EXTENDING CURRENT MODELS OF A PHOSPHOINOSITIDE CODE OF CILIA BY EXAMINING THE EFFECT OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE REDUCTION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Extending current models of a phosphoinositide code of cilia by examining the effect of phosphatidylinositol 4,5-bisphosphate reduction

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Cellular organelles called cilia sense diverse physical and chemical stimuli and transmit them to the cell, facilitating sensory modalities such as light, sound and smell. In humans, cilium dysfunction causes genetic disorders known as ciliopathies whose clinical phenotypes include polycystic kidney disease, congenital heart disease and retinal degeneration. A subset of ciliopathies is associated with mutations in \textit{INPP5E}, which encodes a phosphatase for the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}), preventing its intraciliary build up. This observation has lead to a model where high intraciliary PIP\textsubscript{2} perturbs intraciliary transport in mammals and \textit{Drosophila melanogaster}. Using \textit{Drosophila} as a model, we investigated whether reducing PIP\textsubscript{2} affects cilium formation and function.

We found that reduction of PIP\textsubscript{2} in the \textit{Drosophila} male germline by ectopic expression of the PIP\textsubscript{2} phosphatase SigD and mutation of the type I phosphatidylinositol phosphate kinase (PIPKI) Skittles (Sktl) affected maturation of cilium transition zones (TZs) and induced their hyperelongation. TZ hyperelongation was concomitant with defects in TZ detachment from basal bodies at the onset of axoneme elongation and loss of connections between cilia and the plasma membrane. The \textit{onr} allele of the \textit{Drosophila Exo84}, which encodes a component of the exocyst complex, exhibited loss of cilium-plasma membrane contacts but not TZ hyperelongation, decoupling the two phenotypes.

In ciliated chordotonal neurons, \textit{sktl} knockdown following ciliogenesis induced sensory defects in adults. In cells with \textit{sktl} knocked down, No mechanical potential B (NompB), the
homolog of intraflagellar transport protein IFT88, accumulated at the tips of chordotonal cilia, indicating a defect in its retrieval. Similar to previous studies, we observed an enrichment of PIP$_2$ at the base of chordotonal cilia, but we also found that it was present within the ciliary dilation in these cells, indicating that cilia and PIP$_2$ are not spatially mutually exclusive. Chordotonal neuron clones that are mutant for sktl form truncated cilia, indicating that sktl is required for ciliogenesis in these cells as well.

Our results indicate that PIP$_2$ is important for cilium formation and function, and that PIPKIs might act as regulators of cilia and as genetic modifiers of ciliopathies associated with TZ dysfunction.
For my parents.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ana1</td>
<td>Anastral spindles 1, a core centriolar protein</td>
</tr>
<tr>
<td>Asl</td>
<td>Asterless, a pericentriolar protein</td>
</tr>
<tr>
<td>BB</td>
<td>Basal body</td>
</tr>
<tr>
<td>Cby</td>
<td>Chibby, a conserved transition zone protein</td>
</tr>
<tr>
<td>Cep290</td>
<td>Centrosomal protein of 290 kDa, a conserved transition zone protein</td>
</tr>
<tr>
<td>ChN</td>
<td>Chordotonal neuron</td>
</tr>
<tr>
<td>Exo84</td>
<td>Exocyst complex protein of 84 kDa, yeast ortholog of EXOC8</td>
</tr>
<tr>
<td>F&gt;sktl RNAi</td>
<td>Hairpin RNA against sktl driven by nanchung-GAL4 (ChN-specific)</td>
</tr>
<tr>
<td>(G/R/Y)FP</td>
<td>(Green/Red/Yellow) fluorescent protein</td>
</tr>
<tr>
<td>IFT</td>
<td>Intraflagellar transport</td>
</tr>
<tr>
<td>IFT-A</td>
<td>Retrograde IFT</td>
</tr>
<tr>
<td>IFT-B</td>
<td>Anterograde IFT</td>
</tr>
<tr>
<td>INPP5E</td>
<td>Inositol polyphosphate 5-phosphatase E (phosphatase for PIP$_2$ and PIP$_3$)</td>
</tr>
<tr>
<td>onr</td>
<td>onion rings (an allele of Exo84)</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphoinositide, also known as phosphatidylinositol phosphate</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP$_3$</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PI4P</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIPKI</td>
<td>Type I phosphatidylinositol phosphate kinase</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Sktl</td>
<td>Skittles, one of two Drosophila PIPKIs</td>
</tr>
<tr>
<td>sktl$^{2,3}$</td>
<td>Strongly hypomorphic allele of sktl (276bp intronic deletion)</td>
</tr>
<tr>
<td>Styp</td>
<td>Salmonella typhimurium</td>
</tr>
<tr>
<td>tdT</td>
<td>Tandem dimer Tomato fluorescent protein</td>
</tr>
<tr>
<td>TeTxLC</td>
<td>Tetanus toxin light chain</td>
</tr>
<tr>
<td>Tg737</td>
<td>An allele of mouse IFT88 that causes polycystic kidneys</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential ion channel</td>
</tr>
<tr>
<td>TZ</td>
<td>Transition zone</td>
</tr>
<tr>
<td>$\beta_2$t-SigD</td>
<td>SigD driven by the male germline-specific $\beta_2$-tubulin promoter</td>
</tr>
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Chapter 1

Introduction

1.1 Overview

1.1.1 Summary

Following the description of “incredibly thin feet” that “moved nimbly” in freshwater protists by Leeuwenhoek in the 17th century (Satir, 1995), much of the research on cilia and flagella focused on their motile functions (Bloodgood, 2009). Around the turn of the millenium, the discovery of sensory functions of non-motile or “primary” cilia and their involvement in polycystic kidney disease in Tg737 mice (Pazour et al., 2000) fueled a spectacular resurgence of interest in cilia in the fields of biology and medicine. Since then, numerous developmental signalling pathways have been found to be associated with cilia (Goetz and Anderson, 2010), and various genetic disorders have been linked to aberrant cillum formation (ciliogenesis) or function in humans (Waters and Beales, 2011). Although the number of yearly scientific publications on cilia has plateaued recently*, this is probably due to the difficulty of studying these tiny organelles rather than waning scientific interest — research efforts to advance the current understanding of mechanisms underlying cillum formation and function are limited considerably by techniques to overcome the small size of cilia, and the immense diversity in their assembly, morphology and functions.

*According to “Results by year” results from PubMed with the query “cilia OR cilium”.

1
1.1.2 Thesis structure

This thesis is structured as follows:

- **Chapter 1** provides a summary of literature on cilia and their functions, modes of ciliogenesis, and diseases associated with cilia (ciliopathies), with a focus on *Drosophila melanogaster* and humans. The latter part of this chapter is dedicated to a discussion of the lipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and its relationship to cilia and disease.

- **Chapter 2** describes a role for PIP$_2$ in ciliogenesis in the male *Drosophila* germline, and its effect on cilium transition zone (TZ) maturation.

- **Chapter 3** describes a role for the *Drosophila* type I phosphatidylinositol phosphate kinase (PIPKI) called Skittles (Sktl), which synthesizes PIP$_2$, in proper function of ciliated chordotonal neurons (ChNs), and in cilium assembly and function.

- **Chapter 4** summarizes results from Chapters 2 and 3, proposes experiments to address open questions and describes the relevance of this research.

1.2 Cilia, their structure and functions

1.2.1 Overview

Cilia* are tiny antenna-like organelles that project out from the surface of almost every cell in the human body, receiving extracellular cues and relaying them to the cell interior, and thus facilitating a large number of homeostatic signalling pathways that require extracellular input (Goetz and Anderson, 2010). Many cilia (and all flagella) are motile and generate mechanical force that can propel extracellular fluid or entire cells such as spermatozoa. Ciliary dysfunction can affect fertility, viability and sensory abilities.

*Throughout this thesis, the term “cilia” refers to both cilia and flagella for brevity, unless the distinction is important. Flagella are essentially long motile cilia.
in organisms as varied as protists, fruit flies and humans. Defects in cillum formation and function in humans are associated with a large number of genetic disorders known as ciliopathies, which often manifest as multi-organ developmental abnormalities, with varying phenotypic displays and severities (Gerdes et al., 2009). This chapter (Chapter 1) provides a concise introduction to these topics.

1.2.2 Cilium structure

The morphology and ultrastructure of cilia are specialized to support their functions, and thus vary greatly between tissues and organisms. Generally, axonemes are composed of nine microtubule doublets arranged in a radially symmetrical organization. Motile cilia contain two additional singlet microtubules within the central core of the axoneme that provide substrates for protein-mediated ciliary bending and motility (Satir et al., 2014). Axonemal microtubules are nucleated by a basal body (BB) — a centriole with structural modifications that allow it to anchor to the plasma membrane (PM). BBs and centrioles are made up of microtubule triplets, containing A, B and C tubules, of which only the A and B tubules extend into the cilium to compose axonemal doublets. In most cases, axonemes are ensheathed by a ciliary membrane that is contiguous with the PM but distinct from it in protein and lipid composition, and thus function. Figure 1.1 shows a diagram of a stereotypical cilium.

The transition zone (TZ) generally lies at the base of the cilium, immediately distal to the BB, and acts as a diffusion barrier to regulate transport of proteins in and out of the cilium (Goncalves and Pelletier, 2017). It is composed of a number of conserved protein complexes linking axonemal microtubules to the membrane. TZs are essential for formation, maintenance and composition, and therefore function, of cilia. One set of proteins that depends on TZs for ciliary entry comprises those required for intraflagellar transport (IFT) (Zhao and Malicki, 2011) — a protein trafficking system composed of microtubule motors and adaptor proteins that haul cargo from the ciliary base to the tip.
Chapter 1. Introduction

Figure 1.1: Structure of a cillum (left) and cross-sectional detail (right). For the axoneme cross-section, a 9+2 structure containing dynein arms is shown. In some organisms and tissues, Y-links connecting axonemal microtubules to the membrane can be observed using transmission electron microscopy (not shown). Based on Veleri et al. (2014).

(anterograde IFT or IFT-B), or from ciliary tip to the base (retrograde IFT or IFT-A) by walking along axonemal microtubules (see Figure 1.1) (Hao and Scholey, 2009). The Tg737 mutation, for instance, which causes polycystic kidneys in mice, was found to be an allele of IFT88, which encodes a highly conserved IFT-B protein required for ciliogenesis.
1.2.3 Steps of cillum assembly

The prevalent mode of ciliogenesis in eukaryotes, particularly vertebrates, occurs through the “compartmentalized pathway” (Avidor-Reiss and Leroux, 2015), which is described here. Typically, ciliogenesis begins in G1 phase of the cell cycle when the mother centriole — the older of the two centrioles at interphase — matures into a BB within the cytoplasm, for example, through incorporation of appendages that enable membrane tethering found in some cells (Kobayashi and Dynlacht, 2011). A ciliary vesicle forms at the distal tip of the BB, through the action of small G-proteins Rab8 and Rab11, and EHD (Eps15 homology domain) proteins EHD1 and EHD3 which are important for membrane trafficking and remodelling (Yee and Reiter, 2015; Lu et al., 2015). The microtubule plus-end capping factor CP110 is then removed from the distal tip of the BB, enabling TZ assembly and compartmentalization of ciliary space, followed by PM docking of the BB, initiation of IFT by recruitment of IFT proteins to the ciliary base, and axoneme elongation (Garcia-Gonzalo and Reiter, 2012). Axoneme elongation occurs in tandem with extension of the ciliary membrane, the latter of which is thought to require exocyst, a heterooctameric complex of proteins important for polarized vesicle secretion (Lobo et al., 2017; Seixas et al., 2016).

1.2.4 Functions of cilia and flagella

Broadly speaking, cilia and flagella serve roles in sensory reception, and cell and fluid motility.

Motile functions of cilia and flagella

Ciliary motility, which requires axoneme-associated microtubule motor proteins called dyneins, is powerful enough to drive extracellular fluids such as mucus in the human respiratory tract, or entire cells such as sperm. Motile cilia and flagella are essential for
locomotion in unicellular ciliated protists (Ginger et al., 2008), for fertility in humans and *Drosophila*, for bacterial clearance by the mucociliary escalator in the human airway (Vareille et al., 2011) and for proper left-right asymmetry through nodal flow in vertebrate embryos (Babu and Roy, 2013). Defects in cilium motility are associated with primary ciliary dyskinesia in humans, which leads to chronic respiratory infections, breathing problems and infertility (Kurkowiak et al., 2015). The presence of motile cilia in all extant eukaryotic supergroups indicates that the last eukaryotic common ancestor had a motile cilium (Mitchell, 2007).

**Motile cilia are also sensory**

Although not all cilia are motile, it is likely is that most cilia and flagella are sensory (Bloodgood, 2010). For example, motile cilia of the human nasopharyngeal epithelium regulate their own beat frequency by sensing mucus viscosity through the mechanosensitive TRPV4 (transient receptor potential vanilloid) channel (Shah et al., 2009). Similarly, motile cilia of the mammalian oviduct, which transport oocytes, express a host of hormone and cytokine receptors, as well as the ion channels polycystin-2 and TRPV4, and can modulate their own beat frequency depending on hormone levels (Bloodgood, 2010). Flagella in *Drosophila* sperm are chemosensory and require polycystin-2 for directional motility in the female genital tract (Kottgen et al., 2011). In ChNs of the Johnston’s organ in the *Drosophila* antennae, which form the fly’s auditory center, vibrational ciliary motility allows stereo hearing and non-linear tuning of mechanosensory inputs from sound waves (Boekhoff-Falk and Eberl, 2014). *Chlamydomonas* flagella are not only important for locomotion, but also serve as the first site of contact between gametes during mating leading to intracellular signalling from flagellar polycystin-2 channels (Huang et al., 2007). In *Paramecium*, a similar process occurs during mating, and cilia have other mechanosensory roles that self-modulate beat frequency and directionality or turning (Bloodgood, 2010). In other cases, ciliary motility can alter signalling in neighbouring
cells, such as in the embryonic node in vertebrates (Basu and Brueckner, 2008).

**Primary cilia**

Primary cilia are non-motile cilia that are present on almost all cells in vertebrates. Axonemes of primary cilia generally lack central microtubule singlets and dynein arms that are required for ciliary motility. A number of developmental signalling pathways are linked to cilia, including Sonic hedgehog (Shh) which inextricably requires functional cilia in vertebrates (Caspary et al., 2007). The receptor Patched 1 resides within cilia in the absence of its ligand Shh; Shh-binding causes its translocation out of the cilium to the PM and translocation of the Shh effector Smoothened into the cilium, ultimately leading to pathway activation and transcriptional response (Goetz et al., 2009). Many phenotypes observed in patients with ciliopathies, including polydactyly and defects in limb patterning and neural tube closure, are caused by aberrant Shh signalling (Murdoch and Copp, 2010). In addition to Shh, well-studied signal transduction pathways, such as canonical Wnt (Wingless-related integration site), planar cell polarity, Notch, Hippo and platelet-derived growth factor (PDGF), have been linked to cilia (Wheway et al., 2018). Cilia are also important for sensing light in photoreceptors, odours in the nasal cavity epithelium and sound in the inner ear, where the morphologies and compositions of cilia have been adapted to serve their sensory purposes. In invertebrates such as *Drosophila* and *Caenorhabditis elegans*, non-motile sensory cilia sense the physical and chemical environment (Inglis et al., 2007; Jana et al., 2016). In fact, the primary role of centrioles in *Drosophila* is to template cilia as BBs — development proceeds relatively normally after postembryonic loss of centrioles, but adult flies are severely uncoordinated due to lack of mechanosensory and proprioceptive inputs and die soon after eclosion (Basto et al., 2006).
Cilia release extracellular vesicles

Cilia are also known to secrete extracellular vesicles called ectosomes*. Active G-protein coupled receptors that accumulate at the tips of cilia due to inefficient retrieval are removed in ectosomes in an actin-dependent manner in mammalian cultured cell lines (Nager et al., 2017). In *Chlamydomonas reinhardtii*, ectosomes released from flagellar membranes in an ESCRT (endosomal sorting complexes required for transport)-dependent manner contain various membrane-associated and ubiquitylated proteins (Long et al., 2016), in addition to a protease important for hatching of daughter cells after mitosis (Wood et al., 2013). In *C. elegans*, ciliary ectosomes bud from the ciliary base into the environment, and are able to alter behaviour of males (Wang et al., 2014). Thus, cilia can regulate their own signalling, and signalling of other cells, through ectosomes.

