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The identification and characterisation of conserved ciliary genes expressed in *Drosophila* sensory neurons

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PhD
The University of Edinburgh
2014
Declaration

I certify:

(a) that the thesis has been composed by me, and

(b) either that the work is my own, or, where I have been a member of a research group, that I have made a substantial contribution to the work, such contribution being clearly indicated, and

(c) that the work has not been submitted for any other degree or professional qualification except as specified.

Daniel J. Moore

September 2014
Abstract

*Drosophila* provide an excellent model organism in which to study cilia as there are only two ciliated cell types; the sensory neurons and sperm cells. The chordotonal neuron is one such ciliated cell and is required for hearing, proprioception and gravitaxis. Mechanical manipulation of the cilium that extends from the neuronal dendrite is required for signal transduction. Chordotonal neuronal differentiation is regulated by a transcription factor cascade. Atonal begins the cascade, which is then continued by RFX and Fd3F for ciliary genes (Cachero et al 2011, Newton et al 2012). Genes expressed in developing chordotonal neurons are downstream of these transcription factors and their characterisation can further elucidate how neuronal differentiation is regulated. Ciliary genes are highly enriched in developing chordotonal cells; uncharacterised genes enriched in these cells can therefore be considered candidate ciliary genes (Cachero et al 2011).

A behavioural assay was conducted to identify further genes that could have a role in ciliary formation and function. Candidate genes were identified by combining enrichment data with previous genomic, proteomic and transcriptomic studies of cilia. A climbing assay of RNAi mediated knock down of these genes identified a number of candidates for future work.

One gene found to be highly enriched in developing chordotonal neurons is *CG11253*. *CG11253* P element insertion mutant flies show a mild uncoordinated phenotype in a climbing assay consistent with reduced chordotonal organ function. Male flies are also infertile due to a lack of motile sperm. *CG11253* is expressed in motile ciliated cells and is conserved in organisms with motile cilia. *CG11253* expression is also regulated by RFX and Fd3F, suggesting that it is involved in cilium motility. This was confirmed by electron microscopy, which showed disruption of axonemal dynein arm localisation in chordotonal cilia and sperm flagella. A CG11253::mVenus fusion protein was found to localise mainly to the cytoplasm and to a lesser extent the cilia of chordotonal neurons. Patients with symptoms consistent with Primary Ciliary Dyskinesia (PCD), a condition caused by
cilium immotility, have subsequently been found to have point mutations in ZMYND10, the human homologue of CG11253.

The identification of PCD patients with ZMYND10 mutations showed that investigating cilium motility in Drosophila chordotonal neurons could identify novel PCD genes. It was thought that investigating previously uncharacterised targets of Fd3F could identify novel genes involved in cilium motility and thus candidate PCD genes. CG31320 is a gene regulated by RFX and Fd3F and conserved in organisms with motile cilia. RNAi mediated knock down of CG31320 resulted in both a mild uncoordinated phenotype and male infertility due to a lack of motile sperm. Electron microscopy showed a complete loss of axonemal dynein arms in chordotonal neuron cilia. An mVenus fusion protein of CG6971, an inner dynein arm component, was also mislocalised from the cilia in CG31320 deletion mutant larvae. This shows that CG31320 is required for the appropriate localisation of the axonemal dynein arms and thus cilium motility. This further showed that uncharacterised genes enriched in chordotonal neurons and regulated by Fd3F could be novel ciliary genes required for cilium motility. Our collaborators and Horani et al (2012) showed that the human homologue of CG31320 (HEATR2) is mutated in patients with PCD, further confirming that this method can be used to identify PCD genes.

I have identified two factors required for cilium motility. Disruption of the axonemal dynein arms in both cases results in reduced coordination, and lack of fertility due to sperm immotility. Mutations in the human homologues of these genes have been found to result in PCD. This indicates that further PCD genes could be identified from genes enriched in Drosophila chordotonal neurons that are regulated by Fd3F.
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Abbreviations

α1Tub84B - α-Tubulin at 84B
AC3 – Adenylyl cyclase III
Ana2 – Anastral spindle
ARL - ADP-ribosylation factor-like
ARM - Armadillo
ARMC4 - Armadillo repeat containing 4
Asl - Asterless
Ato - Atonal
AZI1 - 5-azacytidine induced 1
B9D – B9 protein domain
Bam - Bag of marbles
BBIP10 - BBSome interacting protein of 10kDa
BBS – Bardet-Biedl syndrome
BDGP – Berkeley Drosophila genome project
BLD10 – Basal body protein 10
Blu - Beta-catenin in lung
Btv - Beethoven
Bun - Bunched
Cato - Cousin of atonal
CBF Cerebrospinal fluid
Cby - Chibby
CC2D2A - C2 domain containing 2A
CCDC - Coiled coil domain containing
CCT – chaperonin containing TCP-1
CEP – Centrosomal protein
CHE - abnormal chemotaxis
CNG – Cyclic nucleotide gated
CSC - Calmodulin and spoke associated complex
CS domain - CHORD-containing proteins and SGT1 domain
DAF – abnormal dauer formation
Der - Dicer
DEAF-1 - Deformed epidermal autoregulatory factor 1
Dila - Dilatory

DNAAF - Dynein arm assembly factor
DNAHC - Dynein, axonemal, heavy chain
DNAIC - Dynein, axonemal, intermediate chain
DNAL1 - Dynein, axonemal, light intermediate chain
DVL – Dishevelled
DYF – abnormal dye filling
DYX1C1 - Dyslexia susceptibility 1 candidate 1
ELAV - Embryonic lethal, abnormal vision
ES - External sensory organs
FACS - Fluorescence associated cell sorting
FFB1 - (Fas (TNFRSF6))-binding factor 1
Fd3F - Forkhead 3F
FLA – Flagellar associated
FLNA – Filamin A
FOXJ1 - Forkhead box protein J1
FZD – Frizzled
GLI – Glioma
GLIA – GLI activator
GLIR – GLI repressor
HEAT - Huntingtin, EF3, PP2A, TOR1
HEATR2 - HEAT repeat containing 2
HH - Hedgehog
HRP - Horse radish peroxidase
hpRNA - Hairpin RNA
HEK - Human embryonic kidney
HSP – Heat shock protein
HYDIN - Hydrocephalus inducing protein
Iav - Inactive
IDA - Inner dynein arm
IFT - Intraflagellar transport
IHC - Immunohistochemistry
IMCD - Inner medullary collecting duct
INVS – Inversin
JNK - c-Jun N terminal kinase
KAP – Kinesin associated protein
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIF</td>
<td>Kinesin family member</td>
</tr>
<tr>
<td>KLP</td>
<td>Kinesin like protein</td>
</tr>
<tr>
<td>Ktu</td>
<td>Kintoun</td>
</tr>
<tr>
<td>Ktub</td>
<td>King tubby</td>
</tr>
<tr>
<td>LECA</td>
<td>Last eukaryotic common ancestor</td>
</tr>
<tr>
<td>LEPR</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>lch</td>
<td>lateral chordotonal neuron</td>
</tr>
<tr>
<td>L-R</td>
<td>Left-right</td>
</tr>
<tr>
<td>LRRRC</td>
<td>Leucine rich repeat containing</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MKS</td>
<td>Meckel syndrome</td>
</tr>
<tr>
<td>MKSR</td>
<td>MKS related</td>
</tr>
<tr>
<td>mTEC</td>
<td>mouse tracheal epithelial cells</td>
</tr>
<tr>
<td>MYND</td>
<td>Myeloid, nervous and DEAF-1</td>
</tr>
<tr>
<td>Nan</td>
<td>Nanchung</td>
</tr>
<tr>
<td>N-DRC</td>
<td>Nexin-dynein regulatory complex</td>
</tr>
<tr>
<td>Nomp</td>
<td>No mechanoreceptor potential</td>
</tr>
<tr>
<td>NPHP</td>
<td>Nephronopthisis</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OCR</td>
<td>OSM9 and Capsaicin receptor related</td>
</tr>
<tr>
<td>ODA</td>
<td>Outer dynein arm</td>
</tr>
<tr>
<td>ODA-DC</td>
<td>ODA docking complex</td>
</tr>
<tr>
<td>OSEG</td>
<td>Outer segment</td>
</tr>
<tr>
<td>OSN</td>
<td>Olfactory sensory neurons</td>
</tr>
<tr>
<td>OSM</td>
<td>Osmotic avoidance abnormal</td>
</tr>
<tr>
<td>PC</td>
<td>Polycystin</td>
</tr>
<tr>
<td>PCD</td>
<td>Primary ciliary dyskinesia</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCM1</td>
<td>Pericentriolar material 1</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>PKD</td>
<td>Polycystic kidney disease</td>
</tr>
<tr>
<td>PLK4</td>
<td>Polo like kinase 4</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-Protein interactions</td>
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<tr>
<td>PTCH1</td>
<td>Patched 1</td>
</tr>
<tr>
<td>RempA</td>
<td>Reduced mechanoreceptor potential</td>
</tr>
<tr>
<td>RFX</td>
<td>Regulatory factor X</td>
</tr>
<tr>
<td>RHO</td>
<td>Rhodopsin</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelial</td>
</tr>
<tr>
<td>RPGRIP1L</td>
<td>RPGR interacting protein 1 like</td>
</tr>
<tr>
<td>RSPH</td>
<td>Radial spoke homologue</td>
</tr>
<tr>
<td>Sak</td>
<td>SAK Kinase</td>
</tr>
<tr>
<td>SAS</td>
<td>Spindle assembly abnormal</td>
</tr>
<tr>
<td>Sca</td>
<td>Scabrous</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SMO</td>
<td>Smoothened</td>
</tr>
<tr>
<td>SOP</td>
<td>Sense organ precursor</td>
</tr>
<tr>
<td>SPAG</td>
<td>Sperm associated antigen</td>
</tr>
<tr>
<td>SPD-2</td>
<td>Spindle defective</td>
</tr>
<tr>
<td>SSTR3</td>
<td>Somatostatin receptor 3</td>
</tr>
<tr>
<td>SUFU</td>
<td>Suppressor of fused</td>
</tr>
<tr>
<td>SYNE</td>
<td>Specrtrin repeat containing nuclear</td>
</tr>
<tr>
<td>envelope 2</td>
<td></td>
</tr>
<tr>
<td>TilB</td>
<td>Touch insensitive larvae B</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell factor</td>
</tr>
<tr>
<td>TCTN1</td>
<td>Tectonic family member 1</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TMEM</td>
<td>Transmembrane protein</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential channel</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide repeat</td>
</tr>
<tr>
<td>TUB</td>
<td>Tubby</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activating sequence</td>
</tr>
<tr>
<td>Unc</td>
<td>Uncoordinated</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>vch</td>
<td>Ventral chordotonal</td>
</tr>
<tr>
<td>VDRC</td>
<td>Vienna <em>Drosophila</em> RNAi Center</td>
</tr>
<tr>
<td>vtd</td>
<td>ventral tracheal multidendritic neuron</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-type MMTV integration site</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast 2 hybrid</td>
</tr>
<tr>
<td>ZMYND10</td>
<td>Zinc finger, MYND containing</td>
</tr>
<tr>
<td>ZYG-1</td>
<td>Zygote defective embryonic lethal</td>
</tr>
</tbody>
</table>
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Introduction

Cilia and flagella are microtubule-based organelles that protrude from the cell surface whilst enclosed within a plasma membrane. Their structure consists of an axoneme made up of nine microtubule doublets that extends from a modified centriole known as the basal body (Figure 1.1). The axoneme can also contain an additional central microtubule pair; the arrangement of axonemal microtubules is termed 9+2 or 9+0 with the 2 or 0 detailing the presence or absence of the central pair. Cilia and eukaryotic flagella both have a conserved structure and as such are treated as specialised forms of the same organelle.

Cilia and/or flagella are present in organisms of many eukaryotic lineages and are thought to have been present in the LECA (last eukaryotic common ancestor) (Reviewed in Carvalho-Santos et al 2011). Whilst the general ciliary structure is conserved, cilia have diverged to fulfil a number of different roles in various cells and organisms. These roles can be loosely divided into those that require motile cilia and those in which the cilium is immotile. The active movement of motile cilia and flagella can allow the movement of small organisms and cells through their environment or can generate fluid flow by the coordinated beating of many cilia on multiciliated tissues (Ringo 1967, Ibanez-Tallon et al 2004). Immotile cilia are present on a number of different cell types and have been implicated in both sensory and signalling processes (Huangfu et al 2003, Nauli et al 2003).

Motile flagella are present on a number of small organisms such as the algae Chlamydomonas, and the planarian Schmidtea mediterranea (Ringo 1967, Rompolas et al 2009). They are also present on gametes such as human sperm cells (Afzelius et al 1995). The beating of these flagella allows the propulsion of the organism or cell through its environment (Ringo 1967, Rompolas et al 2009). The coordinated beating of motile cilia is required for the movement of fluid; for example in the human respiratory tract for mucosal clearance or on the ependymal cells of the mouse brain.
Figure 1.1 The structure of the cillum
A diagram of the ciliary structure detailing specialised regions and the localisation of some of the cilia-linked proteins and complexes.
ventricles to generate cerebrospinal fluid (CBF) flow (Afzelius 1976, Ibanez-Tallon et al 2004). Axonemes with a 9+2 arrangement are more common in motile cilia and flagella; however 9+0 structured axonemes can also be motile such as the motile cilia of the mouse embryonic node which whilst being of a 9+0 axonemal structure beat to generate an extra-embryonic flow required for the establishment of left-right body asymmetry (Nonaka et al 1998). Motility of a cilium or flagellum is driven by the action of the cilium motility machinery, which contains both the dynein arms that instigate ciliary movement by the sliding of microtubules and the components which regulate their movement such as the nexin-dynein regulatory complex (Brokaw & Kamiya 1987, Heuser et al 2009).

Not all cilia contain the cilium motility machinery; those that do not are considered to be immotile and are known as primary cilia. Immotile cilia play a role in a number of sensory processes including mechanosensation of fluid flow in the vertebrate kidneys and as a hub for the localisation of olfactory receptors and signal transduction components in olfactory sensory neurons of a number of species including mammals and Drosophila (Kulaga et al 2004, Jana et al 2011). Immotile cilia have also been implicated in forming a specialised signaling compartment for a number of developmental signaling pathways; including the Hedgehog (HH) and Wingless-type MMTV integration site (WNT) signaling pathways (Huangfu et al 2003, Gerdes et al 2007). Immotile cilia usually have a 9+0 axonemal ultrastructure however there are exceptions to this as mammalian olfactory neurons have a 9+2 axonemal structure (Menco 1984).

As cilia are required for a number of biological processes in human cells, disruption of their synthesis, structure or function can cause human disease (Reviewed by Badano et al 2006). Conditions caused by a lack of ciliary function are termed ciliopathies and the symptoms of these diseases reflect the various roles that cilia play. Primary Ciliary Dyskinesia (PCD) is one such condition caused by defects in the cilium motility machinery leading to cilium immotility; the symptoms of which include respiratory problems due to poor mucal movement in the respiratory system and randomisation of left-right body axis due to loss of nodal ciliary movement (Afzelius 1976).
As the general ciliary structure is well conserved the cilia and flagella of model organisms have been used to better understand cillum biogenesis, structure function and ciliopathies. Study of the two flagella of *Chlamydomonas reinhardtii* have and continue to reveal key ciliary processes (Ringo 1967, Kozminski et al 1993, Mitchison et al 2012). In this study I use the cilia of the *Drosophila melanogaster* chordotonal neuron and the sperm flagella to identify components involved in ciliary formation. This is possible as despite some specialisation of chordotonal neuron cilia for their role in the hearing, gravitaxis and proprioception, the general structure of the cilium and its components are conserved (Eberl et al 2000, Lee et al 2008).

Formation of a functional cilium includes a number of processes, collectively referred to as ciliogenesis. The basic structure of the cilium requires the formation a basal body from a centriole, its migration to and docking with the plasma membrane and the extension of the ciliary axoneme and the extension of the surrounding membrane (Sorokin 1962). A specialised transport pathway termed intraflagellar transport (IFT) is required for the transport along the cilium for axoneme formation and maintenance (Kozminki et al 1993). In addition the ciliary components necessary for the function of that particular cilium also need to be formed, be that the cilium motility machinery in motile cilia or the signal transduction material in immotile cilia. Regulation of ciliogenesis includes not only the transport of ciliary components to the position within the cilium that they function, but also their assembly in the cytoplasm by chaperones, transport to the ciliary base and entrance into the ciliary compartment (Nachury et al 2007, Williams et al 2011, Mitchison et al 2012). Disruption of any of these processes can result in ciliary dysfunction and can cause ciliopathies in humans.

### 1.1 Cilia structure and formation

#### 1.1.1 Centriole and Basal Body Formation

The microtubule axoneme of the cilium extends from a modified centriole known as the basal body (Anderson 1972). Both centrioles and basal bodies are cylindrical structures made up of nine triplets of microtubules, with a few exceptions such as the centrioles of the early *Drosophila* embryo and those in *Caenorhabditis elegans*.
which are made up of microtubule doublets and singlets respectively (Callaini et al 1997, Pelletier et al 2006). Centrioles and basal bodies have many components; proteomic analyses of *Chlamydomonas* and *Tertahymena* basal bodies identified 61 *Chlamydomonas* (Keller et al 2005) and 97 *Tetrahymena thermophila* (Kilburn et al 2007) candidate centriole proteins.

Much of the mechanism of centriole duplication is conserved between ciliated organisms (Hodges et al 2010). Centriole duplication represents an early step necessary for ciliogenesis as the basal body that nucleates the ciliary axoneme forms from a centriole. It is only then to be expected that disruption of centriole duplication results in ciliary defects (Bettencourt-Dias et al 2005, Basto et al 2006, Rodrigues-Martins el al 2007). In *Drosophila*, mutation of genes implicated in centriolar formation and duplication (*spindle assembly abnormal-4* (*sas-4*) (Basto et al 2006), *spindle assembly abnormal-6* (*sas-6*) (Rodrigues-Martins et al 2007), *asterless* (*asl*) (Blachon et al 2008) or *sak kinase* (*sak*) (Bettencourt –Dias et al 2005)) results in a disruption or loss of the cilia of the sensory neurons and/or sperm flagella. These genes are required for centriole duplication and therefore basal body assembly (Bettencourt-Dias et al 2005, Basto et al 2006, Rodrigues-Martins et al 2007, Blachon et al 2008).

The first steps in the centriole duplication pathway have been well characterised in *Caenorhabditis elegans* (Delattre et al 2006, Pelletier et al 2006). The initial step is thought to be the recruitment of the kinase ZYG-1 by SPD-2 (Delattre et al 2006, Pelletier et al 2006). *Caenorhabditis elegans* embryos display disrupted centriole duplication when *spd-2* is knocked down by siRNA (Pelletier et al 2004). In *Drosophila* centriole formation Asterless is thought to be one of the earliest recruited proteins (Blachon et al 2008). *asterless* mutant flies lack basal bodies and flagella in both sensory neurons and spermatids (Blachon et al 2008). Morpholino mediated knockdown of *cep152*, the zebrafish *asterless* homologue, also resulted in a reduction in basal body and olfactory ciliary formation (Blachon et al 2008). CEP152 has been shown to interact with Polo like kinase 4 (PLK4) (Hatch et al 2010) and Asterless interacts with Sak, the *Drosophila* homologue of PLK4 (Dzhindzhev et al
2010). SPD-2 and Asterless/CEP152 therefore fulfil a similar role in the recruitment of a kinase that has an important role in centriole duplication.

*sak* mutant flies have defects in centriole duplication and the flagella of their sperm cells are missing (Bettencourt-Dias et al 2005). Human osteocarcinoma U2OS cells in which PLK4 has been knocked down by siRNA also display a lack of centriole duplication (Bettencourt-Dias et al 2005). Overexpressing *sak* in fly embryos or *PLK4* in human U2OS cells results in the production of additional centrioles further showing its important role in centriole formation (Habedanck et al 2005, Peel et al 2007).

The *Caenorhabditis elegans* kinase ZYG-1 has been shown to recruit additional proteins that are required for centriole formation (Dellatre et al 2006, Pelletier et al 2006) This includes binding to SAS-6, thereby recruiting it to the centriolar precursor (Lettman et al 2013). SAS-6 has been found to be required for centrosome duplication in *Caenorhabditis elegans*, *Drosophila*, and human osteosarcoma (U2OS) cells (Leidel et al 2005, Rodrigues-Martins et al 2007). In *Caenorhabditis elegans* SAS-6 is first recruited with SAS-5 to form a central tube onto which nine microtubule singlets can later be attached (Dellatre et al 2006, Pelletier et al 2006). In other organisms including *Chlamydomonas*, mammals and *Drosophila* the centriolar precursor instead contains a cartwheel, a central tubule projecting nine spokes with a nine-fold symmetry (Anderson & Brenner 1971, Nakazawa et al 2007, Stevens et al 2010). SAS-6 has been found to localise to the central tube of the cartwheel in both *Drosophila* and *Chlamydomonas* (Nakazawa et al 2007, Gopalakrishnan et al 2010). Additionally in SAS-6 mutant *Chlamydomonas* the cartwheel central tubule is lost (Nakazawa et al 2007). This indicates that SAS-6 is required to form the cartwheel structure of the centriolar precursor that gives centrioles, basal bodies and cilia their nine fold radial symmetry.

In *Caenorhabditis elegans* SAS-5 is also recruited at this stage and is thought to be required for formation of the central tube (Pelletier et al 2006, Dellatre et al 2006). In *Drosophila* anastral spindle 2 (Ana2) is thought to act with Sas-6 as overexpression of both *sas6* and *ana2* together in *Drosophila* spermatocytes resulted in the formation of structures that resembled part of the cartwheel (Stevens et al 2010).
This indicates a conserved role for SAS-6 in the generation of a centriolar precursor, be that with SAS-5 to form a central tube in *Caenorhabditis elegans* or with Ana2 to form a cartwheel in *Drosophila* (Pelletier et al 2006, Stevens et al 2010).

Following the formation of the central tube in *Caenorhabditis elegans* centriole formation, SAS-4 is next recruited and is required to attach the nine microtubule singlets (Pelletier et al 2006). *sas4* mutant *Drosophila* have disrupted centriole duplication such that by late larval stages no centrioles remain (Basto et al 2006). However adult flies are viable and lack both sperm flagella and cilia from sensory neurons (Basto et al 2006).

BLD10 is thought to have a role in cartwheel formation in *Chlamydomonas* (Hiraki et al 2007). BLD10 forms part of the cartwheel spokes and plays a role in the binding of the cartwheel to microtubules (Hiraki et al 2007). CEP135, the human homologue of BLD10, is required for centriole duplication in human U2OS cells and interacts with both SAS-6 and microtubules indicating a potentially similar function (Lin et al 2013). However in *Drosophila* mutation of *bld10* does not affect cartwheel formation and instead it is thought to have a role nucleating the central microtubule pair (Carvalho-Santos et al 2012). As a result the axonemes of *bld10* mutant sperm cells lack the central pair of microtubules (Mottier-Pavie & Megraw 2009).

The basal bodies of primary cilia form from pre-existing centrioles however in multi-ciliated cells the synthesis of many additional centrioles is required (Sorokin 1962, Sorokin 1968, Anderson & Brenner 1971). In multi-ciliated tissues centrioles have been observed forming either by a centriolar pathway, where many centrioles form around a pre-existing centriole or an acentriolar pathway where they form from an electron dense structure known as the deutrosome (Sorokin 1968, Anderson & Brenner 1971). After centriole formation the next step in ciliogenesis in single ciliated and multi-ciliated cells is the migration to and docking with the cell membrane (Sorokin 1962, Sorokin 1968).
1.1.2 Basal body docking and migration

In order for the basal body to be able to dock to the plasma membrane it needs to migrate from either its point of synthesis or its position within the cell. Dawe et al (2009) identified that centrioles failed to migrate in mouse Inner Medullar Collecting Duct (IMCD-3) cells when the either Mks1 or Tmem67 (also known as Mks3 or Meckelin) were knocked down by siRNA. TMEM67 has been found to interact with the actin binding proteins spectrin repeat containing nuclear envelope 2 (SYNE) (Dawe et al 2009) and filamin-A (FLNA) (Adams et al 2012). Actin enrichment at the apical membrane has also been shown to be required for basal body docking and ciliogenesis in mouse tracheal epithelial cells (mTEC) (Pan et al 2007). In both mouse inner medullary collecting duct (IMCD3) cells where Syne has been knocked down by siRNA (Dawe et al 2009) and in the neuroepithelium of Flna mutant mouse embryos (Adams et al 2012) disruption of centriolar migration to the apical membrane was observed; this further indicates that manipulation of the actin cytoskeleton has a role this process (Dawe et al 2009). Mutations in TMEM67 have been found in patients with Meckel syndrome (MKS), a lethal ciliopathy characterised by renal cystic dysplasia, polydactyly and central nervous system defects (Smith et al 2006). Adams et al (2012) identified a patient with an MKS-like ciliopathy that had a mutation in a region of TMEM67 that could disrupt the interaction with FLNA, implicating actin remodeling in the pathology of this disease.

Basal bodies can either directly dock to the plasma membrane following migration (Sorokin 1968) or first bind to an intracellular vesicle at the centrioles distal tip (Sorokin 1962, Sorokin 1968). Sorokin (1962) observed in a number of mammalian and chicken fibroblasts and smooth muscle cells that a vesicle binds the distal tip of the mother centriole generating a ciliary bud. The axoneme is then extended into the ciliary vesicle such that it generates a sheath around the growing axoneme (Sorokin 1962). The ciliary vesicle can then fuse with the cell’s plasma membrane allowing the protrusion of the cilium from the cell surface whilst enclosed within the ciliary membrane (Sorokin 1962). In multi-ciliated tissues, such as the rat respiratory epithelium, centrioles have been observed without a ciliary sheath indicating that they can fuse directly with the plasma membrane (Sorokin 1968). However Sorokin
also observed undocked centrioles with small vesicles at their distal tips indicating that vesicular docking could also be occurring in multi-ciliated epithelia.

Vesicle or membrane docking occurs at the distal appendages of the mother centriole (Schmidt et al 2012). It is the mother centriole that forms the cilium as it takes more than one cell cycle for the centriole to mature and these appendages to form (Vorobjev & Chentsov 1982). CEP164 is a component of the distal appendages which has been found to be required for primary cilium formation and ciliary vesicle docking in human retinal pigment epithelial (RPE) cells (Graser et al 2007, Schmidt et al 2012). Schmidt et al (2012) found that CEP164 interacts with the guanine nucleotide exchange factor RABIN8 and the small GTPase RAB8, which are both implicated in vesicular transport, to mediate vesicular docking to the mother centriole in these cells. Consistent with an important role for CEP164 in ciliary formation, patients with a mutation in CEP164 have been found to suffer from a Nephronophthisis-related ciliopathy with symptoms including cystic kidneys and retinal degeneration (Chaki et al 2012). The role of CEP164 is conserved in other vertebrates as morpholino mediated knock down of cep164 in zebrafish resulted in a number of cilia related defects including cystic kidneys, hydrocephalus, retinal degeneration and the disruption of left-right body axis (Chaki et al 2012). The mechanism of basal body docking in Drosophila has not been characterised, however CG9170, the homologue of CEP164 has been found to be enriched in developing chordotonal neurons, one of the few ciliated cells types in Drosophila (Cachero et al 2011, zur Lage & Jarman unpublished).

CCDC41 is a further distal appendage component that is required for ciliary vesicle docking and therefore ciliogenesis (Joo et al 2013, Tanos et al 2013). Joo et al (2013) found that a knock down of CCDC41 and the intraflagellar transport gene IFT20 resulted in a reduction in ciliary vesicle docking in human RPE cells. Joo et al (2013) also reported that in these cells CCDC41 co-localises with IFT20 both at the mother centriole and the Golgi, indicating a possible role of both these proteins in the recruitment of Golgi vesicles to the mother centriole. CCDC41 is not conserved in Drosophila, however morpholino mediated knockdown of ccdc41 in zebrafish
resulted in a reduction in olfactory cilia formation indicating that this role for CCDC41 is conserved in other vertebrates (Joo et al 2013).

TALPID3 is a centrosomal protein implicated in the regulation of both basal body docking and migration (Yin et al 2009, Stephen et al 2013). In TALPID3 mutant chicken embryos a number of defects associated with disrupted ciliogenesis have been observed, including cystic kidneys and phenotypes associated with disrupted HH signalling such as polydactyly and neural tube defects (Davey et al 2006, Yin et al 2009). talpid\textsuperscript{3} embryos displayed a loss of both primary (Yin et al 2009) and motile (Stephen et al 2013) cilia. In talpid\textsuperscript{3} neural tube cells basal bodies were misorientated, lacked ciliary vesicles and failed to dock with the cell membrane resulting in a lack of cilium formation (Yin et al 2009). In addition the organisation of actin near the apical membranes was also disrupted (Yin et al 2009). Furthermore centrioles in the normally multi-ciliated choroid plexus cells of talpid\textsuperscript{3} embryos failed to migrate to the apical cell membrane (Stephen et al 2013). This implicates TALPID3 in both vesicular docking and basal body migration or orientation possibly via an interaction with the actin cytoskeleton (Yin et al 2009). The role for TALPID3 in ciliogenesis is conserved amongst vertebrates as Talpid3 mutant mice and talpid\textsuperscript{3} morpholino knock down zebrafish both display phenotypes consistent with disrupted ciliogenesis (Bangs et al 2011, Ben et al 2011). This is however no homologue of TALPID3 in either Drosophila or Caenorhabditis elegans (Davey et al 2006).

The formation of a centriole, its migration to the plasma membrane and docking to form a basal body represent some of the first steps in ciliogenesis. Disruption of genes implicated in this process results in a lack of ciliogenesis and can cause a range of defects associated ciliary loss, which in humans can manifest as ciliopathies (Smith et al 2006, Chaki et al 2012). The process of Drosophila centriole migration and docking has not been characterised, the presence of Drosophila homologues of some of the proteins involved in this process in vertebrates indicates that there may be similarities, however the lack of homologues to several others indicates that there are likely major differences.
1.1.3 Intraflagellar Transport

The cilium is extended by the addition of tubulin to the distal tip of the axoneme (Marshall & Rosenbaum 2001). Tubulin is transported to the tip by a process known as intraflagellar transport, a mechanism that is also required to transport other ciliary proteins within the cilium (Marshall & Rosenbaum 2001, Qin et al 2004). Kozminski et al (1993) first identified intraflagellar transport (IFT) in *Chlamydomonas* flagella, where the movement of particles along the flagellum between the axoneme and the ciliary membrane was observed. Anterograde IFT is driven by a kinesin motor, which with the IFTB complex of proteins allows the transport of ciliary cargos to the distal tip of the cilium (Kozminski et al 1995, Cole et al 1998). A dynein motor drives retrograde transport back down the cilium and with the IFTA complex of proteins allows transport back to the ciliary base (Cole et al 1998, Pazour et al 1998).

IFT proteins are well conserved in ciliated organisms and are required to construct and maintain most cilia, with the exception of the *Drosophila* flagellum and *Plasmodium falciparum* flagella (Han et al 2003, Briggs et al 2004, Sarpal et al 2003).

1.1.3.1 Anterograde IFT

Kozminski et al (1995) identified that the kinesin component *FLA10* was required for anterograde transport in *Chlamydomonas* flagella. Cole et al (1998) found that *FLA10* was part of a heterotrimeric kinesin II. In *Drosophila* heterotrimeric kinesin II is composed of kinesin-like protein at 64D (KLP64D), kinesin-like protein at 68D (KLP68D) and kinesin associated protein (KAP) (Sarpal et al 2003). Mutation of *klp64D* or *kap* has been found to result in complete loss of *Drosophila* sensory cilia axonemes (Sarpal et al 2003). Furthermore in *klp64D* mutant flies α-tubulin (α1Tub84B) cannot enter the cilium of *Drosophila* sensory neurons and accumulates at the ciliary base (Avidor-Reiss et al 2004). Mouse in which *Kif3a* (the mouse homologue of *klp64D*) is mutated displayed a loss of cilia indicating that its requirement for ciliogenesis is conserved (Marszalek et al 1999).

In bipartite cilia, which contain microtubule doublet and singlet regions, a homodimeric kinesin has also been found to drive IFT (Snow et al 2004). In
Caenorhabditis elegans sensory neurons the homodimeric kinesin OSM-3 has been found to be required for ciliary formation (Snow et al 2004) OSM-3 acts redundantly with heterotrimeric kinesin II in the proximal region of the cilia but then acts alone for transport along the distal microtubule singlet region (Snow et al 2004). Homodimeric kinesin has also been detected in vertebrates; Kif17 has been found to colocalise with IFT proteins in zebrafish photoreceptors and be required for the formation of the photoreceptor outer segment (Insinna et al 2008).

Kinesin motors do not however transport cargos by IFT alone, the IFTB complex of proteins are also required for anterograde transport towards the ciliary tip (Pazour et al 2002). The Chlamydomonas IFTB complex was elucidated by Cole et al (1998) and found to contain a number of proteins (IFT172, IFT88, IFT81, IFT80, IFT74/72, IFT57/55, IFT52, IFT46, IFT27 and IFT20). Follit et al (2009) identified the mouse homologues to the Chlamydomonas IFT-B proteins and found that they too formed a complex in mouse IMCD3 cells and localised to the cilium. In addition Follit et al (2009) also identified the IFTB complex to contain the novel component IFT25. IFT54, IFT70 and IFT22, the mouse homologues of IFT proteins previously identified in Caenorhabditis elegans (DYF-11, DYF-1 and IFTA-2) were also found to be part of the mouse IFTB complex (Ou et al 2005, Schafer et al 2006, Li et al 2008, Follit et al 2009). The IFTB complex is well conserved with humans and Chlamydomonas both having homologues of all known IFTB proteins (Van Dam et al 2013). Caenorhabditis elegans also contains homologues to all IFTB complex proteins except IFT25 and IFT27 (Van Dam et al 2013).

There are Drosophila homologues to all IFTB complex proteins except IFT81, IFT74, IFT27, IFT25 and IFT22 (Van Dam et al 2013). Oseg2 and Oseg5, the Drosophila homologues of IFT172 and IFT80 have been found to localise to the ciliary base and the axoneme (Avidor-Reiss et al 2004). In flies mutant for oseg2 α-tubulin cannot enter the cilium consistent with Oseg2 being part of the Drosophila IFTB complex (Avidor-Reiss et al 2004). This also highlights that the IFTB complex is required to transport tubulin along the axoneme for ciliary formation and maintenance (Avidor-Reidss et al 2004).
As the IFTB complex is required for anterograde IFT, mutation of its components results in either the loss or shortening of cilia (Pazour et al 2000, Han et al 2003). Pazour et al (2000) demonstrated that mutation of IFT88 results in the loss of flagella from *Chlamydomonas* and that mutation of Ift88 causes a shortening of cilia in mouse kidneys. Mutation of *nompB*, the *Drosophila* homologue of IFT88, also results in the loss and deformation of sensory neurons cilia (Han et al 2003). This indicates that in *Drosophila*, as in mammals and *Chlamydomonas*, IFT is required for the formation of the ciliary axoneme (Han et al 2003).

IFT is also required to transport ciliary proteins and complexes inside the ciliary compartment (Qin et al 2004, Hou et al 2007). Qin et al (2004) identified that the precursors of the radial spokes are transported as cargo by IFT before assembly on the *Chlamydomonas* flagella. Hou et al (2007) also showed that *Chlamydomonas* where *IFT46* was mutated had a less stable IFTB complex and that the outer axonemal dynein arms were mislocalised indicating that these components of the cilium motility machinery require IFT for their proper localisation.

Follit et al (2006) found that an IFT protein may also have a role in transport to the cilium. IFT20, an IFTB component, was found to localise to the Golgi complex in mouse IMCD3 cells, as well as localising to the cilium and ciliary base, and could be observed trafficking in pig kidney epithelial cells (LLC-PK1) (Follit et al 2006). This indicates a possible role in vesicular trafficking for IFT20 (Follit et al 2006). Additionally whilst a strong RNAi mediated knockdown of *IFT20* caused a loss of cilia, a weaker knock down caused a reduction in the amount of the membrane protein polycystin-2 (PC-2), also known as polycystic kidney disease 2 (PKD2), in the ciliary compartment (Follit et al 2006). This indicated that IFT20 had a role in the trafficking of membrane proteins to the cilium (Follit et al 2006).

**1.1.3.2 Retrograde IFT**

A dynein motor is required for driving retrograde IFT rafts back to the ciliary base (Pazour et al 1998). *Chlamydomonas* with mutations in the dynein light chain LC8 or the dyenin heavy chain DHC1b have short flagella that accumulate material at their distal tips consistent with a lack of retrograde transport (Pazour et al 1998, Porter et
al 1999). Dynein motors have also been found to drive retrograde IFT in *Caenorhabditis elegans* and mouse embryos; mutation of dynein motor components in these organisms results in short swollen cilia consistent with defective retrograde transport but normal anterograde transport (Signor et al 1999, Schafer et al 2003, May et al 2005). Mutation of the *Drosophila* dynein *beethoven (btv)* (the homologue of *DYNC2H1*) results in the accumulation of material in the distal ciliary compartment and loss of IFTA protein (Outer segment 1 (Oseg1)) from the cilium indicating that it could be required for retrograde transport (Eberl et al 2000, Lee et al 2008).

The IFTA complex of proteins acts with the dyenin motor to allow retrograde transport (Lee et al 2008, Iomini et al 2009). As IFTA proteins are necessary for retrograde but not anterograde IFT their mutation results in short cilia with bulges of IFTB proteins at their distal tip (Lee et al 2008, Iomini et al 2009). Cole et al (1998) identified the Chlamydomonas IFTA complex to contain IFT144, IFT140, IFT139 and IFT22. In addition to these IFT121 and IFT43 have also been identified (Piperno et al 1998, Blacque et al 2006). All of the six IFTA proteins are conserved in humans and Chlamydomonas, however *Caenorhabditis elegans* lack a homologue of IFT43 and *Drosophila* lack a homologue of IFT139 (Van Dam et al 2013).

Avidor-Reiss et al (2004) found that Oseg1, RempA, Oseg4 and Oseg6 (the *Drosophila* homologues of IFT122, IFT140, IFT121 and IFT144) all localise to the cilium and ciliary base in ciliated sensory neurons. Flies in which *oseg1* was mutated show a disrupted ciliary structure but could still transport α-tubulin to the ciliary tip, consistent with a role in retrograde transport rather than anterograde transport (Avidor-Reiss et al 2004). *rempA* mutant flies also indicate such a role as sensory neuron cilia are shorter than wild type and show accumulated IFTB proteins at the distal ciliary tip (Lee et al 2008). This is a common phenotype of disrupting components of the IFTA complex and is caused by the mutation of IFTA components in other organisms (Iomini et al 2009). Mutation of *IFT139* or *IFT144* in Chlamydomonas also results in the accumulation of IFTB proteins at their distal tip (Iomini et al 2009). IFT is thought to be responsible for the transport of membrane proteins as well as intraflagellar cargos (Qin et al 2005). The *Caenorhabditis elegans*
transient receptor potential vanilloid (TRPV) channels OSM-9 and OCR-2 were transported along the cilium at the same rate as IFT in in mutants of *daf-10* (homologue of *DYNCHI*) and the IFTA component *che-11* (homologue of *IFT122*) their ciliary localisation was disrupted (Qin et al 2005).

IFT is a well conserved transport mechanism specialized to transport cargo along cilia and flagella. Disruption of its components leads to not only disrupted ciliary structure but also disrupted localisation of the factors required for ciliary function.

### 1.1.4 Transport to the cilium – The BBSome

As well as being transported along the cilium by IFT, ciliary proteins also require transport to the ciliary base. The BBSome is a complex of proteins that is required for the trafficking of vesicles and membrane associated proteins into the ciliary compartment (Nachury et al 2007). Nachury et al (2007) first identified a complex containing Bardet-Biedl syndrome protein 1 (BBS1), BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9 in human retinal pigment epithelial (RPE) cells and mouse testis extract. Loktev et al (2008) later found that BBSome interacting protein of 10kDa (BBIP10) was also part of this complex. Nachury et al (2007) found that in RPE cells the BBSome associates with the guanine nucleotide exchange factor RABIN8 and via the GTPase RAB8 facilitates the docking and fusion of vesicles to the ciliary compartment. Jin et al (2010) observed that the BBSome components from bovine retinal extract interacted with ADP-ribosylation factor-like 6 (ARL6) and that GTP bound ARL6 and the BBSome could form a coat complex on synthetic liposomes *in vitro*. Both ARL6 and BBSome components were found to be required for the trafficking of membrane associated proteins to the ciliary membrane (Jin et al 2010). In the cultured hippocampal neurons of *Bbs2* and *Bbs4* mutant mice and cultured hippocampal neurons where *Arl6* has been knocked down by shRNA the ciliary localisation of the G protein coupled receptor somatostatin receptor 3 (SSTR3) is disrupted (Berbari et al 2008, Jin et al 2010). Taken together this suggests that the BBSome could form a coat complex on vesicles and membrane associated proteins to regulate their trafficking to the cilium (Nanchury et al 2007, Jin et al 2010).
Mutations in the BBSome components BBS1 (Mykytyn et al 2002), BBS2 (Nishimura et al 2001), BBS4 (Mykytyn et al 2001), BBS5 (Li et al 2004), BBS7 (Badano et al 2003), BBS8 (Ansley et al 2003), BBS9 (Nishimura et al 2005) and BBIP10 (Scheidecker et al 2014), as well as in ARL6 (Chiang et al 2004) have been found in patients with Bardet-Biedl syndrome (BBS); a ciliopathy with symptoms including obesity, kidney cysts, learning difficulties, polydactyly, anosmia and retinal degeneration. Mouse models of BBS where Bbs2 or Bbs4 are mutated display similar symptoms including obesity, retinal degeneration, renal cysts, olfactory defects and defects in social interaction (Mykytyn et al 2004, Nishimura et al 2004). In most tissues in these mutant mice cilia could still form, indicating that the BBSome’s role in trafficking to the cilium is likely to be responsible for these cilia-linked phenotypes rather than a more general ciliogenesis defect (Mykytyn et al 2004, Nishimura et al 2004). Many of the observed phenotypes of BBS can be explained by the loss of ciliary localisation of membrane proteins (Nishimura et al 2004, Loktev & Jackson 2013). For example Bbip10 mutant mice are obese and display a loss of ciliary localisation for the G protein coupled receptor neuropeptide Y (NPY) in the hypothalamic neurons that regulate food intake (Loktev & Jackson 2013). In Bbs2 mutant mice photoreceptors rhodopsin (RHO) was also mislocalised prior retinal degeneration (Nishimura et al 2004). The mutation of a component of the BBSome can therefore result in the mislocalisation of membrane bound proteins and cause defects in ciliated cell function (Nishimura et al 2004, Loktev & Jackson et al 2013). Even if a cilium appears to have formed normally, if it lacks the components necessary for its biological role then its function may be disrupted and defects can occur.

BBS1, BBS4, BBS5, BBS8 and BBS9 are conserved in Drosophila (Hodges et al 2010). Whilst the Drosophila BBSome has not been characterised, it was found that GAL4 driven from the upstream regulatory regions of the Drosophila homologues of BBS1, BBS4, BBS5 and BBS8 could cause UAS-GFP to be expressed in ciliated sensory neurons, indicating that their role could be conserved (Avidor-Reiss et al 2004). Homologues to all of the BBSome proteins have been identified in Chlamydomonas and a BBSome like complex containing BBS1, BBS4, BBS5, BBS7 and BBS8 has been identified (Lechteck et al 2009). Membrane-associated proteins
linked to phototactic behavior were abnormally accumulated in bbs1, bbs4 and bbs7 flagella, indicating that in Chlamydomonas the BBSome also acts to remove signaling proteins from the flagella (Lechtreck et al 2009). All of the components of the BBSome are also conserved in Caenorhabditis elegans and bbs-1, bbs-2, bbs-7 and bbs-8 have been shown to be expressed in the ciliated chemosensory neurons (Ansley et al 2003, Hodges et al 2010). bbs-7 and bbs-8 Caenorhabditis elegans mutants displayed a disrupted ciliary structure and chemotactic behavioral defects (Blacque et al 2004).

BBSome components have also been observed being transported by IFT through the cilium (Ou et al 2005, Nanchury et al 2007, Lechtreck et al 2009). BBS-7 and BBS-8 have been found to coordinate IFT complexes transported by two different kinesin motors in Caenorhabditis elegans sensory neuron cilia (Ou et al 2005). BBS4 moves at rates consistent with transport by IFT in human RPE cell cilia (Nanchury et al 2007) and in Chlamydomonas flagella BBS4 was been observed on a subset of IFT complexes (Lechtreck et al 2009). This indicates that the transportation of BBSome components by IFT is conserved and may constitute an important part of its function. However whilst in Caenorhabditus elegans bbs-7 and bbs-8 mutants IFT was disrupted, in BBS4 mutant Chlamydomonas IFT was found to proceed normally, indicating that there are difference in the role of the BBSome proteins for IFT (Blacque et al 2004, Lechtreck et al 2009).

Mutations in BBS patients have also been found in BBS6 (Katsanis et al 2000), BBS10 (Stoetzel et al 2006) and BBS12 (Stoetzel et al 2007). However these are not part of the BBSome but were observed to form a complex with chaperonin containing TCP-1 (CCT) chaperonin proteins in human embryonic kidney (HEK293T) cells, as well as mouse eye and testis extract (Seo et al 2010). In both HEK293T cells where BBS10 or BBS12 were knocked down by siRNA and Bbs6 mutant mouse eye and testis extracts BBSome assembly was disrupted indicating that this complex is required for the assembly of the BBSome (Seo et al 2010). This highlights that an important part of ciliogenesis is assembling the protein complexes that are required for ciliary formation and function and that disruption of this assembly process can result in human ciliopathies. BBS6, BBS10 and BBS12 are not
conserved in *Drosophila*, *Caenorhabditis elegans* and *Chlamydomonas* indicating that different mechanisms must be required for the assembly of the BBSome in these organisms (Hodges et al 2010).

