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<td><strong>Authors(s)</strong></td>
<td>Sanders, Anna Antoinette Wilhelmina Maria</td>
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<tr>
<td><strong>Publication date</strong></td>
<td>2014</td>
</tr>
<tr>
<td><strong>Publisher</strong></td>
<td>University College Dublin. School of Biomolecular and Biomedical Science</td>
</tr>
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<td><strong>Link to online version</strong></td>
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Identification and Functional Characterisation of New Ciliary Base Proteins and Investigation of Diffusion Kinetics Across the Ciliary Transition Zone in Caenorhabditis elegans Roundworms

Anna Antoinette Wilhelmina Maria Sanders, MSc

The thesis is submitted to University College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy

UCD School of Biomolecular and Biomedical Sciences, Conway Institute
Head of school: Dr. Keith Murphy
Principal supervisor: Dr. Oliver E. Blacque

Doctoral Studies Panel:
Dr. Margaret McGee
Professor Jeremy Simpson

October 2014
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SUMMARY

Cilia are evolutionarily conserved microtubule based organelles extending from the surface of most cells serving important sensory and signalling functions. Defects in cilia cause a variety of disorders with overlapping phenotypes, termed ciliopathies. The ciliary base, acting as a ciliary gate, plays a key role in regulating ciliary protein composition and cilia-related signal transduction pathways, forming membrane and cytosolic diffusion barriers that prevent exchange between ciliary and non-ciliary compartments. The transition zone at the proximal ~1 μm of the axoneme is part of the ciliary base and ciliopathy proteins localising specifically to the transition zone form two functional modules (MKS and NPHP). Together, these modules are required for ciliogenesis, and are implicated in membrane diffusion barrier function in C. elegans. Active transport across the transition zone diffusion barrier is thought to be facilitated by intraflagellar transport. The protein composition of the ciliary base is not fully known and molecular mechanisms underlying the diffusion barriers are poorly understood.

The work presented in this thesis is focussed on the ciliary base and the transition zone in C. elegans ciliated sensory neurons. Specifically, Chapter III focusses on characterisation of a candidate ciliary component, K04F10.2. Exhaustive phenotypic analysis indicates that K04F10.2 serves ciliary functions. Consistent with a ciliary role, subtle IFT defects were observed, as was a functional interaction with the ciliopathy protein, Joubert Syndrome-associated ARL-13. The interactome of human KIAA0556 (K04F10.2 ortholog) was identified and subsequent analysis in worms revealed novel ciliary localisations and transport properties for RAB-28 and F47G4.5/KATNBL1.

Chapter IV describes the development of a Fluorescence Recovery After Photobleaching (FRAP)-based assay to validate the existence of a transition zone membrane diffusion barrier. The subsequent use of this assay to investigate the molecular mechanisms that establish and maintain this barrier reveals that various MKS- and NPHP-module components are differentially required for diffusion barrier integrity. Additionally, IFT components are required for active transport to overcome the transition zone membrane diffusion barrier. This FRAP assay is the first such described in a multicellular system and allows for in vivo investigation of exchange kinetics across the transition zone membrane diffusion barrier in real time.
STATEMENT OF ORIGINAL AUTHORSHIP

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.
Contributions

Throughout the work presented here, others have contributed data.

**Figure 3.5** TEM sample preparation and imaging was performed by Katarzyna Welzel, Imaging Core Facility, Conway Institute, University College Dublin, Ireland.

**Figure 3.10** Phylogenetic analysis was performed by John van Dam, Centre for Molecular and Biomolecular Informatics, UMC St. Radboud, Nijmegen, the Netherlands.

**Figure 3.11 and 3.12** Analysis was performed by Qianhao Lu, University of Heidelberg, Germany.

**Figure 3.13** TAP experiments were performed by Minh Nguyen and Lisette Hetterschijt, Radboud University Nijmegen, the Netherlands. Mass spectrometry analysis was performed by Karsten Boldt and Marius Ueffing, University of Tuebingen, Germany. Yeast two-hybrid analysis was performed by Erik de Vrieze, Radboud University Nijmegen, the Netherlands.

**Figure 3.20** TEM sample preparation and imaging was performed by Julie Kennedy, Conway Institute, University College Dublin, Ireland.

**Figure 4.3** Experiments were performed by Anita Wdowicz, Conway Institute, University College Dublin, Ireland.

**Figure 4.5** Transgenic strains were generated by Sebiha Cevik and Andrea Mullins, Conway Institute, University College Dublin, Ireland.
ACKNOWLEDGEMENTS

First and foremost I would like to express my gratitude to my thesis supervisor, Oliver Blacque. His guidance and enthusiasm throughout this project have helped me develop as a scientist and I have gained a new appreciation for academic research. Thank you for your support and encouragement to pursue new ideas and avenues of research.

I would also like to thank members of collaborating labs from the Syscilia consortium for their contributions to this work, and the Conway Imaging Core facility for their technical support.

I am very grateful to the past and present members of the Blacque lab. Rachel, Chris, Sebiha, and Oktay, thank you for helping me get settled in the lab and introducing me to the worms. Lara, Julie, Nils, Stefanie, Noémie, and Laura, thank you for all your help during this project, and for all of the fun times inside and out of the lab.

Finally, I’d like to thank my family, who have been very supportive of my choice to pursue a career in science and of my moving to Ireland to do so.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>a.u.</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>ADPKD</td>
<td>Autosomal dominant polycystic kidney disease</td>
</tr>
<tr>
<td>arCRD</td>
<td>Autosomal recessive cone-rod dystrophy</td>
</tr>
<tr>
<td>ARPKD</td>
<td>Autosomal recessive polycystic kidney disease</td>
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<tr>
<td>BB</td>
<td>Basal body</td>
</tr>
<tr>
<td>BBS</td>
<td>Bardet-Biedl Syndrome</td>
</tr>
<tr>
<td>CI</td>
<td>Chemotaxis index</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DA</td>
<td>Distal appendages</td>
</tr>
<tr>
<td>Daf</td>
<td>Dauer defective</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent resistant membrane</td>
</tr>
<tr>
<td>DS</td>
<td>Distal segment</td>
</tr>
<tr>
<td>Dyf</td>
<td>Dye-filling defective</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EM-CCD</td>
<td>Electron multiplying charge coupled device</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cell line</td>
</tr>
<tr>
<td>hFF</td>
<td>Human foreskin fibroblast cell line</td>
</tr>
<tr>
<td>IFT</td>
<td>Intralflagellar transport</td>
</tr>
<tr>
<td>IMCD3</td>
<td>Inner medullary collecting duct cell line</td>
</tr>
<tr>
<td>JBTS</td>
<td>Joubert Syndrome</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cell line</td>
</tr>
<tr>
<td>MKKS</td>
<td>McKusick-Kaufman Syndrome</td>
</tr>
<tr>
<td>MKS</td>
<td>Meckel-Gruber Syndrome</td>
</tr>
<tr>
<td>MS</td>
<td>Middle segment</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode growth medium</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NPHP</td>
<td>Nephronphthisis</td>
</tr>
<tr>
<td>PCD</td>
<td>Primary ciliary dyskinesia</td>
</tr>
<tr>
<td>PCM</td>
<td>Periciliary membrane</td>
</tr>
<tr>
<td>PCMC</td>
<td>Periciliary membrane compartment</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet derived growth factor alpha</td>
</tr>
<tr>
<td>RFX</td>
<td>Regulatory factor X</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Rod outer segment</td>
</tr>
<tr>
<td>RPE1</td>
<td>Retinal pigment epithelial cell line</td>
</tr>
<tr>
<td>sDA</td>
<td>Subdistal appendage</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SF-TAG</td>
<td>Streptavidin-FLAG tag</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TZ</td>
<td>Transition zone</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast two-hybrid</td>
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CHAPTER I

INTRODUCTION
1.1 **Overview of Cilium Structure, Function, and Formation**

Evolutionarily conserved, motile and immotile cilia (also termed flagella) are ubiquitous microtubule-based structures found on single cell organisms (Figure 1.1) such as *Chlamydomonas reinhardtii*, *Trypanosoma brucei*, and *Tetrahymena thermophila*, as well as on most cells of multicellular eukaryotes including commonly used model organisms *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, *Danio rerio*. Cilia are also present on the apical surface of most mammalian cells (Doroquez et al., 2014, Sengupta and Barr, 2014, Oh and Katsanis, 2012, Vincensini et al., 2011, Satir and Christensen, 2007).

Cilia are formed around microtubules extending from the cell body, surrounded by an extension of the plasma membrane, and can be compartmentalised into several canonical parts. At the ciliary base, the mother centriole-derived basal body is a symmetrical ring of nine microtubule triplets, consisting of A-, B-, and C-tubules. The basal body is anchored to the surrounding periciliary membrane via distal appendages, also called transition fibres (Kobayashi and Dynlacht, 2011, Azimzadeh and Marshall, 2010, Benmerah, 2013). Also connected to the basal body are subdistal appendages, which appear to stabilise the organelle (Piel et al., 2000). The axoneme is formed by extension of A- and B-tubules from the basal body. Most motile cilia possess a central pair of microtubules (9+2 arrangement), radial spokes and dynein arms for flagellar beating and motility (Satir and Christensen, 2007, Louvi and Grove, 2011). Primary cilia possess nine doublet microtubules (9+0 arrangement). The axoneme can be subdivided into subcompartments, such as the proximal-most transition zone, the adjacent Inv (Inversin) compartment, and a distal and ciliary tip segment (Figure 1.2).

Due to their ubiquitous nature, motile and immotile cilia serve a plethora of functions. A single cell can possess multiple motile cilia that can generate fluid flow through coordinated beating, and can serve functions related to cell motility, sensory behaviours and signalling (Hirokawa et al., 2006, Nonaka et al., 2002, Watanabe et al., 2003, Morris and Scholey, 1997, Lechtreck et al., 2008). For example, motile cilia are found on
embryonic nodal cells, where fluid flow is required to establish left-right asymmetry (Hirokawa et al., 2006, Nonaka et al., 2002, Watanabe et al., 2003, Sen et al., 1999). Motile cilia are also found on tracheal cells and in protozoans such as *Chlamydomonas* and *Trypanosoma* that possess flagella for cell motility (Vincensini et al., 2011, Engel et al., 2012, Pazour et al., 1995, Lechtreck et al., 2009b). Primary cilia are signalling hubs that host components of sensory and developmental signalling pathways, such as Shh, Wnt, PDGFRα, EGFR, FGFR, IGF-1R, and TGF-β signalling pathways. Many of these components require ciliary localisation for signalling pathway regulation (Gao and Chen, 2010, Basten and Giles, 2013, Neugebauer et al., 2009, Tabler et al., 2013, Christensen et al., 2012, Clement et al., 2013, Lee and Gleeson, 2011, Goetz and Anderson, 2010).

Cilia form during G0/interphase, and ciliogenesis has been observed to occur via two pathways in mammalian cells (Reiter et al., 2012, Pedersen et al., 2008, Sorokin, 1962). In the first pathway, a ciliary vesicle docks to the mother centriole, and the axoneme begins to form at the distal end of the mother centriole. Following this, the ciliary vesicle can fuse with secondary vesicles, while migrating to the cell surface with the centriole. When the centriole and growing axoneme surrounded by a membrane sheath, reach the cell surface, the ciliary vesicle merges with the plasma membrane. The sheath membrane forms the ciliary pocket, and the cilium can elongate. In the second pathway, the basal body (matured mother centriole) docks to the plasma membrane directly and the axoneme extends from the cell surface (Sorokin, 1962, Sorokin, 1968). Axonemal extension relies on cytosolic and membrane trafficking pathways, notably intraflagellar transport (IFT), which delivers ciliary cargo molecules to the cilium and transports ciliary turnover products to the cell body (Rosenbaum and Witman, 2002).
Figure 1.1 Electron micrographs of primary and motile cilia. Top box: primary cilia possess a 9+0 microtubule arrangement. Examples of mammalian primary cilia include olfactory cilia (A, scanning electron micrograph, SEM) and rod outer segment (ROS) photoreceptors (C, transmission electron micrograph, TEM). In retinal pigment epithelial (RPE1, E, TEM) and inner medullary collecting duct (IMCD3) cell lines (B, SEM) ciliation can be induced by serum starvation. C. elegans (D) possess 60 primary cilia, a longitudinal TEM cross-section is shown. Bottom box: SEM images show motile cilia, most of which possess a 9+2 microtubule arrangement. Mammalian examples are nodal cilia (F, possess 9+0 MT arrangement), tracheal cilia (G). Protists with flagella include Trypanosoma brucei (G) and Chlamydomonas reinhardtii (H). All arrows point to the base of the cilium. Images adapted from (McIntyre et al., 2012, Vincensini et al., 2011, Benmerah, 2013, Ishikawa and Marshall, 2011, Rosenbaum and Witman, 2002, Doroquez et al., 2014, Frederick and Watt, 2011, Louvi and Grove, 2011)
Figure 1.2 Overview of canonical *C. elegans* and human primary cilium compartments. Left: Nematode cilia lacking a basal body/centriole, consist of a ciliary base/periciliary membrane compartment (PCMC), followed distally by the transition zone, where Y-links connect nine microtubule doublets to the membrane. In cilia with a bipartite axoneme, adjacent to the transition zone lies the middle segment that consists of nine microtubule doublets (A and B tubules). The B tubule terminates at the middle-distal segment border, and nine microtubule singlets (A tubules) form the distal segment. Right: mammalian canonical ciliary compartments. At the ciliary base lies the basal body, consisting of nine microtubule triplets (A-, B-, and C-tubules). The A- and B-tubules extend distally and form the adjacent transition zone, followed distally by the axoneme, which in a subset of ciliated mammalian cells includes the Inv (inversin) compartment and a distal segment. DA; distal appendage. sDA; subdistal appendage. Image from (Blacque and Sanders, 2014).
1.2 CILIOPATHIES

Due to the ubiquitous presence and diversity of cilia, defects in ciliary structure and function underlie a group of genetic disorders termed ciliopathies, which are rare individually, but taken together the frequency of these disorders approaches that of a common disorder like Down syndrome (Davis and Katsanis, 2012). Ciliopathies exhibit overlapping phenotypes, including mental retardation, hydrocephalus, situs inversus, polydactyly, bone deformation, retinal degeneration, cystic kidneys, and obesity (Fliegauf et al., 2007, Tobin and Beales, 2009, Davis and Katsanis, 2012). Because primary cilia are important signalling hubs during development, many of these phenotypes are related to defective signalling pathway activity. For example, localisation of sonic hedgehog (Shh) pathway components is spatiotemporally regulated by the cilium. Upon binding to the Hh ligand, the Patched receptor exits the cilium, allowing Smoothened (Smo) to enter the cilium, promoting Gli2 and Gli3 activation by antagonizing activity of Sufu, a Gli2 and Gli3 repressor that also localizes to the ciliary tip. Subsequently, activated Gli transcription factors are removed from the cilium and translocate to the nucleus to induce transcription of target genes (Goetz and Anderson, 2010, Haycraft et al., 2005, Huangfu et al., 2003, Murcia et al., 2000, Liu, 2005, Huangfu and Anderson, 2005).

To date, more than 16 genetic disorders have been characterized as ciliopathies. These include autosomal dominant and recessive Polycystic Kidney Disease (ADPKD and ARPKD), Primary Ciliary Dyskinesia (PCD), Nephronophthisis (NPHP), Joubert Syndrome (JBTS), Meckel Gruber Syndrome (MKS), Bardet-Biedl Syndrome (BBS), Leber Congenital Ameurosis (LCA), Senior-Loken Syndrome (SLS), Orofacialdigital Syndrome (OFD), a number of Short Rib Polydactylies (SRPs), Alström Syndrome (ALMS), Usher Syndrome (US), and McKusick-Kaufman Syndrome (MKKS) (Hildebrandt et al., 2011, Davis and Katsanis, 2012, Zaghloul and Katsanis, 2010, Huber and Cormier-Daire, 2012, Cardenas-Rodriguez and Badano, 2009, Lobo et al., 2014). The underlying genes typically encode proteins that localise to ciliary compartments, serving functions relating to cilium formation, maintenance, transport, and signalling.
Ciliopathy phenotypes may be linked to protein localisation and function in specific ciliary compartments. For example, mutations in proteins that localise to the transition zone can lead to Nephronophthisis (NPHP), Joubert Syndrome (JBTS), and Meckel-Gruber Syndrome (MKS), in ascending order of severity (Sang et al., 2011, Hildebrandt and Zhou, 2007, Hildebrandt et al., 2009, Hildebrandt et al., 2011). Indeed, a number of these proteins are thought to form two multimeric functional modules (MKS/JBTS and NPHP) in the transition zone (Sang et al., 2011, Huang et al., 2011, Garcia-Gonzalo et al., 2011, Williams et al., 2011). Additionally, a functional module of NPHP and JBTS proteins has been identified at the basal body and Inversin compartment (Sang et al., 2011). However, various ciliopathies, such as Bardet Biedl Syndrome (BBS) can be caused by mutations in proteins that form a functional module (BBSome) that localises to different compartments, indicating that a clear correlation between protein localisation and ciliopathy is not always present (M'Hamdi et al., 2014). Additionally, non-ciliary functions have been identified for a number of ciliary proteins, suggesting that cilia may not be the only functional site for these proteins, and disease phenotypes could be due to a non-ciliary defect (Delaval et al., 2011, Robert et al., 2007, Finetti et al., 2009, Follit et al., 2006, Deane et al., 2001, Dawe et al., 2007).

Ciliopathies are allelic disorders with large genotypic overlap. For example, patients diagnosed with Bardet Biedl Syndrome can possess mutations in genes previously associated with Nephronophthisis, Joubert Syndrome, and Meckel-Gruber Syndrome (M'Hamdi et al., 2014, Davis and Katsanis, 2012, Zaghloul and Katsanis, 2010), and mutations in CEP290 have been identified in patients diagnosed with Joubert Syndrome, Senior-Loken Syndrome, Nephronophthisis, Bardet Biedl Syndrome, Meckel-Gruber Syndrome, and Leber Congenital Ameurosis (Zaghloul and Katsanis, 2010, Davis and Katsanis, 2012, Tory et al., 2007, den Hollander et al., 2006, Leitch et al., 2008, Baala et al., 2007, Sayer et al., 2006, Valente et al., 2006). Therefore, translation of genotypes to specific phenotypes is challenging, although the data indicates close functional connections between the various different ciliopathy genes and modules.
Autosomal dominant polycystic kidney disease (ADPKD) is the most common, potentially lethal, dominant genetic disorder, with a prevalence between 1:400 and 1:1000 live births (Torres et al., 2007, Bergmann, 2014). Autosomal recessive PKD (ARPKD) is less common, more severe and presents in childhood, while ADPKD is usually adult onset. Symptoms include enlarged kidneys, development of cysts in distal tubules and collecting ducts (ARPKD), or throughout the kidney (ADPKD), and in all ARPKD cases and some ADPKD cases liver deformations and cysts are present (Bergmann, 2014). ADPKD-causing mutations have been identified in \( PKD1 \) (80-85% of patients) and \( PKD2 \) (15-20%) genes encoding a dimeric membrane receptor complex that is involved in renal tubulogenesis (Qian et al., 1997, Tsiokas et al., 1997, Harris and Torres, 2009). PC1 and PC2 (polycystin-1 and -2), the protein products encoded by \( PKD1 \) and \( PKD2 \), respectively, form a heterodimeric membrane complex that localises to the cilium and is thought to regulate signal transduction in response to mechanosensation (Low et al., 2006, Nauli et al., 2003). PC1 functions as a G-protein coupled receptor (GPCR) and has been shown to activate G-proteins (Parnell et al., 1998, Delmas et al., 2002). PC2 is a cation channel sensitive to \( \text{Ca}^{2+} \) and regulates PC1-mediated signal transduction pathway activation through C-terminal interactions with PC1. Mutations in PC1 or PC2 lead to dysfunctional downstream signal pathway activation and eventually to development of cystic kidneys (Delmas et al., 2002, Low et al., 2006, Parnell et al., 1998, Shillingford et al., 2006, Vassilev et al., 2001, Qian et al., 1997, Tsiokas et al., 1997). Additionally, PC1 and PC2 are involved in modulation of downstream canonical (PC1 and PC2) and non-canonical (PC1) Wnt signalling (Lancaster and Gleeson, 2010).

**Nephronophthisis (NPHP), Joubert Syndrome (JBTS), Meckel-Gruber Syndrome (MKS)**

NPHP, JBTS, and MKS are three related ciliopathies with overlapping genetic causes and phenotypes, and varying severity, ranging from kidney cysts leading to renal failure (NPHP) to neonatal lethality in MKS (Sang et al., 2011, Davis and Katsanis, 2012, Lee and Gleeson, 2011, Otto et al., 2011). Nephronophthisis is an autosomal recessive cystic
kidney disorder, and represents the most common genetic cause for end stage renal disease early in life (Hildebrandt et al., 2009, Hildebrandt and Zhou, 2007, Hildebrandt et al., 2011). To date, mutations in 17 genes are shown to cause Nephronophthisis. Patients typically present with kidney cysts; however, unlike in polycystic kidney disease, kidney size is normal or reduced (Hildebrandt et al., 2011, Davis and Katsanis, 2012, Hoff et al., 2013, Failler et al., 2014). Other phenotypes observed in Nephronophthisis patients include retinal degeneration, mental retardation, cerebellar hypoplasia, and hepatic disease (Davis and Katsanis, 2012, Hildebrandt et al., 2011). Joubert Syndrome patients present with retinal degeneration, cystic kidneys, liver disease, polydactyly, *situs inversus*, obesity, and mental retardation and ataxia, due to cerebellar hypoplasia characteristically presented with the molar tooth sign (Hildebrandt et al., 2011, Davis and Katsanis, 2012, Doherty, 2009). Meckel-Gruber Syndrome is a neonatal lethal syndrome, characterized by cleft palate, encephalocele, cystic kidneys, liver disease, polydactyly, *situs inversus*, and skeletal defects (Barker et al., 2014b, Davis and Katsanis, 2012, Hildebrandt et al., 2011).

Mutations in *NPHP1, INVS, NPHP3, NPHP4, NPHP5, CEP290, GLIS2, RPRGIP1L, NEK8, SDCCAG8, TMEM67/MKS3, IFT144, TTC21B, CEP83, CEP164, ANKS6* and *AHI1* cause Nephronophthisis (Davis and Katsanis, 2012, Hoff et al., 2013, Otto et al., 2011, Failler et al., 2014, Hildebrandt et al., 2011, Davis et al., 2011). Joubert Syndrome can be caused by mutations in *AHI1, ARL13B, B9D1, CC2D2A, CEP41, CEP290, INPP5E, KIF7, MKS1, NPHP1, OFD1, RPRGIP1L, TCTN1, TCTN2, TCTN3, TMEM67/MKS3, TMEM138, TMEM216, TMEM231, TMEM237, and TTC21B* (Davis and Katsanis, 2012, Davis et al., 2011, Hildebrandt et al., 2011, Bachmann-Gagescu et al., 2012, Doherty, 2009, Huang et al., 2011, Lee et al., 2012, Romani et al., 2014, Srour et al., 2012, Huppke et al., 2014). To date, 18 genes have been found to be mutated in MKS patients: *B9D1, B9D2, BBS2, BBS4, BBS6, BBS10, CC2D2A, CEP290, KIF7, MKS1, NPHP3, RPRGIP1L, TCTN2, TCTN3, TMEM67/MKS3, TMEM216, TMEM231, and TTC21B*, and *WDPCP* (Davis and Katsanis, 2012, Davis et al., 2011, Hildebrandt et al., 2011, Shaheen et al., 2013, Valente et al., 2010, Smith et al., 2006, Kim et al., 2010b, Huppke et al., 2014). Thirteen identified genes cause at least two of these ciliopathies, four of which have been implicated in all three diseases, highlighting the genotypic overlap and functional relatedness of these three ciliopathies. Additionally, mutations in all genes encoding known components of the transition zone-associated MKS/JBTS module have
been identified in MKS and JBTS patients (Huang et al., 2011, Sang et al., 2011, Garcia-Gonzalo et al., 2011, Cardenas-Rodriguez and Badano, 2009, Davis and Katsanis, 2012, Chih et al., 2012, Shaheen et al., 2013). Genotypic and phenotypic overlap between these three ciliopathies is further emphasized by identification of several mutations in MKS/JBTS module components in NPHP: *NPHP3* null mutations have been identified in MKS-like patients, and JBTS patients have been identified with *NPHP1* mutations, though these may be aggravated by additional mutations in *AHI1* or *CEP290* (Bergmann et al., 2008, Soliman et al., 2012, Tory et al., 2007, Davis and Katsanis, 2012).

A number of distinct functional modules have been identified that include many of the proteins encoded by these genes. Several NPHP-, JBTS-, and MKS-related proteins interact and are thought to form functional modules in the ciliary transition zone and in the adjacent Inversin (Inv) compartment. NPHP5/NPHP6 complexes localize to the centrosome, ANKS6/INVS/NPHP3/NEK8 localize to the Inv compartment, and NPHP1/NPHP4/RPGRIP1L form the NPHP module in the transition zone (Hoff et al., 2013, Sang et al., 2011). Additionally, the MKS/JBTS module in the transition zone is thought to be formed by at least 14 proteins: MKS1, TMEM216, TMEM67, RPGRIP1L, CC2D2A, B9D1, B9D2, TMEM237, AHI1, TCTN1, TCTN2, TCTN3, TMEM17, and TMEM231 (Garcia-Gonzalo and Reiter, 2012, Reiter et al., 2012, Sang et al., 2011, Huang et al., 2011, Barker et al., 2014b). Overlapping localisation patterns of NPHP-, MKS-, and JBTS-proteins suggests a strong role for the transition zone in maintaining cilium function. However, in mammalian cell culture, a number of transition zone-associated proteins localise at non-ciliary sites, such as cell-cell contacts, the plasma membrane, and cytoplasm. Additionally, NPHP4 and SDCCAG8 can localise to the photoreceptor inner segment, suggesting dysfunction of cilia-related roles may not be responsible for the full spectrum of phenotypes observed in NPHP, MKS, and JBTS patients (Dawe et al., 2007, Cui et al., 2013, Patil et al., 2012, Sang et al., 2011).
Bardet-Biedl Syndrome (BBS)

Bardet-Biedl Syndrome is an extensively studied ciliopathy first identified in 1866, characterised by mental retardation, polydactyly, obesity, hypogonadism, cystic kidneys, and retinal degeneration. Hepatic dysfunction, *situs inversus*, diabetes mellitus, hearing loss, and craniofacial defects have been observed in a subset of patients (Davis and Katsanis, 2012, Zaghloul and Katsanis, 2009). Many mutations causing BBS affect subunits of the BBSome, a multimeric module involved in active transport pathways such as intraflagellar transport (IFT, (Wei et al., 2012, Jin et al., 2010, Nachury et al., 2007, Zaghloul and Katsanis, 2009, Blacque and Leroux, 2006, Ansley et al., 2003)). However, several alleles have been identified in NPHP-, JBTS-, and MKS-associated genes, illustrating the genetic heterogeneity in these disorders (M'Hamdi et al., 2014, Davis and Katsanis, 2012). MKKS presents several phenotypes overlapping with BBS; polydactyly, genital abnormalities, and congenital heart defects (Stone et al., 2000). Suspected null mutations in the *MKKS* gene can also cause BBS, emphasizing the genotypic and phenotypic overlap between ciliopathies (Katsanis et al., 2000).

BBS proteins were first characterised as ciliary proteins involved in cilia structure and function, forming a multimeric module involved in IFT (Kim et al., 2004, Blacque et al., 2004, Ansley et al., 2003). A core of BBS-associated proteins forms the BBSome (BBS1/2/4/5/7/8/9) that localises to the ciliary base and along the entire axoneme, and may function in assembly and stabilisation of IFT particles, assisted by other BBS proteins including chaperonin-like BBS6, BBS10, BBS12, and BBIP1 (Lechtreck et al., 2009b, Wei et al., 2012, Zhang et al., 2012b, Nachury et al., 2007, Seo et al., 2010). Additionally, BBSome components are involved in membrane biogenesis and polarised transport of vesicles to the ciliary base (Nachury et al., 2007, Jin et al., 2010). However, BBS proteins are also involved in cell cycle progression, microtubule stability, and retrograde intracellular transport pathways, suggesting Bardet-Biedl Syndrome phenotypes may not be caused exclusively by ciliary dysfunction (Jin et al., 2010, Kaplan et al., 2010, Kim et al., 2004, Loktev et al., 2008, Kaplan et al., 2012, Blacque and Leroux, 2006, Yen et al., 2006).
1.3 CILIARY TRANSPORT

Cilia lack protein synthesis machinery and therefore rely on several intracellular transport mechanisms to deliver proteins required for cilium structure and function. Firstly, an active transport mechanism called intraflagellar transport (IFT) appears to traffic proteins into and out of the cilium. Secondly, polarised vesicle transport mechanisms, including endo- and exocytosis, are used to target proteins to the ciliary base. Thirdly, lateral transport can deliver membrane (-associated) cargo to the cilium (Hsiao et al., 2012, Sung and Leroux, 2013).

1.3.1 INTRAFLAGELLAR TRANSPORT

Intraflagellar transport (IFT) is a bidirectional motility travelling from the ciliary base to tip (antero) and from tip to base (retro) to deliver ciliary proteins to the axoneme or ciliary membrane and recycle turnover products to the cell body (Rosenbaum and Witman, 2002, Blacque et al., 2008, Ishikawa and Marshall, 2011, Qin et al., 2004, Hao et al., 2011b, Wren et al., 2013, Cevik et al., 2013, Marshall et al., 2005). Initially discovered in *Chlamydomonas*, intraflagellar transport is conserved across ciliated species and has been extensively studied *in vivo* in multiple systems (Kozminski et al., 1993, Buisson et al., 2012, Orozco et al., 1999, Signor et al., 1999a, Absalon et al., 2008). The IFT assembly components accumulate at the ciliary base or transition fibres and are present along the axoneme (Deane et al., 2001, Buisson et al., 2012, Ou et al., 2007). These assemblies consist of kinesin-2 motors for anterograde transport, a cytoplasmic dynein complex for retrograde transport, as well as IFT-A, IFT-B, and BBSome complexes, and associated cargo molecules.

ANTEROGRADE IFT

Anterograde intraflagellar transport is driven by kinesin-2 motors, which are required for cilium formation. Travelling together with this motor are the cytoplasmic dynein motor
complex, and IFT-A and –B complexes (Pedersen and Rosenbaum, 2008). In *Chlamydomonas*, these assemblies form 700 nm ‘trains’, consisting of repetitive units suggestive of multiple copies of motors and associated transport machinery (Pigino et al., 2009, Kozminski et al., 1993, Kozminski et al., 1995, Dentler, 2005). From ciliary base to distal tip, anterograde IFT is powered by canonical heterotrimeric kinesin-II that consists of two heterodimerising motor proteins, KIF3A and KIF3B, and an accessory protein, KIFAP3 (or KAP3; kinesin associated protein (Scholey, 1996, Cole et al., 1993)). A variation in the anterograde pathway is observed in some nematode cilia, where kinesin-II cooperates with an accessory kinesin-2 motor (OSM-3/KIF17) to drive intraflagellar transport along proximal ciliary regions (Ou et al., 2005, Snow et al., 2004).

The IFT-B complex is thought to function with the kinesin-2 motor(s) during the anterograde phase of transport. This complex is required for ciliogenesis and is thought to interact with ciliary cargo transport to the cilium via intraflagellar transport. Loss of most IFT-B components, except for IFT25, leads to complete loss of intraflagellar transport and severe ciliary defects (Absalon et al., 2008, Deane et al., 2001, Follit et al., 2006, Fujiwara et al., 1999, Perkins et al., 1986, Haycraft et al., 2003, Haycraft et al., 2001, Kobayashi et al., 2007, Blacque et al., 2005, Pathak et al., 2007, Ishikawa et al., 2014, Hao et al., 2011b, Cevik et al., 2013, Ou et al., 2007, Bell et al., 2006, Murayama et al., 2005, Efimenko et al., 2006, Bacaj et al., 2008, Keady et al., 2012, Huet et al., 2014). In *Chlamydomonas* and mice the IFT-B complex consists of at least 15 subunits: IFT25, IFT27, IFT70, IFT46, IFT88, IFT52, IFT22, IFT74, IFT81, IFT80, IFT57, IFT54, IFT172, and IFT20 form a ~710-760 kDa multimeric complex, together with CLUAP1 and TTC26 (Follit et al., 2009, Taschner et al., 2012, Cole et al., 1998). Within this complex a ~500 kDa core of nine proteins is thought to form, containing IFT25/27/70/46/88/52/22/74/81 that is stable at high salt concentrations (Lucker et al., 2005, Lucker et al., 2010, Taschner et al., 2011). Furthermore, direct interactions have been shown between IFT46/52/88/70, IFT25/27, and IFT81/72/74. Prior to IFT-B complex assembly, the IFT46/51/70/88 complex is thought to associate with IFT25/27, followed by association with IFT72/74/81. Once this core complex is formed, peripheral proteins can associate to fully form the IFT-B particle (Richey and Qin, 2012, Taschner et al., 2011, Lucker et al., 2005, Lucker et al., 2010). Other IFT-B proteins appear to function in the periphery of this core, dissociate at increasing salt concentrations, and are not required for formation of the core complex.
How the IFT-B complex drives anterograde IFT remains unknown, though *C. elegans* DYF-1/IFT70 has been shown to activate the OSM-3/KIF17 kinesin-2 motor (Ou et al., 2005).

