Scrupinizing ciliopathies by unraveling ciliary interaction networks

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Research of cilia has gained significant momentum in the last 15 years, as an increasing number of human genetic diseases were found to be caused by disruption of a protein that localizes to cilia. These ciliopathies are as diverse as the functions of the associated proteins, covering a spectrum of overlapping phenotypes that ranges from relatively mild characteristics in isolated tissues with a late onset, to severe defects of multiple tissues with an onset early in embryogenesis that is incompatible with life. As cilia harbour many receptors and components of key signaling cascades, such as Hedgehog, Wnt, Notch and Hippo signaling, disruption of ciliary function has severe consequences. Recent (affinity) proteomics studies have focused on the composition and dynamics of ciliary protein interaction networks. This has unveiled important knowledge about the highly ordered, interconnected but very dynamic nature of the cilium as a molecular machine. Disruption of the members of the same functional modules of this machine leads to similar phenotypes, and detailed analyses of the binding repertoire, the biochemical properties and the biological functions of these modules have yielded new ciliopathy genes as well as new insights into the pathogenic mechanisms underlying ciliopathies.

INTRODUCTION

The cilium is an intriguing, multifunctional organelle, which projects from the cell surface of many different cells. Cilia are conserved in a wide variety of eukaryotic species throughout evolution, and almost every vertebrate cell type is able to form a cilium under the right circumstances, i.e. a quiescent or differentiated state. The early scientific history of this organelle, first observed by Antoni van Leeuwenhoek in 1675 while observing a protozoan move around a drop of rain water, is chronologically described by Peter Satir (1). Ciliary research gained significant momentum only in the last 15 years, when human geneticists teamed up with cell biologists to identify that dysfunctional cilia can lead to over 20 severe human genetic traits with overlapping phenotypes, the ciliopathies (2,3). The affected tissues are as diverse as different cell types that carry cilia, almost covering every aspect of the human body. Studies describing these clinical connections have been intensively reviewed in recent years (4–12) and have pinpointed numerous important new aspects of ciliary function. These include vital importance in (neuronal) development (5,9,13,14), tumorigenesis (15–22) and cellular homeostasis (4,13,23–25). An overview of the currently known ciliopathies is given in Supplementary Material, Table S1.

Because of the strongly interconnected nature of the ciliary processes, structures and signaling pathways, it is evident that studies that aim to unravel the composition and dynamics of the protein networks of cilia are required to explain the chain of detrimental events that occurs when the robustness of this protein network is affected by a mutation in a ciliopathy gene. In recent years, (affinity) proteomics studies as well as genetic interaction studies at different levels of throughput have informed us about the physical connections and functional hierarchy in ciliary interaction networks, which unveiled important knowledge about the molecular pathogenesis of ciliopathies. This will be the focus of this review.

CILIA STRUCTURE

Cilia can take a wide variety of polarized, longitudinal shapes, which are governed by their tissue-specific function. All cilia contain a ciliary axoneme, a ring-shaped core of nine...
micotubule doublets, which connects the base of the cilium to its tip (Fig. 1). This axoneme is covered by the ciliary membrane and projects from a modified centriole, the basal body. The majority of cells develop a single, non-motile primary cilium (26), which usually only contains a cylinder of nine doublets of microtubules (so called ‘9+0’ configuration).

These primary cilia have evolved to key signaling hubs of the cells during evolution (13,14,27), as the ciliary membranes harbour receptors for crucial signaling cascades, most prominently Hedgehog signaling (28–33), Wnt signaling (12,34–41), planar cell polarity signaling (12,42–46,48), FGF signaling (49–52), Notch signaling (53–57), mTor signaling (58,59), PDGF signaling (60,61) and, most recently, Hippo signaling (62). They catch extracellular cues by their specific set of transmembrane receptors and transduce them to the associated intracellular signaling pathways to exert cellular responses (5,12,27,63). If the cilium is motile, it is sometimes referred to as a flagellum. Motile cilia often appear in bundles on cells, which each can contain up to 200–300 cilia, enabling a powerful motion of the extracellular fluid. The active movement of cilia usually requires an additional pair of microtubules in the center (a 9+2 configuration), and outer and inner arm dynein motors (64).