BBS4 was observed to localise to the centriolar satellites as well as the cilium in human RPE cells (Nanchury et al 2007). The centriolar satellites are cytoplasmic granules that are proposed to play a role cytoplasmic trafficking of proteins to basal bodies and centrosomes (Kubo et al 1999, Dammermann & Merdes 2002). Kubo et al (1999) observed Pericentriolar material 1 (Pcm1) containing centriolar satellites isolated from *Xenopus* epithelial A6 cells moving along microtubules *in vitro*. Nachury et al (2007) found that knocking down PCM1 by siRNA resulted in a reduction of the number of cells that were ciliated indicating a role for PCM1 and the centriolar satellites in ciliogenesis. Other ciliary proteins have also been observed localising the centriolar satellites including Centrosomal protein of 290KDa (CEP290) and 5-azacytidine induced 1 (AZI1) (also known as CEP131), which are both also found to localise to the transition zone (Kim et al 2008, Hall et al 2013). Hall et al (2013) observed AZI1 trafficking along microtubules towards and away from the centrosome in mouse fibroblast (NIH-3T3) cells further implicating the centriolar satellites in the transport of ciliary proteins. PCM1 is not conserved in *Drosophila* indicating that this mechanism of transport may also not be conserved (Hodges et al 2010).

**1.1.5 The ciliary gate**

As shown by proteomic studies of cilia and flagella, the protein population within the ciliary compartment is different to that in the rest of the cell (Ostrowski et al 2002, Pazour et al 2005). This is maintained by a ciliary gate at the base of the cilium which is composed of transition fibres, which link the basal body to the membrane, and the transition zone, at which the axoneme is linked to the membrane via Y-links (Ringo 1967, Anderson 1972, Craige et al 2010, Williams et al 2011). The transition fibres form from the distal appendages of the mother centriole (Tateishi et al 2013). Deane et al (2001) identified that IFT52 could localise to the transition fibres in *Chlamydomonas* flagella suggesting that they act as a docking complex for IFT complexes before ciliary transport. DYF-19, the *Caenorhabditis elegans* homologue
of Fas (TNFRSF6) -binding factor 1 (FBF1), has been found to localise to the transition fibres of *Caenorhabditis elegans* cilia and is required for the ciliary entry of IFTA and BBS proteins (Wei et al 2013). This role was also conserved in mammalian cells as whilst the IFTB protein IFT88 could enter the cilium of human RPE cells in which *FBF1* had been knocked down, the IFTA protein IFT140 could not (Wei et al 2013). The transition fibres therefore form a docking site for the IFT proteins.

Distal to the transition fibres is the transition zone, which is located at the base of the cilium where the microtubule triplets of the basal body meet the microtubule doublets of the axoneme (Ringo 1967). Craige et al (2010) found that mutation of *CEP290* in *Chlamydomonas* resulted in the loss of Y-link structures that project from the microtubules of the transition zone, attaching the microtubule doublets to the ciliary membrane. It was also observed that the loss of the Y links coincided with the dysregulation of the levels of ciliary proteins within the *CEP290* mutant *Chlamydomonas* flagellum (Craige et al 2010). This finding indicates the important role of the Y links in maintaining the ciliary gate.

Two redundant modules of proteins that localise at the transition zone of *Caenorhabditis elegans* sensory cilia have been identified and are required for the maintenance of the ciliary gate and association of the ciliary membrane with the axoneme (Williams et al 2008, Williams et al 2011). The Meckel Syndrome (MKS) module contains the *Caenorhabditis elegans* homologues of Meckel syndrome 1 (MKS1), B9 protein domain 1 (B9D1), B9D2, transmembrane protein 67 (TMEM67) and coiled-coil and C2 domain containing 2A (CC2D2A); MKS-1, Meckel syndrome 1 related 1 (MKSR-1), MKSR-2, MKS-3 and MKS-6 in *Caenorhabditis elegans* (Williams et al 2008, Williams et al 2011). The Nephronophthisis (NPHP) module contained nephronophthisis 1 (NPHP-1) and NPHP-4, whilst MKS-5 (the *Caenorhabditis elegans* homologue of RPGR interacting protein 1 like (RPGRIP1L)) is thought to link both modules (Williams et al 2008, Williams et al 2011).

Mutation of a gene from both modules resulted in a loss of Y links (double mutants of mks-1, mks-3, mks-5, mks-6 or mksr-1 with nphp-4) and dissociation of the ciliary membrane with the transition zone (double mutants of mks-5, mks-6 or mksr-1 with
nphp-4) (Williams et al. 2011). *Caenorhabditis elegans* with individual mutations in mksr-1, mksr-2, mks-5, mks-6, nphp-1 or nphp-4 also caused abnormal protein accumulation within the cilium, highlighting the importance of the transition zone in the function of the ciliary gate (Williams et al. 2011).

Transition zone protein modules have also been identified in mammals (Garcia-Gonzalo et al. 2011, Sang et al. 2011). Sang et al. (2011) used proteomic approaches in mouse fibroblast (NIH-3T3), mouse inner medullary collecting duct (IMCD3) and human RPE cells to identify modules of interacting proteins. One such module containing NPHP1, NPHP4 and RPGRIPL1 was found to localise to the transition zone in mouse IMCD3 cells (Sang et al. 2011). A MKS1 containing module was also identified however it was thought to localise to the ciliary base rather than the transition zone (Sang et al. 2011). Garcia-Gonzalo et al. (2011) identified a complex of proteins from NIH-3T3 cells and COS cells (derived from African Green monkey kidney) which contained homologues to several of the proteins of the *Caenorhabditis elegans* MKS module (MKS1, TMEM67, B9D1, CC2D2A), as well as proteins not previously identified in either *Caenorhabditis elegans* transition zone module (including TMEM216, CEP290, tectonic family member 1 (TCTN1)). The components of this complex were found to localise to the transition zone of human RPE cells (Garcia-Gonzalo et al. 2011). Mouse embryonic fibroblasts from Tctn1 and Cc2d2a mutant mice showed a loss of the ciliary localisation of adenylyl cyclase III (AC3) and polycystic kidney disease 2 (PKD2) (Garcia-Gonzalo et al. 2011). This indicates that, as in *Caenorhabditis elegans*, mammalian transition zone modules can act as part of the ciliary gate (Garcia-Gonzalo et al. 2011).

Further evidence that transition zone proteins are vital for ciliary function comes from their mutation in human ciliopathies. Mutations in NPHP1 (Hildebrandt et al. 1997), NPHP4 (Mollet et al. 2002) and TMEM67 (Otto et al. 2009) have all been found in patients with Nephronopthisis, a ciliopathy characterised by the formation of kidney cysts. Mutations in MKS1 (Kyttala et al. 2006), TMEM67 (Smith et al. 2006), CEP290 (Baala et al. 2007), RPGRIPL1 (Delous et al. 2007), CC2D2A (Tallila et al. 2008), TMEM216 (Valente et al. 2010), B9D1 (Hopp et al. 2011) and B9D2 (Dowdle et al. 2011) and have all been found in to be mutated in Meckel syndrome.
That mutation of many components of the transition zone result in human disease indicates the importance of maintaining and regulating the ciliary gate. In order for a cilium to be functional the cellular machinery required for its function need to be allowed to pass through the ciliary gate into the cilium and maintained within the ciliary compartment.

The composition of the transition zone in *Drosophila* is not known, however a number of candidate genes have been proposed (Baker et al 2004, Ma & Jarman 2011, Enjolras et al 2012). Homologues to several of the MKS module proteins have been identified in *Drosophila* and CG14870, the *Drosophila* homologue of B9D1, has been found to localise to the distal basal body suggesting that the function of the MKS module could be conserved (Hodges et al 2010, Enjolras et al 2012). The NPHP module proteins are not conserved in *Drosophila*, indicating a major difference in the *Drosophila* transition zone (Hodges et al 2010). Chibby (Cby), Uncoordinated (Unc) and Dilatory (Dila) have all been suggested as putative transition zone proteins due to their localisation at the distal basal body (Baker et al 2004, Ma & Jarman 2011, Enjolras et al 2012). Whether ciliary protein localisation is altered in *unc* mutant flies is not known, however it has been found that in *cby* mutant flies NompB accumulates in the sensory cilia, whilst the normally ciliary localised CG11356 (the *Drosophila* homologue of ARL13B) is lost from the cilium (Enjolras et al 2012). Additionally in *dila* mutant flies the HRP labelled glycoproteins that normally localise at the luminal bands are mislocalised (Ma & Jarman 2011). *dila* and *unc* have been shown to genetically interact suggesting that they have a similar function, therefore Cby, Dila and Unc could make up part of the *Drosophila* transition zone (Ma & Jarman 2011, Enjolras et al 2012).

The vertebrate homologues of Dila and Cby (AZI1 and CBY1) have also been implicated in ciliogenesis (Enjolras et al 2012, Steere et al 2012). CBY1 localises to the transition zone of mouse IMCD3 cells and the distal end of the mother centriole in HeLa and RPE1 cells indicating that it could have a role in the ciliary gate of vertebrate cilia (Enjolras et al 2012, Steere et al 2012). AZI1 has been observed in the transition zone of human RPE1 cells, indicating that it too could have a conserved transition zone role (Hall et al 2013).
Ciliogenesis involves a number of processes; from building the general structure of the cilium by basal body docking and the extension of an axoneme, to transporting, assembling and allowing ciliary access to the functional components of the cilium, including signaling proteins or the cilium motility machinery. Disrupting these processes leads to a number of different phenotypes in different organisms, these ciliary phenotypes reflect the various roles that cilia play.

1.2 The roles and functions of cilia

Cilia have historically been categorised as having motile or sensory/primary roles. Motile cilia, with a few exceptions, have a central microtubule pair (9+2) and components of the cilium motility machinery to allow ciliary bending. Primary cilia are usually immotile and lack a central pair (9+0). However there are exceptions to this rule, such as the cilia of the embryonic node, which lack a central pair but remain motile (Nonaka et al 1998). Motile cilia and flagella have been utilised for locomotion by small organisms and for the transport of fluid on several vertebrate epithelia such as the multiciliated respiratory epithelia (Ringo 1967, Afzelius 1976). Immotile or primary cilia cannot beat as they lack the ciliary motility machinery however they are utilised as signalling hubs for sensory signal transduction and signalling pathways (Huangfu et al 2003, Kulaga et al 2004). The sensory and motile properties of cilia are not mutually exclusive (Huang et al 2007, Shah et al 2009). The flagella of Chlamydomonas are motile and utilised for movement but also have sensory roles during mating (Huang et al 2007). The motile cilia of human respiratory epithelium also have the ability to be sensory, the chemosensory detection of bitter compounds allowing an adjustment in ciliary beat frequency (Shah et al 2009).

1.2.1 Immotile cilia

Primary or sensory cilia are immotile and generally lack both the cilium motility machinery and a central pair. They can provide a specialised compartment for the localisation of components of signalling pathways and as such have a role in several sensory and developmental signalling pathways. Here I shall discuss some of the
roles that immotile cilia can play with a focus on the roles of immotile cilia in vertebrates, which can be disrupted in ciliopathies.

1.2.1.1 Olfactory cilia

Olfactory sensory neurons are multiciliated with the olfactory cilia extending from the dendritic knob into the mucous of the external environment (Menco 1984). Mammalian olfactory cilia are of a 9+2 ultrastructure but lack dynein arms and are thought to be immotile (Menco 1984). However the olfactory cilia of some other vertebrates such as rainbow trout (Salmo gairdneri), green frogs (Rana clamitans), and bull frogs (Rana catesbeiana) retain dynein arms and as such are thought to be motile (Rhein & Cagan 1980, Adamek et al 1984, Reese 1985). Olfactory cilia are compartmentalised with the proximal portion being of a 9+2 ultrastructure and a distal portion containing only a few microtubule, at which most of the olfactory signalling pathway components are thought to localise (Kulaga et al 2004, Flannery et al 2006).

The olfactory sensory neurons detect odorants via G-protein coupled receptors, which via adenylyl cyclase III increase cAMP within the cilium and results in the opening of Cyclic Nucleotide Gated (CNG) channels allowing neuronal depolarisation and signal transduction (Buck & Axel 1991, Brunet et al 1996, Wong et al 2000, Kajiya et al 2001). As the machinery for this signalling pathway is found within the cilium, disruption of ciliary structure reduces the ability of it to function (Kulaga et al 2004, Flannery et al 2006, McIntyre et al 2012). For example mice in which the IFTB complex component Ift88 is mutated lose olfactory cilia, have mislocalised components of the olfactory signalling machinery (adenylyl cyclase III, cyclic nucleotide gated channel α 2 (CNGA2) and the G protein Gγ13) and have been found to be anosmic (McIntyre et al 2012).

As a result of the cilia’s role in olfaction, anosmia is a symptom of syndromic ciliopathies (Kulaga et al 2004). Bardet-Biedl syndrome patients have olfactory defects and mouse models of BBS have disrupted olfactory cilia (Kulaga et al 2004, Tadenev et al 2011). Mutations in Bbs1 and Bbs4 result in mice with disrupted olfactory cilia and mislocalisation of olfactory signalling components such as
adenylyl cyclase III (AC3) (Kulaga et al 2004). Tadenev et al (2011) also reported olfactory defects in Bbs8 mutant mice, which had lost most olfactory cilia from their OSNs.

Disfunction of the transition zone can also result in olfactory defects (McEwen et al 2007). Patients with mutations in the transition zone protein CEP290 display anosmia (McEwen et al 2007). Additionally mice with mutated Cep290 have largely normally structured cilia, however they still display olfactory defects as G proteins required for olfactory signalling are unable to enter the cilium (McEwen et al 2007). These findings indicate that the cilium is vital for olfactory function as a hub for the localisation of the olfactory signalling components. Disruption of transport and entry into the cilium of olfactory signalling elements can result in olfactory defects.

Drosophila olfactory sensory neurons are also ciliated; they have been found to contain three different forms of bipartite cilia which vary in structure from having a singlet diatal region to having an elaborate branched structure (Shanbhag et al 1999, Jana et al 2011). The use of immotile cilia for olfactory processes is therefore widely conserved across ciliated organisms. However there is also clearly diversity amongst olfactory ciliary structures.

**1.2.1.2 Cilia and Obesity**

Obesity is a symptom of the Bardet-Biedl Syndrome ciliopathy suggesting that cilia have a role in the regulation of food intake and obesity (Mykytyn et al 2002).

Davenport et al (2007) have previously shown that knocking out Ift88 in the hypothalamic neurons results in mice with obesity and high leptin levels. Seo et al (2009) had also shown that Bbs2, Bbs4 and Bbs6 mutant mice were unable to respond to leptin signals. It was found that BBS1 physically interacted with the leptin receptor (LEPR) (Seo et al 2009). Thus it was thought that the link between obesity and cilia was via leptin signalling.

However an inability to respond to leptin signalling was recently shown to occur as a result of obesity rather than act as a cause of it (Berbari et al 2013). Bbs4 mutant mice were found to be able to respond to leptin signalling prior to the development
of obesity (Berbari et al 2013). Evidence for an alternate pathway came from the work of Loktev and Jackson (2013) that showed that the neuropeptide Y receptors are trafficked to the cilium by the BBSome and as such may link BBS and obesity. The neuropeptide Y receptors are G protein coupled receptors which localise to the cilium (Loktev & Jackson 2013). The binding of the neuropeptide Y ligand in wild type mice was necessary for the regulation of food intake (Loktev & Jackson 2013). However in Bbip10 mutant mice this could not occur resulting in obesity (Loktev & Jackson 2013). This was also found to be the case in tubby (Tub) mutant mice (Loktev & Jackson 2013) tubby is a ciliary localised factor whose mutation in mice has also previously been linked to obesity (Kleyn et al 1996). Thus the trafficking of G coupled receptors to mouse hypothalamic cilia is necessary for the regulation of food intake. king tubby (ktub), the Drosophila homologue of Tub, is also required for the localisation of TRP channels in Drosophila chordotonal neurons cilia (Park et al 2013). Whilst these neurons are not required for the regulation of food intake it does indicate that the role of Tub in the regulating the ciliary entrance of membrane proteins could be conserved.

1.2.1.3 Cilia and the kidney

Cilia have previously been linked to diseases that result in kidney defects including the formation of kidney cysts; as well as Nephronophthisis, Polycystic Kidney Disease (PKD) is thought to be related to ciliary function (Nauli et al 2003). Ift88 mutant mice, which have shortened cilia, have previously been reported to display kidney cysts (Pazour et al 2000), as do mouse mutants which lack Kif3A in the kidneys (Lin et al 2003). These studies indicate that the cilium is needed for normal kidney development. Autosomal dominant polycystic kidney disease has been found to be caused by mutations in the polycystic kidney disease 1 (PKD1), also known as polycystin-1 and polycystic kidney disease 2 (PKD2), also known as polycystin-2 (Rossetti et al 2001, Magistroni et al 2003). PKD1 is involved in G protein coupled signaling and PKD2 is a calcium channel (Gonzalez-Perrett et al 2001, Delmas et al 2002). The mouse homologues of both localise to the cilia of mouse cortical collecting duct cells (Yoder et al 2002).
Bending of the cilium on Madin-Darby canine kidney (MDCK) cells has previously been shown to induce an increase in intracellular calcium (Praetorius & Spring 2001). Nauli et al (2003) showed that loss of Pkd1 in mouse kidney cells results in the bending of the cilium in response to fluid flow no longer eliciting such an effect. Cells with cilia lacking PKD1 can therefore no longer act as mechanoreceptors. The calcium channel PKD2 was also found to be required for the increase in intracellular calcium in response to ciliary bending (Nauli et al 2003). An inability to sense fluid flow and induce intracellular calcium signaling might therefore result in the formation of kidney cysts.

### 1.2.1.4 The WNT pathway

The Wingless-type MMTV integration site (WNT) signalling pathway has also been linked to cilia (Gerdes et al 2007). In canonical WNT signaling the WNT ligand normally binds to its receptor Frizzled (FZD) causing the release of Dishevelled (DVL) (Bhanot et al 1996, Chen et al 2003, reviewed in Clevers & Nusse 2012). Dishevelled can then block the phosphorylation and resulting degradation of Beta-catenin (reviewed in Clevers & Nusse 2012). Beta-catenin can then accumulate and be transported to the nucleus where it regulates the targets of Wnt signaling via the T-cell factor (TCF) transcription factor (Behrens et al 1996, Molenaar et al 1996, Reviewed in Clevers & Nusse 2012).

An increase in WNT signaling due to beta-catenin stabilisation has been reported in bbs1, bbs4 and bbs6 morphant zebrafish embryos and BBS1, BBS4 and BBS6 knock down human embryonic kidney (HEK) cells (Gerdes et al 2007). An increase in beta-catenin has also been observed in mouse embryonic fibroblast cells from mutants of Kif3A (Corbit et al 2008). These finding indicate that cilia could have a role in the control of WNT signalling.

The bbs1, bbs4 and bbs6 morphant zebrafish displayed defects in convergent extension during gastrulation that appeared similar to those observed in zebrafish with morpholinos targeted against components of the planar cell polarity pathway (Gerdes et al 2007). The planar cell polarity (PCP) pathway is a form of non-canonical (beta-catenin independent) WNT signalling which is required to establish
polarity within the plane of the epithelium (Theisen et al 1994, reviewed in Ezan & Montcouquiol 2013). Ross et al (2005) found that Bbs6 mutant mice shared phenotypes previously observed in mice with mutated PCP genes such as Vangl2 (Montcouquiol et al 2003) including defective neural tube formation and disorganized stereociliary bundles. Ross et al (2005) and Gerdes et al (2007) also identified that there were genetic interactions between PCP genes and BBS genes in both mice and zebrafish. The loss of ciliary genes therefore results in the promotion of the canonical WNT pathway and the suppression of the PCP or non-canonical WNT pathway.

However there is some evidence against a role for the WNT and PCP pathways in cilia. Ocbina et al (2009) reported no PCP defects and no difference in expression in canonical WNT reporters in several ciliary (Kif3A, Ift88, Ift72, Dynch2h1) mutant mouse embryos. Huang & Schier (2009) also reported no PCP defects or changes in the expression of canonical WNT reporters in ift88 deficient zebrafish.

Simons et al (2005) identified the link between cilia and the canonical and non-canonical Wnt signaling pathways. The cilia localised protein Inversin (INVS) acts as a switch between forms of WNT signaling by interacting with and causing the degradation of Dishevelled (Simons et al 2005). Inversin had previously been linked to kidney disease as patients with Nephronophthisis (a ciliopathy resulting in kidney cysts) had been found with mutations in INVS (Otto et al 2003). A loss of PCP could be a cause of cystic kidneys in ciliopathies. Fischer et al (2006) identified that the mitotic orientation of cells was disrupted in Tcf2 mutant mice, a mouse model of polycystic kidney disease, indicating that disrupted PCP could play a role in cyst formation. Simons et al (2005) found that Inversin expression was induced by fluid flow over mouse IMCD cells and Lin et al (2003) also found an increase in β-catenin in the cytoplasm of Kif3A mutant mice with kidney cysts. These findings link the mechanosensory kidney cilia to WNT signalling and demonstrate a mechanism by which cystic kidney disease can occur. A reduction in INV expression or INV localisation due to ciliary loss or disfunction could disrupt the PCP of kidney cells and result in the formation of cysts.
1.2.1.5 The Hedgehog pathway

Primary cilia have been implicated in the Hedgehog (HH) signaling pathway (Huangfu et al 2003). In this pathway the Hedgehog (HH) ligand binds its receptor Patched1 (PTCH1), allowing Smoothened (SMO) to block the degradation of Glioma (GLI) transcription factors to GLI repressors (GLIR) that is promoted by Suppressor of fused (SUFU) (reviewed in Briscoe & Therond 2013). Activated Gli (GLIA) can then enter the nucleus and regulate the transcription of the targets of HH signaling (reviewed in Briscoe & Therond 2013).

Huangfu et al (2003) first identified a link between cilia and the HH signalling pathway when screening for neural tube defects in mice. Two of the mutants generated were as a result of disrupting Ifi172 and Ifi88 (Huangfu et al 2003). These IFT genes were required for the proper formation of the ventral cells of the neural tube, indicating that cilia or at least IFT was required for Hh signalling (Huangfu et al 2003). Huangfu and Anderson (2005) found that a double mouse mutant of Ifi172 and Smo had a weaker phenotype than Smo mutants indicating that the requirement for IFT is also downstream of Smoothened. Huangfu and Anderson (2005) also showed that IFT is required for the proteolytic processing of GLI proteins and that its disruption in mice where Ifi172, Kif3a or Dnchc2 are mutanted leads to patterning defects due the loss of GLIR activity but also neural tube defects due to loss of GLIA activity.

HH signalling is required for patterning of the limb bud; defects in this pathway result in abnormal digit numbers (Masuya et al 1995, Mo et al 1997, Litingtung et al 2002). Liu et al (2005) showed that Ifi88 mutant mice display polydactyly as GLIR cannot be proteolytically processed. However a reduction in SHH target gene expression was also observed indicating that GLIA activity was also disrupted (Liu et al 2005). Haycraft et al (2005) showed that adenoviral administered full length GLI1 (GLIA) and the repressor form of GLI3 (GLIR) could still be active in Ifi88 mutant mouse primary limb bud cells, indicating that SHH signaling defects are due to the proteolytic processing of these components rather than by altered activity.
These findings suggest that IFT is required for the proteolytic processing of GLI to both GLIR and GLIA, IFT is required for this part of the signaling process as it occurs within the cilium (Haycraft et al 2005). GLI2, GLI3 and SUFU have been found to localise to the distal ciliary tip of mouse limb bud cell cilia (Haycraft et al 2005). PTCH1 has also been found within the cilium of mouse embryonic fibroblasts (MEF); ligand binding results in its export and the import of SMO into the ciliary compartment (Rohatgi et al 2007). Jia et al (2009) found that Sufu and Ift88 double mutant mouse embryos results in a rescue of the defective SHH signalling observed in Ift88 mutants. The SHH signalling defects associated with ciliary loss were therefore caused by SUFU repressing the activity of GLI proteins (Jia et al 2009). Sufu and Smo double mutants displayed similar spinal cord patterning defects to Sufu mutants indicating that loss of Smo does not affect SHH signalling without Sufu and that SMO may move into the cilium to remove repression by SUFU (Jia et al 2009).

Parts of the HH signalling pathway therefore occur within the cilium and IFT is required for its function. As IFT is necessary for both GLIA and GLIR processing, the disruption of cilia results in defects associated with both increase and loss of HH signalling. Findings in chicken and zebrafish indicate that the requirement of cilia for HH signaling is not restricted to mammals and is conserved in other vertebrates (Davey et al 2007, Huang & Schier 2009).

1.2.1.6 The cilia and signalling in Drosophila

Despite the involvement of cilia in HH and WNT signalling in vertebrates, these pathways are not thought to always require cilia in Drosophila as flies in which the cilia do not form or are disrupted have not been reported to show any phenotypes associated with these pathways (Han et al 2003, Basto et al 2006, Enjolras et al 2012). However it was recently shown that HH signalling does involve cilia in the ciliated olfactory neurons (Kuzhandaivel et al 2014). Both Smo and Patched (Ptc) could localise to the cilia of olfactory sensory neurons (Kuzhandaivel et al 2014). In addition knock down of oseg2 resulted in a reduction of Smo localisation in the cilia and decreased expression of a Hh target gene (Kuzhandaivel et al 2014). This indicates that Hedgehog signalling can occur both via cilia and independent of cilia in Drosophila.
1.2.2 Motile cilia

1.2.2.1 What is a motile cilium?

A motile cilium is one that can move by a force generated within its own structure. Cilia that are moved by external forces such as the bending of mammalian kidney mechanoreceptor cilia in response to fluid flow are not considered to be motile despite their bending. The cilium motility machinery consists of the axonemal dynein arms and the proteins required for their formation and regulation. Not all of the cilium motility machinery needs to be present for a cilium to be motile. An example of this is the mammalian nodal cilia that lack radial spokes and the central apparatus but are still motile (Nonaka et al 1998). Some form of the motility machinery is needed in order to generate ciliary bending by microtubule sliding.

Although motile cilia often have a 9+2 microtubule arrangement, they do not always and this arrangement is not necessarily indicative of a cilium’s motility. The mammalian nodal cilia have a 9+0 microtubule arrangement and yet are motile (Nonaka et al 1998), whilst mammalian olfactory cilia have a 9+2 microtubule arrangement and are immotile (Menco 1984).

A cilium can therefore be considered motile if it firstly can bend and secondly if there is evidence that this bending occurs due to machinery inside the cilium.

1.2.2.2 Respiratory cilia and PCD

The coordinated movement of motile cilia on the human respiratory epithelium is required for mucociliary clearance, disruption of ciliary beating leads to respiratory problems and a condition known as primary ciliary dyskinesia (PCD) (Afzelius 1976, reviewed in Bush et al 2007, Leigh et al 2009). The symptoms of PCD include respiratory infections, bronchiectasis, sinusitis, otitis media, male and female infertility (due to immotility of sperm flagella and fallopian tube epithelia cilia) and laterality defects (due to immotility of nodal cilia) (Afzelius 1976, Halbert et al 1997). Axonemal ultrastructural defects have been seen in many PCD patients, with various components of the ciliary motility machinery being lost in different patients (Shoemark et al 2012).
A number of genes have been found to be mutated in patients with PCD (Figure 1.2), however known PCD genes can only account for 65% of cases (Austin-Tse et al 2013). The known genes represent a number of components of the ciliary motility machinery. Here I shall discuss both the identity of PCD causing genes and the aspects of the ciliary motility machinery that they affect.

1.2.2.3 Axonemal dynein arms

Cilium motility is possible due to the ATPase action of dynein arm motors allowing the sliding of adjacent microtubule doublets (Summers and Gibbons 1971). Attached to each microtubule doublet of a motile cilium or flagellum are outer dynein arms (ODA), which are thought to control beat frequency, and inner dynein arms (IDA), which regulate the waveform the beat takes (Brokaw & Kamiya 1987). The dynein arms are made up of a number of conserved components including heavy, light, intermediate and light intermediate dynein chains (Pazour et al 2006). The composition of dynein arms along human respiratory cilia is known to differ; ODAs more proximal to the ciliary base contain the heavy chains DNAH5 and DNAH11, whilst those more distal contain DNAH5 and DNAH9 (Fliegauf et al 2005).

A number of PCD genes (genes mutated in PCD patients) had been identified when I began this study. The first gene found to be mutated in PCD patients was the intermediate chain ODA component DNAI1 (Pennarun et al 1999, Guichard et al 2001, Zariwala et al 2001). ODA were lost from the respiratory cilia of PCD patients in which DNAI1 was mutated (Pennarun et al 1999, Guichard et al 2001, Zariwala et al 2001). Mutations in DNAI1 were screened for as its Chlamydomonas homologue IC78 is required for flagella motility (Pennarun et al 1999).

The ODA dynein heavy chain DNAH5 was proposed as a PCD gene by linkage analysis in a family displaying immotile cilia and situs inversus (Omran et al 2000). PCD patients with mutations in DNAH5 were found to lack ODA on their respiratory epithelium cilia (Omran et al 2000, Olbrich et al 2002). Mutations in NME8/TXND3 have also been found in PCD patients that have either short or missing ODA (Duriez et al 2007). NME8/TXND3 is a thioredoxin and is thought to be part of the ODA as it is homologous to IC1, a component of the sea urchin sperm ODA (Ogawa et al 1996). Mutations in the ODA intermediate DNAI2 have also been found in PCD.
**Figure 1.2 The localisation of proteins found to be mutated in PCD**

1. The axonemal ultrastructure of human respiratory epithelial cilia.
2. Components of the cilium motility machinery (onto which the localisation of the proteins encoded by genes mutated in PCD are listed).
3. The localisation of cytoplasmic PCD proteins or those for which the specific axonemal localisation is not known. Figure based on that of Kobayashi & Takeda (2012).
patients (Loges et al 2008). Cilia from these patients displayed ODA defects and lack both DNAH5 and DNAH9 (Loges et al 2008).

Bartoloni et al (2002) identified a potential PCD patient with mutations in the ODA heavy chain DNAH11. Whilst this patient had situs inversus, whether they had PCD was not known as they also had mutations in a causative gene for cystic fibrosis (Bartoloni et al 2002). Schwabe et al (2008) confirmed that this gene could be mutated in PCD, however no defects were observed in the ciliary ultrastructure of these patients. DNAH5 and DNAH9 also localised normally suggesting that much of the dynein arm components were present (Schwabe et al 2008). Schwabe et al (2008) do however report that cilia from these patients have an abnormal stiff hyperkinetic beating pattern. Further mutations in DNAH11 have since been identified in PCD patients with normal ciliary ultrastructure showing that whilst mutating DNAH11 does not visibly disrupt axonemal ultrastructure it is still required for respiratory cilia motility (Pifferi et al 2010, Knowles et al 2012).

In addition to those which were known at the beginning of my own work a further mutation in the ODA light chain DNAL1 has been identified (Mazor et al 2011). Mutations in DNAL1 lead to a partial loss of ODA from respiratory cilia (Mazor et al 2011). Mutations in a number of different ODA components have been found in PCD patients, the mutation of several of these results in the loss or disruption of the entire ODA.

All of the dynein genes thus far found to be mutated in PCD patients are components of the ODA. PCD patients are found with only IDA defects however mutations in IDA genes have yet to be identified (Shoemark et al 2012). Mutations in the IDA heavy chain DNAH1 have been detected in infertile male patients with abnormal sperm flagella ultrastructure (Khelifa et al 2014). However despite DNAH1 being expressed in motile ciliated cells, these patients do not have PCD (Khelifa et al 2014). The lack of IDA PCD mutations and that an IDA mutation does not cause PCD suggests that individual components of the IDA have less of a role in respiratory cilia function than ODA components.
1.2.2.4 Dynein Arm Assembly Factors – DNAAFs

Fowkes and Mitchell (1998) have shown that several dynein heavy and intermediate chains can be co-immunoprecipitated from the cytoplasm of *Chlamydomonas* with an antibody against a single heavy chain. This indicated that parts of the dynein arms were preassembled in the cytoplasm before transport into and onto the cilium. The dynein arm assembly factors (DNAAFs) are thought to form a complex with chaperone proteins to ensure proper formation of preassembled dynein arms (Omran et al 2008). When I began this study *DNAAF1/ODA7/LRRC50* (Freshour et al 2007, Duquesnoy et al 2009, Loges et al 2009) and *DNAAF2/ktu/PF13* (Omran et al 2008) had been identified. During the course of my own investigations two more DNAAFs were discovered; *DNAAF3/PF22* (Mitchison et al 2012) and *DNAAF4/DYX1C1* (Tarkar et al 2013). Mutations all of these genes have been found in PCD patients with loss of both ODA and IDA.

1.2.2.5 DNAAF1

Freshour et al (2007) identified that mutation of *ODA7* in *Chlamydomonas* results in a loss of ODA from the flagella. Loss of *ODA7* also resulted in a lack of preassembly of heavy and intermediate chains in the cytoplasm (Freshour et al 2007). ODA7 can therefore be considered a dynein arm assembly factor. Mutations in *DNAAF1*, the human homologue of *ODA7*, have since been found in PCD patients whose respiratory cilia show a lack of both ODA and IDA (Duquesnoy et al 2009, Loges et al 2009). Duquesnoy et al (2009) showed that whilst ODA7 could enter the cilium it mostly localised to the cytoplasm. This is consistent with a role in cytoplasmic preassembly of dynein arm components.

1.2.2.6 DNAAF2

*DNAAF2*, which encodes a cytoplasmically localised protein, was found to be mutated in PCD patients who lacked ODA and IDA (Omran et al 2008). Cilia from PCD patients with *DNAAF2* mutations lacked DNAH5 and DNAI2 from the distal cilium but retained some in the proximal compartment (Omran et al 2008). In mutants of *PF13*, the *Chlamydomonas* homologue of *DNAAF2*, heavy and intermediate chain complexes could not be coimmunoprecipitated as they can in wild
type *Chlamydomonas* and there was a loss of heavy chains from the cytoplasm (Omran et al 2008). This indicated that DNAAF2 was required for not only the preassembly of dynein arm components but also their stability. Further evidence for this came from a protein-protein interaction found by coimmunoprecipitation and GST-tagged pull down between the mouse homologue of DNAAF2 and HSP70 (Omran et al 2008). This indicated that DNAAFs could aid the stability of dynein arm components during cytoplasmic preassembly via interaction with chaperone proteins.

### 1.2.2.7 DNAAF3

In addition to those known when I begun my own investigations, further DNAAFs have been identified. DNAAF3 is a further cytoplasmically localised factor required for the formation of the both ODA and IDA (Mitchison et al 2012). PCD patients whose respiratory cilia lack both ODA and IDA were identified with mutations in DNAAF3 (Mitchison et al 2012). PF22, the *Chlamydomonas* homologue of DNAAF3, was also found to be cytoplasmically localised (Mitchison et al 2012). Mitchison et al (2012) identified that in *pf22, oda7* and *pf13* (the *Chlamydomonas* homologues of DNAAF1, DNAAF2 and DNAAF3) mutants dynein heavy chains were less stable and more susceptible to degradation by trypsin. Preassembled complexes of dyneins could be identified by immunoprecipitation from *pf22* cells, however only by using an alternative antibody than for wild type (Mitchison et al 2012). This indicates that the complexes can form but that they are in an abnormal conformation which hides a normally accessible antigen.

### 1.2.2.8 DNAAF4

*DYX1C1* is a cytoplasmic factor required for ODA and IDA formation and found to be mutated in PCD patients (Tarkar et al 2013). Further evidence for a role in dynein arm preassembly comes via its coimmunoprecipitation with HSP70, HSP90 and DNAAF2 (although not DNAAF1 or DNAAF3) (Tarkar et al 2013). The cytoplasmic localisation of DYX1C1 combined with its interaction with chaperone proteins and a known DNAAF is sufficient evidence to conclude that it is indeed an additional DNAAF (DNAAF4).
1.2.2.9 Further DNAAFs?

DNAAFs are cytoplasmically localised but are required for the formation of both ODA and IDA. SPAG1 is cytoplasmically localised and SPAG1 has been found to be mutated in PCD patients with defects in both ODA and IDA (Knowles et al 2013b). No further evidence has been obtained for the role SPAG1 might play in dynein arm formation, however it is possible that it too is an additional DNAAF.

In addition to those discussed above mutations have also recently been found in HEATR2 (Horani et al 2012, Diggle et al 2014), LRRC6 (Kott et al 2012, Horani et al 2013a, Zariwala et al 2013) and ZMYND10 (Moore et al 2013, Zariwala et al 2013). All of these factors are cytoplasmically localised and are required for the formation of both ODA and IDA making them candidate DNAAFs. These genes are however not discussed here as they are discussed more fully in Chapters 4 (ZMYND10 and LRRC6) and 5 (HEATR2). In addition mutations have been found in PCD patients in the axonemal factor C21orf59 which is discussed in Chapter 6 (Austin-Tse et al 2013).

1.2.2.10 Dynein arm transport

The preassembled dynein arm components are transported into the cilium by IFT (Ahmed et al 2008). Dynein arms have previously been found to coimmunoprecipitate with IFT components (Qin et al 2004). Mutations in IFT46 in Chlamydomonas result in flagella without dynein arms (Hou et al 2007). Dynein arm transport via IFT requires ODA16, which has been found to interact with IFT46 (Ahmed et al 2008). oda16 flagella lack dynein arms however ODA16 was found not to be required for preassembly or docking of the dynein arms (Ahmed et al 2008). Its physical interaction and co-localisation with IFT components identified it as a transport factor required for the IFT based transport of dynein arms into flagella (Ahmed et al 2008).

1.2.2.11 The Docking Complex

The ODA docking complex (ODA-DC) is an axonemal structure required for ODA attachment to the axoneme (Takada & Kamiya 1994). The Chlamydomonas
ODA-DC consists of three components; ODA1 (Takada et al 2002), ODA3 (Koutoulis et al 1997) and ODA14 (Casey et al 2003). Unlike for *Chlamydomonas* strains in which dynein arm components are mutated, in the mutant strains *oda1* and *oda3* wild type dynein arm components are unable to bind the axoneme in vitro (Takada & Kamiya 1994). An additional factor (the ODA-DC) was found to be required for ODA attachment and could be observed in the axonemal ultrastructure of dynein arm component mutants but not those of *oda1* and *oda3* (Takada & Kamiya 1994). The DC has been found to preassemble in the cytoplasm, however this is independent of ODA preassembly (Wakabayashi et al 2001). This highlights that preassembly in the cytoplasm is an important step in the formation of several components of the cilium motility machinery.

Since beginning this thesis mutations in components of the docking complex have been identified in PCD patients. Mutations in the human homologue of *ODA1* (*CCDC114*) have been found in PCD patients that lack ODA from the ultrastructure of their respiratory cilia (Knowles et al 2013a, Onoufriadis et al 2013). CCDC114 was found to localise to the axoneme and is required for ODA attachment indicating that it also acts as a component of the ODA-DC (Onoufriadis et al 2013). No human homologues have been detected for ODA3 and ODA14 suggesting that the ODA-DC has a different structure in humans (Onoufriadis et al 2013).

Mutations in PCD patients with ODA defects have also been found in *ARMC4* (Hjeij et al 2013). Cilia from these patients show loss of DNAH5 from the distal cilium and spread of DNAH9 along the entire cilium (Hjeij et al 2013). ARMC4 is also thought to be part of the human ODA-DC as its axonemal localisation is lost in *CCDC114* mutant cilia (Hjeij et al 2013). Mutations have also been identified in *CCDC103* in patients with disrupted ODA structure (Panizzi et al 2012). CCDC103 localises to the axoneme and mutation of *CCDC103* results in the loss of heavy chains from the distal cilium (Panizzi et al 2012). No evidence of a direct link to the ODA-DC has been found for CCDC103, however its axonemal localisation and ODA specific disruption suggests it too could play a role in this complex.
1.2.2.12 The Nexin-Dynein Regulatory Complex (N-DRC)

Coordinated dynein arm motor activity is required for efficient cilia or flagella bending, this is regulated by the radial spokes, central pair apparatus and the dynein regulatory complex (DRC). The DRC was identified as mutation of its components rescued defective flagella beating phenotypes of mutations in components of the central pair complex or radial spokes (Huang et al 1982). Mutation of DRC components results in altered axonemal beating and affects IDA binding (Brokaw & Kamiya 1987, Piperno et al 1994).

Resistance from structural components is also required to facilitate ciliary bending. The nexin links which join microtubule doublets are thought to play a role in restricting microtubule sliding which results in flagella bending (Bower et al 2013). Heuser et al (2009) used cryoelectron tomography to identify that the DRC and nexin links are the same structure: the nexin-dynein regulatory complex (N-DRC). Since I began work on this thesis mutations have been identified in CCDC164 the human homologue of dynein regulatory complex 2 (DRC1) (Wirschell et al 2013). Cilia from PCD patients with CCDC164 mutations have an increased beat frequency and an altered waveform consistent with a lack of regulation of dynein motor activity (Wirschell et al 2013). Mutations in CCDC65, the human homologue of DRC2, have also been found in PCD patients which have reduced nexin links and IDA in their respiratory cilia (Austin-Tse et al 2013, Horani et al 2013b).

Mutations have also been identified in CCDC39 and CCDC40 in PCD patients that result in the loss of IDA and the N-DRC complex (Becker-Heck et al 2011, Merveille et al 2011, Antony et al 2013). In both CCDC39 and CCDC40 mutant cilia ODA components are retained whilst IDA and N-DRC components are restricted to the cytoplasm (Becker-Heck et al 2011, Merveille et al 2011, Antony et al 2013). Consistent with the loss of these components from the axoneme, CCDC39 mutant cilia are rigid and hyperkinetic (Merveille et al 2011). These two components are required for the formation of the N-DRC and potentially the IDA. Both components localise to the cilium and the apical cytoplasm (Becker-Heck et al 2011, Merveille et al 2011, Antony et al 2013). Furthermore CCDC39 ciliary localisation is lost in CCDC40 mutant cilia suggesting that they act together (Becker-Heck et al 2011).
1.2.2.13 The Radial Spokes and Central Pair Apparatus

Abnormalities in the central pair apparatus and radial spokes also contribute to the disruption of ciliary beating (Castleman et al 2009, Olbrich et al 2012, Kott et al 2013). The radial spokes project from the outer microtubule doublets towards the central pair (Pigino et al 2011). Radial spokes have been found to contain proteins which could have a role in regulatory signal transduction including calmodulin (Yang et al 2001). Heuser et al (2012) identified a calmodulin and spoke associated complex (CSC) in *Chlamydomonas* flagella with contacts to the radial spokes and the N-DRC which could allow signal transduction between the radial spokes and dyenin arms. The regulatory rather than structural role of the radial spokes and the central apparatus was highlighted by Huang et al (1982) who noted that suppressor mutations could rescue motility in *Chlamydomonas* flagella lacking radial spokes without restoring the structures themselves. Furthermore as 9+0 ultrastructured cilia can still be motile, such as those found on the mouse embryonic node, the action of the radial spokes and central pair is not essential for all motility (Nonaka et al 1998). The radial spoke components preassemble in the cytoplasm before transport into the axoneme (Qin et al 2004). Once assembled the stalk attaches to the outer microtubule doublets with the head projecting towards the central pair (Pigino et al 2011).

When I began my own studies mutations in radial spoke head component genes *RSPH9* and *RSPH4A* had already been identified in patients with PCD (Castleman et al 2009). Respiratory cilia from these patients can lack both radial spokes and the central microtubule pair (Castleman et al 2009). Whilst the beat frequency of these cilia is normal, the waveform of the beat is altered showing the importance of the radial spokes for coordination of dynein motor activity (Castleman et al 2009). More recently mutations were also found in *RSPH1* in PCD patients that displayed loss or disruption of the central microtubule pair (Kott et al 2013). These mutations also resulted in both a reduced beat frequency and altered waveform (Kott et al 2013). PCD patients with mutations in radial spoke genes do not display *situs inversus* or heterotaxy as the nodal cilia whose immotility results in these conditions lack a central pair and radial spokes (Nonaka et al 1998).
The central apparatus consists of a number of projections from the central microtubule pair, these have been observed physically contacting the radial spokes and may be required for mechanosensory signalling (Oda et al 2014). Mutations in HYDIN can be found in patients with PCD but without laterality defects (Olbrich et al 2012). This is as cilia from HYDIN mutant cells lack a projection on the central pair, a defect which would not affect respiratory cilia but not nodal cilia. The cilia from HYDIN mutant cells have a normal beat frequency but an abnormal bending pattern indicating a defect in the coordination of the dynein motors (Olbrich et al 2012). HYDIN has previously been found to cause hydrocephalus in mice, however the PCD patients thus far identified do not display this symptom perhaps due to morphological differences between species (Ibanez-Tallon et al 2004, Olbrich et al 2012).

1.2.2.14 Flagella and cilia for locomotion

Flagella or cilia beating caused by the action of the ciliary motility machinery is required for the locomotion of gametes such as mammalian sperm cells, small organisms such as Chlamydomonas, and small multicellular organisms such as the planarian Schmidtea mediterranea (Ringo 1967, Afzelius et al 1995, Rompolas et al 2009). The conservation of flagella structure and the ciliary motility machinery has allowed Chlamydomonas to become an extensively used model organism for the investigation of motile cilia and modelling PCD (Wickstead & Gull 2007, Omran et al 2008, Mitchison et al 2012, Horani et al 2012). Flagellar beating is necessary for the function of human sperm cells. Alterations in beat frequency and beat pattern of the flagellum are required for the sperm to respond to the chemo-attractant cues that guide it through the female reproductive system (Spehr et al 2004). Defects in the ciliary motility machinery can result in male infertility (Khelifa et al 2014), as such many PCD patients also have fertility defects (Afzelius 1976, Omran et al 2000).

1.2.2.15 Nodal cilia

Coordinated movement of motile cilia can result in the movement of fluid. Motile cilia in the embryonic node of mice have been found to generate an extra-embryonic flow that is thought to allow the establishment of L-R (Left-Right) body axis
Unlike most motile cilia these were found to lack the central microtubule pair and therefore be of a 9+0 ultrastructural arrangement (Nonaka et al 1998). The loss of cilia from the in embryonic node of kif3B mutant mouse embryos coincided with a loss of nodal flow (Nonaka et al 1998). Additionally an artificial fluid flow over embryos with immotile cilia can restore L-R patterning showing that the cilia driven flow of extra-embryonic fluid is required to establish the L-R body axis (Nonaka et al 2002). Motile cilia are also thought to be required for the formation of L-R body axis in humans as PCD patients often also display situs inversus (Kennedy et al 2007). A loss of laterality can either result in a complete switch of body axis such as situs inversus totalis or in laterality defects of individual or groups of organs known as heterotaxy, this has been observed in both PCD patients and mouse models of PCD (Kennedy et al 2007, Tan et al 2007).