1.2.5 Properties of cilia that facilitate their sensory functions

Many hypotheses exist for why cilia are so suitable as cellular antennae that participate in numerous signalling pathways in different cell types (reviewed by Nachury, 2014). A prominent idea is that the high surface area to volume ratio allows cells to achieve higher effective protein concentrations within cilia than within the cell. By doing this, cells can enhance their sensitivity to physical or chemical cues, and the effect of protrusion of cilia from the cell surface exposes them to extracellular cues in a more restricted directional manner, such as apically or basally. For example, near-crystalline concentrations of rhodopsin in photoreceptor outer segment allow highly sensitive photon sensing, enabling responses to single photons (Calvert et al., 2001). Cells can also regulate the membrane composition of cilia without altering PM lipid composition, which may facilitate certain signalling processes (Garcia et al., 2018), for example, by regulating protein localization or lateral mobility (Vereb et al., 2003). The vast diversity in cilium length, structure and

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*Ectosomes are derived from PM budding, whereas exosomes are derived from multivesicular bodies that are released during exocytosis. Due to the difficulty in distinguishing between them in some cases, the generic term “ciliary extracellular vesicle” has been used (Wang and Barr, 2016; Wood and Rosenbaum, 2015).
function, even amongst different tissues within an organism (Howell and Hobert, 2017; Nevers et al., 2017; Choksi et al., 2014), suggests that mechanisms of regulating these features may be highly modular, adaptable and versatile.

1.2.6 Evolutionary diversity of cilia and flagella

All extant eukaryotic supergroups contain organisms with motile cilia or flagella, and it is hypothesized that these were present in the last eukaryotic common ancestor (Carvalho-Santos et al., 2011; Mitchell, 2007). Cilia are absent in yeast and most seed plants, and variations on canonical cilium structures, such as deviations from the 9+2/9+0 axoneme structure, which probably represents the ancestral configuration, can be found in some organisms. For example, gametes of *Lecudina tuzetae*, a marine protist, contain 6+0 flagella with very slow undulating motility (Schrevel and Besse, 1975). Apparently, axonemes lacking the two central microtubules display simpler movement patterns than 9+2 axonemes, and therefore energy constraints may dictate axoneme structure, and vice versa, over the course of evolution (Mitchell, 2007). Similar evolutionary constraints may also have dictated centriole and BB structure. In *Drosophila*, for example, centrioles containing triplet microtubules are only found in the ciliated male germline, whereas somatic cells contain doublet or singlet microtubules (Gottardo et al., 2015). *C. elegans*, which lacks motile cilia but has morphologically diverse non-motile sensory cilia (Inglis et al., 2007; Howell and Hobert, 2017), contains centrioles composed of singlets in embryos and sperm, but doublets in ciliated tissues, even though most BBs degenerate after ciliogenesis (Serwas et al., 2017). Organisms lacking cilia, like Angiosperms and fungi, have completely lost centrioles, which is suggestive of coupled evolutionary constraints on cilia and centrioles.
Diversity in transition zones

The structure of the TZ, particularly the Y-links (Y-shaped links connecting the ciliary membrane and axoneme) observed in some cells using electron microscopy (Craige et al., 2010; Williams et al., 2011), as well as its protein composition, are highly evolutionarily divergent. A bioinformatics survey of TZ genes from 52 organisms belonging to different eukaryotic supergroups found considerable diversity among TZ components (Barker et al., 2014). Only six TZ proteins were found to be conserved in more than half of all ciliated organisms in each supergroup, and all of these belong to the “MKS module”, which is implicated in Meckel-Gruber syndrome and other ciliopathies (Williams et al., 2011). Others, like Chibby, are conserved in metazoans only (Enjolras et al., 2012). Some organisms, like Plasmodium falciparum and Giardia intestinalis, lack TZ components entirely, and probably use an entirely different way of achieving cilium compartmentalization, if any (Barker et al., 2014). Thus, TZ proteins have been adapted for the particular needs of a tissue or organism.

Diversity in intraflagellar transport

IFT proteins, including IFT-B (IFT-88, 172, 81, 74, 70), IFT-A (IFT-144, 140, 122, 121), and the BBSome (BBS1–8), an octameric complex of protein associated with Bardet-Biedl syndrome (Nachury et al., 2007), are involved in transport of protein cargo along the axoneme in anterograde and retrograde directions, as well as their turnaround at the ciliary tip (Wei et al., 2012). Most IFT proteins are present in ciliated organisms in all eukaryotic supergroups and were therefore likely to have been present in the last eukaryotic common ancestor. The BBSome is the least conserved of these (van Dam et al., 2013), despite its importance in bridging IFT-B and IFT-A during turnaround at cilium tips, at least in C. elegans (Wei et al., 2012). For instance, although BBS2 and BBS7 are required for proper IFT in C. elegans and mice, Drosophila completely lacks genes encoding these proteins (Blacque et al., 2004; Zhang et al., 2013; Nishimura et al., 2004).
Several organisms have lost subsets of IFT subunits. On the other hand, *Plasmodium* completely lacks IFT proteins despite containing motile flagella in its short-lived gametes (van Dam et al., 2013). Again, this suggests a very modular and adaptable system that, for the most part, correlates well with the presence of cilia and flagella.

**Diversity in cilium morphology and number**

The morphology of cilia and their length are specialized for particular roles or needs in cells. For example, the rod and cone photoreceptor outer segment is a highly modified lamellated cilium capable of storing extremely high concentrations of the photoreceptor rhodopsin (Rachel et al., 2012). Some cells are multiciliated, *i.e.*, a single cell contains multiple cilia. Multicilia are predominantly motile, such as those present in the human nasopharyngeal epithelium; multiciliation allows hydrodynamic coupling of motility and metachrony that enhances fluid movement (Brooks and Wallingford, 2014). Steady-state length of cilia, which refers to the length when rates of assembly and disassembly are equal, can also vary between cells dependent on IFT. This is particularly common in organisms such as vertebrates and *Chlamydomonas* where ciliary lengths vary over the cell cycle due to assembly and disassembly. The physiological relevance of having different cilium lengths remains unclear (Broekhuis et al., 2014, 2013). In *C. elegans*, diversity in cilium morphology is linked to differential expression of a single-pass membrane immunoglobulin protein, with its expression level correlating with cilium elaboration and branching (Howell and Hobert, 2017). In addition, the structure of some odorant cilia in *C. elegans* is dynamic and is remodelled in response to sensory cues produced by pathogenic bacteria (Mukhopadhyay et al., 2008), suggesting that cilium structure can be regulated homeostatically and adaptively.
1.2.7 Ciliated cells in *Drosophila*

*Drosophila melanogaster* is a relatively well-studied model for cilia research due to the availability of powerful genetic tools, well-conserved ciliary genes and presence of both motile and non-motile cilia. *Drosophila* contains two types of ciliated cells — type I sensory neurons and male germ cells.

**Cilia in *Drosophila* chordotonal neurons (ChNs)**

Type I sensory neurons are bipolar neurons that contain a single sensory dendrite with a cilium. They are organized into sensory organs or “sensilla” along with 2-4 accessory cells. Type I sensory neurons include

- macrochaetes or large bristles, which respond to direct touch,
- campaniform organs, which respond to cuticle strain,
- chemosensory neurons, which sense odours and gustatory stimuli, and
- chordotonal neurons (ChNs), which are stretch-sensors that are described in the following paragraph.

ChNs are described in detail in this thesis. Other types of ciliated neurons in *Drosophila* are reviewed in more detail elsewhere (Tuthill and Wilson, 2016).

Macrochaetes, campaniform organs and chemosensory neurons contain non-motile 9+0 cilia, whereas ChNs contain 9+0 cilia with dynein-dependent oscillatory motility. Of these, ChNs are the most well-established as a system for studies of cilia due to their accessibility to imaging and long cilia (approximately 10 µm). A large number of proteins are known to localize to ChN cilia and to be important for different aspects of cilium formation and function (Table 1.1).

ChNs function as proprioceptors and exteroceptors, and are somewhat analogous to amphid neurons in *C. elegans* and vertebrate hair cells (Jarman, 2002). In *Drosophila*
### Table 1.1: Proteins expressed in ChNs important for cilium function or formation. Name aliases are parenthesized. TRP is short for transient receptor potential.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beethoven (Btv)</td>
<td>Ciliary motility</td>
<td>Cilium</td>
<td>Eberl et al., (2000)</td>
</tr>
<tr>
<td>Cep290</td>
<td>Ciliogenesis</td>
<td>TZ</td>
<td>Basiri et al., (2014)</td>
</tr>
<tr>
<td>CG11356/Arl13b</td>
<td>(Unknown)</td>
<td>Cilium</td>
<td>Enjolras et al., (2012)</td>
</tr>
<tr>
<td>Chibby (Cby)</td>
<td>TZ formation/function</td>
<td>TZ</td>
<td>Enjolras et al., (2012)</td>
</tr>
<tr>
<td>Dilatory (Dila)</td>
<td>Regulates IFT</td>
<td>BB</td>
<td>Ma &amp; Jarman (2011)</td>
</tr>
<tr>
<td>Inpp5e</td>
<td>Tulp localization, cilium function</td>
<td>TZ</td>
<td>Park et al., (2015)</td>
</tr>
<tr>
<td>NompB/IFT-88</td>
<td>Anterograde IFT</td>
<td>Entire cilium</td>
<td>Han et al., (2003)</td>
</tr>
<tr>
<td>NompC</td>
<td>Mechanotransduction</td>
<td>Distal ciliary segment</td>
<td>Lee et al., (2010)</td>
</tr>
<tr>
<td>Painless (Pain)</td>
<td>TRPA ion channel required for gravitaxis</td>
<td>(Unknown)</td>
<td>Sun et al., (2009)</td>
</tr>
<tr>
<td>Pyrexia (Pyx)</td>
<td>TRPA ion channel required for gravitaxis</td>
<td>(Unknown)</td>
<td>Sun et al., (2009)</td>
</tr>
<tr>
<td>RempA/IFT-152</td>
<td>Retrograde IFT</td>
<td>Ciliary dilation</td>
<td>Lee et al., (2008)</td>
</tr>
<tr>
<td>Rootletin</td>
<td>Mechanotransduction</td>
<td>Ciliary rootlet</td>
<td>Chen et al., (2015)</td>
</tr>
<tr>
<td>Tulp (King tubby)</td>
<td>Mechnanosensory ion channel trafficking</td>
<td>Cilium</td>
<td>Park et al. (2013)</td>
</tr>
</tbody>
</table>
adults and larvae, ChNs provide proprioceptive feedback to locomotory circuits (Caldwell et al., 2003; Eberl et al., 2000; Tauber and Eberl, 2001). In addition, large arrays of ChNs in the *Drosophila* antennae function as exteroceptors, sensing wind currents, sound and gravity, effectively forming *Drosophila*’s auditory system (Kamikouchi et al., 2009; Gopfert and Robert, 2002). In ChN sensilla, tips of cilia are physically connected to the epidermis through a dendritic cap. Deformation of the epidermis, such as when the fly moves its limbs, induces stretching/shearing forces on cilia due to mechanical coupling between them, leading to activation of stretch-sensitive ion channels. Antennal ChN cilia spontaneously vibrate in a dynein-dependent manner, acting as harmonic oscillators that can help tune input stimuli (Gopfert and Robert, 2002; Karak et al., 2015). Predictably, ChN mutants exhibit sensory defects (Jarman, 2002).

**Cilia in the *Drosophila* male germline**

The *Drosophila* male germline undergoes ciliogenesis that eventually produces the 1.8 μm long sperm tail axoneme. Although the axoneme contains the canonical motile 9+2 structure, only the distal-most ~2 μm is covered by a ciliary membrane cap, whereas the rest of the axoneme lies within the cytoplasm. The *Drosophila* polycystin-2 Ca$^{2+}$ channel Almost there (Amo) localizes to the distal tip of sperm (Watnick et al., 2003), possibly inside this ciliary “cap” (Basiri et al., 2014; Vieillard et al., 2016), which is also the leading edge of swimming sperm in the female genital tract (*i.e.*, these sperm swim backwards, tail-end first) (Kottgen et al., 2011). Mature sperm from *amo* mutants are motile, but unable to enter the female sperm-storage organ following transfer into the uterus due to a defect in sperm chemotaxis (Watnick et al., 2003; Kottgen et al., 2011).

*Drosophila* male germ cells form cilia through a non-canonical process, referred to as “cytoplasmic ciliogenesis” (Avidor-Reiss and Leroux, 2015). Nascent TZs assemble on all four spermatocyte BBs during G2 phase prior to meiosis (Riparbelli et al., 2012). TZ assembly occurs concomitantly with PM docking of BBs (Avidor-Reiss and Leroux, 2015);
there is no conclusive evidence of a ciliary vesicle in these cells. During the prolonged G2 phase, both the ciliary axoneme, consisting mostly of the TZ, as well as the BBs elongate simultaneously, a process I refer to as TZ maturation. These cilia migrate to the cell interior during meiosis to promote spindle formation, remaining linked to the PM through PM invaginations. Following meiosis, BBs dock at the nuclear envelope and axoneme elongation begins in earnest. It is here that the TZ splits off from the BB, and migrates like a sliding ring along the growing axoneme ~2 µm from the tip, tethered onto the ciliary membrane (Basiri et al., 2014; Fabian and Brill, 2012). A description of the process can be found in Figure 2.1. This entire process occurs independently of IFT (Han et al., 2003; Avidor-Reiss et al., 2004). Presumably, the ciliary compartment is sufficiently small for efficient exploration by protein diffusion. Studies of the *cep290* mutant indicate that a compartmentalized ciliary cap is required for proper specification of axoneme structure but not axoneme elongation (Basiri et al., 2014). Defects in ciliogenesis or cilium function in *Drosophila* are associated with sensorimotor defects and infertility (Basiri et al., 2014), which are also present in patients with some ciliopathies.

### 1.3 Ciliopathies

Consistent with the prevalence of cilia in human physiology, cilium dysfunction is associated with a number of genetic disorders known collectively as ciliopathies (Waters and Beales, 2011). Ciliopathies consist mainly of monogenic diseases with considerable allelism, although modifier mutations appear to play an important role in disease severity and clinical presentation (Bachmann-Gagescu et al., 2015). Some types of cancers are also linked to ciliary aberrations, particularly through modulation of Hedgehog signalling (Hassounah et al., 2012). For other diseases like the oculocerebrorenal syndrome of Lowe, the contribution of ciliary defects to clinical phenotypes remains unclear, and as a result, it has been difficult to classify them as ciliopathies (Madhivanan et al., 2012).

Early studies using classical methods of linkage mapping to identify loci associated
with polycystic kidney disease in humans and mice found genes expressed in the renal epithelium that encoded ciliary proteins like cystin, PKD1 and PKD2 (Yoder et al., 2002). A large number of diseases exhibiting heterogeneous sets of clinical phenotypes associated with ciliary malformation or dysfunction have since been identified (Waters and Beales, 2011). Joubert syndrome is a prototypical ciliopathy — patients with Joubert syndrome exhibit malformations of the cerebellum that cause cognitive defects and ataxia, with variable features like retinal, liver and renal anomalies, polydactyly and facial defects (Parisi et al., 2007). At least 20 different forms of Joubert-related diseases caused by mutations in distinct genes are known and grouped under Joubert syndrome related disorders, where Joubert syndrome represents the original characterization five decades ago (Joubert et al., 1969). Further discussion of the various known ciliopathies can be found elsewhere (Waters and Beales, 2011; Badano et al., 2006; Reiter and Leroux, 2017).

