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Investigating the role of R2TP-like co-chaperone complexes during axonemal dynein assembly

Jennifer Lennon



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The University of Edinburgh

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Declaration

I declare:

1. This thesis has been composed by me, and
2. Either that the work is my own, or where I have been a member of a research team, that I have made a substantial contribution to the work, with such contribution being clearly indicated, and
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Jennifer Lennon

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Abstract

Motile cilia are specialised cell-types which in humans have important roles in the linings of the airways, the reproductive system and the brain. The movement, required for this type of cilia to function, is facilitated by structures called axonemal dynein motor complexes. These are large, multi-subunit structures, and so it is crucial that they are assembled correctly. In humans, if the motility of these is defective, it can lead to a disorder called Primary Ciliary Dyskinesia, or PCD. This is a heterogeneous, autosomal recessive disorder – symptoms of which include abnormally positioned organs, chronic respiratory infections and infertility. Therefore, the development and structure of the motile cilia is tightly regulated by multiple proteins including chaperones, dynein axonemal assembly factors (DNAAFs), microtubule inner proteins (MIPs), the outer arm docking complex (ODA-DC) and the nexin-dynein regulatory complex (N-DRC). Chaperones work with co-chaperones to regulate their many functions within the cell. One of these co-chaperones is the R2TP complex, which was originally discovered in yeast but is conserved in higher organisms. This multi-protein co-chaperone is involved in the assembly of multi-subunit complexes such as the axonemal dynein motors. Two of the R2TP subunits, Pontin and Reptin, are involved in many cellular functions both in this co-chaperone complex and independently. It is thought that as some DNAAFs share similar protein domains to the components of the R2TP complex, they may form R2TP-like complexes. However, the specific details surrounding the roles of these complexes during the assembly process remains unclear. The structure of motile cilia is highly conserved throughout evolution and *Drosophila melanogaster* has been shown previously to be an excellent model for furthering understanding into the development and function of these structures as only two cell types in the fly contain axonemal dynein motor complexes. These are the chordotonal neuron, which has a motile ciliated dendrite essential for its mechanosensory function, and the sperm flagellum. In this thesis, I use the *Drosophila* model to further characterise

putative ciliary genes (*Wdr16* and *Dpcd*) identified by a transcriptome analysis previously carried out in the lab. RNAi knockdown experiments as well as expression analysis supported motile cilia functions. The diversity which has been identified regarding the roles of these two putative ciliary genes highlights how proteins can be involved in motile cilia in different ways. I also use this genetically tractable model to further understand the roles of the individual proteins of a previously identified R2TP-like complex (R2DP₃). Electron microscopy, proteomics and investigation into how the localisation of dynein subsets was affected in null mutants (generated using CRISPR/Cas9) allowed for the role of this R2TP-like complex in the dynein assembly process to be further specified. Using co-immunoprecipitation and affinity purification, we identified an additional protein complex featuring Pontin and Reptin of the R2TP complex, alongside the DNAAF Heatr2 and the putative DNAAF Dpcd. As well as a role in dynein assembly, both DNAAFs are additionally expressed in the neuroblasts of the CNS, and disruption to their function results in a late larval lethality. Therefore, we have found these genes to not be specific to the dynein assembly process and hypothesise that *Dpcd* may have an additional function (working with *Pontin*, *Reptin* and potentially *Heatr2*) in the regulation of AKT signalling and therefore impact cell proliferation.

Lay Summary

Motile cilia are finger-like projections on the surface of cells which beat back and forth in a coordinated manner. They are important for many processes in the body including fertility and respiration. If their movement is affected, it can result in a disease called Primary Ciliary Dyskinesia, or PCD. Large, complex molecular motors called dynein arms drive and regulate this motility. These are complicated structures for the cell to make, and how they are assembled is tightly controlled by many other proteins. A group of these proteins, called dynein axonemal assembly factors (DNAAFs) regulate the development of these motor structures inside the cell. There are still a lot of key players in this cellular process which remain unknown. Moreover, it is thought that some of these DNAAFs work together in different multi-subunit groups during the assembly process, and the specific functions of these individual groups remains unclear.

In this project, the fruit fly *Drosophila melanogaster* is used to further understand this process, as the motile cilia genes of the fly share similarity to those of the human. Moreover, the fly only has the motile cilia machinery in two cell types: the auditory nerve cells (required for hearing) and the sperm cells. Therefore, flies lacking motile cilia genes are typically able to survive but are deaf and uncoordinated in movement, and the males are infertile. I use this model system to learn more about genes with a potential job in regulating the function of motile cilia (*Wdr16* and *Dpcd*). This was achieved by several approaches. First, looking at where in the fly these genes are active relative to the cells containing the motile cilia machinery. Next, flies in which these genes were defective were assessed to identify whether they displayed typical characteristics of those with immotile cilia.

I also use this fly model to look at the DNAAFs which work together in groups, focusing on one group (which I refer to as R2DP₃). Using high resolution microscopes, as well as examining how the subunits of the dynein arms are affected when R2DP₃ components are missing, allowed for further insight into the specific function of this group. Through examination of motile cilia-related protein interactions, a novel group of DNAAFs working together was identified featuring the DNAAF *Heatr2* and the potential DNAAF *Dpcd*. By disrupting the function of *Dpcd* and *Heatr2*, I discovered that these genes have another role in addition to dynein assembly. Analysis of the mutant flies suggests that these genes are most likely involved in controlling tissue growth by regulating an important cellular pathway, mTOR.

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Abbreviations

ANOVA	Analysis of variance
APF	After puparium formation
AP-MS	Affinity purification mass spectrometry
ato	Atonal
BSA	Bovine serum albumin
CNS	Central nervous system
dB	Decibel
DII	Distal-less
DNAAF	Dynein axonemal assembly factor
DNAH	Dynein axonemal heavy chain
EDTA	Ethylenediaminetetraacetic acid
fd3F	Forkhead domain 3F
GMC	Ganglion mother cells
GPCR	G-protein coupled receptors
gRNA	Guide RNA
HEK	Human embryonic kidney
Hh	Hedgehog
Hz	Hertz
HRP	Horseradish peroxidase
HSP	Heat shock protein
IAV	Inactive
IDA	Inner dynein arm
INP	Intermediate neural progenitor
IFT	Intraflagellar Transport
IPA	Ingenuity pathway analysis
IPC	Inner proliferating centre
JO	Johnston's organ

LFQ	Label-free quantification
MIP	Microtubule inner protein
mTOR	Mammalian target of rapamycin
ODA	Outer dynein arm
OPC	Outer proliferating centre
ORF	Open reading frame
N-DRC	Nexin dynein regulatory complex
NMD	Nonsense-mediated decay
NPC	Nuclear pore complex
PAM	Protospacer adjacent motif
PCD	Primary ciliary dyskinesia
PNK	Polynucleotide kinase
PNS	Peripheral nervous system
PTC	Premature termination codon
Rfx	Regulatory factor X
RS	Radial spokes
SOP	Sense organ precursor
TEM	Transmission electron microscopy
TF	Transition fibres
TRP	Transient receptor potential
TZ	Transition zone
UAS	Upstream activator sequence
VNC	Ventral nerve cord
WNT	Wingless
WT	Wild type

Chapter 1

General Introduction

Cilia extend from the cell body and are required for many vital physiological functions. Therefore, it is essential that they develop correctly, a process referred to as ciliogenesis (Ishikawa & Marshall, 2011). The importance of this process is highlighted by the number of diseases (termed ciliopathies) which are caused by mutations to genes with roles during it (Hildebrandt et al., 2011). There are two main types of cilia. Primary or non-motile cilia, which mainly function as sensory organelles, and are an essential signalling hub only relatively recently identified (Huangfu & Anderson, 2005). Motile cilia are required for the movement of fluids and found along epithelial surfaces, the reproductive system and the brain (Mitchison & Valente, 2017). This project will focus on motile cilia, and when these are non-functional it can cause the motile ciliopathy Primary Ciliary Dyskinesia (PCD), a condition which is difficult to diagnose due to its heterogeneity. Only ~70% of PCD-causing genes have been identified to date (Takeuchi et al., 2016)

Motile cilia contain axonemal dynein motors, of which there are two types: outer dynein arms (ODAs) and inner dynein arms (IDAs). These are multi-subunit protein complexes that drive the motility of these cilia (Kamiya, 2002; King, 2016; Nicastro et al., 2005; Sakato & King, 2004). The ODAs power the motion, while the IDAs are required to regulate the movement. These complexes are assembled in the cytoplasm, and then transported and docked along the axoneme of the cilia. As the dynein arms are made up of multiple protein subunits, their assembly is tightly regulated by a group of genes referred to as dynein axonemal assembly factors (DNAAFs) (Fowkes & Mitchell, 1998; King, 2016; Omran et al., 2008). This group is essential for motile cilia function, and numerous studies in model organisms, as well the identification of novel PCD-causing mutations in humans have contributed to the growing number of genes classified as DNAAFs. The specific functions of

many of these individual genes remain unknown, and as they are linked to PCD, it is essential to understand further how they facilitate dynein motor assembly (Aprea et al., 2021; Braschi et al., 2022).

Motile cilia genes and the dynein motor assembly process are highly conserved throughout evolution, and this project utilises the *Drosophila* model to gain understanding into putative as well as previously identified DNAAFs. *Drosophila* is chosen as the model of interest in this study as it is extremely genetically tractable, and only the sperm flagellum and chordotonal neurons contain axonemal dynein motors. The chordotonal neurons have axonemal dynein motor complexes along their 'motile zones' to carry out mechanosensitive functions in the mediation of proprioception and auditory reception. Therefore, *Drosophila* can be used as a straightforward system to understand the genes involved in this complex assembly process (Moore et al., 2013; zur Lage et al., 2018, 2019).

1.1 External structure of a cilium

As mentioned above, there are two main types of cilia: immotile cilia which have a sensory function, and motile cilia which beat back and forth to generate movement (Ishikawa & Marshall, 2011). Both cilia forms are composed of an axoneme containing nine microtubule doublets that extends from the basal body, a modified centriole (Fig. 1.1).

The cilium has a general structure shared by both motile and immotile cilia, shown in Figure 1.1. All cilia have the basal body at the proximal region, which develops from a mother centriole and is composed of a ring of nine microtubule triplets. The ciliary axoneme extends from this, and so the basal body is essential for cilia development as it is the foundation (Hodges et al., 2010; Sorokin, 1968). The axoneme is composed of tubulin protein, which is now in the form of microtubule doublets, and surrounded by a ciliary membrane. Primary or immotile cilia typically have a 9+0 axonemal arrangement, while motile cilia typically have a 9+2 axonemal arrangement due to the presence of

an additional microtubule pair in the centre. This pair is thought to be required to regulate the microtubule sliding that leads to ciliary movement, and therefore not needed in primary cilia (Teves et al., 2016). Another important ciliary subcompartment is the transition zone, which is located next to the basal body and functions as part of the ciliary gate, allowing required proteins to pass in and transport along the cilia via intraflagellar transport (IFT). The ciliary necklace and Y-fibres are in this zone, which are required to connect the microtubules to the ciliary membrane (Szymanska & Johnson, 2012).

It is crucial that this ciliary structure develops precisely during ciliogenesis. This process occurs in several stages: basal body development, basal body docking, development of the axoneme and ciliary membrane, as well as intraflagellar transport which is required for the development and maintenance of the axoneme. The BBsome and ciliary gate are also crucial to ciliogenesis to allow important proteins to enter the cilium (Ishikawa & Marshall, 2011). The stages of ciliogenesis will be described in greater detail in the next section.

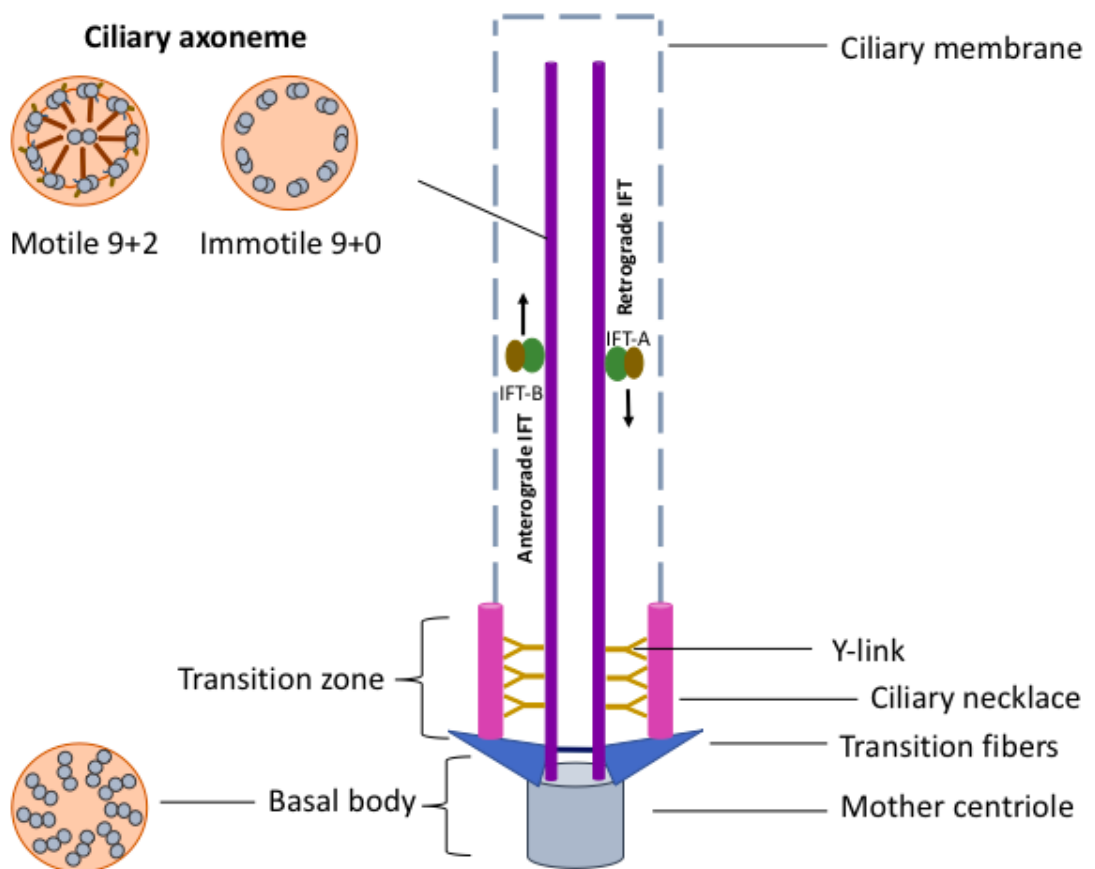


Figure 1.1 Structure of the cilium. Schematic showing the general cilia structure and its specialised regions based on Szymanska & Johnson (2012).

1.1.1 Centriole duplication

The initial step of ciliogenesis is centriole duplication, which is when the basal body of a cilium develops from a centriole (Hodges et al., 2010). As shown in Figure 1.1, this basal body has a highly conserved structure consisting of 9 microtubule triplets. The arrangement of the basal body differs in *C. elegans* and early *Drosophila* embryos, where the microtubules are arranged as singlets and doublets, respectively (Greenan et al., 2018; Ross & Normark, 2015). Regardless of differences in microtubule arrangement, the regulation of centriole duplication is evolutionarily conserved (Hodges et al., 2010; Reiter et al., 2012).

Centrosomes develop when mother centrioles accumulate pericentriolar material and duplicate (Fu et al., 2015). This duplication results in daughter centrioles, formed near the mother centriole during the S-phase of the cell cycle (Conduit et al., 2015). The daughter centriole disassociates from the mother centriole late in mitosis to become a mother centriole itself.

Centrosomes are important for several cellular processes including cell signalling, cell cycle regulation and establishing polarity (Arquint et al., 2014; Darling et al., 2017). In cells that are in G₁ or G₀, the centriole pair moves to the surface of the cell, and the mother centriole becomes the basal body (Fig 1.1). There is an essential crosstalk which takes place between cilium formation and cell division, indicated by the fact that defective division can result in abnormal ciliogenesis and *vice versa* (Avasthi & Marshall, 2012)

Centriole duplication is essential to ciliogenesis, so disruption to this step results in defective cilia. This phenotype was highlighted by loss-of-function studies in *Drosophila*, where genes involved in this process were altered. These genes included *sak kinase (sak)* which when disrupted caused a loss of centriole structures, as well as disordered mitotic spindles and a loss of axonemes (Bettencourt-Dias et al., 2005). The severity of this phenotype highlights the importance of the basal bodies in relation to axoneme development. Once the basal body is formed, it migrates and docks at the plasma membrane (Sorokin, 1968)

1.1.2 Development of the axoneme and ciliary membrane

The axoneme develops from this docked basal body and has a surrounding sheath called the ciliary membrane, shown in Figure 1.1. This structure fuses with the plasma membrane, allowing the developing cilium to be enclosed in an external covering (Sorokin, 1962). The axoneme extends due to the presence of additional tubulin at its distal end, which migrates to this location from the cytoplasm (where protein synthesis takes place) by intraflagellar transport (IFT; Marshall & Rosenbaum, 2001). This process enables the movement of molecules along the cilia between the axoneme and the

surrounding ciliary membrane and is crucial to the development and maintenance of cilia (Kozminski et al., 1993). IFT was discovered by the Rosenbaum group using Differential Interference Contrast (DIC) microscopy on *Chlamydomonas* flagella. Particles were observed moving up (anterograde transport) and down (retrograde transport) the cilium (Kozminski et al., 1993). The two types of IFT, anterograde and retrograde, will be explained further below.

1.1.3 Anterograde IFT

Anterograde IFT is conducted by two kinesin motors (heterotrimeric and homodimeric), that function alongside the IFT-B protein complex to transport IFT trains, which move particles (such as tubulin) to the distal end of the cilium (Pazour & Rosenbaum, 2002). In *Chlamydomonas*, by temperature-shift experiments and high-resolution video-enhanced DIC microscopy, it was shown that *FLA10*, a subunit of heterotrimeric Kinesin-2 is required for anterograde transport (Kozminski et al., 1995). In *Drosophila*, heterotrimeric kinesin II is composed of several proteins: kinesin-like protein at 64D (KLP64D), kinesin-like protein at 68D (KLP68D) and kinesin associated protein (KAP). When these proteins are non-functional, there is a loss of axonemes as tubulin cannot migrate distally along the cilium without anterograde IFT (Sarpal et al., 2003). This finding is highlighted by the loss of *klp64D*, which limits tubulin from entering the cilium and itself accumulates at the ciliary base (Avidor-Reiss et al., 2004)

IFT proteins are evolutionarily conserved and essential to ciliogenesis, except in *Plasmodium falciparum* flagella and *Drosophila* sperm flagella - the latter of which will be discussed in further detail later in this Chapter (Han et al., 2003). As IFT is still required in the other ciliated cells in *Drosophila*, there are homologues for the majority of IFTB complex proteins (van Dam et al., 2013). *Oseg2* and *Oseg5*, the *Drosophila* homologues of IFT172 and IFT80, localise to the ciliary base and the axoneme. *Oseg2* mutants show a lack of tubulin in the cilium, which indicates the importance of the IFTB complex for tubulin transport (Avidor-Reiss et al., 2004). When IFTB proteins are mutated in

Drosophila, it causes a disruption to the development of sensory neuron cilia, further highlighting the critical role of IFT in axoneme development (Han et al., 2003). IFT is also important for the transport of certain ciliary proteins e.g., the radial spokes and axonemal dynein motors within the ciliary membrane (Hou et al., 2007; Qin et al., 2004). The transport to the motile machinery will be discussed in further detail later in this Chapter.

1.1.4 Retrograde IFT

Retrograde IFT is facilitated by the IFT-A protein complex in combination with an IFT dynein motor (cytoplasmic dynein 2) to transport particles back to the base of the cilium along the microtubule (Pazour et al., 1998). The requirement of the dynein motor for retrograde IFT is evidenced by the observation that in *Chlamydomonas*, mutations in the dynein light chain *LC8* or dynein heavy chain *DHC1b* (subunits of cytoplasmic dynein) have shorter flagella and a build-up of particles at the distal ends (Pazour et al., 1998). Similar phenotypes have been observed in additional organisms such as *C. elegans*, mice as well as in *Drosophila*, where the loss of the dynein *beethoven* (*bvt*) causes a build-up of material at the distal end of the cilia and a loss of the IFT-A complex (Lee et al., 2008).

1.1.5 The BBSome

In addition to a requirement for proteins to be transported along the cilia, proteins also need to be transported to the ciliary base from the cytoplasm. This movement is facilitated by the BBSome, a protein complex that forms a surrounding coat on proteins of interest to aid their transport into the ciliary compartment. The protein complex is composed of Bardet-Biedl syndrome proteins 1-9 (BBS1-9), and the BBSome interacting protein (BBIP10) (Nachury et al., 2007; Loktev et al., 2008). The assembly of the BBSome coat onto the proteins is activated by the GTPase BBS3. If the BBSome is not functioning as it should, ciliary proteins may not localise correctly and cause defective cilia function (Lechtreck et al., 2009). In this situation, the cilium may appear to have developed as expected, but may be missing important proteins causing

defective ciliary function, similar to what occurs in the ciliopathy Bardet Biedl Syndrome (BBS) (Singh et al., 2020).

In *Drosophila*, there are fewer proteins in the BBSome complex, as there are no homologues for BBS2 and BBS7 (Klink et al., 2010). Not much information is known regarding the BBSome in *Drosophila*, but a GFP expression study indicated that the *Drosophila* BBS proteins are present in ciliated cells (which in *Drosophila* are specifically the type-I sensory neurons), and so it is likely these conserved proteins have a similar function to those in vertebrates (Avidor-Reiss et al., 2004).

1.1.6 The ciliary gate

As the protein content of the cilia is different from that of the general cytoplasm, it is clear there must be some form of barrier at the base of the cilium. Therefore, the final important development that occurs during ciliogenesis is that of the ciliary gate. This structure is composed of the transition zone (TZ), as well as the transition fibres (TF) that link the basal body to the membrane. These fibres are an important docking area for IFT proteins, which are enriched at this location (Reiter et al., 2012). Y-shaped linkers and the ciliary necklace can be also observed at the ciliary gate, characteristic structures of this ciliary sub-compartment (Szymanska & Johnson, 2012). In addition to allowing important proteins to enter the ciliary membrane (via active transport), the ciliary gate also prevents non-specific proteins travelling along the cilium. It has been observed that the TFs and TZs form pore-like structures like the nuclear pore complex (NPC), to facilitate this selective transport of materials. GTPases such as Rab8 have been linked to this ciliary trafficking (as well as linked to interactions with the BBSome) (Nachury et al., 2007; Westlake et al., 2011). Many human ciliopathies are associated with transition fibre components and disrupted ciliary gating, including Joubert Syndrome (JBTS) and Meckel-Gruber syndrome (MSK) (Sang et al., 2011). Therefore, understanding how the ciliary gate functions *in vivo* will aid understanding of the role of the transition fibres and transition zone in these ciliopathies (Wei et al., 2015).

Overall, it can be concluded that ciliogenesis is a complex process consisting of several important steps. Disruption to any of these steps can result in disease conditions in humans, highlighting the importance of fully functioning cilia - immotile and motile, both of which will be described in greater detail below.

1.2 Primary cilia function

Primary cilia, also known as immotile cilia, are sensory organelles that have a wide range of functions in the body. These functions include olfactory reception, the regulation of food intake, kidney development and mediating cell signalling pathways. Primary cilia generally have a 9+0 axonemal arrangement, lacking the motile cilia apparatus and the central pair - both of which will be described later in this Chapter. As primary cilia have important functions in several cell types, ciliopathies can occur when they are defective. These disorders include Polycystic Kidney Disease (PKD), some forms of cancer and BBS, which was briefly mentioned above and includes symptoms such as retinal degeneration, obesity, diabetes and polydactyl. As this thesis focuses on motile cilia, primary cilia and the associated disorders will only be briefly introduced below to highlight the importance of cilia in the body in both healthy and disease states.

1.2.1 Primary cilia and olfactory reception

While most primary cilia have a 9+0 axonemal arrangement, olfactory cilia have a 9+2 axonemal arrangement. These are considered to be primary cilia as they function as sensory organelles with G-protein coupled receptors (GPCRs), which increase the level of the intracellular signalling molecule cyclic-AMP (cAMP) in the cilia (Takeda et al., 2002).

The partial or complete loss of the sense of smell, known as Anosmia, is a common symptom in ciliopathies. For example, patients with BBS commonly exhibit olfactory defects (Kulaga et al., 2004). This symptom indicates the vital

link between cilia and olfaction, as this organelle is where the necessary signal transduction occurs.

1.2.2 Primary cilia and obesity

Primary cilia are also required for regulating food intake, which when overactive (termed hyperphagia), can cause obesity. Obesity, as well as the related symptom of diabetes, is commonly observed in BBS patients (Mykytyn et al., 2002; Volta & Gerdes, 2017). The hyperphagia was initially thought to be caused by defective leptin signalling and linked to cilia through mouse knockouts of the anterograde IFT component *ift88*. This gene was removed from the mouse hypothalamus, which is the region of the brain responsible for the release of hormones (such as leptin). When *ift88* was non-functional in the hypothalamus, it resulted in mice that had high levels of leptin and were obese (Davenport et al., 2007). When BBSome components *Bbs2*, *Bbs4* and *Bbs6* were non-functional, mice were unable to respond to leptin signals and irregular food intake was observed (Seo et al., 2009). However, before the onset of obesity, *Bbs4* mutant mice were found to be responsive to leptin signalling, suggesting that defective leptin reception was not the cause of the obesity symptom and instead occurred because of it (Berbari et al., 2013).

The primary cilia of the hypothalamus regulate food intake through GPCR signalling (Engle et al., 2021). Neuropeptide Y receptors are G protein coupled receptors, which are transported to the cilium by the BBSome and regulate food levels (Loktev & Jackson, 2013). The Tubby family of proteins are linked to this process. In mice, mutations in *tubby* cause retinal degeneration and obesity - symptoms commonly associated with ciliopathies (Kleyn et al., 1996). In *Drosophila*, the homologue of *tubby*, *dTulp*, is required in the ciliated chordotonal neuron for Transient Receptor Potential (TRP) channel localisation (Park et al., 2013). These mechanosensitive neurons are required for auditory and proprioceptive function in the fly (not the regulation of food intake) and will be described in more detail later in this introduction.

1.2.3 Primary cilia and kidney function

Defective kidney function is a characteristic associated with ciliopathies, with cysts and nephronophthisis commonly observed in patients (Nauli et al., 2003). In mice, mutations in the cilia genes *ift88* and *kif3A* result in shorter cilia and the presence of kidney cysts (Lin et al., 2003). These observations demonstrate the key role of primary cilia in healthy kidney development. The primary cilia are present along the nephrons of the kidneys and bend in response to moving liquid, opening calcium channels to allow ions move into the cells. Polycystic kidney disease was actually the first ciliopathy to be characterised, and mutations in *polycystic kidney disease 1 (PKD1)* and *polycystic kidney disease 2 (PKD2)* hinder the development of these primary cilia, resulting in the occurrence of defective mechanosensation and signal transduction, which causes abnormal cell proliferation and fluid accumulation (leading to cysts) (Wilson, 2004).

1.2.4 Primary cilia and cell signalling pathways

Primary cilia have also been associated with several types of cancer including renal, pancreatic and breast cancer (Higgins et al., 2019). This link is due to primary cilia being important regions for the signal transduction associated with cell signalling pathways (such as WNT and Hedgehog) to occur, which are associated with key cellular processes like cell growth and differentiation.

The crucial relationship between cilia and cell signalling pathways was identified by the Anderson group, who conducted screens in a mouse model to look for genes essential to Hedgehog (Hh) signalling. They found a set of genes that were required for both Hh signalling and primary cilia development (Huangfu et al., 2003).

Once the initial screen conducted by the Anderson group identified genes with an essential role in both Hh signalling and primary cilia, they explored where the components of the Hh pathway were localised to or enriched in the organelle. They found that some of these proteins were upregulated in the primary cilium, such as *Ptch1* (Ocbina & Anderson, 2008).

In the Hh signalling pathway, the Hedgehog (Hh) ligand binds to its receptor Patched1 (PTCH1) releasing Smoothed (SMO). SMO inhibits the degradation of Glioma transcription factors (GLIA) to GLI repressors (GLIR), which is activated by Suppressor of fused (SUFU). GLIA moves to the nucleus and regulates the expression of Hh targets.

Mutations in cilia genes can disrupt this pathway and cause ciliopathy symptoms such as polydactyl, which was observed in mice when the IFT-associated genes *ift172* and *ift88* were mutated (Huangfu et al., 2003; Liu et al., 2005). The defective Hh signalling is caused by a disruption to the GLI transcriptional activator (GLIA) and GLI transcriptional repressor (GLIR), which require IFT for regulation (Goetz & Anderson, 2010; Huangfu & Anderson, 2005)

WNT signalling has also been linked to primary cilia. In this pathway, the ligand (WNT) binds to its receptor, Frizzled (FZD), resulting in the release of the kinase substrate Dishevelled (DVL). This substrate inhibits phosphorylation, resulting in the degradation of the effector, Beta-catenin. This effector accumulates and migrates towards the cell's nucleus to regulate WNT signalling through an interaction with the transcription factor T-cell factor (TCF). When primary cilia are defective, this results in an increase in WNT signalling. This increase is caused by the effector Beta-catenin not being degraded, as shown by experiments in Zebrafish and human embryonic kidney (HEK) cells where the BBSome proteins *bbs1*, *bbs4* and *bbs6* were non-functional (Gerdes et al., 2007). Defective primary cilia can also cause a suppression of the non-canonical WNT pathway, or Planar cell polarity pathway (PCP), which is beta-catenin independent. Interactions between PCP genes and BBS genes have been reported, showing primary cilia have a role in both classes of WNT signalling (Ross et al., 2005).

In *Drosophila*, defective cilia do not generally cause Hh or Wnt defects. However, the ciliated olfactory neuron requires Hh signalling (Kuzhandaivel et al., 2014). When Hh binds to Ptch1 in vertebrates, this is moved from the cilia and Smo is transported into the cilia. In *Drosophila*, most cells do not have

cilia, so Smo moves to the plasma membrane when the Hh signalling pathway is activated. However, it has been discovered that there is more than one Hh pathway in *Drosophila*, and so in the olfactory sensory neurons (OSNs), Smo localises to the cilia, facilitated by IFT (Kuzhandaivel et al., 2014).

1.3 Motile cilia function

Motile cilia and flagella, like primary cilia, are highly conserved organelles required for a variety of essential processes. These include fluid flow and movement within fluids. In humans, motile cilia are found along the epithelial surfaces, the ventricular brain system and the fallopian tubes (Mitchison & Valente, 2017). When these organelles are non-functional, it can result in motile ciliopathies - the most common being Primary Ciliary Dyskinesia (PCD), which is characterised by abnormally positioned organs, chronic respiratory infections, and infertility. This disorder will be described in further detail later in this introduction and highlights the importance of understanding the genetics and physiology of the motile cilia in the body.

As these organelles are highly conserved, a lot of information regarding their function has been obtained by studying protozoans such as *Chlamydomonas* and *Tetrahymena*. These simple organisms are genetically tractable, and so they have been useful in both forward and reverse genetic screens to identify novel motile cilia genes and understand how the cilia move (Bayless et al., 2019).

As shown in Figure 1.2A, motile cilia typically have a 9+2 axonemal arrangement, with nine microtubule doublets surrounding a central pair. Axonemal dynein motor complexes are present along with the outer microtubule doublets and drive the motor function of the cilium. Nodal cilia, which have a 9+0 axonemal arrangement, also contain axonemal dynein motor complexes and are required for left-right determination of organ development in vertebrates. Nodal cilia rotate clockwise due to a lack of a central pair

(Buceta et al., 2005; Essner et al., 2002). This movement contrasts with the other motile cilia, which has a unidirectional flow (Wagner & Yost, 2000).

When the cilia are motile, the dynein motors along one side of the axoneme are activated in response to changing intraciliary Ca^{2+} levels in a waveform towards the apical end of the cilium. Following this, the dyneins along the opposite side are activated similarly to reverse the movement. This mechanism is tightly regulated to ensure only one side is active at a time in what is referred to as the switch point model (King & Sale, 2018). As the axonemal dyneins are AAA+ enzymes, they convert ATP hydrolysis (chemical energy) to mechanical work by powering the microtubule doublets to slide against one another (King, 2016). The sliding motion translates to a bending motion by additional structures of the motile cilia ultrastructure, such as the Nexin-DRC, which will be described in detail below.

1.4 Motile cilia ultrastructure

Ciliary motility is facilitated by axonemal dynein arms, which as described above cause the microtubules doublets of the axoneme to slide, resulting in motion. Along the axoneme is a 96nm repeat unit, which is partially determined by a protein group (CCDC39 and CCDC40) referred to as the molecular ruler (Lin et al., 2015; Oda et al., 2014). This 96nm region, shown in Figure 1.2B, features several important components: outer dynein arms (ODAs) and inner dynein arms (IDAs), as well as the radial spokes (RS) and Nexin-Dynein regulatory complex (N-DRC). These components make up the motile cilia machinery and are all required for successful movement. The details of these structures will be described in this section.

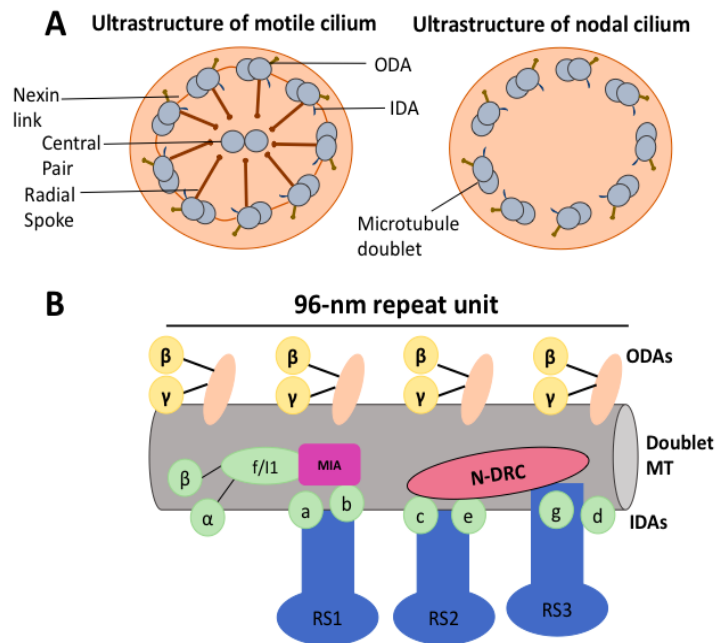


Figure 1.2 Structure of the mammalian motile cilium modelled on schematic of *Chlamydomonas* motile cilium in (Viswanadha et al., 2017). (A) Schematics showing the ultrastructure of a motile cilium (9+2 axonemal arrangement) and a nodal cilium (9+0 axonemal arrangement). Motile cilia machinery labelled on the diagram includes the outer dynein arm (ODA) and inner dynein arm (IDA), which drive the motor function. (B) the 96-nm repeat unit of the motile cilium, highlighting several important components: the ODAs (which have 2 heavy chains β and γ) and IDA subsets (a-g and the two-headed f), the radial spokes (RS1-3), the moderator of inner arms (MIA) complex as well as the nexin-dynein regulatory centre (N-DRC).

1.4.1 Outer dynein arms (ODA)

The outer dynein arm (ODA) is a large motor complex (~2 MDa) that repeats along the axoneme every 24nm, as shown in Figure 1.2 (Nicastro et al., 2005). The function of the ODAs is to drive the motility of the cilium. As shown in Figure 1.3, these structures are composed of heavy chains (2-3 dependent on the species), intermediate chains and several light chains (Sakato & King, 2004). The dynein heavy chains are large structures which contain a microtubule-binding region, as well as an ATPase domain (a globular ring of non-identical AAA+ domains). The microtubule-binding region is ATP-sensitive and is located at the tip of a coiled coil stalk that extends from AAA4 and interacts with an additional coiled-coil stalk that extends from AAA5 (Fig. 1.3).

The intermediate and light chains of the ODA are required for cargo/microtubule binding and regulatory functions (DiBella et al., 2004).

The outer dynein arm docking complex (ODA-DC), identified through studies in *Chlamydomonas*, directly interacts with the ODAs and facilitates the stable binding of these motors to the microtubule doublet. This complex also has a 24nm periodicity along the axoneme and comprises CCDC151, CCDC114 and ARMC4, with TTC25 and Calaxin required for ODA-DC assembly and attachment to the axoneme (Owa et al., 2014; Wallmeier et al., 2016; Gui et al., 2021).

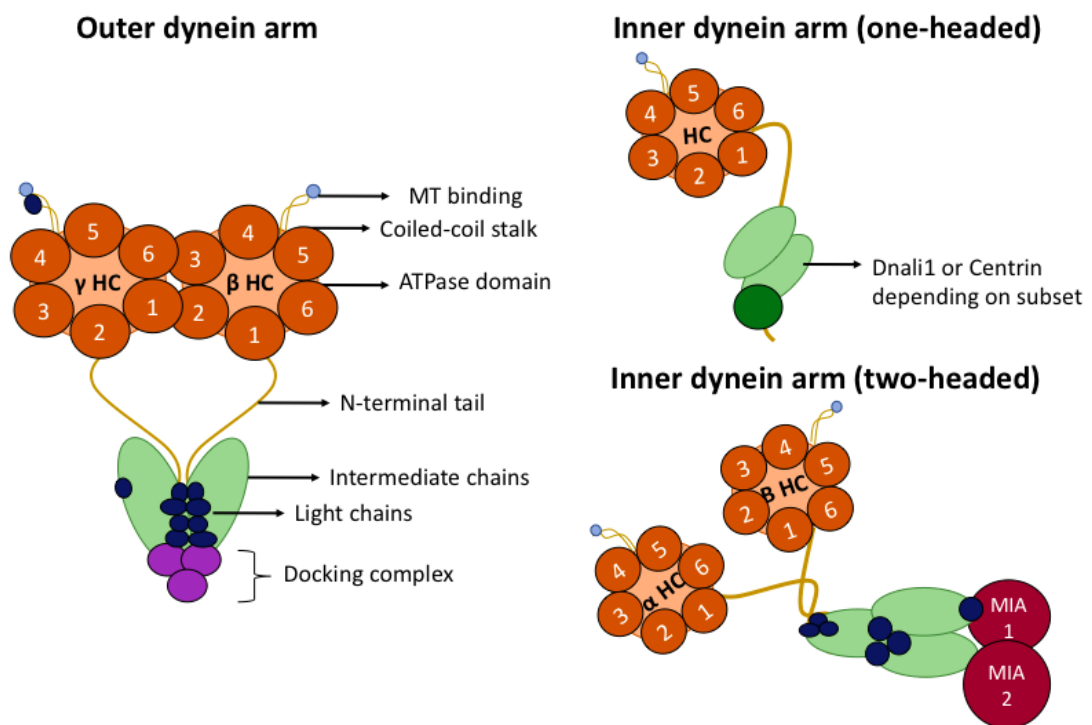


Figure 1.3 Structure of the different axonemal dynein motor complexes (in vertebrates and invertebrates). Outer dynein arms have two dynein heavy chains (β and γ HC). The outer dynein arm is attached to the axoneme by the outer dynein arm docking complex (ODA-DC). There are two different types of inner dynein arms, the first of which is the one-headed (one heavy chain). The monomeric dynein arms usually carry out their functions as pairs, with one dynein arm containing the protein Dnali1 and the other containing the protein Centrin. The two-headed dynein arm contains two heavy chains, α and β . This dynein arm interacts with the moderator of inner arms complex (MIA1 and MIA2) to regulate ciliary movement. Figure adapted from zur Lage et al., (2019).

1.4.2 Inner dynein arms (IDA)

The inner dynein arms (IDAs) are required for regulating ciliary movement and as shown in Figure 1.3 consist of a dynein heavy chain (or 2 heavy chains) which consists of the AAA ring, a coiled-coil stalk and an N-terminal tail, that extends distally from the ring (Burgess et al., 2003). There are several different IDA subsets within the 96 nm repeat region, shown in Figure 1.2. The monomeric subsets (i.e. 1 heavy chain) function as dyad pairs (*a/b*, *c/e*, *g/d*), with one subset in each pair containing the protein Dnali1 and the other containing the Ca^{2+} binding protein Centrin (Fig. 1.3). Analysis of IDA mutants suggest that not all IDAs are required for ciliary motility but that specific combinations of these IDAs are necessary (Kamiya, 2002). Each of these dyads has a corresponding radial spoke in the 96 nm repeat (Bui et al., 2008). Signals are transmitted from these radial spokes and the central pair to the IDAs (King, 2016).

The additional subset, IDA I1/f, has two heavy chains and is located close to the microtubule at the proximal end of the 96 nm repeat (Bui et al., 2008). As shown in Figure 1.3, this subset directly interacts with the moderator of the inner arms (MIA) complex, which is composed of MIA1 and MIA2. IDA I1/f and the MIA complex work together to regulate ciliary movement, most likely by transmitting signals to the ODAs (Yamamoto et al., 2013).

1.4.3 Dynein arm assembly

As described above, outer and inner dynein arms are complex in structure. For ciliary motility to be functional, these structures need to be assembled in a specific manner.

Historically, *Chlamydomonas* has been used as a model system to understand the mechanisms surrounding ciliary motility. Early immunoprecipitation and protein detection experiments in this model indicated that the dynein arms are initially pre-assembled as subcomplexes in the cytoplasm before being transported to the cilium and docking along the axoneme, shown in Figure 1.4 (Fowkes & Mitchell, 1998). As previously mentioned, dynein heavy chains are

quite large proteins (~4500 residues), so the synthesis of these proteins and assembly of the dynein motor complexes can take some time, which increases the likelihood of heavy chain misfolding and the misassembly of complexes (King, 2016). Therefore, this assembly process is tightly regulated by a group of genes called dynein axonemal assembly factors (DNAAFs), which work together with chaperones to facilitate and regulate this process (Desai et al., 2018; Omran et al., 2008). DNAAFs are highly conserved throughout evolution, from unicellular *Chlamydomonas* to vertebrates such as zebrafish, mice and humans.

The first DNAAF identified in humans was *DNAAF2*, mutations in which were found in individuals diagnosed with PCD (Omran et al., 2008). As mentioned before, DNAAFs are closely associated with this ciliopathy, which will be described in greater detail later in this Chapter.

This characterisation of *DNAAF2* was supported by research conducted in *Chlamydomonas*, that found when the *DNAAF2* homologue (*PF13*) was non-functional, dynein assembly was hindered (Omran et al., 2008). Since this discovery, many additional genes with functions in this process have been identified through whole-exome sequencing of PCD patients, as well as analysis conducted in model organisms including *Chlamydomonas* and *Drosophila*. Table 1.1 shows the current list of DNAAFs conserved between *Chlamydomonas*, *Drosophila* and humans.

Table 1.1 List of conserved dynein axonemal assembly factors

Human	<i>Chlamydomonas</i>	<i>Drosophila</i>
<i>DNAAF1/LRRC50</i>	<i>ODA7</i>	<i>CG31623</i>
<i>DNAAF2/KTU</i>	<i>PF13</i>	<i>CG1553</i>
<i>DNAAF3</i>	<i>PF22</i>	<i>CG17669</i>
<i>DNAAF4/DYX1C1</i>	<i>PF23</i>	<i>CG14921</i>
<i>DNAAF5/HEATR2</i>	<i>CrHEATR2</i>	<i>CG31320</i>
<i>DNAAF6/PIH1D3</i>	<i>TWI1</i>	<i>CG5048</i>
<i>DNAAF7/ZMYND10</i>	<i>CrZMYND10</i>	<i>CG11253</i>
<i>DNAAF8/C16orf71</i>	<i>N/A</i>	<i>N/A</i>
<i>DNAAF9/SHULIN</i>	<i>Cre11.g467556 (tbc)</i>	<i>N/A</i>
<i>DNAAF10/WDR92</i>	<i>CrWDR92</i>	<i>CG14353</i>
<i>DNAAF11/LRRC6</i>	<i>MOT47</i>	<i>CG14620</i>
<i>DNAAF12/LRRC56</i>	<i>ODA8</i>	<i>CG14185</i>
<i>DNAAF13/SPAG1</i>	<i>CrSPAG1</i>	<i>CG18472</i>
<i>DNAAF14/PIH1D1</i>	<i>MOT48</i>	<i>CG5792</i>
<i>DNAAF15/PIH1D2</i>	<i>N/A</i>	<i>CG4022</i>
<i>DNAAF16/C21orf59</i>	<i>FBB18</i>	<i>CG18675</i>
<i>DNAAF17/CCDC103</i>	<i>Pr46b</i>	<i>N/A</i>
<i>DNAAF18/DAW1</i>	<i>ODA16</i>	<i>CG7568</i>
<i>TTC12</i>	<i>CrTTC12</i>	<i>CG6980/CG34297</i>
<i>RUVBL1/PONTIN</i>	<i>CrRUVBL1</i>	<i>CG4003</i>
<i>RUVBL2/REPTIN</i>	<i>CrRUVBL2</i>	<i>CG9750</i>

The function of DNAAFs can be quite variable. For example, as shown in Figure 1.4, *DNAAF2*, *DNAAF4* and *DNAAF6*, which will be investigated in this project (Chapters 3,4), have been proposed to have a role in the assembly of the intermediate chain (IC) complex (Omran et al., 2008; Paff et al., 2017;

Tarkar et al., 2013). *DNAAF7* and *LRRC6* have been proposed to have a role in the maturation of the heavy chains (HC) and movement of these proteins to R2TP, R2TP-like complexes (Chapter 3) and Hsp90 for the IC and HC complexes to combine (Mali et al., 2018). An additional mechanism has also been proposed for dynein assembly, featuring a late preassembly complex (*DNAAF1*, *DNAAF3*, *DNAAF4*, *DNAAF11/LRRC6*, *DNAAF7* and *DNAAF6*), suggested by experiments conducted in primary airway cell cultures and iPSCs (Horani et al., 2018). Therefore, further clarity on the specific roles of DNAAFs, step by step during dynein assembly is essential.

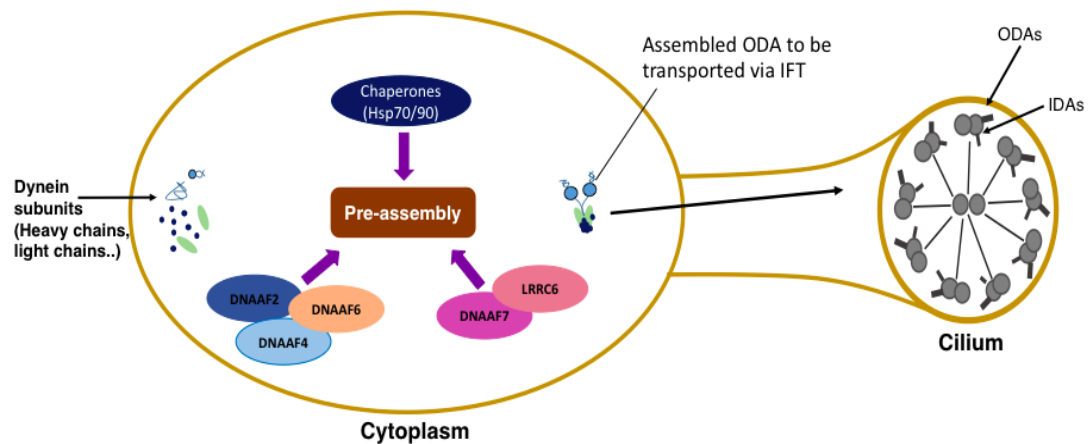


Figure 1.4 Schematic of the axonemal dynein assembly process. Dynein motor complexes are made up of multiple proteins which assemble in the cytoplasm. This is regulated by chaperones, which work with a group of genes called DNAAFs to ensure the correct assembly of the motors. Once assembled, these structures are transported into the cilium by IFT and docked along the axoneme.

Originally genes that were given the nomenclature DNAAF were directly involved in dynein pre-assembly in the cytoplasm. However, the group has been expanded in recent years to include genes that function in the movement of assembled dynein arms from the cytoplasm to the cilia, such as *DNAAF18*, which functions as an IFT adaptor in the trafficking of assembled dyneins (Braschi et al., 2022; Gao et al., 2010). This trafficking process will be described in further detail in Section 1.4.6.

1.4.4 Dynein axonemal particles (DynAPs)

Several studies have suggested that during dynein pre-assembly, DNAAFs and chaperones work together in cytosolic ‘foci’ (Diggle et al., 2014; Horani et al., 2012; Li et al., 2017) and that the different steps take place in what is referred to as a ‘chaperone relay’ (Mali et al., 2018). However, while there have been several proposals regarding this process, the exact regulatory mechanism is yet to be defined.

In multiciliated *Xenopus* embryos, live imaging showed DNAAFs, axonemal dynein subunits and chaperones localise to distinct areas within the cytoplasm that were named Dynein axonemal particles (DynAPs). These are thought to be biomolecular condensates which develop as a result of liquid-liquid phase separation. It was found that dynein subunits remained inside these areas, with the DNAAFs entering rapidly when required for assembly. DynAPs could therefore provide the essential quality control required for the assembly of large protein complexes such as the dynein arms by allowing chaperones and co-chaperones (which are essential to this process) to rapidly enter this specific compartment when required (Huizar et al., 2018). Further analysis revealed that ODAs and IDAs were separated into sub-regions within these DynAPs for assembly (Lee et al., 2020).

However, the exact role of these DynAPs has been considered controversial in the field, with the spatial confinement of the ODAs and IDAs in these foci suggested to be too rigid to be classified as a liquid-like state. Moreover, the heavy chain subunits, which are the largest element of the protein complex and power the motility, have not been reported as being present in these DynAPs to date (King, 2021).

1.4.5 Dynein arm transport (IFT)

DynAPs or no DynAPs, the dynein motor complexes are assembled in the cytoplasm of the cell and then transported to the axoneme via intraflagellar transport (IFT). As discussed in Section 1.1, IFT is facilitated by kinesin-2, cytoplasmic dynein and the IFT A and IFT B complexes.

During ciliogenesis, tubulin directly interacts with the IFT trains (Bhogaraju et al., 2013). However, adaptor proteins have been found to be required for the motile cilia machinery (outer and inner dynein motor complexes, as well as the dynein regulatory complex (DRC) and the Radial Spokes) to associate with the trains for transport to the ciliary compartment.

The first of these, that was identified in *Chlamydomonas*, was ODA16 (DAW1/DNAAF18). Using pull-down assays and co-immunoprecipitation experiments, this protein was found to directly interact with IFT46, a subunit of the IFT B complex and to function as a link between the outer dynein arm (IFT cargo) and the IFT subunit (Ahmed et al., 2008; Dai et al., 2018). It seems that the outer dynein arms require IFT for immediate movement from the cytoplasm to the cilium, and then dock directly along the axoneme. However, it has been suggested that the inner dynein arms and radial spokes differ in this respect and remain associated with the IFT trains until they reach the tip of the cilium, where the trains change in their composition and release the motile cilia machinery (Ahmed et al., 2008). ODA8 (LRRC56/DNAAF12) has also been proposed as a possible IFT adaptor protein, and co-IP experiments in HEK293 cells have shown that it interacts with IFT88 (Bonnefoy et al., 2018).

Studies in *Chlamydomonas* also revealed an IFT adaptor (IDA3) for inner dynein arms, specifically the double-headed I1 dynein complex, so that it can be loaded onto IFT trains and transported into to the cilium (Hunter et al., 2018). Additionally, an IFT adaptor for the Radial Spokes has been recently identified: ARMC2 (Lechtreck et al., 2022). Less is known to date regarding adaptors for the single-headed IDAs and the DRC. However, DRC4 cargos have been found to be associated with IFT trains in *Chlamydomonas* cilia, and so it is likely that there are additional IFT adapter proteins yet to be identified that are required to transport these complexes (Wren et al., 2013).

It was not known until recently how the ODAs managed to be transported along the axoneme without interacting with the microtubules until their final destination. *Shulin* was identified in *Tetrahymena* as a regulator of ODA activity during IFT. This protein inactivates the ODAs by causing the heavy

chains to fuse together, which leads them to have weak affinity for the microtubules until docking is required (Mali et al., 2021).

1.4.6 Nexin-Dynein Regulatory Complexes (N-DRC)

As well as the outer and inner dynein arms, there are several additional components of the motile ciliary machinery required for a successful function. These include several regulatory complexes such as the nexin-dynein regulatory complexes (N-DRC) and radial spoke (RS) complexes.

Using cryo-ET in wild-type and mutant strains of *Chlamydomonas*, it became evident that the dynein regulatory complex (DRC) and nexin link were one structure, and which is now known as the N-DRC (Heuser et al., 2009). This complex comprises 11 proteins (DRC1-11), and was identified by co-immunoprecipitation, iTRAQ labelling and quantitative mass spectrometry experiments (Bower et al., 2013).

The N-DRC regulates dynein motor activity and axonemal bending (Ferkol & Leigh, 2019). It connects the nine outer microtubule doublets, and it is known to be required to regulate the interdoublet sliding that occurs when dynein motors are active. The N-DRC is also connected with other components of the motile cilia ultrastructure and has been suggested to be required to transmit signals from the central pair-radial spoke (CP-RS) complex to the axonemal dynein arms (Heuser et al., 2009).

1.4.7 Radial spoke proteins and central pair apparatus

The radial spokes (RS) connect and transmit messages from the central pair of singlet microtubules to the dynein arms of the outer microtubule doublets and are therefore required for ciliary movement (Oda et al., 2014). The RS is a T-shaped structure (Fig 1.2), which connects to the A-microtubule of the doublet through an elongated stalk. This group of structures (most organisms have 3 of them) repeat every 96 nm along the axoneme (Pigino & Ishikawa, 2012). The central pair (CP) transiently interacts with the RS to facilitate

axoneme stability by controlling the microtubule sliding which occurs in motile cilia (Jeanson et al., 2015; Teves et al., 2016b).

1.5 Motile ciliopathies in humans: Primary ciliary dyskinesia (PCD)

As briefly mentioned above, defective motile cilia are commonly associated with the respiratory disorder Primary Ciliary Dyskinesia (PCD), initially characterised by Kartagener. In 1976, Alfelius first found the link between this disorder and defects in motile cilia ultrastructure (Ferkol & Leigh, 2019).

Symptoms commonly associated with this condition include chronic respiratory infections caused by defective mucociliary clearance, hearing impairments and infertility. Laterality defects such as *situs inversus* are also present in ~50% of patients due to non-functional nodal cilia, and the overall condition is then referred to as Kartagener syndrome. Lung function can be drastically impaired as a result of PCD, and so early diagnosis is crucial (Lucas et al., 2014)

Obtaining a PCD diagnosis is complicated in that there is no specific diagnostic test. Therefore, diagnosis can be delayed or sometimes missed, with one study reporting that 70% of patients attended over 50 doctor appointments before receiving their diagnosis (Sommer et al., 2011). Previously, this disorder was thought to affect one in 10,000-20,000 and was diagnosed by assessing symptoms. However, a recent study has revealed that PCD is much more prevalent than previously thought, affecting 1 in 7554 people. It is also worth noting that this result was based on LOF alleles in a subset of PCD-associated genes, and so may still be a substantial underestimate of the frequency of the condition (Mitchison & Smedley, 2022). Common screening tests used in PCD diagnostics include measuring nasal nitric oxide (nNO) levels (which are typically low in PCD patients), high speed video microscopy to examine ciliary beat frequency as well as TEM analysis of the ciliary ultrastructure (Lucas et al., 2014). As a diagnostic test, TEM analysis is costly

and does not contribute much information regarding the disease phenotype but can be useful in combination with genetic testing (Boon et al., 2014).

PCD is a heterogeneous disorder and mutations in over 50 genes can result in it, with non-functional outer and inner dynein arms being the most common reason (Dunsky et al., 2021). Some of the known PCD-causing genes will be described below.

DNAH5 mutations are the most frequent cause of PCD (Hornef et al., 2006). This protein is an outer dynein arm heavy chain and is therefore a crucial driver of ciliary motility. Mutations to the ODA-DC (such as the subunit *CCDC151*) also causes PCD, as this prevents ODA components such as *DNAH5* from docking at the axoneme (Hjeij et al., 2014). As mentioned above, DNAAFs are a group of genes associated with PCD, and mutations in these genes, such as *DYX1C1* and *SPAG1*, can cause ODA and IDA defects (Knowles et al., 2013; Tarkar et al., 2013). Other causative PCD genes include the molecular ruler components *CCDC39* and *CCDC40*, which when non-functional can cause ultrastructural defects such as microtubule disorganisation and the loss of IDAs. Mutations in radial spoke proteins (such as *RSPH9* and *RSPH4A*) have been shown to cause PCD with defects to the central microtubule pair (Castleman et al., 2009). *RSPH3* mutations have also been shown to cause PCD, with disruptions to the central pair and radial spokes (Jeanson et al., 2015). In addition, N-DRC proteins such as *CCDC65* and *GAS8* cause PCD when their functions are disrupted (Austin-Tse et al., 2013; Jeanson et al., 2016).

Current PCD treatments include using airway clearance therapy (ACT) as well as physiotherapy to improve mucociliary clearance, rapid treatment of bacterial infections with antibiotics, bronchodilators, and inhalation corticosteroids. However, these treatments, as well as others, focus on providing relief to already existing symptoms. Personalised medicine, such as gene therapy and gene editing, is an approach which is currently under investigation as a promising aid to PCD diagnosis (Paff et al., 2021). For example, mutations in *DNAI1* are responsible for 10% of ODA defects, and when functional *DNAI1*

cDNA was administered to human airway epithelial cells in a lentiviral vector, the ciliary beating was partially restored (Chhin et al., 2009). This study was the first attempt at gene therapy for a PCD mutation, and due to the presence of a mosaic of cells (some with ODAs and some without), the ciliary beating pattern was still uncoordinated, and so it was only somewhat successful. As many PCD genes are large, transporting them in vectors is difficult, and while this approach is challenging, gene therapy remains a hopeful treatment for PCD. Gene editing is another treatment currently under investigation, with the first study published by Lai et al in 2016. In this study, the ODA heavy chain *DNAH11* was edited using TALEN and administered to both 293T cells and epithelial cell cultures from PCD patients, resulting in a partial restoration of ciliary motility – a promising result overall (Lai et al., 2016).

1.6 Cilia in *Drosophila*

As *Drosophila melanogaster* contains various forms of cilia (both motile and immotile), it can be utilised as a model to further understand the functions of these organelles in organisms in general. The advantage of using *Drosophila* as a model to study cilia development and function is that unlike mammalian models, most cells do not have cilia, and so *Drosophila* mutants of ciliary proteins do not cause embryonic lethality (Jana et al., 2016). Cilia are specifically required for type-I sensory neurons and sperm function, meaning that when they are non-functional it causes defects in touch, proprioception, olfaction and taste, auditory reception, and male fertility (Dubruille et al., 2002; zur Lage et al., 2019). Therefore, assessing behavioural responses to stimuli and fertility can provide insight into cilia function in *Drosophila*. Sperm cells (spermatocytes) initially have immotile cilia, which develop into motile flagellum as the sperm cell matures. This process (spermatogenesis) will be discussed in greater detail in the next section.