McGrath et al (2003) have suggested that immotile cilia may also be required to establish L-R asymmetry. They noted that polycystic kidney disease 2 (PKD2), also known as polycystin-2 (PC2), was present in mouse nodal cilia and that asymmetric calcium signalling occurred as a result of nodal flow suggesting a possible mechanism for how nodal flow generated by motile cilia is perceived and translated via immotile sensory cilia allowing the establishment of laterality (McGrath et al 2003).

Movement of motile cilia has also been shown to be required for establishment of laterality in zebrafish (Essner et al 2005) and Xenopus (Schweickert et al 2007). However not all vertebrates require motile cilia for this purpose. The establishment of both chicken and pig laterality is not thought to require motile ciliary beating (Gros et al 2009). Stephen et al (2014) have reported that whilst cilia are present on the chicken Hensen’s node, these are likely to be immotile and thus cilium motility is not required for the establishment of laterality in chicken. Therefore some but not all vertebrates require motile cilia to generate extra-embryonic flow in order to establish L-R axis.
1.2.2.16 Ependymal cilia

Hydrocephalus is a condition caused by a build up of cerebrospinal fluid (CSF) in the ventricles of the brain (Ibanez-Tallon et al 2004). This condition has been reported in a number of mouse studies which cause cilia immotility (Sapiro et al 2002, Ibanez-Tallon et al 2004, Lechtreck et al 2008). Sapiro et al (2002) reported that Spag6 mutant mice which had sperm motility defects also displayed hydrocephalus and Lechtreck et al (2008) showed that Hydin mutant mice with largely normal ultrastructured but immotile sperm also developed hydrocephalus. These defects occur due to the immotility of ependymal cilia whose coordinated beating is required to generate CSF flow through the ventricles of the brain (Ibanez-Tallon et al 2004). Ibanez-Tallen et al (2004) showed that a lack of motility in ependymal cilia due to disruption of Dnah5 resulted in a lack of ependymal flow through the ventricles and cerebral aquaduct in mice. Without the ependymal flow the cerebral aquaduct closed causing hydrocephalus.

However ciliary dysmotility may not be the only or primary ciliary mechanism by which hydrocephalus can occur (Banizs et al 2005). Banizs et al (2005) showed that Ifi88 mutant mice begun to develop hydrocephalus before the formation of motile cilia. It was suggested that immotile cilia on the choroid plexus epithelium can play a role in hydrocephalus formation (Banizs et al 2005). Regardless it is clear that ependymal cilia are required for the generation of ependymal flow and that their loss can result in hydrocephalus (Ibanez-Tallon et al 2004).

Patients have been reported which suffer from both PCD and hydrocephalus (reviewed in Lee 2013), however mutation of HYDIN has been found to cause PCD but not hydrocephalus in humans (Olbrich et al 2012). Due to difference in ventricle size it is thought that cilium immotility merely increases the risk of hydrocephalus in humans, unlike in mice where it is a more common phenotype (Olbrich et al 2012).

1.3 Conservation of cilia and flagella

It is thought that the last eukaryotic common ancestor (LECA) possessed a flagellum and as such cilia and flagella are well conserved in eukaryotes (reviewed in Carvalho-Santos et al 2011). The conservation of homologous genes in ciliated
organisms has been used to identify novel ciliary genes (Avidor-Reiss et al 2004, Li et al 2004). The conservation of candidate ciliary genes in organisms with a particular type of cilia has also been used to identify more specialised ciliary genes (Avidor-Reiss et al 2004, Baron et al 2007). During the course of my own study I use the conservation of homologues in organisms with and without motile cilia to implicate genes in cillum motility. In this section I shall introduce some of the organisms used for this study and detail the roles and form of their cilia.

Cilia and flagella are present on many vertebrate cells and fulfil a number of different roles as already discussed. Whilst the roles and structures of many of these genes are conserved there are differences between species. For example motile cilia are required for the function of the zebrafish kidneys but not mammalian kidneys and as a result of this kidney cysts are often formed in zebrafish mutants of ciliary motility genes (Sullivan-Brown et al 2008). Another example has already been discussed whereby motile cilia are required for the establishment of laterality in some vertebrates (humans and mouse) but not others (chicken) (Nonaka et al 1998, Stephen et al 2014). As a number of the roles that vertebrate cilia fulfil have already been covered they will not be discussed in detail here.

*Caenorhabditus elegans* possesses no motile cilia and only immotile cilia in a subset of its sensory neurons, the structure of these cilia varies between cells with some displaying elaborate ciliary structures (Mukhopadhyay et al 2007). The axonemal ultrastructure consists of 9 outer microtubule doublets with several microtubule singlets in the centre of the axoneme (Evans et al 2006). In the distal portions of *Caenorhabditus elegans* cilia only the microtubule singlet’s remain (Snow et al 2004). *Caenorhabditus elegans* has proved to be a useful model organism for studying cilia in its own right, however from an evolutionary conservation perspective it is particularly useful as it only contains immotile cilia and thus it can be compared to organisms with motile cilia.

*Chlamydomonas reinhardtii* is a green alga that has been widely used to study ciliary biology. It possesses two flagella which are of a 9+2 ultrastructure and contain the components of the ciliary motility machinery (Ringo 1967). *Osterococcus tauri* is another green alga, despite it not possessing flagella, it was found to contain
homologues to IDA heavy and intermediate chains suggesting that they have evolved to fulfil a secondary role (Wickstead & Gull 2007).

A number of other organisms have been used to investigate the conservation of ciliary homologues. Unless otherwise stated the axonemal ultrastructure of their flagella is 9+2 microtubule doublets and they contain ODA, IDA and radial spokes (Carvalho-Santos et al 2011). The malarial parasite Plasmodium falciparum possesses flagellated microgametes, however these form without IFT (Briggs et al 2004). The protozoan cat parasite Toxoplasma gondii also has flagellated gametes and lacks the dynein motor required for retrograde IFT (Wickstead & Gull 2007). The protozoan ciliate Tetrahymena thermophila possess many motile cilia with 750 basal bodies per organism (reviewed in Pearson & Winey 2009). The diatom Thalassiosira pseudonana has motile flagellated male gametes, however these are of a 9+0 structure and contain only outer dynein arms (Wickstead & Gull 2007). Phytophthora sojae is an oomycete pathogeneic to soy beans, it utilises flagella for movement (King & Butler 1968). Giardia lamblia is a flagellated parasitic protozoa, known to cause giardiasis (Lenaghan et al 2011). Trypanosomes are also flagellated parasitic protozoa (Broadhead et al 2006). The motility of a trypanosomes single flagellum has been found to be necessary for survival at the bloodstream stage of their life cycle (Broadhead et al 2006). The genomes of all of these organisms have been utilised to investigate whether they contain homologues to candidate ciliary genes that I have investigated in Drosophila melanogaster.

1.4 Drosophila cilia

Drosophila are an excellent model organisms in which to study cilia as they contain only two ciliated cell types (sensory neurons and sperm cells) and are viable even without cilia, meaning the resultant defects can be more easily studied than in a system which requires cilia for viability (Basto et al 2006).

1.4.1 Drosophila sperm flagella

The developing sperm cells are one such cell type. The sperm flagella of Drosophila melanogaster have a 9+2 microtubule arrangement axoneme, which contains the cilium motility machinery to allow sperm to swim by flagella beating (Fuller 1993,
Hoyle et al 2008, Wasbrough et al 2010). Sperm flagella axonemes also contain 9 outer accessory microtubules (Hoyle et al 2008) (Figure 1.3). Sperm flagella extension is independent of IFT, mutants of both an IFTB component (nompB) (Han et al 2003) and kinesin-II subunits (kap & klp64D) (Sarpal et al 2003) disrupt sensory ciliary formation but have no effect on sperm flagella.

1.4.2 Spermatogenesis

Germ line stem cells at the apical tip of the testes asymmetrically divide to form a spermatogonial cell and a stem cell (Fuller 1993, Yamashita et al 2007). The spermatogonial cells are encompassed by two accessory cyst cells, which remain present throughout spermatogenesis (Fuller 1993). Spermatogonial cells then undergo four rounds of mitosis without complete cell division to generate a syncytial cyst of 16 primary spermatocytes joined by intracellular bridges (Fuller 1993). As cells divide they proceed basally through the testes (Figure 1.3). The primary spermatocytes then undergo meiosis resulting in a cyst of 64 spermatids (Fuller 1993). It is during this stage that the mitochondria of the spermatids aggregate and interfold to generate the onion stage nebenkern (Fuller 1993). These bundles of spermatids then undergo IFT independent flagella extension from the basal bodies (Fuller 1993). When the flagella are of full length and the spermatids have matured they are individualised from the cysts; actin based individualisation complexes progress along the spermatid stripping excess cytoplasm and cellular components into waste bags (Fuller 1993, Noguchi & Miller 2003). Once individualised sperm can pass from the terminal epithelium into the seminal vesicle for storage.

1.4.3 Drosophila ciliated sensory neurons

The only somatic ciliated cells in Drosophila are the sensory neurons; the neurons of the external sensory (ES) neurons, chordotonal neurons and olfactory neurons are all ciliated (Uga & Kuwabara 1965, Shanbhag et al 1999, Avidor-Reiss et al 2004). The ES organs, including the external sensory bristles, are required for the perception of touch and proprioception (reviewed in Keil 1997). ES neurons contain a short modified cilium that attaches to a tubular bundle at the base of the bristle (Avidor-
A germ line stem cell at the apical tip of the testes asymmetrically divides to form a spermatogonial cell. These then undergo mitosis four times to generate 16 spermatocytes. Primary spermatocytes then undergo meiosis resulting in the generation of 64 spermatids. Flagella then extend from the spermatids as the cells progress basally.

(B) The progression of spermatogenesis through the testes. As sperm cells divide and develop they progress through the testes. Bundles of spermatids are individualised and motile sperm pass into the seminal vesicle. Figure based on that of Hirst & Carmicheal (2011).

(C) The axonemal structure of sperm flagella. The axonemes are of a 9+2 arrangement with 9 additional accessory microtubules. The cilium motility machinery is present in these flagella including ODA, IDA and radial spokes to allow coordinated flagellar beating.

Figure 1.3 *Drosophila* spermatogenesis
(A) A diagram of the cell divisions that occur to generate spermatids from a germ line stem cell.
Reiss et al 2004). The loss of ciliary components of ES organs has been found to cause the loss of mechanosensory potentials (Kernan et al 1994).

Chordotonal organs are required for hearing, as well as proprioception and gravitacttic behaviour (Eberl et al 2000, Sun et al 2009, Kamikouchi et al 2009). The chordotonal organ (or scolopidium) consists of a ciliated neuron and three accessory cells: a ligament cell, a scolopale cell and a cap cell (Uga & Kuwabara 1965, reviewed in Field & Matheson 1998) (Figure 1.4). The ligament and cap cells join the organ to surfaces at either tip, whilst the scolopale surrounds the chordotonal neuronal dendrite and cilium (Uga & Kuwabara 1965, Chung et al 2001, reviewed in Yack 2004). Chordotonal neurons are internal stretch receptors; mechanical manipulation of the neuronal cilium is thought to result in signal transduction by opening force-gated ion channels (Nadrowski et al 2008, Lehnert et al 2013). I have used the chordotonal neuron as a model to study cilium formation.

1.4.4 Chordotonal neuron development

Chordotonal sense organ precursors (SOPs) form from proneural clusters in the neuroectoderm by the expression of the proneural basic helix-loop-helix transcription factor atonal (Jarman et al 1993, Jarman et al 1995). SOPs then undergo a series of asymmetric divisions to generate a neuron, three accessory cells (ligament, cap and scolopale) and an ectodermal cell that degrades (Figure 1.5) (Brewster & Bodmer 1995, reviewed in Lai & Orgogozo 2004). Atonal (Ato) is the master regulator of chordotonal sense organ fate determination and thus the differentiation of the cells which form from the precursor (Jarman et al 1995).

1.4.5 Organisation of chordotonal neurons

8 chordotonal neurons are located in each embryonic hemisegement (Figure 1.6), which become the chordotonal neurons of the larval body wall that have been implicated in proprioception and hearing (Dambly-Chaudiere & Ghysen 1986, Caldwell et al 2003, Zhang et al 2013, Hope & Jarman unpublished).

In adult flies chordotonal neurons act as proprioceptors to aid coordination during movement, for example the femoral chordotonal neurons allow coordinated limb
Figure 1.4 Chordotonal organ structure.
A diagram showing the placement of the chordotonal neuron and support cells within a single scolopidium. The scolopale surrounds the cilium, whilst the cap and ligament tether the neuron at either end. Figure based on that of Kernan (2007) and Cachero et al (2011).

Figure 1.5 Cell division of the SOP.
A diagram showing the series of asymmetric divisions the SOP undergoes to generate the chordotonal neuron and accessory cells. Figure based on those of of Brewster & Bodmer (1996), Kernan (2007) and Cachero et al (2011).
Figure 1.6 The structure of the embryonic chordotonal neurons

(A) A diagram showing the location of the neurons of the peripheral nervous system in a single hemisegment of a *Drosophila* embryo. The chordotonal neurons are individually labeled and their axons are shown (magenta). The position of the cell bodies of multidendritic (green) and external sensory neurons (black) are also shown. Two of the multidendritic neurons (v’td 1 and 2) are discussed later in this study as they share a lineage with chordotonal neurons, these are also labeled on this diagram. This diagram is based on those of Orgogozo & Grueber (2005) and an image composed by A Jarman in Moore et al (2013).

(B) A diagram showing the structure of the embryonic chordotonal neurons. Cilia extend from the dendrites of each chordotonal neuron.
movement (Shanbhag et al 1992, reviewed in Field & Matheson 1998). The Johnston’s organ (JO) is a group of 240 chordotonal scolopidia in the a2 segment of the Drosophila antennae which is required for hearing and gravitaxis (Eberl et al 2000, Kamikouchi et al 2006, Kamikouchi et al 2009, Sun et al 2009) (Figure 1.7 and 1.8). Each scolopidium contains 2 or 3 chordotonal neurons meaning that the Johnston’s organ contains around 470 chordotonal neurons (Kamikouchi et al 2006).

Particle movement as a result of a sound stimulus results in the vibration of the arista, causing the a3 antennal segment (or funiculus) to rotate within the a2 segment (or pedicel) (Figure 1.7 and 1.8) (Gopfert & Robert 2002). The resulting movement is detected by chordotonal neuron stretch receptors which attach directly to the a3 segment and results in signal transduction (Figure 1.8). The position of the a3 segment within the a2 segment is also thought to be detected by the manipulation of chordotonal neuron cilia (Kamikouchi et al 2009). This allows the perception of gravity to enable flies to have negative gravitactic behaviours (Sun et al 2009, Kamikouchi et al 2009). The neurons in the Johnston’s organ that perceive gravity and sound are thought to be distinct and have different populations of mechanotransducing ion channels (Sun et al 2009, Kamikouchi et al 2009).

1.4.6 Drosophila ciliary genes

A number have ciliary genes had been identified in Drosophila at the time that this study began (Figure 1.9). Kernan et al (1994) had used a forward genetic screen of the X chromosome to identify a number of mutants that caused an alteration in the larval response to touch. Amongst those identified were two that have subsequently been characterised. Unc (Uncordinated) has since been found to be required for the formation of sensory cilia and sperm flagella (Baker et al 2004). Unc localises to the basal body and transition zone, its mutation results in short cilia with axonemal defects (Baker et al 2004).

A further gene identified by the larva behavioural of Kernan et al (1994) is tilB (touch insensitive larvae B). This gene had been found to be required for sound evoked potentials of the Johnston’s organ but not mechanoreceptor potentials (Kernan et al 1994, Eberl et al 2000). TilB is a cytoplasmically localised factor
Figure 1.7 The antennal movements which allow Drosophila hearing. A diagram showing the movements of the antennae in response to a sound stimulus.
1. The arista acts as the sound receiver moving back and forth in response to particle movement.
2. Arista movement results in the rotation of the a3 segment.
3. The “hook” of the a3 segment rotates within the a2 segment. This movement is detected by the Johnston’s organ chordotonal neurons.
This figure is based on that of Nadrowski et al (2008) and Newton et al (2012).

Figure 1.8 The chordotonal neurons of the antennal Johnston's Organ. (A) A diagram showing that movement of the a3 segment within the a2 segment is sensed by the chordotonal neurons of the Johnston’s organ. Over 400 chordotonal neurons form the Johnston’s organ, their mechanical manipulation due to a3 movement results in signal transduction. Figure based on that of Nadrowski et al (2008). (B) The structure of the chordotonal neurons within a Johnston’s organ scolopidium, The scolopidium contains 2 to 3 neurons, each ciliated with a compartmentalised cilium.
Figure 1.9. The chordotonal neuron cilium.
A diagram showing the structure of the chordotonal neuron cilium. The cilium is divided into two compartments with distinct protein populations by the ciliary dilation. The location of known *Drosophila* ciliary proteins are indicated. Figure based on that of Kernan (2007).
required for the formation of axonemal dynein arms in both chordotonal neurons and sperm flagella (Eberl et al 2000, Kavlie et al 2010).

Kernan et al (1994) also conducted a forward genetic screen on the second chromosome for uncoordinated behaviour similar to that found for unc. Mutants identified were then screened for defective mechanosensory bristle potentials (Kernan et al 1994). A number of these were identified which resulted in no mechanosensory potential (Nomp) or a reduced mechanosensory potential (Remp) (Kernan et al 1994). Several of these have subsequently been characterised.

NompA was found to be a component of the cap accessory cell and be required for attachment of cap cells to chordotonal neurons (Chung et al 2001). NompB has since been identified as a component of the IFTB complex (human homologue – IFT88) (Han et al 2003). Mutations in nompB result in a short or absent sensory neuron cilia, however sperm flagella remain unaffected (Han et al 2003). NompC was found to be a Transient Receptor Potential (TRP) channel, which localises to the distal cilium and is required for signal transduction (Lee et al 2010). NompC is amongst a number of ion channels necessary for chordotonal neuron function.

1.4.7 Ion Channels

A number of ion channels have been implicated in chordotonal organ function and may have a role in mechanotransduction (Kim et al 2003, Sun et al 2009, Lee et al 2010). The TRP-Vanilloid (TRPV) channels Nanchung (Nan) and Inactive (Iav) localise to the proximal cilium (Kim et al 2003, Gong et al 2004) whereas the TRP-NompC (TRPN) channel NompC localises to the distal cilium (Lee et al 2010). Iav and Nan are required for both hearing and gravitaxis, while NompC is required only for hearing (Sun et al 2009, Effertz et al 2011). The TRP-Ankyrin (TRPA) channels Painless and Pyrexia are also present in the Johnston’s organ (in the neuron and cap cell respectively) and are required for gravitactic behaviour (Sun et al 2009). Lehnert et al (2013) have suggested that Iav and Nan are required for mechanical transduction, whilst NompC is required for increased sensitivity. This is consistent with the proposed transduction complex model as Nan and Iav co-localise with the axonemal dyneins arms (Kim et al 2003, Gong et al 2004, Kavlie et al 2010).
However others have suggested that NompC has the primary role in mechanotransduction as loss of NompC results in the loss of active amplification in response to low stimuli, whilst loss of the TRPV channels does not (Gopfert et al 2006).

1.4.8 RempA, Btv and ciliary compartmentalisation

rempA, a further gene identified by the screen of Kernan et al (1994), is a component of the IFTA complex and the Drosophila homologue of IFT40. It localises to the ciliary dilation and when mutated results in short cilia in which IFTB proteins aggregate and the loss of IAV::GFP from the cilium (Lee et al 2008).

The chordotonal neuron cilium is divided into two functionally distinct regions by an electron dense ciliary dilation (Eberl et al 2000, Lee et al 2008). As discussed above the ion channel populations in these two compartments differ (Kim et al 2003, Gong et al 2004, Lee et al 2010). Additionally outer and inner axonemal dynein arms are present in the proximal section but not the region distal to the ciliary dilation (Field & Matheson 1998, Lee et al 2008). Components of the IFTA complex have been found to have a role in this compartmentalisation (Eberl et al 2000, Lee et al 2008).

In mutants of beethoven (btv), a dynein motor component (Drosophila homologue of DYNC2H1) IAV::GFP is distally mislocalised suggesting that Btv has a role in maintaining the ciliary compartments (Lee et al 2008). In addition RempA localisation is lost from the ciliary dilation in btv mutants (Lee et al 2008). A btv mutant was originally identified by Eberl et al (1997) and was subsequently found to not be able to produce sound evoked potentials (Eberl et al 2000). Mutation of btv also resulted in an enlarged ciliary dilation (Eberl et al 2000). This suggests that Btv is required to maintain the structure of the ciliary dilation and thus separate the cilium into proximal and distal segments which contain different protein populations.

1.4.9 Evidence for chordotonal neuron cilia motility

The proximal ciliary compartment of Drosophila chordotonal neuron cilia contain much of the cilium motility machinery which allows ciliary movement in other systems. The cilium motility machinery, including the axonemal dynein arms is
required for chordotonal neuron function (Senthilan et al 2012). It is thought that the dynein motors form part of a transduction complex, acting as adaption motors that facilitate conversion of a mechanical force into a signal which can be transduced via coupling to ion channels by a gated spring (Nadrowski et al 2008). The motility machinery is also required for active amplification of weak stimuli to ensure signal transduction (Gopfert & Robert 2003, Gopfert et al 2005). However are these cilia actually motile or do they just require the cilium motility machinery for an adapted function?

Although *Drosophila* chordotonal cilia have not been observed bending, those in the grasshopper (*Melanoplus bivittatus*) femoral chordotonal organs have been observed doing so (Moran et al 1977). Further evidence comes from the spontaneous movement of the sound receiver (a3 segment and arista) in the absence of a stimulus (Gopfert & Robert 2003, Gopfert et al 2005). Mutation of *nompA*, which is required for chordotonal neuron attachment to the cap cell, resulted in the loss of spontaneous movement showing that the chordotonal neurons of the Johnston’s organ are necessary for this process (Gopfert & Robert 2003). Mutation of *tilB*, which is required for the formation of the axonemal dynein arms also resulted in a loss of spontaneous movement of the sound receiver suggesting that the dynein arms are necessary for this movement (Gopfert & Robert 2003).

Monitoring the movement of the sound receiver has shown that mechanical energy is produced by the chordotonal sensory neurons (Gopfert et al 2005). This results in a power gain allowing a non-linear response to weak stimuli (Gopfert et al 2003, Gopfert et al 2005). Again these properties are lost in *tilB* flies showing that the dynein arms are likely required for these attributes of the fly antenna. Gopfert et al (2005) suggest that active amplification of weak signals is occurring to enhance the stimulus received by the sound receiver and allow signal transduction.

There are two possible explanations for these phenomena. The first involves the cilium motility machinery actively moving the cilium at low stimuli to allow power gain and a non-linearity of response. This would also be consistent with TilB, and potentially dynein arm, dependent spontaneous movement of the sound receiver. This is similar to the active movement of the cochlear amplifier in frogs (Martin et al
or that which has been observed in vitro for the gerbil cochlea (Chan & Hudspeth 2005). If this is the case then the cilia could be considered to be motile.

The second possibility is that the motility machinery acts as to adjust stiffness of the cilium as part of a transduction complex. Nadrowski et al (2008) have shown by computer modelling that a process such as this could explain the power gain and non-linearity of stimulus response previously recorded. If this was the case then the cilium would not be motile but would require the cilium motility machinery for its function and as a result could still be used to study cilium motility as these components are required for it elsewhere.

It is also possible that both processes are happening, with a transducer complex also being able to convey ciliary movement. However whilst motility is suggested, based on what is known to date it cannot be said for certain that the chordotonal neuron cilia are motile. However if in this thesis a factor is referred to as being required for cilium motility in Drosophila what is meant is that it is required for the function of the cilium motility machinery; for flagella movement in sperm cells or for either motility or as part of transducer complex in the chordotonal neurons.

All of these phenomena have also only been examined in Johnston’s organ cilia which are required for hearing. Whether dynein arms are vital for the function of the femoral chordotonal organs necessary for proprioceptive function and whether they too bend as those in grasshoppers has not been established.

1.5 Transcriptional control of ciliogenesis

Atonal begins the transcription factor cascade that results in neuronal differentiation, including ciliogenesis (Figure 1.10) (Jarman et al 1993, Jarman et al 1995, Cachero et al 2011). Atonal and its downstream targets are therefore required for the expression of genes necessary to build the chordotonal neuron cilium. A number of ciliary factors are expressed prior to ciliogenesis and prior to neuron terminal differentiation (Cachero et al 2011). Some ciliary genes are direct targets of Atonal, for example dila, which encodes a protein that localises to the basal body (Cachero et al 2011, Ma & Jarman 2011). dila is thought to be directly regulated by Atonal as the expression of an enhancer construct was induced by ectopic atonal expression and
Figure 1.10 The transcriptional cascade controlling the expression of ciliary genes. A diagram detailing the regulation of ciliogenesis in the transcriptional cascade begun by the proneural transcription factor Atonal. RFX and Fd3F represent downstream transcription factors required for the expression of either general ciliary genes or a subset of ciliary genes respectively. Figure based on that of Cachero et al (2011).
lost when the Atonal binding sites (E boxes) were mutated (Cachero et al 2011). However ciliogenesis is also transcriptionally regulated by the transcription factors RFX (regulatory factor X) (Dubruille et al 2002) and Fd3F (forkhead 3F) (a diverged FOXJ1 (forkhead box J1) homologue) (Newton et al 2012). These represent transcription factors that are not only part of the cascade in *Drosophila* but are conserved in other organisms for transcriptional control of ciliogenesis.

### 1.5.1 RFX

RFX is a general transcriptional regulator of ciliary genes. *Rfx* expression has been detected in both ES and chordotonal neurons, as well as in the testes (Vandaele et al 2001). RFX has been found to control the expression of some *Drosophila* ciliary genes (Dubruille et al 2002, Laurençon et al 2007). Differences in expression pattern in chordotonal and ES neurons are thought to have a role in establishing the differences in ciliary specialisation (Cachero et al 2011). The ciliary role of *Rfx* is conserved in metazoans (Chu et al 2010). *Daf-19*, the *Caenorhabditis elegans* *Rfx* homologue, is required for the expression of ciliary genes in ciliated sensory neurons (Swoboda et al 2000, Efimenko et al 2005). 7 RFX homologues are present in vertebrates; 3 of these have been found to regulate the expression of subsets of ciliary genes (Ashique et al 2009, El Zein et al 2009, Bisgrove et al 2012, Chung et al 2012). RFX3 was found to regulate the expression of cillum motility genes, including axonemal dyneins, and *Foxj1* in a mouse ependymal cell culture (El Zein et al 2009). *Rfx4* mutant mice show a reduction in the expression of *Ift172* as well as spinal cord and telencephalon defects associated with defective SHH signalling (Ashique et al 2009). RFX2 is required for ciliogenesis in ciliated zebrafish and *Xenopus* tissues (Bisgrove et al 2012, Chung et al 2012). The RFX family of transcription factors represent conserved general ciliary regulators that are required for expression of components of both primary and motile cilia.

### 1.5.2 FOXJ1

A further transcription factor implicated in transcriptional control of ciliogenesis is FOXJ1 (Blatt et al 1999, Brody et al 2000). *Foxj1* is expressed in motile ciliated cells in mice (Blatt et al 1999). The loss of FOXJ1 has been found to disrupt the formation
of motile cilia in a number of organisms (Chen et al 1998, Brody et al 2000, Yu et al 2008). Foxj1 loss in mice results in a loss of motile airway cilia, however immotile olfactory cilia were still present (Brody et al 2000). The loss of Foxj1 also resulted in laterality defects associated with disrupted nodal cilia even though some nodal cilia remained present (Brody et al 2000). Foxj1 mutant mice also display hydrocephalus suggesting defects in the ependymal cilia (Chen et al 1998) Morpholino mediated knock down of foxj1a in zebrafish results in the loss of motile cilia from the floor plate, Kupffer’s vesicle and kidney (Yu et al 2008). A FoxJ1 homologue has also been found to be required for the formation of motile cilia in Xenopus (Stubbs et al 2008) and flatworm (Scmidtea mediterranea) (Vij et al 2012). FOXJ1 is conserved throughout unikonts, this is demonstrated by the fact that sea urchin FOXJ1 can induce the expression of motility genes in zebrafish (Vij et al 2012).

The loss of foxj1a has been found to cause a reduction in the expression of ciliary motility genes in zebrafish (Yu et al 2008) and ectopic expression can result in the expression of ciliary motility genes in Xenopus (Stubbs et al 2008). The requirement of FOXJ1 for the formation of motile cilia and the effects of its manipulation on motility gene expression suggest that FOXJ1 is a motile cilium specific transcription factor. Indeed a homologue of FOXJ1 (Fd3F) has recently been identified in Drosophila and is required for the expression of motility-linked genes (Newton et al 2012). FOXJ1 has however been implicated in the regulation of more than just the cilium motility genes. Loss of Foxj1 in mice results in defects in centriole migration and docking and ciliated tissues demonstrate a loss of ciliogenesis rather than just a loss of cilium motility (Brody et al 2000). It has been demonstrated that FoxJ1 also regulates the expression of genes required for the docking of the basal body (Gomperts et al 2004). FOXJ1 is a therefore a conserved transcription factor linked to the ciliogenesis and expression of components of motile cilia.

1.6 What is a ciliary gene?

A number of studies have tried to identify “ciliary genes”, as have I in my own investigations. But what is a ciliary gene? One possible definition is the genes that encode proteins that localise to the cilium. The identity of these genes can be elucidated by proteomic approaches. If a protein is localised to the cilium then the
gene that encodes it can be considered a ciliary gene. The selective transport of proteins into the ciliary compartment means that those which are localised there are likely to have a role in ciliary biology. However this excludes genes vital for ciliary function that are localised to the cytoplasm such as the DNAAFs.

Another definition of a ciliary gene is one that encodes a protein that is required for the structure or formation of the cilium or its components. However several proteins that act in structural components of the cilium are not vital for its function. The loss of some components of the Caenorhabditis elegans transition zone alone had no deleterious effect (Williams et al 2008). However when combined with further mutations in secondary genes a ciliary defect was observed. Therefore a gene does not have to be vital for ciliary formation or function, but those that are can be considered ciliary genes.

As cilia fulfil many different biological roles, this means that components of a number of pathways have part of their function linked to cilia. Whether the cilia localised components of the HH signalling pathway can be considered ciliary genes is a further complication. Whilst these genes are vital for the function that cilia play in those cells they are not vital for the formation of the cilium itself. The ciliary genes that I attempted to identify and characterise in Drosophila sensory neurons were considered to be ciliary if they had a role in an aspect of cilium formation or cilium function.

1.7 What is a ciliary motility gene?

A ciliary motility gene encodes a protein that is part of a structure vital for active ciliary or flagellar movement. An obvious example of these would be those that encode components of the cilium motility machinery. The genes required for their assembly are also considered ciliary motility genes. Genes that are required for general ciliary processes are not considered to be motility genes unless they have a specialised function relating to cilium movement. For example an IFTB complex protein would not be considered to be encoded by a cilium motility gene if it caused motility defects by causing general problems in ciliogenesis. However an IFT component with a specialised role in the transport of dynein arms would be
considered a motility gene. A motility gene is therefore one that is required for the formation or function of the ciliary motility machinery that is necessary to drive movement of flagella or cilia.

1.8 Methods of identifying genes required for ciliogenesis

A number of different techniques have been used to identify novel ciliary components. Transcriptional regulation by known ciliary regulators such as RFX would implicate a gene in ciliary processes. Efimenko et al (2005) and Laurençon et al (2007) have identified a number of candidate ciliary genes by the presence of upstream X boxes conserved between closely related species. Comparative genomics has also been used to identify novel ciliary genes. This method relies upon organisms with cilia retaining the genes for ciliary components whilst organisms that have lost cilia losing the genes required for ciliary function. Comparative genomics has been used to compare organisms with and without cilia (Avidor-Reiss et al 2004, Li et al 2004) and organisms with motile cilia and those without (Avidor-Reiss et al 2004, Baron et al 2007). There are however drawbacks of using this approach as some organisms that have lost cilia retain ciliary genes (Hodges et al 2011). Homologues to genes implicated in ciliary processes in other organisms have been found in plant species without cilia, in which they fulfil a secondary role unrelated to cilia (Hodges et al 2011).

Proteomics has also been employed to investigate the components of ciliary and flagella axonemes (Ostrowski et al 2002, Pazour et al 2005). However this approach neglects cytoplasmically localised factors required for ciliary function and formation.

Expression of genes in ciliated cells is a further indicator of a ciliary function. Blacque et al (2005) identified candidate ciliary genes expressed in ciliated Caenorhabditis elegans neurons but not in other non-ciliated tissues. Stolc et al (2005) found a number of Chlamydomonas genes with a potential role in ciliogenesis that were the expressed during reflagellation. Hoh et al (2012) identified expression profiles in FOXJ1-GFP expressing cells during mouse tracheal epithelial cell differentiation, which provides further candidates for ciliary function.
These techniques have been successful in identifying novel ciliary genes and provide candidates for further investigation. However many candidates remain uncharacterised and the different gene profiles identified by different techniques show that these techniques need to be used together to most effectively isolate ciliary genes.

1.9 The identification of novel ciliary genes in Drosophila

*Drosophila* provide a good model organism in which to study cilia. Only two cell types are ciliated and both have readily assessable phenotypes associated with lack of ciliary or flagella function. Additionally *Drosophila* can survive to adulthood without cilia (Basto et al 2006) and are viable without chordotonal neurons (Jarman et al 1995). In addition a great number of genetic tools are available for use in *Drosophila*. For example in this thesis I utilise the VDRC (Vienna *Drosophila* RNAi Center) RNAi library which allows knock down of most genes in the *Drosophila* genome (Dietzl et al 2007) and the BDGP (Berkeley *Drosophila* Genome Project) gene disruption project from which many transposon insertion lines are available which can either be used to generate mutant flies or may alter gene expression themselves (Bellen et al 2004). My aim was to make use of the genetic tools and suitability of *Drosophila* to studies in cilia to identify novel ciliary genes. As many ciliary components are well conserved these findings could be relevant for mammalian ciliopathies. A further resource which made identifying novel ciliary genes in *Drosophila* suitable is the temporal profiles of gene expression during development of ciliated cells generated by Cachero et al (2011) and zur Lage and Jarman (unpublished).

1.10 Temporal profiles of gene expression in atoGFP expressing cells

Cachero et al (2011) have generated temporal profiles of gene expression during the development of chordotonal neurons. Embryos expressing *atoGFP* (*GFP* under to control of the *atonal* promoter) were homogenised and fluorescence associated cell sorting (FACS) used to isolate GFP expressing cells from the rest of the embryo (Cachero et al 2011). mRNA was then isolated from these cells and an Affymetrix 2.0 chip microarray used to compare mRNA level and thus gene expression between
atoGFP expressing cells and the rest of the embryo (Cachero et al 2011). atoGFP is expressed in the SOP and its daughter cells between stages 10 and 12 as well as in the developing bolwigs organ (Figure 1.11) (Cachero et al 2011). Cachero et al (2011) analysed embryos at three time points representing the first three hours of neuronal development (Figure 1.11). Gene expression in atoGFP cells at these time points was compared to that in the rest of the embryo to identify genes that were enriched in the developing chordotonal organs (Cachero et al 2011). Cachero et al (2011) carried out this study in order to discover how the fate determining expression of atonal results in the differentiation of the chordotonal organs (Cachero et al 2011). Part of investigating this was to elucidate the transcriptional regulatory cascade that causes neuronal differentiation down stream of Atonal.

### 1.10.1 Atonal

Atonal is a basic-helix-loop-helix transcription factor that acts as a proneural gene to dictate the fates of chordotonal SOPs and photoreceptors (Jarman et al 1993, Jarman et al 1994, Jarman et al 1995). Atonal is expressed from stage 8 to stage 12, at first in the proneural cluster and later in the SOP (Jarman et al 1993). It is also expressed in the precursors of the bolwigs organ (larval eye) at stages 10 to 12 (Daniel et al 1999). Mutation of atonal results in the loss of most of the chordotonal neurons, with only a few remaining (including two of the lch5 neurons) (Jarman et al 1995). Expression of atonal results in the specification of a sense organ precursor and begins the transcriptional cascade that controls division and differentiation of these precursors into functioning sensory organs (Jarman et al 1995). Cachero et al (2011) carried out their investigation in order to better understand how Atonal regulates this process.

### 1.10.2 Temporal profiles of gene expression in catoGFP expressing cells

An investigation complimentary to that of Cachero et al (2011) was carried out by zur Lage and Jarman (unpublished). This used the same method as for the previous microarray but used the expression of GFP from the cato (cousin-of-atalon) promoter (catoGFP) to mark developing chordotonal cells (zur Lage & Jarman unpublished). catoGFP is expressed in chordotonal organ SOPs and their daughter
Figure 1.11 The developmental time points at which atoGFP and catoGFP cells were isolated.

A diagram showing the timepoints (T1-T4) at which the atoGFP or catoGFP transcriptome was sampled in relation to embryonic stages and the expression of atonal, atoGFP, cato and catoGFP in these cells. Cachero et al (2011) identified the atoGFP linked transcriptome at time points 1, 2 and 3. zur Lage & Jarman (unpublished) identified the catoGFP linked transcriptome at time points 2, 3 and 4. The figure is adapted from that of Cachero et al (2011)
cells from stage 10 to stage 14 and also weakly in the sense organ precursors of the ES organs (Figure 1.11) (zur Lage & Jarman 2010). Within the developing chordotonal organs *catoGFP* is most strongly expressed in the neuron, scolopale and ligament cells (zur Lage & Jarman 2010). Gene expression in *catoGFP* expressing cells was analysed at the later two time points used for the *atoGFP* microarray by Cachero et al (2011), as well as an additional time point representing the fourth hour of neuronal development (zur Lage & Jarman unpublished) (Figure 1.11). This microarray was carried out to compliment that done by Cachero et al (2011). zur Lage and Jarman (unpublished) carried out this second microarray as the later and more neuronally focussed expression of *catoGFP* allowed investigation of the transcriptional regulatory cascade at later time points and also allowed a focus on factors required for neuronal differentiation.

### 1.10.3 Cousin of atonal

Cousin of atonal (Cato) is a basic-helix-loop-helix transcription factor that has a role in the regulation of neuronal development but is downstream of Atonal and thus a later part of the transcriptional regulatory cascade (Goulding et al 2000, zur Lage & Jarman 2010). *cato* is normally expressed in the chordotonal SOPs from stage 10 and the ES SOPs from stage 11 (Goulding et al 2000). Mutation of *cato* results in the duplication of some sensory neurons, whereas a double mutant of *cato* and *atonal* results in the loss of all chordotonal neurons (zur Lage & Jarman 2010).

### 1.10.4 Temporal profiles enriched in ciliary genes

The investigations by Cachero et al (2011) and zur Lage and Jarman (unpublished) identified a number of genes that were enriched in developing chordotonal organs (as marked by *atoGFP* or *catoGFP* expression compared with the rest of the embryo). Both investigations found that many of the genes enriched in *ato/catoGFP* expressing cells were associated with cilia (Cachero et al 2011, zur Lage & Jarman unpublished). This finding was surprising as whilst the chordotonal neurons are ciliated, the time points assayed by the microarrays were before the final cell divisions had taken place and thus before ciliogenesis (Cachero et al 2011, zur Lage & Jarman unpublished). Time point 3 (representing the third hour of neuronal
development or 7 45 - 8 45 hours) was particularly enriched with ciliary genes for both of the microarrays, however many were detected by zur Lage and Jarman (unpublished) at time point 2 (6 45 – 7 45 hours).

As well as genes of known function, both ciliary and non-ciliary, many uncharacterised genes were identified as being enriched in \textit{atoGFP} or \textit{catoGFP} expressing cells (Cachero et al 2011, zur Lage & Jarman unpublished). These could represent novel, previously uncharacterised ciliary genes and as such during this study I shall be investigating enriched genes in order to identify new ciliary genes in \textit{Drosophila}.

1.10.5 \textit{Dila}

Proof that uncharacterised genes enriched in \textit{ato/catoGFP} expressing cells can be novel ciliary genes comes from the characterisation of \textit{dila} by Ma and Jarman (2011). \textit{dila} was the highest enriched gene at time point 2 in \textit{atoGFP} expressing cells (Cachero et al 2011). Mutating \textit{dila} resulted in uncoordinated and infertile flies with short cilia (Ma & Jarman 2011). Dila is required for the formation of sperm flagella and both ES and chordotonal cilia (Ma & Jarman 2011). It is thought to localise to the putative transition zone distal to the basal body (Ma & Jarman 2011). The discovery of a gene vital for ciliogenesis amongst the list of those enriched in \textit{ato/catoGFP} expressing cells validates the approach of using these gene lists to find novel ciliary genes.

1.10.6 \textit{Fd3F}

A further cilia-linked gene indentified from those enriched in \textit{ato/catoGFP} expressing cells is \textit{fd3F} ((Cachero et al 2011, zur Lage & Jarman unpublished). Fd3F is a transcription factor which regulates the expression of ciliary genes, however in contrast to \textit{Rfx}, \textit{fd3F} is only expressed in \textit{Drosophila} chordotonal neurons (Cachero et al 2011, Newton et al 2012). \textit{fd3F} \textit{flies} are deaf and uncoordinated, showing that chordotonal neuron function is defective. \textit{fd3F} \textit{flies} are fertile and \textit{fd3F} is not expressed in the testis indicating that it has no role in sperm flagella formation (Newton et al 2012). Cilia are present in \textit{fd3F} \textit{chordotonal neurons}, however they
lack dynein arms, compartmentalisation and have electron dense tips suggesting defective retrograde IFT (Newton et al 2012).

Newton et al (2012) screened the expression of known ciliary genes and uncharacterised genes highly enriched in ato/catoGFP expressing cells in fd3F\(^-\) embryos to find its transcriptional targets. This was carried out in order to investigate its place within the transcriptional cascade regulating chordotonal organ formation.

Fd3F was found to regulate the expression of a subset of ciliary genes thought to be linked to chordotonal cilia specialisation. These include the TRPV channels required for signal transduction, cilium motility machinery components including axonemal dynein arms and genes linked to retrograde IFT transport such as rempA and btv, which are also required for ciliary compartmentalisation (Newton et al 2012). However a number of genes screened were not regulated by Fd3F such as dila, nompB and CG15161 (IFT46 homologue) (Newton et al 2012). In addition several genes previously unlinked to cilia were found to be regulated by Fd3F such as CG31320 (Newton et al 2012). These genes can be considered good candidate ciliary genes and as such I investigate some of these in this study.

1.10.7 Co-regulation of ciliary genes by RFX and Fd3F

Examination of the upstream region of Fd3F regulated genes showed that forkhead binding sites were often found in close proximity to the X boxes required for regulation by RFX (Newton et al 2012). The expression of these genes was found to be lost in Rfx\(^{49}\) embryos (Newton et al 2012). This indicates that RFX and Fd3F act together to regulate the transcription of shared targets in Drosophila chordotonal neurons. Whilst RFX is required for the expression of a number of different ciliary genes, the subset that are required for chordotonal ciliary specialisation have their expression boosted by Fd3F (Newton et al 2012). It has been suggested that fd3F is a diverged homologue of FOXJ1 (Newton et al 2012). RFX3 and FOXJ1 have also been found to act together for optimal expression of ciliary genes following transfection into human airway basal cells (Didon et al 2013). The presence of either or both of X boxes and forkhead binding sites in the upstream region of enriched genes will be used to implicate enriched genes in a ciliary role.
1.11 Study aims

The aim of this study was to identify and characterise novel ciliary genes. I utilised the temporal profiling data of Cachero et al. (2011) and zur Lage and Jarman (unpublished) to form a candidate list of ciliary genes that could then be investigated. Uncharacterised genes found to be regulated by Fd3F (Newton et al. 2012) were also implicated in a ciliary role and thus included in this candidate gene list. Furthermore identifying factors downstream of the Atonal transcription cascade (Figure 1.10) can shed further light on how this cascade regulates neuronal differentiation and ciliogenesis in Drosophila chordotonal neurons. Combining enrichment in developing chordotonal neurons with evolutionary conservation, transcriptional regulation and expression in ciliated tissues can provide criteria by which novel ciliary genes can be identified. As defective cilia result in a number of human conditions, the homologues of novel Drosophila ciliary genes provide candidate genes for ciliopathies.

Chapter 2 details the formation of the list of candidate ciliary genes and narrowing it down from those enriched in ato/catoGFP expressing cells. This chapter also details a behavioural screen of candidate genes knocked down by RNAi. CG11253 is one gene that was included on the candidate gene list. An expression reducing mutation caused by a P element insertion in it’s transcriptional unit made it particularly convenient to investigate. Chapter 3 discusses the initial characterisation of CG11253 and whether it is required for ciliary function. Chapter 4 summarises investigation into the role of CG11253. CG11253 is required for the localisation of the axonemal dynein arms required for cilium motility. ZMYND10, the human homologue of CG11253, was identified being mutated in PCD. This inspired investigation of further genes on the candidate gene list, including Fd3F targets, to identify additional cilium motility genes whose homologues could be implicated in PCD. Chapter 5 discusses the characterisation of CG31320 in an attempt to identify one such gene. Chapter 6 details the identification of further candidate genes from those enriched in ato/catoGFP cells and investigation into whether they could have a role in cilium motility and thus their homologues can be candidate PCD genes.
Identifying candidate ciliary genes from chordotonal neuron temporal profiles

2.1 Introduction

As described in the introduction, a number of techniques have been used to identify ciliary genes. Differential expression in ciliated tissues or during ciliogenesis has previously identified genes potentially linked to cilia. For example Blacque et al (2005) identified genes expressed in ciliated sensory neurons but not in other Caenorhabditis elegans tissues. Stolc et al (2005) identified genes expressed during Chlamydomonas reflagellation. Cachero et al (2011) and zur Lage and Jarman (unpublished) identified genes that were enriched in developing Drosophila sensory neurons prior to ciliogenesis. A number of ciliary genes were differentially expressed in these cells (Cachero et al 2011, zur Lage and Jarman unpublished). Uncharacterised genes enriched in atoGFP or catoGFP expressing cells can therefore be considered candidate ciliary genes.

