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The study of sensory cilia development in *Caenorhabditis elegans*

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ABSTRACT

Cilia and flagella are widespread eukaryotic subcellular components that are conserved from green algae to mammals. In different organisms they function in cell motility, movement of extracellular fluids and sensory reception. While the function and structural description of cilia and flagella are well established, very little is known about the developmental mechanisms by which cilia are generated and shaped and how their components are assembled into functional machineries. To answer these questions, we used sensory cilia development in the nematode *Caenorhabditis elegans* as a model system.

The work described here developed from the initial discovery of the ciliogenic properties of the gene *daf-19*, which encodes the sole *C. elegans* member of the RFX-type transcription factors. All members of the RFX transcription factor family are characterized by the presence of a conserved DNA binding domain, which recognizes special motifs (X-boxes) in promoters of its target genes. By using a genome search approach for X-box promoter motif-containing genes (*xbx* genes) we identified a list of about 750 *xbx* genes (candidates). This list comprises some already known ciliary genes as well as new genes, many of which we hypothesize to be important for cilia development and functioning.

A computational search for X-box motifs in the *C. briggsae* genome has demonstrated strong conservation of this motif between closely related nematode species. To find out whether RFX-type transcription factors can also regulate ciliogenic pathways in other organisms, we applied a similar search strategy to distant species such as the fruit fly *Drosophila*. Using X-box consensus sequences with varying degrees of refinement and subsequent gene expression analysis, we were able to identify a set of *Drosophila xbx* genes. Intriguingly, the majority of fly *xbx* genes that have homologs in *C. elegans* were down regulated in *dRfx* fly mutants, suggesting an evolutionary conserved role for RFX-type transcription factors in the regulation of ciliary genes.

Using X-box matches as a prediction tool we were able to identify novel ciliary genes, *dyf-2* and *dyf-11*, in the *C. elegans* genome. We cloned these genes by transgenic rescue of mutant phenotypes and by sequencing of mutant alleles. Loss of DYF-2 and DYF-11 functions selectively affects the assembly and motility of different intraflagellar transport (IFT) components, resulting in compromised protein transport within cilia, and subsequently in defective cilia structures and sensory functions. Importantly, the mouse orthologs of DYF-2 and DYF-11 also localize to cilia, pointing to evolutionarily conserved roles for these proteins in cilia biogenesis.

In conclusion, our studies of the regulation of sensory cilia formation demonstrated how contributions of multiple factors are integrated into a developmental module that leads to the formation of the primary sensory organs, cilia. In addition, data obtained during the course of this study provide a useful resource for researchers interested in further identification and study of new genes implicated in cilia biogenesis and will have a significant impact on the understanding and treatment of cilia-based pathologies in humans.

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II Laurençon, A., Dubruille, R., **Efimenko, E.,** Grenier, G., Bissett, R., Cortier, E., Rolland, V., Swoboda, P. and Durand, B. (2007). Identification of novel Regulatory Factor X (RFX) target genes by comparative genomics in Drosophila species. *Genome Biology* **8**, R195.

III **Efimenko, E.,** Blacque, O. E., Ou, G., Haycraft, C. J., Yoder, B. K., Scholey, J. M., Leroux, M. R. and Swoboda, P. (2006). *Caenorhabditis elegans* DYF-2, an orthologue of human WDR19, is a component of the IFT machinery in sensory cilia. *Molecular Biology of the Cell* **17**, 4801-4811.

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RELATED PUBLICATIONS

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1 INTRODUCTION

1.1 CILIA ARE UBIQUITOUS, EVOLUTIONARY CONSERVED ORGANELLES

Cilia and their close relatives flagella are microtubule-based structures that are positioned at the apical surface of the cell. Having arisen early in eukaryotic evolution, cilia can be found across a broad phylogenetic spectrum with just a few exceptions like the plant *Arabidopsis thaliana*, the fungus *Saccharomyces cerevisiae*, and the slime mold *Dictyostelium discoideum* (Cavalier-Smith, 2002).

Although not completely enclosed by a membrane, cilia are reasonably considered as organelles. The basal bodies or their structural equivalents dock cilia on the surface of the cell membrane and serve as the interface between the cilium and the cytoplasm. Basal bodies are analogous to the pre-existing mature centriole in the cell. They possess nine radially oriented microtubule triplets composed of A, B, and C fibers, of which A and B extend into the proximal region of the cilium, known as the transition zone (Figure 1). Beyond the transition zone, the axoneme consists of the middle segment, which maintains nine microtubule doublets either with (9+2) or without (9+0) a centrally located pair of microtubules (central cylinder), followed by the distal segment, a portion of the cilium which is typically composed of nine single microtubule fibers (Davis et al., 2006). Depending on internal structure and function, there are two basic types of cilia: motile and sensory (primary).

Historically, motile cilia (or flagella) have been the most studied of all cilia and are generally found on the epithelial cells of the trachea, ependymal cells in the brain, and on cells lining the reproductive tract. Normally concentrated in large numbers on the cell surface, motile cilia beat in an orchestrated wavelike fashion and are involved in fluid and cell movement such as mucociliary clearance in the lung, cerebrospinal fluid movement in the brain, and ovum and sperm transport along the respective reproductive tracts (Davenport and Yoder, 2006). In motile types of cilia, the internal structure consists of a (9+2) axoneme and associated inner and outer dynein arms, which generate the force for motility (Figure 1). At regular positions along the length of the cilia axoneme, an accessory protein, nexin, cross-links the microtubules together (Figure 1). Molecules of axonemal dynein form bridges between the neighboring doublet microtubules around the circumference of the axoneme. When the motor

domain of the dynein molecule is activated, the dyneins attached to one microtubule doublet attempt to walk along the adjacent microtubule doublet, tending to force the adjacent doublets to slide relative to one another. However, the presence of the nexin links between the microtubule doublets prevents this sliding, and the dynein force is instead converted into a bending motion (Alberts et al., 2002). The outer doublets of motile cilia are also characterized by the presence of multi-protein structures called radial spokes, which project towards the central cylinder (Figure 1). Diverse evidence indicates that the central cylinder / radial spokes system is not required for oscillatory beating of the axoneme (Marschese-Ragona and Holwill, 1980). Rather, the outer doublet microtubules and associated dynein arms appear to be sufficient for the initiation and propagation of bends. Recently, it has been shown that the central cylinder and radial spokes serve as mechano-chemical sensors to control motility (Smith and Yang, 2004; Yang et al., 2006; Lechtreck and Witman, 2007).

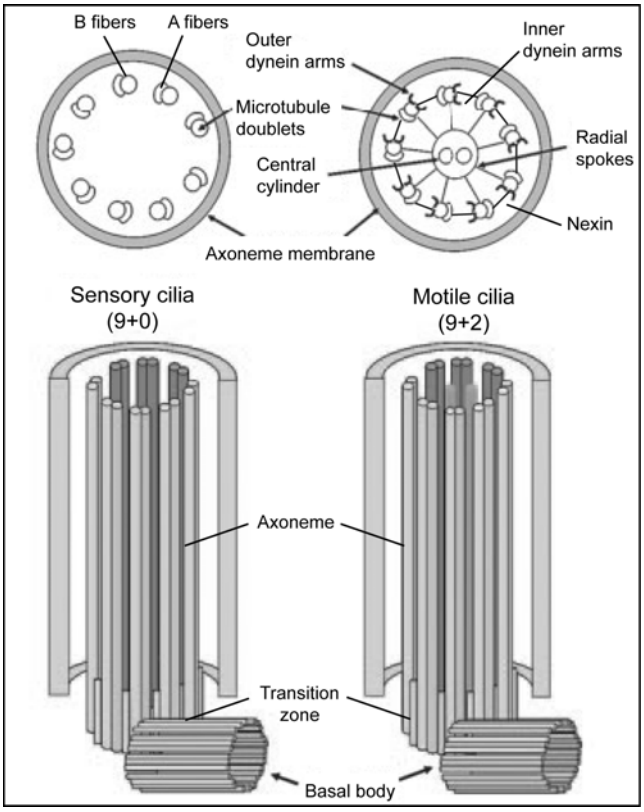

Figure 1. Cross section of sensory and motile cilia axoneme

Structural differences determine functional types of cilia. The axoneme of motile cilia consists of nine microtubule doublets surrounding two inner microtubule singlets (central cylinder) (9+2) and associated dynein molecules, which are used to generate movement. Sensory (primary) cilia are lacking both central cylinder (9+0) and the axonemal dyneins, and typically serve as a place for the exposure of various receptors (adapted from Davenport and Yoder, 2006).

