Dysfunction of ciliary vesicle trafficking in ciliopathies

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Dysfunction of ciliary vesicle trafficking in ciliopathies

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The Volkswagen Transporter T3 is used as a reference to the trafficking, or transport, of ciliary vesicles within the cell. The red and white circle refer to the vesicles itself. The illustration of the cilium is adapted from a figure originally created by Machteld M. Oud, in the review ‘Ciliopathies: Genetics in Pediatric Medicine’, published in the Journal of Pediatric Genetics, volume 6, in 2017.

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Dysfunction of ciliary vesicle trafficking in ciliopathies

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Voorwoord

De grootste drijvende kracht achter wetenschappelijk onderzoek is nieuwsgierigheid, dat gevoed wordt door het verlangen om het leven te begrijpen. Dit fenomeen was ook datgene dat Antonie van Leeuwenhoek, van origine een handelsman geïnteresseerd in de kwaliteit van textiel, ertoe bracht om een druppel slootwater onder zijn eigen uitgevonden en gefabriceerde microscoop te leggen. Hij was de eerste mens die micro-organismen zag rondzwemmen, in het jaar 1674, welke hij beschreef als “een soort van diertgens, versien met verscheide ongelooflijke dunne pootgens, of beentgens, die seer vaerdig bewogen wierde … waermede sij ongelooflijke snelle beweginge teweeg bragten”. Deze kleine ‘pootgens’ zijn nu bekend als beweeglijke, ook wel ‘motievel’, cilia en worden sindsdien wetenschappelijk onderzocht. Ondanks deze ontdekking in 1674 duurde het tot 1900 voordat wetenschappers de niet-motievel solitaire variant van cilia opmerkten, het primaire cilium, en vervolgens dit onderdeel van de cel begonnen te onderzoeken. In de daaropvolgende 100 jaar kreeg de wetenschap langzaam meer inzicht in de structuur en de functie van dit intrigerende celorganel, maar de grootste sprong voorwaarts in het onderzoek naar het primaire cilium werd pas in de afgelopen 15 jaar gemaakt. Deze tijdslijn illustreert dat de functie van het primaire cilium voor lange tijd niet goed werd begrepen. Dit veranderde met de komst van de moleculaire genetica, dat hielp in het verkrijgen van (gemuteerde) diermodellen en het ontdekken van mutaties in genen die voor cilium-eiwitten coderen, welke oorzakelijk zijn voor verschillende erfelijke syndromen. Dit leidde tot een nieuw te onderscheiden groep van erfelijke aandoeningen: de ciliopathiën. De erfelijke syndromen binnen deze nieuwe groep aandoeningen waren al lang daarvoor bekend, zoals het Bardet-Biedl syndroom (BBS, voor het eerst beschreven rond 1900, later klinisch gespecificeerd), het Joubert syndroom (JBTS, voor het eerst beschreven in 1969) en Nephronophthisis (NPHP, voor het eerst beschreven in 1951). Patiënten met deze syndromen lijden onder andere aan obesitas gecombineerd met blindheid (BBS), aan defecten in de hersenontwikkeling (JBTS) en/of aan nierfalen (NPHP). Door deze verscheidenheid aan klinische kenmerken werden deze erfelijke aandoeningen nog niet eerder met elkaar in verband gebracht en niet eerder erkend als gevolg van een defect in hetzelfde celorganel, het primaire cilium. In dit proefschrift beschrijf ik mijn onderzoek waarin ik getracht heb meer inzicht te krijgen in de moleculaire mechanismen die ten grondslag liggen aan ciliopathiën, door enerzijds het identificeren van genen die betrokken zijn bij ciliopathiën, en anderzijds het ontrafelen van het netwerk van eiwit-eiwit interacties waarin de gerelateerde ciliopathie-eiwitten zich bevinden.

---

1 “...myn arbeyt, dien ik veel jaren agter een gedaan hebben, niet is geweest om den Lof dien ik nu geniet, daardoor te bejagen, maar meest uyt een drift van weetgierigheyt; die in my meer woont, gelyk ik merk, als in veel andere Menschen. Ende daar benevens dan iets ontdekt hebbende, het gene aanmerkenswaardig was, hebbe ik het van myn pligt geagt, het ontdekte op het Papier te zetten, opdat het wereltkundig zoude wezen...” Antonie van Leeuwenhoek, in zijn brief gedateerd 12 juni 1716.

2 “...myn geringen arbeyt komt alleen voort uyt een neyginge, die ik hebbe om de beginstelen van de geschapene saaken te ondersoeken, tot soo verre als het my mogelyk was...” Antonie van Leeuwenhoek, in zijn brief gedateerd 22 juni 1716.
Preface

The main driving force behind scientific research is curiosity, fueled by a desire to understand life. This made Antonie van Leeuwenhoek, originally a tradesman interested in the quality of fabrics, to put a drop of pond water underneath his own invented and fabricated microscope.\(^c\)\(^d\) He was the first man to observe microorganisms swimming around, as early as 1674, which he described as “little animals with diverse incredibly thin little feet, or little legs, which were moved very nimbly...and wherewith they brought off incredible quick motions.”\(^1\)\(^;\)^\(^2\) These little legs are now known as motile cilia, and have since then been a topic of investigation. However, it was not until 1900 that scientists reported about non-motile, solitary, primary cilia and started investigating their function.\(^3\) In the following 100 years the scientific community slowly gained insights in the structure and function of this intriguing cell organelle, but a major leap in the understanding of primary cilia was only made in the last 15 years. This illustrates that for a long time, the function of primary cilia was not well understood. This changed with the advances in molecular genetics, that aided the design of (mutant) animal models and the discovery of mutations in genes that encode ciliary proteins for several hereditary syndromes, which lead to the identification of a new class of inherited diseases: ciliopathies.\(^1\) This class comprises diseases which were already long known like Bardet-Biedl syndrome (BBS; first described around 1900, later clinically specified),\(^4\)\(^;\)^\(^5\) Joubert syndrome (JBTS; first described in 1969)\(^6\) and Nephronophthisis (NPHP; first described in 1951).\(^7\) In these syndromes, patients suffer from obesity combined with blindness (BBS), neurodevelopmental defects (JBTS) and/or kidney failure (NPHP). Because of this broad presentation of clinical features, they were never identified as phenotypes arising from malfunctioning of the same organelle, the primary cilium. In this thesis, I describe my research in which I aimed to gain further insights into the molecular mechanisms that underlie the ciliopathies through identifying the genes involved in ciliopathies and unraveling the protein-protein interaction network in which the encoded proteins reside.

\(^c\) “my work, which I’ve done for many a long time, was not pursued in order to gain the praise I now enjoy, but chiefly from a craving after knowledge, which I notice resides in me more than in most other men. And therewithal, whenever I found out anything remarkable, I have thought it my duty to put down my discovery on paper, so that all ingenious people might be informed thereof.” Antonie van Leeuwenhoek, letter dated June 12, 1716.

\(^d\) “...my work springs only from an inclination I have to inquire into the beginnings of created things, in so far as it was ever possible for me to do so...” Antonie van Leeuwenhoek, letter dated June 22, 1716.
Chapter 1

General introduction
Chapter 1.1

The cilium: structure and function
Cilia are cellular organelles with a slender, longitudinal shape that protrude from the cellular membrane of almost all cell types. Two main types of cilia can be distinguished, motile cilia and immotile cilia. Motile cilia often occur as bundles of up to 200-300 hair-like cilia on the cell surface, that are capable of beating in a constant frequency whereby a fluid is propelled (as observed in protozoan ciliates by Antonie van Leeuwenhoek). In cells with immotile or primary cilia, however, only one cilium is generated per cell, and this single cilium has evolved as the cell’s communication center, which allows exchange of molecular information with its surroundings. In this thesis, the main focus will be on the primary cilium that hereafter will be referred to as ‘cilium’.

1.1.1 Structure of cilia
Cilia project from a basal body, a centriole-based structure composed in most vertebrate cells of nine triplets of microtubules, arranged in a nine-fold symmetry, that often become doublets towards the distal ends. These ends are used as a foundation to build the ciliary microtubule skeleton, the axoneme. The axoneme is composed of nine doublets of microtubules, tubular polymers of tubulin, which are tightly wrapped by the ciliary membrane. The plus ends of the microtubules, where growth by polymerization or treadmilling takes place, are situated at the tip of the cilium. The region directly apical to the basal body is specified as the transition zone (TZ), which functions as a gate and regulates the entry and exit of proteins into and out of the cilium. Structurally, the TZ can be recognized by Y-shaped linkers between the axoneme and the ciliary membrane, which together form the ‘ciliary necklace’, a term originating from the similarity of a beaded necklace wrapped around the base of the cilium (Figure 1). Just below the TZ, transition fibers are connecting the basal body to the membrane. These structures are proposed to have a dual function. First they are forming a physical diffusion barrier for vesicles targeted towards the cilium. Transmembrane proteins, which arrive in vesicles at the TZ, are forced to integrate into the ciliary membrane, after which they will be transported (still being within the plane of the ciliary membrane) towards the tip of the cilium by the intraflagellar transport (IFT) proteins. Vesicle delivery to the cilium contributes to the establishment of a highly specialized ciliary membrane that contains various receptors and signaling proteins. The other function of transition fibers is to provide ample docking and assembly sites for the IFT proteins (Figure 1). IFT is essential for ciliary functionality as protein synthesis does not occur within the cilium. There are two types of IFT complexes which bind specific motor proteins to transport proteins in an anterograde direction, from the base to the tip of the cilium (IFT-B powered by cytoplasmic motor dynein-2), or in a retrograde direction, back from the tip to the base (IFT-A powered by the kinesin-2 motor). Finally, there is an invagination of membrane at the base of the cilium, called the ciliary pocket (Figure 1), which is likely to be important for regulation of cilia composition being the site of exocytosis and endocytosis of ciliary proteins.
**Chapter 1.1**

Figure 1: **Schematic structure of the cilium.** Cilia are composed of a basal body (BB) and a ciliary axoneme that protrudes from the apical plasma membrane. These two structures are linked via the transition zone (TZ) that consists of three compartments, i.e., a base, and two compartments filling the space between the microtubules and the axonemal membrane. Structurally the TZ is defined by the presence of Y-shaped linkers, which together form the ‘ciliary necklace’ that is wrapped around the base of the cilium, as shown in the electron microscopy image at the bottom right corner. The TZ acts as a diffusion barrier and regulates ciliogenesis and signaling. Cilia contain a microtubule cytoskeleton that is composed of nine microtubule doublets organized in a ring, with the plus-ends situated at the ciliary tip. A central microtubule pair is present only in motile cilia. These microtubules together with inner and outer dynein arms, radial spokes, and nexin links drive ciliary movement. To generate movement dynein heavy chains of one doublet slide against microtubules of a neighboring doublet thereby orchestrating the beating of the cilium in an ATP-dependent fashion. In both cilium types the ciliary microtubules
1.1.2 Function of cilia

The antenna-like appearance of the primary cillum reveals its function: they are involved in sensing environmental cues coming from other cells or from outside the human body. The signals are received by ciliary receptors and converted into an intracellular signal, and subsequently transduced to the cell body. Because of their function in several developmental signaling pathways, cilia play a pivotal role in vertebrate development. Examples of cilia-dependent pathways are platelet-derived growth factor signaling (e.g. involved in chemotactic-directed migration of fibroblasts),\(^{18}\) Notch signaling (cell proliferation and differentiation),\(^{19}\) Hippo pathway (regulating cell proliferation, survival, and differentiation)\(^{20}\) and Wnt signaling (modulating the cytoskeleton). The latter can be divided into two branches, a canonical β-catenin dependent branch and non-canonical branch or planar-cell polarity (PCP) pathway. The canonical pathway has important roles in the formation of neuronal circuits during development and involves stabilization followed by nuclear localization of β-catenin, resulting in transcription of target genes.\(^{21}\) The cillum is believed to dampen the canonical Wnt pathway by a spatial regulation of Jouberin (AHI1), which normally facilitates the translocation of β-catenin to the nucleus.\(^{22}\) When Jouberin is retained in the ciliary compartment, it impairs the nuclear localization of β-catenin, thereby modulating the pathway activity. The PCP pathway is linked to left-right patterning\(^{23}\) and neural tube closure\(^{24}\) and results in changes in cell morphology (actin dynamics, cell polarity, and cell shape) rather than transcription.\(^{23}\) In the last years, studies of Wnt-signaling in (defective) cilia have shown contradicting results and therefore the exact relationship with the cillum remains under debate.\(^{25}\)

The best described signaling pathway in cilia is the Sonic Hedgehog (Shh) signaling cascade (see Box 1). Defects in the Shh signaling pathway can cause various birth defects, including incomplete separation of the brain hemispheres (holoprosencephaly), polydactyly and craniofacial and skeletal malformations. In studies of mutant animal models with these phenotypes, variants in Shh genes, but also genes encoding IFT machinery have been identified.\(^{26}\) Furthermore, the link with the cillum became clear when several key players for Shh were localized to the cillum.\(^{27}\) In the case of correct neural tube development, a ventrodorsal gradient of Shh protein is essential for a correct spatial differentiation of specific subtypes of neurons.\(^{28}\) The different concentrations of Shh result in different levels of Shh

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(\(^{1}\)Legend Figure 1) also function as tracks for IFT trains that control bidirectional ciliary transport. IFTs dock at the base of the cillum on transition fibers (TF), which firmly attach the BB to the plasma membrane. Cargo vesicles loaded with ciliary signaling receptors are transported with the help of Arf/Rab GTPases (stars), the exocyst and/or the BBSome along microtubule (MT) tracks towards the base of the cillum, where they are endocytosed in a membrane invagination that is known as the cillum pocket. Some ciliary vesicles originate from the trans-Golgi network. Another route of transport is via lateral diffusion after exocytosis (right side). For transport of cytosolic proteins, the presence of a high RanGTP concentration in the cillum (dark gradient) promotes the release of actively transported large cytosolic proteins (dark shaded) from importins (light shaded squares) which act as transport chaperones. Source: the electron microscopy image of cilia in rat tracheal epithelium is obtained from: Gilula and Satir. The ciliary necklace. A ciliary membrane specialization. J Cell Biol 1972;53:494-509. Adapted from: Oud et al. Ciliopathies: Genetics in Pediatric Medicine. J Pediatr Genet 2017;6:18-29.
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pathway activity, and thereby controls (either activating or repressing, depending on the level) the expression pattern of sets of target genes. This mechanism causes specific patterning of progenitor cells during development, which is essential for the correct formation of the central nervous system, as well as the limbs. Because of the differential expression of ciliary signaling proteins in tissues and cellular subtypes, variants can cause specific disturbances of signaling pathways in a subset of cells and thereby lead to a spectrum of phenotypes.

Box 1: The ‘canonical’ ciliary signaling pathway: Sonic Hedgehog (Shh)

One well described signaling pathway related to cilia, is Shh signaling (see figure below). In this pathway, when no Shh ligand is present, the Patched-1 (PTCH1) receptor represses Smoothened (SMO) from activating Gli2 and Gli3 transcription factors and entry of SMO into the ciliary membrane. Furthermore, the Gli transcription factors are bound by SUFU. When Shh ligand is present, PTCH1 is activated and will release SMO, allowing its ciliary entry and translocation to the tip. This will activate and release the Gli transcription factors from SUFU, allowing them to translocate from the cilium to the nucleus and activate downstream target genes important for neuronal and skeletal development. These Gli transcription factors are bi-functional and can also actively repress transcription. When the pathway is not activated, Gli2 and Gli3 are phosphorylated by PKA, CK1 and GSK3β, causing proteolytic cleavage which generates repressor forms (Gli2R and Gli3R). PKA is thought to be activated by increased cAMP levels generated by GPR161. Activation of the Shh pathway promotes the exit of GPR161 from the cilium, and thereby stops Gli2/3 phosphorylation and generates high concentrations of activating (uncleaved) transcription factors. The precise balance of activator and repressor forms of the Gli transcription factors determines the gene expression pattern of the cell and thereby its differentiation into a specific progenitor subtype.
Since cilia are specialized in the perception of extracellular signals, they are also involved in our senses. Specialized cilia are present in our eyes, ears and nose to transduce the specific type of signal received. Olfactory cilia in our nose detect odorants via specific receptors on the ciliary membrane,9 mecano-sensing cilia in the inner ear bend due to vibrations of the air and convert this in neuronal signals,8 and there are highly specialized sensory cilia in the retina of the eye, which form the outer segments of the photoreceptor cells responsible for receiving and transducing light stimuli.29 An essential part of these photoreceptor cells is the ‘connecting cilium’ that bridges the inner and outer segments of the cell to allow continuous transport of proteins that is necessary to support the phototransduction cascade in the outer segments.29 This specialized role of the cilium in the retina will be discussed further in section 1.1.6.

1.1.3 Ciliogenesis
Ciliogenesis or cilium biogenesis is cell cycle-dependent and only takes place in non-cycling or quiescent cells at the G0/G1 stage of the cell cycle. During ciliogenesis, the ciliary axoneme extends from the mother centriole, which is the oldest of the two centrioles of the centrosome inherited during mitosis.31 Normally, centrosomes are involved in organizing the rapid deployment of the microtubule-based spindle assembly required for separating the chromosomes of the cell during mitosis. This implicates that a cell is only able to form a cilium when the centrioles are not involved in cell division. The conserved process of ciliogenesis comprises the following stages. First the mother centriole will dock to a ciliary vesicle and this docking is facilitated by the conversion of a mother centriole to a basal body, because distal and subdistal centriolar appendages transform into transition fibers and basal feet, which act as physical attachments to the vesicular membrane (Figure 1).12 These elements are also required to establish the orientation of the basal body at the plasma membrane.32 After a basal body-ciliary vesicle is formed, this structure will migrate to the plasma membrane, which seems to be dependent on the actin cytoskeleton and two ciliopathy proteins: MKS1 and MKS3 (TMEM67).33, 34 During this migration the axoneme starts to elongate, and invaginates one side of the ciliary vesicle, which creates the ciliary membrane and accompanying TZ. Many TZ proteins have been found to be important for these first steps of ciliogenesis, since disruption of one or more TZ proteins results in defects in cilantrose migration or membrane docking and attachment.35 After the basal body-ciliary vesicle is firmly docked and the ciliary vesicle has fused with the plasma membrane, the final stage of ciliogenesis is that the axoneme is elongating further from the TZ compartment, which is facilitated by the IFT machinery.1

1.1.4 The transition zone
Due to the lack of protein synthesis within the cilium and the requirement of a highly specialized protein composition for signaling, the TZ of the cilium is essential for its maintenance. Several protein complexes have been identified as components of the TZ or have been shown to be essential for TZ functionality, and have been associated to a spectrum of ciliopathies; Meckel-Gruber syndrome (MKS), JBTS, NPHP, Senior-Løken syndrome (SLSN)
Chapter 1.1

and Leber congenital amaurosis (LCA) (See: Chapter 1.2). As mentioned above, some MKS proteins (MKS1 and MKS3) are involved in the first stages of ciliogenesis, however, others are also involved in the barrier function of the TZ. Genetic experiments in C. elegans and proteomic studies in ciliated cells have identified a protein module composed of ~12 proteins (MKS1, TMEM17, TMEM67, TMEM216, TMEM231, AHI1, CC2D2A, TCTN1, TCTN2, TCTN3, B9D1 and B9D2). Because of the identification of variants in these genes in patients with MKS and JBTS, the module was indicated as the MKS/JBTS module. Another identified TZ module is the NPHE protein module, composed of genes that have previously been found mutated in patients with nephronophthisis (NPHP), a ciliopathy sub(phenotype)type characterized by interstitial fibrosis, medullary basement membrane disruption and cysts in the kidneys. This module is composed of ~9 proteins (NPHP1, NPHP4, NPHP8, NPHP3, INVS, NEK8, ATXN10, NPHP5 and CEP290). CEP290 is an intriguing protein as variants have been associated with a wide spectrum of ciliopathies from LCA (non-syndromic blindness), BBS and JBTS to MKS (lethal ciliopathy). It was initially identified as a large centrosomal protein of 290kDa, hence its name, and it is responsible for tethering of the ciliary membrane to axonemal microtubules at the TZ. High resolution imaging in cultured Retinal Pigment Epithelium-1 (RPE1) cells recently revealed its localization just below the Y-links. Combined with the numerous interactions found with other ciliary or ciliopathy proteins like NPHP5 (IQCB1) and the BBSome, PC1-11 and CC2D2A, it is suggested that CEP290 has a scaffolding function for many other TZ components. Indeed, a recent extensive study in C. elegans shows that CEP290 is a central assembly factor during ciliogenesis, specifically for the MKS/JBTS module components. Furthermore, it was shown that this process depends upon RPGRIP1L (MKS5) for proper TZ localization of all protein modules, including CEP290 itself.

Other groups of proteins involved in ciliary compartmentalization are septins and nucleoporins. Septins are a group of GTPases (see: 1.1.5), which together form large-order structures like rings. Studies have shown that septins specifically retain transmembrane proteins within the ciliary membrane, without obstructing the movement of these proteins along the entire axoneme. Interestingly, the ciliary localization of septins is shown to be species, tissue and cell type specific. However, the functionality of this differentiation awaits further explanations. Restriction of entry of large soluble proteins (>50-70 kDa) into the cilia is thought to be regulated by nucleoporins (NUPs), originally identified as members of the nuclear pore complex. Several NUPs have been localized to the base of the cillum or have been identified in ciliary proteome studies, and it has been proposed that active transport into the cilia of large soluble proteins is facilitated by a ciliary pore complex. In an elegant assay, Takao and others showed that dimerization of NUP62, a component of the central channel of the nuclear pore complex, resulted in reduced entry of large cytosolic proteins into the cilium, but not of transmembrane proteins or small cytosolic proteins. Another functional similarity with the nuclear pore complex is the Ran-GTP (GTPase Ras-like Nuclear protein (Ran) bound by GTP) gradient as high Ran-GTP concentrations are mainly present in the nucleus but also in the ciliun, and RanGDP mainly in the cytoplasm. The presence of RanGTP in the cilia promotes the release of actively transported large cytosolic proteins
from importins like the kinesin-2 motor KIF17,\textsuperscript{55} but also Shh transcription factor Gli2.\textsuperscript{56} Thus active maintenance of high RanGTP levels within the cilium drives directionality of transport of large cytosolic proteins.\textsuperscript{47}

1.1.5 Vesicle transport towards the cilium

Several (complexes of) proteins are involved in transport of vesicles towards the base of the cilium (Figure 1). An important family of proteins that drives several branches of ciliary transport are small GTPases and their regulators. Small GTPases function as molecular switches, activated by guanine nucleotide exchange factors (GEFs) that facilitate the binding of GTP. These small GTPases are inactivated by GTPase activating proteins (GAPs), which catalyze the conversion of GTP into GDP and result in an inactive GDP-bound state.\textsuperscript{57} GTP binding causes a conformational change allowing interacting proteins to be able to discriminate between the active or inactive state.\textsuperscript{58} Over the past years several studies have shown that vesicle trafficking coordinated by various small GTPases is essential for ciliary function, and specifically for ciliogenesis.\textsuperscript{59} One of the central players is Rab8, a small GTPase from the Rab family of proteins (Ras-related proteins in brain) that is associated with the ciliary membrane. It is a major component of a mechanism that drives cilia formation; when this protein is constitutively active in the GTP-bound form, elongation of cilia occurs.\textsuperscript{60} Rab11a activates Rab8 trough the GEF Rabin8 and this series of interactions is known as the Rab11-Rabin8-Rab8 ciliogenesis cascade. The Rab8 GTPase plays a crucial role in this cascade and directly interacts with ciliary cargo and proteins, like Polycystin-1,\textsuperscript{61} CEP290,\textsuperscript{42} and the large protein complex consisting of proteins involved in Bardet-Biedl syndrome, the BBSome.\textsuperscript{60} In rod photoreceptor cells, Rab8 directly interacts with rhodopsin.\textsuperscript{62} Another family of GTPase proteins involved in ciliary trafficking consists of the ADP ribosylation factor (Arf) proteins, which cooperate with the Rab proteins. For example, the small GTPase Arf4, its GAP ASAP1, Rab11 and their shared GTPase interacting effector RAB11FIP3, form the ciliary targeting complex which is involved in rhodopsin trafficking from the trans-Golgi network (TGN) towards the connecting cilium in photoreceptor cells, a process that is crucial for vision (explained below).\textsuperscript{63} When Arf4 is activated at the TGN, it binds its cargo, rhodopsin, and ASAP1. This large protein is multi-functional as it is a GAP and effector for Arf4, and it provides a scaffolding function to promote communication between Arf4, RAB11FIP3 and Rab11a.\textsuperscript{64} The interaction with Rab11a initiates the recruitment of Rabin8 and Rab8, which ultimately directs cargo from the TGN to the cilium via this signaling cascade.

Another important protein complex involved in ciliary trafficking is the ‘BBSome’, a well-studied complex of proteins encoded by genes that are mutated in Bardet-Biedl syndrome (BBS) patients.\textsuperscript{65,66} Chapter 3 of this thesis describes the clinical and genetic characterization of five Pakistani families with classic BBS or a BBS-like syndrome. The BBSome coordinates ciliary entry and exit of vesicles with transmembrane proteins. The complex is formed by seven BBS subunits and its assembly occurs in several stages. First an assembly intermediate is formed of three BBSome proteins (BBS2, BBS7 and BBS9), the BBSome core,\textsuperscript{67} with the help of other chaperonin-like BBS proteins (BBS6, BBS10 and BBS12).\textsuperscript{68} Then the remaining four
Chapter 1.1

peripheral subunits (BBS1, BBS4, BBS5 and BBS8) are incorporated to form the holo-complex.\textsuperscript{67} Only one subunit, BBS5, is believed to have direct contact with the ciliary membrane.\textsuperscript{60} BBS3/ARL6, an Arf-like GTPase, is a major effector of the BBSome, and recruits the BBSome to the membrane where it sorts membrane cargos to cilia.\textsuperscript{65} In order to gain access to the ciliary compartment and deliver the cargo, the BBSome needs to cross the TZ. It is believed that NPHP5 and CEP290 are mayor players in this process,\textsuperscript{41} however, the exact mechanism is still under investigation.

Another protein complex involved in ciliary trafficking is the exocyst complex. This octomeric protein complex was first described in \textit{S. cerevisiae} and linked to many cellular processes involving polarized exocytosis to deliver cargo or membrane, and specifically involved in the tethering step of exocytosis.\textsuperscript{66} The first relationship with the cilium was established in 2004 by Rogers and colleagues, when they localized the exocyst complex at the cilium in Madin-Darby canine kidney (MDCK) cells.\textsuperscript{70} This ignited multiple studies, which provided the first clues about a ciliary function for the exocyst complex. Knockdown of Exoc5 disturbed ciliogenesis as well as normal cyst morphogenesis in cultured MDCK cells.\textsuperscript{71} Knockdown in zebrafish caused a polycystic kidney disease (PKD, a ciliopathy, see: 1.2.2) phenocopy.\textsuperscript{72} Work in frog retinas showed that the exocyst complex is involved in delivery of rhodopsin to the outer segments of rod photoreceptor cells, which can be considered specialized cilia (see: 1.1.6).\textsuperscript{73} Rhodopsin is the critical G-protein coupled receptor (GPCR) that activates the phototransduction cascade upon reception of a light photon. Furthermore, interactions with other ciliary transport regulators like Cdc42 (with Exoc5)\textsuperscript{74} and Rab8 with its regulator Rabin8 (with Exoc6) are required for ciliogenesis.\textsuperscript{75} \textit{EXOC8} was identified as a Joubert syndrome candidate gene,\textsuperscript{76} while shortly thereafter, \textit{EXOC4} was found mutated in a patient with Meckel-Gruber syndrome.\textsuperscript{77} These studies confirm the importance of the exocyst complex for proper cilium function, however, the complete mechanism of cargo delivery at the base of the cilium remains to be studied more extensively. In chapter 4 of this thesis, I studied the interaction network of the exocyst to further unravel the ciliary involvement of this protein complex.

1.1.6 A specialized cilium in photoreceptor cells

As mentioned above, the retina contains photoreceptor cells with highly specialized cilia. The retina is the most inner layer of the vertebrate eye, and responsible for the transduction of light stimuli into neuronal impulses, which are transferred to the brain where they are collated into a perceived image. The retina can be divided into two layers, the neurosensory layer and outer pigmented layer. The pigmented layer is a single cell layer composed of retinal pigment epithelium (RPE) cells, directly lining the apex of the photoreceptor cells of the neurosensory layer, which contains several cell types such as bipolar cells, ganglion cells, amacrine cells, horizontal cells, and Müller glial cells.\textsuperscript{78} Photoreceptor cells, either rods or cones, are the most abundant cells of the neurosensory layer and positioned parallel to one another. Rod photoreceptor cells are responsible for highly sensitive light transduction at low light intensities, and in humans present at the periphery of the retina. In the center of the human retina, the ‘macula lutea’ or macula region, cones are more concentrated and
responsible for color vision. This process relies on various types of opsins with different specific spectral sensitivity. Although 100-fold less sensitive then rods, cones facilitate high visual resolution, especially in the fovea centralis, almost solely occupied by cone photoreceptors.

Both rod and cone photoreceptor cell types are highly specialized and polarized neurons with distinct cellular compartments. The outer segments (OS) are stacked with hundreds of membrane disks where the actual phototransduction cascade takes place. The inner segment (IS) contains all cell organelles responsible for all biosynthesis and metabolism processes of the cell. Adjacent to the IS lies the cell body with the nucleus followed by the synaptic terminal, where the generated electrical membrane potential is transmitted to bipolar cells. The IS and OS are connected via a narrow bridge, the so-called connecting cilium (CC). The CC corresponds to the TZ of a primary cilium and the entire OS, CC, and basal body can be considered a highly specialized primary sensory cilium. The CC is critical for retinal function, as it connects the biosynthetic IS to the phototransductive OS. As photoreceptor cells are terminally differentiated, they had to adopt a mechanism to renew their membranous opsin-filled disks, as these continuously suffer from light-induced oxidative damage during the day. This occurs through a continuous light-dependent biosynthesis and stacking of new discs at the OS base, and shedding of old discs at the OS tip, which are subsequently phagocytosed by the adjacent RPE cells. Approximately 10% of the outer discs of rods are recycled in this way every night in humans. This disc recycling process demands optimal functionality of the IS and the CC to be able to provide enough material to maintain the OS, and thereby vision. Several ciliary protein complexes as discussed in earlier sections are located to the CC, and have been shown to be essential for normal photoreceptor function. In chapter 2 we found that disruption of basal body protein POC1B resulted in cone-rod dystrophy.

Similar to a primary cilium, the core of the CC is composed of nine microtubule doublets, which originate from a basal body and is wrapped by a (ciliary) membrane. The OS is an exceptionally large cilium and therefore firmly anchored with a ciliary rootlet in the IS. This rootlet is mainly composed of the large protein rootletin,79 and linked to the centrioles of the basal body by CEP250.29 Like the TZ of the primary cilium, the CC also contains Y-shaped linkers that connect the axoneme with the membrane. Up till now, only CEP290 has been specifically located in this structure.80 Other examples of ciliary proteins and complexes at the CC are the NPHP proteins, Rab GTPases, the IFT machinery and the BBSome.63

Due to the specialization of the photoreceptor cilium, it also contains specialized proteins and complexes that are not present in the ‘general’ primary cilium. A nice example of this is a specific splice variant of RPGR, which includes exon 15, that is only expressed in photoreceptor cells.81 RPGR localizes to the base of the CC in a network with ciliopathy proteins RGRIP1 and NPHP4, and it is believed that RPGR regulates entry or retention of specific sets of proteins in photoreceptor cilia.82 The specific splice variant of RPGR has been shown to interact with whirlin, which is a scaffold protein connected to the USH protein
network. The latter group of proteins is specifically important for normal functioning of photoreceptor cells and ciliated sensory cells in the inner ear. Variants in genes encoding USH proteins can cause hereditary combined deaf-blindness, Usher (USH) syndrome. In the abovementioned sensory tissues, the USH proteins are integrated into membrane adhesion complexes. The proteins regulate ciliary transport and thereby contribute to correct ciliary functioning. In photoreceptor cells specifically, USH proteins are localized to calyceal processes which are actin based microvillus-like extensions from the IS, which surround the basal fifth portion of the OS. The function of calyceal processes is, however, far from understood. Their architecture and spatial arrangements strongly suggest they serve the structural stability of the CC and OS. The USH proteins are thought to form a protein complex required for the adhesion between the microvilli membrane and the OS membrane, thereby involved in the suspected supporting role of the calyceal processes. Another function of the USH proteins in the photoreceptor cells is the regulation of vesicle transport. At the base of the CC surrounding the basal bodies, in the periciliary region (similar to the ciliary pocket of a conventional cilium), USH proteins bridge the CC membrane with the IS membrane and are linked to transport machinery proteins. It is thought that cargo vesicles are targeted to this region of the CC membrane by the USH membrane adhesion complex, which facilitates transport of transmembrane proteins across the CC.

1.1.7 Protein-protein interaction studies
One approach to gain insights into the composition and function of the cilium, including the consequences of variants, is studying protein-protein interactions. In recent years, the techniques have evolved and several comprehensive protein-protein interaction studies have been performed on ciliated tissues or cells. In 2011, Sang and colleagues reported a study in which they performed repetitive tandem affinity purifications followed by mass spectrometry (see box 2) for nine ciliopathy proteins related to NPHP, JBTS and MKS. Since variants in some of these genes can cause all of these phenotypes, they hypothesized that these disorders may link to defects in a specific set of cellular mechanisms and that the proteins may interact and function in common pathways. They generated three different cell lines (fibroblasts, kidney cells and RPE cells) stably expressing one of the nine NPHP/JBTS/MKS proteins of interest as baits. Subsequently, they isolated the protein complexes associated with each overexpressed bait protein. Bioinformatic analysis of the identified proteins revealed that all of these proteins are connected in three distinct modules: NPHP1-4-8, NPHP5-6 and a MKS module. These modules were confirmed by using co-immunoprecipitation and in vitro binding assays. Further analysis revealed that the NPHP1-4-8 module was functioning at cell-cell contacts as well as the TZ, NPHP5-6 functions at the centrosomes and the MKS module was linked to Hedgehog signaling. Interestingly, after generating a list of the most common baits identified in their screens and sequencing these candidates in relevant JBTS and NPHP patient cohorts, they identified two new disease-associated genes: ATXN10 and TCTN2. Studying the Tctn2 mouse knockout showed that this gene is involved in neural tube development and Hedgehog signaling. In another study a similar approach was equally successful in identifying new
ciliopathy associated genes, furthermore, it provided insight into the function of the (up till then) poorly understood ciliopathy protein Jbts17 (C5orf42).\textsuperscript{51} In this study protein interaction studies, \textit{in vivo} imaging and genetic analysis of proteins linked to PCP (Inturned, Fuzzy and Wdpcp) identified a new module, called CPLANE (ciliogenesis and planar polarity effector). Linked to the CPLANE module was ciliopathy protein Jbts17, and together these proteins were shown to be involved in recruiting a subset of IFT-A proteins to the basal body of the cilium. Subsequent analysis of the CPLANE genes in ciliopathy patients identified disease-causing mutations in \textit{WDPCP} and \textit{INTU}. The approach used in these studies is an excellent showcase of how knowledge obtained from protein-protein interaction studies can be used in gene identification studies. In \textbf{chapters 2, 4 and 5}, I used protein-protein interaction studies to unravel the function of disease genes and study the effect of the sequence variants.

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\textbf{Box 2. Techniques to study protein-protein interactions} \\
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Two techniques used in this thesis to study protein-protein interaction networks are Strep/Flag Tandem Affinity Purifications (SF-TAP)\textsuperscript{88} and the GAL4-based Yeast Two-Hybrid (Y2H) system.\textsuperscript{89} While SF-TAP isolates protein complexes and cannot distinguish between direct and indirect interactions, Y2H is applied to specifically study direct protein-protein interactions. Both technologies are illustrated below.

\textit{SF-TAP} \\
To conduct SF-TAP, a protein of interest, or bait, is cloned with a SF-tag of only 4.6 kDa containing a combination of a tandem Strep-tag II and a FLAG-tag. The tagged bait protein is overexpressed in mammalian cells, e.g. human embryonic kidney 293T (HEK293T) cells. The cells are lysed and subsequently subjected to two rounds of affinity purifications. In the first round (illustration I), the bait protein (red sphere) will be bound by Strep-Tactin resin beads via two Strep epitopes in the tag, and unbound proteins (light blue spheres) are washed away. The bound protein complexes (yellow spheres) are eluted from the resin and a second round of affinity purification is applied using the FLAG-tag. In this round (illustration II), the bait protein is bound via the FLAG epitope to anti-FLAG agarose and again unbound proteins are washed away. The protein complexes are eluted and then subjected to mass spectrometry in order to identify the affinity purified proteins.

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I & II \\
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\textbf{Strep purification} & \textbf{FLAG purification} \\
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\textit{SF-TAP principles.} Adapted from https://www.helmholtz-muenchen.de/proteinsience/research/proteomics-technology-development/interaction-proteomics/

\textit{(Box 2 continued on next page)
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**Box 2 continued. Techniques to study protein-protein interactions**

**GAL4-based Y2H**

Modified baker’s yeast (*Saccharomyces cerevisiae*) is used as host to test for binary protein interactions based on a transcription factor complementation assay (illustration III). The coding sequence of the bait protein is cloned with the transcription factor GAL4 DNA binding domain (BD) into one plasmid, and the coding sequence of a prey protein with the activating domain (AD) of the transcription factor is cloned into another plasmid. Both plasmids are co-transformed into yeast. When bait and prey proteins interact (curved arrow) they come into close proximity in the nucleus of the yeast cell, which unites the BD with the AD, promoting the general transcription machinery (G) to transcribe a subset of reporter genes, allowing the yeast cell to grow on selective media, or inducing α- or β-galactosidase activity resulting in green/blue coloration in presence of a substrate. This system can be applied to study the binding of a specific bait-prey combination, or screen large cDNA libraries (illustration IV) fused to AD, with your bait of interest fused to BD. For library screening, yeast cells containing either an AD or BD plasmid are mated together to form diploid clones harboring both plasmids. If the yeast cell survives subsequent selection because of transcription of the reporter genes, PCR and sequencing of the AD-fusion plasmid will identify the coding sequence of the interacting protein. In this way, direct protein interactions in an array of specific tissues can be tested.

**Image 2 continued. Techniques to study protein-protein interactions**

**Y2H principles.** Adapted from Giorgini and Muchowski, Connecting the dots in Huntington’s disease with protein interaction networks, Genome Biol. 6(3): 210, Copyright (2005), with permission from BioMed Central Ltd.

Another technique, based on proximity dependent biotinylation of proteins, has been used to investigate the protein composition of the cilium to gain more insight in its function as well as to assess the consequences of variants found in ciliopathy-associated genes. For example, this method was used to determine the protein composition surrounding various centrosomal and TZ proteins to study the centrosome-cilium interface. The ciliary protein NPHP3 has also been used as bait to specifically identify proteins within the entire ciliary compartment. The latter experiment identified several new proteins that are involved in ciliogenesis (e.g. CEP128, WRAP73) and revealed a key player of the Shh signaling cascade (the GLI2/3-phosphorylating enzyme PKA, see Box 1 in section 1.1.2) to reside inside the ciliary compartment, which was previously not detected there.
Chapter 1.2

Ciliopathies

This section is adapted from:
Ciliopathies: Genetics in Pediatric Medicine

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Ciliopathies are disorders that are caused by defects in motile or immotile cilia. As cilia occur on virtually all cells of the human body, their dysfunction can lead to a broad spectrum of features. Common features of ciliopathies caused by dysfunctional primary cilia are blindness, renal cystic disease, obesity, neural tube defects, intellectual disability (ID), ectodermal defects, and skeletal abnormalities ranging from polydactyly to abnormally short ribs and limbs. These features can occur isolated or may be part of a recognizable syndrome. Here, we describe the different types of ciliopathies, their characteristics, and to what extent they genetically and phenotypically overlap.