2.1.1 Candidate gene selection

The atoGFP and catoGFP enrichment data was used as a starting point to generate a list of candidate genes for a role in the formation or function of cilia in Drosophila. Enrichment alone did not necessarily indicate a ciliary role as enriched genes could be required for chordotonal organ function unrelated to cilia. As a result of this a number of other criteria were also used to select candidate genes from those enriched in developing chordotonal neurons. These included expression pattern, presence in other cilia studies, the roles of their homologues and the domains contained within their predicted proteins.
2.1.1.1 Enrichment

Many genes with known ciliary functions were enriched in *atoGFP* and *catoGFP* expressing cells (Cachero et al 2011, zur Lage & Jarman unpublished) (Table 2.1). Cachero et al (2011) found that 36 of the top 100 enriched genes at time point 3 in *atoGFP* expressing cells were also found in the *Drosophila* Cilia and Basal Body database (Laurençon et al 2007). These included IFT genes such as *nompB* (Han et al 2003) and *rempA* (Lee et al 2008) and motility linked genes such as *tilB* (Kavlie et al 2010) (Table 2.1). In addition the putative transition zone gene *dila* (Ma & Jarman 2011) and the transcriptional regulator *fd3F* (Newton et al 2012) were identified and characterised due to their enrichment in *atoGFP* and *catoGFP* expressing cells. Time point three for *atoGFP* expressing cells showed the highest number of enriched cilia-linked genes, although several were also enriched at earlier time points (Cachero et al 2011). Many known ciliary genes were enriched in *catoGFP* expressing cells from time point two (zur Lage & Jarman unpublished). Genes highly enriched in both *atoGFP* and *catoGFP* expressing cells formed a starting point from which a candidate ciliary gene list could be constructed (Table 2.2). Enriched uncharacterised genes were investigated for other evidence implicating them in ciliogenesis.

Several genes were found to differ in the timing and level of enrichment between *atoGFP* and *catoGFP* cells. This discrepancy in enrichments could be due to the different expression patterns of *atoGFP* and *catoGFP*. As for example *atoGFP* is also expressed in the larval eye, whilst *catoGFP* is expressed in the developing ES at later stages (zur Lage & Jarman 2010, Cachero et al 2011). Alternatively these differences in enrichment could be due to partial differences in time points due to the environment in which embryos were aged (zur Lage & Jarman unpublished).

2.1.1.2 Expression pattern

Genes were selected where there was evidence of a chordotonal or chordotonal enriched expression pattern (Table 2.3). In situ hybridisation had previously been used to detect the RNA expression pattern of a number of enriched genes (Cachero et al 2011). Further evidence for a ciliary role came from genes that were also expressed in the testes. Expression in both motile ciliated cells of *Drosophila* could
Table 2.1 Ciliary gene mRNA enrichment in *atoGFP* and *catoGFP* expressing cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>T1 <em>atoGFP</em></th>
<th>T2 <em>atoGFP/catoGFP</em></th>
<th>T3 <em>atoGFP/catoGFP</em></th>
<th>T4 <em>catoGFP</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fd3F</em></td>
<td>5.55</td>
<td>5.44/35.95</td>
<td>19.72/27.20</td>
<td>19.04</td>
</tr>
<tr>
<td><em>rampA</em> (IFTA)</td>
<td>x</td>
<td>x/3.88</td>
<td>2.71/4.05</td>
<td>4.40</td>
</tr>
<tr>
<td><em>nompB</em> (IFTB)</td>
<td>x</td>
<td>x/2.06</td>
<td>2.35/3.20</td>
<td>6</td>
</tr>
<tr>
<td><em>tilB</em></td>
<td>x</td>
<td>x/2.38</td>
<td>2.30/3.34</td>
<td>3.87</td>
</tr>
</tbody>
</table>

Table 2.2 Candidate gene mRNA enrichment in *atoGFP* and *catoGFP* expressing cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>T1 <em>atoGFP</em></th>
<th>T2 <em>atoGFP/catoGFP</em></th>
<th>T3 <em>atoGFP/catoGFP</em></th>
<th>T4 <em>catoGFP</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CG10064</em></td>
<td>x</td>
<td>x/6.48</td>
<td>8.28/10.8</td>
<td>15.91</td>
</tr>
<tr>
<td><em>CG10339</em></td>
<td>x</td>
<td>x/10.25</td>
<td>7.55/13.09</td>
<td>15.63</td>
</tr>
<tr>
<td><em>CG11253</em></td>
<td>x</td>
<td>2.23/21.77</td>
<td>18.41/20.53</td>
<td>20.82</td>
</tr>
<tr>
<td><em>CG14353</em></td>
<td>x</td>
<td>x/2.29</td>
<td>2.08/2.81</td>
<td>3.58</td>
</tr>
<tr>
<td><em>CG14905</em></td>
<td>x</td>
<td>3.09/32.44</td>
<td>20.39/27.93</td>
<td>33.10</td>
</tr>
<tr>
<td><em>CG15701</em></td>
<td>x</td>
<td>x/10.45</td>
<td>5.86/11.1</td>
<td>10.51</td>
</tr>
<tr>
<td><em>CG18472</em></td>
<td>x</td>
<td>x</td>
<td>x/2.53</td>
<td>6.23</td>
</tr>
<tr>
<td><em>CG18675</em></td>
<td>x</td>
<td>x/11.89</td>
<td>12.31/14.82</td>
<td>19.02</td>
</tr>
<tr>
<td><em>CG3085</em></td>
<td>x</td>
<td>x/10.86</td>
<td>9.26/14.69</td>
<td>19.7</td>
</tr>
<tr>
<td><em>CG31320</em></td>
<td>x</td>
<td>x/2.44</td>
<td>2.77/4.57</td>
<td>7.06</td>
</tr>
<tr>
<td><em>CG32703</em></td>
<td>x</td>
<td>x/9.34</td>
<td>7.90/13.99</td>
<td>13.38</td>
</tr>
<tr>
<td><em>CG4525</em></td>
<td>x</td>
<td>2.43/27.34</td>
<td>10.5/23.05</td>
<td>20.38</td>
</tr>
<tr>
<td><em>CG5359</em></td>
<td>x</td>
<td>x/3.37</td>
<td>x/2.71</td>
<td>2.75</td>
</tr>
<tr>
<td><em>CG6405</em></td>
<td>x</td>
<td>2.09/2.23</td>
<td>2.79/4.89</td>
<td>4.25</td>
</tr>
</tbody>
</table>

Table 2.1 & 2.2 The enrichment of ciliary-linked and candidate gene mRNA at time points 1-4 in cells expressing *atoGFP* (T1-3) (Cachero et al 2011) or *catoGFP* (T2-4) (zur Lage & Jarman unpublished). X indicates that enrichment is less than two fold or that no enrichment is present.
Table 2.3 Candidate gene expression in *Drosophila*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Embryonic expression pattern</th>
<th>Sperm proteome</th>
<th>Testes expression</th>
<th>Testes expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG10064</td>
<td>Ch (B)</td>
<td>Y</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td>CG10339</td>
<td>Ch (C)</td>
<td>N</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>CG11253</td>
<td>Ch (C)</td>
<td>N</td>
<td>Y</td>
<td>Early and late spermatocytes</td>
</tr>
<tr>
<td>CG14353</td>
<td>Ch (S)</td>
<td>N</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td>CG14905</td>
<td>Ch (N)</td>
<td>N</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td>CG15701</td>
<td>ND</td>
<td>N</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>CG18472</td>
<td>ND</td>
<td>N</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CG18675</td>
<td>Ch (M, B)</td>
<td>N</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CG3085</td>
<td>Ch (C)</td>
<td>Y</td>
<td>Y</td>
<td>Early spermatocytes to late elongating spermatids</td>
</tr>
<tr>
<td>CG31320</td>
<td>Ch (C)</td>
<td>N</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td>CG32703</td>
<td>ND</td>
<td>N</td>
<td>Y</td>
<td>No expression detected</td>
</tr>
<tr>
<td>CG4525</td>
<td>Ch enriched (C)</td>
<td>N</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td>CG5359</td>
<td>Pan sensory (B, C)</td>
<td>N</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td>CG6405</td>
<td>ND</td>
<td>N</td>
<td>Y</td>
<td>Early spermatocytes to early elongating spermatocytes</td>
</tr>
</tbody>
</table>

Expression in testes as described in FLYAtlas2, mRNA enrichment as detected by Affymetrix expression array, Chintapalli et al 2007.
Testes expression pattern as shown by FLY TED (Zhao et al 2009). (Ch: Chordotonal, N : No, Y: Yes, ND: not determined)
indicate a role in cilium motility. Evidence for testis expression was provided by the presence of a genes encoded protein in the *Drosophila* sperm proteome (Dorus et al 2006, Wasbrough et al 2010), enrichment of mRNA in the testes (FlyAtlas - Chintapalli et al 2007) or the presence of a testes RNA in situ hybridisation expression pattern (Fly-TED, Zhao et al 2009) (Table 2.3). Genes that were found to be expressed in both chordotonal neurons and the testes were considered good candidates for a role in cilia and several were included in the candidate gene list for further investigation.

### 2.1.1.3 Domain structure

It had previously been found that TPR (TetratricoPeptide Repeats) and WD40 domains were enriched in ciliary datasets (Avidor-Reiss et al 2004, Li et al 2004, Baron et al 2007). As a result highly enriched genes that encode proteins with predicted TPR or WD40 domains were also investigated (Table 2.4). Both of these domains have been found in Drosophila IFT genes (OSEG (Outer Segment)1-6) (Avidor-Reiss et al 2004) and are thought to play a role in protein-protein interactions (Blatch & Lassle 1999, Xu & Min 2011). Enriched genes were scanned for these domains using InterProScan (Quevillon et al 2005) and Pfam (Punta et al 2012). Highly enriched genes containing these domains were considered potential candidate genes for further investigation.

### 2.1.1.4 Genes previously linked to cilia

The many genomic, proteomic or transcriptomic investigations of ciliary genes have identified several ciliary candidates. Enriched genes that had previously been implicated in having a role in cilia but were as yet uncharacterised were identified (Table 2.5). These studies include proteomic analyses of cilia from human bronchial epithelial cells (Ostrowski et al 2002), trypanosome flagella (Broadhead et al 2006), *Chlamydomonas* flagella (Pazour et al 2005) and *Chlamydomonas* basal bodies (Keller et al 2005).

Comparative genomic techniques have also identified a number of potential ciliary genes. Studies by Avidor-Reiss et al (2004) and Li et al (2004) highlighted genes conserved in organisms with cilia. The data they generated was used to select
Table 2.4 Candidate gene human homologues and protein domains

<table>
<thead>
<tr>
<th>Gene</th>
<th>Human homologue</th>
<th>Protein domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG10064</td>
<td>WDR16</td>
<td>WD40</td>
</tr>
<tr>
<td>CG10339</td>
<td>-</td>
<td>Carboxyl esterase</td>
</tr>
<tr>
<td>CG11253</td>
<td>ZMYND10</td>
<td>MYND</td>
</tr>
<tr>
<td>CG14353</td>
<td>WDR92</td>
<td>WD40</td>
</tr>
<tr>
<td>CG14905</td>
<td>CCDC63/CCDC114</td>
<td>-</td>
</tr>
<tr>
<td>CG15701</td>
<td>WDR60</td>
<td>WD40</td>
</tr>
<tr>
<td>CG18472</td>
<td>SPAG1</td>
<td>TPR</td>
</tr>
<tr>
<td>CG18675</td>
<td>C21orf59</td>
<td>-</td>
</tr>
<tr>
<td>CG3085</td>
<td>Tektin 2</td>
<td>Tektin</td>
</tr>
<tr>
<td>CG31320</td>
<td>HEATR2</td>
<td>HEAT</td>
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<td>MAPK15</td>
<td>Kinase</td>
</tr>
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<td>TPR</td>
</tr>
<tr>
<td>CG5359</td>
<td>TCTEX1D2</td>
<td>Tctex-1</td>
</tr>
<tr>
<td>CG6405</td>
<td>FAM92A1</td>
<td>Fam92</td>
</tr>
</tbody>
</table>

Table 2.4 Homologues found by DRSC integrative ortholog prediction (Hu et al 2011). Protein domains as found by Pfam (Punta et al 2012) and InterProScan (Quevillon et al 2005).
<table>
<thead>
<tr>
<th></th>
<th>CG10064</th>
<th>CG10339</th>
<th>CG11253</th>
<th>CG14353</th>
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<th>CG18675</th>
<th>CG3085</th>
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<th>CG4525</th>
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<tr>
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<td></td>
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</tr>
</tbody>
</table>

Table 2.5 Candidate genes found in other studies.

Information sourced from the studies themselves and the DCBB (Drosophila Cilia and Basal Body) database compiled by Laurençon et al (2007).
candidate genes from those highly enriched in \textit{ato/catoGFP} cells. Homologues of two genes found by Baron et al (2007) in their comparison of motile ciliated and non-motile ciliated organisms were found to be enriched in \textit{ato/catoGFP} expressing cells. RNAi knock down of these (\textit{TbCMF1}/\textit{CG14353} & \textit{TbCMF12}/\textit{CG18675}) resulted in trypanosome behaviour consistent with a reduction in ciliary motility (Baron et al 2007). As such these genes were also selected for screening.

\textbf{2.1.1.5 Transcriptional regulation}

Transcriptional regulation can give an indication of a gene's role. As a result the proposed targets of known ciliary master transcriptional regulators have been identified as candidate genes. This includes genes with conserved RFX binding motifs (Efimenko et al 2005, Laurençon et al 2007) and those found to be regulated by Fd3F (Newton et al 2012) (Table 2.5). F Newton had identified a number of uncharacterised genes to be transcriptionally regulated by Fd3F (Newton et al 2012). These were also included among the list of candidate genes.

\textbf{2.1.2 Climbing assay}

The approaches described above were used to narrow down the list of uncharacterised genes enriched in developing chordotonal neurons to a list of ciliary candidate genes. These candidates were then screened to ascertain whether disrupting their expression resulted in a reduction in chordotonal neuron function and thus a potential reduction in ciliary function.

Candidate genes were screened using a behavioural assay to investigate whether they play a role in chordotonal neuron function. The chordotonal neurons are required for proprioception, gravitaxis and hearing. \textit{Ato} \textsuperscript{l} flies which lack most of the chordotonal organs display an uncoordinated phenotype (Jarman et al 1995). Mutations in \textit{Drosophila} ciliary genes have also been reported to cause uncoordinated fly movement (Kernan et al 1994, Eberl et al 2000). A climbing assay would allow the proprioceptive and gravitactic capabilities of flies to be assessed. If proprioceptive and gravitactic function was disrupted, this would suggest that chordotonal and potentially ciliary function are also affected. The behavioural assay allowed the
identification of candidates with functions vital for cell function, which could be investigated further to elucidate their ciliary role.

2.1.3 Climbing assay method (Method A and Method B)

A basic climbing assay had previously been used by members of the group which involved quantifying the number of flies which can pass a threshold in a given time (Ma & Jarman 2011, Newton et al 2012). However whilst this approach can identify severely uncoordinated phenotypes, it does not allow the identification of milder defects. The first climbing assay approach I used (Method A) was to divide the tube in which the flies were assayed into four bins and generate a climbing score based on how many flies were in each bin after 10 seconds. However this approach had some limitations and a second approach was also taken (Method B). Method B involved measuring the individual height each fly was able to climb after 10 seconds. The experimental technique of both Method A and B are similar. The main difference between these methods is in the way the data was collected and analysed.

2.1.4 Positive controls

In addition to the candidate genes a number of positive control genes were also screened. This allowed confirmation that my approach could be used to identify defects in ciliary function. These include the transcriptional regulator fdl3F and the IFT (A and B respectively) genes rempA and nompB.

2.1.5 GAL4/UAS

In order to investigate candidate gene function the UAS/GAL4 system was utilised to drive hairpin RNA (hpRNA) expression to induce knock down expression of genes of interest.

The GAL4/UAS system allows spatiotemporal control of the expression of a construct (Brand & Perrimon 1993, Duffy 2002). GAL4 was originally found in yeast, where it regulates gene expression. The site at which GAL4 binds is known at the Upstream Activating Sequence (UAS). In Drosophila the promoter of a known gene can be used to drive expression of GAL4 in a spatiotemporal pattern matching the normal expression pattern of that gene. GAL4 can then bind UAS sequences
ahead of a gene of interest, driving its expression in this pattern. Outwith the normal area of expression of the chosen promoter the gene of interest is not expressed as GAL4 is not present.

2.1.6 RNAi to knock down candidate gene expression

The GAL4/UAS system can be used to direct RNAi mediated knock down of a gene of interest. GAL4 binding to UAS can stimulate the expression of hpRNA which is then cleaved by the ribonuclease Dicer-2 (Dcr-2) to form short interfering RNA (siRNA) (Hannon 2002, Dietzl et al 2007). These fragments can then bind to homologous mRNA strands, resulting in their degradation by the RNA-induced Silencing Complex (RISC) complex.

This process was used to knock down candidate gene expression in chordotonal neurons. The UAS tagged RNAi constructs (obtained from the Vienna Drosophila RNAi Center) were driven be a Scabrous-GAL4 (Sca-GAL4) driver. Sca-GAL4 is a pan-neural driver, which has been found to drive expression during neurogenesis (Mlodzik et al 1990, Klaes et al 1994). Dcr-2 has previously been shown to increase the efficacy of RNAi mediated knock down (Dietzl et al 2007). As a result of this it was also incorporated into the knock down flies. The control used for comparison was P{attP} (landing site VIE260B), the line which the RNAi lines had originally been injected into when constructed by the Vienna Drosophila RNAi Center (Dietzl et al 2007).

RNAi lines were acquired from the Vienna Drosophila RNAi Center for candidate genes and controls. The genotype of the flies assayed were UAS-Dcr2/+; Scabrous-GAL4/hpRNA, however they will be referred to using the format of GENEConstruct ID.

2.2 Results

2.2.1 Climbing assay (Method A) of candidate genes

2 to 7 day old female flies expressing GAL4 driven hpRNA were assayed for their climbing ability in a climbing assay (Figure 2.1). A climbing score was calculated for four tubes of 17-20 flies for each genotype. nompBKK108656 flies showed a severe uncoordinated phenotype and as a result were not assayed. Positive control
Figure 2.1 Climbing assay (Method A) of candidate ciliary genes.
A graph showing the average climbing score of flies in which candidate genes had been knocked down by RNAi. The flies screened by this assay were the progeny of a cross between UAS-RNAi or control flies and UAS-Dcr; Scabrous-GAL4. 17-20 flies were screened in each assay (N=4). An average climbing score was calculated from each repeat. Two data sets are shown, these are indicated by circles and squares. Error bars show SD. P values: fd3F: 0.0001, CG14353: 0.0128, CG14905: 0.0214, CG18472: 0.0018, CG6405: 0.0455 (calculated by Kruskal-Wallis and Dunns multiple comparisons test).
flies climbed significantly worse than the $P_{\text{attP}}$ negative control flies, indicating disrupted proprioception. Significantly lower climbing scores were also observed for $CG14353^{KK102248}$, $CG14905^{KK100384}$, $CG18472^{KK100470}$ and $CG6405^{KK101426}$. This indicates that disrupting these genes could result in a chordotonal defect, and that they can be considered for further investigation.

$CG11253^{KK100221}$ appeared to have reduced climbing capability, however it was not found to be statistically significant from the negative control flies. At the time that this climbing assay was carried out I had already found that a $CG11253$ P element insertion mutant ($CG11253^{EY10866}$) displayed reduced climbing ability due to disruption of chordotonal neuron function (discussed in Chapter 3). It is possible that $CG11253^{KK100221}$ did not sufficiently disrupt gene function as to show a phenotype. However it appears that there is a reduction in climbing ability, if not a statistically significant one. To investigate this further the climbing assay was repeated using a different method.

### 2.2.2 Climbing assay (Method B) of candidate genes

When repeated, rather than generating a climbing score, the height climbed by each individual fly was measured. Each assay was filmed and an image recorded after 10 seconds. The height each fly climbed from the base of the tube was then measured in these images using ImageJ. It has previously been shown that the density of flies within a tube does not affect their climbing capability (up to 25 flies) (Gargano et al 2005). This suggests that each fly climbs independently of all others assayed at the same time as it. This allows the assessment the height climbed by each fly individually.

Using this method the control $fd3F^{KK110727}$ and $rmpA^{KK100864}$ flies showed significantly reduced climbing capability indicating a proprioceptive defect (Figure 2.2). $CG14353^{KK102248}$ and $CG6405^{KK101426}$, which provided statistically significant reductions in climbing capability in the Method A assay, also did so using Method B. The reduction in climbing capability shown by $CG11253^{KK100221}$ was found to be statistically significant using Method B. In addition $CG31320^{KK102625}$ and
Figure 2.2 Climbing assay (Method B) for candidate genes.

A box-whisker plot showing the heights climbed by flies in which candidate genes were knocked down by RNAi. The flies screened by this assay were the progeny of a cross between UAS-RNAi or control flies and UAS-Dcr; Scabrous-GAL4. The heights climbed by 32-40 flies (N=32-40) of each genotype were calculated using ImageJ. P values: fd3F, rempA, CG11253, CG14353, CG18675, CG31320: <0.0001, CG6405: 0.0021 (calculated by Kruskal-Wallis test followed by Dunn's multiple comparisons test).
CG18675^KK102730^, which had not previously been screened, also showed a statistically significant reduction in climbing capability.

2.3 Discussion

2.3.1 Control genes showed climbing defects

RNAi constructs targeting \( fd3F \) and \( rempA \) were used as positive controls to confirm that the climbing assay can be used to identify ciliary defects. The climbing ability of both \( fd3F^{KK110727} \) and \( rempA^{KK100864} \) flies was statistically significantly different from the \( P\{attP\} \) control. This indicates that ciliary defects can indeed be identified by the climbing assay. It also further indicates that ciliary function is required for the proprioceptive and gravitactic behaviour being assessed by the assay. As knocking down positive control genes resulted in climbing defects, if knocking down previously uncharacterised genes results in reduced climbing ability they too could be required for ciliary function.

2.3.2 Climbing assay considerations

A number of caveats need to be considered when assessing the climbing assay results. The results form a starting point for further investigation rather than confirmation of a role in chordotonal or ciliary function. That the gene in question is knocked down also remains to be confirmed. In situ hybridisation would provide a way of assessing this. Additionally the lack of a reduction in climbing ability does not indicate that the candidate genes are not required for chordotonal or ciliary function. It is possible that a knock down of expression did not occur or that it was not sufficient to elicit a phenotype. The results obtained could be confirmed by assessing further hrRNA constructs or by generating mutants.

2.3.3 Method A Climbing assay

The initial climbing assay using Method A identified that knocking down \( CG14353, CG14905, CG18472 \) or \( CG6405 \) resulted in reduced climbing ability. Knocking down \( CG11253 \) and \( CG15701 \) also appeared to show reduced climbing ability however this was not found to be statistically significant. This conflicted with findings that a mutation which reduced \( CG11253 \) expression caused disrupted
climbing behaviour (discussed in Chapter 3). Whilst a climbing defect is clear for the four genes found to be statistically different from the control, other genes that are required for chordotonal function could be missed using this method. It was thought that a more accurate way to assess climbing ability would be to measure the height each individual fly climbed rather than averaging these measurements by dividing into bins and then generating average climbing scores.

2.3.4 Method B Climbing assay

CG14353\textsuperscript{KK102248} and CG6405\textsuperscript{KK101426} showed a climbing defect using both Method A and Method B. 10 lines were screened in both assays, of those only CG11253\textsuperscript{KK100221} showed a different result. In addition to re-screening genes from the first climbing assay, a number of new lines were screened in which genes from the candidate list were knocked down. Of these a statistically significant reduction in climbing ability was found in CG18675\textsuperscript{KK102730} and CG31320\textsuperscript{KK102635}.

The result for CG11253\textsuperscript{KK100221} using Method B mirrored the climbing defect found in a CG11253 mutant (Chapter 3). It also justified the switch from Method A to Method B as it appeared to be more sensitive for detecting defective climbing ability. As a result of this I used Method B for all future investigations (although some had already been conducted using Method A).

2.3.5 Candidate genes for further investigation

Combining the findings of both climbing assays identifies several candidates for further investigation (Climbing assay method used to identify shown in brackets): CG11253 (B, not A), CG14353 (A & B), CG14905 (A), CG18472 (A), CG18675 (B), CG31320 (B) and CG6405 (A & B). The reduction in the climbing ability associated with these genes highlights them as potentially being required for chordotonal and ciliary function.

Further work on CG11253 (discussed in Chapter 3 and 4), CG31320 (Chapter 5), CG14353 (Chapter 6) and CG18675 (Chapter 6) has been carried out. Characterisation of CG11253 and its role in ciliary function had already begun when this climbing assay was carried out. Early work on CG31320 had also begun,
however the finding that $CG31320^{KK102625}$ had reduced climbing ability inspired its further use to investigate the role of $CG31320$. $CG14353$ and $CG18675$ were selected for further investigation as a direct result of the reduced climbing ability recorded in the climbing assay.

$CG14905$ is a homologue of the *Chlamydomonas* gene *ODA1*, a member of the ODA-docking complex required for ODA assembly to the flagellum (Takeda et al 2002). $CG14353$ and $CG18675$ have also previously been implicated in ciliation motility (Baron et al 2007). This was due to a lack of cytokinesis following an RNAi mediated knock down of homologues of these genes in trypanosomes. $CG18472$ and $CG6405$ are two novel candidate genes with limited or no previously reported links to cilia. $CG6405$ was identified as having a nearby X box sequence for regulation by RFX, however it is not known whether this is functional (Laurençon et al 2007). Whilst I have not been able to follow up and investigate these genes myself in the course of this study they are good candidates for future work.

### 2.3.6 Candidate genes implicated in human disease

Since carrying out these initial investigations several of the homologues of the candidate genes have been implicated in human ciliary diseases. Ciliopathy patients have been identified in which homologues of the *Drosophila* genes screened were found to be mutated. This acts as proof that identifying ciliary candidates by this method can provide candidate genes for human diseases. In addition to the genes discussed below PCD patients have been found in which the human homologues of $CG11253$, $CG31320$ and $CG18675$ are mutated. These are discussed in more detail in Chapters 4, 5 and 6 respectively.

#### 2.3.6.1 $CG18472$/SPAG1 and $CG14905$/CCDC114 are mutated in PCD

Mutations in *SPAG1* (Knowles et al 2013b) and *CCDC114* (Knowles et al 2013a, Onoufriadis et al 2013) have been identified in patients with PCD. The *Drosophila* homologues of these genes ($CG18472$ and $CG14905$) showed a reduced climbing ability suggesting a proprioceptive defect when knocked down by siRNA. Mutations in *SPAG1* resulted in disruption of ODA and IDA (Knowles et al 2013b). *SPAG1* was found to localise to the cytoplasm, leading to the suggestion that it was involved
in assembly or transport of axonemal dynein arms (Knowles et al 2013b). Mutations in CCDC114 resulted in a loss of ODA suggesting a role in ODA attachment (Knowles et al 2013a, Onoufriadis et al 2013).

**2.3.6.2 CG15701/WDR60**

Knocking down CG15701 was not found to cause a significantly reduced climbing capability when assessed using Method A. It was not rescreened using Method B. The level of climbing defect from the first climbing assay (Method A) appears to be similar to that for CG11253KK100221. When CG11253KK100221 was assayed using Method B it proved to have a statistically significant defect in climbing behaviour. This suggests that CG15701KK102519 may also have shown defective climbing ability.

Mutations in WDR60, the human homologue of CG15701, have been found in cases of the skeletal ciliopathies Jeune syndrome and short-rib polydactyly syndrome (McInerney-Leo et al 2013). WDR60 was found to localise to the ciliary base and be required for ciliogenesis. This finding suggests that identifying and characterising genes enriched in ato/catoGFP flies can help identify candidate genes not just for PCD but also for other ciliopathies linked to primary cilia. Additionally as WDR60 is required for ciliary function, CG15701 may also be a ciliary gene. The inability of the Method A climbing assay to identify CG15701 as a candidate, despite a potential reduction in climbing ability, further highlights its deficiencies. Using Method B to assay CG15701KK102519 may have allowed the detection of a significant climbing defect as it did CG11253KK100221.

**2.4 Conclusion**

The list of genes enriched in developing chordotonal cells marked by atoGFP or catoGFP expression was narrowed down to a candidate gene list using additional sources of data. The climbing assay allowed this list to be screened and candidates that showed a potentially defective chordotonal neuron and cilia phenotype when knocked down to be identified. Several of these candidates have since been implicated in human ciliary disease. This data acts as a starting point, suggesting several of these candidates for further investigation. Several of the genes highlighted by the screen will be discussed in the forthcoming chapters.
3

CG11253: a candidate ciliary gene

3.1 Introduction

Temporal profiling of developing chordotonal neurons has allowed the identification of many previously uncharacterised genes (Cachero et al 2011, zur Lage & Jarman Unpublished). As shown in Chapter 2 this list of enriched genes was narrowed down to a candidate gene list for further investigation. One of my aims for this study was to use the genetic tools and techniques available for Drosophila to identify novel ciliary genes. One such tool is P element insertion mutations in which transposons have been inserted into the Drosophila genome in order to disrupt gene function (Bellen et al 2004). One such mutation was available for one of the genes on the candidate gene list: CG11253. A 10.8Kb P(EPgy2) transposon had been inserted into the transcriptional unit, 2 base pairs into the final intron of CG11253 (Figure 3.1).

3.1.1 CG11253 is regulated by Fd3F

In addition CG11253 was amongst the genes found by F Newton to be regulated by Fd3F (Newton et al 2012). Examining mRNA expression of CG11253 in fd3f embryos by in situ hybridisation identified it as a potential target of Fd3F (Newton et al 2012). Genes regulated by Fd3F include the TRPV channels Nan and Iav, and genes associated with retrograde transport; including IFT-A components and dynein motor. Additionally Fd3F regulates the expression of genes linked to cilium motility including both axonemal dynein arm components and genes required for axonemal dynein arm assembly such as tilB (Eberl et al 2000, Kavlie et al 2010). Fd3F does not however regulate the expression of all ciliary genes. A number of genes showed no change in expression in fd3f embryos including IFT-B complex components (Newton et al 2012). Transcriptional regulation by Fd3f is therefore suggestive of a role in retrograde transport or cilium motility.
Table 3.1. Enrichment levels of CG11253 in atoGFP (Cachero et al 2011) and catoGFP (zur Lage & Jarman unpublished) expressing cells during embryonic development.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Embryonic Stage</th>
<th>Enrichment in cells expressing atoGFP</th>
<th>Enrichment in cells expressing catoGFP</th>
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<td>T2 (6.45 to 7.45 Hrs)</td>
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<td>2.23</td>
<td>21.77</td>
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<tr>
<td>T3 (7.45 to 8.45 Hrs)</td>
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<td>18.41</td>
<td>20.53</td>
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<tr>
<td>T4 (8.45 to 9.45 Hrs)</td>
<td>12/13</td>
<td>n/a</td>
<td>20.82</td>
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</table>

Table 3.1. Enrichment levels of CG11253 in atoGFP (Cachero et al 2011) and catoGFP (zur Lage & Jarman unpublished) expressing cells during embryonic development.

Figure 3.1 A P element insertion in CG11253
A diagram showing the position of the P(EPgy2) EY10866 transposon in the CG11253 transcriptional unit in CG11253EY10866 flies.

Figure 3.2 : The predicted MYND domain of CG11253.
The protein structure of CG11253 showing the position of the MYND domain (green) and the LXXLL motifs (purple). An alignment of the domain is also shown with the cysteine and histidine residues thought to be important for domain function highlighted in red.
3.1.2 CG11253 is expressed in the testes

A testes mRNA in situ hybridisation pattern for CG11253 is available in the FLYTED database (Zhao et al 2009). This shows that CG11253 is also expressed in the primary spermatocytes of the adult testes. CG11253 is therefore expressed in two ciliated tissues prior to the maturation of those cells and shortly before the beginning of ciliogenesis. It is worth noting that the two tissues in which CG11253 is expressed are the only two Drosophila cell types to contain motile cilia, perhaps suggesting a role for CG11253 specific to cells with motile cilia. This would be consistent with its regulation by Fd3F.

3.1.3 CG11253 is enriched in developing chordotonal neurons

Cachero et al (2011) found CG11253 mRNA to be enriched in atoGFP expressing cells at time points 2 and 3 (6 45 -8 45) hours of embryonic development at 25 °C) (Table 3.1). CG11253 was the fourth highest enriched gene at time point 3 (equivalent to late stage 12). zur Lage & Jarman (unpublished) found CG11253 to also be enriched in catoGFP expressing cells at time points 2 to 4 (6 45 to 9 45 hours). High enrichment in these cells at these time points suggests that CG11253 could have an important role in neuronal development and differentiation. As already stated genes required for ciliogenesis have been found to be highly enriched in chordotonal cells at these time points despite it being before ciliogenesis occurs. This suggests that highly enriched genes of unknown function could be previously uncharacterised ciliary genes. It is possible therefore that CG11253 has a role in the generation or function of cilia.

3.1.4 Protein structure: MYND domain and LXXLL motifs

The predicted protein encoded by CG11253 is 451 amino acids in length and contains a zinc finger MYND type domain at its C terminus (Figure 3.2). The MYND domain is named after other proteins that also contain this domain, namely Myeloid, Nervy and DEAF-1 (Gross & McGinnis 1996). Although this domain has been found in proteins that can regulate transcription (for example DEAF-1) it is thought to have a role in protein-protein interactions rather than DNA-protein interactions (Gross & McGinnis 1996). Proteins with this domain have previously
been linked to cilia. DAF-25/CHB-3 has been found to localise to sensory cilia in
*Caenorhabditis elegans* (Jensen et al 2010) and is required for the ciliary localisation
of guanylyl cyclases.

In addition to the MYND domain CG11253 contains several LXXLL motifs (L –
leucine, X – any amino acid (identified by collaborator R Emes)). These motifs have
previously been found to be required for protein-protein interactions. LXXLL motifs
have been found in transcriptional co-activators and are required for their
interactions with nuclear receptors (McInerney et al 1998, Savkur & Burris 2004).
However LXXLL motifs have also been found in proteins not linked to
transcriptional regulation. The LDXLL of the scaffold protein paxillin are thought to
be required for protein–protein interactions at focal adhesions (Brown et al 1998).

### 3.1.5 MYND domain conservation

The CG11253 predicted MYND domain is well conserved, with the cysteine and
histidine residues that could form the zinc binding motif being conserved from
humans to *Drosophila* (Gamsjaeger et al 2007) (Figure 3.2). As this domain is well
conserved (45% identity – detected using DRSC Integrative Ortholog Prediction
Tool (Hu et al 2011)) it is likely that it is important for CG11253 function, and
implicates the protein to act in a complex with other proteins due to the domains
function in protein-protein interactions. LXXLL motifs do exist in the human
homologue of CG11253 (ZMYND10), but in different positions to those found in
CG11253. They could however still be required for the function of both proteins.

### 3.1.6 CG11253 is a candidate ciliary gene

*CG11253* is expressed in all *Drosophila* motile ciliated cells and is transcriptionally
regulated by a known regulator of ciliary genes. *CG11253* is therefore a good
candidate to play a ciliary role. The existence of mutant flies in which a P element
has been inserted into the transcriptional unit of *CG11253* could make investigating
it more convenient that for other genes on the candidate gene list. In this chapter I
will describe how I investigated whether *CG11253* had a role in ciliary and
chordotonal neuron function,
3.2 Results

3.2.1 Expression pattern of CG11253

RNA in situ hybridisation was used to examine the expression pattern of CG11253. This was conducted to confirm the previously identified transcriptomic enrichment (Cachero et al. 2011, zur Lage & Jarman unpublished) and to observe whether this gene was expressed in any areas other than the cells of the developing chordotonal organs. CG11253 mRNA is expressed between stages 11 and 17 in developing chordotonal neurons (Figure 3.3). Expression is most clearly observed in the lateral (Ich5) and ventral (v’ch1 and vchA/B) neurons (Figure 3.3E).

Expression from stage 11 is consistent with the enrichment data as time point 2 is comparable with late stage 11 or early stage 12. This again confirms that CG11253 is expressed prior to ciliogenesis and before the final round of cell divisions have occurred. This expression pattern indicates that CG11253 is expressed in a spatiotemporal region consistent with a role in chordotonal neuron development.

3.2.2 Expression of CG11253 is transcriptionally regulated by RFX

As mentioned in the introduction to this chapter, F Newton had previously found that CG11253 was transcriptionally regulated by Fd3F (Newton et al. 2012). The upstream region of CG11253 contains binding motifs for both Fd3F and RFX (Figure 3.4A). The closest to the transcriptional start site contains two X boxes and a forkhead binding site in close proximity. Further upstream (120 bp) there is another pair of binding sites, in this case with one X box and one forkhead-binding site. The presence of these binding motifs suggested that expression of CG11253 was regulated by RFX as well as Fd3F. These two transcription factors are known to co-regulate ciliary genes (Newton et al. 2012). No Fd3F targets have yet been identified that when tested are not also RFX targets (Newton et al. 2012).

In order to test whether CG11253 is also regulated by RFX I used in situ hybridisation to probe for mRNA expression in Rfx^{49} embryos (Figure 3.4B & C, Table 3.2). Expression was investigated in a mixed collection of embryos from Rfx^{49}/TM3 parents. Whilst it was not possible to distinguish between genotypes, it
Figure 3.3: The expression pattern of CG11253.
In situ hybridisation showing the embryonic expression of CG11253 mRNA. CG11253 is expressed in the developing chordotonal neurons from Stage 11 to Stage 17.
(A) CG11253 mRNA is detected in the developing chordotonal organs of a Stage 11 embryo.
(B) CG11253 mRNA is detected in the chordotonal neurons of a late Stage 11/early Stage 12 embryo.
(C) CG11253 mRNA is detected in the chordotonal neurons of a Stage 13 embryo.
(D) CG11253 mRNA is detected in the chordotonal neurons of a Stage 15 embryo.
(E) CG11253 mRNA expression can be detected in the Ich5, v’ch1 and vchA/B neurons (Stage 14).
(F) A diagram showing the arrangement of chordotonal neurons in each embryonic abdominal segment (Based on arrangement at Stage 14).
Scale bars: A-C - 100µm, D - 50µm
**Figure 3.4 : CG11253 is transcriptionally regulated by RFX**

(A): The location of regulatory elements upstream of CG31320

A diagram showing the location of X boxes (green) and forkhead binding sites (purple) in the upstream region of CG11253. The motif locations described by Newton et al. (2012) were combined with those found using GenePalette to search for the consensus sequences of the forkhead binding site (RYMAAYA, Newton et al. 2012) and X box (RYYNYY{1,3}RRNRAC, Laurençon et al. 2007).

(B) In situ hybridisation showing CG11253 mRNA localisation in a Stage 13 Rfx49 heterozygous embryo.

(C) In situ hybridisation showing a lack of CG11253 mRNA localisation in a Stage 13 Rfx49 homozygous embryos.

Scale bars – 100µm

**Table 3.2 : CG11253 expression is reduced in Rfx49 embryos**

<table>
<thead>
<tr>
<th></th>
<th>Embryos with CG11253 expression</th>
<th>Embryos with no CG11253 expression detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rfx49</td>
<td>59 (N=76)</td>
<td>17 (N=76)</td>
</tr>
<tr>
<td>Wild type</td>
<td>66 (N=69)</td>
<td>3 (N=69)</td>
</tr>
</tbody>
</table>

Table 3.2 : The proportion of Rfx49 and wild type stage 13 and 14 embryos with reduced CG11253 mRNA expression. (P value : <0.0001 Fishers exact test).
was expected that 25% of these embryos would be homozygous. No CG11253 expression was detected in 22.37% of stage 13 and 14 embryos from this cross (Figure 3.4B & C, Table 3.2). In a wild type control only 4.35% showed no expression. The proportion of embryos with no CG11253 expression is similar to the presumed proportion of Rfx49 homozygotes. From this I concluded that it is likely that CG11253 expression is reduced in Rfx49 embryos and as a result CG11253 is transcriptionally regulated by RFX.

### 3.2.3 Homologues of CG11253

Comparative genomics has previously been used to identify ciliary genes, looking for either those conserved in organisms with cilia and not those without, or conserved in organisms with a particular ciliary type, such as motile cilia (Avidor-Reiss et al 2004, Li et al 2004, Baron et al 2007). As cilia are present in many organisms, it was thought that if CG11253 had a role in ciliary formation or function it could have homologues in other organisms that had cilia. In order to discover homologues of CG11253 I used a reciprocal best DELTA (domain enhanced lookup time accelerated) BLAST search. The best hit in the genus targeted was used to search the Drosophila genome to ensure that the two genes were homologues. This revealed that the presence of CG11253 homologues correlated with the presence of motile cilia and axonemal dynein arms (Figure 3.5, Table 3.3). Organisms that lack cilia such as Arabidopsis thaliana and organisms that only have non-motile cilia such as Caenorhabditis elegans lack any genes that can be considered homologues of CG11253. Amongst those that did have homologues are single celled organisms with motile flagella such as Chlamydomonas reinhardtii and Trypanosoma cruzi.

Notably there is a homologue in Ostreococcus tauri that does not have cilia but does retain homologues of a component of the axonemal dynein arms (Wickstead & Gull 2007). This suggests that CG11253 is linked to dynein arm function rather than ciliary function. CG11253 is well conserved in vertebrates with 32-33% identity to the zebrafish, mouse and human homologues (Table 3.3).
Figure 3.5: The conservation of CG11253.
The conservation of homologues of CG11253 in a variety of organisms with and without cilia. Homologues of CG11253 are found in most organisms with motile cilia and not in most of those with immotile or no cilia. A CG11253 homologue was however found in Ostreococcus tauri which has no cilia.
A black filled circle indicates that a homologue of CG11253 was detected and a white filled circle shows that no homologue was detected. Homologues were detected by best reciprocal DELTA-BLAST. Figure based on similar homologue conservation tree for TilB from Kavlie et al (2010).
### Table 3.3 : Homologues of CG11253

<table>
<thead>
<tr>
<th>Genus</th>
<th>Accession number/identifier of best match</th>
<th>Name</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homo sapiens</strong></td>
<td>NP_956691.1</td>
<td>ZMYND10</td>
<td>32%</td>
</tr>
<tr>
<td><strong>Mus musculus</strong></td>
<td>NP_444483.2</td>
<td>Zmynd10</td>
<td>32%</td>
</tr>
<tr>
<td><strong>Gallus gallus</strong></td>
<td>XP_422923.3</td>
<td>ZMYND10</td>
<td>32%</td>
</tr>
<tr>
<td><strong>Danio rerio</strong></td>
<td>NP_956691.1</td>
<td>zmynd10</td>
<td>32%</td>
</tr>
<tr>
<td><strong>Xenopus tropicalis</strong></td>
<td>XP_002935679.2</td>
<td>zmynd10</td>
<td>33%</td>
</tr>
<tr>
<td><strong>Chlamydomonas reinhardtii</strong></td>
<td>Cre08.g358750.t1.3</td>
<td>-</td>
<td>31%</td>
</tr>
<tr>
<td><strong>Ostreococcus tauri</strong></td>
<td>XP_003074686.1</td>
<td>-</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Plasmodium falciparum</strong></td>
<td>XP_966061.2</td>
<td>-</td>
<td>24%</td>
</tr>
<tr>
<td><strong>Toxoplasma gondii</strong></td>
<td>EPR62334.1</td>
<td>-</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Tetrahymena thermophila</strong></td>
<td>XP_001026696.1</td>
<td>-</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Thalassiosira pseudonana</strong></td>
<td>XP_002295392.1</td>
<td>-</td>
<td>29%</td>
</tr>
<tr>
<td><strong>Phytophthora sojae</strong></td>
<td>EGZ28483.1</td>
<td>-</td>
<td>27%</td>
</tr>
<tr>
<td><strong>Trypanosoma cruzi</strong></td>
<td>EKF38524.1</td>
<td>-</td>
<td>26%</td>
</tr>
<tr>
<td><strong>Giardia lamblia</strong></td>
<td>XP_001708842.1</td>
<td>-</td>
<td>23%</td>
</tr>
</tbody>
</table>

Table 3.3 : CG11253 homologues found and identity calculated by best reciprocal DELTA-BLAST.
3.2.4 CG11253<sup>EY10866</sup> - a P element insertion mutant

In order to investigate the role of CG11253 further I utilised the P element insertion mutant CG11253<sup>EY10866</sup> generated by the Berkeley Drosophila Genome Project in order to do this (Bellen et al 2004). RNA in situ hybridisation was carried out in order to ascertain whether the insertion of a P element had any effect on gene expression. A homozygous stock of CG11253<sup>EY10866</sup> could not be established so a heterozygous stock was used for the in situ hybridisation. The implication of this was that not all of the embryos probed were of the same genotype however CG11253 expression could be detected in a significantly lower proportion of CG11253<sup>EY10866</sup> embryos than wild type (Table 3.4, Figure 3.6). This indicates that CG11253 expression is reduced in CG11253<sup>EY10866</sup> embryos. It is not known whether insertion of the transposon results in a null mutation or whether levels of expression are simply reduced to an undetectable level. There is some evidence that CG11253<sup>EY10866</sup> is not a null as a few embryos were identified with very low levels of expression (Figure 3.6D).

3.2.5 Behavioural assay of CG11253<sup>EY10866</sup>

If CG11253 is a ciliary gene it could be required for the chordotonal neuron’s function or development. The chordotonal organs, and their ciliated neurons are required for fly proprioception, gravitaxis and hearing. Therefore if CG11253 were vital for function, then reduction of its expression would result in impairment of these behavioural attributes. In order to discover whether this was the case a climbing assay was used to assess the proprioceptive and gravitactic capabilities of CG11253<sup>EY10866</sup> flies. Whilst expression has only been detected in embryonic chordotonal organs the same transcriptional cascade governs adult chordotonal development and differentiation. It is therefore likely that CG11253 is also expressed during the development of adult chordotonal neurons and as such the function of these neurons can be tested.

The climbing assay involved knocking the flies to the bottom of a tube and assessing their ability to climb back up. If chordotonal neuron function is disrupted then the flies will be less able to right themselves, sense gravity and coordinate their limbs to
Figure 3.6: *CG11253<sup>EY10866</sup>* shows reduced *CG11253* expression

(A) In situ hybridisation showing *CG11253* mRNA expression in a wild type Stage 13 embryo.

(B) In situ hybridisation of Stage 13 *CG11253<sup>EY10866</sup>* embryo in which *CG11253* mRNA expression could not be detected.

(C) In situ hybridisation showing *CG11253* mRNA expression in a wild type Stage 13 embryo.