Many of the genetic studies of motile flagella have been done in the unicellular green alga, *Chlamydomonas reinbardtii* (Dutcher, 1995). It has been found that *Chlamydomonas* axonemes contain over 250 polypeptides (Luck et al., 1977). Using mutant strains missing specific parts of the axoneme, more than 100 of those polypeptides have been assigned to particular structures. However, the identity of about half of the polypeptides remains unknown.

In contrast to motile flagella, sensory (primary) cilia are solitary organelles projecting from the surface of cells. These cilia lack the central pair of microtubules and axonemal dyneins needed to generate motile force and are thus described as having a (9+0) pattern (Figure 1) (Davenport and Yoder, 2006). Sensory cilia typically serve as a place for the exposure of various receptors. In vertebrates, sensory cilia are ubiquitously present in different cell types (examples of sensory cilia can be found at http://members.global2000.net/bowser/cilialist.html).

There is a special subtype of primary cilia that are specifically located on the ventral surface of the node of the early mammalian embryo (nodal cilia). Nodal cilia resemble primary cilia but they are unique in that they have an unusual twirling movement that is not seen in other primary cilia. The motility of nodal cilia generates a directional fluid flow across the node, which initiates signaling events that lead to the normal development of left–right asymmetry in the organism (Nonaka et al., 1998).

In humans cilia are near ubiquitous organelles that project from the surfaces of many different cell types to carry out both motile and sensory functions. Cilia have been implicated directly in many developmental processes such as the generation of left-right asymmetry, maintenance of the renal epithelium, or respiratory function. Defects in cilia structure or function lead to a wide range of developmental problems and diseases (ciliopathies). Therefore, the study of cilia biogenesis is now attracting an increasing amount of attention.

1.2 MAIN STAGES OF CILIOGENESIS

Generally, ciliogenesis can be subdivided into four main stages: centriole production; migration of centriole to the apical cell surface to become the basal body; elongation of ciliary axoneme; and formation of accessory structures (Figure 2). The differences between ciliogenesis in cells with primary cilia and in multi-ciliated cells can be observed at the stage of centriologenesis: a primary cilium forms from a pre-existing centriole (centriolar pathway) while cilia of multi-ciliated cells require the

production of many centrioles via a non-templated (acentriolar) pathway (Sorokin, 1968; Dawe et al., 2007b).

In the centriolar pathway, daughter centrioles arise directly from pre-existing templates (mother centrioles) (Figure 2A). A procentriole forms from the side of each centriole and elongates throughout S phase. At the end of G2 phase, the cell contains two pairs of centrioles (Dawe et al., 2007b).

The earliest recognizable event in acentriolar ciliogenesis is the formation of fibrous granules (Figure 2B). These granules form the fibrogranular complex with filamentous material. The next step is the formation of centrosomes, which are large, electron-opaque globular bodies without limiting membranes. Centrosomes are generated by aggregation and condensation of fibrous granules and serve as the core for centriole formation (Figure 2B) (Youson, 1982; Hagiwara et al, 2000). Procentrioles initially occur around centrosomes as a ring structure made from fibrous granules and develop further into centrioles.

In cells that produce a primary cilium, the centriole pair duplicates once per cell cycle during S phase and maturation occurs later, while in cells that will become multi-ciliated, centriole production is differentiation related and centrioles are made mature as they form (Dawe et al., 2007b).

To serve as cilia basal bodies, mature centrioles move toward the apical region of the cell and are aligned perpendicularly to the cell surface (Figure 2). Recent findings suggest that the temporal establishment of an apical web-like structure of actin is necessary for basal body docking and subsequent axoneme growth (Pan et al., 2007). Apical web formation is mediated by small Rho GTPase proteins and requires the activity of hepatocyte nuclear factor-3 / forkhead homologue 4 (HFH-4), a transcription factor expressed in ciliated cells (Brody et al., 2000; Pan et al., 2007).

During the final stages, cilia are formed either directly in the luminal membrane (Figure 2B), or indirectly through special vesicles, which are located at the distal end of basal bodies (Figure 2A) (Hagiwara et al, 2000). Cilia shafts extend at the periphery of the luminal surface of ciliogenic cells. In parallel, accessory structures like cilia rootlets originate from basal bodies and extend proximally toward the cell nucleus (Figure 2). It was recently shown that rootletin, a large protein composed almost entirely of coiled-coil domains, is a structural component of cilia rootlets (Yang et al., 2002).

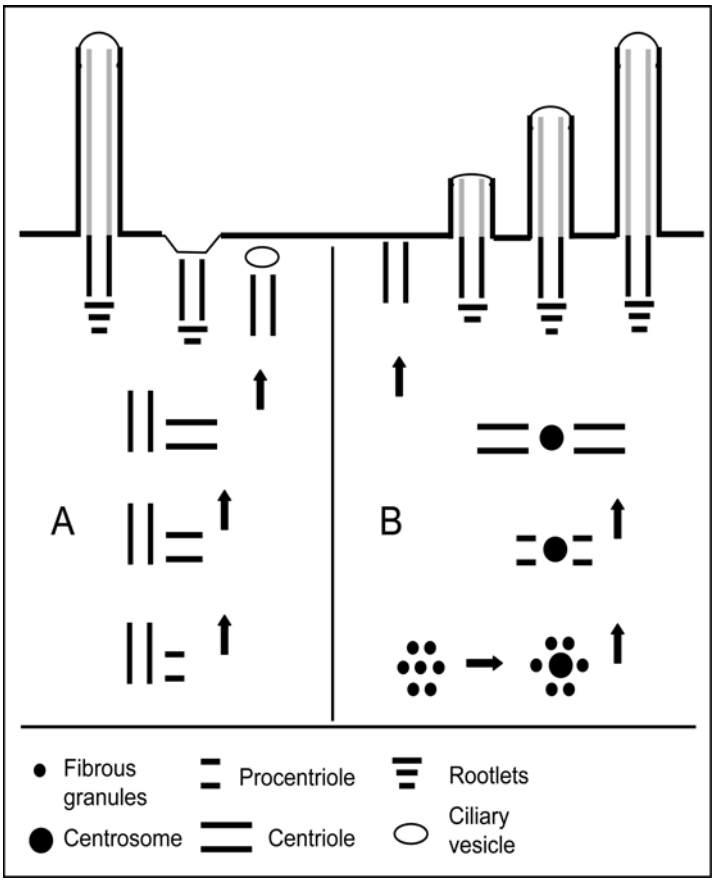

Figure 2. Main stages of ciliogenesis

Ciliogenesis is a fundamental process, which can be subdivided into four main stages: duplication of centrioles; migration of centrioles to the apical cell surface to become basal bodies; elongation of cilia axoneme; and formation of accessory structures like cilia rootlets. Basal bodies (centrioles) can be formed through both centriolar (A) and acentriolar (B) pathways (adapted from Hagiwara et al., 2000).

The cytoskeletal appearance of rootlets suggests a role as critical support structures for cilia, especially in situations where mechanical stress is expected to be high. Sensory and motile cilia that lack rootlets initially exhibit no apparent functional deficits. However, further analyses revealed a striking fragility at the base of cilia leading to cilia abnormalities over time (Yang et al., 2005).

The aforementioned mechanisms of ciliogenesis are common for many different species. In a few cases, however, such as in the sperm cells of *Drosophila* and the flagella of the parasite *Plasmodium yoelii* the entire axoneme is first assembled inside the cytosol and only later either extruded or matured into a flagellum (Tokuyasu et al., 1972; Sinden et al., 1976).

1.3 INTRAFLAGELLAR TRANSPORT (IFT)

At the time of cilia and flagella development, the axoneme is assembled by the addition of new axonemal subunits at its distal tip. Cilia lack the machinery that is necessary for protein synthesis and the site of axoneme assembly is far removed from the site of synthesis of axonemal proteins in the cell body. This poses a problem for the delivery of new axonemal building blocks to their site of assembly. The cell has solved this problem by means of intraflagellar transport (IFT) (Rosenbaum and Witman, 2002). During IFT, non membrane-bound particles (IFT particles) and their associated cargoes are moved continuously along the axonemal doublet microtubules just beneath the cilia membrane, from the base to the tip of the organelle (Kozminski et al., 1993) (Figure 3). The importance of IFT proteins in cilia biogenesis has been demonstrated in many different organisms, including *T. brucei* (Kohl et al., 2003), *D. melanogaster* (Avidor-Reiss et al., 2004) or mammals (Baker et al., 2003). Significant information about the organization of the IFT machinery was obtained from studies of motile flagella in the green alga *Chlamydomonas reinhardtii* and of sensory cilia in the nematode *Caenorhabditis elegans* (summarized in Table 1).

