Regulation of Intraflagellar Transport in the sensory cilia of *Caenorhabditis elegans*

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Regulatie van intraflagellair transport in de sensorische cilia van *Caenorhabditis elegans*

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Copromotor	Dr. G. Jansen

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voor Deborah

Contents

Scope of the thesis

Sensory cilia

It is essential for the survival of all living organisms that they can sense environmental signals and respond to these cues. Many environmental cues are detected by specialized sensory neurons, which have cilia exposed to the environment (Dutcher 1995; Wheatley et al. 1996). Cilia extend as long appendages from the cell. They consist of a microtubular axonemal core surrounded by a membrane and contain the protein machinery required for the detection of extracellular signals (Rosenbaum and Witman 2002; Scholey 2003). Since proteins cannot be synthesized in the cilia, all components have to be transported into and out of the cilia. This is done by a specialized transport system called intraflagellar transport (IFT) (Rosenbaum and Witman 2002; Scholey 2003; Snell et al. 2004). There are several indications that IFT transports both structural components of the cilia and signaling molecules (May et al. 2005; Ou et al. 2005b; Qin et al. 2005). It is very likely that mechanisms exist that regulate IFT. This would provide structure and plasticity to the cilia and moreover targeting of specific signaling molecules (Pan et al. 2004; Qin et al. 2004; Pan and Snell 2005; Qin et al. 2005). However, not much is known about the regulation of IFT. A summary of our current knowledge of IFT is described in Chapter I.

Cilia are highly conserved subcellular compartments and the structural and functional characteristics of the sensory cilia of the nematode *Caenorhabditis elegans* (Fig. 1A) are very similar to those in mammals (Rosenbaum and Witman 2002; Scholey 2003; Snell et al. 2004).

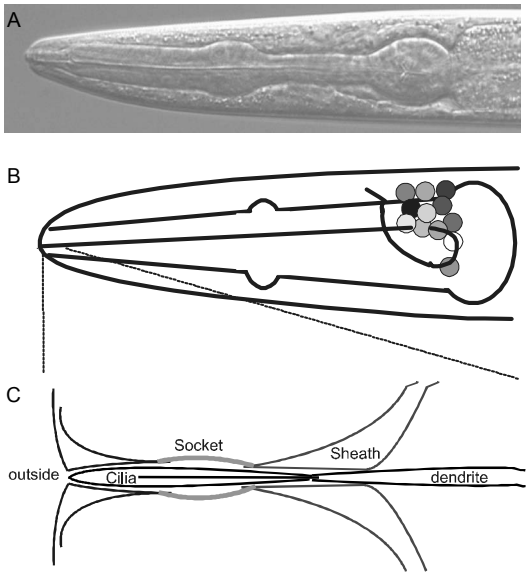

Figure 1. **A.** Head of an adult *C. elegans*. **B.** Graphic representation of the head region of an adult *C. elegans* with the sensory neuron cell bodies and dendrites ending in the cilia. **C.** Magnification of the cilia region, with the dendrite ending in a cilium together with the support cells (sheath and socket cells).

Since in *C. elegans* only the sensory neurons have ciliated endings, mutations that lead to abnormal cilia formation do not influence the viability of the nematode, whereas in mammals they frequently do (Pazour and Rosenbaum 2002; Snell et al. 2004). This makes *C. elegans* an ideal model organism to study cilia development and function. Moreover, molecular genetic analysis of cilia function and IFT are relatively straightforward in *C. elegans* (Scholey 2003).

The nematode *C. elegans* as a model organism for cilia research

C. elegans is a nematode that lives in the soil, where it feeds on bacteria and fungi. In the laboratory it is grown on agar plates with a layer of *Escherichia coli*. *C. elegans* is a small (about 1 mm long as an adult) animal, which is normally grown at 15-25°C. It has a short life cycle depending on the culture temperature. After fertilization embryos will develop in the uterus for approximately 3 hours and for another 18 hours after they have been laid. The embryos will hatch and the young animals will go through four larval stages L1 to L4 before reaching reproductive adulthood. Under harsh conditions like overpopulation or food deprivation, *C. elegans* can adopt an alternative life form, called dauer larvae. A dauer larva can survive severe conditions for about three months. If environmental conditions improve the animal will develop into an L4 larva and eventually into a reproductive adult.

Working with *C. elegans* has several advantages. They are small animals, easy to cultivate and with a short life cycle. Moreover, their relatively small genome has been completely sequenced (The *C. elegans* Sequencing Consortium 1998). In addition, *C. elegans* has transparent soma, which makes examination of gene and protein expression patterns *in vivo* relatively easy (Mello et al. 1991). Furthermore, *C. elegans* are hermaphrodites, which is very convenient in forward genetic screens. Male animals do exist, but they are only present at very low frequencies. Finally, the development and anatomy of *C. elegans* have been very well characterized (Sulston et al. 1983).

An adult hermaphrodite consists of 959 somatic cells of which 302 are neurons (White 1986). 60 of these neurons are sensory neurons with ciliated endings. 11 pairs of ciliated amphid sensory neurons are used for the detection of chemical cues from the environment, 8 of which are directly exposed to the environment (Perkins et al. 1986). Some of the sensory neurons exposed to the environment take up fluorescence dyes (dye filling) (Perkins et al. 1986). This phenomenon has been used to identify mutant animals that cannot take up dyes due to defects in their cilia. Characterization of the genes affected in these mutants have identified many proteins involved in IFT (Perkins et al. 1986; Starich et al. 1995; Haycraft et al. 2001; Qin et al. 2001; Haycraft et al. 2003; Blacque et al. 2005; Murayama et al. 2005; Ou et al. 2005a).

Aim of this study

The aim of the work described in this thesis is to gain more insight into how the transport machinery in the cilia is regulated. In our laboratory we study how sensory signals are processed via heterotrimeric G proteins (discussed in Chapter II). We were intrigued by the finding that a dominant active mutation of the Gα subunit *gpa-3* (*gpa-3QL*) (Zwaal et al. 1997) affects the ability of the sensory neurons of *C. elegans* to take up fluorescent dyes . We surmised that perhaps *gpa-3QL* affects IFT. We have characterised the dye filling defect of *gpa-3QL* animals and uncovered a novel mechanism that regulates the localization of specific signalling molecules in the sensory cilia of *C. elegans* (Chapter IV). To gain more insight into how *gpa-3QL* affects the localization of signalling molecules in the cilia, we performed a forward genetic screen for mutations that suppress the dye filling defect of *gpa-3QL* animals. In addition, we have identified a novel gene *dyf-5*, which when mutated causes a dye filling defect (Chapter V). This gene encodes the homologue of the mammalian MAK kinase (Togawa et al. 2000). Characterisation of the cilia of *dyf-5* animals suggests that this kinase regulates certain aspects of IFT transport, but is not a structural component of the IFT machinery (Chapter V). Furthermore we found that exposure of *C. elegans* larvae to dauer pheromone, a continuously secreted compound that can induce dauer development and that serves as a measure of high population density (Ren et al. 1996), changes the localisation of specific sensory signalling molecules (Chapter IV and VI). We propose that environmental cues, such as the dauer pheromone, can signal via G proteins to regulate IFT and thus regulate the presence or absence of specific signalling molecules. Finally, we selected some candidate genes, based on data from the literature, which could play a role in IFT regulation. Using these approaches, we have found several mutants that suppress the *gpa-3QL* dye filling defect (Chapter VII). We expect that identification of the mutated loci and further characterization of their functions will reveal how GPA-3QL regulates IFT.

Chapter

I

1. Intraflagellar transport

1.1 Transport in cilia and flagella

Cilia and flagella are projected from the surfaces of many eukaryotic cells. Cilia and flagella have a similar internal structure and protein content. Therefore the terms cilia and flagella are used interchangeably. When multiple structures protrude from a cell they are usually referred to as cilia and when they occur singly or in small numbers they are often referred to as flagella. Cilia are highly conserved subcellular compartments and almost all mammalian cells have at least one cilium (Wheatley et al. 1996).

Cilia can have motile or sensory functions. For example, in sperm and unicellular eukaryotes motile cilia propel cells to their destination, while in epithelial cells multiple motile cilia beat synchronously to stir extracellular fluid (Fig. 2A) (Johnson 1995; Porter and Sale 2000; Bray 2001).

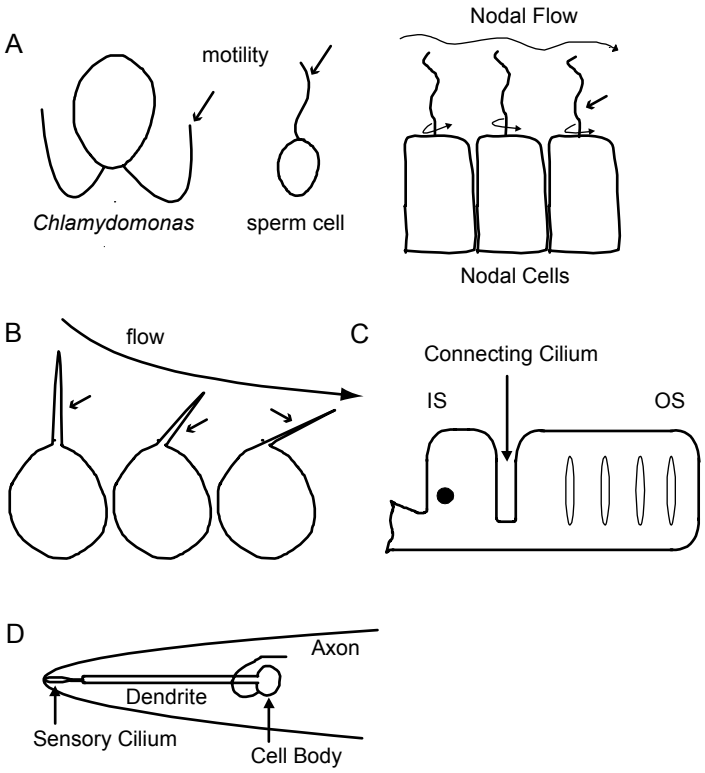

Figure 2. Cilia and flagella are organelles that extend from the cell surface. They are involved in motility and sensory reception. Flagella and cilia are indicated with an arrowhead. Schematic representations of: **A** cilia involved in cell swimming and the nodal cilia that by rotating movement create a gradient of the embryonic fluid across the node, resulting in left-right axis determination.. **B**. Renal epithelial kidney cilia that bend by fluid flow. **C.** Connecting cilium between the inner segment (IS) and the outer segment (OS) of the vertebrate rod. **D.** Head of nematode *C. elegans*, schematically showing one of the chemosensory neuron cell body and dendrite ending in the sensory cilium.

Nodal cilia, which are located on the ventral surface of the node of early mammalian embryos are responsible for the left-right asymmetry (Fig. 2A). In many cases, immotile cilia have a sensory function and provide a subcellular compartment specialized to organize the sensory signaling machinery (Wheatley and Bowser 2000). For example, cilia in the kidney sense the fluid passing through the collecting ducts and tubules (Fig. 2B). In addition, sensory cilia are found in photoreceptors in the vertebrate retina (Fig. 2C) and on the endings of the sensory neurons of *C. elegans* (Fig. 2D) (Perkins et al. 1986; Starich et al. 1995; Marszalek et al. 2000). Various cilia related defects have been described, including respiratory distress, male sterility, polycystic kidney disease, ocular degeneration, Bardet-Beidl syndrome and, due to defects in the nodal cilia, situs inversus or the much more harmful partial reversals, which can lead to internal-organ defects, congenital heart disease and prenatal lethality. (Afzelius 1976; Haycraft et al. 2001; Qin et al. 2001; Pazour and Rosenbaum 2002; Besharse et al. 2003; Afzelius 2004; Avidor-Reiss et al. 2004; Blacque et al. 2004).

All cilia have a similar design, consisting of an array of microtubules surrounded by the cell membrane. Motile cilia contain an array of nine doublet microtubules that surround a central pair of microtubules, also called 9+2 cilia (Fig. 3A). Non-motile, sensory cilia lack the central pair of microtubules (9+0 cilia). All microtubules are arranged with their plus ends pointing towards the flagellar tip (Fig. 3B) and their minus ends towards the cell body.

This 9+2/9+0 arrangement of microtubules is called the axoneme, which is anchored in the cell by the basal body. The end of the basal body and the beginning of the axoneme are called the transition zone (t.z.). During ciliary growth the axoneme is assembled by the addition of new axenomal subunits to its distal tip and subsequently new membrane and flagellar matrix move into the organelle (Rosenbaum and Child 1967; Binder et al. 1975; Witman 1975; Johnson and Rosenbaum 1992; Piperno et al. 1996). Transitional fibers extend from the microtubles of the distal portion of the basal body to the cell membrane. It has been suggested that these fibers form a ‘flagellar pore’, which regulates the flow of flagellar components between the cell body and flagellar compartments (Fig. 3B) (Rosenbaum and Witman 2002).

Since cilia lack the machinery that is necessary for protein synthesis, the site of assembly of the axoneme is distant from the site of synthesis of axonemal proteins in the cell body. This problem was solved by means of intraflagellar transport (IFT), which carries building blocks, proteins and metabolites from the cell soma into the cilia.

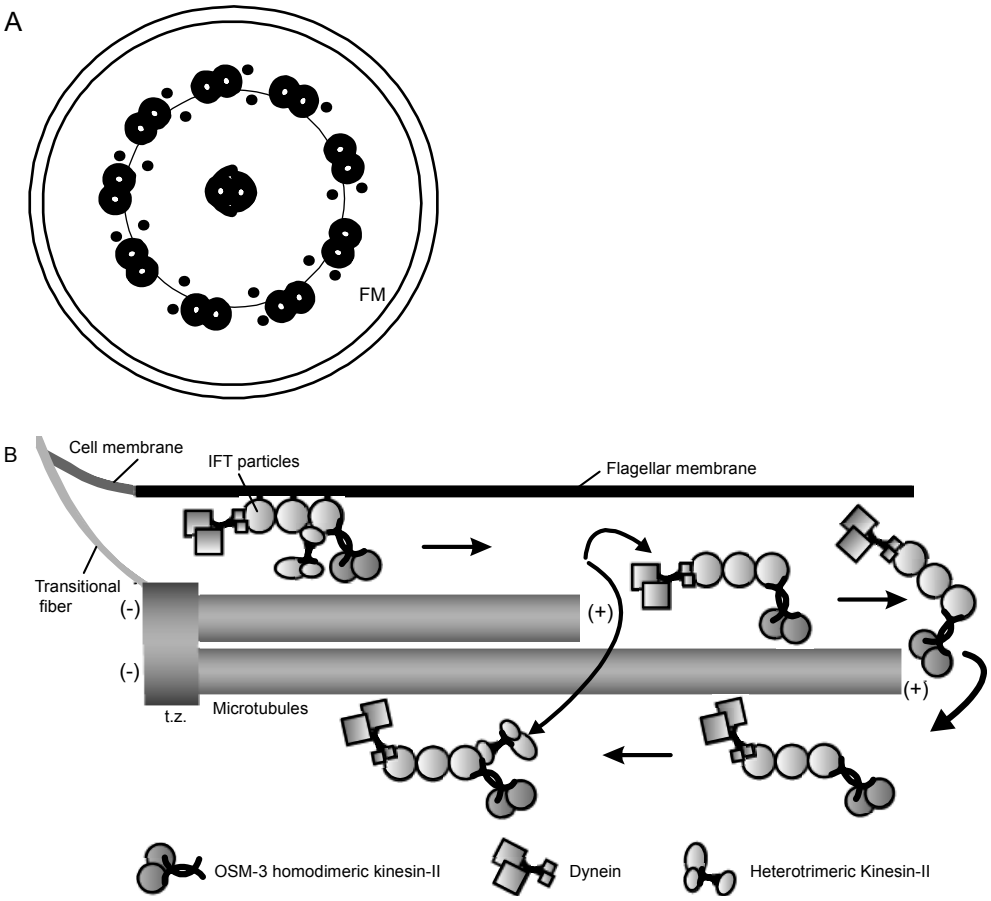

Figure 3. **A** Structure of the axoneme. Motile axonemes usually contain 9 pairs of outer microtubules and 2 pairs of inner microtubules (9+2). Primary nodal and sensory cilia are 9+0 and lack the central pair of microtubules. Representation of a cross-section of a flagellum: CPP: projections from the central pair of microtubules; FM: flagellar membrane. **B** IFT machinery in the cilia of *C. elegans*. The cilia consist of a middle segment with microtubule doublets, and a distal segment consisting of microtubule singlets. The middle segment is anchored in the basal body. The ending of the basal body and the beginning of the middle segment is called the transition zone (t.z.). IFT transports cargo along the middle and distal segment of the ciliary axoneme with the kinesin motors driving anterograde transport and dynein driving retrograde movement.

1.2 Intraflagellar transport

Joel Rosenbaum and collaborators were the first to discover that IFT particles move up and down within the flagella of the biflagellated *Chlamydomonas reinhardtii* (Kozminski et al. 1993). IFT is essential for the assembly and maintenance of cilia (Pazour and Rosenbaum 2002; Scholey 2003). IFT is a rapid process that is responsible for bi-directional transport of cargo proteins packed in

macromolecular complexes (rafts) from the base to the tip of the cilium (anterograde) and back (retrograde). Separate motors exist for anterograde and reterograde IFT movement. Kinesin motors are responsible for anterograde transport and cytoplasmic dynein moves the rafts back in the retrograde direction (Fig. 3B) (Morris and Scholey 1997; Cole et al. 1998; Pazour et al. 1998; Brown et al. 1999; Orozco et al. 1999; Signor et al. 1999b; Wicks et al. 2000). The molecular mechanism of IFT is not well understood, however individual events within this process are becoming clearer. IFT motors, particles and their cargo accumulate at the basal body (Cole et al. 1998; Orozco et al. 1999; Deane et al. 2001), after which the IFT-motor-particle-cargo complexes assemble on the transitional fibers (Deane et al. 2001; Iomini et al. 2001), where they are sorted and translocated to the base of the axoneme.

1.2.1. Anterograde motor

Studies on conditional mutants of the *Chlamydomonas fla10* gene were the first to show that heterotrimeric kinesin-II functions as an anterograde IFT motor (Goodson et al. 1994; Walther et al. 1994; Lawrence et al. 2004). *fla-10* codes for one of the kinesin-II subunits. Later it became clear that all ciliated organisms use at least this kinesin-II motor as an anterograde IFT motor in all cilia and flagella. The kinesin-II holoenzymes contain two distinct motor subunits KIF3A and KIF3B that heterodimerize and an accessory subunit KAP, which is associated with the tail domain of the heterodimer motor complex (Wedaman et al. 1996). KAP contains several armadillo repeats, which are well known for mediating multiple protein-protein interactions (Hatzfeld 1999). Therefore, KAP is expected to be the primary docking site of cargo transported by kinesin-II.

The *C. elegans* sensory cilia use two sequential IFT pathways. The first pathway exists in the middle segment, a 4 µm long domain of the cilia, which extends from the basal body. The second pathway covers the distal segment of cilia, a 2,5 µm long domain that extends from the middle segments to the distal tip (Perkins et al. 1986; Pazour et al. 1998). In the middle segment both heterotrimeric kinesin-II and homodimeric OSM-3 kinesin (KIF17) mediate anterograde transport, whereas the distal segment pathway depends on OSM-3 kinesin alone (Fig. 3B) (Snow et al. 2004). Cargo molecules can either be transported to the middle segment or the middle and distal segment.

1.2.2. Retrograde motor

The retrograde motor for IFT is dynein, which was discovered in sea urchin embryos as a form of cytoplasmic dynein. It is upregulated following de-ciliation, suggesting that cytoplasmic dynein is involved in ciliary assembly (Gibbons et al. 1994). More conclusive results were obtained with the *Chlamydomonas* temperature-sensitive cytoplasmic dynein heavy chain isoform DHC1B (Dhc1)(Pazour et al. 1999). When the temperature-sensitive mutant of *dch1b* was allowed to form flagella at the permissive temperature and was then shifted to the restrictive temperature, the flagella shortened. This indicated that retrograde IFT is

dependent on dynein and is required for the maintenance of flagella. Further evidence for a direct role of cytoplasmic dynein in retrograde IFT was shown by making mutations in genes encoding the IFT-dynein heavy chains, light intermediate chains, or light chain in *Chlamydomonas* or *C. elegans*. These mutations led to the production of short flagella or sensory cilia, which are filled with IFT particle subunits (Fig 4) (Pazour et al. 1998; Pazour et al. 1999; Signor et al. 1999a; Wicks et al. 2000; Perrone et al. 2003; Schafer et al. 2003). These data indicate that following loss of IFT-dynein function, IFT particles are transported only anterogradely and as a result accumulate at the tip of the axoneme (Fig. 4), because the transport to the basal body that normally returns these particles is defective.