1.2.1 Retinal ciliopathies.
Retinal dystrophies, either syndromic or non-syndromic, can be caused by variants in more than 250 genes (https://sph.uth.edu/Retnet) and a large proportion of these genes encode ciliary proteins. This section focuses on non-syndromic retinal dystrophies. The clinical presentation can vary depending on the subset of photoreceptors that are primarily affected by the disease. The most common non-syndromic retinal ciliopathies include retinitis pigmentosa (RP), cone and cone-rod dystrophy (COD/CRD), and Leber congenital amaurosis (LCA). The most common ciliary genes mutated in non-syndromic retinal ciliopathies are listed in table 1. As mentioned previously, retinal dystrophy can also occur as part of a syndromic ciliopathy such as Bardet-Biedl syndrome (BBS, 1.2.3), Joubert syndrome (JBTS, 1.2.4) or Senior-Løken syndrome (SLSN). In the latter syndrome patients present with blindness and nephronophthisis (NPHP, 1.2.2).

RP belonging to the rod-cone dystrophies (RCDs), is the most common inherited retinal degeneration and affects up to 1 in 4,000 individuals. Examination of the fundus shows dark pigmented clumps, hence its name. It is characterized by initial loss of rod photoreceptor cells at 20 years of age (or earlier in recessive and X-linked subtypes) and causes night blindness followed by a slow progression into tunnel vision and decreased central vision. Cone photoreceptors become affected in advanced stages of the disease, which eventually results in blindness. Other ocular symptoms include cataracts, myopia, astigmatism and keratoconus. Subtypes of RP are characterized by its inheritance pattern: autosomal dominant (adRP), autosomal recessive (arRP) or X-linked (XLRP). In OMIM, over 78 genes have been associated with RP and it is estimated that almost 40% represent ciliary genes. Most genes encode proteins that are thought to be structural components of the connecting cilium (CC), or responsible for the trafficking processes traversing the CC. However, our understanding of the molecular pathways is limited. Variants in numerous arRP-associated genes may also cause syndromic ciliopathies, depending on variant type and location. In BBS8, a variant affecting an exon specifically expressed in photoreceptors causes a frameshift and absence of the BBS8 protein in these cells, but not in other tissues, resulting in non-syndromic arRP, while variants in other gene regions have been associated with BBS.

COD and CRD are less common than RP and have a prevalence of ~1:40,000. In COD, solely cone function is lost progressively, causing disturbed color vision and associated visual loss in
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the first or second decade of life. When the initial loss of cones is closely followed by the deterioration of rods, the disorder is referred to as CRD. CRD patients present with photophobia, decreased visual acuity, color vision defects and decreased sensitivity of the central visual field. When rods become affected, patients may present with night blindness and loss of peripheral vision.\textsuperscript{29} Generally, the clinical course of CRD is more severe and rapid than RCDs, and lead to earlier onset legal blindness.\textsuperscript{95} Full field electroretinogram (ERG) recordings, which measure electrical potentials of the entire retina upon specific light stimuli, can distinguish potentials generated by rods or cones and thereby allow discrimination between RP, COD and CRD.\textsuperscript{96} Only a small proportion (\textasciitilde4\% ) of the genes associated with COD/CRD encode ciliary proteins.\textsuperscript{92} These proteins often localize and function at the base of the cilium.\textsuperscript{29} Variants in \textit{RPGR} have been identified in both patients with COD and CRD, emphasizing the considerable overlap between the two diseases.\textsuperscript{97}

Leber congenital amaurosis (LCA) is the most severe retinal dystrophy causing blindness before the age of 1 year, and is characterized by severe retinal degeneration combined with pendular nystagmus. The prevalence ranges from 1:30,000 to 1:81,000.\textsuperscript{98,99} Patients usually present at birth with profound vision loss, nystagmus and severe retinal dysfunction. Characteristic for LCA is the ‘Franceschetti’s oculo-digital sign’,\textsuperscript{100} a specific behavior where patients are pressing, rubbing and poking their eyes with a knuckle or finger. Additional ocular symptoms are photophobia and hyperopia. LCA is mostly inherited in an autosomal recessive manner and 23 different genes have been associated with this disease.\textsuperscript{101} Variants in \textit{CEP290} are the most common cause of LCA, accounting for approximately 20\% of the LCA cases in Western countries. \textit{CEP290} encodes a ciliary protein also implicated in JBS, MKS, BBS and SLSN. Other ciliary genes involved in LCA are \textit{IQCB1}, \textit{LCA5}, \textit{RPGRIP1} and \textit{TULP1}.\textsuperscript{92}

The list of (ciliary) genes involved in these retinopathies will likely expand in the future (Table 1) as a significant percentage of clinical cases remain to be genetically explained. In LCA 20-50\% of cases remains unsolved,\textsuperscript{102-104} in RP 40\%\textsuperscript{104} and in COD/CRD the portion of unexplained cases is the highest with a range of 50-80\%.\textsuperscript{92,105}

1.2.2 Renal ciliopathies

Polycystic kidneys are commonly seen in ciliopathies. The best known renal ciliopathies are nephronophthisis (NPHP), and autosomal dominant and autosomal recessive polycystic kidney disease (ADPKD and ARPKD, respectively). ADPKD is most common with an incidence of \textasciitilde1:1,000, and is characterized by enlarged polycystic kidneys.\textsuperscript{106} Liver cysts and abnormalities of the vasculature and heart may also be present.\textsuperscript{106} \textit{PKD1} and \textit{PKD2} variants explain 85\% and 15\% of ADPKD patients, respectively. Most individuals with ADPKD have a normal renal function during childhood, however, renal insufficiency progresses during life and results in end-stage renal disease (ESRD) in 50\% of affected individuals.\textsuperscript{106} In contrast, ARPKD is usually diagnosed in late pregnancy or at birth by the detection of enlarged cystic kidneys by ultrasound. Grossly enlarged kidneys cause pulmonary hypoplasia and thoracic compression leading to respiratory distress and death in \textasciitilde30-50\% of affected neonates. In the
The majority of remaining patients ESRD evolves in adulthood. In addition, virtually all ARPKD patients show hepatic involvement, i.e. hepatomegaly and liver fibrosis.\(^{107}\) To date, only variants in PKHD1 have been reported and these explain at least 80-87% of ARPKD patients.\(^{108}\) PKHD1 encodes the fibrocystin protein that forms a ciliary protein complex together with PKD1 and PKD2.\(^{109}\) Another monogenetic cause of ESRD in children is NPHP. Three types have been described based on age of onset of ESRD; infantile (<2 years), juvenile (~13 years), and adolescent (~19 years) NPHP.\(^{110}\) Juvenile NPHP is most common and affects 5-10% of the children with ESRD.\(^{111}\) It is estimated to occur in 1:50,000 births in Canada and 1:900,000 in the United States.\(^{111}\) Typical histopathological characteristics are renal cysts, tubular basement membrane disruption, and tubulointerstitial fibrosis. Most features are shared between the different NPHP types; however, tubular basement membrane disruption is usually absent in infantile NPHP. Also, kidneys of patients with infantile NPHP are usually enlarged, while normal-sized or smaller kidneys are seen in patients with other forms of NPHP (Figure 2).\(^{111}\) NPHP can both be isolated and syndromic. Twenty NPHP genes are known so far (Table 1) and explain roughly one-third of the patients. All NPHP proteins reside in the basal body, transition zone or axonemal base of primary cilia; however, they have also been found in other cellular sites such as cell junctions and nuclei.\(^{112;113}\) Variants in NPHP genes perturb a variety of signaling cascades including Wnt-, Hedgehog-, Hippo-, and DNA damage response signaling, which in turn disrupt renal development and tissue homeostasis.\(^{113}\)

### 1.2.3 Isolated and syndromic obesity

Nonsyndromic obesity is a growing problem in the general population and mainly caused by multifactorial factors. Yet, in some cases isolated obesity is a Mendelian disorder that results from ciliary anomalies.\(^{114}\) Previously, ciliary defects had already been described in obesity syndromes such as in Bardet-Biedl syndrome (BBS) and Alström Syndrome (ALMS). Both syndromes display an autosomal recessive inheritance; however, oligogenic inheritance has also been described in BBS.\(^{115;116}\) The prevalence of BBS varies markedly between populations; from 1:160,000 in northern Europe to 1:13,500 births in isolated communities in Kuwait.\(^{117}\) Cardinal BBS features are RCD, obesity, polydactyly, ID, hypogonadism, and renal dysfunction (Figure 2). Secondary features include cardiovascular abnormalities, craniofacial anomalies, psychomotor delay, type 2 diabetes and hearing loss. The clinical diagnosis of BBS is based on the presence of at least four primary features or a combination of three primary plus two secondary features. In general the BBS phenotype evolves during the first decade of life, except for polydactyly which is present at birth; generating an accurate clinical diagnosis may therefore be challenging in infancy and early childhood.\(^{3}\) BBS has an adverse prognosis, with renal impairment being a frequent and important cause of death.\(^{118}\) The phenotypically related ALMS is characterized by CRD, obesity, hearing impairment, cardiomyopathy, type 2 diabetes, hepatic and renal disease.\(^{119}\) A clinical diagnosis is based on the above mentioned cardinal features, and differs from BBS by the presence of hearing impairment and absence of polydactyly and learning difficulties.\(^{117}\) While it may be difficult to make a clinical diagnosis of BBS and ALMS in early childhood, molecular testing clearly distinguishes both disorders. To
date, only one gene, \textit{ALMS1}, is known to be mutated in ALMS (Table 1). In Bardet-Biedl syndrome variants in 19 genes are reported to be causative (Table 1), explaining over 80% of the patients.\textsuperscript{117} The protein encoded by \textit{ALMS1} and most of the proteins encoded by BBS-associated genes localize to the base of the primary cilium. ALMS1 is also found in endosomal structures and is suggested to play a role in the recycling endosome pathway.\textsuperscript{121} The BBSome, composed of BBS1-2, 4-5, 7-9 and BBIP10, plays an important role in trafficking proteins into and out of the cilium, while the chaperonin complex that consists of BBS6 and BBS10-12 is required for assembly of the BBSome.\textsuperscript{117; 122; 123} The remaining BBS proteins have variable functions. Some are thought to act as GTPases or E3 ubiquitin ligases, others have been shown to regulate centriole migration and IFT.\textsuperscript{117}

\textbf{1.2.4 Neurodevelopmental ciliopathies}

Joubert syndrome (JBTS) and Meckel-Gruber syndrome (MKS) are rare neurodevelopmental disorders that genetically and phenotypically overlap. JBTS was first described by Marie Joubert in 1969 and is diagnosed when patients have hypotonia, ataxia, developmental delay and distinctive cerebellar and brain stem malformations that can be recognized as a “molar tooth sign” (MTS) on MRI (Figure 2A).\textsuperscript{6; 124; 125} JBTS patients also often present with kidney cysts, polydactyly, coloboma, retinal dystrophy, respiratory abnormalities (episodic tachypnea or apnea), oral frenulae, and hepatic fibrosis.\textsuperscript{126} The health of JBTS patients largely depends on the occurrence and severity of liver and kidney disease that require appropriate management and treatment.\textsuperscript{124; 127} MKS shows marked clinical overlap with Joubert syndrome, but is more severe and perinatal lethal. This disorder is characterized by occipital encephalocele, polycystic kidneys, polydactyly, and hepatic fibrosis (Figure 2B-D).\textsuperscript{128} MKS is diagnosed when two of these four features are present or when one is present in combination with two other anomalies including cleft lip/palate, cardiac septal defects, holoprosencephaly, agenesis of corpus callosum, Dandy-Walker malformation, microphthalmia, \textit{situs inversus}, ambiguous genitalia, and shortening and bowing of long bones.\textsuperscript{129; 130} The incidence of JBTS ranges from 1:80,000 to 1:100,000 live births,\textsuperscript{127} while the prevalence of MKS is less frequent at 1:140,000.\textsuperscript{131} Exceptions exist as the occurrence of MKS in the Finnish population is as high as 1:9,000 births.\textsuperscript{132; 133}
Albers-Schönberg disease (ASD) is a well-defined clinical syndrome that consists of a spectrum of manifestations including conical peduncles, microbialia of the third and fourth ventricles, and cerebellar hypoplasia. It is caused by mutations in the ALX1 gene. Other less common features include short stature, cataracts, and aortic coarctation.

Figure 2: Phenotypic features of ciliopathies. The following features are commonly seen in ciliopathies. (A) Distinctive cerebellar and brain stem malformation that is known as the “molar tooth sign” in a JBTS patient. Open and filled arrowheads point out a deepened interpeduncular fossa and elongated superior cerebellar peduncles, respectively. (B-D) MKS fetus at the 14th week of gestation showing swollen stomach due to enlarged kidneys (B), occipital encephalocoele (C), and polydactyly of hands (B) and feet (D). (E) Radiograph of an SRTD fetus displaying a short and narrow thorax, horizontally oriented ribs, short tubular bones with smooth ends, short and ovoid tibiae, and postaxial polydactyly. (F) Patient with CED showing a narrow thorax, pectus excavatum, and rhizomelic shortening of limbs. (G) Narrow thorax in a JATD patient. (H, I) Images of an EVC patient with a long narrow chest and shortness of the limbs (H), and hypodontia, i.e. absence of upper and lower conical incisors (I). (J) BBS patient with truncal obesity, micropenis, and apathetic facial features. (K) Cystic kidneys of a patient with infantile NPHP. (L) Postaxial polydactyly of the right hand in a patient with OFDS. Reprinted from Brancati et al., Joubert Syndrome and related disorders, Orphanet J Rare Dis. 8:20, Copyright (2010), with permission from BioMed Central Ltd (A); Tallila et al., Identification of CC2D2A as a Meckel syndrome gene adds an important piece to the ciliopathy puzzle, Am J Hum Genet. 2008;82:1361-7, Copyright (2008) with permission from Elsevier (B-D); El-Hokayem et al., NEK1 and DYNC2H1 are both involved in short rib polydactyly Majewski type but not in Beemer Langer cases, J Med Genet. ;49:227-33, Copyright (2012) with permission from BMJ Publishing Group Ltd (E); Gilissen et al., Exome sequencing identifies WDR35 variants involved in Sensenbrenner syndrome, Am J Hum Genet. 10;87:418-23, Copyright (2010) with permission from Elsevier (F); Schmidts, Clinical genetics and pathobiology of ciliary chondrodysplasias, J Pediatr Genet. ;3:46-94, Copyright (2014) Thieme Publishers (G); Baudet and Le Merrer, Ellis-van Creveld syndrome, Orphanet J Rare Dis, 2, Copyright (2007) with permission from Central Ltd. (H, L); Sahin et al., Two brothers with Bardet-Biedl syndrome presenting with chronic renal failure, Case Rep Nephrol. 764973, Copyright (2015) Sahin et al. (I); Oud et al., Early Presentation of Cystic Kidneys in a Family with a Homozygous INVS Mutation, Am J Med Genet A. ;164A:1627-34, Copyright (2014) with permission from John Wiley and Sons Inc (J); Poretti et al., Delineation and diagnostic criteria of Oral-Facial-Digital Syndrome type VI, Orphanet J Rare Dis. 11;7, Copyright (2012) with permission from BioMed Central Ltd (K).
Both JBTS and MKS syndromes are usually autosomal recessive, but a few JBTS cases with X-linked inheritance have been reported.\textsuperscript{134} JBTS and MKS overlap genetically; 25% of the 25 known JBTS-associated genes have also been found mutated in MKS (Table 1).\textsuperscript{125; 135} It remains elusive why certain variants in a gene lead to JBTS, while other variants in the same gene cause MKS. In some cases the variant type may determine the phenotypic outcome. For example, missense variants in CC2D2A cause JBTS, whereas null alleles are associated with MKS.\textsuperscript{125} Modifier genes have also been proposed to play a role in phenotypic outcome and could explain intrafamilial phenotypic differences.\textsuperscript{125; 135} Genotype-phenotype correlations have been reported and can facilitate choices for specific genetic testing. For example, AHI1 variants often occur in JBTS patients with retinal dystrophy, while NPHP1 and RPRGIP1L variants are most frequent in JBTS with renal dysplasia. Moreover, in 50% of JBTS patients with both retinal and renal phenotypes CEP290 variants are detected.\textsuperscript{124; 127} When considering the protein localization it is evident that most JBTS and MKS proteins reside in the ciliary transition zone (Figure 1, chapter 1.1). When they are mutated the structure of the ciliary transition zone is perturbed causing disruption of its function as a ciliary gate thereby affecting a variety of signaling pathways, including Wnt/β-catenin and Shh signaling.\textsuperscript{125; 135}

### 1.2.5 Ciliopathies with major skeletal involvement

Many ciliopathies are characterized by skeletal abnormalities such as dwarfism, short limbs and ribs, and brachydactyly/polydactyly. Ciliopathies with major skeletal involvement can be divided into four groups: cranioectodermal dysplasia (CED), short-rib thoracic dysplasia (SRTD), Ellis-van Creveld syndrome (EVC), and oral-facial-digital syndrome (OFDS). CED is rare and autosomal recessive. Typical features are sagittal craniosynostosis, narrow thorax with pectus excavatum, rhizomelia, brachydactyly, and ectodermal dysplasia (Figure 2F).\textsuperscript{126} Vital organ dysfunction often occurs and causes death in ~20% of CED patients before the age of seven.\textsuperscript{136} SRTD is clinically and genetically related to CED. This family of disorders includes the following syndromes: short-rib polydactyly syndrome (SRPS), Jeune asphyxiating thoracic dysplasia (JATD) and Mainzer-Saldino syndrome (MZSDS). These disorders are autosomal recessive and frequencies range from 1:100,000 to 1:1,000,000.\textsuperscript{137} The most prominent SRTD features are limb shortening and a small rib cage. These features are most pronounced in perinatal lethal SRPS (Figure 2E). Additional SRPS features may include polydactyly, cleft lip/palate, and anomalies of a variety of organs and tissues including the brain, heart, eyes, kidneys, liver, pancreas, intestines, and genitalia.\textsuperscript{138} Patients with JATD present with similar, but milder skeletal anomalies compared to SRPS. The main complication in JATD patients is the narrow thorax phenotype that causes severe respiratory insufficiency; this phenotype causes lethality in 20-60% of JATD infants (Figure 2G). Besides skeletal abnormalities, JATD patients also occasionally present with NPHP, blindness, liver fibrosis, and ID.\textsuperscript{137} Similar as in CED, MZSDS is also featured by a relatively mild narrow thorax phenotype. Other features of MZSDS are shortened limbs, blindness, NPHP, liver fibrosis and pancreatic abnormalities.\textsuperscript{139} EVC is another ciliopathy that is featured by major skeletal anomalies. Besides dwarfism, polydactyly, and ectodermal dysplasia, congenital heart malformations are commonly seen in
EVC patients (Figure 2H,L). The latter have a major influence on the prognosis for individual patients.140 Finally, a variety of oral-facial-digital syndromes have been reported; however, many are based on single or few cases that are not molecularly tested.141 The best known OFD type is OFD1, which is X-linked and characterized by bifid tongue, oral frenula, cleft lip/palate, dental anomalies, syndactyly, polydactyly, polycystic kidney disease, and central nervous system abnormalities (Figure 2K).

Ciliary chondrodysplasias are genetically heterogenous. EVC is caused by variants in EVC and EVC2. EVC variants have also been reported in patients with autosomal dominant Weyers acrodental dysostosis, which presents with a milder phenotype than EVC.137 Genetic overlap is mostly seen between CED, JATD, SRPS, and MZSDS (Table 1), but also occurs between OFD and JBTS (Table 1).134;141-143 The majority of CED and SRTD variants have been found in genes encoding IFT and associated motor proteins (Table 1). OFDS is also genetically heterogeneous and recent data suggest that a proportion of OFDS proteins reside in centriolar appendages where they regulate ciliogenesis in an antagonistic manner; C2CD3 promotes ciliary growth, whereas OFD1 is a repressor.144;145

1.2.6 Primary Ciliary Dyskinesia

Most phenotypes associated by dysfunction of motile cilia result in primary ciliary dyskinesia (PCD). Cardinal symptoms are neonatal respiratory abnormalities and recurrent airway infections during childhood, which eventually develop into bronchiectasis. Patients also often present with situs inversus, dextrocardia, and infertility.146;147 Diagnosis of PCD is challenging due to the phenotypic overlap with other respiratory diseases such as asthma, immune deficiencies, and bronchomalacia, and the absence of clear diagnostic criteria further complicates making an accurate diagnosis.147 The prevalence of PCD, being 1:10,000 – 1:20,000, may therefore be and underestimate.147 Variants in 30 genes have been associated with PCD (Table 1) and explain 60-70% of the cases.147;148 The inheritance pattern associated with PCD is usually autosomal recessive; however, X-linked or autosomal dominant inheritance have also been reported in a few cases.147 Roughly 60% of proteins that are encoded by PCD genes are found in protein complexes and axonemal structures that are typically found in motile cilia such as inner and outer dynein arms, radial spokes, the central microtubule pair and nexin links (Figure 1, chapter 1.1), while the remaining proteins are cytoplasmic with a role in (pre)assembly and transportation of dynein components.148
### Table 1. Genes mutated in ciliopathies

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Chapter 1.2

(Legend of Table 1, see previous page) This overview shows the genetic and clinical heterogeneity that occurs in the ciliopathy spectrum. Corresponding OMIM identifiers are shown, or ‘NA’ when not available. Acrocallosal syndrome (ACLS), Alström syndrome (ALMS), autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD), Bardet-Biedl syndrome (BBS), cranioectodermal dysplasia syndrome (CED), cone-rod dystrophy (CRD), endocrine-cerebro-osteodysplasia syndrome (ECO), Ellis-van Creveld syndrome (EVC), Jeune asphyxiating thoracic dysplasia (JATD), Joubert syndrome (JBS), Leber congenital amaurosis (LCA), macular dystrophy (MD), McKusick-Kaufman syndrome (MCKS), Meckel-Gruber syndrome (MKS), morbid obesity and spermatogenic failure (MOSPF), nephronophthisis (NPHP), oral-facial-digital syndrome (OFDS), primary ciliary dyskinesia (PCD), retinal dystrophy (RD), retinitis pigmentosa (RP), Senior-Loken syndrome (SLSN), short-rib thoracic dysplasia (SRTD), Simpson-Golabi-Behmel syndrome (SGBS), Von Hippel-Lindau syndrome (VHL), Weyers acroental dysostosis (WAD).

1.2.7 Why is genetic screening important?

The phenotypic spectrum of ciliopathies as a whole is very broad, but phenotypic overlap between certain ciliopathies can be marked, such as in JBTS and MKS or BBS and ALMS. A correct diagnosis can thus not always be made solely based on the clinical phenotype but requires molecular testing. Molecular information facilitates making an accurate diagnosis and is therefore essential for proper genetic counseling, prognosis, recurrence risk estimation and clinical management. In the past years, gene panels and exomes have been analyzed by next-generation sequencing (NGS) technologies. This resulted in the detection of numerous pathogenic variants and dozens of novel disease genes. NGS thus enormously facilitated our ability to make an early diagnosis and to recognize important genotype-phenotype correlations. This knowledge contributes to the clinician’s ability to correctly inform patients about expected disease progression and allows clinicians to form appropriate interdisciplinary teams leading to optimal clinical care and disease management. For example, early diagnosis is highly beneficial to PCD patients in whom progression to irreversible lung disease continues unless treated early on. Gene and variant identification also allows for development of personalized therapies such as targeted gene therapy. This is important as no cures are available for ciliopathies to date. Gene therapy successes have been booked in patients with a variety of hereditary disorders, including retinal degeneration.

1.2.8 Ciliary genes and NGS in research

Since 1994, 143 syndromic ciliopathy genes have been identified (Table 1), initially with linkage studies and positional cloning (1980s-2005), later with Single Nucleotide Polymorphism (SNP) microarrays and candidate gene analysis (2006-2010) and most recently with various NGS methods (2009-2017). Targeted gene panels have been sequenced with NGS methods in dozens or even hundreds of patients for efficient and cheap variant detection in known genes in a variety of ciliopathies, including but not limited to JBTS, BBS, ALMS, NPHP and PCD. Gene panels not only targeted known ciliary disease genes, but also included candidate genes, e.g. genes with a predicted ciliary function. These panels have proven to be successful tools to identify novel disease genes by various groups. Currently, we can genetically explain 45-90% of JBTS and MKS patients, 55-70% of CED and SRTD patients, 84% of EVC patients, 80% of BBS and ALMS patients, 21% of NPHP patients, and 65% of PCD patients with panels. The advantage of gene panel testing versus genomic methods such as whole-exome sequencing (WES),...
whereby all exons of the genome are sequenced, is that panels usually result in higher coverage data at a relatively low cost and with high-throughput. In addition, the chance of identification of incidental findings, which are clinically relevant findings that are unrelated to the clinical problem for which screening was initially intended, is extremely low when only known ciliopathy-specific genes are being sequenced; however, it is difficult to add new genes to existing gene panels. The possibility of detection of incidental findings is an important concern when analyzing WES data, however, WES has proven to be highly successful for the identification of a variant in a gene that has not previously been implicated in ciliary disease. Furthermore, retrospective analysis of the data is possible when WES is used. A recent research study reported that WES provides a molecular diagnosis in 44% of ciliopathy patients.

1.2.9 Diagnostics of ciliopathies

In past and current genome diagnostics for ciliopathies, pre-screening methods like (SNP) microarrays for homozygosity mapping (and copy number variant [CNV] detection) or targeted panels with only known variants in ciliary disease genes have been used to reduce costs by limiting the list of genes to be tested by gene-specific methods like Sanger sequencing. More recently, WES started to be implemented as a diagnostic test for ciliopathies and is considered to be cost-effective when a clinician needs to investigate more than two genes to identify the genetic cause of disease, a scenario that often applies when a ciliopathy patient presents in the clinic considering the enormous genetic heterogeneity in these disorders. While incidental findings are a concern in WES, these can be easily circumvented in diagnostics by first (or only) analyzing known ciliopathy genes and leaving the remainder of WES data concealed. This approach has proven to be an effective tool for gene and variant identification with reasonable throughput and acceptable costs. Diagnostic WES has an estimated success rate of about 30% for ciliopathies in our institute (personal communication, D. Lugtenberg and www.genomediagnosticsnijmegen.nl). Other groups reported general success rates for rare diseases that vary between 20-45% based on various studies that include thousands of patients.
Chapter 1.3

Aim and outline of this thesis
After addition, the POC1B protein mislocalization to vesicle trafficking studies with CEP164, like the base of the heterogeneous CEP164 complex, associated with syndrome-related CEP164, with phenotype studies on retinal ciliopathy. The aim was to understand the Cep164 variants in families with severe ocular defects. These variants showed functional disruption of Cep164, with a specific interaction of Cep164 with the exocyst subunits, EXOC6B and EXOC4, rather than the classic BBSome, in five families with non-syndromic retinal ciliopathy. The introduction of Cep164 was rescuing the exocyst mutants, which is also evidenced in Zebrafish. Since this connection, exocyst function is pivotal for ciliary exocyst subunit interactions, exocyst subunits, and exocyst components, as well as exocyst subunit interactions in vivo. Functional characterization of the exocyst subunits, along with the role of the exocyst subunit interactions, in vivo is critical for understanding the function of the exocyst subunits. The exocyst subunits are involved in the transport of vesicle trafficking, and the exocyst subunits are involved in the transport of vesicle trafficking.
The aim of this thesis was to further decipher the molecular factors that drive ciliopathies, and to specifically understand the role of vesicle trafficking in ciliary disorders.

After the general introduction into cilia structure and function (chapter 1.1) and the spectrum of ciliopathies (chapter 1.2), I describe the identification of variants in basal body protein POC1B in two families with CRD, a non-syndromic retinal ciliopathy, in chapter 2. Zebrafish studies showed a specific ocular phenotype upon morpholino-induced knockdown and this phenotype was (partially) rescued with WT, but not with mutant POC1B mRNA. By using protein-protein interaction studies, I showed that the identified missense variants disrupt the (co-)localization and interaction with FAM161A, which is involved in another non-syndromic retinal ciliopathy, RP. In chapter 3, I report on the clinical and genetic characterization of four Pakistani families with classic BBS, and one family with a BBS-like syndrome. BBS is associated with the BBSome, a protein complex involved in trafficking of proteins to the cilium. In this study we identified variants in five known ciliopathy genes (ARL6, BBS5, BBS9, BBS12 and CEP164), and this chapter further underlines that BBS is both genetically and phenotypically heterogeneous. Specifically, we present another example of the fact that one gene can be associated with multiple related ciliopathy syndromes. We found that variants in the gene CEP164, originally associated with Nephronophthisis-15, are present in a family with a BBS-like syndrome. Another protein complex involved in (ciliary) vesicle trafficking, the exocyst complex, which previously was associated with two syndromic ciliopathies (JBTS and MKS), is discussed in Chapter 4. I studied the protein interactions of each component of the complex and identified several candidate proteins that may be involved in docking of the exocyst at the base of the cilium. These data also identified EXOC6B as an interactor; I propose that this protein may be involved in an alternative assembly of the exocyst complex. Furthermore, based on the results from my protein-protein interaction studies, I predict that the variants reported in ciliopathy patients in two exocyst subunits, EXOC4 and EXOC8, rather disrupt regulation and specific interactions of the complex than the assembly of the entire complex itself. Chapter 5 reports on a ciliopathy-like syndrome, and shows the importance of correct regulation of vesicle trafficking in the development of the brain. I report on the detection of two heterozygous de novo missense variants in RAB11B, a gene encoding a small GTPase protein, in families with severe ID and a distinct brain phenotype on MRI. Functional analysis showed that the variants disrupt the nucleotide binding pocket in RAB11B, cause mislocalization of the protein and enhances the binding with GTPase activating protein SH3BP5. The protein dysfunction leads to misregulation of vesicle trafficking and ultimately neurodevelopmental defects. The exact link with the cilium is unclear and still waits further investigation.

In summary, these chapters emphasize the importance of different aspects of regulated vesicle trafficking for proper human development and ciliary function. The different steps of transport towards the cilium, and consequences of malfunction, are discussed in Chapter 6. In addition, I review new concepts in ciliary structure and function, and I provide a general discussion on the future perspectives for the ciliary field as a whole.
Chapter 1

1.4 References
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Chapter 1


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Chapter 1


Disruption of the basal body protein POC1B results in autosomal recessive cone-rod dystrophy

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Chapter 2

2.1 Abstract

Exome sequencing revealed a homozygous missense mutation (c.317C>G, p.(Arg106Pro)) in POC1B, encoding POC1 centriolar protein B, in three siblings with autosomal recessive cone dystrophy or cone-rod dystrophy, and compound heterozygous POC1B mutations (c.199_201del [p.(Gln67del)] and c.810+1G>T) in an unrelated person with cone-rod dystrophy. Upon overexpression of POC1B in human TERT-immortalized retinal pigment epithelium 1 cells, the encoded wild type protein localized to the basal body of the primary cilium, while this localization was lost for p.(Arg106Pro) and p.(Gln67del) variant POC1B proteins. Morpholino oligonucleotide-induced knock-down of poc1b translation in zebrafish resulted in a dose-dependent small eye phenotype, impaired optokinetic responses and decreased photoreceptor outer segment length. These ocular phenotypes could partially be rescued by wild type human POC1B mRNA, but not by c.199_201del and c.317C>G mutant human POC1B mRNAs. Yeast two-hybrid screening of a human retinal cDNA library revealed FAM161A as a binary interaction partner of POC1B. This was confirmed in coimmunoprecipitation and co-localization assays, which both showed loss of FAM161A interaction of p.(Arg106Pro) and p.(Gln67del) variant POC1B. FAM161A was previously implicated in autosomal recessive retinitis pigmentosa and shown to be located at the base of the photoreceptor connecting cilium where it interacts with several other ciliopathy-associated proteins. Taken together, this study demonstrates that POC1B mutations result in a defect of the photoreceptor sensory cilium affecting the cone and rod photoreceptors.
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2.2 Introduction
Inherited cone disorders are a heterogeneous group of diseases that primarily affect cone photoreceptors and that have an estimated worldwide prevalence of 1:30,000-1:40,000.\textsuperscript{1-3} They can be divided in progressive cone dystrophies and the more stationary disorders, also named cone dysfunction syndromes. The stationary subtypes, like achromatopsia (ACHM, MIM #262300), are congenital and children with ACHM present with a congenital nystagmus, significantly reduced visual acuity, severe photophobia, poor or absent color vision, and normal fundi. Electroretinograms (ERG) show no or residual cone responses with normal rod responses. The cone dystrophies (COD, MIM #613093), on the other hand, start in childhood or early adult life and cause progressive deterioration of visual acuity and color vision, with reduced cone responses on ERG.\textsuperscript{4,5} The fundus examination in COD varies from normal to either a bull’s eye maculopathy, to total atrophy of the macular region.\textsuperscript{1} A considerable amount of persons with COD will also develop rod dysfunction leading to a cone-rod dystrophy (CRD, MIM #120970) with panretinal degeneration. In CRD, the loss of rod function may also be concomitant with the loss of cone function. So apart from the loss of central vision, individuals with CRD will also experience night blindness and loss of peripheral vision, leading to legal blindness at an earlier age.\textsuperscript{1,6}

Molecular genetic studies identified five genes mutated in persons with ACHM, eight genes implicated in COD, and 17 genes in CRD (Retinal degeneration network).\textsuperscript{1,7-9} Cone disorders can follow all modes of Mendelian inheritance and present as non-syndromic and syndromic forms.\textsuperscript{1,2} The cone disease-associated genes encode proteins that fulfill crucial roles in the cone phototransduction cascade, transport processes towards or through the connecting cilium, cell membrane morphogenesis and maintenance, synaptic transduction, and the retinoid cycle.\textsuperscript{1,7-9}

Whole exome sequencing (WES) has proven to be very effective in the discovery of genetic defects in inherited retinal diseases.\textsuperscript{10-14} Employing WES, we here report the identification of mutations in POC1B (MIM *614784), encoding a protein previously associated with basal body stability,\textsuperscript{15} underlying autosomal recessive COD or CRD. In addition, we provide an integrated functional approach to substantiate the causality of the identified mutations.

2.3 Material and methods

2.3.1 Subjects and clinical evaluation
A non-consanguineous family of Turkish origin (Family A) with three siblings affected by COD and CRD, and an isolated Dutch individual (Family B) with ACHM that evolved into a progressive retinal dystrophy, were included in this study (Figure 1A). These families belong to a large cohort of individuals affected by ACHM (n=21), COD (n=110) or CRD (n=112). Most of the probands are the only affected persons in their family, and they were ascertained in various ophthalmic centers in the Netherlands, Belgium, the United Kingdom, and Canada. The individuals that were diagnosed with ACHM had a history of congenital pendular nystagmus, reduced visual acuity, photophobia, and poor or absent color vision on early infancy, and had absent cone function on ERG with normal rod responses. Persons in our
Chapter 2

cohort were classified as COD when they presented with a childhood or early adult onset progressive deterioration of visual acuity and color vision, with reduced cone responses on ERG, and normal rod responses for ≥5 years. Inclusion criteria for CRD were progressive loss of central vision, color vision disturbances, and a reduction of both cone and rod ERG responses, with cones equally or more severely reduced.

After identification of the genetic defect, the medical records of the affected individuals of families A and B were evaluated. Ophthalmologic examinations were performed on several occasions and included best-corrected visual acuity (Snellen chart), slit-lamp biomicroscopy, ophthalmoscopy, color vision testing (Hardy–Rand–Rittler color vision test and Lanthony Panel D-15 tests), and visual field testing using Goldmann kinetic perimetry (targets V-4e, and I-4e to I-1e). Fundus photographs centered on the macular area as well as on the four peripheral quadrants were available for two individuals. Electroretinography (ERG) was performed according to the protocol of the International Society for Clinical Electrophysiology of Vision (ISCEV). Stratus optical coherence tomography (Time-domain OCT, Stratus 3, Carl Zeiss Meditec, Dublin, CA, USA) was obtained in one affected individual (A-II:4) and spectral-domain optical coherence tomography (SD-OCT; Heidelberg Spectralis HRA+OCT, Heidelberg Engineering, Germany; 30° single line scans, 10 frames per line) in proband B-II:1. The acquisition of SD-OCT was limited due to nystagmus. This study was approved by the Institutional Review Boards of the participating centers, and adhered to the tenets of the declaration of Helsinki. All subjects provided written informed consent prior to participation in the study.

2.3.2 Exome sequencing and variant identification
A SOLiD4 sequencing platform (Life Technologies, Carlsbad, CA, USA) was utilized for WES in 12 probands with CRD or COD from families with suspected autosomal recessive inheritance, and the exomes were enriched according to the manufacturer’s protocol using Agilent’s SureSelect Human All Exon v2 Kit (50Mb), containing the exonic sequences of approximately 21,000 genes (Agilent Technologies, Inc., Santa Clara, CA, USA). LifeScope software v2.1 (Life Technologies, Carlsbad, CA, USA) was used to map color space reads along the hg19 reference genome assembly. High-stringency calling with the DiBayes algorithm was used for single-nucleotide variant calling. The small Indel Tool was used to detect small insertions and deletions. For individual A-II:1, 69,686,646 reads were uniquely mapped to the gene-coding regions, with a median coverage of 58.2x and 44,784 sequence variants. For proband B-II:1, 75,493,298 reads were uniquely mapped to the gene-coding regions, with a median coverage of 66.7x and 47,304 sequence variants. To validate the WES sequence variants and to exclude the presence of other POC1B mutations, all coding exons and exon-intron boundaries of POC1B were amplified using primers that were designed using Primer 3 software (Table S1).
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2.3.3 mRNA analysis by RT-PCR
To assess the effect of c.810+1G>T on the POC1B transcript, total RNA was isolated from peripheral blood cells from the affected person B-II:1 and three control individuals according to the manufacturer’s protocol (Qiagen, Venlo, The Netherlands). The peripheral blood cells of B-II:1 and the control individuals were cultured according to standard procedures using phytohaemagglutinine. The leucocytes of the affected individual were grown for 4-6 hours with or without cycloheximide to visualize the effect of the mutation and possible degradation of nonsense-containing mRNAs by nonsense mediated decay (NMD). Using a hypotone osmotic shock and centrifugation, the leucocytes were separated from the erythrocytes. Reverse transcription with iScript (Biorad, Veenendaal, The Netherlands) was performed on 1 µg of total RNA. RT-PCR experiments were performed using 2.5 µl cDNA with primers in exons 5 and 9 (Table S2) (35 cycles) followed by Sanger sequencing using a 3100 or 3730 DNA Analyzer (Applied Biosystems, Bleiswijk, the Netherlands).

