs of control and β_2t-SigD cells showing TZ-distal puncta containing the centriolar protein Ana1 in β_2t-SigD spermatocytes (arrowheads). (D) Treatment of control and β_2t-SigD cells with the microtubule-stabilizing drug Taxol. Images demonstrate variability in Cep290 distribution. Arrowheads mark TZ-distal Ana1. (E) Quantification of Cep290 lengths in Taxol-treated control and β_2t-SigD cells from D (*n*=30–40).

retains cilium–PM association (Basiri et al., 2014). Notably, *EXOC8*, which encodes the human Exo84, has been linked to the ciliopathy Joubert syndrome (Dixon-Salazar et al., 2012), and a similar defect in ciliogenesis might be present in humans with mutations in *EXOC8*.

MATERIALS AND METHODS

Transgenic flies and stocks

Drosophila stocks were cultured on cornmeal molasses agar medium at 25°C and 50% humidity. Stocks expressing β_2t::SigD (chromosome 3) and β_2t::YFP-Sktl (chromosome 2) were described previously (Wei et al., 2008;

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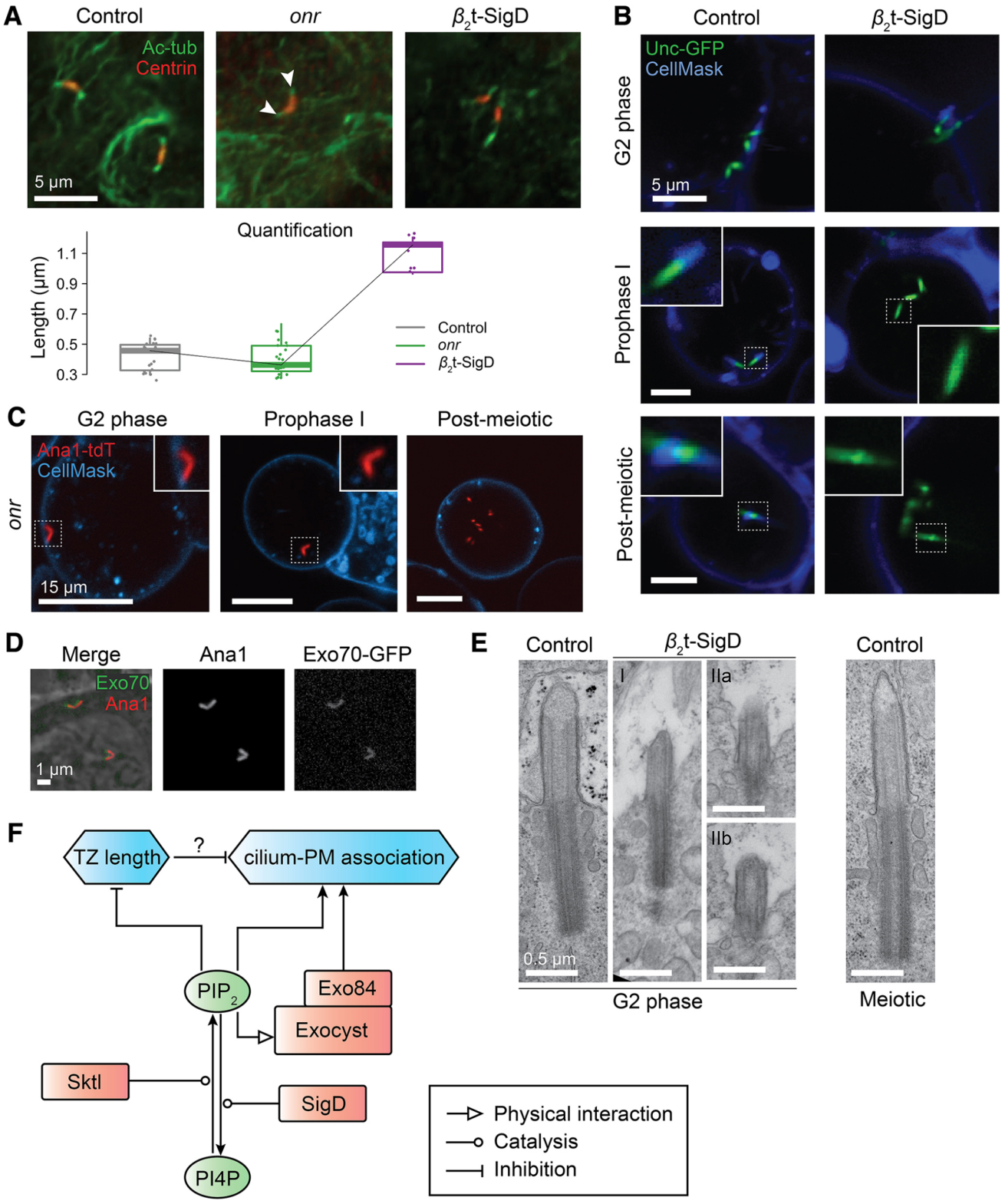