**Figure 1.** Architecture of cilia. Schematic overview of the cilium and the IFT machinery. Cross sections show the microtubule composition of ciliary axonemes and the basal body. A ciliary axoneme consists of an array of microtubule doublets that each consists of an A and a B tubule. Motile and immotile cilia often differ with respect to their microtubule backbones. Motile cilia usually have a ‘9+2’ microtubular configuration, while immotile cilia mainly consist of ‘9+0’ arrays. Motile and immotile cilia also differ in the presence of radial spokes, nexin links and (inner and outer) dynein arms. Ciliary rootlets provide stability, while transition fibers attach the basal body (a centriole-derived structure that consists of triplets of microtubules) to the plasma membrane and represent an important barrier for proteins. Proteins are targeted to the cilium in Golgi-derived vesicles that fuse near the cilium base at the apical membrane and the ciliary pocket. Proteins are transported through the transition zone with IFT. This is a bidirectional transport process that requires activity from kinesin II and dynein motors as well as from IFT A and IFT B complexes.

**CILIARY AXONEME AND INTRAFLAGELLAR TRANSPORT**

Studies of cilia have been pioneered by cell biologists, who used the two flagella of the green algae *Chlamydomonas reinhardtii* as model cilia to discover many functional properties. They identified how ciliary transport is regulated in this organism and called it intraflagellar transport (IFT), which was reviewed recently (65). The multi-subunit IFT particles are key to ciliogenesis (65–69) and localize between the ciliary membrane and the axonemal microtubules. The content and speed of the particles varies with their direction (66). IFT-B particles govern retrograde transport from the ciliary tip back to the base. They contain at least six IFT-A proteins that assemble in a particle and are driven by the cytoplasmic dynein 2/1b motor. All IFT-B proteins that were initially found in the green alga have also physically been identified in mouse by co-immunoprecipitation and mass spectrometry (70). The smaller IFT-A trains govern retrograde transport from the ciliary tip back to the base. They contain at least six IFT-A proteins that assemble in a particle and are driven by the cytoplasmic dynein 2/1b motor. The cargo of IFT particles in different
Cilia contain the protein subunits required for the ciliary assembly, such as subunits of the dynein arms, nexin, the radial spokes and membrane proteins (Fig. 1). Also, the IFT-B particles carry the IFT-A subunits and the inactive retrograde (dynein1b/2) motor subunits as cargo, and vice versa. IFT-A trains in turn carry the ciliary turnover products out of the cilium (71). The IFT proteins are not only involved in ciliary IFT, as the IFT20 protein was found to be dynamically shuttled between the Golgi complex and the cilium (72,73), and IFT20 also assembles an IFT complex, including IFT57 and IFT88, required for the correct assembly of the immune synapse of cells that lack cilia (74).

**CILIogenesis**

Ciliary assembly or ciliogenesis is a conserved, cell-cycle-dependent process; ciliary assembly is initiated in G1 and finalized at G0. For a cell that forms a primary cilium, the entry into a quiescent state is then the cue for the centrosomes to migrate to the cortex of the cell. During this process, Golgi-derived vesicles generate a centriolar vesicle that caps the distal end of the mature or mother cilium, from which the ciliary axoneme grows, directed by IFT (75,76). Upon docking at the cell surface, the extended centriolar vesicle has formed a ciliary membrane, which fuses with the cell membrane, forming a cup-like structure called the ciliary necklace. The mature cilium is then called the basal body, which is anchored to the plasma membrane by the centriolar appendages. At that point, the cilium elongates to its full size.

Many ciliopathy-associated mutations lead to defects in ciliogenesis, most commonly through a loss of protein function. The onset of ciliogenesis seems to be controlled by mechanisms often shared between centrioles and basal bodies. The CP110 protein, vital for centrosome duplication and cytokinesis, interacts with Cep97 and with CEP290 at the mother cilium and basal body to antagonize and thus control ciliary assembly (77,78). Recently, the centriolar kinesin Kif24 was also found to be involved in this control by stabilizing and/or recruiting CP110 at the distal end of the mother cilium and remodeling specifically the centriolar microtubules (79).