The type-I sensory neurons consist of two different cell types. The first is the external sensory (ES) neurons, which are required for olfaction and taste.

These neurons have non-motile cilia, consisting of a 9+0 axonemal arrangement and no axonemal dynein arms (Jana et al., 2011). The second ciliated sensory neuron is the chordotonal neuron, which is required for proprioception, gravitaxis and hearing, and will be described in further detail in Section 1.7.2. This sensory neuron also has a 9+0 axonemal arrangement but is considered to be motile and has axonemal dynein motors present along the proximal end of the cilia, required for the active amplification of vibrations (Kavlie et al., 2010). The chordotonal neuron also has a non-motile 9+0 cilium distal to the ciliary dilation, where several ion channels required for auditory reception are located. This will be discussed in greater detail in the next section, which will focus on the different motile cilia of *Drosophila*, and the requirement of these structures for fertility, proprioception and auditory transduction.

1.7 Motile cilia in *Drosophila*

The structure and function of motile cilia is highly conserved throughout evolution, from protozoans such as *Chlamydomonas* and *Tetrahymena* to metazoans like humans. However, there are evident differences between protozoans and metazoans, including differences in gene function as well as overall dynein motor complex structure. For example, metazoans have no ODA α -HCs but possess a longer radial spoke 3 structure (RS3). Metazoans also have no linker between the nexin-dynein regulatory complex (N-DRC) and the ODAs (Yamaguchi et al., 2018). Therefore, it is essential to also conduct research in higher metazoan models which are also genetically tractable, to gain information that is applicable to humans.

The dynein motor assembly process is highly conserved in *Drosophila melanogaster*, and so understanding how this occurs in this model can provide information translatable to humans and the motile ciliopathy PCD, as almost all motile cilia genes have *Drosophila* homologues (zur Lage et al., 2019).

Many of these genes are restricted in expression to the two cell types that have motile cilia in *Drosophila*: the sperm flagellum and the chordotonal neuron, both of which will be described in greater detail in this section.

1.7.1 Sperm flagellum

The sperm flagellum is one of two ciliated cell types in *Drosophila* that contain axonemal dynein motors. As shown in Figure 1.5A, it has a 9+2 axonemal arrangement, with high power output to beat and propel forward in sinusoidal waveforms (Yang et al., 2011). In addition, the ultrastructure of the flagellum indicates the presence of nine outer accessory microtubules, as well as the presence of a luminal filament in the central pair, most likely at hand to increase microtubule stability (Jamieson, 1998).

1.7.2 Spermatogenesis

As shown in Figure 1.5B, adult *Drosophila* testes contain all of the stages of sperm development within the one structure. Initially in development, which occurs at the apical tip, the germline stem cells divide to form a spermatogonial cell, contained in cyst cells, and a stem cell. The spermatogonial cells mitotically divide four times with incomplete cytokinesis resulting in a cyst of sixteen connected primary spermatocytes. These spermatocytes meiotically divide, resulting in a cyst of sixty-four spermatids (the onion stage). These spermatids then elongate and change shape while the axonemes of the flagellum assemble in their characteristic 9+2 arrangement, with dynein motors present along this structure. The flagellum extends from the basal body rooted in the spermatid nucleus. Once this flagellum has extended fully, mature spermatids undergo individualisation and are transported to the seminal vesicle (Fuller, 1993).

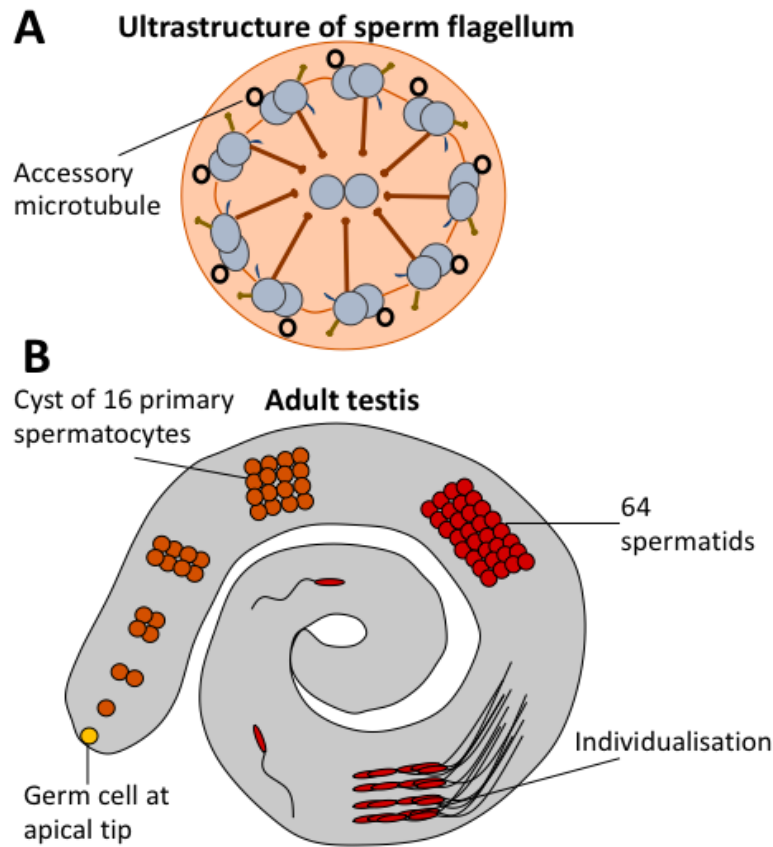


Figure 1.5 Development of the motile sperm flagellum (A) Ultrastructure of sperm flagellum showing 9+2 axonemal arrangement and the additional 9 accessory microtubules. **(B)** Schematic showing the stages of spermatogenesis which take place in the adult testis. Spermatogonial cells mitotically divide four times to develop into a cyst of primary spermatocytes, which then meiotically divide into a bundle of 64 spermatids. These elongate and the axoneme of flagellum develops (cytoplasmically). Once fully extended, individualisation takes place.

1.7.3 Cytoplasmic flagellum formation

Once assembled, axonemal dynein motors are typically transported to the cilium via IFT. However, there is no IFT taking place in the *Drosophila* sperm flagellar compartment (Han et al., 2003). The sperm flagellum is long (approx. 1.9mm in length), and axoneme development occurs in the cytoplasm where the required components are already present (Mendes Maia et al., 2015). This assembly is referred to as a type of cytoplasmic cilia development, a process that allows for the faster assembly of long cilia and flagella, shown in Figure 1.6.

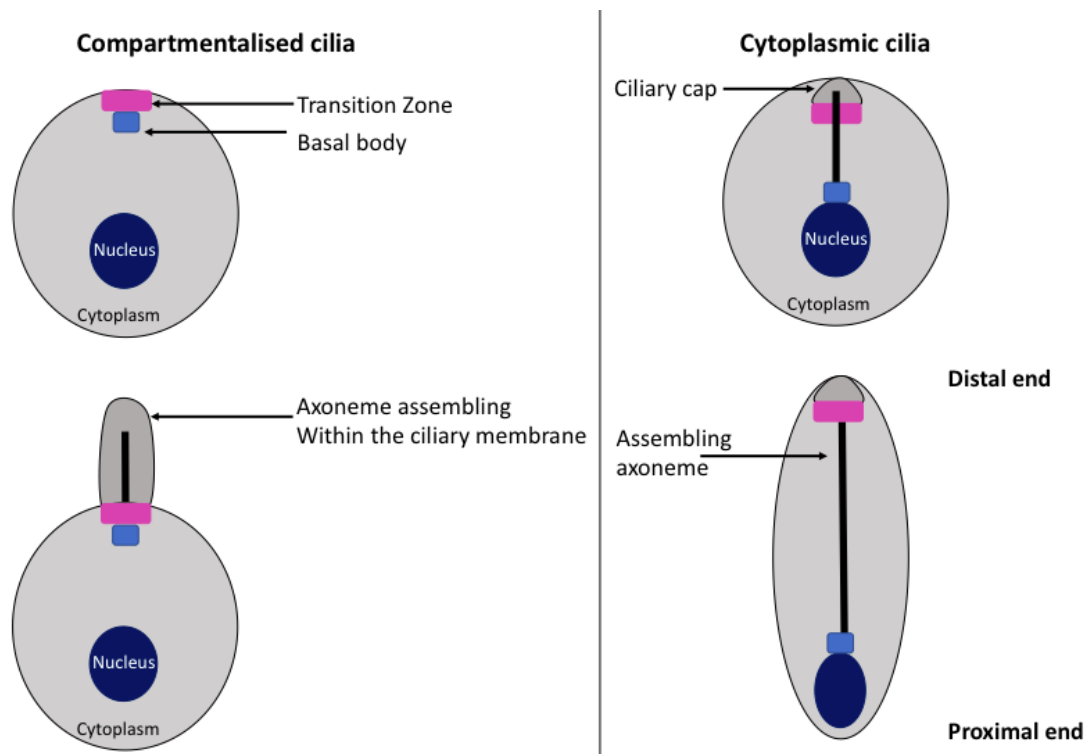


Figure 1.6 Schematic illustrating the two different methods of axoneme development. These are compartmentalised cilium formation, where the axoneme develops in a specialised compartment, and cytoplasmic cilium/flagellum formation, where the axoneme develops in the cytoplasm as this is the location of the required proteins. Adapted from Fingerhut & Yamashita, (2020).

Immunofluorescent staining and small-molecule RNA FISH (smFISH) identified an RNP granule containing the mRNAs for the testis-specific axonemal dynein heavy chains *kl-3* and *kl-5* (ODAs), and *kl-2* and *Dhc98D* (IDAs). These dynein proteins are sub-organised within the granule, with the ODA proteins localised at the core, and the IDA proteins localised peripherally.

The dynein assembly co-chaperones Ruvbl1/2 were found to be co-localised with these heavy chain proteins: initially with *Dhc98D*, and then with the other *kl* mRNAs. This RNP granule is polarised to the distal end of the flagellum and the presence of this structure allows for efficient translation of axonemal dynein during flagellar assembly in the cytoplasm (Fingerhut & Yamashita, 2020).

The assembled dynein motor complexes of the sperm flagellum differ in their ODAs from those of the chordotonal neuron. The heavy chains (HC)

mentioned above, *kl-3* and *kl-5* (which correspond to the human subunits *DNAH8* and *DNAH17*), are specific to the testes and are located on the Y chromosome (Carvalho et al., 2000). The ODA heavy chains *CG9492* and *Dhc93AB*, which are the *Drosophila* homologues of *DNAH5* and *DNAH11*, are exclusively expressed in the chordotonal neurons (zur Lage et al., 2019). The intermediate chain (IC) *CG9313/Dnai1* is common in both cell types, while the intermediate chain *Dnai2* has three orthologues in *Drosophila*: one that is chordotonal-neuron specific (*CG6053*), and two that are testis-specific (*CG1571*, *CG10859*), indicating that there are differences in the ICs as well as the HCs of the two tissues. For light chains (LC), there are two orthologues of *Dnal1*, one which is expressed in both the Ch neurons and the testis and one that is testis-specific. There are several families of light chains and whether they are all specific to one or common in both types is not known. However, evidence suggests that some will be different, for example the Roadblock family have several homologues specific to the testis and one to the Ch neuron (zur Lage et al., 2019). In contrast to the ODAs, the single-headed IDAs are similar in both cell types, while the double-headed IDA is completely absent in the Ch neuron (zur Lage et al., 2019).

1.7.4 The chordotonal organs

As previously mentioned, type-I sensory neurons are the only somatic cells in insects that have cilia, which are necessary for effective sensory transduction. Some of these sensory neurons have retained the apparatus of ciliary motility, namely the chordotonal (Ch) neurons, the cilia of which have a 9+0 axonemal arrangement and are thought to move in a similar rotary manner to that of nodal cilia, which also have a 9+0 arrangement (Fig. 1.7A).

Figure 1.7A shows that the cilia of the chordotonal neurons (in groups of 2-3) are surrounded by a scolopale cell. These cilia are attached by their tip to the extracellular dendritic cap. When stimulated the cap pulls and stretches them, opening the auditory transducer channels and leading to electrical changes and signals transmitting to the brain (Albert et al., 2020).

In the adult fly, chordotonal organs are in the Johnston's Organ (JO) and the leg (Shanbhag et al., 1992). In the leg, chordotonal neurons are specifically located in the femur, where they are contained in 32 scolopidia and are required in the mediation of proprioception and the detection of gravitational forces (Shanbhag et al., 1992). Defective proprioceptive ability (and therefore defective chordotonal neuron function) can be assessed by testing the climbing capacity of adult flies.

The chordotonal neurons of the JO are required for regulating proprioception and hearing (Albert & Gopfert, 2015). This organ is in the second antennal segment (a2), shown in Figure 1.7B, and is the largest mechanosensory organ in *Drosophila*, containing 227 sensory units or scolopidia. Stimulation occurs by the arista moving in response to near-field sound, wind or gravity, causing the 3rd antennal segment (a3) to rotate (Boekhoff-Falk & Eberl, 2014). The neurons of the JO are arranged into several groups: A-E. Neuronal types A and B are required for auditory reception, C and E are associated with gravity and wind reception, and the function of type D remains unknown (Boekhoff-Falk & Eberl, 2014).

In the larvae, chordotonal organs are in the abdominal segment walls and detect vibrations, while also functioning in proprioception (Prahlad et al., 2017; Zhang et al., 2013). These neurons are mechanosensitive in a similar manner to the chordotonal organs of the adult fly, and the ion channels that are involved in the amplification of wind- and sound-associated vibrations will be described below. This is the process where the chordotonal cilium uses energy to increase wind/sound-induced motion.

The proximal zone, which is the motile zone of the chordotonal neuron cilia (see Fig 1.7A) contains the mechanosensitive ion channels Nanchung (NAN) and Inactive (IAV), which work together in auditory transduction (Zhang et al., 2013). The distal zone, which is the sensory zone of the neuron contains the ion channel No mechanoreceptor potential C (NOMPC). *Drosophila NompC* mutants have a loss of the amplification of sound stimuli due to defective

mechanosensation, but the main role of this ion channel is in the sensation of proprioception (Effertz et al., 2011; Yan et al., 2013; Zhang et al., 2013).

Axonemal dynein motors are also required to power this amplification by assisting in transducer adaptation (Karak et al., 2015). This has been indicated by examining mutants of *dnah3* and *dnai2*, both ODA genes. Null mutants of these genes resulted in no electrical sound responses, a characteristic associated with defective amplification (Karak et al., 2015).

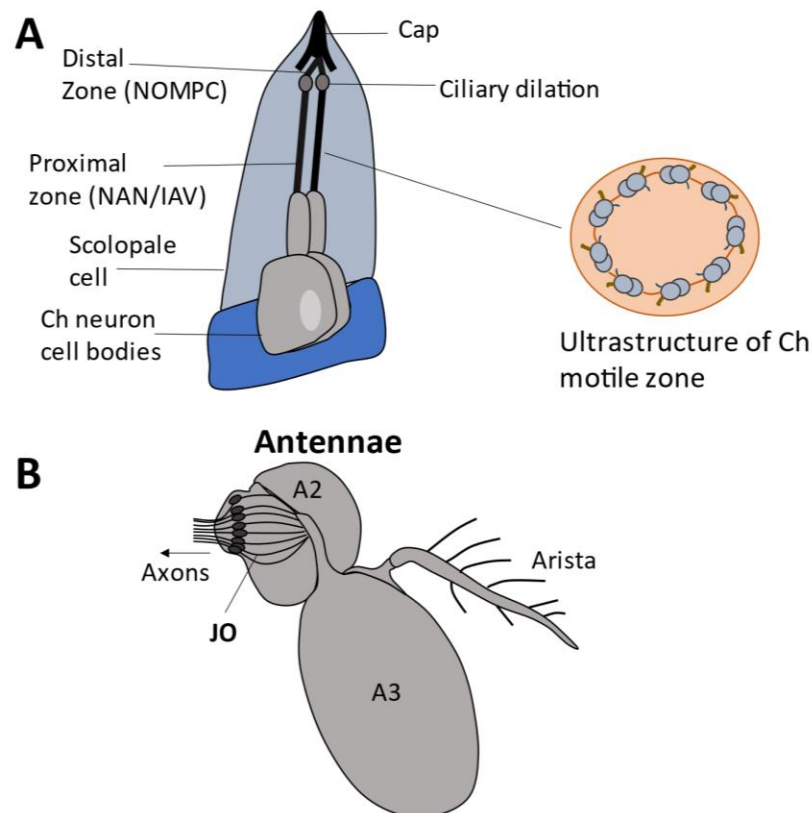


Figure 1.7 Structure of the motile chordotonal neuron (A) Scolopidium, showing 2 chordotonal (Ch) neurons with the motile ciliated proximal zone (9+0 arrangement) and non-motile ciliated distal zone. The location of the ion channels that are required for auditory reception and proprioception are labelled in the diagram: NOMPC is present in the distal zone and Nanchung and Inactive (NAN/IAV) are present in the proximal zone. **(B)** Diagram showing the location of the Johnston's Organ (JO) in the adult antenna a2 segment. The arista is responsible for the stimulation of the mechanosensitive neurons of the JO, which responded to sound, wind or gravity causing the a3 segment to rotate stretching the ch neuron cilia.

The chordotonal neuron is a cell-type that has been successfully utilised to understand the key players in the transcriptional regulation of ciliogenesis (Cachero et al., 2011; zur Lage et al., 2019). The proneural gene *atonal* (*ato*) is essential for cilia development in *Drosophila*, as it is required to activate the downstream genes involved in cilia formation and function (Jarman et al., 1993). *ato* mutant flies exhibit phenotypes associated with defective chordotonal neuron function, including auditory deficits and incoordination. *Atonal* is of similarity to the mammalian gene *atoh* in that it can rescue function in *atoh1* mutant mice and *vice versa* (Ben-Arie et al., 2000; Yan et al., 2013).

Downstream of the proneural gene *atonal* (*ato*) are the *Drosophila* transcription factors *Fd3F* and *Rfx*, which together regulate ciliogenesis and ciliary specialisation (Newton et al., 2012).

Regulatory factor X (*Rfx*) has an X-box DNA binding domain, characteristic of this family of transcription factors (Reith et al., 1990). The *Drosophila* homologue of this transcription factor is present in both external sensory cells (including olfactory neurons) and chordotonal neurons and is regulated differently by proneural genes such as *atonal* depending on the sensory neuron being specialised (Cachero et al., 2011). In *Drosophila* *Rfx* mutants, neuronal cilia are both structurally and functionally defective, supporting a role for *Rfx* in ciliogenesis (Cachero et al., 2011).

The cilia of flies lacking the transcription factor *forkhead domain 3F* (*Fd3f*) are structurally similar to wild-type. However, the mechanosensory function mediated by the chordotonal neuron cilium is defective in these mutants (Newton et al., 2012). Therefore *Fd3f*, which is the *Drosophila* orthologue of the mammalian transcription factor *Foxj1*, is required for chordotonal neuron specialisation. *Fd3f* regulates the genes required for the development of the axonemal dynein motor complexes as well as the transient receptor potential (TRP) V cation channels of the chordotonal neuron described above that are necessary for its mechanosensory role. Transmission electron microscopy (TEM) has shown that in the chordotonal neuron cilia of *fd3f* mutants, both outer and inner axonemal dynein arms are absent, resulting in ciliary immotility

and therefore supporting a role in motile cilia specialisation for this transcription factor (Newton et al., 2012).

Identifying the transcription factors that are essential to chordotonal neuron function has led to the identification of novel ciliary motility genes (i.e., downstream targets). Assessing whether these genes are specifically or highly expressed in the developing chordotonal neurons (and spermatocytes) has refined a list of genes of interest, and two of these genes, *Wdr16* and *Dpcd*, will be characterised in this project. The process of utilising the developing chordotonal neurons of the fruit fly to identify novel genes with a role in motile cilia function will be discussed in greater detail in Chapter 2.

1.7.5 *Drosophila* as a model for PCD

Drosophila is a useful model for studying human disorders due to short generation time, low maintenance costs and the genetic tools available for use in this system such as RNAi and transgenic lines. Ciliary motility genes, including those associated with PCD are almost completely conserved in *Drosophila*, making this a relatively straightforward model to gain information that can be translatable to this human disorder.

There are several advantages of using *Drosophila* to screen and analyse ciliary motility genes, as only two cell types contain the motile cilia apparatus (the sperm flagellum and chordotonal neuron). ‘Flies with PCD’ therefore have easily recognisable phenotypes: they are viable, male infertile and have auditory and proprioceptive deficits. These phenotypes can be detected by relatively simple assays: male fertility assays, adult climbing assays, and larval hearing assays. All three of these assays have been previously shown to be useful in characterising the function of motile cilia genes such as *Zymnd10*, *Heatr2* and *Wdr92*, which will be described in greater detail in the next section. This contribution indicates that *Drosophila* has been and remains to be a useful model for PCD research.

1.8 Project background: novel dynein axonemal assembly factors

1.8.1 Transcriptional control of ciliogenesis in *Drosophila melanogaster*

Drosophila chordotonal organs are completely absent in atonal mutants (Jarman et al., 1995). Atonal is a proneural gene that regulates downstream genes involved in the specialisation of sensory neurons such as the photoreceptor cells and the chordotonal neurons. This process is referred to as sense organ precursor (SOP) specification and occurs in the ectoderm during embryonic development (Jarman & Groves, 2013). During development, atonal is specifically present in the SOPs due to notch-mediated lateral inhibition. Cells divide asymmetrically until the chordotonal organ cells are terminally differentiated. Therefore, the absence of chordotonal organs in atonal mutants is due to the chordotonal SOPs not developing and differentiating. The absence of these structures results in flies exhibiting incoordination and auditory deficits (Jarman & Groves, 2013).

To identify the key players required for chordotonal neuron specialisation downstream of atonal, late-stage *Drosophila* embryos (which are the stages 10-13 i.e., when neural development begins) were used in a transcriptome analysis (Cachero et al., 2011). First atoGFP cells were separated using fluorescence-activated cell sorting (FACS) and then transcriptomic analysis was performed to identify genes differentially expressed between cells expressing atonal, and the cells in the rest of the embryo that do not. The enriched genes expressed in the developing chordotonal neurons were genes associated with ciliogenesis, including genes required for the ciliary rootlet, the IFT-A and the IFT-B complex (Cachero et al., 2011).

A second experiment was conducted using later stages of embryonic development to focus on chordotonal neuron differentiation and identify additional ciliary genes (zur Lage et al., 2019). The transcription factor cousin

of atonal (cato) was used to identify later-stage chordotonal cells in the embryo, and genes that co-expressed with this regulator were added to the list of putative ciliary genes. This extensive list was then refined using RNA in situ hybridisation to check whether the candidate ciliary genes are expressed in ciliated cells.

Analysis of the initial screening (Cachero et al., 2011), identified two transcription factors that are downstream of atonal: Rfx and fd3f - their role in the motile cilia specialisation of the chordotonal neuron is described in 1.7.2 and also shown below in Fig 1.8. As these are cilia-related transcription factors, these downstream targets suggested that ciliary genes were worth investigating further in the *Drosophila* chordotonal neurons.

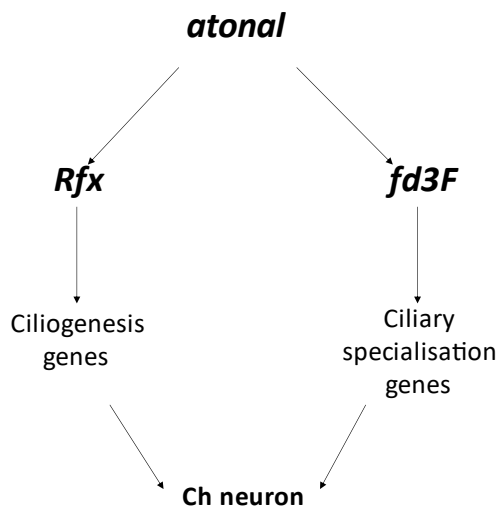


Fig 1.8. Simplified schematic illustrating the transcriptional regulation of chordotonal neuron development. The proneural gene *atonal* regulates the differentiation transcription factors *Rfx* and *fd3F*. *Rfx* has a role in the regulation of ciliogenesis and therefore is involved in the development of the external sensory neurons, as well as the chordotonal neurons. *Fd3F* regulates the genes required for chordotonal neuron specialisation and mechanosensation.

Therefore, this screen identified a long list of motile cilia genes regulated by *Rfx* and *Fd3F*, both known and novel (Cachero et al., 2011). Characterising genes from this list has the potential to identify previously unknown PCD-associated genes. The Jarman lab has aided the discovery and understanding

of several DNAAF genes: ZMYND10, HEATR2, WDR92 and DNAAF3, which will be described in further detail in this section (Diggle et al., 2014; zur Lage et al., 2021, 2018; Moore et al., 2013).

1.8.1.1 ZMYND10

ZMYND10 is a DNAAF and PCD causative gene which TEM and immunofluorescent staining has shown to have a role in the assembly of both inner and outer dynein arms (Moore et al., 2013; Zariwala et al., 2013). In *Drosophila*, *Zmynd10* is specifically expressed in motile cilia cells (indicated by RNA *in situ* hybridisation of whole embryos). Consequently, due to the many benefits of this model described above, *Drosophila* was used to assess the functional role of *Zmynd10*. GFP-tagging *Drosophila Zmynd10* supported expression specifically in motile cilia cells and indicated that this protein is primarily cytoplasmic. A P-element line (deletion mutant) was obtained, which completely disrupted the function of *Zmynd10* in the fly. As *Zmynd10* is specifically expressed in motile cilia cells, flies were viable with no obvious morphological defects. A climbing assay tested whether gene silencing caused a reduction in proprioception and indeed found *Zmynd10* deletion mutants to be uncoordinated. A fertility assay indicated *Zmynd10* males produced no offspring when mated with wild type females, due to immotile sperm. TEM analysis indicated that *Zmynd10* silencing causes both ODA and IDA defects in *Drosophila*, similar to what was observed in PCD patients with mutations in this gene (Moore et al., 2013). This study showed *Drosophila* to be a useful model organism for understanding PCD-related gene function through relatively simple behavioural and fertility assays *in vivo*.

Since its initial identification as a PCD gene, ZMYND10 has been found to interact with several proteins during the assembly of axonemal dynein intermediate and heavy chains: initially with FKBP8 and subsequently with LRRC6 (Cho et al., 2018; Moore et al., 2013).

1.8.1.2 HEATR2

The HEAT-repeat protein HEATR2 is another DNAAF and PCD gene that causes absent dynein arms and a loss of ciliary beating (Horani et al., 2012). Once again, the *Drosophila* system was utilised to further the understanding of HEATR2 function. The *Drosophila* homologue was found to be expressed in differentiating Ch neurons (detected by the transcriptome analysis previously mentioned). This discovery was confirmed through RNA *in situ* hybridisation. A P-element line was generated for *Heatr2*, which showed an absence of homozygous adults - suggesting an essential non-motile cilia function for this gene. Hence, to gain an understanding of the motile cilia function of this gene in *Drosophila*, *Heatr2* was knocked down specifically in motile cilia cells using the UAS/Gal4 system (which will be discussed in greater detail in Chapter 2). These knockdown flies were uncoordinated, indicated by climbing assay performance, as well as deaf, shown by a larval hearing assay. *Heatr2* knockdown males also produced no progeny, thus once again indicating these behavioural and fertility assays in *Drosophila* are effective at understanding function of PCD-associated genes (Diggle et al., 2014).

Both inner and outer arm dyneins were affected in *Heatr2* mutant flies, similar to what was observed in PCD patients with a mutation HEATR2. Only a small number of PCD patients have defects in HEATR2, with the mutations causing the last of ten HEAT repeats and the C-terminus of the ARM-type fold domain to be disrupted. The function of *Heatr2* outside of motile ciliated cells remains unknown, and human HEATR2 mutations have only affected motile cilia to date (the cells with the highest levels of HEATR2) - which may be the most sensitive to alteration in this gene's expression (Diggle et al., 2014).

1.8.1.3 WDR92

In addition to contributing to the understanding of the PCD-causative genes ZMYND10 and HEATR2, *Drosophila* as a model has identified a novel DNAAF, and candidate PCD gene: *Wdr92*.

WDR92 was poorly characterised functionally but known to bind to RPAP3 of the R2TP complex, which is a multiprotein complex linked to dynein assembly (Glatter et al., 2011). Similar techniques utilised in the characterisation of *Zmynd10* and *Heatr2* were once again harnessed: behavioural assays, fertility assay, RNA *in situ* hybridisation, immunofluorescent staining, TEM analysis and the generation of a CRISPR null mutant and mVenus fusion protein line. These experiments indicated that *Drosophila Wdr92* is a cytoplasmic protein expressed specifically in motile ciliated cells and required for ciliary motility. TEM analysis indicated a loss of dynein arms from the axonemes of the *Wdr92* null, with proteomic analysis indicating an effect on the assembly of dynein heavy and intermediate chains. Affinity Purification and co-Immunoprecipitation (co-IP) showed that *Wdr92* associates with the known DNAAF Spag1, as well as RPAP3 (zur Lage et al., 2018). It has been suggested that *Wdr92* binds to the RPAP3_C domain of both SPAG1 and RPAP3, and so may be associated with the R2TP complex as well as novel R2TP-like complexes, both of which will be discussed in greater detail in Chapter 3 (Patel-King et al., 2019). Therefore, this *Drosophila* model successfully identified *Wdr92* as a novel DNAAF that works with other co-chaperones during dynein assembly (zur Lage et al., 2018).

In higher organisms such as humans and mice, *WDR92* is expressed in additional tissues other than those associated with motile cilia, and so may have additional functions. As it is exclusively a motile cilia gene in *Drosophila*, shown through RNA *in situ* hybridisation and reporter line expression experiments, this model is a straight-forward system to help further understanding into this specific function of *Wdr92*, as well as other potential DNAAFs that have wider roles in humans.

1.8.1.4 DNAAF3

Loss-of-function mutations were first identified in the gene *DNAAF3* in individuals which exhibited *situs inversus* as well as outer and inner dynein arm defects. In zebrafish, a knockdown in *Dnaaf3* function resulted in similar observations: disrupted dynein arm assembly and ciliary motility, causing

laterality defects and hydrocephalus (Mitchison et al., 2012). As this protein has no typical chaperone-related domains, the specific function of this DNAAF remains unknown. The examples described above showed that *Drosophila* has been successful at furthering understanding of PCD genes, and so this model was once again utilised to gain insight into the role of *Dnaaf3*. The *Drosophila* gene is expressed in the cytoplasm of motile cilia cells, and when function is disrupted, flies show phenotypes typical of ciliary/flagellar immotility, as well as a loss of both ODAs and IDAs. Proteomic analysis of *Dnaaf3* mutants indicated a reduction in the abundance of dynein motor proteins, with heavy chains particularly affected, providing additional insight into the specific role of this gene during dynein assembly (zur Lage et al., 2021).

As well as motile cilia gene discovery and characterisation, further understanding of the mechanisms involving known PCD genes is required to develop potential therapeutics, such as chaperone therapy, which has been shown to be promising for other conditions such as Alzheimer's Disease (Martín-Peña et al., 2018). As indicated above, *Drosophila* is a useful model for understanding these complex mechanisms, which will be shown throughout this thesis.

1.9 Aims of this thesis

As discussed during this Introduction, there are many protein complexes (ODAs, IDAs, N-DRC etc) that are required to work together for motile cilia to function correctly. To ensure that these subunits (in particular the outer and inner dynein arms) assemble correctly and arrive at their destination, a group of proteins called dynein axonemal assembly factors (DNAAFs) work with chaperones to aid the complex formation of the mature motile cilia apparatus in the cytoplasm. This assembly process is further complicated by the recent evidence that some of these DNAAFs carry out their regulatory functions as part of multi-protein complexes. The specific details of these complexes remain unclear, and the literature indicates that their roles can vary across organisms. This thesis uses a *Drosophila* model to understand the role of

known and new candidate dynein assembly factors. The rationale is that the identification and characterisation of candidate assembly factor genes has proven useful for several DNAAFs so far and so can be extended to further understand the role of one of these dynein assembly complexes, as well as characterise two genes with potential dynein assembly roles identified from temporal profiling experiments conducted by the lab (Cachero et al., 2011; zur Lage et al., 2019).

In Chapter 2, using *WDR92* as a precedent, a potential dynein assembly role for the *Drosophila* homologue of *WDR16* is assessed through expression analysis as well as behavioural and fertility assays on RNAi knockdown flies. In Chapter 3, the function of the *Drosophila* homologue of *DYX1C1* (or *DNAAF4*) is characterised, once again through the analysis of gene expression and behavioural phenotypes, as well as through the analysis of the dynein motor complexes and proteomics, to evaluate whether it has a motile cilia-specific function or a wider role, as the literature suggests being the case in higher organisms. Chapter 4 builds upon our findings from Chapter 3 and details the characterisation of a potential protein partner of *DYX1C1*: *PIH1D3* (*DNAAF6*). This Chapter aims to further clarify whether these proteins interact and if so, what their specific function is as a complex during the assembly process. In Chapter 5, the *Drosophila* *DPCD* homologue (a putative ciliary gene detected from the transcriptomic data) is investigated, to pinpoint its role in motile cilia function through the assessment of null mutant phenotypes and protein interactions. Overall, the aim of this thesis is to shed further light on the specific roles of some key proteins during the dynein assembly process.

Chapter 2

Preliminary investigation of the putative DNAAF, *Wdr16*.

2.1 Introduction

2.1.1 Using transcriptomic data to identify candidate ciliary genes for further research

The general aim of this project was to identify and characterise novel genes involved in ciliary motility. Expression data obtained previously from the lab was used to generate an extensive list of genes with potential roles in ciliogenesis (Cachero et al., 2011; zur Lage et al., 2019). As described in the Introduction, the fly model has two specific cell types with motile cilia apparatus (the chordotonal neuron and sperm flagellum). Therefore, it is a straightforward *in vivo* model to characterise the specific function of candidate cilia genes. RNA *in situ* hybridisation is a useful technique for assessing whether these putative ciliary genes are expressed in *Drosophila* motile cilia cells (Cachero et al., 2011). Additionally, disrupting the function of these genes in tissues that require the motile cilia apparatus can provide insight into whether they are involved in this cellular process.

For example, as the chordotonal neuron mediates auditory reception and proprioception, hearing assays and climbing assays can be utilised to assess whether these processes are affected when genes of interest are non-functional. The sperm flagellum requires motility to fertilise the egg and reproduce successfully. Therefore, a fertility assay examining the number of progeny produced when the function of a potential motile cilia gene is disrupted in the testis can indicate whether it is required for this motor function (Moore et al., 2013). Once it has been confirmed that a gene is expressed in motile cilia cells and required for motile cilia function, further experimentation to provide insight into the specific role of this gene can be conducted. These experiments include examining the overall structure of cilium, as well as the

dynein motor complexes which facilitate ciliary motility, through transmission electron microscopy (TEM) and conducting proteomic analysis to assess whether relative abundances of proteins of interest are affected when a particular protein is non-functional (zur Lage et al., 2018).

This screening process to identify novel genes with a role in ciliogenesis has previously proven to be successful by the lab, resulting in the characterisation of three novel dynein axonemal assembly factors: *Zmynd10*, *Heatr2* and *Wdr92*, described in the Introduction. These novel cilia genes contain binding sites for the transcription factors *fd3F*, which corresponds to vertebrate FoxJ1 and is highly expressed in motile cilia cells (Newton et al., 2012), and the ciliogenesis transcription factor *Rfx* in their promoter regions. As these transcription factors regulate genes involved in ciliogenesis and ciliary specialisation, the presence of these binding sites further supports a motile cilia role for these genes (Diggle et al., 2014; Moore et al., 2013; zur Lage et al., 2018).

As previously discussed, *Zmynd10*, *Heatr2* and *Wdr92* are genes which have been selected from this extensive list and further characterised using the *Drosophila* model (Diggle et al., 2014; Moore et al., 2013; zur Lage et al., 2018). Therefore, this screening has been successful in discovering new genes related to motile cilia function. Motile cilia development and function is complex and there are many more genes to be identified that play a role in these processes. The number of unknown genes involved in motile cilia is highlighted by the fact that in only 70% of cases of the heterogeneous motile ciliopathy PCD is the genetic cause known (Takeuchi et al., 2016). Therefore, it is crucial to identify novel motile cilia genes, for both diagnostics and potential therapeutics. From the extensive list of potential cilia genes, *CG10064*, which is the predicted homologue of the human gene *WDR16*, was selected as a promising candidate for further investigation as it is highly expressed in developing chordotonal neurons (Fold Change = 38.1423) (zur Lage et al., 2019). This candidate was chosen from the list because at the onset of this project, not much information was known regarding its specific function. A few

publications, described below, suggested a motile-cilia role for *WDR16*. However, the specifics of how this gene is involved in motile cilia function had not been deduced, making it an ideal candidate to study using our straightforward model. This screening also detected *CG13901*, the *Drosophila* homologue of *DPCD*. This gene was also selected as a candidate of interest in this project because of the lack of publications regarding its function, as well as its high expression level in chordotonal cells (zur Lage et al., 2019). The role of this gene and whether it has a function in motile cilia will be investigated in Chapter 5.

2.1.2 Mutations in *WDR16* cause laterality defects, hydrocephalus and male infertility and therefore support a motile cilia-related role

Our hypothesis is that *WDR16* is a novel gene involved in motile cilia function, specifically axonemal dynein assembly. Therefore, it was important to initially assess what is known about this gene in other organisms.

In humans, the gene *WDR16* has been associated with *situs inversus*, as it participates in asymmetric gene expression of the developing embryo (Postema et al., 2020). This asymmetry is facilitated by extraembryonic fluid, which moves because of nodal cilia. A whole-exome study was conducted in members of a consanguineous family and a homozygous deletion in *WDR16* segregated with situs anomalies including heterotaxy and *situs inversus*. Therefore, it has been suggested that *WDR16* may have a role in laterality patterning, mediated by nodal cilia (Ta-Shma et al., 2015).

In rats, *WDR16* is expressed in motile cilia-containing tissues such as the testis and not present in primary cilia cells (Hirschner et al., 2007). In the testis, *Wdr16* is localised to microtubule structures such as the manchette and the axoneme of the flagellum. *WDR16* has been suggested to play a role in the stability, maintenance, and motility of the sperm flagellum. The absence of *WDR16* therefore results in the phenotype of male infertility (Tapia Contreras & Hoyer-Fender, 2020).

When *WDR16* is knocked down in zebrafish it results in severe hydrocephalus, a phenotype associated with defective motile cilia function. However, the hydrocephalus observed was not caused by immotile cilia but due to the presence of enlarged ventricles. This could be caused by *WDR16* as a cilia-associated gene having an indirect role in cell polarity, and required for the signal transduction mechanisms which regulate brain fluid homeostasis (Hirschner et al., 2007). Interestingly hydrocephalus is not a common symptom of the motile ciliopathy PCD, suggesting ciliary motility may not be the deciding cause of hydrocephalic conditions, including those caused by a *WDR16* knockdown (Sakamoto et al., 2021).

Thus, these results in other organisms support our motivation to work on *Wdr16*, as it has the potential to be a novel axonemal dynein assembly factor (DNAAF). *Wdr16* contains a WD40 repeat domain, the role of which is to mediate protein-protein interactions and may be important for the role of *Wdr16*. Another WD40 repeat protein, *Wdr92* has been reported to have a role in the axonemal assembly of dynein motor complexes (zur Lage et al., 2018). While *Wdr16* most likely acts in the assembly or stabilisation of protein complexes related to ciliary motility, the specific details of its role in the process remains relatively unknown.

As *Wdr16* is highly conserved throughout evolution, identifying and understanding the role of the *Wdr16 Drosophila* homologue can provide information which can be translated to humans.

2.1.3 Applying the Gal4/UAS system to knockdown the function of the *Drosophila* gene CG10064 to test hypothesis

To investigate the hypothesis that *Wdr16* has a role in the axonemal dynein assembly process, we start by determining whether *Wdr16* is required for ciliary motility. As mentioned above, the motile cilia machinery is present in two specific cells in *Drosophila*: the chordotonal neuron and sperm flagellum

(Cachero et al., 2011). As there are a wide range of genetic tools available for use in the *Drosophila* model, we can disrupt the function of *Wdr16* specifically in these cell types and assess whether motile cilia are affected. Knocking down the function of putative motile cilia genes in the chordotonal neurons and the testis has proven beneficial previously when characterising function (Diggle et al., 2014; zur Lage et al., 2018).

Specifically knocking down gene function is achieved using the well-established Gal4/UAS (upstream activator sequence) system. This system has two key players: the yeast transcription factor Gal4, which is then placed under the control of a tissue-specific promoter, as well as the gene of interest cloned downstream of Gal4 binding sites (UAS). When Gal4 binds to the UAS sequence (i.e., its binding sites), this results in the activation of the gene of interest in cells where the tissue-specific promoter is present (Brand & Perrimon, 1993) . This system is demonstrated below in Fig 2.1.

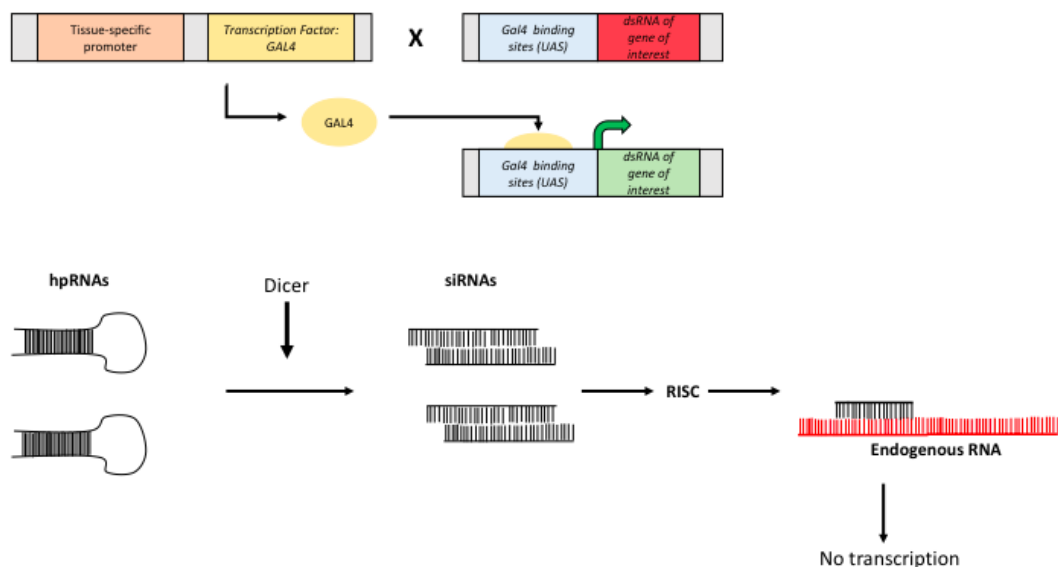


Fig 2.1. Schematic explaining the Gal4/UAS system. Gal4 is under the control of a tissue-specific promoter. Separately, a double-stranded RNA of the gene of interest is cloned downstream of Gal4 binding sites (upstream activator sequence). When these are combined, Gal4 protein binds to the UAS sequence resulting in the successful activation of the hairpin (hp) RNAs of the gene of interest in the tissue of interest (where the promoter is). These are broken down by the enzyme Dicer into small interfering RNAs (siRNAs) which join the RNA-induced silencing complex (RISC) and degrade the endogenous RNA, inhibiting transcription.

To examine the function of *CG10064*, this binary system can be used to observe the effect a knockdown of this gene has during *Drosophila* development. This knockdown is facilitated using RNA interference (RNAi) and silences the gene at mRNA level. The enzyme Dicer degrades double-stranded RNA into small interfering RNAs (siRNAs). These siRNAs then join the nuclease complex RISC (RNA-induced silencing complex), and this complex breaks down the homologous RNA therefore preventing transcription (Agrawal et al., 2003).

The Gal4/UAS system in combination with RNAi technology results in the controlled disruption of a gene in a cell- or tissue-specific manner during different developmental stages. Therefore, this genetic tool is advantageous when investigating genes resulting in lethality or male infertility, as fly lines are viable and fertile when the Gal4 transcription factor is not expressed in tissues crucial to these phenotypes. To knock down the function of a gene, the Gal4 transcription factor binds to the UAS sequences and activates the gene of interest. If this gene is expressing hairpin RNAs, RNAi will begin upon the binding of the Gal4 and result in the degradation of target mRNAs and knockdown the expression of the gene of interest in the tissue of interest (Yamamoto-Hino & Goto, 2013). This system is useful when conducting preliminary experiments to learn more about a particular gene, such as *CG10064*, as genetic function can be assessed in different tissues during development.

2.2 Aims of this chapter

The aim of this chapter is to compare human *WDR16* and the *Drosophila* homologue bioinformatically, and to characterise the role of this gene in relation to motile cilia.

2.3 Results

2.3.1 *CG10064* is the *Drosophila* homologue of WDR16

Motile cilia genes are well conserved in *Drosophila melanogaster* (zur Lage et al., 2019). To confirm that *CG10064* is the *Drosophila* homologue of the human gene *Wdr16*, I conducted a bioinformatic analysis using the DRSC Integrative Orthology Prediction Tool (DIOPT) (Hu et al., 2011). This alignment (Fig. 2.2) shows that *CG10064* is the *Drosophila* homologue of *Wdr16*, with 54% similarity. Therefore, for the remainder of this thesis, the *Drosophila* gene *CG10064* will be referred to as *Wdr16*.

Alignment Length: 643 **Identity:** 232/644 (36%)
Similarity: 350/644 (54%) **Gaps:** 48/644 (7%)

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Human   18 AVIGFNGHVPTGLKCHPDQEHMIYPLGCTVLIQAINTEQNFLQGHGNNVSCLAISRSGEYIASG 82
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly     15 AIFGINGKVENGVQRQHPVTGSIIIFALGNKIGIADYKKNTQEFVAGHTNTISCLDLKSGKYMASG 79
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human   83 QVTFMGFKADIILWDYKNRELLARLSLHKKGKIEALAFSPNDLYLVSLGGPDGSSVVVWSIAKRDA 147
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly     80 QINHMGRFGYVVIWNYDQRKEIARHDLHKVSVQAICFTAQDRCVVSIGGVDDGSSVIVFDMFAFSP 144
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  148 ICGSPAAGLNVGNATNVIFSRCDDEMFTAGNGTIRVWELDLPNRKIWPTTECQTGQLKRIVMSIG 212
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    145 ICQTPASRAISGNALTVRPLHNNPYFFVTAGDRHLRLWSILREEKKLYVQDVLMASTRVFCAR 209
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  213 VDDDDSFYYLGTGTTGDILKM-----NPRTKLLTDVGPADKDFSL 251
      ::.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    210 INKTDEFAYLGTGTGDIVKIVLNCCDRDHVSKQGSTSCILGAFGTHNPR---KPTGRDCNRYVN 270
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  252 GVSAIRCLKMGGLLVGSGAG-----LLVFCKSPG-----YKPIKKIQLQGGSITITLR 299
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    271 GVRALYVLEGGRLLIGAGNGDIELVEERTDVPLINFRDYPGPTWPYLRITLKKTHVSGRISSF-VR 334
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  300 GEGHQFLVGTTESHIYRVSTDFKETLIATCHFDAVEDIVFPFGTAELFATCAKKDIRVWHTSSN 364
      ::.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    335 SKTDMFYICTDTNELIYGLNIKTWVLKLLRCHTKSVYICITFPKNYSGVFATSGKECIRIWSGRK 399
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  365 RELLRITVPNMTCHGIDFMRDGKSIISAWNDGKIRAFAPETGRLMYVINNAHRIGVTAIATSDC 429
      :|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    400 QELLRIMVYNFNCACVRFTHDGTSIVSVWNDGIIRAFTPTITGRLIYAIPNAHNKGCALAVASSG 464
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  430 KRVISGGGEGEVVRWQIGCQTQKLEELKEHKSSVSCIRVKRNNEECVTASTDGTCTIIDLVLRLR 494
      ::.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    465 RLIVTGGIEGQVRVWKIDPYRQDLVGLKDHSGPITSLDINYLDEVISACTDGSQCVIWDIKRMT 529
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  495 RNQMILANTLFQCVCYHPEEFQIITSCTDRKIAYWEVFDGTVIRELEGLSGSINGMDITQEGVH 559
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    530 RKQVVTANTQFMSASYFPTGVQVITCGSDGRIIYWMVYNGALIRELTASKKSSVNCLAINETGDY 594
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Human  560 FVTGGNDHLVKVWDYNEGEVTHVGVGHSGNITRIRISPGNQYIVSVSADGAILRWKYP 617
      |:.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
Fly    595 FITVGSDLQVKLWDYNSGAVVGIGSEHASSVISCAYSPCMTMFTVGSTDGSLIWDVP 652

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Fig 2.2. *CG10064* is the *Drosophila* homologue of *Wdr16* Protein alignment between human *WDR16* and the *Drosophila* gene *CG10064* (*Wdr16*), with 54% similarity. Alignment conducted using the online software DRSC Integrative Orthology Prediction Tool (DIOPT). Alignment indicates degree of similarity between amino acids; with “|” indicating exact match, “:” indicating a strong positive score on the BLOSSUM62 matrix and “.” indicating any other two bases which align.

2.3.2 *Wdr16* is regulated by the transcription factors *Fd3f* and *Rfx* in *Drosophila*

The transcription factor binding sites present in the upstream promoter region of a gene can give an insight into the function of that gene (Boeva, 2016). As mentioned above, *Rfx* is a ciliogenic transcription factor and *Fd3f* is a transcription factor required for the specialisation of motile ciliated cells (Dubruille et al., 2002; Newton et al., 2012). The upstream promoter region of *Wdr16* was examined on the UCSC genome browser for *Rfx* and *Fd3f* binding sites to support a role for this gene in motile cilia function (Kent et al., 2002). The presence of these binding sites, RYYNYY1,3RRNRAC for RFX, and RYMAAYA for FD3F, near the transcriptional start site (Fig. 2.3A) supports the hypothesis that *Wdr16* is regulated by these transcription factors (Laurençon et al., 2007; Newton et al., 2012). Therefore, this result supports the hypothesis that *Wdr16* is a motile ciliary gene that is conserved between humans and *Drosophila*.

2.3.3 *Wdr16* is transcribed in motile cilia specific cells

Assessing what cell types a gene is expressed in, can give insight into its genetic function (Alberts et al., 2002). Location of *Wdr16* in *Drosophila* was examined to identify whether this gene was present in motile cilia cells. FlyAtlas2 (Chintapalli et al., 2007) indicates that *Wdr16* is highly enriched in the adult testes transcriptome (881 units), where motile sperm flagella are located. Additionally, the transcriptome analyses conducted by the Jarman lab previously (Cachero et al., 2011; zur Lage et al., 2019) revealed *Wdr16* to be highly enriched in the chordotonal neurons of developing embryos (38.1423 fold). Together, these results support expression of *Wdr16* in motile cilia cells.

To support the bioinformatic and transcriptomic enrichment data as well as to assay for additional expression in other tissues, the mRNA expression pattern of *Wdr16* was examined using RNA *in situ* hybridisation. Late-stage embryos were assessed, as this is the time-point when motile cilia genes are active (Cachero et al., 2011; zur Lage et al., 2019). Figure 2.3B shows *Wdr16* mRNA to be present in chordotonal neuron precursors. *Wdr16* has a similar

expression pattern to that observed for DNAAFs, including *Zmynd10* and *Wdr92* (Moore et al., 2013; zur Lage et al., 2018). Therefore, this mRNA expression pattern supports a motile cilia role for *Wdr16*.

Binding sites for *Rfx* and *fd3f* are located upstream of *Wdr16*, suggesting that these regulators control its expression. *fd3f* null mutant embryos were examined to assess whether *Wdr16* expression was disrupted. Figure 2.3C shows a loss of *Wdr16* expression in null mutant embryos, supporting the idea that *Wdr16* is a motile cilia gene regulated by the transcription factor *Fd3f* and is expressed in developing chordotonal neurons.

2.3.4 *Wdr16* RNAi knockdown males are infertile, due to immotile sperm

As *Wdr16* is highly expressed in the testis and proposed to be a motile cilia gene, male fertility is predicted to be defective when this gene is non-functional. To test this theory, the function of *Wdr16* was knocked down using RNAi technology and a testis driver, *Bag of marbles (Bam)-Gal4*. The *Wdr16*RNAi knockdown (KD) males were then crossed to wild-type virgin females, and a fertility assay was conducted. As a control, male progeny from the KK control line (the line in which the RNAi constructs were injected, VDRC #60100) crossed to the *Bam-Gal4* line. Results of this assay (Fig. 2.4A) indicated *Wdr16* KD males were infertile, with significantly less progeny being produced.

To confirm infertility was caused by defective sperm motility, the testes were removed from the fertility assay flies post-experiment. A 'testis crush' was performed and samples were observed using brightfield microscopy. Control males (Fig 2.4B) had motile sperm, which were observed moving individually in S-shaped waves (black arrows). No movement of sperm was observed in the *Wdr16* KD testes (Fig 2.4C), with the sperm remaining grouped in large bundles and not moving towards the seminal vesicle, which remained small in size. The observation of immotile sperm supports the significant fertility assay result obtained and a motile cilia role for *Wdr16*.

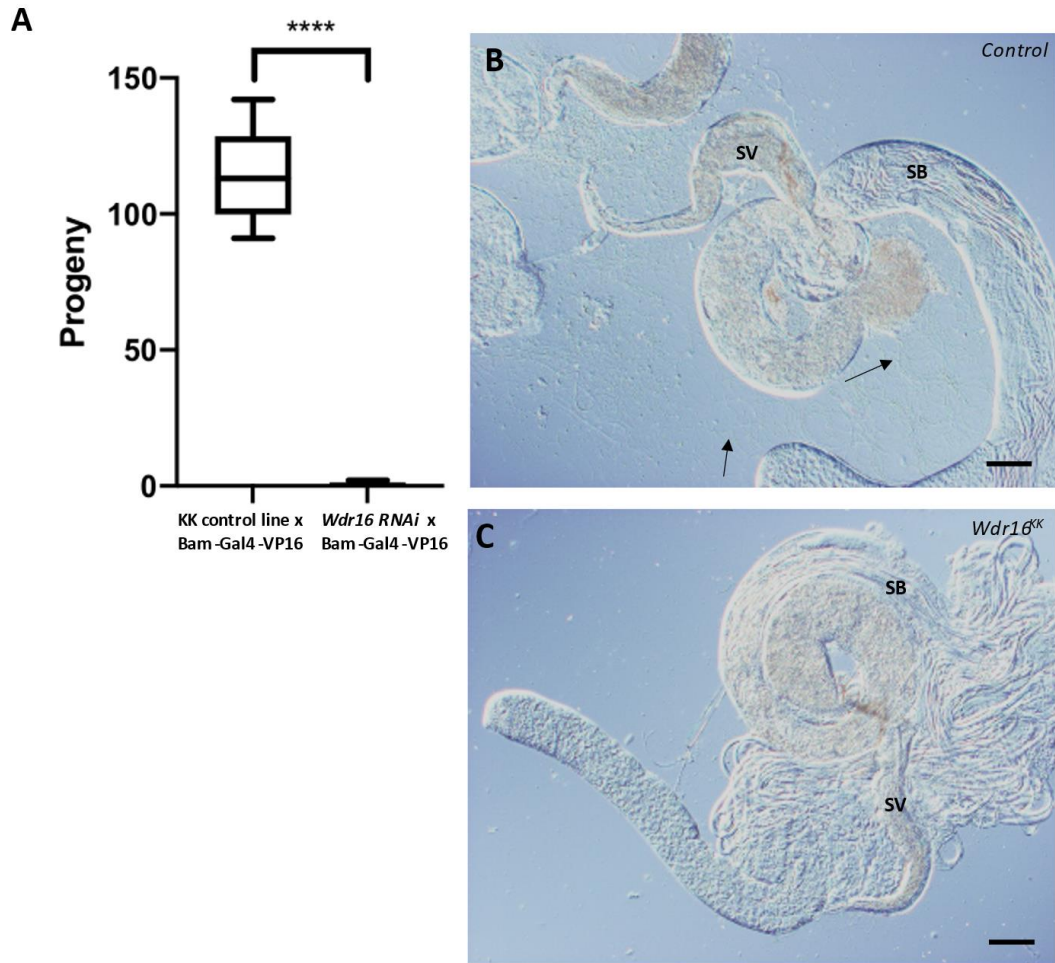


Fig 2.4. RNAi KD of *Wdr16* shows a requirement for functional motility in the sperm (A) Control males generated progeny when mated with WT virgin females. *Wdr16* KD males had a dramatic reduction in the number of progeny when crossed with WT virgin females (**** $P < 0.0001$). $n = 10$ males. (B) Control adult male testis from fertility assay showing individual sperm making curved movements (black arrows) upon release. The Seminal Vesicle (SV) is large. (C) *Wdr16* RNAi testis-KD from fertility assay showing large sperm bundles (SB) but no individual sperm observed moving. Seminal vesicle is small and lacking mature sperm. Bars: 100 μ m. Significance for (A) was determined by Mann Whitney test.

2.3.5 *Wdr16* RNAi knockdown adults do not have defective proprioception

Drosophila are negatively geotactic, and therefore move against the gravitational pull (Linderman et al., 2012). The ciliated chordotonal neuron detects these gravitational forces, and therefore defective ciliary function can result in observable proprioceptive and gravitaxis defects. This defect has been previously found in KDs of many ciliary motility genes such as *Zmynd10*, *Heatr2*, *Wdr92* (Diggle et al., 2014; Moore et al., 2013; zur Lage et al., 2018). As *Wdr16* has a motility role in the testes, the next step was to identify whether a KD of *Wdr16* disrupts the function of the other motile ciliated cell type in *Drosophila* - the chordotonal neuron. This defect can be measured as reduced climbing ability, correlating that poor climbing performance is a phenotype of irregular *Drosophila* ciliary motility.

The function of *Wdr16* was knocked down using the pan-neuronal driver *Scabrous-Gal4* (*Uas-Dcr-2*; *Sca-Gal4*). Batches of 8-12 female flies were placed in a graduated cylinder that was divided into four quadrants. This cylinder was banged on a soft surface 5 times, bringing the flies into the bottom quadrant. As the flies are negatively geotactic, they climb towards the top of the cylinder. After a 10-second recording period of climbing ability, the number of flies present in each quadrant was recorded for each genotype. There was no significant difference in climbing ability between the *Wdr16* KD flies and control group (Fig 2.5A). This result suggests no proprioceptive defect is present when the function of *Wdr16* is knocked down in chordotonal neurons.