![Image](https://example.com/image1.png)

**Figure 1.** POC1B mutations in families with cone dystrophy and cone-rod dystrophy. (A) Sanger sequencing showed the segregation of the homozygous missense mutation M1 (c.317G>C; p.(Gln67Pro)) in family A and the M2 (c.199_201del; p.(Gln67del)) and M3 (c.810+1G>T) mutations in family B. (B) mRNA reverse transcription-PCR studies show a normal product size of 521 bp, and aberrant products of 387 bp and 271 bp lacking exon 7 and exons 6 and 7, respectively. These CDNA deletions result in the predicted truncated POC1B protein products p.(Val226Glyfs*30) (c.677_810del) and p.(Phe188Asps*73) (c.561_810del), respectively. (C) Evolutionary conservation of amino acid residues Gln67 and Arg106 in POC1B. The glutamic acid at position 67 is moderately conserved, whereas the arginine at position 106 is completely conserved among the listed species. Identical amino acids are indicated in **black boxes**, and conserved residues are indicated in **gray boxes**.
2.3.4 Zebrafish morpholino knockdown
Tupfel long fin zebrafish were bred and raised under standard conditions.\textsuperscript{18} All experiments were carried out conforming to European guidelines on animal experiments (2010/63/EU). Zebrafish eggs were obtained from natural spawning. Antisense morpholino oligonucleotides (MO) blocking the translation (5'-GATCCTCCATTACAGCCATGAT-3') or the 5' splice-site of exon 2 (5'-AAGTTCTCTGTCTTTACAGAGA-3') of poc1\textsuperscript{b}\textsuperscript{15} were obtained from GeneTools (Philomath, OR, USA). A MO directed against a human β-globin intron mutation (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a standard negative control. For MO knockdown, one nanoliter of diluted MO (ranging from 2 ng to 10 ng) was injected into the yolk of one- to two-cell stage embryos using a Pneumatic PicoPump pv280 (World Precision Instruments, Hertfordshire, UK). A minimum sample size of 80 larvae was used in each injection experiment. After injection, embryos were raised at 28.5°C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\textsubscript{2}, and 0.33 mM MgSO\textsubscript{4}), supplemented with 0.1% (w/v) methylene blue. At 4 days post fertilization (dpf) embryos were divided into two groups based on the presence of the ocular phenotype as described by Pearson et al.\textsuperscript{15} (< 15 arbitrary units on a stereomicroscope’s ocular scale bar (Zeiss, Oberkochen, Germany)). Embryos from both groups, as well as injected and uninjected controls, were subjected to optokinetic response (OKR) measurement and histological analysis of the retina.

cDNAs encoding human wild type and variant (p.(Gln67del) and p.(Arg106Pro)) POC1B were cloned in pCS2+/DEST using Gateway technology (Life Technologies, Bleiswijk, The Netherlands), linearized and used as template for in vitro transcription. mRNAs were prepared using the mMESSAGE mMACHINE Kit (Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer’s instructions. A dose of 100 pg mRNA was injected with or without the MOs as described above.

2.3.5 Zebrafish optokinetic response assay
The OKR was measured using a previously described method.\textsuperscript{19} Zebrafish larvae were mounted in an upright position in 3% methylcellulose in a small petridish. The petridish was placed on a platform surrounded by a rotating drum of 8 cm in diameter. A pattern of alternating black and white vertical stripes was displayed on the drum interior, each stripe subtending an angle of 36°. Larvae (4 dpf) were visualized through a stereomicroscope positioned over the drum and illuminated with fiber optic lights. Eye movements were recorded while larvae were optically stimulated by the rotating stripes. Larvae were subjected to a protocol of 30 seconds counter clockwise rotation, a 10-second rest, followed by 30 seconds clockwise rotation. Thereafter the larvae were washed out of the methylcellulose and fixed for histological analysis.

2.3.6 Photoreceptor outer segment staining in zebrafish embryos
Larvae (4 dpf) that were tested for OKR were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 24h, hereafter dehydrated through graded ethanol steps from 70% to 100%, and embedded via a standard protocol in glycol methacrylate. The eyes were sectioned (2 μm), stained with boron-dipyrrromethene (BODIPY, 1:10,000 in PBS) for membranes and with
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4’,6-Diamidin-2-phenylindoldihydrochlorid (DAPI, 1:8,000) for nuclei and imaged with a Zeiss Axio Imager Z1 fluorescence microscope (Zeiss, Oberkochen, Germany).

2.3.7 Immunostaining and microscopy
Zebrafish and rat samples for immunohistochemistry (unfixed and 4% paraformaldehyde fixed) were rinsed in 30% sucrose in PBS and directly frozen in Tissue Tek in melting isopentane. Cryosections of unfixed adult zebrafish and rat eyes (7 μm) were stained for Poc1b with anti-hsPOC1B (1:50, generously provided by Chad Pearson and Mary Pinter, University of Colorado, Denver, CO, USA) as described for cultured cells by Pearson et al. 2009.15 Sections were counterstained with GT335 (1:100; mouse monoclonal antibody against polyglutamylated tubulin, kindly provided by Carsten Janke, CNRS Centre de Recherches en Biochimie Macromoleculaire, Montpellier, France). Sections of fixed zebrafish morphants were stained for green/red double cones using a mouse monoclonal (zpr-1, raised against Arr3a, 1:500; ZIRC, Eugene, OR, USA) and for rod with anti-rhodopsin (1:500; Novus Biologicals, Cambridge, UK). Sections were washed with PBS, permeabilized for 20 minutes in 0.01% (v/v) Tween-20 in PBS and washed again. Next, sections were blocked with 10% normal goat serum and 2% bovine serum albumin in PBS and primary antibodies were incubated overnight at 4°C in blocking buffer. After washing with PBS, secondary antibodies were incubated in blocking buffer for 45 minutes at room temperature. Samples were counterstained with DAPI and mounted with Prolong Gold (Life technologies, Bleiswijk, The Netherlands). For all sections, goat-anti-mouse or goat-anti-rabbit Alexa 488, 568 (1:500; Life Technologies, Bleiswijk, The Netherlands) secondary antibodies were used.

2.3.8 cDNA constructs
All expression constructs were created with Gateway technology (Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer’s instructions. These constructs encoded 3xHA-POC1B wild type and variants (p.(Arg106Pro) and p.(Gln67del)) and 3xFLAG-FAM161A for coimmunoprecipitation, and mRFP-POC1B wild type and variants and PalmMyr-CFP-FAM161A for (co)localization studies. cDNA constructs encoding the full-length POC1B protein of 478 amino acids (aa) (POC1B; NCBI Reference Sequence: NM_172240.2 (gene); NP_758440.1 (protein)), or different fragments thereof were generated by Gateway-adapted PCR and subsequently cloned.20 The first fragment (POC1B-WD40) spans aa 1 – 297 and contains the WD40 domain. The second fragment (POC1B-SR) spans aa 298 - 426 and does not hold any known domains. The third fragment (POC1B-CC) spans aa 427 – 478 and contains a single coiled-coil domain (Figure S1). Constructs encoding POC1B variants p.(Gln67del) and p.(Arg106Pro) were generated by site-directed mutagenesis PCR. Constructs encoding the full length FAM161A protein were generated from a full length FAM161A clone.21 The sequence of all entry clones was verified by Sanger sequencing.
2.3.9 Yeast two-hybrid assay

The GAL4-based yeast two-hybrid system (HybriZAP, Stratagene, La Jolla, USA) was used to identify binary protein-protein interaction partners of POC1B. The construct encoding the full length POC1B protein, as well as the three constructs encoding fragments of POC1B, were fused to a DNA binding domain (GAL4-BD), and used as a bait to screen a human oligo-dT primed retinal cDNA library. The yeast strain PJ69-4A, which carried the HIS3 (histidine), ADE2 (adenine) and LacZ (β-galactosidase) reporter genes, was used as a host. Interactions were analyzed by assessment of reporter gene activation via growth on selective media (HIS3 and ADE2 reporter genes) and β-galactosidase colorimetric filter lift assays (LacZ reporter gene). cDNA inserts of clones containing putative interaction partners were confirmed by Sanger sequencing.

2.3.10 Localization in hTERT-RPE1 cells

Human TERT-immortalized Retinal Pigment Epithelium 1 cells (hTERT-RPE1) were cultured as previously described. Cells were seeded on coverslips, grown to 80% confluency and subsequently serum-starved for 24 hours in medium containing only 0.2% fetal calf serum to induce cilium growth. The cells were then co-transfected with constructs encoding mRFP-POC1B (wild type or mutant) and PalmMyr-CFP-FAM161A (wild type) using Lipofectamine 2000 (Life Technologies, Bleiswijk, The Netherlands), according to the manufacturer’s instructions. Cells were fixed in 4% paraformaldehyde for 20 minutes, treated with 1% Triton X-100 in PBS for 5 minutes and blocked in 2% bovine serum albumin (BSA) in PBS for 20 minutes. Cells were incubated with the primary antibody (1:500, GT335; cilium and basal body marker) and α-RPGRIP1L (1:500; SNC039 + SNC040, transition zone marker), diluted in 2% BSA in PBS, for 1 hour. After washing in PBS, the cells were incubated with the secondary antibody for 45 minutes. Secondary antibodies, goat-anti-mouse, goat-anti-guinea pig or goat-anti-rabbit Alexa 488, 568 or 647 (1:500; Life Technologies, Bleiswijk, The Netherlands), were diluted in 2% BSA in PBS. Cells were washed with PBS and briefly with milliQ before being mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). The cellular localization of wild type and variant POC1B proteins was analyzed with a Zeiss Axio Imager Z1 fluorescence microscope equipped with a 63x objective lens. Optical sections were generated through structured illumination by inserting an ApoTome slider into the illumination path, followed by processing using AxioVision (Zeiss, Oberkochen, Germany) and Photoshop CS4 software (Adobe Systems, San Jose, CA, USA).

2.3.11 Coimmunoprecipitation

3xHA-POC1B (wild type and variants) and 3xFLAG-FAM161A were co-synthesized in Human Embryonic Kidney 293T cells (HEK293T). As a negative control, the functionally unrelated p63 was co-synthesized with both POC1B and FAM161A. As positive controls, the previously described interactions between nephrocystin-4 (encoded by NPHP4, MIM *607215) and RPGRIP1, and between lebercilin (encoded by LCA5, MIM *611408) and FAM161A were used. After 48 hours of expression of these genes, cells were lysed on ice in lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100) supplemented with complete protease inhibitor cocktail (Roche, Woerden, The Netherlands). Lysates were incubated with
anti-HA affinity matrix (Roche, Woerden, The Netherlands), or with anti-FLAG M2-agarose from mouse (Sigma-Aldrich, Zwijndrecht, The Netherlands), for 5 hours at 4°C. After incubation, beads with bound protein complexes were washed in lysis buffer and subsequently taken up in 4x NuPAGE Sample Buffer and heated for 10 minutes at 70°C. Beads were precipitated by centrifugation, and supernatant was run on a NuPAGE Novex 4%–12% Bis-Tris SDS–PAGE gel. The interaction of 3xHA-POC1B with 3xFLAG-FAM161A was assessed by immunoblotting, followed by staining with either monoclonal mouse anti-HA or monoclonal mouse anti-FLAG (1:1,000; Sigma-Aldrich, Zwijndrecht, The Netherlands) as a primary antibody and goat-anti mouse IRDye800 (1:20,000; Li-Cor, Lincoln, NE, USA) as a secondary antibody. Fluorescence was analyzed on a Li-Cor Odyssey 2.1 infrared scanner (Li-Cor, Lincoln, NE, USA).

2.4 Results

2.4.1 Identification of POC1B mutations

To localize the genetic defect in a family of Turkish origin with three siblings affected by COD or CRD (Family A; Figure 1A), exome sequencing was performed in A-II-1. Putative causal mutations were selected when present with a frequency <0.5% in dbSNP and our in-house controls (n=2,604 alleles), and when they represented nonsense, frame shift, canonical splice site, or missense mutations with a PhylolP >2.7 (range -14.1 through 6.4). Under the assumption of autosomal recessive inheritance, we identified potential compound heterozygous mutations (present in >20% sequence variant reads) in ASTE1, CNTN3 (MIM *601325) and TUBGCP2, which based on Sanger sequencing did not segregate with the disease. We identified one potential homozygous mutation (present in >80% sequence variant reads; Table S3), c.317C>G (p.(Arg106Pro)) in POC1B (MIM *614784), which upon Sanger sequence analysis was found to be present in a homozygous state in the three affected siblings and in a heterozygous state in the parents and unaffected sibling (Figure 1A). The arginine at position 106 is highly conserved, up to *Chlamydomonas* (Figure 1C). The c.317 position shows a high PhylolP score of 6.1 and the c.317C>G mutation was not identified in 189 ethnically matched controls, nor in the Exome Variant Server (EVS) database (release ESP6500).

Using the same stringent sequence variant filtering as for Family A, exome sequencing in the proband (B-II-1) of Family B (Figure 1A) of Dutch ancestry and diagnosed with atypical ACHM identified three genes with potential compound heterozygous mutations (Table S4). The sequence variants in NUDT14 (MIM *609219) and PIKFYVE (MIM *609414) did not segregate with the disease. In POC1B, we identified a three nucleotide deletion, c.199_201del (p.(Gln67del)), and a mutation affecting a canonical splice site nucleotide, c.810+1G>T. By RT-PCR of this individual’s lymphoblast mRNA, the latter mutation was shown to induce skipping of exons 6 and 7 (c.561_810del; minor mutant product) or exon 7 (c.677_810del; major mutant product) (Figure 1B), resulting in the predicted truncated proteins p.(Phe188Aspfs*73) and p.(Val226Glyfs*30), respectively. Segregation analysis confirmed that both parents are carriers of one of these POC1B mutations (Figure 1A). Both mutations were
not identified in 149 ethnically matched controls and in the EVS database. The glutamine at position 67 is moderately conserved, up to *Xenopus* (Figure 1C).

Genome-wide SNP homozygosity mapping data of >400 unrelated individuals with autosomal recessive CRD, Leber congenital amaurosis (LCA, MIM #611755) and retinitis pigmentosa (RP, MIM #268000) was assessed through the European Retinal Disease Consortium,\textsuperscript{10,26} and allowed the identification of eight probands with a large homozygous region spanning *POC1B*. However, Sanger sequencing of *POC1B* in these probands did not reveal additional pathogenic mutations. Subsequent Sanger sequence analysis of the entire *POC1B* gene in a more specific patient cohort, i.e. in individuals diagnosed with ACHM (n=21), COD (n=110) or CRD (n=112), also did not reveal additional individuals with *POC1B* mutations.

### 2.4.2 Clinical features of affected individuals of families A and B

Table 1 presents a summary of the clinical features of the four individuals with *POC1B* mutations. Fundus and OCT images are depicted in Figure 2. The proband (A-II:1) of family A presented with reduced visual acuity in early infancy and a mild nystagmus. Based on electoretinography at the age of 9, showing absent cone function with normal rod responses, the diagnosis of incomplete ACHM was contemplated. In the following years, however, visual acuity seemed to deteriorate and a few years later her two younger siblings experienced a rapid loss of central vision suggesting COD (Figures 2A, B) in two siblings and CRD in one sibling. No long term data are available on these three persons in contrast to B-II:1 who was followed for more than 40 years. He was also diagnosed with ACHM in childhood based on the classical signs of reduced visual acuity, photophobia, nystagmus, very poor color vision, and matching ERG responses. In the fifth decade, visual acuity began to drop slowly and in his sixth decade degenerative changes were noted in the periphery of the inferior quadrant consisting of RPE atrophy and bone-spicule pigmentations (Figures 2C, D). On OCT, changes at the inner segment ellipsoid zone were observed, suggesting loss of inner/outer segment junctions (Figure 2E). The ERG at the age of 55 showed absent cone and significantly reduced rod responses. Altogether, the diagnosis changed from isolated cone dysfunction into progressive cone-rod disease. Systematically, he was treated for hypertension and had normal renal function.
Disruption of the basal body protein POC1B results in autosomal recessive cone-rod dystrophy

### Table 1. Summary of the clinical data of four individuals with POC1B mutations

<table>
<thead>
<tr>
<th>Family</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject; Gender</td>
<td>A-II:1; F</td>
<td>A-II:3; F</td>
</tr>
<tr>
<td>Nystagmus</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>First documented visual acuity (age)</td>
<td>RE: 0.16, (3 yrs)</td>
<td>RE: 0.8, (10 yrs)</td>
</tr>
<tr>
<td>Last documented visual acuity (age)</td>
<td>RE: CF, (16 yrs)</td>
<td>RE: 0.3, (19 yrs)</td>
</tr>
<tr>
<td>Refraction (D SE) (age)</td>
<td>RE: -0.6 D, (16 yrs)</td>
<td>RE: -1.0 D, (19 yrs)</td>
</tr>
<tr>
<td>Funduscopy (age)</td>
<td>Subtle RPE disturbances in the periphery, otherwise normal (9 yrs)</td>
<td>Relative hypopigmentation in the periphery, otherwise normal (12 yrs)</td>
</tr>
<tr>
<td>OCT (age)</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Color vision (age)</td>
<td>Tritan defect (9 yrs)</td>
<td>Anomaloscope: red shift, both eyes (11 yrs)</td>
</tr>
<tr>
<td>Visual field; Goldmann (age)</td>
<td>Relative central scotoma, mild peripheral constriction (13 yrs)</td>
<td>Relative central scotoma, mild peripheral constriction (11 yrs)</td>
</tr>
<tr>
<td>ERG (age)</td>
<td>Non-recordable cone responses, normal rod responses (9 and 13 yrs)</td>
<td>Severely reduced cone responses, normal rod responses (11 yrs)</td>
</tr>
<tr>
<td>Final diagnosis</td>
<td>Cone dystrophy</td>
<td>Cone dystrophy</td>
</tr>
</tbody>
</table>

CF, counting fingers; D, diopters; ERG, electroretinogram; LE, left eye; NP, not performed; OCT, optical coherence tomography; RE, right eye; RPE, retinal pigment epithelium; SE, spherical equivalent; visual acuity is in Snellen decimals; yrs, years; *, refractive error before cataract extraction at age of 59 yrs.
2.4.3 Localization of POC1B in human hTERT-RPE1 cells

To investigate the effect of the identified POC1B mutations on the subcellular localization of the encoded protein, we synthesized wild type and variant recombinant POC1B proteins, fused to monomeric red fluorescent protein (mRFP), in ciliated hTERT-RPE1 cells. Wild type POC1B localization was concentrated at the ciliary basal body as indicated by co-staining with the anti-polyglutamylated tubulin antibody GT335, although also some diffuse cytoplasmic localization could be observed (Figure 3A and Figure S2A). This confirms previous results of Venoux et al.\(^2^0\) In contrast, variant POC1B proteins, carrying either the p.(Gln67del) (Figure 3B and Figure S2B) or p.(Arg106Pro) (Figure 3C and Figure S2C) amino acid changes, had completely lost their ciliary localization.

2.4.4 Localization of Poc1b in photoreceptor cells

The retinal function of Poc1b was evaluated in zebrafish. Staining with anti-human POC1B showed that the protein is located at the basal bodies of both the inner and outer photoreceptor layer of the adult zebrafish retina (Figure 4A). In a different focus plane compared to the basal bodies, some staining could also be observed at the outer limit of the ONL (data not shown). Poc1b immunostaining could also be detected at the basal body of rat photoreceptors cells (Figure S3).

Figure 2. Clinical presentation of subjects with POC1B mutations. (A,B) Fundus photography of the right eye of individual A-II:3 of family A at age 12. (A) Posterior pole with normal macular region. (B) Peripheral field with relative hypopigmentation (*), but no pathologic RPE changes. (C,D) Fundus photography of the right eye of individual B-II:1 of family B at age 60. (C) Posterior pole with optic nerve pallor (*), attenuated vessels (arrowhead), and no RPE disturbances at the macula. (D) Peripheral inferior field with mild RPE atrophy and bone-spicule pigmentation (arrowhead). (E) Optical coherence tomography of the left eye of individual B-II:1 showing no foveal hypoplasia, but changes at inner segment ellipsoid zone (arrow), and adherent posterior hyaloid membrane (*).
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2.4.5 Poc1b knockdown results in visual impairment in zebrafish

Poc1b morphants display the typical ciliopathy phenotypes previously described by Pearson et al.,\textsuperscript{15} including pericardial edema, small eyes, pigment mislocalization and shortened- and curved body axis (Figure S4A). Small eyes were already observed in larvae treated with 2 ng MO and became more frequent as the dose increased (Figure 4B and Figure S4B). At a dose of 6 ng MO, smaller eyes occurred in 39.8% of morphants eyes and were on average significantly smaller compared to wild types (Figure 4B, Figure S4B), while 92.5% displayed one or more of the phenotypes described above without increased mortality compared to controls. Specificity of the morpholino knockdown was previously verified with a second morpholino targeting the 5’ splice site of poc1b exon 2, and resulted in our hands in 59.1% of morphants with small eyes (Figure S4B).
Figure 4. Morphological and functional effects of morpholino knockdown of Poc1b in zebrafish larvae. (A) Localization of Poc1b (green) in the retina of adult zebrafish. The retina of adult zebrafish typically contains two layers of photoreceptor outer segments and associated basal bodies (OS layer 1; OS layer 2). Poc1b immunoreactivity is observed in both layers, overlapping with, and adjacent to, GT335 staining (red), a marker of the connecting cilium. OS = outer segment, CC = connecting cilium, BB = basal body, IS = inner segment, ONL = outer nuclear layer. Scale bar = 15 µm. (B) Phenotypic analysis of morphant eyes. Injection of 6 ng translation-blocking morpholino oligonucleotides (MO) increased the number of small eyes compared to control injected larvae. The phenotype could be partially rescued by co-injection of wild type POC1B mRNA but not by c.199_201del (p.Gln67del) or c.317C>G (p.Arg106Pro) mutant POC1B mRNA. (C) Analysis of the optokinetic response (OKR; Movies S1 and S2). Larvae with decreased responses were already observed in a pool of 2 ng MO injected larvae (indicated with an accolade). At this dose, there were also morphants that did respond to the visual stimulus. (D) Sections of control and poc1b MO injected eyes stained for outer segments (red) and nuclei (blue). Eyes of morphants that did not respond to the visual stimulus were smaller. Scale bar = 50 µm. (E) Outer segments were decreased in length or absent in non-responsive morphants (OKR -), while responsive larvae (OKR +) had a normal outer segment length. Scale bar = 10 µm.
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The OKR was assessed in control and poc1b MO injected larvae. OKR was decreased or absent in morphants with small eyes compared to wild type and control injected larvae (Figure 4C and Movies S1, S2). Morphants that received the same dose of MO but had normal-sized eyes responded normally to the OKR stimulus. This phenotype was confirmed in larvae treated with a splice-site blocking MO (data not shown). Outer segment length was not affected in control injected larvae and morphants that did show an OKR. Histological analysis of the retina of the morphants subjected to OKR measurement revealed shortened or absent outer segments of the photoreceptors, while lamination appeared normal (Figures 4D, E). We observed a perfect correlation between the small eye phenotype and a diminished or absent OKR. The size of the eyes appears to correlate with outer segment length and responsiveness to visual stimuli. As such, we can quantify the size of the eye to measure the effects of loss of Poc1b function. Indeed, co-injection of 100 pg human wild type POC1B mRNA, but not by c.199_201del (p.(Gln67del)) and c.317C>G (p.(Arg106Pro)) mutant POC1B mRNA, significantly rescued poc1b knockdown (Figure 4B, Figure S4B).

Immunohistochemical staining for typical rod (rhodopsin) and cone (zpr-1) markers was absent from a subset of cells in morphants with smaller eyes (Figure S4C). High magnification pictures show that, while the immunostaining is absent in certain regions of the morphant retina, the nuclei of the photoreceptor cells are still present.

2.4.6 Identification of a retinal protein interacting with POC1B

To identify interaction partners of POC1B in the retina, we employed a GAL4-based interaction trap screen in yeast (yeast two-hybrid system). We screened a library expressing human retinal cDNAs for potential interactors of POC1B. Both a construct expressing full-length POC1B, as well as different POC1B fragments were used as baits (Figure S1). The construct containing the carboxy-terminal coiled-coil domain of POC1B was found to putatively interact with five different proteins, including FAM161A (Figure S5A). The interaction with this known retinal disease-associated protein caught our attention and was confirmed by a coimmunoprecipitation assay using both full length proteins (Figure 5A). In the same assay, we investigated the effect of the identified missense and single aa deletion variants in POC1B on the interaction. Indeed, a significantly lower amount of mutated POC1B coprecipitated with FAM161A compared to the wild type protein, indicating a disrupted physical interaction. The unrelated p63 protein did not coprecipitate with either POC1B or FAM161A, which confirmed specificity of the interaction between POC1B and FAM161A in the coimmunoprecipitation assay. The interaction between wild type POC1B and FAM161A, and the decreased interaction between variant POC1B proteins and FAM161A was confirmed by reciprocal coimmunoprecipitation (Figure S5B). Uncropped images of the immunoblots are shown in Figure S6.
Figure 5. Coimmunoprecipitation and hTERT-RPE1 localization studies of POC1B and FAM161A. (A) Coimmunoprecipitation assay in HEK293T cells. Wild type 3xHA-POC1B efficiently co-precipitated with 3xFLAG-FAM161A (lane 1), which was reduced for variants 3xHA-POC1B-Gln67del and 3xHA-POC1B-Arg106Pro (panel 4, lanes 2 and 3). Specificity was confirmed by including the unrelated p63 protein, which failed to coimmunoprecipitate wild type and variant POC1B. As positive controls, coimmunoprecipitation of lebercilin (encoded by LCA5) by FAM161A, and of RPGRIP1 by nephrocystin-4 (encoded by NPHP4) were used. Immunoblots of the input are shown in panels 1 and 2, immunoblots of the FLAG immunoprecipitates in panels 3 and 4. Size markers are depicted in kDa. (B) Colocalization in hTERT-RPE1 cells. PalmMyr-CFP-FAM161A (green) was targeted to the cell membrane and microtubules and translocated mRFP-POC1B wild type (red) from the cytosol towards the cell membrane and microtubules. (C) This translocation by PalmMyr-CFP-FAM161A (green) is not observed for mRFP-POC1B-Gln67del (red) nor (D) mRFP-POC1B-Arg106Pro (red) which both maintain their cytosolic localization. RPGRIP1L (magenta) was used as transition zone marker of the cilium. Nuclei are stained with DAPI (blue). Scale bars: 10 µm. Additional images are shown in Figure S7.
To validate the loss of interaction of FAM161A with variant POC1B in mammalian cells, we co-transfected hTERT-RPE1 cells with constructs encoding wild type and mutant mRFP-POC1B together with PalmMyr-CFP-FAM161A (Figures 5B-D and Figure S7A-C). The PalmMyr tag provides residues for palmitoylation and myristoylation that induces membrane association of the protein of interest. This can subsequently be visualized by fluorescence microscopy due to expression of the fluorescent CFP tag. Co-expression of FAM161A and POC1B shows complete colocalization of the encoded proteins at the plasma membrane, basal body and association with the microtubule network. When either one of the mutations was present in the POC1B construct, the colocalization with FAM161A was lost completely. PalmMyr-CFP-FAM161A then maintains its membrane, basal body and microtubule association, but the localization of variant POC1B is cytosolic without enrichment at specific subcellular sites (Figures 5C, D and Figures S7B, C).

2.5 Discussion

In this study, we identified three mutations in POC1B that cause autosomal recessive COD or CRD. In two siblings of one family loss of central vision was observed in childhood consistent with progressive cone disease, whereas in another sibling and in one isolated individual, poor visual acuity and nystagmus were present from early infancy suggesting a form of ACHM. In the latter two individuals, visual acuity also deteriorated over time and in one of them peripheral retinal degeneration was observed in the sixth decade. Although there is overlap in genes that are associated with either ACHM or COD due to mutations in CNGA3 (MIM *600053) and CNGB3 (MIM *605080), there are to our knowledge no reports on the natural history of ACHM concerning peripheral degeneration.

POC1B, proteome of centriole 1B (previously Pix1), is one of the two POC1 homologs that function together as a highly conserved core centriole/basal body component in vertebrates, invertebrates and even in Chlamydomonas reinhardtii and in Tetrahymena thermophila. The other POC1 homolog, encoded by POC1A (MIM *614783, previously Pix2), shows a similar protein structure and intracellular localization as POC1B. Studies in Tetrahymena thermophila suggest that POC1 proteins are essential for both structure and stability of the basal body. Depletion studies show that POC1B, unlike POC1A, is necessary for ciliogenesis, and typical ciliopathy-associated developmental defects (e.g. curved body axis, kidney cysts and laterality defects) were described in poc1b morphant zebrafish. Interestingly, they were also reported to exhibit smaller eyes, but a more detailed ophthalmological analysis was not undertaken.

In light of the retinal phenotype we observed in affected persons with POC1B mutations and the reported smaller eyes in poc1b morphant zebrafish, we disrupted poc1b expression using the same translation-blocking MO as used by Pearson et al. An accurate evaluation of Poc1b function in the eyes indeed confirmed that the protein is required for normal vision, as the Poc1b-depleted zebrafish show a severely decreased OKR in combination with smaller eyes (Figure 4C). Analysis of morphant eyes revealed decreased photoreceptor outer segment length in the cone dominated larval retina (Figures 4D, E). Poc1b appears present at all basal bodies of vertebrate photoreceptors, suggesting that loss of function affects both rods and
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ciliopathies (Figures 4A, S3). Indeed, knockdown of poc1b reduced immunoreactivity for important proteins in the light transduction cascade of rod and cones alike (Figure S4C). This corresponds with the decreased visual reponse of poc1b morphants, measured in the OKR assay. Rescue of the smaller eyes associated with this phenotype was achieved using wild type human POC1B mRNA.

The affected aa identified in this study are moderately or highly conserved in evolution (Figure 1C) and both affect the N-terminal WD40 domain (Figure S1). The third mutation alters the splice site of exon 7 and results in a truncation of the protein within the last WD40 repeat. This WD40 domain, but not the C-terminal region of POC1, has been demonstrated to be sufficient for targeting POC1 localization to centrioles. Indeed, while wild type POC1B localized to the basal bodies as previously reported, both p.(Gln67del) and p.(Arg106Pro) POC1B variant proteins revealed a loss of association with the basal body of the cilium (Figure 3). The effect of the variants on Poc1b in zebrafish was addressed by coinjection of a poc1b MO in combination with human POC1B mRNA carrying either one of the mutations. In contrast with wild type mRNA, (partial) rescue of the ocular phenotype could not be obtained by coinjection of the mutated mRNA, confirming the disturbing retinal effect of the variant aa residues (Figure 4B).

To provide further insights into the retinal function of POC1B, we aimed to identify retinal proteins interacting with POC1B using a GAL4-based interaction trap screen in yeast of a retinal cDNA library. Out of the four different bait fragments of POC1B employed, only the coiled-coil region was found to yield one significant interactor: FAM161A. Interestingly, mutations in FAM161A lead to another retinal ciliopathy, autosomal recessive RP (RP28). Binding of FAM161A was validated using communoprecipitation and colocalization studies (Figure 5). Although the interaction was initially detected using a fragment containing the coiled-coil region of POC1B, introduction of the two POC1B variants in de WD40 domain of the full-length protein strongly decreased its interaction with FAM161A, reiterating the structural importance of this domain. As FAM161A was found to be a retinal ciliopathy-associated protein, the decreased interaction we observed with this retina-specific protein may induce rod photoreceptor degeneration due to POC1B mutations in persons with CRD.

Pearson et al. showed that in the absence of Poc1b, zebrafish present with various phenotypes that points towards a syndromic ciliopathy. In contrast, the POC1B mutations identified in this study are associated with a much milder, non-syndromic cone disease phenotype in two families. Although species-specific differences may contribute to the observed phenotypic heterogeneity, based on the type and combinations of mutations identified and the reduced but not absent interaction between the mutant POC1B protein with the retina-specific FAM161A protein, it is plausible to conclude that persons with cone dystrophies have residual POC1B activity. Combinations of more severe and/or loss-of-function POC1B mutations therefore may be associated with syndromic forms of retinal ciliopathies, in line with the wide disease spectrum previously observed for another ciliopathy-associated gene, CEP290 (MIM *160142).
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In conclusion, WES led to the identification of POC1B mutations in two unrelated families with autosomal recessive non-syndromic COD and CRD. These variants disrupt the ciliary basal body localization of POC1B as well as its interaction with a retina-specific, RP-associated protein, FAM161A. As loss of Poc1b in zebrafish furthermore resulted in early-onset retinal dysfunction, this study pinpoints a new basal body protein module of photoreceptors, containing POC1B and FAM161A, which is required for photoreceptor homeostasis.
2.6 References


Chapter 2


2.7 Consortium members

2.8 Web resources
Retinal degeneration network, https://sph.uth.edu/retnet/
Primer 3, http://frodo.wi.mit.edu/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

2.9 Acknowledgements
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2.10 Supplemental data
Supplemental data also contains two movies, which can be found online with the article: http://www.sciencedirect.com/science/article/pii/S0002929714002742

Figure S1. Domain structure POC1B and FAM161A. The POC1B gene encodes a 478 amino acid (aa) 54 kDa protein (UniProt ID: POC1B_HUMAN, Q8TC44) and is predicted to contain an N-terminal WD40 domain with seven WD40 repeats, and a C-terminal coiled-coil domain. All three variants lie within the WD40 domain. For the yeast two-hybrid screen of the retinal cDNA library, three bait fragments were generated containing either the WD40 domain, spacer region or coiled-coil domain. 0.97 x 10^6, 2.25 x 10^6, and 1.56 x 10^6 cDNA clones were screened for binary interactions, respectively. Only the coiled-coil domain yielded in-frame positive clones not previously identified as background. Two overlapping clones of FAM161A were identified, encoding aa 50-660 and aa 65-660 of FAM161A. Four other in-frame clones were identified, but as these did not encode ciliary nor retinal degeneration-associated proteins, none of these were further validated. The POC1B antibody was raised against the spacer region. FAM161A is a 660 aa/ 77 kDa protein (UniProt ID: F161A_HUMAN, Q3BS20) that is predicted to contain three coiled-coil domains and an uncharacterized protein family UPFO564 domain.
Figure S2. Localization of wild type and variant POC1B in hTERT-RPE1 cells. Overlay photomicrographs of additional fields of hTERT-RPE1 cells corresponding to the images depicted in Figures 3A-C. (A) Overlay images of the localization of wild type mRFP-POC1B. Cilia are counterstained with the basal-body and cilium marker GT335 (green) and transition zone marker RPGRIP1L (cyan). (B) Overlay images of the localization of mRFP-POC1B-Gln67del, and (C) mRFP-POC1B-Arg106Pro (both in red). Here, cilia are counterstained with a rabbit polyclonal antibody against ARL13B in green (1:500; Proteintech, Chicago, IL, USA) and centrosomes are counterstained with a mouse monoclonal antibody against centrin in cyan (1:500; Millipore, Billerica, MA, USA). Wild type mRFP-POC1B shows cytosolic localization with enrichment at the basal body region, as seen in the magnifications in the insets. Both variants show similar cytosolic localization but lack the enrichment at the base of the cilium. In all pictures nuclei are stained with DAPI (blue). Scale bars: 10 µm.
Disruption of the basal body protein POC1B results in autosomal recessive cone-rod dystrophy.

**Figure S3. Immunohistochemistry of Poc1b in rat retina.** Immunohistochemistry of Poc1b on a cryosection of an unfixed rat retina. Poc1b immunoreactivity in the photoreceptor is observed overlapping with, and adjacent to, GT335 staining (red), a marker of the connecting cilium. OS = outer segment, CC = connecting cilium, BB = basal body, IS = inner segment, ONL = outer nuclear layer. Scale bar = 10 µm.
Figure S4. Phenotypic analysis of poc1b morphant zebrafish. (A) Morphology of poc1b injected larvae. Morphants had smaller eyes, curved body axis, pigment mislocalization (arrow) and pericardial edema (arrowhead). None of these phenotypes were observed in control injected animals. (B) Overview of the occurrence of small eyes in poc1b morpholino oligonucleotide (MO) knockdown experiments. This phenotype increased dose-dependently with the amount of injected MO. At 8 ng almost all morphants had smaller eyes, but mortality was also increased compared to controls. In all other groups mortality was similar compared to control injected animals. (C) Immunohistochemistry of cone (zpr1, green) and rod (rhodopsin, red) markers in control and morphant (6 ng) eyes. Not only were the eyes smaller, both zpr1 and rhodopsin immunoreactivity was absent in some parts of the morphant retina. Scale bar = 50 µm in low magnification pictures and 15 µm in high magnification pictures.
Disruption of the basal body protein POC1B results in autosomal recessive cone-rod dystrophy

Figure S5. Yeast two-hybrid and coimmunoprecipitation of POC1B and FAM161A. (A) POC1B interacts with FAM161A in a yeast two-hybrid assay. Interactions were analyzed by assessment of reporter gene activation via growth on selective media (-LWHA) and by a colorimetric filter lift assay (-LWHA; β-galactosidase). The POC1B fragment containing the coiled-coil domain spanning amino acids (aa) 427 – 478 (POC1B-CC) was found to bind the FAM161A fragment containing aa 50-660 in one yeast clone (clone 19) and a fragment containing aa 65-660 of FAM161A in three yeast colonies (clones 5, 7, and 26). (B) Coimmunoprecipitation assay in HEK293T cells. Wild type 3xFlag-FAM161A efficiently coprecipitated with 3xHA-POC1B using anti-HA antibodies (panel 4, lane 1). Introduction of the POC1B variants p.(Gln67del) and p.(Arg106Pro) reduced the coimmunoprecipitation efficiency (panel 4, lanes 2 and 3). Specificity was confirmed by including the unrelated 3xFLAG-p63 protein, which failed to coprecipitate significantly with wild type and mutant 3xHA-POC1B, and the 3xHA-p63 protein, which did not coprecipitate with 3xFLAG-FAM161A. As positive controls of the coimmunoprecipitation assay, coprecipitation of 3xHA-lebercilin with 3xFLAG-FAM161A, and of 3xHA-RPGRIP1 with 3xFLAG- nephrocytins-4 were used. Immunoblots of 10% of the input are shown in panels 1 and 2, immunoblots of the HA immunoprecipitates in panels 3 and 4. Size markers are depicted in kDa.
Figure S6. Western blots of coimmunoprecipitation of POC1B with FAM161A. Uncropped immunoblots of the coimmunoprecipitation assay of FAM161A and POC1B. Dashed rectangles indicate cropped immunoblots shown in Figure 5 and S5. The upper blots show 10% of the input used in the coimmunoprecipitation experiment, the middle blots the FLAG precipitates and the lower blots the HA precipitates. The protein bands were visualized by either staining with FLAG-antibody (left blots) or HA-antibody (right blots). Lane numbering as follows, lane 1: 3xFLAG-FAM161A + 3xHA-POC1B-WT; lane 2: 3xFLAG-FAM161A + 3xHA-POC1B-Gln67del; lane 3: 3xFLAG-FAM161A + 3xHA-POC1B-Arg106Pro; lane 4: 3xFLAG-FAM161A + 3xHA-p63; lane 5: 3xFLAG-FAM161A + 3xHA-lebercilin; lane 6: 3xFLAG-p63 + 3xHA-POC1B-WT; lane 7: 3xFLAG-p63 + 3xHA-POC1B-Gln67del; lane 8: 3xFLAG-p63 + 3xHA-POC1B-Arg106Pro; lane 9: 3xFLAG-nephrocystin-4 + 3xHA-RPGRIP1; lane 10: Marker. Size markers are depicted in kDa.
Disruption of the basal body protein POC1B results in autosomal recessive cone-rod dystrophy

**Figure S7. Localization studies of POC1B and FAM161A in hTERT-RPE1 cells.** Overlay photomicrographs of additional fields of hTERT-RPE1 cells corresponding to the images depicted in Figure S5-D. (A) Overlay images of localization upon overexpression in hTERT-RPE1 cells. PalmMyr-CFP-FAM161A (green) was targeted to the cell membrane and microtubules, and translocated mRFPP-POC1B wild type (red) from the cytosol towards the cell membrane and microtubules. (B) This translocation by PalmMyr-CFP-FAM161A (green) is not observed for mRFPP-POC1B-Gln67del (red) nor (C) mRFPP-POC1B-Arg106Pro (red) which both maintain their cytosolic localization. RPRIP1l (cyan) was used as transition zone marker of the cilium. Nuclei are stained with DAPI (blue). Scale bars: 10 μm.