1.2.3. IFT particles and ciliary proteins

Currently about 16 IFT proteins have been identified that make up two complexes: IFT complex A (550 kDa), which contains at least 4 polypeptides and complex B (710-760kDa), that is composed of 12 polypeptides (Table 1) (Cole et al. 1998; Piperno et al. 1998). The connection between these complexes is unknown. During purification the two subcomplexes are always co-purified. However, certain experimental data suggest that they are functionally distinct.

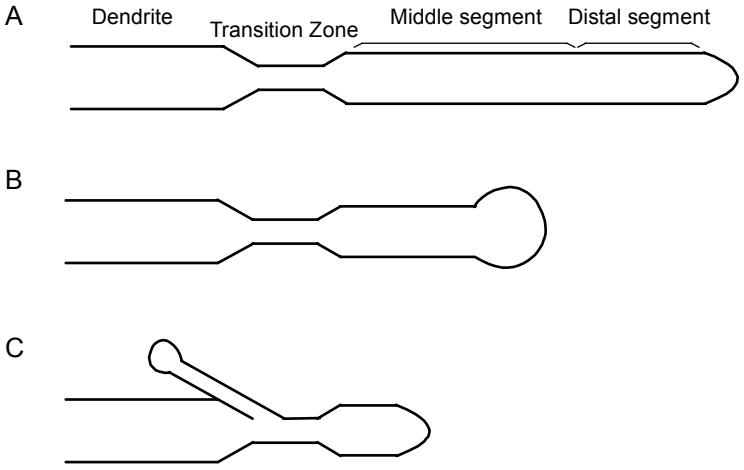

Figure 4. Schematic representation of cilia morphology. **A** Wild type full-length cilia. **B** Complex A IFT subunit mutants have truncated cilia with accumulation of IFT particle subunits at the cilia axoneme end, as a result of the defective retrograde movement. **C** Complex B IFT mutants have severely truncated cilia, caused by defective anterograde movement.

For instance, mutations in IFT-dynein and complex A subunits will lead to less severely truncated cilia and flagella than mutations in kinesin-II and complex B subunits (Fig. 4) (Perkins et al. 1986; Kozminski et al. 1995).

It has been suggested that complex A is specifically engaged in retrograde IFT, while IFT complex B is involved in anterograde transport and flagellar

assembly. The hypothesis is that complex A polypeptides and dynein travel as IFT cargo to the tip where they take over the transport back to the basal body and the kinesin motor becomes cargo (Fig. 3B). This model is supported by the fact that *C. elegans* complex A and B polypeptides have identical motility rates, both in anterograde and reterograde movement (Qin et al. 2001).

Genetic screens in *C. elegans* have been very useful for the identification of molecules involved in IFT. These molecules were identified in genetic screens for animals that can no longer take up fluorescent dyes in the chemosensory neurons exposed to the environment (Dyf, dye filling defective) and screens for animals with various chemosensory defects (Che, chemotaxis defective; Daf, dauer defective; Osm, osmotic avoidance defective) (Perkins et al. 1986; Starich et al. 1995). 25 genes involved in IFT were identified in these mutants: 13 *dyf* genes, 6 *che* genes, 4 *osm* genes, and 2 *daf* genes. These include the OSM-3 kinesin (Perkins et al. 1986; Snow et al. 2004), the dynein heavy chain CHE-3 (Wicks et al. 2000) and several genes that code for IFT particle subunits that have previously been characterized in *Chlamydomonas*, including CHE-2, CHE-11, CHE-13, OSM-1, OSM-5, OSM-6, and DAF-10 (Table. 1) (Fujiwara et al. 1999; Haycraft et al. 2001; Qin et al. 2001; Rosenbaum and Witman 2002; Haycraft et al. 2003; Snell et al. 2004).

The two motor complexes in *C. elegans* travel together along the middle segment with a rate of 0.7 µm/s (Fig. 5). Only the OSM-3 kinesin motor enters the

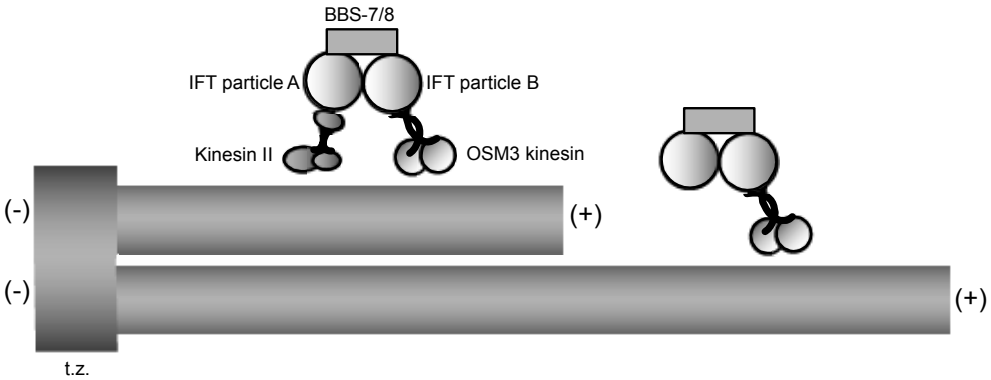

Figure 5. *C. elegans* cilia with a middle segment anchored in the basal body (transition zone, t.z.). The middle segment consists of microtubule doublets, whereas the distal segment are microtubule singlets. The interaction between complexes A and B is stabilized by BBS-7 and BBS-8, which stabilize the heterotrimeric kinesin-II and the homodimeric OSM3 kinesin onto these IFT particles. DYF-1 is required for the activation of OSM-3 kinesin and docking of IFT cargo.

distal segment where it accelerates to a rate of 1.3 µm/s. This suggests that there has to be a control mechanism to coordinate these motors in the middle segment. The first indications for such a mechanism were found in loss-of-function mutants in the *bbs-7* and *bbs-8* genes. BBS-7/8 are associated with the heritable human ciliary disease Bardet-Biedl syndrome, and mutations in these genes in *C. elegans*

Table 1. Protein machinery of IFT in the nematode *C.elegans*					
IFT component	**Subunits**	**Protein**	**Mutant phenotype *C. elegans***	**Mammalian homolog and phenotype**	***Chlamydomonas* homolog**
Anterograde motors					
Heterotrimeric kinesin-II	motor	KRP85	No mutant known	Lack nodal cilia, retinal degeneration, situs inversus and other embryological abnormalities, lethal	FLA10
	motor	KRP95	Wild type	Lack nodal cilia, retinal degeneration, situs inversus and other embryological abnormalities, lethal	FLA10H
	accessory KAP	KLP-11			FLA3
	accessory KAP	KLP-20			
	accessory KAP	KAP-1	Wild type		
Homodimeric kinesin-II		OSM-3	Lack the cilia distal segment	KIF17	
Retrograde motors					
IFT-dynein	heavy chain of cytoplasmic dynein	CHE-3	Short		DHC1b
	dynein light intermediate chain	XBX-1	Short		D2LIC
	family of dynein light chain	XBX-2	No mutant known		
IFT polypeptides					
Complex A		DAF-10	Short		IFT122A
		CHE-11	Short		IFT140
Complex B		OSM-1	Bold		IFT-172
		CHE-2	Bold		
		DYF-3	Bold		
		OSM-5	Bold	Polaris, polycystic kidney disease, lack nodal cilia, retinal degeneration, situs inversus, lethal	IFT-88
		OSM-6	Bold	NGD5	IFT-52
		CHE-13	Bold	Hippi homolog, implicated in neural cell death induced by Huntington's disease	IFT-57
Ciliary components					
		DYF-1	Positive regulator of OSM-3 motility		
		DYF-5	Regulates entry of IFT particles at tz	MAK kinase	
		BBS-8	Lack distal cilia segment	BBS-8 involved in Bardet-Biedl syndrome	
		OSM-12	Lack distal cilia segment	BBS-7 involved in Bardet-Biedl syndrome	
		DYF-13	Lack distal cilia segment		

lead to a partial loss of the ciliary segments (Blacque et al. 2004; Ou et al. 2005a).

It has been shown that BBS proteins serve to stabilize the activities of the IFT particles and motors (Fig. 5). In *bbs-7/8* mutants the two motors kinesin-II and OSM-3 kinesin move separately from each other in the middle segment with a speed of respectively 0.7 µm/s and 1.3 µm/s.

Many IFT genes are regulated by DAF-19, a regulator factor X (RFX) type transcription factor, which regulates the expression of numerous IFT genes in the worm (Swoboda et al. 2000; Efimenko et al. 2005). DAF-19 recognizes a conserved 14bp DNA sequence, which is also present in IFT genes from *Clamydomonas* to human (Avidor-Reiss et al. 2004; Li et al. 2004; Blacque et al. 2005). The regulation in *C. elegans* occurs within the first 150 nucleotides upstream of the translational start (ATG).

Recently, many new ciliary/flagellar proteins were discovered by proteomic and genomic studies. Proteomic analysis of the axoneme of human cilia identified over 200 potentially axonemal proteins (Ostrowski et al. 2002). Some were previously identified, but many are of unknown function. Proteomic analysis of isolated *Chlamydomonas* centrioles also revealed ciliary proteins, together with many new proteins (Keller et al. 2005). Moreover, in genomic approaches many new genes were identified that might encode ciliary proteins (Avidor-Reiss et al. 2004; Li et al. 2004; Blacque et al. 2005). In one report the authors took advantage of the idea that the ancestral eukaryote was ciliated and the organisms that during evolution have lost the cilia/flagella, also lost most of the 400-500 genes that are predicted to be needed for forming and regulating the ciliary apparatus. This has lead to the identification of hundreds of genes involved in cilia structure and functioning (Li et al. 2004). Others used the DAF-19 recognition sequence to identify ciliary genes in *C. elegans* (Blacque et al. 2005; Efimenko et al. 2005).

1.3. IFT cargo

IFT particles are thought to transport sensory ciliary membrane receptors, in addition to structural and force-generating components of the axoneme. Experimental data clearly showed that flagellar precursors are transported to the tip and turnover products to the cell body (Rosenbaum and Witman 2002; Qin et al. 2004). It has been suggested that also signaling molecules are transported by IFT. Currently, this has been proven for three different signaling proteins; i.e. qilin, a polycystic kidney disease (PKD) associated protein (Ou et al. 2005b), OSM-9, a capsaicin receptor homolog involved in sensory signal transduction (Colbert et al. 1997; Tobin et al. 2002; Qin et al. 2005), and Smoothened, a protein involved in hedgehog signaling (May et al. 2005). In addition, an aurora-like protein kinase involved in signaling associated with mating requires kinesin-II for proper localization into *Chlamydomonas* flagella (Pan and Snell 2000; Pan and Snell 2002; Pan and Snell 2003). Furthermore, signaling factors involved in photoreception are transported to the rod outer segments in the vertebrate retina by IFT (Marszalek et al. 2000; Pazour and Rosenbaum 2002).

1.3.1 Targeting signals required for localization to the cilia

The cilium contains specific plasma membrane proteins. It is therefore believed that a mechanism must exist for sorting and targeting these proteins to the ciliary membrane. Several signals required for targeting of proteins to the cilia have been described. It has been shown that the flagellar membrane specific glucose transporter in *Leishmania* requires an amino-terminal leader sequence of 30 amino acids to reach its flagellar site. Another transporter that is normally not located to the cilia was fused to the amino-terminal portion of the glucose transporter. This fusion protein was found to be also localized to the flagellar membrane (Snapp and Landfear 1997; Snapp and Landfear 1999). Similarly, a specific consensus sequence for myristoylated and palmitoylated, of the EF-HAND calcium-binding protein was required for the localization to the flagellar membrane in *Trypanosoma* (Godsel and Engman 1999). Also G proteins have been shown to have a consensus sequence for palmitoylation and myristoylation in the N-terminal part, which are likely to be required for targeting of the G proteins to the cilia. Thus, several different signals exist to target proteins to the cilia.

1.4. Regulation of IFT and signaling

When *Chlamydomonas* cells are deflagellated, they immediately begin to regenerate their missing flagella (Rosenbaum et al. 1969), a process which takes about 90 minutes. In order to generate full-length flagella, cells must turn on the transcription of flagellar genes and synthesize new flagellar proteins (Lefebvre et al. 1978; Lefebvre et al. 1980). It is unknown how cells sense that their flagella are missing and have to upregulate flagellar gene transcription. There are many indications that cells regulate the lengths of their flagella (Berman et al. 2003), and that they also possess mechanisms for the regulated elimination of their flagella. For example, when cells in liquid culture are exposed to a pH of 4.5, they actively and rapidly excise their flagella at the distal ends of the basal bodies (Lewin et al. 1982). During the process of flagellar shortening, the axoneme is disassembled and the entry rate of IFT particles into flagella is increased (Pan and Snell 2005). Moreover, these particles lack cargo. Thus, a cell that is triggered to shorten its flagellum not only activates the disassembly of the axoneme, but also stimulates entry into the flagellum of IFT particles that have empty cargo binding sites, which are free to bring back the disassembled components to the basal body.

It has been shown that CrEB1, a member of the microtubule plus end tracking EB1 protein family, is present at the tips of full length and growing cilia. It also associates with the proximal ends of the basal bodies (Pedersen et al. 2003). It has been suggested that CrEB1 could affect flagellar assembly and turnover rates by promoting the dynamic instability of microtubules at the flagellar tip.

The key effector in flagellar disassembly in *Chlamydomonas* is probably the aurora protein kinase CALK (Pan et al. 2004). Members of the aurora family of protein kinases play crucial roles in regulating several cellular processes dependent on microtubule-containing structures (Adams et al. 2001; Nigg 2001;

Dutertre et al. 2002). Using RNA interference methods it was shown that CALK is required for experimentally induced flagellar excision and for the regulated disassembly of flagella, which occurs upon exposure to altered ionic conditions or low pH. Phosphorylation of CALK was triggered within seconds after flagellar excision and phosphorylation also occurred upon regulated disassembly of flagella. It has been suggested that CALK is a cytoplasmic effector of the disassembly of flagella in a checkpoint system that monitors and responds on environmental condition.

Chapter

II

2. Heterotrimeric G proteins

2.1. G protein signaling

Organisms receive many different signals from their environment. Integration and processing of these signals is essential for a proper response of the organism. The first intracellular step in this chain of signal integration events is mediated by a variety of proteins, including kinases, phosphatases and nucleotide binding proteins. Many of these proteins alternate between an on and an off state to regulate the duration and intensity of the signal. Guanine nucleotide binding proteins or G proteins are among the most ubiquitous of these cellular switches. Two types of G proteins exists, the heterotrimeric G proteins and the small G proteins. They alternate between a GDP-bound off state and a GTP-bound on state. Many eukaryotic organisms employ heterotrimeric G proteins for signal processing and homeostasis. They are involved in a wide range of biological processes, including the regulation of mitotic spindle movements and the transduction of a wide array of extracellular signals received from G Protein Coupled Receptors (GPCRs) (Du and Macara 2004; Hampoelz and Knoblich 2004; Hess et al. 2004; Siderovski and Willard 2005).

Heterotrimeric G proteins consist of an α, β and γ subunit, and are often coupled to GPCRs. Upon binding of a ligand to a GPCR, the receptor acts as a guanine nucleotide exchange factor. The Gα subunit exchanges GDP for GTP, thereby dissociating from the Gβγ subunit; the Gβγ does not dissociate (Fig. 6A). The Gα and βγ subunits can activate a variety of targets, such as adenylyl cyclases, guanylyl cyclase, calcium ion channels and many more. The Gα subunit slowly hydrolyses GTP to GDP, which returns the Gα subunit to its inactive state (Ford et al. 1998; Wall et al. 1998). All effector interactions are terminated by the re-association of Gβγ to the Gα GDP bound state. Based on this cycle, the duration of heterotrimeric G protein signalling is thought to be controlled by the life time of the GTP-bound state of the Gα subunit (Fig. 6A).

There are 16 Gα genes located in the human genome which code for 23 known Gα proteins (McCudden et al. 2005). These proteins can be divided into four major classes based on sequence similarity: the Gαs class, containing Gαs and Gαolf, the Gαi or Gαo class, containing Gαi1, Gαi2, Gαi3, Gαo, Gαt-rod, Gαt-cone, Gαgust and Gαz, the Gαq class, containing Gαq, Gα11, Gα14 and Gα16 and the Gα12 class containing Gα12 and Gα13 (Simon et al. 1991). All four classes of Gα subunits have well-established cellular targets. Gαs was shown to be a stimulator of adenylyl cyclases (Ross and Gilman 1977). In contrast, Gαi inhibits adenylyl cyclases and thus opposes the action of Gαs (Smith and Limbird 1982;

Hildebrandt and Birnbaumer 1983; Hildebrandt et al. 1983; Hsia et al. 1984). The

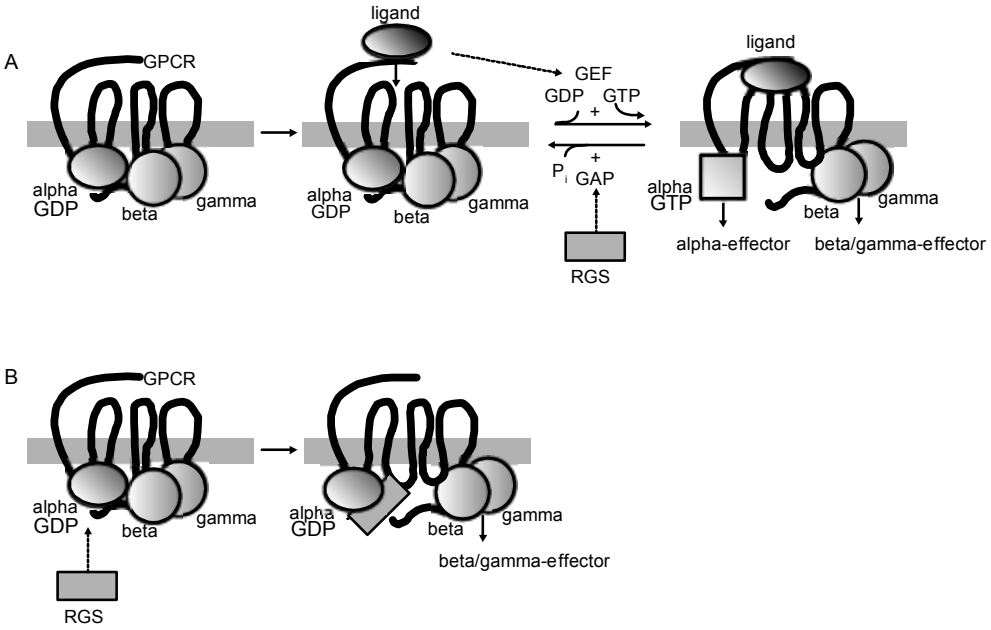

Figure 6 **A**. Model of receptor-dependent activation of G proteins. After binding of a ligand GPCRs act as guanine nucleotide exchange factor by inducing a conformational change in the Gα subunit, allowing it to exchange GTP for GDP. Gβγ dissociates from Gα GTP, and both Gα·GTP subunit and Gβγ subunit can activate various downstream targets. **B**. RGS protein which bind and stabilizes GαGDP and thereby promotes the release of Gβγ.

membrane-bound adenylyl cyclases exhibit a miscellaneous expression pattern and can respond either positively or negatively depending on various regulatory inputs including Gβγ and divalent cations (Hanoune and Defer 2001; Sunahara and Taussig 2002).

Gα protein signaling is also essential in sensory transduction. GPCRs can function as tastant or odorant receptors and couple to Gα subunits such as Gαgust and Gαolf, respectively (Buck 2000; Zhang et al. 2003). Vision also depends on GPCR-mediated phototransduction, which consist of a signaling cascade that regulates a cyclic GMP-gated Na^{+}/Ca^{2+} channel via Gαt and its effector cGMP phosphodiesterase (Arshavsky et al. 2002). G protein subunits of the Gαq class (Gαq, Gα11, Gα14 and Gα16) activate phosphoinositide-specific phospholipase C isozymes (Rhee 2001). Activated Gα12/13 proteins can stimulate the small G protein RhoA via effectors that possess Dbl-homology and pleckstrin-homology domains characteristic of Rho-family guanine nucleotide exchange factors (Worthylake et al. 2000). However, the exact mechanism how Gα12/13 accomplish this is unknown.