An important caveat to note when analysing climbing assay data is that other factors may be influencing climbing ability. These factors include phototaxis, and so conducting these assays in a dark environment has been shown to worsen subtle climbing defects (Sun et al., 2009). This climbing assay was repeated under dark conditions to observe whether subtle proprioceptive deficits would become more severe. However, there was no decreased climbing ability observed for *Wdr16* KD flies, indicating phototaxis was not masking chordotonal defects (Fig 2.5A). This was an unexpected finding, as

the literature suggests *Wdr16* to have a motile cilia role, and therefore contribute to chordotonal neuron function.

2.3.6 *Wdr16* RNAi knockdown larvae do not have hearing defects

The chordotonal neuron also mediates *Drosophila* auditory reception, and so another method of investigating chordotonal neuron function is by conducting a larval hearing assay. The aim of this experiment was to further validate the climbing assay results obtained and to assess whether chordotonal neuron function was disrupted during development.

Drosophila larvae have chordotonal neurons in their abdominal segment walls, the role of which is to detect vibrations through their mechanosensitive structure. These neurons can be observed in the embryo images shown in Figure 2.3 (Jarman et al., 1993; Zhang et al., 2013). In their natural environment, these structures are advantageous as they allow the larvae to sense nearby predators and respond in an act of defence: the ‘startle-freeze’ behavioural response. In a lab environment, this behaviour can be replicated in a controlled manner by playing a tone of a similar frequency to that of a predator (wasp wing - optimally 400-1000Hz) (Zhang et al., 2013; zur Lage et al., 2018). Larvae are placed upon an agar plate on top of a speaker. The number of larvae contracting before and during a 1s 1000Hz sine tone is recorded. If there is a significant difference between the number of larvae contracting before and during the tone, this indicates that a behavioural response was exhibited upon the initiation of the auditory stimulus. If no difference takes place, this may show that no response occurs due to the larvae having hearing deficits. This assay has previously aided the characterisation of several DNAAFs by measuring whether auditory function is affected in these mutants (Moore et al., 2013; zur Lage et al., 2018).

In this experiment, *Wdr16* KD larvae behaved similarly to the control group and had a significant response to the stimulus as shown in Figure 2.5B.

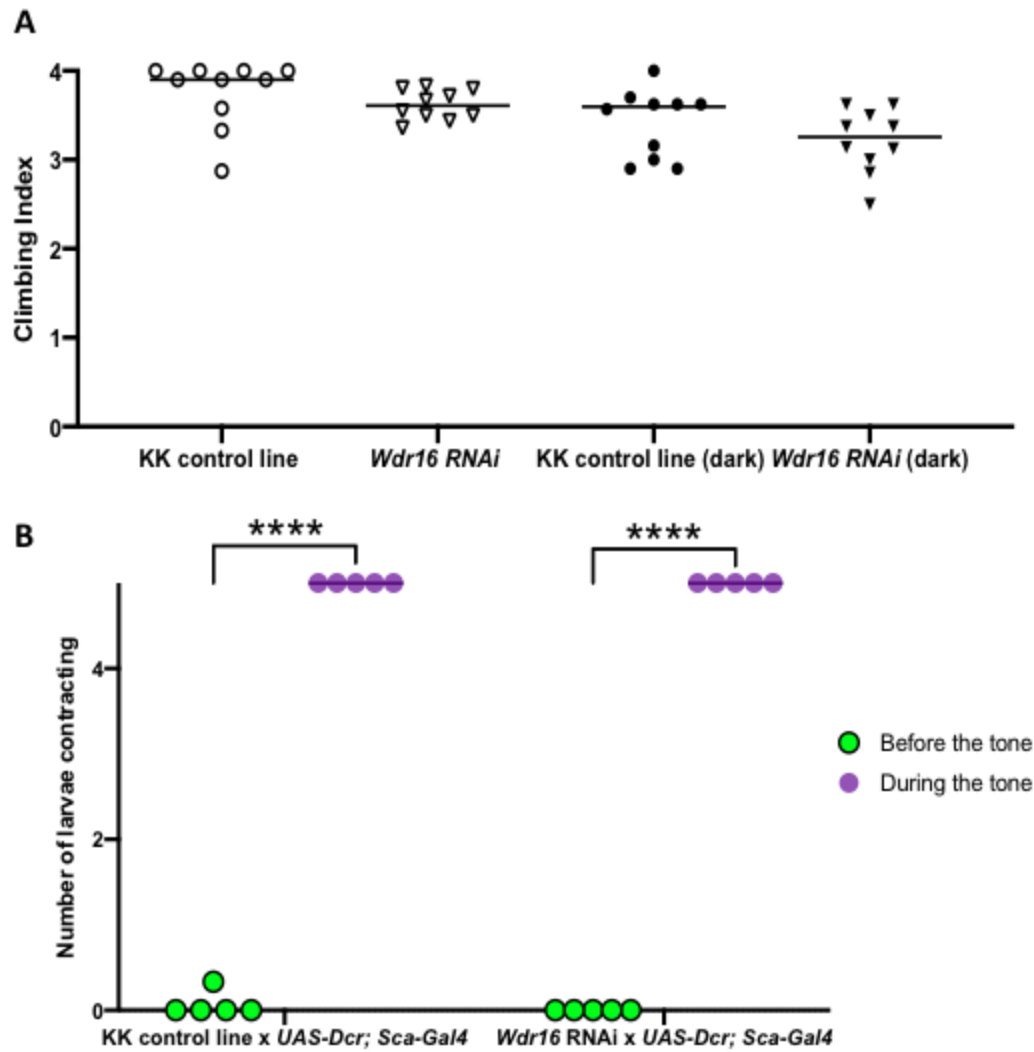


Fig 2.5. RNAi KD of *Wdr16* does not show a requirement for functional motility in the chordotonal neuron. **(A)** Bar graph (with median and individual values) showing *Wdr16* RNAi knockdown flies do not show significant proprioceptive defects in comparison to control in either light or dark environments ($P > 0.9999$). While there is a trend for the *Wdr16* KD to have a lower climbing index than the control, this result is not significant. Both lines were crossed to *UAS-Dcr-2, Sca-Gal4* to achieve KD in Ch neurons. $n = 10$ batches of 8-12 flies. **(B)** Plot (with individual values and median) showing that *Wdr16* KD larvae have no auditory deficits. There is a significant difference ($P > 0.0001$) between the number of larvae contracting before and during the 1000 Hz sine tone for both the control and KD groups. $N = 5$ batches of 5 larvae, exposed to 3 tones 20s apart. Significance was determined by Mann Whitney test (A) and 2-Way ANOVA with Sidak's multiple comparisons (B). Significance on plots is shown using asterisks: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

The hearing and climbing assay results suggest that when *Wdr16* is knocked down in the developing nervous system, chordotonal neuron function is not strongly affected. This was an unexpected finding and leads to several questions. RNAi has previously been revealed to be ineffective at completely knocking down the function of genes, so perhaps in this situation the technology was unsuccessful at knocking down the function of *Wdr16* in the nervous system but effective at knocking down the function in the testis. This could be tested through qPCR analysis, examining and comparing *Wdr16* gene expression levels in the head and the testes. Another potential reason for this result is that *Wdr16* may have a more vital role in the sperm flagella (which has a 9+2 axonemal arrangement) than in the chordotonal neuron (which has a 9+0 axonemal arrangement) and so it is important to gain insight into its function in other organisms to further assess whether this may be the case.

2.4 Discussion

The hypothesis at the onset of this project was that *Wdr16* was a novel gene with a role in axonemal dynein assembly. However, this hypothesis was not supported. The experiments described above supported a ciliary motility role for *Wdr16* in sperm, and the RNA *in situ* hybridisation of whole-mount embryos indicated specific expression in developing chordotonal neurons. Therefore, our next planned step included assessing whether the motile cilia role of *Wdr16* was specifically required during axonemal dynein assembly. These experiments were to include generating a null mutant using CRISPR/Cas9 technology and examining how dynein subunits localised through immunofluorescent staining and confocal microscopy as well as how the entire outer and inner dynein arm structures were affected using transmission electron microscopy (TEM). However, during this project it was published that *Wdr16* is a microtubule inner protein (MIP) (Owa et al., 2019), and so further investigation of its function in *Drosophila* was halted to focus on a more

promising candidate with a potential role in dynein assembly (*CG13901/Dpcc*, Chapter 5).

2.4.1 *Wdr16* is a microtubule inner protein (MIP)

Since the onset of this project, a novel group of proteins that are localised to microtubules have been identified and characterised as microtubule inner proteins (MIPs). MIPs have a role in maintaining the stability of the ciliary axonemal structure and protecting the axoneme from the mechanical stress caused by ciliary movement (Ma et al., 2019).

In *C. reinhardtii*, removal of the *Wdr16* homologue results in the loss of MIPs in B-type microtubules, causing weakness in the overall axonemal structure (Owa et al., 2019; Wallmeier et al., 2020). The location of the *C. reinhardtii* *Wdr16* homologue in the microtubule doublet is shown in Fig 2.6.

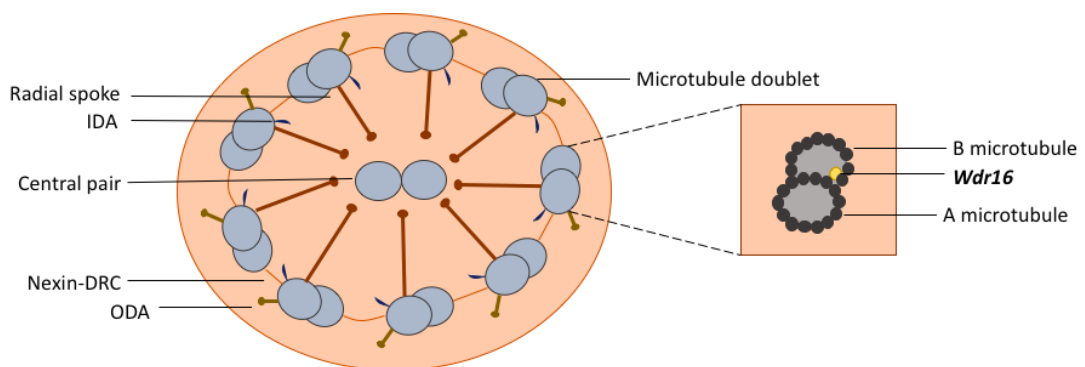


Fig 2.6. Schematic showing the inner structure of a motile cilium. There are 9 microtubule doublets surrounding a central pair. On each of the outer doublets there are outer dynein arms (ODAs) and inner dynein arms (IDAs) which facilitate ciliary motility. The microtubule doublet contains microtubule inner proteins, and the *C. reinhardtii* homologue of *Wdr16* is proposed to function as one of these, located in the B-tubule near protofilament B9 and B10.

In humans defective MIPs have been linked to laterality defects, like those described above observed in patients with *Wdr16* mutations (Ta-Shma et al., 2015). Interestingly, a deficiency in *Wdr16* has been described as causing a motile ciliopathy that does not fulfil the criteria of a complete PCD diagnosis (Dougherty et al., 2020). This indicated that *Wdr16* did not have a function

typical of motile-cilia-related genes characterised previously by our lab (Diggle et al., 2014; Moore et al., 2013; zur Lage et al., 2018).

There are structural differences as well as differences in the molecular architecture of MIPs between organisms (Kirima & Oiwa, 2018). Therefore, it would be interesting to assess what the function of *Wdr16* is in relation to microtubule stability and structure in *Drosophila*. Future work could involve the generation of a *Wdr16* null mutant by homology-directed repair, replacing the open reading frame of this gene with the mini-white gene and selecting for transformants based on eye colour. Cryogenic electron tomography (Cryo-ET) is a technique which has proven to be advantageous in providing high-resolution detail of the microtubule inner structures in other organisms, including *Chlamydomonas*, *Tetrahymena* and sea urchins (Kirima & Oiwa, 2018; Pigino et al., 2012). This technique could be performed on the *Wdr16* null mutant as well as a control group to examine how the axonemal structure is affected, providing insight into the specific function of this gene in *Drosophila* and whether it is also located on the tubular inner walls.

MIPs also have been proposed to interact with the dynein motor complexes that facilitate motility but are not considered to have an assembly factor role as this interaction occurs after the complexes have been transported and docked to the cilium (Ichikawa et al., 2019). When *Wdr16* is knocked down, it results in male infertility caused by sperm immotility. A defect in a MIP may result in the loss of a connection required for ciliary beating, and therefore the role of *Wdr16* solely as a MIP may cause this immotility phenotype.

Wdr16 has been reported to localise to the manchette and flagellum in male germ cells, supporting the role in male fertility which we found the *Drosophila* homologue to have (Tapia Contreras & Hoyer-Fender, 2020). If MIPs interact with outer and inner dynein motor structures, they could provide essential signals to ensure the dynein motor complexes localise to their correct location along the cilium. The generation of a *Wdr16*mVenus fusion protein could aid the identification of the localisation pattern of this protein in the motile cilium, providing further insight into its function during axonemal assembly and

stabilisation by assessing whether it is expressed in the cytoplasm where dynein assembly takes place, or is its sole function as a MIP in the axoneme.

2.4.2 The use of RNAi to knockdown the function of a gene

RNAi technology is widely used to investigate the function of individual genes and the mechanism of how this process works is described in the introduction of this chapter. This technology has been invaluable to research and has illuminated the function of many genes. As hairpins for almost all genes are readily available as well as Gal4 driver lines for many tissues, researchers can knockdown genes of interest both temporally and spatially (Southall & Brand, 2008; Jenett et al., 2020; Weaver et al., 2020).

However, it is important to ensure that gene function has been effectively knocked down by quantifying at both mRNA and protein level. Sometimes the hairpin cannot effectively knock down the target RNA or if the gene's protein has a long half-life, the function can still be conducted despite a reduction in mRNA. Many techniques have aided RNAi validity including Northern blot analysis, RT-PCR, western blot analysis, immunofluorescence, and flow cytometry (Mocellin & Provenzano, 2004). A limitation of this research is that the RNAi efficiency was not followed-up and in the future should be tested using the techniques mentioned above. As effectiveness of RNAi can vary it is important to always take this into consideration when analysing results obtained, and to remember that it is a knockdown of gene function as opposed to a total knockout of a gene. Another method of validating the RNAi experiment is to ubiquitously knockdown the gene in the fly, using a driver line such as tubulin-Gal4. If Wdr16 has a more important role in the testes than in the peripheral nervous system you would expect to observe similar results to that observed in this Chapter.

Another disadvantage of this system is that RNAi can also lead to off-target effects. Activation of the interferon system as well as degradation of closely related mRNAs have been reported (Mocellin & Provenzano, 2004). Therefore, it is important to be aware of this detail when analysing observed phenotypes.

An additional factor to consider is that an ineffective RNAi line or a weak Gal4 line can limit insight into gene function. Overexpression of Dicer-2 (Dcr-2) can cause a stronger knockdown. This enzyme is what cleaves the long dsRNAs into siRNA, resulting in the knockdown of gene function. It has been reported that the addition of Dicer-2 is useful when studying gene function in neuronal cells, as RNAi on its own can be ineffective (Dietzl et al., 2007). Another method of enhancing knockdown efficiency is increasing temperature as the transcription factor Gal4 is more active at higher temperatures (Ni et al., 2008). Both limitations were considered when conducting RNAi knockdown experiments in this chapter. UAS-Dcr-2 was crossed in when using the neuronal driver line and genetic crosses were kept at 28.5°C to increase the efficiency of the knockdown. However, no defective chordotonal neuron function was observed in *Wdr16* KD flies.

Another limitation of RNAi is that this technology generally works better in males, perhaps due to higher Gal4 activity (Ni et al., 2008). As we saw an effect in the fertility assay (male flies, non-neuronal driver) and no effect in the climbing or hearing assays (female flies, neuronal driver), this could indicate that male flies may be advantageous for RNAi experiments in future work. If the RNAi was effective at knocking down the function of *Wdr16* in the developing nervous system and resulted in no observable phenotype, this may indicate that *Wdr16* has a role in the *Drosophila* sperm flagellum (9+2 microtubule doublets) but not in the *Drosophila* chordotonal neurons (9+0 microtubule doublets).

2.4.3 *Wdr16* may have a more important role in 9+2 motile cilia in *Drosophila* than in 9+0 motile cilia

The motility of sperm flagella and cilia relies on coordinated activity of the axonemal dynein motor complexes which are docked on the outer doublet microtubules (Viswanadha et al., 2017). In humans, motile cilia and flagella contain a '9+2' axoneme, with 9 outer microtubule doublets surrounding a central pair. Nodal cilia have a 9+0 axonemal structure, and so the central pair and radial spokes are absent. These cilia move in a different manner to the

9+2 motile cilia, resulting in a whip-like movement rather than a beat (Ma et al., 2019). Even within the group of organelles containing 9+2 microtubule arrangement, there is variation in their beat frequency and the amplitude of this. Therefore, *Wdr16* may be required for specific types of motile cilia.

It has been reported that *Wdr16* may have a role in laterality patterning in humans, which is regulated by nodal cilia (9+0) (Ta-Shma et al., 2015). *Wdr16* has also been reported to play a role in male fertility (9+2) (Hirschner et al., 2007; Tapia Contreras & Hoyer-Fender, 2020). Our results suggest a role for *Drosophila Wdr16* in male fertility (9+2) but not in chordotonal neuron function (9+0). As the motile zone of the chordotonal neuron functions in detecting vibrations and promoting active amplification, it moves in a quite different manner to the beat of the sperm flagella or the whip-like motion of the nodal cilia. Therefore, *Wdr16* may not be required for the chordotonal neuron-type of motion and have a more important role in 9+2 motile cilia and flagella (Karak et al., 2015).

2.5 Conclusion

Wdr16 was identified as a candidate motile cilia gene in *Drosophila* through transcriptome analysis and investigation of mRNA expression in developing motile ciliated cells.

In rats, this gene is expressed in motile cilia tissues such as the testes, specifically localising to microtubule structures including the axoneme of the flagellum. Therefore, it was suggested to play a role in the stability and maintenance of the sperm flagellum. When this gene was disrupted in rats, it resulted in the phenotype of male infertility. MIPs are a novel group of proteins which play a role in the stability of the axoneme. Since the onset of this project, the *Chlamydomonas* homologue of *Wdr16* has been reported to function as a MIP, which supports a role for *Wdr16* in the stability of the flagellar axoneme (Owa et al., 2019; Ma et al., 2019).

In this study, when the function of *Wdr16* was knocked down in the testes, *Drosophila* males were infertile due to immotile sperm. This phenotype supports the results obtained in mammalian models and suggests that *Wdr16* plays a role in the stability of the flagellar axoneme – perhaps acting as a MIP in *Drosophila*. As MIPs vary between organisms it would be interesting to use Cryo-ET to identify the role of *Drosophila Wdr16* in relation to microtubule stability (Fabritus et al., 2021). *Wdr16* could have more than one function in *Drosophila*, perhaps working during motile cilia development and assembly (resulting in the immotile flagella observed) as well as in stability. As MIPs interact with dynein motors, it could also be that a sole function as a MIP caused the phenotype observed.

MIPs play a significant role in protecting the axoneme from mechanical stress. Therefore, *Wdr16* could play a more important role in some axonemes over others. *Wdr16* has been previously associated with *Situs Inversus*, which is facilitated by nodal cilia. These cilia contain dynein motor arms to facilitate their whip-like motion and have a 9+0 axonemal arrangement. In the *Drosophila* chordotonal neuron, which also has a 9+0 axonemal arrangement and contained axonemal dynein motors, no role for *Wdr16* was identified. This may be due to an inefficient knockdown, but also may be because *Wdr16* has an important axonemal protective role against the stress caused by the waveform and its amplitude of specifically the flagella and nodal cilia. As the role of the chordotonal neuron cilia is to detect vibrations and actively amplify, its motile function is quite different to the typical beats performed by cilia with axonemal dynein motors. Perhaps *Wdr16* is not required for this specific type of motion. Therefore, I conclude *Wdr16* is functioning as a MIP essential for the motility of nodal cilia and sperm flagella through the stabilisation of the axoneme.

Chapter 3

The Dyx1c1 homologue in *Drosophila*, although truncated, has a conserved function in dynein assembly.

3.1 Introduction

3.1.1 The role of DYX1C1 in dynein assembly and neuronal migration among other biological processes

DYX1C1 (Dyslexia susceptibility 1 candidate 1) has been characterised as a dynein axonemal assembly factor (DNAAF4) with a role in both outer and inner dynein arm assembly in humans (Tarkar et al., 2013). In mice, deletions of exons 2-4 resulted in primary ciliary dyskinesia characteristics. The nulls which survived exhibited severe hydrocephalus and laterality defects. These laterality defects are caused by defective nodal cilia, which require axonemal dynein motors to carry out function. In *DYX1C1* null mice, TEM of tracheal cilia showed a loss of both ODA and IDA structures, similar to what was observed in human PCD patients with mutations in *DYX1C1* (Tarkar et al., 2013).

Zebrafish morpholino studies further support a ciliary role for *DYX1C1* in cilia growth and function (Chandrasekar et al., 2013). Phenotypes observed included hydrocephalus, kidney cysts and a reduction of cilia length in Kupffer's vesicle, which is important in zebrafish for establishing the left-right axis (Chandrasekar et al., 2013; Tarkar et al., 2013).

To comprehend the specifics of the *DYX1C1* phenotype, an understanding of the different outer and inner dynein subsets is required. As discussed in the Introduction, dynein arms are made up of multiple subunits, including dynein heavy chains (HCs), intermediate chains (ICs) and light chains (LCs) (Fig

3.1A). In mammals, respiratory cilia have two different types of outer dynein arms (ODAs), both of which have a two-headed heavy chain structure. Type 1 ODAs are found in the proximal end of the cilia and type 2 ODAs are in the distal end (Loges et al., 2008). The unicellular flagellate *Chlamydomonas* differs in this respect and has only one type of ODAs, which contains three heavy chain structures (Pazour & Agrin, 2006). There are several different subsets of IDAs which are composed of different proteins and have different roles in facilitating ciliary motility (Fig 3.1B). The IDA subsets that contain one heavy chain are thought to function as dyad pairs: *a/b*, *c/e*, *g/d*. The IDA subset *I1/f* contains two heavy chains and does not carry out its regulatory function as part of a dyad pair (Hirose & Amos, 2012; Kubo et al., 2018).

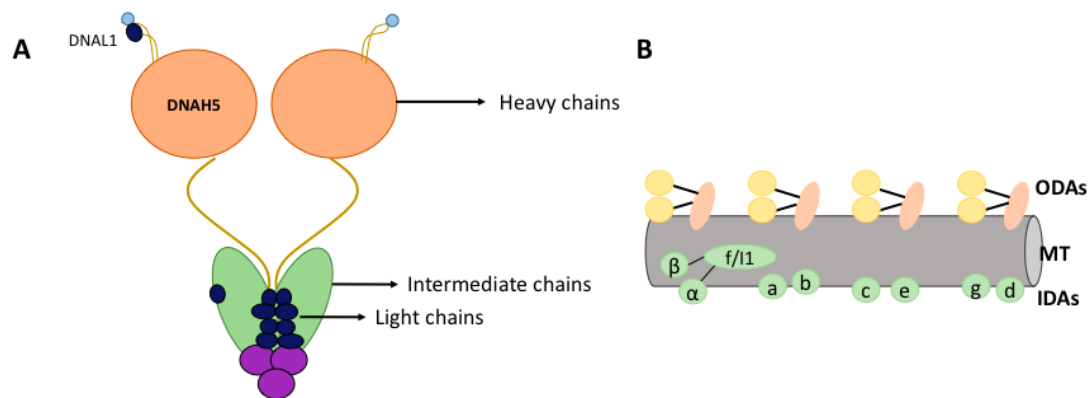


Figure 3.1. Organisation of the dynein motor arms on the axonemal microtubule. (A). The dynein motor arms are composed of several subunits including heavy chains, intermediate chains and light chains. Schematic shown illustrates the structure of a mammalian outer dynein arm, with two subunits (DNAH5, DNAL1) labelled, as these will be mentioned later in the chapter. **(B)** Schematic illustrating how the ODAs and different IDAs are organised along the axonemal microtubule (MT). The IDAs function in dyad pairs: *a/b*, *c/e*, *g/d*, as well as the IDA subset *I1/f* functioning alone.

In a mouse model, *DYX1C1* mutants showed a loss of immunofluorescent staining of ODA and IDA subunits, MDNAH5 and DNALI1, respectively. This result was conserved in humans, as PCD patients with *DYX1C1* mutations had both ODA and IDA defects, shown by TEM analysis of respiratory cilia. Immunofluorescent examination of these cilia indicated a reduction in the localisation of type 1 and type 2 ODA protein subunits (DNAH5, DNAH9 and

DNALI2) as well as IDA subsets, shown by a loss of the localisation of subunit DNALI1, which is present in IDA subsets *a*, *c* and *d*. *DYX1C1* mutations affected type 2 ODA more so than type 1 ODA (Tarkar et al., 2013). Interestingly in *Chlamydomonas*, which only have one type of ODA, the ODA is partially retained when *Dyx1c1* is non-functional. Regarding the inner dynein arms, when the *Chlamydomonas* homologue is absent, assembly of all subsets is affected. Urea-PAGE was used to examine heavy chain composition to determine the severity of the effect on dynein arms. This experiment showed the subsets *b,c,d,e,f,g* were present at less than 20% of WT level, and subset *a* was present at 70% that of WT level. ODAs were present at ~50% of WT level. Therefore, the absence of *Dyx1c1* in *Chlamydomonas* influences the assembly of all IDA subsets and ODAs, with less of an effect on IDA subset *a* and the ODAs (Yamamoto et al., 2017).

Despite its role in dynein assembly, *DYX1C1* was originally identified as a candidate causative gene for dyslexia. This condition is the most common childhood learning disorder (prevalence of 5-12%) and affects reading ability (Schumacher et al., 2007). Although dyslexia is considered to be a neurological condition with genetic susceptibility, *DYX1C1* was actually the first candidate gene to be detected. This link was proposed because of a translocation breakpoint which disrupted *DYX1C1* function co-segregated with dyslexia in one family (Taipale et al., 2003). This was an exciting discovery, as it suggested the study of *DYX1C1* function could provide insight into the complex process of brain development and maturation. However, the association of *DYX1C1* with dyslexia is controversial. Mutations in this gene segregating with dyslexia may be just a coincidence or may be due to linkage disequilibrium (whereby variants in *DYX1C1* could correlate with variants in a nearby dyslexia-causing gene within a population), as the functional *DYX1C1* alleles which co-segregated in this study did not associate with dyslexia significantly in other populations (Marino et al., 2005; Scerri, 2004; Wang et al., 2006). Therefore, the *DYX1C1* alleles of this study may only be linked to dyslexia susceptibility in certain populations (Taipale et al., 2003; Wang et al.,

2006). The human subjects with *DYX1C1* mutations in Tarkar et al. (2013) showed no characteristics common in dyslexia, meaning the precise function of *DYX1C1* remains unclear.

DYX1C1 has also been reported to have an additional role in neuronal migration in the developing neocortex of rodent models. The function of *DYX1C1* was knocked down using RNAi, and this caused a decrease in neuronal migration resulting in neurons accumulating in a multipolar stage of migration (Wang et al., 2006). Other susceptibility candidate genes for dyslexia such as *Kiaa0319* have been shown to have roles in neuronal migration (Paracchini et al., 2006). The identification of a role for *DYX1C1* in neuronal migration supports the possibility of a role in dyslexia.

The literature surrounding *DYX1C1* suggests a complex role in multiple biological processes in higher organisms. This complexity makes it difficult to separate out the different roles and pinpoint what this gene is doing in the different processes, including axonemal dynein assembly. In this chapter, the *Drosophila* model will be utilised to distinguish the primary roles of *DYX1C1* from its other speculative roles.

3.1.2 Chaperone proteins and the R2TP complex

A significant feature of *DYX1C1* concerning dynein assembly is that it has structural features reminiscent of proteins of the Hsp70/90 co-chaperones R2TP. This section introduces R2TP and how it relates to protein complex assembly.

Chaperones such as the Heat Shock Protein (HSP) family of proteins, which includes Hsp70 and Hsp90, regulate protein folding as well as facilitating the assembly of large multi-subunit complexes like the axonemal dynein motors. During this process, Hsp70 initially regulates the openness of protein regions, ensuring the correct molecular interactions occur (Lackie et al., 2017). Hsp70 has two domains: a nucleotide-binding domain responsible for client association, and a C-terminal substrate-binding domain which recognises

hydrophobic sequences and interacts with them in an ATP-regulated manner (Lackie et al., 2017; Mayer & Bukau, 2005). Hsp90 then facilitates protein folding by working with client proteins (i.e. dynein subunits) and co-chaperones. Co-chaperones are important for broadening the functional range of Hsp proteins by controlling their rate of ATP hydrolysis, conformational flexibility, and the binding of specific substrates (Makhnevych & Houry, 2012; Schopf et al., 2017). These co-chaperones can include multi-protein entities, such as the R2TP complex shown in Figure 3.2 (Fabczak & Osinka, 2019).

R2TP (Rvb1, Rvb2, Pih1 and Tah1) was identified as a complex during a screen for interactors of the chaperone Hsp90 conducted in *S. cerevisiae* (Kakihara & Houry, 2012; Zhao et al., 2005). This screen identified 627 Hsp90 cofactors and substrates associated with a wide range of cellular processes (Zhao et al., 2005). Two of these cofactors were previously uncharacterised and so were named Pih1 (Protein interacting with Hsp90) and Tah1 (TPR-containing protein associated with Hsp90). These proteins were found to interact with the AAA+-type DNA helicases Rvb1/2 to form the R2TP complex (R. Zhao et al., 2005).

R2TP is highly conserved, and as shown in Figure 3.2, in mammals consists of PIH1D1 and RPAP3 (homologue of Tah1), which heterodimerise and associate with a heterohexamer made up of RUVBL1 (Pontin) and RUVBL2 (Reptin). The RPAP3:PIH1D1 heterodimer is unique to the R2TP complex, while RUVBL1/2 are involved in several other complexes in the cell, such as INO80 and SRCAP (Maurizy et al., 2021). PIH1D1 acts as an adaptor, linking HSP90 and RPAP3 to RUVBL1/2. HSP90 is recruited to the R2TP complex through the CS domain of PIH1D1 and the TPR domain of RPAP3, which binds to a conserved motif (MEEVD) in HSP90 (Olcese et al., 2017).

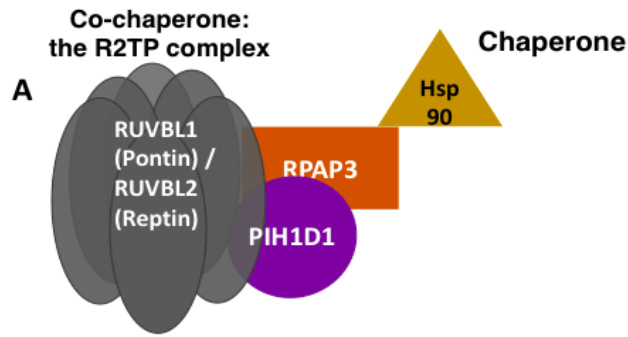


Figure 3.2: The R2TP complex. Schematic showing the components that make up the mammalian co-chaperone complex R2TP. This is composed of RPAP3, PIH1D1, Pontin and Reptin, and interacts with the chaperone Hsp90 during axonemal dynein assembly.

R2TP was initially found to be required for the assembly of small nucleolar ribonucleoproteins (snoRNPs), however additional R2TP substrates have been identified since then including RNA polymerase II and the phosphatidylinositol 3-kinase-related kinases (PIKKs), which are recruited to the R2TP complex by an additional complex referred to as TTT (composed of Tel2p, Ttip and Tti2p) (Hořejší et al., 2010; Kakihara & Houry, 2012). Substrates link R2TP to many biological processes including DNA damage signalling, transcription regulation, nonsense-mediated mRNA decay and nutrient signalling.

The R2TP complex was also shown recently to be involved in axonemal dynein assembly, regulated by Wdr92 (zur Lage et al., 2018). This result was supported by the evidence that a knockdown of either RuvBL1/Pontin or RuvBL2/Reptin in zebrafish and mice results in dynein assembly defects (Li et al., 2017; Zhao et al., 2013). Therefore, the R2TP complex has also been associated with axonemal dynein assembly, with direct interactions occurring between the complex and dynein chains, novel R2TP clients (zur Lage et al., 2018).

3.1.3 TPR domains in relation to DYX1C1 function

TPR domains are present in a wide range of proteins, including RPAP3, and are made up of three or more TPR motifs. The versatility of this protein domain is due to the N-terminal capping helix, the structural plasticity of which leads it to act as the protein-protein interaction site required for cargo binding (Nguyen et al., 2017).

As well as the TPR domain being important for the binding of the R2TP complex to HSP90, as shown in Figure 3.3, this protein domain is also present in several DNAAFs including TTC12, SPAG1 and DYX1C1 (Tarkar et al., 2013; Thomas et al., 2020; zur Lage et al., 2018). TPR repeats are important for facilitating protein-protein interactions, so the observation that this domain is present led to the suggestion that it plays a similar role in the function of these DNAAFs, and that DYX1C1 and the other TPR proteins form R2TP-like co-chaperone complexes with important roles during dynein assembly. The evidence for these complexes will be discussed in greater detail in the next chapter.

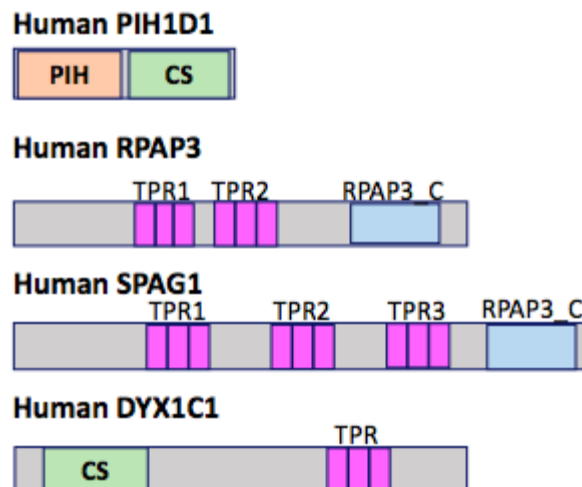


Figure 3.3: Important protein domains of the R2TP complex. Important protein domains present in both the R2TP complex and DNAAFs. These include PIH domains, CS domains and TPR domains, which are all important for mediating protein-protein interactions.

The importance of the TPR domains to DYX1C1 function during dynein assembly is highlighted by the fact that in a study of human DYX1C1 PCD-causing mutations, seven of the nine mutations predicted a premature protein termination - meaning that if the truncated protein is stably synthesised, it would be lacking TPR domains. However, premature protein termination usually leads to the protein being degraded by nonsense mediated decay (NMD) rather than leading to a truncated protein being expressed (Tarkar et al., 2013). In *Chlamydomonas*, a mutant of the *DYX1C1* homologue predicted a loss of function of two TPR motifs and a reduction in the amount of protein present. This finding suggested that either the TPR repeats were required for dynein arm assembly or the dosage of DYX1C1 was important for dynein arm assembly (Yamamoto et al., 2017). Based on the protein structure of DYX1C1 and the presence of these domains, it seems that its specific role is to provide a scaffold for other DNAAFs and dynein subunits to utilise during the dynein motor arm assembly process (Yamamoto et al., 2017).

The TPR domains in the C-terminal of DYX1C1 have also been shown to be crucial for its suggested additional functions (Taipale et al., 2003; Wang et al., 2006). The translocation breakpoint in DYX1C1 which co-segregated with dyslexia in a family was in the TPR-domain coding region and disrupted gene function (Taipale et al., 2003). Moreover, the study suggested a role for DYX1C1 in neuronal migration and found from transfecting C- and N-terminal truncations of the protein that the TPR domains were crucial to determining the intracellular localisation of DYX1C1. Therefore, the C-terminus, which contains these important protein repeats, is necessary for the role of DYX1C1 in neuronal migration (Wang et al., 2006).

Interestingly in *Drosophila*, Dyx1c1 is truncated. The Uniprot database indicates that the homologue (CG14921) contains the N-terminal CS domain, which is also important for protein-protein interactions but lacks the TPR repeats that are required for binding to HSP90. Therefore, it is important to identify whether the function of Dyx1c1 is conserved despite this truncation.

3.2 Aims

Given the multiple functions associated with DYX1C1 in mammals, the aim of this chapter is to use the straight-forward model of *Drosophila* to determine which of these are conserved (if any). This may aid indication of which DYX1C1 roles are likely in humans. The *Drosophila* homologue, CG14921, is truncated and so it is also necessary to first confirm that this gene has a similar function to the human gene, despite this truncation.

3.3 Results

3.3.1 CG14921 is the *Drosophila* homologue of DYX1C1, and it is truncated

As described in the Introduction, motile cilia genes are highly conserved in *Drosophila*, and so we have successfully used this model to identify new and further the knowledge surrounding existing axonemal dynein assembly factors (Diggle et al., 2014; Lage et al., 2021; Moore et al., 2013; zur Lage et al., 2018).

Therefore, to use this model to further the understanding of the specific role of the DNAAF DYX1C1, the *Drosophila* homologue was identified. Bioinformatic analysis was conducted using DIOPT to identify how similar the human and fly proteins aligned. Results in Figure 3.4A show CG14921 as the *Drosophila* homologue of DYX1C1, with 42% similarity in the protein region shown below. CG14921 is truncated in *Drosophila* and lacks the TPR region (located between positions 290-399). The CS domain however (positions 3-87) is highly conserved and the closest to the human gene, indicating that they are orthologs. Therefore, CG14921 will be referred to as *Dyx1c1* hereafter in this thesis.

3.3.2 Phylogenetic tree analysis of the truncated proteins Dyx1c1 and Spag1

To further investigate the loss of the essential TPR protein domain from the Dyx1c1 *Drosophila* homologue, I sought to identify where in evolution this truncation event occurred by generating a phylogenetic tree. Dyx1c1 homologues were identified in different organisms using the databases BLAST, Uniprot and Flybase. Preliminary analyses using these databases indicated that the truncation of Dyx1c1 occurred within the radiation of insects. Therefore, when gathering protein sequences of Dyx1c1 homologues, those belonging to the insect class were the primary focus. Sequences were then aligned, and the phylogenetic trees were generated using the software MEGA7. Bootstrap analysis was also conducted and verified the robustness of the phylogeny predicted. A bootstrap value of >70 indicates a reliable clade within the tree (Zhang et al., 2018).

The phylogenetic tree shown in Figure 3.4B indicates that the TPR truncation is not *Drosophila*-specific, with other Diptera such as *Musca domestica* and *Glossina morsitans* also lacking the protein domain. Interestingly, not all Diptera lack this protein domain. A large divergence event occurs within the order Diptera, with *Aedes aegypti* and *Culex quinquefasciatus* still retaining TPR domains. The loss of the TPR domain causes dramatic divergences to occur within the phylogenetic tree, and this can reduce accuracy of such analyses (Cantarel, 2006). Therefore, an alignment was also conducted using only the CS domain as this domain is present in all Dyx1c1 homologues. This tree (Fig 3.4C) is structured in a similar manner to that of the full protein, indicating that differences occur between species within the conserved CS domain also.

Dyx1c1 is not the only DNAAF truncated in *Drosophila*. Spag1, a TPR-containing DNAAF, is missing its RPAP3 domain (zur Lage et al., 2018). Dyx1c1 and Spag1 are homologous proteins, sharing 42% similarity in *Drosophila*.

To identify whether the truncated Spag1 diverged in a similar manner to Dyx1c1, I constructed a phylogenetic tree of the Spag1 homologues in a similar manner to that of Dyx1c1 above. Spag1 does not contain a divergence within the Diptera order as observed for Dyx1c1, as shown in Figure 3.4D (Black arrow). The timing of the truncation of this protein in evolution differs to that of Dyx1c1, indicating differences between the two DNAAFs.

3.3.3 *Dyx1c1* is transcribed in motile cilia-specific cells

In order to gain insight into the function of *Dyx1c1* in *Drosophila*, I assessed where this gene is transcribed. The FlyAtlas Anatomical Expression data in Figure 3.5.A shows *Dyx1c1* to be most present in the adult testis (293.5 units, Robinson et al., 2013). In a transcriptome analysis previously conducted by the lab, *Dyx1c1* was shown to be 5.2-fold enriched in embryonic chordotonal cells (zur Lage et al., 2019). The expression of *Dyx1c1* in these cells suggests a motile cilia function for this gene as these are the two cell types which contain the motile cilia machinery in *Drosophila*.

To corroborate this transcriptomic data, RNA *in situ* hybridisation was carried out on late-stage whole-mount *Drosophila* embryos and the expression pattern of *Dyx1c1* was analysed. Berkeley Drosophila Genome Project (BDGP) data suggests that *Dyx1c1* mRNA is restricted to the lateral chordotonal organs (lch5), a subset of the chordotonal neurons in late-stage embryos (stages 13-16). This restriction to a subset of chordotonal neurons has not been previously observed for other DNAAFs, which are typically expressed in all embryonic chordotonal neurons (lch5, v'ch1 and vchA and B, illustrated in Fig 3.5D). This *in situ* hybridisation was then replicated by P. zur Lage and as shown in Figure 3.5B, confirmed the restricted expression shown in the BDGP data. Anti-futsch, a neuronal marker, was then used as a counterstain to highlight this restricted expression of *Dyx1c1*mRNA (Fig 3.5C). There was no evidence of general expression in the embryo, which would support wider roles for *Dyx1c1*. Therefore, these experiments support the hypothesis that *Dyx1c1* is expressed in motile ciliated cells.

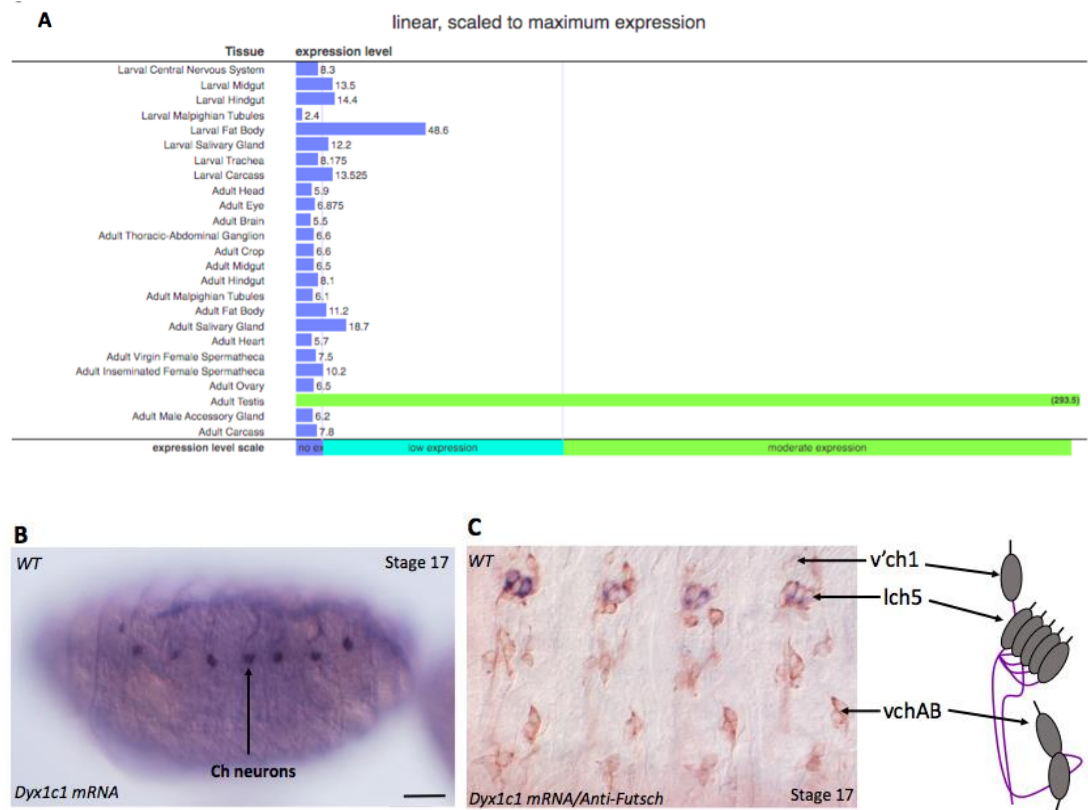


Figure 3.5 *Dyx1c1* is transcribed in motile cilia cells. (A) Screenshot showing FlyAtlas expression data (Robinson et al., 2013), which highlights that *Dyx1c1* is most highly expressed in the adult testes (red square). (B) RNA in situ hybridisation carried out by P. zur Lage on late-stage whole-mount embryos with *Dyx1c1* probe showing a restricted chordotonal (ch) neuron pattern. (C) Higher magnification shows *Dyx1c1* is expressed specifically in a subset of the lateral chordotonal neurons (lch5). The other chordotonal neurons (v'ch1, vch AB) show no *Dyx1c1*mRNA staining (D) Schematic illustrating the chordotonal neurons of the embryonic abdominal segment, based on an image from (zur Lage et al., 2004).

3.3.4 *Dyx1c1*mVenus fusion protein localises to motile cilia cells

3.3.4.1 Generation of *Dyx1c1*mVenus fusion protein

To further investigate the expression pattern of *Dyx1c1* in *Drosophila*, an mVenus fusion protein construct was generated to be expressed under the control of the gene's endogenous promoter and as a result GFP was expressed where *Dyx1c1* is expressed. When designing the construct, an additional 100bp of the upstream region was included, so that the predicted binding sites in the promoter region for the two cilia-associated transcription

factors fd3F (F) and RfX (X), which were located using UCSC genome browser, were present. This construct was initially cloned into the pDONR221 vector and then into the pBID-GV vector, as described in Chapter 7. This was injected into a 2nd chromosome line (attP40). A schematic of this construct is shown in Figure 3.6A.

3.3.4.2 Localisation of *Dyx1c1*mVenus in the embryo

The mVenus fusion protein was first investigated in embryos, to follow on from the *Dyx1c1* mRNA expression pattern that was found in the embryonic chordotonal neurons.

As shown in Figure 3.6B, *Dyx1c1* is expressed in the developing chordotonal neurons in whole-mount embryos. Unlike the restricted expression observed for mRNA, *Dyx1c1* protein is present in all JO chordotonal neurons: lch5, v'ch1 and vchA and B. This suggests that *Dyx1c1* protein is not present in a subset of chordotonal neurons, and that *Dyx1c1* mRNA was present in all embryonic ch neurons at some stage, perhaps an earlier time-point and only transiently. Alternatively, an upstream region may be absent in the reporter line that is required for the repression of *Dyx1c1* expression. Interestingly, this protein is also present in the external sensory (ES) neurons which are ciliated but not motile ciliated. This additional ES neuronal staining was not seen in the *in situ* hybridisation, and so it is most likely the result of a disruption of regulation in the fusion protein, supporting that there is an additional regulator of *Dyx1c1*, which limits expression to motile cilia cell types. Overall, the staining of *Dyx1c1*mVenus in the embryo shows definite expression in the motile cilia cells, supporting a motile cilia role for *Dyx1c1* in *Drosophila*.

3.3.4.3 Localisation of *Dyx1c1*mVenus in pupal antennae

To further examine *Dyx1c1* protein expression, pupal antennae were assessed. The Johnston's Organ (JO), which contains an array of chordotonal neurons, are found in the third segment of the antenna. Additional ciliated neurons, such as the olfactory neurons are also located in the third segment of the antennae (Gomez-Diaz et al., 2018). Analysis of pupal antennal

expression was conducted to indicate whether Dyx1c1 localised to motile-ciliated cells later in development (i.e. chordotonal-neuron specific) or also present in other ciliated neurons (i.e. the olfactory neurons). As shown in Figure 3.6C, investigation of pupal antennae revealed Dyx1c1 to be localised specifically to the cell body of chordotonal neurons, indicating that it is a cytoplasmic protein. No additional neuronal expression was observed in any antennae viewed or imaged in Fig 3.6C, suggesting Dyx1c1 is specific to chordotonal cells. As dynein pre-assembly takes place in the cytoplasm before complexes are transported and docked in the cilium, the expression of Dyx1c1 in the chordotonal neuron cell body suggests a role in this axonemal dynein assembly process.

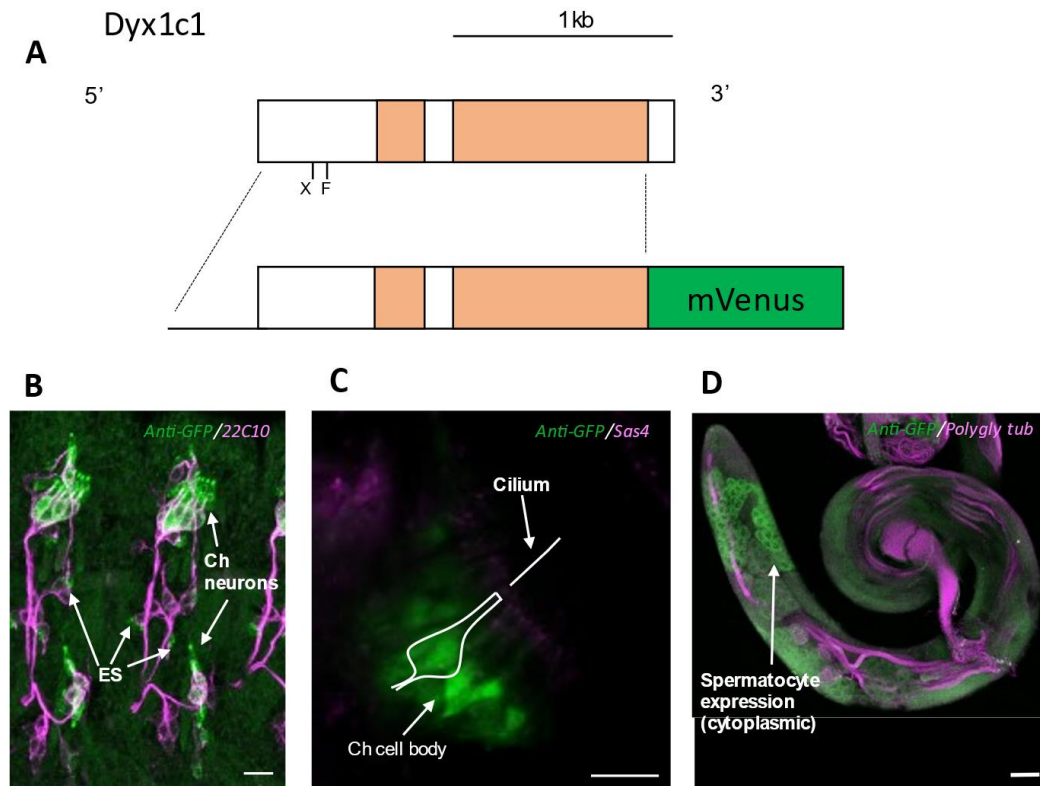


Fig 3.6. Dyx1c1 protein is expressed in motile ciliated cells (A) Schematic showing the generation of the Dyx1c1mVenus fusion protein construct, which includes binding sites in the promoter region for the two cilia-associated transcription factors, Fd3f (F) and Rfx (X). (B) Expression of Dyx1c1mV in the developing chordotonal neurons as well as the external sensory (ES) organs in the embryo (green). Counterstained with anti-futsch (magenta), which is a neuronal marker. (C) Dyx1c1 is expressed in the cell body of chordotonal neurons in the pupal antennae (green) and counterstained with the basal body marker Sas4 (magenta). (D) Dyx1c1mV expression in adult testis, specifically in the cytoplasm of the spermatocytes (green). Counterstained with polyglycylated tubulin, which stains the sperm flagella. Bars: (B) 10 μ m (C) 5 μ m (D) 50 μ m. Number of samples imaged showing phenotype: (B) $n = 7/7$ (C) $n = 9/9$ (D) $n = 8/8$

3.3.4.4 Localisation of *Dyx1c1*mVenus in adult testis

As well as the chordotonal neuron dendrite, the *Drosophila* sperm flagellum contains motile cilia machinery. Immunofluorescent staining was performed on the Dyx1c1mVenus adult male testes to examine the cellular localisation of this protein. As shown in Figure 3.6D, Dyx1c1 is highly expressed in spermatocytes, and is cytoplasmic. There was no Dyx1c1 expression in the flagella in any samples imaged, indicating this protein is not a component of cilia/flagella. As axonemal dynein motor complexes are assembled in the

spermatocyte cytoplasm, expression in this location is consistent with a role in assembly.

3.3.4.5 No localisation of *Dyx1c1mVenus* in the CNS

In mammals, DYX1C1 has been linked to neuronal migration and identified as a candidate dyslexia gene (Taipale et al., 2003; Wang et al., 2006). To assess whether *Drosophila Dyx1c1* has additional roles beyond motile cilia, I dissected the larval CNS to look for additional expression in non-ciliated neuronal tissue. Image analysis shown in Fig 3.7 revealed no additional *Dyx1c1* expression in the CNS and does not support an involvement of this gene in a CNS function in *Drosophila*. As a control, a *UAS-GFP* fly line was crossed to *Elav-Gal4*, so it expresses GFP in all the post-mitotic neurons of the CNS.

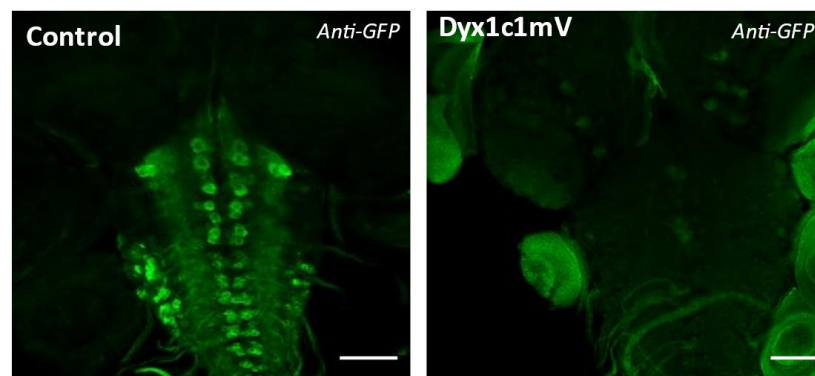


Figure 3.7 *Dyx1c1mV* is not expressed in the larval CNS. Control (*UAS-GFP* x *ElavGal4*) showing expression in neuroblasts of the ventral nerve cord (VNC). *Dyx1c1mV* shows no expression in CNS, supporting a motile cilia specific role for *Dyx1c1* in *Drosophila*. Scale bar = 50um.

Therefore, *Dyx1c1* seems motile ciliated cell-specific in *Drosophila*. Further examination of *Dyx1c1* function through RNAi knockdown experiments and the generation of a null mutant were conducted to determine its function in these cells.

3.3.5 *Dyx1c1* RNAi knockdown experiments support a role in motile cilia

Having confirmed that *Dyx1c1* is expressed in motile ciliated cell types, the phenotype which occurs when function is knocked down in these cells was assessed. Initially the Gal4/UAS system in combination with RNAi technology (described in Chapter 2) was used to knockdown *Dyx1c1* function in the cell types of interest. A *Dyx1c1* RNAi transgenic fly line (Bloomington #102921) was crossed to the pan-neuronal driver *UAS-Dicer-2; Scabrous-Gal4* (*UAS-Dcr; Sca-Gal4*) to assess whether the knockdown of *Dyx1c1* in the chordotonal neurons resulted in a proprioceptive defect. As mentioned in Chapter 2, Dicer aids an efficient gene knockdown, and so it is useful to add this enzyme to genetic crosses when conducting RNAi experiments in neuronal cells (Dietzl et al., 2007). If the motile cilia role of *Dyx1c1* is conserved in *Drosophila*, then defective chordotonal neuron function would be expected to be observed. This connection will be assessed by conducting a climbing assay. After a 10 second climbing period results (Fig 3.8A) indicated a significant difference between the climbing index of flies with a *Dyx1c1* knockdown and the control line. As defective proprioception indicates disrupted function of the chordotonal function, this climbing assay result supports a motile cilia role for *Drosophila* *Dyx1c1*.

Dyx1c1 is highly expressed in the adult testes, so its function was knocked down specifically in this tissue using a testis-specific driver *Bag of marbles* (*Bam*). A fertility assay was performed crossing these males to wild-type virgin females and assessing progeny numbers produced in comparison to control males. Significantly less progeny were produced when *Dyx1c1* was knocked down in the testis (median number of progeny = 113 for control, 0 for knockdown as shown in Fig 3.8B).

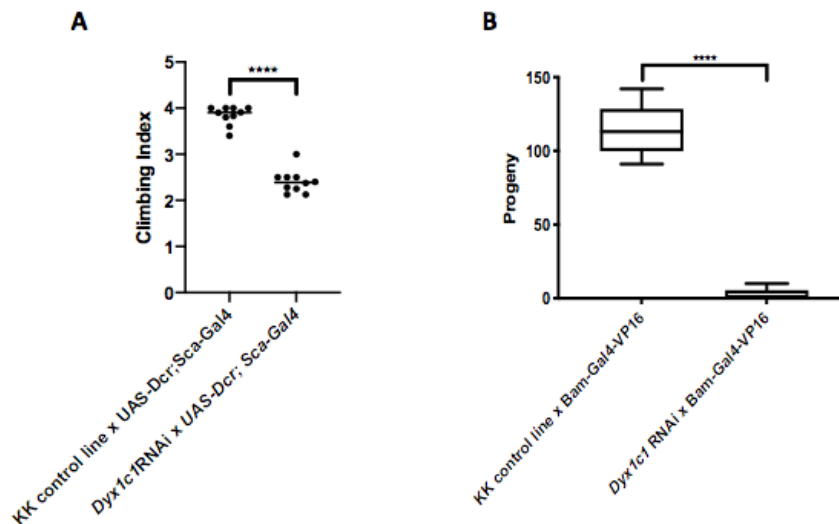


Figure 3.8 RNAi knockdown experiments show *Dyx1c1* has a role in motile ciliated cells in *Drosophila*. (A) Bar graph (with median and individual values) showing that when *Dyx1c1* function is knocked down in the developing nervous system, there is a significant decrease in climbing ability ($P < 0.0001$) $n = 10$ batches of 8-12 females. (B) Box plot indicating when *Dyx1c1* is knocked down in the testis, there is a significant decrease in the number of progeny produced ($P < 0.0001$). $n = 10$ males. Significance was determined by Mann Whitney test. Significance on plots is shown using astericks: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Overall, this preliminary investigation using RNAi technology supported a motile cilia role for *Dyx1c1* in *Drosophila*, resulting in fertility and proprioceptive defects when function was disrupted. Having previously discussed the potential limitations of this technology in Chapter 2, the next aim was to follow up these experiments and further investigate the role of *Dyx1c1* in *Drosophila* by generating a CRISPR/Cas9 null mutant. The creation of a null mutant would identify whether there is a wider role for *Dyx1c1*, by assessing the phenotypes observed beyond those expected for ciliary motility defects, such as whether *Dyx1c1* mutation is lethal.