1.3.1 IFT motors

Kinesin-2 is the motor for anterograde intraflagellar transport. Members of this kinesin family were found in most ciliated organisms, but not in fungi, consistent with the idea that kinesin-2 evolved in parallel with the evolution of the axoneme for use in IFT. However, in higher organisms it is used for other transport functions as well (Marszalek and Goldstein, 2000). Two forms of the kinesin-2 family are known to function as anterograde motors in IFT: heterotrimeric kinesin-2 and homodimeric kinesin-2 (Table 1) (Lawrence et al., 2004). In *Chlamydomonas*, as well as in many other ciliated organisms, anterograde IFT appears to be driven by a single motor complex, heterotrimeric kinesin-2, which consists of two motor subunits, FLA10 and FLA8, as well as a nonmotor subunit, FLA3 (Cole et al., 1993; Walther et al., 1994; Kozminski et al., 1995; Morris and Scholey, 1997; Cole et al., 1998; Miller et al., 2005; Mueller et al., 2005). A null mutant in *FLA10* fails to assemble flagella, indicating that heterotrimeric kinesin-2 is essential for flagellar assembly in *Chlamydomonas* (Matsuura et al., 2002). In sensory cilia of the nematode *Caenorhabditis elegans*, however, two distinct kinesin-2 motor complexes, heterotrimeric kinesin-II and homodimeric OSM-3-kinesin work cooperatively during anterograde IFT (Figure 3). Kinesin-II and OSM-3-kinesin function redundantly to build the proximal and middle

segments of the *C. elegans* cilia axoneme, whereas transport of IFT particles along the distal segment is carried out by OSM-3 alone (Signor et al., 1999a; Snow et al., 2004; Ou et al., 2005a; Evans et al., 2006). *Chlamydomonas* flagella do not appear to contain an OSM-3 ortholog (Table 1), and therefore anterograde IFT in *Chlamydomonas* is probably less complex than in *C. elegans*.

Retrograde shuttling of IFT particles, including kinesin motors, is mediated by the cytoplasmic dynein complex 2 (Figure 3). This dynein complex has a unique role in IFT and is sometimes known as IFT-dynein (Pazour et al., 1999; Porter et al., 1999; Signor et al., 1999b; Wicks et al., 2000; Pfister et al., 2005; Pfister et al., 2006). Current evidence suggests that the cytoplasmic dynein complex 2 is a homodimer of two heavy chains with associated light intermediate chains (Perrone et al., 2003; Schafer et al., 2003). No other subunits have yet been identified for this complex, and it does not appear to interact with dynactin (Pfister et al., 2006). Inactivation of genes for the IFT-dynein complex leads to stumpy cilia and flagella containing large accumulations of IFT particles both in *Chlamydomonas* and in *C. elegans*, consistent with functioning in retrograde IFT (Porter et al., 1999; Signor et al., 1999b; Schafer et al., 2003).

1.3.2 IFT particles

The observation that the movement of IFT particles in flagella requires the activity of kinesin-2 allowed further purification of IFT particle components from *Chlamydomonas*. Matrix containing fractions prepared from flagella of *fla10* mutants grown under permissive conditions were found to contain a 16-17S complex of approximately 16-17 polypeptides that were absent from the flagella of cells grown under restrictive conditions (Piperno and Mead, 1997; Cole et al., 1998). By varying the ionic strength during fractionation, the 16-17S complex was resolved into two complexes: IFT complex A (550 kDa) and complex B (750 kDa) (Table 1) (Cole et al. 1998; Piperno et al., 1998).

Various genetic screens for sensory cilia mutants in *C. elegans* generated a large number of candidate genes. Depending on mutant phenotypes, these genes can be subdivided into one or more of the following classes: *osm* (*osm*otic avoidance defective), *che* and *odr* (*che*motaxis and *od*orant *r*esponse defective), *daf* (*da*uer *f*ormation defective) and *dyf* (fluorescent *dy*e-*f*illing defective) (Culotti and Russell, 1978; Perkins et al., 1986; Bargmann et al., 1993; Malone and Thomas, 1994; Starich et al., 1995; Ou et al., 2007). Sequence comparisons showed that many of the polypeptides present in *Chlamydomonas* IFT particle complexes were homologues of proteins required for sensory cilia assembly in *C. elegans* (Table 1) (Cole et al. 1998).

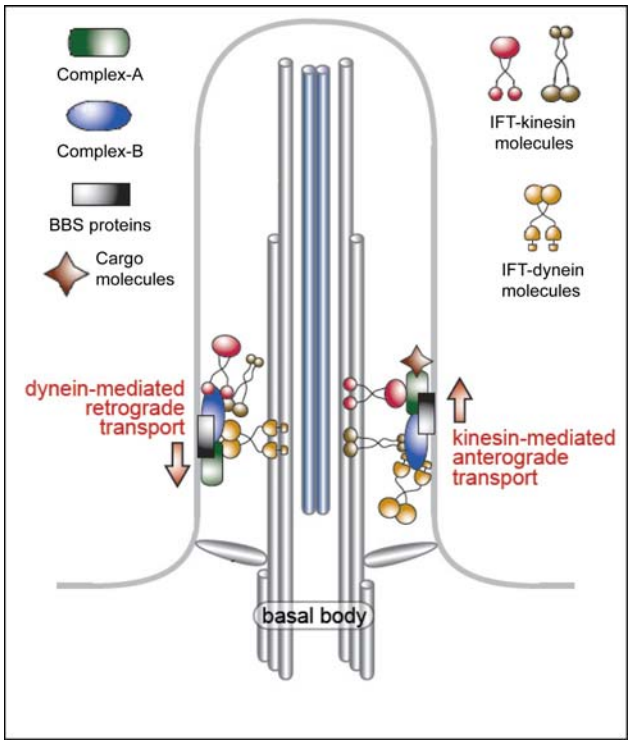

Figure 3. IntraFlagellar Transport (IFT) machinery

The IFT machinery consists of kinesin and dynein molecular motors, IFT particle subcomplexes (A and B) and various accessory components, like BBS proteins, which coordinate anterograde motors. Motor-IFT particles dock near the basal body. From there, cilia cargo (axonemal precursors or receptors) are moved toward the tip of the cilium by one (*Chlamydomonas*) or two (*C. elegans*) IFT-kinesin(s). IFT components are recycled back to the base by IFT-dynein-mediated transport (adapted from Inglis et al., 2006).

While most of *Chlamydomonas* complex A and B mutants exhibit short or bald flagella (Cole, 2003), *C. elegans* IFT-A and IFT-B mutants show different cilia abnormalities. Thus, mutants of complex B (CHE-2, CHE-13, DYF-2, DYF-11, OSM-1, OSM-5, OSM-6) typically show a drastic reduction of cilia length with accumulation of IFT particles near the transition zone (Perkins et al., 1986; Cole et al., 1998; Collet et al., 1998; Fujiwara et al., 1999; Signor et al., 1999b; Haycraft et al., 2001; Haycraft et al., 2003; Efimenko et al., 2006; Li et al., unpublished). In contrast, mutants of complex A (CHE-11, DAF-10 or IFTA-1) have only slightly reduced cilia with massive accumulations of IFT particles along the axoneme (Perkins et al., 1986; Qin et al., 2001; Blacque et al., 2006). These data suggested that complex B proteins are necessary for anterograde directed movement of IFT particles, whereas complex A components function in retrograde transport (Perkins et al., 1986; Qin et al., 2001; Haycraft et al., 2003; Schafer et al., 2003).