2.2. Gα subunit structure

The Gα subunit is composed of two domains: a nucleotide binding domain with high structural homology to Ras-superfamily GTPases, and an all-alpha-helical domain that, in combination with the Ras-like domain, helps to form a deep pocket for binding a guanine nucleotide (Sprang 1997). Gα subunits contain three flexible regions designated switch-I, -II and -III that change conformation in response to GTP binding and hydrolysis (Coleman et al. 1994; Lambright et al. 1994; Sondek et al. 1994; Lambright et al. 1996; Tesmer et al. 1997). The GTP-bound conformation of Gα shows decreased affinity for the Gβγ subunit resulting in dissociation and increased affinity for Gα effectors. Mutations of either a critical arginine (e.g. R178 in Gαi) or glutamine (e.g. Q204 in Gαi), each involved in stabilizing the γ-phosphate leaving group during hydrolysis, are commonly used to make Gα subunits GTPase-deficient and thus constitutively active. This is also the case for the dominant active Gα subunit (GPA-3) used in this thesis, where the glutamine was changed to a leucine (GPA-3QL).

The GDP bound Gα subunit binds tightly to the Gβγ heterodimer, which serves to couple this Gα subunit to the receptor and also to inhibit the spontaneous release of GDP, thus acting as a guanine nucleotide dissociation inhibitor (Brandt and Ross 1985; Higashijima et al. 1987; Robillard et al. 2000; Evanko et al. 2001).

2.3. Gβγ heterodimers

There are 5 human Gβ and 12 Gγ subunit genes (McCudden et al. 2005), resulting in a large number of potential combinations of Gβγ dimers. The Gβγ subunit is a functional heterodimer that forms a stable structural unit. All Gβ subunits contain seven WD-40 repeats, which fold into a seven-bladed β-propeller structure, while the N-terminus forms an α-helix (Wall et al. 1995; Lambright et al. 1996; Sondek et al. 1996). Gγ folds into two α-helices; the N-terminal helix forms a coiled-coil with the α-helix of Gβ, while the C-terminal helix makes extensive contacts with the base of the Gβ propeller. Unlike the conformationally flexible Gα subunit, the Gβγ dimer does not change conformation when it dissociates from the Gα (Sondek et al. 1996). All Gγ subunits are C-terminally prenylated. This lipid modification of the Gγ polypeptide is important for the membrane localization of the βγ dimer.

The Gβγ dimer is not only required to facilitate coupling of Gαβγ heterotrimers to GPCRs and to act as a Gα inhibitor, but the free Gβγ can also activate a large number of effectors (Clapham and Neer 1997; Jones et al. 2004), such as the G protein-regulated inward-rectifier K^+ channels (GIRK or Kir3 channels) (Logothetis et al. 1987). Gβγ subunits can also regulate kinases and small G proteins, such as ERK1/2, JNK and p38 MAPKs (Faure et al. 1994; Yamauchi et al. 1997). In addition, neuronal N- and P/Q-type Ca^{2+} channels are regulated by both Gα and Gβγ subunits (Delmas et al. 2000; Lu et al. 2001). It is becoming clear that Gβγ heterodimers are required for many cellular processes.

2.4 Regulators of G proteins

Signalling mediated by heterotrimeric G proteins in *vivo* is usually extremely rapid and transient, which cannot be explained by the slow intrinsic GTPase activity of the Gα subunit. In 1996 the answer for this enigma came with the discovery of a class of proteins called the regulator of G protein signalling (RGS proteins), which contain a 120 amino acid RGS domain responsible for GTPase activation. The slow intrinsic rate of GTP hydrolysis by Gα subunits can be dramatically accelerated by RGS proteins (Fig. 6A) (De Vries et al. 1995; Dohlman et al. 1996; Druey et al. 1996; Koelle and Horvitz 1996; Siderovski et al. 1996; Yu et al. 1996). The finding of the RGS proteins and their GTPase-accelerating activity on Gα subunits not only resolved apparent timing paradoxes between known GPCR mediated physiological responses, but in addition led to the identification of novel, GPCR independent functions of Gα subunits in cellular processes like mitotic spindle movements.

Many RGS proteins have a Goloco domain that stabilizes GαGDP and thereby promotes the release of Gβγ (Fig. 6B) (Colombo et al. 2003; Srinivasan et al. 2003). Other proteins that interact with and stabilize GαGDP are Partner of Inscuteable (Pins) and locomotion defective (Loco). This type of GαGDP regulation happens during mitotic spindle orientation in various organisms (Schaefer et al. 2000; Du et al. 2002; Gotta et al. 2003; Yu et al. 2005). The RGS, Pins and Loco proteins are asymmetrically localized during cell division, which will likely result in asymmetric activation of the heterotrimeric G proteins and as a consequence asymmetric division of the cell. One additional factor involved in the regulation of the movement of the mitotic spindle is RIC-8. This protein has been shown to behave as a guanine exchange factor for Gαi in mammalian cells and GOA-1 in *C. elegans.* Thus, by stimulating the exchange between Gα bound GDP to GTP, G proteins can be activated independently of GPCRs (Couwenbergs et al. 2004; Hess et al. 2004; Afshar et al. 2005). Moreover, it was recently shown that Ric-8 is also required for cortical localization of the Gα subunit GPA-16 in *C. elegans*, which regulates the spindle orientations during early cell divisions (Tall et al. 2003; Afshar et al. 2005; Wang et al. 2005).

2.5 G proteins and *C. elegans*

The general properties of heterotrimeric G proteins and their signalling pathways are well conserved throughout evolution. Homologs of all four mammalian G protein classes have been identified in the nematode *C. elegans.* This animal is often used as a model organism for understanding G-protein signal transduction.

C. elegans has two Gβ subunits, GPB-1 and GPB-2, two Gγ subunits, GPC-1 and GPC-2 and 21 Gα proteins (Mendel et al. 1995; Segalat et al. 1995; Brundage et al. 1996; Zwaal et al. 1996; Park et al. 1997; Zwaal et al. 1997; Roayaie et al. 1998; Jansen et al. 1999). *C. elegans* has one clear homolog for

each of the four vertebrate classes. Gαs, Gαi/o, Gαq and Gα12 are represented by GSA-1, GOA-1, EGL-30 and GPA-12 (Lochrie et al. 1991; Brundage et al. 1996; Park et al. 1997; Jansen et al. 1999; Cuppen 2003). GSA-1 is broadly expressed and functions in locomotion and egg laying. Gain-of-function of *gsa-1* leads to necrotic cell death (Berger et al. 1998). Moreover, *gsa-1* is essential during development, since loss-of-function mutations in this gene lead to developmental arrest in L1 larvae (Korswagen et al. 1997; Berger et al. 1998; Simmer et al. 2003). GOA-1 and EGL-30 are also broadly expressed. These proteins have opposite regulatory functions in locomotion and egg laying (Mendel et al. 1995; Segalat et al. 1995; Brundage et al. 1996). Furthermore, GOA-1 regulates spindle orientation in early cell divisions together with GPA-16 (Gotta et al. 2003). GPA-12 acts predominantly in feeding behavior and may have minor roles in locomotion and embryogenesis (van der Linden et al. 2003; Yau et al. 2003).

As the other 17 Gα proteins cannot clearly be grouped in any of these classes, they are considered as an outgroup with new Gα subunits. Of these, GPA-16 functions in regulating mitotic spindle movements, as mentioned before (Gotta et al. 2003). GPA-7 is broadly expressed and mutations in this gene have small effects on locomotion. Not much is known about the function of GPA-17. This Gα protein is expressed in the gut, but neither loss nor gain-of-function mutants exist (unpublished results J. Burghoorn and G. Jansen). The other 14 Gα subunits are specifically expressed in the sensory neurons (Fig. 7). Gain and loss-of-function mutants of these genes showed that several are involved in sensory perception, probably both in the detection of various compounds and in more complex processes that modulate the behavioural response of the animals after perception. At least three Gα subunits are involved in dauer formation, GPA-2, GPA-3 and GPA-11 (Zwaal et al. 1997; Ailion and Thomas 2003). GPA-2, 3, 5, 13 and ODR-3 play a role in olfaction (Roayaie et al. 1998; Jansen et al. 1999; Lans et al. 2004). GPA-2, 3, 5, 6, 13 and ODR-3 are involved in the regulation of gene expression in the olfactory neurons. ODR-3 and GPA-3 are required for the detection of aversive compounds (Roayaie et al. 1998; Hilliard et al. 2004). GPA-1 and ODR-3 are involved in gustatory plasticity (Hukema et al. 2006) and GPA-1, 11 and ODR-3 in the regulation of longevity (Lans, *et al,* submitted).

Part of the studies described in this thesis focus on the function of the Gα subunit GPA-3. GPA-3 is expressed in 10 of the 12 pairs of amphid sensory neurons, and functions in avoidance and the dauer response (Hilliard et al. 2004; Lans et al. 2004). Furthermore, GPA-3 acts as a stimulator in the olfactory neurons AWA and AWC, redundantly to ODR-3 (Lans et al. 2004). Expression of the dominant active form of *gpa-3* (*gpa-3QL*) leads to a dye filling defect (Zwaal et al. 1997). We characterized the Dyf phenotype of *gpa-3QL* animals and found a novel mechanism that regulates transport of sensory signaling molecules in the sensory cilia of *C. elegans* (Chapter IV).

Chapter

III

3. Dauer development

3.1 Dauer pathway

Numerous organisms respond by entering a dormant state when they encounter surroundings that are inappropriate for their survival and growth. Well known examples are hibernation of mammals, reptiles and amphibians, diapause of insects, dormant buds of plants and sporulation of fungi and bacteria (Denlinger 2002; Carey et al. 2003).

C. elegans also has a specialized developmental stage for survival under harsh environmental conditions such as starvation, high population density, or high temperatures: the dauer stage (Cassada and Russell 1975). *C. elegans* can sense the population density by detecting a pheromone that is constantly secreted by the nematode, i.e. dauer pheromone. High concentrations of dauer pheromone induce the entry into the dauer larval stage (Albert et al. 1981; Perkins et al. 1986; Vowels and Thomas 1994). This pheromone, a fatty acid derivative, is the most potent activator of the dauer formation pathway (Golden and Riddle 1982; Jeong et al. 2005) and can overcome low temperature and high food concentration signals, which normally would inhibit dauer formation.

After switching to the dauer developmental pathway the pre-dauer larvae (L2d) accumulate fat in preparation for a prolonged period of non-feeding. Dauer larvae have a constricted pharynx, shrunken intestinal lumen, and a specialized cuticle. After improvement of the environmental conditions the larvae resume development to reproductive animals (Riddle 1997). *C. elegans* has 12 pair of ciliated amphid sensory neurons for the detection of the environment (Fig. 7) (Perkins et al. 1986).

Laser ablation of 3 of these sensory neurons, ADF, ASG, and ASI, causes animals to activate their dauer formation pathway (dauer arrest). This indicates that these neurons mediate signals that stimulate growth to normal reproductive adulthood and that lack of these signals causes dauer arrest (Bargmann and Horvitz 1991; Schackwitz et al. 1996). Ablation of the sensory neuron ASJ results in animals that do not form dauers, indicating that ASJ is the dauer promoting sensory neuron.

Genes that regulate dauer larvae formation have been identified and characterized, and can be categorized into two groups: dauer-constitutive (Daf-c) mutants, which form dauers even under non-inducing conditions, and dauer-defective (Daf-d) mutants, which do not form dauer larvae, even under unfavorable environmental conditions.

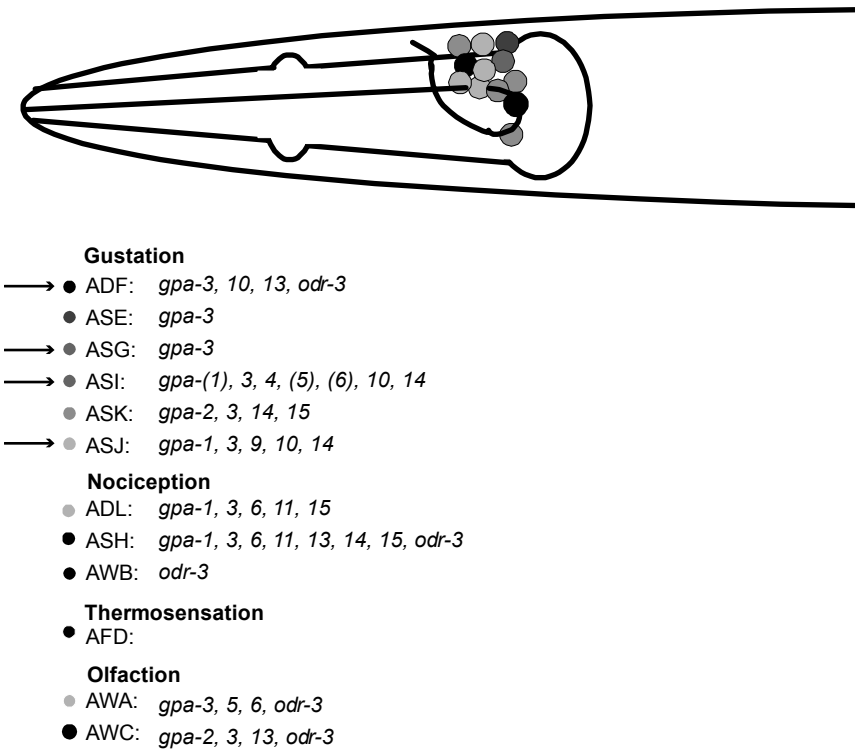

Figure 7. Schematic representation of the *C. elegans* head with the sensory neurons. Below the expression of the different Gα subunits in the various sensory neurons are indicated. The sensory neuron indicated by an arrow are involved in the integration of dauer signals and recovery of the dauer state.

DAF-11, a guanylyl cyclase (Birnby et al. 2000), is one of the first factors of a complex network that regulates the decision between dauer formation versus reproductive growth. DAF-11 positively stimulates two endocrine pathways; the DAF-2 (insulin/IGF I receptor) and the DAF-7 (TGFβ) pathways (Fig. 8) (Riddle et al. 1981; Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Gerisch et al. 2001; Li et al. 2003). It has been suggested that that these two funnel into a third endocrine pathway, which includes DAF-12 (Fig. 8). This nuclear hormone receptor is the key regulator protein in the decision between the normal developmental and the diapause program (Fig. 8) (Antebi et al. 2000).

3.2 DAF-11

daf-11 is expressed in the ciliated sensory neurons and possibly transduces dauer pheromone signals by the production of the second messenger, cyclic GMP, thereby controlling dauer arrest. Loss-of-function mutations in *daf-11* cause a

strong dauer constitutive phenotype in *C. elegans*. It has been shown that DAF-11 regulates the two endocrine pathways that regulate dauer formation. Inhibition of *daf-11* results in a lower expression of *daf-28*, an insulin-like ligand that activates the DAF-2 insulin-like growth receptor pathway, and it results in strongly reduced expression of *daf-7*, a TGF-β growth factor (Murakami et al. 2001; Li et al. 2003). DAF-11 is most likely activated by G proteins, which transduce the dauer pheromone signal (Fig. 8).

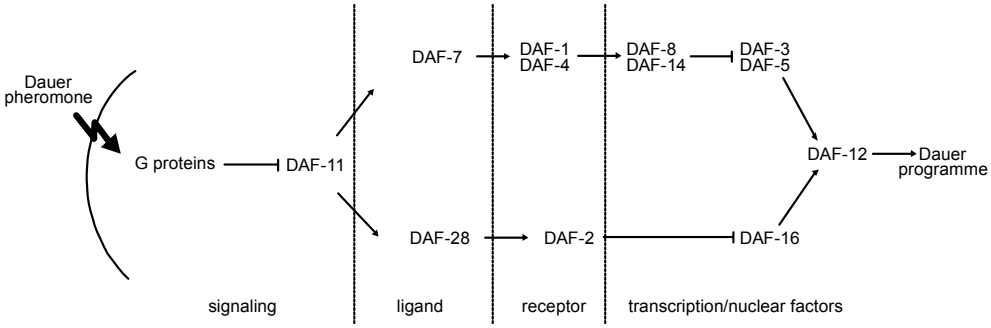

Figure 8. Schematic diagram of the TGF-β and Insulin pathways, which mediate dauer formation together with their upstream factors. G.C: guanylyl cyclase, T.R.: TGF- β receptor type I and II, I.R.: insulin receptor, T.F.: transcription factor, N.H.R.: nuclear hormone receptor.

Previous studies have identified three Gα subunits that are involved in dauer formation, *gpa-2*, *gpa-3* and *gpa-11*. *gpa-2* loss-of-function animals have a Daf-d phenotype, whereas *gpa-2* gain-of-function animals have a Daf-c phenotype (Zwaal et al. 1997). Loss-of-function of *gpa-11* does not influence dauer formation, but a gain of *daf-11* function leads to a Daf-c phenotype when the animals are cultured at 27^{o}C (Ailion and Thomas 2003). Both G proteins are expressed in a subset of sensory neurons that have thus far not been implicated in dauer formation. *gpa-3* is expressed in almost all amphid neurons, including those that regulate dauer formation. Loss-of-function of *gpa-3* reduces the sensitivity to dauer pheromone, and animals that express a dominant active form of GPA-3 (*gpa-3QL*) have a Daf-c phenotype (Zwaal et al. 1997). It is therefore likely that *gpa-3* acts upstream of *daf-11*. Our experiments suggest that *gpa-3* indeed acts upstream of *daf-11* and inhibits DAF-11 activity. However, we propose that GPA-3 does not directly regulate DAF-11 activity, but acts by regulating the localization of other Gα subunits in the sensory cilia, which in turn regulate DAF-11 activity (Chapter VI).

3.3 Insulin pathway

Insulin superfamily genes are present in vertebrates and invertebrates, including insects, mollusks and *C. elegans*. (Kimura et al. 1997; Duret et al. 1998; Gregoire et al. 1998; Smit et al. 1998). The *C. elegans* insulin pathway regulates various processes such as metabolism, growth and longevity. The activity of the sole *C. elegans* insulin receptor DAF-2, which is broadly expressed, promotes growth, whereas lack of activity causes animals to arrest at the dauer stage (Kimura et al. 1997). Down-regulation of DAF-2 signaling during the adult stages causes accumulation of lipids, and greatly extends life span (Kenyon et al. 1993). A decreased signal of the DAF-2 pathway results in nuclear localization and activation of the DAF-16 forkhead transcription factor, which in turn activates DAF-12 and as a consequence the dauer developmental pathway (Henderson and Johnson 2001; Lee et al. 2001; Lin et al. 2001). Because the longevity, dauer arrest and metabolic-shift phenotypes caused by the *daf-2* mutant are fully suppressed in a *daf-16* mutant, DAF-16 is a major output of the DAF-2 signaling pathway (Gottlieb and Ruvkun 1994).

Of the 38 *C. elegans* insulin ligand genes consisting of *ins-1* till *37* and *daf-28*, many are expressed in the sensory neurons (Pierce et al. 2001). Among the *ins* genes, *ins-1* and *ins-18* regulate dauer formation and life span, respectively. Up-regulation of the *ins-1* gene induces dauer arrest, most likely by antagonizing the DAF-2 activity. INS-18 has been implied to be involved in the regulation of life span by activating DAF-2 (Kawano et al. 2000). INS-4, INS-6, INS-7 and DAF-28 are agonists of the DAF-2 pathway, whereas for the other 32 INS proteins no function has been found thus far.

It was suggested that sensory input regulates the *daf-2* pathway by regulating the production and/or secretion of insulin ligands, which in turn are engaged in the *daf-2* pathway. For one insulin-ligand, DAF-28, it was shown that the expression level depends on food signal (Li et al. 2003). DAF-*28* positively activates DAF-2 (Kimura et al. 1997; Li et al. 2003) and its expression is down-regulated during starvation and completely shut-down in dauer animals. The major signal that shuts down *daf-28* expression is probably starvation, since animals that cannot produce dauer pheromone show similar down regulation of *daf-28* expression and dauer formation upon starvation, indicating that the dauer pheromone is not required for down-regulating *daf-28* expression.