Ciliopathies linked to phosphatidylinositol 4,5-bisphosphate (PIP$_2$)

In humans, Joubert syndrome type 1 and MORM (mental retardation, obesity, retinal dystrophy, micropenis) are linked to mutations in the phosphoinositide phosphatase INPP5E. Joubert syndrome type 1 manifests as cognitive defects due to abnormal development of the cerebellum, whereas patients with MORM syndrome display multi-organ defects (Bielas et al., 2009). These distinct phenotypes are associated with distinct alleles of $INPP5E$ — mutations in the 5-phosphatase domain in the case of Joubert, and in the CaaX box motif in the case of MORM syndrome (Table 1.2), the latter of which affects INPP5E localization. INPP5E localizes to endosomes, PM and cilia, and can dephosphorylate PIP$_2$ and PI (3,4,5)-P$_3$ in vivo (Xu et al., 2017; Garcia-Gonzalo et al., 2015). The extra-neuronal phenotypes in MORM might be due to extraciliary roles of INPP5E.

A whole-exome sequencing project identified an $EXOC8$ variant segregating with Joubert syndrome in a single consanguineous family (Dixon-Salazar et al., 2012). This
Table 1.2: Ciliopathies associated with PIP$_2$ regulators or effectors.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Reference</th>
<th>Linked genes</th>
<th>Predicted effect on protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joubert (type 1)</td>
<td>Hampshire et al. (2006)</td>
<td>INPP5E</td>
<td>Reduced 5-phosphatase activity</td>
</tr>
<tr>
<td>MORM</td>
<td>Bielas et al. (2009)</td>
<td>INPP5E</td>
<td>Disruption of CaaX box</td>
</tr>
<tr>
<td>Joubert (unspecified type)</td>
<td>Dixon-Salazar et al. (2012)</td>
<td>EXOC8</td>
<td>Disruption of PH domain</td>
</tr>
</tbody>
</table>

variant is a missense mutation in a highly conserved residue within the pleckstrin homology (PH) domain of EXOC8, human ortholog of the yeast Exo84. Exo84 is a component of the octameric exocyst complex.

1.3.1 Pleiotropy in ciliopathies

A number of ciliopathies are linked to mutations in $CEP290$ (Table 1.3). Approximately 100 different polymorphisms in $CEP290$ have been identified so far, with little correlation between allele and clinical phenotypes, which has limited the prognostic power available purely from gene sequencing (Coppieters et al., 2010). $CEP290$ is a TZ protein which is essential for ciliogenesis in *Drosophila* and mice, and for maintenance of protein composition of *Chlamydomonas* cilia (Basiri et al., 2014; Rachel et al., 2015; Craige et al., 2010). The dissection of the pleiotropy associated with $CEP290$ mutations could be useful for diagnosis and personalized therapy, and might also serve as a model for pleiotropy in ciliopathies in general, as well as related developmental disorders.

Various explanations for the genetic heterogeneity of $CEP290$-associated ciliopathies have been proposed. According to one hypothesis, the large size of $CEP290$ (290 kDa) makes it more susceptible on average to picking up random mutations than a smaller-sized gene (Rachel et al., 2012; Gupta et al., 2015). In addition, second-site modifier mutations likely control cell type specificity, severity and penetrance of disease. Furthermore, although certain cilium mutants are unable to form cilia in cultured cells derived from
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Reference</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joubert (type 5)</td>
<td>Valente et al. (2006)</td>
<td>Neurologic, renal, retinal degeneration</td>
</tr>
<tr>
<td>Senior-Løken (type 6)</td>
<td>Sayer et al. (2006)</td>
<td>Retinal dystrophy, chronic renal disease</td>
</tr>
<tr>
<td>Leber congenital amaurosis</td>
<td>den Hollander et al. (2012)</td>
<td>Congenital retinal dystrophy</td>
</tr>
<tr>
<td>Meckel-Gruber (type 4)</td>
<td>Frank et al. (2007)</td>
<td>Polydactyly, hepatic cysts, CNS malformation, perinatal lethality</td>
</tr>
<tr>
<td>Bardet-Biedl (type 14)</td>
<td>Leitch et al. (2008)</td>
<td>Retinitis pigmentosa, obesity, mental retardation, renal disease</td>
</tr>
</tbody>
</table>

Table 1.3: Ciliopathies associated with *CEP290* mutations.

a particular tissue or cell type, within the context of a whole organism, many cilia mutations can be tolerated.

### 1.4 Phosphatidylinositol 4,5-bisphosphate (PIP2) — functions and synthesis

PIP2 is one of seven phosphoinositides (PIPs) present in eukaryotes and is a minor lipid of the inner PM leaflet, which is also its major cellular repository (Balla, 2013). PIP2 is important for PM identity (Hammond et al., 2012), and is therefore not normally found in other organelles in substantial amounts (see Tan et al., 2015 for a review of non-PM related functions of PIP2). In particular, ciliary membranes in mammalian cultured cells and *Drosophila* ChNs are thought to be devoid of PIP2, and this asymmetry is essential for proper cilium function in these systems (Chavez et al., 2015; Park et al., 2015; Garcia-Gonzalo et al., 2015).

The chemical structure of PIP2 is shown in Figure 1.2. Like all PIPs, PIP2 contains a glycerol backbone linking two membrane-associated fatty acyl chains and a cyclic six-carbon inositol head group exposed to the cytoplasm. The inositol ring in PIP2 is phosphorylated on the D4 and D5 carbons. The D3, D4 and D5 positions on the inositol ring in PIPs can be phosphorylated in all seven possible combinations allowing
interconversion through sequences of phosphorylation and/or dephosphorylation events mediated by specific lipid kinases and phosphatases (reviewed in Balla, 2013). This allows particular PIPs to be enriched on specific subsets of intracellular membranes, serving as membrane identifiers and regulators of organelle biogenesis and trafficking. Note that although PIP$_2$ refers to the 4,5-phosphorylated species of PIPs in this thesis for brevity, both PI(3,4)P$_2$ and PI(3,5)P$_2$ qualify as PIP$_2$ in a strict sense.

![Chemical structure of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and biosynthetic mechanisms of PIP$_2$ production and turnover. Phosphoryl groups are represented with orange circles. The structure of the fatty acyl chains can differ from those shown here in vivo.](image)

Figure 1.2: Chemical structure of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and biosynthetic mechanisms of PIP$_2$ production and turnover. Phosphoryl groups are represented with orange circles. The structure of the fatty acyl chains can differ from those shown here in vivo.
1.4.1 Functions of PIP$_2$

PIP$_2$ functions in three main ways. First, it can act as an anchor for proteins, recruiting them reversibly from the cytoplasm to the membrane either through specific binding of the 4,5-phosphorylated head group to a lipid binding domain or non-specific electrostatic interactions with its anionic head group. Dynamic membrane localization can serve to expose a cytoplasmic protein to its membrane-bound regulators or effectors, or sequester proteins from their cytoplasmic targets. For example, binding to PM-associated PIP$_2$ sequesters TULP3 from the nucleus and its role as a transcriptional regulator in the absence of G$_q$ receptor signalling (Santagata et al., 2001). Other PIP$_2$-binding proteins include N-WASP (neural Wiscott-Aldrich syndrome protein) and ERM (ezrin, radixin, moesin) family proteins, which link PM to cortical F-actin (Sechi and Wehland, 2000), and exocyst components Exo70 and Sec3, which target the exocyst complex to the PM and control polarized vesicle transport (He et al., 2007; Zhang et al., 2008). Generally, PIP$_2$ binding motifs show low affinity and specificity towards PIP$_2$, and stable PM binding requires coincident interactions with other factors in addition to PIP$_2$, such as small G-proteins (McLaughlin et al., 2002).

Second, PIP$_2$ binding can allosterically regulate protein activity. For example, binding of focal adhesion kinase (FAK) to PIP$_2$ at focal adhesion sites induces conformational changes leading to disinhibition of its protein kinase activity, which is important for cell adhesion and migration (Goni et al., 2014). During clathrin-mediated endocytosis, multiple subunits of the heterotetrameric adaptor protein AP-2 interact sequentially with PIP$_2$ for proper stabilization and maturation of clathrin coated pits (Kadlecová et al., 2017). PIP$_2$ can also affect protein activity independently of membrane recruitment, such as for integral membrane proteins like ion channels (Suh and Hille, 2008; Hansen, 2015). In some cases, PIP$_2$ can mediate both stimulatory and inhibitory effects on ion channel activity through different molecular determinants (Jeong et al., 2016).

Third, PIP$_2$ can be a precursor for other signalling molecules. Hydrolysis of PIP$_2$ by
phospholipase C yields the soluble inositol 1,4,5-trisphosphate (IP$_3$) and membrane-bound diacylglycerol (DAG), potent second messengers important for calcium signalling and protein kinase C activation, respectively (Cocco et al., 2015). IP$_3$ can be phosphorylated into higher order inorganic inositol phosphates such as IP$_6$, as well as inositol pyrophosphates that contain high-energy diphosphate groups, which are important for non-canonical protein phosphorylation, bioenergetics and regulation of protein activity (Seeds and York, 2007; Wilson et al., 2013; Park et al., 2018). PIP$_2$ also serves as an important source for PIP$_3$, which regulates AKT-based cell survival and proliferation (Manning and Toker, 2017).

1.4.2 Functional and spatial PIP$_2$ pools

The involvement of PIP$_2$ in disparate processes at the PM suggests that it is present in distinct functional pools — isolated nanoreactors of PIP$_2$ and a distinct subset of effector proteins that orchestrate a specific process, such as actin assembly or endocytosis, in response to some upstream signal. This hypothesis is supported by observations of non-uniform distribution of PIP$_2$ within the PM (Chierico et al., 2014; Ji et al., 2015; Wang and Richards, 2012; van den Bogaart et al., 2011). It is important to note that the various techniques used to infer PIP$_2$ distribution within cells are complicated by artefacts and that the specific nature of PIP$_2$ nanodomains remains a contentious topic within the field. For the purposes of this thesis, we assume the existence of functional and spatial PIP$_2$ pools within cells but refrain from commenting on their size or dynamics.

1.4.3 Synthesis of PIP$_2$ by type I phosphatidylinositol phosphate kinases (PIPKIs)

The majority of cellular PIP$_2$ is generated by phosphorylation of phosphatidylinositol 4-phosphate (PI4P) by type I phosphatidylinositol phosphate kinases (PIPKIs), also known as PI4P 5-kinases (van den Bout and Divecha, 2009). PIPKIs were characterized
first in *Saccharomyces cerevisiae*, which encodes a single PIPKI called Mss4p (multicopy suppressor of the PI 4-kinase STT4), which was found to be essential for viability, proper actin assembly during morphogenesis and growth (Desrivieres et al., 1998; Homma et al., 1998), and for secretion (Mizuno-Yamasaki et al., 2010).

In contrast to *S. cerevisiae*, humans and other vertebrates express three PIPKI isoforms — α, β and γ — that display considerable functional plasticity and redundancy at cellular and developmental levels (Balla, 2013). For example, a single copy of PIPKIγ, the longest and most ubiquitously expressed isoform of the three murine PIPKIs, with multiple splice variants, can support development till adulthood in the absence of other two isoforms PIPKIα and PIPKIβ (Volpicelli-Daley et al., 2010). Nevertheless, the three isoforms have distinct intracellular roles in different tissues. PIPKIα is essential for phagocytosis, PIPKIβ for neutrophil chemotaxis and PIPKIγ for synaptic vesicle trafficking, and all three are important for endocytosis in different types of cells (see Balla, 2013 for a review of functions of mammalian PIPKIs). A mutation in the kinase domain of the human *PIP5KIC*, encoding the γ-isoform, is linked to lethal congenital contractural syndrome (LCCS3), which affects joint and limb development (Narkis et al., 2007).

**PIPKIs in *Drosophila melanogaster***

The *Drosophila melanogaster* genome encodes two PIPKIs — Skittles (Sktl) and PIP5K59B (PIP5K at chromosome location 59B). PIP5K59B is relatively poorly studied, and has been found to supply PIP₂ for actin organization during synaptic growth at neuromuscular junctions (Khuong et al., 2010). This neuronal phenotype is consistent with the relatively high expression of *pip5k59b* in the *Drosophila* nervous system according to tissue-specific RNA-seq data from the modENCODE database (FlyBase 2018). The only other reported role for PIP5K59B in phototransduction (Chakrabarti et al., 2015) has recently been disputed (Liu et al., 2018).

Compared to *PIP5K59B*, *sktl* is better characterized and appears to be expressed
more ubiquitously in Drosophila. sktl was isolated in a study of the not enough muscles (nem) mutant, which exhibited defects in differentiation of somatic mesoderm (Knirr et al., 1997). Although the nem phenotype was found to be linked to the gene inscuteable (insc), examination of the locus showed that another gene bearing homology to the yeast MSS4 was nested within the the first intron of insc. This gene was named “skittles” by Stefan Knirr, the lead author for the original 1997 paper, out of fondness for Skittles candy (personal communication with Stefan Knirr and Renate Renkawitz-Pohl). sktl was cloned by cDNA rescue of embryonic lethality of the fata morgana (fam) complementation group (Hassan et al., 1998), which is a P-element insertion on the second chromosome that disrupts formation of a subset of embryonic ChNs (Kania et al., 1995). fam affects both sktl and insc (Hassan et al., 1998).

Functions of sktl in Drosophila development

sktl is an essential gene — strong hypomorphic alleles exhibit embryonic or early larval lethality (Hassan et al., 1998). Epithelial sktl clones have been reported to be small and rare (Hassan et al., 1998; Gervais et al., 2008), possibly due to a cell polarity defect leading to rejection from the epithelium or defects in cell viability or proliferation. sktl is essential for development of male and female germlines (Hassan et al., 1998; Gervais et al., 2008; Fabian et al., 2010), and for formation of the peripheral nervous system (Prokopenko et al., 2000).

In the female germline, sktl is essential for polarized actin cytoskeleton by regulating activity of the ERM protein Moesin and recruitment of PAR (partitioning defective) polarity proteins to the PM, disruption of which perturbs microtubule assembly and proper localization of mRNAs important for symmetry breaking in the Drosophila embryo (Gervais et al., 2008). Sktl is also important for clathrin-mediated endocytosis (Compagnon et al., 2009). In the follicular epithelium, Sktl is important for regulation of apicobasal polarity and cell shape by controlling asymmetric localization of PAR proteins (Claret
et al., 2014).

In S2 cells, a cell line derived from immortalized embryonic hemocytes, Sktl is important for actin organization and cell shape changes during mitosis via Moesin (Roubinet et al., 2011). Sktl overexpression enhances apical recruitment of the formin Diaphanous and asymmetrical F-actin localization in the tracheal epithelium (Rousso et al., 2013). Thus, Sktl seems to be associated with prototypical PIPKI functions related to regulation of actin assembly and vesicular trafficking, which makes *Drosophila* a good model system to investigate PIPKI regulation and functions in more detail *in vivo*, in the hopes that they will also be applicable to human health and disease.

**1.4.4 Molecular mechanisms of regulating PIPKI localization and activity**

Considering the diversity of functions of PIPKIs, there are probably relatively sophisticated intracellular methods for controlling PIPKI localization and kinase activity. Various small GTPases such as Rac, Rho and Arf can bind PIPKIs and regulate their activity in response to integrin activation (Ren and Schwartz, 1998) and focal adhesion sites during cell migration (Pratt et al., 2005). PIPKIs can also be activated by lipids such as phosphatidic acid (Divecha et al., 2000) and inhibited or activated by phosphorylation of specific residues by protein kinase A (van den Bout and Divecha, 2009). An extensive discussion of molecular interactions of PIPKIs can be found in Choi et al. (2015) and Van den Bout et al. (2009).