Table 1. Protein machinery of IFT in *C. reinhardtii* and *C. elegans*

C. reinhardtii	*C. elegans*	Reference
Anterograde motors		
Heterotrimeric kinesin-2		
FLA10	KLP-11	Walther et al., 1994; Cole et al., 1998; Signor et al., 1999a
FLA8	KLP-20	Signor et al., 1999a; Miller et al., 2005
FLA3	KAP-1	Signor et al., 1999a; Mueller et al., 2005
Homodimeric kinesin-2		
Not found	OSM-3	Signor et al., 1999a
Retrograde motors		
DHC1B	CHE-3	Porter et al., 1999; Signor et al., 1999b; Wicks et al., 2000
LIC	XBX-1	Perrone et al., 2003; Schafer et al., 2003
IFT particle complex A		
IFT144	Not characterized yet	
IFT140	CHE-11	Qin et al., 2001
IFT139	Not characterized yet	
IFT122	DAF-10	Qin et al., 2001; Bell et al., 2006
IFT43	Not characterized yet	
Not characterized yet	IFTA-1	Blacque et al., 2006
IFT particle complex B		
IFT172	OSM-1	Signor et al., 1999b; Pedersen et al., 2005; Bell et al., 2006
IFT88	OSM-5	Pazour et al., 2000; Haycraft et al., 2001
IFT81	F32A6.2	Lucker et al., 2005; Kobayashi et al., 2007
IFT80	CHE-2	Fujiwara et al., 1999
IFT74	C18H9.8	Lucker et al., 2005; Kobayashi et al., 2007
IFT57	CHE-13	Haycraft et al., 2003
IFT55	Not characterized yet	
IFT52	OSM-6	Collet et al., 1998; Deane et al., 2001
IFT46	DYF-6	Bell et al., 2006; Hou et al., 2007
IFT27	Not characterized yet	Qin et al., 2007
IFT20	Y110A7A.20	Baker et al., 2003
Not characterized yet	DYF-2	Efimenko et al., 2006
Not characterized yet	DYF-11	Li et al. (see manuscript IV)

IFT associated proteins		
Not characterized yet	BBS-1	Ou et al., 2007
Not characterized yet	BBS-7	Blacque et al., 2004; Ou et al., 2005a; Pan et al., 2006
Not characterized yet	BBS-8	Blacque et al., 2004; Ou et al., 2005a; Pan et al., 2006
Not characterized yet	DYF-1	Ou et al., 2005a; Pathak et al., 2007
Not characterized yet	DYF-3	Murayama et al., 2005; Ou et al., 2005b
LF4	DYF-5	Berman et al., 2003; Burghoorn et al., 2007
Not characterized yet	DYF-13	Blacque et al., 2005
IFT cargoes		
Not characterized yet	OSM-9	Qin et al., 2005
Not characterized yet	IFTA-2	Schafer et al., 2006; Ou et al., 2007

1.3.3 IFT associated proteins

Bardet-Biedl syndrome (BBS) is a rare genetic disorder characterized by pleiotropic phenotypes that include obesity, rod-cone dystrophy, renal malformations, polydactyly, cognitive impairment, and several other ailments (Blacque and Leroux, 2006) (see also below). It was shown that BBS is caused by defects in basal bodies and / or primary cilia (Ansley et al., 2003). Presently, 11 BBS proteins have been found and at least three of them, BBS-1, BBS-7 and BBS-8, are important for proper IFT (Figure 3; Table 1). It has been demonstrated that *C. elegans* BBS proteins play selective roles in the assembly of IFT particle components at the base of cilia (Blacque et al., 2004). In *bbs-1*, *bbs-7* and *bbs-8* mutants, IFT-A and IFT-B particles can be driven separately along the middle and distal segments of the cilium by means of two distinct kinesin-2 motor complexes, kinesin-II and OSM-3-kinesin, respectively (Ou et al., 2005a; Ou et al., 2007). Recent findings support a mechanical competition model, where IFT particles are moved along sensory cilia by kinesin-II and OSM-3-kinesin acting together, with the slower moving kinesin-II exerting a drag on the faster moving OSM-3, whereas the faster moving OSM-3 tends to pull the slower moving kinesin-II along. This produces a mechanical competition that translates into tension across the IFT particles, leading to their dissociation in the absence of BBS proteins. Thus, BBS proteins antagonize this tension force and maintain the integrity of the IFT particles by stabilizing the association of IFT-A with IFT-B (Pan et al., 2006).

It has recently been demonstrated that seven highly conserved BBS proteins can form a 450 kDa complex, which is called the “BBSome”. Rabin8, a GTP exchange

factor specific for Rab8, helps recruit the BBSome to the centrosome / basal body from the neighboring centriolar satellites and activates Rab8 to promote the docking and fusion of vesicles near the cilia membrane. This event likely allows the movement of $Rab8^{GTP}$ and probably also BBS proteins into cilia, thereby promoting ciliogenesis (Nachury et al., 2007).

The conserved MAP kinase DYF-5 is also important for the constitution and coordination of IFT particles in *C. elegans* (Burghoorn et al., 2007). However, unlike BBS proteins, DYF-5 functions at sites where the IFT particles switch between different motor complexes, such as the transition from the middle to the distal segment and at the distal tip. Failure in removing kinesin-II from IFT particles would explain its entry into the distal segments in *dyf-5* mutants. In addition, a failure in switching from anterograde to retrograde transport would explain the aggregation of IFT proteins in distal segments (Burghoorn et al., 2007). Another conserved *C. elegans* ciliary protein, DYF-1, is specifically required for OSM-3-kinesin to dock onto and move IFT particles, because OSM-3-kinesin is inactive and intact IFT particles are moved by kinesin-II alone in *dyf-1* mutants (Table 1) (Ou et al., 2005a). Intriguingly, it has recently been shown that the zebrafish *dyf-1* homologue, *fleer*, could act as an adaptor protein that links tubulin tyrosine ligase-like (TTLL) enzymes to IFT. Anterograde transport of TTLL enzymes leads to tubulin polyglutamylation all along the length of the axoneme. In the absence of Fleer/DYF-1, tubulin is polyglutamylated only in the basal body region of the cilia and distal microtubule doublets are not polyglutamylated that causes B-subfiber instability, impaired dynein motility, and the arrest of polyglutamylation-dependent kinesin motility (Pathak et al., 2007).

1.3.4 IFT cargoes

The cilium, in spite of its apparent stability, is a dynamic structure in which microtubules and associated proteins are constantly turning over. The continuous assembly and disassembly of axonemal components requires the delivery of precursors and the removal of discarded proteins. One posited role of IFT is to transport cilia precursors to and from the point of their assembly (Figure 3). The formation of radial spokes in *Chlamydomonas* flagella can be taken as an example of such a process (Qin et al., 2004). Radial spokes (axonemal subunits consisting of 22 polypeptides) were found to be partially assembled in the cell body, before being transported to the flagellar tip by anterograde IFT. In contrast, fully assembled radial spokes, detached

from axonemal microtubules during flagellar breakdown or turnover, are removed from flagella by retrograde IFT (Qin et al., 2004).

Sensory cilia in *C. elegans* harbor various channels and receptors involved in sensing of enviromental clues. For example, the transient receptor potential vanilloid (TRPV) channel OSM-9 is located in the membrane of sensory cilia, where it functions in several sensory transduction modalities, including osmosensation, olfaction, and mechanosensation (Colbert et al., 1997). When observed by time-lapse fluorescence microscopy, OSM-9::GFP moved bidirectionally in sensory cilia at a rate similar to IFT. In IFT mutants OSM-9::GFP accumulates in aggregates along the length and at the base of the cilium and is not detected moving (Qin et al., 2005). This study demonstrates that IFT, previously shown to be necessary for transport of axonemal precursors, is also involved in the motility of functional cilia components. Another example of an IFT cargo molecule can be obtained from the study of the *ifta-2* gene, which encodes a *C. elegans* Rab-like protein (Schafer et al., 2006). IFTA-2::GFP localizes to cilia and is transported along the axoneme similar to previously characterized IFT proteins (Ou et al., 2007). However, disruption of IFTA-2 does not alter cilia morphology or transport of other IFT components, suggesting its role as possible IFT cargo (Schafer et al., 2006; Ou et al., 2007).

1.4 CILIA AND CELL SIGNALING

Sensory behavior in different organisms depends on correct recognition and processing of signals from the environment or from within the organism. Primary cilia, exposed on cell surfaces, serve as a perfect antennae receiving and transducing various sensory cues. The role of cilia in sensing of environmental stimuli is best understood with the examples of olfaction and photoreception. In the process of olfaction, an odorant interacts with a G protein–coupled receptor (GPCR) on the cilia membrane of an olfactory sensory neuron, producing the second messenger cyclic adenosine monophosphate (cAMP) within the cilium. Increased levels of cAMP then depolarize the cell by opening a cyclic nucleotide-gated channel also located in the cilia membrane (Singla and Reiter, 2006). The principles of photoreception are similar to those of olfaction. The rod and cone cells of the vertebrate retina possess a primary cilium with an expanded tip called the outer segment. In the outer segment, opsin GPCRs respond to photons of light by increasing hydrolysis of a different cyclic nucleotide, thereby closing cGMP-gated channels (Singla and Reiter, 2006).