3.4 TGF-beta pathway

The TGF-β factor superfamily is essential for multiple developmental decisions in various organisms from *C. elegans* to man (Suga et al. 1999; Massague et al. 2000; Patterson and Padgett 2000; Finnerty et al. 2004). The core of the TGF-β

signaling pathway has been elucidated and reveals a rather straightforward signaling cascade. The ligands transmit the TGF-β signal by binding to the TGF-β transmembrane receptor type II. Once the ligand is bound, the type II receptor activates the type I TGF-β receptor by phosphorylation. The activated Type I receptor phosphorylates downstream mediators, which are referred to as the Smads. The receptor regulated Smads (R-Smads) are now able to interact with another subset of Smads called the common Smads (Co Smads). These are subsequently translocated to the nucleus where they affect target gene transcription (Heldin et al. 1997; Massague 1998; Whitman 1998).

Of the five TGF-β like ligands in *C. elegans*, *dbl-1* and *daf-7* are the best characterized. *dbl-1* mutants have a small body size with an abnormal tail (Morita et al. 1999; Suzuki et al. 1999), while *daf-7* animals have a Daf-c phenotype. DAF-7 ligand regulates development and inhibits dauer formation. It is thought to be secreted by the sensory neuron ASI, and to endorse reproductive growth by binding the widely expressed DAF-4/TGF-β type II receptor. The DAF-4 receptor recruits DAF-1, the TGF-β type I receptor and the downstream targets DAF-8 and DAF-12/Smads are activated, which repress the DAF-3/Smads and the Ski oncoprotein homolog DAF-5 (Fig. 8) (Georgi et al. 1990; Estevez et al. 1993; Patterson et al. 1997; Inoue and Thomas 2000; da Graca et al. 2004).

Dauer pheromone negatively regulates *daf-7* expression. At high concentrations of dauer pheromone *daf-7* expression is completely shut-down (Ren et al. 1996; Schackwitz et al. 1996). As a result DAF-3 and DAF-5 are no longer inhibited and DAF-12 and subsequently the dauer formation pathway will be activated.

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Chapter

IV

G proteins and environmental cues regulate the localization of ciliary signalling molecules

Jan Burghoorn, Suzanne Rademakers, Evgeni Efimenko, Peter Swoboda and Gert Jansen

Summary

Sensory perception requires the proper localization of signalling molecules in sensory cilia. Intraflagellar transport (Rosenbaum and Witman 2002; Scholey 2003), a system essential for the assembly and maintenance of cilia, plays a critical role in trafficking cargo (May et al. 2005; Ou et al. 2005b; Qin et al. 2005). Here we identify a novel mechanism that regulates the localization of signalling molecules in the sensory cilia of the nematode *C. elegans*. We show that a gain-of-function mutation in the Gα protein GPA-3 (*gpa-3QL*)(Zwaal et al. 1997) interferes with entry into the distal segments of the cilia of three sensory Gα proteins, GPA-4, GPA-9 and GPA-15, but does not affect other Gα proteins. In wild type animals GPA-4 and GPA-15 can be transported in the cilia middle segments by both kinesin II and OSM-3 kinesin motor complexes, and in the distal segments by OSM-3. In *gpa-3QL* animals the two Gα proteins are predominantly transported by kinesin II; OSM-3 mediated transport can only be seen in the absence of kinesin II. However, we found no evidence that *gpa-3QL* affects the two kinesin complexes, the retrograde motor complex or several other IFT proteins. In contrast, *gpa-3QL* strongly affects the localization of OSM-5, the *C. elegans* homologue of the mouse Polaris/Tg737 IFT protein (Haycraft et al. 2001; Qin et al. 2001). We propose a model in which G protein mediated signals regulate the localization of specific signalling molecules in the cilia by modulating, via OSM-5/Polaris, the assembly of motor protein - cargo complexes. Finally, we show that a similar effect on the localization of GPA-4 and GPA-15 in the cilia can be accomplished by exposing *C. elegans* larvae to dauer pheromone. We hypothesize that our findings reveal a novel mechanism that allows structural plasticity in response to environmental cues.

Results and discussion

Caenorhabditis elegans has 60 ciliated cells (White 1986), including 12 pairs of sensory neurons, the amphid neurons, which it uses to detect environmental cues (Bargmann 1997). A subset of amphid neurons exposed to the environment take up fluorescent dyes (dye filling) (Perkins et al. 1986). The characterization of dye filling defective (Dyf) mutants has identified many proteins that play important roles

in the IFT machinery (Scholey 2003). We were intrigued by the finding that animals that carry a dominant active sensory Gα protein *gpa-3* (*gpa-3QL*), expressed in almost all amphid neurons, show a Dyf phenotype (Zwaal et al. 1997) (Fig. 1A and B). We surmised that perhaps *gpa-3QL* affects IFT.

Mutations in many genes that encode components of the IFT machinery have severe effects on the microtubular axoneme (Scholey 2003). However, anti-tubulin staining of *gpa-3QL* animals revealed no gross abnormalities of the cilia (Fig. 1G and H). Alternatively, dye filling defective (Dyf) phenotypes can be caused by defects in the sheath cells (Perkins et al. 1986; Perens and Shaham 2005), glia like cells that support the cilia of the sensory neurons (White 1986). However, expression of fluorescent proteins in the amphid sheath cells revealed no obvious morphological defects (data not shown). In addition, *gpa-3* expression has not been observed in the sheath cells (Zwaal et al. 1997; Lans et al. 2004).

After ruling out structural cilia and sheath cell defects, we surmised that perhaps *gpa-3QL* causes a regulatory defect. Therefore, we generated animals that carry a heat shock inducible *gpa-3QL* construct. Induction of *gpa-3QL* expression resulted in a strong dye filling defect, both in larvae and in adult animals (Fig. 1D and E). This induced Dyf phenotype was reversible since dye filling was restored 24 hours after heat shock treatment (Fig. 1F). These results suggest that the effect of *gpa-3QL* is not restricted to a specific developmental time-window, but affects a mechanism important for cilia function throughout *C. elegans* life.

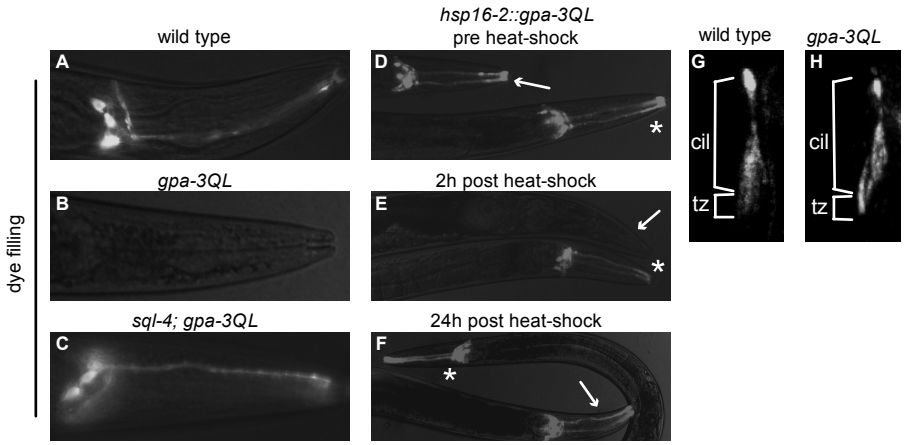

Figure 1**.** *gpa-3QL* interferes with dye filling, but does not affect the cilia microtubular axoneme. a-f, DiO dye filling of wild type animals (**A**), no dye filling of *gpa-3QL* animals (**B**), and restored dye filling of *sql-4; gpa-3QL* animals (**C**). Dye filling of transgenic animals expressing *gpa-3QL* under control of the *hsp16-2* heat-shock promoter (marked with an arrow) before treatment; also a non-transgenic animal is shown (marked with *) (**D**). Two hours after heat-shock treatment *hsp16-2::gpa-3QL* animals showed no dye filling (**E**). 24 hours after heat-shock treatment dye filling was fully restored (**F**). **G-H**, Tubulin immunostaining of wild type (**G**) and *gpa-3QL* animals (**H**) revealed no defects of the microtubular axoneme (cil, cilia; tz, transition zone).

To determine if *gpa-3QL* animals display a more specific IFT defect, we visualized

the localization of five sensory Gα proteins, since they are possible IFT cargo molecules. Three Gα proteins were not properly localized: GPA-4, GPA-9 and GPA-15 (Fig. 2A, B, I and J; results not shown). GFP fusion constructs of these Gα subunits accumulated at the transition zone, where the cilia connect with the dendrites, and stained only the proximal half of the cilia of *gpa-3QL* animals, while they could be detected throughout the cilia of wild type animals. We observed similar effects on GPA-4::GFP and GPA-15::GFP localization in two independent *gpa-3QL* alleles. In addition, the effects of *gpa-3QL* on GPA-4::GFP and GPA-15::GFP localization were confirmed by introducing both GFP fusion constructs in *gpa-3QL* by microinjection. Two other Gα subunits, ODR-3 and GPA-13, were normally localized in the cilia of *gpa-3QL* animals (results not shown), indicating that *gpa-3QL* affects the localization of only specific signalling molecules.

Structurally, the sensory cilia of *C. elegans* can be divided into a middle segment with nine doublet microtubules and a distal segment with nine singlet microtubules (Perkins et al. 1986). Anterograde transport within the middle segment is mediated by two kinesin complexes, OSM-3 kinesin and kinesin II, encoded by *klp-11*, *klp-20* and *kap-1* (Snow et al. 2004). Only OSM-3 mediates transport in the distal segments of the cilia (Snow et al. 2004). Both GPA-4::GFP and GPA-15::GFP were localized throughout the cilia in *kap-1* animals (Fig. 2D and I), but could only be observed in the middle segments and not in the distal segments in *osm-3* animals (Fig. 2F and N), suggesting that GPA-4::GFP and GPA-15::GFP can be transported by both kinesin complexes. The length of the cilia of the *osm-3* animals was very similar to the length of the GPA-4::GFP and GPA-15::GFP staining area in *gpa-3QL* animals (Fig. 2H and P), suggesting that *gpa-3QL* interferes with entry of GPA-4 and GPA-15 into the distal segments.

Since *gpa-3QL* affects the localization of Gα proteins only in the distal segments, we surmised that perhaps *gpa-3QL* affects OSM-3 mediated transport of these proteins. First, we determined the localization of the two anterograde motors, using KAP-1::GFP and OSM-3::GFP, and the retrograde motor, using XBX-1::GFP to visualize the dynein light intermediate chain (Schafer et al. 2003). All three motor complexes showed wild type localization in the cilia of *gpa-3QL* animals (Fig. 3A-F), although we occasionally observed OSM-3::GFP accumulations at the transition zone. In addition, we observed motility of KAP-1::GFP, OSM-3::GFP and XBX-1::GFP particles both in *gpa-3QL* and in wild type animals (Supplementary movies S1-6). These results suggest that *gpa-3QL* does not directly affect the anterograde or retrograde motor complexes.

Our results suggest that GPA-4::GFP and GPA-15::GFP can be transported both by kinesin II and OSM-3. First, we determined if we could see motility of GPA-15::GFP particles. As can be seen in Supplementary movie S7 we observed motility of GPA-15::GFP, very reminiscent of the motility of IFT particles, suggesting that GPA-15::GFP is transported by the IFT machinery. To determine whether kinesin II or OSM-3 mediated transport of the two Gα proteins is affected by *gpa-3QL* we generated double mutants. GPA-4::GFP and GPA-15::GFP staining could only be observed in the cilia middle segments of *osm-3*; *gpa-3QL*

animals (Fig. 2G, H, O and P), indicating that transport in this segment, probably mediated by kinesin II, is not affected by *gpa-3QL*. In contrast, GPA-4::GFP and GPA-15::GFP could be observed both in the cilia middle and distal segments in *gpa-3QL*; *kap-1* animals (Fig. 2E, H, M and P), indicating that the restriction of GPA-4 and GPA-15 to the middle segments in *gpa-3QL* animals requires kinesin II. Taken together, our results suggest that in *gpa-3QL* animals GPA-4 and GPA-15 are predominantly transported by kinesin II and not or only very little by OSM-3 kinesin. However, the two Gα proteins can be transported by OSM-3 kinesin in *gpa-3QL* animals, but this can only be seen in the absence of kinesin II.

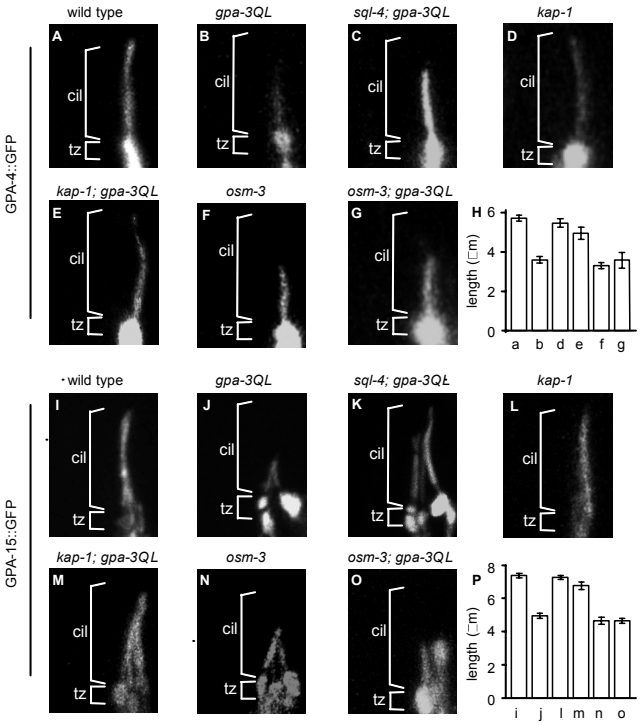

Figure 2. *gpa-3QL* affects the localization of Gα proteins in the cilia distal segments. a-h GPA-4::GFP localization in wild type (**A**), *gpa-3QL* (**B**), *sql-4; gpa-3QL* (**C**), *kap-1* (d), *kap-1; gpa-3QL* (**E**), *osm-3* (**F**), *osm-3; gpa-3QL* (**G**). **H**, Length of GPA-4::GFP staining in the cilia, in µm (**A**) wild type (n=24), (**B**) *gpa-3QL* (n=34), (**D**) *kap-1* (n=23), (e) *kap-1; gpa-3QL* (n=25), (**F**) *osm-3* (n=21) and (**G**) *osm-3; gpa-3QL* (n=27). **I-P**, GPA-15::GFP localization in wild type (**I**), *gpa-3QL* (**J**), *sql-4; gpa-3QL* (**K**), *kap-1* (**L**), *kap-1; gpa-3QL* (**M**), *osm-3* (**N**), *osm-3; gpa-3QL* (**O**). **P**, Length of GPA-15::GFP staining in the cilia, in µm (**I**i) wild type (n=28), (**J**) *gpa-3QL* (n=27), (**L**) *kap-1* (n=22), (**M**) *kap-1; gpa-3QL* (n=25), (**N**) *osm-3* (n=25) and (**O**) *osm-3; gpa-3QL* (n=22). We consistently observed a small difference in the length of GPA-4::GFP and GPA-15::GFP cilia. We think this is due to the stronger GPA-15::GFP expression (cil, cilia; tz, transition zone).

IFT particles consist of at least 17 proteins, ordered in complex A and complex B proteins, involved in retrograde and anterograde transport, respectively (Rosenbaum and Witman 2002; Scholey 2003). We analysed whether these IFT proteins were affected by *gpa-3QL*. Both the complex A protein CHE-11 (Qin et al. 2001) and the complex B proteins CHE-13/IFT-57 (Haycraft et al. 2003) and OSM-1/IFT172 (Signor et al. 1999) showed similar localization in *gpa-3QL* animals as in wild type animals, and we saw similar motility (results not shown). In contrast, *gpa-3QL* severely affected the localization of the complex B protein OSM-5/IFT-88/Polaris (Qin et al. 2001; Rosenbaum and Witman 2002). OSM-5::GFP accumulated at the transition zone and was hardly detectable in the cilia (Fig. 3G and H). Since the expression of OSM-5::GFP was reduced in *gpa-3QL* animals, we

generated additional transgenic strains expressing higher levels of OSM-5::GFP. In these animals, the fusion protein could be detected in the cilia, but we observed disorganized accumulations in the cilia and at the transition zone (Fig. 3I). In addition, we observed no motility of OSM-5::GFP in *gpa-3QL* animals (results not shown). This indicates that *gpa-3QL* interferes with entry of OSM-5 into the cilia, however a fraction of OSM-5 can enter the cilia and travel to the distal end, sufficient for the basic IFT machinery to function. Similarly, studies in mouse mutants have shown that low levels of OSM-5/Polaris are sufficient for cilia development (Taulman et al. 2001).

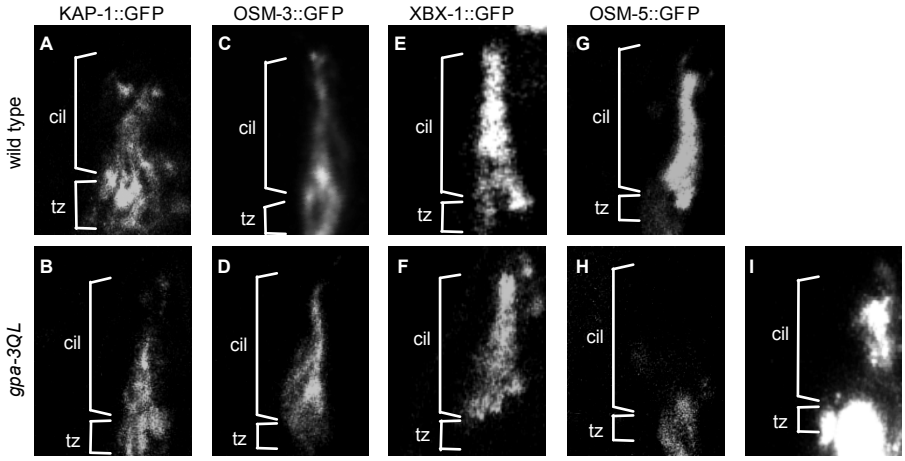

Figure 3. *gpa-3QL* does not affect the localization of IFT motor complexes, but does affect OSM-5::GFP. **A**,**B**, Localization of KAP-1::GFP in wild type animals (**A**) is very similar to that in *gpa-3QL* animals (**B**). **C**,**D**, Localization of OSM-3::GFP in wild type (**C**) and *gpa-3QL* animals (**D**). **E,F**, Localization of XBX-1::GFP in wild type (**e**) and *gpa-3QL* animals (**F**). **G-I**, Localization of OSM-5::GFP in cilia of wild type animals (**G**). OSM-5::GFP entry into the cilia is strongly reduced in *gpa-3QL* animals (**H**); it could only be detected upon over-expression and showed a disorganized localization (**I**) (cil, cilia; tz, transition zone).

To study how *gpa-3QL* regulates the localization of signalling molecules, we performed a genetic screen for suppressors of the *gpa-3QL* Dyf phenotype. We identified four mutants (*sql:* Suppressor of *gpa-3QL*), which suppressed the Dyf phenotype: *sql-1(gj202)III*, *sql-2(gj804)I*, *sql-3(gj823)IV* and *sql-4(gj201)I*. *sql-4* restored dye filling of *gpa-3QL* animals (Fig. 1C) and restored GPA-15::GFP localization in the cilia distal segment, but not the localization of GPA-4::GFP (Fig. 2C and , K). This latter result suggests that different regulatory mechanisms may exist for the localization of different G proteins in the cilia. Although we have not yet identified the suppressor loci, our results confirm the existence of a mechanism that regulates the localization of specific signalling molecules in the sensory cilia of *C. elegans*. This mechanism is clearly affected by the dominant active Gα protein GPA-3QL. However, it is not clear which Gα proteins are involved in wild type

animals. Loss-of-function of *gpa-3* does not affect dye filling, or the localization of GPA-4::GFP and GPA-15::GFP (data not shown). Since *C. elegans* sensory neurons express many Gα proteins (Jansen et al. 1999), it is possible that the function of GPA-3 in the regulation of IFT is masked by functional redundancy.