Fig. 4. The *onr* allele of *Exo84* decouples TZ hyperlongation from loss of plasma membrane contacts. (A) *onr* mutants do not display hyperelongated acetylated tubulin (Ac-tub) at the cilium (arrowheads). Acetylated tubulin marks the axoneme, which colocalizes with the TZ in spermatocytes (Pratt et al., 2016). Boxplots show length quantifications (bottom). (B) Cells expressing β_2t-SigD fail to maintain cilium–PM tethering despite initially anchoring to the PM. The PM is marked with CellMask, a cell impermeable dye. (C) *onr* mutants do not maintain PM–cilium tethering. (D) GFP-tagged Exo70 localizes to BBs in spermatocytes. (E) Transmission electron micrographs of spermatocyte cilia protruding from the cell surface (G2 phase) and after internalization (Meiotic). Panels IIa and IIb are different EM sections from the same cilium, whereas I is a different cilium. (F) Schematic summary showing role of PIP_2 in regulation of TZ length and cilium–PM association. We postulate that TZ hyperelongation inhibits cilium–PM association (question mark) (our data and Vieillard et al., 2016). Note that SigD can dephosphorylate PIP_2 to generate PI5P in addition to PI4P (Norris et al., 1998).

Wong et al., 2005). GFP–Exo70 was cloned into the low-level expression vector *tv3* (Wong et al., 2005), and transgenic flies were generated using standard *P* element-mediated transformation. Ana1–tdTomato- and Cep290–GFP-expressing flies were provided by Tomer Avidor-Reiss (Department of Biological Sciences, University of Toledo, USA) (Basiri et al., 2014). Unc–GFP-expressing flies was originally provided by Maurice Kernan (Department of Neurobiology and Behavior, Stony Brook University, USA) (Baker et al., 2004). Stocks expressing GFP-tagged Cby and Mks1 were provided by Bénédicte Durand (Institut NeuroMyoGéne, Université Claude Bernard Lyon-1, France) (Enjolras et al., 2012; Vieillard et al., 2016). The *Exo84*^{onr} mutant was described previously (Giansanti et al., 2015). Stocks for generating *sktl*$^{2.3}$ clones were originally provided by Antoine Guichet (Institut Jacques Monod, Université Paris-Diderot, France) (Gervais et al., 2008). *w*^{1118} was used as the wild-type control.

Antibodies

The following primary antibodies were used for immunofluorescence at the indicated concentrations: chicken anti-GFP IgY (ab13970, abcam, Cambridge, UK), 1:1000; rat anti-RFP IgG (5F8, ChromoTek, Planegg, Germany), 1:1000; rabbit anti-Centrin (C7736, Sigma-Aldrich, St. Louis, MO), 1:500; mouse anti-acetylated α-tubulin (6-11-B, Sigma-Aldrich), 1:1000. Secondary antibodies were Alexa Fluor 488- and Alexa Fluor 568-conjugated anti-mouse-, anti-rabbit- and anti-chicken-IgG (Thermo Fisher Scientific, Waltham, MA, USA) used at 1:10,000 DAPI (Thermo Fisher Scientific) at 1:1000 was used to stain for DNA.

Fluorescence microscopy

For live imaging, testes were dissected in phosphate-buffered saline (PBS). To stain for DNA, intact testes were incubated in PBS with Hoechst 33342 (1:5000) for 5 min. Testes were transferred to a polylysine-coated glass slide (P4981, Thermo Fisher Scientific) in a drop of PBS, ruptured using a syringe needle and squashed under a glass coverslip using Kimwipes. The edges of the coverslip were sealed with nail polish and the specimen was visualized using an epifluorescence microscope (Zeiss Axioplan 2, Carl Zeiss, Oberkochen, Germany) with an Axiocam CCD camera. Cells were examined live whenever possible to avoid artefacts from immunostaining.

For Taxol treatments, testes from larvae or pupae expressing Ana1–tdTomato; Cep290–GFP were dissected into Shields and Sang M3 medium (S8398, Sigma-Aldrich) supplemented with a predefined concentration of Taxol (T7402, Sigma-Aldrich) in DMSO and incubated overnight in a humidified sterile chamber in the dark at room temperature. These were then squashed with a coverslip in PBS and imaged live.