Recent studies implicate cilia also in several important developmental signaling pathways. Thus, proper development of the kidney requires Wnt signaling (Figure 4). At early stages of kidney development, canonical Wnt signaling induces the formation of metanephric mesenchyme and cell proliferation during branching morphogenesis (Perantoni, 2003). Later, the non-canonical Wnt / planar cell polarity (PCP) signaling pathway is required to align the mitotic orientation of proliferating cells of the renal tubules (Veeman et al., 2003). Mutations of the ciliary protein Inversin (Invs) cause Nephronophthisis type II, an autosomal recessive cystic kidney disease (Davenport and Yoder, 2006). It has recently been reported that Inversin might act as a switch between canonical Wnt signaling and the non-canonical Wnt (PCP) pathway (Figure 4) (Simons et al., 2005). In the canonical pathway, Wnt binding to Frizzled (Fz) leads to the activation of Dishevelled (Dvl1) and the stabilization of cytoplasmic β-catenin, through inhibition of the Apc2/axin/Gsk3 degradation complex (Bisgrove and Yost, 2006). In response to an environmental stimulus such as fluid flow, Inversin forms a complex with Dvl1 and Apc2. This complex targets Dvl1 for proteosomal degradation, resulting in a loss of β-catenin stabilization and inhibition of the canonical Wnt pathway (Figure 4). Subsequently, Inversin and Dvl1 translocate to the plasma membrane, where they associate with PCP proteins and activate the non-canonical Wnt (PCP) pathway (Figure 4) (Simons et al., 2005; Bisgrove and Yost, 2006).

The importance of primary cilia was also described for Sonic Hedgehog (Shh) signaling in vertebrates. Shh signaling culminates in the conversion of Gli transcription factors from repressors to activators. Central to this conversion are two transmembrane proteins, Smoothened (Smo) and the Shh receptor Patched (Ptch) , which can be localized within the cilium. In the absence of Shh signals, Ptch maintains Smo in an inactive state, and Gli transcription factors are processed to their repressor forms. Upon binding Shh, Ptch loses the ability to repress Smo, leading to the generation of Gli transcriptional activators that execute the Shh transcriptional program (Singla and Reiter, 2006). It has been shown that Shh expression is normal in IFT mutants. However, the expression of downstream targets like Patched 1 (Ptch 1) and Gli1 is reduced (Huangfu and Anderson, 2005). The data obtained from different studies indicate that IFT is required downstream of Ptch 1 and Smo. IFT is also required for Gli activation and for the proteolytic processing of Gli3 into Gli3R, but not for the trafficking of Gli proteins to the nucleus (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005). It is still unclear how Smo

activates Shh signaling in the cilium, but the cilium might provide a specialized microtubule-associated domain that coordinates Smo and other components of the pathway to facilitate the activation of Gli transcription factors and the proteolytic processing of Gli3 to generate Gli3R (Bisgrove and Yost, 2006).

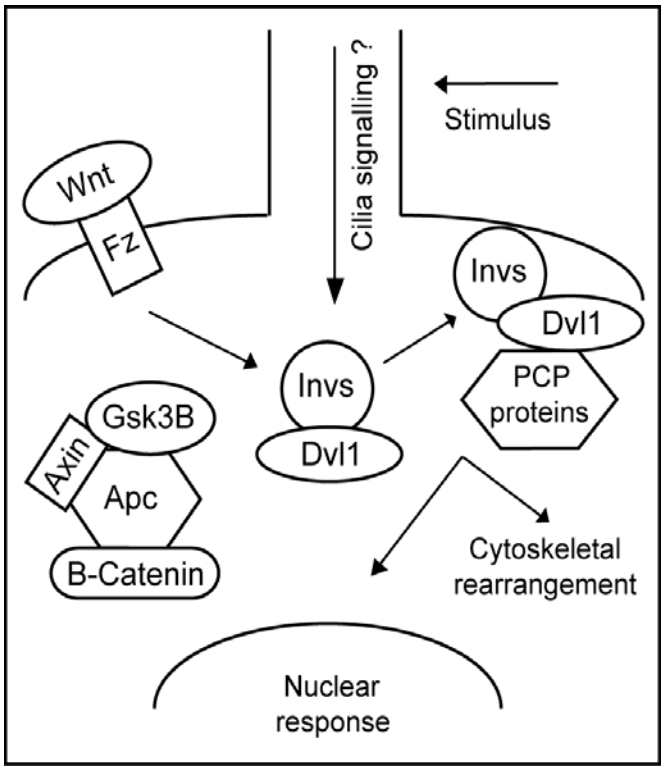

Figure 4. Cilia and Wnt signaling

The conserved ciliary protein Inversin (Invs) acting as a switch between canonical Wnt signaling and the non-canonical Wnt/planar cell polarity (PCP) pathway. In response to an environmental stimulus, Inversin forms a complex with Dishevelled (Dvl1). This complex targets Dvl1 for proteosomal degradation, resulting in a loss of β-catenin stabilization and inhibition of the canonical Wnt pathway. Subsequently, Inversin and Dvl1 translocate to the plasma membrane, where they activate the non-canonical Wnt (PCP) pathway, which is important for mitotic spindle orientation or for cell polarization (adapted from Bisgrove and Yost, 2006).

1.5 CILIOPATHIES

In mammals cilia are near ubiquitous organelles that project from the surfaces of many different cell types to carry out motile and sensory functions. Cilia have been implicated directly in many developmental processes such as the generation of left-right asymmetry, maintenance of the renal epithelium, or respiratory function (Rosenbaum and Witman, 2002). Therefore, defects in cilia function or structure can

lead to a wide range of developmental problems and diseases, among them: Bardet-Biedl syndrome, Alström syndrome, Meckel syndrome, Oral-Facial-Digital syndrome, Kartagener syndrome, Jeune syndrome, Polycistic Kidney Disease, etc.

Bardet-Biedl syndrome (BBS) (OMIM #209900) is a highly pleiotropic human disorder, characterised by a multitude of symptoms, including obesity, retinal degradation, renal abnormalities, polydactyly, male hypogonadism and learning disabilities (Blacque and Leroux, 2006). The first breakthrough in understanding the molecular pathology of BBS came from the study of Ansley and colleagues, where BBS proteins have been found associated with cilia and basal bodies (Ansley et al., 2003). The ciliogenic nature of BBS proteins was later confirmed by different comparative genomics studies (Li et al., 2004; Avidor-Reiss et al., 2004). Thus, *BBS* gene homologs were found only in ciliated organisms, but not in those lacking cilia. Finally, BBS shares significant phenotypic overlaps with other human disorders known to be associated with defects in primary cilia function (see below). Taken together, the above findings firmly established cilia / basal body dysfunction as an underlying cause of BBS. So far, 11 members of the *BBS* gene family have been discovered: *BBS1* (Mykytyn et al., 2002); *BBS2* (Nishimura et al., 2001); *BBS3* (Fan et al., 2004); *BBS4* (Mykytyn et al., 2001); *BBS5* (Li et al., 2004); *BBS6* (Katsanis et al., 2000); *BBS7* (Badano et al., 2003); *BBS8* (Ansley et al., 2003); *BBS9* (Nishimura et al., 2005); *BBS10* (Stoetzel et al., 2006); *BBS11* (Chiang et al., 2006).

Alström syndrome (AS) (OMIM #203800) is a progressive disease characterized by neurosensory deficits and by metabolic defects including retinitis pigmentosa, obesity, and diabetes mellitus. Although this disorder bears many similarities to the Bardet-Biedl syndrome, there are no mental defects, polydactyly, or hypogonadism. Alström syndrome has recently been associated with ALMS1 protein. This protein was shown to localize to the basal bodies of cilia as well as to centrosomes (Hearn et al., 2005). Intriguingly, the proteins associated with diseases that are phenotypically similar to AS, such as BBS, also localize to cilia of ciliated cells, and their disruption lead to cilia structure abnormalities (Ansley et al., 2003). Analyses of ciliated cells in *Alms1* mutant mice reveal that cilia undergo normal assembly. However, disruption of the *Alms1* gene in the retina results in the accumulation of large membrane-bound vesicles within photoreceptor inner segments and mislocalization of rhodopsin to the outer segments, indicating a potential role of ALMS1 as functional, but not structural component of ciliogenesis (Collin et al., 2005).