3.3.6 A *Dyx1c1* null mutant is generated using a CRISPR/Cas9 approach, and analysed for insight into gene function

3.3.6.1 Generation of null mutant

The RNAi data supported a role in motile cilia cells for *Dyx1c1*. However, the generation of a CRISPR null mutant will address whether this is the only function for this gene in *Drosophila* considering *Dyx1c1* in humans has been linked to dyslexia and neuronal migration (Taipale et al., 2003; Wang et al., 2006).

The process of generating the null mutant is described in detail in Chapter 7. The open reading frame (ORF) of *Dyx1c1* was replaced with the mini-white gene using CRISPR/Cas9 homology-directed repair as shown in Figure 3.9A. Interestingly, this null mutant proved to be viable, and resulted in the successful generation of a stock to be used in future experiments. Therefore, *Dyx1c1* does not seem to have an additional general function essential to survival during development, supporting the previous expression experiments.

3.3.6.2 *Dyx1c1* null males are infertile, due to immotile sperm

Using the CRISPR-generated *Dyx1c1* null mutant, the male fertility assay was repeated to verify whether a total removal of the gene resulted in a similar phenotype observed when using RNAi technology. The fly line Oregon-R, which is used to represent wild-type flies and *Dyx1c1* heterozygotes were used as controls. As shown in Figure 3.9B, fertility of the null mutants is strongly affected, with no progeny produced by this line.

To confirm that the testes themselves were developing correctly and that this fertility defect was the result of sperm immotility, testes from the males used in the fertility experiment ($n=10$ pairs for each group) were dissected and examined using a brightfield microscope. Testes developed normally in the *Dyx1c1* KO males (Fig 3.9 C, D), however the seminal vesicle was smaller than in the control as mature sperm were not travelling to it. In contrast to the control groups, no motile sperm were observed under the microscope, and

instead clumped together in large groups. This result supports the hypothesis that *Dyx1c1* has a role in flagella motility in *Drosophila*.

3.3.6.3 *Dyx1c1* null adults have defective proprioception

The climbing assay was repeated on the *Dyx1c1* knockout line to assess chordotonal neuron function. Climbing ability of homozygous mutants was significantly reduced compared to control groups (wild type flies and heterozygotes). The result shown in Figure 3.9E indicates that the *Dyx1c1* null mutant has proprioceptive defects like that observed when the gene function was knocked down using RNAi.

3.3.6.4 *Dyx1c1* null larvae have hearing deficits

As well as mediating proprioception, the chordotonal neuron also regulates auditory reception (Gong et al., 2004). Therefore, a larval hearing assay was performed to gain further insight into how the function of these chordotonal neurons is affected in a *Dyx1c1* null mutant. The number of third-instar larvae that exhibited a behavioural response to a 1000 Hz tone was recorded for each genotype. As shown in Figure 3.9F, no homozygous larvae carried out a response to the auditory stimulus - which was indicated by no difference in behaviour before and during the tone. However, both control groups (wild type and heterozygous) had a significant difference in the number of larvae that contracted before and during the tone, indicating a response to the stimulus. This result shows that *Dyx1c1* null mutants have auditory deficits suggesting defective chordotonal function, which supports a motile cilia role for this gene.

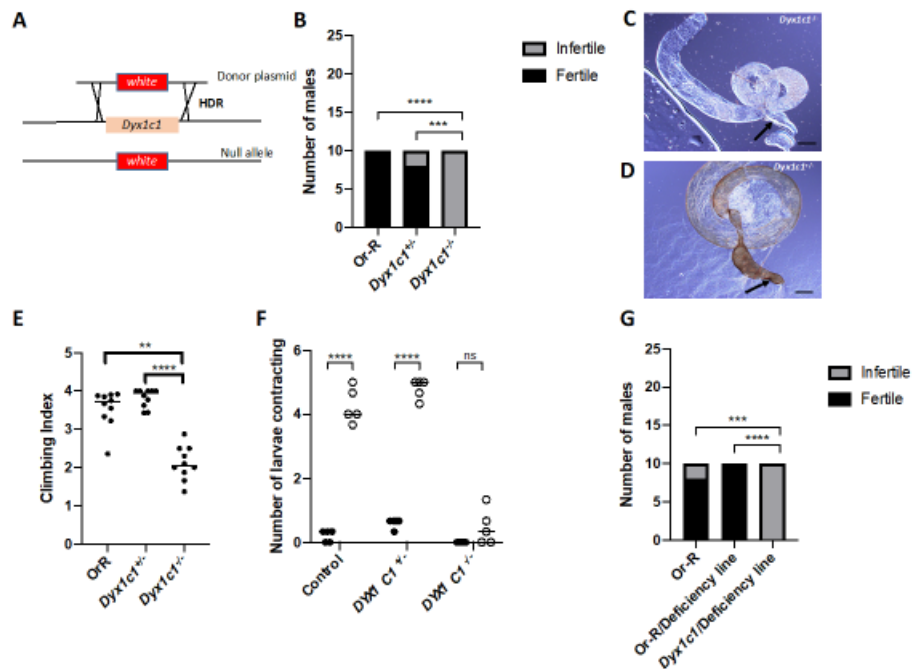


Fig 3.9 Null mutants of *Dyx1c1* are viable and show defective motile cilia function. (A) Generation of *Dyx1c1* mutant by homology directed repair. The ORF was replaced with the *mini-white* gene, allowing selection of null mutants by eye colour. (B) Fertility assay results displayed as bar graph showing a dramatic decrease ($P = 0.0001$) in the number of fertile males for *Dyx1c1^{-/-}* in comparison to control. $n = 10$ males (C-D) Adult male testis from *Dyx1c1* fertility assay. (C) *Dyx1c1^{-/-}* null mutant showing small seminal vesicle (black arrow) due to an absence of motile sperm. (D) *Dyx1c1^{+/+}* testis showing motile sperm (S-wave shapes) surrounding a full seminal vesicle (black arrow). (E) Bar graphs (with median and individual values) of adult female climbing assays for *Dyx1c1*. *Dyx1c1^{-/-}* females had a significant decrease in climbing ability when compared to *Dyx1c1^{+/+}* ($P < 0.0001$) and *Or-R* ($P = 0.0067$) controls. $n = 10$ batches of 8-12 flies for each genotype. (F) Plots (with individual and median values) showing hearing assay performances for *Dyx1c1^{-/-}* larvae in comparison to heterozygote and wild-type controls. Number of larvae contracting before and during a 1000Hz tone was measured. There is a significant difference between the number of larvae contracting before and during the tone ($P < 0.0001$) for control groups genotypes. There is no significant difference between the number of contractions occurring before and during the tone for *Dyx1c1* null mutants, indicating no behavioural response to stimulus exhibited. n for each genotype = 5 batches of 5 larvae, each exposed to 3 tones 30s apart. (G) Fertility assay with the deficiency line (Bloomington #24905) crossed to the *Dyx1c1* CRISPR mutant verified the immotile cilia phenotypes are caused by the expected locus. This fly line has a significant decrease in the number of males producing progeny compared to controls. Significance was determined by Fisher's exact test (B, G) Kruskal-Wallis and Dunn's multiple comparisons test (E) and two-way RM ANOVA and Sidak's multiple comparisons test (F) Significance was determined by Mann Whitney test (A) and 2-Way ANOVA with Sidak's multiple comparisons (B). Significance on plots is shown using astericks: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. Bars, 100 μ m (C, D).

3.3.6.5 Use of deficiency lines confirms the *Dyx1c1* null mutation to the expected locus

Deficiency lines are useful for confirming that the phenotype observed when analysing the homozygous mutant is caused by a mutation in the expected gene region (Ryder et al., 2007). These are fly lines in which a particular region of a chromosome is absent that were generated by the Bloomington Deletion Project using transposable element insertions (Cook et al., 2012). Crossing a deficiency line (Bloomington #24905) that is missing the region where *Dyx1c1* is located (32D4) to the *Dyx1c1* mutant line results in flies which are heterozygous for the CRISPR-generated mutation as well as having the chromosomal deficiency. These flies should behave in a similar manner to that observed for the null mutant if the locus that is absent in the deficiency line is causing the phenotypes observed.

The *Dyx1c1*/Deficiency line flies behaved similarly to the null mutant line described above (Fig 3.9G). Males from this cross were completely infertile, resulting in the production of no progeny. The wild-type flies, as well as wild-type flies crossed to the deficiency line were fertile, resulting in the generation of many progeny. This experiment confirmed that the phenotypes observed in the null mutant were a result of genetic alteration in the expected locus, and further validated the null mutant that was generated.

3.3.7 *Dyx1c1*mVenus successfully rescues *Dyx1c1* null mutation

This experiment aimed to show that the mVenus fusion protein used for the immunofluorescent staining above is functional, so this fly line could be used in proteomic experiments to investigate protein-protein interactions involving *Dyx1c1*. The other aim of this rescue experiment was to validate the null mutant. A fly line was generated containing both the CRISPR-generated *Dyx1c1* null mutant and the GFP-tagged *Dyx1c1* protein through recombination. Based on their positions on the 2nd chromosome, there was a 10% chance of successful recombination, and so several attempts were made before two rescue fly lines were successfully generated and validated by PCR.

As shown in Figure 3.10, similar assays to those described previously were carried out on these fly lines to investigate whether the presence of the Dyx1c1mVenus protein could rescue the phenotypes observed in *Dyx1c1* null mutants. The rescue lines produced flies that climbed and produced progeny in a similar manner to controls. These assays validate the mVenus line for use in proteomic experiments.

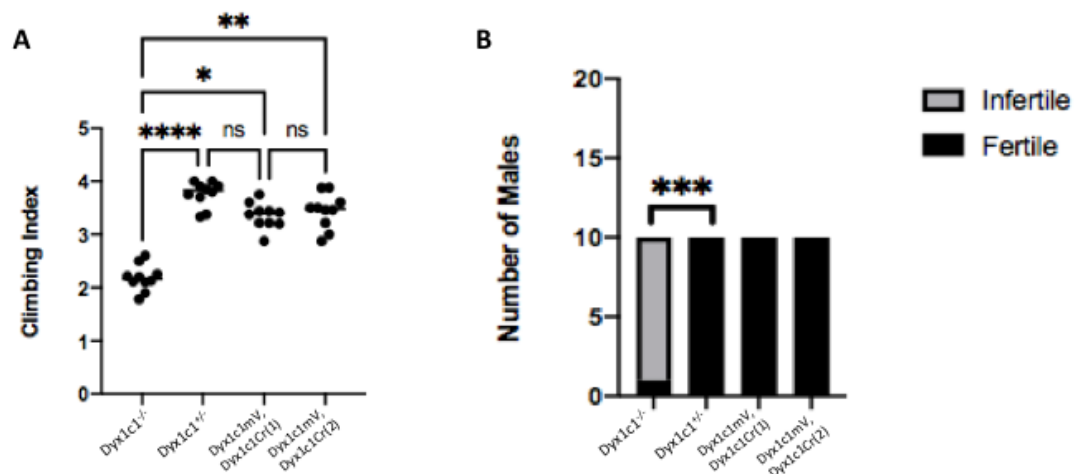


Figure 3.10 Dyx1c1mVenus successfully rescues null mutant phenotype. (A) Both rescue lines have a climbing ability similar to the Heterozygote control. There is a significant difference in the climbing ability of the homozygous *Dyx1c1* null mutant and the rescue lines. $n = 10$ batches of 8-12 flies **(B)** Both rescue lines are fertile, with all males producing progeny. $n = 10$ males. These results show that the *Dyx1c1mV* line successfully rescues the null mutation and validates its use in future experiments. Significance was determined by Mann Whitney test **(A)** and Fisher's exact test **(B)**. Significance on plots is shown using astericks: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

3.3.8 Electron microscopy indicates a loss of both ODAs and IDAs in a *Dyx1c1* null mutant.

Hearing, proprioceptive and fertility defects indicate that *Dyx1c1* has a motile cilia function, consistent with conserved function as a dynein assembly factor. The axonemal dynein motor arms were examined in the null mutant to assess how they were affected. Transmission electron microscopy (TEM) was performed at Newcastle University on adult antennae and as shown in Figure 3.11A-B, and while the cilia are largely normal, both outer dynein arm (ODA) and inner dynein arm (IDA) complexes appeared strongly reduced or absent

in chordotonal neuron cilia. As *Dyx1c1* is expressed in the cytoplasm of motile cilia cells and the dynein motor complexes which drive ciliary motility are absent in a null mutant, it suggests the dynein assembly factor function of *DYX1C1* is conserved in *Drosophila*. This was further investigated by immunofluorescence analysis for dynein subunits.

3.3.9 Investigating the localisation of axonemal dyneins in *Dyx1c1*^{-/-} pupal antennae

3.3.9.1 Initial use of RNAi technology to examine dynein localisation

Immunofluorescent staining was conducted on pupal antennae as the TEM images do not necessarily provide a concrete breakdown of how the dynein motors are affected. TEM is useful for visualising ODA and axonemal defects. However immunofluorescent staining is more useful in assessing molecular defects in the axonemal subunits that are difficult to visualise with TEM such as the IDAs (Aprea et al., 2021). Furthermore, assessing pupal chordotonal neurons allows for insight into these structures during development.

In a preliminary experiment, RNAi technology was used to knockdown the function of *Dyx1c1* in the pupal antennae using pan-neuronal driver *Scabrous-Gal4* (*Sca-Gal4*). The fly lines also contained an mVenus reporter for either the inner dynein arm (IDA) light-intermediate subunit *Dnali1* or the outer dynein arm (ODA) light chain *Dnal1* (*Scagal4, Dnali1mV* | *Scagal4, Dnal1mV* - both lines generated by Fay Newton) Therefore, the aim of this experiment was to see how ODA and IDA subunits are affected in a *Dyx1c1* knockdown.

Dnali1 localised in a similar manner to that observed in the control line (Fig3.11C, D), with strong staining present in the cilium. *Dnal1* also localised in a similar manner in both the knockdown and control (Fig 3.11E, F). These results suggested that ODAs may not be affected during development in a *Dyx1c1* knockdown. However as discussed in Chapter 2, a weak driver line can result in an ineffective knockdown and therefore additional enzyme Dicer-2 can be required when studying neuronal cells such as those in the

chordotonal organ (Dietzl et al., 2007). The genetics of the fly lines used in this experiment did not allow for the inclusion of *Dicer-2*, and so the knockdown may have been ineffective. Climbing ability of RNAi flies crossed to *Sca-Gal4* and *Uas-Dcr-2*; *Sca-Gal4* was assessed. As shown in Figure 3.11G, it appears *Sca-Gal4* can be ineffective at knocking down a DNAAF function on its own and suggests the localisation of ODAs and IDAs to the cilium may not be an accurate representation of the phenotype.

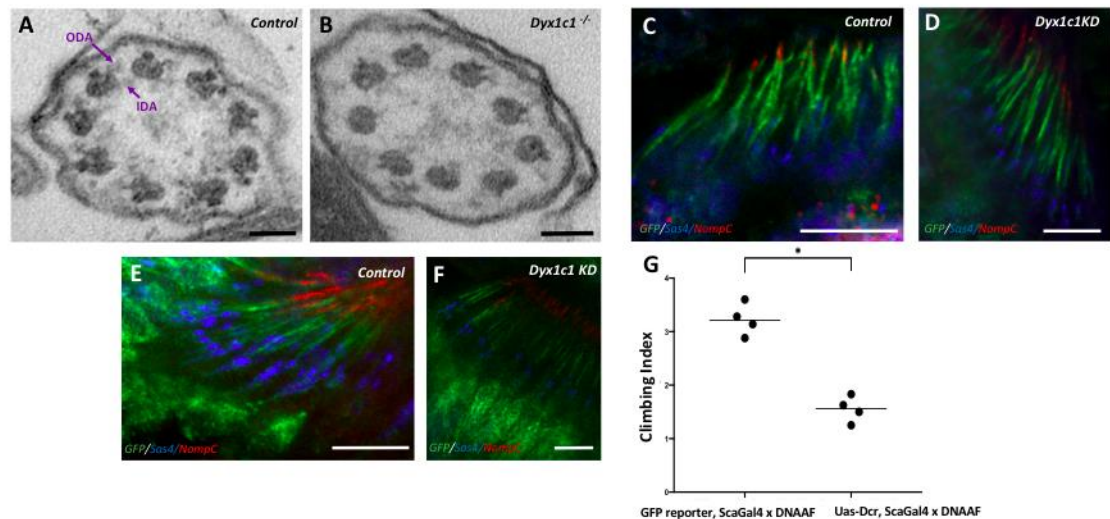


Figure 3.11 TEM reveals a loss of ODAs and IDAs in a *Dyx1c1* knockout, but RNAi knockdown data does not support this loss of localisation of dynein arms – most likely due to an insufficient knockdown of *Dyx1c1*. (A-B) TEM of adult chordotonal neurons, transverse sections. (A) Control chordotonal neuron cilium with characteristic (9+0) axonemal arrangement and outer (ODA) and inner (IDA) dynein arms on each microtubule doublet. (B) Chordotonal neuron cilium of *Dyx1c1*^{-/-} indicating a loss of these ODA and IDA structures from the microtubule doublets. (C) Control (KK control line x *Dnal1mV*, *ScaGal4*) showing expression of IDA subunit in the cell body and the cilium. Counterstained with the basal body marker *Sas4* (blue) and *NompC* (red), the TRP channel present in the distal ciliary zone (D) *Dyx1c1* RNAi x *Dnal1mV*, *ScaGal4* pupal antennae showing when *Dyx1c1* is knocked down in the developing nervous system, IDA subunits are still localising in the cilium. (E) Control (KK control line x *Dnal1mV*, *ScaGal4*) pupal antennae showing ODA subunit is localising in the cell body and cilium. (F) *Dyx1c1* RNAi x *Dnal1mV*, *ScaGal4* pupal antennae showing ODA localisation unaffected when *Dyx1c1* function is knocked down in the cilium. (G) Climbing assay experiment indicates *scabrousGal4* on its own is inefficient at knocking down the function of a DNAAF in the developing nervous system. Bars: (A,B) 50nm (C-F) 5µm. Number of antennae imaged for IF: (C) *n* = 4 (D) *n* = 5 (E) *n* = 4 (F) *n* = 2 (G) *n* = 4 batches of flies. Significance for (G) was determined using Mann-Whitney test.

3.3.9.2 Localisation of Dnali1 (IDA marker) in *Dyx1c1*^{-/-} pupae

Using genetic recombination, a fly line was generated containing both a null mutation for *Dyx1c1*, as well as an mVenus reporter line for the IDA light-intermediate chain Dnali1. Staining was conducted on pupal antennae to assess how this IDA subunit localised in the *Dyx1c1* knockout. As shown in Fig 3.12A-B, there was reduced staining of Dnali1 in the null mutant when compared to control. This reduction was consistently observed throughout samples and suggests incomplete localisation of the IDAs to the cilium. There is a lack of quantitative measurement results in relation to these images due to difficulty in tracking a chordotonal cell body to its corresponding cilium as a result of the orientation of the neurons in the JO. However, from observation, Dnali1 staining is in general stronger in the cell body of the chordotonal neurons of the *Dyx1c1* KO pupal antennae than in the chordotonal neuron cilium.

As mentioned in the introduction, there are different subsets of IDAs. Dnali1 is present in subsets *a*, *c* and *d*. The IDA subset *d* is not conserved in *Drosophila* (zur Lage et al., 2019). Therefore, the partial localisation observed in this experiment suggests that *Dyx1c1* has a crucial role in the assembly of one of the other Dnali1-containing subsets in *Drosophila*, or both moderately.

3.3.9.3 Localisation of Dnal1 and Dnah5 (ODA markers) in *Dyx1c1*^{-/-} pupae

TEM results showed a loss of ODAs in *Dyx1c1* knockout adult chordotonal neurons (Fig 3.11A-B). Using genetic recombination, a fly line was generated which had both a homozygous knockout of *Dyx1c1* and an mVenus reporter line for the *Drosophila* homologue of the ODA light chain Dnal1. *Dyx1c1*^{-/-} pupal antennae were assessed to see how this ODA subunit localised in comparison to control (Dnal1-mVenus line). As shown in Figure 3.12.C-D, Dnal1 expression is in the cell body of the chordotonal neuron, where pre-assembly is taking place, but not localising into the cilium.

An antibody was generated by P. zur Lage in the lab to investigate a different ODA subunit - the *Drosophila* homologue of the ODA heavy chain DNAH5, the

most common gene mutation found in PCD patients (Hornet et al., 2006). Using this antibody on both homozygotes and heterozygotes shows that the subunit DNAH5 is affected when *Dyx1c1* is non-functional. As shown in Figure 3.12E-F, the antibody is observed to be in the cell body but absent in the cilium. These results further validate that *Dyx1c1* has a role in outer dynein arm assembly in *Drosophila*.

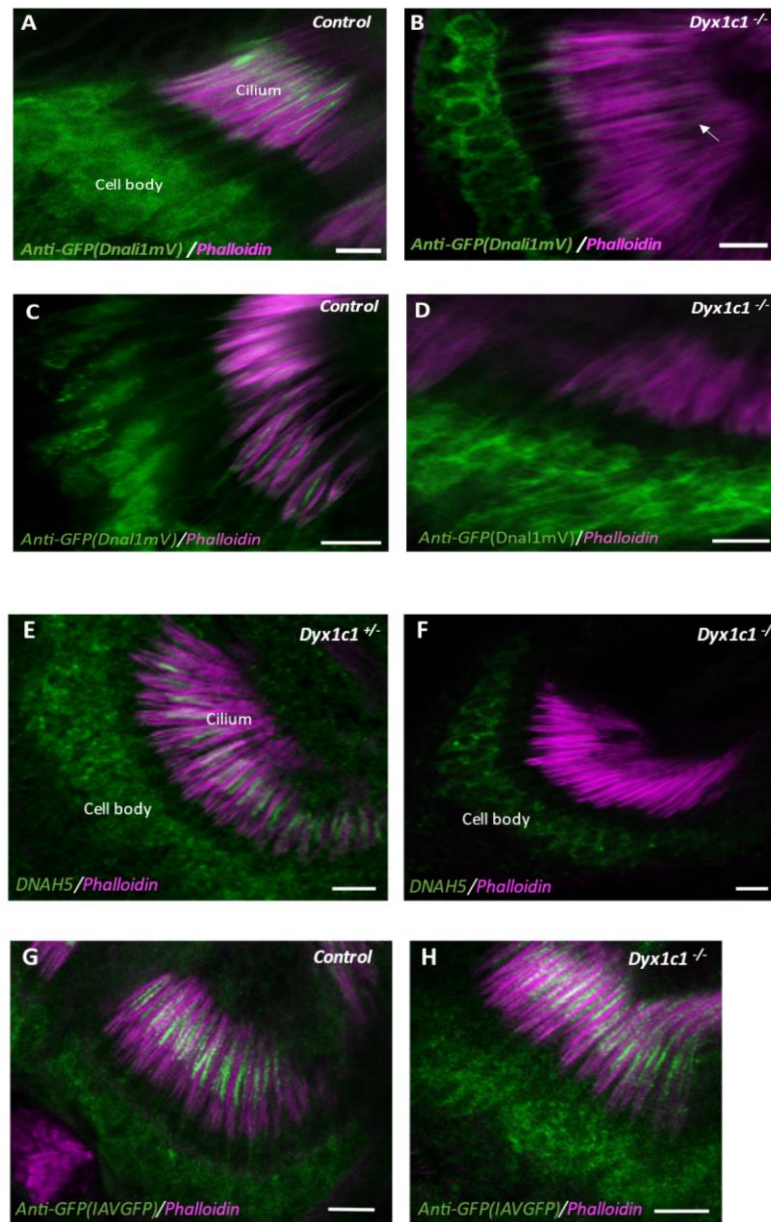


Fig 3.12. Localisation of both ODAs and IDAs are affected in Dyx1c1 knockout pupal antennae (A) Control (Dnal1mV) pupal antennae showing expression of IDA subunit in cell body and cilium. (B) Dyx1c1 null pupae show expression of IDA subunit in cell body and reduced expression in cilium. This result indicates partial localisation (white arrow) of IDA subunits, but that IDAs are affected by a Dyx1c1 KO. (C) Control (Dnal1mVenus) showing expression of ODA subunit in cell body and cilium. (D) Dyx1c1 null pupae have a loss of localisation of Dnal1 in the cilium. (E) Control (Dyx1c1^{+/+}) pupal antennae showing expression of the ODA subunit DNAH5 in both the cell body and cilium. (F) Dyx1c1^{-/-} pupal antennae showing DNAH5 staining in the cell body, but a loss of staining in the cilium. (G) Control (IAVGFP) showing expression of protein in cell body and cilium. (H) IAV localisation to the cilium unaffected in Dyx1c1 null mutant. Bars: (A-H) 5μm. Number of antennae showing phenotype out of those imaged for IF: (A) $n = 5/5$ (B) $n = 9/9$ (C) $n = 5/5$ (D) $n = 10/10$ (E) $n = 7/7$ (F) $n = 7/7$ (G) $n = 6/6$ (H) $n = 7/7$.

3.3.9.4 Localisation of inactive (IAV) in *Dyx1c1*^{-/-} pupae

As mentioned in the Introduction, Inactive (IAV) is a mechanotransduction channel involved in the response to different stimuli including startle, heat and sound and is expressed in the chordotonal neuron cilia (Gong et al., 2004). *Drosophila* with mutations in DNAAFs have previously shown dynein motor defects but typical localisation of the mechanotransduction channels (zur Lage et al., 2018). Therefore, to validate that the formation of the general cilia structure is unaffected in a *Dyx1c1* knockout as it is specifically involved in axonemal dynein assembly, the localisation of IAV was examined.

A GFP reporter line for IAV was crossed into *Dyx1c1*^{-/-} flies and localisation was assessed. As shown in Figure 3.12G-H, when *Dyx1c1* was non-functional, IAV localised in a similar manner to that of the control (IAV-GFP on its own). Therefore, the cilium itself is developing correctly in *Dyx1c1*^{-/-} flies and mechanotransduction channels are unaffected. Hence, a loss of *Dyx1c1* results in ciliary immotility caused by a loss of axonemal dynein motor arms. This phenotype supports the hypothesis that the assembly factor role of *Dyx1c1* is conserved in *Drosophila*.

3.3.10 Proteomic analysis of *Dyx1c1* knockdown testes

3.3.10.1 Optimisation of protocol

The immunofluorescent analysis described above had a limited number of markers which could be utilised. Proteomics allows for greater insight into how dynein subunits are affected when *Dyx1c1* is non-functional. This experiment was conducted on testes, where the sperm flagella are located - the other cell type in the fly that contains axonemal dynein motors. Label-free mass spectrometry was initially carried out on wild-type (Oregon-R) pupal testes to investigate whether there was a difference in the number of proteins and type of proteins detected during sperm development. As dynein pre-assembly takes place in the cytoplasm of the cell, the aim of this optimisation was to identify a stage of pupal development where testes contained many spermatocytes before the differentiation of the sperm flagellum.

For this experiment, male pupae were selected at 24h and 48h after puparium formation (APF). These time-points were selected as they are an early and middle-point of testes development where high levels of spermatocytes would be expected. 20 testes were dissected per biological replicate and there were five replicates per time point. Label-free quantification (LFQ) was performed to determine the relative level of proteins present in each sample. Table 3.1 lists the peptide signals, filtered for motile cilia proteins, detected in this initial mass spectrometry experiment. 75% of known DNAAFs had an LFQ intensity high enough to be detected, while only 19.5% of dynein chains were detected. This result suggests the time-points selected were too early, explaining the lack of dynein chain proteins.

Table 3.1 Peptide signals detected during initial mass spectrometry experiment (combining the 24 and 48 hour timepoints)

	<i>D. melanogaster</i>	<i>H. sapiens</i>
Outer arm dynein		
	<i>CG9313</i>	<i>DNAI1</i>
	<i>CG10859</i>	<i>DNAI2</i>
Inner arm dynein, single headed		
	<i>CG5526</i>	<i>DNAH7</i>
	<i>CG31802</i>	<i>CENTRIN</i>
Inner arm dynein I1/f, double headed		
	<i>CG7051</i>	<i>DIC61B</i>
Light chains		
	<i>CG10751</i>	<i>ROBL</i>
	<i>CG1014</i>	<i>ROBL62A</i>

Protofilament stability and IDA docking		
	<i>CG4767</i>	<i>TEKTIN-A</i>
	<i>CG10541</i>	<i>TEKTIN-C</i>
Radial spoke		
	<i>CG10014</i>	<i>ROPN1L</i>
	<i>CG8336</i>	<i>PPID</i>
	<i>CG17266</i>	<i>PPIH</i>
	<i>CG10578</i>	<i>DNAJ-1</i>
Dynein cytoplasmic preassembly factors		
	<i>CG14353</i>	<i>WDR92</i>
	<i>CG13570</i>	<i>RPAP3</i>
	<i>CG18675</i>	<i>C21orf59</i>
	<i>CG6980</i>	<i>TTC12</i>
	<i>CG17669</i>	<i>DNAAF3</i>
	<i>CG31320</i>	<i>HEATR2</i>
	<i>CG9750</i>	<i>REPTIN</i>
	<i>CG31623</i>	<i>DNAAF1</i>
	<i>CG4003</i>	<i>PONTIN</i>
	<i>CG5048</i>	<i>PIH1D3</i>
	<i>CG10064</i>	<i>WDR16*</i>
	<i>CG13901</i>	<i>DPCD*</i>

As shown in Figure 3.13, a small number of motile cilia-related proteins increased in expression at the later time point of 48h, but overall, most proteins remained at a similar expression. This result potentially indicates that time points may not need to be as stringent as initially thought.

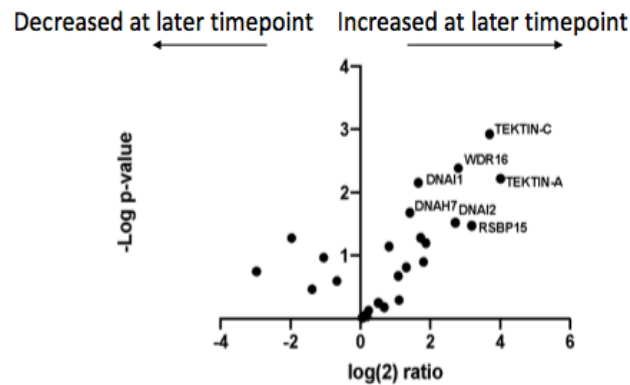


Figure 3.13. Motile-cilia proteins increase in expression at the later time point of 48h. Comparison of *OrR* males at 24h and 48h shown as a volcano plot filtered for motile cilia-related proteins. Proteins with significance ($-\log_{10}(\text{pValue}) > 1.3$) are labelled. Significance was determined using the Empirical Bayes method. $n = 20$ testes/replicate. 5 replicates per timepoint.

Successful LF-MS experiments previously conducted on *Drosophila* testes have detected >3000 proteins, sometimes >5000 (zur Lage et al., 2018; zur Lage et al., 2021). A list of 150 motile cilia accession numbers was compiled by the Jarman lab (zur Lage et al., 2018) and is maintained up-to-date using databases such as Uniprot and Flybase. This list of motile cilia proteins was used to filter the dataset obtained from the experiment and concentrate on proteins of interest. In proteomic experiments conducted by the lab, this filtered sublist typically contains ~75 proteins and so is utilised to focus more specifically on proteins associated with dynein assembly (zur Lage et al., 2018). However, in this preliminary experiment only 900 proteins were detected overall. Of these proteins, only 26 were motile cilia related. This small number of proteins detected suggests samples were at far too low a protein concentration and in future experiments a larger number of testes should be included for each replicate.

Dissecting pupal testes is time intensive. Therefore, to facilitate the collection of larger samples, I moved onto using adult testes. Sperm development continues in adult testes, and so spermatocytes as well as individualised mature sperm are present in a mixed population in these samples, unlike pupal testes, which contain more spermatocytes and less mature sperm. Furthermore, a recent paper has suggested that dynein assembly might take place during sperm individualisation rather than in spermatocytes (Fingerhut & Yamashita, 2020). Adult testes are also larger than pupal testes and contain a higher concentration of protein, verified by spectrophotometry. Therefore, in subsequent mass spectrometry experiments, adult testes were used instead of pupal testes.

3.3.10.2 *Dyx1c1* knockdown showed a reduction in abundance of dynein motor proteins

A loss of *Dyx1c1* results in defective localisation of axonemal dynein motor complexes; however, the specifics of how these structures are affected is unknown. Unassembled or poorly assembled complexes are less stable, and so it is assumed that their abundances will be reduced. Therefore, relative changes to protein abundances of dynein-related proteins were assessed in a *Dyx1c1* knockdown (UAS-*Dyx1c1*RNAi x BamGal4, the testes-specific driver line) to further examine the role of this gene. Proteomic analysis was also conducted on several other dynein related proteins simultaneously (UAS-*Dpcd*RNAi/UAS-*Spag1*RNAi), and as null mutant lines were not available for all of these and time constraints would not allow for the generation of the required CRISPR fly lines, the function of genes were knocked down using RNAi technology, which was effective in the testes as shown previously in Figure 3.8.

For each genotype, 30 pairs of testes were dissected per replicate, of which there were four. The proteomic experiment successfully detected 6171 proteins in total across all samples, and as expected, the relative abundance of *Dyx1c1* was low in the knockdown compared to the control. The reduction of the protein of interest was not as dramatic as those observed in previous

proteomic experiments conducted by the lab (zur Lage et al., 2018). Previous experiments were carried out on null mutants resulting in a complete removal of the gene of interest and greater reductions on protein levels. Therefore, it is important to note that while the RNAi-mediated knockdown was successful and reduced the protein of interest, the reductions are lower and subtle changes could be hiding amongst the noise.

Ingenuity Pathway Analysis (IPA) software was used on the unfiltered dataset to identify potential pathways and networks altered in a *Dyx1c1* knockdown other than dynein assembly. This software uses the ingenuity knowledge base, a source of well curated biological interactions and functional annotations generated from individually modelled relationships between proteins, genes, complexes, cells, tissues, drugs and diseases. This program can be useful in understanding complex proteomic data and identifying previously unknown connections.

As IPA is better suited to mammalian models only 50% of the 6170 protein hits were recognised. I conducted further analysis on the recognised proteins but there are major limitations to the results as it does not represent the dataset as a whole. Ideally this software will be improved in the future to include more information relevant to invertebrate models. However, this software was still utilised to predict what might happen downstream of the *Dyx1c1* knockdown as an effect of the protein expression in the dataset. Encouragingly, this highlighted that the developmental disorder PCD is associated with the affected pathways (Table 3.2). While this result is unsurprising given the literature surrounding *DYX1C1* and the phenotypes exhibited by the *Drosophila* null mutant, it is independent confirmation of this function of *DYX1C1* and the validity of the experiment.

Table 3.2. IPA analysis highlights the disorder PCD is associated with affected pathways in a *Dyx1c1* knockdown

Type of disease	Disease	E value	Proteins in pathway affected
Cardiovascular disease, Developmental Disorder	Kartagener Syndrome	4.09E-03	ARMC4, DNAI1, LRRC6
Connective Tissue Disorders, Developmental disorder	Macrocephaly	2.16E-02	MTOR, TRIT1
Developmental Disorder, Hereditary Disorder	Primary Ciliary Dyskinesia 1	2.61E-02	DNAI1
Developmental Disorder, Hereditary Disorder	Primary Ciliary Dyskinesia 23	2.61-02	ARMC4
Developmental Disorder, Hereditary Disorder	Primary Ciliary Dyskinesia Type 19	2.61-02	LRRC6

The dataset was then filtered to show motile cilia proteins only. This refined list was further filtered to show motile cilia proteins that were significantly depleted or enriched in a *Dyx1c1* knockdown compared to the control. Results are displayed on a volcano plot in Figure 3.14A. This plot shows that in this *Dyx1c1* knockdown, there is a general reduction in both ODA and IDA heavy chain proteins DNAH8 (ODA), DNAH3 (IDA subsets *a,b,c,e*), DNAH6 (IDA subset *g*). TTC25 of the ODA late assembly complex, CCDC43B of the MIA complex and the Nexin-DRC protein IQCD were also significantly depleted. It is important to note that some IDA and ODA heavy chain proteins remain unchanged compared to control, and some Nexin-DRC proteins. Overall, the

result suggests that *Dyx1c1* may have a role in the assembly of outer and inner dynein arms in *Drosophila*.

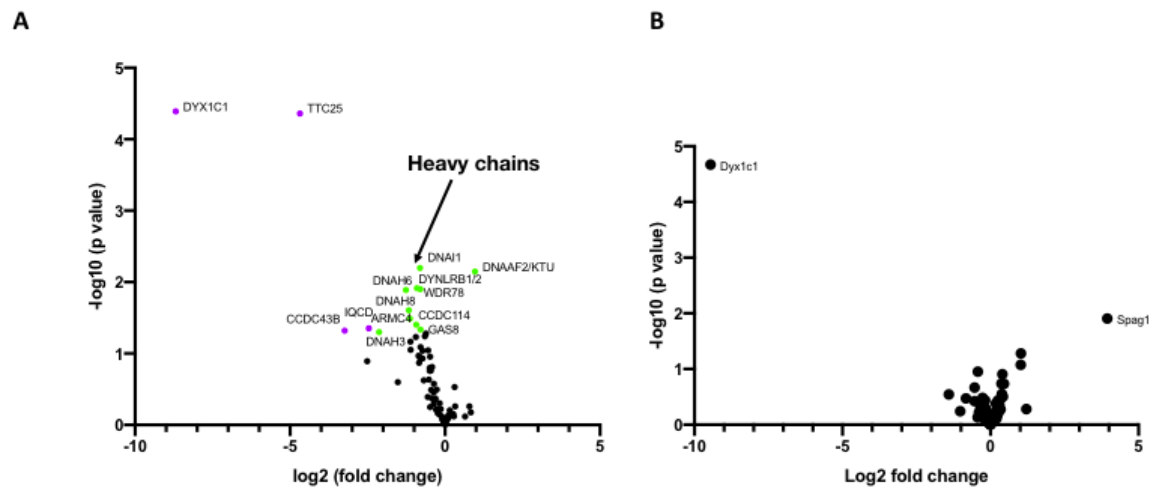


Figure 3.14. Proteomic analysis supports a dynein assembly role for *Dyx1c1* in *Drosophila*. (A) Comparison of *Dyx1c1*RNAi knockdown males (*Dyx1c1*RNAi x UAS-*BamGal4*) with control (KK injection line x UAS-*BamGal4*) shown as volcano plot filtered to show motile cilia-related proteins. Proteins with significance ($-\log_{10}(\text{p value}) > 1.3$) are labelled in green. Proteins of interest with a $\log_2(\text{fold change}) > \pm 1.5$ and a significant p value are labelled in magenta. (B) Comparison of the datasets obtained from *Dyx1c1* knockdown and *Spag1* knockdown supports that these two proteins work in similar complexes as other motile cilia proteins are expressed similarly in both knockdowns. Significance was determined using the Empirical Bayes method. $n = 30$ pairs of testes/replicate. 4 replicates per genotype

3.3.10.3 A *Dyx1c1* knockdown compared to a knockdown of the known DNAAF *Spag1*

Dyx1c1 is truncated in *Drosophila* and lacks the TPR domains required for important protein-protein interactions, including the interaction with the chaperone Hsp90. *Dyx1c1* has a conserved axonemal assembly factor function in *Drosophila* despite this truncation. One hypothesis about how this role is conserved is that another TPR-containing protein works with *Dyx1c1* to compensate for the truncation. As *Spag1* is also a known PCD-causative gene containing TPR repeats that is truncated in *Drosophila*, changes to protein abundances in a knockdown of this gene were also assessed and compared to that of the *Dyx1c1* knockdown. The aims of this analysis were to identify whether similar dynein proteins were downregulated in both knockdowns, and what effect the knockdown of one of these DNAAFs has on the other one.

When comparing datasets of the motile cilia proteins detected in *Spag1* and *Dyx1c1* knockdown experiments shown in Figure 3.14B, there are no proteins other than those expected (*Spag1*, *Dyx1c1*) significantly depleted or enriched. This result indicates that similar proteins are affected in both knockdowns and suggests that *Spag1* and *Dyx1c1* could work in similar complexes, with *Spag1* potentially providing the important TPR domains.

3.3.11 GFP-Trap AP-MS did not identify direct interactions with known DNAAFs *in vivo*

Dyx1c1, although truncated, has a conserved DNAAF role in *Drosophila* with a role in the assembly of both outer and inner dynein arms. To assess whether an additional protein provides the TPR domains missing in the *Drosophila* homologue, A GFP-Trap Affinity Purification Mass Spectrometry (AP-MS) was conducted using the validated *Dyx1c1mVenus* line to identify protein interactors *in vivo* and test the notion that *Dyx1c1* works as part of an R2TP-like complex.

In this form of ‘bottom-up proteomics’, proteins of interest (i.e., *Dyx1c1*), as well as interaction partners are usually isolated during the purification process. 150 pairs of testes were dissected per replicate, and there were three replicates per genotype. The control for this experiment was testes from F1 males of *GAPmVenus* crossed to *BamGal4*, in which GFP is ubiquitously expressed. As shown in Figure 3.15A, which displays all proteins (unfiltered dataset) found to be associated with *Dyx1c1mVenus*, the *Dyx1c1* protein itself was pulled down, as was Pontin of the R2TP complex. This result suggests that the DNAAF *Dyx1c1* works with some or all the components of this co-chaperone complex during the assembly process. Figure 3.15B shows the proteins associated with *Dyx1c1mVenus* but has been filtered using the motile cilia sub list mentioned in the previous proteomics experiments. This analysis showed that *Dpcd*, a putative motile cilia protein of high relevance to this PhD project (see Chapter 5), as well as the DNAAF *Heatr2* are close to significance. Overall, the AP-MS analysis supports the idea that *Drosophila* *Dyx1c1* is part

of an R2TP-like complex, as suggested by the literature, despite the lack of TPRs.

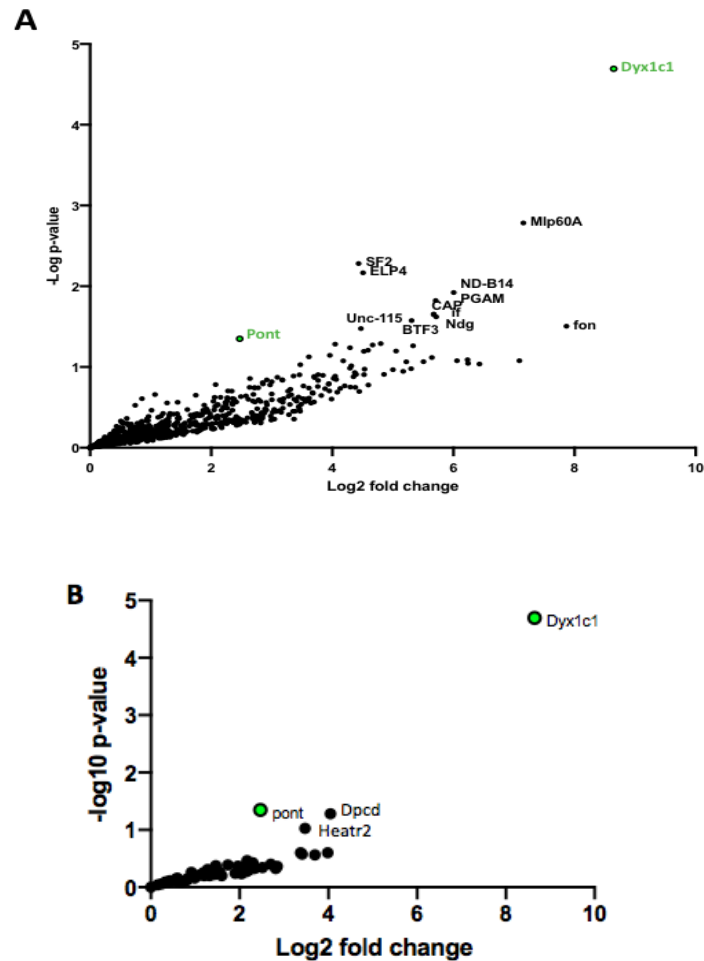


Fig 3.15 GFP-Trap AP-MS detected less motile-cilia related protein interactors for Dyx1c1 than expected. (A) Volcano plot showing protein associated with Dyx1c1-mVenus from AP-MS analysis. Significant proteins ($\log_{10} (p\text{Value}) > 1.3$, $\log_2(\text{fold change}) > 1.5$) are named on graph with motile cilia-related proteins labelled in green. (B) Volcano plot showing filtered dataset from AP-MS analysis – only motile cilia proteins present. Dpcd, a putative motile cilia protein, as well as the DNAAF Heatr2 are close to significance. Pontin of the R2TP complex is an interactor of Dyx1c1. Significance for (A and B) was determined using the Empirical Bayes method. $n = 150$ pairs of testes/ replicate. 3 replicates per genotype

However, no other significant motile cilia protein hits were obtained. One possible cause of this could be a large number of proteins sticking to the negative control, therefore reducing the number of significant hits in the Dyx1c1mV pull-down. These hits are not supposed to be present when the bait protein is absent and may be unspecifically precipitated by the GFP-Trap.

These proteins may be denatured, causing non-specific interactions to occur. A more stringent wash buffer with a higher salt concentration may aid the prevention of non-specific background, and therefore additional protein interactors of Dyx1c1 in *Drosophila*. In the DYX1C1 mouse interactome, no known DNAAF proteins were detected either (Tarkar et al., 2013). However, this does not mean no further interactions occur and highlights that several methods for detecting protein interactions such as co-immunoprecipitation should be utilised.

3.4 Discussion

Overall, this project shows that the DNAAF role of *Dyx1c1* is conserved in *Drosophila* despite its truncation and that it is required for the assembly of both outer and inner axonemal dynein arms.

3.4.1 Dyx1c1 embryonic expression pattern

In the *Dyx1c1*mRNA *in situ* hybridisation, late-stage embryos showed expression specific to a subset of chordotonal neurons (lch5). This expression in a specific subset of chordotonal neurons has not been previously observed for DNAAFs (zur Lage et al., 2021, 2018; Moore et al., 2013).

The immunofluorescent data obtained using the Dyx1c1mVenus protein indicates at pupal stage, this protein is present in all chordotonal neurons and not just a subset. Moreover, at embryonic level, Dyx1c1 protein is expressed in all chordotonal neurons. Therefore, *Dyx1c1*mRNA is most likely transiently expressed at an earlier stage in all embryonic chordotonal neurons and therefore, difficult to detect.

The Dyx1c1mVenus staining in embryos shows additional expression not observed in the *in situ*. Dyx1c1 is also present in the external sensory (ES) cells, which mediate taste and olfaction and while they are ciliated, they do not contain dynein arms and are therefore considered immotile. This expression

pattern is not typical for a motile cilia protein, suggesting regulation was potentially disrupted in the generation of the mVenus fusion stock. There may be an additional regulator of *Dyx1c1*, which the role of is to limit gene expression to motile cilia cell types. This embryonic staining shows expression in type-I sensory organs: both the ES neurons and Ch neurons, which derive from the same original source (a single ectodermal precursor) before asymmetric cell division results in the different sensory organs (Brewster & Bodmer, 1995). This differentiation process is regulated by proneural genes and is described in greater detail in Chapter 2. There are many additional regulators involved in type-I sensory cell specialisation including *cut*, which is a homeodomain protein required for deciding es-ch cell fate (Bodmer, 1987). Cut, or an additional proneural factor may play a role in restricting the expression of *Dyx1c1* to the Ch neuronal lineage. *Jim lovell (lov)*, a gene which has a regulator role during midline development is expressed in ES organs as well as a subset of ch organs (lch 2 and 4). As this protein is nuclear, it is suggested to play a role in transcriptional regulation (Bjorum et al., 2013). Therefore, this is another candidate regulator of *Dyx1c1* expression, and future work includes assessing for binding sites for *lov* upstream of *Dyx1c1*.

Later in development, *Dyx1c1* expression is as expected for its hypothesised role as a motile cilia gene. At pupal stage, there appears to be specific chordotonal expression in the antenna. Therefore, this additional and unusual expression is restricted to the embryonic stage. As future work for this mVenus reporter line included use in a GFP-Trap experiment, it was important for it to be validated. The mVenus reporter line successfully rescued a *Dyx1c1* CRISPR-mediated knockout, indicating that it is functional and can be used in future experimentation.

3.4.2 Dyx1c1 (CG14921) is the *Drosophila* homologue of the DNAAF DYX1C1, despite lacking TPR repeats

Studies in humans, mice, zebrafish and *Chlamydomonas* indicate that when DYX1C1 is non-functional, ciliary defects occur (Chandrasekar et al., 2013; Tarkar et al., 2013; Yamamoto et al., 2017). As dynein motor complexes facilitate ciliary motility, when these structures do not assemble correctly the phenotypes described in a *DYX1C1* mutant occur. When DYX1C1 function is disrupted, both outer and inner dynein arms are affected (Tarkar et al., 2013). Specifically, DYX1C1 is thought to have a role in the assembly of ODAs and all the IDA subsets in varying degrees. In *Chlamydomonas*, a loss of *DYX1C1* resulted in a particularly severe effect on the IDA subsets *b,c,d,e,f* and *g* (Yamamoto et al., 2017).

Our research using a *Drosophila* model supports the data obtained from other organisms. The *Drosophila* homologue is expressed uniquely in motile cilia cells and when function is disrupted it causes proprioceptive, auditory and fertility defects – phenotypes typically observed for motile cilia genes. In *Drosophila*, *Dyx1c1* is specifically expressed in the cytoplasm of motile cilia cells, where axonemal dynein pre-assembly occurs. This localisation has been previously observed for *Drosophila* DNAAFs suggesting that DYX1C1 function is conserved (zur Lage et al., 2021, 2018).

TEM was conducted on adult antennae of the *Dyx1c1* null mutant and showed a loss of both ODAs and IDAs. This was a similar phenotype to that observed in the *DYX1C1*^{-/-} mouse TEM results (Tarkar et al., 2013).

Previous proteomic analyses showed that knockouts of the DNAAFs Zmynd10 and Wdr92 result in a degradation of dynein motor subunits, particularly heavy chains (these are also the subunits where PCD mutations are most located) (Mali et al., 2018; zur Lage et al., 2018). The proteomic results shown in this chapter support that *Dyx1c1* has a similar role to these DNAAFs during dynein assembly in *Drosophila*.

Immunofluorescent staining was performed on pupal antennae to further pinpoint the specific role of Dyx1c1 in *Drosophila*. The localisation of ODA subunits was disrupted, similar to what has been reported in other organisms. As the outer dynein arm drives motor function, the loss of this structure correlates with the severe motile cilia defect observed in the *Dyx1c1* knockout. The localisation of the IDA light-intermediate subunit Dnali1 was also assessed. This chain is present in subsets *a* and *c* in *Drosophila* and partially localised in a *Dyx1c1* knockout. Therefore, in *Drosophila*, *Dyx1c1* could have a role in the assembly of one, or partially in both Dnali1-containing IDA subsets. As *Chlamydomonas* research suggests a less important role for DYX1C1 in IDA subset *a* assembly (Yamamoto et al., 2017), this may be conserved in *Drosophila* and the IDA subset *a* is localising to the cilium as normal. Therefore, from this localisation study we can propose that *Dyx1c1* is specifically required for the assembly of IDA subset *c*, as well as potential additional subsets. To pinpoint what these may be, the lab has generated an antibody against another IDA subunit DNAH7, which is present in subsets *a,b,c* and *e*. By understanding how the localisation of this structure is affected in a *Dyx1c1* null mutant, the specific role of *Dyx1c1* during dynein assembly will be further refined.

The TPR domains of DYX1C1, which mediate protein-protein interactions, have been shown to be important for its multiple function in higher organisms (Taipale et al., 2003; Tarkar et al., 2013; Wang et al., 2006). However, *Dyx1c1* is truncated in *Drosophila*, and lacks these TPR repeats. The role of *Dyx1c1* during dynein assembly is conserved in *Drosophila*, suggesting that the TPR domains are not required in this organism. *Dyx1c1* may have a more restricted role in *Drosophila*, specific to dynein assembly. The TPR repeats may be required for any additional functions of DYX1C1 in higher organisms that may exist, and not for this dynein assembly role.

77% human PCD-causing mutations in *DYX1C1* mapped to the C-terminus where the TPR repeats are located (Tarkar et al., 2013). A mutant gene would match the *Drosophila* truncation due to a premature termination of translation

and therefore lack TPR repeats, and yet it causes PCD. The truncated human gene may not produce a protein because of nonsense-mediated decay - when mRNAs that have premature stop codons are degraded. This is a surveillance mechanism to prevent errors in protein synthesis but may be causing the disease phenotype if the translated truncated protein is sufficient (Miller & Pearce, 2014). These observations suggest a potential route to therapy. As the TPR repeats may not be essential for the DNAAF role of DYX1C1, a potential therapeutic for patients with these mutations is premature termination codon (PTC) suppression. Anticodon engineered transfer RNAs (ACE-tRNA) suppress in-frame premature stop codons and result in a functional truncated protein to be generated. This procedure has already been shown to be a successful treatment for cystic fibrosis and so may be a future direction in PCD therapeutics for Dyx1c1 patients (Lueck et al., 2019).

As described above, the truncated Dyx1c1 lacking TPR repeats may be sufficient for the DNAAF role in *Drosophila*. This could be because they are not essential for dynein assembly, or it could be that in *Drosophila*, an additional protein works with Dyx1c1, providing those vital protein domains.

Dyx1c1 is not the only Rpap3-like protein to be truncated in *Drosophila*, as CG13570 (Spag/RPAP3) and CG18472 (Spag1) are also lacking important protein domains (zur Lage et al., 2018). SPAG1 is a homologue of DYX1C1 and contains TPR repeats. If this similarity is conserved in *Drosophila* these genes could be working together to carry out function, compensating for the lack of TPR repeats. However, while Spag1 could be working with Dyx1c1 in *Drosophila*, the results obtained above from sequence alignment and phylogenetic analysis indicate differences between the two DNAAFs. The truncation events did not occur simultaneously (meaning that in *Drosophila* these are not two parts of the one gene which separated during evolution). However, proteomic analysis suggests similar functions for the two DNAAFs, perhaps working in complexes together during the assembly process. Future directions to further investigate whether Spag1 works in a complex with Dyx1c1, providing the TPR repeats, would be to assess whether they interact

on a protein level through co-immunoprecipitation experiments in S2 cells or to look for evidence of *in vivo* genetic interaction between the two genes. The latter could be achieved through the generation of trans-heterozygotes and examining whether these flies have a reduced climbing ability and fertility when compared to individual heterozygotes.

3.4.3 Potential neural migratory role for DYX1C1 in higher organisms

DYX1C1 has been linked, somewhat controversially, to dyslexia. While this gene co-segregated with dyslexia in one study, its functional alleles did not do so in other populations (Marino et al., 2005; Meng et al., 2005; Scerri, 2004). DYX1C1 has also been reported to be required for neuronal migration in the developing neocortex (Wang et al., 2006) and this has been taken as a possible explanation to the dyslexia link. The TPR domains, which are absent in *Drosophila* Dyx1c1, have been shown to be important for these additional functions in higher organisms. The Dyx1c1mVenus fusion protein shows no expression in the larval CNS and therefore does not support a CNS/neural migratory role in *Drosophila*, suggesting that it is specifically motile cilia in this organism. This specificity is further supported by the *Dyx1c1* null mutant that was generated, as it is viable and shows specifically motile cilia defects. An additional role for Dyx1c1 in the CNS would result in more severe phenotypes than auditory, proprioceptive and fertility deficits.

L-R asymmetry, which is affected in *DYX1C1* zebrafish mutants, is important during embryonic development, and when this does not occur correctly can impact brain structure and connectivity (Chandrasekar et al., 2013; Tarkar et al., 2013). Therefore, the potential role of *DYX1C1* in neuronal migration during brain development may be an indirect effect of its motile cilia function. Several ciliary genes are required for brain development, and many ciliopathies can lead to cognitive defects (Valente et al., 2014). Moreover, neuropsychiatric disorders including schizophrenia, autism, bipolar disorder and dyslexia are linked to L-R asymmetry (Trulioff et al., 2017). Axonemal dynein motors are present in both nodal and motile cilia and are required during nervous system

development for Cerebrospinal Fluid (CSF) flow (Kumar et al., 2021). When this movement is affected, it can result in hydrocephalus and prevent the migration of neural progenitors (Sawamoto et al., 2006; Trulioff et al., 2017). A recent study sequenced the genome of two people with both *situs inversus* and developmental dyslexia. One individual had two disadvantageous mutations in *DNAH5*, and the second individual had a mutation in *DNAH11*. As these genes are not highly expressed in the human brain (Allen Brain Atlas) and are not known to have a role in primary or neuronal cilia, it is most likely that this link between *situs inversus* and developmental dyslexia is due to defective nodal cilia (Bieder et al., 2020). This finding supports the likely situation that DYX1C1 could be required for neuronal migration indirectly because of its role solely as a DNAAF.

3.5 Conclusion

DYX1C1 (DNAAF4) is involved in the assembly of ODA and IDAs. When this gene is non-functional in mice, it results in ciliary immotility phenotypes, including hydrocephalus and *situs inversus*. DYX1C1 is also reported to have a role in dyslexia and/or neuronal migration - suggesting a complex role in multiple biological processes in higher organisms. As both *situs inversus* and dyslexia are both not that uncommon, their link with DYX1C1 could be coincidental. However, this association with brain development could also be an indirect effect of its motile cilia function. As *Drosophila* is a straightforward model to study motile cilia genes, the aim of this chapter was to use this model to gain further insight into the role of this gene during axonemal dynein assembly.

The *Drosophila* homologue is truncated and missing TPR domains. These protein domains are important for binding the R2TP co-chaperone complex to HSP90 during dynein assembly, and it is thought to have a similar role in TPR-containing DNAAFs. However, despite truncation, the *Drosophila* homologue shows similar results to that obtained in higher organisms. Dyx1c1 is

expressed in motile cilia cells and when non-functional, results in viable flies with motile cilia defects. *Drosophila* Dyx1c1 has a role in the assembly of ODAs and IDAs. From assessing the partial localisation of Dnali1, an IDA subunit, it is clear that Dyx1c1 is more important in some IDA subsets than in others. In *Chlamydomonas*, it was suggested that DYX1C1 plays less of an important role in IDA subset a assembly. As Dnali1 is present in IDA subsets a and c (and in higher organisms also d), *Drosophila* Dyx1c1 may be required for the assembly of c. To further pinpoint all the subsets which Dyx1c1 plays a crucial role in assembly in *Drosophila*, additional antibodies will be raised against IDA subunits and the localisation of these will be examined in a *Dyx1c1* knockout.