**Table S1. Primer sequences for POC1B**

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**Table S2. Primer sequences for POC1B mRNA analysis**

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Tabelle 4. Variations in protein coverage from phylogenetic models.

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Genetic and clinical characterization of Pakistani families with Bardet-Biedl syndrome extends the genetic and phenotypic spectrum

Maleeha Maria,1,2,* Ideke J.C. Lamers,2,3,* Miriam Schmidts,2,3,4,5 Muhammad Ajmal,1 Sulman Jaffar,6 Ehsan Ullah,7,8 Bilal Mustafa,2 Shakeel Ahmad,2 Katia Nazmutdinova,4 Bethan Hoskins9, Erwin van Wijk,10,11 Linda Koster-Kamphuis,12 Muhammad Imran Khan,2 Phil L. Beales,6,13 Frans P.M. Cremers,2,11 Ronald Roeperman,2,3 Maleeha Azam,1,# Heleen H. Arts,2,3,14,# and Raheel Qamar1,15,16,#

1 Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan
2 Department of Human Genetics, Radboud University Medical Center, Nijmegen, the Netherlands
3 Radboud Institute for Molecular Life Sciences, Radboud University, Nijmegen, the Netherlands
4 Genetics and Genomic Medicine, UCL Institute of Child Health, 30 Guilford Street, London, UK
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Chapter 3

3.1 Abstract

Bardet-Biedl syndrome (BBS) is an autosomal recessive disorder that is both genetically and clinically heterogeneous. To date 19 genes have been associated with BBS, which encode proteins active at the primary cilium, an antenna-like organelle that acts as the cell’s signaling hub. In the current study, a combination of mutation screening, targeted sequencing of ciliopathy genes associated with BBS, and whole-exome sequencing was used for the genetic characterization of five families including four with classic BBS symptoms and one BBS-like syndrome. This resulted in the identification of novel mutations in BBS-associated genes ARL6 and BBS5, and recurrent mutations in BBS9 and CEP164. In the case of CEP164, this is the first report of two siblings with a BBS-like syndrome with mutations in this gene. Mutations in this gene were previously associated with nephronophthisis 15, thus the current results expand the CEP164-associated phenotypic spectrum. The clinical and genetic spectrum of BBS and BBS-like phenotypes is not fully defined in Pakistan. Therefore, genetic studies are needed to gain insights into genotype-phenotype correlations, which will in turn improve the clinician’s ability to make an early and accurate diagnosis, and facilitate genetic counseling, leading to directly benefiting families with affected individuals.
Characterization of families with BBS extends the genetic and phenotypic spectrum

3.2 Introduction

Bardet-Biedl syndrome (BBS) is a complex, heterogeneous, autosomal recessively inherited disorder. The 19 causative genes that have been identified thus far\(^1\) encode proteins that function at different sites of the primary cilium, a non-motile oblong sensory organelle that protrudes from the surface of most mammalian cells. Within the cilium, BBS proteins are involved in a wide variety of processes ranging from regulation of intraflagellar transport to chaperonin and GTPase activity. On retinal disease information database, RetNet: Summaries of genes and loci causing retinal diseases, more than 20 genes are enlisted as causative of BBS. The BBS phenotype is multi-systemic, its primary features are blindness, renal dysfunction, intellectual disability, polydactyly, obesity and hypogonadism\(^2\). In addition, secondary characteristics include hepatic malfunction, type 2 diabetes mellitus, slow growth, psychomotor delay, delayed speech development, hearing loss and cardiac malformations. A BBS diagnosis can be made with the presence of at least four cardinal features, or a combination of three cardinal plus two secondary features\(^2\). BBS is mostly inherited as an autosomal recessive trait, but there have been a few reports that indicate oligogenic inheritance for BBS\(^3\)\(^-\)\(^5\), however, this mode of inheritance has been under discussion\(^6\)\(^-\)\(^9\).

BBS is a rare disorder with differences in the prevalence of the disease in different populations. For example, among the total live births, in North America and Europe BBS affects 1 out of 140,000-160,000\(^10\)\(^,\)\(^11\), but BBS is more common in Newfoundland (1/17,000)\(^12\) as well as in Kuwaiti bedouins (1/13,500)\(^13\) and the Faroe Islands (1/3,700)\(^14\). These differences can be due to various factors including consanguinity, which is a social norm in countries such as Kuwait, Iran, Saudi Arabia and Pakistan. At the genomic level, about 10% of the total genome has been estimated to be homozygous in such families\(^15\). These homozygous regions generally contain the causative genetic mutation in recessive disorders such as BBS. The genetic defect is usually inherited from a single ancestor and is passed to the father and mother of the affected child who ultimately carries identical disease-causing (homozygous) mutations in both alleles of a gene\(^16\)\(^-\)\(^18\). In Pakistan more than 60% of the total marriages are consanguineous, and of these, about 80% are among first cousins\(^16\), which explains the high frequency of homozygous mutations in families affected with recessive disorders.

The prevalence of BBS in Pakistan is not yet known. To date, there have been only nine reports of 18 Pakistani families with mutations in eight genes already known to be involved in BBS: ARL6 (OMIM # 608845)\(^19\)\(^,\)\(^20\), BBS1 (OMIM # 209901)\(^21\), BBS2 (OMIM # 606151), BBS5 (OMIM # 603650)\(^22\), BBS9 (OMIM # 615986)\(^23\), BBS10 (OMIM # 610148)\(^19\)\(^,\)\(^24\)\(^,\)\(^25\), BBS12 (OMIM # 610683)\(^22\)\(^,\)\(^26\) and TTC8 (OMIM # 608132)\(^27\). Therefore, comprehensive studies are needed to further explore the genetic spectrum of BBS in the Pakistani population.

In this study, four families with classical BBS and one family with a BBS-like phenotype from Pakistan were genetically analyzed. In total, four homozygous mutations were identified,
including mutations in \textit{ARL6} (F01), \textit{BBS5} (F02 and F03), \textit{BBS9} (F04) and \textit{CEP164} (F05). The identified mutations in \textit{ARL6} and \textit{BBS5} are novel.

### 3.3 Methods

#### 3.3.1 Ethics statement
In the current study the recommendations of the Helsinki declaration were followed, and the “Ethics Review Board” of the COMSATS Institute of Information Technology, Islamabad and the contributing hospitals approved the study. The recruited families were informed in detail about the purpose of the study and their written consent was taken prior to blood sample collection and genetic analyses.

#### 3.3.2 Selection and clinical evaluation of BBS families
A total of five families were included in this study, in which the diagnosis of BBS was made based on the criteria described by Beales et al.\textsuperscript{2}, the phenotypes of these families are given in Table 1. Moreover, biochemical tests were performed that included urine (regular examination), serum creatinine and serum urea to evaluate renal function, gonadotropin levels were assessed to find indications for hypogonadism, and thyroid levels were analyzed to diagnose hypothyroidism (Supplementary Table S1). Abdominal and pelvic ultrasonography was also performed to assess anatomy of the vital organs, i.e. liver and kidneys. Cardiac function evaluation was done by electrocardiography (ECG). For the diagnosis of ocular abnormalities refraction testing, visual acuity testing and funduscopy were performed. Magnetic resonance imaging (MRI) for the assessment of brain anomalies were performed for one family (F05).

#### 3.3.3 DNA isolation
Blood samples of the affected and healthy individuals of BBS families were collected in ethylenediaminetetraacetic acid (EDTA) vacutainers. Genomic DNA was extracted from lymphocytes using a previously described standard protocol\textsuperscript{28} and then stored at -20°C until further use.

#### 3.3.4 Targeted mutation screening
BBS probands were first screened for previously reported mutations occurring in BBS-associated genes in Pakistani patients (Supplementary Table S2)\textsuperscript{29}. The exons in which mutations have been reported previously, were amplified and analyzed by Sanger sequencing using dye-termination chemistry (BigDye Terminator, version 3 on a 3730 or 2100 DNA analyzer; Applied Biosystems, Foster City, CA).

#### 3.3.5 Targeted exome sequencing (TES) of BBS genes
DNA of probands of whom no molecular diagnosis could be obtained after targeted mutation screening, were analyzed for mutations in 21 ciliopathy genes associated with BBS using
Fluidigm Technology (Supplementary Table S3). The targeted-exome sequencing (TES) analysis was performed on a next-generation sequencing MiSeq platform as previously reported \textsuperscript{30,31}.

### 3.3.6 Whole exome sequencing and in silico predictions
The DNA of proband V:2 of BBS family F05 in whom no causative mutation was identified by TES was further analyzed by whole-exome sequencing (WES) using an Illumina HiSeq2000 platform. Genomic DNA from the proband was purified with a QIAamp DNA mini kit (cat# 51304) according to the manufacturer’s instructions. WES was performed at the Beijing Genomics Institute (BGI). The Agilent SureSelect version 4 exome kit was used for whole exome capture and a set of Illumina HiSeq 2x100 bp reads was generated. 72,192 reads were uniquely mapped to gene-coding regions and the exome had a median coverage of 50x. Variants were prioritized from WES data by using dbSNP (Feb 2009 build, GRCh37/hg19) and an in-house SNP database consisting of 5,036 exomes. Variants were selected if they occurred at <0.5% in the above-mentioned databases. The data were further prioritized by selecting truncating variants, splice site variation (until positions +6/-6) and missense variants with a Grantham score of ≥80 and/or PhyloP ≥2.7 and/or PHRED scaled Combined Annotation–Dependent Depletion (CADD-PHRED) score >15, which means that the variant is ranked among the top 5% of deleterious variations\textsuperscript{32}. The cut-off values for the Grantham and PhyloP were based on a report of Vissers et al.\textsuperscript{33} The pathogenicity of variants was also assessed by various in silico programs including Polymorphism Phenotyping version 2 (Polyphen-2)\textsuperscript{34}, Sorting Intolerant From Tolerant (SIFT)\textsuperscript{35} and Mutation Taster\textsuperscript{36}. Supplementary Figure S1 summarizes the filtering protocol. Homozygous regions in WES data were identified by using the homozygosity mapper\textsuperscript{37}. CNV analysis was also performed on the WES data using the software Copy Number Inference From Exome Reads “CoNIFER version 0.2.2”\textsuperscript{38}. WES reads with mapping quality (MAPQ) score ≥20 were prioritized and respective binary sequence alignment map (BAM) files were used for CNV detection.

### 3.3.7 Mutation segregation analysis
Prioritized variants were first confirmed by Sanger sequencing followed by segregation analysis in the respective families.

### 3.3.8 Splice site prediction
The ARL6 synonymous mutation (c.534A>G; p.(Gln178Gln)) was identified in family F01 by TES. The effect of this synonymous change on splicing was assessed in silico using Alamut visual Version 2.7.1 from Interactive Biosoftware (http://www.interactive-biosoftware.com) (Supplementary Fig. S2).

### 3.3.9 Minigene splicing assay
The predicted effect of splice site mutation in ARL6 (c.534A>G; p.(Gln178Gln)) was validated by in vitro experiments using a minigene assay as previously described by Cooper\textsuperscript{39}. The amplified DNA fragments of 656 bp carrying ARL6 exon 8 along with its flanking intronic
sequences were cloned between RHO exon 3 and exon 5 in pClneo mammalian expression vector. HEK293T cells in passage 20 were transfected using polyethylenimine (PEI) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% pyruvate and 1% antibiotic mixture of penicillin and streptomycin followed by 48 hours of incubation at 37°C. Nucleospin kit (RNA-MACHERY-NAGEL-05/2014, Rev 16) was used for total RNA isolation, and iSCRIPT (BioRad) RT-PCR kit was used to perform reverse transcriptase (RT) PCR. Rhodopsin exon 3 forward and exon 5 reverse primers (Supplementary Table S4) were used to detect the effect of variant on splicing. The amplified fragments were electrophoretically separated on agarose gel followed by purification and Sanger sequencing (Fig. 1).
3.4 Results
Families F01, F02, F03 and F04 had classical BBS phenotypes, whereas family F05 was diagnosed as having BBS-like symptoms (Table 1).

3.4.1 Pre-screening of known mutations
None of the previously reported mutations in BBS1, BBS2, BBS5, BBS10, BBS12, ARL6 and TTC8 (Supplementary Table S2) in Pakistani BBS patients\textsuperscript{29}, were found in the current panel and were therefore excluded as the causative factor in these families.

3.4.2 TES revealed mutations in ARL6, BBS5 and BBS9
The TES analysis of the 21 previously reported ciliopathy genes associated with BBS (Supplementary Table S3) resulted in the identification of a novel synonymous variant c.534A>G; p.(Gln178Gln) in ARL6 in family F01, and a novel 11 bp deletion c.734_744del; p.(Glu245Glyfs*18) in BBS5 in families F02 and F03 (Fig. 2). A recurrent mutation in BBS9, c.1789C>T; p.(Gln597*)\textsuperscript{40}, was identified in family F04 (Fig. 2; Table 2). All three variants segregated with the disease phenotype.

![Pedigrees showing identified mutations segregating in BBS families](image)

**Fig. 2: Pedigrees showing identified mutations segregating in BBS families.** Squares and circles represent males and females, respectively. Unfilled symbols indicate healthy individuals and filled symbols indicate affected individuals, symbols with a diagonal line represent deceased individuals. ‘M’ represents the mutant allele and ‘+’ represents ancestral allele. Arrows in F05 indicate the individuals analyzed by WES.
Table 1: Clinical features of affected individuals from BBS families.

<table>
<thead>
<tr>
<th>Family ID</th>
<th>Pedigree ID</th>
<th>Diagnosis</th>
<th>Gender</th>
<th>Age (yrs) at diagnosis</th>
<th>BMI</th>
<th>CRD/RP</th>
<th>Polydactyly</th>
<th>Obesity</th>
<th>Intellectual disability</th>
<th>Hypogonadism</th>
<th>Renal anomalies</th>
<th>Additional features</th>
</tr>
</thead>
<tbody>
<tr>
<td>F01</td>
<td>IV:2</td>
<td>BBS</td>
<td>M</td>
<td>32</td>
<td>ND</td>
<td>Yes</td>
<td>Yes</td>
<td>ND</td>
<td>No</td>
<td>Yes</td>
<td>Renal parenchymal disease; deceased due to renal failure</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>IV:3</td>
<td></td>
<td>M</td>
<td>40</td>
<td>ND</td>
<td>Yes</td>
<td>Yes</td>
<td>ND</td>
<td>No</td>
<td>Yes</td>
<td>Elevated liver enzymes, abnormal ECG, gynaeomastia</td>
<td></td>
</tr>
<tr>
<td>F02</td>
<td>IV:1</td>
<td>BBS</td>
<td>M</td>
<td>47</td>
<td>25.2</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Bilateral renal calculi</td>
<td>Hypodontia, syndactyly, brachydactyly, ataxia, speech disability, gall bladder calculi, mild spleno- and hepatomegaly, elevated liver enzymes, abnormally high cholesterol level</td>
</tr>
<tr>
<td></td>
<td>IV:2</td>
<td></td>
<td>M</td>
<td>45</td>
<td>25.1</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Bilateral renal calculi</td>
<td>Speech disability</td>
</tr>
<tr>
<td>F03</td>
<td>IV:2</td>
<td>BBS</td>
<td>F</td>
<td>15</td>
<td>26.6</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Developmental delay, irregular menstruation, low progesterone levels, diabetes, borderline hepatomegaly with fatty infiltration, abnormally high cholesterol level, elevated liver enzymes</td>
</tr>
<tr>
<td>F04</td>
<td>IV:2</td>
<td>BBS</td>
<td>M</td>
<td>15</td>
<td>33.3</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Left kidney: focal calciectasis in upper and interpolar region</td>
<td>Elevated liver enzymes, hypodontia, speech disability, gynaeomastia</td>
</tr>
<tr>
<td>F05</td>
<td>V:2</td>
<td>BBS</td>
<td>M</td>
<td>20</td>
<td>33.8</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Gynaeomastia, cerebral atrophy</td>
</tr>
<tr>
<td></td>
<td>V:4</td>
<td>- like</td>
<td>F</td>
<td>25</td>
<td>32.9</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>ND</td>
<td>Yes</td>
<td>No</td>
<td>Irregular menstruation, Severe depression and psychosis at 26 yrs</td>
</tr>
</tbody>
</table>

Abbreviations: BBS: Bardet Biedl syndrome, BMI: Body mass index, CRD: Cone-rod dystrophy, ECG: Electrocardiogram, ID: Identity, NA: Not applicable, ND: Not determined, RP: Retinitis pigmentosa, yrs: Years. *Also refer to Table S1.
Table 2: Genetic variations identified by targeted exome sequencing of 21 BBS-associated genes in Pakistani BBS families.

<table>
<thead>
<tr>
<th>Family Id</th>
<th>Gene</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Protein variant</th>
<th>ExAC allele frequency (Total)</th>
<th>SIFT² (score)</th>
<th>Polyphen V2²</th>
<th>Mutation Taster² (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F01</td>
<td>ARL6</td>
<td>c.534A&gt;G</td>
<td>c.534A&gt;G</td>
<td>p. = p.(Q178Q)</td>
<td>1/121,316</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>F02</td>
<td>BBS5</td>
<td>c.734_744del</td>
<td>c.734_744del</td>
<td>p.(E245Gfs*18)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>F03</td>
<td>BBS5</td>
<td>c.734_744del</td>
<td>c.734_744del</td>
<td>p.(E245Gfs*18)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>F04</td>
<td>BBS9</td>
<td>c.1789C&gt;T</td>
<td>c.1789C&gt;T</td>
<td>p.(Q597*)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>F05</td>
<td>BBS12</td>
<td>c.2014G&gt;A</td>
<td>+</td>
<td>p.(A672T)</td>
<td>149/119,520</td>
<td>Del (0.0)</td>
<td>DC (1.0)</td>
<td>PrD (1.000)</td>
</tr>
</tbody>
</table>

Abbreviations: DC: Disease causing, Del: deleterious, PrD: Probably damaging, ExAC: Exome aggregation consortium, SIFT: Sorting intolerant from tolerant, NA: Not applicable, Polyphen V2: Polymorphism phenotyping version2. In case of protein truncating mutations these values are not applicable.

Table 3: Filtered variants after WES analysis in family F05.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Gene</th>
<th>Variation</th>
<th>Zygosity</th>
<th>Depth</th>
<th>ExAc frequency Total</th>
<th>phyloP</th>
<th>CADD_ PHRED</th>
<th>Grantham Score</th>
<th>SIFT (score)</th>
<th>Polyphen V2</th>
<th>Mutation taster (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>CEP164</td>
<td>c.277C&gt;T; p.(R93W)</td>
<td>Hom</td>
<td>34</td>
<td>1/121,408</td>
<td>4.165</td>
<td>29</td>
<td>101</td>
<td></td>
<td></td>
<td>DC (1.0)</td>
</tr>
<tr>
<td>4</td>
<td>BBS12</td>
<td>c.2014G&gt;A; p.(A672T)</td>
<td>Het</td>
<td>115</td>
<td>149/119,520</td>
<td>5.869</td>
<td>34</td>
<td>58</td>
<td></td>
<td></td>
<td>DC (1.0)</td>
</tr>
</tbody>
</table>

in the respective families, which means that the affected individuals in these families were homozygous for the detected mutations while their unaffected relatives were not. TES analysis revealed a heterozygous mutation in BBS12, (c.2014G>A; p.(Ala672Thr)) in family F05, which did not segregate in this family (Table 2), in addition no CNVs were detected, therefore family F05 was further analyzed by WES.

3.4.3 Functional validation of the ARL6 synonymous variant c.534A>G; p.(Gln178Gln)

In silico analysis using splice prediction tools predicted that the synonymous change c.534A>G; p.(Gln178Gln) found in ARL6 in F01, could affect the normal splicing of exon 8 (Supplementary Fig. S2). The predicted effect on splicing of exon 8 was validated with a minigene splice assay, which confirmed that the c.534A>G is a variant that causes skipping of exon 8 of ARL6 (Fig. 1). Exon skipping in turn results in a premature stop codon at residue 160 (p.(Cys160*)).

3.4.4 WES identified a CEP164 variant in family F05

The variants identified by WES analysis (Table 3) of family F05 were selected after in silico analyses, out of which the recurrent missense mutation c.277C>T; p.(Arg93Trp) in CEP164 segregated with the disease phenotype in this family (Fig. 2; Supplementary Fig. S1; Table 3).

3.4.5 LOVD mutation updates

The identified variants in the respective families were not found in the control individuals. We have uploaded all the variants in the respective Leiden Open (source) Variation Databases (LOVDs), uploaded data are available at http://databases.lovd.nl/shared/genes/ARL6, http://databases.lovd.nl/shared/genes/BBS5, http://databases.lovd.nl/shared/genes/BBS9 and http://databases.lovd.nl/shared/genes/CEP164. As an alternate LOVD gene specific data can be viewed by typing www.lovd.nl/ followed by gene symbol.

3.5 Discussion

In the current study genetic characterization of five families from Pakistan are reported, four families had classic BBS and one family was diagnosed with a BBS-like syndrome. Two novel causative homozygous variants were identified in ARL6 and BBS5 in three families F01, F02 and F03, and previously reported variants were detected in BBS9 and CEP164 in the remaining two families F04 and F05, respectively.

In family F01, a novel synonymous variant c.534A>G; p.(Gln178Gln) was identified in exon 8 of ARL6. Although this variant does not affect the glutamine codon, in silico analysis predicted this mutation to result in aberrant splicing (Supplementary Fig. S2). As adenine at position 534 is the second last nucleotide in exon 8, and is located in the consensus splice site sequence, the transition of this adenine to a guanine will likely result in exon-skipping during splicing. Result of an in vitro splicing assay confirmed the generation of mutated mRNA and provides support for the pathogenic nature of this novel variant. Exon 8 of ARL6 indeed appeared to be skipped during the splicing process, which results in a frameshift that causes a premature stop codon at position 160 (p.(Cys160*)) that may lead to the production of a truncated ARL6
peptide. However, aberrantly spliced mRNAs containing premature stop codons are usually degraded in a process that is known as nonsense mediated decay (NMD)\(^{42,43}\). However, when analyzing the phenotypes of the affected siblings in F01 (IV:2 and IV:3) having the common genetic defect, it is obvious that besides clinical overlap there are also clinical differences between the siblings. For example, proband (IV:3) had no renal disease, whereas his affected brother (IV:2) died because of renal failure at the age of 35 (Table 1). In addition, the degree of polydactyly varies between the siblings, i.e. while IV:2 had postaxial hexadactyly of both hands and feet, IV:3 only displayed hexadactyly of both feet.

In families F02 and F03, a novel frameshift mutation c.734_744del; p.(Glu245Glyfs*18) was found in exon 9 of BBS5. The frameshift probably creates a null allele since the mRNA is likely to be degraded by NMD. In case of the synthesis of a truncated BBS5 protein, 78 amino acid residues from the C-terminus will be missing, which might affect BBSome assembly\(^{44,45}\) that in turn is likely to disturb normal ciliary transport mechanisms. Both families (F02 and F03) originate from the same region in Pakistan and we hypothesized that these families may be related to each other. However, both families had no information on their possible relationship. Despite having the same mutation, phenotypic variability was apparent within and between both families. Within family F02, proband (IV:1) has post axial hexadactyly on left foot, syndactyly of middle and ring finger on left hand and brachydactyly of both feet, whereas his affected sibling (IV:2) did not (Table 1). With respect to the clinical variation between families, it is apparent that the affected female in F03 did not have intellectual disability or renal anomalies, while these features were reported in both affected individuals of family F02 (Table 1). In addition, Table 1 shows various other phenotypic differences between families and patients. As observed for F01, phenotypic variability within and between F02 and F03 may be attributed to yet unknown modifying factors\(^{21,46}\). Generally, the existence of modifier alleles imposes a great challenge in establishing clear-cut genotype-phenotype correlations and complicates an accurate prognosis for affected individuals.

In family F04 a previously reported nonsense mutation c.1789C>T; p.(Gln597*) was identified in BBS9 by TES. This mutation had previously been reported in a homozygous state as the primary cause of a form of hereditary blindness known as retinitis pigmentosa (RP) in a Latino proband\(^{40}\). Other criteria for BBS in the Latino proband were not determined while the current proband IV:2 from family F04 was fully characterized and diagnosed with BBS (Table 1). With incomplete data of the Latino proband, the phenotypes cannot be compared between the current and the previous study. At the molecular level, as a result of this mutation, BBS9 transcripts in this family (F04) are likely to be degraded by NMD. Alternatively, synthesis of a truncated protein may lead to disrupted BBSome assembly\(^{47}\), ciliary biogenesis and function\(^{48}\).

Based on results from previous studies and our current study, eight out of the 21 BBS-associated genes have been found mutated in Pakistani patients with BBS features (Supplementary Fig. S3). Thus far, 44 patients from 22 BBS-families\(^{19-27,49}\), including four BBS
families from the current study, have been identified in Pakistan. In this group, BBS10 mutations were most common and were identified in 27% of the cases, and mutations in BBS5 were the second most common and explained 18% of the reported BBS families (Supplementary Fig. S3).

This analysis does not include the BBS-like family F05 from the present study. Affected individuals in family F05 were initially suspected of BBS based on the co-occurrence of RP, obesity, intellectual disability and hypogonadism in this family (Table 1). As F05 is a consanguineous family, homozygosity mapping was performed on the WES data, which revealed the presence of candidate gene CEP164 in the fourth-largest homozygous region of 16 Mb (Supplementary Table S5). This was an interesting observation as this gene had previously been associated with Nephronophthisis (NPHP) 15 [OMIM: #614845] that is characterized by various ciliopathy features. WES analysis of proband V:2 indeed identified a homozygous missense mutation c.277C>T; p.(Arg93Trp) in CEP164 in family F05. Although structural information of the CEP164 protein remains absent to date, the affected amino acid is just downstream of the predicted WW domain of the protein (aa 57-89) that is known to mediate protein-protein interactions and might affect its structure. The p.(Arg93Trp) variant had previously been reported in a compound heterozygous state together with a truncating mutation c.1573C>T; p.(Gln525*) on the second allele in a family with three affected individuals of whom two presented with NPHP and blindness, while the third patient also had mild intellectual disability. The F05 probands also had neural defects; V:2 had mild cerebral atrophy. In addition, psychological problem was observed in V:4 who developed obsessive compulsive disorder and psychosis at the age of 26 years. Remarkably, while all but one family with CEP164 defects (c.277C>T; p.(Arg93Trp) and other mutations) reported by Chaki et al. had renal insufficiency during childhood; the affected individuals in F05 did not have any sign of renal anomalies at 21 and 26 years of age (Table 1), respectively. A single patient without renal insufficiency reported by Chaki et al. was described as a nonsyndromic RD (LCA) patient, a mild phenotype compared with the syndromic features of other patients carrying mutations in CEP164 and likely caused by the specific missense mutation of the CEP164 stop codon in this patient. Family F05 in our study was thus diagnosed with BBS-like syndrome instead of NPHP15 or BBS. It is interesting to note that Chaki et al. also reported a patient with a homozygous p.(Arg576*) mutation who showed a broader syndromic phenotype that included cerebellar vermis hypoplasia, bilateral polydactyly, abnormal liver function and obesity. In conclusion, our results confirm that mutations in CEP164 can result in a broadly variable clinical outcome between and within families, varying from non-syndromic retinal degeneration to a BBS-like phenotype, which implies that there are restrictions on making an accurate diagnosis and prognosis in these families. Molecularly, the phenotypic resemblance with BBS is in line with the shared direct molecular association of both CEP164 and the BBSome with Rabin8, which mediates membrane assembly of the primary cilia.
3.6 Conclusion
Our data confirm inter-/intra-familial clinical heterogeneity in patients having common genetic defects in BBS genes, and describe a broader clinical phenotypic spectrum resulting from mutations in \textit{CEP164}, extending it beyond retinal-renal ciliopathies to a BBS-like phenotype. Further studies are needed to establish if certain phenotypic features are associated with dysfunction of specific BBS genes, and if phenotypic differences between and within families can be explained by genetic modifiers. These molecular insights are helpful in genetic counseling of the affected families to prevent disease inheritance in the next generations.
3.7 References

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syndrome family with two mutant genes (BBS2, BBS10), three mutations, but no triallelism.
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21. Ajmal, M. et al. Exome sequencing identifies a novel and a recurrent BBS1 mutation in
23. Khan, M.A., Mohan, S., Zubair, M. & Windpassinger, C. Homozygosity mapping identified a
novel protein truncating mutation (p.Ser100Leufs*24) of the BBS9 gene in a consanguineous
Pakistani family with Bardet Biedl syndrome. BMC Med Genet 17, 10 (2016).

3.8 Acknowledgements
We are thankful to the contributing families. This study was funded by grant no 1082 to R.Q. from the Pakistan Academy of Sciences, Islamabad, Pakistan. H.H.A., P.B., K.N., and R.R. received support from the Dutch Kidney Foundation (CP11.18 “KOUNCIL”). H.H.A and R.R. received an institutional grant from the Radboud Institute for Molecular Life Sciences (RIMLS). M.S. is funded by a Hypatia Tenure Track Fellowship (Radboudumc Nijmegen and Radboud Institute for Molecular Life Sciences) and a Radboud University Excellence Fellowship, and acknowledges funding from the German Research Foundation (DFG, grant SFB-1411, KIDGEM (Kidney Disease from gene to mechanism). This work was financially also supported by the Dutch Organisation for Scientific research (NWO Veni grant 91613008 to H.H.A.; NWO Veni grant 016.136.091 to E.v.W.; NWO Vici grant 865.12.005 to R.R.), the Dutch Organisation for Health Research and Development (ZonMW E-rare grant "Eur-USH" 40-42900-98-1006), the Dutch eye foundations (Stichting Nederlands Oogheelkundig Onderzoek, SAFDOR and Stichting Blindenhulp) to E.v.W. M.M. is thankful to the Higher Education Commission (HEC) of Pakistan for supporting her studies.

3.9 Author contributions
Characterization of families with BBS extends the genetic and phenotypic spectrum

3.10 Supplemental data

Supplementary Fig. S1: WES data filter summary for family F05.

Supplementary Fig. S3: Genetic frequency of eight BBS-associated genes from 22 Pakistani BBS families. Eighteen families were previously reported while 4 BBS families are part of this study, where most of the Pakistani families carry mutations in BBS10.
Supplementary Fig. S2: *In silico* analysis of novel synonymous mutation in *ARL6* (c.534A>G; p.=) identified causative factor in family F01. The changes in scores between upper panel (reference sequence) and lower panel (mutated sequence) indicate an effect on splicing of exon 8. Source: Alamut visual Version 2.7.1 from Interactive Biosoftware (http://www.interactive-biosoftware.com).
Supplementary Table S1: The results of biochemical tests with abnormal values.

<table>
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<th>Individual ID</th>
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<th>Result (Normal)</th>
</tr>
</thead>
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<td>RFT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urea</td>
<td>198 mg/dL (13-43 mg/dL)</td>
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<tr>
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<td>Creatinine</td>
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<td></td>
<td>IV:3</td>
<td>LFT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>ALT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>ALP</td>
<td>321 U/L (98-279 U/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>serum triglycerides</td>
<td>167 mg/dL (&lt;160 mg/dL)</td>
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<td></td>
<td></td>
<td>HDL cholesterol</td>
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</tr>
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<td></td>
<td></td>
<td>Creatinine</td>
<td>0.7 mg/dL (0.7-1.3 mg/dL)</td>
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<td>LFT</td>
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<tr>
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<td>ALT</td>
<td>86 U/l (&lt;40 U/L)</td>
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<td>ALP</td>
<td>283 U/L (98-279 U/L)</td>
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<td></td>
<td></td>
<td>AST</td>
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<td>LPT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Serum triglycerides</td>
<td>350 mg/dL (&lt;160 mg/dL)</td>
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<td>Serum cholesterol</td>
<td>204 mg/dL (&lt;200 mg/dL)</td>
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<td>HDL cholesterol</td>
<td>30 mg/dL (35-40 mg/dL)</td>
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<td>GTPT</td>
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</tr>
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<td></td>
<td></td>
<td>LH</td>
<td>0.58 mIU/mL (1.14-8.75 mIU/mL)</td>
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<td>Testosterone</td>
<td>0.39 ng/mL (1.66-8.77 ng/mL)</td>
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<td>IV:2</td>
<td>Lactate</td>
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<td>Plasma Lactic acid</td>
<td>22.5 mg/dL (4.5-19.8 mg/dL)</td>
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<tr>
<td></td>
<td></td>
<td>Testosterone</td>
<td>1.27 ng/mL (1.66-8.77 ng/mL)</td>
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<td></td>
<td></td>
<td>LFT</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>ALT</td>
<td>50 U/l (&lt;40 U/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALP</td>
<td>368 U/L (98-279 U/L)</td>
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<td>LPT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serum triglycerides</td>
<td>253 mg/dL (&lt;160 mg/dL)</td>
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<td></td>
<td></td>
<td>Serum cholesterol</td>
<td>243 mg/dL (&lt;200 mg/dL)</td>
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<tr>
<td></td>
<td></td>
<td>LDL cholesterol</td>
<td>155 mg/dL (100-130 mg/dL)</td>
</tr>
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<td></td>
<td></td>
<td>GTPT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Progesterone</td>
<td>0.1 ng/mL (1.2-15.9 ng/mL)</td>
</tr>
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<td>F04</td>
<td>IV:2</td>
<td>LFT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALT</td>
<td>40 U/l (&lt;40 U/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALP</td>
<td>462 U/L (98-279 U/L)</td>
</tr>
</tbody>
</table>
### LPT
- LDL cholesterol: 90 mg/dL (100-130 mg/dL)
- HDL cholesterol: 27 mg/dL (100-130 mg/dL)

### GTPT
- Prolactin: 32.63 ng/mL (3.46-19.40 ng/mL)
- Lactate: 329 U/L (125-243 U/L)
- Plasma Lactic acid: 20.1 mg/dL (4.5-19.8 mg/dL)

### F05
<table>
<thead>
<tr>
<th>V:2</th>
<th>RFT</th>
<th>Creatinine: 1.3 mg/dL (0.7-1.3 mg/dL)</th>
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<tr>
<td></td>
<td>LFT</td>
<td>ALT: 55 U/L (&lt;40 U/L) ALP: 340 U/L (98-279 U/L)</td>
</tr>
</tbody>
</table>
|     | LPT | Serum cholesterol: 90 mg/dL (100-130 mg/dL)
Serum triglycerides: 180 mg/dL (100-130 mg/dL)
HDL cholesterol: 34 mg/dL (100-130 mg/dL)
|     | GTPT | Testosterone: 0.0029 ng/mL (1.66-8.77 ng/mL) |

### V:4
| GTPT | Testosterone: 0.0008 ng/mL (1.66-8.77 ng/mL) |

Characterization of families with BBS extends the genetic and phenotypic spectrum

**Supplementary Table S2: Targeted mutations prescreened in five probands from affected families.** These mutations were reported from Pakistani BBS population (Khan et al.\textsuperscript{29}).

<table>
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<tr>
<th>Gene</th>
<th>RefSeq Id</th>
<th>Nucleotide variant</th>
<th>Protein variant</th>
<th>Exons</th>
<th>References</th>
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<td>BBS1</td>
<td>NM_02464.9.4</td>
<td>c.47+1G&gt;T</td>
<td>p.(?)</td>
<td>Ex1</td>
<td>1</td>
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<tr>
<td>BBS1</td>
<td>NM_02464.9.4</td>
<td>c.442G&gt;A</td>
<td>p.(D148N)</td>
<td>Ex5</td>
<td>2</td>
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<tr>
<td>BBS10</td>
<td>NM_024685.3</td>
<td>c.271dup</td>
<td>p.(C911fs*5)</td>
<td>Ex2</td>
<td>3</td>
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<tr>
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<td>c.1091del</td>
<td>p.(N364Tfs*5)</td>
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<td>3</td>
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<td>BBS10</td>
<td>NM_024685.3</td>
<td>c.1958_1967del</td>
<td>p.S653fs*4</td>
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<td>4</td>
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<td>BBS10</td>
<td>NM_024685.3</td>
<td>c.2121dup</td>
<td>p.(K708*)</td>
<td>Ex2</td>
<td>3</td>
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<tr>
<td>BBS12</td>
<td>NM_152618.2</td>
<td>c.1589T&gt;C</td>
<td>p.(L530P)</td>
<td>Ex3</td>
<td>5</td>
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<tr>
<td>BBS12</td>
<td>NM_152618.2</td>
<td>c.2102C&gt;A</td>
<td>p.(S701*)</td>
<td>Ex3</td>
<td>6</td>
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<tr>
<td>BBS2</td>
<td>NM_031885.3</td>
<td>c.1237C&gt;T</td>
<td>p.(R413*)</td>
<td>Ex11</td>
<td>5</td>
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<tr>
<td>ARL6</td>
<td>NM_032146.3</td>
<td>c.123+1119del</td>
<td>p.(?)</td>
<td>Intron 4</td>
<td>7</td>
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<td>BBS5</td>
<td>NM_152384.2</td>
<td>c.2T&gt;A</td>
<td>p.(M1K)</td>
<td>Ex1</td>
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<tr>
<td>TTC8</td>
<td>NM_144596.2</td>
<td>c.1049+2_1049+4del</td>
<td>p.(?)</td>
<td>Ex11</td>
<td>8</td>
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Abbreviations: RefSeq Id: Reference sequence identity
**Supplementary Table S3:** Transcripts and exons of 21 BBS-associated genes sequenced in targeted next generation sequencing panel.

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Characterization of families with BBS extends the genetic and phenotypic spectrum

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Abbreviation: Chr #: Chromosome number
Chapter 3

Supplementary Table S4: Primers used for the Sanger sequencing of RT-PCR products in minigene splicing assay.

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Supplementary Table S5: Homozygosity mapping on WES data of the proband from family F05 using GRCh37/hg19 reference assembly. The cutoff selected for significant homozygous regions is 1Mb.