Most studies on PIPKIs use overexpression, which results in a predominantly PM localization of the enzymes despite both direct and indirect evidence of PIPKI isoforms localizing to Golgi (Jones et al., 2000), intracellular vesicles (Doughman et al., 2003), focal adhesion sites (Di Paolo et al., 2002) and nuclear speckles (Mellman et al., 2008), in addition to the PM. This is likely due to an increase in PM-associated PIP$_2$ in cells overexpressing PIPKIs, since overexpressed kinase-dead PIPKIs do not associate with
PM as strongly (Giudici et al., 2006). This is corroborated by discovery of a positive feedback loop intrinsic to mammalian PIPKIs that links PIP$_2$ synthesis to membrane association due to electrostatic interactions between a membrane-facing cationic patch on PIPKIs and the anionic PIP$_2$ (Fairn et al., 2009). Various studies have discovered protein-intrinsic mechanisms of membrane binding that could regulate PIPKI localization and activation, including PIPKI multimerization (Liu et al., 2016; Lacalle et al., 2015; Hu et al., 2015; Hansen et al., 2017).

1.5 The role of PIP$_2$ in cilium assembly and function

1.5.1 Exclusion of PIP$_2$ from the ciliary membrane

PIP$_2$ is a lipid determinant of PM identity and is essential for various PM-associated processes, such as linking cortical actin to the PM, as well as for endocytosis and exocytosis (Balla, 2013). Since cilia are specialized organelles that perform a different function than the PM, it would be natural to hypothesize that cilia have a different PIP$_2$ composition than the PM. Consistent with this idea, it has been found that although the ciliary membrane is physically contiguous with the PM, it contains very little PIP$_2$ in sensory neurons from *C. elegans* and *Drosophila*, and in cultured human cells. In ciliated sensory neurons in *C. elegans*, PIP$_2$ is enriched in the “periciliary membrane compartment” (equivalent to the ciliary base) and mutation of the TZ protein *mks-5* causes a build-up of PIP$_2$ inside the cilium (Jensen et al., 2015). Similarly, although PIP$_2$ is enriched at the ciliary base, it is absent from within the cilium in *Drosophila* ChNs and mammalian cells due to the action of INPP5E (Park et al., 2015; Garcia-Gonzalo et al., 2015; Chavez et al., 2015). Nakatsu (2015) has proposed a model of a phosphoinositide code of cilia based on these studies in which INPP5E maintains the cilium as an organelle devoid of PIP$_2$, and allowing ciliary signalling (Figure 1.3).
Chapter 1. Introduction

Figure 1.3: A model of the phosphoinositide code of cilia. The cilium resident PIP$_2$ phosphatase INPP5E dephosphorylates PIP$_2$ from the ciliary membrane in wild-type cells. In *INPP5E* knockouts, PIP$_2$ build-up within the cilium causes accumulation of the PIP$_2$-binding ciliary protein transporter TULP3, as well as its cargo proteins. These include Gpr161, a negative regulator of Hedgehog signalling (in mice and humans), and mechanosensory ion channels (in *Drosophila*, recruited by the TULP3 ortholog Tulp). The result is perturbation of normal sensory functions of cilia in *INPP5E* mutants. Reproduced from Nakatsu (2015) by permission of the journal.

1.5.2 Disruption of cilium function by high intraciliary PIP$_2$

Two studies published in 2015 showed that PIP$_2$ removal from the ciliary membrane by INPP5E is crucial for proper Hedgehog signalling in mammals. INPP5E localizes along the cilium in murine neural stem cells, preventing intraciliary PIP$_2$ buildup by dephosphorylating PIP$_2$ to PI4P, the latter of which is relatively enriched within the ciliary membrane (Chavez et al., 2015). Neural stem cell-specific conditional inactivation of *INPP5E* leads to a reduction in cilium length, and a concomitant increase in the levels of PIP$_2$, the PIP$_2$-binding protein TULP3, its IFT-A binding partner IFT122, and
their cargo Gpr161 (Chavez et al., 2015). Gpr161 is a repressor of Hedgehog signalling, and its increased intraciliary localization in INPP5E mutants leads to a reduction in Sonic hedgehog signalling and neurogenesis in vivo. Identical results were found in fibroblasts from INPP5E−/− mice (Garcia-Gonzalo et al., 2015). These studies affirmed the importance of INPP5E in both cilia function and Sonic hedgehog signalling in mammals, and showed that a large part of these defects could be explained by an increase in intraciliary PIP2 and mislocalization of PIP2-binding Tubby proteins, rather than a decrease in PIP3.

**Functions of Drosophila Inpp5e**

In *Drosophila*, mutations in inpp5e cause build-up of the Tubby protein Tulp within ChN cilia, leading to mislocalization of the ciliary TRP ion channels Inactive (Iav) and NompC. Normally, Tulp is present in small amounts inside cilia\(^*\), Iav is present in the proximal ciliary segment and NompC is restricted to the distal ciliary segment. In *tulp* mutants, Iav fails to localize to the proximal ciliary segment whereas NompC redistributes to the proximal segment in addition to the distal segment (Park et al., 2013). In *inpp5e* mutants, which contain excess intraciliary Tulp, Iav is enriched within cilia but NompC has the same distribution as in *tulp* mutants (Park et al., 2015). Together, this data indicate that Tulp regulates the transport of Iav but not NompC to cilia. Overexpression of the PIP2-binding mutant of Tulp in the *inpp5e* background can partially rescue sensory defects and localization of Iav and NompC (Park et al., 2015). These results are summarized in Table 1.4. Interestingly, the PIP2-binding ability of Tulp is important for Iav localization to the proximal ciliary segment but not for Tulp localization (Park et al., 2013), suggesting that Tulp might not directly transport Iav to cilia.

Although *Drosophila tulp* and *inpp5e* mutants show defects in sound-evoked nerve potentials, neither of these exhibit ciliogenesis defects. In contrast, zebrafish morphants

\(^*\text{Tulp is also present in the ChN cell body. Unlike mammalian TULP3, PIP3 binding does not regulate cytoplasmic-nuclear shuttling of Tulp in ChNs, although it does so in salivary glands in overexpression experiments (Park et al., 2013).}\)
## Chapter 1. Introduction

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Intraciliary PIP$_2$</th>
<th>Tulp</th>
<th>Iav</th>
<th>NompC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Distal segment only</td>
</tr>
<tr>
<td>$tulp$</td>
<td>Low</td>
<td>Absent</td>
<td>Absent</td>
<td>Distal + proximal</td>
</tr>
<tr>
<td>$inpp5e$</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Distal + proximal</td>
</tr>
<tr>
<td>$inpp5e + Tulp^AIP2$</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>Distal segment only</td>
</tr>
</tbody>
</table>

Table 1.4: Summary of phenotypes from studies of Tulp and Inpp5e in *Drosophila* (Park et al., 2013, 2015).

and mouse mutants of *INPP5E* show malformed or reduced numbers of cilia in some cell types, indicating a tissue-specific requirement (Xu et al., 2017; Jacoby et al., 2009). In zebrafish, overexpression of the ERM protein ezrin rescues phenotypes associated with *inpp5e* mutants, such as apical docking of BBs, by enhancing F-actin assembly (Xu et al., 2017). *INPP5E* inactivation in murine epithelial fibroblasts affects cilium maintenance (Jacoby et al., 2009; Hardee et al., 2017), possibly due to a mitogenic effect caused by increase in PIP$_3$, similar to those observed in conditional mutants of *INPP5E* in mice (Hakim et al., 2016). *INPP5E*–/– epithelial fibroblasts also show loss of TZ proteins TCTN1, MKS1, B9D1 and septin 2 from the TZ when Hedgehog signalling is perturbed with a Smoothed agonist; nonetheless, cilia are not resorbed (Dyson et al., 2017).

OCRL1, which is linked to oculocephaloretinal syndrome of Lowe, is a 5-phosphatase specific for PIP$_2$ that localizes to cilia in a number of ciliated cultured cell lines (Luo et al., 2012). Zebrafish *ocrl* morphant embryos display shorted cilia in Kupffer’s vesicle, and phenotypes suggestive of ciliary dysfunction such as hydrocephalus and kinked tails, and fibroblasts from patients with Lowe syndrome have shorter cilia (Luo et al., 2012; Coon et al., 2012). Although Lowe syndrome is not generally considered a ciliopathy because renal and ocular phenotypes in patients with Lowe differ from typical ciliopathic phenotypes, the importance of Ocr11 in limiting ciliary PIP$_2$ (Prosseda et al., 2017) indicates that some of these defects may be due to cillum dysfunction. OCRL2/INPP5B, the paralog of OCRL1, also localizes to cilia and has roles in ciliogenesis (Luo et al., 2013). However, relatively little is known about mechanisms of how OCRL proteins regulate
ciliogenesis compared to INPP5E.

1.5.3 Requirement for PIPKIγ in ciliogenesis

In addition to a well-established role for INPP5E in limiting intraciliary PIP₂ levels, there is evidence that mammalian PIPKIγ coordinates the initiation of ciliogenesis along with INPP5E at the centrosome (Xu et al., 2016). In proliferating cell lines, INPP5E generates a pool of PI4P at the mother centriole. This PI4P binds to CEP164 and prevents its association with τ-tubulin kinase 2 (TTBK2) directly, inhibiting CP110 removal and ciliogenesis (Xu et al., 2016). In quiescent cells, INPP5E is removed from the BB and PI4P is phosphorylated to form PIP₂ by BB-resident PIPKIγ, licensing the interaction of CEP164 and TTBK2, and subsequently, CP110 decapping and axoneme assembly. Notice that it is PI4P rather than PIP₂ that plays an active role in this process; PIP₂ is simply a by-product. It is unclear where precisely PI4P and PIP₂ localize at the centrosome and BB, i.e., what membranes or vesicles they localize to. It is unlikely that this mechanism is conserved in invertebrates — C. elegans does not seem to encode a CP110 ortholog while the Drosophila homolog is dispensable for ciliogenesis (Franz et al., 2013). The study by Xu et al. does show that RNAi-mediated knockdown of ppk-1, encoding the sole C. elegans PIPKI, induces a dye filling defect in ciliated sensory neurons, indicating a role in ciliation formation (Xu et al., 2016).

1.6 Rationale

Our lab previously found that reduction of PIP₂ in the Drosophila male germline by ectopic expression of the PIP phosphatase SigD caused complete male sterility (Wei et al., 2008; Fabian et al., 2010). SigD has potent 5-phosphatase activity against PIP₂ in vivo in Drosophila (Sengupta et al., 2013; Wei et al., 2008). SigD expression in the male germline caused defects in cell polarization, morphogenesis and cytokinesis, but also in the structure of the sperm axoneme (Wei et al., 2008). Specifically, a subset of SigD-expressing cells
contained fragmented axonemes, or microtubule triplets in addition to doublets. This phenotype could be rescued by co-expression of wild-type Sktl. We hypothesized that loss of PIP$_2$ in the male germline affected formation of TZs, which enabled some C-tubules to protrude beyond the TZ into the cilium, and that PIP$_2$ might be important for ciliogenesis. To investigate this possibility, we examined whether reduction of Sktl and PIP$_2$ affected cilium formation or function in two types of ciliated cells in *Drosophila melanogaster*, male germline (described in Chapter 2) and chordotonal neurons (described in Chapter 3).
Chapter 2

**PIP$_2$ affects transition zone maturation during ciliogenesis**

### 2.1 Summary

Cilia are cellular antennae that are essential for human development and physiology. A large number of genetic disorders linked to cillum 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 cillum. Despite substantial effort to identify TZ proteins and their roles in cillum 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 by 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.*

*Parts of this chapter were published in the Journal of Cell Science (2018, volume 131(16)). I performed all experiments and analysis described herein.*
2.2 Introduction

Cilia are sensory organelles that are important for signalling in response to extracellular cues, and for cellular and extracellular fluid motility (Satir et al., 2010; Marshall and Nonaka, 2006; Eley et al., 2005; Brooks and Wallingford, 2014). Consistent with their importance, defects in cilium formation (*i.e.* ciliogenesis) are associated with genetic disorders known as ciliopathies, which can display neurological, skeletal and fertility defects, in addition to other phenotypes (Waters and Beales, 2011; Valente et al., 2014; Hammarsjo 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 (Goncalves and Pelletier, 2017), the process of TZ maturation, through which it is converted from an immature form to 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) (Figure 2.1). 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 by an increase in TZ length in the *Drosophila* male germline.

We previously showed that the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is essential for formation of the axoneme 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 PIP$_2$ due to the action of the cilium resident PIP phosphatase INPP5E, the base of the cilium 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 (Chavez 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 the 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).

## 2.3 Methods

### 2.3.1 Transgenic flies

*Drosophila* stocks were cultured on cornmeal molasses agar medium at 25°C and 50% humidity. Stocks expressing $\beta_2$t::Styp\SigD and $\beta_2$t::YFP-Sktl were described previously (Wei et al., 2008; 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 were provided by T. Avidor-Reiss (Basiri et al., 2014). $Sp/CyO$, Unc-GFP was originally provided by M. Kernan (Baker et al., 2004). Stocks expressing GFP-tagged Chibby and Mks1 were
Chapter 2. PIP₂ affects transition zone maturation during ciliogenesis

provided by B. Durand (Enjolras et al., 2012; Vieillard et al., 2016). The Exo₈₄onr mutant was described previously (Giansanti et al., 2015). Stocks for generating sktl².³ clones were originally provided by A. Guichet (Gervais et al., 2008). Clones were induced by heat shock for two hours on days 3, 4 and 5 after egg laying. w¹¹¹⁸ was used as the wild-type control.

2.3.2 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, USA), 1:500; mouse anti-acetylated α-tubulin 6-11-B (6-11-B, Sigma-Aldrich), 1:1000. Secondary antibodies were Alexa 488- and Alexa 568-conjugated anti-mouse, anti-rabbit and anti-chicken IgG (Thermo Fisher Scientific, Waltham, MA, USA) at 1:1000. DAPI (Thermo Fisher Scientific) at 1:1000 was used to stain for DNA.

2.3.3 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 minutes. Testes were transferred to a polylysine-coated glass slide (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) supple-
mented 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 in PBS and imaged live.

For CellMask staining, cells were spilled from testes in M3 medium onto a sterilized glass-bottom dish pre-treated with sterile polylysine solution to enable cells to adhere. CellMask Deep Red (C10046, Invitrogen, Waltham, MA, USA) 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 minutes. Slides were transferred to ice-cold methanol for 5-10 minutes 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-minute washes with PBS, 1 hour incubation with secondary antibodies, and three 5-minute washes with PBS. Samples were mounted in Dako (Agilent, Santa Clara, CA, USA) and imaged with a Zeiss Axioplan 2 epifluorescence microscope or a Nikon A1R scanning confocal microscope (SickKids imaging facility).

2.3.4 FLP/FRT-mediated mitotic recombination

Mitotic clones were generated in flies of genotype $w$, hsFLP/Y ; FRT42B, sktl$^{2.3}$/FRT42B. Clones were induced by heat shock for two hours for three consecutive days three days after egg laying, and males were dissected 1-2 days after eclosion. We expect that this maximized the chances of recovering clones which had undergone mitotic recombination very early during their life cycle (for example, as stem cells or gonial cells).
2.3.5 Statistical methods

Statistical analysis and graphing was performed using R (version 3.4). A Gaussian jitter was applied when plotting results 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 \( a \ priori \) for all classical analyses.

2.3.6 Phylogenetic analysis

Candidate orthologs of Skittles and PIP5K9B 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 to a vector image in Illustrator and colours were added for the purpose of illustration.