**Retrograde IFT**

Retrograde IFT is powered by a cytoplasmic dynein 2-containing motor complex. TEM and videomicroscopy studies in *Chlamydomonas* have shown that retrograde IFT ‘trains’ typically move faster than anterograde trains, are more electron dense, and shorter, measuring 250 nm (Pigino et al., 2009, Engel et al., 2012). Multiple subunits of IFT-dynein have been identified, including the dynein heavy chain 1b motor, as well as multiple light, light-intermediate, and intermediate chains (Pfister et al., 2006). Heavy chain subunits contain the motor domains, and intermediate and light chains may facilitate cargo specificity (Karcher et al., 2002). However, the cytoplasmic dynein complex is poorly understood and not all subunits may be essential to its activity. In *C. elegans* a cell-specific secondary dynein complex may function in retrograde IFT (Hao et al., 2011a).

The IFT-A complex is thought to function with IFT-dynein during the retrograde phase of transport. Evidence for this interaction comes from biochemical studies showing IFT-A components cofractionate with cytoplasmic dynein, but not with kinesin II or IFT-B components, and in IFT-A mutants, IFT motors and associated particles accumulate at the ciliary tip (Rompolas et al., 2007, Ou et al., 2007, Qin et al., 2011). The ~550 kDa IFT-A complex is formed by at least six subunits: IFT140, IFT122, IFT144, IFT121, IFT139, and IFT43, of which IFT140, IFT122, and IFT144 may form a ‘core’ complex (Taschner et al., 2012, Cole et al., 1998, Mukhopadhyay et al., 2010). Consistent with a role in retrograde transport, removal of IFT-A proteins typically results in less severe phenotypes than those observed in IFT-B mutants. Indeed, most IFT-A mutants can form cilia and can exhibit some anterograde transport, but no retrograde transport is observed (Ishikawa and Marshall, 2011, Blacque et al., 2008, Perkins et al., 1986, Schafer et al., 2003, Absalon et al., 2008, Cevik et al., 2013, Tsao and Gorovsky, 2008, Bell et al., 2006, Efimenko et al., 2006, Wicks et al., 2000, Blacque et al., 2006).
IFT regulators

In *C. elegans* and *Chlamydomonas* BBS proteins are implicated in intraflagellar transport, either as cargo (*Chlamydomonas*), or to facilitate associations of the two kinesin-2 motors, as well as IFT-A and -B complexes (*C. elegans*) (Wei et al., 2012, Ou et al., 2007, Lechtreck et al., 2009b). The BBSome associates with the IFT particle and plays important roles in assembly and function of IFT ‘trains’ at the ciliary base and tip (Wei et al., 2012, Blacque et al., 2004, Ou et al., 2005, Lechtreck et al., 2009b). Components of the BBSome co-localise with intraflagellar transport proteins and undergo IFT at similar speeds in wild type systems (Ou et al., 2005, Lechtreck et al., 2009b). Experiments in *C. elegans* and *Chlamydomonas* suggest that the BBSome may not be directly required during transport, but rather is required at the ciliary base and tip for association of complexes to form the IFT assembly (Wei et al., 2012, Lechtreck et al., 2009b). However, in systems with only one ciliary kinesin motor, the BBSome may function as an adaptor facilitating interactions between IFT proteins and ciliary cargo molecules (Lechtreck et al., 2009b, Berbari et al., 2008, Jin et al., 2010).

Intraflagellar transport is also regulated by various other proteins, such as ARL-13/ARL13B and ARL-3, which are required in *C. elegans* for IFT assembly and stability. In mutants of these genes, intraflagellar transport complexes and motors dissociate in the middle segment, with IFT-A and -B complexes travelling with kinesin II, and OSM-3 travelling alone (Cevik et al., 2010, Li et al., 2010). Stability of the IFT assembly is also regulated by GPA-3 (G-protein α subunit) and SQL-1 (GMAP210 orthologue), and in mutants of these genes kinesin II and OSM-3 kinesin motors are uncoupled in the middle segment (Broekhuis et al., 2013, Burghoorn et al., 2010). In mammalian cells FBF1 is required for ciliogenesis and nematode DYF-19/FBF1 is required for IFT assemblies to enter the cilium through interactions with DYF-11/IFT54. In *dyf-19* mutants IFT-B proteins and OSM-3/KIF17 kinesin accumulate at the ciliary tip, while IFT-A proteins, kinesin II and dynein subunits fail to enter the cilium (Wei et al., 2013). Undocking of kinesin II from the IFT assembly is mediated by MAP kinase DYF-5 in worms, and this protein is also required for docking of OSM-3/KIF17 kinesin onto IFT particles (Burghoorn et al., 2007), and DYF-1 regulates activity of OSM-3 (Ou et al., 2005).
Since its discovery in *Chlamydomonas*, IFT has been suggested to function as a transport mechanism for ciliary cargo, including structural subunits of the axoneme and proteins that function in the organelle (e.g. signalling molecules) (Kozminski et al., 1993). Proteins that require intraflagellar transport for ciliary localisation, but are not required for intraflagellar transport to take place, are defined as ciliary cargo. In *Chlamydomonas*, two structural subunits, DRC4 (dynein regulatory complex 4) and radial spokes have been identified as ciliary cargo molecules that are associated with anterograde and retrograde intraflagellar transport machineries (Qin et al., 2004, Wren et al., 2013). Radial spoke precursor complexes are assembled in the cell body prior to ciliary entry. Then, these 12S complexes are transported to the flagellar tip via intraflagellar transport, where the intact 20S radial spoke is assembled. Following turnover from the axoneme, the radial spokes are removed from the flagellum via retrograde transport. In *pf14* mutants lacking radial spokes, dynein intermediate chain FAP133/WDR34 localises to flagella, similar to wild type, suggesting that intraflagellar transport does not require radial spokes, thus making radial spokes true IFT cargo (Rompolas et al., 2007). DRC4 flagellar entry can be mediated by intraflagellar transport and can occur via diffusion, though at a lower frequency. In intraflagellar transport mutants, DRC4 can only enter cilia via diffusion. DRC4 moves together with IFT20 in flagella, but is not present on all IFT assemblies (Wren et al., 2013). However, it is unknown whether DRC4 is required for intraflagellar transport to take place, thus, it is not confirmed that this protein is a true ciliary cargo molecule (Bower et al., 2013, Rupp and Porter, 2003, Wren et al., 2013).

Tubulin subunits interact with IFT74/81 dimers in mammalian cells, and this interaction is required for ciliogenesis, suggesting tubulin subunits are transported to tips of growing axonemes via IFT (Bhogaraju et al., 2013). Indeed, in *C. elegans*, ciliary cargoes include tubulin isotypes TBA-5 and TBB-4 that require IFT for ciliary delivery to maintain ciliary length (Qin et al., 2004, Hao et al., 2011b, Wren et al., 2013). Also, ciliary localisation of OSM-9, OCR-2, CHE-12, and PKD-2/TRPP2 is dependent on intraflagellar transport (Qin et al., 2005, Qin et al., 2001, Bae et al., 2006, Jenkins et al., 2006, Mukhopadhyay et al., 2010, Bacaj et al., 2008). However, PKD-2 does not require IFT for ciliary targeting, but
ciliary abundance of PKD-2 may be regulated by IFT (Bae et al., 2006). Other ciliary cargo molecules include rhodopsin and GC1 in mouse photoreceptors; SSTR3 (somatostatin receptor-3) and MCHR1 (melanin concentration hormone receptor-1) G-protein coupled receptors, and olfactory CNG (cyclic nucleotide gated) channels in mammalian cells (Jenkins et al., 2006, Mukhopadhyay et al., 2010, Trivedi et al., 2012).

Identifying intraflagellar transport cargo molecules is technically challenging, as IFT mutants possess severe ciliary phenotypes, or lack cilia completely. For example, Shh signalling is dysfunctional in intraflagellar transport mutants (Liem et al., 2012, Zhang et al., 2012a, Ocbina and Anderson, 2008). This does not necessarily mean that components of this pathway are IFT cargoes, because the phenotype could be due to a defect in ciliogenesis or maintenance of cilium integrity rather than a specific Shh pathway-related defect.

Cooperation between multiple mechanisms is required for efficient targeting and delivery of ciliary cargo. These mechanisms include post-Golgi trafficking to the ciliary base, IFT complex assembly and association with cargo molecules, ciliary gate function regulating ciliary entry and exit, and intraflagellar transport in the cilium. The far-reaching synergy between these processes is highlighted in targeting of opsin to outer segments (highly modified cilia) in zebrafish photoreceptors. First, opsin is transported from the Golgi to the base of the outer segment via post-Golgi vesicles, which fuse with a specialised domain region of the rod inner segment (Deretic and Papermaster, 1991, Deretic, 1997). Then, ciliary localisation of axonemal Inversin/NPHP-2 requires interactions between IFT-B subunit Fleer/DYF-1 and transition zone protein B9D2/MKSR-2. Finally, Inversin interacts with NPHP-5 and B9D2/MKSR-2 in these cells to facilitate ciliary transport of opsin, but not other photoreceptor membrane proteins (Zhao and Malicki, 2011, Keady et al., 2011).
1.3.2 Vesicle trafficking

Upstream of IFT-mediated delivery of proteins within the cilium, membrane trafficking pathways such as endo- and exocytosis are involved in transport of ciliary vesicles and associated cargo. Ciliary membrane cargo may be sorted at post-Golgi sites into vesicles, which travel to the ciliary region and subsequently fuse with the periciliary membrane (Follit et al., 2006, Papermaster et al., 1985). Endocytic events at the ciliary base are implicated in ciliary membrane recycling and cilium-based signalling (Kaplan et al., 2012, Hu and Wittekind, 2007, Olivier-Mason et al., 2013, Molla-Herman et al., 2010, Clement et al., 2013).

In mammalian cell culture, IFT20 dynamically exchanges between the Golgi and the cilium, and ciliary levels of polycystin-2 are reduced in cells with moderate IFT20 knockdown, suggesting that IFT20 is required for ciliary targeting of membrane proteins (Follit et al., 2006, Keady et al., 2011). Additionally, in C. elegans, PKD-2, CHE-11 and ODR-10 are found in dendritic vesicles moving towards cilia (Dwyer et al., 2001, Kaplan et al., 2010, Bae et al., 2006).

Components of the exocytic pathway have been identified in targeting vesicles to the ciliary base (Das and Guo, 2011). Ciliary targeting and transport of rhodopsin and polycystin-1 require RAB11, FIP3, ARF4, and ASAP1 (ARF4 GTPase activating protein) that sorts ciliary cargo at the trans-Golgi network (TGN) (Mazelova et al., 2009, Ward et al., 2011, Wang et al., 2012). ARF4 recognizes ciliary targeting sequence motif VxPx (or RVxP), which has been identified in polycystin-1 and -2, rhodopsin, cyclic nucleotide gated channel subunit 1b (CNG1b) and nematode ARL-13/ARL13B (Mazelova et al., 2009, Geng et al., 2006, Jenkins et al., 2006, Kaplan et al., 2012, Ward et al., 2011, Tam et al., 2000). However, this motif has not been identified in the ciliary targeting sequence (CTS) of fibrocystin, which requires ARF4 for efficient ciliary targeting, suggesting the VxPx motif is not the only ciliary targeting sequence motif (Follit et al., 2014, Follit et al., 2010). Indeed, other targeting motifs have been identified in ciliary targeting (Madhivanan and Aguilar, 2014), such as motifs consisting of a hydrophobic and basic residue; FR in
rhodopsin and nematode ODR-10, YR in nematode STR-1, FK in SSTR3 (somatostatin receptor 3) and HTR6 (serotonin receptor 6), and WR in Smoothened (Dwyer et al., 2001, Corbit et al., 2005, Madhivanan and Aguilar, 2014). ASAP1 interacts with activated ARF4 and RAB11, which then activates RABIN8 and subsequent RAB8 recruitment to the ciliary base to direct polarised membrane transport (Hattula et al., 2002, Westlake et al., 2011, Nachury et al., 2007). The RAB11/RABIN8/RAB8 cascade is required for ciliogenesis, and RAB11 and RAB8 can interact with exocyst components, also required for cilia formation (Nachury et al., 2007, Westlake et al., 2011, Chiba et al., 2013, Das and Guo, 2011, Hehnly et al., 2012, Rogers et al., 2004). Interestingly, RABIN8 also interacts with BBS1, an effector of ARL6/BBS3 and component of the BBSome, and is required for ciliary sorting of G protein coupled receptors SSTR3 and MCHR1 (Nachury et al., 2007, Jin et al., 2010, Berbari et al., 2008).

Endocytic pathway components play a role in ciliary membrane trafficking and recycling at the ciliary base, and clathrin coated pits (CCPs) have been found at the ciliary pocket of mammalian cells (Hu and Wittekind, 2007, Olivier-Mason et al., 2013, Molla-Herman et al., 2010). An example of endocytic pathway activity at the ciliary base is found in human foreskin fibroblasts (hFF), where TGF-β receptors accumulate at the ciliary base upon stimulation, where SMAD2/3 and ERK1/2 are phosphorylated. SMAD2/3 phosphorylation and subsequent translocation of SMAD4 and phosphorylated SMAD2/3 from the ciliary base to the nucleus requires clathrin dependent endocytosis (Clement et al., 2013). Also, in C. elegans, LOV-1 (polycystin-1 homolog) and PKD-2 (polycystin-2 homolog) are targeted for lysosomal degradation through the STAM (signal-transducing adaptor molecule) and HRS (hepatocyte growth factor regulated tyrosine kinase substrate) complex that sorts endocytosed proteins on endosomes to the multivesicular body (Hu and Wittekind, 2007, Lloyd et al., 2002, Mizuno et al., 2003). Additionally, nematode endocytosis components dynamin, RAB-5, and AP2 clathrin adaptor complex regulate periciliary and ciliary membrane retrieval at the ciliary base, possibly counterbalanced by ciliary membrane delivery via exocytic pathways (Kaplan et al., 2012).
1.3.3 LATERAL TRANSPORT

Two types of lateral transport along the plasmamembrane can be employed to deliver membrane (-associated) cargo to the cilium: post-Golgi vesicles can merge with the plasma membrane, after which cilium-bound membrane proteins can move laterally to the periciliary membrane. Then, proteins can undergo lateral transport (active transport or diffusion) across the transition zone membrane and into the ciliary membrane. Vesicles are present at the periciliary membrane compartment of nematode ciliated cells (Cevik et al., 2013), but few vesicles have been observed in the transition zone and in the cilium (Doroquez et al., 2014), suggesting that most ciliary membrane proteins must undergo a form of lateral transport to enter the cilium.

One example that employs both types of lateral transport is flagellar targeting of SAG1, an agglutinin involved in flagellar adhesion in *Chlamydomonas* (Belzile et al., 2013). SAG1-C65 is present on the plasma membrane, and upon flagellar adhesion induced signalling, rapidly translocates to membranes at the flagellar base prior to flagellar entry. Then, SAG1-C65 translocates from the membrane at the flagellar base to the flagella membrane in *Chlamydomonas*. This lateral transport is facilitated by cytoplasmic microtubules and is independent of the intraflagellar transport kinesin-2 motor (Belzile et al., 2013).

Lateral transport has also been proposed as a ciliary entry mechanism for Smoothened upon Shh pathway activation (Milenkovic et al., 2009). Ciliary entry of Smoothened is mediated by intraflagellar transport, requiring IFT-A subunit IFT144, which suggests that Smoothened cannot diffuse into the ciliary membrane, but rather relies on active transport mechanisms (Milenkovic et al., 2009, Liem et al., 2012).
1.4 CILIARY GATING

Ciliary protein transport and restriction are tightly controlled processes, as many ciliary proteins require precise localisations to function properly, and a number of pathways, such as the sonic hedgehog signalling pathway, employ the cilium as a spatiotemporal regulatory mechanism (Goetz and Anderson, 2010, Huangfu and Anderson, 2005). At the ciliary base, a size-dependent diffusion barrier is present to regulate ciliary access of cytosolic proteins, and ciliary entry and exit of membrane (-associated) proteins are suggested to be controlled by membrane diffusion barriers (Williams et al., 2011, Cevik et al., 2013, Dishinger et al., 2010, Kee et al., 2012, Hunnicutt et al., 1990, Lin et al., 2013, Hu et al., 2010, Hu and Nelson, 2011, Hurd et al., 2011).

1.4.1 MEMBRANE DIFFUSION BARRIER

A functional barrier at the flagellar base was first shown in Chlamydomonas, where activated agglutinin was found in the flagellum, but not on cell body membranes (Hunnicutt et al., 1990, Musgrave et al., 1986). Studies in nematodes and mammalian cell culture have shown that MKS- and NPHP-module components at the transition zone, and a septin ring at the ciliary base, respectively, are involved in membrane diffusion barrier function. When these proteins are removed, non-ciliary proteins abnormally enter the cilium and ciliary membrane proteins are no longer retained at the ciliary membrane (Williams et al., 2011, Cevik et al., 2013, Chih et al., 2012, Hu et al., 2010, Hu and Nelson, 2011). Nematode studies have suggested the transition zone forms a membrane diffusion barrier for a number of ciliary proteins (RPI-2, TRAM-1, ARL-13). This barrier requires the function of a number of MKS and NPHP proteins, which localise at the transition zone and form two distinct functional modules (‘MKS’ and ‘NPHP’ modules), consisting of at least eight and two proteins, respectively (Williams et al., 2011, Huang et al., 2011, Jauregui and Barr, 2005, Jauregui et al., 2008, Williams and Winkelbauer, 2008, Winkelbauer et al., 2005). Consistent with roles in defining ciliary composition, loss of MKS- and NPHP-modules causes defects in cilium structure, function, and transition zone Y-link assembly (Williams et al., 2011, Jauregui and Barr, 2005, Jauregui et al., 2008,
How the MKS and NPHP modules form the transition zone membrane diffusion barrier is unknown. Components of the MKS- and NPHP-modules could be part of Y-link structures, found only in transition zones, connecting microtubule doublets to the transition zone membrane. These Y-links are thought to terminate in or organise the ciliary necklace, which could be involved in the membrane diffusion barrier through regulation of lipid composition and fluidity of the transition zone membrane (Garcia-Gonzalo and Reiter, 2012, Gilula and Satir, 1972, Reiter et al., 2012). Based on domain analysis of mammalian MKS- and NPHP-module components it was suggested that transmembrane proteins TMEM216/MKS-2, TMEM67/MKS-3, and TMEM237/JBTS-14 may anchor the Y-links to the membrane. Proteins lacking membrane (-association) domains, for example RPGRIP1L and CEP290 could form the stalk of Y-links. Additionally, NPHP-1 and NPHP-4 possess microtubule-binding domains and may form the Y-link connections to the microtubule doublets of the transition zone (Garcia-Gonzalo and Reiter, 2012). Whether these proteins are part of the Y-links or ciliary necklace remains unknown, but efforts are ongoing using immuno-electron and superresolution microscopy approaches to elucidate exact localisation of transition zone components.

How ciliary proteins overcome the transition zone membrane diffusion barrier is not fully known. The transition zone membrane diffusion barrier prevents leakage of ciliary ARL-13 into the periciliary membrane, and active transport in the form of intraflagellar transport is required for ARL-13 to enter and exit the cillum (Cevik et al., 2013), suggesting IFT association is required for this membrane-associated protein to overcome the transition zone diffusion barrier. However, not all ciliary membrane proteins require intraflagellar transport for ciliary entry, because in *Chlamydomonas*, agglutinin SAG1-C65 can enter the cillum independent of intraflagellar transport, but is facilitated by cytoplasmic microtubules (Belzile et al., 2013).
In mammalian cells, Septin2 is part of a membrane diffusion barrier at the ciliary base. Septins are a family of GTPases that can form filaments and rings and can function as membrane diffusion barriers (Mostowy and Cossart, 2012). Septin 2 was identified at the ciliary base, and plays a role in ciliary membrane protein retention, including components of the Shh signalling pathway, but is not required for diffusion of IFT proteins between cilium and cell body (Chih et al., 2012, Hu et al., 2010). Additionally, septins have been shown to be required for ciliogenesis, regulate ciliary length, and are important in developmental signalling (Fliegauf et al., 2014, Ghossoub et al., 2013, Kim et al., 2010b, Zhai et al., 2014, Dash et al., 2014). Also, retention signals can prevent ciliary entry through interactions with the actin cytoskeleton (Francis et al., 2011).

1.4.2 CYTOSOLIC DIFFUSION BARRIER

At the ciliary base a cytosolic diffusion barrier is present, regulating ciliary access of cytosolic proteins. This barrier is size-dependent, though reports differ on the size of components that can diffuse across the barrier without requiring an active transport mechanism. Kee et al. reported that dextrans with a relative molecular mass of 40,000 (40K) or greater were unable to enter the cilium. However, proteins with a relative molecular mass of up to 41K were able to pass the cytosolic diffusion barrier (Kee et al., 2012). A later report by Lin et al. found that proteins with a radius of up to 7.9 nm were able to enter the cilium, suggesting that the size-dependence of the barrier relates to protein size, rather than mass, of the protein (Lin et al., 2013).

Ciliary entry of cytosolic proteins may be regulated by nucleocytoplasmic transport machinery. Importin β2 localises to the centrosome and cilium, and is required for RP2 ciliary targeting and RanGTP mediated ciliary entry of KIF17 (Dishinger et al., 2010, Kee et al., 2012). RanGTP localises to cilia and basal bodies, and can drive ciliogenesis. RanGTP is also required for biochemical interactions between Importin β1 and cilium-localised Crumbs3, but because these proteins colocalise in multiple cellular regions, whether RanGTP regulates the interaction at the cilium is unknown (Fan et al., 2007). RanBP1, a negative regulator of RanGTP, localises to the cytoplasm and the basal body,
preventing RanGTP localisation and activity at these sites, and is required for KIF17 ciliary localisation (Fan et al., 2011). Finally, Kee et al. also showed that a subset of nucleoporins localise to the ciliary base. When the nuclear pore machinery was inhibited using antibodies, diffusion of cytosolic proteins into the cilium was reduced, suggesting nucleoporins form a pore complex at the ciliary base, allowing size-dependent diffusion of proteins into the cilium (Kee et al., 2012).

1.5 DEFINING CILIARY COMPONENTS

The advent of high throughput techniques, the ‘omics’ era, has provided insight into the components that make up the cilium (Diniz et al., 2012, Inglis et al., 2006). Genomics, transcriptomics, proteomics, and bioinformatics approaches have led to identification of disease candidate genes and novel ciliary functions of candidates. High throughput ‘omics’ experiments may prove to be the final key to identifying the complete list of ciliary components. Cooperative efforts to elucidate cilium composition, structure, and function have resulted in online resources, such as CilDB and the SYSCILIA gold standard (van Dam et al., 2013, Arnaiz et al., 2009).

1.5.1 GENOMICS

Genomics approaches, such as exome sequencing, have been used to map pathogenic mutations and identify novel ciliopathy genes. As different mutations in one gene can have vastly different consequences, sequencing and mapping mutations has become more important and may develop into a predictive tool used in a clinical setting (Bacino et al., 2012, Coussa et al., 2013, Halbritter et al., 2013, Roosing et al., 2013, Schmidts et al., 2013, Zaghloul and Katsanis, 2010). In addition, forward and reverse genetics in model organisms and in silico genomic analysis have identified multiple novel candidate ciliary and ciliopathy genes (Kim et al., 2010a, Chih et al., 2012, Sun et al., 2004, Baron et al., 2007, Liang and Pan, 2013, Laurençon et al., 2007). For example, forward genetics screens in C. elegans to identify worms with altered sensory behaviours have identified a large
number of genes functioning in cilium structure-function (Perkins et al., 1986, Culotti and Russell, 1978, Starich et al., 1995). In a whole genome siRNA screen using human RPE (retinal pigmented epithelial) cells, multiple novel ciliary genes were identified that are involved in ciliogenesis and cilium length regulation (Kim et al., 2010a).

Comparative genomics studies have provided a novel approach to identifying novel ciliary genes (Li et al., 2004, Avidor-Reiss et al., 2004). By ‘subtracting’ the genome from non-ciliated species from the genome of ciliated species, several novel ciliary genes were identified, including BBS5 and OSEGs, outer segment genes required for ciliogenesis in Drosophila (Avidor-Reiss et al., 2004, Li et al., 2004). A comparative genomics experiment was performed across multiple Caenorhabditis species to identify genes with promoter elements that could bind ciliogenic transcription factor DAF-19. This resulted in the identification of 93 known and putative ciliary genes, and mapped the dyf-5 gene (Chen et al., 2006).

1.5.2 TRANSCRIPTOMICS

Whilst genomics approaches screen for presence of cilia-related genes in the genome, transcriptomics provide insight into expression levels of these genes. A number of studies have been performed to identify genes regulated by ciliogenic RFX transcription factors. Transcriptomic analyses during flagellar regeneration and disassembly in Chlamydomonas have revealed conservation of ciliopathy genes and uncovered novel genes involved in these processes (Stolc et al., 2005, Chamberlain et al., 2008, Albee et al., 2013). Studies using mammalian tissue have identified genes related to cilium function in a cell-type specific manner, such as MDMI and DYXIC1 in tracheal epithelial cells (Ivliev et al., 2012, Farkas et al., 2013, Hoh et al., 2012, Nickell et al., 2012, Song et al., 2009). In C. elegans only one such RFX transcription factor is present, DAF-19 (Senti and Swoboda, 2008, Swoboda et al., 2000). Transcriptomic comparison of wild type and daf-19 mutants lacking cilia has provided several novel ciliary candidate genes whose expression is regulated by this transcription factor (Phirke et al., 2011, Blacque et al., 2005).
1.5.3 BIOINFORMATICS

The X-box promoter element is a well characterised motif that can bind to ciliogenic RFX transcription factors. In *C. elegans* this is a 14 bp element with consensus sequence RYHNYYWTRSHDAY (Blacque et al., 2005, Efimenko et al., 2005, Swoboda et al., 2000). Analysis of the *C. elegans* genome for the presence of X-box promoter sequence motifs that can bind DAF-19 (RFX transcription factor) has revealed novel ciliary genes, such as *dylt-2* (dynein light chain) and *mks-1* (MKS module component) (Efimenko et al., 2005, Swoboda et al., 2000, Fan et al., 2004, Henriksson et al., 2013, Blacque et al., 2005). Additionally, by analysing DAF-19 regulated genes, a novel enhancer element, the C-box, was uncovered (Burghoorn et al., 2012). This motif is found more often in genes with expression patterns that span most ciliated sensory neurons than in genes that exhibit a more restricted expression pattern (Burghoorn et al., 2012). Analysis of this promoter sequence motif in *Drosophila* identified 11 novel genes regulated by the RFX transcription factor, including orthologs of IFT46 (IFT-B component), rootletin, and TRPV4 (transient receptor potential vanilloid channel 4) (Laurençon et al., 2007). Bioinformatic approaches have identified a subset of transition zone genes that is conserved across numerous ciliated species, and suggests different evolutionary development of MKS- and NPHP-module genes (Barker et al., 2014a).

1.5.4 PROTEOMICS

Proteomics of isolated cilia and flagella, and proteomic analysis of basal bodies in several species have identified a number of novel ciliary proteins (Ishikawa et al., 2012, Jakobsen et al., 2011, Lechtreck et al., 2009a, Pazour et al., 2005, Broadhead et al., 2006, Ostrowski et al., 2002, Smith et al., 2005, Boesger et al., 2009, Subota et al., 2014, Keller et al., 2005, Keller and Marshall, 2008, Kilburn et al., 2007, Jakobsen et al., 2013). For example, orthologs of OFD1 and NPHP4 were identified in proteomic analysis of *Chlamydomonas* centrioles (Keller et al., 2005), and analysis of *Trypanosoma* flagella uncovered eight novel flagellar proteins, FLAM1-8 (Subota et al., 2014). Proteome wide screening combined with bioinformatics approaches has also revealed novel centriole components that are required for ciliogenesis (Jiang et al., 2012). Recently, the membrane proteome of
olfactory cilia was identified and revealed four novel ciliary proteins, annexins ANXA1/2/5 and S100A5, a calcium binding protein (Kuhlmann et al., 2014). A variation in these experiments involves comparisons of different proteomes, such as wild type and mutated proteins of interest. This can inform on function of a protein of interest, as well as associated pathomechanisms, because altered interactions can be quantified by TAP (tandem affinity purification) and SILAC (stable isotope labelling with amino acids in cell culture) approaches (Boldt et al., 2011, Cevik et al., 2013, Sang et al., 2011, van Reeuwijk et al., 2011, Texier et al., 2012). Combination of these proteomic efforts has resulted in an online ciliary proteome database (http://www.ciliaproteome.org) (Gherman et al., 2006).

1.6 CAENORHABDITIS ELEGANS AS A MODEL TO STUDY CILIA

1.6.1 GENERAL FEATURES

*C. elegans* has proven to be a very powerful model organism since Sydney Brenner’s seminal publication, and worm research has been awarded with Nobel prizes on three separate occasions (Brenner, 1974). The nematode has been an important model organism in cell biology, neuroscience, aging, and more recently, genomics. In 1998 *C. elegans* was the first multicellular organism to have a fully sequenced genome, which is constantly updated and further annotated (Consortium, 1998, Hillier et al., 2005). This allows for detailed genomic and transcriptomic analysis, which has proven invaluable in identifying and characterizing novel ciliary genes (Efimenko et al., 2005, Inglis et al., 2009, Blacque et al., 2005, Chen et al., 2006). Many cilia-related and ciliopathy genes are conserved in *C. elegans*, including most intraflagellar transport genes. This allows for detailed study of deleterious mutations, as cilia are non-essential organelles in the worm.

Another advantage of *C. elegans* as a model organism is the ease of genetic manipulation. Forward genetics approaches have resulted in the availability of mutant alleles for many ciliary genes (Perkins et al., 1986, Starich et al., 1995, Wei et al., 2012, Ou et al., 2007, Hao et al., 2011b). Large scale projects such as the Million Mutation Project have provided
many novel mutations and new mutations are starting to be generated by evolving techniques such as the CRISPR/Cas9 system for targeted mutagenesis (Jinek et al., 2012, Waaijers and Boxem, 2014, Thompson et al., 2013). Transgenic animals can be produced easily by micro-injection of genetic material; combined with the transparency of the nematode, this facilitates direct investigation of fluorescently tagged proteins in vivo. Fluorescence imaging-based assays, such as intraflagellar transport and FRAP (Fluorescence Recovery after Photobleaching) assays have been used extensively to study protein dynamics, active transport, and diffusion events (Cevik et al., 2013, Ou et al., 2005, Pan et al., 2006, Signor et al., 1999a, Signor et al., 1999b, Snow et al., 2004, Hao et al., 2011b).

*C. elegans* is a small, soil-dwelling roundworm that feeds on bacteria and in a laboratory setting can be maintained on agar plates or in liquid culture in the presence of *E. coli*. Self-fertilizing hermaphrodites produce mostly hermaphroditic progeny, but males can be induced through crossing or heat-shock methods. Mating strategies facilitate isolation of mutations and introduction of transgenic material. Hermaphrodites can produce large numbers of progeny in a short period of time; under optimal conditions (20°C incubation, no starvation) the nematode reproducing life cycle spans 3 days. Nematode hermaphrodites consist of 959 somatic cells; its cell lineage has been fully mapped, and cell specific promoters are available for many ciliated cells to investigate cell-specific cilium structure and function.

302 cells form the neuronal network, whose connections have also been fully mapped, and 60 highly polarized neurons possess primary cilia at the distal dendrite tips. These ciliated sensory neurons are non-essential for survival, allowing for study of mutations that cannot sustain life in other systems. Most of these cells are present in the head of the animal, and dendrites extend anteriorly from the cell bodies around the pharyngeal bulbs, with most cilia environmentally exposed at the nose of the animal through pores in the cuticle. Cilia extending from eight and two neurons are present in bilateral amphid and phasmid pores in the head and tail of the worm, respectively. The spatial resolution provided by amphid and phasmid neurons with a long dendrite and a cilium extending from it allows for detailed *in vivo* study of ciliary mechanisms with relative ease compared to other systems. Amphid
ASE, ASG, ASH, ASI, ASJ, ASK, and phasmid PHA and PHB ciliated neurons possess single rod-like cilia; ADF and ADL contain two rod-like cilia each. All amphid and phasmid cilia perform chemosensory functions, but recognize different substrates (Bargmann et al., 1993, Troemel et al., 1997, Inglis et al., 2007). Also, ASH cilia are involved in mechano- and osmosensing. Amphid cilia all possess rod-like shaped cilia; chemosensory neurons AWA, and AWC possess wing-shaped cilia and AWB possesses fan-like cilia that mediate chemo-attraction to volatile odours, and thermosensory AFD possesses a single cilium surrounded by microvilli-like structures (Inglis et al., 2007, Altun and Hall, 2012).

Behavioural analysis of *C. elegans* ciliary mutants has identified many novel ciliary genes and can provide information on ciliary function (Bargmann et al., 1993, Perkins et al., 1986, Starich et al., 1995). For example, chemo-attraction to benzaldehyde and isoamylalcohol is specifically mediated by the bilateral AWC cilia. Also, a number of ciliary gene mutants exhibit Daf (dauer defective) phenotypes (Ailion and Thomas, 2000, Ailion and Thomas, 2003, Burghoorn et al., 2010, Schafer et al., 2006). Wild type animals can survive long periods under extreme conditions (high temperature, starvation) by entering the dauer developmental stage. Osmosensory, chemosensory, and other behavioural assays allow for screening of large numbers of animals in a short period of time.