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Abbreviations: Chr: Chromosome, Mb: Megabases, WES: Whole exome sequencing
3.11 Supplemental references

The exocyst interactome unveils potential plasma membrane docking sites at the base of the cilium

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Manuscript in preparation.
Chapter 4

4.1 Abstract
Defects in primary cilia, the slender signaling organelles protruding from most eukaryotic cell surfaces, result in severe hereditary disorders with overlapping phenotypes often affecting multiple organs. Various studies have shown that the octameric exocyst complex, that regulates polarized exocytosis by tethering post-Golgi vesicles to the plasma membrane, also plays a role in the cilium. Recently, mutations in EXOC4 and EXOC8 have been found in ciliopathy patients with Meckel-Gruber and Joubert syndrome. As the molecular relationship between the exocyst and the cilium remains unexplored, we set out to elucidate the ciliary involvement of the exocyst complex with a proteomics approach. The ciliary localization of all exocyst components was studied in ciliated retinal pigment epithelium cells (hTERT-RPE1) which confirmed enrichment at the basal bodies/centrioles for all components. No effects of the published mutations were detected on localization in hTERT-RPE1 cells. To analyze the interactome of the exocyst complex, individual exocyst components were overexpressed in renal cells (HEK293T) after which tandem affinity purification was performed followed by mass spectrometry. This yielded a protein network of 309 proteins that were linked to several functional categories and subcellular sites, including the cilium. Identification of EXOC6B with a subset of the exocyst complex members suggests that this non-canonical exocyst component is part of an alternative exocyst assembly. Yeast two-hybrid screening to identify binary interactors of the ciliopathy-associated exocyst members EXOC4 and EXOC8 identified 57 candidate interactors, of which three are structural components of the transition zone at the ciliary base. An integrated view of the data supports the ciliary role of the exocyst and identified several protein interactions that allow for a model in which the exocyst organizes a specific plasma membrane docking site at the base of the primary cilium.
4.2 Introduction

Polarized exocytosis, or the fusion of vesicles at specific sites of the plasma membrane, is essential for a wide range of biological functions in the cell, such as cell growth and migration. The initial contact between the vesicle and the plasma membrane (referred to as tethering) is mediated by the exocyst complex, a 750 kDa protein assembly consisting of eight subunits named EXOC1 till EXOC8.\(^1\) \(^2\) Six of the complex members were originally identified in *Saccharomyces cerevisiae*, while their homologues were later found in multicellular eukaryotes, indicating the high conservation of exocyst genes throughout evolution.\(^3\) Recent elegant work of Picco et al. has generated a structural 3D model of the assembled complex *in vivo*, where they show it is composed of rod-shaped subunits which together have the conformation of an open hand where EXOC5 and EXOC6 bind the vesicular membrane and EXOC1 and EXOC7 bind the plasma membrane.\(^4\) In this way, the complex is able to tether the vesicle close to the plasma membrane, but still allowing space for the SNARE proteins to perform the actual fusion of the membranes (Figure S1).

One organelle linked to polarization of cells and tissues is the primary cilium, an antenna-like microtubule based protrusion from the plasma membrane which in quiescent cells projects from a membrane-docked mother centriole, the ciliary basal body. Cilia are involved in sensing environmental and extracellular cues, and thus are essential for processes such as embryogenesis, neuronal development, renal function, and sensory functions (vision, taste, smell).\(^5\) Since cilia are present on virtually all polarized cells in the human body,\(^6\) defects in ciliary genes can cause severe hereditary phenotypes ranging from isolated congenital retinal degeneration to renal cysts to complex phenotypes where multiple organ systems are affected.\(^6\) \(^8\) Interestingly, two ciliopathies have recently been associated with mutations in a single exocyst complex subunit. Whole exome sequencing of Arab families with Meckel-Gruber syndrome (MKS) identified a missense mutation, p.(Gln578Arg), in EXOC4 [MIM: 608185],\(^9\) and a missense mutation in EXOC8 [MIM: 615283], p.(Glu265Gly), has been found in a family with Joubert syndrome (JBTS).\(^10\) These two neurodevelopmental disorders are genetically and clinically related. JBTS is characterized by hypotonia, ataxia and developmental delay combined with a distinctive cerebellar and brain stem malformation. MKS is more severe and perinatally lethal, and is characterized by occipital encephalocele, renal cysts, polydactyly, and hepatic fibrosis.\(^11\) Several exocyst complex members have been found to localize to the primary cilium or basal bodies/centrioles,\(^12\) \(^15\) and some individual members are essential for ciliogenesis *in vitro*.\(^16\) \(^17\) The mother centriole appendage protein centriolin interacts with EXOC6,\(^18\) and an homologue of EXOC4 is involved in basal body docking in *Xenopus laevis* multi-ciliated cells.\(^19\) A study in zebrafish showed that antisense morpholinos against Sec10 (EXOC5 in human) gave rise to a ciliopathy phenotype, characterized by a curled up tail, left-right asymmetry defects, small eyes and edema.\(^20\)

The general model of the ciliary involvement of the exocyst which arises from these studies, is that the exocyst complex is involved in polarized transport and docking of vesicles carrying ciliary proteins at the base of the cilium. Moreover, it is proposed that the exocyst is involved
in the early stages of ciliogenesis when the basal bodies are docking at the plasma membrane before axonemal elongation occurs. However, the mechanistic details of the ciliary involvement of the exocyst complex remains largely unknown. In this study, we aim to elucidate the protein interaction network of the exocyst complex and specifically its binary interactions with ciliary proteins, in order to further dissect the role of the exocyst complex in the primary cilium.

4.3 Materials and methods

4.3.1 cDNA constructs

All expression constructs were created with Gateway technology (Life Technologies) according to the manufacturer’s instructions. These constructs encoded each exocyst subunit (EXOC1, EXOC2, EXOC3, EXOC4, EXOC5, EXOC6, EXOC7, and EXOC8), EXOC6B, or CEP89, with either N-terminal eCFP tag, C-terminal eYFP tag, N- or C-terminal Strep-tag II FLAG tag for tandem affinity purification tag (SF-TAP-tag), N-terminal 3xHA tag or N-terminal 3xFLAG tag. All cDNA constructs encoded full length human proteins and were generated by Gateway-adapted PCR and subsequently cloned. Constructs encoding EXOC4 with variant p.(Gln578Arg) and EXOC8 with variant p.(Glu265Gly) were generated by site-directed mutagenesis PCR. The sequence of all entry clones was verified by Sanger sequencing.

4.3.2 Strep/FLAG Tandem Affinity Purification (SF-TAP)

Human Embryonic Kidney 293T cells (HEK293T) were transfected with constructs encoding EXOC1, EXOC2, EXOC3, EXOC4, EXOC5, EXOC6, EXOC7, EXOC8, or EXOC6B fused to a SF-TAP-tag using polyethyleneimine (PEI, PolySciences) as a transfection reagent. At 48 h after transfection the cells were lysed in lysis buffer containing 0.5% NP-40 in TBS (30mM Tris-Cl pH7.4, 150mM NaCl), freshly supplemented with protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail 2 (Sigma-Aldrich) and PhosphataseAssrest III (Gbiosciences) for 20 min at 4°C. Cell debris and nuclei were removed by centrifugation at 10,000g for 10 min. The streptavidin- and FLAG-based tandem affinity purification steps were performed as previously described. In short, the cleared supernatant was incubated for 2h at 4°C with Strep-Tactin superfloow (IBA). Next, the resin was washed three times in wash buffer (0.1% NP-40 in TBS supplemented with the same set protease inhibitors). Protein baits were eluted with Strep-elution buffer (2mM desthiobiotin in TBS). For the second purification step, the eluates were transferred to anti-Flag M2 agarose (Sigma-Aldrich) and incubated for 2h at 4°C. The beads were washed three times with wash buffer and proteins were eluted with FLAG peptide (200 µg/ml, Sigma-Aldrich) in TBS. The final elute was subjected to protein precipitation with chloroform and methanol, and protein precipitates were subsequently subjected to mass spectrometry analysis and peptide identification as previously described.

4.3.3 Yeast two-hybrid assay

The GAL4-based yeast two-hybrid system (HybriZAP, Stratagene) was used to identify binary protein-protein interaction partners of EXOC4 and EXOC8. The construct encoding the full length protein was fused to a DNA binding domain (GAL4-BD), and used as a bait to screen
the following libraries: human oligo-dT primed retinal-, kidney- and fetal brain cDNA library. The yeast strain PJE69-4A, which carried the HIS3 (histidine), ADE2 (adenine) and LacZ (β-galactosidase) reporter genes, was used as a host. Interactions were analyzed by assessment of reporter gene activation via growth on selective media (HIS3 and ADE2 reporter genes) and β-galactosidase colorimetric filter lift assays (LacZ reporter gene). cDNA inserts of clones containing putative interaction partners were confirmed by Sanger sequencing.

4.3.4 Localization in hTERT-RPE1 cells
Human TERT-immortalized Retinal Pigment Epithelium 1 cells (hTERT-RPE1) were cultured as previously described. Cells were seeded on coverslips, grown to 80% confluency and subsequently serum-starved for 48 hours in medium containing only 0.2% fetal calf serum to induce cilium growth. The cells were then transfected using Effectene Transfection Reagent (Qiagen) or Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. After 24 hours, cells were fixed in 4% paraformaldehyde for 20 minutes, treated with 1% Triton X-100 in PBS for 5 minutes and blocked in 2% bovine serum albumin (BSA) in PBS for 20 minutes. Cells were incubated with primary antibody GT335 (1:500) as cilium and basal body marker, diluted in 2% BSA in PBS, for 1 hour. After washing in PBS, the cells were incubated with the secondary antibody for 45 minutes. Secondary antibodies, goat-anti-mouse Alexa 488 or 568 (1:500; Life Technologies), were diluted in 2% BSA in PBS. Cells were washed with PBS and briefly with milliQ before being mounted in Vectashield containing DAPI (Vector Laboratories). The cells were analyzed with a Zeiss Axio Imager Z2 fluorescence microscope equipped with a 63x objective lens. Optical sections were generated through structured illumination by inserting an ApoTome slider into the illumination path, followed by processing using Zen 2012 (Zeiss) and Photoshop CC 2015 software (Adobe Systems).

4.3.5 Co-immunoprecipitation
EXOC4 (wild type or variant p.(Gln578Arg)) and CEP89 with an N-terminal 3xHA or 3xFLAG tag respectively, or vice versa, were co-synthesized in HEK293T cells. As a negative control, the functionally unrelated p63 with a 3xFLAG tag was co-synthesized with both 3xHA-EXOC4 and 3xHA-CEP89. As positive controls, the previously described interactions between nephrocystin-4 (encoded by NPHP4) and RPRGIP1,24 and between SF-TAP-EXOC3 and 3xHA-EXOC4 were used. After 24 hours of expression of the constructs, cells were lysed on ice in lysis buffer (50 mM Tris–HCL (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol) supplemented with complete protease inhibitor cocktail (Roche). Lysates were incubated with anti-FLAG M2-agarose from mouse (Sigma-Aldrich), for 2.5 hours at 4°C. After incubation, beads with bound protein complexes were washed in lysis buffer and subsequently taken up in 1x NuPAGE Sample Buffer and heated for 10 minutes at 70°C. Beads were precipitated by centrifugation, and supernatant was run on a NuPAGE Novex 4%–12% Bis-Tris SDS–PAGE gel. The interaction of EXOC4 with CEP89 was assessed by immunoblotting, followed by staining with either polyclonal rabbit anti-HA or polyclonal rabbit anti-FLAG (1:1,000; Sigma-Aldrich) as a primary antibody and goat-anti rabbit IRDye800 (1:20,000; Li-Cor) as a secondary antibody. Fluorescence was analyzed on a Li-Cor Odyssey 2.1 infrared scanner (Li-Cor).
4.4 Results

4.4.1 All exocyst subunits enrich around basal bodies

We first examined the localization of all exocyst complex subunits in relation to the cilium by transfecting constructs encoding N-terminally eCFP tagged proteins in ciliated hTERT-RPE1 cells. All recombinantly expressed exocyst subunits showed enrichment around the basal bodies, however, we could not detect localization along the ciliary axoneme when a cilium was present (Figure 1). Generally, all overexpressed fusion proteins are dispersed throughout the cytosol, but EXOC5, EXOC6 and EXOC7 also show higher intensities in aggregates or specific areas in the cell. These localization patterns were replicated with C-terminally tagged constructs (Figure S2).

Figure 1. Localization of all eight exocyst members in ciliated cells. Ciliated hTERT-RPE1 cells were transfected with N-terminally eCFP tagged constructs of the following individual exocyst members: (A) EXOC1, (B) EXOC2, (C) EXOC3, (D) EXOC4, (E) EXOC5, (F) EXOC6, (G) EXOC7 or (H) EXOC8 (all in green). In all pictures, GT335 is used as basal body and cilium marker (red) and DAPI for the nuclei (blue). Magnifications of the basal body region are shown in the insets. Additional pictures with C-terminally eYFP tagged constructs are shown in Figure S2.

4.4.2 The exocyst interaction network

To further unravel the molecular connectivity of the exocyst with the cilium, we performed affinity proteomics (Strep/FLAG tandem affinity purifications (SF-TAP)\(^\text{25}\) followed by mass spectrometry) for all individual exocyst subunits in HEK293T cells. These cells generate a primary cilium under the conditions used in our experiments, as shown before.\(^\text{22}\)
The exocyst interactome unveils potential plasma membrane docking sites at the base of the cilium

Figure 2. The exocyst interaction network. All protein complex members identified in TAP experiments with all individual exocyst complex members used as baits. Color codes are shown in the legend. After filtering out common proteins identified in many of our unrelated TAP experiments, we show 309 proteins in total (including all exocyst members), functionally annotated with UniProt27 over the following groups: 10 ciliary proteins, 13 motor proteins, 16 GTPases and interacting proteins, 30 vesicle transport proteins, 12 cell cycle proteins, 27 transcriptional proteins, 29 mitochondrial proteins and 164 with other functions of which the 31 preys uniting with 22 exocyst members are depicted here. # Interactors identified: EXOC1 = 133, EXOC2= 55; exoc3 = 32; exoc4 = 58; exoc5 = 46; exoc6 = 39; exoc7 = 18; exoc8 = 52
Chapter 4

After two independent purification experiments for each exocyst subunit and excluding commonly identified proteins (interactors that are identified with ≥30 baits in a dataset of >800 SF-TAP experiments performed with >226 baits in our lab), mass spectrometry identified 309 interactors in total (Table S1). Out of these, 145 interactors were consistently found in both bait replications with each exocyst subunit and 58 co-purified with multiple exocyst proteins. All identified interactors were grouped based on functional annotation and information from UniProt\textsuperscript{26} and visualized using Cytoscape\textsuperscript{27} as shown in Figure 2 and Figure S3. EXOC1 pulled down the most interactors (133), where EXOC7 only yielded 18 interactors (Table S1 and Figure S3A). Each individual exocyst subunit pulled down several other members of the complex in the TAP experiments (Table S1 and Figure S3), suggesting that despite overexpression of the bait protein subunits, the exocyst complex is still able to at least partially assemble.

4.4.3 Identification and characterization of EXOC6B
EXOC6B was consistently identified in SF-TAP data from four other exocyst subunits (Figure 3A), so we decided to study this protein further. Localization experiments of recombinant expressed EXOC6B in hTERT-RPE1 cells confirmed the ciliary link that was reported earlier,\textsuperscript{28,29} since both N- and C-terminally tagged constructs showed enrichment at the base of the cilium, and a weak signal along the ciliary axoneme (Figure 3B-C). Next, we performed four independent SF-TAP experiments with EXOC6B to identify its interactome, and grouped them based on functional annotation as described above (Figure 3D). EXOC6B pulled down 25 proteins of which six exocyst members.

4.4.4 Binary interactors of ciliopathy related proteins EXOC4 and EXOC8
To further understand the consequences of the reported missense mutations on EXOC4 and EXOC8, we first performed localization studies of the mutant recombinant proteins in hTERT-RPE1 cells. When comparing the localization of both WT and mutant proteins, we could not detect any obvious differences (Figure 4A, B). To understand the effects on binary protein interactions and identify direct binding partners of both proteins, we performed yeast two-hybrid (Y2H) library screens of the WT constructs with cDNA libraries of affected tissues in the described individuals. The tissues affected in the individual with MKS were the eyes (microphthalmia), kidneys (polycystic kidneys) and the brain (occipital encephalocele).\textsuperscript{9} In case of the individual diagnosed with JBTS, there was only neurological involvement confirmed with a molar tooth sign on MRI.\textsuperscript{10} Screening for direct interactors in retinal, renal, and neuronal cDNA libraries resulted in 42 unique interactors for EXOC4, and 19 for EXOC8, of which four were shared (Figure 4C and Table S2). When analyzing the Y2H interaction dataset of EXOC4, it is striking that three proteins residing at the base of the cilium are found as binary interactors: CEP89, JBTS17 and NUP62. The interaction with CEP89 was validated by co-immunoprecipitations from HEK293T cells (Figure 4D), and we observed no effect of the mutation in EXOC4 on the interaction with CEP89 in the conditions we used.
The exocyst interactome unveils potential plasma membrane docking sites at the base of the cilium

**Figure 3.** EXOC6 is a putative member of an alternative assembly of the mammalian exocyst complex. (A) A visualization of all interactions found in SF-TAP experiments between all canonical exocyst complex subunits and EXOC6. (B) Localization of EXOC6B constructs fused to an N-terminal eCFP tag or (C) C-terminal eYFP tag, both in green. In both panels, GT335 is used as ciliary/basal body marker in red and nuclei are stained with DAPI in blue. Magnifications of the basal body region are shown in the insets. (D) SF-TAP experiment results with EXOC6B as bait. Identified preys and corresponding average amino acid sequence coverage are shown. Four replications were performed, with either an N-terminal SF-TAP tag (N-TAP N=1-3) or an C-terminal SF-TAP tag (CTAP N=1) and corresponding sequence coverage is shown. The number of times this prey was found in the EXOC6B SF-TAP replicates, or in the SF-TAP experiments of the canonical exocyst (EXOC1, -2, -3, -4, -5, -6, -7 and -8) are printed in the last two columns, respectively. Color coding indicates functional groups as described in Figure 1. Note that EXOC6 did not pull down EXOC6B (A) or vice versa (D).

**4.5 Discussion**

This study provides further insight into the molecular relationship of the exocyst with the cilium, pinpointing to potential plasma membrane docking sites at the base of the cilium. Our localization data confirms the findings of individual exocyst members studied before, and underpins the association of the entire exocyst complex with the cilium, specifically at the cilium base. Furthermore, proteomic approaches encompassing both SF-TAP studies as well as Y2H library screens identified several interacting proteins residing at the base of the cilium.
Figure 4. Analysis of localization and binary interactions of MKS and JBT3 proteins EXOC4 and EXOC8. Localization of WT and mutant EXOC4 (A) and EXOC8 (B) in hTERT-RPE1 cells. GT335 was used as ciliary basal body marker, nuclei are stained with DAPI. (C) Summary of the results of yeast two-hybrid library screens with EXOC4 and EXOC8 as bait in the upper table. The lower table shows the ciliary proteins identified. (D) Immunobots (IB) of lysates of HEK293T cells transfected with combinations of constructs as indicated, and subjected to immunoprecipitation (IP) with FLAG beads. The reciprocal interaction of EXOC4 with CEPI9 was confirmed, and the mutation did not have an effect on the strength of the interaction. The mutations are indicated with single amino acid letter codes. NTAP: N-terminal SF-TAP tag.

Besides the ciliary link, our protein interaction dataset harbors information about the composition of the exocyst complex as well. SF-TAP studies identified far more interactors for EXOC1 then for the other complex members, e.g. EXOC7 (Table S1 and Figure S3A). This could be explained by the structural architecture of the complex, where in comparison EXOC7 is more buried and EXOC1 is more exposed, therefore having intrinsic differences in the number of interactors. Strikingly, this is in line with the recently reconstructed in vivo architecture of the complex by Picco and colleagues (Figure S1). Furthermore, EXOC1 seems to
predominantly associate with proteins related to vesicular transport, (regulators of) GTPases and motor proteins (Figure S3). This fits in a model were EXOC1 is the responsible subunit for interaction with these protein modules that enable translocation of the exocyst complex to targeted sites, whereas the other subunits are involved in the target membrane recognition and complex stability.

One protein complex identified in the SF-TAP data is the BLOC-1 (biogenesis of lysosome-related organelles complex-1) complex, which is described to be involved in the biogenesis of melanosomes and controls the formation of recycling endosomal tubules from sorting endosomes. Five of the eight subunits are present in the dataset of EXOC1; BLOC1S2, BLOC1S3, BLOC1S4, SNAPIN and DTNB1P1 (Figure 2 and Table S1). A recent paper showed that the BLOC-1 complex is specifically necessary for PKD2 delivery (from the recycling endosome) to the cilium, but not for transmembrane proteins smoothened and fibrocystin. In the same study, the exocyst was shown to be involved in fibrocystin as well as PKD2 delivery, validating that our SF-TAP approach is successful and also picking up cargo as PKD2 was identified as prey of EXOC3 (Figure 2 and Table S1). Altogether, their study showed that different transport pathways, e.g. via the exocyst and BLOC-1 complex, are involved in the delivery of specific transmembrane proteins to the cilium. Further analysis of our SF-TAP data and subsequent experimental testing for delivery to the cilium could reveal other candidates as ciliary cargo specifically transported by the exocyst complex.

Another protein that was consistently identified in SF-TAP data from four other exocyst subunits (Figure 3A) is EXOC6B. EXOC6B has previously been associated with the cilium, as it is annotated as a ciliary gold standard protein, based on ciliogenesis defects after siRNA knock down and detection with mass spectrometry analysis in the photoreceptor sensory cilium. Our dataset suggests that EXOC6B is part of the mammalian exocyst complex, and probably replacing EXOC6 in an alternative assembly. This is supported by the fact that EXOC6B does not pull down EXOC6 and vice versa (Figure 3A, D), although both bind EXOC4, EXOC5, EXOC7 and EXOC8 (Table S1). Furthermore, a BLAST (Basic Local Alignment Search Tool) analysis using UniProt showed that EXOC6B has 72% amino acid sequence identity with EXOC6, suggesting comparable functionality. The assembly of either one of the two complexes could be cell/tissue specific, or depending on spatiotemporal factors. This theory is supported by the work of Brymora and colleagues, who initially identified EXOC6B in neuronal tissue together with other exocyst subunits. Over the last decade, several ID patients have been described with micro deletions spanning (part of) EXOC6B [MIM: 607880], suggesting that EXO6B is important for brain development and cognitive function. Interestingly, EXO6B haploinsufficiency does not seem to affect ciliary function, despite of the clear ciliary localization, since the patients did not present with ciliopathy phenotypes. Just recently, Girisha and colleagues have described two consanguineous patients with a novel multiple joint dislocation syndrome without an ID phenotype, having a homozygous truncating variant (p.(Tyr302*)) in EXOC6B. They provide a rationale for the earlier described Intellectual Disability (ID) patients with microdeletions including the EXOC6B gene: these microdeletions
also disrupt the spatial organization of two brain enhancers (11270 and 5866) and the neurodevelopmental transcription factor EMX1 [MIM: 600034]. The physical separation of these enhancers with the transcription factor by the deletions likely alters the expression of the transcription factor and thereby affects brain development, resulting in ID in the patients. The function of EXOC6B is possibly redundant in the brain, since EXOC6 has 72% sequence identity and the truncating mutation found does not give rise to severe (neuro-)developmental phenotypes, in contrast to the missense mutations in EXOC4 and EXOC8 resulting in MKS and JBTS.9 10 Taken together, although recombinant EXOC6B localization confirms a ciliary link, disruption of the protein does not seem to cause a ciliopathy phenotype in humans, which may be explained by redundancy of the protein.

When studying EXOC4 and EXOC8, we found that the ciliopathy associated missense mutations did not cause any obvious differences in localization patterns when compared to WT. This could indicate that the mutations do not affect the localization of the protein, but rather have an effect on the functionality of the protein itself, or of the entire exocyst complex. The domain structures of both proteins are poorly studied, however, it is known that EXOC8 has a PH-domain which is involved in the binding ofRal GTPases.42 It is suggested that EXOC8, positioned centrally within the core of the exocyst complex (Figure S1),4 has a regulatory role for the entire complex. However, the PH-domain and sites that are phosphorylated by Cdk14 are exposed and available for interactions. Although there is no structural model of the entire protein, the PH domain and its interaction with RALA has been modeled by Jin and colleagues (PDB entry 1zc3).43 The p.(Glu265Gly) mutation in EXOC8 is located within this PH domain, but the affected residue is not part of the interaction surface with RALA. Strikingly, the glutamic acid residue at position 265 is actually on another surface of the PH domain and clearly exposed (Figure S4), indicating it could be involved in other interactions which would be affected when it is mutated into a glycine, rather than the mutation of this residue affecting the structure of the PH domain or entire EXOC8 protein. This could mean that the mutation in EXOC8 causes a specific dysregulated assembly or function of the exocyst complex, thereby affecting neurodevelopmental pathways, resulting in JBTS. Interestingly, when studying the architecture of the entire exocyst complex proposed by Picco et al (Figure S1), EXOC4 does not interact with the vesicle or plasma membranes, but is positioned on the opposite site of the exocyst complex. Furthermore, the C-terminal tail of EXOC4 is protruding out of the complex, thereby being exposed for interactions with other proteins. From this model it seems that the affected amino acid (Gln578) in the C-terminal end of EXOC4 is present in the exposed portion of the protein. It is tempting to speculate that this mutation, like the mutation in EXOC8, rather affects specific interactions of the EXOC4 subunit instead of disrupting the folding or assembly of the complex.

Three direct interaction partners identified in the Y2H library screens of EXOC4 are bona fide ciliary base proteins: CEP89, JBTS17 and NUP62. CEP89 is a component of the distal appendages of the mother centriole and during the first stages of ciliogenesis, distal appendages transform into transition fibers.44 These final structures are thought to be part of
The exocyst interactome unveils potential plasma membrane docking sites at the base of the cilium

the ciliary gate/pore, working together with the transition zone to control entry and exit of proteins into the cilium. Direct interactions with EXOC4 could implicate a docking site of the exocyst complex for delivery of vesicles with ciliary cargo. We could not identify an effect of the mutation in EXOC4 in the co-immunoprecipitation experiment in the conditions we used, however, this effect cannot be ruled out based on our data. JBTS17, encoded by C50RF42 [MIM: 614571], is a member of the C-PLANE complex and localizes at the base of the cilium.\(^4\) When mutated it can cause either classic JBTS or the closely related ciliopathy Orofaciodigital syndrome type VI (OFD6) which is characterized by oral frenulae, tongue hamartomas, midline cleft lip, central polydactyly and cerebellar abnormalities including the molar tooth sign.\(^4\) In an earlier study, exocyst complex subunits co-purified with three other CPLANE proteins (Fuzzy, Inturned and Wdpcp).\(^4\) In our Y2H fetal brain cDNA library screen we found a direct interaction of EXOC4 with a C-terminal stretch of JBTS17, starting at aa2629 till the end. In another study, the basal body localization domain of JBTS17 was narrowed down to aa1770-2318.\(^4\) This could implicate a docking site for the exocyst at the base of the cilium since JBTS17 simultaneously can facilitate its own localization at the basal bodies next to direct binding of the exocyst complex via EXOC4. The third direct interactor at the base of the cilium identified for EXOC4 is NUP62. Originally defined as a nuclear pore complex protein, however, recently also identified as a potential ciliary pore protein.\(^4\) Taken together, these three identified proteins are interesting candidates for exocyst-mediated vesicle docking at the base of the cilium, specifically via direct EXOC4 interactions.

In our SF-TAP interaction data set of EXOC4 we also identified IQCB1 and CEP290 (Figure 2 and Table S1), both established ciliopathy-associated proteins. Mutations in IQCB1 [MIM: 609237] can lead to two related ciliopathies: Leber congenital amaurosis (LCA), isolated congenital blindness due to retinal degeneration, or Senior-Løken syndrome, which is a combination of retinal degeneration and renal failure.\(^7\) \(^4\) Mutations in CEP290 [MIM: 610142] can give rise to a spectrum of phenotypes, often involving abnormalities of the retina, ranging from isolated LCA to Bardet-Biedl syndrome (BBS), JBTS or even the perinatal lethal MKS.\(^8\)\(^4\) IQCB1 and CEP290 are part of the transition zone of the cilium. This specific area resides at the base of the cilium, where multi-subunit protein complexes regulate restricted entry and exit of proteins into the ciliary compartment.\(^5\) Specific vesicles carrying ciliary transmembrane proteins are docked here with the help of the BBsome (comprised of eight BBS proteins), and it is described that IQCB1 and CEP290 together regulate this process.\(^5\) The fact that we find these two proteins in our interaction data, underlines the association of the exocyst with the base of the cilium, and provides two additional candidates for exocyst-mediated docking of vesicles at the transition zone, next to the three direct interaction partners identified with yeast two-hybrid experiments described earlier.

4.6 Conclusion

In conclusion, our data provides further insight into the interaction network of the exocyst complex, and the specific interactions with ciliary proteins reveal candidate docking proteins of the exocyst complex at the base of the cilium. EXOC6B is identified as a member of an
(alternative) mammalian assembly of the exocyst complex, where EXOC6B likely is redundant for ciliary function. Finally, we propose that the effect of EXOC4 and EXOC8 mutations lie in disrupting specific subsets of ciliary interactions rather than disturbing exocyst complex assembly, ultimately resulting in JBTs or even MKS.
4.7 References

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from exocyst and retinoic acid pathway due to a recurrent microdeletion of 2p13.2. Orphanet J Rare Dis 8, 100.
4.8 Supplemental data

Figure S1. Architecture of the assembled exocyst complex in *Saccharomyces cerevisiae* as defined by Picco and colleagues. Representative model for the 3D architecture of the exocyst in *Saccharomyces cerevisiae* in three views (top row). The exocyst subunits are represented as strings of beads according to their structural features. N- and C-termini are indicated. Subunits are color coded as indicated, where the mammalian homologue of Sec3 is EXOC1, Sec5 is EXOC2, Sec6 is EXOC3, Sec8 is EXOC4, Sec10 is EXOC5, Sec15 is EXOC6, Exo70 is EXOC7 and Exo84 is EXOC8. The C-terminal tail of EXOC4 (in pink) is pointing out of the complex, and EXOC8 (in green) forms the core of the complex, with the C-terminal part exposed. In the lower composition, color coding of the exocyst is the same as above, and interaction with target membrane and vesicle are modeled including SNARE complex members Sec9, Sso1, Snc2. This figure is adapted from Picco et al. (2017).
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Figure S2. Localization of all eight exocyst members in ciliated cells. Ciliated hTERT-RPE1 cells were transfected using Effectene (Qiagen) with C-terminally eYFP tagged constructs of the following individual exocyst members: (A) EXOC1, (B) EXOC2, (C) EXOC3, (D) EXOC4, (E) EXOC5, (F) EXOC6, (G) EXOC7 or (H) EXOC8 (all in green). In all pictures, GT335 is used as basal body and cilium marker (red) and DAPI for the nuclei (blue). Magnifications of the basal body region are shown in the insets.
Chapter 4

A  Number of preys identified per exocyst subunit divided in functional groups

B  Relative distribution of identified preys per functional group
The exocyst interactome unveils potential plasma membrane docking sites at the base of the cilium.

**Figure S3.** Subdivision of identified preys in the SF-TAP experiments in functional groups per subunit. (A) Absolute numbers of preys identified per subunit TAP, divided in functional groups. (B) Relative contribution of functional groups of preys for each exocyst subunit. (C) Relative contribution of preys identified per exocyst subunit for each functional group.

**Figure S4.** The affected glutamic acid residue of EXOC8 is positioned on the PH-domain surface, however not involved in the interaction with RALA. The 3D protein structure of the PH domain of rat Exo84 (green) and its interaction with RALA (red) has been modeled by Jin and colleagues (PDB entry 1z3). Exo84 is the homologue of EXOC8 in humans. The affected glutamic acid residue in the Joubert syndrome patient (mutation p.(Glu265Gly)) corresponds to Glu256 in the Rat sequence of Exo84, and its sidechain is shown in magenta (black arrow). (A) The affected amino acid is positioned at the top of the PH-domain, not within the interaction surface with RALA in red. (B) When the side chains of all residues is shown, it is clear that the side chain of the affected residue is still exposed and available for other interactions.
Chapter 4

Table S1. Results of SF-TAP experiments with all canonical exocyst subunits. All preys identified in SF-TAPs, after filtering for unspecific binding as specified in the text are shown. Sequence coverage of preys are given for all separate experiments and averages. Color coding of preys is similar as figure 1. Preys in bold are identified by multiple exocyst baits. (Table S1 is available as a separate excel file on request.)

Table S2. Results of yeast two-hybrid library screens with EXOC4 and EXOC8. All preys identified in yeast two-hybrid library screens of EXOC4 and EXOC8 fused to pAD with three cDNA libraries from adult kidney, adult retina and fetal brain (all human), with corresponding numbers on identified clones and unique clones. B-galactosidase scoring is shown for the originally identified clone in the library screen, and for a subset of clones used in a validation assay using co-transformation, where 0 = no activation, 3 = maximum activation. When preys are identified previously in SF-TAP experiments with exocyst subunits, the corresponding baits are indicated (SF-TAP bait). (Table S2 is available as a separate excel file on request.)

4.9 Supplemental references


Tabel 1. Results of SF‐TAP experiments. All preys identified in SF‐TAPs, after filtering for unspecific binding as specified in the text, are shown. Sequence coverage of preys are given for all separate experiments and averages. Color coding of preys is similar as figure 1. Preys in bold are identified by multiple exocyst baits. (Table S1 is available as a separate excel file on request.)

Tabel 2. Results of yeast two‐hybrid library screens of EXOC4 and EXOC8 fused to pAD with three cDNA libraries from adult kidney, adult retina and fetal brain (all human), with corresponding numbers on identified clones and unique clones. β‐galactosidase scoring is shown for the originally identified clone in the library screen, and for a subset of clones used in a validation assay using co‐transformation, where 0 = no activation, 3 = maximum activation. When preys are identified previously in SF‐TAP experiments with exocyst subunits, the corresponding baits are indicated (SF‐TAP bait). (Table S2 is available as a separate excel file on request.)
Chapter 5

Recurrent de novo mutations disturbing the GTP/GDP binding pocket of RAB11B cause intellectual disability and a distinctive brain phenotype

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5.1 Abstract
The Rab GTPase family comprises of ~70 GTP-binding proteins, functioning in vesicle formation, transport and fusion. They are activated by a conformational change induced by GTP-binding, allowing interactions with downstream effectors. Here, we report five individuals with two recurrent de novo missense mutations in RAB11B; p.(Val22Met) in three individuals and p.(Ala68Thr) in two individuals. An overlapping neurodevelopmental phenotype, including severe intellectual disability with absent speech, epilepsy, and hypotonia was observed in all affected individuals. Additionally, visual problems, musculoskeletal abnormalities, and microcephaly were present in the majority of cases. Re-evaluation of brain MRI images of four individuals showed a shared distinct brain phenotype, consisting of abnormal white matter (severely decreased volume and abnormal signal), thin corpus callosum, cerebellar vermis hypoplasia, optic nerve hypoplasia and mild ventriculomegaly. To compare the effects of both variants with known inactive GDP- and active GTP-bound RAB11B mutants (p.(Ser25Asn) and p.(Gln70Leu), respectively), we modeled the variants on the three-dimensional protein structure and performed subcellular localization studies. We predicted that both variants alter the GTP/GDP binding pocket, and show that they both have localization patterns similar to inactive RAB11B. Evaluation of their influence on the affinity of RAB11B to a series of binary interactors, both effectors and guanine nucleotide exchange factors (GEFs), showed induction of RAB11B binding to the GEF SH3BP5, again similar to inactive RAB11B. In conclusion, we report two recurrent dominant mutations in RAB11B leading to a neurodevelopmental syndrome, likely caused by altered GDP/GTP binding that inactivate the protein, and induce GEF binding and protein mislocalization.
5.2 Short report

The application of whole exome sequencing (WES) as a genetic test for intellectual disability (ID) and developmental delay has increased the number of genetic causes to 1500.\textsuperscript{1,2} This has significantly enhanced our knowledge of molecular processes that regulate learning and memory as well as brain development, although the roles of many genes involved in these processes have not yet been defined. Using trio-based WES in diagnostic and research settings,\textsuperscript{1,2} we identified two different heterozygous \textit{de novo} missense mutations in \textit{RAB11B} (GenBank: NM_004218.3; MIM: 604198), c.64G>A; p.(Val22Met) and c.202G>A; p.(Ala68Thr), in five unrelated individuals with severe ID (Figure 1A, B; Table 1). Written consent was obtained from the legal guardians for all individuals and the study was given IRB approval. Following identification of the \textit{RAB11B} mutations of the first two individuals at Radboud University Medical Center, Nijmegen (individual 1: p.(Val22Met); individual 4: p.(Ala68Thr)), a query via GeneMatcher\textsuperscript{3} resulted in the identification of individual 2 at Haukeland University Hospital, Bergen, and individual 3 at Seoul National University College of Medicine, Seoul. Both individuals harbored the variant p.(Val22Met), similar to individual 1. A fifth individual (individual 5; DECIPHER ID: 263643), reported before as part of a study sequencing a large ID cohort,\textsuperscript{4} was identified through international collaboration. Interestingly, this individual had the same variant, p.(Ala68Thr), as individual 4 (Figure 1B). Both identified missense mutations were not present in the ExAC database or in-house control databases and affect highly conserved amino acids in 90% of Rab family members\textsuperscript{5} and among different species (Figure 1B). Additionally, the ExAC database has shown that \textit{RAB11B} is significantly depleted of rare missense variants (z-score 3.48) in healthy controls.\textsuperscript{6}

\textit{RAB11B} is a member of the large Rab GTPase protein subfamily of RAS GTPases, consisting of almost 70 small \textasciitilde{}21kDa monomeric GTP-binding proteins. They serve as molecular switches that function in vesicle formation, transport, tethering and fusion.\textsuperscript{7,8} The tightly regulated spatiotemporal activity of Rabs is controlled by guanine nucleotide exchange factors (GEFs) that catalyze the GDP/GTP-exchange, and GTPase activating proteins which catalyze the hydrolysis of GTP into GDP.\textsuperscript{9-11} Rabs mainly interact with downstream GTPase effector proteins in their GTP-bound active conformation.\textsuperscript{10,12} Rab GTPases and their effector proteins have been described to play a role in neuronal development and the shaping of cognitive functions,\textsuperscript{13} and several genes within this family have been associated with neurodevelopmental disorders and micro- or macrocephaly; such as \textit{RAB18} [MIM: 602207] and \textit{RAB3GAP1} [MIM: 602536] in Warburg micro syndrome [MIM: 600118], and \textit{RAB39B} [MIM: 3007744] in Waismann syndrome [MIM: 311510] and Mental Retardation, X-linked 72 [MIM300271].