Meckel syndrome (MKS) (OMIM #249000) is an autosomal recessive disorder reported in most populations and characterized by a combination of renal cysts and variably associated features including developmental anomalies of the central nervous system, hepatic ductal dysplasia and cysts, and polydactyly. Recent studies allowed to identify two genes, *MKS1* and *MKS3*. (Kyttälä et al., 2006; Smith et al., 2006). MKS1 is a novel protein containing a conserved B9 domain. The expression of *MKS1* was especially prominent in ciliated tissues such as brain, liver, kidney and digits of the upper limbs (Kyttälä et al., 2006). *MKS3* mapping was refined to a 12.67-Mb interval (8q21.13-q22.1) that is syntenic to the *Wpk* locus in rat, which is a model with polycystic kidney disease and hydrocephalus. Positional cloning of the *Wpk* gene suggested a *MKS3* candidate gene, *TMEM67*, which encodes a seven-transmembrane receptor protein (meckelin) (Smith et al., 2006). Comparative genomics and proteomics data implicate both *MKS1* and *MKS3* in cilia functions (Li et al., 2004; Keller et al., 2005). Further evidence of the ciliary nature for those proteins comes from the *C. elegan*s homologs of *MKS1* and *MKS3*, *xbx-7* and F35D2.4, respectively. Both genes contain X-box motifs in their promoters and at least one of them, *xbx-7*, is expressed in ciliated sensory neurons (Efimenko et al., 2005). Finally, siRNA-mediated reduction of *Mks1* and *Mks3* expression in a ciliated epithelial cell-line blocks centriole migration to the apical membrane and consequent formation of the primary cilium (Dawe et al., 2007a). Altogether, these results strongly suggest that MKS proteins play crucial roles in cilia development.

The oral-facial-digital type I (OFD I) syndrome (OMIM #311200) is a human developmental disorder that leads to craniofacial and digital abnormalities and polycystic kidney. The disease is inherited as an X-linked dominant male-lethal trait. The locus for *Ofd1* was mapped by linkage analysis to the Xp22 region. Analyses of several transcripts mapping to that region allowed to identify the *Ofd1* gene, which encodes a protein containing coiled-coil α-helical domains (Ferrante et al., 2001). *Ofd1* knockout mice show failure of left-right axis specification in mutant male embryos, and ultrastructural analysis showed a lack of cilia in the embryonic node (Ferrante et al., 2006). The formation of cilia was also defective in cystic kidneys, implicating ciliogenesis as a mechanism underlying cyst development. Finally, altered expression of the 5' *Hoxa* and *Hoxd* genes was found in the limb buds of mice lacking *Ofd1*. Upregulation of these genes in cells that lack *Ofd1* could be due to a direct effect, but it would also be compatible with a perturbation of Gli3 function. The regulation of Gli proteins function by IFT has been demonstrated (Liu et al., 2005), and therefore, it

might suggest a functional interaction between OFD1 and the Gli proteins in cilia (Ferrante et al., 2006).

Kartagener syndrome (OMIM #244400) or Primary Ciliary Dyskinesia (PCD) is an autosomal recessive disorder characterized by bronchiectasis, situs inversus, and infertility. It was found that sperm and the cilia of the respiratory tract are immotile in patients with Kartagener syndrome. Electron microscopy analysis revealed an absence of axonemal dynein arms in cilia (Afzelius et al., 1976). It has later been demonstrated that different cases of Kartagener syndrome are caused by mutations in a set of axonemal dynein genes, including: axonemal intermediate chain dynein type 1 (*DNAI1*) (Guichard et al., 2001); axonemal heavy chain dynein type 5 (*DNAH5*) (Olbrich et al., 2002); and axonemal heavy chain dynein type 11 (Bartoloni et al., 2002).

Jeune asphyxiating thoracic dystrophy (JATD) (OMIM #208500), an autosomal recessive chondrodysplasia, often leads to death in infancy because of a severely constricted thoracic cage and respiratory insufficiency. A bioinformatic approach allowed to identify the *IFT80 (WDR56)* gene as a potential candidate for Jeune syndrome (Beales et al., 2007). The *C. elegans* ortholog of *IFT80*, *che-2*, is important for proper cilia development and is associated with IFT complex B (Fujiwara et al., 1999). It has been demonstrated that *IFT80* mutations underlie a subset of JATD cases, establishing the association of a defective intraflagellar transport protein with a human disease. Knockdown of *ift80* in zebrafish resulted in cystic kidneys, and knockdown in *Tetrahymena thermophila* produced shortened or absent cilia (Beales et al., 2007).

Polycystic kidney disease (PKD) (OMIM #173900) is a disease of the nephron, characterized by the formation of multiple renal tubular cysts. The most common form is autosomal dominant PKD (ADPKD). Rare forms include autosomal recessive PKD (ARPKD) and nephronophthisis (Wilson, 2004). ADPKD has been shown to result from loss-of-function mutations of either of two molecules, polycystin-1 (PKD1) and polycystin-2 (PKD2). (Davenport and Yoder, 2006). *PKD1* and *PKD2* encode a large 11-transmembrane spanning receptor and transient receptor potential (TRP) channel, respectively (Clapham, 2003). *C. elegans* homologs of PKD1 and PKD2 localize on the cilia membrane and are required for proper male sensory behaviors (Barr et al., 2001). Mammalian PKD1 and PKD2 also localize to sensory cilia of kidney epithelial cells, pointing to an evolutionary conserved role for those proteins in cilia biogenesis (Yoder et al., 2002). Both molecules form a complex, which is implicated in the transduction of environmental signals (i.e. luminal tubular fluid flow and composition)

into cellular events, such as epithelial cell growth. Thus, tubular flow induces Ca^{2+} influx through PKD2 and maintains transcription factor STAT6 in a complex bound to the tail of PKD1. In the absence of flow or in *PKD1* mutants, STAT6 translocates to the nucleus and activates transcription of target genes (Low et al., 2006).

1.6 VARIOUS WAYS TO SEARCH FOR CILIARY GENES

1.6.1 Proteomic studies

One of the first efforts to find the molecular composition of cilia was the characterization of proteins from purified cilia axonemes (so called ciliary proteome). In a pioneer study, cilia from human bronchial epithelial tissue culture cells have been used as starting material (Ostrowski et al., 2002). Subsequent mass spectrometric analysis of axonemal peptides revealed a total of 214 proteins. While many of the proteins identified were previously uncharacterized, some of them represented well known axonemal components, including tubulins, dyneins and radial spokes proteins (Ostrowski et al., 2002). However, most of the proteins obtained in this work were identified on the basis of only one or more peptides and probably include many false positives matches. Also, this study was compromised by the presence of other cellular structures in the axonemal preparation. More accurate and detailed proteomic data have been obtained from the study of isolated flagella in *Chlamydomonas reinhardtii* (Pazour et al., 2005). Unlike in other organisms, flagella of *Chlamydomonas* can be easily isolated and treated biochemically to obtain different sub-fractions, including extracts of membrane plus axoneme, membrane plus matrix (the flagellar matrix is enriched for components such as IFT proteins that are not tightly associated with either the axoneme or membrane) and the axonemal fraction alone. In total, 360 proteins have been identified from different preparations. All matches were represented by five or more peptides, suggesting a high level of confidence (Pazour et al., 2005). Intriguingly, apart from well known components of cilia structure, a significant number of receptors and signal transduction molecules was uncovered, pointing toward the capacity for sensory perception by motile flagella. Recently, this capacity was experimentally proven by the example of the flagellar-adhesion-activated signaling pathway (Wang et al., 2006).

1.6.2 Comparative genomics

The availability of genomic sequence information from multiple species has provided an independent approach for filtering out the cilia component of proteomes. In

one of these approaches, the authors subtracted the non-flagellated proteomes of *Arabidopsis* from the shared proteome of ciliated / flagellated organisms like *Chlamydomonas* and human (Li et al., 2004). As a result, 688 proteins that are exclusively present in organisms with flagella and basal bodies have been identified. In a similar study, the nonciliated *Arabidopsis*, *Dictyostelium* and *Saccharomyces* genomes have been compared with those of five ciliated eukaryotes, by using *Drosophila* as the anchor species (Avidor-Reiss et al., 2004). This approach yielded about 200 genes that were conserved across ciliated organisms, but absent in nonciliated species. Additional functional and genetic tests validated the enrichment of the obtained lists for ciliary proteins and made possible the characterization of previously unknown genes, including *BBS5* (Li et al., 2004) and a novel family of outer segment (OSEG) genes (Avidor-Reiss et al., 2004). The weak point of this approach is that *bona fide* ciliary genes might be excluded because they are members of conserved multigene families with similar but non-redundant functions. On the other hand, the advantage of comparative genomic studies is that proteins supporting cilia function but are not specifically localized to the organelle (e.g. cannot be detected in proteomic analysis) can be identified (Inglis et al., 2006).