For CellMask staining, cells were 'spilled' from testes in M3 medium onto a sterilized glass-bottom dish pretreated with sterile polylysine solution to enable cells to adhere. CellMask Deep Red (C10046, Invitrogen, Waltham, MA) solution (20 µg/ml) was added to the medium dropwise immediately before visualization under a confocal microscope.

For immunocytochemistry, testes were dissected in PBS, transferred to a polylysine-coated glass slide in a drop of PBS, ruptured with a needle, squashed and frozen in liquid nitrogen for 5 min. Slides were transferred to ice-cold methanol for 5–10 min for fixation. Samples were then permeabilized and blocked in PBS with 0.1% Triton-X and 0.3% bovine serum albumin, and incubated with primary antibodies overnight at 4°C, followed by three 5-min washes with PBS, a 1-h incubation with secondary antibodies, and three 5-min washes with PBS. Samples were mounted in Dako (Agilent, Santa Clara, CA) and imaged with a Zeiss Axioplan 2 epifluorescence microscope, a Nikon A1R scanning confocal microscope or Zeiss Elyra PS1 microscope with Andor iXon3 885 for structured illumination microscopy (SIM) (SickKids imaging facility). Raw fluorescence images were manipulated in ImageJ version >1.50.

Transmission electron microscopy

TEM was performed as in Fabian et al. (2010) using a JEM 1011 microscope (JEOL USA) with a 5 megapixel AMT CCD camera (Model XR50, AMT).

FLP/FRT-mediated mitotic recombination

Mitotic clones were generated in flies of genotype *w*, hsFLP/*Y* ; FRT42B, *ubi*::GFPnls, *sktl*$^{2.3}$/FRT42B. *sktl*$^{2.3}$ is a strongly hypomorphic allele of *sktl* (Gervais et al., 2008). Mitotic recombination was induced by heat shock for 2 hours at 30°C on 3 consecutive days starting on the 2nd day after egg laying, and males were dissected 1–2 days after eclosion to examine mutant cells lacking nuclear GFP. This regimen was chosen to maximize chances of recovering clones that had undergone mitotic recombination very early during their life cycle.

Statistical methods

Statistical analysis and graphing was performed using R software (version 3.4). A Gaussian jitter was applied when plotting results in Figs 1 and 2 for clearer visualization of trends, but raw data was used for all analyses. Statistical tests for 'absence of phenotype' were computed using a binomial test under the assumption that the probability of the phenotype occurring was fixed. All *t*-tests were unpaired and two-sided with Welch's correction for unequal variances. *n* represents the pooled number of samples (individual cilia) from multiple flies. A significance level of 0.01 was fixed in advance for all classical analyses. All raw data and code for analysis and plotting can be found online at http://www.github.com/alindgupta/germline-paper/. For results shown as box plots, the box represents the 25–75th percentiles, and the median is indicated. The whiskers extend to a maximum of 1.5 times the interquartile range above the upper quartile and below the lower quartile. Data points beyond the whiskers are outliers.

Phylogenetic analysis

Candidate orthologues of Skittles and PIP5K59B were queried from Inparanoid (version 8.0) and FlyBase (version FB2017_05). Poorly annotated protein sequences were confirmed to encode type I phosphatidylinositol phosphate kinases using reciprocal BLAST search. Phylogeny.fr (http://www.phylogeny.fr) (Dereeper et al., 2008) was used for phylogenetic reconstruction with T-Coffee for multiple alignment and MrBayes for tree construction. The output was converted into a vector image in Illustrator (Adobe) and colours were added for the purpose of illustration.

Acknowledgements

We thank Brian Ciruna for insightful discussions, Bénédicte Durand, Tomer Avidor-Reiss, Antoine Guichet and Maurice Kernan for fly stocks, and Bénédicte Durand and Bill Trimble for critical comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.G., J.A.B.; Methodology: A.G.; Validation: A.G.; Formal analysis: A.G.; Investigation: A.G., L.F.; Resources: A.G., L.F.; Data curation: A.G., L.F.; Writing - original draft: A.G.; Writing - review & editing: A.G., L.F., J.A.B.; Visualization: A.G.; Supervision: J.A.B.; Project administration: J.A.B.; Funding acquisition: J.A.B.

Funding

We gratefully acknowledge funding from the Canadian Institutes of Health Research (MOP-130437 to J.A.B) and a University of Toronto Open Fellowship and Ontario Graduate Scholarship (to A.G.).

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