**Ciliary Disassembly**

Disassembly of cilia occurs when cells re-enter the cell cycle prior to mitosis (80). The centrioles then duplicate in S-phase and the basal body detaches from the plasma membrane, reparticipating in mitosis as the microtubule-organizing center. Control of ciliary disassembly involves interactions between the scaffolding protein HEF1 and the Aurora A kinase at the basal body of cilia. These interactions induce phosphorylation and thereby activation of HDAC6, a tubulin deacetylase. Subsequent deacetylation of tubulin then triggers cilia disassembly (81). Recently, another protein was identified that acts in a related way. The basal body and ciliary necklace protein Pitchfork (Pifo) activates Aurora A, resulting in ciliary disassembly. Pifo is also associated with vesicular ciliary transport, and Pifo mutations cause a unique cellular phenotype: cilia retraction and basal body liberation failure and over-replication of cilia, hence its name (82). Other mechanisms that are involved in control of ciliary disassembly include ubiquitination of cilia, hence its name (83). Inhibition of ciliogenesis by the Nde1 protein that accelerates cell cycle re-entry (84) and the activity of Tctex-1/DYNLT1, a cytoplasmic dynein subunit, which controls ciliary disassembly and S-phase entry when uncoupled from the dynein complex.

**CONTROL OF CILium SIZE**

Ciliary length is controlled by many different mechanisms, but a balance between anterograde and retrograde IFT trafficking seems of vital importance, as continuous assembly and disassembly at the tip of the cilium is thought to result in a steady state of continuous turnover (65). Alteration in the levels of specific molecules transported by IFT, such as several tubulin isotypes in C. elegans, can modulate ciliary length (85), but also soluble tubulin levels in the cytosol may be a factor (86), as are proteins specifically modifying ciliary microtubules, such as α-TAT1, the major α-tubulin K40 acetyltransferase (87). Activity of small GTPases, their guanine nucleotide exchange factors and GTPase activating proteins are also of crucial importance in the control of cilium length. An example of this is the regulation of IFT integrity by coordinated ARL-3 and ARL-13 action, which in turn is mediated by an HDAC6 deacetylase-dependent pathway (88). Also, the small GTPases Rab8, Rab11, Arl6, Arf4 and Ran enable ciliary entry of proteins (89–93), which in turn may affect the IFT/turover steady state. Regulation of ciliary entry and sorting of ciliary (membrane) protein content are thus vital for ciliary length and function. The role of Ran and importin-β2 (92) indicates that mechanisms similar to nuclear import play a role in this, and multiple ciliary targeting sequences have been identified (90,94–101). The BBSome, a complex of Bardet–Biedl Syndrome (BBS) proteins (89), is capable of assembling a coat that sorts ciliary membrane proteins, using their ciliary targeting signals, for lateral transport into the cilium (93), thereby passing the septin diffusion barriers that maintain ciliary membrane protein distribution (102). It is also of key importance that structures such as microtubule-membrane linkers at the transition zone remain intact to maintain a correct protein composition. The CEP290 protein (103) and likely the associated Meckel–Gruber syndrome (MKS)/neurophthalmophthisis (NPHP)/Joubert syndrome (JBTS) protein modules (104) play an important role in this.

**LESIONS OF CILIOlARY FUNCTIONAL MODULES CAUSE CILIOPATHIES**

Ciliogenesis, ciliary disassembly and ciliary homeostasis depend on a great number of different factors that all have to be optimally tuned and aligned in order to generate fully functional cilia of the right size (65,76,80,105). Proteins encoded by genes that are mutated in ciliopathies have been described to be associated with mechanisms that involve the building of a cilium, the maintenance of cilium structure and
function and the control of cillum length. Disruption of cillum function in a single tissue may lead to a tissue-restricted phenotype, while disruption of cillum function at early embryonic stages, e.g. affecting Wnt or Hedgehog signaling cascades, may cause severe malformations leading to embryonic lethality. All different levels of severity of the ciliopathy disease spectrum have been observed, and the spectrum is continuously expanding (5–13,48,106,107).