1.6.3 Cilia-specific transcriptomes

Similar to comparative genomics, the analysis of cilia-specific transcriptomes allows to uncover genes important for the formation or function of cilia, regardless of the subcellular localization of the encoded protein. A significant amount of data about the cilia-specific transcriptome was obtained from studies of ciliated sensory neurons (CSN) in the nematode *Caenorhabditis elegans*. In one of those studies, the authors obtained mRNA from CSN of *C. elegans* using an mRNA-tagging method, in which poly(A) RNA was co-immunoprecipitated with an epitope-tagged poly(A)-binding protein specifically expressed in sensory neurons. Subsequent cDNA microarray analyses led to the identification of a panel of CSN-specific genes (Kunitomo et al., 2005).

In a different study, a transcriptome enriched for ciliary genes was obtained by comparison of the transcription profiles of ciliated cells with those lacking cilia (Blacque et al., 2005). In this approach all CSN were labelled with a specific GFP marker, isolated by disrupting the embryo and fluorescence-activated cell sorting, and subjected to serial analysis of gene expression (SAGE). Similarly, specific subsets of non-ciliated cells expressing pan-neuronal, intestinal or muscle-specific GFP markers were analyzed by SAGE. In total, 1282 genes have been identified with 1,5-fold or

greater level of expression in ciliated cells versus each of the non-ciliated cell transcriptomes (Blacque et al., 2005).

A similar approach was used to identify *C. elegans* sensory neuron type-specific genes (Colosimo et al., 2004). In this study, the authors performed microarray analysis using RNA from sorted AWB olfactory (ciliated) and AFD thermosensory (non-ciliated) neurons. Using an arbitrary 2-fold or higher ratio, 58 genes have been found differentially expressed in the AWB neuron (Colosimo et al., 2004).

Finally, valuable data were obtained from the study of the flagellar regeneration transcriptome in *Chlamydomonas reinhardtii*. Regeneration of *Chlamydomonas* flagella leads to substantial upregulation of genes required for building a functional organelle. Microarray analysis of this process allowed to identify 220 genes that had more than two fold induction during flagellar regeneration, including 85 genes previously known to encode cilia components (Stolc et al., 2005).

1.6.4 Computational search for the X-box promoter motifs

It has previously been shown that the expression of some genes required for cilia biogenesis is controlled by regulatory factor X (RFX)-type transcription factors (Swoboda et al., 2000; Dubruille et al., 2002). All members of the RFX transcription factor family are characterized by the presence of a conserved DNA binding domain (DBD). The RFX - DBD binds to special motifs (X-boxes) in promoters of its target genes. Many proteomics and comparative genomics studies used computational searches for the X-box promoter motif as an additional filtering tool that allowed to identify numerous novel ciliary genes (Li et al., 2004; Avidor-Reiss et al., 2004; Efimenko et al., 2005; Blacque et al., 2005; Chen et al., 2006; Laurençon et al., 2007).

1.6.5 Ciliome resources

Various search approaches for cilia components have yielded partially overlapping ciliary, basal body and centrosomal protein collections, reflecting both the discrete composition of each subcellular compartment as well as false positives and false negatives unique to each method. Recently, all existing ciliary and basal body proteomics data have been integrated into ciliary proteome (ciliome) databases: http://www.ciliaproteome.org and http://www.ciliome.com (Inglis et al., 2006; Gherman et al., 2006). These databases provide the means to search the ciliome with user-defined criteria and to extract genomic, genetic and basic functional information, as well as biological links to human diseases (ciliophaties).

Cilia and flagella are now capturing an increasing amount of attention given their ubiquity, participation in a variety of physiological functions and links to different disorders. Recent progress in compilation and cross-comparison of several ciliary data sets can now be used to focus on previously unrecognized, putative ciliary proteins for functional characterization. In addition, these resources can be useful for researchers interested in identifying and studying new genes implicated in known or suspected ciliopathies.

1.7 STRUCTURE OF CILIATED SENSORY NEURONS IN *C. ELEGANS*

Caenorhabditis elegans is a small (1 mm long) free-living nematode found in temperate regions. In *C. elegans*, 60 of the 302 neurons of the hermaphrodite are ciliated sensory neurons (CSN) (White et al., 1986). The 60 CSN comprise 24 distinct neuron classes with structurally different types of sensory cilia, which function in the reception of chemosensory stimuli (Troemel, 1999). The amphids, a pair of lateral sensilla in the head, are the principal chemosensory organs of nematodes (Figure 5). Each amphid comprises the ciliated dendrites of 12 sensory neurons plus two support cells called sheath and socket cells (Perkins et al., 1986). Of the 12 amphid neurons, 8 are evidently chemosensory in that their rod-like cilia extend into the channel of the socket cells and are thereby exposed to the external environment. The rod cilia are about 7.5 μm long and consist of the transition zone, the middle and the distal segments (Figure 5). The wing-like cilia of three additional neurons are separate from the others, and invaginate individually into the sheath cell, proximal to where the fascicle of rod cilia enters the socket cell. The dendrite of the AFD thermosensory neuron remains separate from the fascicle of wing and channel cilia (Figure 5) (Perkins et al., 1986).

Various genetic screens for structural and functional mutants of sensory cilia in *C. elegans* generated a large collection of candidate genes. Depending on mutant phenotypes, these genes can be subdivided into one or more of the following classes: *osm* (*osm*otic avoidance defective), *che* and *odr* (*che*motaxis and *od*orant *r*esponse defective), *daf* (*da*uer *f*ormation defective) and *dyf* (fluorescent *dy*e-*f*illing defective) (Culotti and Russell, 1978; Perkins et al., 1986; Bargmann et al., 1993; Malone and Thomas, 1994; Starich et al., 1995; Ou et al., 2007). All of these mutants have different defects of cilia structure and / or function and thus, represent a valuable tool box that can be used to illuminate different aspects of cilia biogenesis.

Grounded in an extraordinary database (e.g. complete genome sequence, cell lineage, and nervous system structure are known), as well as having an advantageous cilia properties (cilia are extremely large and unlike in many other organisms are not essential for viability, so mutant strains lacking cilia can be isolated and maintained easily), the *C. elegans* model system is readily suited for a multi-faceted approach that is based on mutational analyses of genes involved in sensory cilia formation. A variety of genetic, genomic, molecular, cell biological, biochemical, and ultrastructural methods are available to allow detailed characterization of mutant phenotypes. Finally, because the structural and functional characteristics of *C. elegans* sensory cilia are very similar to those in mammals, results obtained with the *C. elegans* model system will have general significance.

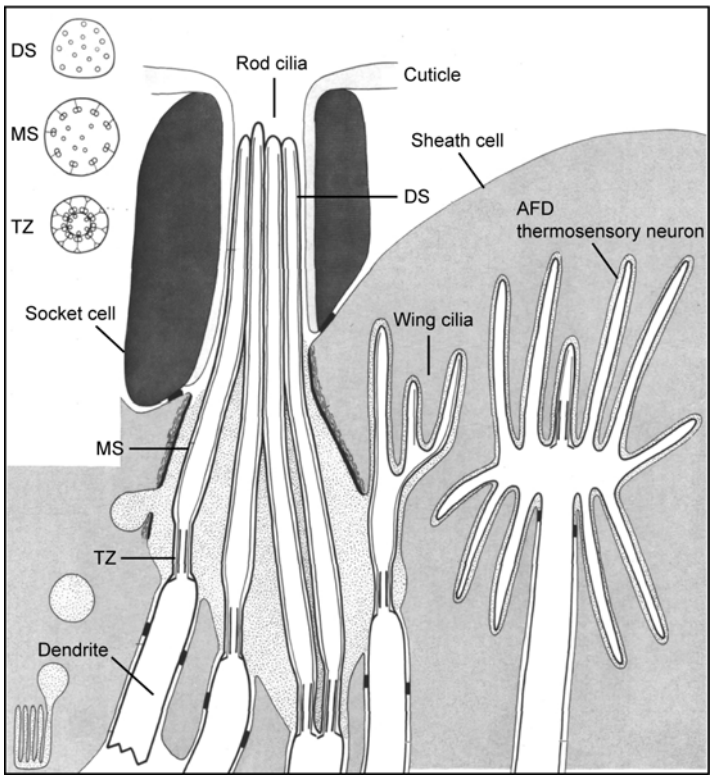

Figure 5. Structure of the amphid sensilla in *C. elegans*

The amphids, a pair of lateral sensilla in the head, are the principal chemosensory organs of nematodes. Each amphid comprises the ciliated dendrites of 12 sensory neurons plus two support cells called sheath and socket cells. 8 out 12 neurons process their single or double rod-like cilia into the channel formed by the socket cells. The wing-like cilia of three additional neurons are separate from the others and invaginate individually into the sheath cell. The dendrite of the AFD thermosensory neuron remains separate from the fascicle of wing and channel cilia. The rod cilia have segmented structure and consist of the transition zone (TZ), the middle (MS) and the distal (DS) segments (adapted from Perkins et al., 1986).