As the function of Dyx1c1 is conserved in *Drosophila*, the TPR domains may not be required in this organism. It could also be that another TPR-containing DNAAF, such as Spag1, works with Dyx1c1 and provides the important protein domains. Another possibility is that the TPR repeats are not needed for the dynein assembly function of DYX1C1. Human DYX1C1 PCD-causing mutations are mostly found in the C-terminal where the TPR domains are located. However, if a truncated protein lacking these domains is sufficient, perhaps this protein is not being produced due to nonsense-mediated decay. Therapeutics are currently underway for other disorders, suppressing this surveillance mechanism and allowing a functional truncated protein to be generated - and could be a potential therapeutic for this form of PCD.

Chapter 4

CG5048, the *Drosophila* homologue of the PIH protein PIH1D3, forms an R2TP-like complex with CG14921 (DYX1C1).

4.1 Introduction

4.1.1 The PIH proteins and dynein assembly

As mentioned in Chapter 3, PIH1D1 is a component of the R2TP co-chaperone complex, which works with Hsp90 during the assembly of various protein complexes including axonemal dyneins. This protein contains a CS and PIH domain, which are important for its Hsp90 regulatory role (Kakihara & Houry, 2012). Several other proteins contain related domains, referred to as the PIH proteins. Interestingly, many of these have DNAAF roles and form the basis of the proposed R2TP-like complexes. It is not known what the precise roles these different PIH proteins (and the complexes they are a part of) play during dynein assembly.

All organisms with motile cilia have 3-4 PIH proteins, whereas those that do not have motile cilia, such as *S. cerevisiae* and *C. elegans*, only have the widely expressed PIH1D1 (Yamaguchi et al., 2018). Therefore, the PIH proteins are important for motile cilia function. There is a lack of comparison of the PIH proteins across organisms. Table 1 below illustrates the different gene names for them, and how they relate across species.

Table 4.1 Homologues of the human PIH proteins

Human	Zebrafish homologue	Chlamy. homologue	Fly homologue
PIH1D1	PIH1D1	MOT48	CG5792
PIH1D2	PIH1D2	N/A	CG4022
DNAAF2	KTU	KTU/PF13	CG1553
PIH1D3/DNAAF6	TWISTER	TWI	CG5048

As shown in Table 4.1, in the vertebrate genome there are four PIH proteins. These are PIH1D1 (of the canonical R2TP complex), PIH1D2, DNAAF2 and PIH1D3 (also known as DNAAF6) (Yamaguchi et al., 2018). The zebrafish homologues are PIH1D1, PIH1D2, KTU and TWISTER, respectively (Yamaguchi et al., 2018). *Chlamydomonas* has three PIH proteins: MOT48, KTU/PF13 and TWI, as there is no PIH1D2 equivalent. Research has been conducted in all these model organisms to gain more insight into the individual functions of these PIH proteins during assembly, as this is yet to be fully elucidated.

For example, studies in medaka, human and *Chlamydomonas* have revealed that mutations in the PIH protein DNAAF2 lead to PCD-like phenotypes caused by defective outer and inner dynein arm assembly (Cheong et al., 2019; Omran et al., 2008; Sun et al., 2020). As discussed in the Introduction, this DNAAF was one of the first to be classified as an assembly factor, when it was found not to be a dynein subunit and instead to be required for the assembly process that takes place in the cytoplasm (Omran et al., 2008).

PIH1D3 has also been shown to be required for the cytoplasmic assembly of axonemal dynein arms and is an X-linked PCD-associated gene causing defects to both type 1 and type 2 ODAs. This DNAAF is also required for IDA assembly, as well as for stabilising and facilitating the formation of the ODA intermediate-heavy chain complex. DNALI1, a subunit commonly used as an

indicative marker of IDA dynein localisation, was found to be greatly reduced or absent in PIH1D3 mutant respiratory ciliary axonemes - further supporting defective cytoplasmic preassembly of IDAs (Dong et al., 2014; Olcese et al., 2017; Paff et al., 2017).

Some studies have sought to compare the roles of different PIH proteins in dynein assembly. In zebrafish, the four PIH proteins were shown to be required for the assembly of different dynein subtypes. If required for context, diagrams showing the position of the ODAs and different IDA subsets within the motile cilia 96nm repeat unit can be found in Chapters 1 and 3.

Null zebrafish embryos were generated for each of the different PIH proteins: PIH1D1 and PIH1D2 by TALEN, and KTU (DNAAF2 homologue) and TWISTER (PIH1D3 homologue) by CRISPR/Cas9 (Yamaguchi et al., 2018). The resulting mutant spermatozoa were examined to observe their axonemal dyneins using cryo-ET and subtomogram averaging. Different dynein subsets were affected depending on the PIH protein that was absent as shown in Figure 4.1. It was found that PIH1D1 has a role in ODA and IDA subset *c* assembly. PIH1D2 and KTU, which have partially overlapping functions in zebrafish, have roles in the assembly of ODAs and IDA subsets *b*, *c* and *e*. TWISTER, the PIH1D3 homologue, has a role in the assembly of ODAs and IDA subsets *c*, *d* and *g*. These results were supported with biochemical data obtained through immunoblotting, using specific antibodies raised against Zebrafish axonemal dynein components. DNALI1, which was mentioned above as being heavily reduced or absent in PIH1D3 mutant respiratory ciliary axoneme, is the orthologue of *Chlamydomonas* p28, which is present in IDA subsets *a*, *c* and *d*. In this study, DNALI1 was slightly reduced in *pih1d1*^{-/-}, *ktu*^{-/-} and the double mutant *pih1d2*^{-/-}; *ktu*^{-/-}, supporting the loss of one of these IDAs (IDA *c*) in these mutants. In a TWISTER mutant, DNALI1 reduction was more substantial as two IDA subsets (*c* and *d*) were lost (Yamaguchi et al., 2018).

The specific function of the PIH proteins differs between zebrafish and *Chlamydomonas*. In *Chlamydomonas*, spectral counting and HPLC chromatography were used to measure how dynein subunits were affected in

PIH protein mutants. MOT48, which is the putative orthologue of PIH1D1, was identified through the analysis of a *Chlamydomonas* mutant (*ida10*) and as shown in Figure 4.1, found to have a primary role in the assembly of ODAs and IDAs *b*, *c*, *d* and modestly IDAs *e* (Yamamoto et al., 2010, 2020). KTU/PF13, the second of the three PIH proteins in *Chlamydomonas*, was found to have a primary role in the assembly of ODAs and IDA subset *b*, *c*, and *g*, suggesting some element of functional redundancy in IDA assembly (Omran et al., 2008; Yamamoto et al., 2010). TWI, the third conserved PIH protein in *Chlamydomonas* and the PIH1D3/TWISTER homologue, has a primary role in the assembly of IDA subset *c* (Yamamoto et al., 2020). Using gel electrophoresis and ion exchange column chromatography, dynein composition in the axoneme was observed and results were verified through western blot analysis using antibodies against dynein subunits. As *Chlamydomonas* is a useful model organism to carry out genetic manipulation in, double mutants of the PIH gene were able to be isolated to observe whether the PIH proteins work together in similar pathways during dynein assembly. These experiments revealed partially overlapping functions for the PIH proteins in *Chlamydomonas*. However, this model also has limitations in terms of identifying exact functions. As the three-headed ODA assembly relies on the presence of certain ODA subunits, the precise role of the individual PIH proteins during ODA heavy chain assembly is unknown. A similar limitation exists in relation to the IDA subunit *f/I1*. In this study no PIH protein was identified as having a primary role in the preassembly of IDA subset *a* and *f/I1*, and so these subsets may be assembled by additional factors (Yamamoto et al., 2020). However, Electron Tomography (ET) of human *PIH1D3* PCD patients did show a loss of IDA subset *f/I1*, as well as subset *g* (Olcese et al., 2017).

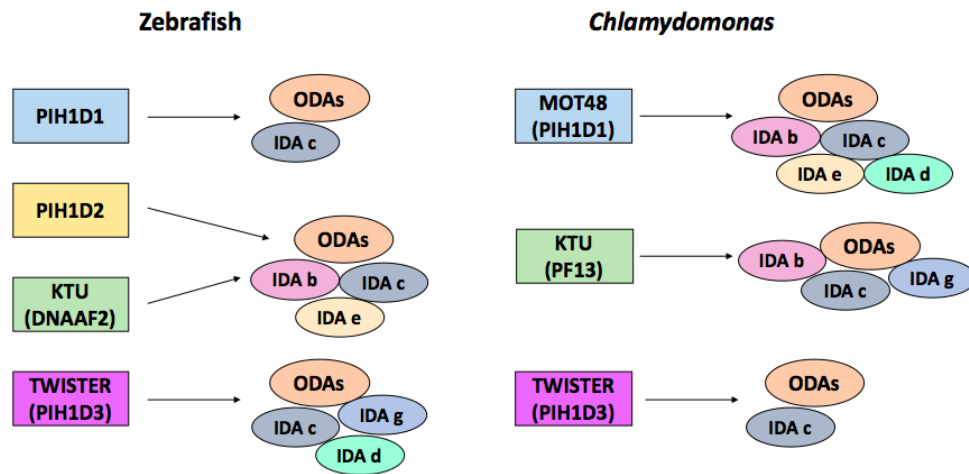


Figure 4.1 The PIH proteins have different roles during the axonemal dynein assembly process, and this varies between organisms. Mutant zebrafish spermatozoa were examined to pinpoint the roles of the individual PIH proteins. PIH1D1 has a role in the assembly of ODAs and IDA subset c. PIH1D2 and KTU (DNAAF2) have overlapping functions in the assembly of ODAs and IDA subsets b, c and e. TWISTER (PIH1D3) has a role in the assembly of ODAs and IDA subsets c, d and g. In *Chlamydomonas*, there are three PIH proteins and using spectral counting and HPLC chromatography it is evident that their individual roles differ from those found in zebrafish. MOT48 (PIH1D1) has a role in the assembly of ODAs and IDA subsets b, c, d and e. KTU is involved in the assembly of ODAs and IDA subsets b, c and g. TWISTER (PIH1D3) has a role in the assembly of ODAs and IDA subset c.

Overall, these experiments in Zebrafish and *Chlamydomonas* show a loss of specific axonemal dynein subsets depending on the PIH protein affected. The ODAs (and IDA subset c) were the most affected by PIH mutants, supporting the suggestion that ODA assembly takes place in several stages, each mediated by a different PIH protein working as part of an R2TP-like complex (see next section). Interestingly IDA subset a and f were unaffected in the null mutants in both Zebrafish and *Chlamydomonas*, suggesting that these dynein subsets are assembled independently. Alternatively, different PIH proteins are functionally redundant in the assembly of these subsets (Yamaguchi et al., 2018; Yamamoto et al., 2020).

The experiments described above in zebrafish and *Chlamydomonas* indicate that the different PIH proteins play individual roles during the assembly process and suggest that these diverge between organisms. Therefore, it is

not yet known in *Drosophila* what the specific functions of the different PIH proteins are. As shown in Figure 4.2, the DRSC Integrative Ortholog Prediction Tool (DIOPT) software indicates that the PIH protein homologues in *Drosophila* are CG5792 (Pih1d1), CG1553 (Dnaaf2) and CG5048 (Pih1d3). While CG4022 shows homology to Pih1d2, the lack of transcriptomic data suggests that this may not be a functional gene and instead be a pseudogene. Therefore, *Drosophila* seems to lack a functional *Pih1d2*.

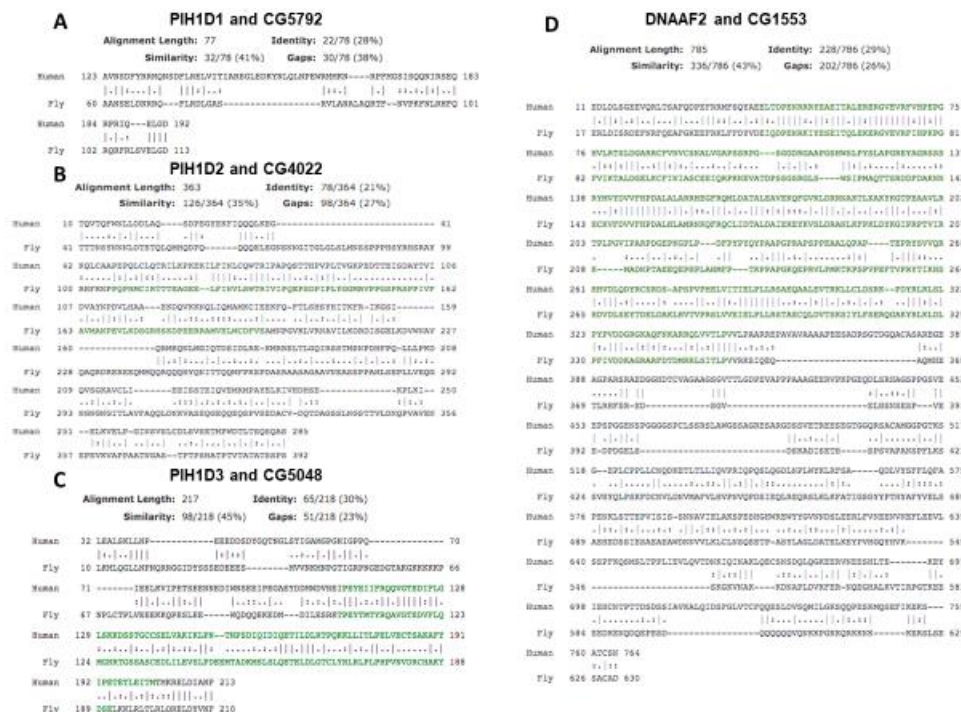


Figure 4.2 DIOPT software detects *Drosophila* homologues of the human PIH proteins. (A) Alignment of the p23-like domain of PIH1D1 and the *Drosophila* homologue CG5792 (Pih1d1) which has 28% identity. (B) Alignment of PIH1D2 and the *Drosophila* homologue CG4022, which is non-functional. (C) Alignment of PIH1D3 and the *Drosophila* homologue CG5048 (which will be referred to as Pih1d3 for the rest of this thesis). The conserved p23-like domain is highlighted in green. (D) Alignment of the PIH protein DNAAF2 and its *Drosophila* homologue CG1553 (Dnaaf2) showing 43% similarity and 29% identity. The highly conserved PIH domain is highlighted in green (42% identity). Alignment key: “|” indicates exact amino acid match, “:” indicates a strong positive score on the BLOSSUM62 matrix and “.” indicates any other two bases which align.

The *Drosophila Pih1d1* homologue has been reported as having a role in motile cilia cells (zur Lage et al., 2018). Additionally, the *Drosophila Dnaaf2* homologue has been described as showing expression in motile cilia cells

(albeit moderately) and is predicted to be required for motile cilia function (zur Lage et al., 2019).

This chapter will focus on the *Drosophila* homologue of *Pih1d3*, *CG5048*, which has been shown to be highly expressed in motile cilia cells, consistent with a role in dynein assembly (zur Lage et al., 2019).

4.1.2 The role of R2TP-like complexes in axonemal dynein assembly

As some DNAAFs share similar protein domains (TPR, CS) to the individual components of the R2TP complex, it is thought that they form R2TP-like complexes with RuvBL1/2 to carry out specific roles during the dynein assembly process (Maurizy et al., 2018; Pal et al., 2014). There are multiple R2TP-like interactions involving the different PIH proteins. As shown by Maurizy et al., (2018), these include SPAG1-DNAAF2, DYX1C1-DNAAF2 and DYX1C1-PIH1D3 shown below in Figure 4.3. SPAG1, an ancient duplication of RPAP3 and described in the previous chapter, contains 3 TPR repeats as well as a PIH binding domain and an RPAP3 C-like terminal. SPAG1 has been proposed to form an R2TP-like complex with HSP90, PIH1D2 and RUVBL1/2 termed the R2SP complex, which has a role in the early stages of dynein assembly (Maurizy et al., 2018). Co-IP experiments in *Drosophila* S2 cells as well as in HEK293 cells support the presence of this R2SP complex (Smith et al., 2022; zur Lage et al., 2018).

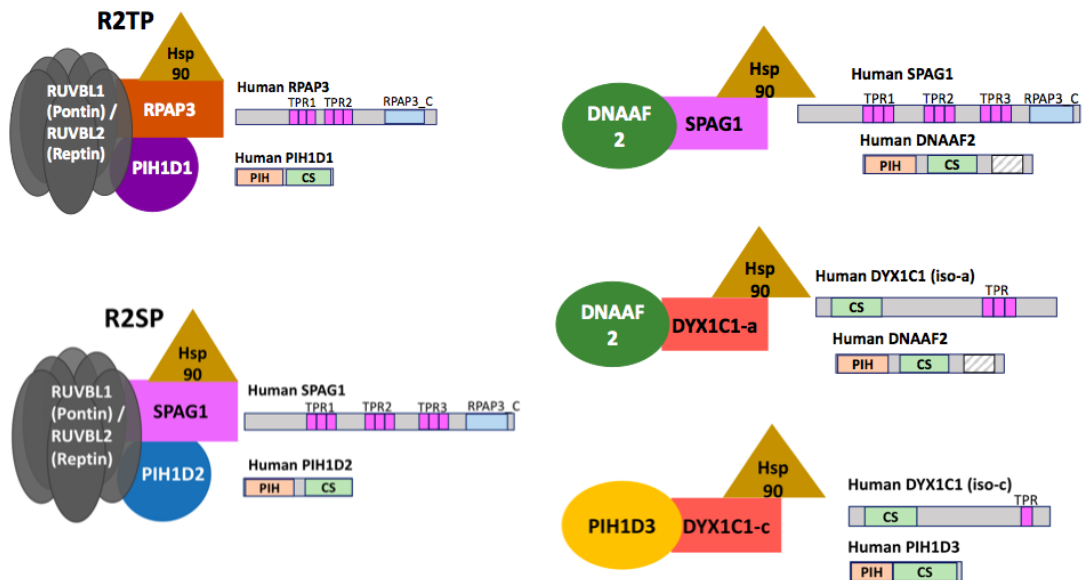


Figure 4.3 Potential R2TP-like interactions that have been previously reported. RPAP3 of the R2TP complex contains TPR repeats important to its interaction with Hsp90 (triangles). The DNAAFs DYX1C1 and SPAG1 are also TPR proteins (rectangles). PIH1D1 of the R2TP complex contains PIH and CS domains, as do the other DNAAF PIH proteins discussed in this chapter (circles). As these DNAAFs contain protein domains important for mediating protein-protein interactions, and like those of the individual components of the R2TP complex, it is thought that these proteins form R2TP-like complexes with each other.

There is high sequence similarity between the CS domains of both PIH1D3 and DNAAF2. This is the protein domain of PIH1D1 of the endogenous R2TP complex which interacts with the TPR repeats of RPAP3. Therefore, it is suggested that the PIH proteins form R2TP-like complexes by interacting with TPR domains of DNAAFs such as DYX1C1, shown in Figure 4.3 (Olcese et al., 2017). This DNAAF is described in Chapter 3 and contains a CS domain and TPR repeats, which vary in number depending on the isoform. There are 3 DYX1C1 isoforms in humans and 2 in mice. These TPR domains (Fig. 4.3) are crucial for the DYX1C1 R2TP-like interaction with PIH protein DNAAF2 (isoform-a), with which it has a strong association. DYX1C1 also has a strong interaction with PIH1D3 (isoform-c), but with an IP efficiency of 5-10% less than that of DNAAF2 (Maurizy et al., 2018).

DYX1C1 therefore interacts with both DNAAF2 and PIH1D3, PIH proteins which have been associated with the assembly of IDA subsets *b,c,d*, *e* and *g* (Yamaguchi et al., 2018; Yamamoto et al., 2020). However, when DYX1C1 is absent in *Chlamydomonas* the assembly of all IDA subsets is affected, suggesting additional functions of DYX1C1 that are independent of these PIH proteins (Yamamoto et al., 2017). This observation further highlights the complexity of the dynein assembly process, as well as the precise roles of these R2TP-like complexes during it.

These roles remain unclear, as does whether they are specific to dynein assembly, required for specific steps in the assembly process, or required for specific subsets of dyneins. This process is further complicated in higher organisms such as mammals, where some DNAAFs have wider roles. Thus, *Drosophila* is commonly used as a straightforward model to gain insight into the dynein assembly function of these genes in humans.

4.1.3 Pih1d3 in *Drosophila*

This chapter focuses on *Pih1d3* as this gene is predicted to potentially interact with *Dyx1c1*, which was characterised in the previous chapter. In that chapter, a *Drosophila Dyx1c1* null mutant was generated and showed that its assembly factor function is conserved despite truncation. Therefore, in this part of the project a null mutant of the putative protein partner *Pih1d3* was also generated to follow-up preliminary experiments conducted by the lab into its general function.

It is not yet known whether this interaction is conserved in *Drosophila* and what its specific role is during dynein assembly. As both DYX1C1 and PIH1D3 have been suggested to be late pre-assembly proteins, so it is possible that this complex carries out its role at a late stage in the assembly process (Horani et al., 2018). They could also have a role in the assembly of an intermediate complex, suggested by Mali et al., (2018).

4.2 Aims

This chapter will examine the PIH proteins of *Drosophila* and assess how similar they are to the PIH homologues of other organisms. The PIH protein PIH1D3, (also known as DNAAF6) will be the focus of this chapter. This DNAAF has been shown in other organisms to have an R2TP-like interaction with DYX1C1 - the *Drosophila* homologue of which was characterised in Chapter 3. In this chapter, a *Pih1d3* null mutant is generated to further elucidate the function of this proposed R2TP-like complex during the dynein assembly process.

4.3 Results

4.3.1 Preliminary investigation of *Drosophila Pih1d3*

Preliminary characterisation of the *Drosophila* homologue of PIH1D3 (CG5048 but will be referred to as *Pih1d3* for this thesis) was conducted by the lab before the onset of this project and is shown below in Figure 4.4. RNA in situ hybridisation, as well as the generation of an mVenus construct found this homologue to be specifically expressed in motile-cilia cells. TEM carried out on *Pih1d3* RNAi knockdown antennae showed a loss of inner dynein arms and reduced outer dynein arms.

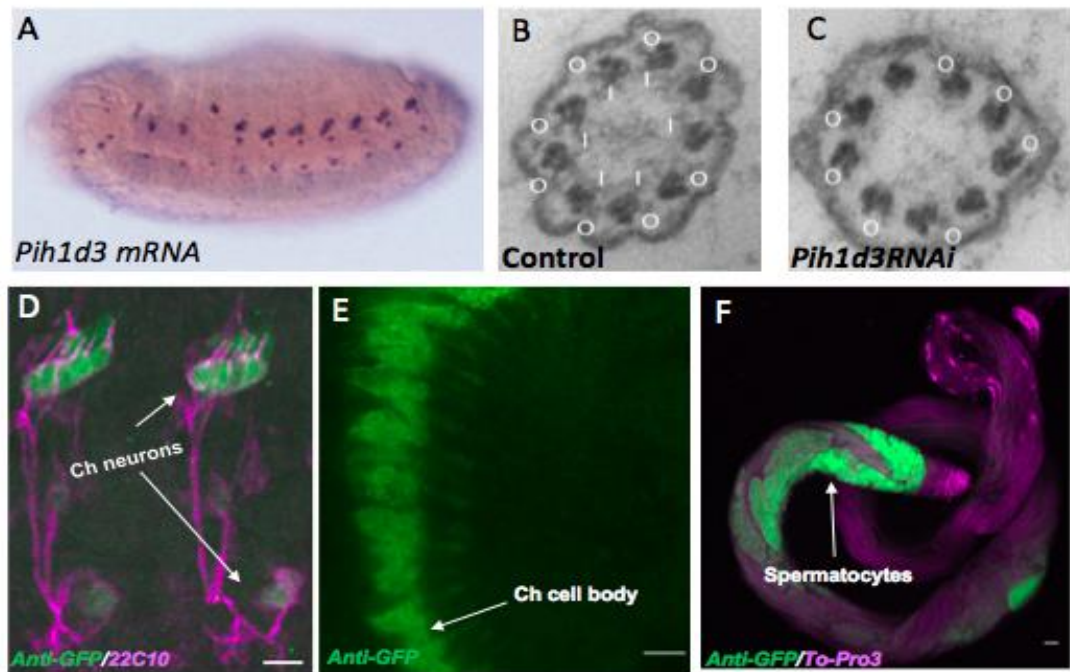


Figure 4.4 Preliminary investigation conducted before the onset of this project into the function of *Drosophila* Pih1d3 indicates expression in motile ciliated cells and a proposed function in inner dynein arm assembly. (A) RNA *in situ* hybridisation carried out on WT whole-mount embryos using a *Pih1d3*mRNA probe shows expression in developing chordotonal neurons. **(B-C)** TEM of adult antennae showing chordotonal neuron ciliary ultrastructure. **(B)** Control group showing 9+0 axonemal arrangement, with outer and inner dynein arms (O, I) on each microtubule doublet. **(C)** RNAi knockdown of *Pih1d3* in the developing nervous system results in a loss of inner dynein arms. Outer dynein arms (O) are still present along the axoneme. **(D-F)** Generation of *Pih1d3*mVenus fusion protein supports a role in cytoplasm of motile ciliated cells, where dynein assembly takes place. **(D)** *Pih1d3* protein is expressed in the developing chordotonal neurons of the embryo. **(E)** *Pih1d3* is expressed in the cell body of the pupal chordotonal neurons, as expected for a protein involved in dynein assembly. **(F)** *Pih1d3*mVenus is expressed in the adult testes in the spermatocytes.

This preliminary data suggested that the function of *Pih1d3* in motile cilia may be conserved in *Drosophila*, and further investigation is justified to assess the specific role of this known DNAAF, as well as its potential interaction with *Dyx1c1*.

4.3.2 A *Pih1d3* null mutant is generated and tested using a CRISPR/Cas9 approach

4.3.2.1 Generation of *Pih1d3* knockout by CRISPR/Cas9

In Chapter 3, *Drosophila Dyx1c1* null mutant line was generated and used to characterise its role in ODA and IDA assembly. As *Pih1d3* is a proposed protein partner of *Dyx1c1*, a knockout line was generated to follow up previous preliminary RNAi knockdown experiments and assess whether *Pih1d3* has a similar function to *Dyx1c1*. Using CRISPR/Cas9 technology, the open reading frame (ORF) of *Pih1d3* was removed and replaced with the mini-white gene, as shown in Figure 4.5A. Homozygous null mutant flies are viable, in common with most other DNAAF mutations. This finding suggests that *Pih1d3* does not have vital functions. Flies were therefore analysed for motile cilia phenotypes. It is important to note that null mutants are observed to be not as healthy as heterozygotes, and struggle to compete in a crowded stock for food.

4.3.2.2 *Pih1d3* null mutant males are infertile, caused by immotile sperm

A fertility assay was conducted in a similar manner to that described previously. Homozygotes were mated to wild-type virgin females, and the number of males that produced progeny was recorded. This number was compared to heterozygous and wild-type controls. Similar to what was found for *Dyx1c1*, the *Pih1d3* null mutant males also produced no progeny at all, in comparison to both control groups - which contained mainly fertile males (Fig. 4.5B).

Testes from the males used in the fertility assay ($n=10$ males/genotype) were dissected to assess morphology and sperm motility. The testis of the homozygous group (Fig 4.5C), was as expected - apart from the seminal vesicle, which was smaller due to the lack of mature individualised sperm able to move there. In the controls (Fig 4.5D), sperm were visible moving in S-shaped waves. Sperm motility is absent in the null mutants, clarifying that the infertility of the *Pih1d3* homozygotes is caused by immotile sperm.

4.3.2.3 *Pih1d3* knockout adults have defective proprioception

To assess whether this motile ciliated cell type was affected in the *pih1d3* null mutant, a climbing assay was conducted similarly to that described in previous chapters. As shown in Figure 4.5E, there was a significant difference between how far the *Pih1d3* null mutants could climb in comparison to both control groups. As this genotype had a reduced climbing ability, this result in combination with the expression pattern obtained previously, supports a proprioceptive defect caused by abnormal chordotonal neuron function.

4.3.2.4 *Pih1d3* knockout larvae have hearing deficits

As the chordotonal neuron mediates auditory reception, a larval hearing assay was conducted on *Pih1d3*^{-/-} larvae similarly to that described in Chapter 2. As shown in Figure 4.5F, there is no significant difference between the number of *Pih1d3*^{-/-} larvae that contracted before and during the tone, indicating no response to the auditory stimulus. However, both control groups had a significant increase in the number of larvae contracting when the tone was played - supporting normal hearing function in these groups. Together, this result and the reduction in climbing ability indicate that *Pih1d3* homozygotes have defective chordotonal neuron function, which supports a motile cilia role for this gene in *Drosophila*.

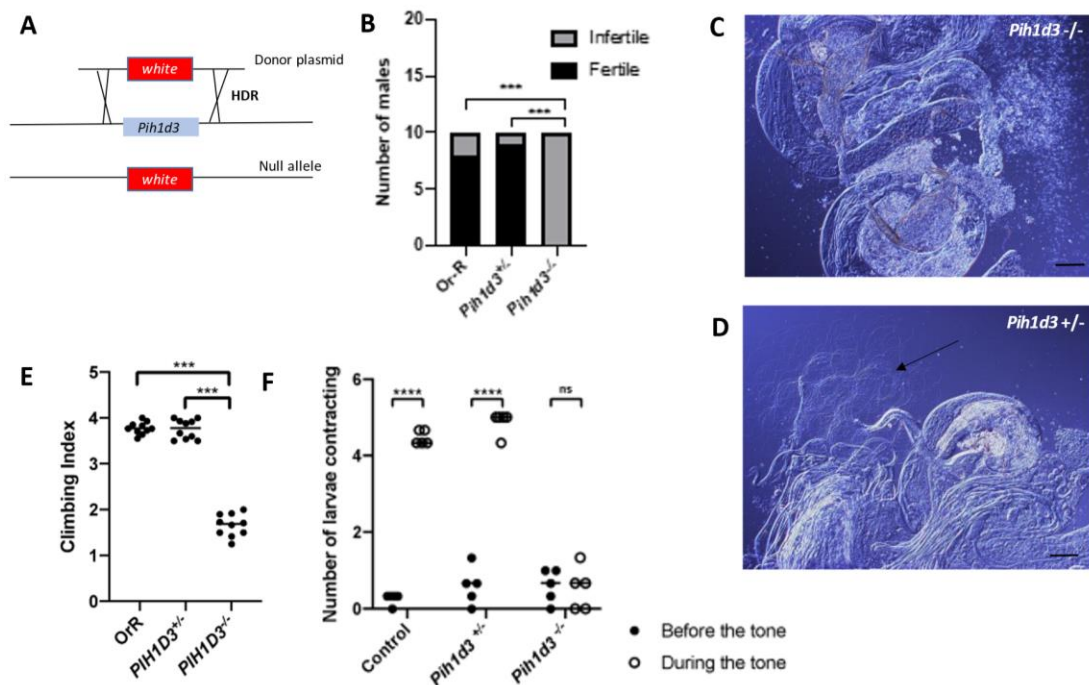


Figure 4.5. *Pih1d3* null mutant shows defective motile cilia function. (A) Generation of *Pih1d3* null mutant by homology directed repair. Open reading frame was replaced with the mini-white gene, allowing mutants to be selected by eye colour. (B) Fertility assay results showing a decrease in the number of fertile males in the null mutant when compared to control groups ($P=0.0001$). $n = 10$ males per genotype. (C-D) Adult male testes from fertility assay showing infertility caused by a loss of sperm motility. (C) *Pih1d3* homozygous male showing small seminal vesicle with an absence of motile sperm. (D) *Pih1d3* heterozygotes showing motile sperm (with characteristic S-shaped waves) being released from the seminal vesicle. (E) Bar graph (with median and individual values) of female adults climbing assay. *Pih1d3* null mutants had a significant decrease in climbing ability compared to control groups: *Pih1d3* heterozygotes ($P=0.0006$) and Or-R ($P=0.0003$). $n = 10$ batches of 8-12 flies per genotype. (F) Plot (with individual and median values) showing hearing assay results for *Pih1d3* homozygous larvae in comparison to control groups. Significant difference for number of larvae contracting before and during tone ($P<0.0001$) for all control groups. No significant difference between number of contractions before and during tone for *Pih1d3* homozygotes, indicating no response exhibited. N for each genotype = 5 batches of 5 larvae, each exposed to 3 tones 30s apart. Significance was determined by Fisher's exact test (B) Kruskal-Wallis and Dunn's multiple comparisons test (E) and 2-Way ANOVA with Sidak's multiple comparisons (F). Significance on plots is shown using astericks: *, $P\leq 0.05$; **, $P\leq 0.01$; ***, $P\leq 0.001$; ****, $P\leq 0.0001$. Bars, 100 μ m (C-D).

4.3.2.5 Use of a deficiency line confirms the null mutation to the expected locus

As mentioned in the previous chapter, the aim of using a deficiency line is to confirm the *Pih1d3*^{-/-} phenotypes observed are a result of a disruption in the expected chromosomal region (or locus). When the *Pih1d3* null mutant line was crossed to the deficiency line, it resulted in similar phenotypes to that of the homozygous null mutant - shown below in Figure 4.6. Male flies were infertile, producing no progeny like the result shown above in Fig 4.5B. This result supported that the phenotype observed in null mutants resulted from a mutation in the expected locus, where *Pih1d3* is present.

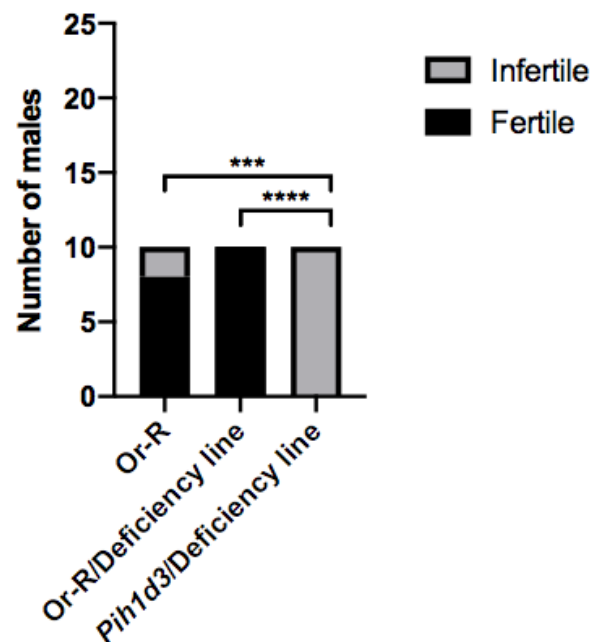


Figure 4.6 Use of the deficiency line supports null mutant phenotypes as a result of a disruption to the expected locus. Fertility assay indicates that *Pih1d3* null heterozygote in combination with a deficiency line for that gene region results in similar phenotypes observed for null homozygotes. These male flies produce no progeny, unlike control groups ($P < 0.0001$). $n = 10$ males for each genotype. Significance determined by Fisher's exact test.

4.3.3 Pih1d3mV partially rescues the null mutation

A Pih1d3mVenus construct was generated by Petra zur Lage before the onset of this PhD project. Immunofluorescent staining and confocal microscopy on this fly line (Fig 4.4) showed Pih1d3 to be expressed in the cytoplasm of motile cilia, as expected for a DNAAF. To validate this mVenus protein, as well as the null mutant generated for future use in experiments such as GFP-Trap AS-MS, a rescue experiment was conducted. A fly line containing both the Pih1d3mVenus and the *Pih1d3*^{-/-} was generated to assess whether the fusion protein could rescue the null mutant phenotypes observed. However, the mVenus line only partially rescued the null mutant function. In the fertility assay results indicate that the rescue line did not function similarly to control, with the production of zero progeny from rescue males (Fig. 4.7A). The testes from these flies were dissected, and Figure 4.7C shows the sperm in the rescue line to be immotile - unlike that of the control line (Fig. 4.7B). The rescue line is indeed able to climb better than the null mutant but cannot climb to the ability of the heterozygote control group (which would have supported a full rescue of function) (Fig. 4.7D). With the inability of the mVenus line to successfully rescue function, it is not wholly beneficial to use this protein fusion line in future experiments such as GFP trap. However, the partial rescue observed in the chordotonal neurons validates the mVenus fusion protein and null mutant line to some extent.

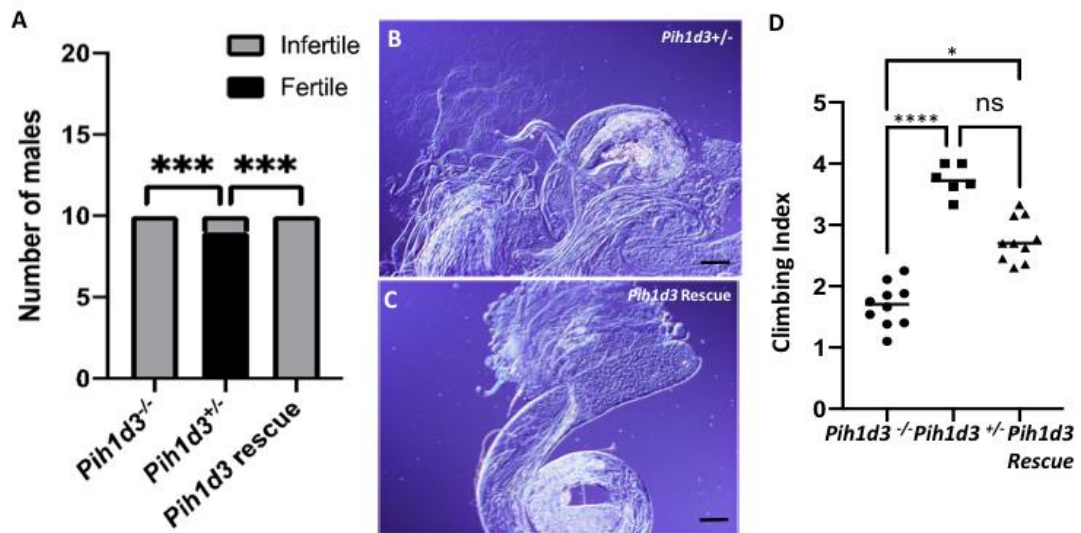


Figure 4.7 *Pih1d3mVenus* fusion protein line only partially rescued null phenotype. (A) *Pih1d3* rescue does not produce progeny, indicating male infertility is still present ($P < 0.0001$), like that of the homozygous null mutants $n = 10$ males. (B-C) When testes were dissected, the rescue line showed no motile sperm travelling, unlike those observed in the heterozygote control group. (D) Climbing assay results indicate a partial rescue of proprioception, but the rescue line does not have the climbing ability of heterozygous controls. $n = 10$ batches of 8-12 flies. Significance is determined by Fisher's exact test (A) and Kruskal-Wallis, Dunn's multiple comparison test (C). Bars 100um (B-C).

4.3.4 Investigation of the localisation of dyneins in *Pih1d3*^{-/-} pupal antennae

4.3.4.1 The outer dynein arm heavy chain DNAH5 does not localise to the cilium in *Pih1d3* null mutant pupal antennae

Preliminary TEM analysis into the function of *Pih1d3* in *Drosophila* suggested a role primarily in the assembly of dynein arms. As RNAi is not always effective at knocking down gene function, the null mutant generated in this project was used to assess how dynein arm subunits were localising in the cilium.

Both *Pih1d3* homozygote and heterozygote mutant antennae were dissected and stained with the DNAH5 antibody that was utilised in Chapter 3. The general morphology of the chordotonal scolopidia appears normal for both genotypes, and DNAH5 was expressed in the chordotonal neuron cell bodies,

indicating that the protein is expressed in both homozygotes and heterozygotes. However, when comparing the two genotypes, a loss of staining in the cilium is observed in homozygous mutants (Fig 4.8A-B). This result indicates that localisation of outer dynein arms in the cilium is affected in *Pih1d3* null mutants, like what was observed in *Dyx1c1* null mutants. As it is hypothesised that *Dyx1c1* and *Pih1d3* work together in an R2TP-like complex, it is expected they would have similar phenotypes, and so this result supports that thought. The immunofluorescent staining shows a more severe defect on the ODAs than that observed in the knockdown TEM, indicating that TEM analysis should be repeated on the null mutant antennae. Ongoing experiments include assessing whether this CRISPR mutant mirrors the previous arm defects shown or exhibits a more severe defect.

4.3.4.2 The inner dynein arm light-intermediate chain DNALI1 partially localises to the cilium in *Pih1d3* $-/-$ null mutant antennae

Preliminary research by the lab showed that when TEM was conducted on an RNAi knockdown of *Pih1d3*, there was a complete loss of IDAs. Therefore, to follow-up this research, immunofluorescent staining was conducted on pupal antennae to assess how the localisation of Dnali1 (IDAs a and c) is affected during development.

A fly line was generated which had a mVenus reporter line for Dnali1 as well as a homozygous null mutation for *Pih1d3*. The tissue was stained with GFP to enhance endogenous expression. In the control group (Dnali1mVenus stained with anti-GFP, Figure 4.8C, C'), staining was present in both the cell body and the cilium. In the homozygous knockout (Fig 4.8D, D'), Dnali1 staining was present in the cell body, but also partially in the cilium (although reduced when compared to control). Dnali1 is present in several subsets of IDAs, and so this result suggests that *Pih1d3* is involved in the assembly of some but not all these subsets.

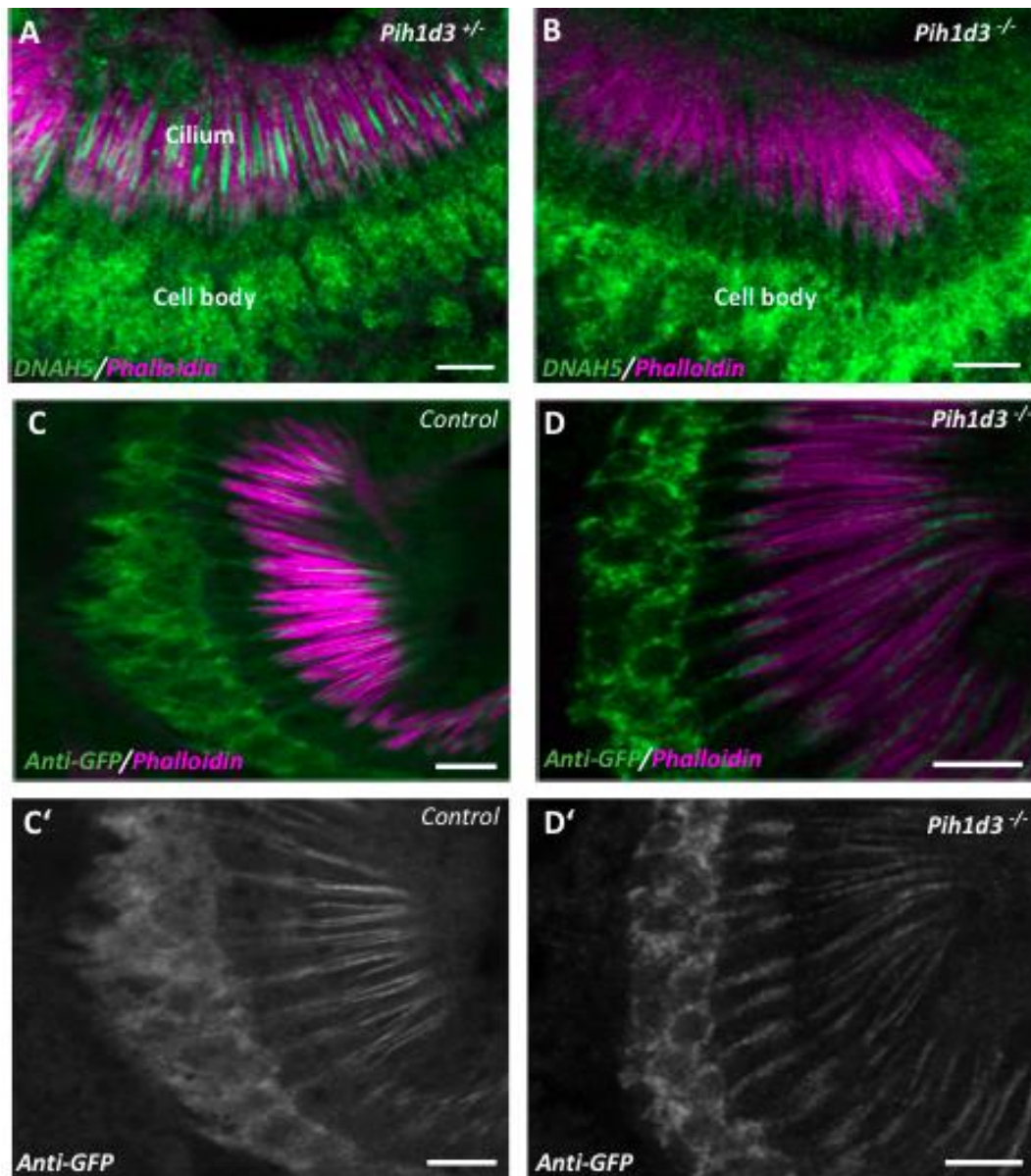


Figure 4.8 Immunofluorescent staining indicates Pih1d3 has a role in the assembly of ODAs and a subset of IDAs. (A) Heterozygous control showing expression of the ODA subunit DNAH5 in both cell body and cilium. (B) *Pih1d3* homozygous pupal antennae showing a loss of DNAH5 localisation in the cilium, but presence still in the cell body. (C) *Dnali1-mVenus* control line showing expression of the IDA subunit in the cell body and the cilium. (D) *Pih1d3* null mutant antennae showing *Dnali1-mVenus* expression in the cell body, and a slight reduction and defective localisation of the IDA subunit in the cilium when compared to control. Due to subtle reduction in IDA subunit localisation in the null mutant, the GFP channels for C and D are shown in greyscale (C', D') which indicates an accululation of IDA at the inner segment of the mutant, and the reduced localisation in the cilium. Bars: (A-D) 5µm. Proportion of antennae showing phenotype: (A) = 6/6 (B) 5/5 (C/C') 8/8 (D/D') 9/9.

An observation in Figure 4.8D/D', is that there is an accumulation of protein at the inner segment, as if the inner dynein arms are assembling and transporting to the base of the cilium, but something is preventing them from entering and localising as normal.

4.3.5 *in vivo* functional interactions between *Dyx1c1* and the *Pih* proteins

The experiments in this thesis are consistent with conservation of assembly factor roles for *Dyx1c1* and *Pih1d3*. As these DNAAFs are hypothesised to form an R2TP-like complex, I sought to gain *in vivo* evidence for them functioning together by analysing genetic interactions. Petra zur Lage has conducted co-immunoprecipitation experiments in S2 cells that suggest that *Dyx1c1* and *Pih1d3* interact (data not shown). A standard method in *Drosophila* of identifying evidence for physical association of proteins, is to combine heterozygous mutations of genes of interest and assess whether a more severe phenotype is observed than for the individual heterozygotes. In humans, trans-heterozygous interactions for the PCD genes DNAH6 and DNAI1 or DNAH5 have been shown to cause heterotaxy, suggesting heterozygous mutations in multiple PCD genes can cause disease phenotypes (Li et al., 2016).

To further investigate whether *Dyx1c1* and *Pih1d3* work together in a complex *in vivo*, a climbing assay was conducted on individual heterozygotes, as well as on the double heterozygotes for both genes. As these DNAAFs have been found to interact by co-IP, it was predicted that the double heterozygotes would not perform as well in the climbing assay as the individual heterozygotes. However, no difference was observed between the groups. So, the climbing assay was repeated in a dark environment to assess whether there were subtle differences (Fig. 4.9A). Again, no statistically significant difference was found to be present, so this assay did not detect evidence of these DNAAFs working together *in vivo*. A limitation to this experiment is the lack of a wildtype control. As shown in previous climbing assay data (see Fig 4.5E), healthy flies typically climb to the 4th quadrant in the 10s interval. For future experiments

investigating dose-dependent effects, a wildtype control should always be included for comparison.

A fertility assay was also conducted on these heterozygotes, and the number of progeny produced by the three groups was recorded to identify any potential small cumulative effects. No difference was observed (Fig 4.9B), testes dissections revealed the double heterozygote sperm to be as motile as that of the individual heterozygotes - suggesting no effect on fertility in this experiment. Therefore, these experiments found no evidence for a genetic interaction between *Dyx1c1* and *Pih1d3*.

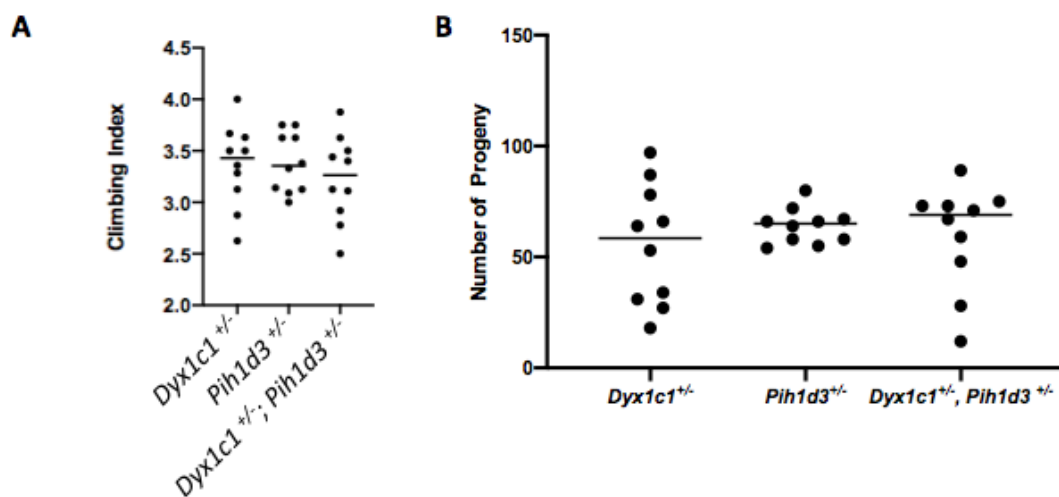


Figure 4.9 First experimental attempt at investigating R2TP-like interaction *in vivo* proved unsuccessful. (A) Climbing assay on *Dyx1c1*, *Pih1d3* double heterozygotes (in dark) showed no decrease in climbing ability when compared to individual *Dyx1c1* and *Pih1d3* heterozygotes. *n* = 10 batches of 8-12 flies (B) Fertility assay where progeny produced was recorded indicates that the double heterozygote flies show no significant decrease in numbers. *n* = 10 males per genotype. Significance for both A-B determined by Kruskal-Wallis and Dunn's multiple comparison test.

The experiment described above may not be sensitive enough to show a genetic interaction between *Pih1d3* and *Dyx1c1*. Therefore, a new experiment was designed combining an RNAi knockdown of *Pih1d3* with a heterozygous mutation of *Dyx1c1* and assessing whether this combination causes a more severe knockdown than the RNAi knockdown alone.

A fly line was then generated through genetic recombination that contained a *Dyx1c1* heterozygous mutation as well as *Scabrous*-Gal4. This line was then crossed to a *UAS-Pih1d3RNAi* line. The PIH protein DNAAF2 has also been proposed as a *Dyx1c1* partner, and so the UAS-RNAi line for this *Drosophila* homologue was also crossed to the *Dyx1c1*, Sca-Gal4 recombinant line. As shown below in Figure 4.10, when the function of *Pih1d3* and DNAAF2 is knocked down in the PNS, climbing deficits are observed for both genotypes. However, the addition of a heterozygous copy of a *Dyx1c1* mutation does not cause a more severe defect, as there is no significant difference between the individual knockdowns of these genes with and without the *Dyx1c1* defect. For *Pih1d3*, the spread of data almost indicates a partial rescue when *Dyx1c1* function is affected. However, this difference is not significant ($P>0.9999$). There is also a trend towards a difference for DNAAF2, and perhaps a larger scale experiment with a higher n number is required for these DNAAFs based on these findings.

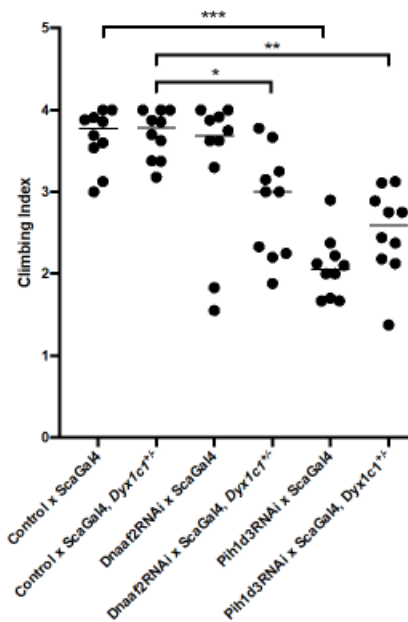


Figure 4.10 The addition of a heterozygous mutation of *Dyx1c1* does not cause a more severe phenotype to be observed in this type of assay. Both *Pih1d3* and *Dnaaf2*, PIH proteins which are known assembly factors and proposed to form R2TP-like complexes with *Dyx1c1* have a reduced climbing ability when compared to KK control line (*:P=0.0255, **: P=0.0055,***=0.0001). However, the addition of a heterozygous mutation to *Dyx1c1* does not cause the climbing defect to become more severe. *n* = 10 batches of 8-12 flies per genotype. Significance determined by Kruskal-Wallis and Dunn's multiple comparison test.

4.4 Discussion

4.4.1 The role of *Drosophila* Pih1d3 in axonemal dynein assembly

PIH1D3 is a known DNAAF and PCD-causative gene, with a role in the assembly of ODAs and a subset of IDAs (Paff et al., 2017). In *Chlamydomonas*, the *PIH1D3* homologue is involved in ODA and primarily IDA subset c assembly (Yamamoto et al., 2020). Using the inner dynein arm light-intermediate chain DNALI1 as a marker, it was shown that in Zebrafish both IDA subsets c and d were affected in a *PIH1D3* mutant (Yamaguchi et al.,

2018). Electron Tomography (ET) on human respiratory cells also suggest a role for *PIH1D3* in the assembly of IDA subsets *g* and *l1/f* (Olcese et al., 2017).

In *Drosophila*, the motile cilia role of *Pih1d3* is conserved, with null mutants exhibiting proprioceptive, auditory and fertility defects. TEM and dynein localisation studies show a defective assembly of ODAs and IDAs. As *Pih1d3mVenus* is expressed in the cytoplasm of motile ciliated cells, where dynein preassembly takes place, it is likely that its dynein assembly factor role is conserved in *Drosophila*.

Using Dnali1 staining, like that described above in zebrafish, IDA localisation was assessed in *Pih1d3* knockout pupae. This subunit is common in IDA subsets *a,c* and *d* (zur Lage et al., 2019). If *Drosophila Pih1d3* plays a similar function to that of other organisms, perhaps subset *c* is affected in a knockout and subset *a* is localising correctly - explaining the partial localisation observed. Interestingly an accumulation of Dnali1 was observed at the inner segment, suggesting the inner dynein arms are assembling and transporting to the base of the cilium but are unable to enter and localise along the axoneme. This could be a quality control mechanism. Chaperones play a role in facilitating the assembly of large multi-subunit complexes such as the dynein motor arms. However, they are also important for monitoring for misfolded proteins when maintaining proteostasis. They can achieve this by sequestering misfolded proteins to specific cellular locations (Chen et al., 2011). The build-up of Dnali1 at the inner segment of the chordotonal neuron could be that these subunits are not assembling correctly and the chaperone proteins act as quality control surveyors, preventing the movement of these complexes into the cilium.

The generation of additional IDA markers, as well as carrying out label-free MS on homozygous mutant testes and assessing what dynein subunits are depleted, may further pinpoint the role of *Pih1d3* in *Drosophila*.

4.4.2 The *Drosophila* R2TP-like complex: R2DP₃

Both *Dyx1c1* and *Pih1d3* have conserved DNAAF roles in *Drosophila*, involved in the assembly of ODAs and a subset of IDAs. It is thought that DYX1C1 works with both PIH1D3 and DNAAF2 in distinct 'R2TP-like complexes' during dynein assembly.

GFP-Trap AP-MS is a useful technique when investigating *in vivo* protein-protein interactions. However, when this was conducted on the *Dyx1c1mVenus* rescue line it was unsuccessful. The *Pih1d3mVenus* line used in this chapter did not fully rescue function (only a partial rescue in the chordotonal neuron), so it may not be informative to use this line in a GFP-Trap experiment. However, evidence of interaction between these proteins is important to verify that they function as part of an R2TP-like complex.

Co-immunoprecipitation experiments have been conducted by the lab on *Drosophila* S2 cells. These showed that the *Drosophila* *Dyx1c1* protein binds to *Pih1d3* despite missing the protein interaction TPR domains. As well as binding to each other, these also co-precipitate with *Ruvbl1/2* of the canonical R2TP complex. Therefore, these *in vitro* experiments support the formation of this R2TP-like complex, which will be referred to as R2DP₃ in *Drosophila*.

Additionally, the dynein localisation data supports this hypothesis. Yamaguchi et al., 2018 found a greater reduction of *Dnali1* in a *Twister* mutant than in *pih1d1*^{-/-}, *ktu*^{-/-} and the double mutant *pih1d2*^{-/-};*ktu*^{-/-} as more dynein subsets are affected in a *Twister*^{-/-}. Therefore, as it is thought *Dyx1c1* carries out its functions in multiple R2TP-like complexes, a more severe defect to IDA localisation is expected. In *Drosophila* too, I found that *Dnali1* localisation is more severely affected in a *Dyx1c1* knockout than in a *Pih1d3* knockout. Preliminary evidence for a possible genetic interaction between *Dyx1c1* and *Dnaaf2* further supports this.

Dyx1c1 and *Pih1d3* may carry out a role on IDA subset c and *Dyx1c1* and DNAAF2 carry out a role on another IDA subset such as subset a. All IDA subsets were found to be affected in a *Dyx1c1* knockout in *Chlamydomonas*,

so that *Dyx1c1* may have a role in additional R2TP-like complexes unaccompanied by PIH proteins (Yamamoto et al., 2017). Therefore, the IDA subunit *Dnali1* expression is less in a *Dyx1c1* null as more R2TP-like complexes are non-functional and therefore more IDA subsets are not assembling correctly. One method of further investigating these additional R2TP-like complexes involving *Dyx1c1*, is characterising the role of *DNAAF2* in *Drosophila* and assessing how IDA subsets are affected during assembly in a *Dnaaf2* null mutant.