1.6.2 NEMATODE CILIARY SUBCOMPARTMENTS

Compartmentalisation of cilia is conserved in nematodes, consisting of a ciliary base/periciliary membrane compartment, followed distally by the transition zone, and in a subset of cells, a bipartite axoneme with a microtubule doublet containing middle segment, and a distal segment formed around singlet microtubules (Figure 1.2 and (Blacque and Sanders, 2014)).
The periciliary membrane surrounding the ciliary base is a dedicated membrane patch and targeting site for vesicles carrying ciliary cargo, analogous to the ciliary pocket found in other systems (Benmerah, 2013, Molla-Herman et al., 2010). Periciliary membrane volume homeostasis is tightly regulated by maintaining a balance between fusion of vesicles carrying ciliary-bound cargo on one side, and endocytosis on the other hand, recycling membrane back to the cell body and targeting ciliary proteins for degradation (Kaplan et al., 2012). At the ciliary base, BBS-8 and endocytic pathway components, including dynamin DYN-1, OSTA-1 (organic solute transport alpha-like 1), RAB-5, RAB-8, and AP-2 µ2 subunit DPY-23, are required for periciliary membrane volume regulation (Kaplan et al., 2012, Kaplan et al., 2010, Dwyer et al., 2001, Olivier-Mason et al., 2013). Furthermore, STAM-1 (signal-transducing adaptor molecule 1) and HGRS-1 (hepatocyte growth factor regulated tyrosine kinase substrate 1) target LOV-1/PKD-2 (polycystin-1/-2) receptor-channel complexes for degradation, preventing abnormal accumulation of these proteins at the ciliary base (Hu and Wittekind, 2007).

Several transport and signalling proteins accumulate in the periciliary membrane region, possibly to prevent depletion of receptors upon stimulation. Accumulating at the periciliary membrane for example, are G protein coupled receptors SRBC-66 and ODR-10, involved in dauer pheromone and odorant signalling, respectively (Kaplan et al., 2012, Dwyer et al., 2001). Also accumulating at the ciliary base are transmembrane glycoprotein TRAM-1 (translocating chain-associated membrane protein 1) and membrane-associated RPI-2/RP2 (retinitis pigmentosa 2); mammalian RP2 functions as an ARL3 GTPase activating protein (GAP) and is involved in ciliary targeting of G proteins, NPHP3 and possible other myristoylated cargo (Schwarz et al., 2012a, Schwarz et al., 2012b, Wright et al., 2011). However, unlike SRBC-66 and ODR-10, TRAM-1 and RPI-2 are excluded from the cilium, possibly by the membrane-diffusion barrier formed by the transition zone (Williams et al., 2011).

The nematode ciliary base lacks a centriole, appendages, and transition fibres, but serves similar functions to the basal body found in other species (Doroquez et al., 2014, Reiter et al., 2012). Intraflagellar transport particle and BBSome subunits, as well as DYF-19/FBF1 (Fas binding factor 1) accumulate at the ciliary base below the transition zone prior to
ciliary entry (Blacque et al., 2008, Nachury et al., 2007, Blacque et al., 2004). DYF-19 is required for ciliary entry of assembled intraflagellar transport particles, and the BBSome is required for intraflagellar transport complex assembly and stability in *C. elegans* (Wei et al., 2012, Wei et al., 2013).

Adjacent to the periciliary membrane and ciliary base region lies the transition zone. This compartment houses multiple ciliopathy proteins that are proposed to form two functional modules, cooperating to maintain cilium integrity and barrier function. The MKS module (ortholog of MKS/JBTS module) consists of MKS-1, MKS-2/TMEM216, MKS-3/TMEM67, MKS-5/RPGRIP1L, MKS-6/CC2D2A, MKSR-1/B9D1, MKSR-2/B9D2, and JBTS-14/TMEM237; the NPHP module consists of NPHP-1 and NPHP-4 (Williams et al., 2011, Williams et al., 2010, Jauregui and Barr, 2005, Jauregui et al., 2008, Bialas et al., 2009). These modules function redundantly to assemble Y-links and restrict protein entry (TRAM-1, RPI-2) or exit (ARL-13) (Cevik et al., 2013, Williams et al., 2011, Huang et al., 2011). Y-links extend from the junction between A and B tubules to the plasma membrane, where they possibly organise the ciliary necklace, a membrane specialisation that could act as the transition zone membrane diffusion barrier (Gilula and Satir, 1972, Reiter et al., 2012).

The axoneme extending from the transition zone in amphid and phasmid cilia possesses a bipartite microtubule structure, where doublets do not extend to the ciliary tip. Instead, the B-tubules terminate at the approximate midpoint, forming a proximal doublet-containing middle segment (MS), followed distally by a microtubule singlet-containing distal segment (DS) (Doroquez et al., 2014, Perkins et al., 1986). Middle segment biogenesis is driven by two redundant kinesin-2 motors, travelling at ~0.7 µm/s (Pan et al., 2006, Orozco et al., 1999, Signor et al., 1999a, Signor et al., 1999b, Khan et al., 2000, Ou et al., 2005, Snow et al., 2004, Evans et al., 2006). Canonical heterotrimeric kinesin II, consisting of KLP-11, KLP-20, and KAP-1, possesses an intrinsic speed of ~0.5 µm/s, and homodimeric kinesin-2, OSM-3/KIF17 that travels at ~1.3 µm/s, power anterograde intraflagellar transport in the middle segment (Ou et al., 2005, Ou et al., 2007, Pan et al., 2006, Snow et al., 2004). The IFT-B subunits associated with anterograde transport function are conserved in *C. elegans*, with the exception of IFT25 and IFT27 (Wang et al., 2009). Powering retrograde
transport, *C. elegans* cytoplasmic dynein 2 travels at ~1.1 µm/s and consists of heavy chain CHE-3/DYNC2H1, intermediate chain DYCI-1/WDR34, light intermediate chain XBX-1/DYNC2LI1, and light chains DLC-1/DYNLL1 and DYL1T-2/TCTE3 (Signor et al., 1999a, Efimenko et al., 2005, Wicks et al., 2000, Schafer et al., 2003, Blacque et al., 2005). The IFT-A subunits associated with retrograde transport function are conserved in *C. elegans*, with the exception of IFT43. The conserved BBSome is required for assembly and stability of the IFT complexes, mediated by possible interactions between IFT-A component DYF-2/IFT144 and BBSome components BBS-1, BBS-7, and BBS-9 (Wei et al., 2012).

The middle segment appears analogous to the mammalian Inversin (Inv) compartment. INV/NPHP-2 (Inversin), Joubert Syndrome ARL13B/ARL-13, NPHP3, NPHP9/NEK8, and ANKS6 all localize to this compartment, and Inversin and ARL13B are shown to dynamically exchange at the membrane (Shiba et al., 2010, Shiba et al., 2009, Cevik et al., 2013, Hoff et al., 2013, Larkins et al., 2011). In *C. elegans* middle segment cilia, ARL-13 also dynamically exchanges at the membrane (Cevik et al., 2013). Additionally, nematode NPHP-2, as well as cyclic-nucleotide gated (CNG) cation channel subunits CNG-3, TAX-2, and TAX-4 localizations are restricted to the middle segment (Wojtyniak et al., 2013, Warburton-Pitt et al., 2012). However, TAX-2 and TAX-4 do not seem to exchange at the membrane (Wojtyniak et al., 2013).

Not all nematode cilia possess the middle-distal segment distinction and in these cilia microtubule doublets extend to the ciliary tip (Inglis et al., 2007). For those cilia possessing a bipartite microtubule arrangement, a 2-5 µm long distal segment is observed (Doroquez et al., 2014, Perkins et al., 1986). Kinesin II dissociates from the IFT assembly at the middle-distal segment transition, thus, homodimeric kinesin-2 OSM-3 drives distal segment biogenesis (Snow et al., 2004, Ou et al., 2005). EBP-2, a microtubule binding protein related to end binding protein 1 (EB1), accumulates at the middle and distal segment tips, suggesting a role for this protein in stabilizing microtubules (Hao et al., 2011b). At the distal tip the IFT assembly dissociates, and reforms with cytoplasmic dynein complexes attaching to microtubule singlets for retrograde transport (Snow et al., 2004, Wren et al., 2013, Wei et al., 2012).
1.7 Thesis aims

The work presented here focuses on the ciliary base and transition zone in *C. elegans* ciliated sensory neurons. Specifically, this thesis aims to characterise a novel conserved cilia-related gene K04F10.2/KIAA0556. The second aim is to develop an imaging-based assay to validate the existence of a transition zone membrane diffusion barrier, and then use this assay to investigate the molecular mechanisms that establish and maintain this barrier.

Chapter III presents a thorough investigation of a candidate ciliary gene, K04F10.2, which was chosen for further investigation based on previous publications showing its expression was regulated by the DAF-19 transcription factor. However, it was not known if this gene is truly a ciliary gene, and if so, what its function is relating to cilium structure-function. To investigate the role of K04F10.2 in cilium structure and function, expression, localisation, and functional approaches were employed. Transcriptional and translational GFP-reporter assays were used to assess K04F10.2 expression and localisation, fluorescence and transmission electron microscopy to examine cilium (ultra-) structure, and sensory behaviour assays to assess cilium function, as well as double mutant analysis to investigate genetic interactions. Finally, to further investigate the role of K04F10.2 in other organisms, human KIAA0556 (K04F10.2 orthologue) and candidate interactors were identified using proteomics approaches, and the interactors were subsequently investigated in *Caenorhabditis elegans* for cilia-related roles. This work ultimately identifies K04F10.2/KIAA0556 as a novel ciliary base component with subtle roles in cilium structure, function, and transport. Also, new insight is provided for cilia-related roles of RAB-28, and katanin subunit F47G4.5.

Chapter IV focuses on the development and implementation of a novel *in vivo* FRAP assay to functionally investigate the transition zone membrane diffusion barrier. Until now, this barrier had only been investigated in cultured cell systems, which requires extensive cell manipulation. To investigate the barrier *in vivo*, a FRAP approach was employed to show
that ciliary membrane-associated ARL-13 is mobile at the membrane and is retained by the presence of a ciliary diffusion barrier. To functionally investigate this diffusion barrier, the requirement of transition zone-associated genes and intraflagellar transport genes for ARL-13 ciliary retention was further investigated. The work presented here shows that the transition zone diffusion barrier is dependent on the function of various transition zone-associated genes, but not most examined intraflagellar transport genes. Instead, intraflagellar transport appears to actively transport ARL-13 across the barrier. This is the first such work in a multicellular system to investigate membrane diffusion kinetics across the transition zone.
CHAPTER II

METHODOLOGY
2.1 Strains

All strains were maintained at 15°C or 20°C using standard techniques (Brenner, 1974). Mutant strains were obtained from the C. elegans Genetics Centre or the Japanese National Bioresource Project. Wild type strain: N2 (Bristol).

2.1.1 Mutant Alleles

Mutant alleles: K04F10.2(tm1830), K04F10.2(gk112869), arl-13(tm2322), F47G4.5(ok2667), osm-5(p813), klp-11(tm324), mks-5(tm3100), arl-3(tm1703), ifta-1(nx61), nphp-4(tm925); him-5(e1490), and che-11(e1810).

Standard genetic crossing methods were employed to generate double and triple mutants and to introduce transgenes into the mutant background. PCR genotyping was used to follow tm1830, gk112869, tm2322, ok2667, tm324, tm3100, tm925, and tm1703 mutations. The dye-filling assay was employed to identify strains homozygous recessive for p813, nx61, or e1810.

2.1.2 Transgenic Strains

Strains expressing cell-specific promoter GFP-reporters: N2;Ex[P\text{K04F10.2::GFP + P\text{unc-122::DsRed}}], N2;Ex[P\text{mei-1::GFP + P\text{unc-122::DsRed}}], N2;Ex[P\text{mei-2::GFP + P\text{unc-122::DsRed}}], N2;Ex[P\text{F47G4.4::GFP + P\text{unc-122::DsRed}}], N2;Ex[P\text{F47G4.5::GFP + P\text{unc-122::DsRed}}], dpy-5(e907); Ex[P\text{rab-28::GFP + pCeh361}], lin-15(n765); kyIs104[P\text{str-1::GFP + lin-15(+)}], N2;kyIs164[P\text{gye-5::GFP}].

Transgenes encoding fluorescently labelled IFT proteins: N2;\text{myEx10[che-11::GFP + pRF4]}, N2;\text{lqIs2[osm-6::GFP]}, N2;\text{ejEx[osm-3::GFP + pRF4]}, N2;\text{Ex[rpi-2::GFP + xbx-1::tdTOMATO + pRF4]}, N2;\text{Ex[ifta-2::GFP + pRF4]}. Transgenes encoding other fluorescently labelled ciliary proteins: N2;Ex[P\text{K04F10.2::K04F10.2::GFP + pRF4}] (injected at 5.0 ng/µl), N2;Ex[P\text{K04F10.2::K04F10.2::GFP + P\text{unc-22::DsRed}}] (injected at 0.5 ng/µl), dpy-5(e907);
Ex[P\textsubscript{rab-28::GFP::rab-28} + pCeh361 + P\textsubscript{unc-122::GFP}], dpy-5 (e907);
Ex[P\textsubscript{F47G4.5::GFP::F47G4.5} + pCeh361 + P\textsubscript{unc-122::DsRed}], N2;kyIs156[P\textsubscript{str-1::odr-10::GFP}], dpy-5(e907); nxEx[bbs-7::GFP + pCeh361], N2;ogEx58[Parl-13::arl-13::GFP + pRF4], N2;Ex[mks-2::GFP + tram-1::tdTOMATO + pRF4], N2;nxEx[mks-5::tdTOMATO + pRF4], N2;ogEx[P\textsubscript{arl-13::arl-13::tdTOMATO} + pRF4].

2.2 GENERATION OF TRANSGENIC, DOUBLE, AND TRIPLE MUTANT STRAINS

2.2.1 GENOMIC DNA PREPARATION

WORM LYSIS
Wild type genomic DNA was prepared from 4 plates of starved L1 larvae. Animals were transferred to Eppendorf tubes with 1.5 ml M9 buffer (42 mM Na\textsubscript{2}HPO\textsubscript{4}, 22 mM KH\textsubscript{2}PO\textsubscript{4}, 8.6 mM NaCl, 19 mM NH\textsubscript{4}Cl) and pelleted using a microcentrifuge (1 min at 3000 rpm). Supernatant was discarded and pellet resuspended in 400 µl worm lysis buffer (10 mM Tris HCl pH8.2, 50 mM KCl, 2.5 mM MgCl\textsubscript{2}, 2.25% Tween-20, 0.05% gelatin), supplemented with 1:100 proteinase K (Qiagen). Samples were incubated overnight at -80°C, transferred to a PCR thermocycler (T3000, Biometra), incubated 90 minutes at 65°C for optimal proteinase K activity, followed by 30 min at 95°C to inactivate the enzyme, and stored at 4°C prior to DNA extraction.

DNA EXTRACTION
Equal volume of buffer-saturated phenol:chloroform:isoamylalcohol (25:24:1) were added to DNA solutions. After shaking for 15 seconds samples were centrifuged for 3 minutes at maximum speed in a cooled microcentrifuge (4°C). The aqueous layer was transferred to a separate Eppendorf tube, and extraction steps were repeated until interface was no longer visible. Samples were kept on ice prior to ethanol precipitation.
2.2.2. ETHANOL PRECIPITATION

1:10 v/v 3M sodium acetate pH5.2 was added to adjust salt concentration. 2 volumes cold 100% EtOH were added, mixed, and incubated on ice for 20 min. Precipitated DNA was subsequently pelleted using a cooled microcentrifuge (4°C, 15 min. at maximum speed). Following careful removal of the supernatant, the pellet was resuspended in 1 ml cold 100% EtOH. After pelleting by centrifugation (4°C, 30 sec. at maximum speed), supernatant was decanted and pellet was allowed to air dry. Pellet was then resuspended in ddH₂O and aliquots stored at -20°C.

2.2.3 GENERATION OF TRANSGENES

Transgenes were generated by fusion PCR as previously described (Hobert, 2002). Briefly, for transcriptional GFP-reporters, a GFP (containing a nuclear localisation signal) and unc-54 3’ UTR DNA fragment (amplified from pPD95.67) was stitched to a DNA fragment containing the 5’ UTR and the first 14-20 bp of the gene’s coding sequence (using genomic DNA as template). For the latter fragment, the reverse primer was designed to mutate the gene’s start codon so that translation in the fused product occurred from the start codon of GFP (Figure 2.1). See Table 2.1 for primers. For C-terminal translational fusions, a GFP (no nuclear localisation signal) and unc-54 3’ UTR fragment (amplified from pPD95.77) was stitched to a fragment containing the 5’ UTR and entire genomic sequence without the stop codon (amplified from genomic DNA, Figure 2.2). For N-terminal translational fusions, a GFP fragment lacking the stop codon was first stitched to a fragment containing the entire genomic DNA sequence of the gene of interest (including some 3’ UTR sequence). The resulting stitched product was then fused to a fragment containing the 5’ UTR promoter region of the gene of interest (Figure 2.3). For primers see Table 2.2.
Figure 2.1 Generation of transcriptional GFP-reporters. 1. Amplification of the 5’ UTR and the first 14-20 bp of the gene’s coding sequence from genomic DNA. 2. Amplification of a GFP and *unc-54 3’ UTR* DNA fragment from pPD95.67. 3. Fusion PCR of the two fragments to generate transcriptional GFP-reporter.
Figure 2.2 Generation of C-terminal translational GFP-reporters. 1. Amplification of 5’ UTR and the entire genomic sequence with a mutated stop codon of the gene of interest. 2. Amplification of a GFP and *unc-54* 3’ UTR DNA fragment from pPD95.77. 3. Fusion PCR of the two fragments to generate translational GFP-reporter.
Figure 2.3 Generation of N-terminal translational GFP-reporters. 1. Amplification of a DNA fragment containing the entire genomic sequence of the gene of interest, including some 3’ UTR. 2. Amplification of a GFP fragment lacking the stop codon from pPD95.77. 3. Fusion PCR of the two amplified fragments. 4. Amplification of the 5’ UTR promoter region of the gene of interest. 5. Fusion PCR of fragment from (3) and (4) to generate N-terminal translational GFP-reporter.
Table 2.1 Primer sequences for transcriptional GFP-fusions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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</thead>
<tbody>
<tr>
<td>K04F10.2 Forward</td>
<td>GCT GGT GAT TGT ACA AAG TAA CTT TTG</td>
</tr>
<tr>
<td>Nested forward primer</td>
<td>CTT CAC AAT CCA AAA ATA GAA AAT CC</td>
</tr>
<tr>
<td>Reverse primer with</td>
<td>GTA CAA TGA TGG ACT ACA CGG CAG T</td>
</tr>
<tr>
<td>GFP overhang</td>
<td></td>
</tr>
<tr>
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<td>AGG CGA CAT CGG TGA TTG QAA AQA G</td>
</tr>
<tr>
<td>Nested forward primer</td>
<td>GTA CAA TGA TGG ACT ACA CGG CAG T</td>
</tr>
<tr>
<td>Reverse primer with</td>
<td>AGT CGA CCT GCA GGC ATG CAA GTG CGG TGG TCA ACG CTT CGC TCA CGT TTA QTT G</td>
</tr>
<tr>
<td>GFP overhang</td>
<td></td>
</tr>
<tr>
<td>mast.2 Forward primer</td>
<td>GAT ATT CCT TGG CAA CCA AGA AGC</td>
</tr>
<tr>
<td>Nested forward primer</td>
<td>GCG CTA CGG AGT GCA TGG TTT CT TG</td>
</tr>
<tr>
<td>Reverse primer with</td>
<td>AGT CGA CCT GCA GGC ATG CAA GTG CGG TGG TCA ACG CTT CGC TCA CGT TTA QTT G</td>
</tr>
<tr>
<td>GFP overhang</td>
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Table 2.2 Primer sequences for translational GFP-fusions.

<table>
<thead>
<tr>
<th>N-terminal fusion</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
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<td>Gene</td>
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<tr>
<td>K04F10.2 Forward</td>
<td>GTA TAG CTG TTA CTT ACC TTT CGG</td>
</tr>
<tr>
<td>Nested forward primer</td>
<td>ATG TCC GAT TCT GAC TGG AAA G</td>
</tr>
<tr>
<td>Reverse primer with</td>
<td>GAG TCG ACC TGG AGG CAT CGA ACC TTA ATG ATT AAG TTA ATC TGG AG</td>
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<table>
<thead>
<tr>
<th>C-terminal fusion</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>Gene</td>
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</tr>
<tr>
<td>F47G4.5 Forward</td>
<td>GCA AAG TCG TGA GTT GSG AGG TGT G</td>
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<tr>
<td>Forward primer</td>
<td></td>
</tr>
<tr>
<td>Nested forward primer</td>
<td>GCA AAG TCG TGA GTT GSG AGG TGT G</td>
</tr>
<tr>
<td>Reverse primer with</td>
<td>GCA AAG TCG TGA GTT GSG AGG TGT G</td>
</tr>
<tr>
<td>GFP overhang</td>
<td></td>
</tr>
<tr>
<td>Gene and 3’UTR reverse primer</td>
<td>GAG TCG ACC TGG AGG CAT CGA ACC TTA ATG ATT AAG TTA ATC TGG AG</td>
</tr>
<tr>
<td>Promoter Forward</td>
<td>GCA AAG TCG TGA GTT GSG AGG TGT G</td>
</tr>
<tr>
<td>Forward primer</td>
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<tr>
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</tr>
<tr>
<td>Promoter_reverse with</td>
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<td>GFP overhang</td>
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<tr>
<td>Gene and 3’UTR reverse primer</td>
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<tr>
<td>Promoter Forward</td>
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<tr>
<td>Gene and 3’UTR nested reverse primer</td>
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<td>Promoter_reverse with</td>
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<td>GFP overhang</td>
<td></td>
</tr>
<tr>
<td>Gene and 3’UTR reverse primer</td>
<td>GAG TCG ACC TGG AGG CAT CGA ACC TTA ATG ATT AAG TTA ATC TGG AG</td>
</tr>
<tr>
<td>Promoter Forward</td>
<td>GCA AAG TCG TGA GTT GSG AGG TGT G</td>
</tr>
<tr>
<td>Forward primer</td>
<td></td>
</tr>
<tr>
<td>Gene and 3’UTR nested reverse primer</td>
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</tr>
<tr>
<td>Promoter_reverse with</td>
<td>GCA AAG TCG TGA GTT GSG AGG TGT G</td>
</tr>
<tr>
<td>GFP overhang</td>
<td></td>
</tr>
</tbody>
</table>
2.2.4 CROSSING STRATEGY TO GENERATE MUTANT ANIMALS EXPRESSING TRANSGENES

10 wild type males were crossed with three transgenic hermaphrodites on a ‘drop’ plate (NGM agar with a drop of *E. coli* OP50 at the centre of the plate). From this cross, 12-14 wild type transgenic males were crossed with 5-6 homozygous mutant hermaphrodites (P0 generation) on a drop plate incubated at 20°C for 24 (fluorescent co-injection marker) or 48 hours (roller co-injection marker), after which P0 hermaphrodites were transferred to individual seeded NGM plates. F1 progeny inheriting the transgene (and therefore heterozygous for the mutant allele) were transferred to individual seeded NGM plates and incubated at 20°C until F2 progeny was produced. ~25% of F2s will be homozygous recessive for the mutation of interest. The above is summarised in **Figure 2.4**. Homozygous recessive mutants were identified by PCR-based genotyping or a visible phenotypic characteristic. For mutations causing a dye-filling phenotype (e.g. IFT mutants), the population of F2 animals were screened for dye-filling defective (Dyf) animals. For alleles identified through PCR genotyping, 16 transgenic F2 hermaphrodites were transferred to individual seeded NGM plates, incubated at 20°C for 48 hours (until transgenic F3 progeny was produced), after which single worm PCR was employed to determine the genotype of the F2 parents. Following identification of a homozygous recessive mutant F2 worm, F3 progeny were genotyped or phenotyped (e.g. dye-filling) to confirm the line.

2.2.5 CROSSING STRATEGY TO GENERATE DOUBLE MUTANT ANIMALS

10 wild type males were crossed on a drop plate with three hermaphrodites homozygous mutant for gene A, but wild type for gene B. 12-14 heterozygous males produced from this cross were crossed with 5-6 hermaphrodites homozygous mutant for gene B for 24 hours at 20°C (P0 generation). Subsequently, P0 hermaphrodites were transferred to individual seeded NGM plates. Before reaching the adult stage, 12-16 F1 hermaphrodite cross progeny (heterozygous for mutation B; heterozygous or wild type for mutation A) were transferred to individual seeded NGM plates and incubated at 20°C for 48 hours. Once F2 self progeny were produced, the F1 parents were subjected to PCR genotyping to identify
those heterozygous for mutation A. 16 F2 hermaphrodite progeny from a double heterozygote (mutations A and B) were transferred to individual plates and incubated at 20°C for 48 hours until F3 progeny was produced. F2 parents were subsequently genotyped for mutations A and B. If no animals were identified to be homozygous mutant for both mutations, 16 F3 hermaphrodites were transferred to individual plates, grown for 48 hours, and re-genotyped, until animals homozygous mutant for both genes were identified. Following identification of double mutant animals, hermaphrodites of the next generation were lysed and to confirm the genotype. Figure 2.5 outlines the crossing strategy employed to generate double mutant animals.
Figure 2.4 Crossing strategy to generate transgenic mutant animals. Wild type males are crossed with wild type transgenic hermaphrodites to produce wild type transgenic males. These males are crossed with homozygous mutant hermaphrodites (P0 generation). Resulting F1 transgenic progeny are allowed to self-fertilise. ~25% of F2 progeny will be homozygous recessive for the mutation of interest. Red boxes indicate selected genotypes.
Figure 2.5 Crossing strategy to generate double mutant animals  Wild type males are crossed with hermaphrodites mutant for gene A, but wild type for gene B. The resulting males are crossed with hermaphrodites mutant for gene B. F1 progeny are allowed to self-fertilise and produce F2 progeny. ~16% of F2 progeny will be homozygous mutant for both genes. Red boxes indicate selected genotypes.
2.2.6 DYE-FILLING ASSAY TO IDENTIFY HOMOZYGOUS MUTANT ANIMALS

Animals were transferred to an Eppendorf tube with 1.5 ml M9 buffer and pelleted using a microcentrifuge (1 min., 3000 rpm). Supernatant was discarded and animals resuspended in DiI (1:200 in M9, Invitrogen) and incubated for 30 minutes at room temperature. 1 ml M9 buffer was added and worms pelleted. Most of the supernatant was discarded; animals were resuspended in remaining supernatant and transferred to a seeded NGM plate. Dye-filling defective animals were identified using a dissecting microscope fitted with epifluorescence, and transferred to individual plates.

2.2.7 PCR STRATEGY TO IDENTIFY HOMOZYGOUS MUTANT ANIMALS

WORM LYSIS
Animals were transferred to the caps of individual tubes (250 µl volume), into 10 µl worm lysis buffer, supplemented with 1:100 proteinase K (Qiagen). Animals were pelleted using the microcentrifuge (1 min., 6500 rpm), and incubated at -80°C for at least 60 minutes. Tubes were transferred to a PCR thermocycler (T3000, Biometra) and incubated 90 minutes at 65°C for optimal proteinase K activity, followed by 30 min at 95°C to inactivate the enzyme, and stored at 4°C prior to polymerase chain reaction (PCR) genotyping.

PCR GENOTYPING
PCR mix was prepared of 0.5 µM forward and 0.5 µM reverse primers, 0.2 mM dNTPs, GoTaq Green buffer (10x stock, Promega), 0.2 µl GoTaq polymerase (Promega), and 1 µl of DNA template. Tubes were transferred to a PCR thermocycler (T3000, Biometra) and following PCR program was run:

1. 94°C, 2 minutes
2. Denaturing step: 94°C, 20 seconds
3. Annealing step: 30 seconds, temperature dependent on primers
4. Extension step: 72°C, 2 minutes, 15 seconds
5. Repeat steps 2-4 35 times
6. Final extension step: 72°C, 10 minutes

7. Store at 4°C

Following PCR, samples were loaded onto a 2% agarose gel for gel electrophoreses, and bands were visualised using UV light.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
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<td>TGA CTG AGT ACA CTG GAA GTC TCG</td>
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<td>gk112859</td>
<td>AAACCTGACCTTATCAGATTCTTGGG</td>
<td>AAACCTGACCTTATCAGATTCTTGGG</td>
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<td>tm2322</td>
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<td>AAC GTT TTG AAT CCT TTG TAT CCG GC</td>
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<td>ok2667</td>
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<td>GTG AGC TGA TGA GTG TCT GA</td>
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</table>

Table 2.3 Primers used for PCR genotyping
2.3 Nematode Egg Preparation Using a Bleach Protocol

To synchronise a population of nematodes, a bleach protocol was used to obtain an egg preparation. A plate of gravid adult nematodes was transferred to a microcentrifuge tube using 1.5ml M9 buffer (42 mM Na2HPO4, 22 mM KH2PO4, 8.6 mM NaCl, 19 mM NH4Cl) and pelleted using a microcentrifuge (1 min at 3000 rpm). Supernatant was discarded and 1 ml bleaching solution (20% bleach, 0.5 M NaOH) added, followed by vigorous shaking (or vortexing) of the tube for 5 minutes to ensure shearing of the nematode cuticles. Supernatant was discarded and carcasses were washed first with M9 buffer supplemented with Tween-20 and then with M9 buffer only. The carcasses and egg pellet were plated on the edge of the bacterial lawn of an NGM plate and incubated until the desired worm stage was reached (~60 hours at 20˚C or until hatched larvae reach early adulthood). Before preparing slides transgenic animals were transferred to a new seeded plate.

2.4 Behavioural and Dye-Uptake Assays

For all behavioural and dye-uptake assays animal populations were synchronised by bleaching and early adult animals were assayed.

2.4.1 Osmotic Avoidance Assay

Osmotic avoidance assays were performed as described previously (Culotti and Russell, 1978). Animals were transferred to non-seeded NGM plates prior to the assay to remove bacterial traces. Using the lid of a laboratory (permanent ink) marker, ~1 cm diameter rings of 8M glycerol (Sigma) supplemented with Bromophenol Blue (Alfa Aesar) were applied to non-seeded NGM plates. When liquid had dried into the plate, 5 animals were transferred to the centre of the ring and monitored for 10 minutes. If a worm crossed the glycerol barrier, the time of crossing was recorded and the animal removed from the assay plate. Results were plotted as percentage of worms that crossed the high osmotic barrier.
2.4.2 CHEMOTAXIS (VOLATILE ATTRACTANT) ASSAY

Chemotaxis assays were performed as described previously (Bargmann et al., 1993). Chemotactic attraction to benzaldehyde (Sigma) was assayed on 2% minimal medium agar plates, supplemented with 5% potassium phosphate pH6.0, 1mM CaCl₂, and 1mM MgSO₄, poured 12-36 hours before the assay. 5 mm from the edge of the plate, on opposite sides, 1 µl of benzaldehyde, diluted 1:200 in EtOH, or 1 µl EtOH (control) was pipetted. Animals were washed three times with M9 buffer and once with deionized water and 50-100 animals were pipetted onto the centre of the plate. At 30 and 60 minutes animals at both spots were counted and chemotaxis index (CI) was calculated: \[ i = (a - b)/n \], where \( a \) is the number of worms within 1.5 cm of the attractant spot, \( b \) the number of worms within 1.5 cm of the control spot, and \( n \) the total number of worms on the plate.

2.4.3 DYE-FILLING ASSAY TO EXAMINE CILIUM INTEGRITY

Animals were prepared as described in section 2.2.6. Following incubation in fluorescent DiI, 1 ml M9 buffer was added to the worms; animals were pelleted, and transferred to NGM plates seeded with \textit{E. coli} OP50 to recover for 30 minutes. For mutant analysis, animals were mounted on 4% agarose pads, anaesthetised with 40 mM levamisole (Sigma) and covered with a glass coverslip. Animals were imaged using a Leica DM5000B microscope, fitted with epifluorescence and a 20x 0.50NA objective (Leica). Images were acquired using a charge-coupled device camera (iXon+ EM-CCD, Andor Technology), controlled by Andor Technology iQ 1.9 software. For co-localisation experiments with promoter GFP-reporters, animals were imaged using the spinning disk confocal microscope set up outlined below.
2.5 FLUORESCENCE IMAGING AND IFT ANALYSIS

2.5.1 PROMOTER ACTIVITY

Transgenic animals expressing promoter GFP-reporters were immobilised on 10% agarose pads with polystyrene microspheres (0.1 μm, Polysciences), and covered with a glass coverslip. Worms were imaged using a Nikon Eclipse Ti microscope, fitted with a 20x 0.75NA CFI Plan Apo objective (Nikon), 50 mW 488 nm and 561 nm lasers, and CSU-X1 spinning disk unit (Yokogawa). Images were acquired using a charge-coupled device camera (iXon EM-CCD, Andor Technology) controlled by Andor Technology iQ 2.6 software. Z-stacks were acquired and NIH ImageJ 1.48 open software was used to generate maximum intensity Z-projections.