(D) In situ hybridisation of Stage 13 *CG11253<sup>EY10866</sup>* embryo in which *CG11253* mRNA expression could be detected in the chordotonal neurons at low levels (labeled by arrows). Scale bars - 100µm

<table>
<thead>
<tr>
<th></th>
<th><em>CG11253</em> expression</th>
<th>No <em>CG11253</em> expression detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CG11253&lt;sup&gt;EY10866&lt;/sup&gt;</em></td>
<td>41 (N=57)</td>
<td>16 (N=57)</td>
</tr>
<tr>
<td>Wild type</td>
<td>59 (N=60)</td>
<td>1 (N=60)</td>
</tr>
</tbody>
</table>

Table 3.4: A statistically significant reduction in observable *CG11253* expression was seen in *CG11253<sup>EY10866</sup>* stage 13/14 embryos. (P value: 0.0001 Fishers exact test).
climb up the wall of the tube resulting in a reduced climbing height. This has been demonstrated by the climbing capabilities of flies in which \( jd3F \) and \( rempA \) were knocked down in the climbing assay described in Chapter 2.

\( \text{CG11253}^{\text{EY10866}} \) homozygous flies climbed significantly shorter distances than \( \text{CG11253}^{\text{EY10866}} \) heterozygous flies (Figure 3.7). \( \text{CG11253}^{\text{EY10866}} \) flies can still climb to an extent, signifying that chordotonal neuron function is not completely removed but is significantly disrupted. \( \text{at}^{+} \) flies which lack chordotonal neurons are very uncoordinated and would likely have very disrupted climbing behaviour (Jarman et al 1995).

**3.2.6 \( \text{CG11253}^{\text{EY10866}} \) is not a null mutation**

\( \text{CG11253}^{\text{EY10866}} \) displays a partial reduction in climbing capability. As it is not clear whether some gene expression remains in these flies the partial reduction in climbing capability may be due to only a partial reduction in gene expression. In order to assess this, it is necessary to know whether the allele is a null and whether a further reduction in the amount of expression would result in further depletion of climbing capability due to further reduction in chordotonal organ function. To test this the \( \text{CG11253}^{\text{EY10866}} \) flies were crossed to a deficiency line, \( \text{Df(3L)}^{\text{ED4486}} \), which covered the area (3L 12,507,519 – 13,025,585) in which \( \text{CG11253} \) was located (3L 13,017,863-13019537). A climbing assay was performed to compare the climbing capabilities of \( \text{CG11253}^{\text{EY10866}} \) and \( \text{CG11253}^{\text{EY10866}}/\text{Df(3L)}^{\text{ED4486}} \) flies (Figure 3.7). \( \text{CG11253}^{\text{EY10866}}/\text{Df(3L)}^{\text{ED4486}} \) flies have a significant reduction in climbing capability compared to \( \text{CG11253}^{\text{EY10866}} \) flies (P value – 0.0255). This gives genetic evidence that \( \text{CG11253}^{\text{EY10866}} \) is a not null mutation. For further proof of the level of \( \text{CG11253} \) expression RT PCR could be carried out to compare expression levels in \( \text{CG11253}^{\text{EY10866}} \) and wild type embryos.

The reduction in climbing ability signifies that \( \text{CG11253}^{\text{EY10866}} \) is not a complete null however it does still show a significant climbing defect meaning it can be used to analyse \( \text{CG11253} \) function. A further caveat is that a control consisting of \( \text{Df(3L)}^{\text{ED4486}/+} \) flies was not carried out. This would allow us to see if any reduction in climbing was due to a reduction in any of the other 64 genes that the
Figure 3.7: CG11253$^{EY10866}$ shows reduced climbing ability
A climbing assay (Method A) showing firstly a significant difference between the climbing ability of heterozygous (balanced over the TM3 Sb balancer) and homozygous CG11253$^{EY10866}$, which indicates that the transposon insertion results in reduced climbing ability. Secondly a significant difference was seen between the climbing capabilities of homozygous and hemizygous (over the Df(3L)ED4486 deficiency) CG11253$^{EY10866}$ indicating that CG11253$^{EY10866}$ is not a null. 17-20 flies were screened in each assay (N=3). An average climbing score was calculated from each repeat. P value - *** : 0.0004, ** : 0.0088, * : 0.0255 (calculated by ANOVA and Tukeys multiple comparisons test).
deficiency line covers. Combining this data with the in situ expression reduction it appears that expression is reduced to a considerable extent.

### 3.2.7 P element mobilisation

As a small amount of gene expression may still remain in *CG11253*<sup>114</sup> I attempted to generate a deletion mutant by imprecise P element excision. This process would also allow the generation of a revertant line where the P element has been mobilised but no deletion has taken place. This revertant line would be almost identical to *CG11253*<sup>114</sup> except for the presence of the P element and would show that the defect in chordotonal function is due to loss of *CG11253*. Additionally it would make an ideal control for any future experiments using *CG11253*<sup>114</sup>.

*CG11253*<sup>114</sup> flies were crossed to a line carrying delta 2-3 transposase (Robertson et al 1988) to facilitate P element excision (Figure 3.8A). Flies that carried chromosomes with the P element removed were then identified by eye colour before a further cross to generate a balanced stock. These were then screened by PCR using one primer upstream of the P element and three downstream to look for deletions of different lengths. However after screening over 300 revertant lines no deletion could be detected. In spite of the lack of a deletion or null mutant I could still use *CG11253*<sup>114</sup> to further investigate *CG11253* as it had shown a phenotype as a result of reduced gene expression.

The P element “hop out” crosses generated a number of revertant lines. In order to assess whether these could be used as a wild type control I needed to assess whether chordotonal neuron function still appeared to be disrupted even without the presence of the P element. In order to do this I used a climbing assay of two of the revertant lines as well as *CG11253*<sup>114</sup> homozygotes and heterozygotes (Figure 3.8B). There was no significant difference between the two revertant lines and the *CG11253*<sup>114</sup> heterozygote, however all three lines were significantly different from the homozygote. This signifies that the revertant lines act as wild type in the climbing assay and that chordotonal organ function is no longer affected now that the P element has been removed. This indicates that it was the transposon that was responsible for the reduced climbing phenotype.
Figure 3.8: Excision of transposon from $CG11253^{EY10866}$ restores climbing ability.
(A): The crossing scheme undertaken to mobilise the $P\{EPgy2\}$ EY10866 P element. $CG11253^{EY10866}$ was crossed to a line containing $\Delta 2-3$ transposase in order to excise the P element transposon. Flies in which the transposon had been excised were subsequently balanced over the TM3 Sb balancer.

(B): A climbing assay (Method A) showing that excision of the P element results in a restoration of climbing ability. Revertant lines in which the transposon has been excised climbed significantly better than homozygous $CG11253^{EY10866}$ flies. 18-20 flies were screened in each assay ($N=3$). P values: $CG11253^{EY10866}$ heterozygote 0.012, Revertant 1 0.007, Revertant 2 0.0176. (calculated by ANOVA and Tukey’s multiple comparisons test).
It must be kept in mind that this behavioural assay is rather crude and disrupting a variety of different systems can result in a poorer climbing assay performance. However combining the expression data and the fact that revertant climbing is unaffected these data suggests that \textit{CG11253} is required for the proper function of chordotonal neurons and perturbation of its expression results in reduced proprioceptive/gravitactic capabilities.

\textbf{3.2.8 Construction of a CG11253 fusion protein}

In order to confirm that the behavioural defect observed was due to disruption of \textit{CG11253} expression, a rescue construct was generated. This construct would also be tagged with the fluorescent protein mVenus in order to discover the cellular and subcellular localisation of the protein.

The gateway cloning system was used in order to generate the construct. Gateway cloning utilises bacteriophage lambda biology to allow the recombination of attachment (att) flanking regions. Primers were designed to include the region upstream of \textit{CG11253} (361 bp) in the construct allowing expression to be directed from its own promoter (Figure 3.9B). The 3’ primer was targeted to the final sequence of the transcript but removing the stop codon to allow read through into the \textit{mVenus} gene of the destination vector. An additional base was also added to this primer between the gene sequence and the attB (bacterial attachment) site to ensure the \textit{mVenus} gene is in frame with \textit{CG11253}. \textit{CG11253} sequence was amplified and tagged with attB flanking fragments by PCR. The construct was then cloned into the donor vector pDonr221, before transfer to the destination vector pBID-UASC-GV (Wang et al 2012). The resulting vector (Figure 3.9A) was then injected into syncytial blastoderm stage embryos expressing \textit{phiC31} intergrase, which is utilised to incorporate the transgene into the \textit{Drosophila} genome at an attP40 site (in this case chromosome 2, 25C6). Flies that had incorporated the transgene were identified by their eye colour and subsequently balanced over a CyO balancer. The presence of \textit{CG11253::mVenus} was confirmed by PCR of single-fly genomic DNA preparations. These flies could now express \textit{CG11253::mVenus} generated the tagged protein (Figure 3.9B).
Figure 3.9: The construction of CG11253::mVenus fusion gene.

(A) A vector map of the pBID-UASC-CG11253::mVenus vector which was injected into blastoderm stage embryos. Vector map generated using Serial Cloner 2.6.1.

(B) A diagram of the CG11253::mVenus insert which was incorporated into the genome of those flies. The CG11253::mVenus fusion gene was designed to include the upstream region of CG11253 to allow expression from its own promoter.
3.2.9 CG11253::mVenus rescues CG11253<sup>EY10866</sup> phenotype

CG11253::mVenus flies were crossed into CG11253<sup>EY10866</sup> (Figure 3.10A). A climbing assay was then carried out to ascertain whether CG11253::mVenus could act as a rescue construct (Figure 3.10B). CG11253::mVenus; CG11253<sup>EY10866</sup> flies and revertant flies were not significantly different from each other in terms of the height that an individual fly can climb after ten seconds. Both of these lines are however significantly different from CG11253<sup>EY10866</sup>, confirming that CG11253::mVenus can rescue CG11253<sup>EY10866</sup> (Figure 3.10B). This adds further evidence that it is loss of CG11253 that is causing the reduction in climbing capability in CG11253<sup>EY10866</sup>. It also confirms that the mVenus tag has not interfered with CG11253 function and thus should also not alter its localisation.

3.2.10 CG11253::mVenus localisation

The CG11253::mVenus fusion protein can be used to confirm the previously identified mRNA expression pattern and also to investigate its subcellular localisation. It also allows co-localisation with markers to confirm neuronal localisation. CG11253::mVenus localisation was first examined in fixed embryos. A GFP antibody was used to detect CG11253 localisation via the mVenus tag as the fixation process reduces mVenus’s fluorescence.

3.2.10.1 CG11253::mVenus localisation in fixed embryos

CG11253::mVenus localisation was found to largely confirm the expression pattern discovered by in situ hybridisation (Figure 3.11A). CG11253::mVenus was found to localise to the chordotonal neurons such as lch5, v’ch1 and vchA and B in the abdominal segments (Figure 3.11A). No localisation was found in the neurons of the external sensory organs. CG11253 expression occurs before the final cell division, as a result of this CG11253::mVenus is found to localise to both the neuron and the scolopale, the neuron’s sibling accessory cell (Figure 3.11C&D).

Aside from its expression in chordotonal neurons CG11253::mVenus was also expressed in the unciliated multidendritic v’td (ventral tracheal dendritic) neurons (Figure 3.11A). Expression in these cells was rarely observed in in situ hybridisation.
Figure 3.10: **CG11253::mVenus rescues CG11253<sup>EY10866</sup> climbing defect.**

(A). The crossing scheme used to cross CG11253::mVenus into CG11253<sup>EY10866</sup>.

(B). A box and whisker plot showing the heights individual flies climbed in a climbing assay (Method B). The climbing ability of CG11253::mVenus; CG11253<sup>EY10866</sup> flies (N=28) was not significantly different from revertant flies (N=44), but was significantly different from CG11253<sup>EY10866</sup> (N=42). This indicates that CG11253::mVenus can rescue the defective climbing ability of CG11253<sup>EY10866</sup>. P values < 0.0001 (calculated by Kruskal Wallis & Dunn's multiple comparisons test).
Figure 3.11: CG11253::mVenus embryonic localisation.

(A) Abdominal sections of Stage 17 embryo showing that CG11253::mVenus (green) localises to the lch5, v’ch1 and vchA&B chordotonal neurons and the v’td neurons. Neurons are labeled with Futsch (magenta).

(B) A diagram of a single chordotonal neuron from an lch5 cluster detailing the localisation of the antibody stains used to characterise CG11253::mVenus’s embryonic localisation.

(C) A diagram showing the relative localisation of the neuron and scolopale in a single neuron from an lch5 cluster. This diagram demonstrates that any ciliary localisation of CG11253::mVenus would be masked by its localisation in the scolopale which surrounds the outer ciliary segment.

(D – F) lch5 neuron cluster from a Stage 16 embryonic abdominal segment which shows that CG11253::mVenus (green) is cytoplasmically localised. Any ciliary localisation of CG11253::mVenus cannot be distinguished from CG11253::mVenus localisation in the scolopale (labelled with arrow). Neurons are labeled with Futsch and cilia with polyglutamylated tubulin (magenta).

(G) Lch5 neuron cluster from an embryonic abdominal segment showing that CG11253::mVenus (green) does not enter the nucleus (labeled with ELAV – magenta)

Scale bars – A – 25µm, D-G 5µm.
As with expression in the scolopale, the cause of transgene expression in v’td neurons is likely to be shared cell lineage with chordotonal cells (Figure 3.12). Cells which form the vchA and B chordotonal organs undergo a further division in the sister cell of the scolopale to generate the chordotonal neuron and the v’td multidendritic neurons (Brewster & Bodmer 1996). The v’td neurons can therefore contain some of the chordotonal neuron mRNA that was expressed before cell division. It is possible that in both v’td neurons and the scolopale, CG11253 may normally be degraded. It can also be speculated that the mVenus tag may stabilise CG11253 allowing it to persist longer than normal and thus making it detectable.

Within the neuron CG11253::mVenus localises across most of the cytoplasm but doesn’t appear to enter the nucleus (Figure 3.11G). Single imaging slices show no co-localisation between CG11253::mVenus and the nuclear protein ELAV (Figure 3.11G). CG11253::mVenus could not be observed localising to the cilia; however any localisation here may be masked by the accessory cell expression around the dendrite (Figure 3.11D-F).

3.2.10.2 CG11253::mVenus localisation in live embryos

Live embryos were also investigated for CG11253::mVenus localisation using mVenus as a fluorophore rather than as a protein tag. The same general pattern as in fixed tissue was observed with CG11253::mVenus most strongly localising to the cell body and further localisation within the dendrites. However it was also observed that CG11253::mVenus could enter the cillum late in development (Stage 17) (Figure 3.13).

3.2.10.3 CG11253::mVenus localisation in larval pelts

The pelts of third instar CG11253::mVenus larvae were dissected in order to observe CG11253::mVenus subcellular localisation in the chordotonal neurons of the larval body wall. Unlike embryonic chordotonal neurons those in larvae have fully matured and thus it was possible that localisation might differ to the developmental stages seen in embryos.
Figure 3.12 : CG11253::mVenus localisation during chordotonal cell divisions.
A diagram showing the cell divisions of the SOP that can given rise to the ventral chordotonal neurons, accessory cells and the vt’d neuron. Cells types where CG11253::mVenus has been detected are shown in green.

Figure 3.13 : Live imaging of CG11253::mVenus localisation in the embryonic chordotonal organs.
The lch5 and vch1 chordotonal neurons from the abdominal segment of a stage 17 embryo. Live imaging revealed CG11253::mVenus localisation (via mVenus fluorescence) in both the cytoplasm and cilia. Scale bar size: 10µm.
CG11253::mVenus localised most strongly to the cell body, with slightly lower levels in the dendrites (Figure 3.14 A-C). CG11253::mVenus is excluded from the cilia with localisation stopping abruptly at the dendritic tip (Figure 3.14 D-F).

### 3.2.10.4 CG11253::mVenus localisation in pupal antennae

The antennae of CG11253::mVenus pupae were dissected in order to examine CG11253::mVenus localisation in the chordotonal neurons of the Johnston’s organ. Localisation again was most strongly cytoplasmic with lower levels in the dendrite (Figure 3.15). However there appears to be a focus of localisation at the end of the dendrite from which the cilia extend. CG11253::mVenus also appears able to enter the cilium but does not localise here as strongly as in other areas. A focus of localisation is visible covering an area slightly proximal and distal to the ciliary dilation (Figure 3.15). This localisation extends distally past the end of polyglutamylated tubulin localisation in the proximal ciliary compartment and partially overlaps with the most proximal localisation of NompC, which marks the distal ciliary compartment (Figure 3.15).

### 3.2.11 Cellular and ciliary morphology of CG11253<sup>EY10866</sup> chordotonal neurons

In order to assess what was responsible for the behavioural defect previously described I used immunohistochemistry (IHC) to observe the cellular and ciliary structure of chordotonal neurons in embryos, the larval body wall and pupal antennae. As CG11253 is regulated by Fd3F it may harbour a similar phenotype to <sup>fd3F<sup>l. fd3F</sup> mutants show an accumulation of matter at the ciliary tip and a lack of ciliary compartmentalisation. Both of these phenotypes are likely to be due to the role of Fd3F in the regulation of the IFTA component genes. If these are unaffected in CG11253<sup>EY10866</sup> it could suggest that CG11253 has a role more similar to the other group of genes regulated by Fd3F, those required for ciliary motility.

No gross morphological defects were detected in CG11253<sup>EY10866</sup> embryonic lch5 chordotonal neurons (Figure 3.16A-C). Mouse antibody 22c10 binds the microtubule-associated protein Futch and shows that the cell body and dendrite are normally formed. Anti-HRP (horse radish peroxidase) staining shows that the cilia
Figure 3.14: CG11253::mVenus localisation in larval chordotonal neurons

(A & D) Third instar CG11253::mVenus larvae were dissected and the localisation of CG11253::mVenus (green) in the larval pelt chordotonal neurons was observed. The chordotonal neurons are stained with a Futsch antibody and the cilium marked with an antibody for NompC (magenta). CG11253::mVenus localises mainly to the cell body, with some localisation also seen in the dendrite (labeled with arrow in D-F).

(B & E) CG11253::mVenus only.

(C & F) CG31320::Futsch and NompC only.

Scale bars: 10µm
Figure 3.15 : CG11253::mVenus localisation in the pupal Johnston’s Organ.

(A) CG11253::mVenus (green) localisation in the chordotonal neurons of the pupal Johnston’s organ. CG11253::mVenus can be seen to localise in the cell body, dendrite and within the cilium distal to the proximal ciliary compartment (marked by polyglutamylated tubulin – magenta).

(B) CG11253::mVenus (green) localisation in the chordotonal neurons of the pupal Johnston’s organ. CG11253::mVenus can be seen to localise in the cell body, dendrite and within the cilium proximal to the distal ciliary compartment (marked by NompC – magenta).

(C) A diagram showing the localisation of polyglutamylated tubulin and NompC within the cilia of pupal chordotonal neurons. A diagram of CG11253::mVenus localisation is also shown indicating that there is a focus of localisation within the cilium where the proximal and distal ciliary compartments connect.

Scale bar size : 10µm
Figure 3.16: No gross morphological defects in CG11253EY10866 neurons. 
(A-B) Wild type (CG11253EY10866/TM3 Kr-GFP) (A) and CG11253EY10866 (B) embryonic abdominal lch5 chordotonal neurons with normal neuronal (Futsch – magenta) and ciliary (anti-HRP – green) morphology. (C) A diagram showing the localisation of anti-HRP (green) and Futsch (magenta) antibody staining in embryonic chordotonal neurons. 
(D & E) Wild type (CG11253EY10866/TM3 Kr-GFP) (D) and CG11253EY10866 (E) larval pelt chordotonal neurons with normal dendritic (anti-HRP – green), ciliary (NompC - magenta) morphology and ciliary compartmentalisation. (F) A diagram showing the localisation of anti-HRP (green) staining at the dendrites and luminal band and NompC in the distal ciliary compartment in larval pelt chordotonal neurons. 
(G & H) Wild type (CG11253EY10866/TM3 Kr-GFP) (G) and CG11253EY10866 (H) pupal Johnston’s organ chordotonal neurons with normal localisation of anti-HRP (green) at the proximal cilium and NompC (magenta) at the distal cilium indicating normal ciliary compartmentalisation. (I) A diagram showing the localisation of HRP in the proximal cilium and NompC in the distal cilium. Scale bars: A, B, D & E - 10µm, G & H - 5µm.
are present (Figure 3.16A-C). The morphology of the larval body wall chordotonal neurons was also examined (Figure 3.16D-F). Unlike the embryonic neurons shown previously these have fully developed. Anti-HRP in this case shows the cell body and dendrite are normally formed in CG11253<sup>EY10866</sup> as well as showing that the luminal band near the ciliary dilation is present. NompC, a TRP channel and marker of the distal ciliary segment is also normally localised (Figure 3.16D & E). This shows that the cilia are present, and normally formed. Compartmentalisation of the cilia also appeared to be normal, suggesting that CG11253 does not have a role in IFTA or compartmentalisation. These findings were confirmed by NompC and anti-HRP staining in the Johnstons organ chordotonal neurons of the developing pupae, which also appeared as wild type (Figure 3.16 G-I). In all of these cases I used a Kruppel-GFP TM3 balancer to identify heterozygotes and homozygotes. These data show that the observed climbing assay defect is not due to any major defects in chordotonal neuronal morphology or due to decompartmentalisation of the cilia. Fd3F is known to regulate both components of the IFTA complex and genes associated with cilium motility. The lack of a defect in ciliary structure or compartments suggests that CG11253 may instead play a role in cilium motility.

### 3.3 Discussion

CG11253 was previously found to be highly enriched in developing chordotonal cells and transcriptionally regulated by Fd3F. I investigated CG11253 to ascertain whether there was any further evidence for a role as a ciliary gene. I found that CG11253 was expressed in ciliated cells and disruption of expression results in a phenotype consistent with disrupted chordotonal neuron function. I also found that CG11253 homologues were conserved in organisms with motile cilia. The human homologue of CG11253 is ZMYND/Blu (herein referred to as ZMYND10). The function of ZMYND10 may give a clue the role of CG11253.

#### 3.3.1 CG11253 – a role in transcriptional regulation?

CG11253 is predicted to contain a MYND domain. MYND containing proteins have been associated with transcriptional regulation however the domain itself is not thought to directly be involved in DNA-Protein interactions (Gross & McInnis
1996). In other cases MYND containing proteins have been linked to transcriptional regulation. ETO acts as a corepressor by recruiting histone deacetylases and transcription factors for transcriptional regulation (Lutterbach et al. 1998a, Lutterbach et al. 1998b, Wang et al. 1998, Melnick et al. 2000). The MYND domain of ETO is necessary but not sufficient for interactions with corepressors (Hildebrand et al. 2001). Nervy, the *Drosophila* homologue of ETO, is expressed in ES SOPs and is thought to interact with Daughterless to regulate transcription of targets of the proneural genes *ascute* and *scute* (Wildonger & Mann 2005). The LXXLL motif has also been implicated in transcriptional regulation. It has been found on co-activators of nuclear receptors (McInerney et al. 1998, Savkur & Burris 2004).

Further evidence of a link between CG11253 and transcriptional regulation comes from its human homologue. The human homologue of *CG11253*, known as *ZMYND10* or *Blu* (β-catenin in lung) has previously been implicated as a tumour suppressor gene. The *ZMYND10* promoter has been found to be hypermethylated resulting in reduced expression in a number of tumour cell lines and cancers (Agathanggelou et al. 2003, Hesson et al. 2007). In NPC (Nasopharyngeal carcinoma cells) ZMYND10 has been found to regulate the cell cycle by changing JNK (c-Jun N-terminal Kinase) activity (Zhang et al. 2012). The reduction in JNK activity coincides with a reduction in the phosphorylation of the JNK target transcription factor c-Jun and a reduction in expression of its target *Cyclin-D*. ZMYND10 has also been reported to interact with sMEK1 (a component of protein phosphatase 4) via its C-terminus (Dong et al. 2012). This interaction could occur via the MYND domain as it is localises within a 139 amino acid region found to interact with sMEK1. The complex formed by these proteins is thought to invoke apoptosis via the transcriptional regulation of the apoptotic machinery and an increase in caspase-3 activity. These two roles describe two different mechanisms as to which ZMYND10 can act as a tumour suppressor gene but both suggest that the protein acts to regulate transcription, perhaps from within a complex.

Despite tumour suppression being the ultimate result of both of these mechanisms it does indicate that ZMYND10 is capable of acting to cause different outcomes by interacting with different proteins. In both of these cases ZMYND10 interacts with
other proteins to control gene expression. This is consistent with some of the previous roles described for a MYND containing proteins which have been found to act as co-repressors to mediate transcription. Protein-protein interactions are important for the function of both ZMYND10 and MYND containing proteins. Any interactors of CG11253 may therefore give clues to its function.

ZMYND10 and other MYND containing proteins have been found to play a role in transcriptional regulation. It is possible that CG11253 could play a similar role. However the main evidence against this comes from the localisation of CG11253::mVenus, which is cytoplasmic rather than in the nucleus.

3.3.2 CG11253::mVenus localisation

CG11253::mVenus has been found to localise to the chordotonal neurons in three stages of the Drosophila life cycle. Senthilan et al (2012) also confirmed that CG11253 is expressed in adult JO neurons by CG11253-Gal4 driven GFP expression. CG11253::mVenus is mainly cytoplasmic at all three stages with the main focus of localisation occurring in the cell body and no clear localisation to the nucleus. Additionally CG11253::mVenus has been found to localise to the cilia of late embryonic and pupal chordotonal neurons.

It is unlikely that the high copy rate of CG11253 (with a fly homozygous for CG11253::mVenus having four copies of the gene) is masking further subcellular specificity as no difference in localisation was seen between homozygous and heterozygous flies. Further evidence that a high level of protein is not masking the true localisation is that the transgene gene is transcriptionally controlled by its own promoter, with no over expression.

That the transgene is expressed in the same pattern as seen in the mRNA in situ hybridisation also confirms that the elements for transcriptional control are indeed contained within the cloned upstream region. The cloned upstream region contained both of the X box and forkhead-binding site clusters. Disrupting individual binding motifs by site directed mutagenesis of the CG11253::mVenus vector would allow analysis of which motifs are bound when transcription is being controlled.
CG11253::mVenus localisation was also found in the cilia and at the end of dendrites in Johnston’s organ neurons. It is possible that the localisation at the end of the dendrites is a pool of protein which has not yet been transported into the cilium, however the levels appear to be higher here that in the cilium itself. The intensity of fluorescence appears to be lower in the cilium than the cell body and dendrite suggesting that transport into the cilium of CG11253 is restricted. The absence of this localisation in larvae could also suggest that CG11253 does not enter the cilium of fully matured neurons. Ciliary localisation is also not ubiquitous in Johnston’s organ neurons. Only some neurons showed localisation of CG11253::mVenus in the proximal cilium, whilst most showed localisation where the proximal and distal compartments meet. This could suggest that CG11253::mVenus is either shuttling in and out of the cilium, or that the proximally localised CG11253::mVenus is being transported to its location between the proximal and distal compartments.

The extent to which CG11253::mVenus is located to the cilium in embryonic chordotonal neurons cannot be confirmed. Live imaging showed that it could localise to the cilium in a few late stage embryos, however for confirmation more comprehensive live imaging of embryos would be necessary. It could also be the case that CG11253 does localise to the cilium but only quite late on in ciliogenesis. This would however not persist in fully matured neurons as I have shown that there is no ciliary localisation in larval chordotonal neurons. In fixed embryos a ciliary localisation was not clearly observed. Ciliary localisation may not have been detected if levels of localisation were low, as in antennae.

In spite of the complications and limitation of observing CG11253::mVenus in the cilium we know that it can localise there at least in late embryos (Stage 17) as it has been observed to enter the cilium in live imaging. It should be noted that the live imaging conducted was not extensive and thus all that can be concluded is that the fusion protein can enter the cilium at this stage. Live imaging was carried out in order to see if the subcellular localisation was altered by the fixation process and whether any protein transport was visible. However no movement of CG11253::mVenus or shuttling into the cilium was observed. This may however been due the technical limitations. Embryos were mounted in hydrocarbon oil, which
meant using high magnification (x60) often lead to the embryo moving on the slide. The result of this was a loss of resolution from using lower magnifications. Movement within the embryos themselves also caused problems. The ciliary localisation was only seen in late stage embryos, which have some muscle contraction, making taking stacks of images unfeasible. Ciliary localisation was also most clearly seen in very late embryos. Such embryos were very close to hatching meaning that they moved quite considerably within the egg. Live imaging of Johnston’s organ chordotonal neurons may be more informative as there did not appear to be any expression in the scolopale.

### 3.4 Conclusion

In situ hybridisation has shown that the insertion of a P element into the transcriptional unit of CG11253 results in reduced CG11253 expression. As a result of this reduction in expression CG11253\textsuperscript{EY10866} flies have reduced climbing capability. This is likely to be as a result of a defect in proprioception or gravitaxis due to disruption of chordotonal neuron function as this is where expression has been detected. Both the revertant and rescued flies have normal climbing capability indicating that it is indeed the P element and lack of CG11253 expression that causes the climbing phenotype.

However when morphology has been investigated the neuronal and ciliary markers investigated thus far appear normal. CG11253 is transcriptionally controlled by Fd3F, it is therefore likely that the defect caused by reducing CG11253 expression is within the realms of the defect caused by f\textit{d}3\textit{F} depletion. One such defect observed in f\textit{d}3\textit{F} mutants is a loss of ciliary compartmentalisation; this is likely to be due to a reduction in levels of IFTA proteins as Fd3F regulates their expression (Newton et al 2012). As reducing levels of CG11253 does not result in a loss of compartmentalisation it is likely to be involved in the other group of genes that Fd3F regulates; those involved in ciliation motility.

Further evidence that CG11253 could be involved in ciliation motility comes by the fact that it is only expressed in cells that develop motile cilia, chordotonal neurons and the spermatocytes. Expression found in the scolopale and the vtd neurons is
likely to be as a result of shared cell lineage with cells that have motile cilia. CG11253 is only conserved in organisms that have motile cilia, or in the case of *Ostreococcus* motile ciliary machinery. A cillum motility defect would also be consistent with the climbing assay result observed, with the cilium motility machinery being required for the proper function of the chordotonal neurons but not being necessary for the neurons to have any functionality at all. The next step in investigating *CG11253* is therefore to test the hypothesis that it is involved in cilium motility.


4

CG11253: a cilium motility gene

4.1 Introduction

The purpose of this chapter is to investigate the hypothesis that CG11253 has a role in cilium motility. The formation of this hypothesis is based on a number of factors discussed in Chapter 3. This includes its conservation in organisms with motile cilia, its expression in cells with motile cilia and that its expression is regulated by Fd3F and RFX. These two transcription factors are known to act together to regulate genes involved in cilium motility and the IFTA complex (Newton et al. 2012). However, the normal morphology of CG11253EY10866 cilia suggests against a role in retrograde transport.

Cilium motility linked genes regulated by Fd3F include components of the axonemal dynein arms, both inner (Dhc16F/DNAH6, Dhc62B/DNAH12, CG6971/DNALII) and outer: (Dhc93AB/DNAH9/11/17, CG9313/DNAI1, CG8800/DNALI), and factors associated with them (tilB, CG14905) (Newton et al. 2012). Genes that are associated with ciliary motility could be required for the function or formation of the axonemal dynein arms, nexin links between microtubules, radial spokes or the central microtubule pair. However, Drosophila chordotonal neuron cilia lack both radial spokes and a central microtubule pair so Fd3F is unlikely to regulate the expression of genes related to these components.

4.1.1 Homologues of CG11253 are implicated in cilium motility

In addition to my findings in Drosophila, there is further evidence for a role in cilium motility for homologues of CG11253. ZMYND10, the human homologue of CG11253, is expressed in human bronchial epithelial cells and Zmynd10, its mouse homologue, in mouse tracheal epithelial cells during ciliogenesis stimulated by culture on an air liquid interface (Ross et al. 2007, Hoh et al. 2012).
Both of these studies implicate \textit{ZMYND10} as possibly having a ciliary role as it is expressed during ciliogenesis in cell types that have motile cilia.

\textit{ZMYND10} was also identified as a possible ciliary motility gene by a co-evolution analysis comparing genes conserved in organisms with and without motile cilia (Kwan et al 2010). A \textit{ZMYND10} homologue was found in organisms with motile cilia (zebrafish, humans \textit{Chlamydomonas reinhardti} and \textit{Physcomitrella patens}) and not in those without (\textit{Arabidopsis thaliana}, \textit{Caenorhabditis elegans}, \textit{Oryza sativa} and \textit{Saccharomyces cerevisiae}) (Kwan et al 2010). This confirms the earlier conservation analysis I carried out which identified that CG11253 was conserved in organisms which had either motile cilia or utilised the cillum motility machinery.

Sammeta et al (2007) found \textit{ZMYND10} mRNA to be enriched in mouse olfactory sensory neurons (OSNs) compared to the rest of the olfactory epithelium. Mammalian olfactory cilia are thought to be immotile and lack dynein arms (Menco 1984). This would appear to be evidence against \textit{ZMYND10} having a role in cilium motility. However Sammeta et al (2007) also found a number of other motility-associated factors to be enriched in OSNs. These included components of the dynein arms (\textit{Dnahe2}, \textit{Dnahe9}, \textit{Dnahe11}, \textit{Dnaic1} & \textit{Dnali1}), genes associated with dynein arm assembly (\textit{Ccdc39}, \textit{Ccdc40}, \textit{Lrrc6} & \textit{Dnaaf2}) and \textit{Foxj1}. Expression in the OSNs therefore does not prohibit a role in cilium motility elsewhere (Sammeta et al 2007). Whilst it is possible that genes required for cilium motility could play some role in OSNs, this enrichment could represent contamination of genes expressed in the motile ciliated cells of neighbouring respiratory epithelia. Further evidence that this may be the case comes from the finding that FOXJ1 protein does not localise to mouse OSNs (Blatt et al 1999).

### 4.1.2 \textit{CG11253}\textsuperscript{EY10866} flies have defective hearing

I have previously identified that disrupting \textit{CG11253} expression results in a behavioural defect consistent with defective proprioceptive or gravitactic chordotonal neuron function. The chordotonal neurons of the Johnston’s organ
are required for *Drosophila* hearing. Senthilan et al (2012) have shown that \( CG11253^{EY10866} \) flies also have defective hearing. \( CG11253^{EY10866} \) had reduced potentials in response to sound and a reduction in sensitivity to a sound stimulus (Senthilan et al 2012). An amplification gain of antennal resonance at lower levels of stimulus requires the mechanical action of the axonemal dynein arms (Gopfert & Robert 2003, Gopfert et al 2005). In \( CG11253^{EY10866} \) flies this amplification is reduced, further implicating \( CG11253 \) in the function of the cilium motility machinery (Senthilan et al 2012). The hearing phenotype observed is similar to that of axonemal dynein arm component mutants \( CG9313^{PADEF334P} \) and \( Dhc36C^{LA00085} \), with \( Dhc93AB^{MB05444} \) showing more extensive defects.

4.1.3 Interaction with TilB

An additional factor implicating CG11253 with a role in cilium motility is a yeast-2-hyrid interaction previously recorded with TilB in a screen for protein-protein interactions (Giot et al 2003). TilB is a factor known to be required for axonemal dynein arm formation (Eberl et al 2000). \( tilB \) mutants are uncoordinated and have defective hearing, matching the phenotype of \( CG11253^{EY10866} \) (Eberl et al 2000). The cilia of the Johnston’s organ and the sperm flagella have been shown to have reduced inner and outer axonemal dynein arm localisation in \( tilB \) mutants (Eberl et al 2000, Kavlie et al 2010). \( tilB \) mutant sperm flagella also display axonemal ultrastructural abnormalities (Eberl et al 2000). TilB is thought to be mainly cytoplasmically localised; Kavlie et al (2010) show a similar localisation of TilB::GFP and mVenus::TilB as I have shown for CG11253::mVenus. Whilst further evidence of the interaction between CG11253 and TilB would be required before it is confirmed, this potential interaction shows another possible link between CG11253 and cilium motility.

Reducing the expression of \( CG11253 \) results in defects consistent with a loss function of the cilium motility machinery. To test whether \( CG11253 \) played a role in cilium motility I investigated its relationship with components of the cilium motility machinery in *Drosophila* motile ciliated cells.
4.2 Results

4.2.1 CG11253<sup>EY10866</sup> Fertility Assay

As discussed in Chapter 3, CG11253 is expressed in *Drosophila* testes (Zhao et al 2009) as well as in chordotonal cells. Other than chordotonal neuron cilia, the sperm flagella are the only other cilia containing the cilium motility machinery in *Drosophila*. In order to test whether CG11253 is also required for sperm flagella function a fertility assay was set up. Individual CG11253<sup>EY10866</sup> and revertant male flies were kept with female wild type flies for 5 days and the rate at which crosses produced progeny was recorded. CG11253<sup>EY10866</sup> flies were observed mating with female flies, but did not produce any progeny (Table 4.1). This is significantly different from revertant flies all of which did produce progeny. atonal<sup>i</sup> flies, in which chordotonal neurons do not form but sperm generation is unaffected, were also assayed and displayed no fertility defect. This indicates that CG11253<sup>EY10866</sup> infertility is not due to its lack of proprioception. A subsequent fertility assay also showed that CG11253<sup>EY10866</sup> infertility can be rescued by CG11253::mVenus (Table 4.2). That rescued and revertant flies both do not show fertility defects confirms that CG11253<sup>EY10866</sup> infertility is as a result of the disruption of CG11253 expression and not due to a neighbouring gene or a factor in the genetic background of the line.

4.2.2 Dissection of CG11253<sup>EY10866</sup> Testes

To further investigate the cause of CG11253<sup>EY10866</sup> infertility, testes of two day old male flies were dissected and observed with bright field microscopy (Figure 4.1, Table 4.3). In wild type testis sperm develop in bundles of 64 spermatids before individualising and moving into the seminal vesicle (Fuller 1993). Squashing the testis results in the release of motile sperm that can be observed oscillating on the slide. In contrast with this no motile sperm were observed from a squash of CG11253<sup>EY10866</sup> testes. CG11253<sup>EY10866</sup> also show abnormal twisting or bending of sperm bundles, small or empty seminal vesicles and significantly more often have an increased rate of coiled individualised sperm at the terminal epithelium at the junction with the seminal vesicle (Figure 4.1D). Flagella
Table 4.1 CG11253\textsuperscript{EY10866} male flies are infertile

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of crosses that produced progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revertant</td>
<td>20 (N=20)</td>
</tr>
<tr>
<td>CG11253\textsuperscript{EY10866}</td>
<td>0 (N=20)</td>
</tr>
<tr>
<td>atonal\textsuperscript{I}</td>
<td>9 (N=9)</td>
</tr>
</tbody>
</table>

Table 4.1: Individual male flies were each crossed to a wild type female virgin fly. The number of these crosses which produced progeny was recorded. CG11253\textsuperscript{EY10866} male flies do not produce progeny from crosses with wild type females. Revertant flies and atonal\textsuperscript{I} flies which lack chordotonal neurons can still produce progeny. CG11253\textsuperscript{EY10866} is significantly different from revertant (P : < 0.0001, Fisher’s exact test). There is no significant difference between revertant and atonal\textsuperscript{I} fertility.

Table 4.2 CG11253::mVenus can rescue CG11253\textsuperscript{EY10866} fertility defect

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of crosses that produced progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revertant</td>
<td>19 (N=20)</td>
</tr>
<tr>
<td>CG11253\textsuperscript{EY10866}</td>
<td>0 (N=20)</td>
</tr>
<tr>
<td>CG11253::mVenus; CG11253\textsuperscript{EY10866}</td>
<td>20 (N=20)</td>
</tr>
</tbody>
</table>

Table 4.2: Individual male flies were each crossed to a wild type female virgin fly. The number of these crosses which produced progeny was recorded. Rescuing CG11253\textsuperscript{EY10866} flies with a CG11253::mVenus restores fertility. The fertility of rescued and revertant male flies was not significantly different. However both were significantly different from CG11253\textsuperscript{EY10866} (P : <0.0001, Fisher’s exact test).
Figure 4.1: Testes physiology of two day old CG11253\textsuperscript{EY10866} flies.
The testes of 2 day old CG11253\textsuperscript{EY10866} and control (heterozygous CG11253\textsuperscript{EY10866}) flies were dissected. (A & B) A schematic showing the phenotypes of control (A) and CG11253\textsuperscript{EY10866} (B) testes. (C) Control testis showing full seminal vesicle and normal amount of individualised sperm. Motile sperm can be seen exiting the seminal vesicle. (D) CG11253\textsuperscript{EY10866} testis with a small/empty seminal vesicle and an accumulation of individualised sperm at the terminal epithelium.
Table 4.3 Testes physiology of 2 day old **CG11253**\(^{EY10866}\) and revertant flies

<table>
<thead>
<tr>
<th>2 days old</th>
<th>Female flies present</th>
<th>Sperm bundle disruption</th>
<th>Empty/Small Seminal Vesicle</th>
<th>Accumulation of individualised sperm</th>
<th>Motile sperm detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CG11253</strong>(^{EY10866})</td>
<td>N</td>
<td>25 (N=30)</td>
<td>30 (N=30)</td>
<td>24 (N=31)</td>
<td>0 (N=32)</td>
</tr>
<tr>
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<td>0 (N=22)</td>
<td>10 (N=32)</td>
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<td>2 (N=16)</td>
<td>3 (N=25)</td>
<td>12 (N=12)</td>
</tr>
</tbody>
</table>

Table 4.3 : The frequency of two-day old fly testes observed that showed defective phenotypes. Instances of sperm bundle disruption, small seminal vesicles and high levels of accumulated individualised sperm at the terminal epithelium were recorded during testis dissection of two day old **CG11253**\(^{EY10866}\), mated revertant and unmated revertant flies. The number of testes in which motile sperm could be observed after testes squash is also shown. There was a significant difference in bundle disruption, seminal vesicle size and motile sperm presence between **CG11253**\(^{EY10866}\) and revertant : P value : < 0.0001. There was also a significant difference in the level of individualised sperm accumulation: P value 0.0004 (Fisher's exact test). No significant difference was found between mated and unmated revertant flies.

Table 4.4 Testis physiology of 7 day old **CG11253**\(^{EY10866}\) and revertant flies

<table>
<thead>
<tr>
<th>7 days old</th>
<th>Female flies present</th>
<th>Sperm bundle disruption</th>
<th>Empty/Small Seminal Vesicle</th>
<th>Accumulation of individualised sperm</th>
<th>Motile sperm detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CG11253</strong>(^{EY10866})</td>
<td>N</td>
<td>14 (N=15)</td>
<td>15 (N=15)</td>
<td>14 (N=15)</td>
<td>0 (N=14)</td>
</tr>
<tr>
<td>Revertant</td>
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<td>1 (N=23)</td>
<td>0 (N=23)</td>
<td>6 (N=22)</td>
<td>19 (N=19)</td>
</tr>
<tr>
<td>Revertant</td>
<td>Y</td>
<td>1 (N=24)</td>
<td>0 (N=24)</td>
<td>3 (N=24)</td>
<td>17 (N=18)</td>
</tr>
</tbody>
</table>

Table 4.4 : The frequency of seven-day old fly testes observed that showed defective phenotypes. Instances of sperm bundle disruption, small seminal vesicles and high levels of accumulated individualised sperm at the terminal epithelium were recorded during testis dissection of seven day old **CG11253**\(^{EY10866}\), mated revertant and unmated revertant flies. The number of testes in which motile sperm could be observed after testes squash is also shown. There was a significant difference in bundle disruption, seminal vesicle size and motile sperm presence between **CG11253**\(^{EY10866}\) and revertant : P value : < 0.0001. There was also a significant difference in the level of individualised sperm accumulation: P value 0.0001 (Fishers exact test). No significant difference was found between mated and unmated revertant flies.
motility is thought to be required for sperm to move into the seminal vesicle, the phenotype observed is therefore consistent with a defect in flagella motility (Fuller 1993).

The testes physiological phenotypes described are similar to those previously recorded for flies in which the dynein intermediate chain and proposed axonemal dynein Dic61B was mutated (Fatima 2012). These flies also display abnormal sperm bundle bending and empty seminal vesicles (Fatima 2012). The testes defects observed could show how mutations in motility genes manifest in Drosophila testes. This could indicate that CG11253 has a role in flagella motility.

4.2.3 Does inability to mate replicate CG11253EY10866 testes phenotypes?

I hypothesised that the CG11253EY10866 testes defects observed were secondary effects of immotile sperm not being able to exit the testis resulting in their aggregation in the terminal epithelium and disruption of sperm bundles. To test whether this was the case I investigated whether stopping turnover and movement of sperm through the testes could increase the likelihood of such defects occurring. To test this I compared revertant male flies that had mated (and thus sperm could move through the reproductive system) with those which could not mate (to try to induce a back up of sperm) (Table 4.3). However there was no significant difference between testes phenotypes from mated and unmated revertant flies. In case two days was not sufficient time to develop such an effect I also examined seven day old flies (Table 4.4). However no significant difference was seen between these flies either and thus no additional evidence for this hypothesis was recorded.

4.2.4 Genetic interaction with axonemal dynein arm components

CG11253 is required for the function of both of the motile ciliated cells of Drosophila. To investigate the relationship between CG11253 and the axonemal dynein arms I investigated whether double heterozygotes of CG11253EY10866 and dynein arm component mutants resulted in reduced function of cilia or flagella.
This also required the characterisation of axonemal dynein arm component mutants. \(CG9313^{MB06913}\), \(Dhc36C^{LA00085}\) and \(Dhc93AB^{MB05444}\) represent lines with transposons inserted into the transcriptional units of the ODA genes \(CG9313\) and \(Dhc93AB\) and the IDA gene \(Dhc36C\). \(CG9313^{MB06913}\) and \(Dhc93AB^{MB05444}\) have minos transposons inserted into exonic regions. \(Dhc36C^{LA00085}\) has a P element insertion in the third intron of \(Dhc36C\). Defects in hearing have been previously been detected in \(Dhc36C^{LA00085}\) and \(Dhc93AB^{MB05444}\) as well as in an alternative \(CG9313\) allele \(CG9313^{PADEF334P}\) (Senthilan et al 2012). This makes these lines ideal candidates in which to investigate the impact of mutating axonemal dynein components in proprioceptive chordotonal organs. All three genes have human homologues (\(Dhc93AB – DNAH9/11/17\), \(Dhc36C – DNAH7\), \(CG9313 – DAI\)). \(DNAI\) (Pennarun et al 1999, Guichard et al 2001, Zariwala et al 2001) and \(DNAHI\) (Schwabe et al 2008, Knowles et al 2012) have previously been found to be mutated in patients with PCD.