To support the *in vitro* evidence of these DNAAFs interacting, and provide evidence of them functioning together *in vivo*, genetic interactions were investigated *in vivo* using fertility and climbing assays on double knockdown/knockouts. As the ciliopathy PCD is a heterogeneous disease, motile cilia formation and function involves many proteins working together during this complex process. Therefore, digenic mutations in PCD genes are likely to cause subtle PCD-like phenotypes (Horani et al., 2016). However, digenic PCD mutations have not yet been thoroughly investigated, most likely due to the difficulty at observing these *in vivo* functional interactions as shown in the experiments above, which proved unsuccessful. This result is most likely due to incorrect experimental design when looking for these potentially very subtle defects. An assay which may be worthwhile to optimise would be a fertility assay looking at sperm competition like that described in (Karak et al., 2015): assessing whether sperm that are double heterozygotes for *Dyx1c1* and *Pih1d3* can compete and fertilise to the same extent as individual *Dyx1c1* and *Pih1d3* heterozygotes. A phenotype such as eye colour is required to aid the distinguishment of the different progeny (i.e., which progeny came from an egg fertilised by a double heterozygote, which is from a single heterozygous). This experiment would require complicated genetics but could spot subtle defects to sperm motility - if they are not able to move as efficiently as the single heterozygous controls, providing evidence of genetic interaction and epistasis.

As the protein interaction is shown to be conserved, this thesis proposes that R2DP₃ has a role in the assembly of ODAs and IDA subset c in *Drosophila*. CryoEM on *Drosophila* sperm flagellum would be a beneficial technique to verify this prediction, as it has successfully done so in other organisms (Lai & Doe, 2014; Lin et al., 2019).

4.5 Conclusion

PIH1D3 is a known DNAAF with a role in the assembly of ODAs and IDAs. This role is conserved in *Drosophila*, where a non-functional *Pih1d3* causes a loss of dynein arms and phenotypes associated with ciliary immotility. It has been proposed that this DNAAF works with DYX1C1 in an R2TP-like complex during dynein assembly, which is also conserved in *Drosophila*. Both DNAAFs are involved in the assembly of ODAs and a subset of IDAs, and Co-IP experiments show that they directly interact in an R2TP-like complex.

Chapter 5

The role of DPCD as a co-chaperone during cellular processes

5.1 Introduction

5.1.1 Discovery of DPCD as a motile cilia gene

Initially, a study showed that DNA polymerase λ -deficient mice (*poll*^{-/-}) exhibited hydrocephalus, situs inversus, chronic sinusitis and male infertility. These phenotypes are associated with the respiratory disorder (PCD), caused by defective motile cilia. These *poll*^{-/-} mice also lacked axonemal inner dynein arms in their respiratory epithelium, structures which facilitate ciliary motility (Kobayashi et al., 2002). This was an unexpected result as a DNA polymerase gene would not be predicted to have a role in motile cilia function. Further analysis revealed that the first exon of a previously unknown gene was also affected in the *poll*^{-/-} mice (Zariwala et al., 2004). When the *poll* catalytic domain is disrupted, no PCD-like phenotypes are observed (Bertocci et al., 2002). This result suggested that the novel gene was causing the defective ciliary motility and lack of inner dynein arms, leading to this gene to be called Deleted in Primary Ciliary Dyskinesia (DPCD). DPCD expression was shown to increase during ciliogenesis, supporting a role in cilia formation and function (Zariwala et al., 2004).

The development of situs inversus in mice with disrupted transcription of *dpcd* was explored (Vogel et al., 2010). These mice were smaller than littermates and were present in lower numbers than those expected according to Mendelian genetics. This supports the previous results reporting the *poll*^{-/-} mice (now known due to disrupted DPCD function) having a high mortality rate after birth as well as delayed development (Kobayashi et al., 2002). DPCD is

conserved in humans; however, no PCD patient has been classified to date as having a mutation in this gene (Zariwala et al., 2004).

The specific function of DPCD remains unknown (Zariwala et al., 2004). However, the human DPCD protein is known to bind the AAA+ proteins Reptin and Pontin (Dafinger et al., 2018; Zhao et al., 2013). Reptin and Pontin are part of many complexes in the cell, including the R2TP complex described previously. As there is a loss of inner dynein arms in the *Dpcd* mutant in mice, this gene may play a role in axonemal dynein arm assembly.

5.2 Aims

DPCD has been shown to have a role in motile cilia function and when disrupted in mice, causes an absence of axonemal inner dynein arms. However, the specific function of this gene remains unknown. The aim of this chapter is to use the *Drosophila* DPCD homologue, CG13901, to gain further information about its function through the analysis of null mutant phenotypes and interactions with other proteins.

5.3 Results

5.3.1 CG13901 is the *Drosophila* homologue of DPCD

To identify the *Drosophila* homologue of *Dpcd*, DIOPT bioinformatic software was used. CG13901 was identified as the homologue of DPCD with 53% similarity and 36% identity, indicating that this gene is highly conserved in *Drosophila* (Fig. 5.1A). Therefore, CG13901 will be referred to as *Dpcd* for the rest of this thesis.

5.3.2 *Dpcd* is transcribed in motile cilia specific cells

In mice, *Dpcd* expression increased during ciliogenesis, indicating an association of this gene with cilia function (Zariwala et al., 2004). Expression of *Dpcd* in *Drosophila* was assessed to gain insight into its function. The transcriptome previously conducted in the lab showed *Dpcd* to be 3-fold enriched in embryonic chordotonal neurons (i.e. *ato* expressing cells) compared to non-*ato* expressing cells (zur Lage et al., 2019). FlyAtlas expression data showed that *Dpcd* is most highly expressed in the testis at 379 units. To further investigate *Dpcd* expression, an mRNA probe was generated to show where *Dpcd* is transcribed in wild-type whole-mount embryos. As shown in Figure 5.1B, the *Dpcd* mRNA probe was shown to be chordotonal neuron specific at late embryonic stages. While there may be some background present, it is difficult to confirm this, as no specific additional staining is observed. This chordotonal neuron expression pattern appears at the point in development where motile cilia specialisation occurs, supporting a motile cilia role for *Dpcd*.

As described previously, *Rfx* and *fd3F* are transcription factors which regulate downstream motile cilia genes. The *Dpcd* mRNA probe was used on *fd3F* null mutant embryos to assess whether expression of this gene was disrupted when the motile cilia transcription factor was non-functional. Figure 5.1C shows the chordotonal neuron expression pattern that was observed in wild-type embryos is absent in *Fd3f* embryos, suggesting *Dpcd* is regulated by this transcription factor. This supports the hypothesis that *Dpcd* plays a role in motile cilium formation or function.

5.3.3 A DpcdmVenus fusion construct shows expression in motile cilia cells

5.3.3.1 Generation of DpcdmVenus fusion protein

To investigate the expression of *Drosophila* Dpcd on a protein level, a DpcdmVenus fusion protein was produced. The construct is shown in Figure 5.2A and resulted in a fly line that exhibited endogenous fluorescence under the control of the Dpcd promoter.

5.3.3.2 Dpcd protein is expressed in embryonic chordotonal neurons

The RNA *in situ* hybridisation on wild-type whole-mount embryos showed that *Dpcd* mRNA is expressed in the developing chordotonal neurons. Using the DpcdmVenus fly line generated, protein expression was assessed in embryos. As shown in Figure 5.2B, in late-stage embryos, a chordotonal neuron-specific pattern was observed, consistent with the results obtained from the *in situ* hybridisation. Therefore, Dpcd is both transcribed and translated in the ciliated chordotonal neuron.

5.3.3.3 Dpcd protein is expressed in the chordotonal neuron of the pupal antennae

Dpcd expression was assessed at a later stage of *Drosophila* development. In the pupal antennae, Dpcd mVenus fusion protein was expressed in the chordotonal neurons of the JO, specifically in the cell body and inner segment (Fig. 5.2C). As dynein preassembly takes place in the cell's cytoplasm, before assembled complexes are docked in the cilium, localisation of Dpcd in the chordotonal cell body specifically suggests a role in dynein assembly for this gene. In this experiment, samples were counterstained with Phalloidin, which labels the actin of the scolopale cell that surrounds the cilium. This finding further highlights that Dpcd is located where dynein assembly occurs, as opposed to in the cilium where assembled dynein complexes are docked.

5.3.3.4 DpcdmVenus is expressed in the adult testis, specifically in the spermatocytes

In addition to the chordotonal neuron, FlyAtlas data indicates that Dpcd is most highly expressed in the testis. Immunofluorescent staining in adult testis shown in Figure 5.2D using the DpcdmVenus line showed protein localisation in the spermatocytes. Expression in this cell type is typical for a protein with a role in dynein assembly, as previously described in Chapters 3 and 4. Samples were counterstained with polyglycosylated tubulin, which highlights where the flagella of the mature sperm are located in the testis. This counterstain shows that Dpcd is expressed earlier in sperm development when the motile machinery of the sperm flagellum is being assembled. At the higher magnification shown in Figure 5.2E, the Dpcd expression in the spermatocytes is specifically cytoplasmic, where axonemal dynein assembly takes place.

Overall, the expression pattern observed for DpcdmVenus in these motile ciliated tissues is consistent with previous results for known DNAAFs (zur Lage et al., 2021, 2018, Chapters 3,4). Therefore, these results support the role of Dpcd in motile cilia, specifically where axonemal dynein assembly takes place.

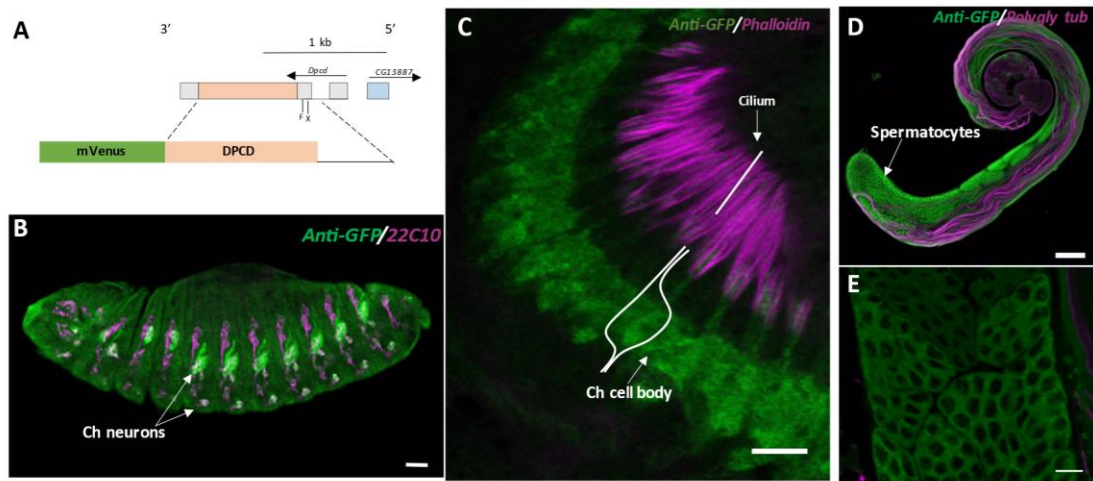


Figure 5.2. *Drosophila* Dpcd protein is expressed in motile cilia cells. (A) Schematic of DpcdmVenus fusion protein construct: a GFP-tagged 3' end of Dpcd, resulting in GFP-expression where the protein is expressed. Construct includes binding sites in the promoter region for two cilia-associated transcription factors, Fd3f (F) and Rfx (X) **(B-E)**. **(B)** Expression of Dpcd-mVenus (green) in developing chordotonal neurons of late-stage embryos. **(C)** Expression of Dpcd-mVenus (green) in pupal antennal chordotonal neurons, specifically expressed in the cell body. **(D)** Expression of Dpcd-mVenus (green) in the adult testis, specifically in the spermatocytes. **(E)** Higher magnification of the spermatocytes of the adult testis indicates that Dpcd expression is cytoplasmic. Bars: (B-C) 100µm (B) 20µm (C) 5µm (D) 100µm (E) 20µm. Number of samples imaged = (B) $n = 15/15$, (C) $n = 4/4$, (B) $n = 8/8$, (C) $n = 4/4$, (D,E) $n = 8/8$

5.3.4 RNAi knockdown experiments show *Dpcd* is required in motile ciliated cells

5.3.4.1 RNAi knockdown of *Dpcd* in the developing nervous system resulted in defective chordotonal neuron function

To assess whether *Dpcd* has a role in chordotonal neuron function, climbing ability of a *Dpcd* knockdown was assessed as a measure of proprioception. The UAS/Gal4 system was harnessed to knockdown the function of Dpcd using RNAi technology (explained in more detail in Chapter 2). *Uas-Dicer2*, *Scabrous-Gal4* was used as the driver line to knock down *Dpcd* function in the developing nervous system. A climbing assay was conducted as previously described to measure the proprioceptive ability of *Dpcd* knockdown flies compared to the control group. As shown in Figure 5.3A, the climbing ability of

*Dpcd*RNAi knockdown flies was significantly reduced, suggesting defective chordotonal neuron function.

The knockdown of *Dpcd* using the *Uas-Dicer2*, *Scabrous-Gal4* driver resulted in phenotypes more severe than previously observed for motile cilia-specific genes. Mature flies struggled to eclose, suggesting there may be a wider function for *Dpcd*.

5.3.4.2 RNAi knockdown of *Dpcd* in the testis results in infertile males, due to sperm immotility

As *Dpcd* is highly expressed in the testis, a testis-specific driver (*Bam-Gal4*) was used to knockdown *Dpcd* function and assess the effect. A male fertility assay was conducted as described in the previous chapters. Figure 5.3B shows that *Dpcd* knockdown males produce no progeny. To ensure the lack of progeny produced was a motility defect, testes from both genotypes were dissected. In the control group, motile sperm were observed, and the seminal vesicle was large due to mature individualised sperm successfully moving to this location (Fig. 5.3C). In the *Dpcd* knockdown however, the seminal vesicle was small and lacked mature individualised sperm, which were not observed moving in their characteristic S-shaped waves (Fig. 5.3D). Therefore, *Dpcd* has a role in facilitating sperm motility as well as chordotonal neuron function, indicating a requirement for this gene in cells that have motile cilia.

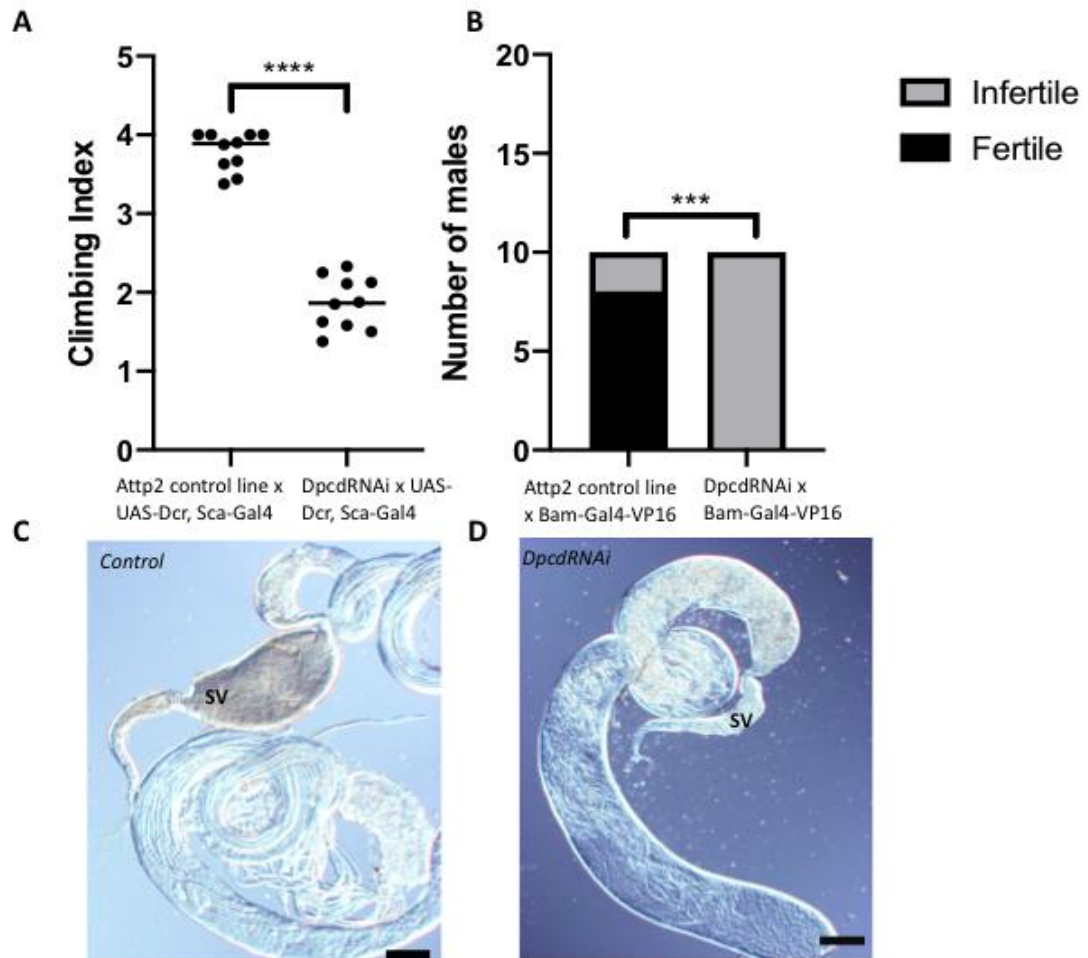


Figure 5.3: *DpcdRNAi* knockdown experiments show that *Dpcd* is required for functional motile ciliated cells (A) Bar graph (with median and individual values) of adult female climbing assay for a *DpcdRNAi* line crossed to UAS-Dcr-2, *Sca-Gal4* driver. Climbing assay is significantly reduced ($P \leq 0.0001$) in comparison to the control group. $N = 10$ batches of 8-12 flies. (B) Fertility assay results displayed as a bar graph for *Dpcd* RNAi flies crossed to a testis specific driver, *BamGal4*. Dramatic decrease ($P = 0.0007$) in the number of fertile males compared to control. $n = 10$ males. (C) Control adult male testis from fertility assay showing large sperm-filled seminal vesicles (SV). (D) Adult male testis of *Dpcd* RNAi-testis-knockdown from fertility assay. Seminal vesicle is smaller, lacking mature motile sperm. The Mann Whitney test (A) or Fisher's exact test (B) determined significance. Significance was determined by Mann Whitney test (A) and 2-Way ANOVA with Sidak's multiple comparisons (B). Significance on plots is shown using astericks: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. Bars, 100 μ m (C-D).

5.3.5 Transmission Electron Microscopy (TEM) indicates a loss of both outer and inner dynein arms

As *Dpcd* is required in motile ciliated cells and the axonemal dynein motor complexes facilitate this ciliary motility, these structures were assessed in a *Dpcd* knockdown. In adult antennae, both outer and inner dynein arms were absent from the 9+0 axonemal arrangement in the *Dpcd* knockdown (Fig. 5.4A-C). Transverse images shown in Figure 5.4A,C indicate the JO and individual cilia structure is forming as expected, suggesting that *Dpcd* does not play a role in JO development or general ciliogenesis and supporting an axonemal dynein-specific function for *Dpcd* in motile cilia.

TEM was also performed on *Dpcd* knockdown adult testes and the sperm flagellum, and as shown in Figure 5.4 D-G there was a loss of both outer and inner dynein arms. Therefore, in *Dpcd* knockdown adults, both motile cilia cell types are lacking outer and inner dynein arms.

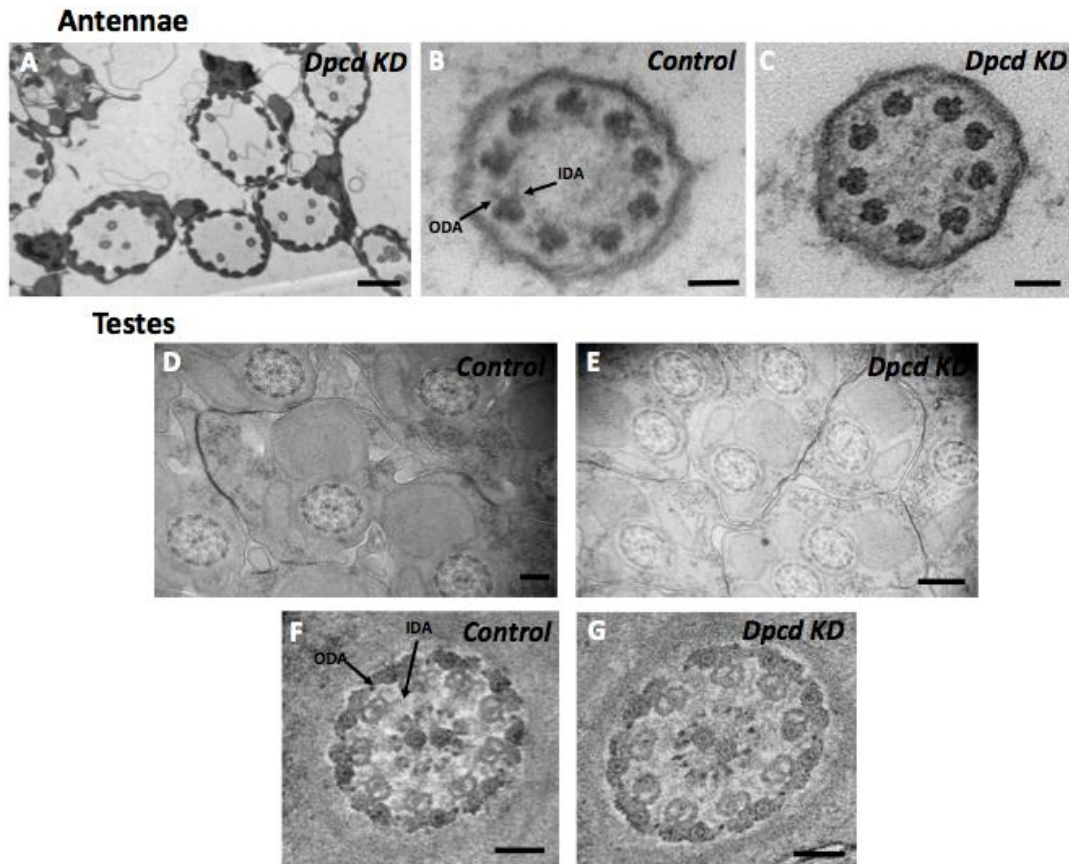


Figure 5.4. TEM of *Dpcd* knockdowns in motile ciliated cells shows a loss of localisation of both ODA and IDA dynein motor complexes (A) TEM of adult antennal chordotonal neurons in *Dpcd* RNAi knockdown flies. Overview showing both the Johnston's organ and individual cilia structure is normal. (B-C) TEM of adult chordotonal neurons, transverse sections (A) Control chordotonal neuron cilium, showing 9+0 axonemal arrangement with attached outer (ODA) and inner dynein arms (IDA). (B) Chordotonal neuron cilium for *Dpcd*RNAi crossed to *UAS-Dcr-2*, *ScaGal* shows the 9+0 axonemal structure remains intact, but lacking both ODAs and IDAs. (D-E) TEM overview of sperm bundle in the control (D) is similar to that in a *Dpcd* knockdown (E). (F-G) TEM of adult testes in *Dpcd* RNAi knockdown flies. (F) Control flagellum showing the 9+2 axonemal structure with outer and inner dynein arms on each microtubule doublet. (G) *Dpcd* knockdown indicating overall axonemal structure remains intact, but there is a loss of ODAs and IDAs. Bars: (A) 1µm (B-C) 50nm (D-E) 200nm (F-G) 50 nm

5.3.6 The localisation of both outer and inner dynein arm subunits is affected in a *Dpcd* knockdown in pupal chordotonal neurons

5.3.6.1 The outer dynein arm subunit DNAH5 does not localise in *Dpcd* knockdown pupal antennae

TEM in adult antennae has shown a loss of ODAs in *Dpcd* knockdown adult antennae. To assess how these structures are affected earlier in development, *Dpcd* knockdown pupal antennae were stained with the *Dnah5* antibody (used in Chapters 3 and 4) and ODA localisation was observed. In the control group (Fig. 5.5A), ODA subunits were found to localise to the cell body (where dynein assembly takes place) and in the cilium (when correctly assembled). However, in the *Dpcd* knockdown (Fig. 5.5B), the ODAs were only present in the cell body, where the assembly is occurring. ODA assembly is therefore defective in a *Dpcd* knockdown, preventing assembled complexes from localising in the cilium.

5.3.6.2 The inner dynein arm subunit DNALI1 does not localise in *Dpcd* knockdown pupal antennae

TEM on adult antennae also showed a loss of IDAs in a *Dpcd* knockdown. To investigate how inner dynein arm assembly is affected during development, a fly cross was conducted to allow for the expression of IDA subunit *Dnali1* to be assessed in a *Dpcd* knockdown background. Figure 5.5C shows *Dnali1* expression in the cell body and cilium of the control. In the *Dpcd* knockdown shown in Figure 5.5D, *Dnali1* was found to be expressed as normal in the cell body but reduced in the cilium when compared with controls. This result indicates a disruption to IDA assembly when the function of *Dpcd* is affected.

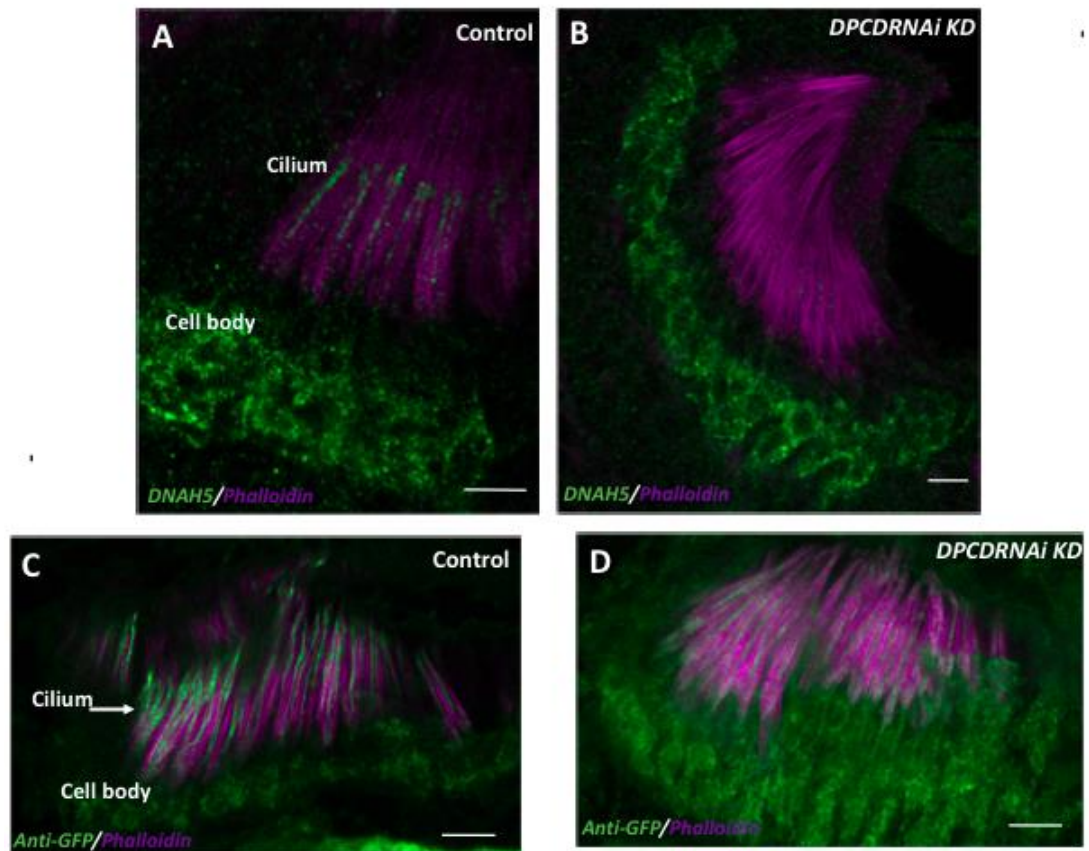


Figure 5.5. *Dpcd* knockdown results in defective localisation of both ODA and IDA subunits (A) Control pupal antennae showing expression of the outer dynein arm subunit DNAH5 in both the cell body and the cilium. (B) *Dpcd RNAi* knockdown showing expression of DNAH5 in the cell body, but significantly reduced in the cilium indicating defective localisation. (C) Control pupal antennae showing expression of this inner dynein arm subunit in the chordotonal neuron cell body and cilium. (D) *Dpcd RNAi* knockdown showing expression of *Dnali1* in the cell body but reduced in the cilium compared with control. Bars: 5µm Number of antennae showing phenotype out of those imaged: (A) $n = 5/5$ (B) $n = 9/9$ (C) $n = 5/5$ (D) $n = 8/8$

5.3.7 Proteomic analysis of *Dpcd* knockdown adult testes

5.3.7.1 *Dpcd* knockdown results in a decrease in abundances of dynein subunits

TEM and the dynein localisation investigation indicated that both the outer and inner dynein arms were affected in a *Dpcd* knockdown in the antennae. For further insight into how these structures are affected, label-free mass spectrometry analysis was conducted on *Dpcd* knockdown adult testes to assess what proteins are depleted and enriched compared to the control

group. The proteomics was conducted in a parallel manner to the analysis described for *Dyx1c1* in Chapter 3. As shown in Figure 5.6A, a *Dpcd* knockdown affected both outer and inner dynein arm subunits, as these proteins were reduced in the knockdown when compared to the control group. Dynein heavy chain proteins were affected, which suggests instability from the inaccurate assembly due to irregular protein folding. Instability is a typical phenotype observed when dynein assembly factors are non-functional, suggesting a DNAAF role for *Dpcd* (Mali et al., 2018).

IPA software was used to identify additional pathways and networks in which *Dpcd* may have a role. As mentioned in Chapter 3, this software is more tailored to mammalian cell work, so analysis was conducted on the *Drosophila* proteins that were recognised by the system. IPA detected several proteins differentially abundant in the *Dpcd* knockdown dataset which had a role in the movement of cilia and whose expression has been altered (Fig. 5.6B).

Overall, the proteomic results support the TEM and dynein localisation data previously described, suggesting that when *Dpcd* is non-functional, the ODAs and IDAs are not localising to the cilium correctly. This defective localisation is due to incorrect dynein assembly, resulting in unstable protein complexes which are degraded.

5.3.7.2 Comparing *Dpcd* knockdown proteomic results against *Dyx1c1* knockdown.

A knockdown of *Dpcd* shows a reduction in abundance of dynein subunits, particularly dynein heavy chains which have been linked to defective assembly previously. Moreover, these are the structures most affected in PCD patients (Mali et al., 2018). This observation suggests a role for *Dpcd* in dynein assembly. The results obtained from a *Dpcd* knockdown were compared with that of a known DNAAF, *Dyx1c1* (described in Chapter 3), to assess whether similar proteins were affected in both knockdowns.

As shown in Figure 5.6C, several motile cilia proteins were less abundant in a *Dpcd* knockdown when compared to the *Dyx1c1* knockdown, including LRRC6 and IQCD. However, most motile cilia proteins were similarly expressed in both knockdowns, suggesting involvement in related motile cilia functions, potentially in similar protein complexes.

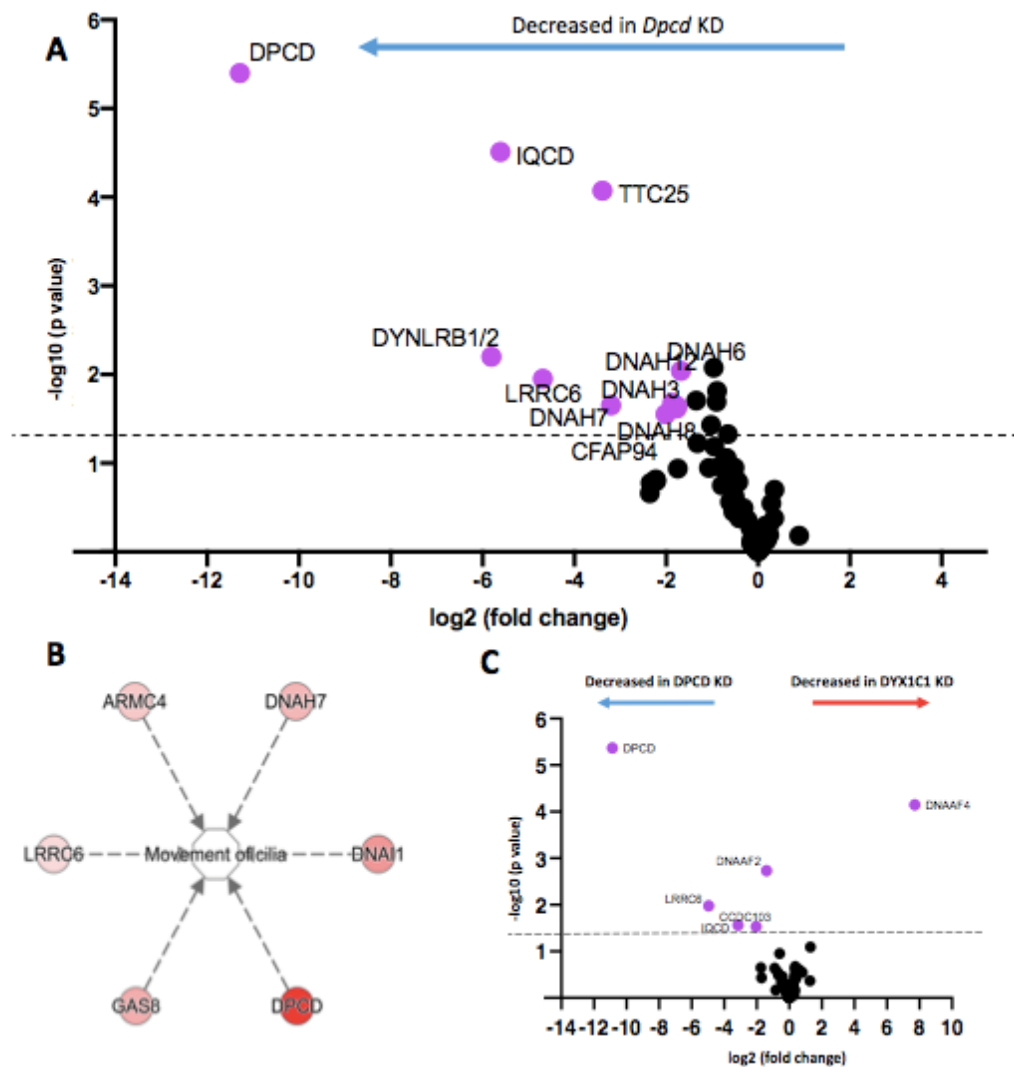


Figure 5.6. Quantitative proteomic analysis of *Dpcd* kd adult testes shows reduction of abundance of dynein motor proteins, especially heavy chains. (A) Volcano plot comparing motile cilia proteins of *Dpcd*RNAi kd males with control. Significant proteins of interest (magenta) are labelled and have a log2(fold change)>+/-1.5 and -log10(p value)>1.3. **(B)** Screenshot from IPA analysis indicating multiple proteins with motile cilia function are depleted in *Dpcd* kd, the darker the circle the greater the effect on regulation. **(C)** Comparing *Dpcd*RNAi kd with a *Dyx1c1* RNAi kd. Volcano plot showing motile-cilia proteins that are depleted or enriched in *Dpcd* kd compared to *Dyx1c1*. Proteins of significance (-log10 pValue>1.3) in magenta. Significance for (A, C) determined using Empirical Bayes method. *n*=30 pairs of testes/replicate. 4 replicates/genotype.

5.3.8 GFP Trap AP-MS suggests Dpcd interacts with DNAAFs

The knockdown evidence suggests that *Drosophila Dpcd* has a role in the outer and inner dynein arm assembly of motile ciliated cells. It has been reported that DNAAFs can interact with one another and carry out their assembly roles as part of complexes (Li et al., 2017; Maurizy et al., 2018; Olcese et al., 2017; zur Lage et al., 2018). To identify potential protein interactors of *Drosophila Dpcd*, GFP Trap AP-MS was conducted on adult testes from the *DpcdmVenus* line. This experiment was carried out in parallel under the same conditions as the GFP Trap AP-MS experiment described in Chapter 3.

As shown in Figure 5.7, the significant protein interactors of *Dpcd* included Pontin and Reptin of the canonical R2TP complex, previously associated with dynein assembly (zur Lage et al., 2018). In addition, the DNAAF Heatr2 was identified as a potential interactor. Heatr2 is suggested to form a scaffold early in the process to facilitate outer and inner dynein assembly (Moore et al., 2013). This proteomic data shows evidence for *Dpcd* having a role in R2TP or R2TP-like complexes during dynein assembly, as well as linking the known DNAAF Heatr2 to these R2TP complexes for the first time.

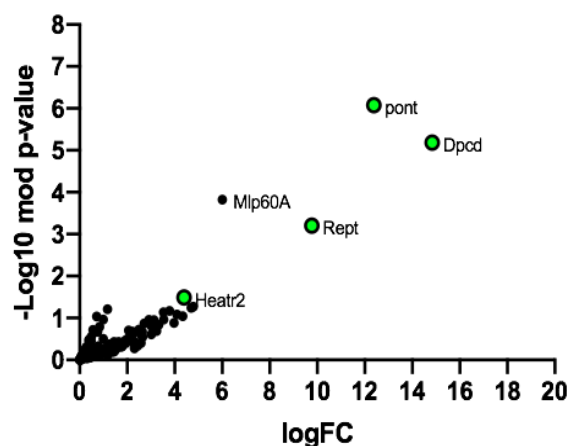


Figure 5.7 GFP-Trap AP-MS detects dynein assembly protein interactors for *Dpcd*. Volcano plot showing proteins associated with *Dpcd*-mVenus from AP-MS analysis compared to control (F1 males of *GAPmVenus* x *BamGal4*). Significant proteins ($\log_{10}(\text{p-value}) > 1.3$, $\log_2(\text{fold change}) > 1.5$) are labelled and those motile cilia-related are in green. Significance for (A) determined using the Empirical Bayes method. $n = 150$ pairs of testes/ replicate. 3 replicates/genotype

5.3.9 Co-IP data shows Dpcd and Heatr2 interact both on their own and in a complex with Pontin and Reptin

To verify the GFP Trap findings, co-immunoprecipitation (co-IP) was conducted with Petra zur Lage using *Drosophila* S2 cells. Tagged proteins were heterologously expressed, and the extracts were analysed by co-IP to assess direct protein interactions (zur Lage). These proteins were detected using a Western blotting process, where the membrane was incubated in anti-HA and anti-FLAG antibodies (to interact with the HA-tagged proteins and anti-FLAG beads used in the co-IP). The protein detected by this experiment was imaged using a Li-COR scanner and is shown in Figure 5.8A below.

This co-IP experiment supports the proteomic data described above, showing that Dpcd and Heatr2 directly interact (in the absence of Pontin and Reptin). Moreover, all four motile cilia proteins identified in the AP-MS (Pontin, Reptin, Heatr2, Dpcd) interact together in a novel protein complex. Dpcd also interacts on its own with Pontin and Reptin, as does Heatr2, an interaction which has not been previously described in the literature. Therefore, this protein interaction study has identified several novel complexes and interactions, shown in Figure 5.8B, with potential roles during axonemal dynein assembly.

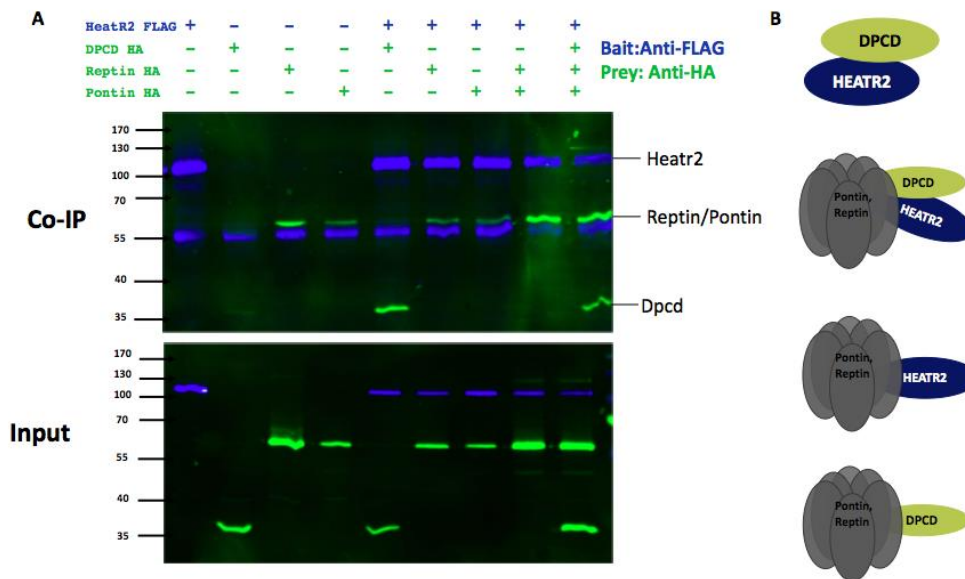


Figure 5.8. Co-IP data indicates that Dpcd and Heatr2 interact both independently and in a complex with Ruvbl1/2. The co-IP fifth lane shows that Dpcd is pulled down with Heatr2 (CG31320) on its own. 8th shows that Heatr2 interacts and pulls down Reptin and Pontin. 9th lane shows that Heatr2 pulls down Dpcd, Ruvbl1/2 and therefore directly interacts in a complex. Not displayed on this co-IP, but it has also been shown by P. zur Lage that Dpcd interacts with Reptin and Pontin on its own.

5.3.10 The generation of a Dpcd null mutant using CRISPR/Cas9 technology

To further investigate the specific function of *Dpcd* in a *Drosophila* model, a null mutant was generated similarly to that previously described in Chapters 3 and 4. As shown in the schematic in Figure 5.9A, the ORF of *Dpcd* was replaced with the mini-white gene using CRISPR/Cas9 homology directed repair. This resulted in an unexpected phenotype of late-larval lethality in homozygotes, where no adult structures develop during pupation (Fig. 5.9B). Knockouts of *Dyx1c1* in Chapter 3 and *Pih1d3* in Chapter 4 in *Drosophila* resulted in viable flies with defective chordotonal neuron function and male infertility. Throughout the cloning of the construct, samples were sent for Sanger Sequencing at Edinburgh Genomics to check for single nucleotide polymorphisms (SNPs) and to verify the correct gene (*Dpcd*) was being removed. Therefore, the lethality of homozygous *Dpcd*^{-/-} flies suggests a wider role for *Dpcd*. However, it is also still possible that the CRISPR engineering

impaired the function of other genes, either adjacent to *Dpcd* or as an off-target deletion elsewhere in the genome.

5.3.10.1 The neighbouring gene *CG13887* is unaffected in the *Dpcd* null mutant line

The use of the deficiency line (data not shown) indicated that the lethality is caused by the gene region containing the *Dpcd* gene. *CG13887* is a neighbouring gene of unknown function, but if this was affected by the deletion may explain the lethality observed. As a means of troubleshooting and ensuring that in the generation of a *Dpcd* knockout the regulation of this neighbouring gene was unaffected, a *CG13887* P-element line was obtained. Homozygotes of this fly line have disrupted *CG13887* function, and I found that they do not have the late lethal phenotype observed for the *Dpcd* null larvae. *CG13887* homozygotes develop similarly to wild type at pupal stage (Fig. 5.9B)

To further verify that this neighbouring gene is unaffected in the *Dpcd* knockout line, a *CG13887mRNA* probe was generated. The predicted expression pattern of *CG13887* in wild-type whole mount embryos was obtained from the Berkeley Drosophila Genome Project (BDGP) website. This showed strong expression for *CG13887* at the different stages of embryonic development. An RNA *in situ* hybridisation was conducted on *Dpcd*^{-/-} embryos. The expression of *CG13887* was as expected in the different developmental stages of the *Dpcd* null embryos. This result, shown in Figure 5.9C, verified that the expression of the neighbouring gene *CG13887* was unaffected in this genetic background, making it more likely that the lethality is due to a non-functional *Dpcd*.

5.3.10.2 A ubiquitous knockdown of *Dpcd* function results in late larval lethality

The *Dpcd* null mutant fly line that was generated caused a late larval lethality. To further support that this phenotype was the result of non-functional *Dpcd*, the UAS/Gal4 system and RNAi technology were used to knockdown gene function in the whole organism. As described earlier in the chapter, RNAi

knockdowns of *Dpcd* function using tissue-specific drivers were successful in gaining insight to the role of this gene in motile cilia. A ubiquitous driver line (Tubulin-Gal4) was obtained and crossed to the UAS-*Dpcd*RNAi line, with the aim of knocking down gene function in all cells. This process resulted in a similar phenotype to that observed in the *Dpcd*^{-/-} fly line. When *Dpcd* function is knocked down in all cells, larvae get to the third-instar stage but no further (Fig. 5.9B). A puparium forms around the larva but no adult structures develop, resulting in eventual lethality. This observation suggests a wider role for *Dpcd*, that is essential for viability.

5.3.10.3 The DpcdmVenus fusion protein successfully rescues the lethality observed in *Dpcd*^{-/-} larvae

As shown in Chapter 3 and 4, mVenus fusion proteins have fully rescued or partially rescued null mutations of DNAAFs previously. To test for the rescue of the *Dpcd* null mutation, I crossed in the *DpcdmVenus* fusion line which surprisingly, did not rescue the lethality. Upon examining the gene annotation on GBrowse, it was found that this construct did not include a potential upstream start site. So, another construct was generated by Petra zur Lage containing an additional 100 base pairs in the promoter region (Fig. 5.9D). This fusion protein successfully rescued the lethal phenotype, producing viable and healthy adult flies.

The climbing ability of the rescue line was assessed and shown to be like that of the control line, further demonstrating that the mVenus fusion protein successfully rescued. This rescue experiment, shown in Figure 5.9E, indicates that the lethality of the *Dpcd* null mutant line is caused by the expected gene, and that there are additional binding sites for *Dpcd* regulators upstream of those for *Rfx* and *fd3F*. This finding suggests that the longer construct supports vital expression of *Dpcd* in locations beyond the ch neurons and testes, as these were expressed in the shorter construct that did not rescue

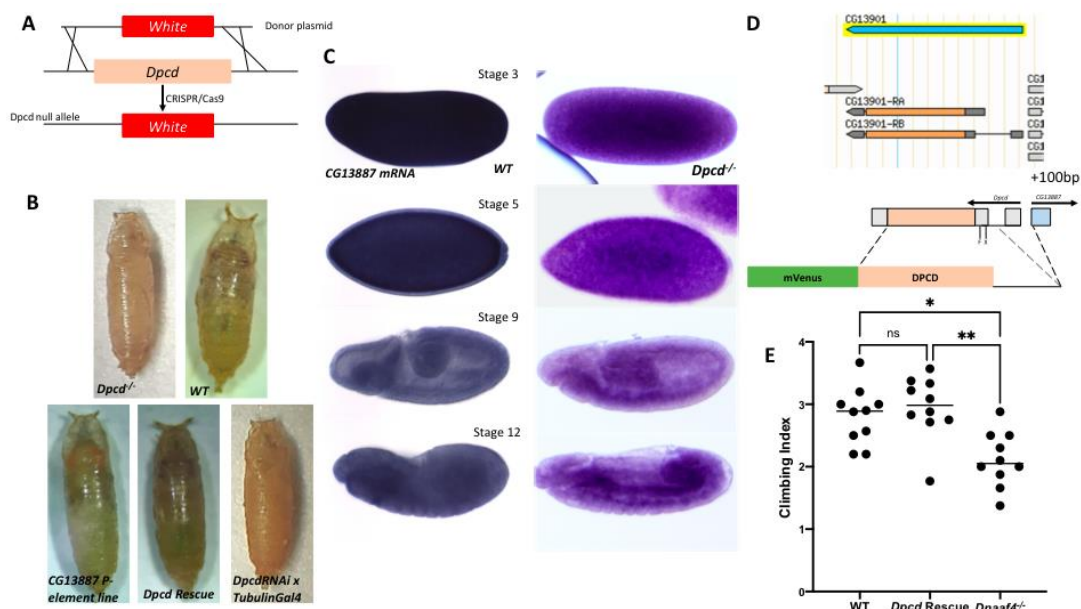


Figure 5.9 Generation of DPCD null mutant causes a late-larval lethality, unexpected for PCD-specific gene. (A) Generation of *Dpcd* null mutant by replacing the ORF of *Dpcd* with the *mini-white* gene using CRISPR/Cas9 homology-directed repair. **(B)** *Dpcd*^{-/-} causes a late-larval lethality, where no adult structures are formed during pupation like those observed in a WT control. A similar phenotype is observed when *Dpcd* is ubiquitously knocked down with tubulin gal4. **(C)** RNA *in situ* hybridisation of whole-mount embryos with *CG13887* probe. WT images obtained from BDGP website. The staining of *CG13887* is unaffected in *Dpcd* null embryos, indicating this gene is unaffected in the generated mutant. Pupal image of *CG13887* deletion mutant in (B) supports this. **(D)** Screenshot from GBrowse (Donlin, 2007) showing the two transcripts and schematic of longer *DpcdmVenus* construct including an additional 100bp upstream of the binding sites for motile cilia transcription factors, *fd3F* and *Rfx*. Dotted lines indicate the region that the initial constructs and the new longer construct include. This mVenus fusion protein successfully rescued the lethal phenotype, shown in (B). **(E)** Climbing assay displayed as a graph (with individual values and median) showing that the climbing ability of the *Dpcd* rescue line is like the wild-type control. $n = 10$ batches of 8-12 flies. ($p = 0.4466$). *Dnaaf4* null mutant performance ran alongside the result (n.d). Significance for (E) was determined by a Kruskal-Wallis test. Number of samples imaged: (B) $n = 9-15$ per genotype (C) $n = 1-2$ per developmental stage.

5.3.11 Anatomical Expression Data and Tissue Expression Data indicate *Dpcd* is expressed in additional tissues

The lethality of the *Dpcd*^{-/-} mutants suggests a wider role for this proposed DNAAF. As discussed earlier in the chapter, *Dpcd* is expressed in *Drosophila* motile cilia cells. To identify whether *Dpcd* is highly expressed in additional cell types which may contribute to the lethal phenotype, both FlyAtlas Anatomical

Expression Data and modENCODE Tissue Expression Data were re-examined. These datasets, shown in Figure 5.10, indicated that *Dpcd* is also expressed in the larval CNS, imaginal discs and adult ovary, supporting a wider function for *Dpcd* in *Drosophila*.

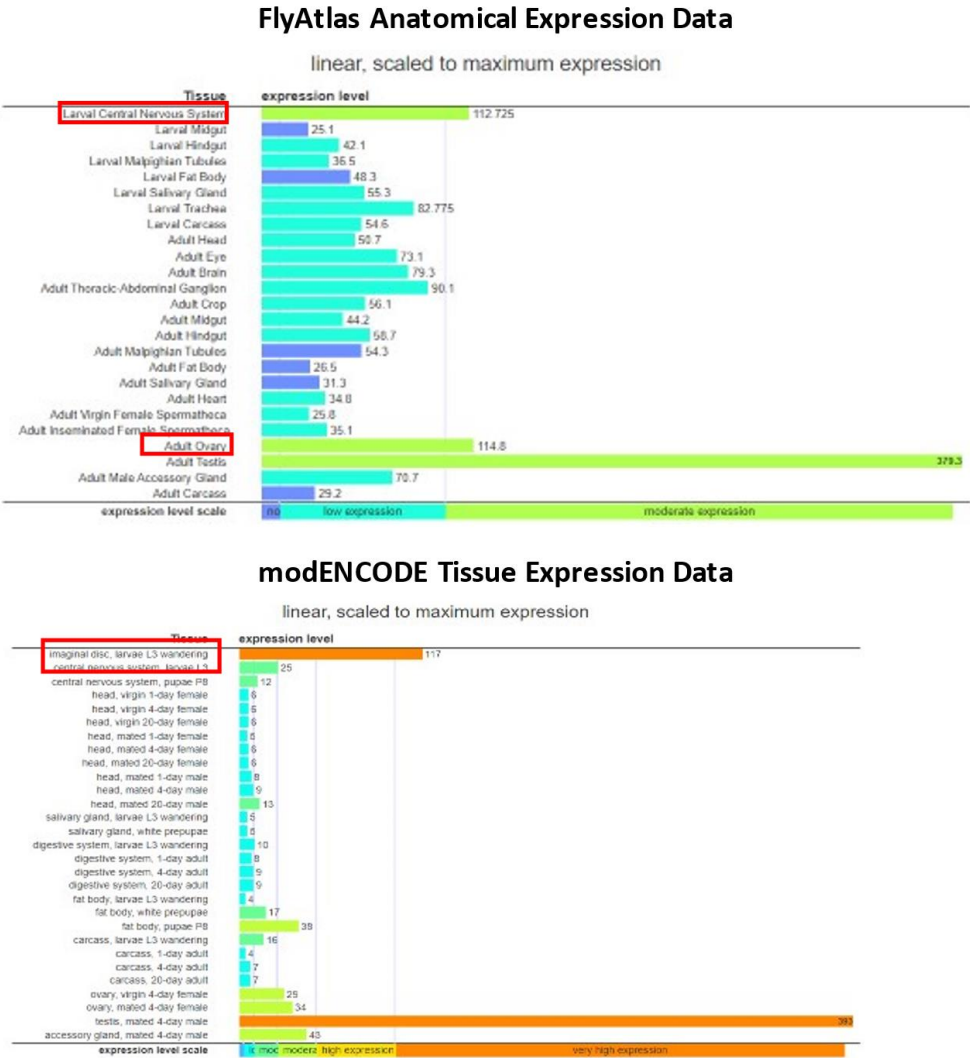


Figure 5.10 *Dpcd* is expressed in the larval CNS, imaginal discs and the adult ovary. (A) Screenshot showing FlyAtlas expression data which highlights that as well as high expression in the testis, *Dpcd* is expressed in the larval CNS. **(B)** Screenshot showing modENCODE tissue expression data that indicates high expression of *Dpcd* in the larval imaginal discs, as well as very high expression in the adult testis.

5.3.12 Expression of *Dpcd* in ovary suggests maternal contribution

The expression of *Dpcd* in the adult ovary, as well as the lethal phenotype at larval stage suggests that there may be a maternal component which aids development until pupation. In *Drosophila*, as well as more complex organisms, maternal gene products are important for regulating the early development of progeny, which have a stockpile of transcripts for general cell functions such as cell division. These transcripts are produced in the nurse cells (in the adult ovary) before being distributed to the oocyte via nurse cell dumping (Gutzeit & Koppa, 1982). If *Dpcd* is essential for viability, maternal contribution may be sufficient to get through the initial stages of development before the next stage of growth and development: metamorphosis.

As the *Dpcd* null mutant line is viable until the onset of pupation, third-instar larvae could be assessed for dynein assembly phenotypes. A larval hearing assay previously successfully identified auditory deficits in CRISPR/Cas9 null mutants of DNAAFs (Chapters 3, 4). This hearing assay was performed on *Dpcd*^{-/-} larvae, which responded to the stimulus and showed no auditory deficits. This result, shown in Figure 5.11A, is unexpected for a DNAAF and suggests that *Dpcd* may be a maternal effect gene, providing a sufficient dose (albeit of variable amounts, suggested by 5.11A) to get the homozygous null mutants through initial stages of development. This hearing assay result is further supported by immunofluorescent staining conducted by Petra zur Lage (unpublished, Fig.5.11B). The DNAH5 antibody was applied to third-instar larval pelts to assess how the ODA are affected in this *Dpcd*^{-/-} genetic background. DNAH5 protein was expressed in the cell body and localised in the cilia of the chordotonal organs of the *Dpcd* homozygous mutant, like that of the control. This result indicates that dynein localisation is unaffected in *Dpcd*^{-/-} third-instar larvae, explaining the preserved auditory function and supporting the notion of a maternal contribution.

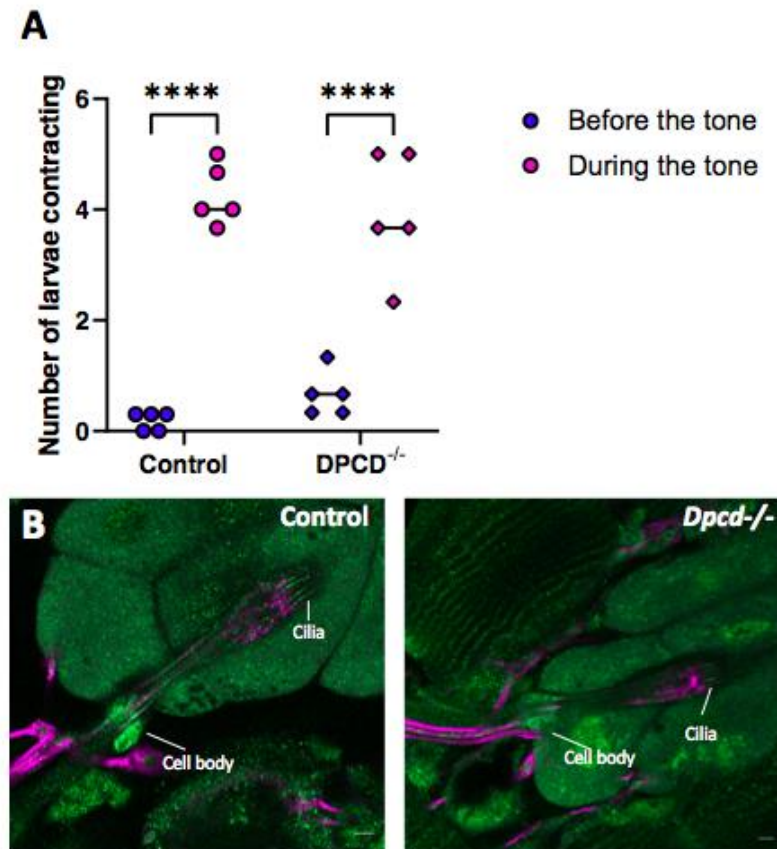


Figure 5.11: DPCD KO larvae do not have defective motile cilia, or axonemal defects (A) Plot showing larval hearing assay performance for *Dpcd*^{-/-} and control. Number of larvae which contracted before and during a 1000Hz tone was measured. *n* for each genotype = 5 batches of 5 larvae, each exposed to 3 tones 30s apart. Significance for (A) was determined using a 2way repeated measures ANOVA and Sidak's multiple comparisons test. **(B)** DNAH5 staining conducted by Petra zur Lage showing staining in the cell body and cilia of larval chordotonal neurons. This indicates ODAs are localising in a similar manner between the *Dpcd* knockout and control.

5.3.13 *Drosophila Dpcd* does not have a role in ciliogenesis

When *Dpcd* function was knocked down in the developing nervous system it resulted in a more severe defect than typically observed for DNAAFs, suggesting a wider role for *Dpcd*. Collaborators of the lab have suggested that *Dpcd* may have a role in ciliogenesis (Personal communication). *dRfx* (the ciliogenesis transcription factor discussed in the Introduction) mutants show more extreme phenotypes than that observed for motile cilia-specific genes, with lethality occurring in most homozygotes at the larval stage (Dubruille et al., 2002). The previous TEM analysis of *Dpcd* knockdown chordotonal

neurons showed that the cilia are forming as expected (Fig. 5.4). Immunofluorescent staining was conducted using anti-HRP to further support that ciliogenesis is unaffected in a *Drosophila Dpcd* knockout (Fig. 5.12). Anti-HRP binds to neuronal membranes, such as those present in the chordotonal neuron (Jan & Jan, 1982).