2.5.2 PROTEIN LOCALISATION

All protein localisation and AWB and ASER cilium structure experiments, except for intraflagellar transport assays were performed on the Leica DM5000B setup outlined above. Animal populations were synchronised by bleaching and transgenic animals at the early adult stage were anaesthetised with 40 mM levamisole, mounted on 4% agarose pads, covered with a glass coverslip, and imaged. Post-acquisition analysis of cilium length was performed using NIH ImageJ 1.48 open software, and Student’s t-test was performed to determine statistical significance.

2.5.4 INTRAFLAGELLAR TRANSPORT ASSAY

IFT assays were performed on the spinning disk confocal microscope setup outlined above, fitted with a 100x 1.4NA Plan APO VC objective (Nikon), and additional magnification obtained through a 1.5x Optovar optical zoom adaptor. Synchronised animals were mounted on 10% agarose pads, immobilised by 0.1 μm polystyrene microspheres
(Polysciences), and covered by a glass coverslip. Amphid and phasmid cilia were used to record IFT movement. All strains were imaged using 200ms exposure time with varying EM gain for different transgenes.

2.5.5 Kymograph Analysis

Generating Kymographs
Multi-TIFF files were imported into NIH ImageJ 1.48 and substacks were isolated where cilia did not show significant movement. The StackReg and TurboReg plugins were used to correct for any drift that occurred (Thévenaz et al., 1998). Multi-TIFF files were then imported into Icy open software, and the Kymograph Tracker plugin was used to generate anterograde and retrograde kymographs (Chenouard et al., 2010).

Speed Measurements
Anterograde and retrograde kymographs were imported into NIH ImageJ 1.48. Lines were drawn over particles and added to the ROI manager. Length and angle measurements were imported into Microsoft Excel, speeds were calculated as follows:

Anterograde particles:
- Time: $\Delta t = \sin(\text{angle}(\text{rad}) \times -1) \times \text{length}$
- Distance: $\Delta s = \cos(\text{angle}(\text{rad}) \times -1) \times \text{length}$
- Speed: $V(\text{pixels/frame}) = \frac{\Delta s}{\Delta t}$

Retrograde particles:
- Time: $\Delta t = \sin(\text{angle}(\text{rad})) \times \text{length}$
- Distance: $\Delta s = \cos(\text{angle}(\text{rad})) \times \text{length}$
- Speed: $V(\text{pixels/frame}) = \frac{\Delta s}{\Delta t}$
Speeds were converted to microns/second:

\[ V(\mu m/s) = V(pixels/frame) \times framerate \times scale(\mu m/pixel) \]

Data was imported into GraphPad Prism 5.0 to generate speed distribution histograms, calculate mean, standard deviation, and perform Student’s t-test analysis.
CHAPTER III

IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF K04F10.2/KIAA0556,
A NOVEL COMPONENT OF THE CILIARY BASE
3.1 Abstract

Multiple proteomics and genomics approaches have been used to identify the molecular parts list of cilia and flagella. However, the specific cilia-related functions of many of these components remain unknown. Previously, *C. elegans* K04F10.2 was shown to possess an X-box promoter motif, indicative of regulation by the ciliogenic RFX transcription factor DAF-19. Here, a thorough investigation of K04F10.2 in nematodes reveals ciliated cell-specific expression and accumulation of K04F10.2 specifically at the ciliary base, and to a lesser degree at the ciliary proximal region. K04F10.2 null mutants possess grossly normal cilium structure and function, although subtle ultrastructural defects in microtubule number and organisation are observed in distal segments. Although ciliary localisations of various ciliopathy, transport–associated (e.g. intraflagellar transport; IFT) and membrane proteins are normal in K04F10.2 mutant worms, a subtle defect in antero-IFT transport rates is observed for OSM-6/IFT52. A cilia-related role for K04F10.2 is supported by a specific genetic interaction with the IFT-regulator, Joubert Syndrome-associated *arl-13/Arl13b*.

Affinity proteomics of human KIAA0556 (K04F10.2 orthologue) further supports a ciliary function, revealing biochemical interactions with the IFT-B complex, various ciliary GTPases, and katanin subunits associated with microtubule severing. It was found that in *C. elegans*, K04F10.2 is co-expressed in ciliated cells with F47G4.5 (p80 katanin subunit) and *rab-28*, and both encode ciliary proteins: F47G4.5 localises near the ciliary base and RAB-28 localises along the entire axoneme where it undergoes IFT. Similar to K04F10.2 mutants, loss of F47G4.5 causes subtle ciliary ultrastructural and IFT defects, yet cilium morphology and function are grossly normal. However, mutant analysis did not reveal genetic interactions or localisation interdependency; thus, the functional relationship between these genes remains elusive.

These data identify a novel component of the ciliary base, K04F10.2, suggested to functionally interact with Joubert Syndrome *arl-13/Arl13b*. Furthermore, the KIAA0556 interactome was identified, and subsequent analyses in worms revealed new ciliary localisations and transport properties for RAB-28 and F47G4.5.
3.2 INTRODUCTION

Functional characterisation of novel ciliary genes and ciliopathy components in the nematode *Caenorhabditis elegans* has provided insight into their function and related pathomechanisms. For a number of these genes, initial evidence has come from bioinformatics, genomics, and transcriptomics studies (Fan et al., 2004, Blacque et al., 2005, Efimenko et al., 2005, Colosimo et al., 2004, Chen et al., 2006).

The nematode gene K04F10.2 (GenBank ID: 71988281) is reported in a number of bioinformatics and transcriptomics datasets as a candidate cilia-related gene (Colosimo et al., 2004, Blacque et al., 2005, Phirke et al., 2011). K04F10.2 was originally identified in a bioinformatics screen to uncover genes with an X-box promoter element, indicative of regulation by the ciliogenic RFX transcription factor DAF-19 (Blacque et al., 2005). Identification of X-box promoter elements has been used to uncover many novel ciliary genes, as ciliated cell-specific expression of many ciliary genes is DAF-19 dependent (Efimenko et al., 2005, Murayama et al., 2005, Phirke et al., 2011, Senti and Swoboda, 2008). K04F10.2 was found to possess an X-box promoter element 171bp upstream of the gene (Blacque et al., 2005), and Phirke et al. found K04F10.2 expression was reduced in *daf-19* mutants, predicting that K04F10.2 could be a novel cilia-related gene (Phirke et al., 2011). Additionally, Colosimo et al. identified K04F10.2 as a novel gene with upregulated expression in AWB and AFD ciliated cells compared to all cells (Colosimo et al., 2004).

Following identification of novel candidate ciliary genes, validation and characterisation experiments need to be performed. In nematodes several approaches can be used to validate and characterise candidate ciliary genes: first, promoter GFP-reporters are used to examine promoter activity. Often, these experiments are performed on top candidates to validate the experimental approach used to predict novel ciliary genes (Blacque et al., 2005, Efimenko et al., 2005, Swoboda et al., 2000, Fan et al., 2004). Second, proteins are fluorescently tagged to investigate localization patterns. Protein localisation can inform on its function; for example, *xbx-2/dylt-2* and *xbx-7/mks-1* were identified in the same screen for X-box promoter elements, *xbx-2* was found to encode a dynein light chain subunit, and
undergoes IFT-like movement in the cilium, and \textit{xhx-7} was later found to localize to the transition zone (TZ), and is thought to function as part of the MKS-module (Efimenko et al., 2005, Williams and Winkelbauer, 2008). Additionally, if mutant alleles are available, genetic analysis can be performed to assess candidate gene requirement for cilium integrity and function. Experiments can include transmission electron microscopy (TEM) of amphid pore cilia to assess ciliary ultrastructure, investigating ciliary protein localisation, and sensory behaviour assays, such as osmotic avoidance, chemotaxis, and dauer entry assays. Finally, double mutant analysis is performed to investigate possible genetic interactions with known ciliary genes. For example, MKS module components cooperate with NPHP module components to maintain transition zone (TZ) integrity, and double mutants of both modules possess severely defective cilium integrity, more extensive than in either MKS- or NPHP-module single mutants (Williams et al., 2011, Williams et al., 2010, Huang et al., 2011, Williams and Winkelbauer, 2008). These experiments can provide insight into the function of novel candidate ciliary genes, and can inform on pathomechanisms of ciliopathies caused by mutations in these genes.

Another approach to identify ciliary roles of candidate genes is to perform affinity proteomics experiments to identify biochemical interactors. Tandem affinity purification (TAP) approaches, followed by mass spectrometry (MS) have been employed to identify biochemical interacting partners, or the interactome, of ciliary candidates (Cevik et al., 2013, Boldt et al., 2011, Sang et al., 2011). Using mammalian cells stably expressing a tagged protein of interest, complexes are eluted after binding to a column and MS is used to identify co-precipitated proteins (Christian Johannes et al., 2007, Gloeckner et al., 2009). Possible interactions can be validated and further mapped to regions of the interacting proteins by performing yeast-2-hybrid (Y2H) experiments. These experiments can inform on candidate function by elucidating its interacting partners and possible functional connections.

In this work, K04F10.2/KIAA0556 is identified as a novel cilia-related gene that encodes a protein at the ciliary base, with subtle roles in the maintenance of cilium integrity by functioning in a redundant manner with Joubert Syndrome-associated \textit{arl-13/Arl13b}. Validation of the KIAA0556 interactome in \textit{C. elegans} identified two new ciliary genes,
*rab-28* and F47G4.5/KATNB1; the latter was not previously linked to primary cilia in metazoans. However, this work was unable to identify a clear functional association between K04F10.2 and these genes.
3.3 RESULTS

3.3.1 K04F10.2 IS EXPRESSED IN CILIATED SENSORY NEURONS

Previously, sequence analysis revealed that the K04F10.2 promoter region contains a weak X-box promoter element 171 bp upstream of the start codon, suggesting it could be a novel cilia-related gene (Blacque et al., 2005). To assess the expression pattern of K04F10.2 in *C. elegans*, a promoter GFP-reporter was constructed, consisting of 675 bp of K04F10.2 5’ UTR sequence (intergenic region between K04F10.3 and K04F10.2) and the first 18 bp of the gene with a mutated start codon fused to GFP (containing an NLS sequence for ease of cell identification). This construct was injected into wild type worms to generate transgenic animals expressing the P_{K04F10.2}::GFP construct as an extrachromosomal array. The K04F10.2 promoter is active almost exclusively in ciliated neurons (Figure 3.1), which are easily identifiable by their position in the animal and by their dendritic structures that extend to the nose and tail regions. Consistent with expression in most ciliated sensory neurons, all five ciliated tail neurons show expression, namely the bilateral pairs of phasmid ciliated sensory neurons (PHAR/PHAL/PHBR/PHBL) and the right-hand only PQR neuron. Activity can also be seen in a few intestinal cells, most prominently in cells of the first and last two intestinal rings (Figure 3.1). GFP expression is notably absent in the pharynx, muscle cells, and the reproductive system. These data confirm that K04F10.2 expression is mostly restricted to ciliated cells.

3.3.2 K04F10.2 ACCUMULATES AT THE CILIARY BASE AND LOCALIZES TO THE PROXIMAL CILIAM

To address the subcellular localization of the K04F10.2 protein in ciliated cells, transgenic worms were generated expressing a translational K04F10.2::GFP construct, encoding a 568 aa K04F10.2 protein product of 64 kDa C-terminally fused to GFP (as an extrachromosomal array). Briefly, the entire intronic and exonic K04F10.2 genomic sequence together with 5’ UTR sequence was fused in frame with GFP. Initially, two lines were generated by injecting different concentrations of the K04F10.2::GFP construct.
Figure 3.1 The K04F10.2 gene promoter is active in ciliated neurons and some intestinal cells. Shown are fluorescence images from a worm expressing a transcriptional GFP reporter under the control of the K04F10.2 promoter. GFP is expressed in head and tail neurons, including amphid and phasmid ciliated sensory neurons. Expression is also observed in several intestinal cells, including cells of the first (Int1) and last two intestinal rings (Int8 and Int9). Scale bar; 50 µm.
In worms injected with a relatively high concentration (5 ng/µl), a dye-filling defect was observed, indicative of a cilium structure abnormality (Figure 3.2A). This phenotype could be due to high copy number of the X-box-containing K04F10.2 in the extrachromosomal array thus leading to possible sequestration of the DAF-19 transcription factor from endogenous ciliogenic gene promoters. However, the second line generated by injection of a lower concentration (0.5 ng/µl) showed no such defect, and subsequently this line was used for protein localisation experiments (Figure 3.2A).

In ciliated sensory neurons in the head and tail of the worm, GFP-tagged K04F10.2 accumulates at the ciliary base and localizes diffusely throughout the rest of the cell (Figure 3.2B). The signal anterior to the ciliary base accumulation measures 3.43 ± 0.69 µm (129 phasmid cilia in 42 animals), suggesting K04F10.2 is excluded from the distal segment. To determine more precisely which ciliary base subcompartment K04F10.2 localises to (transition zone, basal body, or periciliary membrane compartment), double transgenic strains expressing GFP-tagged K04F10.2 and tdTomato-tagged MKS-5 or ARL-13 were generated. Figure 3.2D shows juxtaposition of the K04F10.2 accumulation and the MKS-5 signal in the transition zone, suggesting that K04F10.2 accumulates immediately below the transition zone at the ciliary base. This is confirmed by co-expression of K04F10.2::GFP and ARL-13::tdTOMATO, showing a gap between the K04F10.2 accumulation and ARL-13, which our lab previously reported is excluded from the transition zone and restricted instead to the neighbouring middle segment compartment (Cevik et al., 2013). The axonemal K04F10.2 signal perfectly overlaps with ARL-13, confirming that K04F10.2 (like ARL-13) is excluded from the distal segment. To investigate whether K04F10.2 can undergo IFT-like motility in the cilium, kymograph analysis of the ciliary K04F10.2 signal in the proximal cilium was performed. Figure 3.2C shows that the protein does not undergo IFT-like movement, suggesting that while the localisation overlaps with IFT-complex components, K04F10.2 is not a part of this complex, and is not carried as IFT-cargo into and out of the cilium.

Taken together, these data show that K04F10.2 strongly accumulates at the ciliary base and localises to a lesser extent to the proximal axoneme, including the middle segment. However, similar to ARL-13, K04F10.2 is excluded from the distal segment.
Figure 3.2 The K04F10.2 protein localises at the ciliary base and within the proximal ciliary axoneme. Shown are fluorescence images from worms expressing a translational GFP reporter for K04F10.2. A. K04F10.2::GFP injected at high concentration (5.0 ng/µl) induces dye-uptake defect. At low concentration (0.5 ng/µl) K04F10.2::GFP does not induce a cilium integrity defect and dye-uptake is normal. Shown are images of K04F10.2::GFP expressing worms stained with DiI. Scale bars; 20 µm. B. GFP-tagged K04F10.2 localises throughout phasmid sensory neurons, excluding the nucleus, and strongly accumulates at the ciliary base region of phasmid and amphid ciliated neurons. C, cilium; D, dendrite; CB, cell body. Scale bars; 3 µm. C. K04F10.2 does not undergo processive movement along the ciliary axoneme. Shown is a kymograph (time vs distance plot) derived from a time-lapse recording of GFP-tagged K04F10.2 in a phasmid ciliary axoneme. Horizontal scale bar; 2 µm. Vertical scale bar; 5 s. D. The K04F10.2 protein accumulates below the transition zone and is excluded from the distal segment. Shown are fluorescence images of double transgenic worms expressing K04F10.2::GFP and either MKS-5::tdTomato or ARL-13::tdTomato. Scale bar; 3 µm.
A number of transport pathways cooperate to target and restrict proteins to ciliary compartments, including IFT, membrane transport pathways, and ciliary gating mechanisms. To investigate how K04F10.2 is targeted to the ciliary base, K04F10.2 localisation was assessed in mutant alleles of ciliary transport and ciliopathy-associated genes. Specifically, K04F10.2::GFP localisation was analysed in osm-5 (IFT-B complex), bbs-8 (BBSome), mks-5 (MKS module), nphp-4 (NPHP module), and arl-13 (Joubert Syndrome-associated) gene mutants. In all mutants GFP-tagged K04F10.2 accumulated at the ciliary base, and signals extended distally into the proximal ciliary region, suggesting that K04F10.2 ciliary targeting and localisation is independent of these genes (Figure 3.3).

Interestingly, a number of the images suggest that K04F10.2 associates with the cilia-associated membranes. mks-5/RPGRIP1L mutants possess severe structural defects at the ciliary base, including a lack of most TZ-components (Jensen et al., submitted). In this mutant the K04F10.2::GFP ciliary base signal is split, forming two signals consistent with periciliary membrane localisation (arrow in figure 3.3). However, this signal does not appear to cover the entire periciliary membrane region and seems to be concentrated in the region immediately below the transition zone. More convincing evidence of ciliary membrane-association is observed in arl-13/Arl13b mutants, where K04F10.2 decorates ciliary membrane expansions (arrow in figure 3.3). These data suggest that K04F10.2 localisation does not require various ciliopathy and transport genes, and that GFP-tagged K04F10.2 may be membrane-associated.

![Figure 3.3 K04F10.2 localisation is independent of IFT-B, BBSome, MKS/NPHP modules, and arl-13.](image-url)

Shown are fluorescence images of phasmid cilia from worms expressing translational GFP reporter for K04F10.2 in the indicated genetic background. Arrows; possible membrane association of K04F10.2::GFP. Scale bar; 3 µm.
3.3.4 Structural analysis of amphid and phasmid cilia in K04F10.2 mutants

One of the major experimental advantages of *C. elegans* as a model organism is the availability of mutant alleles for most genes. For K04F10.2, 31 alleles have been identified, most of which are silent or missense mutations acquired through the Million Mutation Project (Thompson et al., 2013). One of these alleles (*gk112869*) is a nonsense mutation, resulting in a premature stop codon at amino acid position 133. A deletion mutant is also available: *tm1830* possesses a frameshift 347 bp deletion spanning exon 4 and intron 4 and is likely a null mutant (*Figure 3.4A*). For the genetic analysis of K04F10.2 requirement for cilium structure and function the *tm1830* allele was used.

An indirect measure of cilium structure is the ability of the nematode to incorporate a lipophilic fluorescent dye, DiI, into the plasma membrane of 6 environmentally exposed amphid and 2 phasmid neurons on either side of the animal (Inglis et al., 2007). Although the relationship between dye-filling and cilium structure is not fully clear, many ciliopathy and IFT gene mutants with cilium integrity defects exhibit a reduced or absent dye-filling phenotype (Dyf, (Starich et al., 1995)). Dye uptake levels in both *tm1830* and *gk112869* mutants were comparable to wild type, suggesting normal cilium integrity in at least these eight dye-filling neurons in the mutant animals (*Figure 3.4B*). To directly assess cilium structure in greater detail, ciliated cell-specific transcriptional GFP-reporters were employed to illuminate entire neuronal structures including the cilium. In *tm1830* mutants, AWB cilium length was normal and the fan structure intact, as were the length and rod-shape structure of the ASER cilium (*Figure 3.4C*). Thus, disruption of K04F10.2 does not lead to any gross alteration of cilium structure, although subtle defects cannot be ruled out in those cilia not analysed.
Figure 3.4 K04F10.2 mutants possess normal cillum structure and morphologies and exhibit normal sensory behaviours. A. K04F10.2 gene structure, showing genomic positions of gk112869 and tm1830 alleles. B. K04F10.2 mutants are dye-filling normal. Shown are fluorescence images of head and tail regions stained with DiI. Scale bars: 20 µm. C. AWB and ASER cillum morphologies are unaffected in tm1830 mutants. Fluorescence images are shown of worms expressing transcriptional GFP reporters for str-1 (AWB) or gcy-5 (ASER). AWB and ASER cillum lengths are not significantly different in tm1830 mutants compared to wild type. AWB: p=0.92, n=44 (wild type) and n=71 (tm1830). Scale bars: 3 µm. D. Osmotic avoidance behaviour is unaffected in tm1830 mutants. Avoidance behaviour to 8M glycerol measured over 10 minutes for wild type, osm-5(p813), and K04F10.2(tm1830) animals is shown. At least 6 rings with 5 worms were assessed for each strain. E. Chemotaxis towards benzaldehyde is unaffected in tm1830 mutants. Chemotaxis indices (CI) measured at 30 and 60 minutes are shown for wild type (N2), che-11 (e1805), and K04F10.2(tm1830). Assays were repeated 4 times for control strains, 6 times for K04F10.2(tm1830). Asterisk; p<0.05.
3.3.5 Ultrastructural analysis of amphid channel cilia

To assess cilium structure in even greater resolution, the ultrastructure of amphid pore cilia in adult worms was examined via transmission electron microscopy (TEM). TEM is a frequently used technique to study cilium ultrastructure in the amphid channels in the head of the nematode and has provided insight into the requirement of many genes for cilium formation and ultrastructure (Perkins et al., 1986, Cevik et al., 2010, Kaplan et al., 2012, Li et al., 2010, Hao et al., 2011, Kaplan et al., 2010, Williams et al., 2011, Evans et al., 2006, Murayama et al., 2005, Doroquez et al., 2014). Cilia of eight sensory neurons are environmentally exposed through the amphid pore. Single cilia extend from six neurons (ASE, ASG, ASH, ASI, ASJ, and ASK) and pairs of cilia extend from two neurons (ADF, ADL). These cilia possess a bipartite microtubule arrangement, where the middle segment possesses nine microtubule doublets, consisting of A- and B-tubules. The B-tubule terminates at the approximate axoneme midpoint, and the nine A-tubules extend to form the distal segment (Doroquez et al., 2014, Perkins et al., 1986).

In wild type animals, ten distal segments are observed at the extreme distal tip of the pore (Figure 3.5A, Y). On average eight microtubule singlets are observed, indicating that not all A-tubules extend to the extreme distal tip (Figure 3.5C, D, Y). More proximally, in the middle segment, microtubule numbers increase to approximately 9 microtubule doublets per axoneme (Figure 3.5M, N, Y) and some smaller, inner singlet microtubules are observed (0.6 ± 0.9 inner MT/axoneme). In the transition zone, Y-links connect nine doublet microtubules to the membrane and approximately 4 smaller, inner singlet microtubules are observed per axoneme (Figure 3.5 Q, R, Y).

In tm1830 mutants, the pores do not contain a full complement of axonemes at the extreme distal tip (8.5 axonemes/pore). Also, distal segment microtubules are defective in number and organisation (Figure 3.5E-G, J-L, Y). Specifically, three microtubule singlets are observed per axoneme at the distal tip (versus 8 in wild type). In more proximal regions of the distal segment, singlet microtubule numbers increase to 3.4 (versus 8.5 in wild type). Also, microtubule doublets are observed in the distal ciliary segments of tm1830 mutants.
These data indicate that in \textit{tm1830} mutants 1-2 cilia that are truncated or mispositioned, and some axonemes possess defects in microtubule number and organisation. However, the middle segment, transition zone, and periciliary membrane compartments of the \textit{tm1830} mutants appear unaffected in relation to microtubule number and arrangement (\textbf{Figure 3.5O, P, S, T, W-Y}).

\subsection*{3.3.6 Investigation of Cilium Function in K04F10.2 Mutants}

Next, cilium function was assessed using assays for osmotic avoidance and chemotaxic behaviours, which are mediated by the ciliated ASH and AWC sensory neurons in the head of the worm, respectively (Inglis et al., 2007). For the osmosensory assay, 5 worms are placed in an area of low osmolarity, inside a ring of high osmolarity (8M glycerol) and assessed as a population. Worms with reduced function (Osm phenotype) will cross this barrier, whereas wild type animals will retreat from the barrier upon contact. \textit{tm1830} mutant animals showed normal osmosensory behaviour, and did not cross the high osmolarity barrier, suggesting that K04F10.2 is not required for this cilia-related function (\textbf{Figure 3.4D}). AWC cilia serve chemosensory functions, sensing volatile attractants, such as isoamylalcohol and benzaldehyde (Inglis et al., 2007). For the assay, worms are placed equidistant between an attractant spot and a control spot, and assessed as a population to determine the chemotaxis index (CI) over time. \textit{tm1830} mutants do not show a significantly different response than wild type controls, suggesting normal AWC cilia function (\textbf{Figure 3.4E}).

These data show that two cell-specific sensory behaviours appear normal in K04F10.2 mutants: osmosensory behaviour, mediated by ASH neurons, and chemotaxis mediated by AWC neurons.
Figure 3.5 K04F10.2(tm1830) mutants possess ultrastructural defects in distal segments of amphid cilia. Transmission electron micrographs of serial cross sections of amphid pores from wild type (N2) and K04F10.2(tm1830) animals. A-B Extreme distal tip of the pore showing 10 axonemes in wild type (A) and 7 axonemes in tm1830 mutants (B). C-G 2 µm proximal to A-B tm1830 mutants (E-G) possess reduced microtubule numbers and microtubule doublets that are not observed in wild type animals (C-D). H-L tm1830 mutants exhibit reduced microtubule numbers and abnormal B-tubule extension in more proximal regions of the distal segment. 2 µm proximal to C-G distal segments of tm1830 mutants possess reduced microtubules and microtubule doublets are observed (J-L). M-P Middle segment ultrastructure is unaffected in tm1830 mutants. 2 µm proximal to H-L axonemes of both wild type (M-N) and tm1830 mutants (O-P) consist of nine microtubule doublets, and several smaller inner singlet microtubules are observed. Q-T Transition zone ultrastructure is normal in tm1830 mutants. 2 µm proximal to M-P transition zones of wild type (Q-R) and tm1830 mutants (S-T) possess nine microtubule doublets, connected to the membrane via Y-link structures (arrows). Also observed are several smaller inner microtubule singlets. U-X Periciliary membrane compartments (PCMC) are unaffected in tm1830 mutants. Wild type (U-V) and tm1830 mutant (W-X) PCMCs show similar morphologies. Y Quantifications of axonemes, microtubule number and arrangement in middle (+6 µm; M-P) and distal segments (+2 µm; C-G, and +4 µm; H-L). Scale bars; 200 nm. Sample preparation and imaging performed by Katarzyna Welzel, University College Dublin.
Despite the lack of gross alterations of cillum structure and function in K04F10.2 mutants, it remains possible that K04F10.2 subtly regulates cilia-related protein targeting and transport pathways. To assess if K04F10.2 is required for ciliary protein localisation and transport, \textit{tm1830} mutants expressing fluorescently tagged components of the IFT complex, the BBSome, transition zone-localised modules (MKS and NPHP modules), and several membrane (i.e. associated) proteins were generated. IFT particle components OSM-3/KIF17 (homodimeric kinesin II), XBX-1/DYNC2LI1 (cytoplasmic dynein light intermediate chain), CHE-11/IFT140 (IFT-A), and OSM-6/IFT52 (IFT-B) all localised along the entire axoneme and accumulated at the ciliary base, comparable to wild type controls (Figure 3.6A). The BBSome subunit BBS-7 showed characteristic ciliary localisation in \textit{tm1830} mutants, accumulating below the cilium and localising along the entire axoneme. The MKS-module component MKS-2/TMEM216 is restricted to the transition zone membrane, suggesting the MKS module is mostly intact, as MKS-2 localisation has been shown to be affected in most MKS module gene mutants (Huang et al., 2011).

Also, membrane-associated ARL-13 shows normal localisation to the Inv compartment in \textit{tm1830} worms (Figure 3.6A). K04F10.2 function is also not required for ciliary targeting and localisation of several transmembrane and membrane-associated proteins (Figure 3.6A). These include RPI-2 (retinitis pigmentosa gene 2) and TRAM-1 (orthologue of the TRAM translocon), which localise at the periciliary membrane, but are excluded from the cilium. The unaffected localisation of these proteins in the K04F10.2 mutants provides further evidence that the transition zone is functioning normally, because in MKS and NPHP module mutants, these proteins abnormally leak into the cilium (Williams et al., 2011). The localisation of the 7 transmembrane odorant receptor ODR-10 to the AWB cilium is also unaffected in K04F10.2 mutants. ODR-10 ciliary targeting is dependent on several membrane trafficking pathway components, including UNC-101 (AP1 µ1 subunit), CHC-1 (clathrin), and RAB-8 (Kaplan et al., 2010, Dwyer et al., 2001). Thus, the normal localisation of this protein in K04F10.2 worms indicates normal polarised membrane transport required for ODR-10 targeting to the cilium.
Figure 3.6 K04F10.2(tm1830) mutants exhibit normal ciliary protein localisations and OSM-6 MS anterograde speeds are reduced. A. Fluorescence images are shown of indicated labelled proteins in wild type and tm1830 AWB (ODR-10 only) or phasmid (all other proteins) cilia. Ciliary localisations of indicated proteins are not affected in tm1830 mutants. Scale bar; 3 µm. B-C. tm1830 mutants exhibit reduced OSM-6 MS anterograde speeds. Kymograph analysis of indicated markers in wild type and tm1830 amphid and phasmid cilia shows OSM-6 (IFT-B) anterograde speeds are reduced in middle segments, but are not significantly different from wild type in distal segments and in retrograde direction. Also shown are sample kymographs (time vs. distance plots) derived from a time-lapse recording of indicated GFP-tagged proteins in a phasmid ciliary axoneme, and anterograde and retrograde particles have been separated. Kymograph dimensions: Horizontal axis (distance); 5 µm. Vertical axis (time); 25 seconds. Particle speed distributions for kymographic analysis are shown in C. MS; middle segment. DS; distal segment. Retro; retrograde.
Although localisation of intraflagellar transport proteins was unaffected in K04F10.2 mutants (see above), it remained possible that functionality of the intraflagellar transport machinery could be subtly affected. To investigate intraflagellar transport kymographic analysis of moving particles in the cilium was performed. Specifically, for OSM-3 (kinesin II), CHE-11 (IFT-A), and OSM-6 (IFT-B), the middle and distal segment anterograde speeds were measured, as were retrograde rates. It was found that in middle segments of wild type animals OSM-3, CHE-11, and OSM-6 moved in the anterograde direction at speeds between 0.65-0.73 µm/s (Figure 3.6B). In K04F10.2 mutants OSM-3 and CHE-11 speeds were comparable to wild type (0.71 µm/s and 0.73 µm/s, respectively), but OSM-6 moved significantly slower (0.65 µm/s, p<0.001). However, anterograde speeds in the distal segment were unaffected for all three markers (Figure 3.6B). Specifically, OSM-3 moved at 1.53 µm/s in wild type distal segments and at 1.44 µm/s in K04F10.2 mutants; CHE-11 moved at 1.45 µm/s and at 1.40 µm/s in wild type and mutant animals, respectively; and OSM-6 moved at 1.31 µm/s in wild type and at 1.30 µm/s in mutants. In the retrograde direction, OSM-3, CHE-11, and OSM-6 speeds were comparable in wild type and K04F10.2 mutants (Figure 3.6B). OSM-3 travelled at 1.31 µm/s in wild type and at 1.34 µm/s in mutant animals; CHE-11 moved at 1.49 µm/s and at 1.40 µm/s in wild type and mutant animals, respectively; and OSM-6 moved at 1.31 µm/s in wild type and at 1.35 µm/s in K04F10.2 mutants.

Together, these analyses show that kinesin II, and IFT-A components travel at comparable speeds in wild type and tm1830 mutant animals (Figure 3.6B and C). However, in the middle segment OSM-6/IFT52 (IFT-B component) was statistically significantly slower in tm1830 than in wild type animals.
Although cilium structure and function are grossly normal in K04F10.2 mutant animals, K04F10.2 may function redundantly with other ciliary/ciliopathy genes and pathways to build and maintain the organelle. For example, previous work has shown that several transition zone-associated MKS- and NPHP-module single gene mutants have grossly normal cilium structure and function, but when combined, MKS-NPHP double mutants possess severe defects in cilium structure and function (Williams et al., 2011, Williams and Winkelbauer, 2008, Williams et al., 2010). To test this hypothesis, double mutant analyses were performed to test for genetic interaction between K04F10.2 and various genes involved in IFT, TZ function, and ciliopathies.

A combination of the K04F10.2 mutation (tm1830) and a kinesin II mutant (klp-11), IFT-A (ifta-1/WDR35), or mutants of either the MKS- or NPHP-module (mks-5 and nphp-4, respectively) did not alter the dye-filling and osmotic avoidance phenotypes observed in single mutants. For example, a double mutant of K04F10.2 and nphp-4 was dye-filling normal, as were both single mutants (Figure 3.7). This suggests K04F10.2 does not genetically interact with any of these genes for dye-filling (cilium structure) or osmotic avoidance (cilium function). However, a double mutant of K04F10.2 and arl-13 (tm2322 allele) exhibited a synthetic dye-filling phenotype (SynDyf), with DiI uptake severely reduced or absent in both head and tail neurons (Figure 3.7). This synthetic interaction was not seen in the osmotic avoidance assay, suggesting that the K04F10.2 mutation does not additionally disrupt ASH function in the arl-13 mutant. The tm2322 allele was previously shown to be an in-frame deletion, with the truncated protein product localising mostly to the cilium and periciliary membrane, indicating that the allele may not be a full null but may retain some level of functionality (Cevik et al., 2010). It is possible that arl-13 and K04F10.2 function in the same pathway and the K04F10.2 mutation deregulates the remaining functioning ARL-13, resulting in a synthetic dye-filling phenotype. Another possibility is that these proteins have a similar ciliary function, but in separate pathways, and by altering ARL-13 function and removing K04F10.2 a synthetic structural defect
occurs. Interestingly, K04F10.2 and ARL-13 both localise to the middle segment and are excluded from the distal segment.