All five individuals that carried \textit{de novo} mutations in \textit{RAB11B} showed severe ID with motor delay and absent speech. A variety of neurological problems were present in all affected individuals, including hypotonia (4/5), epilepsy (3/5), spasticity (2/4), dystonia (2/4), broad-based (3/3) and ataxic gait (2/3) and nystagmus (2/3) (Table 1). Re-evaluation of brain MRI images of four individuals showed similar brain imaging abnormalities (Figure 2 and Table S1).
Chapter 5

Figure 1. RAB11B mutations in five individuals with severe ID. (A) cDNA composition of RAB11B and location of exons (dark grey) and identified mutations (green stars). (B) Amino acids sequence of exon 2 of RAB11B. Both identified mutations (green) localize at one of the two GTP binding sites (red stripes) and mutation p.(Ala68Thr) also involves the switch 2 region (black stripe). Both identified mutations affect highly conserved amino acid residues among different species (light grey box). The known inactive variant p.(Ser25Asn) and known active variant p.(Gln70Leu) are marked orange. (C-E) Structural characterization of mutations. In all panels, the overall Protein DataBank structure 2f9m of RAB11B14 is colored grey and shown in ribbon representation. GNP (phosphoaminophosphonic acid-guanylate ester, a non-hydrolyzable analog of GTP) is multicoloed in a spacefill representation with the guanidine ring in blue shades and the phosphates in red and yellow. The magnesium molecule is represented as a purple sphere. The side chains of the affected amino acids in the affected individuals are green and for the GDP/GTP-locked mutants are orange. (C) Overview of RAB11B bound to GNP and magnesium and the affected amino acids are highlighted. (D) Close up of the p.(Val22Met) mutation shows the side chains of valine at position 22 in green and the methionine in red. (E) Close up of the p.(Ala68Thr) mutation shows the side chains of alanine at position 68 in green and threonine in red, and the mutated glutamine at position 70 which is mutated in the GTP-locked mutant is in orange.
De novo mutations disturbing RAB11B cause ID and a distinctive brain phenotype

All had severely decreased white matter volume (cerebral cortex more severely affected than cerebellum), thin corpus callosum, and mild ex vacuo lateral ventriculomegaly affecting the frontal horns and body of the ventricles more than the occipital and temporal horns. Other features that were scorable in a subset of images included cerebellar vermis hypoplasia (3/3), thin brainstem (3/3), early global white matter signal abnormalities consistent with delayed myelination (2/2), later patchy white matter signal abnormalities consistent with injury (4/4),

Figure 2. Brain imaging features and facial photographs of individuals with RAB11B-associated intellectual disability. (A,F,G,J) Markedly decreased cortical white matter volume in individual 1,3,4 and 5. (B,H,K) Markedly thin corpus callosum (arrows), mildly thin brainstem, and mildly small, atrophic-appearing cerebellar vermis (bracket) in individual 1,4 and 5. (C) Increased T2/FLAIR signal in the cortical white matter (asterisks) in individual 1. (D,E) Atypical partial rhombencephalosynapsis in individual 1 (arrowheads). (A,D,F,G and H) are T2-weighted axial images. (B and H) are T1-weighted sagittal images. (C) is a T2/FLAIR-weighted axial image. (E) is a reformatted inversion-recovery coronal image. (K) is a T2-weighted sagittal image (I, L) Facial photographs show upward slanted palpebral fissures, periorbital fullness, full nasal tip and hypotonic face in individual 4 and upward slanted palpebral fissures, deep set eyes and short philtrum in individual 5.
## Chapter 5

### Table 1: Clinical features of individuals with de novo mutations in RAB11B

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### Mutation (NM_004218.3)

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### Development

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</tr>
<tr>
<td>Abnormal gait</td>
<td>Yes – ataxic; broad-based</td>
<td>NR</td>
<td>NR</td>
<td>Yes – ataxic; broad-based</td>
</tr>
<tr>
<td>Nystagmus</td>
<td>Yes</td>
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### Ophthalmological abnormalities

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<tbody>
<tr>
<td>Refraction abnormality</td>
<td>Hypermetropia – Mild</td>
<td>NR</td>
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<td>Hypermetropia</td>
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<td>Strabismus</td>
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<td>NR</td>
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<tr>
<td>Other</td>
<td>Delayed visual maturation</td>
<td>Reduced vision</td>
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### Musculoskeletal abnormalities

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<tbody>
<tr>
<td>Developmental hip dysplasia</td>
<td>Yes – mild, non-progressive</td>
<td>Yes – right-sided, non-progressive</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Tapering fingers</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Other</td>
<td>Pes cavus; shortened achilles tendons; prominent steloideus ulnae</td>
<td>2 cm anisometria; adducted thumbs; bilateral club foot</td>
<td>None</td>
<td>No</td>
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</table>

### Other

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</thead>
<tbody>
<tr>
<td>Drooling; Simean crease; neonatal feeding difficulties</td>
<td>Bilateral palsy nervous laryngeus recurrens, Diabetes Mellitus Type 1, hydrocephalus</td>
<td>Acanthotic skin, Epidermal nevus in face, neck, trunk; Short neck; Obstructive sleep apnea; cryptorchidism</td>
<td>Simean crease</td>
<td>Bruxism</td>
</tr>
</tbody>
</table>

Abbreviations: NR = Not Reported; SD = Standard Deviation
optic nerve hypoplasia (2/2), and what appears to be atypical partial rhombencephalosynapsis (1/3). Although two other affected individuals did not have rhombencephalosynapsis (partial or complete absence of the cerebellar vermis with fusion of the cerebellar hemispheres), the width of the cerebellar vermis was subjectively narrower than typical. Besides the neurological phenotype, ophthalmological and musculoskeletal abnormalities were present in the majority of individuals. Microcephaly was observed in three individuals (Table 1).

To study the effects of the identified mutations on the well described three-dimensional protein structure of RAB11B, the substitutions were modeled on Protein Data Bank entry 2f9m,\(^1\) using YASARA software,\(^5\) and compared to the described active, GTP-locked RAB11B mutant p.(Gln70Leu) and inactive, GDP-locked RAB11B mutant p.(Ser25Asn) (Figure 1C).\(^6\) The p.(Ser25Asn) mutation was shown to disrupt the binding of a magnesium molecule which is essential for GTP binding, consequently locking the GTPase in an inactive GDP bound, non-membrane-associated state.\(^17-20\) In contrast, the p.(Gln70Leu) mutation affects a conserved residue in the flexible switch II region, which is essential for catalysis, and therefore this mutation is predicted to fix the GTPase in a GTP-bound state, which is constitutively active (Figure 1B,C).\(^19,26\) Both mutations identified in this study are situated in close proximity to the binding pocket for GTP/GDP and specifically on the side where the phosphate groups of GTP/GDP are positioned (Figure 1C). The valine residue at position 22 is located at the base in the middle of the GTP/GDP binding pocket, thereby being responsible for its shape (Figure 1B,D). Substitution into methionine brings in a bigger amino acid which causes a predicted reorganization of the region around this residue, altering the shape of the binding pocket. This likely disrupts binding of GDP and GTP, resulting in a nucleotide-free, inactive state of RAB11B. The second mutation affects an alanine at position 68 and is located within a flexible loop of the switch II region (Figure 1B,E), which together with the switch I region specifically interacts with effector proteins when GTP is bound.\(^12\) This flexible loop provides more space for a bigger amino acid residue, however, the mutant residue is positioned closely to the magnesium molecule, as well as the phosphate groups of GTP/GDP, and thereby alters the binding pocket and is predicted to disrupt nucleotide binding. Furthermore, the location within the switch region suggests that the mutation causes altered binding with effector proteins. Taken together, both identified mutations are predicted to alter the GTP/GDP binding pocket of RAB11B, hence it is likely that the functionality of the protein is affected.

**RAB11B** is one of three genes in the Rab11 GTPase subfamily, together with **RAB11A** [MIM: 605570] and **RAB25** [MIM:612942], and this subfamily specifically associates with recycling endosomes.\(^7,21\) **RAB11A** is the best characterized family member and is ubiquitously expressed,\(^22\) in contrast to specific expression of **RAB25** and **RAB11B** in epithelial tissue\(^23\) or in heart, testis and brain,\(^24\) respectively. The proteins have been implicated in the regulation of vesicular trafficking between the recycling endosome compartment and early endosomes to the trans Golgi network and plasma membrane.\(^17,25\) Furthermore, it has been shown that RAB11 is essential for ciliogenesis,\(^26-28\) and that the proteins are localized to peri-centrosomal recycling endosomes concentrated at the base of the cilium.\(^29\)
Figure 3. Localization of wild type and mutant RAB11B in hTERT-RPE1 cells. Expression of recombinant 3xFLAG-RAB11B wild type (A, G) and 3xFLAG-RAB11B variants p.(Gln70Leu) (B, H), p.(Ser25Asn) (C, I), p.(Val22Met) (D, J), p.(Ala68Thr) (E, K) in hTERT RPE1 cells, detected using anti-FLAG antibodies (all in green). N-terminal tagging was performed to not disturb the prenylation of the C-terminus of RAB11B which is required for membrane association. The Golgi apparatus was visualized with staining of Golgi marker GM130 in red (G-K). Co-localization with PCM1 (F) or GM130 (L) was quantified by calculating the Pearson’s correlation coefficient (PCC) using the JACoP plugin in ImageJ (N=12 cells for each construct, mean in red and error bars represent the standard deviation). The significance of difference with WT was calculated with a Student’s t-test (ns: not significant (P>0.05); *:
To evaluate if the identified mutations affect the sub-cellular localization of RAB11B, potentially at the cilium, we transfected human TERT-immortalized retinal pigment epithelium 1 (hTERT RPE1) cells with cDNA expression constructs encoding WT or mutant RAB11B, fused to an N-terminal 3xFLAG tag. A marker for the peri-centrosomal region (Pericentriolar Material 1; PCM1) was used to assess the effect on the peri-centrosomal localization (Figure 3A-F), and the marker acetylated tubulin to assess cilium morphology (Supplemental Figure S1). Wild type RAB11B localization was scattered throughout the cell with a punctuated pattern as shown in Figure 3A. For some cells, puncta were enriched, as expected, at the pericentrosomal region. We observed that the constitutively active mutant RAB11B-p.(Gln70Leu) had a strong organization in puncta throughout the cytoplasm and a consistent localization at the peri-centrosomal region in all cells (Figure 3B). In contrast, GDP-bound inactive RAB11B-p.(Ser25Asn) showed a generally dispersed cytosolic localization, losing its association with the peri-centrosomal region, but with enrichment near the nucleus suggesting Golgi localization (Figure 3C). This was confirmed using Golgi marker GM130 (Figure 3G-L). Our results upon expression of recombinant WT, p.(Ser25Asn), and p.(Gln70Leu) RAB11B confirmed earlier studies on the effect of these mutations on the localization of this GTPase. Localization of both RAB11B-p.(Val22Met) (Figure 3D) and RAB11B-p.(Ala68Thr) (Figure 3E) showed homogenous localization in the cytoplasm, no association with the peri-centrosomal region and co-localization with Golgi marker GM130 (Figure 3J,K), similar to the GDP-bound inactive mutant p.(Ser25Asn). Quantification of the co-localization patterns with PCM1 and GM130 confirmed our observations (Figure 3F,L). The morphology of the cilium appeared normal in cells transfected with each of the four mutant constructs (Supplemental Figure S1). We reproduced the localization patterns at the base of the cilium or at the Golgi for all RAB11B variants in a different cell line: mouse inner medullary collecting duct 3 (IMCD3) cells (Supplemental Figure S2). In short, introduction of either one of the identified RAB11B mutations results in a similar localization pattern as the GDP-bound inactive form, without an obvious difference between the two. Interestingly, previous studies showed that GDP-locked GTPases lose their membrane association. For the p.(Ser25Asn) mutant of RAB11B, we indeed observe a localization pattern that is similar to that of the published RAB11B-ΔC mutant where five C-terminal amino acids were substituted to abolish prenylation, and consequently disturbed vesicular membrane association. The fact that both identified mutations result in a comparable RAB11B localization pattern as the p.(Ser25Asn) mutant, suggests that in the affected individuals, the association of RAB11B with (vesicular) membranes is affected, contributing to the pathogenic effects of the identified mutations.

To further assess the effect of the identified mutations on the functionality of RAB11B, we screened for binary interaction partners of RAB11B by using a GAL4-based yeast two-hybrid...
screen of cDNA libraries from neuronal tissues (brain and retina) as previously described. In physiological conditions, RAB11B is either GDP or GTP bound. Therefore we used both p.(Ser25Asn) and p.(Gln70Leu) mutant constructs as baits to screen for potential interactors, which identified two (p.(Ser25Asn)) and eight (p.(Gln70Leu)) different potential interactors (Figure 4 and Supplemental Figure S3). All interactions were confirmed using independent co-transformation assays with validation of reporter gene activity (Supplemental Figure S3). The identified interactors were all previously reported as RaI/Rac or Rab11 specific interacting partners (Supplemental Table S2), validating our assay. Interestingly, three direct interactors identified in our yeast screen are encoded by genes where loss of function mutations are associated with neurodevelopmental disorders: CNKS2R [MIM: 300724] with X-linked Intellectual Disability, MYOSA [MIM: 160777] with Griscelli syndrome type 1 [MIM: 214450], and TRAPP9 [MIM: 611966] with Recessive Mental Retardation 13 [MIM: 613192] (Supplemental Table S2).

<table>
<thead>
<tr>
<th>Interactor</th>
<th>type of interactor</th>
<th>selection on -LWH + 5mM 3AT</th>
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<tr>
<td></td>
<td></td>
<td>p.(Val22Met)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>growth</td>
</tr>
<tr>
<td>BSHBP5</td>
<td>GEF</td>
<td>0</td>
</tr>
<tr>
<td>TRAPP9</td>
<td>GEF subunit</td>
<td>0</td>
</tr>
<tr>
<td>CNKS2R</td>
<td>Rac regulator</td>
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</tr>
<tr>
<td>RAB11FIP3</td>
<td>adapter/effecter</td>
<td>3</td>
</tr>
<tr>
<td>RAB11FIP2</td>
<td>adapter/effecter</td>
<td>3</td>
</tr>
<tr>
<td>RAB11FIP4</td>
<td>adapter/effecter</td>
<td>2</td>
</tr>
<tr>
<td>Rab8b</td>
<td>effecter</td>
<td>2</td>
</tr>
<tr>
<td>Myosin-Va</td>
<td>effecter</td>
<td>2</td>
</tr>
<tr>
<td>Myosin-Vb</td>
<td>effecter</td>
<td>2</td>
</tr>
<tr>
<td>RAB11FIP3</td>
<td>adapter/effecter</td>
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</tr>
<tr>
<td>RAB11FIP5</td>
<td>adapter/effecter</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 4. Identified interactors of RAB11B using yeast two-hybrid cDNA library screening and their affinity for RAB11B variants. Co-transformations were performed in Pj69-4a yeast strains with the identified clones from the library screens fused to pAD together with WT or mutant RAB11B constructs fused to pBD. The clones are sorted according to the library in which they have been identified, as indicated in the far left column. The quantification of the selection of the growth of two-hybrid clones grown on medium lacking leucine, tryptophan and histidine with 3mM 3AT is quantified in the ‘growth’ column. α-Galactosidase reporter gene activation (α-gal column) or β-galactosidase reporter gene activation (β-gal column) are quantified as well. Quantifications are on a scale from 0-3; with 0 for no reporter gene activation and 3 for highest reporter gene activation. Details of the identified clones in the cDNA library screens can be found in Table S2, including quantification of reporter gene activation on ~LWHA medium. Original images are shown in supplemental Figure S2.

To assess whether the identified mutations affect the affinity of RAB11B to any of the interactors, we co-expressed p.(Val22Met) and p.(Ala68Thr) mutant constructs with each interactor and compared the binding affinities semi-quantitatively by evaluation of the reporter gene activation levels (Figure 4, Supplemental Figure S3, and Table S2). Wild type, GDP-, and GTP-bound RAB11B were taken along as controls. Since GTP is more abundantly present in the cytosol than GDP, the WT RAB11B construct is expected to be predominantly GTP-bound. We indeed observed that the interaction pattern of the WT was comparable to the constitutively active mutant (Figure 4 and Supplemental Figure S3). When either one of the identified mutations was introduced, the binding pattern was mostly similar to that of WT.
De novo mutations disturbing RAB11B cause ID and a distinctive brain phenotype

RAB11B, but one important difference could be distinguished: both mutant proteins were able to bind to the one bona fide GEF identified in our screen, SH3BP5, while WT RAB11 could not (Figure 4 and Supplemental Figure S3).

The affinity of the p.(Val22Met) mutant protein to SH3BP5 was strong and comparable to the GDP-locked mutant protein p.(Ser25Asn), while the affinity of the p.(Ala68Thr) mutant protein was somewhat lower. Based on the 3D modeling and our localization assay, we already suggested that the p.(Val22Met) mutation results in a nucleotide-free state by the inability of the protein to bind GDP or GTP.\textsuperscript{11, 19} A somatic substitution affecting the adjacent conserved glycine residue (RAB11B position 23; Supplemental Figure S4) has been described in RAS-GTPase RHOA (p.(Gly17Val)),\textsuperscript{38-40} resulting in a nucleotide-free state of RHOA. As a result, mutant RHOA acts in a dominant-negative manner since it sequesters GEFs, which prohibits that these GEFs are available to activate wild type RHOA.\textsuperscript{41} With (1) the position of the p.(Val22Met) mutation adjacent to this reported RHOA substitution, (2) the 3D modeling suggesting a nucleotide-free state of RAB11B (Figure 1C), and (3) the strong affinity of the mutant to bind the RAB11B GEF SH3BP5 (Figure 4 and Supplemental Figure S3), it is likely that the identified p.(Val22Met) mutation acts in a dominant-negative manner as described for RHOA. Interestingly, we observed that this mutant RAB11B is still able to interact with effectors as well as GEFs (Figure 4 and Supplemental Figure S3). Also, for the p.(Ala68Thr) mutation, the binding to SH3BP5 is much less pronounced and only detectable under less stringent assay conditions (-LWH + 5mM 3AT, Figure 4 and Supplemental Figure S3). It has been described that a specific dominant-negative mutant of Ras (Ras15A), member of the RAS-GTPase family as well, has stronger affinities for GEFs with more defective nucleotide binding, compared to another dominant-negative mutant of Ras (Ras17N) where a neighboring residue is affected.\textsuperscript{41} This highlights the possibility of variable effects between mutations in the GTP/GDP binding pocket on the affinity for GEFs and nucleotides. We argue that, despite these slight differences in affinity, both mutations have similar consequences on RAB11B function, since individuals identified with mutation p.(Ala68Thr) have strong phenotypic overlap with individuals carrying the p.(Val22Met) mutation. Furthermore, both mutations caused a similarly disturbed localization (Figure 3). Therefore, we hypothesize that mutation p.(Ala68Thr) could act in a dominant-negative manner as well, with the same phenotypic consequences as the p.(Val22Met) mutation.

The second GEF for RAB11B identified in our screen is TRAPPC9, member of the TRAPP II complex. This group of proteins is involved in intracellular membrane trafficking processes\textsuperscript{10, 42} and acts as a GEF for RAB11B homologues in yeast.\textsuperscript{43-45} The fact that the p.(Val22Met) and p.(Ala68Thr) mutant proteins are not able to associate with TRAPPC9 seems to contradict the results with the GEF SH3BP5. However, SH3BP5 was shown to be a bona fide GEF for RAB11,\textsuperscript{46} while TRAPPC9 requires the other subunits of the TRAPP II complex to act as a GEF. As the
Chapter 5

RAB11B patient mutations do not induce the TRAPPC9 binding, our data also suggest that TRAPPC9, as a single protein, does not act as a RAB11 GEF in contrast to SH3BP5.

It is likely that RAB11B disruption also has molecular consequences other than disturbed protein interactions that may disturb several cellular mechanisms such as calcium influx, synaptic function and neuronal migration,\(^\text{13}\) that could contribute to the underlying pathology. One explanation could be found in the mislocalization of RAB11B mutant proteins, which were able to bind effector proteins based on our yeast two-hybrid data. As a result of the membrane unbound situation suggested by the localization data, mislocalization of mutant RAB11B may therefore result in mislocalization of bound effectors, sequestering them from their usual sites of function.\(^\text{41}\) Our data also show that RAB11B localization is altered at the basal body of the cilium (Supplemental Figure 1), and RAB11B is a direct interactor of the ciliary RAB Rabin8 (Figure 4 and Supplemental Table S2),\(^\text{47}\) although we observed no morphological changes of the cilium in hTERT-RPE1 cells. More subtle changes in cilium morphology could have been missed in our assay, or cilia may have an altered signaling function without morphological abnormalities. Although speculative at this point without an animal model, cilia could be mainly affected in brain tissue, given the predominant expression of RAB11B in the brain.\(^\text{13; 24}\)

In conclusion, we identified two recurrent heterozygous \textit{de novo} missense mutations in RAB11B in five unrelated individuals with severe ID and specific brain abnormalities. We show that (1) both mutations affect the GDP/GTP binding pocket of this small GTPase, (2) the association of the mutant proteins with (vesicular) membranes is affected in localization studies, and (3) both mutations have limited effect on RAB11B effector protein binding, but can enhance the affinity to the GEF SH3BP5. We propose that these effects together cause distinct defects in several neuronal developmental processes, a combination that results in a neurodevelopmental syndrome in human.
5.3 References


11. Cherfils, J., and Zeghouf, M. (2013). Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiological reviews 93, 269-309.


5.4 Web resources
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
GeneMatcher, https://genematcher.org/
ExAC database: http://exac.broadinstitute.org/gene/ENSG00000185236/

5.5 Acknowledgements
This work was supported by the Netherlands Organization for Scientific Research (NWO Vici-865.12.005) to R.R., and by a TOP grant from The Netherlands Organization for Health Research and Development (ZonMw, grant no. 912-12-109) to BdV and SJ. The DDD study presents independent research commissioned by the Health Innovation Challenge Fund [grant number HICF-1009-003] see Nature 2015;519:223-8 or http://www.ddduk.org/
Chapter 5

5.6 Supplemental note: case reports

Individual 1 is the second of two children of non-consanguineous parents of Dutch ancestry. Family history was non-contributory. She was born after 37 weeks of gestation with a birth weight of 3600 gram (+1.5 SD). As neonate, she was hypotonic and had feeding difficulties. She was carrier of hemophilia type A. Both motor and language development were delayed. Since the age of 3 years, she was able to walk short distances with support. For longer distances, she needed a wheelchair. At age 13 years, her developmental age is 6-7 months. She is not able to speak. Neurological examination revealed a nystagmus, ataxic movements and a broad-based gait. At the age of 13 years, she was suspect to have epilepsy (absences), but this was not confirmed on EEG. No regression of achieved milestones has been observed. MRI of the brain showed decreased cortical white matter volume, thin corpus callosum, thin brain stem, atrophic cerebellar vermis and atypical partial rhombencephalosynapsis. Her vision was impaired by delayed visual maturation, mild hypermetropia and strabismus. She had a variety of musculoskeletal abnormalities, including pedes cavus (requiring surgery at the age of 1 year), shortened achilles tendons, prominent steloideus ulnae and mild, non-progressive developmental hip dysplasia (Reimers migration index 33%). Physical examination showed a normal height of 152 cm (-1 SD), normal weight of 47.8 kg (+1.5 SD) and (non-progressive) microcephaly with OFC of 49 cm (-3 SD). Facial dysmorphisms, including prominent antihelix, tapering fingers and Simean crease left, were present. Previous investigation consisting of analysis of FMR1, UBE3A and MECP2 were normal. Affymetrix 500k SNP array revealed a small de novo duplication (46,XX.arr snp Xq13.2(CN_976225->SNP_A-8343339)x3 dn), of unknown clinical significance. Extensive screening of blood and urine metabolites, enzymes and hormones showed no abnormalities.

Individual 2 is the first child of two children of non-consanguineous parents of Norwegian ancestry. Hydrocephaly was found by ultrasound in pregnancy at gestational age of 26 weeks. Caesarean section was performed at gestational age 38 weeks and 6 days. Her birth weight was 3130 gram, her length 49 cm, and OFC 36 cm. The Apgar score was 7-9-9. Bilateral club foot was present. Cerebral MRI at the age of 5 days old showed hypoplasia of corpus callosum and dilated ventricles. Venticulo- peritoneal shunting was performed at 9 days old, 3 months later the shunt failed for the first time. Afterwards, there have been multiple shunt revisions because of infections and shunt failure. Her vision was impaired, possible caused by pressure damage on the visual cortex. Anisomelia of 2 cm (right limb shorter than left) was observed, as well as right-sided hip dysplasia. The latter was diagnosed at the age of 2,5 years, was non-progressive, and had no need for surgery. She developed diabetes mellitus, treated with insuline. From the age three years she was able to stand with support, using a walker. She was non-verbal, and she communicated through body language. Physical examination at the age of 4.5 years old, her height was -2 SD, OFC was -4 SD (decreasing from birth, currently stabilized), and weight was -1,5 SD. Mild facial dysmorphism, including deep set eyes and tented mouth were present. Hearing and olfactory sense were normal. There was normal dentition, normal skin and nails. No seizures were observed. Previous investigation with
Affymetrix 6.0 SNP array was normal. Screening of blood and urine metabolites have not been performed.

**Individual 3** is a 8-year old boy, born after an Caesarean section at GA 37\(^\circ\) with birth weight of 3.14kg from non-consanguineous Korean parents. He had a healthy older brother. At his age of 4 months he visited ophthalmologic clinic due to poor eye contact and abnormal nystagmoid eyeball movements, but showed normal fundus and normal optic nerve. During follow up developmental delay was noticed from his age of 5-6 months. His development was getting better very slowly, he could sit up at his age of 5 years and could walk with holding from his age of 6 years. At his age of 8 years, he could neither walk alone nor speak meaningful word ‘mama’ but could perform a few simple task.

At his age of 9 months, irregular shaped hyperpigmented maculo- papules were detected from his neck, which extent was increased to face, abdomen, arms and legs with thickening and transformed to verrucous papules. On skin pathology of this pigmentation revealed hyperkeratotic acanthosis and papillomatosis with increased basal lamina. At his age of 2 years he got a surgery of orchiopexy due to bilateral cryptorchidism. On physical examination at his age 8 years, his height was 124 cm (0 SD) weight 33.65 kg (+0.75 SD), and head circumference 50.3 cm (-0.75 SD). From his age of 7 and half years, seizures were detected during sleep. On continuous EEG monitoring with video, he showed both arm and leg tonic seizures after sudden arousal from sleep, several times at night with periodic spike discharges on the left fronto- temporal area and spreading to right fronto temporal area. Now his seizures are well controlled after a combination of valproate, oxcarbazepine and clobazam.

**Individual 4** is a 12-years-old boy of Dutch ancestry, born after an uncomplicated pregnancy and delivery at 41+3 weeks with a birth weight of 3825 gram. Family history was unremarkable. He had 2 healthy sisters. The neonatal period was uncomplicated. At the age of 8 months developmental delay was noticed. He could roll over at the age of 7 months, could sit without support at the age of 1 year and could walk independently at the age of 3 years and 2 months. He spoke several words until the age of 1-1.5 years. At the age of 12 years he did not speak, but he did perform simple tasks. He showed stereotypic behaviour (hand flapping). Epilepsy started at the age of 7 years for which he was initially treated with valproic acid, which was later effectively switched to clobazam. He had hypermetropia and constipation. He had normal hearing and did not have sleeping problems. Physical examination at the age of 11.5 years showed a height of 141 cm (-1.5 SD), weight of 34.5 kg (+0.7 SD) and head circumference of 50 cm (-2.2 SD). He walked with a broad-based walking pattern. He had a hypotonic face, upslanting palpebral fissures, full upper eye lids, prominent anti-helix of both ears and folded helices. He had slender fingers, clinodactyly of the fifth fingers and variant simian crease. His feet showed clinodactyly of the fourth and fifth toes and he had overriding second toes. MRI of the brain showed cerebellar vermis atrophy, decreased white matter volume, thin optic nerves, mild thin brainstem and a hypoplastic corpus callosum. SNP-array showed a paternally inherited ~450kb deletion of 10q23.31 which was
considered not to be significant clinically. DNA analysis of *EHMT1, TCF4, FMR1*, methylation of 15q11-13 and metabolic screening were normal.

**Individual 5** is now a 15 year old girl, born at 38 weeks gestation by emergency caesarean section for breech presentation after a pregnancy complicated by hyperemesis. Birth weight was 2550 g, head circumference 9-25\textsuperscript{th} centile. Parents are healthy and non-consanguineous and have an older healthy child. There were no neonatal problems until concerns arose from age 13 weeks with visual inattention, variable strabismus, horizontal nystagmus (later diagnosed as pendular nystagmus with optic atrophy worse on the right and abnormal VEPs), fisting movements and slowing head growth (0.4\textsuperscript{th} centile). Tone was commented to be “quite good, possibly increased” with some head control and normal reflexes. MRI brain at age 4 months noted marked delay in myelination thought to be an evolving leukodystrophy. Repeat MRI a year later showed generalized atrophy of cerebrum and cerebellum, with generalized reduction of white matter and abnormally deep sulcation but no definite change from the previous MRI and no calcification. (Repeat scans taken age 4 are shown). Development was delayed globally but there was no regression. At 2 years she was starting to attempt, but could not sustain, a crawling posture, mouthed persistently and had no babble. She had difficulty swallowing semi-solid foods, with gagging. Relatively long tapering fingers, with generally small hands and feet (not oedematous), were noted clinically but she was not facially dysmorphic. She displayed brief episodes, not thought to be epileptic (normal EEG) and never associated with loss of consciousness, of dystonic posturing with extension of her trunk, neck and arms, associated with hyperventilation. She tended to keep her hands clenched at all times. Truncal tone was increased and tendon reflexes were slightly increased. All neurometabolic investigations were normal, including long chain fatty acids, white cell enzymes, phytanic acid, carnitine and acylcarnitine levels, urine and blood for organic and amino acids, lumbar puncture. Skeletal survey was normal with no evidence of dysostosis multiplex. At age 6 she vocalized but still had no words, though her parents felt she had some simple comprehension. She remains non-verbal but with good hearing and has a happy disposition. She will clap hands when asked. Bruxism, drooling and stereotypic behaviours including hand-flapping, spontaneous paroxysmal laughter and breath-holding are noted features. With support, she can walk with a broad based gait. Problems with gagging on semi-solid food persist. She has no urinary or bowel control. Growth parameters are on the 2\textsuperscript{nd} centile except head circumference at -3 standard deviations. Hip problems arose age 6 with her leg ‘giving way’ with recurrent dislocations. There was significantly decreased abduction of the right hip (10-20 degrees), milder on the left. Hip X-ray showed dysplasia, with deficient moulding of the hip socket, thought to be due to lack of weight-bearing. Surgical hip adductor and psoas release was undertaken bilaterally. Her ankles adopt an inverted equinus position with involuntary spasm. She has had one prolonged generalized seizure age 10 concurrent with urinary tract infection but was afebrile. Later EEG was normal. Genetic investigations including microarray, MECP2, Angelman and TCF4 analyses were all normal. She was therefore recruited to the Deciphering Developmental Disorders (DDD) study (DECIPHER ID 263643). 

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5.7 Supplemental data

Figure S1. Cilium morphology of hTERT-RPE1 cells transfected with wild type or mutant RAB11B constructs. Expression of recombinant 3xFLAG-RAB11B wild type (A) and 3xFLAG-RAB11B variants p.(Gln70Leu) (B), p.(Ser25Asn) (C), p.(Val22Met) (D), p.(Ala68Thr) (E) in hTERT-RPE1 cells, detected using anti-FLAG antibodies (all in green). Recombinant protein expression, immunocytochemistry and image capture was performed as described previously. The following transfection efficiencies were obtained per 3xFLAG-RAB11B construct: wild type, 14%; p.(Gln70Leu), 19%; p.(Ser25Asn), 9%; p.(Val22Met), 10%; p.(Ala68Thr), 14%. Cilia were stained with axonemal marker acetylated tubulin (red) and magnifications are shown in the insets. Wild type and GTP-bound active RAB11B (p.(Gln70Leu)) are showing punctuated localization with an enrichment of puncta at the base of the cilia. Both identified mutations p.(Val22Met) and p.(Ala68Thr) show similar localization as GDP-bound inactive RAB11B (p.(Ser25Asn)) with dispersed localization throughout the cytoplasm. Cilium morphology appeared normal for all constructs. In all pictures, nuclei were stained with DAPI (blue). Scale bars represent 10μm.
Figure S2. Localization of wild type and mutant RAB11B in IMCD3 cells. Expression of recombinant 3xFLAG-RAB11B wild type (A, F) and 3xFLAG-RAB11B variants p.(Gln70Leu) (B, G), p.(Ser25Asn) (C, H), p.(Val22Met) (D, I), p.(Ala68Thr) (E, J) in IMCD3 cells, detected using anti-FLAG antibodies (all in green). Recombinant protein expression, immunocytochemistry and image capture was performed as described previously.1 The following transfection efficiencies were obtained per 3xFLAG-RAB11B construct: wild type 18%; p.(Gln70Leu) 19%; p.(Ser25Asn) 15%; p.(Val22Met) 11%; p.(Ala68Thr) 13%. Cilia were stained with axonemal marker acetylated tubulin (red) and magnifications are shown in the insets (A-E). The Golgi apparatus was visualized with staining of Golgi marker GM130 in red (F-J). Wild type and GTP-bound active RAB11B (p.(Gln70Leu)) are showing punctuated localization with an enrichment of puncta at the base of the cilium. Both identified mutations p.(Val22Met) and p.(Ala68Thr) show similar localization as GDP-bound inactive RAB11B (p.(Ser25Asn)) with dispersed localization throughout the cytoplasm and enrichment at the Golgi apparatus, although less pronounced for p.(Val22Met). Co-localization with GM130 (K) was quantified by calculating the Pearson’s
De novo mutations disturbing RAB11B cause ID and a distinctive brain phenotype

correlation coefficient (PCC) using the JACoP plugin in ImageJ (N=12 cells for each construct, mean in red and error bars represent the standard deviation).\textsuperscript{2,4} The significance of difference between the constructs is shown in the table and was calculated with a Student’s t-test (ns: not significant (P>0.05); *: P<0.05; **: P<0.005; ***: P<0.0005). In all pictures, nuclei were stained with DAPI (blue). Scale bars represent 10µm.
### Chapter 5

#### -LWHA selection

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Controls pos neg controls pos neg controls pos neg controls pos neg
De novo mutations disturbing RAB11B cause ID and a distinctive brain phenotype

Figure S3. Identified interactors of RAB11B using yeast two-hybrid cDNA library screening and their affinity for RAB11B variants. Co-transformations were performed in PJ69-4a yeast strains with the identified clones from the library screens fused to pAD together with WT or mutant RAB11B constructs fused to pBD. Both panels show selection of the two-hybrid clones grown on medium lacking leucine, tryptophan, histidine and adenine (LWHA, upper left panel) or medium lacking leucine, tryptophan and histidine with 3 mM 3AT (lower left panel). α-Galactosidase reporter gene activation (left side) or β-galactosidase reporter gene activation (right side) will cause blue coloration. Controls are shown at the bottom; for the positive control, yeast cells were co-transformed with pAD-WT and BD-WT constructs (pos), or with pLaminC fused to pBD with pAD-WT constructs as negative control (neg). Details of the identified interactors in the cDNA library screens can be found in supplemental Table S2.

Figure S4: Alignment of RAB11B and RH0A. Mutation identified in our study, and a dominant-negative mutation described in RH0A<sup>-/-</sup> are indicated. Conservation of residues is indicated in grey scale, identical residues are indicated with an asterisk.
Table S1. Brain imaging feature of individuals with de novo mutations in RAB11B

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Summary of brain abnormalities observed after re-evaluation of available MRI images of individual 1, 3, 4 and 5 and abnormalities clinically reported for individual 2. For the latter individual, no brain images were available for re-evaluation. Abbreviations: L=Left, mo=months, NA = Not Available, NR=Not Reported, R=Right, yr=years

Table S2. Detailed results of yeast two-hybrid cDNA library screening with RAB11B and the affinity of the identified mutants for these interactors. This table contains extended information on the identified interactors as well as the results of reporter gene activation on −LWH + 3AT and −LWHA selection. Quantification of growth and reporter gene activation is on a 0-3 scale, with 0 = no activation, 3 = strongest activation. (Table S2 is available as a separate excel file on request.)
5.8 Supplemental acknowledgement
The DDD study presents independent research commissioned by the Health Innovation Challenge Fund [grant number HICF-1009-003], a parallel funding partnership between the Wellcome Trust and the Department of Health, and the Wellcome Trust Sanger Institute [grant number WT098051]. The views expressed in this publication are those of the author(s) and not necessarily those of the Wellcome Trust or the Department of Health. The study has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). The research team acknowledges the support of the National Institute for Health Research, through the Comprehensive Clinical Research Network.

5.9 Supplemental references
Chapter 6

General discussion
6.1 The expanding phenotypic spectrum of ciliopathies

Genetic defects in ciliary genes can lead to a wide spectrum of clinical manifestations, as discussed in chapter 1, and can affect the development or function of a single organ or multiple organ systems. The genetic and clinical heterogeneity complicated the recognition that these disorders arise from malfunctioning of the primary cilium in the past, and the term ‘ciliopathies’ was first used to coin this group of disorders in 2006.\(^1\) In this thesis, I focused on studying trafficking processes of proteins towards the cilium. The results described in this thesis show that defects in genes involved in transport towards and docking of cargo at the cilium are associated with a variety of ciliopathies, ranging from isolated blindness to complex, perinatal lethal disorders. An example of a non-syndromic ciliopathy that only affects a single organ is presented in chapter 2. Here, I described families with variants in POC1B and a form of isolated blindness (cone-rod dystrophy - CRD). In chapter 3, families with BBS are presented with a more complex phenotype consisting of retinal dystrophy, obesity, polydactyly, intellectual disability (ID), hypogonadism and/or renal dysfunction. In this chapter, it is clear that phenotypic difference can also exist between BBS patients from the same family. Possible causes of this phenomenon will be discussed further in section 6.2. In chapter 4, I explored the function of the exocyst complex, which is known to be an important regulator of ciliary trafficking. I gained insights into the normal function of this complex and also studied the effects of exocyst variants that are associated with JBTS or MKS. The latter disorder is part of the severe end of the ciliopathy spectrum and is perinatal lethal. While we have a fair comprehension of the width of the ciliopathy spectrum, new ciliopathies are still being uncovered.\(^2\) In chapter 5, I discuss various families with a new neurodevelopmental disorder that is caused by heterozygous de novo variants in RAB11B. Rab11 is a small GTPase and key regulator of slow endocytic recycling through recycling endosomes.\(^3\) Three Rab11 proteins are encoded in the mammalian genome: RAB11A, RAB11B and RAB25, with differential expression patterns. RAB11B is mainly expressed in the brain, whereas its closest homologue RAB11A, sharing 90% amino acid identity,\(^4\) is ubiquitously expressed.\(^5\) Rab11 has proven to be important for ciliogenesis in cultured cells\(^6\)–\(^8\) and localizes at the base of the cilium in peri-centrosomal recycling endosomes.\(^9\) The phenotype of the patients is closely related to other neurodevelopmental ciliopathies, including (but not limited to) severe ID, nystagmus, hypotonia and cerebellar vermis hypoplasia and ventriculomegaly on MRI. However, MRI analysis did not show the hallmark clinical signs of ciliary dysfunction in the brain such as a molar tooth sign or Dandy-Walker malformation.\(^1\)–\(^10\) As fibroblasts of the patients were not available, future experiments with CRISPR-Cas9 generated mutant cell lines that could provide insights into the degree of disruption of the recycling endosome and ciliary function. Since the phenotype of the patients with RAB11B variants is not a classic ciliopathy, the molecular defect possibly only affects general vesicle trafficking in recycling endosomes while leaving cilium function intact. This hypothesis is based on the idea that RAB11A and RAB11B likely have overlapping functions; since RAB11A is ubiquitously expressed it could quench the impact of RAB11B dysfunction. Another example of a complex of proteins with a dual function (in the cilium and lysosome biogenesis) is the biogenesis of lysosome-related
organelles complex (BLOC). When this complex is dysfunctional this can result in a disease that may not necessarily be caused by impaired cillum functioning. As the name implies, BLOC proteins are required for normal biogenesis of specialized organelles of the endosomal-lysosomal system, such as melanosomes and platelet dense granules, and are organized in three complexes; BLOC-1, BLOC-2, and BLOC-3.\textsuperscript{14} Variants in genes encoding BLOC complex proteins are implicated in Hermansky-Pudlak syndrome [OMIM 203300], which is characterized by oculocutaneous albinism combined with hemorrhages due to platelet storage pool deficiency, and lysosomal ceroid storage.\textsuperscript{12} Recently, BLOC-1 has been shown to be important for the trafficking of PKD2 and possibly other ciliary transmembrane proteins to the cilium.\textsuperscript{13} Interestingly, mice with BLOC-1 variants have mildly cystic kidneys,\textsuperscript{13} a characteristic phenotype of ciliary dysfunction, however, this feature is not observed in patients with Hermansky-Pudlak syndrome. Likely, there are other proteins with functions that overlap those of BLOC-1 in human kidneys, which may prevent cyst development in this organ system, whereas there are no compensational mechanisms to rescue the function of BLOC-1 in melanosome and platelet dense granules biogenesis. In conclusion, our understanding of the width of the ciliopathy spectrum is likely incomplete as there may be additional disorders that are fully or in part caused by ciliary disruption. In particular disorders that are only partly caused by cilium dysfunction and non-syndromic manifestations may not easily be identified as ciliopathies.