2.4 Results

2.4.1 PIP\(_2\) is essential for transition zone maturation

To investigate how reduction of cellular PIP\(_2\) affects ciliogenesis in the \textit{Drosophila} male germline, we used transgenic flies expressing the Salmonella PIP phosphatase SigD under control of spermatocyte-specific \( \beta_2 \)-tubulin promoter (hereafter \( \beta_2 t \)-SigD) (Wei et al., 2008). To examine whether axoneme defects in \( \beta_2 t \)-SigD (Wei et al., 2008) were caused by aberrant TZ function, we examined localization of fluorescently-tagged versions of the core centriolar/BB protein Ana1 (CEP295 homolog) (Goshima et al., 2007; Blachon et al.,
2009) and the conserved TZ protein Cep290 (Basiri et al., 2014) during early steps of ciliogenesis. Cep290 distribution appeared similar in control and β₂t-SigD in early G2 phase, when TZs are still immature. In contrast, Cep290-labelled TZs were significantly longer in β₂t-SigD compared to controls by late G2 phase, following the period of TZ maturation (Figure 2.1B and 2.1C). Unlike Drosophila cep290 mutants, which contain longer than normal BBs (Basiri et al., 2014), Ana1 length was not affected in β₂t-SigD, and we did not observe a strong correlation between Cep290 and Ana1 lengths (Figure 2.1D). Consistent with this result, the ultrastructure of BBs in β₂t-SigD is normal, and localization of the centriolar marker GFP-PACT (Basto et al., 2006) is similar, in controls and β₂t-SigD (Wei et al., 2008). In contrast, TZ proteins Chibby (Cby) (Enjolras et al., 2012) and Mks1 (Vieillard et al., 2016; Pratt et al., 2016) exhibited hyperelongation in β₂t-SigD (Figure 2.1E), indicating that this phenotype is not unique to Cep290. TZ hyperelongation was a highly penetrant phenotype (>70%) and showed high correlation (>0.95) within syncytial germ cell cysts, suggestive of a dosage-based response to a shared cellular factor, presumably SigD. Despite persistence of hyperelongated TZs through meiosis, axonemes were able to elongate normally in post-meiotic cells (Figure 2.1F). 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).
Figure 2.1. SigD expression induces transition zone hyperelongation.

(A) Schematic diagram of ciliogenesis in the *Drosophila* male germline. Stages in parentheses correspond to Cenci et al. (1994).

(B) $\beta_2$t-SigD expression induces Cep290 hyperelongation in cilia at late G2 phase (arrowheads).

(C) Quantification of paired Ana1-Cep290 lengths in early and late G2 spermatocytes ($n > 30$ and $> 65$ cilia, respectively). Ana1-tdTomato and Cep290-GFP lengths from individual single cilia are connected by purple line segments.

(D) Lengths of Ana1-tdTomato versus Cep290-GFP in control and $\beta_2$t-SigD cells at late G2 from (C) showing negligible correlation. Regression lines (red) and Pearson correlation coefficients ($r$) are shown.

(E) $\beta_2$t-SigD expression induces hyperelongation of TZ proteins Chibby (Cby) and Mks1 in late G2 (arrowheads). Boxplots (right) show quantifications of lengths ($n > 25$ cilia).

(F) TZ hyperelongation in $\beta_2$t-SigD persists through meiosis (white arrowhead) but does not prevent axoneme outgrowth. Acetylated tubulin (AcTub) labels the axoneme.
2.4.2 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., 2013), SigD can dephosphorylate multiple PIPs in vitro (Norris et al., 1998). To address whether TZ hyperelongation observed in β$_2$t-SigD represented a physiologically relevant phenotype due to decreased PIP$_2$, we attempted to rescue this phenotype by co-expressing β$_2$t-SigD with fluorescently-tagged Skittles (Sktl) under control of β$_2$-tubulin promoter. We found that Sktl expression was able to...
suppress TZ hyperelongation in a cilium-autonomous manner (Figure 2.2A and 2.2B). Furthermore, the BB/TZ protein Unc-GFP (Baker et al., 2004; Wei et al., 2008), exhibited TZ hyperelongation at a low penetrance in sktl<sup>2.3</sup> mutant clones (Figure 2.2C), indicating that Sktl is important for TZ maturation.

Vertebrate type I PIP kinase PIPKIγ has previously been shown to be important for cilium formation in cultured cells (Xu et al., 2016). The two Drosophila PIPKIs, Sktl and PIP5K59B, arose from recent duplication of the ancestral PIPKI gene, and are not orthologous to specific vertebrate PIPKI isoforms (Figure 2.2D). Sktl has diverged more than its paralog PIP5K59B and seems to be functionally related to PIPKIγ and the C. elegans PPK-1 in having roles at cilia (Xu et al., 2014). However, unlike the 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. Notably, neither inactivation nor overexpression of cp110 affects cilium formation in Drosophila, and Cp110 is removed from BBs in early primary spermatocytes (Franz et al., 2013).
Figure 2.2. Sktl is important for transition zone maturation.

(A) Expression of Sktl suppresses $\beta_2$-t-SigD-induced TZ hyperelongation in a cilium-autonomous manner. Images demonstrate varying levels of rescue of Cep290-GFP length in $\beta_2$-YFP-Sktl; $\beta_2$-t-SigD. Arrowheads mark fully rescued Cep290 distribution.

(B) Quantification of Cep290 and Ana1 lengths from control, $\beta_2$-t-SigD and $\beta_2$-YFP-Sktl; $\beta_2$-t-SigD from (A) ($n = 100$ cilia).

(C) Cilia in sktl$^{2.3}$ clones exhibit TZ hyperelongation (arrowheads) marked by Unc-GFP (left). Quantification of Unc-GFP lengths in control ($n = 53$ cilia), sktl$^{2.3}$ ($n = 31$ cilia) and $\beta_2$-t-SigD ($n = 51$ cilia) spermatocytes at late G2 (right). Note that the elongation in sktl clones is not as pronounced as in $\beta_2$-t-SigD.

(D) Phylogenetic tree of PIP5Ks showing evolutionary conservation of cilium-associated functions. Scale bar (bottom) represents expected amino acid substitutions per site. Branch support values are shown in red (a value of 1 indicates maximum support). Black arrows represent previous evidence of involvement in cilium-associated functions (from (Xu et al., 2016)). Black arrowhead indicates Sktl. Abbreviations: 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).
Chapter 2. PIP$_2$ affects transition zone maturation during ciliogenesis

2.4.3 Hyperelongated transition zones exhibit functional defects

We next sought to examine whether TZ hyperelongation due to SigD expression affected TZ function, i.e., whether TZs in $\beta_2$t-SigD exhibited functional defects. Following meiosis in the Drosophila male germline, TZs detach from the BB and migrate along the growing axoneme, maintaining a ciliary compartment at the distal-most $\sim$2 $\mu$m where tubulin is incorporated into the axoneme (Basiri et al., 2014; Fabian and Brill, 2012). As shown by Unc and Cep290 localization, TZs in $\beta_2$t-SigD were frequently incapable of detaching from
BBs and migrating along axonemes despite axoneme and cell elongation (Figures 2.1F, 2.3A and 2.3B). Indeed, the previously reported “comet-shaped” Unc-GFP localization in \( \beta_2\)-t-SigD (Wei et al., 2008) persists during cell elongation after meiosis (Figure 2.3A, bottommost panel) despite elongation of the axoneme (Figure 2.1E).

In *Drosophila* and humans, BBs consist of microtubule triplets (Jana et al., 2016; Lattao et al., 2017), whereas axonemes contain microtubule doublets due to obstruction of C-tubules at the TZ (Gottardo et al., 2013). Consistent with a defect in this barrier and the presence of microtubule triplets in axonemes in \( \beta_2\)-t-SigD (Wei et al., 2008), a subset of cilia (<5%) in \( \beta_2\)-t-SigD contained puncta of Ana1 at the distal tips of TZs (Figure 2.3C). Treatment of germ cells with the microtubule-stabilizing drug Taxol increased the penetrance of this phenotype from <5% in untreated cells to >25% in cells treated with 4 \( \mu\)M Taxol (arrowheads in Figure 2.3D) without significantly affecting Cep290 length (Figure 2.3E). Taxol-treated controls did not exhibit TZ-distal Ana1 puncta (\( p < 0.01 \) at 5% penetrance). Fluorescently-tagged Asterless (CEP152 homolog), a pericentriolar protein (Blachon et al., 2008; Dzhindzhev et al., 2010), did not localize to TZ-distal puncta in \( \beta_2\)-t-SigD (\( p < 0.01 \)) suggesting that 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). Similar to \( \beta_2\)-t-SigD, Taxol-treated male germ cells assemble extremely long axonemes that contain triplet microtubules (Riparbelli et al., 2013), further supporting a functional relationship between PIP\(_2\) and microtubule reorganization in TZ maturation.
Figure 2.3. Hyperelongated transition zones display functional defects.

(A) Unc-GFP is unable to split in spermatids expressing $\beta_2$t-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 Unc-GFP to split in $\beta_2$t-SigD was highly penetrant (>90%, $n = 63$ cilia).

(B) Cep290 is unable to detach and migrate from the basal body at onset of axoneme assembly in $\beta_2$t-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 $\beta_2$t-SigD cells showing TZ-distal puncta of centriolar protein Ana1 in $\beta_2$t-SigD (arrowheads).

(D) Treatment of control and $\beta_2$t-SigD cells with the microtubule stabilizing drug Taxol. Images demonstrate variability in Cep290 distribution. Yellow arrowheads mark TZ-distal Ana1.

(E) Quantification of Cep290 lengths in Taxol-treated control and $\beta_2$t-SigD cells from (D) ($n = 30$-$40$ cilia).
2.4.4 The *onion rings* (*onr*) mutant decouples defects found in cells with reduced levels of PIP$_2$

Male flies homozygous for the *onion rings* (*onr*) mutant of *Drosophila Exo84* are sterile and exhibit defects in cell elongation and polarity similar to $\beta_2$-t-SigD (Wei et al., 2008). Exo84 is a component of the octameric exocyst complex, which binds PIP$_2$ and regulates...
membrane trafficking 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 $\beta_2$-t-SigD, onr did not display hyperelongated TZs (Figure 2.4A), suggesting that Exo84 is dispensable for TZ maturation.

Due to involvement of the exocyst in trafficking at the PM, we examined whether cilium-associated membranes were affected in $\beta_2$-t-SigD or onr mutants in a manner similar to 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). Whereas TZs in $\beta_2$-t-SigD and onr cells were able to dock at the PM initially, they were unable to maintain membrane connections, and were rendered cytoplasmic (Figure 2.4B and 2.4C), similar to TZs in dil; cby mutants. We found that fluorescently-tagged Exo70, a PIP$_2$-binding exocyst subunit, localized to BBs (Figure 2.4D). Our results suggest that the exocyst, and Exo84 in particular, regulates cilium-PM associations, similar to PIP$_2$, and that TZ hyperelongation and loss of cilium-PM association are genetically separable phenotypes.
Figure 2.4. The *onion rings* (*onr*) allele of *Exo84* decouples TZ hyperelongation from loss of plasma membrane contacts.

(A) *onr* mutants do not display hyperelongated acetylated tubulin 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 $\beta_2$t-SigD fail to maintain cilium-PM tethering despite initially anchoring to the PM. 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) 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 PI5P in addition to PI4P (Norris et al., 1998).
Figure 2.4: The *onion rings* (*onr*) allele of *Exo84* decouples TZ hyperelongation from loss of plasma membrane contacts
2.5 Discussion

The process of maturation of a TZ from a nascent form 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; Goncalves 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. Similar to \(\beta_2\)t-SigD, *Drosophila dila; cby* and *cby* mutants display hyperelongated TZs (Enjolras et al., 2012; Vieillard et al., 2016), whereas *mks1* mutants have shorter TZs (Pratt et al., 2016). Because both Cby and Mks1 display longer than normal lengths in \(\beta_2\)t-SigD cells, PIP\(_2\) regulates TZ length independently of an effect on Cby or Mks1 recruitment.

We also 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 \(\beta_2\)t-SigD, albeit with aberrant ultrastructure (Wei et al., 2008), despite the lack of functional TZs or membrane association. The presence of TZ-distal Ana1 puncta in \(\beta_2\)t-SigD cells, without the increase in BB length seen in *cep290* mutants lacking a functional TZ barrier, suggests that \(\beta_2\)t-SigD expression selectively disrupts the ability of TZs to restrict C-tubules and Ana1 without abolishing the TZ barrier entirely. CEP295, the human Ana1 ortholog, regulates post-translational modification of centriolar microtubules (Chang et al., 2016), which may explain the presence of Ana1 along with supernumerary microtubules in \(\beta_2\)t-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 the dynamics of Ana1 and Asl loading onto centrioles (Fu et al., 2016; Saurya et al., 2016) or the more peripheral nature of Asl 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). In this study, we showed that mutation of the PIPKI Sktl induced hyperelongated TZs and that expression of Sktl could suppress TZ hyperelongation in \(\beta_2\)t-SigD, with
some cells showing cilium-autonomous suppression, suggesting Sktl might function \textit{in situ} to regulate TZ length. In humans, \textit{PIPKI}\textsubscript{γ} 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 LCCS1-associated GLE1 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 disease.

Members of the exocyst complex such as Sec10 and Sec8 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 plasma membrane through a direct interaction with PIP\textsubscript{2} (He et al., 2007; Zhang et al., 2008). We previously showed that the \textit{onr} allele of \textit{Drosophila Exo84} phenocopies defects in cell polarity and elongation observed in \textit{β2t-SigD} (Fabian et al., 2010). Here, we show that the \textit{onr} mutation phenocopies the loss of cilium-membrane contacts in \textit{β2t-SigD}, similar to \textit{dila; cby} mutants (Vieillard et al., 2016), but not TZ hyperelongation. Thus, TZ hyperelongation is not a prerequisite for the 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 association. This result is supported by the \textit{Drosophila cep290} mutant, which lacks a functional TZ but retains cilium-PM association Basiri et al. (2014). Notably, \textit{EXOC8}, which encodes the human Exo84, has been linked to the ciliopathy Joubert syndrome (Dixon-Salazar et al., 2012), and a similar process might underlie defects in humans with mutations in \textit{EXOC8}. 
Chapter 3

PIP$_2$ regulates chordotonal cilia

3.1 Abstract

Cilia are antenna-like sensory organelles whose malfunction underlies several human genetic disorders. Recent studies have shown that proper cilium function requires maintenance of low levels of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) within the ciliary membrane by the phosphoinositide 5-phosphatase INPP5E. Here, we show that the Drosophila type I phosphatidylinositol phosphate kinase (PIPKI) Skittles (Sktl) that synthesizes PIP$_2$ is required for cilium formation and proper localization of intraciliary proteins in chordotonal neurons (ChNs). Consistent with a previous report of sktl as a major quantitative trait locus associated with variability in adult fly gravitaxis, ChN-specific sktl knockdown perturbs ability of flies to climb. Unlike sktl overexpression, sktl knockdown does not perturb the localization of Tubby domain protein Tulp. Instead, it causes a build-up of NompB/IFT88 at the tips of the cilia. Intriguingly, PIP$_2$ was enriched within the ChN ciliary dilation, providing evidence that spatially restricted pools of PIP$_2$ can exist within cilia. Our observations reveal unappreciated complexity in the functional relationship between PIP$_2$ and cilia*.

3.2 Introduction

Cilia are tiny antenna-like organelles that project from the cell surface and sense diverse physical and chemical stimuli in various cell types (Satir et al., 2010; Marshall and Nonaka, *A manuscript based on the research described in this chapter has been submitted. I performed all the experiments and analysis described herein.*
2006). Defects in cilium formation (ciliogenesis) and function are associated with multiple developmental disorders known collectively as ciliopathies (Waters and Beales, 2011). A subset of ciliopathies are linked to mutations in the human \textit{INPP5E} gene (Bielas et al., 2009; Jacoby et al., 2009), which encodes a 5-phosphatase for phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$). Removal of PIP$_2$ from the ciliary membrane by INPP5E is essential for proper ciliary localization of PIP$_2$-binding Tubby domain proteins and their protein cargoes that are important for cillum function in mammalian cultured cells and \textit{Drosophila melanogaster} (Garcia-Gonzalo et al., 2015; Chavez et al., 2015; Park et al., 2015). Thus, maintenance of low intraciliary PIP$_2$ is essential for normal cillum function (Nakatsu, 2015), but little is known about whether reduction of PIP$_2$ synthesis affects cilia.