Figure 3.7 K04F10.2 genetically interacts specifically with \textit{arl-13}. A. Shown are fluorescence images of head (top row) and tail (bottom row) regions stained with DiI. K04F10.2;\textit{arl-13} exhibit a synthetic dye-uptake defect (SynDyf) more severe than in either single mutant. Other double mutant combinations tested did not show a SynDyf phenotype. Dye-uptake was assessed by eye. Scale bars; 20 µm. B. Summary of assays used to identify possible genetic interactions. A SynOsm phenotype is not observed in K04F10.2;\textit{arl-13} mutants. ++; normal. +; slightly reduced. +/-; moderately reduced. -; severely reduced. -/- absent.
To further examine the association between K04F10.2 and arl-13, a possible genetic interaction was investigated between K04F10.2 and arl-3, because Li et al. previously showed that arl-3 and arl-13 genetically interact (Li et al., 2010). More specifically, they showed that arl-13(gk513) null mutants possess dye-filling and IFT defects, which were partially rescued by an arl-3 mutation. Like arl-13, arl-3 encodes a small GTPase shown to regulate intraflagellar transport and trafficking of ciliary proteins into the cilium (Li et al., 2010, Humbert et al., 2012, Schwarz et al., 2012, Wright et al., 2011). Different combinations of three alleles (K04F10.2 (tm1830), arl-13 (tm2322), and arl-3 (tm1703)) were generated to investigate if an additional arl-3 mutation could (partially) rescue the SynDyf phenotype observed in arl-13;K04F10.2 double mutants. K04F10.2;arl-3 double mutants did not show a more severe phenotype than either single mutant, and the SynDyf phenotype in the arl-13;K04F10.2 mutant was not rescued in the arl-13;K04F10.2;arl-3 triple mutant (Figure 3.8). This might suggest that K04F10.2 and arl-3 do not genetically interact and that the association between arl-13 and K04F10.2 is not related to the genetic association between arl-13 and arl-3. However, this interpretation is problematic, because we initially assumed that the arl-3 mutation would rescue the Dyf phenotype of arl-13(tm2322) mutants, similar to what was found for the arl-13(gk513) mutants (Li et al., 2010). However, subsequent analysis showed that the arl-3(tm1703) mutation does not rescue the dye-filling defect of arl-13(tm2322) worms (Figure 3.8). Thus, the functional relationship between arl-13 and arl-3 can only be observed with the gk513 null allele. It is possible the tm2322 allele results in a deregulated protein product, with a phenotype that cannot be rescued by the arl-3 mutation, whereas the absence of a protein product in the gk513 genetic background used in other studies can be compensated by an additional arl-3 mutation.
Figure 3.8 K04F10.2 does not genetically interact with arl-3. Shown are fluorescence images of head (top row) and tail (bottom row) regions stained with DiI. K04F10.2;arl-3 show similar dye-uptake levels as corresponding single mutants. K04F10.2;arl-13;arl-3 mutants show a SynDyf phenotype similar to that observed in K04F10.2;arl-13 double mutants. Scale bar; 20 µm.
As a complementary approach to nematode studies to reveal the molecular basis of K04F10.2 ciliary function, bioinformatics approaches were taken. Specifically, evolutionary conservation and domain architecture were assessed. A tBLASTn algorithm querying the human genome using the translated K04F10.2 sequence produced a single hit, which was candidate homolog KIAA0556 (E-value 2E-17). A reverse tBLASTn algorithm using human KIAA0556 to query the nematode genome produced K04F10.2 as the only hit (E-value 1E-74). The nematode 568 amino acid long K04F10.2 protein is shorter than mammalian KIAA0556, aligning with the C-terminal region of the 1618 amino acid long KIAA0556 sequence (Figure 3.9).

Next, we collaborated with the Syscilia network (Centre for Molecular and Biomolecular Informatics, UMC St. Radboud, the Netherlands) to analyse the evolutionary conservation of K04F10.2 by performing a phylogenetic analysis. Several, but not all, species with a ciliated stage or lifecycle contain a copy of K04F10.2/KIAA0556 (Figure 3.10). *Dictyostelium discoideum* is the only non-ciliated species where a homolog was found, further suggesting that K04F10.2/KIAA0556 has a cilia-specific function.

Sequence analysis of K04F10.2/KIAA0556 homologs identified three (short version, K04F10.2) or four (long version, KIAA0556) globular domains (Pfam ID DUF4457) that are part of a galactose-binding domain protein family (Figures 3.10 and 3.11). Based on predicted secondary structure of KIAA0556, these domains show structural homology with the galactose-binding domain of IFT25 (PDB ID 2yc2). Additionally, by circular permutation of the first 25 amino acids of each KIAA0556 repeat, the alignment with the interaction interface of IFT25 is improved, providing further evidence for homology between IFT25 and KIAA0556 (Figure 3.12, analysis performed by Russell lab, University of Heidelberg). IFT25 is an IFT-B component required for Hedgehog signalling, but not ciliogenesis, in mammalian systems (Keady et al., 2012). It forms a heterodimer with small ciliary GTPase IFT27, and is required for IFT27 localisation (Bhogaraju et al., 2011, Keady et al., 2012, Wang et al., 2009).
Figure 3.9 Nematode K04F10.2 aligns with C-terminal human KIAA0556. Shown is a ClustalW sequence alignment of the human tBLASTn hit when queried with nematode K04F10.2 protein sequence. E-value: 2E-17.
Figure 3.10 K04F10.2 is evolutionarily conserved in species with a ciliated stage/cycle. Evolutionary analysis shows that orthologs of K04F10.2/KIAA0556 are present in several species with a lifecycle or stage with cilia or flagella. Non-ciliated species, Dictyostelium discoideum excluded, do not contain a copy of this gene. Two forms are present, of which the longer, KIAA0556-like form is suggested to be the ancestral form. Internal repeats found through sequence analysis are part of a galactose-binding domain protein family, of which C. reinhardtii IFT25 is also a member. Analysis performed by John van Dam, Centre for Molecular and Biomolecular Informatics, NCMLS, UMC St. Radboud, Nijmegen, the Netherlands.
Figure 3.11 KIAA0556 domains show sequence homology in multiple species. Alignment of the domains (DUF4457) found in KIAA0556 homologs shows sequence homology and conservation across several species. BFLO: Branchiostoma floridae. DRER: Danio rerio. MMUS: Mus musculus. TRUB: Trypanosoma brucei. XTRO: Xenopus tropicalis. Analysis performed by Qianhao Lu, University of Heidelberg, Germany.
Figure 3.12 KIAA0556 domains show homology with IFT25 galactose binding domain. Shown are alignments of IFT25 to DUF4457 domains of several KIAA0556 homologs. By circular permutation, the first 25 amino acids of DUF4457 domains are moved to the end of each domain, improving the alignment. 2yc2A; IFT25 galactose binding domain. Has; Homo sapiens. Cfa; Canis familiaris. Rno; Rattus norvegicus. Mmu; Mus musculus. Xla; Xenopus laevis. Pte; Paramecium tetraurelia. Ddi; Dictyostelium discoideum. Cel; Caenorhabditis elegans. Analysis performed by Qianhao Lu, University of Heidelberg, Germany.
Here, phylogenetic analysis shows evolutionary conservation of K04F10.2/KIAA0556 in species with a ciliated stage or lifecycle, and globular domains in the protein show homology to IFT25. Taken together, a hypothesis is formed that KIAA0556/K04F10.2 is a functional paralog of IFT25, and acts in an IFT25-like manner to regulate ciliary GTPases.

3.3.11 Identification of the Human KIAA0556 (K04F10.2 Orthologue) Interactome

Proteomic experiments can inform on ciliary protein function by mapping interaction networks (Cevik et al., 2013, Boldt et al., 2011). In collaboration with colleagues within the Syscilia network (Roepman & Kremer lab; Radboud University Nijmegen, Ueffing lab; University of Tubbingen) interacting partners of human KIAA0556 were identified through a tandem affinity purification (TAP) approach. Importantly, GFP-tagged KIAA0556 also localizes to the ciliary base, indicating that KIAA0556 is likely the functional homologue of K04F10.2 (Figure 3.13A). For the TAP experiments KIAA0556 was N-terminally tagged with a Strep/FLAG tag (SF-TAG) and expressed in human embryonic kidney cells (HEK293 cells). Cells were lysed, and KIAA0556 purified by binding to a Strep-Tactin matrix. Bound proteins were subsequently eluted, and LC-MS/MS used to identify co-precipitating proteins (Gloeckner et al., 2009, Christian Johannes et al., 2007). After exclusion of likely false positive hits (hits that occur in >15% of all investigated bait proteins), 50 proteins (n=2 independent experiments) were found to form the interactome of KIAA0556 (Figure 3.13B, full list of hits in Table 3.1), of which 33 were identified in both experiments. In both replicates the IFT-B complex is identified almost in its entirety. With the exception of IFT54, which was only identified in one experiment, all core proteins and peripheral subunits are identified (Figure 3.13B). In Chlamydomonas reinhardtii these proteins form a 500kDa complex required for anterograde IFT (Lucker et al., 2005, Lucker et al., 2010). Interestingly, in the list of 33 proteins no IFT-A, BBSome, MKS- or NPHP-module components were identified in these experiments, suggesting the interaction between KIAA0556 and the IFT-B complex may be specific and related to KIAA0556/K04F10.2 function.
Figure 3.13 Interactome of human KIAA0556. **A.** KIAA0556 localises to the base of the cilium and the ciliary tip. Fluorescence images are shown of hTERT-RPE1 cells expressing GFP-tagged KIAA0556 (green), and stained for RPGRIP1L/MKS5 (red), polyglutamylated tubulin (GT335, magenta), and nuclei are stained with DAPI (blue). Data contributed by Erik de Vrieze (Radboud University Medical Center, Nijmegen, the Netherlands). **B.** KIAA0556 complexes include most IFT-B components, CEP290, katanin subunits and ciliary GTPases RABL5 and RAB28. All proteins were identified in both TAP (tandem affinity purification) experiments, except for RAB28, CEP290, and IFT54. TAP experiments were performed by Minh Nguyen and Lisette Hetterschijt, Radboud University Medical Center, Nijmegen, the Netherlands. MS analysis was performed by Karsten Boldt and Marius Ueffing, University of Tuebingen, Germany. **C.** KIAA0556 interactions with katanin and IFT-B complexes map to KATNBL1 and IFT88. Shown are interactions found in a yeast two-hybrid analysis using various KIAA0556 fragments against a panel of 196 cilia-related proteins, and an extrapolation of these interactions to worm homolog K04F10.2. Y2H analysis was performed by Erik de Vrieze, Radboud University Nijmegen, the Netherlands.
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<td>Q15370</td>
<td>4</td>
<td>0.170</td>
<td>4</td>
<td>0.390</td>
</tr>
<tr>
<td>FLNA</td>
<td>filamin A, alpha</td>
<td>P2133</td>
<td>3</td>
<td>0.016</td>
<td>3</td>
<td>0.016</td>
</tr>
<tr>
<td>HSPB11</td>
<td>heat shock protein family (beta) member 11</td>
<td>Q8Y547</td>
<td>3</td>
<td>0.330</td>
<td>3</td>
<td>0.330</td>
</tr>
<tr>
<td>IFT22</td>
<td>(Chlamydomonas)</td>
<td>Q9H7X7</td>
<td>4</td>
<td>0.250</td>
<td>2</td>
<td>0.140</td>
</tr>
<tr>
<td>KIF11</td>
<td>kinesin family member 11</td>
<td>P02732</td>
<td>3</td>
<td>0.26</td>
<td>3</td>
<td>0.26</td>
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<tr>
<td>SF3B3</td>
<td>splicing factor 3b, subunit 3, 130kDa</td>
<td>Q6F393</td>
<td>3</td>
<td>0.26</td>
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Table 3.1 Biochemical interactome of human KIAA0556 identified using a tandem affinity purification (TAP) approach. The KIAA0556 interactome (n = 2 independent experiments) consists of 50 proteins, 33 were identified in both experiments. Hits that occur in >15% of interactomes of all investigated bait proteins were removed as probably false positive hits.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Full name</th>
<th>protein ID</th>
<th>peptide cov_E1</th>
<th>Seq cov_E1</th>
<th>peptide cov_E2</th>
<th>Seq cov_E2</th>
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<tr>
<td>SUGT1</td>
<td>SG11, suppressor of G2 allele of SKP1 (S. cerevisiae)</td>
<td>Q9Y220</td>
<td>3</td>
<td>0.093</td>
<td>3</td>
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<td>AHCPY</td>
<td>adenosylhomocysteinase</td>
<td>P23525</td>
<td>2</td>
<td>0.029</td>
<td>3</td>
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<tr>
<td>SFPQ</td>
<td>splicing factor proline/glutamine-rich</td>
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<td>2</td>
<td>0.015</td>
<td>3</td>
<td>0.059</td>
</tr>
<tr>
<td>ASAP2</td>
<td>ArfGAP with SH3 domain, ankyrin repeat and PDZ domains 2</td>
<td>C142150</td>
<td>2</td>
<td>0.053</td>
<td>3</td>
<td>0.053</td>
</tr>
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<td>C15078</td>
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<td>0.004</td>
<td>3</td>
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<td>3</td>
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<tr>
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<td>KIAA1781</td>
<td>Q9COB2</td>
<td>2</td>
<td>0.030</td>
<td>3</td>
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</tr>
<tr>
<td>KMT2D</td>
<td>lysine (K)-specific methyltransferase 2D</td>
<td>C14686</td>
<td>2</td>
<td>0.015</td>
<td>3</td>
<td>0.015</td>
</tr>
<tr>
<td>MAPK1</td>
<td>mitogen-activated protein kinase 1</td>
<td>P28482</td>
<td>2</td>
<td>0.050</td>
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<td>MCCC2</td>
<td>methylcrotonyl-CoA carboxylase 2 (beta)</td>
<td>Q9HCC0</td>
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<td>3</td>
<td>0.064</td>
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<tr>
<td>PPM1B</td>
<td>protein phosphatase, Mg2+/Mn2+ dependent, 1B</td>
<td>C75888</td>
<td>2</td>
<td>0.046</td>
<td>3</td>
<td>0.046</td>
</tr>
<tr>
<td>PTPRU</td>
<td>protein tyrosine phosphatase, receptor type, U</td>
<td>Q92729</td>
<td>2</td>
<td>0.034</td>
<td>3</td>
<td>0.034</td>
</tr>
<tr>
<td>RAD50</td>
<td>RAD50 homolog (S. cerevisiae)</td>
<td>Q92878</td>
<td>2</td>
<td>0.010</td>
<td>3</td>
<td>0.010</td>
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<tr>
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<td>semenogelin II</td>
<td>Q02383</td>
<td>2</td>
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<td>3</td>
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</tbody>
</table>
Furthermore, katanin proteins were identified in the TAP experiments (Figure 3.13B, full list of hits in Table 3.1). Katanin is proposed to be a microtubule severing enzyme complex consisting of an enzymatic subunit (three paralogs: KATNA, KATNALA1, KATNAL2) and a regulatory subunit (two paralogs: KATNB, C15Orf29/KATNBL1) (Roll-Mecak and McNally, 2010). Katanin was originally purified from sea urchin eggs and identified as an ATPase that severs microtubules after a previous study had reported microtubule disassembly activity in Xenopus laevis egg extracts (McNally and Vale, 1993, Vale, 1991). In Chlamydomonas reinhardtii katanin has been implicated in stability of the flagellar central pair microtubules and in flagellar severing. (Dymek et al., 2004, Lohret et al., 1998, Lohret et al., 1999, McNally et al., 1996, Rasi et al., 2009) To date, no reports have been published on katanin function in primary cilia, but the data presented below suggest that this microtubule-severing complex may play a cilia-related role.

Finally, the KIAA0556 interactome was enriched for small GTPases, namely IFT27, RABL5/IFT22, and RAB28 (Figure 3.13B, full list of hits in Table 3.1). The identification of GTPase interaction with KIAA0556 is particularly interesting given the aforementioned hypothesis that this protein is IFT25-like (by structural homology) and thus may interact with IFT27 or an IFT27-like GTPase. In photoreceptors, RAB28 localizes to the basal body and ciliary rootlet and mutations in RAB28 were recently shown to be causative for autosomal-recessive cone-rod dystrophy (arCRD), a progressive retinal dystrophy characterised by early vision impairments (Roosing et al., 2013). In Trypanosoma brucei RAB28 is involved in endosomal trafficking pathways and mediates lysosomal targeting (Lumb et al., 2011). C. elegans ifta-2/Rabl5 expression is controlled by the RFX transcription factor daf-19 that binds to the X-box sequence upstream of the gene (Chen et al., 2006). IFTA-2 localizes to the basal body region and cilia where it undergoes IFT with the IFT-B complex (Ou et al., 2007). However, unlike IFT particle subunits, ifta-2 mutants do not show severe IFT and cilium structure defects. Rather, mutant animals exhibit increased longevity and defective dauer phenotypes, suggesting a function for IFTA-2 in signal transduction rather than ciliogenesis and cilium maintenance (Schafer et al., 2006). In Chlamydomonas RABL5 depletion leads to reduced cellular pools of IFT-A and –B complex subunits, but IFT particles in the flagellum increase and still undergo IFT (Silva et al., 2012). In Trypanosoma brucei RABL5 undergoes IFT in the flagellum, and is essential for flagellum assembly: RNAi knockdown of RABL5 results in
short flagella with accumulated IFT material, suggesting a role for RABL5 in retrograde trafficking or regulating entry of IFT proteins to the flagellum (Adhiambo et al., 2009). IFT27 is a subunit of the IFT-B complex and plays a role in cell division and ciliogenesis and requires IFT25/HSBP11 for its localization (Bhogaraju et al., 2011, Wang et al., 2009, Keady et al., 2012, Qin et al., 2007). IFT25/27 orthologs are absent in the nematode, but based on domain similarities between IFT25 and KIAA0556 (Figure 3.12) it is possible that KIAA0556/K04F10.2 functions in a similar manner to IFT25 to regulate one or more ciliary GTPases.

Syscilia collaborators (Roepman & Kremer lab; Radboud University Nijmegen) further mapped the KIAA0556 interactions by performing one-on-one yeast two hybrid (Y2H) screens of various fragments of KIAA0556 against a panel of 196 cilia-related protein baits that includes IFT and ciliopathy proteins. Specifically, four KIAA0556 fragments were employed, each containing one or more galactose binding domains (GBD). Consistent with the TAP data, Y2H experiments identified interactions with IFT-B protein IFT88 (fragments containing GBD1 and 2), as well as KATNBL1 (fragments containing GBD4, Figure 3.13C). Nematode K04F10.2 consists of the C-terminal three domains of KIAA0556 (Figure 3.10) and as the corresponding KIAA0556 domains (GBD 2-4) house the interaction with the katanin subunit KATNBL1, this interaction may be conserved in C. elegans. Additionally, several cilia-related proteins not present in the TAP interactome were identified as KIAA0556 Y2H interactors, including the TZ proteins NPHP4 and RPGRIP1L/MKS-5, and cilia-related proteins EFCH1, NINLisoB, SPAG5, SPATA4, and SPEF1 (Chan et al., 2005, Kersten et al., 2012, Albee et al., 2013).

In summary, biochemical interactors of human KIAA0556/K04F10.2 are identified, which include IFT-B components, katanin proteins, and small GTPases. Y2H experiments confirmed direct interactions with IFT88 and KATNBL1. In previous sections, extensive analysis was performed to identify a functional connection between nematode K04F10.2 and intraflagellar transport. These experiments showed that K04F10.2 does not undergo IFT-like transport in the cilium, intraflagellar transport is grossly normal in K04F10.2 mutants, and double mutant analysis combining K04F10.2 mutations with IFT gene mutants did not reveal genetic interactions. Therefore, further analysis will focus on
investigation of nematode homologs of ciliary GTPases and katanin proteins. Based on the hypothesis that KIAA0556/K04F10.2 could be an IFT-like GTPase regulator, and identification of three small GTPases in the KIAA0556 interactome, a functional interaction between nematode K04F10.2 and these GTPases will be further investigated. Also, the KIAA0556 interactome revealed biochemical interactions with katanin subunits. Katanin subunits have not been shown to function in primary cilia, and this novel finding was subsequently investigated in *C. elegans*.

### 3.3.12 KIAA0556 Interactor *rab-28* Encodes a Ciliary GTPase That Undergoes IFT in *C. elegans*

Three small GTPases were identified in affinity proteomics experiments to biochemically interact with human KIAA0556/K04F10.2. Two of these GTPases, *ifta-2/Rabl5* and *rab-28* are conserved in *C. elegans*. Here, functional associations between these GTPases and K04F10.2 are investigated. Previous reports have shown that *ifta-2* shows co-expression with K04F10.2 in ciliated sensory neurons, and *ifta-2* expression is regulated by the ciliogenic DAF-19 transcription factor (Blacque et al., 2005, Efimenko et al., 2005, Colosimo et al., 2004). IFTA-2 undergoes intraflagellar transport in cilia, and may regulate specific cilia-related signalling events (Schafer et al., 2006, Ou et al., 2007). However, no studies have been performed to date on *rab-28* in *C. elegans*.

First, expression and localisation of RAB-28 was investigated to determine if it was co-expressed with K04F10.2. A promoter GFP-reporter construct was generated, consisting of 431 bp of *rab-28* 5’ UTR sequence and the first 14 bp of the gene with a mutated start codon fused to GFP (containing an NLS sequence for ease of cell identification). This construct was micro-injected into wild type worms to generate transgenic animals expressing this construct as an extrachromosomal array. *rab-28* promoter activity was observed in head neurons at the first and second pharyngeal bulbs, with dendritic processes leading to the tip of the nose, suggesting these are ciliated cells. Several of these neurons showed dye-uptake, proving that the *rab-28* promoter is active in at least these six ciliated
sensory neurons. Promoter activity was also found in dye-filling positive tail neurons, which proves these are the PHA/B ciliated sensory neurons (Figure 3.14).

Next, the subcellular localisation of RAB-28 was investigated. Transgenic animals expressing a translational Prab-28::GFP::rab-28 as an extrachromosomal array were generated. RAB-28 localised to axonemes, where it underwent IFT-like movement (Figure 3.15A). Kymograph analysis showed that RAB-28 moved at speeds comparable to IFT components in anterograde and retrograde directions (Figure 3.15B and C). RAB-28 MS anterograde speeds were 0.73 ± 0.18 µm/s (0.73-0.77 µm/s for IFT components, Figure 3.6), DS anterograde speeds were 1.20 ± 0.22 µm/s (1.31-1.53 µm/s for IFT components, Figure 3.6), and retrograde speeds were 1.36 ± 0.27 µm/s (1.31-1.49 µm/s for IFT components, Figure 3.6). These data indicate RAB-28 is co-expressed with K04F10.2 in ciliated sensory neurons in C. elegans, where it undergoes IFT movement. Interestingly, these features are similar to those observed for IFTA-2 (Schafer et al., 2006, Ou et al., 2007). However, it was noticed that unlike IFTA-2 (and other IFT proteins), RAB-28 does not accumulate at the ciliary base (Figure 3.15A), indicating a potential functional distinction.

![GFP, Merge, Dil](image)

**Figure 3.14 The rab-28 gene promoter is active in ciliated neurons.** Shown are fluorescence images from a worm expressing a transcriptional GFP reporter under the control of the rab-28 promoter and stained with Dil. GFP is expressed in head and tail neurons, including amphid, labial (head), and phasmid (tail) ciliated neurons, some of which are co-stained with Dil. Scale bar; 10 µm.
Figure 3.15 The RAB-28 protein localises to cilia and undergoes IFT. **A.** A GFP::RAB-28 fusion protein localises along the entire length of amphid and phasmid channel cilia and within the periciliary membrane compartment (PCMC). Diffuse signals also observed in the distal dendrite region (DD) and throughout the neuron (data not shown). DS; distal segment, MS; middle segment, TZ; transition zone. Bars; 3 µm. **B-C.** GFP::RAB-28 undergoes IFT-like motility. Shown in C are split kymographs (anterograde, retrograde) and kymograph schematics denoting processive movement of GFP::RAB-28 particles along the middle (MS) and distal (DS) segments of a phasmid cilium. X-axis (distance) = 6 µm, Y-axis (time) = 22 seconds. Kymographic analyses of particle speed profiles shown in C.
Based on the hypothesis that IFT25-like K04F10.2 could functionally interact with an IFT27-like GTPase, K04F10.2 requirement for localisation of ciliary GTPases RAB-28 and IFTA-1 was investigated. IFT25 regulates small GTPase IFT27 and is required for its localisation (Keady et al., 2012), therefore assessing the requirement of K04F10.2 for ciliary localisation of RAB-28 and IFTA-2 tests this hypothesis. RAB-28 localisation and mobility in the cilium is unaffected in K04F10.2 (Figure 3.16), suggesting RAB-28 localisation is independent of K04F10.2. Previously, IFTA-2/RABL5 was shown to accumulate at the ciliary base and axoneme, where it undergoes IFT associated with the IFT-B complex (Ou et al., 2007, Schafer et al., 2006). IFTA-2 localisation in the cilia of tm1830 mutants is comparable to wild type, indicating that K04F10.2 is not required for IFTA-2 function in the cilium (Figure 3.16).

Taken together, the small GTPase RAB-28 is co-expressed with K04F10.2 and IFTA-2 in ciliated sensory neurons, where both undergo intraflagellar transport, but K04F10.2 is not directly required for localisations of RAB-28 and IFTA-2. The results presented here may disprove the hypothesis stated above that K04F10.2 acts as a regulator of ciliary GTPases. However, roles for K04F10.2 in regulating GTPase function, but not localisation, are still possible.

![Figure 3.16 Ciliary GTPase localisation is unaffected in K04F10.2 mutants. Shown are fluorescence images of phasmid cilia expressing the designated protein tagged with GFP. Scale bar; 3 µm.](image)
3.3.14 KIAA0556 INTERACTOR KATNBL1 (P80 SUBUNIT) IS EXPRESSED IN CILIATED SENSORY NEURONS AND LOCALISES TO THE CILIARY BASE

Similar to the approach taken to investigate RAB-28 expression and localisation, nematode homologs of katanin subunits were investigated. *mei-1* and *mei-2* were originally described as essential genes, encoding the catalytic and regulatory subunits, respectively. These protein products are required for meiotic spindle formation, but need are down regulated by MEL-26 and CUL-3 before mitosis (Srayko et al., 2000, Johnson et al., 2009). Two other katanin subunit homologs, F47G4.4 and F47G4.5 are homologs of regulatory subunit *mei-2*, but no obvious phenotypes were found after RNAi knockdown of these genes (Srayko et al., 2000). Promoter GFP-reporters were generated for the four known nematode katanin subunits and GFP expression relative to dye-uptake in head and tail ciliated neurons was examined (Figure 3.17). All four subunits are expressed in at least a subset of head neurons: the *mei-1/KATNA1*-promoter is expressed in several dye-filling neurons (arrowheads in 3.17A), and some weak GFP signals can be seen in the dendrites of ciliated neurons (arrows in 3.17A). However, most GFP-positive neurons in the head and tail do not show overlap with dye-filling ciliated cells and do not have extended dendrites characteristic of ciliated amphid and phasmid sensory neurons. *mei-2/KATNBL1* (F47G4.5 paralog) is expressed in few cells in the head neuron region, none of which overlap with dye-filling neurons, and is not expressed in the phasmid ciliated neurons (Figure 3.17B). F47G4.4*/KATNB1* is expressed in many neurons in the head, some of which have dendrites extending anteriorly, suggesting they could be ciliated (arrow and arrowhead in 3.17C) and in some intestinal cells (asterisk in 3.17C), but is not expressed in phasmid ciliated neurons. F47G4.5*/KATNBL1* (*mei-2* paralog) is expressed in most ciliated sensory neurons in the head and tail (Figure 3.17D). All amphid and phasmid dye-filling neurons show GFP expression indicative of F47G4.5 promoter activity. Several neurons of the labial sensilla show promoter activity as well, as do some intestinal cells (arrows and asterisk in 3.17D, respectively). Because only one of the katanin subunits shows strong co-expression with K04F10.2 in most ciliated neurons, this gene, F47G4.5, was chosen as a candidate ciliary gene and efforts were directed towards elucidating its localisation and function.
Figure 3.17 The F47G4.5 promoter is active in ciliated neurons. Fluorescence images are shown of worms expressing a transcriptional GFP-reporter under the control of the designated promoters and stained with Dil. A. The *mei-1* promoter is active in several cells in head and tail of the worm. GFP signals are observed in several cells in the head and tail of the worm. Weak GFP signals are observed in dendritic structures leading to the tip of the head (arrow) and two ciliated cells exhibit GFP expression and are stained with Dil (arrowheads). B. The *mei-2* promoter is active in cells in the head of the worm. GFP signals are observed in one cell that is also stained with Dil (arrowhead). GFP signals are not observed in phasmid ciliated cells in the tail region. C. The F47G4.4 promoter is active in several cells in the head of the worm, but not in phasmid ciliated cells in the tail region. Weak GFP signals are observed in dendritic structures leading to the tip of the head (arrow) and one ciliated cell exhibits GFP expression and Dil staining (arrowhead). GFP expression is also observed in some intestinal cells (asterisk). D. The F47G4.5 promoter is active in ciliated neurons in the head and tail of the worm. Ciliated cells stained with Dil also express GFP in head and tail regions (arrowheads). GFP is also observed in dendritic structures leading to the tip of the head (arrows), some of which overlap with Dil staining. Weak GFP signals are observed in intestinal cells (asterisk). Scale bars; 20 µm.
To determine F47G4.5 subcellular localization, a transgenic line expressing GFP-tagged F47G4.5 expressed under its own promoter was generated and expression and localization in ciliated sensory neurons was examined. In amphid cilia F47G4.5 accumulates in the ciliary base region in a ring like structure (asterisk in Figure 3.18A). In labial neurons there is an accumulation of F47G4.5 at the ciliary base region (arrowheads) and it localizes throughout the cilium (brackets). In phasmid cilia GFP spots are found in the cilium and F47G4.5 accumulates at the ciliary base region (arrowhead in Figure 3.18B), but it is unclear if this is a ring-like structure similar to the amphid sensory neuron signal. Using time-lapse microscopy the phasmid ciliary signals were found to be partially mobile, but did not appear to undergo IFT-like movement. Loss and recovery of ciliary GFP signals suggest partial removal and re-incorporation of F47G4.5 in the axoneme (asterisks in Figure 3.18C). Several male tail ray cilia showed expression of GFP-tagged F47G4.5 (Figure 3.18D). Specifically, in the R9 ciliated sensory neurons F47G4.5 accumulates in the ciliary region and localizes throughout the rest of the cell (asterisks in Figure 3.18D).

Table 3.2 Katanin subunits in *C. elegans* and *Homo sapiens*.

<table>
<thead>
<tr>
<th>Subunit</th>
<th><em>C. elegans</em></th>
<th><em>Homo sapiens</em></th>
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<tbody>
<tr>
<td>p80 regulatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canonical</td>
<td>F47G4.4</td>
<td>KATNB1</td>
</tr>
<tr>
<td>WD40-less</td>
<td>MEI-2</td>
<td>KATNB1</td>
</tr>
<tr>
<td></td>
<td>F47G4.5</td>
<td></td>
</tr>
<tr>
<td>p60 catalytic</td>
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<td></td>
</tr>
<tr>
<td>Canonical</td>
<td>MEI-1</td>
<td>KATNA1</td>
</tr>
<tr>
<td>LisH</td>
<td></td>
<td>KATNAL1</td>
</tr>
</tbody>
</table>

Taken together, these data show that F47G4.5/KATNB1L and K04F10.2 are co-expressed in ciliated sensory neurons. F47G4.5 appears to localise to the ciliary base of amphid and phasmid cilia, and axonemes of labial, phasmid, and male tail ray cilia, and is partially mobile in the cilium.
Figure 3.18 The F47G4.5 protein localises at the ciliary base region and within the ciliary axoneme.

A. F47G4.5 localises to ciliary base regions of amphid and quadrant neurons and ciliary axonemes (quadrant neurons only). Shown is a fluorescence image of the head region of a worm expressing a translational F47G4.5 GFP-reporter. In amphid neurons F47G4.5 localises to ciliary base regions in a ring shape (asterisk). Quadrant neurons show GFP localising to the ciliary base region (arrowhead) and the axoneme (bracket). Scale bar; 5 µm.

B. F47G4.5 localises to the ciliary base region and axoneme in phasmid neurons. A translational F47G4.5 GFP-reporter accumulates at the ciliary base region (arrowhead) and localises to axonemes. Scale bars; 3 µm.

C. F47G4.5 is mobile in phasmid cilia. Shown is a kymograph (time vs. distance plot) of GFP-tagged F47G4.5 in a phasmid cilium. Some GFP-signals are mobile and can disappear and re-appear (asterisks). Horizontal scale bar (distance); 2 µm. Vertical scale bar (time); 10 seconds.