Regulation of the localization of sensory signalling molecules would allow plasticity in response to environmental or developmental changes. An important developmental change is the dauer response (Riddle 1997). When exposed to harsh environmental conditions larvae can develop into dauer larvae. This is accompanied by many morphological and behavioural changes, including changes in cilia structure (Golden and Riddle 1982) . Since mutations in GPA-3 affect dauer development (Zwaal et al. 1997), we surmised that perhaps dauer inducing conditions also affect the localization of GPA-4 and GPA-15. We exposed L1 larvae to dauer pheromone, a continuously secreted compound that serves as a measure of population density. Strikingly, GPA-4::GFP was undetectable in the distal segment of the cilia of L2d larvae exposed to dauer pheromone for 24-28 hours (Fig. 4A and B), even though the cilia were still exposed to the environment as determined by dye-filling (results not shown). These results indicate that environmental signals can modulate the localization of ciliary signalling molecules. Interestingly, in dauer animals obtained after prolonged exposure to dauer pheromone we observed strong staining of GPA-4::GFP throughout the cilia (Fig. 4C).

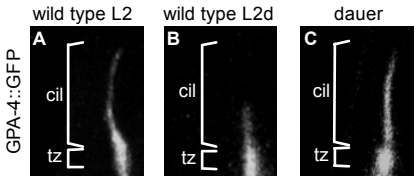

Figure 4. Dauer pheromone affects localization of GPA-4::GFP in the cilia of wild type animals. **A-C**, GPA-4::GFP is localized throughout the cilia of L2 larvae, not exposed to dauer pheromone (**A**), only in the middle segments, but not in the distal segments in L2d larvae, exposed to dauer pheromone for 24-28 hours (**B**) and throughout the cilia in dauer animals (**C**) (cil, cilia; tz, transition zone).

In these animals, the structure of the cilia of the *gpa-4* expressing ASI neurons has changed such that they are no longer exposed to the environment (Golden and Riddle 1982). We hypothesize that perhaps GPA-4::GFP can be transported into the cilia distal segment in dauer animals because the ASI neurons, which play an important role in dauer formation, no longer sense the dauer pheromone.

We propose a model in which G proteins integrate environmental signals such as dauer pheromone to regulate the localization of signalling molecules in sensory cilia. We hypothesize that G proteins affect the distribution of cargo between the two motor complexes, kinesin II and OSM-3 kinesin, probably by regulating the assembly of motor protein-cargo complexes. Our results indicate that OSM-5/Polaris plays an important role in this process. This mechanism would allow regulation of the presence of specific signalling routes in the sensory cilia, enabling the cell to quickly adapt its sensory capacity in response to environmental or developmental changes.

Methods

Strains and constructs used.

All strains were grown at 20°C. Strains and alleles used: wild type Bristol N2, CB4856, *gpa-3QL(syIs25)X*, *gpa-3QL(syIs24)IV*, *kap-1(ok676)II* and *osm-3(p802)IV*. GFP reporters used: *gpa-4::gfp* (Jansen et al. 1999), *gpa-9::gfp* (Jansen et al. 1999), *gpa-13::gfp* (Jansen et al. 1999), *gpa-15::gfp* (Jansen et al. 1999), *vap-1::rfp* (a gift from B. Westlund), *che-11::gfp* (Qin et al. 2001), *osm-1::gfp* (Signor et al. 1999), *osm-3::gfp* (Ou et al. 2005a), *kap-1::gfp* (Orozco et al. 1999), *che-13::gfp* (Haycraft et al. 2003), *osm-5::gfp* (Haycraft et al. 2001) and *xbx-1::gfp* (Schafer et al. 2003). All GFP reporters were crossed into the *gpa-3QL(syIs25)* background. A heat shock inducible *gpa-3QL* construct (*hsp16-2::gpa-3QL*) was generated by subcloning *gpa-3QL* into the pPD49.78 vector (Mello 1995).

Immunofluorescence and microscopy.

Animals were fixed, permeabilized and stained with anti GPA-3, GPA-13, ODR-3 and tubulin antibodies as described previously (Finney and Ruvkun 1990; Lans et al. 2004). Monoclonal rat antibody against tubulin was purchased from Abcam (ab6160). Secondary antibodies were goat-anti-rabbit alexa-594-conjugated and goat-anti-rat alexa-488-conjugated (Molecular Probes). The localization of fluorescent proteins and cilia morphology was examined using a Zeiss confocal microscope CLSM510. Dye filling (Perkins et al. 1986) was performed using DiI (Molecular probes). Cilia lengths were measured using a Zeiss Imager Z1 microscope. Live imaging of the GFP tagged IFT particles was carried out as described previously (Efimenko et al. 2005).

gpa-3QL suppressor screen

gpa-3QL(syIs25) animals were mutagenized with EMS. Dye filling F2 and F3 progeny was cloned and re-analyzed. Presence of the *gpa-3QL* transgene was confirmed by PCR and using anti GPA-3 antibodies. Four suppressor alleles, *sql-1(gj202)III, sql-2(gj804)I*, *sql-3(gj823)IV* and *sql-4(gj201)I* were mapped using SNP mapping (Wicks et al. 2001).

Dauer analysis

Dauer pheromone was isolated as described previously (Golden and Riddle 1982). Adult animals were allowed to lay eggs on plates with inducing concentrations of dauer pheromone, and removed after three hours. The localization of GPA-4::GFP and GPA-15::GFP was determined in L2d larvae after 24 to 28 hours at 25°C or after 52 hours in dauer animals. To analyze whether the ASI cilia were still in contact with the environment the worms were subjected to dye filling.

Supplementary information

Movies can be requested at: Jan.Burghoorn@biosci.ki.se

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Chapter

V

DYF-5 encodes a conserved MAP kinase and has a novel function in the regulation of intraflagellar transport in *C. elegans* sensory cilia

Jan Burghoorn, Suzanne Rademakers and Gert Jansen

Summary

The assembly, maintenance and function of cilia require a specialized transport system, called intraflagellar transport (IFT) (Rosenbaum and Witman 2002; Scholey 2003). The nematode *Caenorhabditis elegans* has 60 ciliated neurons (White 1986). A subset of these neurons can take up fluorescent dyes through their cilia (dye filling) (Perkins et al. 1986). This phenomenon has been used to identify genes that affect cilia development (Perkins et al. 1986; Starich et al. 1995), many of which are components of the IFT machinery (Haycraft et al. 2001; Qin et al. 2001; Haycraft et al. 2003; Murayama et al. 2005; Ou et al. 2005). In this report we describe the identification of a novel IFT regulatory protein *dyf-5*, which encodes a predicted serine threonine kinase, homologous to the mammalian male germ cell associated kinase (Miyata et al. 1999), which belongs to a small group of mitogen-activated protein (MAP) kinases (Fu et al. 2005). Both loss- (l.o.f.) and gain-of-function (g.o.f.) of *dyf-5* cause dye filling defective (Dyf) phenotypes. Expression of *dyf-5::gfp* fusion constructs could be seen in many neurons of *C. elegans*, in the cell bodies, dendrites and axons, but not in the cilia. We used anti-tubulin antibodies and GFP fusion constructs to visualize the cilia and several IFT and cargo proteins. *dyf-5* l.o.f. animals have full-length cilia, but the cilia are not properly aligned into the amphid pore. In addition, we observed excess entry of cargo and IFT proteins into the cilia of *dyf-5* animals. In contrast, we observed reduced entry of cargo and IFT proteins into the cilia of *dyf-5* g.o.f. *(dyf-5XS)* animals. Based on these results we cannot classify the DYF-5 protein in either the complex A or complex B proteins, involved in retrograde or anterograde transport, respectively (Rosenbaum and Witman 2002; Scholey 2003). We propose that DYF-5 functions in a gate keeping process that regulates entry of IFT and cargo proteins into the cilia.

Results and discussion

Forward genetic screens have identified many *C. elegans* mutant strains that

display dye filling defects, including Dyf (dye filling defective), Che (chemotaxis defective), Osm (osmotic avoidance defective) and Daf (dauer larvae formation abnormal) animals (Perkins et al. 1986; Starich et al. 1995). Identification of several of these genes has revealed that many are involved in intraflagellar transport (IFT) (Rosenbaum and Witman 2002; Scholey 2003). Also reverse genetic studies have identified several genes involved in IFT, such as the BBS genes and XBX genes (Schafer et al. 2003; Blacque et al. 2004; Blacque et al. 2005; Efimenko et al. 2005; Ou et al. 2005).

We characterized the phenotype of another dye filling defective mutant, *gpa-3QL*, which expresses a constitutively active GPA-3 Gα protein (chapter IV) (Zwaal et al. 1996). We performed a genetic screen for suppressors of the *gpa-3QL* Dyf phenotype and identified four suppressor alleles, *sql-1* to *sql-4* (Suppressor of *gpa-3QL*). Using SNP mapping (Wicks et al. 2001) *sql-4(gj204)* was mapped to a 280 kb interval on chromosome I (Fig. 1A).

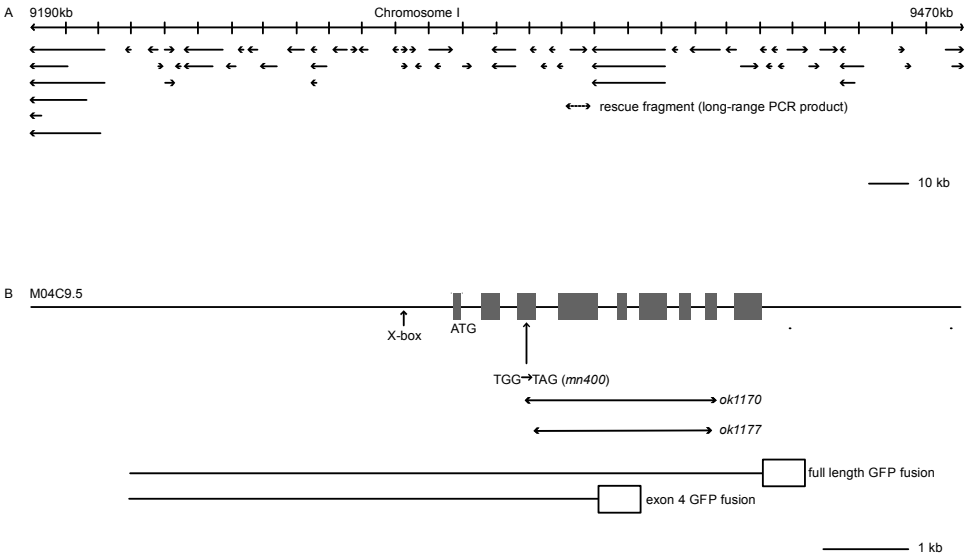

Figure 1. **A**. Schematic representation of the 280 kb *sql-4* mapping interval (based on www.wormbase.org). Rescue of the *dyf-5* Dyf phenotype was obtained with a 8 kb long range PCR fragment containing M04C9.5. All predicted genes and the rescuing fragment are indicated with arrows **B**. Gene structure of *dyf-5*, M04C9.5. Exons are indicated as black boxes; the predicted transcription start and X-box are indicated. Arrows indicate the position of the G to A substitution in *dyf-5(mn400)* animals, and the regions deleted in the *ok1170* and *ok1177* alleles. Finally, the two *dyf-5::gfp* fusion constructs have been indicated.

This interval also contains *dyf-5*, a dye filling defective mutant isolated in a forward genetic screen (Starich et al. 1995). We hypothesized that perhaps *dyf-5* and *sql-4* are alleles of the same gene. Transgenic rescue and candidate gene sequencing identified *dyf-5* as M04C9.5 (Fig. 1A and B). However, we did not find a mutation in M04C9.5 in *sql-4* animals. In addition, neither loss-of-function (l.o.f.) nor gain-of-

function (g.o.f.) mutations in *dyf-5* suppressed the *gpa-3QL* Dyf phenotype (results not shown), indicating that *dyf-5* and *sql-4* are different genes.

dyf-5 encodes a protein of 471 amino acids, which shows highest homology to the mammalian male germ cell associated kinase MAK (Matsushime et al. 1990). MAK belongs to a small subfamily of Ser/Thr protein kinases with modest similarity to mitogen-activated protein (MAP) kinases and cyclin dependent kinases (Cdks). The human MAK subfamily consists of MAK, ICK (Intestinal cell kinase) (Togawa et al. 2000) or MRK (MAK-related kinase (Yang et al. 2002) and MOK (MAPK/MAK/MRK overlapping kinase) (Miyata et al. 1999), with highly conserved N-terminal catalytic domains and more divergent C-terminal non-catalytic domains. All these kinases and DYF-5 contain a TXY motif in their activation loop, characteristic for all MAP kinases (Jinno et al. 1993) (results not shown). The functions of the human MAK, MOK and ICK proteins remain to be determined. MAK is expressed in mammals in male germ cells at and after meiosis, in photoreceptor cells of the retina and in olfactory receptor cells, in the epithelia of the trachea and lung and in the choroid plexus (Togawa et al. 2000). A MAK knock out mouse is viable and fertile (Shinkai et al. 2002). ICK is expressed in most human tissues. Highest expression was found in the intestine, in the intestinal crypt, and in testis and this kinase has a nuclear localization (Togawa et al. 2000; Yang et al. 2002; Fu et al. 2005). MOK is widely expressed in mammalian tissues and localizes to the cytoplasm (Miyata et al. 1999).

dyf-5(mn400) contains a G to A transition in exon 3 (Fig. 1B), which introduces a stop codon at amino acid position 49. In addition, we characterized two deletion alleles of *dyf-5*, *ok1170* and *ok1177*, generated by the *C. elegans* gene knock out consortium. Both deletion alleles remove most of the *dyf-5* coding region and are very likely null alleles (Fig. 1B). All *dyf-5* l.o.f. alleles show Dyf phenotypes (Fig. 2A, B and data not shown). Dye filling could be restored by introducing low levels of an 8 kb genomic fragment containing the M04C9.5 gene as a transgene in *dyf-5*(*ok1170*) animals (Fig. 2C), confirming that this gene indeed encodes *dyf-5*. Also a full length *dyf-5::gfp* fusion construct could restore dye filling in *dyf-5* (*ok1170*) animals (data not shown). Expression of higher levels of the *dyf-5* gene (*dyf-5XS*) or the full length *dyf-5::gfp* construct induced dye filling defects in wild type animals (Fig. 2D), suggesting that the levels of DYF-5 are crucial for its function.

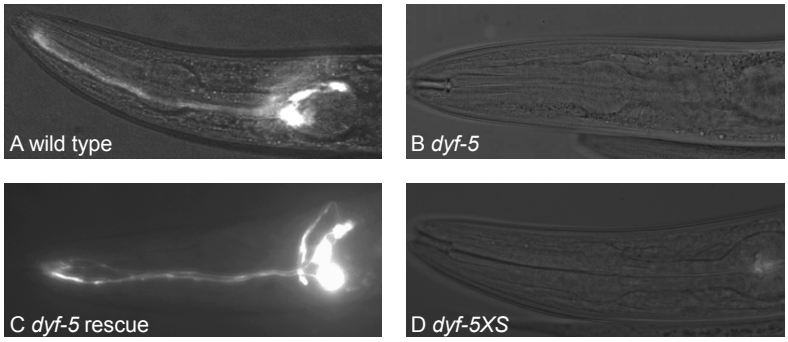

Figure 2. DiO dye filling of (**A**) wild type, (**B**) *dyf-5(mn400)*, (**C**) *dyf-5(ok1170) gjEx824(dyf-5)* rescue strain and (**D**) *dyf-5XS(gjEx817)* animals.

The expression pattern of *dyf-5* was examined using two *dyf-5::gfp* fusion constructs: a fusion construct in which GFP is fused in frame to the fourth exon of *dyf-5* (*dyf-5^{ex4}::gfp*) and a full length, functional *dyf-5::gfp* fusion construct (Fig. 1B). Both *dyf-5::gfp* reporter constructs showed the same expression pattern. We observed DYF-5::GFP expression in many neurons in the head, including the amphid sensory neurons (Fig. 3A). In addition, DYF-5::GFP could be detected in the CAN cells (Fig. 3B), neurons associated with the excretory canal (White 1986), in a pair of neurons in the posterior lateral ganglion and in several neurons in the tail, probably including the phasmid sensory neurons (Fig. 3C). Surprisingly, the full length DYF-5::GFP fusion protein could not be detected in the cilia, but was clearly present in the transition zones, the structures that connect the cilia with the dendrites (Fig. 3D). In addition, we observed uniform expression of the full length DYF-5::GFP in the axons, cell bodies and dendrites (results not shown). The DYF-5^{ex4}::GFP fusion construct showed strong accumulation in the cell bodies, perhaps in the endoplasmic reticulum or in the Golgi system. *dyf-5::gfp* expression could first be observed in embryos between comma and 1.5 fold stage (Fig. 3E), around the time when ciliogenesis starts (Sulston et al. 1983) and continues throughout adulthood.

The expression of many components of the IFT machinery is regulated by the DAF-19 transcription factor (Swoboda et al. 2000). This regulation occurs through an X-box promoter element, which is generally located within the first 150 nucleotides upstream of the translational start. Genomic analyses have identified many genes that contain an X-box element and several of these have indeed been shown to be regulated by DAF-19 (Blacque et al. 2005; Efimenko et al. 2005). Also *dyf-5* contains an X-box element, 193 bp upstream of the SL1 trans splice site and 271 bp upstream of the translational start (Fig. 1B). We expressed the *dyf-5::gfp* construct both in wild-type and in *daf-19* backgrounds and found that the expression of the reporter was similar to wildtype, suggesting that *dyf-5* expression is not regulated by DAF-19.

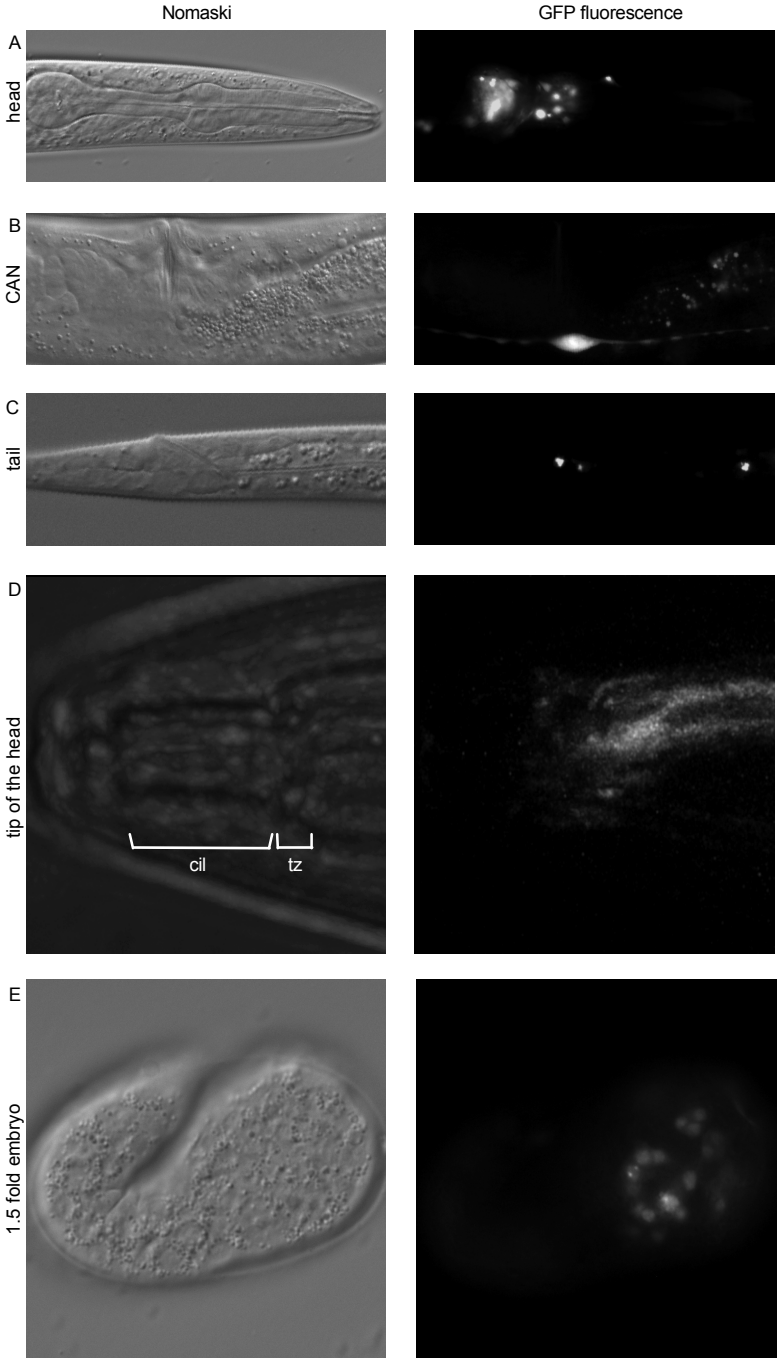

Figure 3. (**A**) Expression of *dyf-5::gfp* in the head of *C. elegans* can be seen in many neurons, amongst others in amphid sensory neurons and inner labial sensilla, (**B**) in the CAN cells (neurons associated with the excretory canal), (**C**) in several neurons in the tail. (**D**) DYF-5::GFP expression in the tip of the head where cilia (cil) and the transition zone (tz) are indicated. DYF-5::GFP does locate to the transition zone, but does not enter the cilia. (**E**) DYF-5::GFP could first be observed in embryos between comma and 1.5 fold stage. Right panel: Nomaski, Left panel: GFP fluorescence.