6.2 Modifiers contribute to clinical heterogeneity

Since 1994,\textsuperscript{14} over 140 syndromic ciliopathy-associated genes that are mutated in 19 clinically distinct disorders have been identified (chapter 1), illustrating the enormous clinical and genetic heterogeneity in ciliopathies. Allelic heterogeneity, which refers to the situation when variants in one gene can lead to different disorders, is also common in the ciliopathy spectrum. For example, variants in CEP290 can cause LCA, SLSN, BBS, JBTS and MKS.\textsuperscript{15} Interestingly, variants can cause clinical variability within and between families, and this phenomenon has previously been described in BBS.\textsuperscript{16} This intrafamilial clinical variability could be explained by variants in other closely related BBS genes. In the past few years, several studies have shown that the mutational load of ciliopathy genes could cause phenotypic diversity.\textsuperscript{17-20} In chapter 3, I describe a family with a BBS-like phenotype without renal involvement that carries a homozygous missense variant p.(Arg93Trp) in CEP164, however, this gene has previously been reported in NPHP related ciliopathy patients.\textsuperscript{21} Unfortunately, in this study of Chaki and colleagues, a clear diagnosis for these families with CEP164 variants, e.g. BBS or JBTS, is not provided. Although a final diagnosis is missing, it is clear from their clinical data that there is a genotype-phenotype correlation for this gene. Families with a homozygous missense variant p.(Gln11Pro) have NPHP combined with retinal degeneration (RD) and possibly obesity, but in families with a truncating variant p.(Gln525*) in combination with a heterozygous missense variant p.(Arg93Trp) the phenotype is more complex and involves NPHP with RD and ID or developmental delay. One family with a homozygous truncating mutation presented with the most severe phenotype; including NPHP, RD, brain
abnormalities, polydactyly and obesity, which can be classified as BBS. Interestingly, the family in our study with a homozygous missense variant in CEP164 also carries a heterozygous BBS12 missense variant, predicted to be damaging according to various algorithms (see chapter 3, table 3). This BBS12 variant could contribute to the severity of the phenotype that was seen in our family and may explain why our family presents with a BBS-like syndrome. It remains unclear why our family does not present with a renal phenotype, however, others have also described families with CEP164 variants without renal manifestations. For example, in the same study of Chaki et al., they reported on a family with isolated LCA, thus no renal insufficiency, with a homozygous variant that mutates the original stop codon, which is predicted to lead to an extension of 57 amino acids to the protein (p.*(1460TrpextX57)).

Taken together, the family reported in chapter 3 seems to fit in the spectrum of phenotypes that arise when CEP164 is mutated, combined with an additional variant in BBS12. With respect to phenotypic variability and modifier effects in other ciliopathies then BBS, I describe families with non-syndromic CRD in chapter 2 with variants in POC1B, which shortly thereafter was identified in a patient with JBTS. Interestingly, this patient carried the exact same variant p.(Arg106Pro) as reported in our families but presented with a complicated JBTS phenotype comprising of a MTS on MRI combined with LCA, PKD resulting in ESRD, and lung hypoplasia. This is in contrast to additional families reported with non-syndromic CRD, again carrying the same variant. Considering the large consanguineous family of the JBTS patient, it is likely that other variants in ciliopathy genes contribute to the severe phenotype. However, in a recent study where a systematic analysis of the contribution of modifier alleles in JBTS genes to the phenotypic diversity of a large JBTS cohort (386 affected individuals) was performed, they found little support for modifier alleles in JBTS genes in these JBTS patients. If this also holds true for other (syndromic) ciliopathies, like BBS, is unclear, and similar studies applied to other phenotypes of the ciliopathy spectrum would provide advanced understanding of the contribution of modifier alleles. Additionally, the possibility of modifier alleles in other ciliary genes then JBTS-related genes, in case of the study in JBTS, needs to be explored. Taken together, the exact contribution of the mutational load to the clinical phenotype in ciliopathies remains to be further characterized, and other influences on phenotypic outcome should still be considered, e.g. environmental factors. In the future, systematic analysis of additional variants in ciliary genes and their functional characterization should shed more light on the exact mechanism of ciliary dysfunction, as well as their contribution to the clinical heterogeneity of ciliopathies.

6.3 Different disruptions in transport processes cause different phenotypes

It is clear that malfunctioning transport is related to a wide spectrum of ciliopathy phenotypes. Variants in genes that encode proteins that are part of the BBSome complex, which are involved in membrane protein transport towards and within the cilium, cause a different phenotype compared to disease-associated variants in genes that encode proteins that are involved in cargo docking and selection at the transition zone. In this section, I discuss
Figure 1. Schematic overview of the different steps of ciliary transport as discussed in section 6.3. Legend see next page.
the different steps of ciliary transport, the proteins involved and related ciliopathies, and possible disruptions in these mechanisms that cause ciliary dysfunction.

### 6.3.1 Ciliary targeting sequences

The prerequisite for a protein to be incorporated in the cilium, is to be recognized by the cilium directed transport machineries. Like for cargo directed towards the nucleus, it has been shown that cargo that is transported towards the cilium also depends on a specific signal sequence, the ciliary targeting sequence (CTS). Several different CTSs are described but lack a consensus sequence, which indicates that proteins targeted for the cilium can be transported via different routes, and/or that each step in the process relies on other signals. For example, different signal sequences for transmembrane proteins have been described, where the tag is positioned either at the cytoplasmic side of the protein or at the N- or C-terminus of the protein. Furthermore, only two sequences have been described for cytoplasmic proteins. The presence of a CTS allows specific interactions with transport-related proteins. For example, there is an interaction between BBS proteins and the CTS of the transmembrane protein SSTR3, where the CTS is positioned in the third intracellular loop of the protein, which results in the formation of a polymerized coat complex at the peri-ciliary membrane and translocation of SSTR3 across the TZ into the ciliary axoneme. Another example is the interaction of Arf4 with the C-terminal CTS in rhodopsin and this complex subsequently binds ASAP1, which activates the Rab11/Rabin8/Rab8 complex and results in transport of rhodopsin into the outer segments of photoreceptor cells. The importance of CTSs is highlighted by the fact that variants affecting the CTS of rhodopsin have been associated with RP [MIM: 613731]. In this case, RP likely results because of impaired rhodopsin transport, which results in accumulation of rhodopsin in the inner segments of photoreceptor cells.

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(Legend Figure 1.) Five processes related to transport of cargo towards the cilium are indicated. Number 6.3.1, and the accompanying panel in the bottom, refers to the importance of a CTS (ciliary targeting sequence) that is recognized by the transport machinery before a protein is transported to the cilium, or transported into the cilary compartment (grey gradient). In the panel, the three possible positions of a CTS in a transmembrane protein are indicated. A subsequent step in the process of transport towards the cilium is the actual movement towards the cilary base along microtubules or actin filaments, with the help of motor proteins, and motor-cargo adapters like the IFT-complexes (section 6.3.2). In the accompanying panel below, three different motor proteins identified in the exocyst protein interaction dataset, and the subdivision of IFT proteins in three complexes are indicated. After arrival at the cilium, the cargo is handled at the cilary base (6.3.3). The panel below shows a magnification of the base of the cilium and (candidate) proteins involved in this step are indicated, as discussed in section 6.3.3. For the tethering of the exocyst (EXOC), the identified candidate proteins for the initial recognition/tethering of the vesicle at the cilary base, before the actual fusion with the cilary pocket, are shown (CEP290, POC1A, IQCB1, CEP89 and NUPL). POC1B and FAM161A co-localize at the basal bodies, and FAM161A has been shown to associate with microtubules. Another protein complex involved in transport towards and into the cilium is the BBSome (6.3.4). It forms a coat-complex together with a cargo receptor in the peri-ciliary membrane and subsequently facilitates entry (and exit) of proteins in the cilary compartment. A final step for transport into the cilary compartment is crossing the transition zone (6.3.5). Two examples of GTPases assisting in this process are shown: Rab8 forming a complex with Importin-1 to facilitate the entry of fibrocystin (not specified), or the lateral transport of D1-type dopamine receptors (not specified) with the help of Rab23. Numbers refer to the different sections in the text where the transport step is discussed, see text for detailed explanations. All elements are not drawn to scale. TF: transition fibers.
6.3.2 Motor proteins in cilium directed transport

Transport machineries directed towards the cilium comprise of motor proteins that drive transport along microtubules or actin filaments, and the adaptor proteins that connect the motor proteins to their cargo. Motor proteins involved in general (vesicle) transport processes in the cell, next to cilium directed transport, like dyneins and kinesins, are often essential for cellular survival in general and not primarily associated with ciliopathies. There are many protein subfamilies that are associated with a specific inherited disorder, e.g. myosins to cardiomyopathies or bleeding disorders, and kinesins and dyneins to neuropathies. Remarkably, the motor proteins involved in transport of vesicles from the trans-Golgi network (TGN) towards the base of the cilium, as well as to the connecting cilium (CC) in photoreceptor cells, are poorly described, and research has mainly focused on the IFT processes and related motors within the cilium compartment. Considering the orientation of minus-ends of microtubules at the base of the cilium, as the basal body still acts as a microtubule organizing center, one would suggest that the minus-end directed motor dynein is a candidate. One study could implicate the involvement of dynein-1 in cilium directed transport, as Rab11-FIP3 was shown to interact with a component of the cytoplasmic dynein-1 motor complex. The same group showed that this interaction mediates the transport of vesicles towards the endosomal-recycling compartment. Only recently, Rab11-FIP3 is also shown to be regulating the transport of rhodopsin towards the cilium, in concert with ASAP1, Rab11 and Rabin8. Whether the interaction of Rab11-FIP3 with dynein-1 is also relevant for cilium directed transport remains to be tested. On the contrary, anterograde transport could also be implicated in transport of vesicles towards the cilium, since the Golgi apparatus also acts as a microtubule organizing center, nucleating microtubules with the plus-ends pointed outward. This would implicate that kinesins, plus-end directed motors, could also be involved in transport of specifically Golgi-derived vesicles towards the cell surface, probably near the cilium. Interestingly, the kinesin-3 family member involved in plasma membrane directed transport of VEGFR2, KIF13B, has been shown to regulate TZ composition and ciliary SMO accumulation, a critical component of the Shh pathway (see chapter 1.1, box 1), linking the cytoplasmic kinesin-3 motor to ciliary function. Another interesting clue can be found in the protein interaction dataset of chapter 4, where I studied the exocyst complex. This octomeric protein complex is involved in polarized vesicle trafficking, ciliogenesis and associates with cargo vesicles, and two complex members are found to be mutated in JBTS and MKS. In the protein-protein interaction dataset of the exocyst complex members presented in chapter 4, three different motor protein families are identified; actin-based myosins (myosin heavy chain 15, myosin X, myosin-IB, myosin-VC, and myosin IXA), microtubule based kinesins (kinesin family member 5A, 5B and 5C, and kinesin light chain 2), and microtubule based dyneins (axonemal dynein heavy chain 7, dynactin 1, dynactin 2 (p50), dynactin 3 (p22), and α-centractin) (details, see chapter 4 Table S1). Of these motor protein families, only the interaction with myosins has been described before. Which of these motor protein families, together with the exocyst
complex, is also involved in the transport of vesicles from the TGN towards the cilium, remains to be further explored.

Closely connected to the actual motor proteins involved in cilium directed transport are the ‘interlinking’ proteins or adaptors, connecting motor proteins with their cargo. Well described complexes within the cilium are the IFT particles A and B,\textsuperscript{54, 55} however, not much is known about these type of complexes involved in transport directed towards the cilium. To date, one IFT-B component, IFT20, has been established in transport of cargo from the TGN towards the cilium. IFT20 localizes both to the Golgi and basal bodies, and loss of IFT20 blocks ciliogenesis, whereas reduced IFT20 activity causes a reduction of PKD2 in the ciliary membrane,\textsuperscript{56} showing that IFT20 is involved in transport of vesicles between the Golgi and the cilium. GMAP210/TRIP11 anchors IFT20 to the Golgi membrane,\textsuperscript{57} and VPS15 mediates the release of IFT20 from the Golgi compartment,\textsuperscript{58} allowing transport of vesicles towards the cilium. Furthermore, IFT20 has been shown to be part of the IFT-B2 complex, which is distinct from the IFT-B1 complex.\textsuperscript{59, 60} Another component of this IFT-B2 complex, IFT57, has also been related to transport of dopamine receptors into the cilium from the periciliary membrane,\textsuperscript{61} as well as being important for transporting of ciliary motility related proteins towards the cilium.\textsuperscript{62} Interestingly, GMAP210/TRIP11 and IFT57 have both been identified in the interaction data set of the exocyst (chapter 4, figure 2). It is tempting to speculate that the exocyst, together with IFT-B2, is responsible for cilium directed vesicle transport, under control of GMAP210 and VPS15 at the Golgi. Interestingly, IFT57 and VPS15 have been identified as ciliopathy genes, IFT57 being related to an unclassified OFD subtype\textsuperscript{63} and VPS15 to an unclassified ciliopathy phenotype characterized by RP, renal failure, mild skeletal developmental features and ID.\textsuperscript{58} Considering the central role of motor proteins in cilium directed transport, it is not surprising that such syndromic ciliopathies are related to dysfunction, because many different (signaling) processes can be affected. However, many factors remain to be explored related to the motor proteins (and their adaptors) involved in cilium directed transport.

### 6.3.3 Cargo handling at the base of the cilium

When vesicles arrive at the base of the cilium, they need to be recognized, docked, and eventually fused with the ciliary pocket. As this requires a combination of several different events, an array of proteins is involved in these steps of cilium directed transport. One complex involved in the recognition of the target membrane during exocytosis events is the exocyst complex, which is known for polarized vesicle trafficking in various cellular events where directed membrane delivery is necessary, like neurite outgrowth, and the leading edge in migrating cells.\textsuperscript{64} As discussed in chapter 4, several exocyst complex members have been found to localize to the primary cilium or basal bodies/centrioles,\textsuperscript{65-68} and some individual members are essential for ciliogenesis in vitro.\textsuperscript{5, 69} By using both SF-TAP and Y2H library screen approaches (see chapter 1.1 box 2), I have identified different sets of proteins interacting with exocyst components. Which interactions are specifically related to the cilium function remains to be investigated. However, I have identified several components localized at the base of the
ciliary (CEP290, IQCB1, POC1A, CEP89, and several NUPs), both in the indirect protein interaction experiments (SF-TAP) as well as using the binary interaction trap screen (Y2H). All of these proteins are candidate proteins for the initial recognition and/or tethering of vesicles at the base of the cilium mediated by the exocyst, either at the transition fibers (CEP89), basal bodies (POC1A), basal side of the TZ (CEP290, IQCB1), or the membrane of the TZ (NUPS). After the initial recognition by the exocyst, other proteins like SNARE proteins could mediate the actual fusion with the ciliary pocket. Future experiments based on knock-down strategies (either CRISPR-Cas9 or siRNA mediated) should discriminate if these candidates are essential for exocyst tethering at the base of the cilium. Besides these TZ and basal body proteins I also identified PKD2, indicating that using an SF-TAP approach, cargo of these vesicles can also be detected, albeit with modest sequence coverage. Future experiments to further discriminate between interacting proteins or cargo proteins would help to extent our understanding of the ciliary involvement of the exocyst. The conditions of these experiments should be adjusted as such, that either vesicle components (e.g. by cellular fractionation) or membrane proteins (adjusted lysis conditions and/or cellular fractionation) can be detected more specifically.

In chapter 2, I studied the basal body protein POC1B, identified as a causative gene in the non-syndromic retinal ciliopathy CRD. Previously, POC1B was shown to be essential for centriole integrity. To further understand the ciliary link of POC1B and study its binary interactors, we performed a Y2H library screen that identified FAM161A. This ciliary protein was previously also found to be associated with an inherited retinal dystrophy, RP, and therefore caught our attention. Interestingly, the interaction with FAM161A was affected by the variants in POC1B identified in the families, suggesting a functional link between these two proteins which is disrupted in the CRD families. FAM161A is also localized to the base of CCs in photoreceptor cells, just like POC1B, and was found to interact directly with the ciliopathy-associated proteins lebercilin (LCA5), CEP290, OFD1 and SDCCAG8. Furthermore, the protein was found to associate to microtubules, similar to lebercilin. Interestingly, FAM161A-interactor SDCCAG8 is shown to be indirectly required for Shh signaling, and is suggested to be involved in ciliary vesicle docking because of its basal body localization and interactions with endosomal sorting complex proteins (RABEP2, ERC1 and ERC2). Further characterization of direct interaction partners of FAM161A revealed a clear link with the Golgi-centrosomal network, suggesting that FAM161A is involved in the formation of a protein scaffold required for MT-based transport from Golgi to the centrosome, and/or to the ciliary basal body. The fact that variants in both FAM161A and POC1B are associated with non-syndromic retinal dystrophies and not affecting other organs, although being ciliary proteins, could be explained by retina-specific expression. Additionally, photoreceptor cells are highly metabolic cells, since 10% of the photosensitive membranous disks in the outer segments are renewed every night because of light-induced oxidative damage. This disc recycling process demands optimal functionality of the inner segment and the CC to provide enough material, making photoreceptor cells more sensitive for changes in transport efficiency due to variants. Taken together, the knowledge obtained about FAM161A suggests a role for its binary interactor POC1B in vesicle trafficking at the base of the cilium.
6.3.4 The BBSome
A subsequent step in transmembrane protein transport towards the cilium is the actual fusion of the cargo vesicle with the plasma membrane, and translocation of the transmembrane protein from the ciliary pocket across the TZ into the ciliary membrane compartment. The BBSome is thought to play an important role in this process, localizing both at the base of the cilium as well as along the ciliary axoneme.76 The BBSome has been suggested to function as a coatamer complex, assembling an electron dense coat on to membranes.27 This functionality is underlined by the fact that the BBSome, together with the IFT-A and IFT-B particles, originates from a common IFT-B-like complex with a coatamer complex ancestry.77 The BBSome is recruited to the membrane by GTP-bound Arl6 (BBS3), and complexes are formed together with the receptor or cargo protein (containing a CTS) that needs to be transported into the ciliary compartment. The exact mechanism hereafter is still unclear. This BBS-Arl6-cargo protein complex might stay within the membrane and mediate active lateral transport across the TZ, or the BBS-Arl6-cargo protein complex buds off as a coated vesicle where after it fuses with the ciliary membrane.78 Either way, the interaction of the BBS with IFT complex proteins is essential for this final entry into the ciliary compartment,79-81 suggesting that IFT-trains “drag” the BBS with its cargo through the TZ, like an icebreaker.78,82 Besides ciliary entry of proteins, the BBSome is also involved in ciliary exit of proteins,83 probably by similar interactions with the IFT proteins. Correct functioning of the BBSome also relies on CEP290 and NPHP5, positioned at the TZ and involved in formation of the TZ diffusion barrier. These two proteins coordinate correct assembly of the BBSome holocomplex, and allow its selective passage into the cilium.85 When either CEP290 or NPHP5 is knocked down using siRNAs, there is no proper BBSome holocomplex assembly, and the incomplete sub-complexes localize into the cilium due to a defective diffusion barrier. In contrast, a loss of any BBSome subunits also prevents the assembly of the holocomplex but the incomplete subcomplexes cannot enter the cilium because the TZ diffusion barrier remains intact. Variants in BBS genes do not necessarily result in ciliogenesis defects, but affect the receptor composition of the cilium,79;80;86 thereby affecting ciliary function. A link with the BBS-specific obesity phenotype is made via the ‘satiety factor’ hormone leptin. It has been shown that the BBSome controls energy homeostasis because it mediates the transport of the leptin receptor to the plasma membrane.57,88 When leptin receptor expression on the cell surface is reduced, e.g. by BBS variants, the regulation of decreased feeding behavior and increased energy expenditure is disturbed, resulting in obesity.89 However, some studies suggest that the leptin resistance is a secondary consequence to the obesity phenotype,90 thus the exact link between ciliary dysfunction and obesity in BBS patients remains a topic of investigation.91

The variants identified in BBS-associated genes in chapter 3 disturb the function of the BBSome via different manners, however, resulting in a similar effect. In case of the splice site variant in BBS3 (encoding Arl6), it causes exon skipping, inducing a frameshift that results in a premature stop codon. This probably will lead to degradation of the mutant mRNA via nonsense-mediated decay (NMD), abrogating protein expression. Since Arl6 is essential for the BBSome to be recruited to the membrane,27;80 proper transport of transmembrane
proteins by the BBSome is disturbed. A similar variant affecting splicing and thereby causing NMD, was identified in BBS5. Likely, this variant also causes unsuccessful association of the BBSome with the membrane as well, since BBS5 is believed to be the only BBSome subunit in direct contact with the ciliary membrane.\textsuperscript{76} Another truncating variant was identified in BBS9, likely activating NMD as well, which encodes one of the three BBSome core complex proteins.\textsuperscript{92} Loss of BBS9 causes disturbed formation of the entire BBSome holocomplex. All these variants in Arl6, BBS5 and BBS9 result in disturbed binding of the BBSome with the target membrane, either because of loss of membrane interactions (Arl6 or BBS5) or because of unsuccessful assembly of the BBSome holocomplex (BBS9). One family carried pathogenic missense variants in CEP164 (homozygously), as well as BBS12 (heterozygously). BBS12 is part of the chaperonin complex, facilitating the formation of the holocomplex, so one could assume that a variant in BBS12 causes a disturbed formation of the holocomplex. Simultaneously, the CEP164 variant could cause improper formation of the basal bodies and TZ, since CEP164 is a centriolar appendage protein important for ciliogenesis,\textsuperscript{93} and specifically for docking of the initial ciliary vesicle before axoneme elongation occurs.\textsuperscript{94} Interestingly, CEP164 is a prey in affinity-capture experiments using BBS7,\textsuperscript{95} which could suggest that there is indeed an (indirect) interaction between CEP164 and the BBSome, although this is not reported earlier. Having variants in both CEP164 as well as BBS12 could result in disturbed assembly of the BBSome, possibly in combination with affected docking at or passing of the TZ. Taken together, variants in the BBSome result in altered receptor composition of the cilium, rather than ciliogenesis defects. This disturbs specific signaling pathways that eventually lead to the complex BBS phenotypes.

\section*{6.3.5 Ciliary entry mechanisms}

The entry of IFT proteins into the ciliary compartment might provide clues for the ciliary entry mechanisms of cargo proteins. It was already described that IFT-B proteins accumulate at the base of the cilium, on the transition fibers.\textsuperscript{96} However, what triggered their release and entry into the cilium remained unknown,\textsuperscript{55} until recently. While studying the centrosomal protein CEP19, previously related to obesity,\textsuperscript{97} the lab of Peter Jackson at Stanford discovered the first known mechanism directing ciliary entry of IFT complexes. They first found that CEP19 is tethered at the base of the cilium by CEP350 and FOP, and most interestingly, it recruits GTP-bound RABL2B which in turn is able to bind the IFT-B complex. Upon GTPase activity of RABL2B, hydrolyzing GTP into GDP, the IFT-B complex is released into the cilium.\textsuperscript{98} Other related mechanisms involved in regulating the entry of cargo proteins at the base of the cilium are Rab GTPase mediated as well. Transportin-1, originally identified as part of the nucleocytoplasmic transport machinery crossing the nuclear pore, was shown to assemble into a complex with Rab8 and a GFP molecule cloned with the CTS of fibrocystin (PKHD1), under the control of the nucleotide binding status of Rab8, thereby facilitating entry of the GFP-CTS construct into the cilium.\textsuperscript{99} Another example is provided by a study focusing on the transport mechanism by lateral diffusion from the periciliary membrane into the ciliary membrane, similar as SMO. This study showed that D1-type dopamine receptors are
delivered to the ciliary plasma membrane with the help of IFT-B proteins (IFT57 and IFT172), ciliary kinesin KIF17 and Rab23, again under the control of the nucleotide binding status of the Rab GTPase. These studies show that the identified entry mechanism is not universal for all receptors, indicating that each subset of receptors needs different transport machineries. This principle can also be related to the specific phenotype of BBS, because a particular subset of receptors fails to reach the ciliary membrane, causing specific ciliary dysfunction.

### 6.4 Vesicle trafficking via recycling endosomes

Over the last years, several studies have revealed a new indirect trafficking route for membrane associated proteins targeted towards the cilium via recycling endosomes (REs). REs are highly dynamic endocytic compartments in the cell for sorting and re-exporting of internalized membrane constituents. They are composed of multiple tubulovesicular recycling pathways, likely for distinct trafficking routes, and are responsible for the assembly and maintenance of specialized apical membrane compartments, like the cilium. It was already shown that there is a tight connection between centrosomes and REs and that during ciliogenesis vesicles originating from REs fuse with the initial basal-body-ciliary-vesicle complex to provide membrane for the growing cilium. Several studies have shown that REs are not just solely involved during ciliogenesis, but also when the cilium is fully formed and needs to be maintained. Involvement of REs as a relay station for cargo directed towards the cilium was already suggested in 2010, however, it was not clear which proteins would be transported towards the cilium via this route. From then on, it was shown that the cytoplasmic tail of fibrocytin (PKHD1) contained a CTS that could direct GFP towards the cilium, and that this construct also resided in REs. Other studies have shown that PKD2 is transported via the RE towards the cilium, under control of the RE proteins SDCCAG3 and PI3K-C2α, which both are also essential for ciliogenesis or ciliary extension. Another specified cargo protein traveling via the RE is newly synthesized rhodopsin. Furthermore, RE trafficking is also involved in the regulation of two ciliary signaling pathways, Shh and NOTCH. Finally, two ciliopathy proteins (ARL13B and ALMS1) are also shown to be involved in RE trafficking. All these studies clearly show the involvement of the RE as a relay station of cargo directed towards the cilium, and now the specific cargo subtypes that travel via this route need to be further identified.

Just recently, a link was made between the BLOC-1 complex proteins and the trafficking of PKD2, via the RE, towards the cilium. As described earlier in section 6.1, the BLOC-1 complex is required for normal biogenesis of specialized organelles of the endosomal-lysosomal system, and consists of eight subunits. Interestingly, in chapter 4, I have identified six of the eight BLOC-1 proteins in the protein interaction dataset of the exocyst complex, either by SFTAP experiments or Y2H library screens. In the study of Monis and colleagues, where they identified the importance of the BLOC-1 complex for PKD2 delivery to the cilium, they also found that this is in concert with the exocyst and IFT20. An interaction of a BLOC-1 subunit DTNBP1 and the exocyst was already identified earlier. IFT20, as discussed earlier in section 6.3.2, is responsible for the transport from the Golgi compartment towards the cilium. When
analyzing the protein interaction dataset of the exocyst in chapter 4 it contains all previously separately identified components for ciliary transport: the BLOC-1 complex, IFT20 related proteins IFT57 and GMAP210 (although IFT20 itself is not identified), as well as cilium directed cargo PKD2. This expands the proposed model of Monis and colleagues, were PKD2 is transported to the cilium via the RE by IFT-B2 (containing IFT20 and IFT57) under control of GMAP210 at the Golgi compartment, interacting with the exocyst and BLOC-1 complexes for targeted exocytosis at the ciliary base. Future studies should validate and further specify these interactions to better understand all the components of the pathway for cilium directed transport via the RE.

6.5 New insights in ciliary function

Over the last 15 years, research about cilia has mushroomed, causing new concepts for ciliary function to emerge which have initiated new fields of research. One important intriguing finding involves submerged primary cilia, which are not directly exposed to the outside cellular environment. The existence of submerged cilia has long been recognized and visualized using electron microscopy,\textsuperscript{110} however, although the ciliary membrane is buried in a deep membranous pit, it was always assumed that these cilia were still responsive to signals.\textsuperscript{111} Just recently, an elegant assay was developed to be able to distinguish between submerged or exposed cilia using fluorescent microscopy,\textsuperscript{112} providing a more practical tool than electron microscopy to better recognize this phenomenon. Interestingly, another recent study found that the presence of subdistal appendages controls if cilia are exposed on the cell surface. Loss of subdistal appendages (together with C-Nap1) causes dissociation of the mother centriole from the daughter centriole, of which the latter is firmly associated with the Golgi apparatus.\textsuperscript{113} Since this is the first report where they actually manipulated the state of the cilium, being exposed or submerged, the responsiveness of these cilia could be tested and compared. Strikingly, while submerged cilia need Shh pathway activation for accumulation of SMO and Gli transcription factors in the cilium to convey the signal, exposed cilia exhibited SMO and Gli accumulation without stimulation of the pathway.\textsuperscript{113} This raises the question if submerged cilia could be a quick pathway to temporarily ‘turn-off’ cilia for signaling, instead of going through the time consuming process of cilium disassembly and re-assembly. Consequently, the finding that the state of the cilium (submerged or exposed) has an effect on the signaling activity, highlights the importance to have caution during the interpretation of data of ciliary signaling experiments. The reported fluorescence microscopy-based assay could help to determine if the state of the cilium has an effect on the readout of pathways.\textsuperscript{112}

Another interesting finding is the fact that the cilium is actively able to form ectosomes,\textsuperscript{114;115} small extracellular vesicles or microvesicles of 100-350 nm in size that are ectocytosed from the plasma membrane.\textsuperscript{116} This finding raises a new concept of cilia being involved in sending signals, instead of solely receiving them. Ectosomes are already proposed to be important for intercellular communications,\textsuperscript{117} and the fact that they could attach to and possibly interact with the cilium,\textsuperscript{115;118;119} suggests that that ectosomes provide the cilium with more means to send and receive signals.\textsuperscript{120} In a recent study of Nager and colleagues, they found that
ectocytosis from the ciliary tip is an alternative pathway to shed activated G protein-coupled receptors from the ciliary membrane, next to retrograde transport back to the cell body. This process of ectocytosis from the ciliary tip turned out to be mediated by actin and actin-regulators. When retrograde transport and ectocytosis was blocked simultaneously (by using a IFT27 knock-out cell line treated with actin poisons), Shh signaling failed, which was not the case when either one of the two processes were still intact. The involvement of actin in ciliary function has been underlined by another study utilizing proximity labeling and subsequent mass spectrometry analysis of the ciliary membrane, where many actin components were identified. Furthermore, it is already known that actin is involved in membrane shaping and endo/exocytosis. Interestingly, in the interaction dataset of the exocyst complex in chapter 4, I have identified several actin components, actin regulators, as well as actin-based motors (components: Actin gamma1 (ACTG1); regulators: DPNBP1, IQGAP1, CCDC42, POI1B, SIPA1L, SYNE2, TPM1, TPM3, TRIM27; actin-based motors: MYH15, MYO10, MYO1B, MYO5C, MYO9A). Combined with the fact that in urine samples the exocyst was identified in exosomes, another type of small extracellular vesicles, suggests that the exocyst complex could also be involved in the membrane shaping events in the cilium. The fact that the exocyst has also been found to be localized along the ciliary axoneme, further supports the possibility of the exocyst being involved in ectocytosis events from the ciliary membrane. Whether the interactions of the exocyst with actin components and regulators is cillum-specific awaits further clarification. In photoreceptor cells, the process of membrane ectocytosis at the distal end of the CC is important for OS disk formation. In fact, the retinal dystrophy associated protein peripherin actually suppresses the formation of ciliary ectosomes, causing membrane expansions to stay attached to the membrane at the CC, ultimately forming the disks of the OS. When peripherin is mutated, it causes the developing OS of photoreceptor cells to solely release ciliary ectosomes, and therefore disk formation fails. All together, these studies show the importance of ectocytosis in ciliary functioning, expanding the list of processes that are important for the cilium.

Recent studies have also revealed the unique phosphoinositide composition of the ciliary membrane, besides the enrichment of specific ciliary receptors for various signaling pathways, e.g. Notch, Wnt and Shh signaling. The ciliary membrane contains phosphatidylinositol 4-phosphate (PI(4)P) whereas the plasma membrane contains phosphatidylinositol 4,5-biphosphate (PI(4,5)P$_2$). The presence of the latter PI(4,5)P$_2$ in the plasma membrane recruits actin regulators and thereby modulates actin polymerization that is required for membrane shaping events. In the cilium, the JIBTS related phosphatase INPP5E converts PI(4,5)P$_2$ into PI(4)P, maintaining a unique phosphoinositide composition in the ciliary membrane, and thereby suppressing actin related processes. As described above, ectocytosis is an actin-driven process, and recently it has been shown that the loss of INPP5E activity causes an increase in PI(4,5)P$_2$ in the ciliary membrane. This induces dramatic actin-driven ectocytosis events at the ciliary tip, generating large ectosomes that significantly reduced the ciliary length, which is referred to as ciliary excision or decapitation. In the same study, they showed that ciliary decapitation precedes cillum disassembly, and that these
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ectocytosis events are required by quiescent cells for timely entry into the G1 phase, re-entering the cell cycle. Furthermore, it has been shown that phosphoinositide composition and INPP5E activity is related to Shh signaling, however, the exact mechanism and consequences for this relationship are still unclear.\textsuperscript{128, 132, 133}

Taken together, all these findings show that the processes of ectocytosis and actin dynamics play important roles in ciliary and photoreceptor function, providing new ways to explain some of the missing links in ciliopathy research. Furthermore, the specific composition of the ciliary membrane, and the state of the cilium being submerged or exposed, are modulators for ciliary signaling. This indicates the complex relationship of all these processes, and shows that caution should be undertaken when analyzing data and interpreting results. Future studies are needed to provide further insight into the molecular pathways involved in these processes, and the involvement in ciliary function.

6.6 Challenges for the future

The topics discussed in section 6.5 about submerged/exposed cilia, ciliary ectosomes and ciliary membrane composition, already give examples of new hurdles to be taken in ciliary and ciliopathy research. When considering the genetic aspects of ciliopathy research regarding identification of new causative genes and particular variants in those genes in ciliopathy patients, many challenges lay ahead as well. WES revolutionized the identification of new genes but will not be able to detect (structural) variations in non-coding regions. Whole genome sequencing (WGS) provides this improved mutation detection,\textsuperscript{134} however, brings along the challenge of variant interpretation, specifically those in non-coding regions. Although our understanding of variation in non-coding DNA is still poor, future research will allow for improved interpretation of variants in these regions, probably in particular in conserved regulatory elements such as promoters, enhancers, insulators, and other regulators.\textsuperscript{135} In this thesis, I have used WES to identify causative variants for several families with CRD (chapter 2), BBS (chapter 3), or a neurodevelopmental disorder (chapter 5). And in a recent study, a combination of sequencing approaches including SNP array for structural changes, gene panel sequencing, and eventually WES, resulted in the identification of causative variants for 85% of 265 families with various ciliopathy phenotypes.\textsuperscript{136} This illustrates that the mutation identification rate is high, however, for a significant number of families the genetic cause is unknown. Implementing and improving WGS for ciliopathy research in the future, including the interpretation of variants, will hopefully increase the success rate of variant identification for these genetically unexplained families.

Another challenge for the future is the functional characterization of newly identified ciliopathy-associated genes, and the interpretation of the effect of specific variants on ciliary function. Using model systems (both cell lines and animal models) and combining protein-protein interaction techniques provides further insight in the mechanism of disease, as shown in chapter 2, 4 and 5. Bringing the output of these techniques to a higher level by incorporating the interaction datasets into one general network, provides even further
insights in the complex processes within the cilium, and could even provide the basis to identify ‘new’ ciliopathies.\textsuperscript{2} Combining datasets will overcome the limitations of individual techniques (as discussed in section 1.1.7 and box 2), as different approaches to identify protein-protein interactions will give different results; e.g. applying either TAP or Y2H with exocyst members generated two different datasets, however, both carrying valuable information (\textit{chapter 4}). Another aspect to be clarified further in the future would be to determine the different transport processes involved in cilium directed transport to better understand the disturbance of ciliary protein composition. This also involves the identification of the specific cargos that are related to these different transport processes, e.g. PKD2 travels via the REs, although SMO reaches the cilium via lateral diffusion.

Eventually, a comprehensive understanding of all genes, proteins, and processes involved in ciliary function will be fundamental in achieving the ultimate objective of the ciliary research field: to develop effective treatments for ciliopathy patients. These therapies can be designed as such to, for example, correct or compensate for a genetic defect by gene therapy,\textsuperscript{137} or to interfere with altered signaling pathways by small molecules.\textsuperscript{138} Developing therapy for ciliopathy patients is a challenging tasks, given the wide spectrum of ciliopathy phenotypes (both syndromic and non-syndromic), the increasing number of genes involved, and the congenital nature of many phenotypes that already start early in development. Up to date, there are no successful therapies for ciliopathies in the clinic. Nevertheless, gene therapy has booked some successes for retinal degeneration in some trials.\textsuperscript{36}

\textbf{6.7 Concluding remarks}

In this thesis, I have discussed the broad spectrum of ciliopathies that is related to ciliary dysfunction, with a focus on the aspects of (disturbed) ciliary transport. It has been clear that several distinct transport mechanisms are important for ciliary function, leading to different types of ciliopathies when disturbed. Furthermore, new pathways of ciliary transport are still being identified in the last years, increasing the complexity of this intriguing organelle. Scrutinizing the protein assemblies involved in these processes, understanding the consequences of their malfunction, as well as the systematic integration of all gathered data into relational databases and networks will provide critical new insights. These will eventually help us understand the complex genotype-phenotype correlations in the still expanding group of ciliopathies, optimizing genetic diagnosis and disease management, and presenting new targets for therapeutic interventions.
6.8 References


General discussion


Chapter 6


Chapter 6


Summary

Cilia are cellular organelles with a slender, longitudinal shape that protrude from the plasma membrane of almost all cell types in the human body. They can occur as motile cilia, often in bundles of several hundred that move in a coordinated fashion to propel liquid, but the far majority of cells form a single non-motile cilium, referred to as the primary cilium. The antenna-like appearance of primary cilia reveals their function: they are involved in sensing environmental cues coming from other cells or from outside the human body. Several processes are necessary to maintain the signaling function of the cilium (chapter 1.1). Variants in genes involved in ciliary function can cause a disruption of crucial signaling pathways that play a pivotal role in the correct development of various tissues, resulting in specific phenotypes that are coined ‘ciliopathies’ (chapter 1.2). The genetic and phenotypic heterogeneity of ciliopathies underscores the complexity of this organelle. This also shows the necessity of unraveling the pathways associated with ciliary homeostasis and function, and the molecular and physiological consequences of ciliary dysfunction. The aim of this thesis was to further decipher the molecular factors that underlie ciliopathies, and specifically to understand the role of vesicle trafficking in ciliary disorders.

A first step in studying the mechanism of disease of a ciliopathy is to identify the genetic cause. Whole exome sequencing (WES) and gene-panel based next-generation sequencing was used to determine the genetic cause of ciliopathy-related phenotypes in several families (chapters 2, 3 and 5). This led to the identification of a new ciliopathy gene (chapter 2), new genotype-phenotype correlations (chapter 3), and a gene that is associated with a ciliopathy-like syndrome (chapter 5). Furthermore, proteomic analysis was employed to identify the protein interaction networks within which candidate ciliopathy proteins reside, to further understand their potential role in cilium biology, and the consequences of genetic variants in the associated genes (chapters 2, 4 and 5).