PIP$_2$ is one of seven phosphoinositides (PIPs) found in eukaryotes. It localizes primarily to the plasma membrane, where it regulates processes such as actin assembly and vesicle trafficking (Balla, 2013). Most cellular PIP$_2$ is generated by the action of type I phosphoinositide kinases (PIPKIs), of which the $\gamma$-isoform has been shown to be important for cillum formation in mammalian cells by licensing assembly of the transition zone (TZ) (Xu et al., 2016). The TZ is present at the base of cilia, distal to the basal body (BB), where it regulates bidirectional transport of ciliary proteins (Reiter et al., 2012; Szymanska and Johnson, 2012). Proper TZ function is essential for cillum formation and function. For example, \textit{Drosophila} Inpp5e localizes to the TZ and prevents buildup of PIP$_2$ and the Tubby protein Tulp within the cillum (Park et al., 2015), thereby ensuring proper transport of mechanosensory ion channels Inactive (Iav/TRPV) and NompC (TRPN1), in chordotonal neuron (ChN) cilia (Park et al., 2015). Furthermore, mutants of the conserved TZ protein Cep290 are unable to form cilia and display severe uncoordination associated with defective sensory reception (Basiri et al., 2014).

Assembly of the TZ is followed by initiation of intraflagellar transport (IFT), an intraciliary trafficking system composed of microtubule motors and adaptor proteins that
carry protein cargoes along the axoneme in both anterograde (IFT-B) and retrograde (IFT-A) directions (Rosenbaum and Witman, 2002; Lechtreck, 2015). In addition to its requirement for ciliogenesis in most cell types, IFT is also important for regulating transport of proteins involved in ciliary signal transduction pathways, such as Hedgehog signalling (Liem et al., 2012). Human TULP3 and Drosophila Tulp bind to IFT-A proteins involved in retrograde trafficking and require the action of INPP5E in maintaining low intraciliary PIP\(_2\) for proper localization (Mukhopadhyay et al., 2010; Park et al., 2015; Chavez et al., 2015; Garcia-Gonzalo et al., 2015). In contrast, INPP5E is not required for IFT-B or cilium formation in Drosophila (Park et al., 2015), but whether loss of PIP\(_2\) affects IFT or ciliogenesis is not known.

Here, we show that the Drosophila PIPKI Skittles (Sktl) is important for cilium function and formation in ChNs. RNAi-mediated sktl knockdown in ChNs perturbs climbing ability in adults, indicating that Sktl is important for ChN function. Chordotonal organs mutant for sktl assemble shorter cilia, and sktl RNAi causes NompB/IFT88 enrichment at the tips of cilia without drastically affecting the localization of Tulp, NompC or RempA/IFT140. We also report enrichment of PIP\(_2\) within the ChN ciliary dilation and show that this enrichment is dependent on Sktl. Our data reveal a requirement for Sktl in ChN function and in regulating intraciliary protein transport and ciliogenesis.

### 3.3 Methods

#### 3.3.1 Transgenic flies

All Drosophila stocks were cultured on cornmeal-molasses-yeast medium. Crosses for experiments were performed at 25°C with 50% humidity. \(P\{nan-GAL4.K\}2\) (#24903), \(P\{TRiP.JF02796\}attP2\) against \(sktl\) (#27715), \(P\{VALIUM20-mCherry\}attP2\) against \(mCherry\) (#35785), \(P\{UAS-TeTxLC.tnt\}E2\) (#28837), \(P\{UAS-mCD8-RFP.LG\}18a\) (#27398) and \(L^1/CyO; P\{UAS-PLC\(\delta\)-PH-EGFP\}3/\(TM6B\), Tb (#39693) were obtained from the Bloomington Drosophila Stock Center. Cep290-GFP and Ana1-tdTomato were
provided by T. Avidor-Reiss (Basiri et al., 2014). Mks1-GFP-myc and Cby-GFP were provided by B. Durand (Vieillard et al., 2016). Iav-GFP, RempA-YFP and NompB-GFP were provided by D. Eberl (Gong et al., 2004; Lee et al., 2008; Han et al., 2003). UAS-Sktl-RFP and UAS-Sktl\textsuperscript{KD}-RFP were provided by P. Raghu (Raghu et al., 2009). Stocks for generating \textit{sktl}\textsuperscript{2.3} clones were originally provided by A. Guichet (Gervais et al., 2008). For generating clones, flies were heat-shocked in a water bath at 30°C for 1.5 hours for three consecutive days starting two days post egg-laying.

### 3.3.2 Antibodies

The following primary antibodies were used for immunofluorescence at the indicated concentrations — rabbit anti-Tulp (Ronshaugen et al., 2002), 1:500; rabbit anti-NompC (Cheng et al., 2010), 1:500; chicken anti-GFP (abcam, Cambridge, United Kingdom), 1:1000; rat anti-RFP IgG 5F8 (ChromoTek, Planegg-Martinsreid, Germany), 1:1000; mouse anti-22C10 IgG (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), 1:500; FITC- and Alexa 594-conjugated anti-horseradish peroxidase (Santa Cruz, Dallas, TX, USA), 1:50. Secondary antibodies were Alexa 488- and Alexa 568-conjugated anti-mouse, anti-rabbit and anti-chicken IgG (Thermo Fisher Scientific, Waltham, MA, USA) at 1:1000. DAPI (Molecular Probes, Eugene, OR, USA) was used to label DNA at 1:1000.

### 3.3.3 Fluorescence microscopy

Antennae were dissected 28-30 hours after puparium formation in 4% paraformaldehyde in PBS supplemented with 0.001% Triton X-100 to disrupt surface tension, and fixed for 20-30 minutes at room temperature. For visualization of fluorescence without immunostaining, fixed antennae were mounted in ProLong Diamond mounting medium (Thermo Fisher) and imaged within 20 minutes. For immunostaining, fixed antennae were first permeabilized and blocked for 1 hour in PBS with 0.1% Triton and 3% normal goat serum. They were incubated with primary antibodies in the permeabilization/blocking solution for 48 hours.
at 4°C, followed by three 10-minute washes in PBS with 0.1 % Triton at room temperature, and then incubated with secondary antibodies for 1 hour at room temperature. This was followed by three 10-minute washes and mounting in ProLong Diamond. All incubations were performed with gentle shaking in a humidified chamber.

Confocal images were captured using a Nikon A1R point scanning confocal microscope (SickKids imaging facility). Images were processed using ImageJ 1.50 (Schneider et al., 2012) or MATLAB (MATLAB and Image Processing Toolbox, 016b). MATLAB plugins for reading .nd2 files were made by Bio-formats (Open Microscopy Environment). Adobe Illustrator (Adobe Creative Cloud) was used for arranging figure panels and creating diagrams.

3.3.4 Behavioural experiments

All parental stocks used for crosses for behavioural assays excluding TRiP lines were introgressed into the w^1118 stock for 4-5 generations. Crosses were set up in vials with equal numbers of parents, and turned every three days to maintain culture density at an appropriate level. All males of the correct genotype that had eclosed by the twelfth day post egg-laying were collected in a separate vial under mild CO₂ anaesthesia, and allowed to recover for 2-3 days. All behavioural experiments were performed 4-6 hours after dawn (lights on). For the assay, male flies were tapped into an empty 18-inch plastic vial and connected to another plastic vial. For stressed testing, vials were tapped down to synchronize flies at the bottom, and flies were allowed to climb for 10-15 seconds in the dark. The number of flies in each section of the testing vial was counted under red light at the end of the trial. This stressed assay is similar to published methods for examining ChN function (Pratt et al., 2016; Sanchez et al., 2016; Chen et al., 2015; Sun et al., 2009). For unstressed testing, flies were tapped down and allowed to climb to the top in ambient white light, and the testing vial was then inverted gently in the dark so that flies were synchronized at the bottom. They were then allowed to climb up for 10-15 seconds in the
dark before quantification. We developed this assay to be more sensitive to defects in gravitaxis-responsive ChNs, similar to the vertical Y-maze assay.

### 3.3.5 Hierarchical Bayesian model for behavioural analysis

For the purpose of this analysis, a trial represents a single round of synchronization and measurement, and an experiment represents the group of trials performed at one time (usually 8-12 trials).

For statistical analysis of tube-climbing ability, we consider \( \theta_{ijk} \) to represent the distribution of flies in section \( i \) of the vial from trial \( j \) and experiment \( k \) where \( i \in \{i_{\text{top}}, i_{\text{middle}}, i_{\text{bottom}}\} \). Therefore, \( \sum_{i=1}^{N=3} \theta_{ijk} = 1 \) where \( N = 3 \) represents the three sections of the vial. Since trials are grouped by experiments, we represent our model as a hierarchical Bayesian model, where the lowest-level (vector-valued) parameters \( \theta_{jk} \) represent trial-level parameters, \( \mu_k \) represents experiment-level parameters, and \( \alpha \) represents the top-level parameter grouping experiment-level parameters together.

For trial-level parameters,

\[
p(\mathbf{X} | \theta) \propto \prod_{i=1}^{3} \theta_{i}^{x_i} \text{ such that } \sum_{i=1}^{3} \theta_i = 1, \sum_{k} x_k = N
\]

For the hierarchical Bayesian model,

\[
Data \sim \text{Multinomial}(\theta_{jk})
\]

\[
\phi_{jk} \sim \mathcal{N}(\mu_k, \Omega), \quad \text{where} \quad \theta_{ijk} = \frac{\exp(\phi_{ijk})}{\sum_{i=0}^{3} \exp(\phi_{ijk})}, \quad i = 1, 2, 3
\]

\[
\mu_k \sim \mathcal{N}(\alpha, \Gamma)
\]

The graphical representation is shown below. Rectangles represent replication.

- \( \alpha \) was drawn from a normal distribution with mean 0 and scale 0.5, and covariance
matrices $\Omega$ and $\Gamma$ were generated from Cholesky decompositions of correlation matrices drawn from LKJ distributions with the hyperparameter set to 5 and component-wise standard deviations set to 1. Visual checks of simulations from the posterior distribution indicated that the choice of hyperparameters did not affect the posterior density very much and therefore the data generally dominates the posterior density for our model.

### 3.3.6 Classical tests of statistical significance

Kruskal-Wallis test and Dunn’s post hoc test with Benjamini-Hochberg correction for multiple comparisons were used to test for significance, with a significance level of 0.05.

### 3.3.7 Quantification of pixel linescans

For quantification, image processing toolbox from MATLAB (version R2016b) was used. Line segments along the proximodistal axis from the ciliary base to the tips were manually selected using mouse input from multiple cilia in a single image. These line profile signals were programmatically aligned to maximize cross-correlation using the `finddelay` function, and mean and standard deviations were calculated based on these alignments. For post-processing, values were scaled homogeneously for both channels if pixel values for the normalization channel (mCD8, HRP or Futsch) were sufficiently different in intensity from control. Figures were plotted using R (≤version 3.5.0).
3.3.8 Statistical modelling of pixel profiles

Consider a chain-structured graphical model containing a latent layer of \( N \) locally connected nodes \( \mathbf{y} = \{y_1, y_2, ..., y_N\} \) with the assumption of Markov independence, i.e. \( y_i \perp \perp y_j \setminus U \mid U \) where \( U = \{i - 1, i + 1\} \) represents the Markov blanket for any node \( y_i \) except for the ones at the ends of the chain. These \( N \) nodes are connected to evidence nodes \( \mathbf{x} = \{x_1, x_2, ..., x_N\} \) representing observed pixel values for a one-dimensional pixel profile. This represents a Markov random field prior over the pixel profiles. We assume quadratic potentials representing the contextuality that neighbouring pixels should have similar values.

For the posterior distribution \( p \)

\[
\log p(\mathbf{y}) \propto \sum_{i=1}^{N} (y_i - x_i)^2 + \tau \sum_{i=1}^{N-1} (y_{i+1} - y_i)^2
\]

where \( \tau \) is a hyperparameter that controls the strength of the smoothing prior on pixel values. We chose to compute the posterior predictive distribution in Stan, and \( p(x_{mat} \mid x_{control}) \) as follows: \( p(x_i \mid \mathbf{y}) \sim \mathcal{N}(y_i, \sigma_i) \) and \( p(y_i \mid \mathbf{y}^{-i}) \sim \mathcal{N}(y_{i+1}, \tau) \) where \( p(y_i) \sim \mathcal{N}(0, 10) \).

The graphical representation is shown below.

\[\sigma_i \] was sampled from a diffuse Gaussian prior and \( \tau \) was specified as a hyperparameter based on visual checks of smoothness in samples drawn from the posterior distribution.
3.3.9 Cluster analysis of published microarray data

The raw microarray dataset was obtained from NCBI GEO (accession number GSE21520) (Cachero et al., 2011). Probe intensity values were normalized using Robust Multi-array Averaging (RMA) using the R package `affy`. Linear model fitting and empirical Bayesian shrinkage were performed using the `limma` package. For clustering, we chose genes with high expression in atonal-GFP+ cells (higher than 50\textsuperscript{th} percentile) and high variance across the three time points (higher than 60\textsuperscript{th} percentile). These were the highest cutoffs that retained sktl. Reducing the dataset in this way helped minimize noise for unsupervised learning. Affinity propagation (AP) clustering with default parameters (R package `apclust`) was used to cluster genes. AP was chosen because it does not require specification of the number of clusters required \textit{a priori}. Gene ontology analysis was performed using `topGO` and statistical significance was calculated using Fisher’s exact test. Clusterings had appropriate gene ontology enrichments for exemplar genes of known functions. For visualization, principal components analysis (PCA) was used.

3.3.10 Computational analysis

Code and documentation for algorithms, as well as raw data and plot generation, used in this study can be found at http://www.github.com/alindgupta/ and are available for use under the MIT license. Statistical analysis and Monte Carlo sampling using Stan were performed in R. All plots were generated in R.

3.4 Results

3.4.1 Chordotonal neuron-specific sktl knockdown perturbs climbing in adults

sktl is a major quantitative trait locus associated with variation in adult \textit{Drosophila} gravitaxis, and heterozygous sktl\textsuperscript{Δ20}/+ mutant flies perform worse than wild-type controls.
in vertical Y-maze assays (Desroches et al., 2010). Because gravity is sensed by ChNs of Johnston's organ within the *Drosophila* antennae (Kamikouchi et al., 2009) (Figure 3.1A), we examined whether behavioural defects in sktl\(^{D20}/+\) flies were due in part to a ChN-intrinsic requirement for sktl. To do this, we drove expression of a hairpin RNA directed against sktl from the *Drosophila* Transgenic RNAi Project (Perkins et al., 2009) using the ChN-specific nanchung-GAL4 driver (also known as nan-GAL4 or F-GAL4) (Kim et al., 2003) to knock down sktl function (referred to hereafter as F\(>\)sktl RNAi). F-GAL4 is expressed in the majority of mature ChNs (Sun et al., 2009; Kamikouchi et al., 2009), but not in ChN precursors prior to differentiation (Cachero et al., 2011). This is consistent with the function of Nanchung as an ion channel required for mechanosensation but not ciliogenesis (Kim et al., 2003; Sun et al., 2009). Thus, F\(>\)sktl RNAi allowed us to examine effects of sktl knockdown on cilium function rather than formation.

To determine whether sktl was required cell-autonomously for ChN function, we subjected F\(>\)sktl RNAi flies to a simple tube-climbing assay to assess climbing ability (Pratt et al., 2016; Sanchez et al., 2016; Chen et al., 2015; Sun et al., 2009). We tested these flies alongside positive controls expressing tetanus toxin light chain (TeTxLC) and negative controls expressing a hairpin RNA directed against mCherry, also driven by F-GAL4. By counting the proportion of flies that were able to climb to the top of the testing vial under red light (Figure 3.1B, see Methods), we found that flies expressing F\(>\)sktl RNAi showed a climbing defect that was intermediate between, yet significantly different from, positive and negative controls (Figure 3.1C and D), and that we could rescue this defect with expression of wild-type Sktl, but not significantly with kinase-dead Sktl (Figure 3.1C). These transgenes are not RNAi-resistant, and it is possible that a part of the rescue we observed is due to titration of the RNAi machinery. Importantly, sktl is dispensable for neurotransmission (Hassan et al., 1998). Thus, our results suggest that sktl is required for ChN function cell-autonomously.
Figure 3.1. Chordotonal neuron-specific *sktl* knockdown induces climbing defects.