D. F47G4.5 localises to a subset of ray cilia in male tails. Shown is a fluorescence image of a male worm expressing a translational F47G4.5 GFP-reporter. Strong GFP signals are observed in the ciliary regions of R9 ray neurons (arrows). Scale bar; 5 µm.
3.3.15 CILIUM STRUCTURAL AND FUNCTIONAL INVESTIGATION IN F47G4.5/KATNBL1 MUTANTS

Previous investigations of katanin subunits in C. elegans have focused on their roles during meiosis; cilia-related functions have not been investigated (Srayko et al., 2000). To investigate F47G4.5 requirement for cilium structure and function the ok2667 allele was used (Figure 3.19A). This allele possesses a frameshift 570 bp deletion spanning most of exon 2 and exon 3, intron 2, and most of intron 3; it is unknown if this is a null mutant. To indirectly assess cilium integrity, the DiI uptake assay was employed. Uptake of DiI into ciliated amphid and phasmid neurons in ok2667 mutants was comparable to wild type animals, suggesting cilium integrity was unaffected in the mutant (Figure 3.19B).

Next, cilium ultrastructure was investigated through analysis of the amphid cilia by TEM. In ok2667 mutants, one axoneme was missing throughout one pore (Figure 3.20C). Also, at the distal tip, distal segments are defective in microtubule number (Figure 3.20 C-D). In more proximal regions of the distal segment microtubule numbers were still reduced (Figure 3.20G-H). These data indicate that ok2667 mutants lack one cilium completely in one pore, and some cilia possess defects in microtubule number. However, middle segment and transition zone compartments of the cilia present in ok2667 mutants appear like wild type in relation to microtubule number and arrangement. Only one animal was examined in this study, and at least one more will need to be studied to validate these findings.

Investigation of cilium integrity by dye-filling revealed no defects in ok2667 mutants. Functionally, these mutants possess normal osmotic avoidance behaviour to glycerol and chemoattraction to beanzaldehyde is also normal, suggesting F47G4.5 function is not required for these cell-specific sensory behaviours (Figure 3.19C-D).

Also, the localisation of several IFT components and ARL-13 was normal in F47G4.5 mutants (Figure 3.21). Interestingly, like in K04F10.2 mutants, an IFT defect was observed. However, this defect appeared to be more severe. Varied IFT defects were observed in anterograde and retrograde trafficking for some, but not all components.
Figure 3.19 F47G4.5 mutants possess normal cillum structure and morphologies and exhibit normal sensory behaviours. A. F47G4.5 gene structure, showing genomic positions of the ok2667 deletion. Scale bar; 100 bp. B. F47G4.5 mutants are dye-filling normal. Shown are fluorescence images of head and tail regions stained with DiI. Scale bars; 20 µm. C. Osmotic avoidance behaviour is unaffected in ok2667 mutants. Avoidance behaviour to 8M glycerol measured over 10 minutes for wild type, *osm-5(p813)*, and F47G4.5(ok2667) animals is shown. At least 6 rings with 5 worms were assessed for each strain. E. Chemotaxis towards benzaldehyde is increased at 30 minutes and unaffected at 60 minutes in ok2667 mutants. Chemotaxis indices (CI) measured at 30 and 60 minutes are shown for wild type (N2), *che-11 (e1805)*, and F47G4.5(ok2667). Assays were repeated 4 times for control strains, 6 times for F47G4.5(ok2667). Asterisk; p<0.05.
Figure 3.20 F47G4.5(ok2667) mutants possess ultrastructural defects in distal segments of amphid cilia. Transmission electron micrographs of serial cross sections of amphid pores from wild type (N2) and F47G4.5(ok2667) animals. **A-D** 2 μm proximal from the extreme distal tip of the amphid pore ok2667 mutants (C-D) is missing an axoneme and possesses reduced microtubule numbers compared to wild type (A-B). **E-H** ok2667 mutant is missing an axoneme and exhibits reduced microtubule numbers in more proximal regions of the distal segment. 2 μm proximal to A-D distal segments of ok2667 mutants possess reduced microtubules and one axoneme is absent. **I-L** Middle segment ultrastructure is unaffected in ok2667 mutants. 2 μm proximal to E-H axonemes of both wild type (I-J) and ok2667 mutants (K-L) consist of nine microtubule doublets, and several smaller inner singlet microtubules are observed. **M-P** Transition zone ultrastructure is normal in ok2667 mutants. 2 μm proximal to I-L transition zones of wild type (M-N) and ok2667 mutants (O-P) possess nine microtubule doublets, connected to the membrane via Y-link structures (arrows). Also observed are several smaller inner microtubule singlets. **Q-T** Periciliary membrane compartments (PCMC) are unaffected in ok2667 mutants. Wild type (Q-R) and ok2667 mutant (S-T) PCMCs show similar morphologies. DS; distal segment. MS; middle segment. TZ; transition zone. PCMC; periciliary membrane compartment. Scale bars; 200 nm. Sample preparation and imaging performed by Julie Kennedy, University College Dublin.
Whilst OSM-3 (kinesin-2) and CHE-11 (IFT-A) MS anterograde speeds were normal, OSM-6 (IFT-B) speeds were significantly slower in the middle segment (Figure 3.21). In the distal segment OSM-6 moved at normal speeds, and CHE-11 and OSM-3 moved significantly slower (Figure 3.21). In the retrograde direction, all three IFT components moved significantly slower, suggesting that in F47G4.5 mutants intraflagellar transport takes place, but may not function optimally (Figure 3.21).

Because of the biochemical interactions identified in mammalian cells between KIAA0556 and KATNBL1, functional associations between nematode K04F10.2 and F47G4.5 were investigated. First, K04F10.2 mutants expressing GFP-tagged F47G4.5 were investigated. In these animals, F47G4.5 localised to the ciliary base region, comparable to wild type (Figure 3.22A), suggesting K04F10.2 is not required for F47G4.5 localisation. Second, K04F10.2::GFP localisation was investigated in F47G4.5 mutants. In these animals, K04F10.2 localisation was also similar to wild type (Figure 3.22B), suggesting there is no interdependency between K04F10.2 and F47G4.5 relating to ciliary localisation. Finally, a double mutant was generated and cilium integrity was assessed by the dye-uptake assay. Both K04F10.2 and F47G4.5 single mutants show similar dye-uptake levels to wild type. Figure 3.22C shows that in K04F10.2;F47G4.5 double mutants dye-uptake levels are similar to those observed in either single mutant, indicating there is no interaction between K04F10.2 and F4G4.5 related to cilium integrity of the dye-filling ciliated neurons.

Taken together, these data suggest cilium structure appears normal in F47G4.5 mutants. However, when assessed in higher resolution using TEM, one cilium is missing and some, but not all amphid cilia possess ultrastructural defects, suggesting F47G4.5 may be required in these cells to maintain cilium integrity. DiI uptake, as well as osmo- and chemosensory behaviours are normal in these mutants, suggesting that the ultrastructural defect may be cell-specific and F47G4.5 is required for cilium integrity of some, but not all, amphid pore cilia. Additionally, an IFT defect is observed in F47G4.5 mutants that is more severe than observed in K04F10.2 mutants. Despite overlapping expression patterns and biochemical interactions of mammalian homologs, K04F10.2 and F47G4.5 are not interdependent for ciliary localisations, and double mutant analysis revealed no genetic interaction.
Figure 3.21 F47G4.5(ok2667) mutants exhibit reduced IFT speeds. A. Fluorescence images are shown of indicated labelled proteins in wild type and ok2667 phasmid cilia. Ciliary localisations of indicated proteins are not affected in ok2667 mutants. Scale bar; 3 µm. B-C. ok2667 mutants exhibit reduced IFT speeds. Kymograph analysis (time vs. distance) of indicated markers in amphid and phasmid cilia shows reduced OSM-6 MS anterograde speeds, reduced DS anterograde speeds of OSM-3 and CHE-11, and reduced retrograde speeds for all three IFT proteins in ok2667 mutants. Particle speed distributions for kymographic analysis are shown in C. MS; middle segment. DS; distal segment. Retro; retrograde.
Figure 3.22 F47G4.5 and K04F10.2 do not genetically interact to maintain cilium integrity. A. F47G4.5 localisation is K04F10.2-independent. Shown are fluorescence images of phasmid tail regions of worms expressing a translational F47G4.5 GFP-reporter. Scale bar; 3 µm. B. K04F10.2 localisation is F47G4.5-independent. Shown are fluorescence images of phasmid tail regions of worms expressing a translational K04F10.2 GFP-reporter. Scale bar; 3 µm. C. F47G4.5 and K04F10.2 do not genetically interact to maintain cilium integrity. Shown are fluorescence images of head and tail regions stained with DiI. Dye-uptake in K04F10.2;F47G4.5 double mutants is comparable to either single mutant. Scalebar; 20 µm.
3.4 DISCUSSION

In this chapter, a new component of the ciliary base, K04F10.2, is identified. K04F10.2 exhibits subtle functions relating to cilium structure and intraflagellar transport, and is suggested to functionally interact with Joubert Syndrome-associated arl-13/Arl13b. Sequence analysis of K04F10.2/KIAA0556 shows conservation across multiple ciliated species and suggests structural homology with IFT25. Affinity proteomics revealed the KIAA0556/K04F10.2 interactome is enriched for IFT-B proteins, GTPases, and katanin subunits. Furthermore, novel nematode ciliary localisations and transport properties are described for RAB-28 and F47G4.5/KATNBL1, two biochemical interactors of human KIAA0556/K04F10.2.

3.4.1 K04F10.2 IS EXPRESSED IN MOST CILIATED CELLS SUGGESTING BROAD CILIARY ROLES

Analysis of the K04F10.2 expression pattern shows almost exclusive promoter activity in ciliated neurons: of non-ciliated cells, only a few intestinal cells exhibit low levels of K04F10.2 promoter activity. Similar expression patterns have been observed for many ciliary genes, such as genes encoding intraflagellar transport proteins, components of the BBSome, and MKS- and NPHP-module components (Blacque et al., 2005, Li et al., 2004, Fan et al., 2004, Efimenko et al., 2005, Jauregui and Barr, 2005, Williams and Winkelbauer, 2008, Collet et al., 1998, Haycraft et al., 2003, Bell et al., 2006, Phirke et al., 2011, Chen et al., 2006, Murayama et al., 2005). Expression of many of these known ciliary genes is regulated through the ciliogenic DAF-19 RFX transcription factor that can bind X-box promoter motifs (Efimenko et al., 2005, Blacque et al., 2005, Phirke et al., 2011, Swoboda et al., 2000). Previous work has identified such an X-box motif upstream of K04F10.2 and found K04F10.2 expression was downregulated in daf-19 mutants (Blacque et al., 2005, Colosimo et al., 2004, Phirke et al., 2011). Because of expression of K04F10.2 in most ciliated cells, combined with its regulation by DAF-19 and presence of an X-box promoter element, it is likely that K04F10.2 has a broad ciliary role.
K04F10.2 accumulates strongly at the ciliary base and localises to a lesser extent to proximal regions of the cilium, including the transition zone and middle segment. Evidence was also found of membrane association at the ciliary base and axoneme of mks-5 and arl-13 mutants, respectively.

K04F10.2 accumulates at the ciliary base and localises to proximal regions of the cilium, including the transition zone and middle segment. The accumulation at the base of the cilium is adjacent to the transition zone, and does not extend proximally into the entire periciliary membrane compartment. This ciliary base localisation is distinct from that of intraflagellar transport and BBSome proteins, and the recently identified ciliary base-localised IFT regulator DYF-19/FBF1, which exhibited wider crescent-like accumulations at the ciliary base, whereas K04F10.2 forms a narrower, slightly flared signal (Collet et al., 1998, Haycraft et al., 2003, Bell et al., 2006, Orozco et al., 1999, Fujiwara et al., 1999, Schafer et al., 2003, Ou et al., 2007, Blacque et al., 2004). Furthermore, K04F10.2 localisation at the ciliary base differentiates from CHE-10/Rootletin localisation, whose signals form a lollipop-like signal, where CHE-10 extends more proximally (than K04F10.2) into the periciliary membrane compartment (Ou et al., 2007, Mohan et al., 2013). MKS-5 strongly localises to the transition zone and is excluded from the ciliary base, whereas K04F10.2 accumulates at the ciliary base, and weak signals are found in the transition zone. Several endo- and exocytosis pathway components localise to the ciliary base, such as DYN-1, RAB-5, RAB-8, AP-2 subunits, STAM-1, and HGRS-1, where these proteins localise diffusely and more broadly throughout the periciliary membrane compartment (Hu and Wittekind, 2007, Kaplan et al., 2012). K04F10.2 localisation is distinct from these proteins, because it is concentrated immediately beneath the transition zone. Finally, K04F10.2 localisation does not overlap with membrane (-associated) proteins at the periciliary membrane compartment. OSTA-1 (organic solute transporter anion-1), TRAM-1 (orthologue of the TRAM translocon) and RPI-2 (retinitis pigmentose gene 2) exhibit membrane localisation throughout the periciliary membrane compartment (Olivier-Mason et al., 2013, Blacque et al., 2005, Bae et al., 2006), whereas this is not the
case for K04F10.2. Thus, the localisation profile of K04F10.2 does not overlap with that of known ciliary base proteins and appears to be unique.

In addition to the ciliary base accumulation, K04F10.2 also localises to the proximal parts of the axoneme, though this signal is weaker than the ciliary base accumulation. By comparing ARL-13 and K04F10.2 localisation patterns, it is shown that K04F10.2 is excluded from the distal segment. Several proteins have been shown to localise almost exclusively to middle segments of nematode cilia with a bipartite configuration. These include ARL-13, NPHP-2, and TAX-2, as well as TAX-4 and CNG-3 in ASK cilia, but not in AWB cilia (Cevik et al., 2013, Warburton-Pitt et al., 2012, Wojtyniak et al., 2013). However, these proteins do not show transition zone or ciliary base localisation, thereby distinguishing K04F10.2 from these proteins based on localisation patterns. Like K04F10.2, heterotrimeric kinesin II subunit KAP-1 also localises to the ciliary base and the middle segment. However, K04F10.2 does not undergo IFT, and like other IFT proteins, kinesin-II displays a crescent shape localisation at the ciliary base versus the narrower signal of K04F10.2 (Snow et al., 2004).

Whether K04F10.2 localises to ciliary membranes or associates with axonemal microtubules lying beneath these membranes remains unknown. In arl-13 mutants K04F10.2 appears to decorate expanded ciliary membranes, suggesting K04F10.2 associates with ciliary membranes rather than microtubules in these mutants. Additionally, whether the K04F10.2 ciliary base signals decorate the periciliary membrane or are perhaps associated with microtubule ‘flares’ that run just under this membrane remains unknown (Doroquez et al., 2014). To further decipher if K04F10.2 associates with microtubules or with the membrane at the ciliary base, superresolution microscopy or immuno-EM approaches could be employed.
3.4.3 CILIARY LOCALISATIONS OF K04F10.2 OCCUR INDEPENDENTLY OF IFT, BBSOME, AND TRANSITION ZONE-ASSOCIATED CILIOPATHY MODULES

Analysis of K04F10.2 localisation in various mutant alleles of ciliary transport (e.g. IFT) and ciliopathy-associated genes revealed K04F10.2 does not require these genes for its targeting and localisation at the ciliary base or proximal axoneme. No defect in K04F10.2 localisation was observed in IFT mutants, indicating that K04F10.2 localisation is IFT-independent. Also not required is the BBSome, because it was found that K04F10.2 accumulates at the ciliary base and localises to the proximal cilium in bbs-8 mutants. Most transition zone components can be assigned to two distinct functional modules (MKS and NPHP modules) that cooperate to maintain transition zone integrity and control ciliary composition by facilitating diffusion barrier functions at the transition zone (Huang et al., 2011, Shiba and Yokoyama, 2012, Williams et al., 2011, Williams et al., 2010). In mutants of genes from either module K04F10.2 accumulates at the ciliary base and localises to the proximal axoneme, indicating that its localisation is independent of these modules.

How K04F10.2 is targeted to the ciliary base and proximal axoneme therefore remains unknown. This work shows that K04F10.2 may decorate periciiliary and ciliary membranes (see above). The localisation and ciliary entry of K04F10.2 appear to be IFT independent, which was shown previously for Chlamydomonas SAG1-C65, a transmembrane agglutinin and for C. elegans ODR-10, which can enter cilia independent of IFT (Dwyer et al., 2001, Belzile et al., 2013). Therefore, if K04F10.2 is associated with membranes, perhaps it is not surprising that its localisation is not affected in IFT mutants. A number of ciliary membrane proteins have been shown to require the BBSome for ciliary targeting, such as SSTR3 and MCHR1 in mammalian systems, and OSM-9 in C. elegans (Perciliz et al., 2007, Berbari et al., 2008), but K04F10.2 ciliary targeting appears to be independent of the BBSome, suggesting another transport mechanism must be required for K04F10.2 targeting and localisation.

If K04F10.2 is cytosolic rather than membrane-associated, it could be targeted to the ciliary base similar to Rabin8. Upon serum starvation of mammalian cells, Rabin8 quickly
redistributes to vesicles, most of which accumulate at the centrosome (Westlake et al., 2011). Also, if K04F10.2 is cytosolic, it could be expected that ciliary entry and localisation are dependent on IFT, and potentially the BBSome also. IFT has been shown to be required for localisation of various cytosolic components of the cilium, such as tubulin subunits TBB-4 and TBA-5, as well as CHE-12 in *C. elegans* (Hao et al., 2011, Bacaj et al., 2008), and DRC4 in *Chlamydomonas* (Wren et al., 2013). Another mechanism for ciliary entry could involve diffusion across the transition zone. While a cytosolic diffusion barrier at the ciliary base has been shown in mammalian cells (Lin et al., 2013, Dishinger et al., 2010, Fan et al., 2007, Fan et al., 2011, Hurd et al., 2011, Kee et al., 2012), this has not yet been shown in nematode cilia. It is possible that cytosolic K04F10.2 could enter the cilium via diffusion, rather than active transport. In this scenario ciliary entry would be independent of IFT.

To further investigate mechanisms targeting K04F10.2 to the cilium, mutants of transport pathway components, such as *ift-20* could be analysed (Follit et al., 2006, Keady et al., 2011). Additionally, analysis of K04F10.2 truncations may help to identify putative ciliary targeting sequences.

3.4.4 K04F10.2 SERVES SUBTLE CILIA-RELATED FUNCTIONS

All assays performed in this work are strongly indicative that K04F10.2 plays minor roles in ciliary structure and function. First, uptake of a fluorescent dye into the plasma membranes of a subset of ciliated neurons and cilium morphology of AWB and ASER neurons is unaffected in K04F10.2 mutants. Many ciliary gene mutants possessing ciliogenic or cilium integrity defects exhibit reduced dye-uptake, thereby making this assay a useful investigative tool to assess cilium integrity. However, normal dye-uptake, and thus grossly normal cilium structure, does not always correlate to intact cilium function. For example, *ifta-2* mutants exhibit normal dye-uptake, yes possess defects in cilia-related signalling pathways (Schafer et al., 2006). Also, *mks-3, mksr-1, mksr-2* (MKS module components), and *nphp-1* (NPHP module) mutants possess normal cilium structure, yet exhibit defects in cillum-related behaviours (Williams et al., 2011, Williams
and Winkelbauer, 2008, Williams et al., 2010). Therefore, it remains possible that K04F10.2 serves a ciliary function unrelated to ciliogenesis or cilium structure.

Second, ciliary function was assessed via cell-specific behavioural assays. Consistent with normal dye-uptake and unaffected cilium morphology, osmotic avoidance and chemotaxis behaviours were found to be normal. A plethora of osmo- and chemosensory behaviours are mediated by sensory cilia in the worm (Inglis et al., 2007). Therefore, responses to other substances and other sensory behaviours may be altered in K04F10.2 mutants, and future investigation of these behaviours could provide further insight into cilia-related functions of K04F10.2.

Third, ultrastructure of amphid channel cilia is only subtly affected, whereby defects in microtubule number and organisation in the very distal end of amphid channel cilia are observed. Defects in B-tubule extension have been identified in nphp-4 mutants. However, cilia in these worms possess prematurely terminated B-tubules, rather than abnormally extended ones that were observed in K04F10.2 (Jauregui et al., 2008). Combined with dissimilar ciliary localisation profiles of K04F10.2 and NPHP-4, and a lack of genetic interaction between K04F10.2 and MKS-module genes, it is unlikely that K04F10.2 and nphp-4 function in a similar pathway. Abnormal B-tubule extension and reduced microtubule number in amphid pore distal segments that were observed in K04F10.2 mutants, have not been found in other ciliary gene mutants, suggesting K04F10.2 may serve a novel function relating to microtubule stability and B-tubule termination at the middle-distal segment border.

Fourth, transition zone integrity is not affected in K04F10.2 mutants. To investigate transition zone integrity, localisation of MKS-module component MKS-2 was assessed. MKS-2 mislocalises in many MKS gene mutants, and is therefore sensitive to alterations in MKS-module integrity (Huang et al., 2011). The unaffected localisation of MKS-2 in the transition zone of K04F10.2 mutants suggests that the MKS-module is intact. Combined with the normal transition zone ultrastructure found in these mutants, this indicates that transition zone formation and integrity does not require K04F10.2.
Consistent with these findings, localisation of transmembrane TRAM-1 and membrane-associated RPI-2 was unaffected. TRAM-1 and RPI-2 decorate the periciliary membrane and are excluded from the cilium in wild type animals. Transition zone mutants have been shown to exhibit abnormal ciliary localisations of these proteins, suggesting that the transition zone functions as a barrier, preventing TRAM-1 and RPI-2 ciliary entry (Williams et al., 2011). Ciliary exclusion of these proteins provides further evidence that the transition zone is unaffected in K04F10.2 mutants.

Finally, another piece of evidence for a subtle ciliary function is that defects in ciliary transport pathways were not observed in K04F10.2 mutants. Polarised vesicle transport is required for ciliary targeting of transmembrane ODR-10 (Dwyer et al., 2001, Kaplan et al., 2010). K04F10.2 mutants exhibit normal ODR-10 localisation, indicating that ODR-10 is targeted independently of K04F10.2. Ciliary targeting and retention of membrane-associated ARL-13 requires function of various ciliary genes, including intraflagellar transport genes, and associated BBSome components, and MKS- and NPHP-module components at the transition zone. Taken together, K04F10.2 is not required for targeting of these membrane (-associated) proteins, but could facilitate or regulate localisation of other ciliary proteins. The focus of future work could be cell- and protein-specific investigation of K04F10.2 function.

Summarising this work, K04F10.2 appears to serve subtle roles in cilium structure and function. The possibility remains that K04F10.2 functions redundantly with another ciliary gene and loss of K04F10.2 does not induce extensive ciliary defects. Consistent with a redundant ciliary role for K04F10.2 is the observation of a genetic interaction with arl-13, which is discussed in the next section.

3.4.5 K04F10.2 IS A POTENTIAL IFT REGULATOR

K04F10.2 could serve functions relating to intraflagellar transport. K04F10.2 accumulates at the ciliary base, where it partially overlaps with IFT component accumulations. Also,
Kymographic analysis revealed that in middle segments, OSM-6 (IFT-B) anterograde speeds are significantly reduced. Also, a genetic interaction between K04F10.2 and IFT regulator arl-13 appears to be required for cilium integrity. Finally, proteomic analysis of the KIAA0556/K04F10.2 interactome revealed biochemical interactions with most IFT-B components. These interactions were further mapped to IFT88 using a yeast two-hybrid approach.

If K04F10.2 serves functions related to intraflagellar transport, what could these be? It is unlikely that K04F10.2 is a component of the IFT core, because it does not undergo intraflagellar transport in the cilium and K04F10.2 null mutants exhibit no ciliogenesis defects, and only subtle ultrastructural defects were observed in these mutants.

K04F10.2 could be a regulator of intraflagellar transport, similar to proteins like DYF-1, DYF-5, DYF-19, SQL-1, GPA-3, ARL-3 and ARL-13 (Cevik et al., 2010, Li et al., 2010, Broekhuis et al., 2013, Burghoorn et al., 2007, Burghoorn et al., 2010, Wei et al., 2013, Ou et al., 2005, Follit et al., 2008). Indeed, some of these mutants, such as sql-1 (GMAP210 orthologue) show only minor ciliary defects, while others such as dyf-1 (IFT70), dyf-5 (ICK), dyf-19 (FBF1), and arl-13 show more extensive ciliary structural and functional defects. The phenotype in K04F10.2 is more subtle than expected for an IFT regulator, and more subtle than what has been observed in any of these known IFT regulator mutants. The anterograde MS speed reduction observed for OSM-6 was not observed for OSM-3 (kinesin) or CHE-11 (IFT-A). Also, OSM-6 localisation at the ciliary base and ciliary distributions are normal in K04F10.2 mutants, and the reduced speed phenotype does not seem to affect ciliogenesis, cilium structure, or function, suggesting that the OSM-6 phenotype may not be physiologically relevant.

How to explain the slower OSM-6 MS velocities in K04F10.2 mutants? Broekhuis et al. suggested that in addition to IFT assemblies containing both kinesin-2 motors (kinesin-II and OSM-3), subpopulations of these motors can travel independently along the middle segments of gpa-3 (G protein α subunit 3) and sql-1 (GMAP210 ortholog) mutants (Broekhuis et al., 2013, Burghoorn et al., 2010). Possibly, K04F10.2 could function in a
similar manner, and in null mutants, some, but not all IFT-B complexes shift from an intact IFT assembly to a slower, independently moving kinesin-II motor, reducing the overall speed of OSM-6. To test this hypothesis further, more IFT components need to be analysed, including kinesin-II subunits.

Another possibility is that K04F10.2 is an IFT cargo. This seems unlikely, because periciliary and ciliary localisation of K04F10.2 is independent of IFT. Additionally, K04F10.2 accumulation only partially overlaps with localisation of IFT components at the ciliary base, though it remains possible that K04F10.2 is loaded onto IFT assemblies in a subcompartment of cargo-loading sites at the ciliary base.

Finally, K04F10.2 genetically interacts with arl-13, a known IFT regulator. However, the IFT phenotype observed in K04F10.2 mutants is distinct from that of arl-13 null mutants, where IFT-A and –B complexes dissociate and a number of IFT components have speed defects (Li et al., 2010). Thus, a functional connection between arl-13 and K04F10.2 remains enigmatic. Interestingly, consistent with a possible functional connection, K04F10.2 and ARL-13 co-localise at the middle segment.

In summary, it is unlikely that K04F10.2 functions as a core IFT protein or IFT cargo, but IFT regulatory functions for this gene are possible. Further experiments such as those discussed above, will need to be performed to determine if K04F10.2 regulates IFT and if so, whether this regulation is direct or indirect, possibly through an arl-13 related pathway.

3.4.6 K04F10.2 COULD FUNCTION AS A GTPASE REGULATOR

In addition to ARL-13, K04F10.2 could function as a regulator of other cilia-related GTPases. Bioinformatics approaches have revealed that galactose binding domains in KIAA0556 (human K04F10.2 ortholog) possesses structural homology to IFT-B component IFT25, which regulates ciliary GTPase IFT27 localisation and function.
IFT25 and IFT27 are not conserved in *C. elegans*; potentially K04F10.2 serves a similar function in worms. This is supported by the observation that, unlike other IFT-B components, *Ift25* is not required for ciliogenesis in mice, though it is required for transport of components of the Shh pathway within cilia (Keady et al., 2012).

Also, affinity proteomics of human KIAA0556 revealed an interactome enriched for ciliary GTPases that includes IFT27, RABL5/IFTA-2, and RAB28, providing further evidence that K04F10.2/KIAA0556 could be involved in regulation of GTPases. One of the identified GTPases, RAB-28 was previously shown to function in the endosomal pathway of *Trypanosoma*, localising to the photoreceptor basal body and rootlet in mice, and mutations can cause autosomal-recessive cone rod dystrophy (Roosing et al., 2013, Lumb et al., 2011). This work shows that in *C. elegans*, similar to K04F10.2, RAB-28 is expressed in ciliated neurons. Protein localisation studies in the worm show that RAB-28 undergoes IFT in the cilium, which has not been shown in other systems.

Interestingly, both RAB-28 and IFTA-2 undergo IFT in cilia, possibly as cargo molecules (Schafer et al., 2006, Ou et al., 2007). Localisation profiles of these GTPases are distinct to and independent of K04F10.2, and suggest that K04F10.2 does not regulate these ciliary GTPases in an IFT25-like manner, because IFT25 is required for ciliary localisation of IFT27 GTPase (Keady et al., 2012). However, other regulatory mechanisms, such as regulation of associated GAPs (GTPase activating proteins) and GEFs (guanine exchange factors) cannot be discounted.

3.4.7 Possible functional associations between K04F10.2 and katanin subunits

A possible functional connection was found between K04F10.2 and the katanin complex, and this connection could be microtubule-related. Evidence for this connection is provided by biochemical interactions between KIAA0556 and katanin subunits and co-expression

First, proteomics experiments identified biochemical interactions between KIAA00556 and katanin subunits, that were mapped to the p80 regulatory subunit KATNBL1 using yeast two-hybrid analyses. This indicates that KIAA00556 and KATNBL1 directly interact, and because KIAA00556 is a ciliary protein, this interaction could be cilia-related.

Second, in *C. elegans*, K04F10.2 and F47G4.5/KATNBL1 are co-expressed in ciliated neurons. The katanin complex subunits are partially conserved in the worm. One p60 regulatory subunit MEI-1/KATNA is present in the nematode, and three p80 regulatory subunits, F47G4.4/KATNB, and MEI-2 and F47G4.5, both of which are homologs of KATNBL1 (Roll-Mecak and McNally, 2010). mei-1 is expressed in some ciliated neurons, but mei-2, and F47G4.4 are expressed mostly in non-ciliated neurons in the head of the worm, while F47G4.5 shows strong expression in ciliated cells in the head and tail of the worm. F47G4.5 localises to the ciliary region, and possibly accumulates at the ciliary base, overlapping with K04F10.2. Further studies, such as co-localisation experiments with IFT, transition zone proteins, and K04F10.2 can elucidate the exact localisation profile of F47G4.5. Localisations of F47G4.5 and K04F10.2 are not mutually dependent, but this does not mean a functional association is not possible.

Third, similarly subtle ultrastructural and IFT defects were observed in F47G4.5 and K04F10.2 mutants. Consistent with microtubule-related functions of katanin, phenotypes observed in F47G4.5 and K04F10.2 mutants were microtubule related, with reduced microtubule numbers observed in distal segments of amphid cilia. Additionally, in both mutants, defects were found in intraflagellar transport, but cilium integrity and sensory behaviours were unaffected. The observed ultrastructural and IFT defects were more severe in F47G4.5 mutants, however, and may reflect different requirements of K04F10.2 and F47G4.5 in cilium structure and function.
Biochemical interactions between KIAA0556 and KATNBL1, co-expression and possible co-localisation of these proteins in *C. elegans*, and phenotypic overlap of K04F10.2 and F47G4.5/KATNBL1 mutants indicate that there is a cilia-related functional connection between these genes. Previous reports have shown that in *Chlamydomonas* and *Tetrahymena* the katanin complex is required for central pair assembly and assembly of motile cilia, respectively (Dymek et al., 2004, Dymek and Smith, 2012, Rasi et al., 2009, Sharma et al., 2007). Because *mei-1* is expressed in some, but not all ciliated neurons, it is possible that F47G4.5 can function in complex with MEI-1, but also independently, or with K04F10.2. Another possibility is that F47G4.5 forms a complex with an as of yet unidentified katanin-like protein in cells where MEI-1 is absent. If a katanin complex of MEI-1 and F47G4.5 is formed in the ciliary region, this could serve to regulate microtubule growth and length in and below the cilium, which could be facilitated by K04F10.2 at the ciliary base and in the proximal axoneme.

### 3.4.8 K04F10.2 REPRESENTS A GOOD CILIOPATHY CANDIDATE

The work presented here points to a subtle, non-essential ciliary role for K04F10.2/KIAA0556. Indeed, collaborative work with the Yoder lab (University of Alabama) has identified a mouse null mutant of KIAA0556, which also possesses subtle ciliary defects, resulting in a hydrocephalus phenotype. Interestingly, like the worm mutant, the KIAA0556<sup>−/−</sup> mouse appears to have normal ciliary function (e.g. beat pattern). Also, the mutant mouse did not present with phenotypes in kidney, liver, and lungs, which are often affected in mouse mutants of ciliary genes. The subtle ciliary roles in *C. elegans* and restricted phenotype in mice make K04F10.2/KIAA0556 a good candidate ciliopathy gene, and sequencing efforts should be directed towards identifying patients with mutations in KIAA0556. Based on the work presented here and the mouse data, it would be expected that patients carrying mutations in KIAA0556 would present with mild cilia-related phenotypes.
3.4.9 Final Conclusions

The work presented in this chapter identifies a novel component of the ciliary base, K04F10.2, that serves subtle functions in cillum structure and function, possibly through functional connections with ARL-13 and katanin subunit F47G4.5. Conserved across many ciliated species, K04F10.2/KIAA0556 possesses domains that exhibit structural homology to IFT25, and KIAA0556 biochemically interacts with several ciliary GTPases, indicating this novel ciliary protein could function as a GTPase regulator. The molecular mechanisms of K04F10.2 are not fully elucidated, but based on subtle phenotypes relating to cillum structure and intraflagellar transport, KIAA0556 presents a good candidate ciliopathy gene.
CHAPTER IV

A novel Fluorescence Recover After Photobleaching-based assay to investigate ciliary membrane diffusion barrier function in Caenorhabditis elegans sensory neurons
4.1 ABSTRACT

Many ciliary proteins require a specific (sub) ciliary localisation for their function, with some localised almost exclusively to cilia. Active transport mechanisms and diffusion barriers are vital for protein localisation and function. The transition zone at the proximal end of the cilium is thought to act as a diffusion barrier to restrict membrane (-associated) proteins to a ciliary compartment and to prevent entry of non-ciliary proteins. Intraflagellar transport (IFT) is thought to facilitate active transport across this membrane diffusion barrier.