Apart from the 80 proteins encoded by 'ciliopathy genes', the cillum consists of about a thousand other proteins that have been described in numerous bioinformatic, genomic and proteomic studies. These data have been stored in different ciliary databases, such as the Ciliome and CiliaProteome databases that were published in 2006 (108,109). More recently, Centrosomedb (110) and Cildb (111) were launched. Centrosomedb is primarily focused on providing insights into the centrosomal proteome, while Cildb is an extensive database in which it is possible to search for information in 18 different proteomes and which can be used to link genetic disease to model organisms. By studying the genetic and physical interactions of selected ciliary genes and proteins, a wealth of information has been unveiled in the last decade regarding the functional modules that underlie ciliary homeostasis and upon disruption lead to ciliopathies. This information can be integrated into a descriptive interaction network, which instantly pinpoints the functional relationships between the proteins. To gain better insights into the disease mechanisms that underlie the ciliopathies and to acquire knowledge about the general importance of the cillum for cellular homeostasis, we constructed a model of a dynamic ciliopathy protein network using data from public databases and published ciliary protein–protein interaction studies, and connecting them to the different ciliopathy phenotypes (Fig. 2 and Supplementary Material, Table S1). This network contains protein–protein interaction information with different levels of accuracy and depth. There is information about binary protein–protein interactions, such as those identified hypothesis-free using yeast two-hybrid screening of designated cDNA libraries (112,113) or hypothesis-driven by testing whether selected pairs of proteins interact (114). It also contains information about protein complexes that were identified by affinity purification, again either hypothesis-driven or hypothesis-free. In the hypothesis-free way, tandem affinity purification is followed by mass spectrometric identification of the interacting protein complexes (115,116).

The combination of such protein–protein interaction information with subcellular localization data, information of gene knockdown studies in different cells and species and ciliary proteomics enables the generation of models of interacting functional modules potentially associated with certain signaling cascades, developmental events or specific ciliary functions such as IFT. When particular interactions are lost due to a gene mutation in a ciliopathy-associated gene, disruption of a physically connected functional module or molecular machine within the network is likely the cause of the molecular pathogenesis, and potentially highlights the disrupted

![Figure 2. Ciliopathy disease protein interaction network.](https://academic.oup.com/hmg/article-abstract/20/R2/R149/639745/14597658) The network illustrates the high degree of connectivity of proteins (red spheres) known to be associated with phenotypically overlapping human ciliopathies (cyan spheres). This protein–protein interaction information is very valuable in the identification of functional modules to scrutinize the molecular machines associated with ciliogenesis, ciliary signaling or specific functions such as IFT. Protein–protein interactions, direct and complex, (grey lines) were derived from the literature. The network was visualized by Cytoscape software (http://www.ncbi.nlm.nih.gov/pubmed/14597658). Supplementary Material, Table S1 lists all known genes associated with a human ciliopathy.
pathway(s). Other members of such a functional module are, by association, also candidates for the same or a very similar ciliary disorder. Such an approach revealed that both the retinitis pigmentosa GTPase regulator (RPGR) interacting protein 1 (RPGRIP1) and the RPGRIP1 like (RPGRIP1L) proteins are in a shared module with the RPGR, involved in X-linked retinal degeneration (117–120). These interactors are both involved in (retinal) ciliopathies: RPGRIP1 mutations lead to Leber’s congenital amaurosis (LCA) and RPGRIP1L mutations lead to JBTS or Meckel syndrome, depending on the level of protein inactivation (121,122). Both proteins also interact with NPHP4 (114,122), involved in the ciliopathy nephronphthisis and sometimes also including retinal degeneration (Senior–Loken syndrome) (123). These interactions further integrate this protein module in the ciliopathy protein network.