(A) Schematic diagram of a ChN from the 2nd antennal segment (not to scale). ChNs are activated by stretching of cilia, which is mechanically coupled to movement of the arista and the 3rd antennal segment.

(B) Scheme for climbing assay. Flies were synchronized at the bottom of the testing vial before being allowed to climb up for approximately 10-15 seconds, and the number of flies in each section of the testing vial was recorded. Experiments were performed under dim red light to minimize effects of phototaxis.

(C) Distribution of flies within the testing vial for stressed (upper graphs) and unstressed (lower graphs) conditions (see Methods for a description of the assays). Error bars represent standard deviation. *F>mCherry* RNAi represents the negative control and *F>Tetanus toxin light chain (TeTxLC)* represents the positive control.

(D) Estimated distribution of $\alpha_{\text{top}}$, representing the highest group-level parameter of our Bayesian model, proportional to the fraction of flies that partition to the top section of the testing vial (refer to the Methods section for the statistical model). Bold curves represent the kernel densities of Monte Carlo samples, and dashed vertical lines represent the mean values.
Chapter 3. PIP$_2$ regulates chordotonal cilia

3.4.2 Localization of PIP$_2$ and F-actin in chordotonal neurons

Previous studies using antibodies showed that PIP$_2$ is enriched at the ciliary base but not within the ciliary membrane in ChNs (Park et al., 2015) and cultured mammalian cells (Sanchez et al., 2016). To visualize PIP$_2$ with higher sensitivity, we examined the PIP$_2$ sensor PLCδ-PH in fixed but impermeabilized cilia (see Methods). We co-expressed the cell membrane marker mCD8 to normalize the PLCδ-PH signal for quantification. By averaging multiple linescans across cilia, we show that, as described previously (Park...
et al., 2015), the base of ChN cilia contains relatively high PIP$_2$ levels whereas the ciliary membrane is relatively devoid of PIP$_2$ (Figure 3.2A and B). However, as revealed by mCD8 localization, and as reported previously (Vieira et al., 2006), the ciliary base is enriched in membranes in general. Interestingly, the correlation between PLC$\delta$-PH and mCD8 signals is low in control cells but rises dramatically in $F>sktl$ RNAi (Figure 3.2B, bottommost graph), suggesting that PIP$_2$ exists in discrete enrichment pools that directly or indirectly depend on the presence of Sktl. The PLC$\delta$-PH sensor also revealed differential enrichment at the ciliary dilation (Figure 3.2A and B), a chordotonal neuron-specific structure that is important for compartmentalization of the proximal and distal ciliary segments (Lee et al., 2008). This ciliary dilation-associated PIP$_2$ enrichment is lost in $F>sktl$ RNAi (Figure 3.2B). Our observations suggest that PIP$_2$ might localize in tightly restricted intraciliary pools in certain differentiated cell types, possibly through localized synthesis by a PIPKI. This is in contrast to a known role for intraciliary PIP$_2$ and actin in promoting cilium excision in cultured mammalian cell lines undergoing mitosis (Phua et al., 2017). Similar to PIP$_2$, we found enrichment of F-actin at the base of cilia, which was reduced in $F>sktl$ RNAi (Figure 3.2C and D). No F-actin enrichment was found within cilia.
Figure 3.2. Localization of PIP$_2$ and F-actin is altered in $F>sktl$ RNAi antennae.

(A) Localization of the PIP$_2$ marker PLC$\delta$-PH-GFP (green) and the membrane marker mCD8-RFP (red) in ChNs. Insets show magnified cilia, with the ciliary dilations marked by white arrowheads. Quantification plots show median aligned line intensity values measured over multiple cilia, with standard deviation in pixel values (shaded region).

(B) Quantification of pixel profiles along multiple cilia from (A). Bold lines represent median value and shaded regions represent standard deviation. Regions demarcated by tick marks along the $x$-axis represent approximate positions of the ciliary base, proximal ciliary segment, ciliary dilation and distal ciliary segment. Bottommost panel shows the Pearson correlation between PLC$\delta$-PH-GFP and mCD8-RFP in control and $F>sktl$ RNAi. $n$ represents number of cilia.

(C) Localization and quantification of the F-actin marker LifeAct-Ruby and neuron marker horseradish peroxidase (HRP) in ChNs. Insets show magnified cilia, with the ciliary dilations marked by white arrowheads.

(D) Quantification of pixel profiles from multiple cilia from (C). $n$ represents number of cilia.
Figure 3.2: Localization of PIP$_2$ and F-actin is altered in $F>skt$ RNAi antennae.

3.4.3 $F>skt$ RNAi perturbs NompB/IFT88 localization

In mice and *Drosophila*, mutations in *INPP5E* affect localization of Tubby domain proteins TULP3 and Tulp, which transport proteins into cilia (Badgandi et al., 2017; Mukhopadhyay and Jackson, 2011). Specifically, the build-up of intraciliary PIP$_2$ due to loss of INPP5E increases the levels of these Tubby proteins within cilia, leading to aberrant localization of Smoothened (in mice) and the mechanosensory ion channels NompC and Inactive (Iav) in flies (Park et al., 2015). In *Drosophila*, expression of a Tulp mutant incapable of binding to PIP$_2$ can partially rescue chordotonal neuron function in *inpp5e* mutants (Park et al., 2013). We examined whether $F>skt$ RNAi affected the localization of ciliary proteins known to be important for mechanotransduction. We found subtle
defects in the localization of Tulp in $F>sktl$ RNAi, with slightly higher enrichment in cilia relative to controls (Figure 3.3). Our results are consistent with the finding that the PIP$_2$ binding ability of Tulp is largely dispensable for its proper localization (Park et al., 2013). Similarly, NompC localization was only mildly affected. We observed the strongest localization defect for the *Drosophila* IFT88 ortholog No mechanical potential B (NompB), where $F>sktl$ RNAi caused an increase in distally-extended NompB signal and a higher intraciliary enrichment (Figure 3.3). This was recued by co-expression of wild-type Sktl (3.4). IFT88 is part of the anterograde (IFT-B) machinery, which transports cargo into cilia. RempA/IFT140, a component of the retrograde (IFT-A) system, was not affected severely (3.5), which is in agreement with our observations for Tulp, which requires proper retrograde IFT for trafficking. Because *nompB* mutants cannot make cilia, we expect that the NompB localization defect we observe does not affect NompB activity severely. Alternatively, because *sktl* knockdown occurs after cilia are built, a NompB defect at this stage may not immediately manifest as a ciliary phenotype.
Figure 3.3. Defects in localization of ciliary proteins in $F>sktl$ RNAi.

(A) Localization and quantification of NompC/TRPN, Tulp and NompB/IFT88 (green) versus mCD8-RFP or neuronal marker Futsch (red) in control and $F>sktl$ RNAi in pupal ChNs.

(B) Quantification of pixel profiles from (A). $n$ represents number of cilia.

(C) Heatmap showing the log-likelihood of a pixel value from one dimensional linescans from (A) representing the probability of the pixel value from $F>sktl$ RNAi being derived from a model trained on control images (see Methods for the statistical model).
Figure 3.3: Defects in localization of ciliary proteins in $F > sktl$ RNAi.
3.4.4 $F \text{> sktl} \text{ RNAi}$ does not alter TZ formation

The TZ maintains cilium composition by regulating bidirectional trafficking of proteins at the base of cilia (Breslow et al., 2013). Drosophila mutants of the conserved TZ protein Chibby (Cby), for example, show increased intraciliary NompB localization (Enjolras et al., 2012). In the Drosophila male germline, we find that reduction of PIP$_2$ causes TZ hyperelongation that is concomitant with defects in TZ function (Gupta et al., 2018). Therefore, we wanted to test whether TZs were affected in $F \text{> sktl} \text{ RNAi}$. To do this,
Figure 3.5: Localization of RempA in ChNs.

(A) Fluorescence micrographs of control and $F>sktl$ RNAi pupal ChNs expressing RempA-YFP.

(B) Quantification of fluorescence from (A). Regions demarcated by tick marks along the $x$-axis represent approximate positions of the ciliary base, proximal ciliary segment, ciliary dilation and distal ciliary dilation. $n$ represents number of cilia.
we examined the localization of the core centriolar/basal body protein Ana1 and the conserved TZ proteins Cep290 (Basiri et al., 2014), Chibby (Cby) (Enjolras et al., 2012) and Mks1 (Pratt et al., 2016; Vieillard et al., 2016). We did not observe a significant difference in the distribution of Ana1, Cby or Mks1 between controls and $F>\text{sktl}$ RNAi (Figure 3.6A and B). Although we found a significant increase in Cep290 length in $F>\text{sktl}$ RNAi, the effect size was rather small (Cohen’s $d \approx 0.55$). We conclude that $F>\text{sktl}$ RNAi does not generally affect ChN TZs.

### 3.4.5 $\text{sktl}$ is essential for cilium formation in chordotonal neurons

Next, we examined whether $\text{sktl}$ was important for cilium formation. The human PIPKI$\gamma$ localizes to centrioles, where it promotes axoneme assembly by converting centriolar PI4P to PIP$_2$ (Xu et al., 2016). PI4P normally binds the $\tau$-tubulin kinase and prevents CP110 uncapping by inhibiting the interaction of TTBK2 and CEP164 (Xu et al., 2016). According to these results, PIPKI$\gamma$ licenses ciliogenesis through PI4P removal rather than PIP$_2$ generation. We suggest that this particular model does not apply to Drosophila, where cells do not undergo multiple rounds of ciliogenesis. Furthermore, unlike vertebrates, ciliogenesis in Drosophila is unaffected in both $\text{cp110}$ mutants and flies overexpressing $\text{cp110}$ five- to tenfold (Franz et al., 2013).

To generate clones of $\text{sktl}$ mutant ChNs, we used heat shock to induce FLP/FRT-mediated mitotic recombination with $\text{sktl}^{2,3}$, a strongly hypomorphic allele. By staining ChNs using an antibody against horseradish peroxidase (HRP), we found that $\text{sktl}$ mutant cells from large clones contained truncated cilia (Figure 3.7A and B). The size of the mutant cells was similar to controls and the mutant cells showed reduced HRP intensity at the ciliary dilation. Smaller $\text{sktl}$ clones, however, did not exhibit a defect in cilium length, suggesting that $\text{sktl}$ is required during an early step for normal ciliogenesis (Figure 3.8). This suggests that $\text{sktl}$ is needed during differentiation of chordotonal precursors for cilium formation and that the Sktl paralog PIP5K59B is unable to support ciliogenesis in
Figure 3.6: Transition zones are not drastically affected in $F>sktl$ RNAi antennae.

(A) Confocal sections of pupal antennae expressing GFP-tagged TZ proteins Cep290, Cby and Mks1 (green or grayscale). The centriole/BB marker Ana1-tdTomato (red) (Basiri et al., 2014) is also shown for reference. Insets show magnified TZ protein distributions.

(B) Quantification of TZ protein and Ana1 lengths in control and $F>sktl$ RNAi antennae. Error bars represent standard deviation. $n$, representing the number of cilia, was $>30$.

To elucidate possible functions of Sktl during ciliogenesis in ChNs, we mined microarray datasets generated by Cachero et al. (Cachero et al., 2011) from developing atonal-positive embryonic chordotonal precursors, subject to the hypothesis that genes showing similar differential expression profiles over time are more likely to be functionally related. Using
affinity propagation to cluster gene profiles in a reduced dataset (see Methods), we found that sktl clustered with genes that were significantly enriched for GO terms associated with actin regulation (Figure 3.7C, D and Table 3.1), which is intimately tied to PIP$_2$. Interestingly, inpp5e clustered with sktl and had a very similar temporal profile. Defects in cilium length in sktl mutant cells may be due to aberrant actin organization, which has been found to be important for ciliogenesis in *Danio rerio* (Ravanelli and Klingensmith, 2011; Xu et al., 2017) and in IFT recruitment and ciliary entry in *Chlamydomonas reinhardtii* (Avasthi et al., 2014).

Figure 3.7. *sktl* is important for cilium assembly in developing ChNs.

(A) Two different confocal sections from the 2$^{nd}$ segment of a pupal antenna showing a large *sktl*$_{2.3}$ clone marked by the absence of nuclear GFP (GFPnls) (demarcated by dashed white line). ChNs are labelled with an antibody against HRP (red) and nuclei are labelled with DAPI (blue).

(B) Magnified images of ChNs from (A) showing that *sktl*$_{2.3}$ clones contain truncated cilia and fainter HRP intensity at the ciliary dilation.

(C) Principal components analysis (PCA) plots of differential expression profiles of genes from embryonic ChN precursors (dataset from (Cachero et al., 2011)). Clusters generated by affinity propagation are grouped by colour showing that they cluster in the PCA space, with the cluster containing *sktl* shown in bright red. Loading vectors (grey arrows) for the three genes with highest variance in differential expression across time points are shown for comparison.

(D) The top ten gene ontology (GO) terms associated with the *sktl* cluster from (C) represented as bar graphs in decreasing order of significance according to a hypergeometric test.
Figure 3.7: *sktl* is important for cilium assembly in developing ChNs.

### 3.5 Discussion

Sensory reception is vital for life. In humans and *Drosophila*, several stimulus modalities require the function of cilia, organelles that function as cellular sensory antennae. Here, we
Figure 3.8: Small $sktl^{2.3}$ clones can assemble cilia of normal length. Confocal sections from a pupal antenna showing a small $sktl^{2.3}$ clone lacking nuclear GFP (green) (demarcated by dashed white line). ChNs are labelled with an antibody against HRP and nuclei are labelled with DAPI (blue). Inset shows the GFP signal from the $sktl$ clone and neighbouring cells. Right: Inverted colour image of ChNs shows no difference in cillum lengths between mutant cells (marked by green arrowheads) and control cells.

show that the PIPKI Sktl is important for cilium assembly and function in *Drosophila* ChNs. ChN-specific $sktl$ RNAi perturbs the ability of flies to climb, indicating a defect in ChN function. At the cellular level, Sktl is important for localization of the IFT-B protein NompB/IFT88 in cilia, as well as proper distribution of PIP$_2$ and F-actin in ChNs.

Antennal ChNs are the primary gravity-sensing organs in *Drosophila* (Kamikouchi et al., 2009). A previous report using vertical Y-mazes identified $sktl$ as a major quantitative trait locus underlying variability in adult gravitaxis (Desroches et al., 2010). Consistent with reports of $sktl$ expression in the peripheral nervous system (Hassan et al., 1998), bioinformatics analysis showed that $sktl$ is relatively highly expressed in embryonic ChN
Chapter 3. PIP$_2$ regulates chordotonal cilia

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Table 3.1: *Drosophila* genes that cluster with sktl during embryonic ChN precursor development.

RNAi-mediated *sktl* knockdown in ChNs using *nan*-GAL4 induced tube climbing defects in adult *Drosophila*, suggesting that *sktl* is required cell autonomously in ChNs for proper function. Sktl, and PIPKIs in general, localize largely to the plasma membrane where they regulate a number of cellular functions such as actin regulation and vesicle transport (Balla, 2013; Fabian et al., 2010). Therefore, the effect on ChN function in *F>sktl* RNAi could be due to an effect on various cellular pathways, excluding neurotransmission (Hassan et al., 1998).