Previous studies have shown that IFT mutants can be categorized in two classes, encoding complex A and complex B proteins (Perkins et al. 1986; Rosenbaum and Witman 2002; Scholey 2003). Complex A mutants have stunted cilia and show massive accumulation of tubulin and IFT proteins at the distal tip of the cilia (Fig. 4B, F). Complex A proteins probably have a role in retrograde movement (Rosenbaum and Witman 2002; Scholey 2003). The cilia in mutants with an IFT complex B defect are severely stunted (Fig. 4C, F), possibly due to a flaw in anterograde movement (Rosenbaum and Witman 2002; Scholey 2003). Visualisation of the cilia structures of *dyf-5* and *dyf-5XS* mutant animals using anti-tubulin antibodies revealed a novel cilia defect. We observed strong staining of the microtubular axonemes of *dyf-5* animals (Fig. 4D). The cilia appeared full length, but misdirected (Fig. 4D). In *dyf-5XS* animals we observed weak anti-tubulin staining, and the cilia appeared shorter (Fig. 4E). Since the anti-tubulin staining of both *dyf-5* l.o.f. and g.o.f. mutants did not resemble either complex A or B mutant staining patterns, we were unable to classify DYF-5 in one of these IFT complexes.

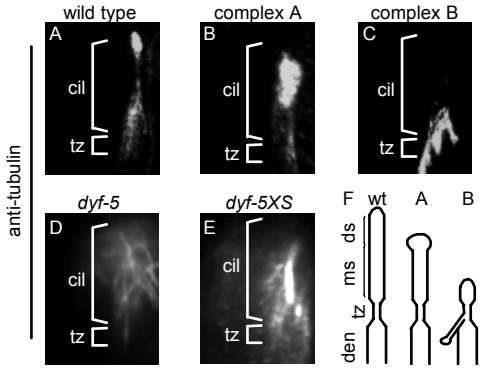

Figure 4. Cilia morphology was visualised using anti-tubulin staining of (**A**) wild type, (**B**) *che-11* (complex A mutant), (**C**) *osm-6* (complex B mutant), (**D**) *dyf-5*, (**E**) *dyf-5XS*. The cilia (cil) and transition zone (tz) are indicated. (**F**) Schematic representation of cilia in wild type, complex A and complex B animals. The dendrite (den), transition zone (tz), cilia middle segment (ms) and distal segment (ds) are shown. The *dyf-5* animals cannot be classified as complex A or B mutant.

Anterograde transport in *C. elegans* cilia is mediated by two kinesin motor complexes and the complex B proteins. In the cilia middle segments transport is mediated by both the heterotrimeric kinesin II, encoded by *klp-11*, *klp-20* and *kap-1*, and the homodimeric OSM-3 kinesin (Snow et al. 2004). In the distal segment, transport is only mediated by OSM-3 (Snow et al. 2004). Retrograde transport is mediated by the dynein motor complex, which contains the CHE-3 dynein heavy chain (Wicks et al. 2000) and XBX-1 dynein light intermediate chain (Schafer et al. 2003), and complex A proteins. We analysed the localization of the OSM-3 kinesin (Snow et al. 2004), the dynein component XBX-1 (Schafer et al. 2003), and the complex B IFT proteins CHE-13/IFT46 (Haycraft et al. 2003) and OSM-5/IFT172 (Haycraft et al. 2001; Qin et al. 2001). In wild type animals, GFP tagged motors and IFT complex B factors show some accumulation at the transition zone, and staining of the cilia (Fig. 5A-D and data not shown). In life microscopy motility of these GFP particles can be seen (supplementary movie 1 and data not shown). In *dyf-5* animals however, we observed no accumulation of the GFP fusion proteins at the transition zone, but instead the GFP fusion proteins accumulated in the cilia

directly after the transition zone (Fig 5E-H and data not shown). In addition, we could hardly detect motility of GFP tagged OSM-5, CHE-13, OSM-3 and XBX-1 in *dyf-5* animals (supplementary movie 2 and data not shown). However, we could still detect the GFP fusion proteins at the distal tips of the cilia (Fig. 5E-H). Interestingly, in *dyf-5XS* animals we observed strong accumulation of OSM-3::GFP, XBX-1::GFP, CHE-13::GFP and OSM-5::GFP at the transition zone (Fig. 5I-L). OSM-3 and XBX-1 GFP fusion proteins could still be detected at the distal end of the cilia (Fig. 5I and J), whereas CHE-13::GFP and OSM-5::GFP showed reduced expression in the cilia and could not be detected in the distal parts of the cilia (Fig. 5K and L). The motility of most of these IFT factors was hardly detectable in *dyf-5XS* animals, except for OSM-3::GFP (supplementary movie 3 and data not shown).

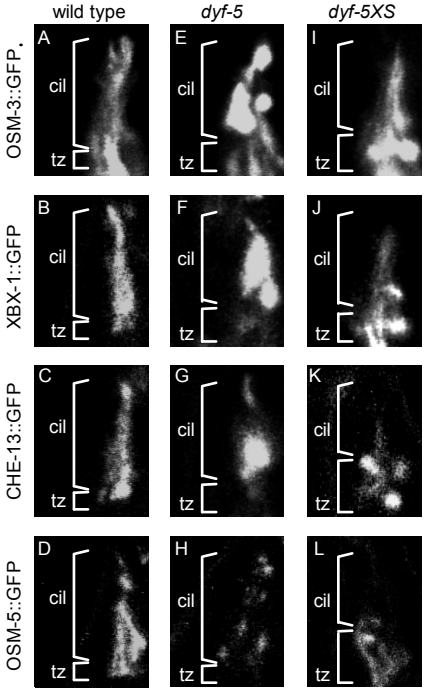

Figure 5. DYF-5 regulates entry of IFT proteins into the cilia. Visualisation of OSM-3::GFP, XBX-1::GFP, CHE-13::GFP and OSM-5::GFP in the cilia of (**A-D**) wild type, (**E-H**) *dyf-5(ok1170)* and (**I-L**) *dyf-5XS* animals showed enhanced entry of IFT proteins in the cilia of *dyf-5* l.o.f. animals, but reduced entry in *dyf-5XS* animals. The cilia (cil) and the transition zone (tz) are indicated.

Finally, we determined the localisation of a cargo protein, the Gα subunit GPA-15 (Jansen et al. 1999) (chapter IV), in the cilia of *dyf-5* and *dyf-5XS* animals. In wild type animals we observed GFP staining along the length of the cilia and small accumulations of GPA-15::GFP at the transition zone (Fig. 6A). In *dyf-5* animals we observed very strong GPA-15::GFP staining along the length of the cilia (Fig. 6B), suggesting that there is increased entry of this Gα subunit into the cilia, and that transport along the length of the cilia still occurs. In contrast, we observed only very weak GPA-15::GFP expression in the cilia of *dyf-5XS* animals and bright

accumulations at the transition zone (Fig. 6C), suggesting that entry of GPA-15 into the cilia is blocked in these animals.

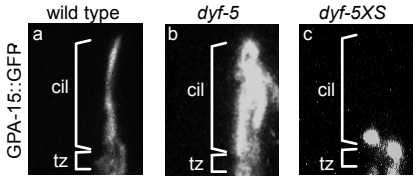

Figure 6. GPA-15::GFP localization in (**A**) wild type, (**B**) *dyf-5* and (**C**) *dyf-5XS* animals. Also GPA-15::GFP shows excess entry into the cilia in *dyf-5* animals, but reduced entry in dyf-5XS animals. The cilia (cil) and the transition zone (tz) are indicated

In conclusion, we have identified the gene that is mutated in *dyf-5* animals. *dyf-5* encodes a conserved ser/thr MAP kinase, with highest homology to the mammalian MAK kinase. DYF-5 is expressed in many neurons, including ciliated neurons, but could not be detected in the cilia of these neurons. Both loss- and gain-of-function of *dyf-5* affect cilia morphology. Loss-of-function of *dyf-5* causes excess entry of various IFT proteins and the cargo protein GPA-15 into the cilia, while gain-of-function of *dyf-5* reduces entry of these proteins into the cilia. Together, our results suggest that DYF-5 regulates entry into the cilia of the IFT machinery. We propose that DYF-5 functions in a gate keeping process at the transition zone, which regulates assembly and docking of IFT factors and cargo and the entry of these complexes into the ciliary axoneme. The putative mammalian homologue of DYF-5, MAK, belongs to a small group of MAP kinases of unknown function. Members of this group are expressed in tissues where cilia play important roles in sensing environmental cues and regulating cell proliferation and differentiation, including sensory neurons, kidney and intestine (Matsushime et al. 1990) (Koji et al. 1992; Jinno et al. 1993; Miyata et al. 1999; Togawa et al. 2000; Shinkai et al. 2002; Yang et al. 2002). We propose that one or more of these kinases play a similar role as DYF-5 in the regulation of IFT in mammals.

Materials and Methods

Strains and constructs

All strains were grown at 20°C. Strains used; Bristol N2 (wild type)*, gpa-3QL(syIs25)X, osm-6(p811)V, che-11(e1810)V, dyf-5(mn400)*, *dyf-5(ok1170)* and *dyf-5(ok1177)*. *dyf-5(ok1170)* contains a 2058 bp deletion (deletion starting with ATTCTGAAAATCCGTTCAAA, ending with TTTCTATTTCTTTCAATTTT) and a 583 bp insertion originating from the X chromosome; dyf-5(ok1177) contains a 1720 bp deletion (deletion starts with GTTGTACAACAAATAAATAA, and ends with ATTATTTATTCAGTAAAATG). *dyf-5* overexpressing animals (*dyf-5XS*) were generated by injecting 75 ng/µl of a 8 kb long range PCR fragment, using forward primer: 5' ATTTCCTCCTGATAACCTTCCATTTGC, and reverse primer 5' CCACTTTCTTCCCATTTTCTTCTCCC. GFP reporters used: *vap-1::rfp* (a gift from B. Westlund)*, gpa-15::gfp* (Jansen et al. 1999)*, osm-3::gfp* (Snow et al. 2004)*, che-13::gfp* (Haycraft et al. 2003)*, osm-5::gfp* (Haycraft et al. 2001) *and xbx-1::gfp*

(Schafer et al. 2003). All GFP reporters were crossed into the *dyf-5* and *dyf-5XS* backgrounds.

dyf-5::gfp reporter constructs were generated using a fusion PCR protocol (Hobert 2002). One fusion PCR fragment contained 3 kb upstream region and the first four exons fused in frame with GFP (forward primer 5' ATTTCCTCCTGATAACCTTCCATTTGC and reverse primer 5' TGGCCAATCCCGGGGATCCTCCGATCACGATCTTTCATTAGCTC). A full length GFP fusion was generated using the same forward primer and reverse primer 5' TGGCCAATCCCGGGGATCCTCACCACCCCCAGAATATTGGGAAGAAAGGC.

These fragments were fused with a PCR fragment containing the GFP open reading frame and the *unc-54* 3' UTR generated with primers 5' GAGGATCCCCGGGATTGGCCA and 5' GCCGACTAGTAGGAAACAGT on plasmid pPD95.75 (a gift from A. Fire). DAF-19 dependent transcription of *dyf-5* was tested by crossing the reporter constructs in the *daf-19(m86);daf-12(sa204)* strain. *daf-12 (sa204)* was used as wild type control. Expression of at least 60 worms was analyzed.

Cloning and characterization of dyf-5

dyf-5(mn400) or *dyf-5(ok1170)* mutant animals were injected with 5 ng/µl of the 8 kb long range PCR fragment containing M04C9.5, or the full length *dyf-5::GFP* construct. Rescue was analyzed using dye filling. We performed RT-PCR to characterize the open reading frame of *dyf-5*.

Microscopy

The localization of GFP tagged proteins and cilia morphology were determined using a Zeiss confocal microscope CLSM510. Dye filling was performed using DiI (Molecular probes)(Perkins et al. 1986). Live imaging of the GFP tagged IFT particles was performed as described previously (Efimenko et al. 2005).

Supplementary information

Movies can be requested at: Jan.Burghoorn@biosci.ki.se

Acknowledgements

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Chapter

VI

Contribution of localization of signaling molecules to dauer formation in the nematode *C. elegans*

Jan Burghoorn and Gert Jansen

Summary

Under harsh environmental conditions, the nematode *Caenorhabditis elegans* can enter an alternative developmental stage, the dauer larva. One of the cues that can induce dauer development is a continuously secreted pheromone. We recently found that exposure of larvae to dauer pheromone reduces the entry into the cilia of a sensory G protein α subunit, GPA-4. Also a gain-of-function mutation in the Gα protein GPA-3 (GPA-3QL) reduces the entry of Gα proteins into the cilia, probably by affecting loading of cargo onto the intraflagellar (IFT) transport machinery. In addition, *gpa-3QL* animals constitutively form dauers (Daf-c phenotype), suggesting that the localization of signaling molecules in the cilia might contribute to dauer development. Here we show that the altered localisation of GPA-4 upon exposure to dauer pheromone is mediated by GPA-3 and the anterograde motor protein kinesin II. In addition, *gpa-3* affects dauer formation by regulating the expression of DAF-7/TGFβ, by decreasing cGMP levels, most likely by inhibiting the guanylyl cyclase DAF-11. However, mutations in *daf-7* and *daf-11* do not affect Gα localization in the cilia, indicating that the altered localization of sensory signalling molecules is not the result of activation of the dauer pathway. Moreover, loss-of-function of *gpa-4* results in hypersensitivity to dauer pheromone and reduced *daf-7* expression. Finally, we show that g.o.f. of the IFT regulatory protein DYF-5, which reduces entry of IFT and cargo proteins into the cilia, induces a Daf-c phenotype and reduced *daf-7* expression. We hypothesize that the regulated localization of GPA-4 and perhaps other signalling molecules contributes to the dauer response. We propose that, upon exposure to dauer pheromone, GPA-3 will be activated and reduce entry of GPA-4 into the cilia by influencing the IFT machinery. The absence of GPA-4 in the cilia will lead to decreased DAF-11 activity and *daf-7* expression, and activation of the dauer pathway.

Introduction

The translation of information from the environment is essential for the survival of all organisms. Exterior signals need to be integrated and processed before a cell or organism can decide upon the appropriate developmental and behavioural changes. Sensory neurons often use cilia to sense environmental cues. Since cilia have no protein synthesis, they are dependent on a specialized transport system, known as intraflagellar transport (IFT). IFT is a motility system, which bidirectionally transports bulky protein complexes, called IFT particles, between the base of the cilia, often called the transition zone, and the tip of the cilia (Rosenbaum and Witman 2002; Scholey 2003; Snell et al. 2004). Cilia are found on most mammalian cells, while in *Caenorhabditis elegans* they are only present on the sensory neurons (White et al). Cilia in *C. elegans* consist of a middle segment with nine doublet microtubules and a distal segment with nine singlet microtubules (Perkins et al. 1986). Anterograde transport within the middle segment is mediated by two kinesin complexes, OSM-3 kinesin and kinesin II, whereas transport in the distal segments is mediated by OSM-3 kinesin alone (Snow et al. 2004).

Previously, we have identified a novel mechanism that regulates the localization of specific signal transduction molecules in the sensory cilia of *C. elegans*. We found that a gain-of-function (g.o.f.) mutation in the Gα protein GPA-3 (*gpa-3QL*) impedes the entry into the distal segments of the cilia of several Gα proteins, including GPA-4 and GPA-15. In wild type animals these Gα proteins can be transported by both kinesin II and OSM-3 kinesin, however in *gpa-3QL* animals they are predominantly transported by kinesin II and not by OSM-3. *gpa-3QL* seems not to affect motility or localization of the IFT motor complexes (chapter IV). These data suggest that GPA-3 can mediate signals to regulate the assembly of motor protein - cargo complexes, thereby affecting the localization of specific signalling molecules in the cilia. In addition, we identified a novel IFT regulatory protein DYF-5, which plays a role in regulating the entry of IFT and cargo proteins into the cilia (chapter V). Loss-of-function (l.o.f.) of *dyf-5* results in excess entry of IFT and cargo proteins into the cilia, while g.o.f. of *dyf-5* results in reduced entry into the cilia.

Also environmental signals can affect the cilia structure. Changes in cilia structure are very important in the dauer response of *C. elegans* (Riddle 1997). Upon starvation or overcrowding *C. elegans* arrests its development prior to reproductive maturity, as a specialized L3 diapause form or the dauer larva (Riddle 1997). After improvement of the environmental conditions, the dauer larva progresses to a normal L4 larva and resumes its development into a reproductive animal. Dauer development can be induced by high temperature, low food concentrations and high dauer pheromone concentrations. The pheromone is a continuously secreted compound that serves as a measure of high population density. These cues are detected using four pairs of amphid sensory neurons: ADF, ASG and ASI, which inhibit dauer formation, and the ASJ neurons, which

stimulate dauer formation (Riddle 1997). The decision between dauer versus reproductive growth is controlled by at least two endocrine processes: the DAF-2 (insulin/IGF I receptor) and the DAF-7 (TGFβ) pathway (Riddle et al. 1981; Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Gerisch et al. 2001; Li et al. 2003). When larvae are exposed to high pheromone concentrations *daf-7* expression is down regulated and remains off in dauer animals. When environmental conditions improve and the worm resumes development, *daf-7* expression is restored (Ren et al. 1996). Downstream components of the DAF-7 pathway include the DAF-1/TGFβ type I receptor and DAF-4/TGFβ type II receptor (Georgi et al. 1990; Estevez et al. 1993). These factors will subsequently activate DAF-8 and DAF-14/Smads, which will repress the DAF-3/Smad, and the Ski oncoprotein homolog DAF-5 (Georgi et al. 1990; Estevez et al. 1993; Patterson et al. 1997; Inoue and Thomas 2000; da Graca et al. 2004). In dauer animals, also the expression of the insulin-like protein *daf-28* is down regulated. DAF-28 is a ligand of the insulin/IGFI like receptor DAF-2 (Kimura et al. 1997; Li et al. 2003). It has been suggested that the two endocrine pathways funnel into a third pathway, which includes DAF-12. This nuclear hormone receptor influences the decision between normal development and the diapause program (Antebi et al. 2000).

daf-7 and *daf-28* expression is regulated by DAF-11, a guanylyl cyclase (Birnby et al. 2000). In *daf-11* l.o.f. animals *daf-28* expression is reduced (Li et al. 2003) and *daf-7* expression is completely shut-down (Murakami et al. 2001). *daf-11* is expressed in the sensory neurons and probably controls dauer arrest in response to dauer pheromone signals by the production of cGMP. L.o.f. mutations in *daf-11* cause a strong dauer-constitutive **(**Daf-c) phenotype (Birnby et al. 2000). Surprisingly, the Daf-c phenotype of *daf-11* animals is bypassed by defects in the cilia structure (Thomas et al. 1993), suggesting that DAF-11 actitvity suppresses an environmental signal which stimulates dauer formation.

C. elegans expresses 14 heterotrimeric G protein α subunits specifically in its amphid neurons (Jansen et al. 1999). Mutations in the Gα subunit *gpa-3* affect dauer formation: l.o.f. of *gpa-3* leads to a defect in dauer formation, while g.o.f. of *gpa-3* (*gpa-3QL*) leads to constitutive dauer formation (Jansen et al. 1999). Since *gpa-3QL* affects both the localization of ciliary signalling molecules and dauer development, we hypothesized that the regulated localization of signalling molecules in the cilia could play a role in dauer development. In a previous study, we reported that also exposure to dauer pheromone affects the localization of GPA-4 in the sensory cilia (Chapter IV), suggesting that GPA-4 might play a role in dauer development, and that its activity could be regulated by mechanism that controls its localization in the cilia.