EXPANDING THE EXPERIMENTAL HORIZONS USING AFFINITY PROTEOMICS

Using affinity proteomics procedures, the same principle was recently employed in multiple studies of different ciliopathies, indeed uncovering crucial clues about disease pathogenesis, ciliopathy genes and ciliary function. The BBSome, containing most BBS proteins, and its crucial role in multiple aspects of ciliogenesis and ciliary homeostasis were unveiled using tandem affinity purification followed by mass spectrometric identification of the protein complex members, and subsequently this complex was further dissected (89,93,124,125). Similar procedures in combination with siRNA knockdown were used to identify the association of THM1 and TULP3 with the IFT-A particle. TULP3 was found to be required to bridge this particle to the membrane (126). Using a large scale, simultaneous approach was undertaken to dissect and molecularly connect the clinically overlapping ciliopathies NPHP, JBTS and MKS, by targeting nine proteins with affinity proteomics (127). This yielded a network of 850 proteins, from which 38 candidates were selected as potentially encoded by ciliopathy genes, and after sequence analysis of DNA from multiple ciliopathy patient collections, two new genes were identified: ATXN10, encoding ataxin 10, is involved in NPHP, and TCTN2, encoding tectonic 2, is involved in JBTS. The network/functional curation and subsequent subcellular localization and cell biological analysis allowed separation of this network into three major functional modules. The module that localized to the ciliary transition zone (containing NPHP1, NPHP4 and NPHP8/RPGRIP1L) was found to be involved in apical organization, whereas the two basally localizing modules were found to be involved in cilium integrity (NPHP5/IQCB1, NPHP6/CEP290 and ataxin 10) and Hedgehog signaling (MKS1, a B9 domain containing protein, MKS6/CC2D2A and tectonic 2), respectively. A recent study also included the B9 proteins B9D1 and B9D2 in this module and identified that the complex is disrupted upon mutation of B9D2 in a MKS patient (128). Inversin/NPHP2 and AH11 seemed to bridge the latter two modules, and NPHP9/NEK8, NPHP3 and inversin/NPHP2 were identified in a more apical ‘inversin’ region with a thus far unclear role (129). In line with these results, affinity proteomics of Tctn1 yielded a module of JBTS and MKS proteins at the transition zone, involved in ciliary assembly and trafficking (130). The subsequent discovery of a mutation in TCTN1 in a JBTS family emphasized the importance of this subspeciality region in cilium function. None of the two studies, however, identified a physical link with the IFT particles, despite the reported involvement in ciliary trafficking. A study in zebrafish, however, recently found that missing link, reiterating the importance of ciliary cargo transport (131).

Similarly, initial studies using tandem affinity purification yielded links of lebercilin with a ciliary protein complex, but not directly with the IFT particle (132). It required a much more sensitive, quantitative approach employing stable isotope labeling of amino acids in cell culture (SILAC) to detect the association of lebercilin with a near-complete IFT particle (133). The same quantitative procedure also allowed the analysis of the effect of the LCA-associated mutations on the functional lebercilin module, which showed that mutated lebercilin was disconnected from the IFT particle, hence disrupting transport across the photoreceptor connecting cilium (133). Finally, tandem affinity purification was also used to compare the protein modules of the RPGRIP1 and RPGRIP1L homologs and identified a mutual connection with Nek4 serine/threonine kinase that was partly localizing to the ciliary rootlets (134). In combination with the ciliogenesis defects observed after down-regulation of the gene, this led to the hypothesis that RPGRIP1 and RPGRIP1L can recruit a Nek4 signaling network that is involved in cilium integrity (134).

CONCLUSION AND FUTURE DIRECTIONS

The core structure and function of the cilium has largely been unveiled in the last 50 years, demonstrating a dynamic and multifunctional molecular machine that is tightly connected with the regulation of cellular development and homeostasis. Proteomics studies have suggested a discrete repertoire of ∼1000 proteins within the organelle, which equals ∼5% of the human proteome. Most of these proteins, however, are still in need of organization into pathways and networks, and recent affinity proteomics studies demonstrated their usefulness for that task. Although important new insights into the function of ciliopathy-associated proteins were uncovered, as well of their role in the disease pathogenesis of ciliopathies, the sensitivity and thus accuracy of such analyses are variable. As such, only part of the story has been told. New quantitative proteomics approaches have the potential to dramatically increase this sensitivity to a level where the effects of gene variants and therapeutic applications on the composition of the ciliary modules can be accurately assessed. Further expansion of the throughput of such interaction network analyses, and their combination with resources harboring data on gene expression and down-regulation, protein structure and localization, genetic variation and disease phenotype, will open avenues for broader, systems-based approaches that may
allow a more detailed view on the many functions of this intriguing organelle.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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