Chapter 2 reports two unrelated families with cone-rod dystrophy (CRD), a non-syndromic ciliopathy, caused by variants in POC1B that encodes a basal body protein. WES analysis revealed a homozygous missense variant (p.Arg106Pro) in one family, and a heterozygous single amino acid deletion (p.Gln67del) combined with a heterozygous splice site mutation (c.810+1G>T) in the other family. We found that POC1B localizes to basal bodies in cultured cells as well as the connecting cilium of photoreceptor cells. Searching for binary protein interactors expressed by retinal cDNAs using yeast two-hybrid screening technology resulted in the identification of FAM161A, a protein that was previously associated with retinitis pigmentosa. CRD-associated variants in POC1B disturbed the localization of POC1B at basal bodies as well as the interaction with FAM161A. Morpholino knock-down in zebrafish resulted in smaller eyes and retinal degeneration leading to vision loss. This phenotype could be rescued using wild type POC1B mRNA, but less efficiently when CRD-associated variants were present. This study unveiled a basal body protein photoreceptor module that contains POC1B and FAM161A that is required for photoreceptor homeostasis.
Summary

Chapter 3 covers the phenotypic and genetic description of five unrelated families that are diagnosed with Bardet-Biedl syndrome (BBS), a ciliopathy that is caused by variants in genes encoding subunits of the BBSome, or proteins affecting its activity. The BBSome is a protein complex involved in trafficking of transmembrane proteins towards and into the cilium. Targeted exome sequencing of 21 ciliopathy genes associated with BBS resulted in the identification of causative homozygous variants in the following genes: BBS3, BBS5 and BBS9, for four out of five families. For the fifth family, subsequent WES analysis identified a homozygous missense variant in CEP164, previously reported to be associated with the renal cystic ciliopathy nephronophthisis 15. The family in our study, however, did not show any signs of renal cysts or renal dysfunction. The results of this study confirm both inter- and intrafamilial clinical heterogeneity in patients having common genetic defects in BBS genes, and describe a broader clinical phenotypic spectrum resulting from mutations in CEP164, extending it beyond retinal-renal ciliopathies to a BBS-like phenotype.

The exocyst complex is another protein complex involved in trafficking of vesicles towards the cilium. Variants in two members were previously associated with ciliopathies, EXOC8 with Joubert syndrome and EXOC4 with Meckel-Gruber syndrome. Therefore, we employed protein-protein interaction studies to decipher the protein interaction network of the exocyst complex, to understand its molecular relationship with the cilium (Chapter 4). Tandem Affinity Purification followed by mass spectrometry with all exocyst members identified EXOC6B, which we propose to be part of an alternative mammalian assembly of the exocyst complex. Additionally, two ciliary proteins that both reside at the base of the cilium (CEP290 and IQCB1) were identified in the exocyst interactome. When yeast two-hybrid library screens were performed with EXOC4 and EXOC8 we identified three binary interacting proteins localizing at the ciliary base: CEP89, JBTS17, and NUP62. The interaction of EXOC4 with CEP89, a distal appendage protein of the mother centriole, was confirmed using co-immunoprecipitations. Taken together, an integrated view of the protein-protein interaction dataset supports the ciliary role of the exocyst and identified several proteins creating a model in which the exocyst organizes a specific plasma membrane docking site at the base of the primary cilium.

In Chapter 5, five families are described with a neurodevelopmental syndrome that resembles a ciliopathy, characterized by severe intellectual disability, microcephaly, and a distinct brain phenotype on MRI. WES analysis identified two dominant heterozygous de novo missense variants (p.(Val22Met) and p.(Ala68Thr)) in RAB11B, encoding one of the three members of the RAB11 GTPase protein family. GTPases are molecular switches that are essentially required for cellular vesicle trafficking, and are involved in the endosomal membrane recycling system (recycling endosomes). Effects of the mutations on general cilium morphology in cultured cells were not apparent and patient material was not available for analysis, therefore the direct ciliary involvement remains unclear. However, analysis of the 3D protein structure of RAB11B did reveal that both missense variants disturbed the GTP-binding pocket. Localization studies of constructs with the identified variants showed a similar
localization pattern as GDP-locked inactive RAB11B. Yeast two-hybrid cDNA library screens of neuronal tissues identified interactions with known GTPase interactors, effectors, and guanidine nucleotide exchange factors (GEFs), and showed that most interactions remain unaffected, except for an enhanced affinity for the GEF SH3BP5. We propose that these data together suggest a dominant-negative effect that results in distinct defects in vesicle trafficking in several neuronal developmental processes, leading to the identified neurodevelopmental syndrome in human.

In chapter 6, the findings of this thesis are discussed in light of recent literature on vesicle trafficking towards the cilium. First, modulators for the genetic and phenotypic heterogeneity in ciliopathies are discussed, with a focus on Bardet-Biedl syndrome. An overview is presented of the different steps in ciliary transport, of the proteins involved and related ciliopathies including the presented proteins/complexes in this thesis, and of the possible mechanisms that cause ciliary dysfunction. This shows that many defects ultimately cause disturbed receptor composition of the ciliary membrane, thereby resulting in ciliary dysfunction. It is clear that several distinct transport mechanisms are important for ciliary function, and new pathways are continuously being reported, extending the complexity of this intriguing organelle. Scrutinizing the interactome involved in these processes, identifying the molecular consequences of their malfunction, as well as systematic integration of all gathered data into relational databases, will allow more detailed modelling of ciliary function in health and disease. Ultimately, this will provide valuable information to improve diagnostics and identify potential therapeutic targets for ciliopathies.
processen cilium, alle homozygote eiwit van verstoring cilium, correlaties Sciliopathieën de bank, aminozuur celltypes in proefschrift resulteerde met‐om syndromische de stap (h de genetische set Varianten in de genetische ‐t variant syndroom potentiële van een genen in een familie cellen in nieuwe genetische‐t rol varianten in het genetische ‐t voorkomen genen in het geassocieerd alsmede hetgeen een ‐t waar het motiel cilium (CRD) van het plasmamembraan van het heterogeniteit het gewone of het motiel cilium en dystrofie van het fenotype: exome as het homeostase het doel ‐t motivatie gen of het spinale site gen van het is meerderheid naar het genetische screenningstechnologie dat de ciliaire en het horeastase en het ciliopathie te bewegen een enkel, een ‐t begrijpen, een ‐t te".
Samenvatting

Cilia zijn organellen met een slanke langwerpige vorm, die uit het plasmamembraan van bijna alle celtypes in het menselijk lichaam steken. Sommige celtypes hebben bundels van een paar honderd beweeglijke of motieke cilia, die door gecoördineerd te bewegen een vloeistofstroom kunnen creëren. Een overgrote meerderheid van de cellen heeft echter een enkel, non-motiel cilium, aangeduid als het primaire cilium. De antenne-achtige verschijning van een primair cilium onthult zijn functie: het is betrokken bij het ontvangen en doorgeven van signalen uit de omgeving die afkomstig zijn van andere cellen of van buiten het menselijk lichaam. Verschillende processen zijn nodig om de signaalfunctie van het cilium te handhaven (hoofdstuk 1.1). Varianten in genen die betrokken zijn bij die functie kunnen leiden tot verstoring van cruciale cellulaire signaleringsprocessen die een essentiële rol spelen bij de correcte ontwikkeling van verschillende weefsels, wat resulteert in specifieke fenotypen: de ciliopathieën (hoofdstuk 1.2). De genetische en fenotypische heterogeniteit van ciliopathieën onderstrept de complexiteit van dit organel. Dit laat ook de noodzaak zien om de cellulaire processen die verband houden met de homeostase en functie van het cilium te ontrafelen, evenals de moleculaire en fysiologische gevolgen van een verstoorde ciliumfunctie. Het doel van dit proefschrift was om de moleculaire factoren in ciliopathieën te ontcijferen, en specifiek om de rol van membraanvesikel transport in ciliaire stoornissen te begrijpen.

Een eerste stap in het bestuderen van het ziekte mechanisme van een ciliopathie is het identificeren van de genetische oorzaak. Whole exome sequencing (WES) en de sequentie analyse van een set van genen werden gebruikt om de genetische oorzaak van ciliopathie-gerelateerde fenotypen in verschillende families te bepalen (hoofdstukken 2, 3 en 5). Dit leidde tot de identificatie van een nieuw ciliopathie-geassocieerd gen (hoofdstuk 2), nieuwe correlaties tussen genotypes en fenotypen (hoofdstuk 3) en een gen dat geassocieerd is met een ciliopathie-achtig syndroom (hoofdstuk 5). Verder werd proteomische analyse gebruikt om de eiwitinteractie-netwerken waarbinnen kandidaat-ciliopathie-ewitten zich bevinden te identifieren, om hun potentiële rol in de ciliumbiologie verder te begrijpen, alsmede de gevolgen van de genetische varianten in de bijbehorende genen (hoofdstukken 2, 4 en 5).

Hoofdstuk 2 rapporteert over twee niet-verwante families met kegel-staaf-dystrofie (CRD), een niet-syndromische ciliopathie, veroorzaakt door varianten in POC1B, hetgeen voor een eiwit van het basaallichaampje van het cilium codeert. WES analyse onthulde een homozygote missense variant (p. (Arg106Pro)) in één familie en een heterozygote deletie van één aminozuur (p. (Gln67del)) gecombineerd met een heterozygote splice site mutatie (c.810+1G>T) in de andere familie. We vonden dat het POC1B eiwit zich bevindt in de ciliaire basaallichaampjes in gekweekte cellen evenals het verbindende cilium van fotoreceptorcellen. Het zoeken naar binaire eiwitinteractoren die in de retina tot expressie komen, met behulp van de “yeast two-hybrid” screeningstechnologie met een retinale cDNA bank, resulteerde in de identificatie van FAM161A, een eiwit dat eerder geassocieerd was met retinitis pigmentosa. CRD-geassocieerde varianten in POC1B verstoren de lokalisatie van
Samenvatting

POC1B in de basaalichaampjes evenals de interactie met FAM161A. Het verlagen van Poc1B eiwit in zebravissen m.b.v. morfolino’s resulteerde in kleinere ogen en retina degeneratie, hetgeen leidt tot zichtverlies. Dit fenotype kan worden tegengegaan door het wild-type POC1B mRNA tot expressie te brengen, en dit was minder efficiënt wanneer CRD-geassocieerde varianten in dit mRNA aanwezig waren. Deze studie onthulde een fotoreceptorbasaalichaam-gerelateerde eiwitmodule, waar POC1B en FAM161A onderdeel van zijn, en welke essentieel is voor fotoreceptor homeostase.

Hoofdstuk 3 beschrijft de fenotypes en genetische afwijkingen van vijf niet-verwante families met Bardet-Biedl syndroom (BBS), een ciliopathie die wordt veroorzaakt door varianten in genen die coderen voor onderdelen van het BBSome, of eiwitten die haar activiteit beïnvloeden. Het BBSome is een eiwitcomplex dat betrokken is bij het transport van transmembraneeiwitten naar en in het cilium. Gerichte exome sequencing van 21 ciliopathie-geassocieerde genen die gemuteerd kunnen zijn in BBS resulteerde voor vier van de vijf gezinnen in de identificatie van oorzakelijke homozygote varianten in BBS3, BBS5 en BBS9. M.b.v. WES analyse werd in de vijfde familie een homozygote missense variant gevonden in CEP164, wat eerder werd geassocieerd met de cystenier gerelateerde ciliopathie nefronofftie 15. De betreffende familie in onze studie vertoonde echter geen tekenen van niercysten of nierdysfunctie. De resultaten van deze studie bevestigen zowel inter- als intrafamiliaire klinische heterogeniteit bij patiënten met gemeenschappelijke genetische defecten in BBS genen, en beschrijft een breder klinisch fenotypisch spectrum dat voortvloeit uit mutaties in CEP164, wat zich uitbreidt van retina-renaal ciliopathieën naar een BBS-achtig fenotype.

Het exocystcomplex is een ander eiwitcomplex dat betrokken is bij het transport van membraanvesikels naar het cilium. Varianten in twee onderdelen waren eerder geassocieerd met ciliopathieën: EXOC8 met Joubert syndroom en EXOC4 met Meckel-Gruber syndroom. Daarom hebben we eiwit-eiwit interactie studies gebruikt om het eiwit-interactienetwerk van het exocyst complex te ontcijferen en zo de moleculaire relatie met het cilium te kunnen begrijpen (hoofdstuk 4). Een dubbele affiniteitszuivering (“tandem affinity purification”) gevolgd door massaspectrometrie met alle eiwitten van het exocyst complex leidde tot identificatie van EXOC6B, een eiwit waarvan we veronderstellen dat het deel uitmaakt van een alternatief samengesteld exocystcomplex in zoogdieren. Daarnaast werden twee ciliaire eiwitten in het exocyst-interactienetwerk geïdentificeerd, die zich beide in de basis van het cilium bevinden (CEP290 en IQCB1). Wanneer interactiescreens met behulp van “yeast two-hybrid” CDNA banken werden uitgevoerd met EXOC4 en EXOC8, identificeerden we drie directe interacties met eiwitten die ook deel uitmaken van de basis van het cilium: CEP89, JBTS17 en NUP62. De interactie van EXOC4 met CEP89, een eiwit dat onderdeel is van het “aanhangsel” van het moedercentriool, werd bevestigd met co-immunoprecipitatie. Samengevat, ondersteunt de geïntegreerde weergave van de eiwit-eiwitinteractie dataset de ciliaire rol van het exocyst en werden verschillende interacterende eiwitten geïdentificeerd, wat een model mogelijk maakt waarin het exocyst een specifieke plasmamembran-koppelingsplaats organiseert aan de basis van het primaire cilium.
Samenvatting

In hoofdstuk 5 worden vijf gezinnen beschreven met een neurologisch ontwikkelingsstoornis syndroom dat lijkt op een ciliopathie, gekenmerkt door ernstige intellectuele beperking, microcefalie en een duidelijk hersenenfenotype op MRI. WES analyse identificeerde twee dominante heterozygote de novo missense varianten (p. (Val22Met) en p. (Ala68Thr)) in RAB11B, een gen dat codeert voor één van de drie leden van de RAB11 GTPase eiwit familie. GTPases zijn moleculaire schakelaars die essentieel zijn voor de logistiek van cellulaire vesikels, en zijn betrokken bij het stelsel van endosomale membraan recycling. Effecten van de mutaties op de algemene ciliarmorfologie in gekweekte cellen waren niet duidelijk en patiëntmateriaal was niet beschikbaar voor analyse, waardoor de directe ciliaire betrokkenheid van dit gen onduidelijk blijft. Echter, uit analyse van de 3D eiwitsamenstelling van RAB11B bleek dat beide missense varianten het GTP-interactie-oppervlak verstoorden. Lokaliseringsstudies van constructen met de geïdentificeerde varianten lieten een vergelijkbaar lokalisatiepatroon zien als GDP-gebonden inactief RAB11B. “Yeast two-hybrid” interactiescreens met cDNA banken van neuronale weefsel cellulaire interacties met bekende GTPase-associaties, effecten en guanidine-nucleotide-uitwisselingsfactoren (GEFs), en toonden aan dat de meeste interacties onaangetast bleven, behalve voor een verhoogde affiniteit voor de GEF SH3BP5. Op basis van de onderzoeksresultaten veronderstellen wij dat de varianten een dominant-negatief effect hebben dat resulteert in verschillende gebreken in membraanVESikel transport in verschillende neuronale ontwikkelingsprocessen, wat uiteindelijk leidt tot het geïdentificeerde neuroloogisch ontwikkelingsstoornis syndroom bij mensen.

In hoofdstuk 6 worden de bevindingen van dit proefschrift besproken binnen de context van recente literatuur over membraanvesikel transport naar het cilium. Ten eerste worden modulatoren voor de genetische en fenotypische heterogeniteit in ciliopathieën besproken, met een focus op Bardet-Biedl syndroom. Er wordt een overzicht gegeven van de verschillende stappen in ciliaire transport en de betrokken eiwitten en verwante ciliopathieën, inclusief de gepresenteerde eiwitten/complexen in dit proefschrift, en van de mogelijke mechanismen die ciliaire interacties verstoren. Deze analyse laat zien dat veel defecten uiteindelijk een verstoorde receptor samenstelling van het ciliaire membraan veroorzaken. Het is duidelijk dat verschillende afzonderlijke transportmechanismen belangrijk zijn voor een goed functionerend cilium, en er worden voortdurend nieuwe biochemische routes ontdekt, waardoor de complexiteit van dit intragende organel alleen maar toeneemt. Het interpreteren van de eiwitinteractienetwerk dat bij deze processen betrokken is, het identificeren van de moleculaire gevolgen van hun verstoring, alsmede de systematische integratie van alle verzamelde data in relationele databases, zal een meer gedetailleerde modellering van de ciliaire functie mogelijk maken. Dit verschaf eveneens inzicht in hoe een verstoorde ciliaire functie leidt tot een erfelijke ziekte. Uiteindelijk zal dit waardevolle informatie bieden om de diagnostiek te verbeteren en potentiële therapeutische doelwitten voor ciliopathieën te identificeren.
Eenvoudiger gezegd

De informatie die in ons DNA ligt vastgelegd bepaalt onze aangeboren uiterlijke kenmerken. Bijvoorbeeld onze haarkleur, oogkleur, of vorm van de oorschelp, maar ook een zesde vinger of verstandelijke beperking. De specifieke samenstelling van onze erfelijke informatie in het DNA wordt het genotype genoemd en is voor iedere persoon uniek. Dit is de mix van erfelijke informatie die je van je vader en moeder hebt gekregen. De specifieke samenstelling van onze aangeboren uiterlijke kenmerken, ofwel je uiterlijk, wordt het fenotype genoemd. Elk genotype (de samenstelling van onze erfelijke informatie) zorgt voor een ander fenotype (de samenstelling van aangeboren uiterlijke kenmerken). In mijn geval zorgt mijn specifieke genotype, samengesteld uit de erfelijke informatie die ik heb gekregen van mijn vader en mijn moeder, voor mijn specifieke fenotype, bestaande uit onder andere blond haar en blauwe ogen.

Erfelijke aandoeningen worden veroorzaakt door schrijffoutjes, of mutaties, in ons erfelijk materiaal. Ons DNA is als een encyclopedie reeks, opgebouwd uit verschillende boeken (chromosomen) en verschillende hoofdstukken (genen). De encyclopedie reeks van de mens bevat 46 boeken (chromosomen) en in totaal wel 20.000 hoofdstukken (genen). Elk gen beschrijft de bouw van een eiwit, en eiwitten zijn zowel de bouwstenen als actieve onderdelen (bijv. enzymen) van de cellen in ons lichaam. Als je drager bent van een mutatie in een bepaald gen, zorgt de mutatie voor een constructiefout in het bijbehorende eiwit, wat er voor zorgt dat dit eiwit niet goed zijn werk kan doen in de cel. Wanneer een kindje wordt geboren met een mutatie in een gen dat een netvlies-eiwit beschrijft, zorgt de mutatie ervoor dat dit netvlies-eiwit niet goed wordt gemaakt. Hierdoor kan dit netvlies-eiwit niet goed zijn werk doen waardoor de netvliescellen uiteindelijk afsterven. Het kindje met die mutatie zal op jonge leeftijd blind worden.

Ons menselijk lichaam bestaat uit miljarden cellen. Elke cel bevat verschillende compartimenten, of organellen, en deze organellen zijn opgebouwd uit eiwitten, vet en water. Trilhaartjes, ook wel cilia, zijn organellen van de cel met een antenne-achtige vorm die uit het cel-oppervlak steken van bijna alle cellen in ons menselijk lichaam. Cilia kunnen in twee vormen voorkomen: als beweeglijke cilia (trilhaartjes) of onbeweeglijke cilia. De beweeglijke cilia komen vaak voor in bundels van een paar honderd cilia, die op gecoördineerde wijze bewegen om een vloeistofstroom te creëren. Een voorbeeld hiervan is dat cilia ervoor zorgen dat het slijm uit je longen omhoog wordt gewerkt. De overgrote meerderheid van de cellen heeft echter de andere vorm: een enkel niet beweeglijk cillum, aangeduid als het ‘primaire cilium’. De antenne-achtige verschijning van een primair cilium onthult zijn functie: hij is betrokken bij het ontvangen van signalen uit de omgeving van de cel. Die signalen kunnen afkomstig zijn van andere cellen of van buiten het menselijk lichaam. Voorbeelden hiervan zijn de onderlinge communicatie tussen cellen tijdens de ontwikkeling van het embryo (welk groepje cellen wordt het hoofd, welk groepje cellen wordt linkerbeen etc.), of het meten van de vloeistofstroom in de nierbuisjes voor het reguleren van de werking.
van de nier, of het ontvangen van licht door netvliescellen in het oog. Verschillende processen in de cel zijn nodig om de signaalfunctie van het cilium te handhaven (uitgelegd in hoofdstuk 1.1).

Als er een mutatie zit in het gen wat een cilium-eiwit beschrijft dat betrokken is bij de werking van het cilium, kan de mutatie leiden tot een verstoorde werking van het cilium. Dit heeft verstrekkelijke gevolgen, omdat het cilium onder andere betrokken is bij de ontwikkeling van het embryo en de aanleg van verschillende organen. Als het cilium dus niet goed werkt door een mutatie in een cilium-gen, kan dat zorgen voor verschillende aangeboren afwijkingen, ofwel ciliumziekten. Deze ciliumziektes worden cilipathieën genoemd (uitgelegd in hoofdstuk 1.2). Tot nu toe zijn er al veel verschillende mutaties gevonden die een cilipathie veroorzaken en zijn er ook veel verschillende soorten cilipathieën ontdekt. Onderzoek van de afgelopen 15 jaar heeft laten zien dat het cilium veel complexer is dan wij dachten, en dat elke ontdekking tot meer vragen leidt. Om het cilium steeds beter te begrijpen is het niet alleen nuttig om de functie van het cilium zelf verder te onderzoeken, maar ook te kijken naar de processen in cel die nodig zijn om het cilium te onderhouden. Daarnaast kan de functie van het cilium verder verduidelijkt worden door te kijken naar de effecten van mutaties in cilium-genen die leiden tot cilipathieën.

Het doel van het beschreven onderzoek in dit proefschrift was om genetische oorzaken (mutaties) van cilipathieën te ontcijferen, te begrijpen welke eiwitten bij de cilium functie betrokken zijn, en om de rol van het transport (binnen de cel) van blaasjes gevuld met cilium-eiwitten naar het cilium beter te begrijpen.

Een eerste stap in het verklaren van het ziektemechanisme van een cilipathie is het identificeren van de genetische oorzaak (mutatie). Hiervoor werden de nieuwste DNA-analyse technieken gebruikt (o.a. Whole Exome Sequencing) om de code van alle 20.000 genen van cilipathie patiënten uit te lezen om ziekmakende mutaties te vinden (hoofdstukken 2, 3 en 5). Dit leidde tot de identificatie van een nieuw cilipathie-gen (hoofdstuk 2), nieuwe verbanden tussen genotypes en fenotypes (hoofdstuk 3) en de identificatie van mutaties in een gen bij patiënten met een cilipathie-achtig syndroom (hoofdstuk 5). Deze nieuw ontdekte cilium-genen coderen voor cilium-eiwitten. Om de functie van een nieuw ontdekt cilium-eiwit te bepalen, kan je gebruik maken van het ‘facebook-principe’: als jij mij jouw vrienden laat zien, zal ik jou vertellen wie jij bent. Wanneer je kunt bepalen met welke andere eiwitten een nieuw cilium-eiwit bindt (of vrienden is) kun je daaruit afleiden wat voor een functie dit nieuwe cilium-eiwit waarschijnlijk heeft. Als het nieuwe cilium-eiwit veel binding aangaat met transporteiwitten, heeft het hoogstwaarschijnlijk ook een rol in transport. Met eiwit-bindingsanalyses kun je het vriendennetwerk rondom een eiwit bepalen, wat het interactie-netwerk wordt genoemd. In het onderzoek in dit proefschrift werden naast de genetische analyses dergelijke eiwit analyses gebruikt om de eiwit-interactie-netwerken van nieuwe cilipathie-eiwitten te bepalen. Deze informatie werd gebruikt om hun rol in het cilium verder te begrijpen. Daarnaast werden deze interactienetwerken gebruikt om de
gevolgen van de mutatie in deze nieuwe cilium-eiwitten te analyseren (hoofdstukken 2, 4 en 5).

Hieronder wordt per hoofdstuk uitgelegd welke onderzoeken zijn gedaan, en tot welke nieuwe inzichten dit heeft geleid.

In hoofdstuk 2 worden twee niet-verwante families beschreven waarvan meerdere familieleden een vorm van erfelijke blindheid hebben. Hierbij sterven eerst de kegeltjes en vervolgens de staafjes van het netvlies af. De kegeltjes en staafjes zijn gespecialiseerde cellen in je netvlies die d.m.v. hun cilium licht kunnen omzetten in een signaal naar de hersenen, waardoor we kunnen zien. Door het uitlezen van alle 20.000 genen van deze familieleden, ontdekten wij mutaties in het gen ‘POC1B’. Wanneer wij dit gen in zebravissen uitschakelden, veroorzaakte dit ook in deze vissen vergelijkbare blindheid. Het gen POC1B codeert voor een eiwit met dezelfde naam. Dit POC1B eiwit bleek zich aan de basis van het cilium te bevinden van de kegeltjes en staafjes in het netvlies. Verder bleek uit onze eiwit-interactie-analyses dat de gevonden mutaties de binding van POC1B met een ander belangrijk kegeltjes en staafjes-eiwit verstoorde (FAM161A). Het onderzoek in dit hoofdstuk laat zien dat wij een nieuwe oorzaak van erfelijke blindheid hebben gevonden, hebben bewezen dat dit eiwit belangrijk is voor de kegeltjes en staafjes in ons netvlies, en dat dit eiwit zich aan de basis van het cilium in deze cellen bevindt waar het samenwerkt met een ander belangrijk kegeltjes en staafjes-eiwit.


Een groepje van eiwitten dat intensief samenwerkt, zoals een hechte vriendengroep, wordt ook wel een eiwitcomplex genoemd. Hoofdstuk 4 beschrijft een eiwitcomplex dat belangrijk is voor het transport van eiwitten naar het cilium. Dit is het exocyst complex en het bestaat uit acht eiwitten. Eerder werden al twee patiënten beschreven met ernstige ciliopathieën die mutaties in een eiwit van dit exocyst complex hadden. De ene patiënt had ernstige hersenafwijkingen, en de andere patiënt was door verschillende organaalafwijkingen niet levensvatbaar bij de geboorte. Om beter te begrijpen waarom mutaties in exocyst eiwitten
Eenvoudiger gezegd

zo’n ernstig ziektebeeld veroorzaken, hebben we de binding van het exocyst complex met andere eiwitten bestudeerd en specifiek gekeken met welke cilium-eiwitten dit complex bindt. Door het gebruik van verschillende technieken om de binding met andere eiwitten te bestuderen (‘Tandem Affinity Purification’ en ‘Yeast Two-hybrid’ analyses), hebben we de samenstelling van dit exocyst complex beter kunnen bepalen. Daarnaast vonden we eiwitbindingen met verschillende belangrijke cilium-eiwitten die zich in de basis van het cilium bevinden. Dit gaf meer inzicht in de specifieke locatie aan de basis van het cilium, waar we denken dat het exocyst complex de eiwitten dat het transporteert aflevert.

In hoofdstuk 5 worden vijf niet-verwante families beschreven met een ernstige hersenafwijking die lijkt op een ciliopathie. Het fenotype van de aangedane familieleden wordt gekenmerkt door ernstige verstandelijke beperking, een kleine hoofdromtrek en specifieke afwijkingen van de hersenen die op de MRI zijn te herkennen. Door het uitlezen van het DNA van deze families, vonden wij mutaties in het gen RAB11B. Dit gen codeert voor een eiwit met dezelfde naam, wat als een ‘aan/uit’-schakelaar werkt voor verschillende processen in de cel, waaronder het transport van blaasjes met eiwitten. Wanneer het RAB11B eiwit ‘aan’ staat, activeert het andere eiwitten waardoor het transport van deze blaasjes wordt geregeld. Om te begrijpen waarom de mutaties van deze patiënten leidden tot ernstige hersenafwijkingen, hebben wij eerst gekeken naar het effect van de mutaties op de vouwing van het RAB11B eiwit met gebruik van 3D-computermodellen. Verder onderzochten wij wat de locatie van het RAB11B eiwit was in de cel, en wat de verschillende eiwitten waren waarmee het RAB11B eiwit bindt. Hieruit bleek dat de patiëntmutaties specifiek de vouwing van de ‘aan/uit’ schakelaar van het RAB11B eiwit verstoorden waardoor het eiwit waarschijnlijk alleen ‘uit’ kan staan. Wanneer het RAB11B eiwit de patiëntmutatie bevatte, bevond het gemuteerde eiwit zich op dezelfde plekken in de cel als wanneer een gezond RAB11B eiwit ‘uit’ staat. Vervolgens bleek dat in eiwit-bindingsanalyses het gemuteerde RAB11B eiwit zich ook groeide alsof het ‘uit’ stond. Dat bracht ons tot de conclusie dat de patiëntmutaties ervoor zorgen dat het RAB11B eiwit altijd ‘uit’ staat, zich daardoor op de verkeerde plek in de cel bevindt, en niet meer goed andere blaasjes-transporteiwitten kan activeren. Omdat het RAB11B eiwit voornamelijk belangrijk is in de hersenen, zorgt dit voor verstoord transport van blaasjes in hersencellen tijdens de ontwikkeling, wat uiteindelijk zorgt voor ernstige hersenafwijkingen zoals wij in de patiënten met de RAB11B-mutaties zien. Het fenotype van deze RAB11B patiënten (de ernstige verstandelijke beperking, een kleine hoofdromtrek, en specifieke afwijkingen van de hersenen die op de MRI zijn te herkennen) lijkt op dat van andere patiënten met een ciliopathie, maar we kunnen nog niet zeggen of de RAB11B patiënten ook ciliopathie patiënten zijn. De vraag of het RAB11B eiwit belangrijk is voor transport van blaasjes naar het cilium konden wij namelijk nog niet beantwoorden.

In hoofdstuk 6 worden de bevindingen van dit proefschrift besproken binnen de context van recente literatuur over blaasjes transport naar het cilium. De oorzaken voor de genetische en fenotypische verscheidenheid in ciliopathieën wordt besproken, met een focus op Bardet-Biedl syndroom. Uit analyse van recente literatuur en de resultaten van dit proefschrift, blijkt
dat veel defecten in blaasjes transport naar het cilium uiteindelijk een verstoorde samenstelling van het cilium veroorzaken, waardoor het cilium niet goed functioneert. Verder blijkt dat verschillende afzonderlijke transportmechanismen belangrijk zijn voor de functie van het cilium, en dat er nog steeds nieuwe transport-routes in de cel worden ontdekt, waardoor de complexiteit van dit interessante orgaan alleen maar groter blijkt te zijn. Verder wordt in dit hoofdstuk besproken dat een uitgebreide analyse van de eiwitbindingen van cilium-eiwitten zal zorgen voor een beter begrip van het functioneren van het cilium in gezondheid en in het geval van een ciliopathie. Dit geeft ook meer inzicht voor de patiënten in hun eigen ziektebeeld, en in het verloop van het ziektebeeld in de tijd. Uiteindelijk zullen genetische analyses en eiwit-bindingsanalyses waardevolle informatie bieden om de diagnostiek te verbeteren en mogelijke medicijnen of genezende behandelingen voor ciliopathie-patiënten te ontwikkelen.
Recurrent de novo heterozygous mutations disturbing the GTP/GDP binding pocket of RAB11B cause intellectual disability and a distinctive brain phenotype. American Journal of Human Genetics 2017;101:824–32.


List of publications


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Ideke Lamers was born on the 27th of December 1987 in Zevenaar, the Netherlands. After finishing her high school with a focus on 'Nature and Health' in 2006, she started her bachelor studies in Biomedical Sciences at the Radboud University of Nijmegen. She performed her bachelor internship at the Laboratory of Pediatrics & Neurology of the Radboud university medical center (Radboudumc), where she sequenced the DPM genes in patients with Congenital Disorders of Glycosylation, Walker-Warburg or (alpha‐)dystroglycanopathy. This project sparked her interest in genetics and specifically in studying the consequences of mutations on a protein level. She continued with her master studies in Biomedical Sciences with a major in Pathobiology in 2009 at the same university. For her first master internship she worked under supervision of Prof. dr. Ronald Roepman and Dr. Karlien Coene at the Department of Human Genetics of the Radboudumc in Nijmegen, on the characterization of the Lebercilin‐like protein, a close homologue of the ciliary protein Lebercilin that is associated with congenital blindness. For her second master internship she visited the lab of Prof. dr. Stefan Heller at the Department of Otolaryngology at Stanford University in Palo Alto, California, USA. During this visit, she focused on developing and characterizing a tagged construct of synaptic vesicle protein VGLUT3 to study synaptic vesicle dynamics in cochlear inner hair cells. After finishing her master studies in 2012 with the distinction 'Bene Meritum', she returned to the Department of Human Genetics of the Radboudumc in Nijmegen to start as a PhD student under supervision of Prof. dr. Ronald Roepman, Prof. dr. Frans Cremers and Dr. Heleen Arts. Her project was funded by a Radboudumc‐Radboud Institute for Molecular Life Sciences (RIMLS) PhD grant. The results of these studies are described in this thesis. She recently joined the group of Prof. dr. Ronald Roepman as a postdoctoral researcher to study photoreceptor cell proteostasis in inherited retinal dystrophies, to identify targets for therapy.
About the author

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Dankwoord

Daar is het dan! Na het schrijven van ~65.000 woorden is mijn ‘boekje’ eindelijk af! De 4 jaar en 8 maanden die ik aan dit onderzoek heb besteed, waren een werkelijke achtbaan aan ervaringen, op zowel professioneel als persoonlijk vlak. Het eindresultaat mag er wezen, en daar ben ik ook trots op. Maar natuurlijk had ik mijn proefschrift niet kunnen schrijven zonder de samenwerking met vele mensen, en daarvoor wil ik hen bedanken. Maar ook alle hulp, (emotionele) steun, gezelligheid, aanmoedigingen en vertrouwen van velen om mij heen hebben ervoor gezorgd dat ik mijn promotieonderzoek tot een goed eind heb kunnen brengen. Mijn dank is groot!

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Dankwoord

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Dankwoord

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Dankjewel voor jullie aanmoedigingen, het vertrouwen in mijn kunnen en grenzeloze liefde!

Daarnaast vond ik het elke keer weer heerlijk om thuis te komen om mijn batterij op te laden. Tegenwoordig woon ik samen met Sietse weer 'thuis', en ik geniet elke keer weer enorm van de weekendjes met jullie allemaal op de boerderij!

Lieve Sietse, ik weet dat ik niet mag zeggen dat dit zonder jou niet was gelukt, maar vooruit, zonder jou was mijn PhD een hele stuk zwaarder geweest. Dankjewel voor je luisterend oor, je kritische vragen, je aanmoedigingen, je pep Talks, je humor, je liefde en je eindeloos vertrouwen in mijn kunnen. Jij was de rots in de branding, waar ik weer even op kon uitrusten als alles mij boven het hoofd groeide. Ik ben je intens dankbaar dat je er altijd voor me bent, ook al liet ik het zelfs soms afweten door al die experimenten. Ik voel mij intens gelukkig met jou samen, en ik kijk er naartoe uit om samen nog meer avonturen aan te gaan, in ons mooie busje!
**TRAINING ACTIVITIES**

<table>
<thead>
<tr>
<th>Year(s)</th>
<th>ECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
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</tr>
<tr>
<td>Courses &amp; Workshops</td>
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</tr>
<tr>
<td>‐ Graduate course (RIMLS)</td>
<td>2013</td>
</tr>
<tr>
<td>‐ Forum ‘Scientific Integrity’ (Radboud University)</td>
<td>2013</td>
</tr>
<tr>
<td>‐ Workshop “How to write a medical scientific paper” (IGMD/IfO/N4i)</td>
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<tr>
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<tr>
<td>‐ Winterschool Nierstichting</td>
<td>2015</td>
</tr>
<tr>
<td>‐ Workshop “UCSC Genome Browser” (department)</td>
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<tr>
<td>‐ Technical Forums (RIMLS)</td>
<td>2016</td>
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<tr>
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<td>2016</td>
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| b) |      |
| Seminars & Lectures | |
| ‐ Seminars and Lectures (RIMLS) | 2013‐2017 | 3.1 |

| c) |      |
| (Inter)national Symposia & Congresses | |
| ‐ RIMLS New Frontiers* | 2013 | 3.25 |
| ‐ RIMLS PhD Retreat**# | 2014 | 4.0 |
| ‐ Nederlandse Federatie voor Nefrologie* (Utrecht, Netherlands) | 2015 | 0.5 |
| ‐ NVHG* (Arnhem, Netherlands) | 2016 | 0.75 |
| ‐ ERA ‐ EDTA* (Amsterdam, Netherlands) | 2015 | 1.0 |
| ‐ Cilia Meeting* (Paris, France) | 2016 | 1.25 |
| ‐ Benelux Kidney Meeting* (Eindhoven, Netherlands) | 2015 | 1.5 |
| ‐ Science Day Dept. Human Genetics* (Nijmegen, Netherlands) | 2016 | 0.5 |
| ‐ Cilia Meeting# (Amsterdam, Netherlands) | 2016 | 0.5 |

| d) |      |
| Other | |
| ‐ Literature discussion department of Human Genetics### | 2013‐2016 | 4.0 |
| ‐ Theme discussion department of Human Genetics## | 2015 | 2.0 |
| ‐ Organizing New Frontiers (RIMLS) | 2015 | 2.0 |
| ‐ Organizing Technical Forum (RIMLS) | 2015 | 0.5 |

| e) |      |
| Lecturing | |
| ‐ Assistance ‘lab practice’, (bio)medical BSc students | 2016 | 0.1 |
| ‐ Workgroups (bio)medical BSc and MSc students | 2014‐2016 | 1.0 |
| ‐ Lecture ‘Ciliopathies’, (bio)medical BSc students | 2016 | 0.1 |
| ‐ Designing/organizing course elements, (bio)medical BSc students | 2015‐2016 | 4.0 |
| ‐ Supervision ‘Writing a grant proposal’, medical BSc students | 2017 | 0.5 |

| f) |      |
| Other | |
| ‐ Supervision internship MSc Medical Biology (H. Kessels) | 2014 | 2.5 |

**TOTAL** 40.7
# RIMLS Portfolio

**Name PhD student:** I.J.C. Lamers  
**Department:** Human Genetics  
**Graduate school:** Radboud Institute for Molecular Life Sciences  
**PhD period:** 01-01-2013 until 05-09-2017  
**Promotor(s):** Prof. dr. ir. R. Roepman, Prof. dr. F.P.M. Cremers  
**Co-promotor(s):** Dr. H. Arts

## Training Activities

<table>
<thead>
<tr>
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## Teaching Activities

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**Total** 40.7

* Poster presentation  
# Oral presentation

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Poster presentation  
Oral presentation
Dysfunction of ciliary vesicle trafficking in ciliopathies

Ideke Lamers

Dysfunction of ciliary vesicle trafficking in ciliopathies

Ideke Lamers

Institute for Molecular Life Sciences
Radboud umc