Here, ARL-13 is shown to be mobile at the ciliary middle segment membrane, and it is shown that IFT components retard ARL-13 in the middle segment. At the transition zone a membrane diffusion barrier is identified, preventing ARL-13 ciliary exit. Using a novel \textit{in vivo} Fluorescence Recovery After Photobleaching (FRAP) assay transition zone-associated genes are found to be required for this barrier. ARL-13 localises to periciliary membranes in transition zone-associated gene mutants and can exchange between ciliary and periciliary compartments. Furthermore, IFT is found to be required for active transport of ARL-13 across the transition zone diffusion barrier. IFT gene mutants exhibit abnormal periciliary localisation of ARL-13, but unlike in transition zone mutants, exchange between ciliary and periciliary pools is limited. Finally, IFT and transition zone components genetically interact to maintain the ARL-13 diffusion barrier. Based on these date, we conclude that IFT and transition zone components play differential roles in establishing and transport across the membrane diffusion barrier that regulates ciliary entry and exit of ARL-13.

This novel FRAP assay is the first such described in a multicellular organism. It allows for \textit{in vivo} investigation of the transition zone membrane diffusion barrier and roles of ciliary transport and ciliopathy genes towards barrier establishment versus active transport across the barrier.
4.2 INTRODUCTION

Transport and restriction of ciliary proteins is tightly regulated. Some of the components involved are the transition zone and periciliary membrane, functioning to regulate entry and exit of membrane and cytosolic proteins (Reiter et al., 2012, Nachury et al., 2010). The molecular mechanisms involved in function of this ciliary ‘gate’ are unknown, although the transition zone Y-links and specialisations of periciliary and transition zone membranes are thought to be involved (Garcia-Gonzalo and Reiter, 2012, Gilula and Satir, 1972, Reiter et al., 2012). This chapter describes a novel fluorescence recovery after photobleaching (FRAP)-based assay in C. elegans to functionally assess the transition zone as a membrane diffusion barrier, restricting ARL-13 to the middle segment membrane.

4.2.1 THE CILIARY GATE REGULATES MEMBRANE PROTEIN COMPOSITION OF THE CILIAM

Ciliary membrane protein composition is tightly controlled and is proposed to involve ciliary gating mechanisms at the base of the organelle that regulate lateral transport into and out of the cilium. Ciliary membrane proteins can be targeted to the cilium through a number of pathways, which all converge on the periciliary membrane region immediately below the transition zone (Nachury et al., 2010, Sung and Leroux, 2013). Once ciliary membrane proteins have been delivered to the periciliary membrane, they can enter the cilium, regulated by mechanisms in the transition zone and the adjacent periciliary membrane (Garcia-Gonzalo et al., 2011, Olivier-Mason et al., 2013, Williams et al., 2011a, Huang et al., 2011, Chih et al., 2012, Hu et al., 2010, Besharse and Wetzel, 1995, Li et al., 1996). In one of the first studies describing a functional barrier at the base of Chlamydomonas flagella, activated agglutinin was identified at the flagellar membrane, but not in the cell body, and this pool could be manipulated independently of the cell body pool (Hunnicutt et al., 1990, Musgrave et al., 1986). Later studies identified lipid and protein components of this barrier, though the mechanisms involved in ciliary membrane protein entry and exit are not fully understood (Vieira et al., 2006, Chih et al., 2012, Hu et al., 2010, Garcia-Gonzalo and Reiter, 2012, Williams et al., 2011a). An example of the transition zone membrane diffusion barrier is found in C. elegans, where MKS- and
NPHP-module mutants exhibit ciliary entry of periciliary membrane (-associated) proteins, suggesting the membrane diffusion barrier at the transition zone may be defective in these worms (Williams et al., 2011a).

4.2.2 Structure of the membrane diffusion barrier

At the transition zone, microtubule doublets are connected to the membrane through Y-link structures (Perkins et al., 1986, Doroquez et al., 2014). These Y-link connectors terminate in or organise the ciliary necklace, a membrane specialisation at the transition zone (Gilula and Satir, 1972, Garcia-Gonzalo and Reiter, 2012). Y-links and the ciliary necklace could be involved in the membrane diffusion barrier through regulation of lipid composition and fluidity of the transition zone membrane (Vieira et al., 2006). In *C. elegans*, two functional modules are present at the transition zone, MKS and NPHP, which are composed of at least eight and two genes, respectively (Williams et al., 2011a, Williams and Winkelbauer, 2008, Williams et al., 2010, Jauregui and Barr, 2005, Jauregui et al., 2008, Bialas et al., 2009, Huang et al., 2011). These modules cooperate to maintain the transition zone, and components may be part of the Y-links, though their exact localisation and function within the transition zone compartment is unknown. Defects in components of the MKS/JBTS and NPHP modules underlie a number of ciliopathies, such as Meckel-Gruber Syndrome, Nephronophthisis, and Joubert Syndrome, emphasizing the importance of these modules in proper cilium function (Huang et al., 2011, Sang et al., 2011, Chih et al., 2012).

Other mechanisms controlling ciliary membrane protein composition include a mammalian septin ring, and the lipid environment of the ciliary pocket and transition zone membranes. In mammalian cells a septin ring at the ciliary base forms a diffusion barrier, regulating lateral transport of membrane proteins. Partial depletion of SEPT2 resulted in reduced diffusion barrier integrity and increased mobility of a number of ciliary receptors into the cilium (Hu et al., 2010). Additionally, SEPT2 is required for B9D1 and CC2D2A localisation to the transition zone. These proteins are part of the MKS/JBTS module, which was shown to be required for function of the membrane diffusion barrier in the transition zone (Chih et al., 2012, Sang et al., 2011). The lipid environment at the ciliary base may
also play a role in formation and function of the ciliary gate: a membrane region of condensed lipids found at the ciliary base in MDCK cells could function as a diffusion barrier, preventing ciliary entry of apical glucosphatidylinositol-anchored protein (FP-GPI) (Vieira et al., 2006). Finally, the lipid environment of the cilium itself may be important in regulating its protein composition through lipid raft-dependent localisation of flagellar membrane proteins (Tyler et al., 2009, Incardona and Eaton, 2000), and, in quail oviduct cilia, the membrane contains higher concentration of filipin-cholesterol complexes than other domains of the plasma membrane, suggesting local reorganisation of the membrane (Chailley and Boisvieux-Ulrich, 1985).

4.2.3 TRANSPORT ACROSS THE CILIARY MEMBRANE DIFFUSION BARRIER

Ciliary membrane proteins can enter the cilium through several lateral transport pathways. Active transport mechanism IFT can be employed to facilitate ciliary entry and exit of membrane proteins. Indeed, in *Chlamydomonas* IFT is required for ciliary exit of PKD2 (Huang et al., 2007), and in *C. elegans* IFT has been shown to be required for ciliary entry of ARL-13 (Cevik et al., 2013). The molecular mechanisms of IFT-mediated active transport of ciliary membrane proteins across the transition zone are unknown, but could involve local interactions with the barrier, allowing regulated lateral transport into and out of the cilium. Membrane proteins can also enter the cilium via IFT-independent pathways. For example, upon adhesion induced signalling, *Chlamydomonas* SAG1 can enter flagella of mutants lacking IFT (Belzile et al., 2013).

4.2.4 ASSESSMENT OF MEMBRANE DYNAMICS IN *C. ELEGANS*

Nematode cilium research benefits from a number of technical advantages over other systems. These include the spatial resolution found in ciliated sensory neurons in the head and tail of the worm, the animal’s transparency, and relatively straightforward methods to introduce genetic material. Combined, these benefits have made *C. elegans* very well suited to study cilia in live animals and have provided insight into cilium structure and function. However, steady state protein localisation assays do not provide insight into the
dynamics of the fluorescently labelled protein of interest, and other techniques, such as FRAP (fluorescence recovery after photobleaching) are employed to investigate protein dynamics.

Several approaches can be taken to investigate protein dynamics in live cells. For example, intraflagellar transport and vesicle transport can be analysed using time lapse microscopy, where a series of images are recorded and subsequent kymograph analysis is used to calculate speeds of moving assemblies and vesicles, respectively (Inglis et al., 2009). However, dynamics of diffusing proteins cannot be assessed using time lapse microscopy, because these proteins establish equilibrium, and thus appear to be static when imaged using time lapse microscopy. To observe this dynamic exchange of fluorescently labelled proteins along the membrane, FRAP can be employed.

Developed in the 1970’s, FRAP was originally performed using dyes coupled to lipids and proteins (Axelrod et al., 1976, Edidin et al., 1976). The isolation of genetically encoded fluorophores such as GFP (green fluorescent protein) and subsequent cloning into vectors has revolutionised live cell imaging and facilitated direct investigation of protein dynamics in living cells (Chudakov et al., 2005, Chalfie et al., 1994, Prasher et al., 1992, Shimomura et al., 1962). Using high power lasers, fluorescence is irreversibly quenched in a region of interest, and the recovery of fluorescence in this quenched area is recorded. Two examples of fluorescence recovery curves are shown in figure 4.1. A protein pool that is completely mobile will recover to its pre-quenching levels, given by a normalised fluorescence ratio between quenched and non-quenched pools. A partially mobile pool will not fully recover, as a fraction of the pool is immobile, and the fluorescence ratio will not recover to its pre-quenching level. This method informs on two properties of the mobility of a fluorescently labelled protein: the fraction of the total pool that is mobile, and its rate of mobility. The mobile fraction can be determined by calculating the maximum recovery level; any signal that is not recovered compared to pre-quenching levels represents the immobile fraction. The rate of mobility is given by the time by which half of the final signal recovers (half-time recovery, $t_{1/2}$). This rate is dependent on free diffusion and active transport rates, interactions with other proteins and membranes, and membrane diffusion barriers or membrane domains such as lipid rafts which can alter protein mobility (Reits and Neefjes,
2001). Using this approach, dynamics of many cellular transport events can be monitored, including active and passive lateral transport of proteins across a membrane diffusion barrier.

4.2.5 CONFOCAL SPINNING DISK FLUORESCENCE MICROSCOPY TO INVESTIGATE MEMBRANE PROTEIN DYNAMICS

To perform FRAP experiments a microscope fitted with laser excitation and quenching sources is required; to minimise photobleaching and phototoxicity, a spinning disk confocal setup is preferred. In widefield microscopy emitted light from every focal plane is captured by the detector, leading to a low signal-to-noise ratio. This can be improved by inserting a pin hole before the detector, allowing only light emitted from the focal plane to pass on to the detector, called confocal laser scanning microscopy (CLSM, figure 4.2A and B and (Ishikawa-Ankerhold et al., 2012)). This technique greatly improves signal-to-noise ratio, preventing out of focus light from reaching the detector.

Development of a spinning disk system has further advanced the field of live cell fluorescence microscopy by reducing photobleaching and phototoxicity effects. Inserted between the excitation source and the sample, the spinning disk unit consists of two spinning disks, with a dichroic mirror placed between the disks. Closest to the excitation and quenching source, a microlens disk splits the laserbeam into many parallel beams, focused precisely on the pinhole (Nipkow) disk that is placed closest to the sample. These two disks spin at the same speed and microlenses are aligned exactly with the pinholes. Emitted light from the sample travels through the pinhole disk, and is deflected by the dichroic mirror towards a detector (Figure 4.2C and (Nakano, 2002)). Photobleaching and –toxicity effects are greatly reduced by using a spinning disk system, because multiple beams excite the sample in low doses, minimising exposure of the sample to excitation light, making spinning disk confocal microscopy a well suited system to investigate protein dynamics in live cells.
Figure 4.1 Examples of fluorescence recovery after photobleaching (FRAP) curves. FRAP-curves inform on two properties of the mobility of a protein. The fraction of a fluorescent pool that is mobile is determined by the maximum recovery level (plateau), and the rate of mobility is given by the half-time recovery ($t_{1/2}$).

Figure 4.2 Spinning disk confocal microscopy. A. Widefield microscopy has a low signal-to-noise ratio. Samples are excited and emitted light is captured by a detector, including out of focus light. B. Confocal laser scanning microscopy (CLSM) improves signal-to-noise ratio. A pinhole is place before the detector, allowing only light from the focal plane to be captured by the detector. C. Mechanism of the spinning disk unit. The unit consists of two spinning disks and a dichroic mirror, and is placed between the excitation source and the sample. A microlens disk splits the laserbeam into parallel beams that excite the sample after passing through the pinhole array (Nipkow disk). Emitted light travels through the pinhole array and is deflected towards the detector. Only emitted light originating in the focal plane can travel through the pinhole array, improving signal-to-noise ratio compared to widefield microscopy. Compared to CLSM exposure to the excitation light is reduced, and phototoxicity and photobleaching effects are reduced.
4.2.6 Chapter Focus

The research presented in this chapter examines the membrane diffusion barrier at the transition zone of *C. elegans* sensory cilia, by using an *in vivo* imaging approach to determine exchange kinetics across this barrier. Specifically, the mechanisms involved in ciliary retention of ARL-13 were investigated. It was found that membrane-associated ARL-13, restricted to the ciliary middle segment, is retained by a diffusion barrier at the transition zone. Using a FRAP assay, it was found that transition zone proteins contribute differentially to the integrity of this barrier. In contrast, IFT components were found to be required for active transport of ARL-13 across the transition zone diffusion barrier.
4.3 METHODOLOGY

4.3.1 STRAINS

All strains were cultured and maintained at 16 and 20°C using standard techniques (Brenner, 1974). Mutant alleles were obtained through the Caenorhabditis Genetics Centre or the Japanese National Bioresource Project. Wild type strain: N2 (Bristol). Mutant alleles: che-11(e1810), mks-5(tm3100), mks-2(nx111), nphp-4(tm925), dyf-13(mn396), xbx-1(ok279), dyf-6(m175), and che-2(e1033). Transgenic strains: N2; oqEx58[P_{arl-13::arl-13::GFP + pRF4}].

Standard genetic crossing methods were employed to cross the oqEx58[P_{arl-13::arl-13::gfp + pRF4}] transgene into mutant allele backgrounds, described in Chapter II. The dye filling assay outlined in Chapter II was employed to identify single mutant strains homozygous recessive for e1810, mn396, ok279, m175, or e1033. Dye filling was also used to identify a double mutant strain homozygous recessive for both mks-2(nx111) and nphp-4(tm925). PCR genotyping, described in Chapter II, was used to follow the tm3100 mutation.

4.3.2 NEMATODE EGG PREPARATION USING A BLEACH PROTOCOL

To synchronise a population of nematodes, a bleach protocol was used to obtain an egg preparation, described in Chapter II. Before preparing slides transgenic animals were transferred to a new seeded plate.

4.3.3 FRAP; IMAGING SETUP AND ACQUISITION

5-10 transgenic animals were mounted on a 10% agarose pad, immobilised in 0.1 µm polystyrene microspheres (Polysciences), and covered with a glass coverslip. For these
experiments a Nikon Eclipse Ti microscope was used, fitted with a 100x 1.4NA Plan APO VC objective (Nikon), a 1.5x Optovar optical zoom adaptor, a 50 mW 488 nm laser, and a CSU-X1 spinning disk unit (Yokogawa). Images were acquired using a charge-coupled device camera (iXon EM-CCD, Andor Technology) controlled by Andor Technology iQ 2.6 software. To excite the sample 50% laser power was used; images were acquired using 50ms exposure time and 20x EM gain. Images were acquired pre-bleach, and at post-bleach timepoints of 0s, 15s, 30s, 60s, 120s, 180s, 240s, 300s, 900s, and 1200s. To quench fluorescent signals, a region of interest (ROI) is first drawn in an image acquired before starting the experiment. Subsequently, a pre-bleach image is acquired, followed immediately by photobleaching of the selected ROI using a single 100 ms pulse of the 50 mW 488 nm laser (100% power), and image acquisition of the 0s post-bleach time point.

4.3.4 POST-ACQUISITION ANALYSIS

An image-stack was generated in ImageJ of all images recorded. Regions of interest (ROIs) were drawn around the bleached (B) and non-bleached (NB) sections. N, NB, and background intensity levels for each ROI were recorded at each time point. Measurements were imported into Microsoft Excel and background levels subtracted to produce \( I_{\text{bleached}} \) \( I_{\text{non-bleached}} \) and \( I_{\text{total}} \) \( \text{B} \) \( \text{NB} \). For each time point ratio \( R(I_{\text{bleached}}:I_{\text{total}}) \) was calculated, \( t=0 \) set to 0, and \( R \) normalised to the pre-bleach ratio. The resulting values were plotted in GrahpPad Prism 5.0 and half-time recovery \( (t_{1/2}) \), plateau and \( R^2 \) values were calculated by fitting a one-phase association curve:

\[
Y = Y_{\text{max}} \ast (1 - e^{-k \ast x})
\]

\[
Y = R(I_{\text{bleached}}:I_{\text{total}})
\]

\( Y_{\text{max}} \) = plateau

\( k = t_{1/2}(s) \)

\( x = t(s) \)
4.4 RESULTS

4.4.1 ARL-13 IS MOBILE AT THE CILIARY MEMBRANE

To determine if GFP-tagged nematode ARL-13 is a useful marker for assessment of transition zone membrane diffusion barrier dynamics, its mobility was first assessed in the sensory cilia of wild type worms. Previous experiments have shown that *C. elegans* and mammalian ARL13B/ARL-13 associates with the ciliary membrane via N-terminal palmitoylation motifs (Cevik et al., 2010, Larkins et al., 2011), and that mouse ARL13B dynamically exchanges in the cilium (Larkins et al., 2011). Also, in the amphid and phasmid channel cilia of nematodes, ARL-13 localises exclusively to the middle segment (Inv domain) (Cevik et al., 2013, Cevik et al., 2010, Li et al., 2010). Regions of the middle segment were photobleached and fluorescence recovery was measured. When the entire ARL-13::GFP ciliary signal was quenched, very little fluorescence recovery was measured (Figure 4.3A), indicating that ARL-13 does not rapidly exchange between ciliary and non-ciliary membrane compartments. Photobleaching the proximal ~40% of ciliary signal resulted in fluorescence recovery with half the signal recovering in 124 seconds (t<sub>1/2</sub>). Figure 4.3B shows that this recovery manifests as a wave originating from the non-bleached region, re-establishing uniform fluorescence levels across the middle segment.

![Figure 4.3](image)

**Figure 4.3 ARL-13 mobility at the ciliary middle segment membrane.** A. Fluorescence recovery after photobleaching (FRAP) curves after quenching 100% or proximal-most 40% of ARL-13::GFP ciliary signals in wild-type phasmid neurons. Signal ratio (au; arbitrary units) calculated from the average intensity of ARL-13 signal in the photobleached region compared to the non-photobleached region. All measurements are background subtracted and normalised to a pre-bleach ratio of 1.0. Each data point reports mean ± SEM. B. Phasmid cilia of worms expressing ARL-13::GFP showing fluorescence recovery after photobleaching ~ 40% of proximal ciliary signal. Scale bar; 1 µm. Data was generated by Anita Wdowicz, University College Dublin. Figure adapted from Cevik et al. (2013).
These experiments demonstrate that *C. elegans* ARL-13 rapidly exchanges at the ciliary membrane, but not between ciliary and non-ciliary membranes. Also, these data provide direct evidence for the existence of a membrane diffusion barrier at the neighbouring transition zone, because ARL-13::GFP fluorescence recovery only occurs within the region of the quenched signal. Thus, a membrane diffusion barrier at the transition zone restricts ARL-13 mobility to the ciliary middle segment.

### 4.4.2 IFT retards ARL-13 mobility at the ciliary membrane

Next, ARL-13 mobility was assessed in IFT and ciliopathy-related gene mutants to determine if these genes are required for ARL-13 mobility at the ciliary membrane. IFT mutants possess shortened cilia and shortened ARL-13 compartments (Cevik et al., 2013, Perkins et al., 1986), so to ensure comparable fluorescence levels were photobleached in each strain, data was considered only from cilia with similar area, length, and ARL-13::GFP fluorescence intensity ((Figure 4.4D). Compared with wild type control worms, *che-11/IFT140* (IFT-A) and *dyf-6/IFT46* (IFT-B) mutants exhibited faster fluorescence recovery, with half-time recoveries of 23 s and 43 s, respectively (Figure 4.4A-B). In wild type animals, some fluorescence recovery can be seen at 30 seconds post-bleach and starts to plateau at 480 seconds, while in IFT mutants (*che-11* and *dyf-6*) recovery occurs much faster, and is stronger at 30 seconds and is almost complete at 60 seconds. *mks-5* mutants exhibit similar fluorescence recovery speeds as wild type animals: some recovery can be seen at 30 seconds post-bleach, and fluorescence recovery starts to plateau around 480 seconds, with near equal fluorescence levels in bleached and non-bleached regions (Figure 4.4C). These data show that ARL-13 mobility is increased in anterograde (*dyf-6*) or retrograde (*che-11*) IFT mutants, suggesting that IFT normally retards the mobility of ARL-13 at the ciliary membrane. In contrast, ARL-13 ciliary mobility was not affected in *mks-5* mutants, indicating that the associated transition zone-localised MKS module does not regulate ARL-13 exchange dynamics at the ciliary membrane.
Figure 4.4 ARL-13 intraciliary mobility at the ciliary membrane in IFT and transition zone-associated gene mutants. A. FRAP curves after quenching proximal-most 40% of ARL-13::GFP ciliary signals. Signal ratio (au; arbitrary units) calculated from the average intensity of ARL-13 signal in the photobleached region compared to the non-photobleached region. All measurements are background subtracted and normalised to a pre-bleach ratio of 1.0. Each data point reports mean ± SEM. B. Quantification of the fluorescence recovery curves depicted in A. C. Phasmid cilia of worms expressing ARL-13::GFP in designated genetic backgrounds showing fluorescence recovery after photobleaching ~40% of proximal ciliary signal. Scale bars; 1 µm. D. Box and whisker (min to max) distribution plots showing the % area, length and intensity of photobleached ARL-13::GFP. Figure adapted from Cevik et al. (2013).
4.4.3 ARL-13 ABNORMALLY LOCALISES TO CILIARY AND PERICILIARY MEMBRANES IN IFT AND TZ GENE MUTANTS.

If the transition zone at the ciliary base functions as an ARL-13 membrane diffusion barrier restricting ciliary entry and exit, dysfunction of the barrier should result in abnormal diffusion between ciliary and non-ciliary membrane compartments. Also, a membrane diffusion barrier at the transition zone implies the existence of mechanisms (e.g., active transport) that enable ciliary membrane (-associated) proteins such as ARL-13 to cross the barrier during transport to and from the organelle. Consistent with these hypotheses, ARL-13::GFP was found to be mislocalised at the ciliary and periciliary membranes of various IFT and transition zone gene mutant worms (Figure 4.5 and (Cevik et al., 2013)). In IFT mutants, although ARL-13::GFP localised to the cilium, a significant fraction of the total signal accumulated very specifically at the periciliary membrane and not elsewhere in the cell. However, signal was absent from the transition zone region and from other regions of the neuron. This suggests dysfunctional ciliary entry or retention and could reflect a role for IFT in actively transporting ARL-13 into cilia, across the transition zone diffusion barrier. Alternatively, it is possible that IFT is important for establishment of an ARL-13 diffusion barrier. In the case of transition zone gene mutants, ARL-13::GFP localisation patterns were qualitatively similar to IFT mutants, with signals observed at the ciliary and periciliary membranes, but not at other parts of the neuron. A subset of transition zone mutant animals also showed mislocalised ARL-13::GFP signals at the transition zone membrane. This suggests that in these mutants a restriction mechanism, like a diffusion barrier at the transition zone membrane may be defective, as was suggested previously for ciliary exclusion of C. elegans RPI-2 and TRAM-1 (Williams et al., 2011a). However, the above data only provide a snapshot of ARL-13 steady state localisation, and cannot conclude on the contribution of IFT and transition zone genes to barrier establishment. To directly address this issue, a FRAP assay was employed to determine ARL-13::GFP exchange kinetics between the ciliary and periciliary membranes of various IFT and TZ mutant animals. These findings are presented in the following sections.
4.4.4 ANALYSIS OF ARL-13::GFP EXCHANGE BETWEEN CILIARY AND PERICILIARY MEMBRANES OF TRANSITION ZONE GENE MUTANTS

Worms with mutations in transition zone MKS and NPHP module genes were examined. Specifically, mks-5 and nphp-4 single mutants, as well as mks-2;nphp-4 double mutants, were selected on account of robust pools of ARL-13::GFP signal at their periciliary and ciliary membranes. As outlined above, C. elegans MKS and NPHP module components interact genetically to maintain transition zone and cilium integrity; MKS-5 may be a central component by serving as a molecular bridge to link both modules (Williams et al., 2011a, Williams and Winkelbauer, 2008, Williams et al., 2010, Huang et al., 2011). When the ciliary membrane pool is photobleached in mks-5 and mks-2;nphp-4 mutants, ARL-13 signals recover quickly, with half-time recovery rates of 20s and 18s, respectively (Table 4.1 and Figure 4.6A-B). In nphp-4 mutants recovery occurs more slowly (t_{1/2}=132 seconds). When the reverse experiment is performed and the periciliary membrane ARL-13 pool is photobleached, similar results were obtained (Figure 4.6C-D). These findings indicate that in nphp-4 single mutants the transition zone may retain more of its
Figure 4.6 FRAP analysis of ARL-13 exchange dynamics between ciliary and periciliary membrane compartments in transition zone mutants. A and C. FRAP curves (background subtracted) derived from bleaching entire ciliary (A) or periciliary (C) ARL-13::GFP signals in phasmid cilia. For ease of comparison, pre-bleach ratios are normalised to 1.0, and bleach ($t_{0 \text{sec}}$) time points normalised to 0. B and D. Representative images from ciliary (B) and periciliary (D) ARL-13::GFP photobleaching experiments in phasmid cilia. Brackets denote bleached regions. All images identically imaged and scaled. Scale bars; 2 µm. Figure adapted from Cevik et al. (2013).
Figure 4.7 Additional ARL-13 FRAP curves (bleaches of periciliary or ciliary regions) in transition zone mutants. Each graph shows fluorescence intensity plots of periciliary and ciliary signals. All curves are subtracted for background photobleaching and any very low level recovery from total (PCM+cilium) photobleaching experiments (shown in Figure 4.8). Data shows that fluorescence recovery in one pool correlates with a signal reduction in the other pool; thus, recoveries come from the non-bleached pool. Figure adapted from Cevik et al. (2013).

Figure 4.8 FRAP analysis of ARL-13 fluorescence recovery after quenching all periciliary and ciliary signals in transition zone mutants. FRAP curves (background subtracted) derived from bleaching entire periciliary and ciliary ARL-13::GFP signals in phasmid cilia. For ease of comparison, pre-bleach ratios are normalised to 1.0, and bleach (t0 sec) time-points normalised to 0. Figure adapted from Cevik et al. (2013).
functionality as a membrane diffusion barrier, compared to mks-5 and mks-2;nphp-4 mutants that exhibit more severe ciliary phenotypes (Cevik et al., 2013, Williams et al., 2011a, Huang et al., 2011).

In mks-5 and mks-2;nphp-4 worms, ARL-13-fluorescence recovery plateaus close to pre-bleach levels, recovering to 83% and 71% for cilium quenching experiments, and 88% and 74% for periciliary membrane quenching experiments. Importantly, recovery stems from the non-bleached periciliary or ciliary membrane pools (depending on the experiment) and not from other cellular pools, because when the ciliary and periciliary membrane pools ARL-13::GFP were photobleached, little or no recovery was observed (Figure 4.8). The same conclusion is reached from plots of fluorescence intensity profiles (Figure 4.7), which show that as fluorescence recovers in the bleached region, it reduces in the non-bleached region (periciliary or ciliary membrane), suggesting that the fluorescence recovery in bleached compartments is due to dynamic exchange with the non-bleached compartment.

Taken together, these data show that the transition zone possesses a membrane diffusion barrier that is required for ARL-13 middle segment sequestration. When transition zone genes are mutated, this barrier is deregulated, ARL-13 mislocalises to the periciliary membrane and can dynamically exchange between ciliary and periciliary membranes.

4.4.5 ANALYSIS OF ARL-13::GFP EXCHANGE BETWEEN CILIARY AND PERICILIARY MEMBRANES OF IFT GENE MUTANTS

Next, IFT gene mutants were examined. Mutants were chosen to analyse requirement of both anterograde (che-2/IFT80) and retrograde (xbx-1/DYNC2LI1) trafficking pathways for retention of ARL-13 at the ciliary membrane. Additionally, dyf-6/IFT46 (IFT-B) and dyf-13/TTC26 (IFT-B associated) mutants were chosen, because these mutants showed usable ciliary and periciliary ARL-13::GFP levels for photobleaching experiments (Figure 4.5 and (Cevik et al., 2013)).
Compared to transition zone gene mutants, quenching of ARL-13 ciliary signals in IFT mutants resulted in limited fluorescence recovery, and this recovery occurred more slowly and to a lesser degree. In *xbx-1*, *dyf-6*, *dyf-13*, and *che-2* mutants, half-time recovery rates of ARL-13 fluorescence recovery were 60s, 112s, 50s, and 53s, respectively (Table 4.1). Additionally, pre-bleach fluorescence levels were not reached: in *xbx-1* mutants fluorescence plateaued at 19% of prebleach levels; in *dyf-6* mutants 50% of original levels were recovered, and 33% of pre-bleach fluorescence intensity was recovered in *che-2* mutants. Interestingly, in *dyf-13* mutants, the calculated fluorescence recovery plateau level was 92% of pre-bleach levels, suggesting pre-bleach fluorescence levels were almost completely recovered (Table 4.1 and Figures 4.9A-B). The reverse periciliary membrane photobleaching experiments show similar results (Figure 4.9C-D). Figure 4.9A and B show that in *dyf-13* mutants fluorescence levels are almost completely recovered (plateau: 0.87) and that this recovery is much faster than the limited fluorescence recovery seen in *xbx-1* and *dyf-6* mutants (t_{1/2}=78s in *dyf-13*, 137s in *xbx-1*, 181s in *dyf-6*). Consistent with these data, the pool of ARL-13::GFP in the non-quenched compartment decreases in *dyf-13* worms but not in *xbx-1* worms (Figure 4.10) and when both ciliary and periciliary signals were quenched, very little fluorescence recovery was observed, indicating the pools cannot be replenished from outside the ciliary region (Figure 4.11). Together, these data show that in most examined IFT mutants little ARL-13::GFP exchange occurs between ciliary and periciliary membrane compartments, suggesting the diffusion barrier is mostly intact. An exception is *dyf-13*, encoding a putative IFT-B associated protein (Ishikawa *et al.*, 2014, Blacque *et al.*, 2005, Franklin and Ullu, 2010), which prevents ARL-13 exchange between compartments.

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Table 4.1 Half-time recoveries and plateau recovery levels for graphs in Figures 4.6, 4.9, 4.12. Table from Cevik *et al.*, (2013).
Figure 4.9 FRAP analysis of ARL-13 exchange dynamics between ciliary and periciliary membrane compartments in IFT mutants. A and C. FRAP curves (background subtracted) derived from bleaching entire ciliary (A) or periciliary (C) ARL-13::GFP signals in phasmid cilia. For ease of comparison, pre-bleach ratios are normalised to 1.0, and bleach (t0 sec) time points normalised to 0. B and D. Representative images from ciliary (B) and periciliary (D) ARL-13::GFP photobleaching experiments in phasmid cilia. Brackets denote bleached regions. All images identically imaged and scaled. Scale bars; 2 µm. Figure adapted from Cevik et al. (2013).
Figure 4.10 Additional ARL-13 FRAP curves (bleaches of periciliary or ciliary regions) in IFT mutants. Each graph shows fluorescence intensity plots of periciliary and ciliary signals. All curves are subtracted for background photobleaching and any very low level recovery from total (PCM+cilium) photobleaching experiments (shown in Figure 4.11). Data shows that fluorescence recovery in one pool correlates with a signal reduction in the other pool; thus, recoveries come from the non-bleached pool. Figure adapted from Cevik et al. (2013).