In this study, we show that the altered localisation of GPA-4 upon exposure to dauer pheromone is mediated by GPA-3 and the anterograde motor protein kinesin II. In addition, *gpa-3* affects dauer formation by regulating the expression of DAF-7/TGFβ, by decreasing cGMP levels, most likely by inhibiting the guanylyl cyclase DAF-11. However, mutations in *daf-7* and *daf-11* do not affect Gα localization in the cilia, indicating that the altered localization of sensory signalling

molecules is not the result of activation of the dauer pathway. Moreover, l.o.f. of *gpa-4* results in hypersensitivity to dauer pheromone and reduced *daf-7* expression. Finally, we show that g.o.f. of the IFT regulatory protein DYF-5, which reduces entry of IFT and cargo proteins into the cilia, induces a Daf-c phenotype and reduced daf-7 expression. We propose a model in which the regulated localization of GPA-4 and perhaps other signalling molecules contributes to the dauer response.

Results and discussion

Mutation of *gpa-3* affects dauer development by changing *daf-7* expression levels

Zwaal and colleagues (Zwaal et al. 1997) have shown that mutation of *daf-5*, a coactivator of the DAF-3/Smad transcription factor (da Graca et al. 2004), suppresses constitutive dauer formation of *gpa-3QL* animals. This suggests that GPA-3QL functions upstream of DAF-5 in the DAF-7/TGFβ pathway. We examined the expression of GFP tagged DAF-7 in *gpa-3* and *gpa-3QL* animals. *gpa-3* animals showed wild type levels of *daf-7::gfp* expression under normal growth condition (Fig. 1A, B, E). However, we found no expression of *daf-7::GFP* in *gpa-3QL* animals (Fig. 1C, E).

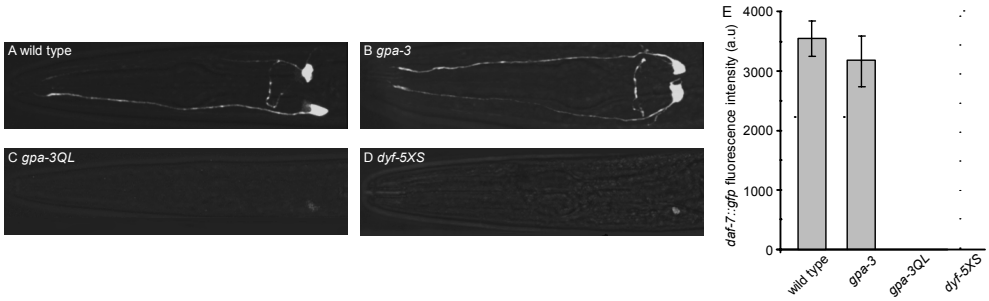

Figure 1. *daf-7::gfp* expression in (**A**) wild type, (**B**) *gpa-3*, (**C**) *gpa-3QL* and (**D**) *dyf-5XS* animals. **E**. The measured fluorescence intensity (a.u) in wild type, *gpa-3*, *gpa-3QL* and *dyf-5XS* animals (n > 20). The expression of *daf-7::gfp* is severely decreased in *gpa-3QL* and *dyf-5XS* animals.

Next, we exposed wild type and *gpa-3* L1 larvae expressing *daf-7::gfp* to dauer-inducing concentrations of dauer pheromone and measured the DAF-7::GFP fluorescence intensity. As shown before, exposure of wild type larvae to dauer pheromone resulted in reduced levels of DAF-7::GFP (Fig. 2A) (Ren et al. 1996). However, *gpa-3* mutants grown in the presence of pheromone showed significantly less reduction of *daf-7::gfp* expression (Fig. 2A). Together, these results confirm that GPA-3 functions in the DAF-7/TGFβ pathway and regulates *daf-7* expression.

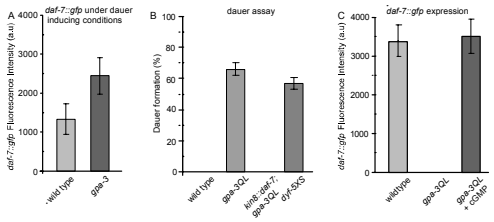

Figure 2. **A**. Quantification of *daf-7::gfp* expression in wild type and *gpa-3* mutant animals grown in the presence of dauer pheromone. **B**. Percentage dauer formation in wild type, *gpa-3QL, kin-8::daf-7 gpa-3QL* and *dyf-5XS* animals grown at 25°C without pheromone. **C**. *daf-7::gfp* expression in *gpa-3QL* animals cultured in the presence or absence of 8-Bromo-cGMP.

To confirm that the Daf-c phenotype of *gpa-3QL* animals is caused by reduced *daf-7* expression, we expressed *daf-7* under the control of a dauer-independent promoter (*kin-8::daf-7*) in *gpa-3QL* animals. We found, that expression of *daf-7* completely suppressed the Daf-c phenotype of *gpa-3QL* animals (Fig. 2B), suggesting that GPA-3 regulates dauer formation by regulating *daf-7* expression.

The transmembrane guanylyl cyclase DAF-11 is required for normal *daf-7* expression (Murakami et al. 2001). First, we tested whether DAF-11 localization in the cilia of *gpa-3QL* animals was changed. We found that DAF-11::GFP was normally localized throughout the cilia of *gpa-3QL* animals (data not shown). We wondered whether GPA-3 acts via DAF-11 to regulate *daf-7* expression. It has been described that the Daf-c phenotype of *daf-11* animals can be rescued by supplementing these animals with the membrane permeable cGMP analogue 8-bromo-cGMP (Birnby et al. 2000). *gpa-3QL* mutants expressing *daf-7::gfp* were cultured in the presence or absence of 8-Bromo-cGMP. When grown on 8-Bromo-cGMP, *gpa-3QL* animals displayed similar *gpa-7::gfp* fluorescence intensity as wild type animals (Fig. 2C). These results indicate that GPA-3 reduces *daf-7* expression and, as a result, induces dauer development by reducing cGMP levels. We hypothesize that *gpa-3* regulates cGMP levels by inhibiting DAF-11 activity.

GPA-4 localization is dependent on GPA-3 and KAP-1

Gain-of-function of *gpa-3* causes constitutive dauer formation by decreasing cGMP levels resulting in suppression of *daf-7* expression. In addition, *gpa-3QL* reduces the entry of the Gα subunits GPA-4 and GPA-15 into the cilia distal segments (Chapter IV; Fig. 3B, J and 4B). This effect of *gpa-3QL* requires the heterotrimeric kinesin II subunit KAP-1 (Chapter IV). Moreover, a similar relocalization of GPA-4::GFP from the distal tip of the cilia to the middle segment could be induced by exposing larvae to dauer pheromone for 24-28 hours (Chapter IV; Fig 3C). We hypothesized that GPA-4 relocalization to the middle segments of the cilia under dauer inducing conditions is dependent on GPA-3 and KAP-1. Indeed, we could not detect relocalization of GPA-4::GFP to the middle segments in *gpa-3* or *kap-1* l.o.f. animals under dauer inducing conditions (Fig 3D-G and J). *osm-3* animals,

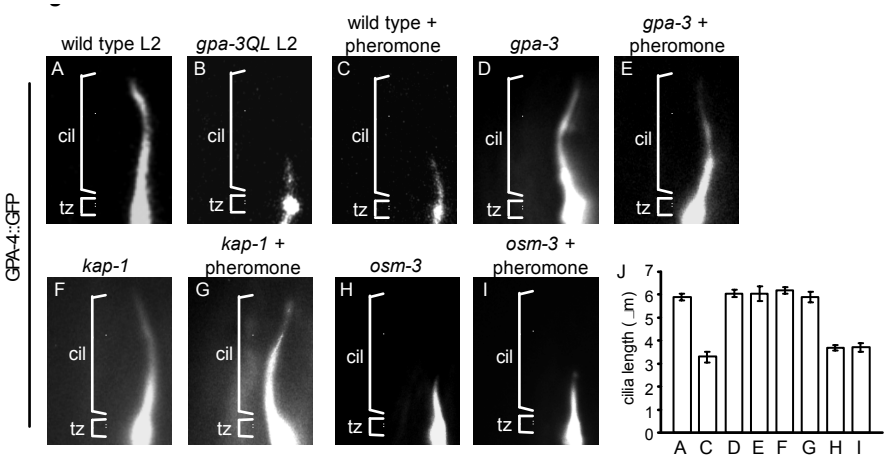

Figure 3. Localization of GPA-4::GFP in the cilia of (**A**) wild type L2, (**B**) *gpa-3QL* L2, (**C**) wild type hatched and grown under dauer inducing conditions for 24 hours (L2d), (**D**) *gpa-3* L2, (**E**) *gpa-3* L2d, (**F**) *kap-1* L2, (**G**) *kap-1* L2d, (**H**) *osm-3* L2 and (**I**) *osm-3* L2d. **J**. Localization of GPA-4::GFP in the middle or distal segment of the different animals. GPA-4::GFP relocalized to the middle segment under dauer inducing conditions, similar to the localization of GPA-4::GFP in *osm-3,* which lacks the distal segment. The cilia (cil) and transition zone (tz) are indicated.

which lack the distal cilia, showed a similar localization pattern of GPA-4::GFP under inducing and non-inducing conditions (Fig 3H and I). These data indicate that the relocalization of GPA-4 upon exposure to dauer inducing signals is regulated by GPA-3 and KAP-1.

Altered localisation of Gα subunits is not an effect of dauer development

We wondered if the altered localization of GPA-4 and GPA-15 is regulated by the dauer response proteins. First, we determined whether mutation of *daf-7* affects the localization of GPA-4::GFP and GPA-15::GFP in the cilia. However, we found no effects of *daf-7* l.o.f. on GPA-4::GFP and GPA-15::GFP localization (Fig. 4D and data not shown). In addition, we examined the localization of GPA-15::GFP in the cilia of *gpa-3QL* animals expressing *daf-7* under control of a dauer independent promoter (*kin-8::daf-7*). In these animals GPA-15::GFP localization was not restored and could only be detected in the cilia middle segments (Fig. 4E), while the Daf-c phenotype could be rescued (Fig. 2B). Next, we looked at GPA-4::GFP and GPA-15::GFP localization in *daf-11* animals. Also in these animals we observed wild type localisation of the Gα subunits throughout the cilia (Fig. 4F and data not shown). In addition, exposure of *gpa-3QL* animals to 8-Bromo-cGMP, which restores *daf-7::gfp* expression, did not restore GPA-15::GFP localization in the cilia (Fig. 4C). Together, these results suggest that the altered localisation of GPA-4 and GPA-15 is not the result of the activity of the DAF-11/DAF-7 signalling pathway. We hypothesize that rather the relocalization of these Gα subunits might cause inhibition of DAF-11 activity and, as a result, activation of the dauer

developmental pathway.

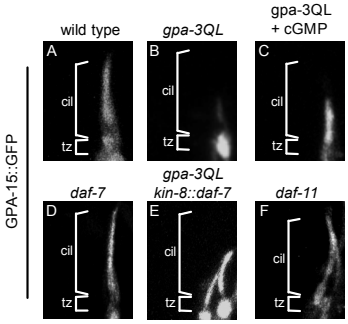

Figure 4. Localization of GPA-15::GFP in (**A**) wild type, (**B**) *gpa-3QL*, (**C**) *gpa-3QL* grown in the presence of cGMP, (**D**) *daf-7*, (**E**) *gpa-3QL* expressing *daf-7* under control of the *kin-8* promoter, (**F**) *daf-11* animals. The cilia (cil) and transition zone (tz) are indicated. cGMP and *kin-8* dependent *daf-7* expression do not affect the localization of GPA-15::GFP. Also *daf-7* and *daf-11* do not influence the localization of GPA-15::GFP.

GPA-4 influences daf-7 expression and dauer development

daf-7 is specifically expressed in the ASI neurons, which play a crucial role in dauer development (Ren et al. 1996). Only one Gα protein is specifically expressed in these neurons: *gpa-4* (Jansen et al. 1999). To further investigate whether GPA-4 plays a role in dauer formation, we determined the dauer formation capacity of *gpa-4* l.o.f. animals. We found that *gpa-4* animals exposed to low concentrations of dauer pheromone form much more dauers than wild type animals (Figure 5A).

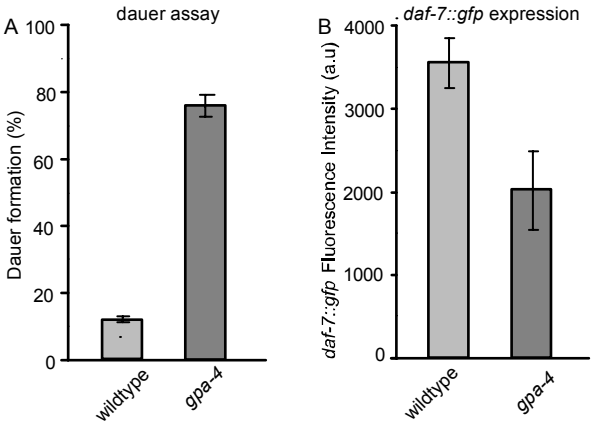

Figure 5. **A**. Percentage dauer formation in presence of low concentrations of dauer pheromone in wild type and *gpa-4* animals. **B**. *daf-7::gfp* expression in wild type and *gpa-4* mutants. *gpa-4* is hypersensitive to dauer pheromone and has a lower expression of *daf-7::gfp*.

Next, we generated *gpa-4* animals expressing the *daf-7::gfp* construct and found an approximately 45% reduction of *daf-7::gfp* expression in *gpa-4* animals (Figure 5B). These data indicate that GPA-4 inhibits dauer development by stimulating *daf-7* expression.

Overexpression of the MAK kinase DYF-5 affects dauer development

Based on the above-described results we propose that regulating the localization of the Gα protein GPA-4 contributes to the decision to become a dauer. We wished to investigate this hypothesis using mutant animals with defects in the IFT machinery that regulates the localization of GPA-4. However, most IFT protein mutants have no cilia, and do not form dauers, because they cannot detect the dauer pheromone. We recently identified the gene mutated in *dyf-5* animals (Chapter V). These animals show excessive entry of IFT and cargo molecules in the cilia, as a result of mutation of a MAK kinase (Chapter V). In contrast, animals that overexpress *dyf-5* (d*yf-5XS*) show reduced entry in the cilia of IFT and cargo molecules, including GPA-4 (Chapter V). Since DYF-5 regulates entry of IFT and cargo molecules in the cilia we wondered if mutations in the *dyf-5* gene affect dauer development. *dyf-5* animals are Daf-d (Starich et al. 1995), like *gpa-3* animals. However, *dyf-5XS* animals show a Daf-c phenotype, very similar to *gpa-3QL* animals (Fig. 2B). In addition, *dyf-5XS* animals showed strongly reduced expression of *daf-7::gfp*, similar to *gpa-3QL* animals (Fig. 1D and E). We suggest that *dyf-5XS* affects *daf-7* expression by reducing entry into the cilia of signalling molecules, including GPA-4.

Dauer pheromone integration

Together the data described in this paper lead us to the model described in figure 6, which is based on the model proposed by Murakami et al. (Murakami et al. 2001). We suggest that one of the mechanisms used to regulate dauer formation is re-localization of various signalling proteins in the cilia. We propose that under normal growth conditions transport of GPA-4 and perhaps other signalling molecules is mediated by both kinesin II and OSM-3 kinesin, delivering GPA-4 into the cilia distal segments. Under these circumstances GPA-4 can activate DAF-11, resulting in cGMP production, ultimately leading to *daf-7* expression and suppression of dauer development. When exposed to dauer pheromone, GPA-3 will be activated and increase loading of GPA-4 and perhaps other cargo onto kinesin II transported particles, thereby blocking transport of GPA-4 by OSM-3 kinesin into the cilia distal segments. The absence of GPA-4 in the distal segments will lead to a decreased DAF-11 activity, resulting in decreased *daf-7* expression and activation of the dauer pathway. A similar effect can be achieved by g.o.f. mutations in DYF-5 or GPA-3, which block transport of GPA-4 into the cilia distal segments

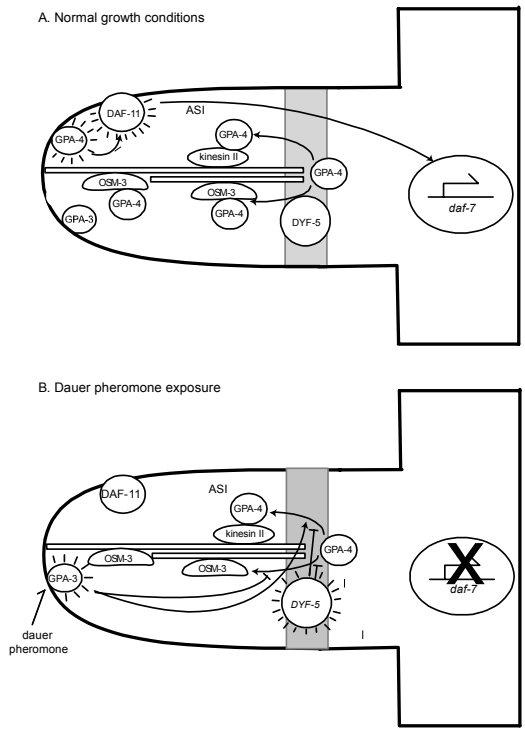

Figure 6. Model for regulation of dauer development by relocalization of signalling molecules in the cilia. We propose (**A**) that under normal growth conditions transport of GPA-4 and perhaps other signalling molecules is mediated by both kinesin II and OSM-3 kinesin, allowing activation of DAF-11, ultimately leading to *daf-7* expression and suppression of dauer development. (**B**) When exposed to dauer pheromone, GPA-3 will be activated and increase loading of GPA-4 and perhaps other cargo onto kinesin II transported particles, and block transport by OSM-3 into the distal segments, leading to a decreased DAF-11 activity and activation of the dauer pathway.

Materials and Methods

Strains and culture conditions

All strains were grown at 20 °C unless stated otherwise. Strains used in this study were: wild type Bristol N2, *gpa-3QL(syIs25)X*, *gpa-3(pk35)V*, *gpa-4(pk381)IV*, *dyf-5(mn400), daf-11(m47)V, daf-7(m62)III*. GFP reporters used: *gpa-4::gfp* (Jansen et al. 1999)*, gpa-15::gfp* (Jansen et al. 1999)*, daf-7::gfp* (Murakami et al. 2001)*, daf-11::gfp* (Birnby et al. 2000).

Creation of transgenic animals

daf-7::gfp, gpa-4::gfp or *gpa-15::gfp* were introduced in the various backgrounds by crossing. To create the *kin-8::daf-7 gpa-3QL* strain we microinjected *gpa-3QL* animals with 50 ng of pK8ETB-daf-7c (gift from Ohshima). *dyf-5XS* animals expressing *gpa-4::gfp* or *daf-7::gfp* were created by injecting 100 ng/µl of an 8kb long range PCR fragment containing *dyf-5* into *daf-7::gfp* or *gpa-4::gfp* expressing animals.

Microscopy

Identification of ciliated neurons expressing GFP was facilitated by Zeiss CLSM510 microscope and Zeiss software. The intensity of DAF-7::GFP was quantified with Zeiss software. Images prepared for quantification were obtained at 63x optics and the brightest focal plane was focused at each animal. At least 25 *gpa-3QL daf-7::gfp* or *dyf-5XS daf-7::gfp* animals were examined. At least 40 *gpa-4 daf-7::gfp* were examined. To measure the DAF-7::GFP fluorescence intensity in wild type and *gpa-3(pk35)* animals exposed to dauer pheromone, we allowed them to grow till late L1 stage, after which they were picked and measured as described above.

Pheromone extract, dauer assay and 8-bromo-cGMP assay

A crude pheromone extract was prepared as described previously (Golden and Riddle 1982), after which the substance was resuspended in distilled water and stored at -20°C. The dauer pheromone was calibrated such that 10 µl gave almost 100% dauer formation in the wild type strain. To test hypersensitivity to dauer pheromone we used 0.5 µl of extract. All dauer assays were performed at 25°C. For each strain presented in this report we performed 6 assays at three different time points. The 8-bromo-cGMP assay was performed as described (Birnby et al. 2000). Briefly, we used 5 mM of 8-bromo-cGMP in NGM agar plates. Fifteen hermaphrodites were picked and allowed to lay eggs at room temperature for up to 3 hours and then removed. The worms were grown at 25°C till the L1 stage after which we measured the *daf-7::gfp* expression. The localization of GPA-4::GFP was determined in L2d larvae after 24 to 28 hours at 25°C.