2 AIMS

The specific aims of the study described here were to:

- Establish a list of potential ciliary genes in *C. elegans* and other species.
- Find and characterize novel ciliary genes.
- Study the mechanisms of cilia assembly and maintenance.

3 RESULTS AND DISCUSSION

3.1 PAPER I: ANALYSIS OF *XBX* GENES IN *C. ELEGANS*

Whereas many ciliary mutants are available in *C. elegans*, there is only one known gene mutation that completely eliminates all classes of sensory cilia and all functional components of cilium structure. This gene is *daf-19,* and it encodes the sole *C. elegans* member of the RFX-type transcription factors, found widely in the eukaryotic kingdom. All members of the RFX transcription factor family are characterized by the presence of a conserved DNA binding domain (DBD). The RFX - DBD binds to special motifs (X-boxes) in promoters of its target genes. Genes containing the X-box promoter motif are called *xbx* genes. The discovery of X-boxes in promoter regions of certain ciliary genes prompted us to analyze the whole *C. elegans* genome for the presence of these motifs. In order to perform this analysis of all *C. elegans* promoters we implemented an in-house searching algorithm – the X-box searcher, which searches for all possible matches to a defined motif sequence. Using X-box consensus sequences with varying degrees of refinement, together with cross-species comparisons between *C. elegans* and *C. briggsae* and subsequent gene expression analysis, we were able to identify a large number of bona fide *xbx* genes, a significant part of which we expect to also be cilia or ciliated sensory neuron (CSN) specific genes.

The expression data from 22 *xbx*-gene GFP fusions that are expressed in CSN and show dependence on DAF-19 function and actual X-box sequence allowed us to derive an in vivo "refined" consensus for *C. elegans xbx* genes. Most of the X-boxes matching this consensus are located in immediate proximity to the gene start (in the range of 50-250 bp upstream of the ATG). Lack of DAF-19 function or changes in the actual X-box sequence lead to drastic reduction or variation of gene expression patterns, suggesting a crucial role of the given motif sequence in the regulation of target genes.

Our previous model associated DAF-19 regulation with only a certain group of genes functioning in cilia morphogenesis and architecture. The data obtained in this study suggest that the repertoire of DAF-19-dependent genes is much broader, including genes for cilia structure, transport machinery, receptors and other factors. Considering the above we propose a model where DAF-19 regulates the development

of a "ciliary module" during the differentiation of sensory neurons in *C. elegans*. According to this model, DAF-19 is a key factor for the general development of cilia. At the same time, together with other factors, it can drive the expression of genes required for specialized functions in cilia.

3.2 PAPER II: IDENTIFICATION OF NOVEL REGULATORY FACTOR X (RFX) TARGET GENES BY COMPARATIVE GENOMICS IN *DROSOPHILA* SPECIES

A computational search for X-box motifs in the *C. elegans* genome allowed us to identify a group of genes important for cilia biogenesis. Subsequent X-box motif searches in the *C. briggsae* genome have demonstrated strong conservation of this motif between closely related nematode species. To find out whether RFX-type transcription factors can also regulate ciliogenic pathways in other organisms, we applied a similar search strategy to a distant species such as the fruit fly *Drosophila*. Using X-box consensus sequences with varying degrees of refinement, together with cross-species comparisons between *D. melanogaster* and *D. pseodoobscura* (closely related *Drosophila* species) and subsequent gene expression analysis, we were able to identify a set of *Drosophila xbx* genes. Intriguingly, the majority of fly *xbx* genes that have homologs in *C. elegans* were down regulated in fly *dRfx* mutants, suggesting an evolutionary conserved role for the RFX-type transcription factors in the regulation of ciliary genes.

3.3 PAPER III: *CAENORHABDITIS ELEGANS* DYF-2, AN ORTHOLOGUE OF HUMAN WDR19, IS A COMPONENT OF THE IFT MACHINERY IN SENSORY CILIA

The assembly and further structural and functional maintenance of cilia and flagella are dependent on the process of intraflagellar transport (IFT). During IFT, non-membrane-bound particles (IFT particles) and their associated cargo molecules are moved continuously along the axoneme by means of kinesin-2 and IFT-dynein molecular motors that mediate their anterograde and retrograde transport, respectively.

In order to improve our understanding of the IFT machinery, we are identifying the full repertoire of molecules involved in this process. Various genetic screens for

sensory cilia mutants in *C. elegans* generated a large number of candidate genes. Mutations that reduce fluorescent dye filling of the amphid and phasmid ciliated sensory neurons, which are directly exposed to the environment, are indicative of general defects in cilia structure (Starich et al., 1995). So far, mutations in all identified IFT genes result in a Dyf (fluorescent *dy*e *f*illing defective) phenotype.

In this paper we describe a novel WD repeat (WDR) containing IFT protein from *C. elegans*, DYF-2, which was identified as a candidate *xbx* / ciliary gene in our previous work. We determined the identity of the *dyf-2* gene by transgenic rescue of mutant phenotypes and by sequencing of mutant alleles. Loss of DYF-2 function selectively affects the assembly and motility of different IFT components and leads to defects in cilia structure and chemosensation in the nematode. Based on these observations, and the analysis of DYF-2 movement in a Bardet-Biedl syndrome mutant background with partially disrupted IFT particles, we conclude that DYF-2 can associate with IFT particle complex B. At the same time, mutations in *dyf-2* can interfere with the function of complex A components, suggesting an important role of this protein in the assembly of the IFT particle as a whole. Importantly, the mouse ortholog of DYF-2, WDR19, also localizes to cilia, pointing to an important evolutionarily conserved role of this WDR protein in cilia development and function.

3.4 PAPER IV: CENTRAL ROLE FOR DYF-11/MIP-T3 IN ASSEMBLING KINESIN MOTOR-INTRAFLAGELLAR TRANSPORT COMPLEXES

MIP-T3 is a human protein previously found to associate with microtubules, but whose cellular function remains unknown. In this study we demonstrate that the *C. elegans* MIP-T3 ortholog DYF-11 is an intraflagellar transport (IFT) protein that plays a critical role in assembling functional kinesin motor-IFT particle complexes. Several known components of the IFT machinery, including Kinesin-II, as well as IFT subcomplex A and B proteins, fail to enter cilia axonemes and / or mislocalize in the *dyf-11* mutant we cloned, resulting in compromised cilia structures and sensory functions. Analyses in different mutant backgrounds further suggest that DYF-11 functions as a novel component of IFT, which can associate with subcomplex B. Consistent with an evolutionarily conserved cilia-associated role, mammalian MIP-T3 localizes to basal bodies and cilia. Intriguingly, the zebrafish MIP-T3 ortholog

interacts genetically with the Bardet-Biedl syndrome basal body / ciliary gene *bbs4* to ensure correct gastrulation. Our findings therefore implicate MIP-T3 in a previously unknown but critical role in cilia biogenesis and further highlight the emerging role of the vertebrate cilium in morphogenetic signaling.

4 CONCLUSIONS

- Through several genome-wide sequence searches for the X-box regulatory promoter motif in *C. elegans* and other organisms like *Drosophila*, we collected many candidate X-box containing (*xbx*) genes.
- We successfully tested a subset of them for cilia specific expression and function, providing evidence that RFX-type transcription factors that bind to the X-box stand at the top of a developmental cascade, that is, they regulate the "ciliary module" of effector genes necessary for generating structurally and functionally intact cilia.
- Using the obtained X-box matches as a prediction tool we were able to identify many novel ciliary genes in both the *C. elegans* and *Drosophila* genomes.
- Finally, we characterized two novel ciliary genes in *C. elegans*, *dyf-2* and *dyf-11*. Loss of functions of these genes selectively affects the assembly and motility of different intraflagellar transport (IFT) components, resulting in compromised cilia structures and sensory functions. The localization of DYF-2 and DYF-11 in cilia across different species points to the evolutionarily conserved roles for these proteins in cilia biogenesis.

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