4.2.4.1 Climbing Assay

A climbing assay was conducted to assess the proprioceptive and gravitactic capabilities of \(CG9313^{MB06913}\), \(Dhc36C^{LA00085}\) and \(Dhc93AB^{MB05444}\) flies. \(CG9313^{MB06913}\) and \(Dhc36C^{LA00085}\) homozygotes climbed significantly less well than their respective heterozygotes signifying a role for these genes in chordotonal neuron function (Figure 4.2). Both \(Dhc93AB^{MB05444}\) homozygotes and heterozygotes displayed defective climbing capabilities, but as they were not significantly different from each other it cannot be concluded that disruption of \(Dhc93AB\) results in a lack of chordotonal neuron function. It is unlikely that heterozygous \(Dhc93AB^{MB05444}\) causes a defective climbing phenotype as a double heterozygote of \(Dhc93AB^{MB05444}\) and \(CG11253^{EY10866}\) (discussed below) did not show the same effect.

Double heterozygotes of \(CG9313^{MB06913}\), \(Dhc36C^{LA00085}\) and \(Dhc93AB^{MB05444}\) with \(CG11253^{EY10866}\) were also assayed and compared to single heterozygotes in order to establish whether a genetic interaction could be identified. However none of the double heterozygotes were significantly different from their
Figure 4.2: Climbing assay (Method A) to screen for CG11253 genetic interactions

A climbing assay (Method A) was used to investigate genetic interactions between CG11253 and axonemal dynein arm components. 17-20 flies were screened in each assay (N=3). An average climbing score was calculated from each repeat. Homozygous and heterozygous Dhc36C<sup>LA00085</sup>, CG9313<sup>MB06913</sup> and Dhc93AB<sup>MB05444</sup> flies as well as those double heterozygous with CG11253<sup>EY10866</sup> were assayed for climbing capability. Dhc36C<sup>LA00085</sup> and CG9313<sup>MB06913</sup> homozygotes climb statistically less well than heterozygotes (P: 0.0065 and 0.0122 respectively, calculated using Holm-Sidak multiple comparison test following ANOVA). Dhc93AB<sup>MB05444</sup> homozygotes and heterozygotes were not significantly different. There was no significant difference between heterozygous CG11253<sup>EY10866</sup> (control) and any double heterozygotes of CG11253<sup>EY10866</sup> and either Dhc36C<sup>LA00085</sup>, CG9313<sup>MB06913</sup> or Dhc93AB<sup>MB05444</sup>. N = 3 climbing scores per line each generated from 17-20 flies.
respective single heterozygotes indicating that a genetic interaction could not be detected (Figure 4.2).

4.2.4.2 Fertility Assay

In order to investigate the role played by these axonemal dyneins in sperm flagella motility a fertility assay was conducted. Double heterozygotes were again investigated to assay for a genetic interaction between dynein arm components and CG11253EY10866 (Figure 4.3). Males were allowed to mate to wild type females for 5 days before removing both parent flies. The resulting progeny were then counted.

CG9313MB06913 and Dhc36CL4A0085 homozygous male flies showed significantly reduced fertility compared to their respective heterozygotes. Dhc93ABMB05444 male flies also showed reduced fertility but as with the climbing assay this was not significantly different from its heterozygote. Fertility is not completely lost in any of these cases contrasting with previous findings for CG11253EY10866.

There was no significant difference between double heterozygotes and their respective single heterozygotes, again signifying that no genetic interaction could be detected.

4.2.5 TEM of CG11253EY10866 Johnston’s organ axonemal ultrastructure

Transmission electron microscopy (TEM) was used to observe the axonemal ultrastructure of CG11253EY10866 chordotonal cilia to identify any defects in the motility machinery. For all subsequent TEM (and that in Chapter 5) tissue fixation was carried out by A P Jarman, TEM was conducted by EM research services, Newcastle University Medical School and I dissected the tissue for fixation and analysed the images generated.

fd3F1 ciliary axonemes revealed a complete loss of both inner and outer axonemal dynein arms (Newton et al 2012). As Fd3F regulates CG11253 I hypothesised that a similar phenotype could be responsible for the behavioural defects previously observed in CG11253EY10866 flies. Examining the axonemal
A fertility assay was carried out to investigate genetic interaction between CG11253 and axonemal dynein arm components. Individual male flies (N=9-10) were each allowed to mate with one wild type virgin female fly for two days. They were then transferred to a new vial and allowed to mate for a further five days. The number of progeny produced in this vial were counted after eclosion.

The amount of progeny produced in crosses with homozygous and heterozygous male Dhc36CA00085, CG9313MB06913 and Dhc93ABMB05444 flies, as well as those double heterozygous with CG11253EY10866 was counted. Dhc36CA00085 and CG9313MB06913 homozygotes produced statistically less progeny than heterozygotes (P : 0.0008 and 0.02 respectively). Dhc93ABMB05444 homozygotes and heterozygotes were not significantly different. There was no significant difference between heterozygous CG11253EY10866 and any double heterozygotes of CG11253EY10866 and either Dhc36CA00085, CG9313MB06913 or Dhc93ABMB05444. Statistical significance calculated using Dunn’s multiple comparison test following Kruskal-Wallace test.
 ultrastructure of adult antennal chordotonal cilia revealed a loss of dynein arms in $CG11253^{EY10866}$ compared to those of revertant flies (Figure 4.4, Table 4.5). ODA were lost from 46.9% of microtubule doublets and IDA lost in 79% of cases (Table 4.5). This finding indicates that $CG11253$ is required for proper formation of the axonemal dynein arms and therefore is a gene associated with cilium motility. A lack of axonemal dynein arms would result in a loss of cilium motility or reduced function of the cilium motility machinery and could explain the defects observed in the function of chordotonal neurons.

4.2.6 Electron microscopy of $CG11253^{EY10866}$ sperm flagella

$CG11253^{EY10866}$ flies showed a phenotype consistent with disrupted flagella motility. TEM was used to examine flagella ultrastructure to see if dynein arms were lost as had been detected in chordotonal cilia.

$CG11253^{EY10866}$ sperm flagella had lost both outer and inner dynein arms (Figure 4.5A & B). As in chordotonal neurons, $CG11253^{EY10866}$ sperm flagella showed higher levels of IDA loss (77.77%) than ODA loss (55.00%) (Table 4.6). The disruption of dynein arm localisation would result in reduced motility of the flagella and could explain both the lack of motile sperm after testis squash and $CG11253^{EY10866}$ male infertility.

I examined the structure of whole sperm bundles and counted the number of cells present in order to ascertain whether any meiotic defect could be detected in $CG11253^{EY10866}$. However in most cases of both revertant and $CG11253^{EY10866}$ flies there were 64 sperm cells per bundle (average cell number : $CG11253^{EY10866}$ – 63.6, revertant – 64.4, n=5) showing that the early cell divisions in sperm cell development were occurring normally.

Disruptions in axonemal structure were also observed in $CG11253^{EY10866}$ flagella in which one or more microtubule pairs were mislocalised away from the axoneme (Figure 4.5D). Microtubule pairs are mislocalised statistically more often in $CG11253^{EY10866}$ flagella (9.4%, N=372) than those of revertant flies (1.24%, N=324) (P value <0.0001 , Fishers exact test). A further structural defect that was observed was that of ectopic luminal densities. The central pair and
Microtubule doublets for which the ODA were visible.

42 (N=45)  

Microtubule doublets for which the IDA were visible.

39 (N=45)  

Microtubule doublets for which the IDA were visible.

17 (N=81)

Table 4.5 Axonemal dynein arm loss in CG11253EY10866 Johnston’s organ cilia

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<tr>
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<th>Revertant</th>
<th>CG11253EY10866</th>
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<tbody>
<tr>
<td>Microtubule doublets</td>
<td>42 (N=45)</td>
<td>43 (N=81)</td>
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<td>for which the ODA were</td>
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<td>visible</td>
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<tr>
<td>Microtubule doublets</td>
<td>39 (N=45)</td>
<td>17 (N=81)</td>
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<td>for which the IDA were</td>
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<tr>
<td>visible</td>
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Table 4.5 : The proportion of microtubule doublets from CG11253EY10866 and revertant Johnston's organ cilia which have visible inner and outer axonemal dynein arms. Dynein arm presence calculated from images obtained by TEM of the antennae of 3 flies. Differences in frequency are significant with a P value less than 0.0001, calculated with Fisher’s exact test.
Figure 4.5: Transmission electron microscopy of CG11253\textsuperscript{EY10866} sperm flagella axonemal ultrastructure

(A) The axonemal ultrastructure of a revertant sperm flagellum. Dynein arms are labeled with red arrows. The electron dense lumens of the central pair and accessory microtubules are labeled with a blue arrow.

(B) The axonemal ultrastructure of a CG11253\textsuperscript{EY10866} sperm flagella. These flagella display a partial loss of axonemal dynein arms (absent dynein arms are labeled with white arrows). Ectopic luminal densities have also been identified in the outer microtubule doublets (blue arrow). These are normally only found in the central pair and accessory microtubules of sperm flagella.

(C) A diagram detailing the defects observed in CG11253\textsuperscript{EY10866} sperm flagella ultrastructure compared to revertants.

(D) CG11253\textsuperscript{EY10866} flagella also show structural disruption, with microtubule pairs being displaced (labeled with yellow arrow). Tissue fixed by A Jarman as per Newton et al (2012), TEM conducted by EM research services, Newcastle University Medical School.

Scale bars: 100nm
Table 4.6: Dynein arm loss in CG11253^EY10866 and revertant flies

<table>
<thead>
<tr>
<th></th>
<th>Revertant</th>
<th>CG11253^EY10866</th>
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<tbody>
<tr>
<td>Microtubule doublets for which the ODA were visible.</td>
<td>43 (N=45)</td>
<td>20 (N=45)</td>
</tr>
<tr>
<td>Microtubule doublets for which the IDA were visible.</td>
<td>41 (N=45)</td>
<td>10 (N=45)</td>
</tr>
</tbody>
</table>

Table 4.6: The number of microtubule doublets from CG11253^EY10866 and revertant sperm flagella which have visible inner and outer axonemal dynein arms. Axonemal structure was observed by TEM of the testes of 3 flies. Differences in frequency are significant with a P value <0.0001, (Fisher's exact test).
outer accessory microtubules are the only microtubules to have electron-dense centres in revertant flies (2.22% microtubule pairs had ectopic luminal densities, N=135). However these have been observed in the nine outer microtubule doublets of $CG11253^{EY10866}$ flagella (36.73%, N=98, P value <0.0001, Fishers exact test).

$CG11253$ is required for proper axonemal dynein arm formation in sperm flagella as in chordotonal neuron cilia. However sperm flagella display a number of other axonemal defects which could contribute to the lack of functional sperm being found in $CG11253^{EY10866}$ testes. It is not known whether these additional defects, such as disruption of axonemal structure, occur as a result of or contribute to the loss of axonemal dynein arms.

### 4.2.7 CG6971::mVenus Construction and Localisation

CG6971 is an inner dynein arm component with chordotonal specific expression in the embryo (Newton et al 2012). In order to further investigate how $CG11253$ regulates axonemal dynein arm localisation a $CG6971::mVenus$ fusion gene was generated. Observing the localisation of this gene in $CG11253^{EY10866}$ could elucidate the mechanism by which $CG11253$ regulates dynein arm localisation.

CG6971 has also been found in the $Drosophila$ sperm proteome (Dorus et al 2006, Wasbrough et al 2010) meaning that it is expressed in the same cell types as $CG11253$. Whilst CG11253 was not detected in the sperm proteome (Dorus et al 2006, Wasbrough et al 2010) we already have evidence of its role in sperm development from the FLY-TED in situ hybridisation pattern (Zhao et al 2009) and the sperm development defects that I have characterised.

The fusion gene was generated using the same techniques as when generating $CG11253::mVenus$. The upstream region that was incorporated ahead of $CG6971::mVenus$ contained binding sites for RFX and Fd3F which should allow expression from its own promoter (Figure 4.6).

In developing chordotonal neurons CG6971::mVenus can be seen to localise both cytoplasmically and to the cilium in late stage embryos (Figure 4.7E). CG6971::mVenus localisation does not appear to be compartmentalised at this
Figure 4.6: The construction of the CG6971::mVenus fusion gene.

(A) A vector map of the pBID-UASC-CG6971::mVenus vector which was injected into blastoderm stage embryos. Vector map generated using Serial Cloner 2.6.1.

(B) A diagram of the CG6971::mVenus insert which was incorporated into the genome of those flies. The CG6971::mVenus fusion gene was designed to include the upstream region of CG6971 to allow expression from its own promoter. Within this promoter the localisation of potential regulatory elements are noted. The diagram shows the location of an X box (green) and forkhead binding sites (purple) in the upstream regions of CG6971. The motif locations were found using GenePallete to search for the consensus sequences of the forkhead binding site (RYMAAYA, Newton et al 2012) and X box (RYNNYY{1,3}RRNRAC, Laurençon et al 2007).
Figure 4.7: The localisation of CG6971::mVenus in wild type flies.

CG6971::mVenus localises to the chordotonal neurons in embryos, larvae and pupae.

(A) In the chordotonal neurons of the larval body wall CG6971::mVenus (green) localisation is concentrated to the proximal ciliary compartment. Dendrites are stained with Futsch antibody and the distal ciliary compartment with NompC (both magenta).

(B) As 4.7A but showing CG6971::mVenus localisation only.

(C) A diagram showing the localisation of CG6971::mVenus in relation to NompC and Futsch in the larval pelt chordotonal neuron cilia.

(D) CG6971::mVenus (green) is localised to the proximal ciliary compartment and the cytoplasm of chordotonal neurons of the pupal Johnston’s organ. Neurons are marked with Futsch and the distal ciliary compartment with NompC (both magenta). This figure also includes a diagram depicting the staining of Futsch, and NompC antibodies in relation to the localisation of CG6971::mVenus

(E) CG6971::mVenus localises to the cytoplasm and the cilia of Ich5 embryonic chordotonal neurons

Scale bar sizes A, B and E: 5µm D: 25µm
CG6971::mVenus strongly localises to the proximal ciliary compartment in larval pelt chordotonal neurons (Figure 4.7A-C). The borders of expression are clearly defined at the beginning of the distal compartment (marked by NompC). CG6971::mVenus localisation is also observed in the neuronal dendrites. In developing pupal antennae CG6971::mVenus can again be seen to localise to the proximal ciliary compartment and to the cytoplasm (Figure 4.7D).

### 4.2.8 CG6971::mVenus localisation in CG11253^EY10866

CG6971::mVenus was crossed into CG11253^EY10866 in order to further understand the relationship between CG11253 and axonemal dyneins (Figure 4.8A). Preliminary imaging data suggests that CG6971::mVenus is present at lower levels in CG11253^EY10866 than wild type antennae (Figure 4.8B & C). CG6971::mVenus can still localise to the cilium but also at very low levels (Figure 4.8C’). The implication of this is that CG11253 is required for appropriate levels of axonemal dynein arm components to be present. However a caveat for this preliminary finding is that thus far CG6971::mVenus localisation has only been observed without a secondary marker.

### 4.2.9 Interaction with TilB

CG11253 has previously been found to interact with TilB in a yeast-2-hybrid screen for protein-protein interactions (Giot et al 2003). In order to provide further evidence for the interaction between CG11253 and TilB indicated by Giot et al (2003) I conducted a climbing assay of tilb^2 and CG11253^EY10866 double heterozygotes (Figure 4.9). However no significant difference was found between the heights climbed by double heterozygotes and heterozygous tilb^2 flies indicating that no genetic interaction was found.

### 4.2.10 ZMYND10 – a regulator of ciliary length?

Albee et al (2013) have recently investigated the role of ZMYND10 in human retinal epithelial cells. They observe that knocking down the levels of ZMYND10
Figure 4.8: CG6971::mVenus localisation in CG11253EY10866 pupal antennae.

(A) The crossing scheme used to cross CG6971::mVenus into CG11253EY10866.

(B) Wild type pupal chordotonal neurons showing CG6971::mVenus (green) localisation in the cytoplasm and the cilia.

(C) CG11253EY10866 pupal chordotonal neurons showing CG6971::mVenus is reduced in level. CG6971::mVenus (green) can still localise to the cytoplasm. This image was taken under the same conditions as (B).

(C') The same image as shown in (C), however the brightness and contrast has been adjusted to reveal that a low level of CG6971::mVenus is still able to localise to the cilium in CG11253EY10866 chordotonal neurons.

Scale bars: 10µm.
A climbing assay to test for genetic interaction between *CG11253* and *tilB*.

A climbing assay (Method B) shows no significant difference (Mann Whitney U test) in climbing capability of heterozygous *tilb*²/+ (N=86) and double heterozygous *tilb*²/+; *CG11253*<sup>EY10866</sup>+ (N=89). The heights climbed by flies of each genotype were calculated using ImageJ. Therefore no evidence of a genetic interaction was detected.
results in shortened cilia and occasionally an increase in basal body/centriole number. The cilia in these cells are not motile so ZMYND10 must have a role not linked to cillum motility. In order to investigate whether cillum length was altered in \textit{CG11253}\textsuperscript{EY10866} I calculated the length of larval chordotonal cilia in revertant and \textit{CG11253}\textsuperscript{EY10866} flies (Figure 4.10). Cillum lengths for each neuron in a lch5 cluster were averaged, generating an average cillum length for each cluster. Cillum length was marked by measuring from the distal tip of NompC staining to the tip of the dendrite marked by Futsch. No significant difference was observed between \textit{CG11253}\textsuperscript{EY10866} and revertant cillum lengths therefore this function of ZMYND10 is not conserved in \textit{Drosophila}. However this finding by Albee et al (2013) work does indicate that ZMYND10 also has role in non-motile cilia.

\textbf{4.2.11 CG11253\textsuperscript{p.Val14Gly}::mVenus as a rescue construct}

In parallel with my investigations into the role of CG11253, A Onoufriadis and H Mitchison had identified PCD patients with mutations in ZMYND10 (further details in discussion, Moore et al 2013). In order to investigate the point mutations observed in PCD patients further the p.Val16Gly mutation (p.Val14Gly is \textit{Drosophila}) was incorporated into the pBid-UASC-CG11253::mVenus construct using the Quickchange mutagenesis kit (work carried out by P zur Lage) (Figure 4.11). This was then injected into blastoderm stage embryos (by P zur Lage) and crossed into \textit{CG11253}\textsuperscript{EY10866} as for \textit{CG6971}::mVenus.

A fertility assay was carried out to compare the ability of a wild type rescue construct and the \textit{CG11253}\textsuperscript{Val14Gly}::mVenus construct to rescue the fertility defect of \textit{CG11253}\textsuperscript{EY10866} (Figure. 4.12). Whilst \textit{CG11253}\textsuperscript{Val14Gly}::mVenus rescued the fertility defect it did so significantly less well than \textit{CG11253}::mVenus. This shows that CG11253 with this amino acid substitution has reduced functionality but did not lose protein function altogether.
Figure 4.10: Larval cilium length in CG11253\textsuperscript{EY10866}.
The average cilium lengths of Ich5 chordotonal neurons in larval body walls were compared in CG11253\textsuperscript{EY10866} homozygotes and heterozygotes (N=9). No significant difference was observed between cilium lengths (Mann-Whitney U) indicating that reducing CG11253 expression had no effect on cilium length. This contrasts with the findings of Albee et al (2013) for ZMYND10.
Figure 4.12: A fertility assay shows that Val14Gly reduces CG11253::mVenus function.

A fertility assay showing the ability of CG11253::mVenus; CG11253EY10866 flies to rescue the fertility defect of CG11253EY10866. Individual male flies (N – 40) were each allowed to mate with one wild type virgin female fly for two days. They were then transferred to a new vial and allowed to mate for a further five days. The number of progeny produced in this vial were counted after eclosion. CG11253Val14Gly::mVenus; CG11253EY10866 flies produced significantly less progeny than CG11253::mVenus; CG11253EY10866 flies.

P value : 0.0385, calculated by Mann Whitney U test.

Figure 4.11: p.Val14Gly in CG11253.

The location of the p.Val14Gly amino acid substitution in CG11253 which mimics the p.Val16Gly amino acid change found in ZMYND10 by A Onoufriadis and H Mitchinson in patients with PCD.
4.3 Discussion

*CG11253* is required for the proper formation of the axonemal dynein arms in *Drosophila*. As a result mutation of *CG11253* causes defective cilium motility or loss of function of the cilium motility machinery resulting in defective motile ciliated cells, be that chordotonal neurons or sperm flagella.

4.3.1 The role of cilium motility in proprioceptive neurons

The loss of axonemal dynein arms and consequent loss of motility in the chordotonal cilia can explain the hearing loss previously described for *CG11253<sup>EY10866</sup>* (Senthilan et al 2012). Cilium motility or at least the action of the cilium motility machinery is known to be required for amplification gain and increased sensitivity at lower sound levels (Gopfert et al 2003, Gopfert et al 2005). It is not known how the cilium motility machinery is utilised for proprioception, it could be required to amplify weak stimuli as for hearing or as part of a transduction complex with force gated ion channels (Nadrowski et al 2008). From the proprioceptive defects I have observed for *CG11253, CG9313* and *Dhc36C* mutants it is clear that axonemal dyneins are required for the proper function of proprioceptive chordotonal neurons.

Sperm flagella and chordotonal cilia display similar rates of outer and inner dynein arm loss in *CG11253<sup>EY10866</sup>*. However the extent of the effect that this had on cellular function differed. Whilst sperm cells were no longer functional with no motile sperm being observed, chordotonal neurons could still function, just not at wild type levels. This reflects the importance of ciliary motility and dynein arm function to each cell types function. Sperm flagellum beating is vital for its function however individual dyneins are not necessary for flagella beating. The moderate uncoordinated phenotype of *CG11253* mutant flies as indicated by the climbing assay was milder than those reported for *atonal* mutants, which lack chordotonal neurons (Jarman et al 1995). This indicates that cilium motility may well be required for a specialised aspect of chordotonal neuron function such as amplification gain. Alternatively the dynein arms that remain in these mutants
could be sufficient for a low level of transduction complex function to be retained.

### 4.3.2 Testis physiology

There are similarities between the testis phenotype I observed for \( CG11253^{EY10866} \) and that recorded for mutations in \( Dic61B \) (Fatima 2012). These include small or empty seminal vesicles and abnormal bending in sperm bundles. Fatima (2012) also reports defects in individualisation with the individualisation complex only passing part way along the axoneme. Mutations in \( yuri \), a component of the actin cone required for individualisation also show empty seminal vesicles, showing that defects such as this can occur as a result of a failure of sperm to individualise (Texada et al 2008). However bundle bending was not recorded for \( yuri \) mutants. Fatima (2012) identifies that the failure to individualise is a secondary effect as abnormally bent bundles block the progression of the actin cones of the individualisation complex. Defects in \( CG11253^{EY10866} \) could be more similar to those observed for the tubulin mutant \( B2t^6 \), which shows normally formed sperm which are immotile due to a loss of ODA attachment (Raff et al 2008).

It is not known how a lack of motility results in the observed phenotypes. One model could be that immotile sperm cannot pass into the seminal vesicle, causing a back up of material that includes individualised sperm, which then disrupts bundle placement. An attempt to show that the phenotypes were caused by an inability of sperm to pass through the reproductive system was ultimately unsuccessful with testis from mated and unmated flies not appearing significantly different. It could be that this is not the mechanism by which the defects occur and that abnormal bending and coiled sperm build up occurs due to the axonemal abnormalities observed.

### 4.3.3 Flagella ultrastructure

The defects observed in axonemal ultrastructure could be linked to motility or could represent a secondary role for \( CG11253 \) in sperm flagella. \( tilB \) mutants, which also effect dynein arm localisation, display sperm flagella with disrupted axonemal ultrastructure (Eberl et al 2000). This indicates that either the motility
machinery plays a role in stabilisation of the axoneme or that structural components have also been lost from the axoneme. Ectopic luminal densities have also previously been linked to loss of cilium motility. B2t6 flies, which have a single amino acid change in β-tubulin, are both missing outer dynein arms and display ectopic luminal densities in their sperm flagella (Raff et al 2008).

Disruption of CG11253 expression results in a number of flagellar defects, whilst these cannot all necessarily be attributed to ciliary motility defects it is clear that CG11253 does modulate cilium motility and that it is essential for proper dynein arm formation.

4.3.4 PCD patients with mutations in ZMYND10

Whole exome sequencing (by A Onoufriadis and H Mitchison) has identified a number of mutations in ZMYND10 in patients with symptoms consistent with PCD (Moore et al 2013) (Figure 4.13). These represent three point mutations that result in amino acid substitutions (p.Val16Gly, p.Leu39Pro and p.Leu266Pro) and two deletions that result in frame shifts and protein truncations (p.Phe22Serfs*21and p.Val198Glyfs*13). p.Leu266Pro occurs in one of the LXXLL repeats found in ZMYND10. This provides the first example of where mutation of one of these domains has been found to be disease causing.

Mutations in ZMYND10 have been found to result in inner and outer axonemal dynein arm loss; this was confirmed both by electron microscopy and immunofluorescence (A Onoufriadis and H Mitchison – Moore et al 2013). Both DNAH5 (ODA) and DNALI1 (IDA) were found to be lost from the cilia of respiratory epithelial cells obtained from patients with ZMYND10 mutations by nasal brushing (A Onoufriadis and H Mitchison – Moore et al 2013).

This finding shows that the function of CG11253 is conserved between flies and humans as mutation of the human homologue of CG11253 also results in loss of dynein arms. However there are some functional differences between CG11253 and ZMYND10 as of the three residues found to be effected by point mutations in PCD patients (and therefore required for protein function) only p.Val16Gly was conserved in Drosophila.
ZMYND10 is mutated in patients with PCD. The location of the amino acid differences caused by the mutations found by A Onoufriadis & H Mitchison in ZMYND10 in patients with primary ciliary dyskinesia.
4.3.5 Modelling PCD mutations in *Drosophila*

In order to investigate the pathogenicity of the p.Val16Gly causing mutation found in patients with PCD a comparable mutation was introduced to a *CG11253* rescue construct (work carried out by P zur Lage). *CG11253<sup>Y10866</sup>* flies which had been rescued with the *CG11253<sup>Val14Gly::mVenus</sup>* showed a restoration of fertility but not to wild type (rescue with *CG11253::mVenus*) levels.

Most mutations in ZMYND10 were found in patients with immotile cilia on their respiratory epithelia (Moore et al 2013, Zariwala et al 2013). *ZMYND10<sup>Val16Gly</sup>* patient cilia were still motile, however they beat more slowly and stiffly (A Onoufriadis and H Mitchison – Moore et al 2013). The point mutations causing the p.Val14Gly change in *CG11253* and the p.Val16Gly change in ZMYND10 both result in reduced protein function without removing it completely. This demonstrates the first instance of using *Drosophila* to model the effects of PCD causing mutations. It demonstrates a system by which the effects of potential PCD causing mutations on protein function can be assessed in the future.

4.3.6 Further ZMYND10 mutations found in PCD patients

In addition to the mutations identified by Onoufriadis and Mitchison (Moore et al 2013), further mutations in *ZMYND10* have been identified which also cause PCD (Zariwala et al 2013). These include two additional point mutations resulting in an amino acid substitution (p.Ser29Pro and p.Tyr379Cys) and seven mutations resulting in protein truncation (p.Phe101Serfs*38, p.Ser163Ilefs*20, p.Thr205Alafs*3, p.Trp228, p.Gln323, p.Gly347Glnfs*30 and p.Gln266*) (Zariwala et al 2013).

4.3.7 Genetic interactions

Double heterozygotes of *CG11253* and dynein mutants were assayed for climbing and fertility defects to investigate if there was a genetic interaction between *CG11253* and dynein arm components. Despite a lack of genetic evidence TEM has shown that CG11253 is required for dynein arm formation.
This suggests that assaying double heterozygotes is not an efficient way to find interactions of associations with other genes.

Double heterozygote genetic interaction was also used to investigate the interaction between *CG11253* and *tilB*. Whilst I identified no genetic evidence for this interaction, a previous high throughput yeast-2-hybrid protein-protein interaction screen suggested that a physical interaction between the two proteins might take place (Giot et al 2003). As assessing double heterozygotes has not proved an efficient way to assess interactions these two proteins could potentially still interact.

### 4.3.8 Interaction between ZMYND10 and LRRC6

Further evidence for the interaction has come from investigation into the human homologues of these genes. LRRC6 (human TilB homologue) and ZMYND10 have been found to interact both by yeast-2-hybrid analysis and GST-tagged pull down (both experiments carried out by P. zur Lage). Further work (P. zur Lage) has identified that the interaction between the two proteins occurs through the MYND domain (interacting fragment 394-430). Zariwala et al (2013) also confirmed the interaction between LRRC6 and ZMYND10 and showed that the predicted HSP-20 like chaperone/CS domain is sufficient to pull down ZMYND10 from rat lung lysate. The CS domain is conserved in TilB (Figure 4.14) indicating that the CG11253 and TilB interaction could occur via the MYND domain and CS domain in *Drosophila*.

Mutations in *LRRC6* have also been found in patients with PCD (Kott et al 2012, Horani et al 2013a, Zariwala et al 2013). DNAH7, DNAI2 and DNALI1 have been shown to be either lost or at reduced levels in respiratory cells from these patients, whereas DNAI1 was mislocalised from the axoneme (Kott et al 2012, Horani et al 2013a). A reduction in the expression of *DNAI1* and *DNAH7* has been recorded in nasal cells of PCD patients with mutations in *LRRC6* (Horani et al 2013a).

The similarity of the phenotypes in patients with ZMYND10 and LRRC6 mutations and the evidence for their physical interaction suggest that they form a
Figure 4.14: The predicted protein domains of TilB and LRRC6.

A diagram showing the protein structure of TilB and LRRC6. Both contain a number of domains: LRR (Leucine rich repeats (Four in each highlighted region)), LRRCT (LRR C terminal domain), coiled coil and CS (CHORD containing proteins and SGT1). Zariwala et al (2013) found LRRC6 to interact with ZMYND10 via its CS domain. Its conservation in TilB indicates that this interaction could potentially persist in Drosophila. All domains as annotated by Uniprot.
complex in human cells. Similarities are also found in the phenotypes found when CG11253 or tilB are mutated. This includes axonemal dynein arm loss in both chordotonal neurons and sperm flagella as well as disrupted flagella axonemal structure (Eberl et al 2000, Kavlie et al 2010). The interaction between their human homologues and the similarities in the phenotypes of their mutants suggests that an interaction between CG11253 and TilB could also be occurring in Drosophila. However this remains to be confirmed either by further physical interaction data such as a GST-tagged pull down or by investigating a genetic interaction using double mutants.

4.3.9 Localisation of CG11253 homologues

The localisation of homologues of CG11253 differs across organisms perhaps reflecting the different roles that homologues play. In Drosophila as already described CG11253 localises predominantly to the cytoplasm with some ciliary localisation. Zariwala et al (2013) have found ZMYND10 to show some co-localisation with SAS-6 and PCM-1 in cytoplasmic puncta suggesting localisation at centriolar satellites in rat trachea. A basal body localisation has also been recorded for human ZMYND10 expressed in Xenopus (Zariwala et al 2013). These differences in localisation may suggest variation in the role that CG11253 plays in different organisms and tissues. Developmental differences in CG11253::mVenus localisation were observed, with no ciliary expression being found in mature neurons. It could be that differences in localisation reflect differences in developmental stage.

4.3.10 A role for CG11253 in transcriptional regulation?

As discussed in the previous chapter proteins containing a MYND domain or LXXLL motifs have been implicated in transcriptional regulation. It is possible that CG11253 and ZMYND10 could be involved in the transcriptional regulation of axonemal dynein genes.

Zariwala et al (2013) detected experimental evidence for this role for ZMYND10. A reduction in mRNA expression of DNAH5 and DNALI1 in human tracheal epithelial cells in which ZMYND10 had been knocked down by shRNA
was detected (Zariwala et al 2013). Additionally a reduction in the mRNA expression of DNAH7 and DNAI1 in nasal cells of PCD patients with LRRC6 mutations has been identified (Horani et al 2013a). Therefore the complex of LRRC6 and ZMYND10 could be acting to regulate transcription of dynein arm genes and thus be required for cilium motility in this manner.

However evidence against a direct role in transcription is that CG11253::mVenus is cytoplasmically localised and has not been observed in the nucleus. Furthermore ZMYND10 has also been reported as cytoplasmically localised (Albee et al 2013, Zariwala et al 2013). Therefore any role of CG11253 in the transcriptional regulation of dynein arms would have to be indirect. To investigate whether CG11253 does have a transcriptional role in Drosophila the expression of dynein arm components in CG11253^{EY10866} embryos is being investigated by in situ hybridisation and RT PCR by other lab members.

### 4.3.11 Transcriptional changes in PCD patients may be a common occurrence

The reduction in expression of dynein arm components in cells in which ZMYND10 was knocked down does not necessarily mean that ZMYND10 and CG11253 have a role in transcriptional regulation. Changes in the level of transcription of ciliary genes may be a common feature of PCD that is not always identified.

Onoufriadis et al (2013) found that cells from patients with mutations in CCDC114 displayed reduced expression of DNAH5. CCDC114 is thought to be a part of a microtubule docking complex required for ODA docking to the axoneme and as such localises along the cilium (Onoufriadis et al 2013). That a further PCD gene causes alterations in gene expression suggests that this effect is not specific to the ZMYND10/LRRC6 complex.

The findings of Geremek et al (2011) also indicate that changes in gene expression are widespread in PCD. Gene clustering analysis of bronchial biopsies from six PCD patients showing a variety of ultrastructural defects identified a cluster of genes with similar expression which was enriched with ciliary genes.
(Geremek et al 2011). The same analysis on control biopsies did not detect a ciliary enriched cluster of similarly expressed genes. Recent work by Geremek et al (2014) has directly shown that mRNA levels are reduced for a number of ciliary motility genes in these biopsies when compared to the controls. This indicates that there are changes in ciliary gene expression in a number of PCD patients. Those found in ZMYND10 knocked down cells are therefore not unique nor should they be taken as an indication of ZMYND10 and CG11253’s function.

### 4.3.12 CG11253 : a DNAAF?

An alternative role for CG11253 could be as a dynein arm assembly factor (DNAAF) required for the pre-assembly of dynein arm components before their transport to into the cilium. The cytoplasmic localisation of ZMYND10 and LRRC6 combined with the lack of dynein arms when they are mutated has made them candidates to also be involved in the preassembly of dynein arms. Cytoplasmically located factors required for dynein assembly have previously been identified (Mitchison et al 2012, Tarkar et al 2013). DNAAF1-4 are thought to be required to act with chaperone proteins for the pre-assembly of dynein arm components (Mitchison et al 2012, Tarkar et al 2013).

LRRC6 also contains a CS domain (CHORD-containing proteins and SGT1), which has previously been found in proteins that interact with chaperones such as DNAAF4/DYX1C1 (Tarkar et al 2013). A yeast-2-hybrid interaction between CG11253 and HSP68, which could have a role as a chaperone, has been identified in the interaction screen of Giot et al (2003). However as with the TilB interaction further confirmation is required before we can presume that this interaction is occurring. ZMYND10 was also not found to interact with DNAAF1-3 suggesting that it is not part of the dynein preassembly complex containing these components (Zariwala et al 2013).

### 4.3.13 CG6971::mVenus localisation in CG11253<sup>EY10866</sup>

The localisation observed for CG6971::mVenus in <sup>CG11253<sup>EY10866</sup> could be consistent with either a transcriptional or a preassembly role for CG11253. The
level of CG6971::mVenus is strongly reduced yet still localises to both the cytoplasm and the cilium. An overall reduction in the levels of dynein arm components does not suggest that CG11253 has a role in transport to the cilium or access to the ciliary compartment as in this instance it would be expected that CG6971::mVenus would be excluded from the cilium and potentially mislocalised elsewhere. It is also unlikely that CG6971::mVenus is being degraded purely due to mislocalisation as it can be seen to localise to the cytoplasm in wild type flies. However misfolding could result in subsequent degradation. The phenotype observed is more consistent with a role for CG11253 in stability of the protein as there appears to be a reduction in protein level and yet it can still localise normally. This is consistent with the TEM findings that some dynein arms could still form in CG11253 mutant flies.

4.3.14 Further work to clarify the role of CG11253

From the evidence available a role for CG11253 in the cytoplasmic assembly of dynein arms appears most likely. However in order to conclude this a number of further investigations need to be carried out. The interaction between CG11253 and TilB needs clarification in order to be able to transcribe any role designated to the ZMYND10/LRRC6 complex. Additionally further interactors either with CG11253 alone or this complex need to be identified to elucidate its role. Further evidence needs to be collected to find whether the potential interaction of CG11253 with a heat shock protein is occurring. At least one of the LXXLL motifs in ZMYND10 is vital for its function (A Onoufriadis and H Mitchison, Moore et al 2013), however what interacts with these motifs in ZMYND10 or CG11253 is unknown. Discovering what interacts with these motifs could again give clues to whether CG11253 does indeed act as a DNAAF.

Aside from interaction data further investigation into the behaviour of axonemal dyneins in CG11253 mutants could allow the clarification of CG11253’s role in dynein arm formation. The generation of further tagged dynein fusion genes, (particularly ODA components as thus far I have only examined IDA in CG11253 mutant flies) could identify if CG11253 is required for dyenin arm preassembly or another step the formation of axonemal dynein arms.
4.4 Conclusion

*CG11253* has been shown to be required for cilium motility. Its expression is necessary for the proper formation and axonemal localisation of inner and outer dynein arms. Loss of dynein arms results in cilium motility defects that in *Drosophila* manifest as behavioural and fertility defects. There is evidence from interactions in homologues that CG11253 could form a cytoplasmic complex with TilB, however further confirmation of this is required. The role of these genes in cilium motility has been conserved in humans and mutation of both of these genes results in PCD.

That a homologue of CG11253 has been implicated in PCD shows that using *Drosophila* as a model organism to better understand ciliary function can have consequences for understanding human disease. Whilst 28 genes that contribute to PCD have been identified they do not account for all occurrences of the disease. Regulation of expression by Fd3F formed an important part of hypothesising that CG11253 was involved in cilium motility. Other genes regulated by Fd3F can also be considered good candidates to have a role in cilium motility and secondly to be candidate PCD genes. Further attributes which helped identify *CG11253* that can be used to identify PCD genes were expression in motile ciliated tissues, conservation in organisms with motile cilia and defects in motile ciliated cell function (hearing, proprioception, fertility). The identification of *ZMYND10* as a PCD gene gives proof of concept that these techniques can be used to identify further PCD genes.

A transcriptional role for CG11253 was considered due to the functions of previously identified proteins with similar domains and findings of reduced dynein arm expression in human cells in which *ZMYND10* was knocked down (Zariwala et al 2013). However CG11253 is cytoplasmically localised and was not observed to enter the nucleus suggesting against a direct role in transcriptional regulation. Additionally alterations in gene expression may be common in cells which have lost cilium motility as changes in gene expression have been recorded in other PCD patients (Geremek et al 2011, Onufriadis et al 2013).
An alternative role for CG11253 may be as a dynein arm assembly factor (DNAAF). LRRC6, the interaction partner of ZMYND10, contains a domain previously found in DNAAFs and proteins known to interact with chaperones (Tarkar et al 2013). DNAAF1-4 are all cytoplasmically localised factors whose mutation results in a loss of dynein arm assembly (Mitchison et al 2012, Tarkar et al 2013). The cytoplasmic localisation and loss of dynein arms from ciliated cells in which CG11253 or ZMYND10 are mutated would be consistent with such a role. The general reduction of the level of CG6971::mVenus in CG11253^EY10866 chordotonal neurons could be consistent with reduced protein stability due to improper assembly of the dynein arms. Further evidence, as discussed, would be required to confirm such a role however with the evidence currently available indicates that a role for CG11253 in the cytoplasmic assembly of dynein arms is more likely than that of transcriptional regulation.
CG31320 – a novel cilium motility gene

5.1 Introduction

The identification of CG11253 showed among the genes expressed during Drosophila chordotonal neuron differentiation are those required for the formation of the cilium motility machinery. If these motility genes are conserved in humans they can be considered candidate genes for PCD. The techniques used to identify CG11253 can be replicated to discover new candidates for involvement in ciliary motility from those highly enriched in developing chordotonal cells.

A number of factors indicated that CG11253 could have been a cilium motility gene. These include regulation of its transcription by Fd3F and its evolutionary conservation in organisms with motile cilia. CG11253’s potential interaction with TilB, a previously identified ciliary motility factor, also suggested such a role. These characteristics can be utilised to identify new cilium motility genes from those found to be enriched in ato/catoGFP expressing cells.

5.1.1 Interolog walk

A fellow lab member, Giuseppe Gallone, devised a bioinformatic technique (Bio::Homology::InterologWalk) that allowed the generation of networks of putatively interacting proteins (Gallone et al 2011). G. Gallone used experimentally demonstrated interactions in orthologues to suggest potential interactions in an organism of interest. For example, an experimentally identified interaction between human protein A and human protein B could be used to predict an interaction between the Drosophila homologues of A and B. This technique could divulge groups of proteins that are required for similar processes. It could also be utilised to implicate previously uncharacterised proteins with biological roles due to their putative interaction with proteins known to function in those processes. This
technique was particularly applicable to investigating ciliogenesis as it requires a number of protein complexes made up of well-conserved components, such as the IFT complex.

G. Gallone used this technique to identify an interaction network containing the proteins produced by genes enriched in the catoGFP expressing cells previously described (Gallone 2012, zur Lage & Jarman, unpublished). This network was then divided into a number of clusters of putatively interacting proteins. One such network cluster was highly enriched in genes that are regulated by Fd3F, suggesting a role for these genes in cilium motility (Figure 5.1). This network contained CG11253 and TilB, which have been previously described in Chapters 3 & 4. It also contained CG14905, the Drosophila homologue of ODA1 and CG16984, a homologue of Enkur (Enkurin). ODA1 is a ODA-DC component, mutation of which results in a loss of ODA from Chlamydomonas flagella (Takada et al 2002). ENKUR is a calmodulin-binding protein that also interacts with TRPC channels (Sutton et al 2004). It has been found to co-localise with TRPC channels in the mouse sperm, flagella (Sutton et al 2004).

CG31320 was a further member of this network, which was not previously linked to cilia. It was highly enriched in developing chordotonal neurons. The microarray analysis previously described found CG31320 to be 7.06 times enriched in cells expressing catoGFP at time point 4 (zur Lage & Jarman Unpublished) (Table 5.1). Its position within the network predicted by G.Gallone highlighted it for further investigation as it lay between CG11253/TilB and the rest of the cilia-linked genes in the network (Gallone 2012).

5.1.2 Transcriptional regulation of CG31320

Work by fellow lab members F. Newton and P.zur Lage on the transcriptional co-regulation of genes by RFX and Fd3F has also suggested that CG31320 has a ciliary function. F. Newton has previously shown by RNA in situ hybridisation that CG31320 expression is reduced in fd3F1 embryos (Newton et al 2012). P. zur Lage has shown that mRNA expression is reduced to an undetectable level in Rfx49 embryos (Newton et al 2012). Additionally the 5’ UTR of CG31320 contains well
Figure 5.1: Putative protein-protein interaction network containing CG31320.
This diagram shows part of a putative protein-protein interaction network containing a number of proteins found to be enriched in catoGFP expressing cells, including CG31320 (diagram and network by G Gallone). G Gallone generated this network based on experimental interactions and putative interactions inferred by those of orthologous proteins (Gallone 2012). Black circles indicate enrichment in catoGFP expressing cells at time point 4 (zur Lage & Jarman unpublished). Their experimental interactors are shown in grey and putative interactors as inferred by orthology in red. The network was generated by G Gallone using Bio::Homolog::InterologWalk (Gallone et al 2011). Proteins discussed in the text are labeled by arrows.

Figure 5.2: The location of regulatory elements upstream of CG31320
A diagram showing the location of X boxes (green) and forkhead binding sites (purple) in the 5'UTR and upstream regions of CG31320. The motif locations described by Newton et al (2012) were combined with those found using GenePallete to search for the consensus sequences of the forkhead binding site (RYMAAYA, Newton et al 2012) and X box (RYYNY{1,3}RNRAC, Laurençon et al 2007).
### Table 5.1 The enrichment of CG31320 mRNA in \textit{ato/catoGFP} expressing cells

<table>
<thead>
<tr>
<th>Time point</th>
<th>Embryonic stage</th>
<th>Enrichment in cells expressing \textit{atoGFP}</th>
<th>Enrichment in cells expressing \textit{catoGFP}</th>
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<tr>
<td>T2 (6 45 to 7 45 Hrs)</td>
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<td>-</td>
<td>2.49</td>
</tr>
<tr>
<td>T3 (7 45 to 8 45 Hrs)</td>
<td>12</td>
<td>2.77</td>
<td>4.57</td>
</tr>
<tr>
<td>T4 (8 45 to 9 45 Hrs)</td>
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<td>n/a</td>
<td>7.06</td>
</tr>
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</table>

Table 5.1. Enrichment levels of CG31320 in \textit{atoGFP} (Cachero et al 2011) and \textit{catoGFP} (zur Lage & Jarman unpublished) expressing cells during embryonic development.
conserved X boxes and Forkhead binding sites (Figure 5.2). These findings show that RFX and Fd3F co-regulate the expression of CG31320, as they have been found to do for a number of cilium motility genes (Newton et al 2012). This further suggests that CG31320 may have a role in the function of the cilium.

5.1.3 CG31320 expression can be knocked down by RNAi

During the course of this investigation flies in which CG31320 was knocked down by RNAi (UAS-Dcr-2/+; CG31320^KK102625/Sca-GAL4) were screened in a climbing assay (described in Chapter 2). Expression of the CG31320^KK102625 RNAi construct resulted in reduced climbing capability. A subsequent in situ hybridisation carried out by P. zur Lage confirmed that expression of this RNAi construct (CG31320^KK102625) knocked down CG31320 expression (Figure 5.3). CG31320^KK102625/Sca-Gal4 (UAS-Dcr-2/+; CG31320^KK102625/Sca-GAL4) embryos showed a reduction in CG31320 expression compared to the control (UAS-Dcr-2/+; P{attP}/Sca-GAL4) (Figure 5.3), showing that CG31320 is knocked down by the RNAi construct (work by P. zur Lage, Diggle et al 2014). This indicated that the CG31320^KK102625 line could be used to knock down CG31320 expression and therefore could be used to investigate CG31320 function.