We found that *sktl* RNAi caused an increase in colocalization between mCD8 and PLC$\delta$-PH in cilia, which label total membrane and PIP$_2$, respectively. PIP5K59B, the *Drosophila* paralog of Sktl, can presumably maintain most cellular PIP$_2$ pools in the absence of *sktl*, as vertebrate PIPKIs possess tremendous functional redundancy and plasticity (Balla, 2013). In addition to PLC$\delta$-PH, F-actin was reduced at the base of the cilium in *F>sktl* RNAi, and localization of ciliary proteins NompC and Tulp was slightly altered as well. We saw a dramatic increase in the enrichment of NompB at the tips of cilia, reminiscent of an IFT-A defect (Mukhopadhyay et al., 2010). Although we did not see an obvious defect in IFT-A protein RempA, it is possible that other IFT-A proteins...
are affected. Since PIP$_2$ was enriched at both the ciliary base and the ciliary dilation, $F>\text{sktl}$ RNAi might cause defects in either or both of those locations. For example, there might be a defect at the base of the cilium in IFT packaging in the absence of sktl that causes defective IFT88 turnaround at the ciliary tips.

We previously showed that reduction of PIP$_2$ in the Drosophila male germline induces TZ hyperelongation (Gupta et al., 2018). In the male germline, PIP$_2$ reduction during ciliogenesis caused elongated distribution of all TZ proteins and markers examined, including Cep290, Cby, Mks1, Uncoordinated (Unc) and acetylated tubulin. Here, we found that $F>\text{sktl}$ RNAi does not affect TZ lengths of Cby or Mks1 in ChNs. Although we observed a significant increase in Cep290 length, we are unsure whether this is physiologically relevant, since no precedent exists for TZ length regulation during ciliogenesis in ChNs, unlike the Drosophila male germline (Pratt et al., 2016; Vieillard et al., 2016). This difference between the male germline and ChNs could be due to various reasons, such as the difference in timing of PIP$_2$ reduction (during or after ciliogenesis) or distinct modes of ciliogenesis (cytoplasmic or compartmentalized, see (Avidor-Reiss and Leroux, 2015)).

Because sktl is an essential gene and is required for development of the peripheral nervous system, we generated clones of the hypomorphic sktl$^{2.3}$ allele and examined their effect on pupal ChNs. We found that sktl mutants from a large clone contained truncated cilia but appeared normal otherwise morphologically, whereas smaller clones did not contain any obvious defects. We expect this is due to slow titration of the sktl mRNA during development of ChN precursors that reveals a requirement for ciliogenesis only when removed early. Our bioinformatics analysis suggests that sktl might be involved in regulation of actin-associated processes, and that it might link actin assembly to proper IFT during ChN formation.
Chapter 4

Discussion and Future directions

4.1 Summary of results

The following is a summary of the results described in Chapters 2 and 3 of this thesis:

- PIP$_2$ is essential for TZ maturation — Reduction of PIP$_2$ levels by ectopic expression of SigD causes hyperelongation of developing TZs in the Drosophila male germline, which can be rescued by expression of Sktl. Hyperelongated TZs are unable to detach from BBs during axoneme elongation, and show other functional defects consistent with presence of microtubule triplets within axonemes that were previously described by our lab. $\beta_2$t-SigD and onr mutations both cause loss of cilium-PM attachment.

- sktl is essential for ChN function — ChN-specific sktl RNAi after ciliogenesis using F-GAL4 perturbs climbing ability in adults. Although cilium length is unaffected in these cells, NompB/IFT88 accumulates at ciliary tips. ChNs from sktl clones induced early during ciliogenesis look morphologically intact, but have shorter distal ciliary segments, indicating a ciliogenesis defect. In ChNs, PIP$_2$ is enriched at the ciliary base, corroborating previous studies, as well as at the ciliary dilation.

4.2 Future directions - regulation of ChN cilia by sktl

I begin with a discussion of possible experiments that can be used to gain mechanistic insight into how Sktl and PIP$_2$ regulate ChN cilia, and address open questions from Chapter 3.
4.2.1 Characterization of ciliary defects in ChN sktl clones

I found that ChNs mutant for sktl, made by FLP/FRT-based mitotic recombination, contained shorter distal ciliary segments. However, we were unable to investigate the ultrastructure of these truncated cilia, or the cellular mechanisms underlying this defect due to the rarity of clones. Studies of mutant ChNs can be facilitated by generating positively-marked (for example, with RFP) clones rather than negative using MARCM (mosaic analysis with a repressible cell marker) (Wu and Luo, 2006) and use of a stringent heat shocking regimen to maximize chances of recovering large clones more frequently. A heat-shock regimen using carefully synchronized embryos to retrieve macrochaete MARCM clones has been described (Murphy et al., 2015) and can serve as a starting point for optimizing recovery of ChN clones. It may be possible to screen for the presence of RFP-positive ChNs in intact early stage pupae and adults for translucent tissues such as wings. Furthermore, our lab has recently been able to recover homozygous viable sktl\textsuperscript{2.3} adults which can be used instead of mosaic flies to simplify cell biological analysis with the caveat of using a fully mutant fly, rather than a mosaic with only a few mutant cells.

To analyze the ultrastructure of cilia in mutant ChNs, correlative light and electron microscopy (CLEM) can be used. CLEM can be used to examine rare cellular events marked with fluorescence using the high ultrastructural resolution provided by electron microscopy. This technique can reveal ultrastructural defects in the ciliary dilation or axoneme architecture of positively-marked ChNs, if any are present. We can also examine whether markers of the BB (Ana1), TZ (Cep290), axoneme (Arl13b) and ciliary dilation (RempA) localize properly in mutant ChNs using immunofluorescence. Most of these markers are fluorescently-tagged proteins and therefore, unique stocks will need to be generated for each of these. If none of these are affected drastically, it might be useful to examine CLEM data for identifying other targets for study, such as the ciliary rootlet or membranes at the ciliary base if they are different between normal cells and mutants. It is important to assess whether ChN-specific expression of wild-type and kinase-dead
Sktl can rescue the defects in mutant ChNs. This will reveal whether mutant support cells contribute to a cilium phenotype, since a ChN and the associated three support cells are derived from the same precursor through asymmetric cell divisions (Jarman, 2014). These sets of experiments can elucidate cellular defects that are affected by loss of sktl and PIP₂.

4.2.2 Examination of IFT using time-lapse imaging in $F^{sktl}$ RNAi

I found the IFT-B protein NompB to be enriched at the tips of cilia in $F^{sktl}$ RNAi. This could be due to either

- excess anterograde transport,
- reduced retrograde transport,
- defective IFT turnaround at ciliary tips

or any combination of these.

To understand whether $F^{sktl}$ RNAi alters the movement of IFT particles, pseudopuncta of fluorescently labelled IFT proteins such as NompB and RempA can be tracked over time in order to measure their velocities. This has recently been achieved in antennal ChNs (Lee et al., 2018). This same report found that NompB travels at a slower rate during retrograde transport in the distal segment than in the proximal segment using time-lapse imaging. Antennal ChNs co-expressing NompB-GFP and a membrane marker such as mCD8-RFP can be visualized using confocal microscopy, and fluorescence kymographs of NompB can be examined in order to visualize trajectories over time in both control and $F^{sktl}$ RNAi pupae, and possibly even mutant ChNs. Quantification of NompB velocities along the cilium in anterograde and retrograde directions in these two conditions should enable us to distinguish among the possibilities listed above. Similar approaches for visualizing IFT particles have been used in *C. elegans, Chlamydomonas*
and cultured mammalian cell lines (Dentler et al., 2009; Ott and Lippincott-Schwartz, 2012). Software for automated construction of kymographs from image sequences is available as ImageJ macros and stand-alone tools, facilitating post-acquisition analysis (Mangeol et al., 2016).

4.2.3 Elucidating where PIP$_2$ acts within ChN cilia

Within ChNs, PIP$_2$ was enriched at the ciliary base and the ciliary dilation, and so it is possible that defects in NompB localization in $F>sktl$ RNAi, and in ciliogenesis in $sktl$ mutants, were caused by disruption of a Sktl-dependent process at either or both of these locations, or within certain pools of PM-associated PIP$_2$. To determine the site of action of Sktl that underlies these phenotypes, we can target a PIP$_2$ phosphatase by tagging it to proteins that localize selectively to one of these three sites. The 5-phosphatase domain of INPP5E has been widely utilized for this purpose (Hammond et al., 2012; Varnai et al., 2006). As long as it is tethered to an integral membrane protein, the INPP5E 5-phosphatase domain can potently desphosphorylate PIP$_2$. The problem with such an approach is finding transmembrane proteins that localize specifically to the PM, ciliary base or ciliary dilation. For example, mCD8 is a single pass membrane protein that normally localizes to the PM, but we found it to also localize to the ciliary membrane and intracellular membranes. There are no known integral membrane proteins in ChNs that localize to the ciliary dilation or ciliary base, but if these are discovered, it may be possible to perform such an assay. Other similar approaches using optogenetics to target a soluble 5-phosphatase specifically to the ciliary dilation and ciliary base may be useful for ChNs in translucent tissue, depending on the phenotypes examined and the control over timing for sustained PIP$_2$ removal through optical stimulation (Idevall-Hagren et al., 2012).
4.2.4 Identify proteins that act downstream of PIP$_2$

Because PIP$_2$ acts in the cell through effector proteins, it is important to identify what proteins might be acting downstream of PIP$_2$ to regulate ciliogenesis and cillum function. Although finding the PIP that a protein of interest binds to can be accomplished with liposome-binding assays or “PIP strips”, it is very challenging to find proteins that interact with a PIP of interest and underlie a particular phenotype. This is because there are no high-throughput assays with enough sensitivity to elucidate proteins that interact with a PIP of interest in vivo due to the low affinity and specificity of PIP-protein interactions. An unbiased method for finding proteins involved in ciliogenesis or cillum function that might underlie the defects seen in $F>sktl$ RNAi or sktl clones can be a genetic modifier screen in a sensitized background such as $F>sktl$ RNAi using an easily measurable phenotype like tube-climbing ability. Because adult flies expressing $F>sktl$ RNAi show an intermediate climbing defect compared to controls, it may be possible to cross them into a large panel of genetic backgrounds containing heterozygous mutations in known genes. If a heterozygous allele enhances the $F>sktl$ RNAi defect, but does not cause climbing defects by itself, we could assume that it interacts genetically or functionally with sktl in ChN function. Such an assay would require a large amount of time, but it is considerably simpler than examining NompB localization by immunostaining for each mutant. Genetic modifiers identified in this way can then be analyzed using bioinformatics to assess whether they might be able to interact with membranes and anionic lipids, and experimentally tested for binding to PIP$_2$ (Bhardwaj et al., 2006).

4.3 Regulation of male germline cilia by Sktl and PIP$_2$

Here, I talk about possible ways of investigating how PIP$_2$ regulates TZ maturation and attachment of the PM to the cillum in the *Drosophila* male germline.
4.3.1 Identify spatial pools of PIP$_2$ important for TZ length

With regard to ciliogenesis, there are three important pools of PIP$_2$ in the male germline — PM, ciliary pocket and TZ. Each of these pools can be tested to address whether their depletion can phenocopy ciliary defects found in $\beta_2$-t-SigD. For example, we can drive expression of INPP5E phosphatase conjugated directly to a PM marker to induce efficient PM PIP$_2$ dephosphorylation (Varnai et al., 2006). To limit the expression of these transgenes to the male germline, we can use GAL4 drivers or promoters of germline-specific genes like nanos or bag of marbles.

The inducible rapamycin assay can be used to control PIP$_2$ turnover spatiotemporally within the cell (Varnai et al., 2006). This bipartite system requires a PIP$_2$ recruiter, which is an integral membrane protein with a very specific localization within the cell. The phosphatase domain of the human INPP5E, which can efficiently dephosphorylate PIP$_2$ when recruited to the membrane but not when expressed in its soluble state in the cytoplasm, is targeted to the membrane-bound recruiter upon addition of a rapamycin analog. However, it is unclear what these recruiters might be in the *Drosophila* germline since no such proteins are known. Specifically, no integral membrane proteins that localize specifically to the ciliary pocket are known in *Drosophila*. The small transmembrane TZ protein TMEM216 localizes to BBs in addition to TZs in the male germline (personal communication with B. Durand, University of Lyon), and is therefore not specific to the TZ. This might not be a problem in spermatocytes. Even the commonly used PM recruiter Lyn11-FRB localizes to a variety of subcellular structures in addition to the PM when expressed at low levels in the male germline using $\beta_2$-tubulin promoter (not shown). Therefore, we would have to identify proteins that localized specifically to membranes in these areas, and ascertain experimentally that they can be used to deplete PIP$_2$ to a measurable extent, by using the PLC$\delta$-PH marker for instance, for this experiment to be meaningful. Furthermore, we would need to identify what phenotypes to measure, and ensure we have different fluorescent proteins to tag the recruiter, the phosphatase and
the protein marker of interest. For example, we could examine whether depleting TZ-associated PIP$_2$ by using TMEM216 as a recruiter affects TZ elongation and PM-cilium attachments.

4.3.2 Characterization of TZ-distal Ana1

As described in Chapter 2, we found the peculiar phenotype of TZ-distal Ana1 puncta in a subset of $\beta_2$-t-SigD cells. To understand what this phenotype represents, we can use CLEM to characterize the ultrastructure of cilia displaying TZ-distal Ana1. This will elucidate what the TZ-distal site represents, for instance, if this TZ-distal Ana1 is associated with microtubules or is a part of a more amorphous protein complex. Cross-sections of cilia with or without TZ-distal Ana1 can be used to test the hypothesis that TZ-distal Ana1 is found in cilia with triplet microtubules within the cilium. We can also examine whether core centriolar proteins like Bld10 (Mottier-Pavie and Megraw, 2009) localize to TZ-distal sites in $\beta_2$-t-SigD, which would corroborate our hypothesis that this phenotype is due to specification of this site as core centriole-like. This characterization can provide insight upon which mechanistic studies can be based. For example, our observation that Taxol treatment can increase the penetrance of TZ-distal Ana1 in $\beta_2$-t-SigD suggests that alleles of microtubule associated proteins might be good candidates for screening for an increase in the penetrance of TZ-distal Ana1 in $\beta_2$-t-SigD.

4.3.3 Evolutionary conservation of PIP$_2$

One long-term aim of this research is to investigate whether PIPKIs are required for ciliogenesis and function in humans, as has previously been hypothesized (Xu et al., 2016), which could then be used for diagnosis and personalized prognosis in the future. Human PIPKI$\gamma$ is required for ciliogenesis in cultured cells. Using an animal model, such as mice, it would be possible to examine whether ciliary defects are observed in $PIPKI$ mutants and if they mimic phenotypes identified in this study. None of the three $PIPKI$ genes is
essential for embryogenesis in mice, so it is possible to examine cilia from individual null or double mutants, using electron microscopy or fluorescence microscopy of TZ and IFT proteins.

4.4 Functional interactors of sktl

Although ciliopathies are generally monogenic disorders, genetic pleiotropy can make it difficult to predict phenotype given a single allele, particularly in the case of CEP290. For better diagnosis and prognosis of disease, it may be valuable to include additional data, such as gene expression, basal exon skipping data (Drivas et al., 2015) and cell biology data from visualizing cilia (using epithelial fibroblasts) either using fluorescence staining or electron microscopy. Drosophila can be a good model of dissecting genetic pleiotropy, given that mutations of known ciliogenesis genes have different tissue-specific effects, and the genetic background can be easily controlled. By scoring phenotypes such as TZ hyperelongation or behavioural defects, we can define genetic interactions that enhance or suppress sktl-associated defects. As a starting point, microtubule regulators like Klp59D (Vieillard et al., 2016), and TZ proteins such as Chibby and Dilatory, all of which cause an increase in TZ length, could be examined for their ability to enhance β2-t-SigD ciliary defects.

4.5 Relevance of this research

The current model of a phosphoinositide code of cilia based on recent research in Drosophila and mammals suggests that cilia are organelles devoid of PIP₂, and that high levels of intraciliary PIP₂ perturb intraciliary trafficking. Our observations in Drosophila show that PIP₂ plays a supportive role in a tissue-dependent manner during cilium assembly and function, and that PIP₂ can localize within cilia. We hope that the research described in this thesis will refine the current understanding of the functional relationship between cilia
and PIP₂, and spur mechanistic investigations underlying this relationship in \textit{Drosophila}
and mammals.
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