Larval pelt dissections of the *Dpcd* null mutant showed ciliated chordotonal neurons developing similarly to that of the control (Fig. 5.12A). Moreover, in the pupal antennae shown in Figure 5.12B, HRP staining is localised to the cilium of the chordotonal neuron in both the control and *Dpcd* RNAi knockdown, suggesting that *Dpcd* does not have a direct role in ciliogenesis.

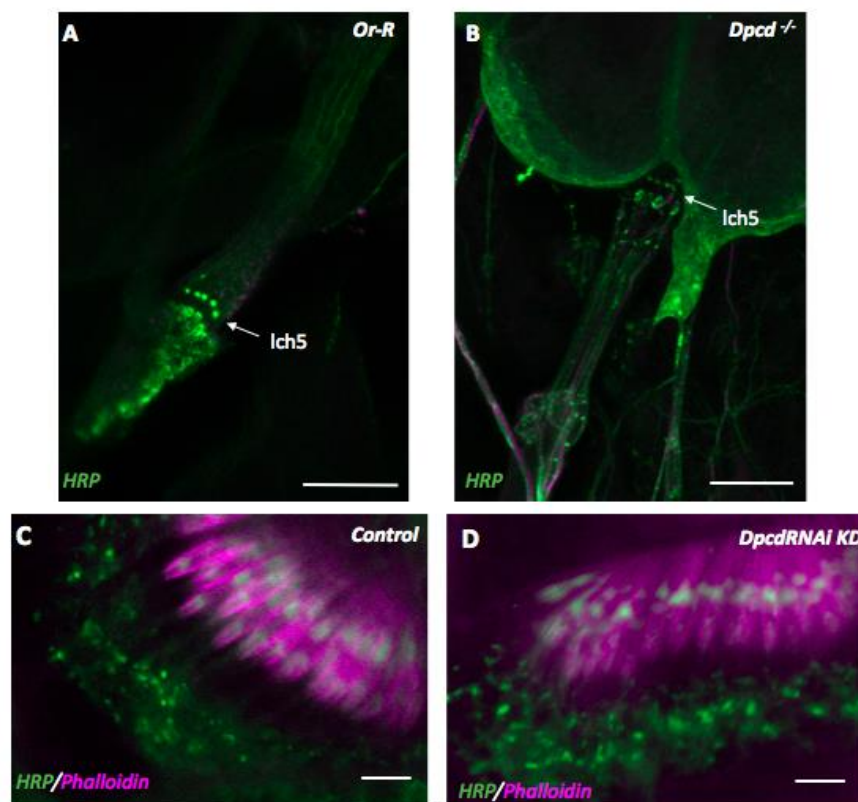


Fig 5.12 Analysis of chordotonal neuron structure does not suggest ciliogenesis defects (A,B). Larval pelt dissection showing structure of presence of chordotonal neurons (lch5) in wild-type (A) and *Dpcd*^{-/-}(B). **(C,D)** anti-HRP staining in pupal antennae showing localisation to the cilium of the chordotonal neuron in both control (C) and *Dpcd* knockdown (D). Bars: (A-B) 15µm (C-D) 5µm. Number of samples imaged for IF:(A) *n* = 7 (B) *n* = 4 (C) *n* = 5 (D) *n* = 6

5.3.14 *Dpcd*^{-/-} have poorly developed imaginal discs, but no chromosomal defects

modENCODE Tissue Expression Data indicates that *Dpcd* is expressed in the imaginal discs. These structures are crucial during metamorphosis, as they become the adult structures of the fly (Aldaz & Escudero, 2010). The late lethal phenotype observed for *Dpcd*^{-/-} larvae is like that described by the Gatti group (Gatti & Baker, 1989). When screening for genes involved in cell-cycle regulation, a 'late lethal' phenotype was reported that survived until the larval-pupal transition due to maternal contribution. Several reasons for this lethality were described, including poorly developed imaginal discs, defective chromosomal integrity and incomplete chromosomal segregation (Gatti & Baker, 1989). To assess whether *Dpcd* has a role in one of these cellular processes, the imaginal discs and polytene chromosomes were examined.

5.3.14.1 Third-instar *Dpcd*^{-/-} larvae show a significant reduction in imaginal disc size

As poorly developed imaginal discs have been shown to cause the late lethal phenotype observed, *Dpcd* homozygous larvae were dissected by Andrew Jarman, and the tissues examined. In the heterozygous control group, the wing, leg and eye-antennal discs are clearly visible and morphologically as expected. However, in the homozygous larvae, shown in Figure 5.13A, the eye-antennal disc is very reduced, and the wing and leg discs appear absent. This result suggests that the imaginal discs are severely affected in a *Dpcd* knockout, resulting in lethality.

5.3.14.2 Imaginal discs are present in *Dpcd*^{-/-} embryos

The imaginal discs which appear to be absent in *Dpcd*^{-/-} null larvae could be due to failure in formation during patterning or in failure of growth of the primordium. Therefore, it is necessary to assess whether they are present earlier in development at the embryonic stage. *Distal-less (Dll)* is a homeobox gene involved in limb development and is expressed in embryonic imaginal discs (Panganiban & Rubenstein, 2002). To identify whether imaginal discs are present early in development, an anti-*Dll* antibody was obtained and used

to stain *Dpcd*^{-/-} and *Dpcd*^{+/-} whole-mount embryos. As shown in Figure 5.13B, immunofluorescent staining indicated that while imaginal discs appear absent at the third-instar larval stage, these tissues are present earlier in development. Therefore, the imaginal discs have a reduction in growth in the homozygous null mutant.

5.3.14.3 Polytene chromosomes are narrower in a *Dpcd*^{-/-} mutant, but no chromosomal defects are visible

Chromosomal defects including defective integrity and incomplete segregation can cause a late lethal phenotype like that observed in *Dpcd*^{-/-} larvae (Gatti & Baker, 1989). To assess whether the chromosomes of *Dpcd*^{-/-} mutants are defective, the polytene chromosomes of the salivary glands were dissected by Andrew Jarman (shown in Figure 5.13C). These are massive super-chromosomes, which have proven to be a useful genetic tool for *Drosophila* researchers to understand chromosome biology (Stormo & Fox, 2017). The polytene chromosomes of *Dpcd* heterozygote and homozygote third-instar larvae were dissected. The polytene chromosomes contain dark bands of chromatin, and these were unaffected in the null mutant. While the chromosomes appeared to be segregating and forming as expected, they were thinner than the heterozygote control. This observation may be due to a general nutrition defect, which can result in small larvae in general, as opposed to defective chromosomal integrity (Ahmad et al., 2018). Overall, these observations suggest a role for *Dpcd* in regulation of tissue growth rather than chromosome biology, which will be further explored later in this chapter.

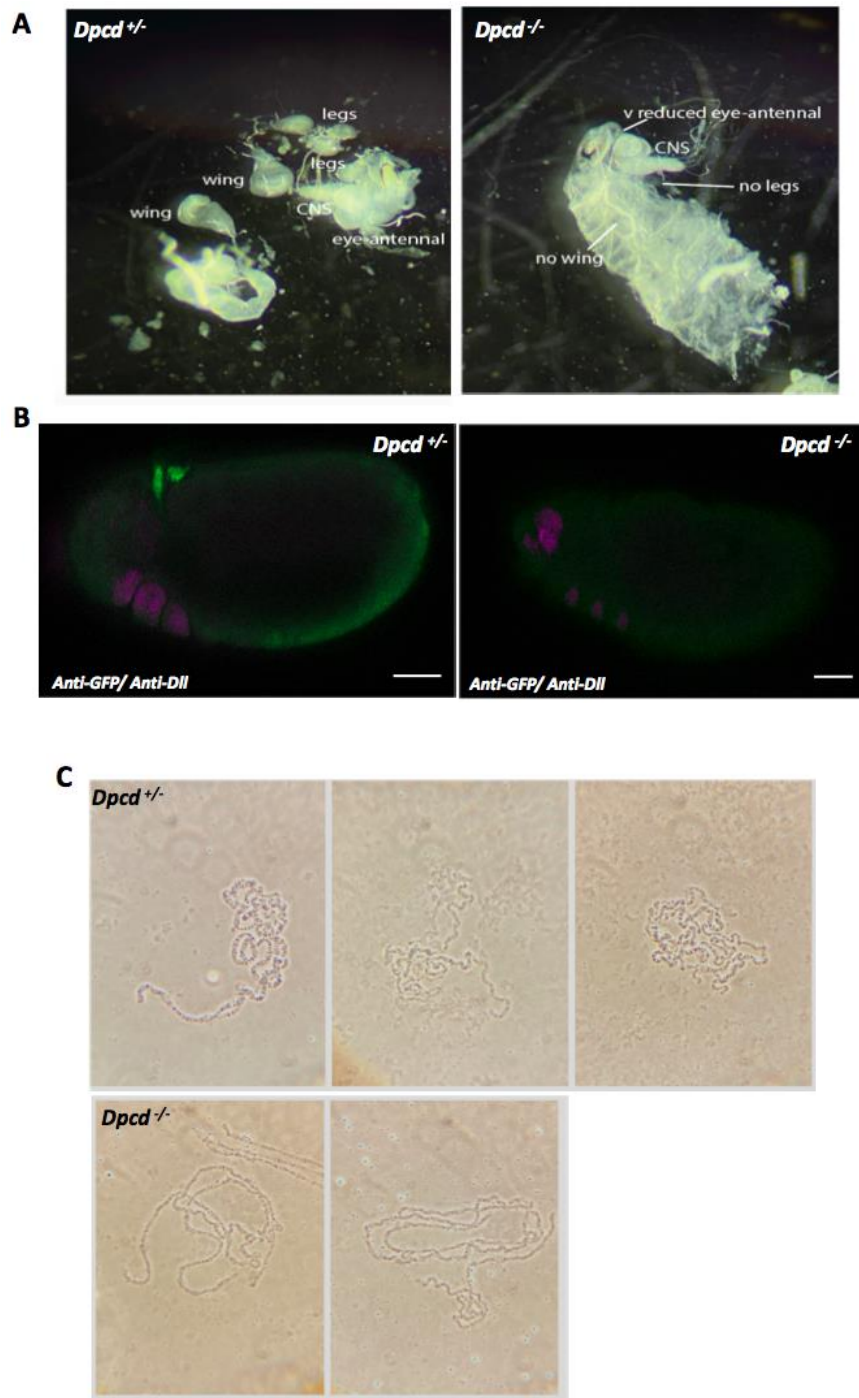


Figure 5.13 *Dpcd*^{-/-} mutants have poorly developed imaginal discs, but no chromosomal defects. **(A)** Dissection of third-instar larvae (conducted by Andrew Jarman) indicates a significant reduction in imaginal disc size in homozygous mutants compared to heterozygous controls. **(B)** *Distal-less* (*Dll*) staining in *Dpcd*^{-/-} embryos indicate progenitor cells for adult fly structures are present early in development. **(C)** Dissection of the salivary glands (conducted by Andrew Jarman) of the larvae indicate that a *Dpcd*^{-/-} null mutation does not result in a chromosomal defect. Bars: (A-B) 50 nm. Number of embryos imaged for IF: (A) *n* = 8 (B) *n* = 8.

5.3.15 *Heatr2* deletion mutants have a late lethal phenotype

The GFP AP-MS analysis, as well as the co-IP experimentation, indicated that *Dpcd* and *Heatr2* directly interact, as well as in a complex with Pontin and Reptin. Therefore, it is our hypothesis that this novel protein complex has a role in both outer and inner dynein assembly. As mentioned in Chapter 3, Pontin and Reptin have many cellular functions both on their own and as part of the R2TP complex (Izumi et al., 2012; Kakiyama & Houry, 2012), including roles in tissue growth. *Dpcd* has an additional broader role in the cell besides dynein assembly, as a null mutation results in lethality. As *Heatr2* is a protein interactor of *Dpcd*, Pontin and Reptin, I investigated whether it too has an additional essential function.

Heatr2 (also known as DNAAF5) was discovered in PCD patients who lost dynein arms and ciliary beating (Horani et al., 2012). As discussed in the Introduction, the function of this gene was validated in *Drosophila* and found to have a role in both outer and inner axonemal dynein assembly (Diggle et al., 2014). Heat-repeat proteins usually function as scaffolds, and as this DNAAF is cytoplasmic, it has been suggested to work as a scaffold and facilitate protein-protein interactions early in dynein assembly. As well as expression in embryonic chordotonal neurons, *Heatr2* was found to be transiently expressed in a mesodermal pattern, suggesting a wider role for this DNAAF (Diggle et al., 2014). A P-element deletion mutant was previously generated by our lab, in which the transcriptional and translational start sites were disrupted. This P-element line lacked viable homozygote adult flies, and it was noted that homozygote larvae were present but smaller than the heterozygotes. It was proposed that *Heatr2* may have an additional essential role, potentially involving nutrition. *Heatr2* is expressed in the midgut of developing embryos (Diggle et al., 2014). However, this phenotype was not followed up further.

To assess whether the phenotypes described for the *Heatr2* deletion line were like that observed for *Dpcd*^{-/-} null mutants, third-instar larvae, pupal and adult flies (or lack thereof) from this P-element line were observed. Third-instar

larvae were thinner than heterozygous controls, similar to *Dpcd*^{-/-} larvae. At the pupal stage, a late lethal phenotype was observed (shown in Figure 5.14A). As homozygotes die at the larval-pupal transition, homozygous adult flies are absent in the *Heatr2* deletion mutant.

modENCODE Tissue Expression Data was assessed for the *Heatr2* *Drosophila* homologue was found to be most highly expressed in the imaginal discs of third-instar larvae (Fig. 5.14B). These are tissues which undergo vast changes during metamorphosis due to cells rapidly proliferating (Handke et al., 2014). As *Dpcd* is also present in the imaginal discs, and these tissues are reduced in null mutants, these DNAAFs may have similar additional functions outside of dynein assembly.

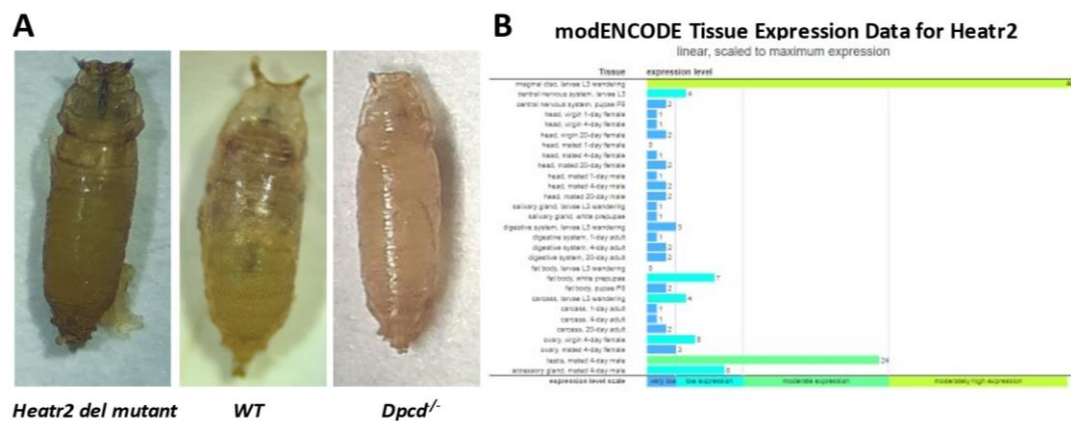


Figure 5.14 *Heatr2* p-element deletion mutants have a late lethal phenotype and is highly expressed in imaginal discs (A) *Heatr2* deletion mutant causes a late-larval lethality, with no adult structures developing during pupation. Similar phenotype to that observed when *Dpcd* is non-functional (B) Screenshot of modENCODE RNA-Seq data indicating very high expression of *Heatr2* in the larval imaginal discs

5.3.16 DpcdmVenus and Heatr2mVenus fusion proteins are expressed in additional tissues

5.3.16.1 Dpcd and Heatr2 are expressed in imaginal discs

ModENCODE Tissue Expression Data indicated both *Dpcd* and *Heatr2* are expressed in imaginal discs. A Heatr2mVenus fusion protein was previously generated by the lab and used to characterise its dynein assembly role (Diggle et al., 2014). When surveying for additional Dpcd expression, the larger mVenus construct which successfully rescued the null mutant lethality was used.

The imaginal discs from the Heatr2mVenus and DpcdmVenus third-instar fly lines were dissected and stained with anti-GFP to enhance fluorescence. A Pih1d3mVenus fly line was used as a control, as this gene's function is specific to dynein assembly. Therefore, any additional expression observed in Heatr2 or Dpcd is linked to their wider functions. Oregon-R larvae were also used as a control for general background.

No fluorescence was observed in the Pih1d3mV and Or-R controls (Fig. 5.15A, B). However, DpcdmV and Heatr2mV imaginal discs showed general expression (Fig. 5.15C, D), indicating cell autonomous roles for these proteins outside dynein assembly.

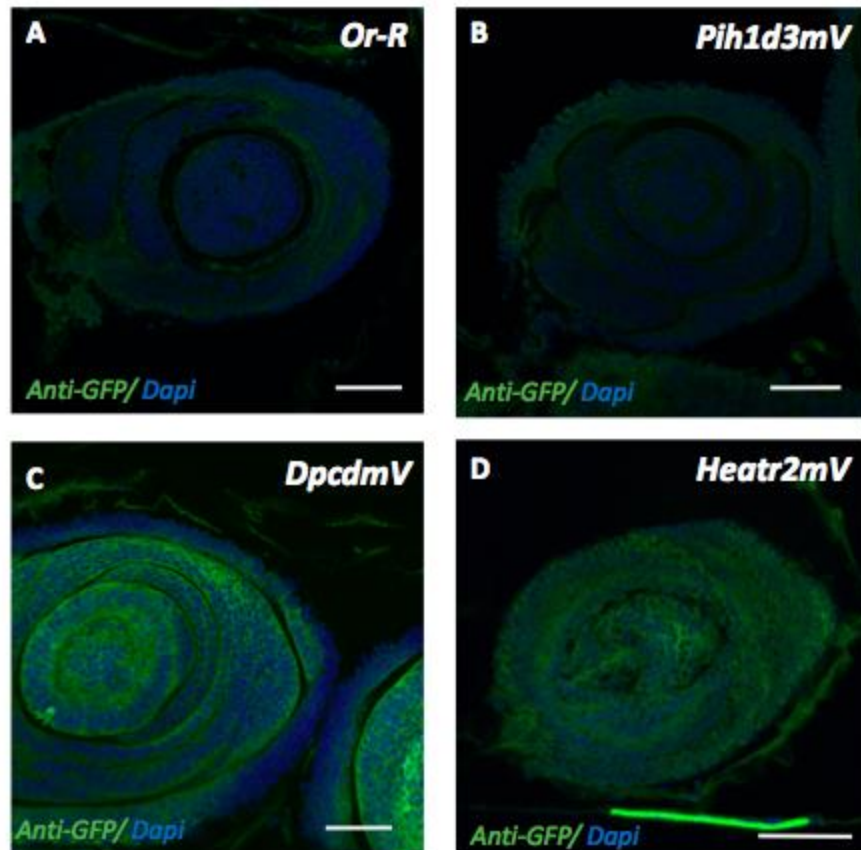


Figure 5.15 Dpcd and Heatr2 have general expression in imaginal discs (A) Control (Or-R) shows no specific GFP expression **(B)** A DNAAF control, Pih1d3mV, is not expressed in imaginal discs **(C)** Dpcd is generally expressed in the imaginal discs **(D)** Heatr2 also shows additional expression in the imaginal discs

5.3.16.2 Dpcd and Heatr2 have additional CNS expression

As well as the imaginal discs, the larval CNS of DpcdmV and Heatr2 fly lines were examined for evidence of additional expression. Control groups Pih1d3mV and Or-R showed no specific expression in the CNS, indicating any additional expression is not a DNAAF characteristic (Fig. 5.16A, B). As shown in Figure 5.16C, DpcdmVenus showed additional expression in the neuroblasts of the VNC and the brain lobes. Heatr2mVenus also showed additional expression in the VNC and the brain lobes of the CNS (Fig. 5.16D). Neuroblasts are cells in the larval CNS in which proliferation and division are crucial for the development of the complex adult brain (Bello et al., 2008). Additional expression of *Dpcd* and *Heatr2* in neuroblasts and imaginal discs suggests roles for these genes in the regulation of cell proliferation.

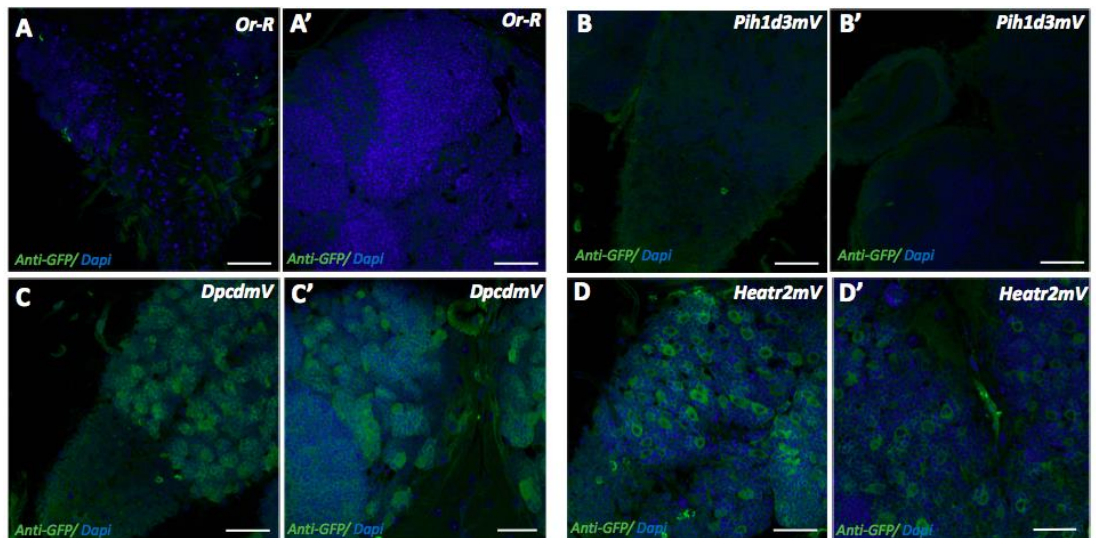


Figure 5.16. Dpcd and Heatr2 have additional expression in the neuroblasts of the larval CNS. (A, A') Control VNC and brain lobes show no specific GFP expression. (B, B') Pih1d3 shows no specific GFP expression pattern, indicating that additional expression in the CNS is not a DNAAF characteristic. (C, C') Dpcd shows additional expression in the neuroblasts of the VNC and brain lobes. (D, D') Heatr2 shows additional expression in the VNC and brain lobes of the CNS, but in a different pattern to that observed for Dpcd. Bars: 30µm. Number of samples imaged: (A) $n = 12/12$ (B) $n = 5/5$ (C) $n = 7/7$ (D) $n = 10/10$

5.3.17 Immunofluorescence in *Dpcd*^{-/-} larvae supports a role in cell proliferation

To further investigate the additional function of *Dpcd* in the CNS, *Dpcd*^{-/-} mutants were stained with DAPI and anti-*asense* (*anti-ase*), an antibody against the neural precursor gene expressed in developing type-I neuroblasts (Brand et al., 1993). *Asense* staining of the neuroblasts appears similar in the *Dpcd* null mutant and control, however in the *Dpcd*^{-/-} brains, there appears to be a reduced optic lobe (indicated by DAPI staining). The inner proliferating centre (IPC) is absent, and the outer proliferating centre (OPC) appears to be less than that of the control. The optic lobe is a region where neurogenesis is actively taking place, and so this preliminary result suggests that there is a cell proliferation defect in *Dpcd*^{-/-} mutants.

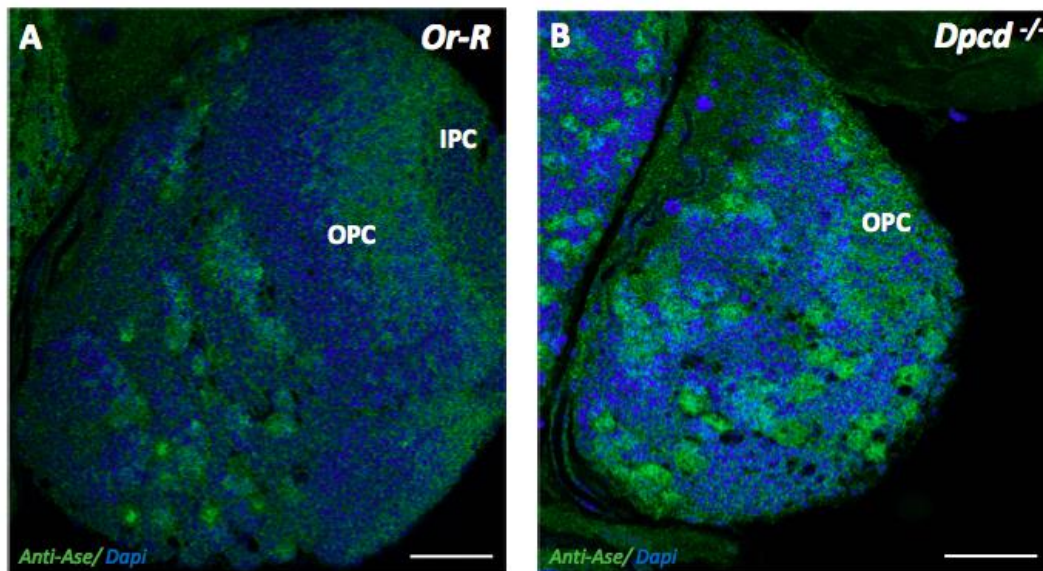


Figure 5.17. The neural precursor gene *Asense* (*ase*) stains neuroblasts in *Dpcd*^{-/-} larvae and Or-R control. (A) *ase* staining of neuroblasts present in both controls, outer (OPC) and inner proliferating centres (IPC) of the optic lobe are present. **(B)** *ase* staining of neuroblasts in *Dpcd* knockout appears similar to control. However, some optic lobe (IPC, partial OPC) is absent in the mutant. Bars (A, B): 30µm. Number of images showing neuroblast staining: (A) *n* = 6/6 (B) *n* = 8/8

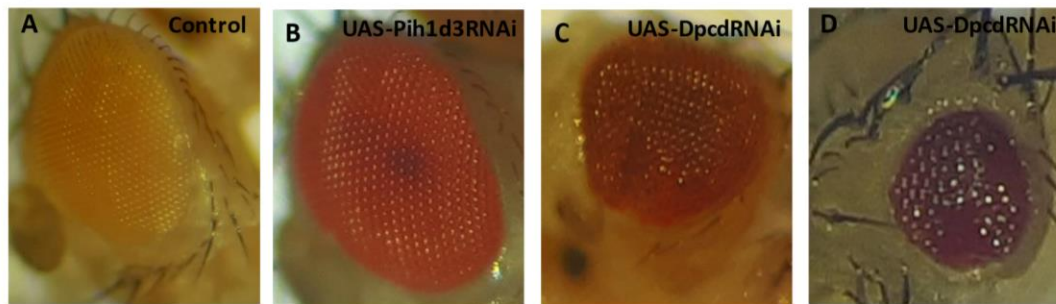
5.3.18 Eye development assay supports cell proliferation role for *Dpcd*

As described previously, the eye-antennal disc is very reduced in *Dpcd*^{-/-} null larvae, a phenotype which can be caused by both cell proliferation and cell death defects. The *Drosophila* eye is a tissue commonly used for studying these cellular processes (Baker, 2001) and so was utilised to determine which cell activity was affected. *Dpcd* function was initially knocked down in the eye using an *Eyeless-Gal4* line. *Eyeless* is a transcription factor expressed very early in eye disc development (Halder et al., 1995). Controls included this Gal4 driver crossed to the UAS line that the RNAi construct was injected into (Fig. 5.18A), as well as an additional control knocking down *Pih1d3* function, to assess whether any phenotype observed is typical for a DNAAF (Fig. 5.18B). In the flies that eclose, there is a reduction of eye size in the *Dpcd* knockdown, however this is not representative of the phenotype. Few flies of the desired genotype eclosed from this genetic cross, indicating that the eclosed flies shown in Figure 5.18C are likely outliers to the actual phenotype. Mature flies that did not successfully eclose were dissected from the pupal case, and as shown in Figure 5.18D, the phenotype was much more severe than that initially observed for the outliers.

To examine whether cell death was affected, *Dpcd* function was also knocked down in the eye using the driver line *GMR-Gal4*, which is expressed after most tissue growth has occurred. This resulted in no severe phenotype as adult flies successfully eclosed and appeared similar to the control (Fig. 5.18E, F). To confirm that the *GMR-Gal4* is functioning as expected, this line was crossed to UAS-HID as a control. This gene promotes cell death, and so as shown in Figure 5.18G, this cross resulted in a severe loss of ommatidia in the eye, validating the driver line. In the eye disc, there is a wave of differentiation called the morphogenetic furrow, which moves anteriorly from the posterior edge. Therefore, the anterior region is where cells are proliferating, and the posterior region behind the furrow is where cells are differentiating to form ommatidia (Fan & Bergmann, 2008). *Eyeless-Gal4* is expressed in proliferating cells and *GMR-Gal4* is expressed in mature differentiating cells. Thus, this eye

phenotype assay indicates that *Dpcd* is required for the promotion of cell proliferation in dividing cells before the onset of differentiation.

Eyeless-Gal4 driver



GMR-Gal4 driver

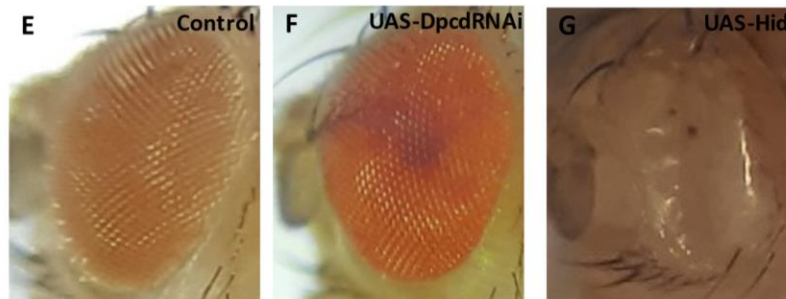


Figure 5.18. Eye phenotype assay supports a tissue growth defect in *Dpcd*-/- (A, B) Controls show eye area unaffected when crossed to Ey-Gal4 driver line (C) Eye area is reduced when the function of *Dpcd* is knocked down using Ey-Gal4. Not representative of phenotype, as few flies eclose. (D) *Dpcd* knockdown fly pulled from pupal case which failed to eclose. Phenotype is much more severe with head structures severely deformed. (E, F) When *Dpcd* function is knocked down using GMRGal4, there is no severe phenotype, and flies appear similar to controls. (G) To show that the GMR gal4 line is functioning as expected, this was crossed to UAS-HID (which causes cell death), which resulted in a loss of ommatidia. Number of flies imaged: A ($n = 16$), B ($n = 13$), C ($n = 5$), D ($n = 4$), E ($n = 15$), F ($n = 15$) G ($n = 5$)

5.4 Discussion

5.4.1 *Dpcd* function in *Drosophila*: dynein assembly

When the *Dpcd* gene was non-functional in mice, it resulted in classic PCD phenotypes including *situs inversus* and male infertility. TEM indicated that ciliary motility resulted from inner dynein arms (Kobayashi et al., 2002; M. Zariwala et al., 2004). This research project found that the motile cilia role of *Dpcd* is conserved in a *Drosophila* model. RNA *in situ* hybridisation indicated that the *Drosophila Dpcd* homologue is expressed in the developing chordotonal neurons of the embryo, a motile ciliated cell type. When the function of *Dpcd* was knocked down using the UAS/Gal4 system and RNAi technology, motile cilia defects were observed. *Dpcd* knockdown flies had a reduced climbing ability, and males produced no progeny due to a lack of mature individualised motile sperm. Axonemal dynein motors, which facilitate ciliary motility, also have an indirect secondary role in spermatogenesis. These structures hold the axoneme together until sperm individualisation occurs, and so when the dynein arms are absent, the axoneme can split and collapse - preventing the development of individualised sperm (zur Lage et al., 2021).

TEM analysis and dynein localisation assessment indicated a loss of ODAs and IDAs in a *Drosophila Dpcd* knockdown. This is a more severe phenotype than that described in the mouse model, which suggested an IDA-specific role for *Dpcd*. However, in the original mouse study, which proposed the IDA defect was caused by the *Poll* gene, the first exon of *Dpcd* was removed as well as the initial ATG start site, causing the observed phenotypes (Kobayashi et al., 2002; Zariwala et al., 2004). There may be a variation of *Dpcd* function between organisms, or as the *Drosophila* study is a complete knockdown of function, perhaps this is more representative of the role of *Dpcd* in axonemal dynein assembly. Label-free MS indicated a reduction of dynein motor complexes in a *Dpcd* knockdown, with ODA and IDA heavy chains particularly affected - subunits that are essential for the motor function of dynein

complexes. Similar results have been described in the literature for the DNAAFs Wdr92 and Zmynd10 (Mali et al., 2018; zur Lage et al., 2018). Moreover, similar results were obtained in Chapter 3 when assessing dynein subunits affected in a *Dyx1c1* knockdown.

A mVenus fusion protein generated in this project indicated that *Dpcd* is expressed in the cytoplasm of motile ciliated cells - where dynein preassembly occurs. Therefore, the results of this project support a role for *Dpcd* as a novel DNAAF, required for both outer and inner dynein arm assembly. This is further supported by the similar results obtained when investigating the putative motile cilia role for *Dpcd* to those obtained when further characterising the function of known DNAAFs *Dyx1c1* and *Pih1d3* in Chapters 3 and 4. DNAAFs such as *DYX1C1* and *PIH1D3* have been identified as PCD-causative genes previously, as well as *HEATR2*, *ZMYND10* and *TTC12* (Horani et al., 2012; Thomas et al., 2020; Zariwala et al., 2013). However, no PCD-causing human mutations in *DPCD* have been identified yet. As the research in this project proposes additional essential functions for *Dpcd*, perhaps mutations in the human gene may not be compatible with life and are therefore not present in the human PCD patients.

5.4.2 *Dpcd* function in *Drosophila*: regulation of tissue growth

In chapter 3 and 4, mVenus fusion proteins fully rescued or partially rescued null mutations of the DNAAFs *Dyx1c1* and *Pih1d3*. The original *DpcdmV* fusion protein supported expression in motile cilia cells (like that observed for the other mVenus proteins used in this project) but did not rescue the lethality. After that, the gene annotation was rechecked, and it was recognised that further upstream of the gene might need to be included. A second mVenus construct was designed, which included an additional 100 base pairs, meaning this construct ended close to the neighbouring gene, *CG13887*. The longer construct successfully rescued function and resulted in viable, healthy adult flies. Therefore, in the 100 bp upstream of the original construct, there are important binding sites for unknown regulators crucial to this wider *Dpcd* role.

To determine what the additional expression of *Dpcd* is, expression data from FlyAtlas and modENCODE databases was re-examined which suggested high expression in imaginal discs and the CNS. This wider expression was verified using the longer mVenus fusion construct, where *Dpcd* was found to be expressed generally in the imaginal discs and in the neuroblasts of the larval CNS, cells which are required for the development of the adult brain (neurogenesis). This additional expression is not expected to be observed for the shorter mVenus construct, as this line did not rescue the lethality caused by the wider role of *Dpcd*.

Overall, the results from the generation of the two mVenus lines suggest that there are alternative promoters for *Dpcd*, resulting in two isoforms with alternative transcription and translation that are required in different cell types: the short transcript is required for dynein assembly and the longer transcript is required for the wider role. Interestingly human *DPCD* cDNA sequences predict 13 spliced variants with 3 potential alternative promoters, suggesting this complex regulation of function is conserved.

To assess whether *Dpcd* has a role during neurogenesis, preliminary staining was conducted on the CNS of *Dpcd*^{-/-} larvae using DAPI and *asense* (*ase*). This staining indicated that the brain lobes of the *Dpcd* mutant were smaller than that of the control due to a reduced optic lobe. The outer and inner proliferating centres of the optic lobe are reduced in a *Dpcd* knockout, suggesting a defect in cell proliferation. The *ase* staining was inconclusive, and it was challenging to determine whether neuroblasts were affected in comparison to control.

There are two different types of neuroblasts. Type- I asymmetrically divides, producing one clone and one ganglion mother cell (GMC) (Doe, 2008). Type II also asymmetrically divides but produces an intermediate neural progenitor (INP) cell, which also asymmetrically divides 4-8 more times, producing a GMC at each division (Boone & Doe, 2008). GMCs divide one more time, with each one producing two mature neurons for the adult nervous system (Lai & Doe, 2014).

Asense is specifically expressed in type-I neuroblasts, however *Deadpan* (*Dpn*) is in both type-I and type-II neuroblasts, and so using an antibody to stain for the *Dpn* transcription factor will give insight into how all neuroblasts are affected in a *Dpcd* knockout. *Asense* is expressed in cells towards the end of their divisions and so later in neurogenesis. *Deadpan* is expressed in areas of the optic lobe with high levels of cell division (Wallace et al., 2000). Therefore, anti-*Dpn* will be a useful progenitor marker to use in future experiments to provide insight into *Dpcd* function and whether cell proliferation and ganglion mother cells (GMCs) and clones are affected in a *Dpcd* knockout. At the third-instar larval stage, photoreceptor neurons begin to project in the optic lobe (Néric & Desplan, 2016). Therefore, assessing how differentiated mature neurons are affected in a *Dpcd* null mutant, *anti-elav* which is present in all neurons will also be used as a counterstain in this future work.

This potential role in cell proliferation/ tissue growth is further supported by the results of the eye phenotype assay, which also ruled out a potential role in apoptosis. The tissue growth defect was so severe that the head structure of the fly did not develop fully (the eye disc forms part of the head capsule), resulting in a lack of eclosion.

Collaborators at the University of Toronto have found that through the use of siRNA technology in Retinal Pigment Epithelium (RPE) primary cilia, *Dpcd* has a potential role in ciliogenesis, as well as an essential role in AKT signalling (Personal communication). In *Drosophila*, HRP staining in larval and pupal chordotonal neurons, as well as TEM analysis, suggest that a *Dpcd* knockdown does not result in a ciliogenesis defect. This proposed ciliogenesis role for *Dpcd* in RPE cells may be an indirect effect of a role in tissue growth.

The late lethal phenotype observed in a *Dpcd* knockout is similar to that described previously in a screen conducted by the Gatti group. These late lethal phenotypes are typically observed in mutants for genes involved in maintaining mitotic chromosome integrity, chromosomal segregation, spindle formation or cytokinesis. In this screening, when dissected, late lethals had poorly developed imaginal discs, like those observed in the *Dpcd* knockout

(Gatti & Baker, 1989). These knockouts had severely reduced imaginal disc size at the third-instar larval stage. *Distal-less* expression in *Dpcd*^{-/-} embryos indicated that these structures were present earlier in development, and so cell proliferation and tissue growth was disrupted, resulting in the imaginal discs appearing absent in larvae. *Dpcd* mutants can most likely survive until the larval-pupal transition due to maternal contribution. Metamorphosis requires the zygotic genome, suggesting *Dpcd* mutants can get through the initial developmental stages until the imaginal cells (adult progenitors) are required to replace the larval cells (Gatti & Goldberg, 1991).

Therefore, *Dpcd* may have an essential role in cell proliferation, which may be specifically linked to the regulation of AKT signalling that was observed by our collaborators (Personal communication).

5.4.3 Potential molecular mechanisms involving *Dpcd*

Many DNAAFs, such as ZMYND10, WDR92, HEATR2, TTC12 contain protein domains that are important for mediating protein-protein interactions (Horani et al., 2012; Thomas et al., 2020; Zariwala et al., 2013; zur Lage et al., 2018). Interestingly, *DPCD* is predicted to have p23/sHsp homology, which is a co-chaperone of HSP90 that is also required for the regulation of protein-protein interactions (Garcia-Ranea et al., 2002). The human DPCD protein has been shown to bind to Reptin and Pontin of the R2TP complex (Dafinger et al., 2018; Zhao et al., 2013). This is a result supported by our collaborators, where they have indicated that *Dpcd* binds to Pontin directly (unpublished data). co-IP experiments conducted by the Jarman lab in parallel to this project indicate that through interactions with Reptin and Pontin, *Dpcd* can bind to the R2TP complex and Wdr92, a DNAAF previously characterised by the lab (zur Lage et al., 2018). As Pontin and Reptin are also a part of R2TP-like complexes with roles during axonemal dynein assembly, such a R2DP₃ discussed in Chapter 4, it is likely that *Dpcd* can bind to these complexes also. As *Dpcd* has a role as a DNAAF, as well as a wider role in cell proliferation, potentially in the regulation of PIKK signalling through an interaction with Akt, it could carry out these functions as part of different complexes with Pontin and Reptin. For

instance, the essential role of *Dpcd* in cell proliferation could be via the R2TP complex, and its DNAAF role could be via R2TP-like complexes such as R2DP₃. Pontin and Reptin also work as part of many complexes in many essential cellular functions, and the *Dpcd* interaction with these AAA+ proteins could be independent of R2TP and R2TP-like complexes.

The GFP-Trap and co-IP data described in this project also propose an interaction between *Dpcd* and *Heatr2*. This is a heat-repeat containing protein, which is a protein domain that commonly functions as a scaffold aiding the stability of protein interactions and cellular processes (Horani et al., 2018). *Heatr2* was characterised by the lab in 2013 as a DNAAF, however *Heatr2* homozygous mutants exhibit a late larval lethality suggesting an additional unknown function for this gene. The cellular role of HEATR proteins can vary, and several proteins from this family have been linked to cell proliferation. For example, the PIKK family of proteins are heat-repeat containing proteins, and the related protein, *Heatr1*, has recently been shown to interact with Pontin and Reptin to facilitate the activation of mTOR during cell proliferation, by acting as a scaffold for the dephosphorylation of AKT (Nakamura et al., 2021). Therefore, this additional function for *Heatr2* could be as part of a complex with *Dpcd*, in the regulation of cell proliferation. Immunofluorescent staining conducted in this project indicated that the expression pattern of *Heatr2* in the neuroblasts is different to that of *Dpcd*. Therefore, these genes may carry out their functions at different stages during metamorphosis, acting at different stages of neurogenesis. The use of neuronal markers on *Dpcd* and *Heatr2* null mutants will provide further insight into the specific roles of these genes during cell proliferation, and whether they work together or independently in their additional widespread functions. Overall, the GFP-Trap and co-IP experiments in this project have identified several novel protein complexes which have potential roles in dynein assembly and cell proliferation. The GFP-Trap analysis detected an additional putative protein interactor of *Dpcd*: *mlp60A*, a protein which is involved in the development and maintenance of *Drosophila* flight muscles. A recent preprint discussed that there are two isoforms of this protein, with functionally distinct roles: the short isoform, expressed

ubiquitously during development, having a role in larval body wall muscle formation and the longer isoform being required for adult flight function (Wishard et al., 2021). While this protein interaction seems unlikely to be linked to the dynein assembly role of *Dpcd*, it may be worthwhile to follow this up in future studies to assess whether it is a valid protein interaction and whether it can shed light on the complex role of *Dpcd*.

The R2TP complex (as well as 'R2' on its own) has been associated with several cell proliferation pathways, one of which is the Pi3K/AKT/mTOR signalling pathway (von Morgen et al., 2015). When activated, AKT is linked to downstream processes including cell survival, growth and proliferation through the phosphorylation of intracellular proteins (Liu et al., 2014). As our results suggest *Dpcd* may be involved in cell proliferation, and AKT is important to this process, AKT signalling (as proposed by our collaborators) may be mediated by *Dpcd* in *Drosophila*. If *Dpcd* is involved in regulating the Pi3K/AKT/mTOR pathway, which is known to be crucial to cell growth and survival in both health and disease conditions (e.g. cancers) it may be a putative chemotherapeutic target (Porta et al., 2014).

In this project it is proposed that the known DNAAF *Heatr2* also has a potential role outside of dynein assembly in the regulation of cell proliferation. This additional function could be together with *Dpcd*, possibly in the regulation of the Pi3K/AKT/mTOR pathway. Our current proposed models are shown below in Figure 5.19.

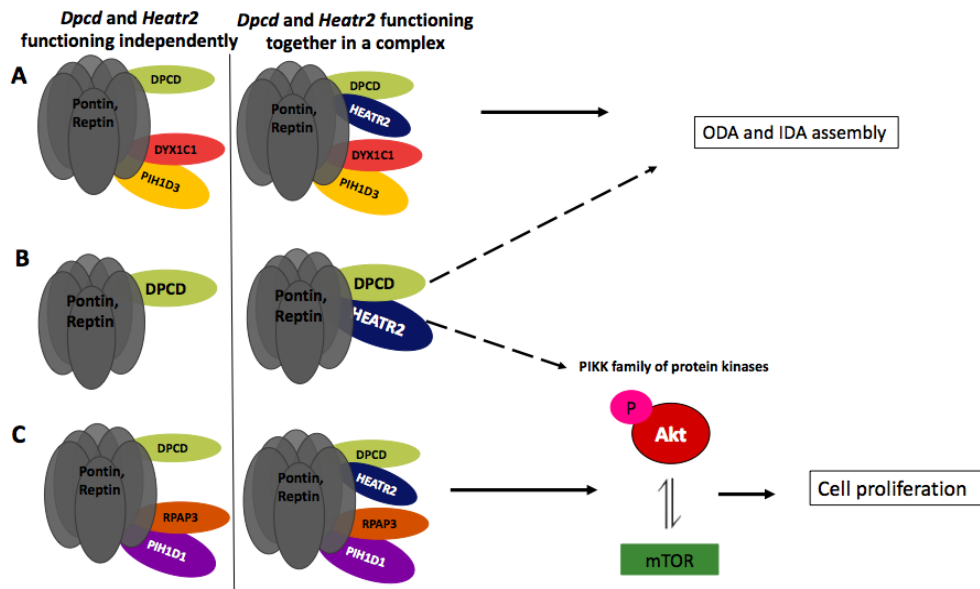


Figure 5.19 Potential protein complexes Dpcc carries out its different functions within. Dpcc interacts with Pontin and Reptin (as well as Heatr2) and has been shown to bind to the R2TP complex, which is involved in dynein assembly and regulation of the PIKK family of proteins. Therefore, Dpcc may work with the R2TP complex (independently or with Heatr2) for the essential cellular role in cell proliferation (C). As Pontin and Reptin are a part of R2TP-like complexes, Dpcc is likely to also bind to these complexes, such as R2DP3 discussed in Chapter 4, during dynein assembly (A). As Pontin and Reptin have cellular roles independent of the R2TP and R2TP-like complexes, Dpcc could be functioning with these AAA+ proteins on their own (B).

As *Dpcc* (and *Heatr2*) binds to Reptin and Pontin, this cell proliferation role may be part of the R2TP complex, or the R2TP-TTT super complex, which regulates several PIKK proteins such as mTOR (Mao & Houry, 2017). *Morgana* (*Mora*) is a CHORD-containing protein proposed to function as an *Hsp90* co-chaperone. *Mora* mutants have a late larval lethality, like that observed in *Dpcc* mutants. *Mora* protein is also co-purified with the R2TP-TTT super complex. Therefore, *Dpcc* may have a similar role to this co-chaperone (Ferretti et al., 2010). *Wacky* (*WAC*) is also an interactor of Pontin/Reptin and associated with the R2TP-TTT complex. This gene has been linked to cellular growth and the regulation of mTOR activity. To assess gene functions, whole eye clones were imaged to observe the phenotype (David-Morrison et al., 2016). The eye assay used in this project could be utilised similarly to assess whether *Dpcc* is linked to the activity of the Pi3K/AKT/mTOR pathway. In this project, the eye assay confirmed a tissue growth role for *Dpcc* as opposed to

a cell death role. Fly lines are currently being generated by the lab to use this eye phenotype assay to investigate interactions with candidate proteins (mTOR, Akt1 etc). It will be interesting to assess whether overexpression of Akt1 rescues the severe eye phenotypes observed when *Dpcd* function is knocked down using *eyelessGal4*. Fly lines can also be obtained to assess whether other cell signalling pathways like Wnt and Hippo are affected in a *Dpcd* knockdown. Future work using this eye phenotype assay will confirm interactions between *Dpcd* and cell signalling pathways associated with cell proliferation. One caveat is that the eye phenotype of a *Dpcd* knockdown is so severe that very few flies eclosed. Therefore, it will be difficult to measure synergistic effects caused by knockdowns of *Dpcd* and changes to components of the cell signalling pathways. The use of clones may be beneficial in observing harmful phenotypes while producing viable flies. *Eyeless*, which is expressed in the eye-antennal disc, regulates the development of the eye and the head structures, and so severe disruption to its regulation results in lethality. Specifically altering the wing discs, and the structures which will become the adult wing may not result in as severe a defect. Thus, using a wing disc-specific driver (such as *Pdm2*) instead of a driver in the eye disc for these genetic crosses may result in more viable adults eclosing and the ability to assess more severe phenotypes.

The lab is also currently assessing whether a *Dpcd* null mutant affects the expression and phosphorylation level of the Pi3K/AKT/mTOR pathway. Western Blot analysis is being conducted on the larval CNS, to assess whether there is an increase or decrease to phospho-Akt1 levels in a *Dpcd*^{-/-} background. Alterations to the expression level of this protein would be further support of our current hypothesis regarding the wider role of *Dpcd*.

5.5 Conclusion

A mouse study proposed Deleted in PCD (DPCD) as a novel motile cilia gene with an effect on inner dynein arms, however the specific function of the gene remained unknown. When this gene was non-functional it resulted in PCD-like phenotypes in mice, however no human PCD patients with mutations in this gene have been identified to date. By studying the *Drosophila* Dpcd homologue, further insight into the function of this gene has been gained. Dpcd is expressed in motile cilia cells, specifically in the cytoplasm where axonemal dynein preassembly occurs. When the Dpcd function is knocked down, it results in immotile cilia due to defective dynein arm assembly. Similar results were observed for knockdowns of dynein axonemal assembly factors (DNAAFs). Dpcd was found to directly interact with the characterised DNAAF Heatr2 and Pontin and Reptin of the R2TP complex (which has also been associated with dynein assembly). Therefore, this project proposes Dpcd as a novel DNAAF, with a role in both outer and inner dynein assembly. This project also proposes a novel complex with a potential role during dynein assembly composed of Dpcd, Heatr2, Pontin and Reptin.

When generating a null mutant to further investigate the motile cilia role of Dpcd, an unexpected phenotype was observed: late larval lethality. This phenotype is indicative of an additional general role for Dpcd. The late larval lethality observed in this project is similar to that described by the Gatti group in their classical screen for genes involved in the cell-cycle. Further investigation of the lethality suggests a role for Dpcd in the regulation of cell proliferation. Imaginal discs are poorly developed in a null mutant and eye growth is severely disrupted when *Dpcd* function is knocked down in the developing eye. Heatr2, the DNAAF found to interact directly with Dpcd, also exhibits a late larval lethality and expression data suggests that this gene may also have an additional function outer dynein assembly. Both DNAAFs are expressed in the imaginal discs and the neuroblasts of the CNS, cells which proliferate during morphogenesis. Neuronal markers will be used to stain the

CNS to gain further insight into these genes' specific functions in cell proliferation. It is most likely that these genes work with Pontin and Reptin to regulate cell signalling pathways associated with this cellular process, such as the Pi3K/AKT/mTOR pathway.

Chapter 6

General Discussion

6.1 Summary of key results

One aim of this PhD project was to use the *Drosophila* model to pinpoint the specific role of a known R2TP-like complex involving the DNAAFs *Dyx1c1* and *Pih1d3*. Chapters 3 and 4 indicated that both *Dyx1c1* and *Pih1d3* have conserved dynein assembly functions (despite the truncation of *Dyx1c1* which lacks TPR repeats). co-IP analysis has shown that their R2TP-like interaction is also conserved in *Drosophila*, and so overall this project proposes that this protein complex has a role in the assembly of ODAs and a subset of IDAs.

Additionally, this study aimed to characterise two genes, *Wdr16* and *Dpcd*, that were detected by the lab as having potential roles in motile cilia function.

At the onset of this PhD project, it was hypothesised that *Wdr16* was a novel DNAAF. RNA *in situ* hybridisation in *Drosophila* embryos indicated that *Wdr16* is expressed in developing chordotonal neurons, and male fertility assays demonstrated that *Wdr16* is required for sperm motility - both results supporting a role in motile cilia. However, since the beginning of this study, it has been reported that *Wdr16* is actually a microtubule inner protein (MIP), which are a novel group of proteins required for axonemal maintenance and stability and are therefore important for motile cilia function (Ma et al., 2019; Owa et al., 2019). The results in Chapter 2 support this role for *Drosophila Wdr16* in the stability of the axoneme required for flagellar motility (most likely as a MIP). As this project focuses specifically on axonemal dynein assembly, the characterisation of this gene in *Drosophila* was stopped at this point to focus on another gene with a putative role in dynein assembly: *Dpcd*.

At the onset of this project, it was also hypothesised that *Dpcd* was a novel DNAAF, as its specific function was unknown. Chapter 5 indicates that *Dpcd* is indeed a DNAAF, with a role in both outer and inner dynein assembly. GFP

Trap AP-MS and co-IP experiments revealed *Dpcd* to form part of a novel complex, interacting with Pontin and Reptin of the R2TP complex as well as the known DNAAF Heatr2, and so may work with these proteins to carry out its role in dynein assembly. This project also highlighted an additional, essential function for *Dpcd* in cell proliferation, and proposes that it works with Pontin and Reptin (and potentially Heatr2) to regulate signalling pathways such as Pik3/AKT/mTOR, which are required for this important cellular process.

Overall this project highlights how proteins can be required for motile cilia function in different ways, summarised in the figure below.

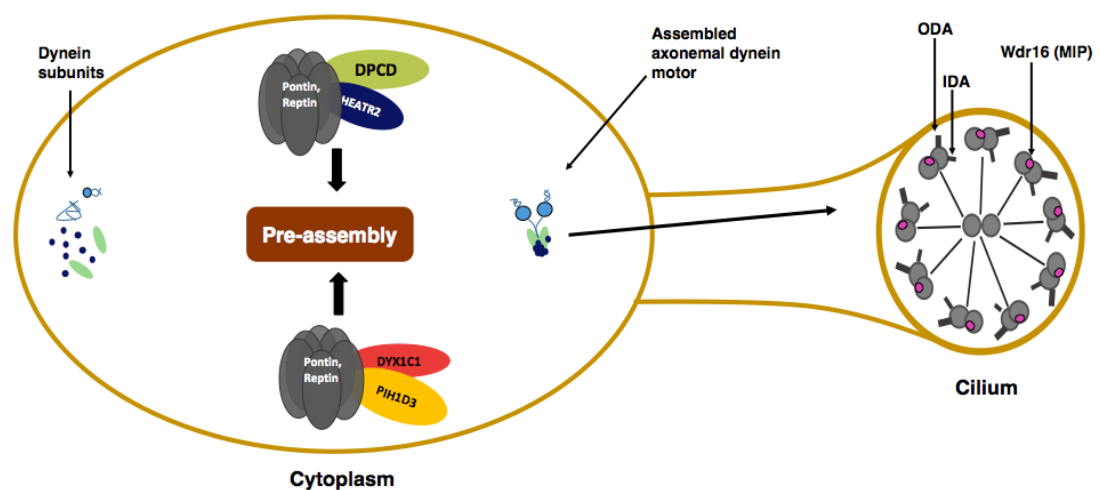


Figure 6.1 Schematic illustrating the individual roles of the genes of interest to this project in regard to motile cilia. *Dyx1c1* and *Pih1d3* (Chapters 3 and 4) were found to have a conserved R2TP-like interaction, as well as conserved roles in the assembly of outer and inner dynein arms. *Dpcd* (Chapter 5) was also found to have a role in dynein arm assembly, and a novel interaction with the known DNAAF Heatr2, as well as the AAA+ proteins Pontin and Reptin was identified. *Wdr16* (Chapter 2) was hypothesised to be a novel dynein assembly factor at the onset of this project. Since then, it has been published that this WD-repeat protein is a MIP, which is important for axonemal stability.

6.2 *Drosophila* as a model for studying PCD

As discussed in the Introduction, obtaining a PCD diagnosis is difficult, and the disorder is much more prevalent than initially thought (Mitchison & Smedley, 2022). While there are to date over 50 genes associated with PCD, many more remain unknown. Identifying and characterising candidate PCD genes will aid diagnosis, as patients can be screened for mutations in these genes. As discussed throughout this thesis, *Drosophila* has been a useful model for providing information that is translatable to humans and has aided the characterisation of several PCD genes, including *Zmynd10* and *Heatr2* (Diggle et al., 2014; Moore et al., 2013). *Drosophila* has also been useful in the characterising novel DNAAFs, *Wdr92* (zur Lage et al., 2018) and *Dpcd* (Chapter 5), in which no human PCD mutations have been identified to date but may aid diagnoses in the future.

As so many genes are associated with PCD, it is considered a heterogeneous disease. Therefore, digenic mutations can occur in PCD patients (Horani et al., 2016). For example, patients with mutations in *DNAH6* and either *DNAI1* or *DNAH5* have been reported (Li et al., 2016). However not much is known about digenic mutations in relation to PCD, as these interactions are difficult to study *in vivo*. *Drosophila* has been previously shown to be a useful model to study genetic interactions *in vivo*, combining heterozygous mutations of genes and assaying for synergistic phenotypes (de la Pompa, 1994; Shearn et al., 1987). Therefore, *Drosophila* could be used as a model system for further understanding these multigenic interactions. As discussed in Chapter 4, future work of this study includes optimising a sperm competition assay to examine DNAAF interactions *in vivo*. This assay would be a more sensitive interaction assay than just assessing straightforward fertility, so could be utilised to further investigate digenic mutations identified in PCD patients which may cause subtle symptoms, therefore contributing to the knowledge that could be beneficial for potential therapeutics.