5.1.4 Protein structure of CG31320

CG31320 contains an ARM (Armadillo)-type fold super family domain across most of its length (Figure 5.4). HEATR2, the human homologue of CG31320 (24% identity, 42% similarity) also contains such a domain. These domains are made up of a number of repeats such as ARM or HEAT repeats. 11 HEAT repeats have been identified in HEATR2 using Pfam (Punta et al 2012), Interpro (Quevillon et al 2005) and MyHits Motifscan (Pagni et al 2007). Only 5 HEAT repeats have been found in CG31320, however I have identified a number of repeats that could represent divergent HEAT repeats (HHrepID – Biegert & Soding 2008). Both CG31320 and HEATR2 have been predicted to form α-solenoid secondary structure with numerous helices (αrd2, Fournier et al 2013).

Proteins containing HEAT repeats are thought to play a role in protein-protein interactions (Andrade et al 2001a, Andrade et al 2001b). They are named after four
Figure 5.3: **CG31320 mRNA expression is reduced in CG31320KK102625 embryos.** In situ hybridisation carried out by P zur Lage showing that CG31320 mRNA expression is reduced in CG31320KK102625/Sca-GAL4 flies (B) compared to control P(attP)/Sca-GAL4 flies (A). Scale bars – 100µm.

Figure 5.4: **The protein structure of CG31320 and HEATR2.** Both HEATR2 and CG31320 contain an ARM-like fold domain (Blue). 11 HEAT repeats (Pink) can be found in HEATR2 and 5 in CG31320 (Pfam, Uniprot, Motif Scan). Further repeats (Green) have been identified in CG31320 which may represent divergent HEAT repeats (HHrepID).
of the genes known to contain HEAT repeats: Huntingtin, Elongation factor 3, Protein phosphatase 2A and TOR1 (Andrade & Bork 1995).

CG31320 is implicated in ciliary function by its enrichment in ciliated cells (Cachero et al 2011, zur Lage & Jarman unpublished), its transcriptional regulation by Fd3F and RFX (Newton et al 2012) and the putative interactions of CG31320 with cilia-linked proteins (Gallone 2012). In order to examine whether CG31320 has a role in ciliary motility I investigated whether it shared any of the other characteristics that initially implicated CG11253 in cilium motility: expression in tissues with motile cilia and conservation in organisms with motile cilia. CG31320 was then further investigated to elucidate whether it had a role in motile cilia function and to understand the nature of that role.

5.2 Results

5.2.1 Embryonic expression pattern

In situ hybridisation was conducted using a CG31320 probe in order to further investigate the expression pattern of CG31320 (Figure 5.5). CG31320 mRNA is expressed in chordotonal neurons from stage 12 until stage 17 (Figure 5.5 A-C). Chordotonal neuron expression of CG31320 at these stages of embryogenesis is consistent with a role in chordotonal neuron differentiation. CG31320 mRNA is expressed in the lch5, v’ch1 and vchA & B neurons (Figure 5.5D). At stage 12 and 13 expression can also be detected in the mesoderm (Figure 5.5A & B). The mesodermal expression pattern shows that CG31320 could also play a role in non-ciliated cell types.

5.2.2 Evolutionary distribution of CG31320 homologues

In Chapter 3 I demonstrated that CG11253 was conserved in organisms with motile cilia. This has also been shown to be the case for tilB and a number of axonemal dynein arm components (Wickstead & Gull 2007, Kavlie et al 2010). I used reciprocal DELTA-BLAST of CG31320 protein sequence to identify homologues of CG31320 in a number of organisms. I searched a number of different genera that either contain motile, non-motile or no cilia (Figure 5.6, Table 5.2). CG31320
Figure 5.5. The expression pattern of CG31320.

In situ hybridisation has shown that CG31320 is expressed from stage 12 to stage 17 in Drosophila embryos. 

(A) At early stage 12 expression of CG31320 mRNA can be detected in the mesoderm, this reduces after stage 13.

(B) CG31320 mRNA is detected in the chordotonal neurons of a stage 13 embryo.

(C) CG31320 mRNA expression in the chordotonal neurons of a stage 16 embryo.

(D) Expression can be detected in the Ich5, v’ch1 and vchA/B neurons (stage 15).

Scale bars: A-C - 100µm, D - 50µm
The conservation of homologues of CG31320 in a variety of organisms with and without cilia. Homologues of CG31320 are found in most organisms with motile cilia but not in those with immotile or no cilia. Homologues detected by best reciprocal DELTA-BLAST. Presence of CG31320 homologue indicated by a black circle, HEATR2 homologue but not CG31320 homologue indicated by a grey, dashed circle and a white circle shows no homologue could be detected. Figure based on similar homologue conservation tree for TilB from Kavlie et al (2010).
### Table 5.2: Homologues of CG31320

<table>
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<th>Genus</th>
<th>Accession number/identifier of best match</th>
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<th>Identity</th>
</tr>
</thead>
<tbody>
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<td><em>Homo sapiens</em></td>
<td>NP_060272.3</td>
<td>HEATR2</td>
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<td><em>Mus musculus</em></td>
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<td>HEATR2</td>
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<td>heatr2</td>
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<td>EKF30439.1</td>
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</tbody>
</table>

Table 5.2: Homologues of CG31320 found and identity calculated by best reciprocal DELTA-BLAST.
homologues were found in most organisms with motile cilia and not in those with only non-motile cilia or those lacking cilia. No homologues were found in *Plasmodium* or *Giardia* when searching with CG31320 despite the presence of motile cilia on organisms of these genera. However homologues were found when searching with HEATR2 protein sequence. This suggests that a homologue was present however one of the genes could be more diverged from the others.

### 5.2.3 CG31320 is a candidate ciliary motility gene

CG31320 was expressed in motile ciliated cells and not in cells with a non-motile cilium (although it was expressed in cells without cilia altogether). CG31320 was transcriptionally regulated by Fd3F (Newton et al 2012) and was conserved in organisms with motile cilia. CG31320 fulfilled several of the criteria used to identify CG11253 as a cilium motility gene and was therefore implicated in cilium motility itself. The next step was to investigate if phenotypes consistent with a lack of cilium motility machinery function were displayed in CG31320 mutants.

### 5.2.4 CG31320*EY06677* shows no proprioceptive defect

CG31320*EY06677* contains a P{EPgy2} transposon inserted in close proximity to the 5’ end of the 5’ UTR of CG31320 (Figure 5.7A). Whilst this insertion is not within the transcriptional unit it was thought that it might disrupt gene expression. Homozygous and heterozygous CG31320*EY06677* flies were assayed for proprioceptive defects in order to assess whether this line can be used to further analyse the role of CG31320. The average climbing score for four tubes of 17-20 flies was calculated, however no significant difference between heterozygotes and homozygotes was observed. This suggests that either gene function and expression were not affected or that CG31320 is not required for chordotonal function, contrasting with the results for CG31320*KK102625* (Figure 5.7B).

### 5.2.5 Generation of CG31320 deletion mutant

In order to be able to analyse the phenotype caused by mutating CG31320 I generated CG31320 deletion mutants. I used the same crossing scheme previously described for CG11253*EY10866* to mobilise the CG31320*EY06677* transposon (Figure
**Figure 5.7 : P{EPgy2}CG31320EY06677 P element insertion**

(A) A P{EPgy2} transposon (P{EPgy2}CG31320EY06677) is inserted upstream of the CG31320 5’ UTR.

(B) A climbing assay (Method A) showed no significant difference between the climbing abilities of homozygous and heterozygous CG31320EY06677 flies. 17-20 flies were screened in each assay (N=4). An average climbing score was calculated from each repeat. P value – 0.8286 (ns), calculated by Mann-Whitney U test.
5.8A). Stocks were established from flies in which the P element had excised. These were then screened for deletions occurring by imprecise excision using PCR primers at varying intervals from the P element site to amplify fragments from single-fly PCR DNA samples. Two stocks were found with such a deletion: \textit{CG31320}^{27}, which has a 992bp deletion, and \textit{CG31320}^{233}, which had a 759bp deletion (Figure 5.8B). Deletion size was calculated in collaboration with P.zur Lage (D. Moore – single-fly PCR and interpretation of results, P. zur Lage – sequencing reaction). Both of these deletions encompassed the transcriptional and translational start site for \textit{CG31320}. In situ hybridisation revealed that expression of \textit{CG31320} is reduced to an unobservable level in \textit{CG31320}^{27} embryos (Figure 5.9, Table 5.3).

### 5.2.6 \textit{CG31320}^{27} and \textit{CG31320}^{233} complementation analysis

\textit{CG31320}^{27} and \textit{CG31320}^{233} were both found to be lethal at an embryonic or early larval stage. Very few third instar larvae were detected in \textit{CG31320}^{27} and \textit{CG31320}^{233} fly stocks. Both lines were out crossed (crossed to wild type flies) in order to remove any deleterious background, however the lethality still remained. The lethality observed in these lines was not due to a lack of chordotonal organ function as \textit{atonal}^1 mutants (which lack most chordotonal neurons) still survive to adulthood (Jarman et al 1995). The lethality could be caused by the loss of \textit{CG31320} mesodermal expression. Alternatively \textit{CG31320} is located within another gene: \textit{NK7.1} (Figure 5.10). It could be that \textit{CG31320}^{27} and \textit{CG31320}^{233} also disrupt \textit{NK7.1} function.

In order to investigate whether this was the case I utilised two deficiency lines: \textit{Df(3R)Exel6267} in which a large deletion encompasses both \textit{CG31320} and \textit{NK7.1}, and \textit{Df(3R)BSC487} which deletes the translational start site of \textit{NK7.1} but does not delete \textit{CG31320} (Figure 5.10B). The deficiency lines were crossed to the deletion lines I had generated to investigate whether they would complement one another. Whilst \textit{Df(3R)Exel6267} did not complement the mutations, \textit{Df(3R)BSC487} did (Table 5.4). This shows that the lethality is caused by loss of \textit{CG31320} and not \textit{NK7.1} as the deletion in \textit{Df(3R)BSC487} encompasses the translation start site of \textit{NK7.1} but not \textit{CG31320}. A caveat of this experiment is that \textit{NK7.1} expression was not assessed by in situ hybridisation, however the scope of the deficiency deletion
Figure 5.8 The generation of CG31320 deletion mutants.

(A) The crossing scheme used to mobilise the P\{EPgy2\}CG31320\textsuperscript{FY06677} P element and generate deletion mutants

(B) A diagram showing the sizes of two deletions generated using this crossing scheme, both of which delete the transcriptional and translational start sites of CG31320 (Deletion size calculated in collaboration with P zur Lage)
Table 5.3 Reduction of CG31320 mRNA expression in CG31320²⁷ embryos

<table>
<thead>
<tr>
<th></th>
<th>CG31320 expression</th>
<th>No CG31320 expression detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG31320²⁷</td>
<td>57</td>
<td>22</td>
</tr>
<tr>
<td>Wild type control</td>
<td>58</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5.3 A cross of CG31320²⁷/TM3 Sb flies produced statistically more progeny without CG31320 expression than a cross of wild type flies. Embryos at stages 13 and 14 were counted. P value = <0.0001 (Fishers exact test).

Figure 5.9: CG31320²⁷ deletion results in loss of CG31320 mRNA expression.  
(A) In situ hybridisation shows the CG31320 mRNA expression pattern in a wild type stage 13 embryo.  
(B) CG31320 mRNA cannot be detected in CG31320²⁷ stage 13 embryos, demonstrating a loss of expression. Scale bars:100µm
**Figure 5.10 : Map of deficiency lines used for complementation.**

(A) *Df(3R)BSC487* and *Df(3R)Exel6267* are both deletions on the third chromosome. This diagram shows the full extent of these deletions.

(B) The position of the deficiency line deletions compared to *CG31320* is shown in this diagram. Whilst *Df(3R)Exel6267* encompasses both *CG31320* and *NK7.1*, *Df(3R)BSC487* deletes entire ORF of *NK7.1* but not *CG31320*. A & B based on FlyBase (St Pierre et al 2014).

**Table 5.4: Complementation of deficiency lines and CG31320 deletions**

<table>
<thead>
<tr>
<th>Progeny genotype:</th>
<th><em>Df(3R)Exel6267</em> (NK7.1 and CG31320)</th>
<th><em>Df(3R)BSC487</em> (NK7.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Df/deletion</td>
<td>Df/balancer or deletion/ balancer</td>
</tr>
<tr>
<td><em>CG31320^27</em></td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td><em>CG31320^233</em></td>
<td>0</td>
<td>73</td>
</tr>
</tbody>
</table>

Table 5.4. Heterozygous *Df(3R)BSC487* and *Df(3R)Exel6267* flies were crossed to heterozygous *CG31320^27* and *CG31320^233* flies. The phenotypes of the progeny were recorded in order to identify if the deficiency lines could complement the deletions. As all four stocks were balanced, complementation was identified by a lack of balancer marker. *Df(3R)BSC487* complemented both deletion lines, whilst *Df(3R)Exel6267* did not.
should include the entire *NK7.1* open reading frame leaving only a portion of the 5’UTR.

This data shows that just *CG31320* and not *NK7.1* is affected by the deletions. However the deletion lines cannot be used to assess the function of chordotonal cilia and sperm flagella in adult flies. Additionally I would not be able to ascertain whether any defects that occurred in larval behaviour were due to chordotonal *CG31320* reduction or by the same mechanism that causes reduced viability. An alternative approach was needed in order to investigate whether ciliary or flagella function was impaired.

**5.2.7 *CG31320*\(^{KK102625}\) climbing assay**

*CG31320*\(^{KK102625}/\text{Sca-Gal4}\) had previously been shown to have reduced climbing capability in a climbing assay screen of ciliary candidate genes (Chapter 2). A further climbing assay of *CG31320*\(^{KK102625}\) (UAS-Dcr-2/+; *CG31320*\(^{KK102625}/\text{Sca-GAL4}\)) and control (UAS-Dcr-2/+; P\(^{attP}\)/Sca-GAL4) flies alone was used to confirm the effects of knocking down *CG31320* (Figure 5.11). Although direct evidence of a knock down of *CG31320* in adult *CG31320*\(^{KK102625}\) has not been obtained, the reduction in expression in embryonic chordotonal neurons previously identified by P. zur Lage implies that a reduction in adult chordotonal neurons would also occur. *CG31320*\(^{KK102625}/\text{Sca-Gal4}\) flies had a significantly poorer climbing performance than the control confirming that knock down of *CG31320* results in a proprioceptive defect.

**5.2.8 *CG31320*\(^{KK102625}; \text{Bam-GAL4}\) male flies are infertile**

In addition to the chordotonal neurons, motile cilia are also present on sperm cells. To investigate whether *CG31320* knock down disrupted ciliary function in these cells a different GAL4 driver was required. Bag-of-marbles (Bam)-GAL4-VP16\(^\dagger\) drives expression in late spermatogonia and early spermatocytes (White-Cooper 2012). Although this expression pattern is early in sperm development cillum

\(^\dagger\) The VP16 element is an additional attachment isolated from herpes simplex virus that increases transcription activation activity (Sadowski et al 1988)
Figure 5.11: CG31320\textsuperscript{KK102625} flies show reduced climbing capability. A climbing assay (Method B) shows a reduction in climbing capability of CG31320\textsuperscript{KK102625}/Sca-GAL4 flies (N=64) compared to control \textit{P(attP)/Sca-GAL4} flies (N=43). The heights climbed by flies of each genotype were calculated using ImageJ. (P value - <0.0001 Mann Whitney U Test)
motility factors have also been found that are expressed at this stage: CG11253 and CG9313 expression begins in the primary spermatocytes (Zhao et al 2009 (Fly TED)). It would appear that as in chordotonal neurons, cilium associated genes can be expressed before flagellum formation (Cachero et al 2011).

A fertility assay showed that whilst CG31320\textsuperscript{KK102625}, Bam-GAL4 male flies (CG31320\textsuperscript{KK102625}/+; Bam-GAL4-VP16/+\textsuperscript{2}) were observed mating they did not produce progeny (Table 5.5). This contrasts with control males (P\textsuperscript{attP}/+; Bam-GAL4/+\textsuperscript{2}) which did produce progeny.

5.2.9 CG31320\textsuperscript{KK102625} testes physiology

In order to investigate the cause of CG31320\textsuperscript{KK102625}, Bam-GAL4 male infertility testes of two-day-old males were dissected. CG31320\textsuperscript{KK102625}, Bam-GAL4 testes displayed abnormal sperm bundle bending and a higher rate of accumulation of individualised sperm at the terminal epithelium (Table 5.6, Figure 5.12). The seminal vesicles of these flies appeared empty and no motile sperm was observed following dissection or testes squash (Figure 5.12).

CG31320\textsuperscript{KK102625} displays defects in both sperm and chordotonal neuron function (as shown by infertility and climbing assay respectively). The phenotypes observed are similar to those previously observed for CG11253 suggesting that CG31320 might also have a role in cillum motility.

5.2.10 CG31320\textsuperscript{KK102625} neuronal and ciliary morphology

Knocking down CG31320 causes defects in the two motile ciliated cell types in Drosophila. To investigate the causes of these defects I used immunohistochemistry to observe chordotonal neuron and ciliary structure. No defects were observed in neuronal structure in CG31320\textsuperscript{KK102625}/Sca-Gal4 larval pelt chordotonal neurons (Figure 5.13A & B). NompC antibody showed that the cilia are present and that the distal ciliary compartment is normally formed. These results were also replicated in the chordotonal neurons of CG31320\textsuperscript{KK102625}/Sca-Gal4 pupal antennae (Figure 5.13C & D). These findings showed that the behavioural defect previously observed for

\textsuperscript{2} Note Dcr-2 is not present
Table 5.5 Fertility assay of male CG31320KK102625 flies

<table>
<thead>
<tr>
<th></th>
<th>% of crosses which produced progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(attP); BAM-GAL4-VP16</td>
<td>100% (n=16)</td>
</tr>
<tr>
<td>CG31320KK102625; BAM-GAL4-VP16</td>
<td>0% (n=20)</td>
</tr>
</tbody>
</table>

Table 5.5 CG31320KK102625 and P(attP) male flies were allowed to mate with wild type female flies for five days. It was recorded whether these crosses produced any progeny.

Table 5.6 Testes physiology of CG31320KK102625 flies

<table>
<thead>
<tr>
<th></th>
<th>Sperm bundle disruption</th>
<th>Empty/Small Seminal Vesicle</th>
<th>Accumulation of individualised sperm</th>
<th>Motile sperm detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG31320KK102625; BAM-GAL4-VP16</td>
<td>23 (n=23)</td>
<td>23 (n=23)</td>
<td>19 (n=22)</td>
<td>0 (n=23)</td>
</tr>
<tr>
<td>P(attP); BAM-GAL4-VP16</td>
<td>1 (n=25)</td>
<td>0 (n=23)</td>
<td>0 (n=22)</td>
<td>23 (n=23)</td>
</tr>
</tbody>
</table>

Table 5.6 The number of fly testes observed that showed defective phenotypes. Sperm bundle disruption, small seminal vesicles and high levels of individualised sperm in testis dissection of five day old P(attP); BAM-GAL4-VP16 and CG31320KK102625; BAM-GAL4-VP16 flies was recorded. The number of testes in which motile sperm could be observed after testes squash is also shown. P value: < 0.0001 (Fishers exact test).
Figure 5.12: Testes physiology of five day old CG31320\textsuperscript{KK102625} flies.
The testes of 5 day old CG31320\textsuperscript{KK102625}; BAM-GAL4-VP16 and \textit{P(attP); BAM-GAL4-VP16} control flies were dissected.

(A and B) A schematic showing the phenotypes of control (A) and knock down (CG31320\textsuperscript{KK102625}; BAM-GAL4-VP16) (B) testes.

(C) Control testis showing a full seminal vesicle and normal amount of individualised sperm.

(D) CG31320\textsuperscript{KK102625}; BAM-GAL4-VP16 testis with an empty seminal vesicle and an accumulation of individualised sperm at the terminal epithelium.

(E) Motile sperm are released from a dissected control seminal vesicle.

(F) No motile sperm are observed following dissection of CG31320\textsuperscript{KK102625}; BAM-GAL4-VP16 seminal vesicle.
Figure 5.13: Morphology of CG31320KK102625 chordotonal neurons.

(A & B) Third instar larval pelt chordotonal neurons.
No difference was seen between control P{attP}/Sca-GAL4 (A) and CG31320KK102625/Sca-GAL4 (B) larvae. This indicates that the neurons and cilia formation and ciliary compartmentalisation are occurring normally. Anti-HRP stains the dendrite and luminal band (green) and NompC labels the distal ciliary compartment (magenta).

(C & D) Chordotonal neurons from the pupal Johnston’s organ.
No difference is observed between CG31320KK102625/Sca-GAL4 (D) and P{attP}/Sca-GAL4 (C) pupae. Anti-HRP labels the cell body and proximal cilium (green) and NompC labels the distal cilium.

(E) A diagram showing the localisation of anti-HRP and NompC stains in larval and pupal chordotonal neurons.
CG31320^KK102625/Sca-Gal4 was not as a result of gross structural neuronal or ciliary defects.

5.2.11 CG31320^KK102625 axonemal ultrastructure

TEM of adult Johnston’s organ ciliary ultrastructure was carried out in order to investigate whether CG31320^KK102625/Sca-Gal4 flies had ultrastructural defects that could not be identified by IHC. Both inner and outer dynein arms are lost from CG31320^KK102625/Sca-Gal4 cilia (Figure 5.14, Table 5.7). This is significantly different from control fly cilia (Figure 5.14). Dynein arm loss appears to be greater in CG31320^KK102625/Sca-Gal4 (ODA – 88.9% missing, IDA – 96.9% missing) than in CG11253^EY10886 (ODA – 56.0% missing, IDA – 77.8% missing) cilia. CG31320^KK102625/Sca-Gal4 axonemes also appeared to be misshapen with the microtubules doublets forming an oval shape rather than that of a regular circle (Figure 5.14). This could suggest a loss of further axonemal components that are required for maintaining axonemal shape such as the nexin links.

Electron microscopy has shown that CG31320 is required for the proper localisation or formation of the axonemal dynein arms. The observed behavioural and fertility defect is likely to be as a result of a lack of ciliary motility. This confirms that CG31320 is a gene required for the function or formation of the cilium motility machinery.

4.2.12 CG31320::mVenus transgene generation

To investigate the role of CG31320 further a CG31320::mVenus fusion protein was generated using the same techniques used to generate CG11253::mVenus and CG6971::mVenus. The proposed binding sites for Fd3F and RFX are located within the 5’UTR, as a result only a small region upstream of the transcriptional start site was incorporated in the construct (Figure 5.15). A fragment containing this upstream region and CG31320 was amplified and tagged with attB flanking regions by PCR. It was then incorporated into a Pdonr221 donor vector be recombination between the attB regions and attP sequences of the vector (catalysed by integrase). This forms attL sites either side of the CG31320 sequence that can then be recombined with attR sites in a pBID-UASC-GV destination vector (catalysed by integrase and
Figure 5.14. Axonemal dynein arms are lost from \textit{CG31320^{KK102625}} antennal cilia.

(A) Electron microscopy of axonemal cross sections from adult \textit{P(attP)/Sca-GAL4} Johnston's organ cilia. ODA and IDA are labeled by red arrows.

(B) Electron microscopy of axonemal cross sections from adult \textit{CG31320^{KK102625}/Sca-GAL4} Johnston's organ cilia. ODA and IDA are lost from many microtubule doublets (labeled with white arrows). The axoneme also appear to be misshapen. Scale bars - 100nm. Tissue fixed by A Jarman as per Newton et al (2012), TEM conducted by EM Research Services, Newcastle University Medical School.

| Table 5.7  Axonemal dynein arm loss from \textit{CG31320^{KK102625}/Sca-GAL4} cilia |
|-----------------|-----------------|-----------------|
| Outer Dynein Arms visible | \textit{P(attP)/Sca-GAL4} (N=45 microtubule doublets) | \textit{CG31320^{KK102625}/Sca-GAL4} (N = 63 microtubule doublets) |
| Inner Dynein Arms visible | 42 | 2 |

Table 5.7 The number of microtubule doublets for which the axonemal dynein arms were visible in \textit{P(attP)/Sca-GAL4} and \textit{CG31320^{KK102625}/Sca-GAL4} as observed in images obtained by TEM. P value - <0.0001 (Fisher’s exact test).
Figure 5.15: CG31320::mVenus fusion gene.

(A) A vector map of the pBID-UASC-CG31320::mVenus vector which was injected into blastoderm stage embryos. Vector map generated using Serial Cloner 2.6.1.

(B) A diagram of the CG31320::mVenus insert which was incorporated into the genome of those flies. The CG31320::mVenus fusion gene was designed to include the upstream region of CG31320 to allow expression from its own promoter.
exisionase). The resulting vector (Figure 5.15) was then injected into blastoderm stage embryos.

### 5.2.13 CG31320::mVenus localisation

Examination of \textit{CG31320::mVenus} embryos revealed that CG31320::mVenus localises to the chordotonal neurons from stage 12 (Figure 5.16). This confirms that the regulatory sequences for chordotonal expression were contained in the 5'UTR. CG31320::mVenus can be seen in the lch5, v'ch1 and vchA &B neurons (Figure 5.16A). As with CG11253::mVenus, CG31320::mVenus was observed in non-ciliated v'td1 and 2 neurons, however it was at much lower levels than chordotonal neurons (Figure 5.16A). This contrasts the expression pattern seen in RNA in situ hybridisation where expression in the v’td neurons was not observed. As these neurons derive from ventral SOPs CG31320::mVenus localisation could be due to a stabilising effect of the mVenus tag allowing the protein to persist where it is normally degraded. Alternatively the upstream region amplified may not contain the sequences necessary to suppress expression in the v’td neurons. CG31320::mVenus was also detected in the scolopale support cells, however this was at relatively low levels. CG31320::mVenus could not be detected in the mesoderm in high levels (Figure 5.16C & D). The transcription factor binding motifs required for mesodermal expression may be outwith the upstream region amplified with \textit{CG31320}.

The subcellular localisation of CG31320::mVenus was also observed. CG31320::mVenus is cytoplasmically located and was not detected in the cilium (Figure 5.16B). CG31320::mVenus expression in the scolopale was at low levels allowing the absence of ciliary localisation to be observed. This could more easily be detected for CG31320::mVenus than had been the case for CG11253::mVenus, which was expressed strongly in the scolopale cell, making detection of ciliary localisation difficult. As with v’td localisation, scolopale expression of CG31320::mVenus could be due to abnormal protein stabilisation by the mVenus tag. CG31320::mVenus localisation was diffuse within the cytoplasm with some granular localisation (Figure 5.16B).
Figure 5.16: CG31320::mVenus embryonic localisation.

A) Abdominal sections of a stage 17 embryo showing that CG31320::mVenus (green) localises to the Ich5, v’ch1 and vchA&B chordotonal neurons and the v’td multidendritic neuron. Neuronal nuclei are labeled with ELAV (magenta).

B) Ich5 neuron cluster from the abdominal segment of a stage 15 embryo. CG31320::mVenus (green) is cytoplasmically localised and does not enter the cilium. The neurons are labelled with Futsch (magenta).

C & D) Early (C) and late (D) stage 12 embryos. CG31320::mVenus (green) can be detected in developing chordotonal organs but not in the mesoderm.

Scale bars – A & B - 10µm , C & D - 50µm.
CG31320::mVenus is also present is fully differentiated larval pelt chordotonal neurons. As in embryos localisation remains mainly cytoplasmic, with some localisation in the dendrite (Figure 5.17). Some puncta within the cytoplasm and dendrite are visible. This is not seen when using the same anti-GFP antibody to label CG11253::mVenus and CG6971::mVenus, so is unlikely to be an artifact of the antibody. As in embryonic chordotonal neurons, CG31320::mVenus could not be observed in the cilium. CG31320::mVenus can also be seen to localise to the developing Johnston’s Organ (Figure 5.18A). As at other developmental stages it remains mainly cytoplasmic and no clear staining was observed in the cilium (Figure 5.18C).

CG31320 is therefore a cytoplasmic factor required for axonemal dynein arm assembly or localisation, which is present in both developing (embryonic and pupal) and mature (larval) neurons.

5.2.14 CG31320::mVenus and CG6971::mVenus rescue CG31320^{KK102625}

The CG31320::mVenus construct was recombined with CG31320^{KK102625} to attempt to rescue the behavioural defects previously observed and show that they are caused by loss of CG31320. This would also show that the localisation observed for CG31320::mVenus is that of functional protein. It was not known whether such an attempt would be successful as RNAi would also target CG31320::mVenus. However as well as the CG31320 promoter, UAS was present upstream of CG31320::mVenus allowing expression to be driven by GAL4. This could increase the levels of CG31320::mVenus being expressed and potentially allow it to escape RNAi-mediated knock down.

A climbing assay of CG31320::mVenus, CG31320^{KK102625}/Sca-GAL4 and CG31320^{KK102625}/Sca-GAL4 flies climbed statistically higher than CG31320^{KK102625}/Sca-GAL4 (P value - <0.0001 Kruskall-Wallis test & Dunn’s multiple comparisons test). This suggested that CG31320::mVenus could rescue the climbing defect caused by knocking down CG31320. However an additional CG6971::mVenus control indicated that this perhaps did not represent the rescue of
Figure 5.17. CG31320::mVenus localisation in larval pelt chordotonal neurons.

(A & D) Third instar CG31320::mVenus larvae were dissected and the localisation of CG31320::mVenus (green) in the larval pelt chordotonal neurons observed. The chordotonal neurons are stained with a Futsch antibody and the cilium marked with an antibody for Polyglutamylated tubulin (magenta). CG31320::mVenus localises mainly to the cytoplasm and the dendrite. Puncta of localisation can be observed in the dendrite.

(B & E) Futsch and Polyglutamylated tubulin only.

(C & F) CG31320::mVenus only

Scale bars : 10μm
Figure 5.18: Localisation of CG31320::mVenus in pupal antennae.

(A) CG31320::mVenus localisation in the pupal antennae. CG31320::mVenus (green) can be seen to localise in the neurons of the Johnston’s organ (JO) but not the olfactory sensory neurons (OSN). Neurons marked with Futsch antibody and the proximal ciliary compartment with polyglutamylated tubulin (magenta).

(B) A diagram showing the localisation of CG31320::mVenus and antibody stains in pupal chordotonal neurons.

(C) CG31320::mVenus (green) localises to the cytoplasm of Johnston’s organ chordotonal neurons. CG31320::mVenus is not observed in the cilium. Neurons are marked with Futsch antibody and the proximal ciliary compartment with polyglutamylated tubulin (magenta).

Scale bars – A - 50µm, C - 10µm.
A climbing assay (Method B) which showed that CG31320::mVenus, CG31320\(^{KK102625}/\text{Sca-GAL4}\) (N=39) and CG6971::mVenus, CG31320\(^{KK102625}/\text{Sca-GAL4}\) (N=37) could climb statistically higher than CG31320\(^{KK102625}/\text{Sca-GAL4}\) flies (N=36). This shows that both mVenus constructs can rescue the climbing defect caused by knocking down CG31320.

P value – <0.0001 (calculated using Kruskall-Wallis test & Dunn’s multiple comparisons test).

**Figure 5.19**: CG31320::mVenus and CG6971::mVenus rescue CG31320\(^{KK102625}/\text{Sca-GAL4}\).
that it at first appeared to. \textit{CG6971::mVenus} was recombined with \textit{CG31320KK102625} in order to better understand the role of \textit{CG31320} in dynein arm assembly. The climbing capability of \textit{CG6971::mVenus}, \textit{CG31320KK102625/Sca-GAL4} was also assessed and proved to be statistically better climbing than \textit{CG31320KK102625/Sca-GAL4} (Figure 5.19).

It was possible that both constructs rescued the effect of knocking down \textit{CG31320}. \textit{CG31320::mVenus} by lessening the level of the knock down and \textit{CG6971::mVenus} by another previously unknown mechanism. However another possibility is that the apparent rescue was due to a reduction in the expression of \textit{UAS-hpRNA}. The additional UAS sequences associated with the mVenus fusion proteins could have caused a relative reduction in activation of expression by GAL4. This may account for the reduction in RNAi-mediated knock down. This could be tested by trying to rescue \textit{CG31320KK102625} using mVenus alone being driven from the UAS sites or by removing the UAS sites altogether from the \textit{CG31320::mVenus} rescue construct.

5.2.15 \textit{CG6971::mVenus} localisation in \textit{CG31320}\textsuperscript{27} larval chordotonal neurons

\textit{CG31320}\textsuperscript{27} flies are not viable, however some third instar \textit{CG31320}\textsuperscript{27} larvae can be obtained from the stock for investigation. \textit{CG6971::mVenus} was crossed into \textit{CG31320}\textsuperscript{27} to investigate the role of \textit{CG31320} in axonemal dynein arm formation (Figure 5.20A). \textit{CG6971::mVenus} localised to the proximal ciliary compartment in wild type larval chordotonal neurons (Figure 5.20B). However in \textit{CG31320}\textsuperscript{27} larvae \textit{CG6971::mVenus} was retained in the dendrites but not localised to the cilium (Figure 5.20C). This confirms the axonemal dynein loss of \textit{CG31320KK102625} cilia and further supports the hypothesis that \textit{CG31320} is a factor required for dynein arm assembly.

5.3 Discussion

\textit{CG31320} was found to be a conserved factor required for correct formation of the axonemal dynein arms. \textit{CG31320} was predicted to be involved in cilium motility by its conservation, expression pattern and its transcriptional regulators. The loss of dynein arms observed by TEM and the change in \textit{CG6971::mVenus} localisation
Figure 5.20: CG6971::mVenus localisation in CG31320\textsuperscript{27} larval pelt chordotonal neurons.

(A) The crossing scheme used to cross CG6971::mVenus into CG31320\textsuperscript{27}.

(B) CG6971::mVenus localisation in wild type larval pelt chordotonal neurons. CG6971::mVenus is present in both the proximal cilium and in the dendrite (labeled by arrows). Neurons are marked by Futsch antibody (magenta).

(C) CG6971::mVenus localisation in CG31320\textsuperscript{27} larval pelt chordotonal neurons. CG6971::mVenus is lost from the proximal cilium but remains in the dendrite (labeled by arrows). Neurons are marked by Futsch antibody (magenta).

Scale bars: 10µm
shows that CG31320 is required for the formation of components of the cilium motility machinery. Although a lack of motility in either chordotonal cilia or sperm flagella was not directly observed it is clear from the lack of dynein arms that the motility machinery is disrupted. This further validates the criteria previously mentioned for identifying new genes involved in cilium motility.

The defects observed in climbing ability and testis morphology for $CG31320^{KK102625}$ (crossed to $Sca$-$GAL4$ and $Bam$-$Gal4$ respectively) were similar to those observed for $CG11253^{EY10866}$. This suggests that these phenotypes are characteristic of mutations affecting the cilium motility machinery and can be used to identify cilium motility genes in the future. This is not to say that these phenotypes would only occur in defects in cilium motility genes but that they are a feature of cilium motility gene mutants in Drosophila.

Electron microscopy of $CG31320^{KK102625}/Sca$-$Gal4$ ciliary ultrastructure and the localisation of CG6971::mVenus in $CG31320^{27}$ have suggested that axonemal dynein arm loss is responsible for the proprioceptive defect observed in the climbing assay. Direct evidence of dynein arm loss from sperm flagella was not obtained however it can be inferred that motility is disrupted from the testis morphology. However as with $CG11253^{EY10866}$ other defects (such as axonemal splitting) may occur in sperm flagella that do not occur in chordotonal cilia. Electron microscopy of flagella axonemes could be carried out to find out whether this is the case.

### 5.3.1 Putative protein-protein interaction network

Part of the evidence linking CG31320 with a ciliary role was its presence in a putative PPI network derived by G.Gallone containing other ciliary proteins including CG11253 (Gallone 2012). The connections that held CG31320 to CG11253 were via interactions of their homologues with homologues of Bun (Bunched). However bun is not differentially expressed across the embryo and mutants do not display climbing assay defects (O’Connell, zur Lage & Jarman – unpublished). This suggests that the interactions inferred by this branch of the network are not occurring in Drosophila. However the interolog PPI network was useful for predicting that $CG31320$ was a ciliary gene. It might therefore be the case
that whilst interolog PPI networks do not show the exact interactome, they can suggest genes that are involved in common processes as their homologues interact in other systems.

5.3.2 HEATR2 is mutated in PCD

As CG31320 is required for the formation of the cilium motility machinery, mutation of its human homologue HEATR2 could potentially cause PCD. An investigation carried out simultaneously with my own by C. Diggle and E. Sheridan discovered that a mutation in HEATR2 was responsible for PCD in a consanguineous family (Diggle et al 2014) (Figure 5.21). Whole genome SNP autozygosity mapping identified a G>C point mutation in a splice site which results in the use of an alternate splice site incurring a 2 base pair deletion at the HEATR2 C-terminus (Figure 5.21) (Diggle et al 2014). The consequent frame shift causes the final 44 amino acids of HEATR2 to be replaced with 77 from the shifted reading frame. The changes in amino acid sequence can be described as pGlu811GlyfsTer78, which indicates that point mutation and alternate splicing causes Glycine to be present at position 881 instead of Glutamic acid (pGlu811Gly) and causes a frame shift (fs) which terminates after 78 amino acid residues (Ter78). Western blot analysis found that HEATR2 protein levels were greatly reduced in family members homozygous for this mutation and halved in those heterozygous (Work carried out by G Mali, Diggle et al 2014).

TEM of nasal respiratory cilia from these patients showed a lack of both inner and outer dyneins (Diggle et al 2014). This phenotype was confirmed by IHC showing a loss of DNAH5 (ODA) and DNALI1 (IDA) from patient nasal cell cilia (work carried out by M Schimidts and H Mitchison, Diggle et al 2014). Mutation of HEATR2 therefore results in similar defects in the cilia of these cells as found when disrupting CG31320 expression in Drosophila chordotonal neurons. This indicates that the role of CG31320 in motile cilia formation is well conserved.

Further evidence for this comes from work by G Mali and P Mill who found that the CG31320 mouse homologue Heatr2 is expressed in the multiciliated ependymal, oviduct and airway epithelium cells of mouse embryos (Diggle et al 2014). G Mali
**Figure 5.21 : HEATR2 is mutated in PCD.**

(A) C Diggle and E Sheridan identified PCD patients with a point mutation in *HEATR2* which causes a 2 base pair deletion due to mis-splicing.

(B) This deletion results in a frame shift mutation with the final 44 amino acids being replaced by a new 77 amino acid C terminus. This disrupts the C terminal of the ARM-type fold and the final HEAT repeat.
and P Mill also found both Heatr2 in multiciliated mouse cells and HEATR2 in human nasal epithelial cells to be cytoplasmically located (Diggle et al 2014). This shows that the cytoplasmic localisation of CG31320 is conserved as well as its role in dynein arm formation. The identification of a mutation in HEATR2 in PCD patients further confirms that homologues of factors required for the formation and function of the ciliary motility machinery in *Drosophila* chordotonal neurons can be considered PCD candidate genes. Taken together these findings show that CG31320 is a conserved cytoplasmic factor required for the formation of the motile ciliary machinery in flies and humans.

### 5.3.3 A further HEATR2 point mutation also causes PCD

During the course of this work a report appeared of another point mutation in HEATR2 linked to PCD (Horani et al 2012). Exome sequencing identified a point mutation resulting in a Leu795Pro amino acid substitution in another family containing individuals with PCD (Horani et al 2012). TEM of nasal epithelial cilia from these patients show ultrastructural defects; lacking ODA and often IDA (Horani et al 2012). However IHC identified that whilst DNAI1 (ODA) was found to be lost from nasal epithelial cells, DNAH7 (IDA) localised normally in nasal epithelia cells from PCD patients (Horani et al 2012). Horani et al (2012) identified further evidence for a lesser role of HEATR2 in IDA formation by RNAi mediated knock down of one of the HEATR2 Chlamydomonas homologues in which ODA were lost from the flagella but IDA were not (Horani et al 2012).

This contrasts with both the findings of C Diggle and E Sheridan and my own data, which suggests that both ODA and IDA are lost by CG31320 or HEATR2 mutation. This may suggest a separation of function for HEATR2 in ODA and IDA formation; with a p.Leu795Pro mutation affecting IDA formation to a lesser extent than ODA formation and p.Glu811Glyfs78 mutation affecting both. However both mutations result in considerable loss of protein making this unlikely (Horani et al 2012, Diggle et al 2014). Additionally Horani et al (2012) reported that most (91.9%) microtubule doublets in pLeu795Pro patient cilia lacked complete IDA suggesting that both mutations in HEATR2 might well have the same level of defect on inner and outer dynein arms. It is therefore likely that in nasal epithelial cilia whilst DNAH7 can
localise normally (as shown be Horani et al (2012)) other IDA components such as DNALI1 cannot (as shown by Diggle et al 2014). Chlamydomonas has two HEATR2 homologues; it could be the case that redundancy of these genes is the cause of IDA not being affected by knock down of only one of these homologues.

5.3.4 A non-ciliary role for CG31320

In situ hybridisation identified CG31320 expression outwith the chordotonal neurons. This expression at stage 12 of embryonic development appears to be mesodermal. This conclusion was made as the expression pattern appears to be similar to other genes recorded as having mesodermal expression such as gleeful (Furlong et al 2001). Directly comparing any co-localisation of CG31320 mRNA and antibodies against markers for the embryonic mesoderm is one way to investigate this further. It was not possible to investigate CG31320 expression in this area with the CG31320::mVenus fusion protein as it was not expressed outside of the chordotonal neurons. Additionally CG31320^{27} and CG31320^{KK102625} combined with a mesodermal Gal4 driver could be used to investigate whether knocking down or out CG31320 affects muscle development.

The pattern of evolutionary conservation of CG31320 largely matched the presence of motile cilia. This could suggest that CG31320 is primarily required for its function in construction of the motile ciliary components, and that CG31320 has evolved to fulfill a secondary role in Drosophila. A number of ciliary genes have secondary roles outwith cilia, with even IFT proteins having non-ciliary functions (Baldari & Rosenbaum 2009). However if ciliary genes are required for roles not linked to cilia then conservation of a gene in organisms with cilia may not necessarily be good evidence for a ciliary role. This has indeed been shown to be the case with cilia linked genes being conserved in plant species that do not have cilia (Hodges et al 2011). Therefore from conservation data alone it is not clear whether the non-ciliary role of CG31320 is unique to Drosophila. Further evidence that this may be the case is that mutations in HEATR2 have thus far only been found in patients with symptoms of PCD and not in genome-wide approaches targeting other conditions. Furthermore mutations that result in greatly reduced protein levels only manifests with PCD symptoms (Horani et al 2012, Diggle et al 2014).
5.3.5 The role of CG31320/HEATR2 in dynein arm formation

The loss of dynein arms from \(CG31320^{27}\) and \(CG31320^{KK102625}\) cilia indicates that CG31320 is required for dynein arm formation. The loss of HEATR2 in PCD patients shows that this role is conserved (Horani et al 2012, Diggle et al 2014). Formation in this instance is used to describe several aspects required for the generation of functional axonemal dynein arms including assembly, stability and transport of dynein arms and their components. However the particular aspect in which CG31320/HEATR2 are involved in is not as clear.

The exclusion of CG6971::mVenus (an IDA component) from the cilium could be consistent with a role for CG31320 in dynein arm assembly, stability or transport. Whilst CG6971::mVenus was clearly excluded from the cilia, an overall reduction in the level of CG6971::mVenus was not observed. This suggests against a role in transcriptional regulation that may have been suggested by the putative connections between CG31320, CG11253 and Bunched (Gallone 2012).

5.3.5.1 CG31320/HEATR2 : a dynein arm assembly factor?

CG31320 and HEATR2 are cytoplasmically localised (Horani et al 2012, Diggle et al 2014). A lack of axonemal dynein arm localisation combined with a cytoplasmic localisation suggests that HEATR2 may be involved in the preassembly of dynein arms in the cytoplasm. As discussed in Chapter 4 a number of cytoplasmically localised dynein arm pre-assembly factors (DNAAFs) have already been identified (DNAAF1-4) and others have been proposed (SPAG1, LRRC6, ZMYND10). Mutations in all of these genes results in PCD and mislocalisation of dynein arm components from the cilia. The lack of CG31320 localisation in the cilium also suggests that CG31320 could be involved in cytoplasmic preassembly. However this proposal is often made without much supportive evidence. SPAG1, LRRC6, ZMYND10, DYX1C1 and HEATR2 have all been found to localise cytoplasmically and as such been suggested as potential DNAAFs (Horani et al 2012, Horani et al 2013a, Knowles et al 2013b, Kott et al 2013, Tarkar et al 2013, Zariwala et al 2013). However without further evidence for this role it cannot be concluded. DYX1C1 can be considered a potential DNAAF (DNAAF4) as a number of protein-protein
interactions with chaperone proteins and DNAAF2 have been identified (Tarkar et al 2013). Mutation of pf13, oda7 or pf22 (the *Chlamydomonas* homologues of DNAAF1-3) causes a reduction in stability and an increase of proteolytic sensitivity of dynein heavy chains (Mitchison et al 2012). Without similar findings for these other genes a role as a DNAAF cannot be concluded.

5.3.5.2 CG31320/HEATR2 : required for transport to or entry into the cilium?

Horani et al (2012) reported puncta within the cytoplasmic localisation of HEATR2 in human multiciliated airway epithelial cells and nasal epithelial cells. G Mali and P Mill also found HEATR2 to appear granular within the cytoplasm of nasal epithelial cells (Diggle et al 2014). This cytoplasmic punctate or granular localisation for HEATR2 matches that seen for CG31320 in larval chordotonal neurons. CG31320::mVenus was cytoplasmically localised in embryonic, larval and pupal chordotonal neurons, with a granular or punctate localisation visible at the larval stage.