Figure 4.11 FRAP analysis of ARL-13 fluorescence recovery after quenching all periciliary and ciliary signals in IFT mutants. FRAP curves (background subtracted) derived from bleaching entire periciliary and ciliary ARL-13::GFP signals in phasmid cilia. For ease of comparison, pre-bleach ratios are normalised to 1.0, and bleach (t0 sec) time-points normalised to 0. Figure adapted from Cevik et al. (2013).
4.4.6 *DYF-13* AND *NPHP-4* GENETICALLY INTERACT TO RESTRICT ARL-13 TO THE MIDDLE SEGMENT

Potential weakening of the ARL-13 membrane diffusion barrier in *dyf-13* mutants could suggest a functional relationship between this IFT-B associated component and transition zone genes. To examine this further, FRAP-assays were conducted on a *dyf-13;nphp-4* double mutant. *dyf-13;nphp-4* double mutants showed rapid fluorescence recovery after ciliary or periciliary pools were photobleached (t$_{1/2}$=14s and t$_{1/2}$=34s, respectively, Table 4.1 and Figure 4.12), which was faster than observed in either single mutant. Fluorescence recovery levels plateaued close to pre-bleach levels in *dyf-13;nphp-4* and *dyf-13* single mutants in periciliary (92% and 87%) and ciliary (85% and 92%) photobleaching experiments (Table 4.1 and Figure 4.12). However, in *nphp-4* mutants, fluorescence levels plateaued at 43% in experiments where ciliary ARL-13::GFP signals were photobleached. Consistent with rapid fluorescence recovery due to ARL-13 exchange between ciliary and periciliary membranes, fluorescence intensity plots show that in *dyf-13;nphp-4* animals, ARL-13::GFP signals decrease in the non-quenched compartment. As was shown for transition zone and IFT single mutants, when both ciliary and periciliary ARL-13::GFP signals were quenched, very little fluorescence recovery was observed (Figure 4.14), showing fluorescence recovery could not originate from other ARL-13 pools in the neuron. Taken together, these data show that the phenotype observed in *dyf-13;nphp-4* double mutants is different than either single mutant, and is comparable to what was described for *mks-5* and *nphp-4;mks-2* transition zone mutants above, suggesting that a functional interaction between *dyf-13* and *nphp-4* regulates the ARL-13 membrane diffusion barrier.
Figure 4.12 FRAP analysis of ARL-13 exchange dynamics between ciliary and periciliary membrane compartments in dyf-13; nphp-4 double mutants. A and C. FRAP curves (background subtracted) derived from bleaching entire ciliary (A) or periciliary (C) ARL-13::GFP signals in phasmid cilia. For ease of comparison, pre-bleach ratios are normalised to 1.0, and bleach ($t_{0}$ sec) time points normalised to 0. B and D. Representative images from ciliary (B) and periciliary (D) ARL-13::GFP photobleaching experiments in phasmid cilia. Brackets denote bleached regions. All images identically imaged and scaled. Scale bars; 2 µm. Figure adapted from Cevik et al. (2013).
Figure 4.13 Additional ARL-13 FRAP curves (bleaches of periciliary or ciliary regions) in dyf-13; nphp-4 double mutants. Each graph shows fluorescence intensity plots of periciliary and ciliary signals. All curves are subtracted for background photobleaching and any very low level recovery from total (PCM+cilium) photobleaching experiments (shown in Figure 4.14). Data shows that fluorescence recovery in one pool correlates with a signal reduction in the other pool; thus, recoveries come from the non-bleached pool. Figure adapted from Cevik et al. (2013).

Figure 4.14 FRAP analysis of ARL-13 fluorescence recovery after quenching all periciliary and ciliary signals in dyf-13; nphp-4 double mutants. FRAP curves (background subtracted) derived from bleaching entire periciliary and ciliary ARL-13::GFP signals in phasmid cilia. For ease of comparison, pre-bleach ratios are normalised to 1.0, and bleach (t0 sec) time-points normalised to 0. Figure adapted from Cevik et al. (2013).
4.5 DISCUSSION

In this chapter a novel *in vivo* FRAP assay is developed and implemented to functionally investigate the transition zone membrane diffusion barrier. ARL-13 is found to be mobile at the middle segment membrane, and this mobility is retarded by IFT. It is also shown that ARL-13 is retained at the middle segment membrane by a membrane diffusion barrier at the transition zone, regulated by MKS and NPHP modules. IFT actively transports ARL-13 across the transition zone barrier, but is not required for establishing the barrier. This is the first such ‘barrier assay’ in a multicellular system and has provided insight into membrane diffusion kinetics across the transition zone.

4.5.1 ARL-13 EXCHANGES AT THE MEMBRANE

ARL-13 is highly mobile at the membrane of the middle segment. After photobleaching part of the ciliary signal, fluorescence recovers in a wave-like form in proximal and distal directions. The observed mobility is slower than that observed for human Arl13b in cell culture (Larkins et al., 2011). These differences could be due to differences in membrane fluidity, allowing faster diffusion in more fluid membranes. For example, nematode ciliary membranes could possess a higher fraction of lipid rafts formed by cholesterol and sphingolipids, resulting in stiffer membranes (Incardona and Eaton, 2000, Levental et al., 2010, Simons and Ikonen, 1997). Another possibility is that transient interactions with other ciliary proteins, such as components of the IFT particle, affect diffusion kinetics of ARL-13 or Arl13b in different manners. For example, ARL-13 has been shown to undergo IFT in developing nematode cilia, but this has not been found in other systems (Cevik et al., 2013). It is possible that the interaction observed between ARL-13 and the IFT machinery in worms is stronger than in other systems, and may retard diffusion of ARL-13 at the nematode ciliary membrane more so than in other systems, resulting in lower mobility of the protein. Mobility at the ciliary membrane is not observed for all ciliary proteins. For example, *C. elegans* TAX-2 and -4 cyclic nucleotide gated (CNG) channel subunits and ODR-10 are mostly immobile at the ciliary membrane (Wojtyniak et al., 2013), and could be anchored in place through interactions with the axoneme.
When the entire ARL-13::GFP ciliary signal is quenched, very little fluorescence recovery was observed, indicating a lack of exchange with non-ciliary compartments. This was also observed in photobleaching experiments of human Arl13b (Larkins et al., 2011), and for other ciliary membrane (-associated) proteins, including Sstr3 (somatostatin receptor 3), Htr6 (serotonin receptor 6), the ciliary targeted C terminal tail of PKHD1 (fybrocystin), and Smo (Chih et al., 2012, Hu et al., 2010). This suggests that turnover levels of these proteins are very low, and that these proteins are very stable.

4.5.2 IFT RETARDS ARL-13 MOBILITY AT THE CILIARY MEMBRANE

Further investigation of ARL-13 mobility at the ciliary membrane revealed that in IFT gene mutants, but not transition zone mutants, ARL-13 fluorescence recovery occurs faster than in wild type, suggesting IFT retards ARL-13 mobility. In contrast, flagellar mobility Chlamydomonas PKD2 is reduced in IFT mutants (Huang et al., 2007), suggesting that IFT regulates mobility of nematode ARL-13 and Chlamydomonas PKD2 in different ways. This difference may be due to different requirements for IFT in ciliary restriction of ARL-13 and PKD2. In IFT mutants PKD2 levels were increased in the flagellar membrane, possibly due to defective ciliary removal of this protein (Huang et al., 2007). However, in C. elegans IFT mutants, ARL-13 maintains ciliary localisation, but also mislocalises to the periciliary membrane, suggesting a role for IFT in transport into cilia, as well as ciliary retention, rather than removal, of ARL-13 (Cevik et al., 2013).

IFT machinery could retard ARL-13 mobility through several mechanisms. One possibility is through direct interactions with ARL-13, and indeed, biochemical associations between human IFT-B components and ARL13B were observed (Cevik et al., 2013). If ARL-13 interactions with the IFT machinery are conserved in the nematode, loss of these interactions in IFT mutants may allow ARL-13 to diffuse faster along the ciliary membrane. Indeed, as mentioned before a subpopulation of ARL-13 can undergo IFT-like transport in developing nematode cilia (Figure 4.15), providing evidence that interactions
between IFT machinery and ARL13B in mammalian cells are conserved in the worm. It is also possible that loss of IFT alters ciliary membrane composition, making the membrane more fluid and allowing faster diffusion of ARL-13 to occur. Indeed, lipid rafts have been identified in *Trypanosome* flagellar membranes, and may associate with IFT particles (Tyler et al., 2009). Lipid rafts increase membrane rigidity, and if these IFT-associated rafts are conserved in *C. elegans*, ARL-13 could be associated with these rafts through its interaction with the IFT machinery. Investigation of nematode ciliary membrane lipid composition and the presence of lipid rafts may provide further insight into molecular mechanisms regulating ARL-13 mobility.

4.5.3 AN ARL-13 DIFFUSION BARRIER AT THE TRANSITION ZONE

The FRAP work presented here shows that ARL-13 is mobile at the membrane, but this mobility does not extend into the transition zone. This work, combined with a previous report suggesting a membrane diffusion barrier at the transition zone preventing ciliary entry of RPI-2 and TRAM-1, shows that the transition zone functions as a bidirectional membrane diffusion barrier, retaining ciliary proteins (ARL-13) and preventing entry of non-ciliary proteins (RPI-2 and TRAM-1) (Williams et al., 2011a). ARL-13 mobility also does not extend into the distal segment, suggesting the presence of a second membrane.
diffusion barrier at the middle-distal segment border. Unlike the transition zone, which has a defined ultrastructural composition, consisting of Y-link structures that may terminate in the ciliary necklace, there is no such structure at the border between middle and distal segments. Thus, the membrane diffusion barrier at this site is not mediated by Y-links, which indicates that Y-links are not universal components or regulators of ciliary diffusion barriers. The middle/distal segment diffusion barrier could be regulated through different lipid composition and fluidity of the membranes of these segments. For example, in *Trypanosoma* flagella, detergent resistant membrane (DRM) patches were found more abundantly in distal regions of the membrane than in proximal regions (Tyler et al., 2009).

4.5.4 MKS/NPHP MODULES REGULATE THE ARL-13 TRANSITION ZONE DIFFUSION BARRIER

In MKS/NPHP module gene mutants ARL-13 localises to ciliary and periciliary membranes, and ARL-13 can exchange between these two compartments. Quenching either ciliary or periciliary signals resulted in fluorescence recovery in the photobleached compartment, indicating that ARL-13 dynamically exchanges across the transition zone in both directions. This provides direct proof that the transition zone functions as a membrane diffusion barrier, restricting ARL-13 to the ciliary membrane. Previously, steady state imaging showed that RPI-2 and TRAM-1 can enter the cilium in MKS- and NPHP-module gene mutants, but exchange of these proteins across the transition zone was not determined (Williams et al., 2011a).

One of the mechanisms that may be involved in the diffusion barrier is the connection between Y-links with the membrane. Indeed, studies on a *Chlamydomonas* CEP290 mutant suggested that Y-links form part of the diffusion barrier at the transition zone (Craige et al., 2010). Y-links are exclusively found in the transition zone and extend from the seam of microtubule doublets and connect to the membrane (Perkins et al., 1986). In this compartment, the membrane-inserted Y-links may help to organise the ciliary necklace, a membrane specialisation that may be important in transition zone function (Gilula and Satir, 1972).
It has been suggested that Y-links may be formed by components of the MKS- and NPHP-modules (Craig et al., 2010, Garcia-Gonzalo and Reiter, 2012). Possibly, the barrier relies more on the membrane (-associated) components of these modules than non-membrane components. Based on domain analysis of MKS- and NPHP-module proteins, it was suggested that membrane proteins act as connectors to the stalks of Y-links, which themselves are suggested to consist of non-membrane proteins such as CEP290 and RPGRIP1L/MKS-5. The Y-link microtubule attachments could be formed by MKS- and NPHP-module proteins possessing microtubule binding domains, such as NPHP1 and NPHP4 (Garcia-Gonzalo and Reiter, 2012). In the current work mks-5 and nphp-4 single mutants were examined and results indicate that the transition zone membrane diffusion barrier is more severely disrupted in mks-5 mutants than in nphp-4 mutants. It is possible that disruption or loss of Y-link microtubule connections in nphp-4 mutants leaves the membrane diffusion barrier mostly intact, but loss of a component of the stalk in mks-5 mutants, results in more severe barrier dysfunction. Contrasting with the idea that the membrane diffusion barrier is physically formed by Y-links is the notion that in several gene mutants of MKS-module components, Y-links are present, but the diffusion barrier is compromised (Williams et al., 2011a, Huang et al., 2011, Jauregui et al., 2008, Bialas et al., 2009). This would suggest that Y-links are not a physical part of the membrane diffusion barrier, but rather regulate the barrier, possibly by recruiting or retaining components of the barrier.

To further investigate differential roles of membrane and microtubule-associated MKS- and NPHP-module components, ARL-13 dynamics across the transition zone should be investigated in mutants of genes encoding MKS-module membrane proteins. Additionally, superresolution and immuno-EM approaches can provide insight into the precise localisations of MKS- and NPHP-module components that potentially could be correlated to a requirement for membrane diffusion barrier function.

Other mechanisms that could regulate the diffusion barrier include a septin ring at the ciliary base. This ring was identified in mammalian systems and shown to form a membrane diffusion barrier regulating ciliary entry of Sstr3, Htr6, and Smo (Chih et al., 2012, Hu et al., 2010). It remains unclear if this ring is at the basal body or at the transition
zone. Also, a potential barrier at the ciliary base of MDCK cells has been described, consisting of condensed lipids that prevent ciliary entry of a GPI-anchored protein (Vieira et al., 2006). Another diffusion barrier present at the ciliary base regulates ciliary entry of cytosolic proteins. This barrier is size-dependent, though whether this relates to mass or size is unclear, and reports differ on the maximum size of proteins that can freely diffuse into and out of the cilium (Kee et al., 2012, Lin et al., 2013). Although this barrier is thought to be regulated by nucleocytoplasmic transport machinery, it remains unknown if it is also involved in regulation of ciliary entry and exit of membrane (-associated) proteins. Ciliary entry and exit of ARL-13 requires IFT machinery, which at least in cilia is attached to both membrane and microtubules (Pigino et al., 2009). Thus, it remains possible that cytosolic diffusion barriers are also involved in regulating ciliary entry and exit of membrane (-associated) proteins, because IFT assemblies may need to pass a cytosolic barrier into and out of the cilium.

4.5.5 IFT ACTIVELY TRANSPORTS ARL-13 ACROSS THE TRANSITION ZONE BARRIER BUT IS NOT REQUIRED FOR ESTABLISHING THE BARRIER

In IFT gene mutants ARL-13 localises to ciliary and periciliary membranes and exchange between the two pools of ARL-13 signal is limited. When exchange is observed, it appears to occur in both directions. In IFT mutants ARL-13 exchange across the transition zone occurred slower and to a lesser degree than in transition zone-associated gene mutants. In both directions, recovery speeds and plateau levels were almost two-fold lower in IFT mutants than in transition zone-associated gene mutants. Notwithstanding these limited levels of ARL-13 exchange between ciliary and periciliary membranes, we observed changes in different IFT mutants. This exchange may be due to different levels of remaining IFT activity in these mutants. For example, in dyf-6/IFT46 mutants, for which the allele is a premature stop codon towards the end of the gene, IFT may not be completely abrogated (Ou et al., 2007). For xbx-1/DYNC2LI1 mutants, for which the allele used is likely a severe loss-of-function or null mutant, IFT-B proteins and kinesin-2 motors can still enter the cilium, though it is unclear if anterograde IFT occurs (Schafer et al., 2003). Our conclusion is that anterograde (dyf-6 mutant) and retrograde (xbx-1 mutant) IFT are not required for establishment of the transition zone membrane diffusion barrier, but
rather for active transport across the barrier in both directions. This is consistent with the
observation that in developing cilia, ARL-13 can undergo IFT (Figure 4.15), and with the
biochemical interactions found between human ARL13B and IFT46/DYF-6 and IFT74
(Cevik et al., 2013).

Finally, DYF-13 appears to serve a separate role to other IFT mutants tested here, relating
to ciliary restriction and retention of ARL-13. In dyf-13 mutants ARL-13 can exchange
between ciliary and periciliary compartments. DYF-13 appears to be associated with the
IFT-B complex (Blacque et al., 2005), but does not seem to have a role as a core IFT
component. Mutant worms exhibit reduced, but not absent, dye-uptake and intraflagellar
transport can take place, though it may be defective (unpublished data, Blacque lab). In
Chlamydomonas knockdown of TTC26/DYF-13 resulted in short cilia, but IFT occurred
normally, suggesting that this protein may be a cargo-interacting protein, but is not
required for IFT (Ishikawa et al., 2014). This is consistent with the observation that in dyf-
13 mutants ARL-13 exchange occurs between ciliary and periciliary membrane
compartments. The rate of exchange is slower in this mutant than in transition zone-
associated gene mutants, possibly because IFT-mediated active transport across the
transition zone may occur slower than diffusion across a defective barrier. Also, dyf-13 and
transition zone-associated nphp-4 genetically interact to maintain the ARL-13 diffusion
barrier. In dyf-13;nphp-4 double mutants ARL-13 exchanges faster between ciliary and
periciliary compartments, indicating that a genetic interaction between IFT and transition
zone-associated genes is required to maintain ARL-13 ciliary restriction.

4.5.6 MODEL: ACTIVE TRANSPORT V. DIFFUSION BARRIERS FOR ARL-13
COMPARTMENTALISATION

Based on differential roles for transition zone and IFT components in targeting and
restriction of ARL-13 at the ciliary middle segment membrane, a model is developed
(Figure 4.16). MKS and NPHP modules are required to form an intact membrane diffusion
barrier that prevents ARL-13 leaking out of the cillum. In transition zone gene mutants this
barrier is weakened or absent, and ARL-13 can diffuse along the membrane across the
transition zone and into the periciliary membrane compartment. IFT is unaffected in these
mutants (Williams et al., 2011a), but cannot fully compensate the loss of the diffusion
barrier, and ARL-13 accumulates outside the cilium at the periciliary membrane. IFT is required for active transport across the transition zone barrier, and through interactions with the IFT-B complex ARL-13 is retarded at the ciliary membrane. In IFT gene mutants, where the transition zone diffusion barrier is intact, ARL-13 cannot efficiently enter the cilium, resulting in periciliary accumulations, and limited exchange between ciliary and periciliary ARL-13 pools. The notion of a protein being restricted to cilia using dual mobilities (diffusion and IFT-mediated active transport) applies to other proteins, such as *Chlamydomonas* DRC4 (Wren et al., 2013). However, DRC4 is cytosolic whereas ARL-13 associates with membranes, thus, diffusion barriers that need to be overcome to enter and exit cilia may be different.

It is important to note that this model does not apply to all ciliary membrane (-associated) proteins, because a recent study in *Chlamydomonas* showed that SAG1, an agglutinin involved in adhesion induced signalling, can overcome the membrane diffusion barrier at the flagellar base and enter the flagellum independent of IFT (Belzile et al., 2013). In addition to MKS/NPHP modules at the *C. elegans* transition zone, barriers are found across multiple systems, but these are composed of different proteins, such as the septin ring at the base of mammalian cells (Chih et al., 2012, Hu et al., 2010).

### 4.5.7 Conclusions

This chapter describes a novel fluorescence recovery after photobleaching (FRAP)-based assay in *C. elegans* to functionally investigate the transition zone membrane diffusion barrier. Using ARL-13, which is mobile at ciliary membranes, differential roles are ascribed to components of the transition zone and intraflagellar transport machinery. This FRAP assay allows for *in vivo* investigation of the transition zone membrane diffusion barrier and roles of ciliary transport and ciliopathy genes towards barrier establishment versus active transport across the barrier.
Further developments of this assay could include the use of other ciliary proteins, as this barrier seems to function differently for periciliary membrane (-associated) RPI-2 and TRAM-1 than for ARL-13 (Williams et al., 2011a), thus barrier function may be protein-specific. Furthermore, to investigate size-dependency of the membrane diffusion barrier at the ciliary base, exchange kinetics of a number of membrane (-associated) proteins could be investigated. Additionally, investigation of exchange kinetics of a membrane protein that localises specifically to both ciliary and periciliary membranes in wild type animals can inform on the rate of exchange in non-defective cilia. A good candidate protein for this is SRBC-66, a transmembrane receptor that localises to both ciliary and periciliary membranes in ASK neurons, but is absent in non-ciliary compartments (Kim et al., 2009). Also, use of photoswitchable and photoactivated fluorophores may provide information on establishment of the exchange equilibrium across the transition zone.

Figure 4.16 Model of differential roles for IFT and transition zone modules in defining the C. elegans ARL-13 ciliary membrane subdomain. (A) In wild type cilia, MKS and NPHP modules ensure an intact transition zone (TZ) barrier to ARL-13 diffusion (blue arrows), preventing ARL-13 exchange between periciliary (PCM) and middle segment (MS) membranes. Through interactions with the IFT-B complex, IFT facilitates ARL-13 ciliary restriction by trafficking PCM-localised ARL-13 (derived from upstream transport or leakage out of the cilium) across the TZ barrier into the middle segment (MS). Alternatively, IFT trains in the MS could prevent ARL-13 from exiting cilia (double arrow). (B) In TZ module mutants, the TZ barrier is weakened (e.g., via loss of Y-link structures) and ARL-13 readily exchanges between PCM and MS membranes. Although IFT remains intact (Williams et al., 2011b), it cannot fully compensate for the weakened barrier, thus ARL-13 accumulates at the TZ and PCM membranes. Low level ARL-13 signals (pink shade) are found in distal segment (DS) regions, suggesting a partial defect in the diffusion barrier at the MS/DS boundary. (C) In IFT gene mutants, the TZ barrier to ARL-13 diffusion is mostly intact; however, loss of IFT causes reduced entry of ARL-13 into cilia (or increased exit from cilia), resulting in PCM accumulation of ARL-13. IFT mutants also display increased ARL-13 diffusion kinetics (thick blue arrows) in the cilium. Figure taken from Cevik et al. (2013).
CHAPTER V:

GENERAL DISCUSSION
The work presented in this thesis is focused around two main themes: the characterisation of a novel component of the ciliary base, K04F10.2/KIAA0556, and the functional investigation of a membrane diffusion barrier at the ciliary transition zone regulating entry and exit of ciliary membrane (-associated) proteins. Although K04F10.2 and the transition zone membrane diffusion barrier do not appear to be directly linked, several functional connections to the intraflagellar transport machinery were found. First, this work suggests an IFT-related role for K04F10.2 based on biochemical and functional experiments and because the protein product localises strongly at the ciliary base, partially overlapping with accumulations of IFT components. K04F10.2 mutants possess a subtle IFT phenotype, further suggesting a role in maintaining stability of IFT assemblies. Second, the functional investigation of the membrane diffusion barrier at the transition zone revealed a requirement for IFT components in ciliary entry and retention of membrane-associated ARL-13.

IFT ASSEMBLY FORMATION AND CARGO-LOADING

The data presented in Chapter III suggests that K04F10.2 could serve functions related to the intraflagellar transport (IFT) machinery. Loss of K04F10.2 induces a subtle anterograde IFT phenotype in C. elegans cilia. Furthermore, a genetic interaction between nematode K04F10.2 and known IFT regulator arl-13 is required to maintain cilium integrity. Finally, KIAA0556 (human homolog of K04F10.2) biochemically interacts with most components of the IFT-B complex, further suggesting an IFT-related role for this novel ciliary base component. Although K04F10.2 does not appear to be required for ciliary entry or exit of IFT assemblies and does not undergo IFT in the cilium, it is possible K04F10.2 function is related to formation and stability of IFT assemblies prior to ciliary entry, based on the strong localisation at the ciliary base.

How IFT assemblies are formed and enter the cilium is not fully understood. One possibility is that in nematodes at the ciliary base IFT-A, -B, and BBSome complexes form an intermediate complex interacting with inactive dynein and possibly with ciliary cargo molecules, though work in Chlamydomonas has shown that not all IFT assemblies appear
to be cargo-loaded (Wren et al., 2013). Then, this complex can form the intact IFT assembly at the ciliary base through interactions with microtubule-bound kinesin-2 motors that will power anterograde IFT. The intact IFT assembly can then enter the cilium. Alternatively, the entire IFT assembly can be formed prior to microtubule binding of the kinesin-2 motors. Upon formation of the IFT assembly, microtubule binding of the kinesin-2 motors can take place, and the intact IFT assembly can enter the cilium. In both models, ciliary entry of kinesin-2 motors would need to be regulated to ensure that only intact IFT assemblies enter the cilium. Recently, the Chlamydomonas KIF3B/KLP-11 homolog FLA8 was found to be inactivated by phosphorylation of a conserved serine residue resulting in IFT-B dissociation from the kinesin-II subunit and prevented ciliary entry (Liang et al., 2014). Phosphorylation of kinesin motors provides a possible regulatory mechanism for ciliary entry of IFT assemblies, though it is likely that additional regulatory mechanisms are involved in formation of the IFT assembly. Indeed, Wei et al. showed that FBF1/DYF-19 is required at the ciliary base for ciliary entry of IFT assemblies (Wei et al., 2013). Furthermore, several kinases have been identified that play IFT-related roles in ciliary length regulation (Burghoorn et al., 2007, Tam et al., 2007, Niwa et al., 2012, Omori et al., 2010, Broekhuis et al., 2014). IFT assembly formation and ciliary entry, cargo-loading, and ciliary length regulation may be related and therefore may be regulated through similar mechanisms. Thus, identification of the phosphorylation targets of these kinases may provide further insight into the molecular mechanisms underlying regulation of IFT assemblies entering the cilium.

Based on the ciliary base localisation of both nematode and human K04F10.2/KIAA0556 and biochemical interactions between IFT-B components and human KIAA0556, it is possible that K04F10.2/KIAA0556 plays a role in formation of the IFT assembly prior to ciliary entry. This could involve mediating interactions that are required to form the IFT assembly, or involve prevention of these interactions to regulate how many IFT assemblies can enter the cilium. If K04F10.2 plays a negative regulatory role in IFT assembly formation, loss of K04F10.2 would be expected to result in increased concentration of ciliary IFT assemblies, which was not observed. As loss of nematode K04F10.2 induces subtle anterograde IFT defects, it therefore seems more likely that K04F10.2 is somehow involved in formation or stability of the IFT assembly. This function could be related to or redundant with that of F47G4.5, a nematode homolog of KATNBL1, which is a p80
CILIARY ENTRY OF IFT ASSEMBLIES AND ASSOCIATED CARGO

Prior to ciliary cargo delivery or removal from the cilium, the intact IFT assembly has to traverse the transition zone. At the transition zone membrane and cytosolic diffusion barriers are thought to regulate entry and exit of ciliary proteins (Garcia-Gonzalo and Reiter, 2012). Once the IFT assembly has formed and kinesin-2 motors are docked onto microtubules, the intact assembly has to pass these diffusion barriers. As IFT components are cytosolic, but can interact with the membrane (Pigino et al., 2009), it is likely that both the membrane and the cytosolic diffusion barriers play important roles in regulating entry and exit of IFT assemblies. Several publications have shown the presence of components of the nucleocytoplasmic transport machinery at the ciliary base (Breslow et al., 2013, Dishinger et al., 2010, Fan et al., 2007, Kee et al., 2012, Hurd et al., 2011). The cytosolic diffusion barrier appears to be size dependent, allowing diffusion of small molecules and restricting ciliary access of large multimeric complexes such as the IFT assembly (Lin et al., 2013, Kee et al., 2012). How the IFT assembly overcomes this diffusion barrier remains unknown, but importin-β2 and a RanGTP gradient were identified to be required at the ciliary base for ciliary entry of kinesin-2 motor KIF17 (Dishinger et al., 2010). One possibility is that the IFT assembly can enter the cilium through biochemical interactions between KIF17 and importin-β2, which can then translocate the IFT assembly through the ciliary pore complex. If ciliary entry is mediated by an interaction with the KIF17 kinesin-2 motor, additional regulatory mechanisms will need to be in place to prevent incomplete IFT assemblies entering the cilium. One such mechanism could be that the IFT assembly is formed prior to microtubule binding of the kinesin-2 motor, and ‘empty’ motors cannot bind to microtubules that extend into the cilium. It is also possible that additional ciliary localisation signals (CLS) are present on components of the IFT assembly and interactions between these components and importin proteins are required for ciliary entry. Indeed, Dishinger et al. suggest that the CLS they identified in KIF17 may not be the only one present in this protein (Dishinger et al., 2010), and similar sequence motifs could be
present in other components of the IFT assembly. Identification of ciliary targeting sequence motifs and further of biochemical interactions between components of the IFT assembly and the ciliary pore complex and importins may provide further insight into ciliary entry of IFT assemblies.

The IFT machinery is also important for ciliary entry and retention of some membrane (-associated) proteins. To overcome the membrane diffusion barrier at the transition zone, *C. elegans* ARL-13 requires IFT components. Data presented in chapter IV shows that ARL-13 accumulates at periciliary membrane compartments of IFT mutants and exchange across the transition zone is limited in these mutants. This suggests that although ARL-13 is not a true IFT cargo molecule as defined in chapter I, it requires IFT machinery for ciliary entry and restriction. The molecular mechanisms underlying ferrying of ciliary membrane (-associated) proteins across the transition zone membrane diffusion barrier remain unknown. The IFT assembly components are cytosolic, but BBSome subunit BBS5 can bind to phosphoinositides and may be responsible for membrane association of the BBSome (Nachury et al., 2007). It is possible that transient interactions between components of the IFT assembly and the membrane at the transition zone allow ciliary proteins to overcome the membrane diffusion barrier and enter the cilium. The work presented in chapter IV identifies a genetic interaction between NPHP-module component *nphp-4* and IFT-B component *dyf-13* that is required for ARL-13 ciliary targeting and restriction. Further investigation of the functional connection between these two genes as well as that between IFT and transition zone genes previously found to interact (Zhao and Malicki, 2011) could reveal how the IFT assembly and the transition zone components cooperate to regulate ciliary protein composition. Another possibility is that IFT assemblies facilitate ciliary membrane (-associated) proteins overcoming the transition zone by regulating entry into the transition zone. The ciliary necklace at the transition zone could be important in regulating ciliary entry of membrane proteins (Gilula and Satir, 1972). This membrane specialisation is formed by distinct membrane particles arranged in a helical pattern, which could suggest that membrane (-associated) proteins cannot cross the transition zone directly, but rather need to travel along the ciliary necklace to enter the cilium. Additionally, it is possible that Y-link structures arrange or terminate in the ciliary necklace. Possibly, IFT assemblies facilitate membrane (-associated) protein entry into this helix by interacting with components of the Y-links or the necklace itself. Recent
superresolution studies performed by members of this lab have revealed that transmembrane proteins of the transition zone localise in distinct spots in the transition zone membrane, suggesting they could be part of the membrane-inserted ends of Y-links or of the ciliary necklace, if this structure is conserved in *C. elegans* ciliated sensory neurons. Further investigation using superresolution microscopy approaches could reveal specific localisations of transition zone components and identify the composition and structure of Y-links and the ciliary necklace in nematode cilia. Complementary immuno-EM approaches could validate light microscopy work and provide further insight into the composition of the ciliary necklace and Y-links, as well as localisation and function of transition zone components.

**CILIARY CARGO DELIVERY AND REMOVAL**

How IFT assemblies deliver cargo and how dissociation of cargo molecules is regulated once they reach their destination remains unclear. Several lines of research have aimed to understand these processes and have revealed interactions between IFT components and specific cargo molecules, but a clear, general model remains absent (Wren et al., 2013, Qin et al., 2005, Bhogaraju et al., 2013). For example, biochemical interactions were identified between IFT74/81 and tubulin subunits (Bhogaraju et al., 2013). These interactions are required for normal cilium formation, but how tubulin subunits dissociate from the IFT assembly at the ciliary tip is unknown. Possibly, dissociation and reformation of the IFT assembly in the retrograde configuration allows for dissociation of cargo molecules, but this does not provide a mechanism for other ciliary cargo molecules that need to dissociate along the axoneme before reaching the very tip. A ciliary cargo that needs to dissociate before reaching the ciliary tip is DRC4, a structural component of *Chlamydomonas* flagella (Heuser et al., 2009). DRC4 was described in *Chlamydomonas* as an IFT cargo that can undergo both active transport and diffusion in the cilium until an incorporation site is reached (Wren et al., 2013). These observations suggest that as the IFT assembly is travelling along the axoneme cargo can be released or taken up, but the underlying mechanism remains enigmatic. It is possible that cargo release is regulated through post translational modifications altering the binding affinity of cargo molecules and IFT components, and that this can be reversed to attach cargo to an IFT assembly. K04F10.2
could play a role in this process, though its function may be specific to a cargo that was not investigated in this work, such as ciliary transmembrane proteins OSM-9 or PKD-2. K04F10.2 localises to middle segments of nematode cilia and loss of K04F10.2 results in middle segment anterograde IFT defects. If K04F10.2 plays a role in formation of IFT assemblies or cargo-loading onto assemblies at the ciliary base, as suggested above, it is also possible it is required for similar processes in the cilium. It seems likely that if K04F10.2 plays a role in cargo-loading at the ciliary base, its role in the middle segment of nematode cilia would be related to cargo association to the IFT assembly, possibly facilitating recycling of ciliary proteins, rather than cargo delivery and dissociation from the IFT assembly. Further investigation of known ciliary cargoes such as OSM-9 and PKD-2 in K04F10.2 mutants and studies of interactions between IFT components and ciliary cargo molecules may reveal the molecular mechanisms of K04F10.2 function. Wei et al. published a very elegant method to show IFT assembly stability by using a bimolecular fluorescence complementation (bIFC) assay to study interactions of IFT components and BBSome subunits (Wei et al., 2013). This method could be adapted to study associations of IFT components and associated cargo and could provide insight into ciliary cargo delivery and recycling.

In summary, K04F10.2/KIAA0556 function and function of the transition zone membrane diffusion barrier do not appear to be directly related, but are both linked to the intraflagellar transport machinery. Future work should focus on elucidating the molecular mechanisms underlying formation of the IFT assembly, cargo-loading onto these assemblies, interactions between IFT components and the transition zone facilitating ciliary entry, and IFT-mediated cargo delivery and recycling, and the roles played in these processes by K04F10.2.
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