As discussed in the Introduction, therapeutics for PCD include those which treat already existing symptoms such as airway clearance therapy, antibiotics for infections and inhalation corticosteroids (Paff et al., 2021). PCD treatments currently under investigation include those which target specific genetic mutations as opposed to symptoms. Chapter 3 discusses *Dyx1c1*, a TPR-containing protein which has been associated with several biological processes in higher organisms: mainly dynein assembly, neuronal migration and dyslexia (Taipale et al., 2003; Tarkar et al., 2013; Wang et al., 2006). This project found that the dynein assembly function of *Dyx1c1* is conserved in *Drosophila* and did not identify any evidence to support additional functions in the CNS. As *Dyx1c1* is truncated in *Drosophila*, and lacking the TPR repeats, it is possible that this protein domain is not required for the dynein assembly role. PCD-associated mutations in *Dyx1c1* are most often found in the region containing the TPR repeats, truncating the protein. If these repeats are not essential for the DNAAF function, perhaps PCD is caused by this truncated protein being degraded (at RNA level) through nonsense-mediated decay (NMD). Treatments which inhibit NMD and allow functional truncated proteins to be translated are under investigation for other conditions such as Cystic Fibrosis (de Poel et al., 2021). If human *DYX1C1* is like the *Drosophila* homologue and the truncated protein is sufficient, perhaps this area could be a therapeutic target for PCD caused by mutations in this gene. PCD is a cilia-specific protein-misfolding condition (Mali et al., 2018), one potential therapeutic is chaperone therapy. This approach introduces chemical chaperones to aid protein folding. The addition of manufactured Hsp70 has been effective at preventing symptoms in a *Drosophila* model of Alzheimer Disease, and so perhaps could have a similar effect in a *Drosophila* model of PCD (Martín-Peña et al., 2018).

Overall, this project highlights that *Drosophila* has been and remains to be a useful model for contributions to PCD research by being a straight-forward system to obtain translatable information.

6.3 Non-dynein functions amongst DNAAFs

Some DNAAFs have been reported as having additional non-dynein functions including *SPAG1* and *WDR92*. *SPAG1*, which is well characterised as having a chaperone function, has several isoforms - one of which is widely expressed and while it remains uncharacterised, has been suggested to have a potential role in cell proliferation (Hu et al., 2017; Smith et al., 2022). In *Drosophila* however, *Spag1* is motile cilia specific. *Wdr92* is also exclusively expressed in motile cilia in *Drosophila*, but like *SPAG1*, *WDR92* is widely expressed in humans and mice and no human PCD mutations in this DNAAF have been identified to date (zur Lage et al., 2018). This expression indicates that some DNAAFs which have wider essential roles in mammals have restricted functions (specific to dynein assembly) in *Drosophila*, making it a straightforward model to understand that particular function of the gene in question. Other DNAAFs that were discussed in this thesis with potential wider roles are described below.

6.3.1 Dyx1c1

As discussed in Chapter 3 and briefly above, *Dyx1c1* has also been loosely reported to have a wider role outside dynein assembly in neuronal migration, and potentially as a causative dyslexia gene (Taipale et al., 2003; Wang et al., 2006). In this thesis, the *Drosophila* model was utilised to determine what specific functions of *Dyx1c1* are likely in humans. It was found to be specific to motile cilia in *Drosophila*, like *Spag1* and *Wdr92*, but in this case I suggest that the proposed wider role of mammalian *DYX1C1* is a secondary effect of its primary dynein assembly role.

6.3.2 DNAAF2

Drosophila DNAAFs are not always motile cilia specific. The *Drosophila* homologue of *Dnaaf2* that was mentioned in Chapter 4 is only moderately expressed in motile cilia cells and widely expressed in adults. RNA *in situ* hybridisation indicated general expression of *Dnaaf2* in the embryo,

suggesting it has additional cellular functions outside of dynein assembly (zur Lage et al., 2019). Similarly in mice, *Dnaaf2* homozygotes have been reported to be lethal due to a role during embryogenesis (Cheong et al., 2019). While *DNAAF2* was the first dynein assembly factor to be characterised, it is only associated with a small number of PCD patients, suggesting that some mutations in this gene may not be compatible with life, most likely due to a wider role (Omran et al., 2008).

6.3.3 *Heatr2*

As discussed in Chapter 5, as well as in the publication by Diggle et al., (2014), additional expression was observed in the mesoderm of *Drosophila* embryos when characterising the *DNAAF Heatr2*. This expression, in combination with the lack of viable homozygote adults in the P-element line, supports a wider role outside dynein assembly for this gene. Unlike *WDR92* (and *DPCD*), human *HEATR2* PCD mutations have been detected, although only a small number when compared to other PCD genes. One of these is a splice acceptor site mutation of the final exon, causing a frameshift in exon 13 that disrupts the last HEAT repeat and the C-terminus of the ARM-domain (Diggle et al., 2014). The other PCD-causing mutation in *HEATR2* which has been reported is a missense mutation in exon 12 (Horani et al., 2012). As human motile cilia cells have the highest expression levels of *HEATR2*, these may be the most sensitive to mutations, explaining the few PCD cases associated with this gene (Diggle et al., 2014).

6.3.4 *Dpcd*

When the *Drosophila Dpcd* null mutant was generated by CRISPR/Cas9-mediated homology directed repair in Chapter 5, it resulted in the absence of viable adult homozygous flies. This finding suggests a wider role for *Dpcd* in *Drosophila* and, if conserved, perhaps explains why no human mutations have been identified to date. The *Dpcd* mouse mutation described in the literature affected the first exon of *Dpcd* and the predicted ATG start site (Kobayashi et al., 2002; Zariwala et al., 2004). It is not clear why the mouse mutation only causes PCD symptoms. As there are two *Dpcd* isoforms this mutation might

specifically disrupt the regulation of the DNAAF function of *Dpcd* (short transcript) resulting in PCD-like phenotypes, but not the lethality observed in the *Drosophila* null (longer transcript). This could also be due to the presence of a potential cryptic start site, which would provide an alternative transcription start point activated by changes to the nucleotide sequence and result in the transcription and translation of a peptide (Colin et al., 2011).

This project proposes that as well as a motile cilia role, *Dpcd* has a function in regulation of cell proliferation in tissue growth, most likely through the regulation of a cell signalling pathway such as Pik3/AKT/mTOR. Ciliary genes have been associated with important cell signalling pathways previously as the primary cilia are essential hubs for the signal transduction of the WNT and Hh pathways, which are also required for regulating cell proliferation (Berbari et al., 2009). Collaborators have proposed that *Dpcd* has a function in ciliogenesis (Personal communication). As mentioned in the Introduction, cell division and ciliogenesis are tightly linked, and when one process is disrupted, it can affect the other (Avasthi & Marshall, 2012). This project found no evidence to support a ciliogenesis role for *Dpcd* in *Drosophila* and suggests that the phenotype is an indirect effect of its role in cell cycle/proliferation. Comparing *Dpcd* to the other DNAAFs highlights that additional functions outside dynein assembly do exist for these genes in *Drosophila* as well as higher organisms, and that these additional functions can be quite variable.

6.4 Future directions

6.4.1 Examining the dynamics of axonemal dynein motor Assembly

As discussed in the Introduction, it is well-established that dynein preassembly takes place in the cytoplasm of the cell, but the specific details of this process remain unclear. It has recently been suggested that DNAAFs work with chaperones to facilitate assembly in specialised liquid-like organelles called DynAPs (Huizar et al., 2018). Building upon what knowledge has been gained in this project regarding R2TP-like complexes, future directions could include

assessing the dynein assembly process in high resolution in the *Drosophila* model, to identify whether these complexes carry out their functions in a ‘chaperone relay’, as has been proposed in the literature (Mali et al., 2018).

Snap-tagging proteins of interest (such as Dyx1c1, Pih1d3, Dpcd, Dnah5, Dnali1) has been shown to be effective in the *Drosophila* system and would allow for *in vivo* visualisation of protein localisation at high resolution (Erdmann et al., 2019). Organic fluorophores such as these SNAP-tags have been shown to be more specific in live imaging, as they yield more photons and provide a strong stable fluorescent signal (Erdmann et al., 2019). To visualise the dynamics, live-cell stimulated emission depletion (STED) microscopy has been shown to be successful in the study of the molecular arrangement of primary cilia, which like motile cilia, are below the diffraction of light (Yang et al., 2016). Moreover, super-resolution time-lapse live imaging has already been performed to visualise subcellular structures in *Drosophila* testes and could be optimised in the future to investigate dynein assembly in this tissue. This result was achieved using Airyscan super-resolution imaging, which can visualise 140 nm (Ranjan & Chen, 2021). The other ciliated cell type (Ch neuron) would be more difficult to visualise at high resolution, as it is surrounded by a thick cuticle. However, a protocol (FlyClear) has been developed which facilitates high resolution imaging of the *Drosophila* nervous system (on fixed tissue). This approach showed clear staining of the pupal and adult antennae, as well as the antennal lobe (Pende et al., 2018), and may be optimised in the future to observe specifically the chordotonal neurons of the JO.

Using high-resolution microscopy, dynamics between DNAAFs of interest and the dynein subunits would provide insight into the assembly process and how the dynein chains are assembled into motor complexes, as well as confirming the presence of DynAPs, and whether they act as hubs for R2TP-like interactions to occur in.

6.4.2 Investigation of DNAAF interactions: Proximity labelling

One of the aims of this project was to investigate whether a particular R2TP-like interaction, between the DNAAFs Dyx1c1 and Pih1d3, is conserved in *Drosophila*. In Chapter 3, GFP-Trap AP-MS was conducted to examine protein interactors of Dyx1c1 *in vivo*. However, this process was problematic, as not a lot of significant motile cilia protein hits were obtained. This result was due to many proteins sticking in the negative control (non-specific interactions), which reduced the number of hits in the Dyx1c1mV pull-down. co-IP experiments were also conducted in S2 cells; however, this type of analysis only confirms direct protein interactions. Therefore, another method is required in future work to detect the weak/transient protein-protein interactions missed by AP-MS and co-IP (Gingras et al., 2019)

Proximity-labelling is a method where a biotin ligase, such as TurboID, is fused to a protein of interest (hypothetically Dyx1c1). When biotin is added, proteins which interact with this protein of interest are then biotinylated (meaning that they are tagged). These biotinylated proteins are then gathered by streptavidin-based affinity purification and analysed through mass spectrometry. This technique has been validated in a *Drosophila* model, where biotin was introduced through the fly food (Branon et al., 2018).

In future experiments, this approach could be utilised to detect interactions between the DNAAFs proposed to be involved in R2TP-like complexes. It may also be useful for aiding the assessment of whether Spag1 (which contains TPR repeats) works in a complex with the truncated Dyx1c1 in *Drosophila*, as discussed in Chapter 3.

6.4.3 Viewing the 3D ultrastructure of the axoneme

As discussed in Chapter 2, *Wdr16* has recently been characterised as a MIP. MIPs can vary between organisms regarding their molecular architecture (Kirima & Oiwa, 2018), and so future work could include assessing the function of *Wdr16* as a MIP in *Drosophila* in more in-depth detail. Cryo-ET has been useful in providing high-resolution images of the ciliary ultrastructure in other

organisms including *Chlamydomonas*, *Tetrahymena* and sea urchins (Ichikawa et al., 2019; Nicastro et al., 2006; Owa et al., 2019). Therefore, future directions of this project could include generating a *Drosophila Wdr16* null mutant and performing Cryo-ET on motile cilia of this fly line. Cryo-ET would be a difficult technique to perform on chordotonal neurons due to the surrounding thick cuticle, and so has not been successfully reported to date. Similarly, Cryo-ET of *Drosophila* sperm flagella has not been reported. However due to the success of this technique on sperm flagella of mammals (Leung et al., 2021), it is predicted to be achievable. Cryo-ET has been performed on the centriole in *Drosophila* S2 cells, which visualised MIPs along the axoneme required for microtubule stability (Greenan et al., 2018). Therefore, conducting Cryo-ET analysis on *Drosophila* sperm flagella may provide insight into how the axonemal structure is affected in this null mutant, illuminating the specific function of the MIP *Wdr16* in *Drosophila*. As discussed in Chapter 4, the specific function of the R2TP-like complex involving *Dyx1c1* and *Pih1d3* remains unknown, and this project proposes that in *Drosophila* it has a role in the assembly of ODAs and IDA subset c. Cryo-ET may be a useful technique in providing enough resolution to confirm this prediction by examining how the ciliary ultrastructure is affected when this R2TP-like complex is disrupted.

6.5 Conclusion

Overall, this project concludes that the motile cilia roles for *Wdr16*, *Dyx1c1*, *Pih1d3* and *Dpcd* are conserved in *Drosophila*. *Wdr16* has been identified as a novel MIP, a function which is predicted to be conserved in *Drosophila*. *Dyx1c1* and *Pih1d3* form an R2TP-like complex, required for the assembly of ODAs and a subset of IDAs. *Dpcd* is a novel DNAAF, and has a wider role linked to cell proliferation, most likely through the regulation of the *Pik3/AKT/mTOR* cell signalling pathway. A novel protein complex has also been identified involving *Dpcd*, *Pontin*, *Reptin* and *Heatr2*, a known DNAAF which this project proposes to also have a wider role in regulating cell

proliferation. Dpcd most likely interacts with these proteins for one or both recently identified functions, highlighting R2TP-like interactions are not necessarily dynein-specific.

Chapter 7

Materials and Methods

7.1 Fly husbandry

Fly stocks were kept at 18°C or 21°C in vials containing cornmeal-agar and were tipped into fresh vials every 4-6 weeks. Genetic crosses for experimentation were stored at 25°C unless stated otherwise. When required, *Drosophila* embryos were collected on red wine agar plates.

7.2 Genomic DNA extraction

Fifty flies were gathered and frozen in lysis buffer (100mM Tris-HCl pH9, 100mM EDTA, 1% SDS). Once thawed and homogenised, additional lysis buffer was added, and samples were incubated at 70°C for 30 mins. Then, 150µl cold 8M KOAc was added, and samples were incubated on ice for 20 mins. Samples were centrifuged (14,000 rpm, 20 mins at 4°C) and supernatant was retained, 0.9 volumes of cold isopropanol were added to this and then centrifuged for 10 mins. Supernatant was discarded and the pellet was washed in 70% ethanol. Pellet was resuspended in 50µl TE (10mM Tris, 1mM EDTA) buffer. Equal part phenol-chloroform was added to the sample and briefly vortexed, followed by centrifugation (14,000 rpm, 10 mins). The upper phase of the supernatant was reserved and 5µl 3M NaOAc (pH5.5) and 250µl 100% ethanol were added. This mix was stored at -20°C overnight. Specimens were centrifuged (14,000 rpm, 10-15 minutes) and the pellet was retained, washed and resuspended in 100µl TE buffer.

7.3 Single fly DNA extraction

Flies were kept on ice, and individually homogenised with a pipette tip in a 0.5ml tube containing squishing buffer (10mM Tris-Cl pH 8.2, 1mM EDTA, 25mM NaCl, and 200µg/ml Proteinase K (Thermo Fisher, cat. EO0491)) for 10s. The squished flies were kept at 37°C for 25 mins and then 95°C for 5 mins (which inactivates the enzyme). Samples were then stored at 4°C until needed.

7.4 Polymerase chain reaction (PCR)

The reaction mix was prepared on ice (31.5µl dH₂O, 0.5µl Taq Polymerase (5U/ µl) (Sigma-Aldrich, cat. 4728874001), 5µl 10x buffer, 5 µl primer 1, 5 µl primer 2, 1µl dNTP (4728874001), 2µl DNA (100 ng/ µl). When 'single fly DNA' was used for PCR, the reaction mix was halved. PCR was conducted using a set program on a Techne thermal cycler resulting in a 30 cycle PCR (step 4 feeds back to step 2 x29):

1. 2 mins at 94°C
2. 30 secs at 94°C
3. 30 secs at 55°C (annealing temp sometimes increased to 57°C to eliminate background)
4. 1 min at 72°C (extension time = 1min/kb: for bigger fragments 90s-2min)
5. 10 mins at 72°C
6. hold at 4°C

All primers were generated by Sigma-Aldrich (Acquired by Merck) and were used at 10 µM concentration. Primer sequences are listed in the appendix.

7.5 Gel electrophoresis

Agarose gel (1%) was set using 0.5g agarose (Melford, cat. 9012-36-6) in 50ml 1xTAE (40mM Tris, 2mM EDTA, 20mM Acetic acid). After being heated in the microwave, Gel Red (Biotium, cat. 41003) was added (7µl/100ml dissolved agarose solution) and then mixture was left for 60 mins to solidify in a tray. The gel tray was placed in a gel tank and submerged in 1xTAE. 4µl GeneRuler 1 kb DNA ladder (ThermoScientific cat.SM0311) was added to the first gel well. 1.5µl PCR products were mixed with 10.5µl of 1x loading buffer (LB) (diluted from 6x LB stock: 30% glycerol (v/v), 0.25% bromophenol blue (w/v) and 0.25% xylene cyanol FF (w/v)) and samples were loaded into subsequent wells. Gels were run at 80V for 1 hour and imaged using a UV transilluminator (UVP).

7.6 Sanger sequencing

The sequencing reaction mix consisted of 500-600ng of DNA, 2µl 5X sequence buffer, 0.9µl Big Dye (Applied Biosystems cat. 4337454), 2µl relevant primer (at 1.6mM concentration) and was made up to 10µl with dH₂O. The reaction was conducted in a thermocycler under the following conditions.

1. 1 min at 96°C
2. 10 secs at 96°C
3. 5 secs at 50°C x25 cycles
4. 75 secs at 60°C
5. hold at 4°C

Samples were sent to Edinburgh Genomics, University of Edinburgh for processing and analysed using 4 Peaks software (RRID:SCR_000015) upon return.

7.7 Immunohistochemistry

7.7.1 Embryos

Late-stage embryos were collected over a 20–24-hour period at 25°C on red wine agar plates and rinsed to remove yeast. Embryos were then dechorionated in 50% bleach for 4 mins and rinsed. In a scintillation vial, embryos were fixed (3.25ml dH₂O, 3.75ml 1x PBS, 1.25ml 37% formaldehyde (Sigma-Aldrich cat. F1635), 5ml n-Heptane (Sigma-Aldrich cat. H2198) for 20 minutes. The bottom phase of the liquid was removed and 10ml of methanol was added and shaken for devitellinisation to occur. Embryos settled to the bottom of the vial and were removed to be washed twice in Methanol. Embryos were rinsed 3x in PBT (1x PBS, 0.3% Triton X-100 (Sigma-Aldrich cat. T-8787)) and then washed 3x in PBT. Blocking solution of 2% Bovine serum albumin (BSA, Roche, cat. 10735078001) was added to samples and incubated overnight at 4°C. Primary antibodies of interest were added to PBT and block solution and incubated overnight at 4°C. Primary antibodies were removed, and samples were rinsed 4x PBT and washed 3x PBT. Secondary antibodies were added and incubated for 2 hours at room temperature (RT). All secondary antibodies used in this thesis are Alexa fluorochrome conjugated Molecular Probes and were incubated under dark conditions. This incubation period was followed by 4x rinses in PBT and 3x washes in PBT. Samples were then mounted on slides in Vectashield (Vector labs, cat. H-1000) and coverslip was sealed with nail varnish.

7.7.2 Larval CNS and imaginal discs

Anterior half of the larva was turned inside out by rolling onto a forceps (like a sleeve) in a watch glass filled with 1x PBS. Excess tissue (e.g., fat bodies) was removed to allow the brain lobes and ventral nerve cord (VNC) to be separated from the mouth parts. Once settled to the bottom of an Eppendorf filled with PBS, the supernatant was removed and samples were fixed (900µl 1x PBT, 100µl 37% formaldehyde) for 20 minutes. Samples were rinsed x2 in PBT, washed x3 in PBT and incubated in 5% BSA for 30 mins. Primary antibodies

were added, and samples were left to incubate at 4°C. 4x rinses in PBT and 3x washes in PBT were conducted, followed by secondary antibody application for 2 hours at RT under dark conditions. This incubation was followed by 4x PBT rinses and 3x PBT washes. Samples were then arranged on a slide in Vectashield mounting medium, and the coverslip was sealed using nail varnish.

7.7.3 Larval pelts

Wandering third-instar larvae were dissected in a Sylgard dish containing 1x PBS with the ventral side facing up. Head and tail ends of the larva were pinned so that the animal was stretched. The ventral midline was longitudinally cut, and small horizontal incisions were made on either side of the pins. This resulted in 4 corners which were then tacked out, revealing the inner part of the larva. Internal organs were carefully removed with forceps. Samples were fixed in 4% formaldehyde for 25 mins followed by 5x 5 min washes in PBS. Fillets were unpinned and transferred to 1.5ml Eppendorf. 2x 15 min PBT washes were performed followed by a blocking step in 2% BSA for 40 minutes. Samples were incubated overnight at 4°C in primary antibody solutions. After 5 PBT washes, the secondary antibody solution was applied and incubated for 2 hours at RT in the dark. Larvae were washed 5x PBT. For mounting, the head and tail ends of animals were removed. The inner surface of the fillet was oriented to face upwards to the coverslip.

7.7.4 Pupal antennae

Late-stage pupae were pinned in a Sylgard dish containing 1xPBS. The outer membrane encompassing the head structure was removed. Pupae were fixed in 3.7% formaldehyde for 30 mins, rinsed 4x in PBT and then washed 3x in PBT. The pupal head was cut with scissors just below where the antennae are located. This tissue was placed in 3% BSA to block for a minimum of 2 hours. Primary antibody solution was added and incubated for two days at 4°C. After 5x10 min washes in PBT, the secondary antibody solution was added to the samples, and these were incubated overnight at 4°C. Samples were washed 5x PBT and then mounted on a slide in Vectashield.

7.7.5 Adult testes

Adult testes were dissected between 0-3 days post-eclosion in 1xPBS. Samples were then transferred to a polylysine coated microscope slide (Thermo Scientific cat. J2800AMNZ). A coverslip was applied, and the slide was immersed in liquid nitrogen for 5 minutes. The coverslip was removed, and the slide was placed in pre-chilled methanol for 15 minutes at -20°C. The slide was then moved to pre-chilled acetone for 30 seconds. Sample was dried and a surrounding border was drawn using a wax-coated marker. After a 1-minute incubation in PBS, slides were transferred to a chamber for the blocking stage (3% BSA for at least 45 minutes). Blocking solution was removed and primary antibody solution was added to slides. This was covered with parafilm and incubated overnight at 4°C. Slides were washed 3x in PBT and the secondary antibody solution was added for 2 hours at RT (or overnight at 4°C). This solution was removed through 3x washes in PBT and 3x washes in PBS. Mounting media was applied to sample, and a coverslip was sealed atop with nail varnish.

7.8 RNA *in situ* hybridisation

7.8.1 Probe generation

PCR primers were designed using Primer3 software (Untergasser et al., 2012) and included a T7 binding site before the 3' primer. The PCR product was cleaned using the GeneJet purification kit (Thermo scientific, cat. K0692). The RNA probe reaction consisted of 3.5µl dH₂O, 1µl 10x transcription buffer, 0.5µl RNase inhibitor, 100-200ng purified DNA, 1µl T7 polymerase (Roche, cat. 10881767001), 1µl DIG dNTP labelling mix (Roche, cat.11277073910). This was incubated overnight at 37°C. Probe reaction was stopped with 2µl of 0.2M EDTA and the product was purified using the RNAeasy kit (Qiagen, cat. 74104). Purified probes were stored at -20°C.

7.8.2 *In situ* hybridisation on whole mount embryos

Fixed embryos were re-hydrated in incrementally more concentrated PBTw: ethanol solution with a final wash in 100% PBTw (1x PBS, 0.1% Tween (Sigma-Aldrich cat. P-2287). Samples were then post-fixed in a formaldehyde/PBTw mix, washed in PBTw and prepared for hybridisation in a 50:50 mix of PBTw and hybridisation buffer (composed of 50% deionised formamide, 5x saline-sodium citrate, tRNA 100mg/ml (Sigma-Aldrich, cat. 10109495001), heparin 50 mg/ml (Sigma-Aldrich, cat. H3393), 0.1% Tween, dH₂O - pH6.5). Embryos were then pre-hybridised in 500µl hybridisation buffer at 70°C for 4 hours. The RNA probe was diluted in 200µl hybridisation buffer and heat shocked at 94°C for 3 mins and chilled on ice. Probe was transferred to embryos and kept at 70°C overnight.

Embryos were then washed in pre-heated solutions (1 x 100% hybridization buffer, 1 x 50% hybridisation buffer in PBTw, 4 x 100% PBTw). One 10-minute PBTw wash was conducted at RT. The anti-DIG alkaline phosphate conjugate (1:2000, Roche, cat. 11093274910) was then added and samples were incubated overnight at 4°C. Samples were washed in PBTw and then rinsed in a microtiter plate in reaction solution x3 (100mM Tris pH9.5, 100mM NaCl). 20µl NBT/ BCIP solution (Roche, cat. 11681451001) was added per 1ml reaction solution and this mixture was added to samples for staining to develop (in dark). The colour reaction was stopped when staining was observed by washing several times in PBTw and then samples were mounted in 70% glycerol.

7.9 Male fertility assay

7.9.1 Recording the number of fertile males

10 male flies (2-5 days post-eclosion) with the function of 'gene of interest' either knocked-down or knocked-out were individually mated with two Or-R virgin females for 48 hours (pre-lay) at 25°C. Control group was set up at the same time under the same conditions. Adult flies were then tipped into fresh vials and left to mate for 5 days. After this time, adults were removed from vials and males were retained for testes dissection. Once progeny (if any) developed in vials, the number of progeny produced and the number of males which produced progeny was recorded.

7.9.2 Testes dissection to examine sperm motility

Testes were dissected from adult males on a slide in a drop of PBS. A cover slip was placed on top of samples and then pressure was added using a pipette tip. This breached the testes allowing sperm (motile or immotile) to leak out. Sample was imaged using an Olympus Provis AX-70 microscope and whether the sperm was motile or immotile was recorded. General structures (sperm bundles, seminal vesicles) of the testes were also assessed.

7.10 Climbing assay

Groups of approx. 8-12 mated female flies (1-3 days post-eclosion) were placed in vials and left overnight to recover from CO₂ exposure. Vials were then placed in the 25°C behaviour room 2 hours before the assay (which was conducted mid-afternoon) to adapt. Each group of flies was transferred to a graduated cylinder with markings dividing it into four quadrants. After 60s of recovery and adaptation, the cylinder was banged five times onto a cushioned pad. The flies were allowed to climb for 10s and the number of flies present in each quadrant was recorded. Climbing assays were imaged or videoed for the subsequent analysis conducted using QuickTime. Each vial was counted as

$n=1$ and was given a climbing index. The climbing index was calculated by the following equation:

$$\frac{\Sigma(\text{number of flies in quadrant} \times \text{quadrant climbing score})}{\div \text{total number of flies}}$$

7.11 Hearing assay

Groups of five 3rd-instar wandering larvae were selected and placed on a red wine agar plate “hearing arena”. This plate was placed on top of a speaker (Samsung SMS-2200) connected to a laptop in the behaviour room. After a 30s habituation period, the larvae were exposed to a 1s 1000 Hz sine tone played through the speaker at ~70 db. In total, three 1 second tones were played 30 seconds apart. Behaviour exhibited by larvae was recorded from a camera stationed above the arena. These videos were analysed using iMovie, assessing the number of larvae contracting (responding) to the stimulus in a binary manner.

7.12 Generation of Crispr/Cas9 null mutants

7.12.1 Cloning of guideRNAs

Fly Crispr (Gratz et al., 2014) was used to identify optimal cutting sites on genes of interest. Sense and antisense primers were designed using Primer3: a 3nt PAM site (NGG) was added to the sequences and restriction sites for the enzymes to be used subsequently. Genomic DNA from Cas9 flies were used for the amplification process. gRNAs were phosphorylated (T4 PNK, NEB) and annealed in a thermocycler. A ligation reaction involving the guide RNAs and the vectors pBFV-U6.2 and pBFV-U6.2B (digested with *Bbs*I, NEB) was performed (LigaFast Rapid DNA Ligation System, Promega, M8221). Schematic of these vectors is shown below in Figure 7.1. A bacterial transformation (see 7.12.3) was conducted on two vectors: pBFV-U6.2-gRNA1, pBFV-U6.2B-gRNA2 (Kondo & Ueda, 2013).

Ampicillin plates were screened for colonies which were sent for sequencing to identify successful clones. The U6.2-gRNA1 vector was digested and DNA was purified. The U6.2B-gRNA2 vector was digested and dephosphorylated (Antarctic phosphatase, NEB cat. M0289). This dephosphorylated digest was also purified, and a ligation reaction was performed with both vectors. A bacterial transformation of the ligation product was carried out and successful clones were selected. These plasmids were isolated by either bulk prep (see 7.12.4) or miniprep (Qiagen, cat. 27104) to be used in microinjection (see 7.12.5).

7.12.2 Cloning of homology arms

Primers were designed using the Primer3 software which would generate homology arms of 1.5-2kb to be located along the sides of the gRNAs. Additional few nucleotides were added to the primer sequences to include restriction sites for the NEB enzymes. Homology arms were digested and ligated onto either side of the *mini-white* gene in the pRK2 vector (Huang et al., 2008, shown in Figure 7.2).



Figure 7.2 Map of the pRK2 vector used in the cloning of homology arms. Homology arms were cloned into either side of the mini-white gene. Map generated using SnapGene software.

7.12.3 Bacterial transformation

S.O.C media, 30-50µl competent cells (which are DH5α *E. coli* cells generated by Petra zur Lage using the Inoue method) and 1-5µl of reaction products were used in bacterial transformations. Cells were initially thawed on ice, then the DNA of interest was added, and the sample was incubated for 20 mins. These were then heat shocked at 42°C for 30 secs and moved to the shaker for 1 hour at 37°C. Transformation mix was diluted 1:10 into LB medium and spread onto an antibiotic-containing agar plate (kanamycin or ampicillin depending on the vector of interest). This was incubated overnight at 37°C and then inspected for the presence of colonies. These were then selected to generate cultures that were shaken in LB at 225 rpm overnight at 37°C.

7.12.4 Bulk preps

Overnight cultures were centrifuged (4500rpm, 15 mins) and resuspended in solution 1 (50mM Glucose, 25mM Tris pH 8, 10mM EDTA, 5mg/ml lysozyme). This solution was centrifuged (4500rpm, 10 mins) and 4ml solution 2 was added (0.2M NaOH, 1% SDS). After 10 mins incubation on ice, 3ml of solution

3 (3M KOAc, 1.3M HCOOH) was added and the mixture was kept on ice for an additional 15 mins. This mixture was centrifuged (4500rpm, 15 mins, 4°C). The supernatant was retained and 0.6 vol isopropanol was added and incubated on ice for 5 mins. Solution was centrifuged (4500rpm, 10 mins) and the pellet was washed in 70% EtOH and dissolved in TE. Equal volume cold 5M LiCl was added and incubated on ice for 5 mins. This mixture was centrifuged (4°C, 5 mins) and the supernatant was retained. Equal volume isopropanol was added to the supernatant and incubated on ice for 10 mins. The solution was centrifuged (4°C, 5 mins) and the supernatant was discarded. The pellet was dissolved in 1xTE, DNase-free RNase was added, and solution was incubated at 37°C for 1hr. On ice, equal vol PEG/NaCl was added to the mixture and incubated for 5 mins. After centrifugation, the pellet was retained and resuspended in TE. PhOH extraction was performed, and the aqueous phase retained. 1/20 vol 3M NaOAc and 2 vol 100% EtOH was added to the solution and mixed. This was kept at -20°C overnight and then centrifuged. Pellet was washed in 70% EtOH and resuspended in H₂O for injection.

7.12.5 Microinjection of embryos

Embryos from the injection line of interest were collected for 30 min on red-wine agar plates, washed and 50% bleach was used for dechoriation. Once the bleach solution was removed, embryos were lined up posteriorly to anteriorly (so that the micropyle was to the right) on agar squares. Double-sided tape was used to pick up embryos and place them on slides. Embryos were left to dry for 9 mins in a desiccator chamber. Series 700 halocarbon heavy oil (Sigma-Aldrich cat. H3393) was applied to the embryos and 2 µl of DNA (concentration of 200ng/ml - measured on Nanodrop 1000 Spectrophotometer, Thermo Fisher Scientific) was pipetted into a glass needle. Once the needle was focussed on the microscope (Olympus CX41), embryos were then put into focus and pushed into the needle to allow the DNA (which was released through the controlled use of a gas pedal) to insert into germ cells. Once injected, embryos were placed in a weighing boat covered in a mixture of series 700 and series 95 halocarbon oil (Sigma-Aldrich cat. H8773) in a wet tray, coverslip facing down, at 21°C. This tray was checked for

the following 24-72 hours and developing larvae were collected. Once grown, transformants were selected by eye colour. These were outcrossed to *w¹¹¹⁸* flies for several generations before crossing to a balancer line.

7.13 Generation of mVenus fusion protein construct

This was achieved using Gateway technology (Thermo Fisher Scientific). Primers were designed using Primer3 to clone from 500bp upstream of the C-terminus (where Rfx and Fd3f transcription sites are) to include the whole gene until the stop codon. Restriction sites, *attB* tags were added to sequences.

A BP reaction was performed between the *attB*-flanked DNA fragment and an *attP*-containing vector (The following was added at RT: attB-PCR product, attB expression clone, pDONR221 vector (Thermo Fisher Scientific), Gateway BP clonase II Enzyme reaction (Thermo Fisher Scientific, cat. 11789020), TE buffer). After 1 hour incubation time, proteinase K was added, and the sample was incubated for a further 10 mins at 37°C.

After a bacterial transformation was conducted, the LR reaction was performed with the pBID-GV vector which is shown below in Figure 7.3 (based on (Wang et al., 2012), but with the original UAS promoter deleted - originally pBID-UASC-GV) to generate the expression clone.

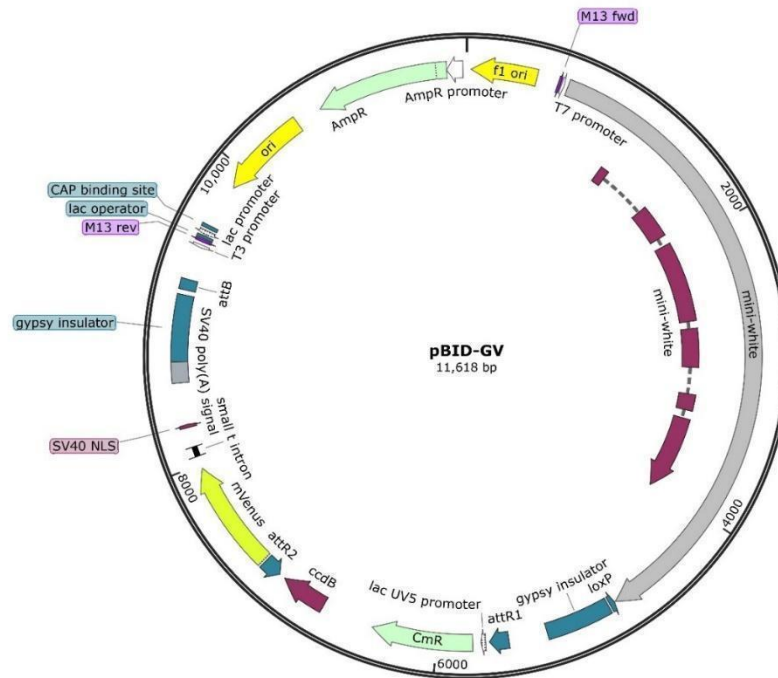


Figure 7.3 pBID-GV vector used in the generation of mVenus protein constructs. Schematic of the destination vector used in the Gateway cloning protocol when creating mVenus fusion proteins in this project. Map generated using SnapGene software.

At RT, the following was added to the reaction mix: *attL*-entry clone (supercoiled, 100-300ng), *attR*-destination vector (supercoiled, 150ng/ul) Gateway LR clonase II Enzyme (Thermo Fisher Scientific, cat. 11791020), and TE buffer. This was then kept at 25°C for 1 hour. Proteinase k was added, and the sample was incubated for an additional 10 mins. Another bacterial transformation was performed, and plasmids of successful colonies were isolated using either bulk prep or miniprep and then prepared for microinjection.

7.14 Label-free mass spectrometry

7.14.1 Sample preparation

The UAS-Gal4 system was used to knock down the function of genes of interest using RNAi technology specifically in the testes. Adult male flies were dissected in cold PBS 1-3 days post-eclosion. Samples were then snap-frozen in liquid nitrogen before being sent to IGMM Mass Spectrometry facility, University of Edinburgh for processing. $n = 30$ pairs of testes/ replicate. 4 replicates per genotype.

7.14.2 Data analysis

Normalised expression data was filtered to show significant proteins. Thresholds used were $\log_2(\text{fold change}) > \pm 1.5$ and $-\log_{10}(\text{pValue}) > 1.3$. Significance was determined using the Empirical Bayes method. Data was analysed through the use of Ingenuity Pathway Analysis (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>) to identify potential pathways and networks altered in knockdowns (such as dynein assembly). The original list was then filtered to look specifically at motile cilia related proteins. These proteins of interest were then displayed as volcano plots comparing the relative protein abundances of the RNAi knockdown to the control group for further analysis. Raw data of proteins and expression levels are available on the ProteomeXchange Consortium with the dataset identifier PXD033608.

7.15 GFP Trap AP-MS

150 pairs of testes/ replicate (3 replicates/genotype) were dissected from *Dyx1c1-mVenus*, *Dpcd-mVenus* and control (*UAS-GAP43-mVenus* x *Bam-Gal4*) fly lines in cold PBS and snap-frozen using liquid nitrogen. Lysis buffer (Tris-HCl pH7.5 50mM, NaCl 100mM, Glycerol 10%, EDTA 5mM, sodium deoxycholate 0.5%, Complete Mini protease inhibitor (Sigma-Aldrich, cat.

4693124001)) was added to samples and homogenised on ice for 2 mins. Samples were then rotated and incubated in lysis buffer at 4°C for 30 mins. Testes were then centrifuged (14000 rpm, 30 mins, 4°C) and transferred to fresh tubes before being sent to IGMM Mass Spectrometry at the University of Edinburgh facility for processing.

When data was returned for analysis, results were displayed as a volcano plot indicating the proteins associated with the mVenus line of interest from AP-MS analysis. Significant proteins ($\log_{10}(\text{pValue}) > 1.3$, $\log_2(\text{fold change}) > 1.5$) were named on graphs with motile cilia-related proteins labelled in green. Significance was determined using the Empirical Bayes method. Raw data of proteins and expression levels are available on the Consortium with the same dataset identifier as the label-free MS experiment (PXD033608).

7.16 Transmission Electron Microscopy

7.16.1 Chordotonal neuron

10 freshly eclosed flies/ genotype of interest were anaesthetised and pinned through the side of the thorax in 0.1M phosphate buffer (PB) diluted from 0.2M stock (for 50ml: 0.302g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.424g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$). Proboscis was held with forceps and cut with scissors. Head was then cut off and placed in Eppendorf containing EM grade fixative (2.5% Glutaraldehyde (TAAB cat. G011/2), 16% Formaldehyde (TAAB cat. F017/3), 0.2M phosphate buffer stock) and this was incubated overnight at 4°C. 4x 0.1M PB rinses at room temperature were conducted, followed by 3x20 min PB washes. Samples were sent to Electron Microscopy Research Services at Newcastle University Medical School for processing and imaging.

7.16.2 Testis

10 male flies/ genotype of interest were anaesthetised and dissected in 0.1M PB 1-3 days post-eclosion. 10 pairs of testes were dissected out and then fixed, washed and sent for processing as described for the chordotonal neuron above.

7.17 Eye phenotype assay

The UAS-Gal4 system was used to cause knockdowns of genes of interest specifically in the eye using the driver lines ey-Gal4 and GMR-Gal4 and UAS-RNAi transgenic lines. Once eclosed, flies were anaesthetised and placed on a white background next to a ruler with mm markings. Images were taken of flies next to this scale with a phone camera (Samsung Galaxy) mounted onto an Olympus SZ-CTV stereomicroscope (Olympus lifescience, Japan) using an adapter. These images were analysed in Fiji (ImageJ), where the scale was set using the ruler, and then a perimeter drawn surrounding the eye area (where the ommatidia are located). This calculated the area of the eye in μm^2 .

7.18 Transfection and co-IP of S2 cells

Constructs for transfection were generated by Petra zur Lage. Transfection of the plasmid constructs into S2 cells was carried out using the X-tremeGENE HP DNA transfection reagent (Sigma-Aldrich, cat. 6366244001). After 3 days, cells were harvested for co-IP which was conducted using the method described in the FLAG Immunoprecipitation kit (Sigma-Aldrich) and briefly below.

Cells were initially centrifuged (420 rcf, 4°C) and the supernatant removed. Pellet was then (2x) resuspended in PBS and centrifuged to ensure all growth media was removed. Washed pellet was resuspended in lysis buffer (50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100) and the solution was incubated for 30 minutes before centrifugation (12,000rcf, 10 mins). Supernatant was retained (lysate). The ANTI-FLAG beads (Sigma-Aldrich, cat. A2220) were centrifuged and localised to the bottom of Eppendorf, supernatant was removed.

Beads were washed in a wash buffer (10x stock: 0.5M Tris HCl, 1.5M NaCl) and the lysate was added for IP reaction. Solution was rotated on a wheel for

a minimum of 2 hours and then centrifuged (8200 rcf). Supernatant was discarded and beads were washed x3 in the wash buffer. Protein was eluted in the 2x sample buffer (125mM Tris HCl, 4% SDS, 20% glycerol and 0.004% bromophenol blue) and incubated at 95°C for 3 mins. After centrifugation, supernatant was retained for western blotting.

After the western blotting process, the membrane was incubated in mouse anti-FLAG M2 antibodies (1:1,000; F1804; Sigma-Aldrich) and rabbit anti-HA (1:4,000; ab9110; Abcam) antibodies overnight at 4°C. After several washes in PBTween, secondary antibodies (IRDye 680RD and IR Dye 800CW, Li-COR) were added and incubated overnight at 4°C. After PBTween washes, protein detection was analysed using a Li-COR Odyssey scanner and ImageStudio v5.2 software.

7.19 Microscopy

Fluorescently stained tissues were initially imaged using a Zeiss Axioskop-2 Mot Plus (Zeiss, Germany) and later in the project using a Leica SP8 lightning confocal microscope (Leica microsystems, Germany) at the IMPACT imaging suite, University of Edinburgh. Brightfield microscopy was conducted using an Olympus AX-70 Provis microscope (Olympus lifescience, Japan). Images were processed using Fiji (Image J).

7.20 Bioinformatics and software analysis

The DRSC integrative ortholog prediction tool (DIOPT) analysed similarity and identity percentages between genetic orthologs (Y. Hu et al., 2011). The UCSC genome browser was used to identify upstream binding motifs for transcription factors of interest (Kent et al., 2002). BLAST (Altschul et al., 1990), Uniprot (Bateman et al., 2021) and Flybase (Larkin et al., 2021 vFB2022_1) databases identified homologues in different organisms for phylogenetic tree generation. These protein sequences were aligned using CLUSTALW/MUSCLE on

MEGA7(S. Kumar et al., 2016), the software which was also used to generate the phylogenetic trees. Ingenuity Pathway Analysis software was utilised to identify proteomic data molecular interactions, cellular functions, and associated human disorders.

7.21 Statistics

Statistical analysis was conducted using GraphPad Prism 9 (GraphPad Software Inc, CA, USA). If the data was parametric, an ANOVA was performed followed by Sidak's multiple comparisons test. If the data was non-parametric, a Mann-Whitney U test or a Kruskal-Wallis test was conducted followed by Dunn's multiple comparisons test. Fisher's exact test was used for the analysis of contingency tables.

Appendix A: Fly Stocks

Table AA: Fly lines used in this study

Genotype	Description	Source
<i>P{CaryP}attP2 control line</i>	Control line	Groth et al., 2004
<i>KK control (RNAi-TK)</i>	Control line	VDRC (#60100)
<i>w[*]; P{w[+mC]=UAS-GFP.S65T}eg[T10] / P{UAS-2xEGFP}AH2</i>	GFP expression under UAS control	Bloomington (#1522/#6874)
<i>W1118</i>	Control line	Bloomington (#3605)
<i>w[*]; ry[506] Dr[1]/TM6B, P{w[+mC]=Dfd-EYFP}3, Sb[1] Tb[1] ca[1]</i>	Balancer	Bloomington (#8704)
<i>yw; Ly/TM3, Sb</i>	Balancer	Abbott, 1986
<i>w*; Sco/Cyo, P{w[+mC]=Dfd-EYFP}2; ry[506] Dr[1]/TM6B, P{w[+mC]=Dfd-EYFP}3, Sb[1] Tb[1] ca[1]</i>	Balancer	Bloomington (#8578)
<i>yw; Pin/ Cyo</i>	Balancer	Bloomington (#1571)
<i>w*; Sco/CyO, P{w[+mC]=Dfd-EYFP}2</i>	Balancer	Gift from Barry Denholm
<i>CG14921ΔCR/Cyo, P{w[+mC]=Dfd-EYFP}2</i>	CRISPR/Cas9 del (2nd)	Generated in this project
<i>CG13901ΔCR/TM6B, P{w[+mC]=Dfd-EYFP}3, Sb[1] Tb[1] ca[1]</i>	CRISPR/Cas9 del (3rd)	Generated in this project
<i>CG5048ΔCR/ TM6B, P{w[+mC]=Dfd-EYFP}3, Sb[1] Tb[1] ca[1]</i>	CRISPR/Cas9 del (3rd)	Generated in this project
<i>w[1118]; Df(3L)BSC632/TM6C, cu[1] Sb[1]</i>	Deficiency line for <i>Dpcd</i>	Bloomington (#25723)
<i>w[1118]; Df(2L)BSC242/CyO</i>	Deficiency line for <i>Dyx1c1</i>	Bloomington (#24905)

w[11118]; Df(3L)ED4543, P{w[+mW.Scer\FRT.hs3]=3'. RS5+3.3'}ED4543/TM6C, cu[1] Sb[1]	Deficiency line for Pih1d3	Bloomington (#8073)
<i>Dnali1-mVenus</i>	Endogenous FP expression	Generated by Fay Newton
<i>Dnal1-mVenus</i>	Endogenous FP expression	Generated by Fay Newton
<i>Dpcd-mVenusJ</i>	Endogenous FP expression	Generated in this project
<i>Dpcd-mVenusL</i>	Endogenous FP expression	Generated by Petra zur Lage
<i>Dyx1c1-mVenus</i>	Endogenous FP expression	Generated in this project
<i>IAV-GFP (3rd chromosome)</i>	Endogenous FP expression	Generated by Fay Newton
w[*]; P{w[+mC]=UASp-Venus.GAP43}7	Endogenous FP expression	Bloomington (#30897)
<i>Heatr2-mVenus</i>	Endogenous FP expression	Generated by Daniel Moore
<i>Pih1d3-mVenus</i>	Endogenous FP expression	Generated by Petra zur Lage
<i>Dyx1c1ΔCR, Dnal1-mVenus/CyO, Dfd-EYFP</i>	Endogenous FP expression in Dyx1c1Cr background	Generated in this project
<i>Dyx1c1ΔCR,Dnali1-mVenus/CyO, Dfd-EYFP</i>	Endogenous FP expression in Dyx1c1Cr background	Generated in this project
<i>Dnal1-mVenus/Cyo,Dfd-EYFP; Pih1d3ΔCR/TM6,Dfd-EYFP, Sb[1] Tb[1] ca[1]</i>	Endogenous FP expression in Pih1d3Cr background	Generated in this project
<i>DNali1-mVenus/Cyo,Dfd-EYFP; Pih1d3ΔCR/TM6, Dfd-EYFP, Sb[1] Tb[1] ca[1]</i>	Endogenous FP expression in Pih1d3Cr background	Generated in this project
<i>Scabrous-Gal4, 6971-mVenus</i>	Endogenous FP expression Gal4 driver	Generated by Fay Newton
<i>fd3^{f1}</i>	<i>fd3F</i> deletion allele	Generated by Fay Newton (Newton et al., 2012)

w[*]; P{w[+m*]=GAL4-ey.H}4-8/CyO	Gal4 driver	Bloomington (#5535)
w[*]; P{w[+mC]=GAL4-ninaE.GMR}12	Gal4 driver	Bloomington (#1104)
P{sca-GAL4.U}	Gal4 driver	Egger et al., 2002
y[1] w[*]; P{w[+mC]=tubP-GAL4}LL7/TM3, Sb[1] Ser[1]	Gal4 driver	Bloomington (#5138)
w; tft/cyo; Bamgal4:VP16	Gal4 driver	gift from Helen White-Cooper
<i>Dyx1c1ΔCR</i> , <i>Sca-Gal4</i> / <i>Cyo</i> , <i>Dfd-EYFP</i>	Gal4 driver in <i>Dyx1c1</i> Cr background	Generated in this project
P{UAS-Dcr-2.D};P{sca-GAL4.U}	Gal4 driver with dicer	Generated by Lina Ma
<i>Dyx1c1ΔCR/Cyo</i> , <i>Dfd-EYFP</i> ; <i>IAV-GFP</i> / <i>TM6</i> , <i>Dfd-EYFP</i> , <i>Sb[1] Tb[1] ca[1]</i>	IAV-GFP expression in <i>Dyx1c1</i> CR background	Generated in this project
<i>W1118 y1M{vas-Cas9} ZH-2A/FM7c</i>	Injection line (Cas9)	Bloomington (#51323)
y w M(eGFP, vas-int, dmRFP)ZH-2A; P{CaryP}attP40	Injection line (mVenus)	Cambridge microinjection facility
P{GawB}elav[C155];UAS-Dicer-2	iRNA overexpression system	Generation K. Kiser, Recombination L. Ma
y[1] w[67c23]; P{y[+mDint2] w[+mC]=EPgy2}CG13887[E Y21004]/TM3, Sb[1] Ser[1]	P element line	Bloomington (#22441)
CG3132027	P element line	Generated by Daniel Moore
<i>Dpcd-mVenus/Cyo</i> , <i>Dfd-EYFP</i> ; <i>DpcdΔCR/TM6B</i> , <i>Dfd-EYFP</i> , <i>Sb[1] Tb[1] ca[1]</i>	Rescue line	Generated in this project
<i>Dyx1c1ΔCR</i> , <i>Dyx1c1-mVenus/CyO</i> , <i>Dfd-EYFP</i>	Rescue line	Generated in this project
<i>Pih1d3-mVenus/Cyo</i> , <i>DfYd-EFP</i> ; <i>Pih1d3ΔCR/TM6B</i> , <i>Dfd-EYFP</i> , <i>Sb[1] Tb[1] ca[1]</i>	Rescue line	Generated in this project
P{UAS-hid.Y}	UAS gene (on Y)	Gift from Alessandro Scopelliti

<i>P{KK100997}</i>	UAS-CG10064 RNAi	Bloomington (#106172)
<i>P {TRiP.HMC06270} attP2</i>	UAS-CG13901 dsRNAi	Bloomington (66336)
<i>P{KK111069}</i>	UAS-CG14921 RNAi	Bloomington (#102921)
<i>P{KK109842} VIE-260B</i>	UAS-CG1553 RNAi (over Cyo, DfYFP)	VDRC (#104806)
<i>P{KK108561}</i>	UAS-CG5048 RNAi	VDRC (#101352)
<i>P{KK100806}</i>	UAS-CG5792 RNAi	Bloomington (#106988)
Oregon-R-P2	Wild type	Bloomington (#2376)

Appendix B: Antibodies

Table AB: Antibodies used in this study

Antibody	Dilution	Source
Mouse anti-futsch	1:200	Developmental Biology Hybridoma Bank, Iowa (DSHB)
Mouse anti-GFP	1:500	Abcam
Mouse anti-pan-polyglycylated tubulin	1:100	Merck Millipore
Rabbit anti-DNAH5	1:2000	Generated by Petra zur Lage
Rabbit anti-distalless	1:100	Donated by Barry Denholm
Rabbit anti-GFP	1:500	Invitrogen
Rabbit anti-HRP	1:500	Jackson ImmunoResearch laboratories
Rabbit anti-sas-4	1:350	Donated by Johnathan Howard
Rabbit anti-asense	1:1000	Generated the Jarman lab
Sheep anti-DIG-AP	1:2000	Roche
Conjugated Phalloidin	1:2000	Sigma-Aldrich
DAPI	1:1000(of 14.3 mM stock)	Invitrogen
Mouse anti-FLAG M2	1:1000	Sigma-Aldrich
Rabbit anti-HA	1:4000	Abcam

Appendix C: Primers

Table AC: Primers used in this study

Oligo Name	Sequence (5'- 3')	Use of primer
(FN) CG10064 5'	ATCCTGCGTGAGGAGAAGAA	RNA in situ
(FN) CG10064 3'T7	GTAATACGACTCACTATAGGGCTTGG GAAACGTGATGCAGTA	RNA in situ
(JL) CG14921 5 probe	TGGCCTGAGATGTTCCAGAA	RNA in situ
(JL) CG14921 3 plus T7 probe	GTAATACGACTCACTATAGGGCCATA TGCCTTCTCCATAGCGG	RNA in situ
(JL) CG14921 attB fw	GGGGACAAGTTTGTACAAAAAAGCAG GCTGCAACCAGAGGACGCTGAATAT GGATAC	Generation of mVenus line
(JL) CG14921 attB rev	GGGGACCACTTTGTACAAGAAAGCTG GGTCTTCATCGACGCTCTCCATCGGC GACTG	Generation of mVenus line
(JL) CG14921 Rev	ATAGCGCCGACTTAGTGTTG	Sequencing Cas9 flies - amplifying genomic DNA
(JL) CG14921 CRISPR 3prime sense	CTTCGTATTCATCGACGCTCTCCAT	Generation of gRNAs
(JL)CG14921 CR 3prime antisense	AAACATGGAGAGCGTCGATGAATAC	Generation of gRNAs
(JL) CG14921 5prime antisense	AAACTCCTATTGTCAGTCGCAGAC	Generation of gRNAs
(JL) CG14921 5prime sense	CTTCGTCTGCGACTGACAATAGGA	Generation of gRNAs

(JL) CG14921 LA 5' EcoR1	GGGGAATTCTCTGCGTACGAAATGTG CTG	Generation of homology arms
(JL) CG14921 LA 3' Kpn1	GGGGGTACCGGAAGGAAGCTTATGTTT TTGAGCTGGC	Generation of homology arms
(JL) CG14921 RA 5' BglII	GGGAGATCTGAGAGCGTCGATGAAT AAGCGTT	Generation of homology arms
(JL) CG14921 RA 3' Xho1	GAAGTCGAGGATCGCAGGGCACTAT GTTG	Generation of homology arms
(JL) MidHA CG14921 noncod3 primer	TCGGTTTCAGGTGGGTACTC	Checking for CRISPR knock-out of gene
(PzL) Coding white gene downstream	GGGTCTGGCCATTCTCATC	Checking for CRISPR knock-out of gene
(JL) CG5048 CRISPR check left	ATTCGGTCGAGCTTAGCCTA	Sequencing cas9 flies - amplifying genomic DNA
(JL) CG5048 CRISPR check right	CGTATGCGCAATTTGGTAGC	sequencing Cas9 flies - amplifying genomic DNA
(JL) CG5048 5prime sense	CTTCGTTTATGGACGGACTATCAC	Generation of gRNAs
(JL) CG5048 5prime antisense	AAACGTGATAGTCCGTCCATAAAC	Generation of gRNAs
(JL) CG5048 3prime sense	CTTCGTATGTCTTTTAATATTATA	Generation of gRNAs
(JL) CG5048 3prime antisense	AAACTATAATATTAAGACATAC	Generation of gRNAs
(JL) CG5048 5prime left	CTGGAATTCCGAGCCCAAGGTGAAA GTTC	Generation of homology arms (EcoR1)
(JL) CG5048 5prime right	GCAGGTACCCACTGGATCTCACACTT TTTC	Generation of homology arms (Kpn)

(JL) CG5048 3prime left	GGGAGATCTATATGGCCATTTACTTT GTG	Generation of homology arms (BglII)
(JL) CG5048 3prime right	GATCTCGAGAGGGTGTGGTTGATCAT CGT	Generation of homology arms (Xho1)
(JL) CG5048 3prime midHA NC	TGAGGCAAAACATAACCGCA	Checking for CRISPR knock-out of gene
(JL) CG13887 5prime probe	AATAGCCACTAGACCGTCCG	RNA in situ
(JL) CG13887 3prime T7	GTAATACGACTCACTATAGGGCCGGT GTTCTTGTCTCCAGC	RNA in situ
(JL) CG13901 left	AATGGCCGAGGAGTACAACA	RNA in situ
(JL) CG13901 right T7	GTAATACGACTCACTATAGGGCGTGT TGAAGTGGTGGTGGAC	RNA in situ
(JL) CG13901 5prime left ATTB	GGGGACAAGTTTGTACAAAAAAGCAG GCTCAAGACAAATCGATCATAAGG	Generation of mVenus line
(JL) CG13901 3prime ATTB	GGGGACCACTTTGTACAAGAAAGCTG GGTCTTTGGCCATCAGACCCTTCAG	Generation of mVenus line
(PzL) CG13901 5prime left ATTB long	GGGGACAAGTTTGTACAAAAAAGCAG GCTTCACCGGCCACGTCAACTCTGTT TT	Generation of mVenus line
(JL) CG13901 CRISPR check left	CAACTGTAAATTGGGTAGCCAGA	Sequencing cas9 flies - amplifying genomic DNA
(JL) CG13901 CRISPR check right	CCCTCCTTCGATTACAGCCT	Sequencing cas9 flies - amplifying genomic DNA
(JL) CG13901 5primesense	CTTCGCTTGTTTACATCGCAAGCC	Generation of gRNAs

(JL) CG13901 5prime antisense	AAACGGCTTGCGATGTAAACAAGC	Generation of gRNAs
(JL) CG13901 3primesense	CTTCGAGATCATCCATGTCTGGTTT	Generation of gRNAs
(JL) CG13901 3prime antisense	AAACAAACCGACATGGATGATCTC	Generation of gRNAs
(JL) CG13901 3prime left	GCTACTAGTCCGACATGGATGATCTG CTG	Generation of homology arms (Spe1)
(JL) CG13901 3prime right	TGACTGCAGAGGCCTTTGTGAGATCA GCT	Generation of homology arms (Pst1)
(JL) CG13901 5prime right	TACGGTACCCAAGCCGGGTTACCAA GGCA	Generation of homology arms (Kpn1)
(JL) CG13901 5prime left	GTAGCGGCCGCATACTTAGGACACTC GGGCG	Generation of homology arms (Not1)
(JL) CG13901 midHA 3prime NC	CTCATTTCACGAGCGAAGG	Checking for CRISPR knock-out of gene
(JL) CG14921 midgene 2	ACGTTACGATAACAAGCGGC	Checking successful recombination including mVenus line
(PzL)3' GFP237	AAGTCGTGCTGCTTCATGTG	Checking for successful recombination including mVenus line
(PzL) 8800mV 5Prime	GTGGCTGAAACTCTGGAGGA	Checking for successful recombination
(FN) qPCR primer 2 fw 6971	GAACAGGCAGAGCGTCGTAA	Checking for successful recombination including mVenus line

(SC) Gal4 Fw primer	CCGAATTTGGTGGTCTGTCT	Checking for successful recombination including Gal4 line
(SC) Gal4 Rev primer	GCGGTCTCGTTATTCTCAGC	Checking for successful recombination including Gal4 line

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