The punctate localisation of HEATR2 (Horani et al 2012, Diggle et al 2014) could indicate a role for CG31320 in transport to the cilium. At developmental (embryonic and pupal) stages CG31320::mVenus localisation was diffuse across the cytoplasm, however some granular localisation could also be observed. In mature (larval) neurons puncta of localisation can more clearly be seen. One factor previously implicated in axonemal dynein transport is ODA16 in *Chlamydomonas* (Ahmed et al 2008). ODA16 is required for transport of dynein arms to the cilium and has been found to interact with the IFT complex. Alternatively the lack of dynein arms in the axoneme could suggest that CG31320 has a role in allowing access to the ciliary compartment. The HEAT containing protein Importin-β2 has previously been found to be required for entrance to the ciliary compartment of kinesin motor proteins (Dishinger et al 2010). CG6971::mVenus is excluded from the cilium of *CG31320* larval chordotonal neurons, however it persists in the dendrites. This localisation could be consistent with disrupted transport to the ciliary base or entrance into the cilium. However the lack of CG6971::mVenus aggregates at the base of the cilium and the fact that CG31320::mVenus does not localise to the ciliary base gives more
weight to the argument that CG31320::mVenus is required for transport to the cilium rather than entrance into it.

To investigate this role for CG31320 further protein-protein interactions with known cytoplasmic factors should be investigated as α-solenoid proteins have previously been found to have a role in protein-protein interactions. Furthermore live imaging of CG6971::mVenus and ODA fusion tags would allow further elucidation of the role of CG31320. This would allow the visualisation of the transport of dynein arm components to the ciliary base and into the cilium in both wild type and CG3132027 or CG31320Kk102625 neurons.

5.3.6 CG31320 is required for more than dynein arm formation

The disrupted axonemal shape of CG31320Kk102625 pupal chordotonal cilia suggests that knocking down CG31320 expression affects more than just the axonemal dynein arms. CG31320 could also be required for the formation of factors required for the maintenance of axonemal shape. The nexin links between microtubule doublets could have a role in maintaining axonemal structure. The nexin links cannot be observed in chordotonal cilia by electron microscopy. However homologues of N-DRC components (CG10958 and CG30259) are enriched in embryonic chordotonal neurons (zur Lage & Jarman unpublished). Alternatively another factor required for maintaining axonemal shape could be affected. Examining the localisation of antibodies or fusion proteins for N-DRC components in CG31320Kk102625 pupal chordotonal cilia is one way to investigate this.

5.3.7 CG11253 and CG31320 have different roles in dynein arm formation

CG11253 and CG31320 are both cytoplasmically located factors required for dynein arm formation. However there are a number of differences between them. CG31320::mVenus localises to the cytoplasm whereas CG11253::mVenus localises to both the cytoplasm and the cilium. Additionally CG11253::mVenus strongly localised to the embryonic scolopale cell contrasting with the low levels of CG31320::mVenus observed. This could reflect differences in expression level at earlier developmental stages.
There were also differences in axonemal phenotypes. Whilst disruption of both CG31320 and CG11253 expression resulted in a loss of axonemal dynein arms, the level of loss was more for CG31320\textsuperscript{KK102625} (CG31320\textsuperscript{KK102625} – 11.11\% ODA and 3.14\% IDA visible, CG11253\textsuperscript{EY10866} – 44.00\% ODA and 22.20\% IDA visible). The axonemal shape defects observed in CG31320\textsuperscript{KK102625} cilia were also not found in those of CG11253\textsuperscript{EY10866}. Preliminary results indicate that CG6971::mVenus is reduced in expression level from CG11253\textsuperscript{EY10866} cilia and neuronal cytoplasm. This contrasts with its localisation in CG31320\textsuperscript{27} where it is excluded from the cilium but persists in the dendrite. The different phenotypes suggest that CG31320 and CG11253 have roles in different aspects of axonemal dynein arm formation or stability.

Despite a common role in axonemal dynein arm assembly there are differences between the function of CG11253 and CG31320. This is further evidence that a common role for genes cannot be presumed due to cytoplasmic location and axonemal dynein arm loss.

### 5.4 Conclusion

CG31320/HEATR2 is a conserved factor required for axonemal dynein arm formation. The criteria that were used to identify CG31320, of conservation, expression pattern and transcriptional regulation mechanism, can be used to identify further motility genes. Uncharacterised Fd3F targets in particular provide a shortlist for cillum motility genes and homologues of PCD candidates. Whilst several PCD genes have been identified, the causative mutated gene has not been isolated in many cases. The identification of CG31320 demonstrates the use of Drosophila as a model for identifying new cillum motility genes and thus PCD candidate genes.
6

CG14353 and CG18675

6.1 Introduction

The characterisation of CG11253 and CG31320 showed that novel cillum motility factors could be identified from genes enriched in developing chordotal neurons. The finding of mutations in ZMYND10 and HEATR2 in PCD patients also indicated that the human homologues of Drosophila cillum motility genes could be considered candidate PCD genes.

The climbing assay discussed in chapter two implicated a number of genes from my candidate gene list as being required for ciliary function in chordotal neurons. It was thought that these could also potentially be cillum motility genes. I sought to investigate some of these genes further to find out whether they proved to be good candidate cillum motility genes and thus their human homologues could be considered candidate PCD genes. The identities of likely Drosophila cillum motility genes could then be passed on to collaborators who could screen PCD patients for mutations in the human homologues of these genes.

CG14353 and CG18675 were selected from those highlighted by the climbing assay screen as their homologues had previously been implicated in cillum motility (Baron et al 2007). This was due to a lack of cytokinesis following an RNAi mediated knock down of these genes in trypanosomes. However the behaviours observed by Baron et al (2007) could also be explained by other factors. Theses two genes were investigated further in order to provide further evidence to assess whether they represented candidate ciliary motility genes and thus whether their human homologues could be considered candidate PCD genes.
6.2 Results

6.2.1 Evolutionary conservation of CG14353 & CG18675

Baron et al (2007) had identified homologues of CG14353 and CG18675 to be present in organisms with motile cilia (Trypanosoma brucei, humans, Chlamydomonas reinhardtii, Drosophila) but not in those without (Arabidopsis thaliana and Caenorhabditis elegans). To investigate their conservation further I used a reciprocal best BLAST technique to identify homologues of these genes in other organisms. This would further test the hypothesis that these genes are required for motile ciliary function. Homologues of CG14353 and CG18675 are generally found in organisms with motile cilia, but not in those without cilia or with immotile cilia (Figure 6.1, Tables 6.1 and 6.2). There were exceptions to this, a homologue of CG14353 was found in Ostreococcus, as was the case for CG11253. A homologue to CG14353 was also found in Dictyostelium, which lacks cilia. It is possible that a homologue does exist in this genus as the homology did meet the reciprocal best match criteria. However it is noteworthy that the identity between CG14353 and the Dictyostelium homologue is much lower than for other homologues, even those at a further evolutionary distance. This could be evidence of the gene being conserved after ciliary loss from the lineage and has diverged further due to different selective pressures. Cilia are thought to have been lost in this lineage as it is thought that the last eukaryotic common ancestor (LECA) possessed cilia (Carvalho-Santos et al 2011).

6.2.3 Transcriptional regulation

As shown previously transcriptional regulation of genes by RFX and Fd3F can implicate a gene in having a ciliary role. Not being regulated by these transcription factors does not necessarily mean that a gene does not have a ciliary role. However if a gene is regulated by RFX and Fd3F it is a good indication that it is involved in ciliary formation or function. I used GenePalette to search the upstream regions of these genes for X boxes (RYYNYY13RRNRAC – Laurençon et al 2007) and Forkhead binding sites (RYMAAYA – Newton et al 2012). CG14353 contains both of these binding motifs within it’s 5’ UTR (Figure 6.2A). CG18675 also contains
Figure 6.1 CG14353 and CG18675 homologues throughout evolution.
The conservation of homologues of CG14353 and CG18675 in a variety of organisms with and without cilia. Homologues were detected by reciprocal BLAST. CG18675 is conserved only in organisms with motile cilia, whereas CG14353 is conserved in organisms with motile cilia and two without (Ostreococcus and Dictyostelium). Presence of homologues indicated by a black circle, a white circle shows no homologue could be detected. Figure based on similar homologue conservation tree for TilB from Kavile et al (2010).
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Table 6.1. Homologues of CG14353 and identity detected by reciprocal BLAST.
# Table 6.2 Homologues of CG18675

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</table>

Table 6.2. Homologues of CG18675 and identity detected by reciprocal BLAST
these motifs in its upstream region (Figure 6.2B). The presence of these binding motifs indicates that RFX and Fd3F could regulate these genes transcription. As these transcription factors both regulate the expression of cilium motility genes this further suggests that CG14353 and CG18675 could have a role in cilium motility. The presence of these motifs alone however does not indicate that they are functional. Analysis of expression in Rfx and fd3F mutants would be required to confirm that they do indeed regulate gene expression.

6.2.4 Testes defects

If CG14353 and CG18675 are required for cilium motility they may also be necessary for motile sperm development. In order to assess this UAS-hpRNA flies were crossed to BAM-GAL4-VP16 in order to express the siRNA against CG14353 and CG18675 in developing sperm cells. Testes were dissected from five day old male CG14353\(^{KK102248}\), CG18675\(^{KK102730}\) and P\{attP\} flies (Figure 6.3, Table 6.3). CG14353\(^{KK102248}\) and CG18675\(^{KK10273}\) testes showed abnormal bundle bending, an increased frequency of high levels of individualised sperm in the terminal epithelium and empty or small seminal vesicles. Upon testis squash no motile sperm were detected. These findings are consistent with CG14353 and CG18675 having a role in cilium motility.

6.3 Discussion

Homologues of CG14353 and CG18675 had previously been implicated in cilium motility (Baron et al 2007). Identifying further homologues in organisms with motile cilia generated further evidence that these genes are involved in cilium motility. CG14353\(^{KK102248}\) and CG18675\(^{KK102730}\) display testes defects similar to those observed in CG31320\(^{KK102625}\) and CG11253\(^{EY10866}\). This further suggests that they have a role in similar processes to these two genes and thus be required for flagellum motility.

To investigate the roles of these genes further electron microscopy could be used to examine the axonemal ultrastructure and the presence of the dynein arms and motility machinery. Additionally crossing in genes for tagged proteins such as CG6971::mVenus could allow further elucidation of their role. The
Figure 6.2 The binding motifs for RFX and Fd3F upstream of CG14353 and CG18675. A diagram showing the location of X boxes (green) and forkhead binding sites (purple) in the 5'UTR or upstream regions of CG14353 (A) and CG18675 (B). Motifs were found using GenePallete to search for the consensus sequences of the forkhead binding site (RYMAAYA, Newton et al 2012) and X box (RYYNYY{1,3}RRNRAC, Laurençon et al 2007).

Table 6.3 Testes physiology of CG14353KK102248 and CG18675KK102730

<table>
<thead>
<tr>
<th></th>
<th>5 days old</th>
<th>Sperm bundle disruption</th>
<th>Empty/Small Seminal Vesicle</th>
<th>Higher level of individualised sperm</th>
<th>Motile sperm detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>p[attP]</td>
<td>p[attP]</td>
<td>0 (N=18)</td>
<td>0 (N=18)</td>
<td>0 (N=17)</td>
<td>17 (N=17)</td>
</tr>
<tr>
<td>CG14353KK102248</td>
<td>CG14353KK102248</td>
<td>16 (N=20)</td>
<td>15 (N=17)</td>
<td>12 (N=17)</td>
<td>0 (N=19)</td>
</tr>
<tr>
<td>CG18675KK102730</td>
<td>CG18675KK102730</td>
<td>17 (N=17)</td>
<td>15 (N=17)</td>
<td>14 (N=17)</td>
<td>0 (N=16)</td>
</tr>
</tbody>
</table>

Table 6.3 The number of fly testes observed that showed defective phenotypes. The frequency of sperm bundle disruption, small seminal vesicles and high levels of individualised sperm in a testis dissection of five day old p[attP] ; BAM-GAL4-VP16 , CG14353KK102248 ; BAM-GAL4-VP16 and CG18675KK102730 ; BAM-GAL4-VP16 flies. The number of testes in which motile sperm could be observed after testes squash is also shown. P value : < 0.0001 (Fishers exact test).
Figure 6.3 Testis dissection of CG14353\(^{KK102248, 8}\); BAM-GAL4-VP16 and CG18675\(^{KK102730, 8}\); BAM-GAL4-VP16.

The testes of 5 day old CG14353\(^{KK102248, 8}\); BAM-GAL4-VP16, CG18675\(^{KK102730, 8}\); BAM-GAL4-VP16 and \(P(\text{attP})\); BAM-GAL4-VP16 control flies were dissected.

(A and B) A schematic showing the phenotypes of control (A) and knock down (CG14353\(^{KK102248, 8}\); BAM-GAL4-VP16 or CG18675\(^{KK102730, 8}\); BAM-GAL4-VP16) (B) testes.

(C) Control testis showing normally sized seminal vesicle and amount of individualised sperm. Motile sperm can be seen exiting the seminal vesicle.

(D) CG14353\(^{KK102248, 8}\); BAM-GAL4-VP16 testis with a small seminal vesicle, an accumulation of individualised sperm. No motile sperm are released from the dissected seminal vesicle.

(E) CG18675\(^{KK102730, 8}\); BAM-GAL4-VP16 testis showing small seminal vesicle and an accumulation of individualised sperm. No motile sperm are observed.
CG6971::mVenus flies that I previously generated are unsuitable for this as shown in the previous chapter. However if the UAS sites were removed from the CG6971::mVenus construct it may prove useful. Additionally constructing an ODA::mVenus construct would allow observation of any differing roles for IDA and ODA formation. mVenus fusion genes for both CG14353, CG18675 and CG8800, an ODA component, were designed in collaboration with K Styczynska-Soczka, who is currently characterising these fusion proteins following injection into blastoderm stage embryos.

6.3.1 Transcriptional regulation by RFX and Fd3F

I identified binding motifs for RFX and Fd3F upstream of both of these genes. Avidor-Reiss et al (2004) also identified X boxes upstream of both CG14353 and CG18675. However the presence of binding sites alone does not confirm that these transcription factors regulate their expression. Fellow lab members have obtained further evidence for this mechanism of transcriptional regulation of CG18675. CG18675 expression has been found to be reduced in both fd3F and Rfx flies (in situ hybridisation carried out by P. zur Lage and S. Mukherje respectively). This indicates that CG18675 expression is indeed regulated by RFX and Fd3F. CG14353 expression in these mutants has not been tested so it is not yet known whether the binding motifs are functional.

6.3.2 CG18675/C21orf59 is mutated in PCD

C21orf59, the human homologue of CG18675 has since been found to be mutated in identified several patients with PCD (Austin-Tse et al 2013). This gene was identified as a PCD candidate gene by a knock down screen for motile ciliary phenotypes in zebrafish (Austin-Tse et al 2013). The candidate gene list in this instance was generated from previously published ciliary proteomic data (Austin-Tse et al 2013). This indicates a similar approach to my own if only using a different method to generate the candidate list and different model organisms.

Mutations in C21orf59 resulted in disruption of ODA and IDA. Immunofluorescence demonstrated that DNAH5 and DNALI1 were lost from the cilium in respiratory epithelial cells obtained from PCD patients with C21orf59
mutations (Austin-Tse et al 2013). C21orf59 localised in cytoplasmic puncta in rat trachea (Austin-Tse et al 2013). As some of these puncta co-localised with SAS6 it was proposed that C21orf59 had a role at the basal body or centriolar satellites. However the *Chlamydomonas* homologue, FBB18, was detected in the flagellar matrix (Austin-Tse et al 2013).

### 6.4 Conclusion

Further evidence of a role in cilium motility was obtained for CG14353 and CG18675. Both were mainly conserved in organisms with motile cilia although a CG14353 homologue was also present in two organisms without. Knocking down these genes generated phenotypes in testes similar to those I had found previously for CG11253 and CG31320. This appears to be how a motility defect manifests in *Drosophila* testis. This gives further evidence that *CG14353* and *CG18675* could be cilium motility genes. The identities of these genes were passed on to collaborators and the human homologues of these genes were considered candidate PCD genes. The human homologue of *CG18675* was confirmed as a PCD gene by an alternate group searching PCD patients for genes identified via motility defects in zebrafish. This gives proof of concept that PCD genes can be identified in this way and as such investigations into the conservation, transcriptional regulation and requirement for sperm motility should be carried out for all genes on the candidate gene list.
7

General Discussion

7.1 Identification of novel ciliary genes

The aim of this study was to identify and characterise novel ciliary genes in *Drosophila*. The behavioral assay in Chapter 2 indicated a number of potential ciliary genes that were required for chordotonal neuron function. *CG11253* and *CG31320* were characterised and identified as being required for the assembly of the cilium motility machinery. This indicates that novel ciliary genes could indeed be identified from those enriched in *atoGFP* and *catoGFP* expressing cells. Both of these genes were identified from time points before ciliogenesis, the extension of the microarrays conducted by Cachero et al (2011) and zur Lage and Jarman (unpublished) to include further time points during ciliogenesis could potentially identify further candidate ciliary genes. Furthermore the candidate gene list compiled was done as to have a manageable number of genes to investigate. Additional candidate ciliary genes could well have not been included on it.

Newton et al (2012) identified a number of Fd3F target genes. That *CG11253* and *CG31320* were transcriptionally regulated by Fd3F implicated them in ciliary motility. Further Fd3F targets could therefore be candidate ciliary motility genes, such as *CG10339*. The Fd3F targets identified by Newton et al (2012) were selected for testing by whether they were known ciliary genes or enriched in *atoGFP* and *catoGFP* expressing cells. The identification of further Fd3F targets in a less restrictive way, such as a microarray comparing wild type and *fd3F* embryos, could allow the identification of further motility genes.

Additionally I focused this study on the genes required for cilium motility. However genes required for ciliary roles not related to motility could also be identified from those enriched in *ato/catoGFP* expressing cells. An example of this is the
identification of the human homologue of CG15701 as mutated in Jeune syndrome patients (McInerney-Leo et al 2013).

The list of candidate genes was compiled using resources available from within our laboratory such as the zur Lage and Jarman (unpublished) temporal profiles or the identity of Fd3F targets (Newton et al 2012). However I would not have been able to narrow down the candidate list without utilising many other studies including proteomic, transcriptomic and genomic investigations into cilia (listed in Chapter 2). This highlights the importance of combining data from different studies and different organisms to better understand cilia.

7.2 CG11253

CG11253 was found to be a mainly cytoplasmic factor required for the formation of the axonemal dynein arms. Its selection from the candidate gene list was based on the presence of a transposon insertion mutant that could be used to investigate its function. This highlights the use of the genetic resources available in Drosophila.

It was thought possible that CG11253 had a role in the transcriptional regulation of dynein arm genes. This hypothesis was formed as MYND and LXXLL containing proteins had previously been implicated in transcriptional regulation and as ZMYND10 had been linked to pathways that regulated transcription in other tissues (Zhang et al 2012, Dong et al 2012). Additionally the mRNA level of dynein arm components were found to be reduced in cells where ZMYND10 or its interactor LRRC6 were disrupted (Horani et al 2013a, Zariwala et al 2013). However CG11253::mVenus was not found to enter the nucleus in chordotonal neurons suggesting that any role in transcription cannot be direct. Further evidence that a role in transcriptional regulation is not likely comes from the findings of Geremek et al (2014) that large scale transcriptional changes are common in PCD patient ciliated cells. This suggests that a reduction in dynein arm expression would be found in cells with cilia with motility defects. It is therefore not likely that CG11253 has a role in transcriptional regulation.

What then is the role of CG11253? What is clear is that CG11253 is required for dynein arm formation. The loss of dynein arms observed by TEM and the reduction
of ciliary CG6971::mVenus in \textit{CG11253^{EY10866}} shows that CG11253 is essential for dynein arm formation. The general reduction in level of CG6971::mVenus in both the cilium and the cytoplasm suggests a defect in the stability of this component. This and the cytoplasmic localisation of CG11253::mVenus have led to the suggestion that it is a potential new DNAAF. Whilst this is the most likely role from the evidence available it is far from confirmed and further evidence will be required to investigate whether this is the true role of CG11253 (discussed in 7.4).

### 7.3 CG31320

\textit{CG31320} was also found to be a cytoplasmically localised factor required for the formation of both inner and outer dynein arms. Its identification as a ciliary candidate gene was aided by the putative protein-protein interaction network constructed by G. Gallone (2012). This indicates the use of bioinformatic techniques to help mine candidates from large datasets such as the temporal profiles of gene expression and shows that interolog walking can be used to implicate previously uncharacterised genes in a process.

In addition to its role in the chordotonal neurons \textit{CG31320} was expressed elsewhere in the embryo. Its mesodermal expression is likely to be responsible for the lethality of \textit{CG31320^27} flies. I did not investigate this role of CG31320, however it could be useful to better understand CG31320’s ciliary function.

CG31320 is certainly required for dynein arm formation, but which aspect of formation is less clear. The localisation of CG6971::mVenus in \textit{CG31320^27} larval chordotonal neurons gives some clues. CG6971::mVenus was found to be excluded from the cilium but not reduced in level from the dendrites. This may indicate that CG31320 is required for axonemal dynein arm transport to or entry into the cilium. Clear puncta of CG31320::mVenus are seen within chordotonal neurons. Observing whether this colocalises with axonemal dyneins is one way to proceed with this investigation. An axonemal dynein would need to be tagged with an alternative protein tag to allow localisation of both it and CG31320::mVenus to be observed at the same time. Live imaging may also allow the visualization of any movement of
CG31320::mVenus to the cilium or lack of movement in tagged axonemal dyneins when \textit{CG31320} is mutated.

### 7.4 Comparing CG11253 and CG31320

There are many similarities between the CG11253 and CG31320. Both are cytoplasmically localised and are required for inner and outer dynein arm formation. It could be that they both play similar roles in dynein arm formation. However there are differences between them. Whilst CG6971::mVenus is lost from the cilium of mutated \textit{CG31320} neurons but retained in the dendrites, mutation of \textit{CG11253} results in a general reduction in level. They are therefore likely to carry out different roles in the formation of CG6971::mVenus containing dynein arms. However the proposed roles for both of these proteins are largely based on the localisation of one dynein arm component. Different dynein arm components may behave differently, for example in \textit{DNAAF2} mutant respiratory cells DNALI1 is lost from the cilia, whilst DNAH5 is lost from the distal cilium but only reduced in level in the proximal cilium (Omran et al 2008). Therefore to further elucidate the roles of these genes and to understand the part they both play in dynein arm formation the localisation of further tagged dynein arms needs to be investigated. The proposed roles for each of these genes seem the most likely with the information available however the specific role they play in dynein arm formation needs further investigation. Only that they are both required for part of axonemal dynein arm formation is known for certain.

Additionally investigating the interactome of these proteins could give clues to their role. For example an interaction with a heat shock protein could confirm a role in dynein arm pre-assembly. Such an interaction was detected by Giot et al (2003) in a high throughput interaction screen, however this needs to be repeated and confirmed by alternate methods before it is proved. For already identified DNAAFs the evidence which allowed confirmation of their role was a reduction in protein stability, an increase in proteosomal degradation and a lack of immunoprecipitation of preassembled dynein arm components (Mitchison et al 2012). These studies were carried out in \textit{Chlamydomonas}, so either the \textit{Chlamydomonas} homologues of CG11253 and CG31320 need to be investigated with the antibodies and techniques already available or a more detailed investigation into \textit{Drosophila} preassembly is
necessary to be able to identify which dynein arm components preassemble in wild type and potential DNAAF mutant flies.

7.5 What is a ciliary motility gene?

A ciliary motility gene is one that encodes a protein necessary for active ciliary movement. The ciliary motility machinery is required for ciliary motility and as such its components are encoded by ciliary motility genes. The investigations into CG11253 and CG31320 highlight the importance of cytoplasmic factors for the assembly of the ciliary motility machinery and shows that ciliary motility genes are not restricted to those that encode proteins which localise to the cilium. The lack of ciliary motility in PCD patients with mutations in ZMYND10 and HEATR2 shows that ciliary motility genes can be identified in Drosophila despite it not being known whether the Drosophila chordotonal cilium is motile. The ciliary motility machinery, and the components that assemble it are vital for the function of chordotonal neurons and as such they can be used to investigate the machinery that allows ciliary motility in other organisms.

7.6 Drosophila melanogaster as a model of PCD

Mutations in the human homologues of CG11253 and CG31320 were found in PCD patients. My findings indicate the first use of Drosophila as a model to investigate PCD. Much of the focus of PCD research has been on identifying new disease causing mutations. Drosophila can be used to identify further PCD candidate genes through the homologues of those found to be necessary for chordotonal motile ciliary function. However Drosophila can also be used to characterise and investigate the function of homologues of PCD genes. Additionally the reduction in fertility of CG11253Val14Gly::mVenus flies demonstrated the capability of Drosophila to model disease causing mutations.

However Drosophila could not be used to investigate all PCD causing mutations as they lack both the central apparatus linked to the central microtubule doublets and the radial spokes. Mutations in genes related to these complexes have been found in PCD patients (Castleman et al 2009, Olbrich et al 2012, Kott et al 2013). These could
be investigated in Drosophila sperm but could not be identified by the chordotonal neuron based methods that I have used. Additionally the role of the dynein arms in the function of chordotonal cilia could result in some structural differences to respiratory cilia. If they do form a transduction complex there may be a limit to the conservation of the cilium motility machinery in these two cell types. However much of the cilium motility machinery has been found to be expressed in ciliated Drosophila cells (Cachero et al 2011, zur Lage & Jarman unpublished). Additionally homologues of the two genes that I identified have been found to be mutated in PCD patients indicating that there is homology between the cilium motility machinery of chordotonal neurons and respiratory cells and that Drosophila can be used to investigate PCD.

ZMYND10 mutations were identified by A Onoufriadis and H Mitchison at the same time as my own investigations into CG11253. C Diggle and E Sheridan also found mutations in HEATR2 at the same time as my own investigations into CG31320. A novel ciliary gene from Drosophila has not yet directly resulted in the discovery of a novel PCD causing gene. However from CG11253 and CG31320 we know it is possible. The identities of further candidate PCD genes identified in Drosophila have already been passed on to collaborators.

Furthermore Drosophila can be used to investigate the pathogenicity of PCD causing mutations as shown for the Val16Gly causing mutation in ZMYND10. However of the three mutations identified by A Onoufriadis and H Mitchison in ZMYND10 only one was conserved to Drosophila. This indicates another limitation of Drosophila; the evolutionary distance may mean that certain aspects may not be conserved. However ciliary function is readily assayable in Drosophila and this could still be utilised in these incidences; if ZMYND10 could rescue CG11253^{EY10866} then the ability of mutated ZMYND10 to rescue CG11253^{EY10866} and therefore the pathogenicity of the mutations could be assessed.

Drosophila provides a useful addition to the model organisms which can be used to investigate PCD and my investigations thus far have identified a number of candidates for further investigation.
7.7 Proprioception requires dynein arms

Mutations in dynein arm components and in cytoplasmic factors required for their formation (including \textit{CG11253}) have previously been shown to result in hearing defects (Senthilan et al 2012). The requirement of \textit{CG11253}, \textit{CG31320} and dynein arm components for proprioceptive behavior indicates that not only the antennal chordotonal neurons, required for hearing, need the cilia motility machinery for their function. Mutations in both axonemal dynein arm components and their cellular regulators result in reduced proprioceptive and/or gravitactic function. These two properties are likely to be linked, as both require awareness of the position of body parts. Gravitactic behaviour can therefore be considered an extension of proprioceptive behaviour. The exact role of the cilium motility machinery in proprioceptive chordotonal neurons is not known. It is possible that as in hearing the cilium is involved in active amplification or as part of a transduction complex.

7.8 Transcriptional cascade

\textit{CG11253} and \textit{CG31320} represent downstream target genes of the Atonal transcriptional cascade. Fd3F acts in this cascade to regulate the expression of the dynein arm components (Newton et al 2012). During differentiation it is important to regulate not only the complexes that will function in a fully differentiated cell but also the cellular regulators required for their formation. In this transcriptional cascade Fd3F appears to not only regulate the expression of the axonemal dynein arm components but also the factors required for dynein arm formation such as \textit{CG31320} and \textit{CG11253}.

7.9 Further screening

A number of different techniques could be used to identify further candidate ciliary genes. As already stated further potential candidates could be identified by the examination of temporal profiles at later time points or by identifying more Fd3F targets. The testes phenotypes observed for disruption of \textit{CG11253}, \textit{CG31320}, \textit{CG14353} and \textit{CG18675} all appear similar. This could perhaps be used to screen candidate genes, either by examining testes physiology in RNAi knocked down male
flies or simply by assessing fertility. However if using testes physiology care needs to be taken to identify the aspects of physiology that are specific to a loss of motility rather than other spermatogenesis defects. The climbing assay I carried out was able to identify a number of candidate ciliary genes that showed proprioceptive defects when knocked down. This approach could be extended to screen P element insertion mutants to try to identify proprioceptive defects.

Crossing mVenus tagged axonemal dyneins into a GAL4 driver could allow a high throughput assessment of whether a gene is required for axonemal dyenin assembly. This GAL4 line could be crossed to UAS-hpRNA and the localisation of axonemal dynein in the chordotonal neurons could then be observed. My own CG6971::mVenus construct would not be suitable due to the potential reduction in knock down caused by increased UAS sites. Generating a number of tagged axonemal dynein genes would allow the formation of both ODA and IDA to be assessed.

I used Drosophila to identify and characterise a number of ciliary genes. Several of the approaches mentioned above could be used to identify further ciliary motility genes from those enriched in atoGFP and catoGFP expressing cells.
Materials and Methods

8.1 Fly husbandry

Stocks were kept at 18°C and tipped to new food every four-six weeks. Crosses were conducted in vials or bottles of food at 25°C. Crosses for injection or embryo collection were conducted in cages with a red wine agar plate at the bottom.

8.2 Climbing assay

Two methods for the climbing assay were conducted. They differ only in the number of flies assayed and the analysis of the results. In both cases 2-7 day old flies were transferred to the behavioural room (21°C) at least one hour before the assay was conducted. Flies had been kept in a 25°C incubator with a 12-hour light – dark cycle. Assaying flies at times reflecting dawn and dusk in the light cycle was avoided. A light sheet was used to ensure the environment was consistent during the assay. Flies were transferred to a 25ml measuring cylinder and given 1 minute to acclimatise. Flies were then knocked to the bottom of the cylinder. Flies were filmed climbing for 10 seconds. Video analysis was conducted using Quicktime and ImageJ.

Method A consisted of assaying 4 x 20 flies which were then divided into four bins depending on the height they had climbed after 10 seconds. A climbing score was then calculated for each tube of 20 flies based on how many flies were in each bin. Flies that climbed less than 5cm scored 1 point, 5-10 cm : 2 points, 10-15 cm : 3 points, 15-20cm: 4 points. This was then divided by the number of flies assayed to provide the climbing score.

Method B consisted of assaying 4 x 10 flies. Instead of dividing the flies into bins, the height that each individual fly climbed was measured using ImageJ.
8.3 Fertility assay

Individual 2 to 5 day old male flies were placed in a vial with a wild type female (OregonR or W^{1118}). Flies were allowed to mate for two days before being transferred to a new vial and allowed to lay eggs for three-five days. Either the resulting progeny were counted, or the presence of progeny was scored.

8.4 Testes dissection

Testes were dissected and mounted Ringers solution (3mM calcium chloride, 182mM potassium chloride, 46mM sodium chloride, 10mM Tris-HCl, (pH 7.2)). Accessory glands were removed from the reproductive organs where possible leaving the testis, seminal vesicle and ejaculatory duct intact. A cover slip was placed over the slide but not sealed. Testes were then squashed by pressing down the cover slip, allowing the observation of motile sperm in wild type flies.

Testes for confocal microscopy were prepared as for bright field examination except they were mounted in Vectashield (Vector labs) and the coverslip was sealed with nail varnish.

8.5 Extraction of genomic DNA

50 flies were frozen (-20°C) in 200µl Lysis buffer (100mM Tris-HCl (pH 9), 100mM EDTA, 1% SDS). The sample was then homogenised with a motor driven plastic pestle before adding a further 600µl Lysis buffer. To this was added 150µl of cold potassium acetate (8M) before incubating on ice for 20 minutes. The mixture was then spun for 20 minutes at 14,000 rpm in a centrifuge chilled to 4°C. The supernatant was then added to 0.9X isopropanol. After a further 5 minutes spin the supernatant was discarded. The pellet was washed in 70% ethanol, air-dried and resuspended in 50µl TE (10mM Tris, 1mM EDTA buffer).

8.5.1 Phenol chloroform extraction

Equal volume of Phenol-chloroform was added, before 15 seconds vortex and 10 minutes spin (14,000 rpm). The upper layer was then removed and placed in a fresh tube.
8.5.2 Precipitation of DNA

5µl sodium acetate was added to the removed liquid and was subsequently mixed. 250µl cold 100% ethanol was then added before incubating at 4°C overnight. The sample was then spun for 15 minutes (14,000 rpm) and the pellet washed, dried and resuspended in TE as done previously.

8.6 Single Fly DNA extraction

A single fly was placed in a 0.5ml tube and mashed with a pipette tip containing 50 µl of squishing buffer (Stock solution of 1mM EDTA, 25mM NaCl, 10mM Tris-HCl pH 8.2 and freshly added 200µg/ml Proteinase K). Squishing buffer was only released after 20 seconds of mashing. The tube was then incubated at 37°C for 30 minutes. Tubes were then heat shocked at 95°C for 2 minutes to inactivate Proteinase K. 5µl of this preparation would be used in a PCR reaction instead of the standard 2µl.

8.7 Gel electrophoresis

0.8% agarose gels were made with 1 x TAE buffer (diluted from 50 x stock solution of 40mM Tris, 2mM EDTA, 20mM Acetic acid) and Gel Red (7 µl of 10,000x stock in 100ml liquid gel (Biotium)). DNA and loading buffer (diluted from 6x stock of 30% glycerol (v/v), 0.25% bromophenol blue (w/v) and 0.25% xylene cyanol FF (w/v)) were mixed and loaded into the gel wells. Gels were run at 80hz in TAE buffer.

8.8 PCR

A polymerase chain reaction mix contained 2µl DNA, 1µl dNTP mix (Roche), 5µl 10x buffer (Roche), 5µl each of 5’ and 3’ primers (10µM) (Sigma Aldrich) and 0.5µl Taq polymerase (Roche) and made up to 25µl with dH₂O. This was mixed and placed in a PCR machine. The following programme was run: 94°C 2 mins, 30 cycles of (94°C 30 secs, 55°C 30 secs, 72°C 1 min/kb fragment), 72°C 10 mins and then held at 4°C. For amplifying constructs to generate fusion genes PfuUltra II DNA polymerase
and PfuUltra II buffer (Agilent technologies) were used in place of Taq polymerase and 10x buffer.

### 8.9 Sequencing of DNA

Fragments amplified by PCR were sequenced using the Big Dye Terminator kit (Applied Biosystems). A reaction mix was made of 2µl 5x sequencing buffer, 1µl Big Dye, 10 µM primer, 200ng DNA and was made up to 10µl with dH2O. This mix was then put in a PCR machine and run on a programme consisting of 1 minute at 96ºC, then 25 cycles of (96ºC 10 secs, 50ºC 5 secs, 60ºC 75 secs) before being held at 4ºC. The reaction mix was then sent to The GenePool, Edinburgh for Sanger sequencing.

### 8.10 Immunohistochemistry

#### 8.10.1 Embryos

Embryos were collected from overnight cages and washed off the red wine plates into a sieve. They were then rotated at 50rpm for 4 minutes in 50% bleach to remove the chorion membrane. Embryos were then washed and transferred to a scintillation vial containing 3.75ml PBS (Phosphate buffered solution), 1.25ml 3.7% formaldehyde and 5ml heptane. Embryos sit between the two phases and were shaken at 200rpm for 20 minutes. The bottom phase was then removed and 10ml methanol added. Shaking removed the vitelline membrane. Once the membrane had been lost embryos sank to the bottom of the vial and could be collected. These were then washed twice with methanol. Embryos were washed three times for 5 minutes on a rotating wheel in PBT (0.3 % Tritron X-100 in PBS) and then blocked for two hours with 2% BSA (bovine serum albumin (in PBT)) (Roche). BSA was then removed and the primary antibody mix added. This included 125µl BSA, 25µl NGS (Normal Goat Serum) and appropriate quantities of the primary antibodies. This was then made up to 450µl with PBT. The mix was incubated overnight at 4ºC. The following day the primary antibody mix was removed and saved for repeated use. The embryos were then rinsed four times, and washed three times for fifteen minutes in PBT. The secondary antibody mix was added and then incubated for 2 hours at room temperature. This contained 25µl NGS and appropriate levels of secondary
antibodies. The mix was then made up to 450µl with PBT. Tubes containing secondary antibodies were covered in aluminium foil to stop any reaction with light. Following secondary antibody incubation the embryos were rinsed four times and washed three times for fifteen minutes in PBT. Embryos were then mounted in Vectashield (Vector Labs).

8.10.2 Larval pelts

Pelts were dissected and pinned onto a Sylgrad dish in 9ml PBS. 1ml 37% formaldehyde was then added and the dish rotated at 50 rpm for 30 minutes. Pelts were washed on the dish four times for 15 minutes in PBT. Pins were then removed and the pelts placed in a 1.5ml eppendorf with 1 ml 2% BSA. The IHC protocol was then followed as for embryos.

8.10.3 Antennae

Whole pupae were pinned to a Sylgard dish. The pupal case was removed and an incision made behind the pupal head. Pupae were then fixed as for larvae. However before mounting the pupae were repined and the antennae or a portion of the head containing the antennae dissected and mounted in Vectashield (Vector labs).

8.11 RNA in situ hybridisation

Embryos were collected and fixed as for immunohistochemistry. After the final methanol wash the embryos could either be stored at -20°C or used immediately. Embryos were then washed 3 times for 5 minutes in decreasing dilutions (70%, 50%, 30%) of methanol in PBTween (0.1% Tween in PBS) followed by two rinses and a 5-minute wash in PBTween. Embryos were then post-fixed for 15 minutes in 3.7% formaldehyde (in PBTween) followed by one rinse and four 5 minutes washes in PBTween. Embryos were washed in a mix of 400µl PBTween and 400µl Hybridisation buffer (50% formamide, 5x SSC, tRNA 100mg/ml, Heparin 50mg/ml, 0.1% Tween 20 pH6.5 (pH adjusted with hydrochloric acid)) for 10 minutes, followed by a 10 minute wash in 800µl Hybridisation buffer. Embryos were prehybridised at 70°C for at least two hours in 500µl hybridisation buffer. RNA probes were diluted in 100µl hybridisation buffer (or reused after collection at end of
the procedure). CG11253 RNA probe was designed and generated by F Newton, CG31320 RNA probe was designed and generated by G Mali and P zur Lage, both were used in a dilution of 1:200. The probe was the heat shocked at 94°C for 3 minutes and chilled on ice. Hybridisation buffer was removed from the embryos and replaced with the probe. Embryos were then incubated at 70°C overnight in a dry block.

Embryos were then washed six times (once with hybridisation buffer, once with 400μl hybridisation buffer, 400μl PBTween, four times with PBTween) for 30 minutes in the 70°C dry block followed by a 5 minute wash in PBTween at room temperature. Embryos were then incubated with 1:2000 anti-Digoxigenin-alkaline phosphatase for two hours at room temperature. Embryos were washed three times for 20 minutes in PBTween and transferred to microtitre plates. This was followed by two 2 minute washes in reaction solution (100mM sodium chloride, 100mM Tris pH 9.5) at 50 rpm. 20μl of NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-indolyphosphate) was then added to 1 ml of reaction solution, mixed and added to the embryos. Embryos were incubated in the dark for between 5 hours and overnight (based on observation of staining). The reaction was then stopped with PBTween and embryos subsequently rinsed three times and washed three times for 15 minutes. Embryos were then mounted in 70% glycerol in PBS.

8.12 mVenus fusion protein generation

8.12.1 Primer design

Primers were designed to amplify and tag with attB the gene of interest (GOI) and upstream region. The primer targeting the C terminus of the GOI removed the stop codon but added an extra base pair to ensure the mVenus tag would be in frame. Fragments were amplified using PfuUltra II DNA polymerase. A sample of the PCR fragment was run on a 0.8% agarose gel to observe its purity. The PCR fragments were purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). DNA concentration was calculated using Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific).
8.12.2 BP reaction

150ng of attB-PCR product was added to 150ng of pDonr221 (Invitrogen) (Figure 8.1) and TE buffer added up to 8µl. BP Clonase II was then thawed for two minutes, vortexed and added to the mix. This was in turn vortexed then incubated at 25°C overnight. The following day the reaction was stopped by the addition of 1µl Proteinase K and incubation at 37°C for 10 minutes.

8.12.3 Transformation of bacteria

A vial of DH5α E. coli (previously made competent by P zur Lage using the Inoue method (Sambrook & Russell (2006)) was thawed on ice before adding 1µl of the BP reaction mix. The vial was then mixed gently and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 30 seconds before being placed back on ice for 2 further minutes. 250µl of LB medium was then added and the vial shaken at 225rpm for 1 hour at 37°C. Cells were then plated onto 50µg/ml Kanamycin LB agar plates and incubated overnight at 37°C. Individual colonies were used for small overnight cultures. A pipette tip was used to transfer a colony into 5ml 50µg/ml Kanamycin LB medium. Cultures were shaken overnight at 225rpm and 37°C. Plasmids were then isolated from cultures by the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). Plasmids were purified and concentration measured as for PCR products.

8.12.4 LR reaction

50-150ng of entry vector was added to an equal quantity of pBid-UASC-GV (1-7µl) (Figure 8.2) (Wang et al 2012) and TE buffer added up to 8µl. LR Clonase II was then thawed for two minutes, vortexed and added to the mix. This was in turn vortexed then incubated at 25°C overnight. The following day the reaction was stopped by the addition of 1µl Proteinase K and incubation at 37°C for 10 minutes. The resultant plasmids were then transformed, cultured and isolated as for the BP reaction. However the LB broth and agar contained 100µg/ml Ampicillin rather than Kanamycin. After isolation of plasmids by the GeneJET Plasmid Miniprep Kit
Figure 8.1 PDonr221 Vector (Map generated by Serial Cloner)

Figure 8.2 pBID-UASC-GV Vector (Map generated by Serial Cloner)
(Thermo Fisher Scientific) a Phenol-Chloroform extraction was carried out. Plasmid DNA was resuspended up in water rather than TE in the final step.

**8.12.5 Injection and transformant isolation**

A cage was set up from a cross containing Nos-integrase (yw P(Nos-integrase-NLS)) and an attp40 landing site (yw; p((CaryP)attp40)). On the day of injection the cage plate was changed every 45 minutes. Embryos were washed off of the plate and dechorionated in 50% bleach for 4 minutes. They were then washed and lined up on squares of red wine agar. Embryos were then transferred to a cover clip by double-sided sticky tape. Cover slips were attached to slides by halocarbon oil and then dried for 8 minutes on pre-heated silica gel pellets. The embryos were then covered with a strip of halocarbon oil (700). Syncytial blastoderm stage embryos were injected with 500ng/µl DNA. Once injected the cover slip was removed and kept in halocarbon oil (95) for two days (overnight at 18°C and then 21°C). Larvae were then collected and placed in separate vials of food. Transformants were identified by eye colour. A series of crosses and back crosses to w1118 and Pin/Cyo balancer flies allowed removal of the intergrase and the establishment of a balanced stock.

**8.13 Recombination**

CG6971::mVenus and CG31320::mVenus flies were crossed to CG31320\textsuperscript{KK102625}. Female virgins were isolated and crossed to males of the Pin/Cyo balancer line. CyO flies were isolated from the resultant progeny and again crossed to Pin/Cyo. The parent flies were then screened by PCR for mVenus and the RNAi construct.

**8.14 Microscopy**

Confocal microscopy of fixed tissue was carried out using a Zeiss LSM5 Pascal laser-scanning microscope. Bright field microscopy was conducted using an Olympus AX-70 Provis microscope. Live imaging was carried out at the using a Nikon A1R confocal microscope at the IMPACT imaging facility, Edinburgh.
8.15 Software & bioinformatics

Homologues were detected by the best reciprocal BLAST (either BLASTp or DELTA-BLAST) against the NCBI non-redundant protein sequence database. The exception to this was for *Chlamydomonas* homologues, which were detected by reciprocal BLAST against the Phytozome 9.1 genome database (Goodstein et al 2012). The DRSC integrative ortholog prediction tool was also used to identify homologues (Hu et al 2011). Both of these methods were also used to calculate % identity.

Repeat domains were found using HHrepID (Biegert & Soding 2008). ARD2 was used to predict α-solenoid structure (Fournier et al 2013). Predicted protein domains were detected using Pfam (Punta et al 2012), InterPro (Quevillon et al 2005) and Myhits Motifscan (Pagni et al 2007).

Binding motifs were detected by searching for consensus sequences using Gene pallete (Rebeiz & Posakony 2004). UCSC genome browser was also used to observe conserved sequences in *Drosophila* species (Kent et al 2002).

8.16 Statistics

All statistics were calculated using Graph pad prism 6. A D’Agostino’s normality test was first used to assess whether data was normally distributed. If it was then a parametric approach was taken, if the data was not normally distributed then the statistical significance was calculated in a non-parametric manner. Parametric tests used were the students T test or ANOVA followed by a post-hoc test for comparing multiple groups. The parametric approaches taken were a Mann Whitney U test or a Kruskal-Wallis test followed by a Dunn’s test for multiple comparisons. A Fisher’s exact test was used to compare distributions.

8.17 TEM

Tissues for TEM were dissected form 2-day old flies and rinsed in 0.5% triton x-100. They were then fixed in 2.5% glutaldehyde and 2% paraformaldehyde in a phosphate buffer. They were then post fixed in Osmium tetroxide and embedded in polybed 812.
resin. Ultrathin sections were then stained with aqueous uranyl acetate and lead citrate.
## Appendix

Table A1: Antibodies used during this study

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<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Mouse anti Futsch</td>
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<td>DSHB</td>
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<tr>
<td>Mouse anti NompC</td>
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<td>Gift from J Howard, Max Planck Institute Dresden</td>
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<td>Jackson Labs</td>
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<tr>
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<td>Adipogen</td>
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<td>Goat anti Rabbit 568</td>
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Table A1: DSHB – Developmental Studies Hybridoma Bank
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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>CG11253 5’ (Deletion detection)</td>
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<td>CG11253 3’1</td>
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<td>CG11253 3’2</td>
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<td>CG11253 3’3</td>
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<td>SV2- For identification of RNAi construct (VDRC)</td>
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<td>5’ CG11253 P element</td>
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Table A3: Antibodies used during this study

Table A3: DSHB – Developmental Studies Hybridoma Bank
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