Acknowledgements

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Chapter

VII

Identification of genes that regulate intraflagellar transport in the sensory cilia of *Caenorhabditis elegans*

Jan Burghoorn, Suzanne Rademakers and Gert Jansen

Summary

The development, maintenance and function of cilia require a specialized transport system, called intraflagellar transport. We recently identified a novel mechanism by which environmental signals regulate the localization of signal transduction molecules in the cilia. This mechanism requires the G protein α subunit GPA-3; a dominant active mutation of GPA-3 (GPA-3QL) causes reduced entry of the Gα proteins GPA-4 and GPA-15 in the cilia, and a defect in the uptake of fluorescent dyes in the cilia (Dyf, dye filling defective). Here we identify several mutants that suppress the *gpa-3QL* dye filling defect. These mutants were identified in a forward genetic screen and based on data from the literature. These approaches thus far identified four genes that probably function downstream of *gpa-3QL*: *sql-1*, *sql-4*, *kin-29* and *qui-1*. *sql-1* encodes for the *C. elegans* homologue of the mammalian GMAP-210, a Golgi microtubule associated protein that plays a role in the assembly and maintenance of the *cis*-Golgi around the centrosome. *sql-4* encodes a protein of unknown function with some homology to a mammalian microtubule associated protein, MAP1. KIN-29 is a protein kinase, distantly related to the family of MARK or EMK kinases, which modulate microtubule organization. QUI-1 encodes for a protein with unknown function; loss-of-function of *qui-1* affects avoidance of several compounds, suggesting that it functions in sensory signaling. Further characterization of the functions of these proteins, and the identification of other *gpa-3QL* suppressor genes will unravel the molecular mechanisms by which environmental signals, transduced via heterotrimeric G proteins, regulate IFT.

Introduction

Cilia have essential functions in cell motility or the detection of environmental cues. Recent studies have revealed that cilia dysfunction can lead to a variety of human disorders including polycystic kidney disease and Bardet–Biedl syndrome (BBS), a syndrome associated with obesity, hypertension, and diabetes (Haycraft et al. 2001; Qin et al. 2001; Blacque et al. 2004; Snell et al. 2004). Since cilia have no protein synthesis, their development, maintenance and function rely on transport of ciliary components into and out of the cilia. This is mediated by a specialized transport system, called intraflagellar transport (IFT) (Rosenbaum and Witman

2002; Scholey 2003; Snell et al. 2004). Kinesin motor complexes transport ciliary precursors and signaling molecules bound to IFT particles from the base of the cilium, called the transition zone, to their site of action (anterograde transport). Dynein motors mediate retrograde transport along the microtubular axoneme, back to the transition zone (Rosenbaum and Witman 2002; Scholey 2003). Although studies in *Chlamydomonas* and *C. elegans* have identified many components of the IFT particles (Fujiwara et al. 1999; Rosenbaum and Witman 2002; Haycraft et al. 2003; Schafer et al. 2003; Scholey 2003), the molecular mechanisms of IFT are not well understood. In addition, there are several indications that IFT is regulated, however not much is known about the signals that mediate this process (Pedersen et al. 2003; Pan et al. 2004; Pedersen et al. 2005).

We use the nematode *Caenorhabditis elegans* to study cilia function, since mutations that lead to abnormal cilia formation or even absence of cilia are not lethal in this model organism, whereas they frequently are in mammals. Moreover, cilia are highly conserved organelles and the structural and functional characteristics of *C. elegans* cilia are very similar to those in mammals (Rosenbaum and Witman 2002; Scholey 2003). In *C. elegans* only 60 sensory neurons have ciliated endings (White 1986), of which 11 pairs of chemosensory neurons in the head are used to sense chemical cues from the environment (Perkins et al. 1986). Most of these amphid cilia are directly exposed to the external environment and will take up lipophilic fluorescent dyes such as DiO (dye filling). In the past, mutant strains have been isolated that showed no dye filling (Dyf, dye filling defective) (Perkins et al. 1986; Starich et al. 1995). Identification of the genes affected in these mutants revealed many components of the IFT machinery (Rosenbaum and Witman 2002; Scholey 2003; Murayama et al. 2005; Ou et al. 2005). Most of these proteins are probably structural components of the IFT particles that facilitate loading of cargo or binding to the motor complexes. Thus far, only very few regulatory proteins have been identified. Functional characterisation of the *bbs-7* and *bbs-8* genes, the *C. elegans* homologues of the human *bbs-7* and *8* genes involved in Bardet–Biedl syndrome, and the *dyf-1* gene has shown that these proteins function in the process that coordinates transport using multiple different anterograde and retrograde motors (Blacque et al. 2004; Ou et al. 2005).

We recently identified a novel mechanism that regulates the localization of signalling molecules in the sensory cilia of *C. elegans*. Exposure of *C. elegans* larvae to dauer pheromone, a continuously secreted compound that can induce development into an alternative developmental stage, the dauer larva, can strongly reduce entry of the Gα subunit GPA-4 into the cilia. This process requires the Gα subunit GPA-3 and the kinesin II motor complex. We have proposed a model in which the dauer pheromone activates the Gα protein GPA-3, which can modulate loading of cargo to either of the two kinesin motor complexes, kinesin II or OSM-3 kinesin. Animals that carry a constitutively active GPA-3 (*gpa-3QL* animals) show a similar reduction of entry of GPA-4 into the cilia. In addition, these animals do not take up fluorescent dyes. Although it is not clear how the sensory neurons take up

fluorescent dyes, we expect that the two processes, dye filling and regulation of IFT, are related. To identify novel proteins that function in the same pathway that regulates IFT as GPA-3 we performed a forward genetic screen for suppressors of the *gpa-3QL* Dyf phenotype. Thus far, this has identified two genes that probably also function in the regulation of IFT, and two unidentified genes that suppress the dye filling defect. In addition, we have shown that two other genes, previously characterized by others, also function in the *gpa-3QL* pathway that regulates IFT.

Results

Genetic screen for suppressors of the *gpa-3QL* Dyf phenotype
We performed a genetic screen to identify mutants that suppressed the Dyf phenotype of *gpa-3QL* animals (Fig. 1).

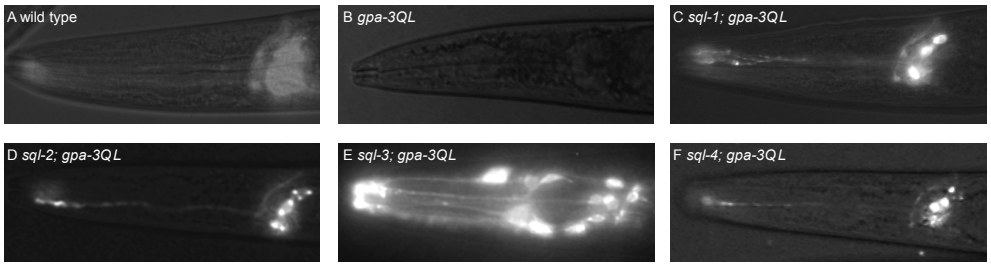

Figure 1. A subset of cilia are exposed to the environment and can take up fluorescence dyes. Dye filling of **A**. Wild type, **B**. *gpa-3QL*, **C**. *sql-1; gpa-3QL* **D**. *sql-2; gpa-3QL*, **E**. *sql-3; gpa-3QL* and **F**. *sql-4; gpa-3QL* animals.

gpa-3QL animals were mutagenized using ethyl methanesulfonate (EMS) to generate random mutations. 100 cultures were started with 12 EMS-treated animals each. Each P_0 animal gave approximately 40 progeny. The F2 and F3 progeny of the mutagenized animals were subjected to dye filling and 12 animals that took up fluorescent dye were individually picked onto new culture dishes. The progeny of these putative suppressor mutants was subjected to dye filling. This procedure identified nine independent suppressor mutants. Using SNP mapping (Wicks et al. 2001) we roughly mapped 8 of these suppressor loci. The mapping suggested that our mutants map to four different loci. Representative mutants for these four loci, called *suppressor of gpa-3QL* (*sql-1* to *sql-4*), were subsequently mapped to regions of approximately 120-500 kb. Two loci are located on the left and right arm of chromosome I, one we have mapped to the right arm of chromosome III and one is positioned at the middle of chromosome IV (Fig. 2A).

sql-1

sql-1(gj202) suppressed both the Dyf phenotype of *gpa-3QL* animals (Fig. 1C) and the effect of *gpa-3QL* on the localization of GPA-15::GFP in the cilia (Fig. 3C). *sql-1* was mapped to a region of approximately 120 kb of chromosome III, which

contains 11 predicted genes (http://www.wormbase.org) (Figure 2B). We performed transgenic rescue to identify the gene mutated in *sql-1*. Micro-injection of a mixture of four long range PCR products containing the predicted genes Y111B2A.4, 5 and 26 (Figure 2B) rescued the dye filling of *sql-1 gpa-3QL* animals, suggesting that these three genes might actually be one. We used RT-PCR to confirm that these three genes are indeed one gene (results not shown). In addition, we identified a mutation in exon 7 of this gene in *sql-1* animals, which introduces an early stop codon, confirming that this gene is *sql-1*. *sql-1* encodes the *C. elegans* homolog of mammalian GMAP-210 (Golgi microtubule-associated protein 210), a 210 kD protein with a very long central coiled-coil domain, an N-terminal domain that binds to Golgi membranes and a C-terminal microtubule binding domain (Infante et al. 1999). GMAP-210 probably functions in the assembly and maintenance of the *cis*-Golgi around the centrosome (Rios et al. 2004). We hypothesize that *C. elegans* SQL-1/GMAP-210 might function in organizing the Golgi around the transition zone, and play a role in the transfer or assembly of IFT particles from the Golgi into the cilia.

sql-2

sql-2(gj804) suppressed the Dyf phenotype of *gpa-3QL* animals (Fig. 1D), but it did not suppress the effect of *gpa-3QL* on the localization of GPA-15::GFP in the cilia (Fig. 3D). *sql-2* was mapped to a region of approximately 500 kb of chromosome I (Fig. 2A). This region contains approximately 60 predicted genes (http://www.wormbase.org) (Fig. 2C). The Y18H1A.6 gene (DNA helicase), located within this region, was described in a genomic study that predicted many new ciliary proteins (Li et al. 2004). We tested whether in *sql-2* animals this DNA helicase gene was affected using transgenic rescue. We injected *gpa-3QL sql-2* animals with a long-range PCR fragment containing the Y18H1A.6 gene. Unfortunately, we found no rescue; transgenic animals showed similar dye filling as non-transgenic *gpa-3QL sql-2* animals. From crosses performed with the *gpa-3QL sql-2* animals it was not clear if the *sql-2* mutation was recessive or dominant. We expect that it would not have been possible to rescue the *sql-2* phenotype, if this mutation was dominant. A possible approach would be to use a similar long range PCR fragment generated using *sql-2* DNA for microinjection into the *gpa-3QL* mutant, and test if this can suppress the dye-filling defect.

sql-3

sql-3(gj823) suppressed both the Dyf phenotype of *gpa-3QL* animals (Fig 1E) and the effect of *gpa-3QL* on the localization of GPA-15::GFP in the cilia (Fig. 3E). *sql-3* was mapped to a region of approximately 200 kb of chromosome IV, which contains approximately 60 predicted genes (http://www.wormbase.org) (Fig. 2A and D). A possible candidate for *sql-3* is the aurora kinase. Members of the aurora family of protein kinases have been implicated in the regulation of several cellular processes dependent on microtubule-containing structures (Adams et al. 2001; Nigg 2001; Dutertre et al. 2002). In addition, the key effector in flagellar

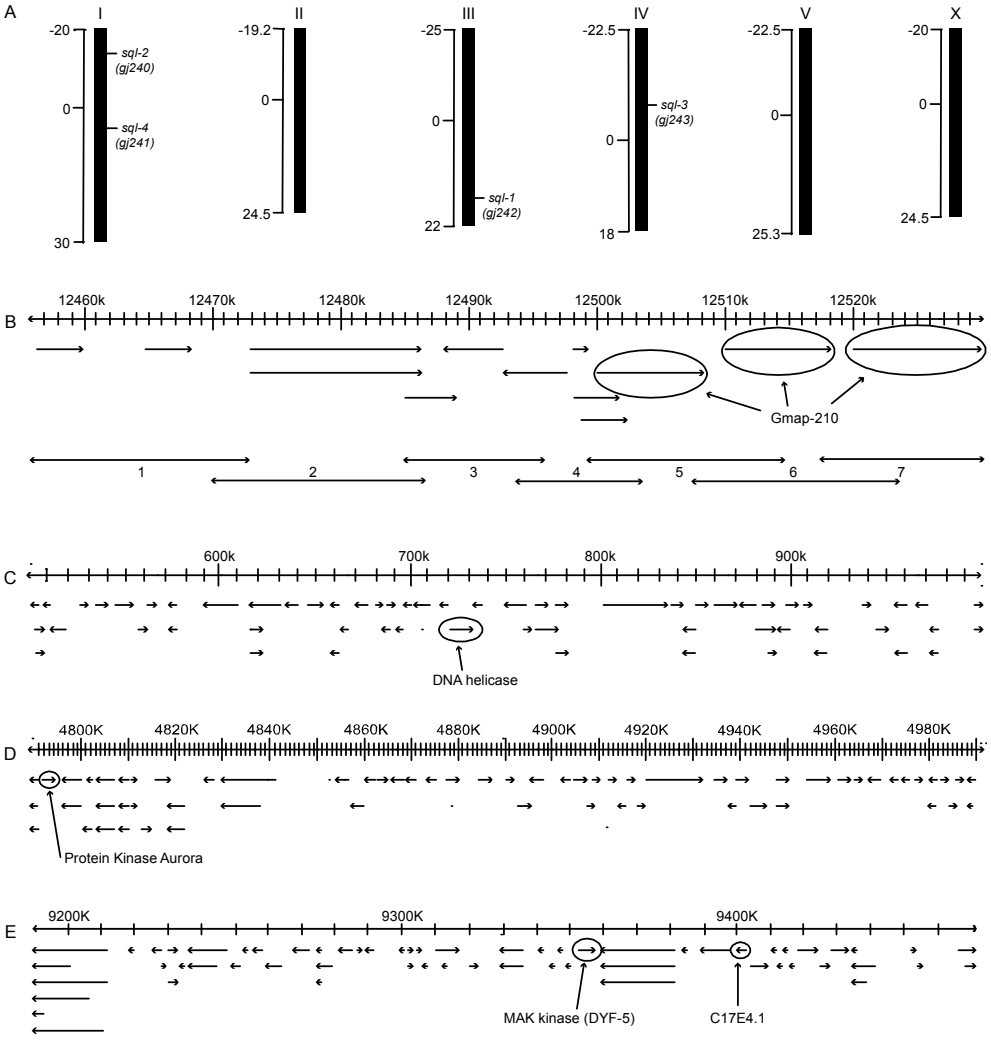

Figure 2. Suppressor mutants of *gpa-3QL*. **A**. Localization of the four mutant genes, *sql-1*, *sql-2*, *sql-3* and *sql-4* on the chromosomes. **B**. *sql-1* maps to a 120 kb region on chromosome III. Predicted gene structures are indicated. The arrows show the location of the different long-range PCR fragments. The three predicted genes Y111B2A.4, 5 and 26 together form SQL-1/GMAP-210 (adapted from www.wormbase.org). **C**. *sql-2* maps to a 500 kb region on chromosome II. A possible candidate gene, a DNA helicase is indicated (adapted from www.wormbase.org). **D**. *sql-3* maps to a 200 kb region on chromosome IV. A possible candidate gene, the protein kinase Aurora, is indicated (adapted from www.wormbase.org). **E**. *sql-4* maps to a 240 kb region of chromosome I. The MAK kinase DYF-5 and the *sql-4* candidate gene C17E4.1 are indicated (adapted from www.wormbase.org).

disassembly in *Chlamydomonas* is the aurora kinase CALK (Pan et al. 2004). *sql-3* is a dominant mutation and thus cannot be rescued using microinjection of wild type long range PCR fragments containing genes from this region of chromosome IV. Alternatively, long-range PCR fragments generated using *sql-3* genomic DNA as template could be microinjected into *gpa-3QL* animals to determine which fragment can suppress the *gpa-3QL* dye filling defect.

sql-4

sql-4(gj201) suppressed both the Dyf phenotype of *gpa-3QL* animals (Fig. 1F) and the effect of *gpa-3QL* on the localization of GPA-15::GFP in the cilia (Fig. 3F).

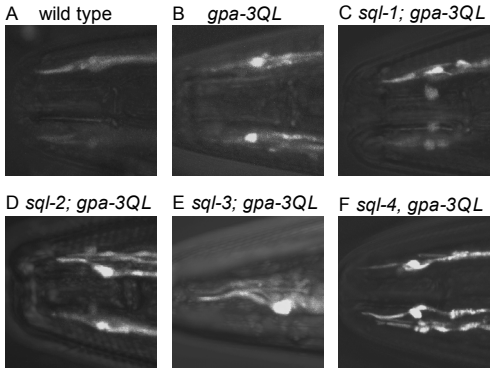

Figure 3. GPA-15::GFP localization in (**A**) wild type, (**B**) *gpa-3QL*, (**C**) *sql-1; gpa-3QL*, (**D**) *sql-2; gpa-3QL*, (**E**) *sql-3; gpa-3QL* and (**F**) *sql-4; gpa-3QL* animals. Full-length cilia can be seen in wild type, *sql-1; gpa-3QL*, *sql-3; gpa-3QL* and *sql-4; gpa-3QL* animals. In *gpa-3QL* and *sql-2; gpa-3QL* GPA-15::GFP staining is restricted to the cilia middle segments.

sql-4 was mapped to a region of approximately 240 kb of chromosome I. This region contains approximately 45 predicted genes (http://www.wormbase.org) (Fig. 2A and E). We performed transgenic rescue to identify the gene mutated in *sql-4* animals. Micro-injection of a long range PCR product containing the gene M04C9.5 rescued the dye filling of *gpa-3QL sql-4* animals, suggesting that *sql-4* might be M04C9.5. However, sequencing of the 8 kb rescuing fragment did not identify a mutation in M04C9.5 in *sql-4* animals. Fortunately, the *dyf-5* mutation was mapped to this same region, and we could identify a mutation in M04C9.5 in these animals (Chapter V). Recently, we found rescue of dye filling after injection of *gpa-3QL sql-4* animals with a long range PCR fragment containing gene C17E4.1. Sequencing of this gene in *sql-4* animals is ongoing. C17E4.1 encodes a protein with some homology to the mammalian microtubule-associated protein MAP1B. However, since the similarity between C17E4.1 and MAP1B is quite low, the function of this protein is unclear.

KIN-29 and QUI-1 act in the gpa-3QL pathway

Various large-scale proteomic and genomic studies have been performed to identify novel IFT factors. We have searched the literature to find possible IFT regulators that we could test in our system. We identified two genes, *kin-29* and *qui-1*, that play a role in the *gpa-3QL* pathway.

C. elegans kin-29 encodes a novel Ser/Thr kinase, which is related to the MARK family of kinases (Lanjuin and Sengupta 2002). It has been shown that the activity of MARK kinases is important for neuronal polarity. Moreover, MARK kinases affect the cytoskeleton by regulating microtubule dynamics (Matenia et al. 2005). Furthermore, the *kin-29* mutation in *C. elegans* affects the dauer pheromone signaling pathway and *kin-29* is expressed in sensory neurons. We generated animals that express *gpa-3QL* under the control of a heat-shock promoter. Induction of *gpa-3QL* expression after heat-shock in wild type animals resulted in a clear dye filling defect, whereas expression of *gpa-3QL* in *kin-29* animals did not affect dye-filling (data shown), suggesting that loss-of-function of *kin-29* suppresses the *gpa-3QL* dye filling defect.

Mutation of *qui-1* affects avoidance of certain repellents (Hilliard et al. 2004). However, since *qui-1* encodes a novel protein its function in avoidance is not known. We tested if mutation of *qui-1* could restore dye filling of *gpa-3QL* animals. Surprisingly, *qui-1* could rescue the dye-filling defect observed in *gpa-3QL* animals. Moreover, the distribution of GPA-15::GFP is affected in *qui-1* animals. We observed that GPA-15::GFP does not localize to the distal tip (Figure 4A).

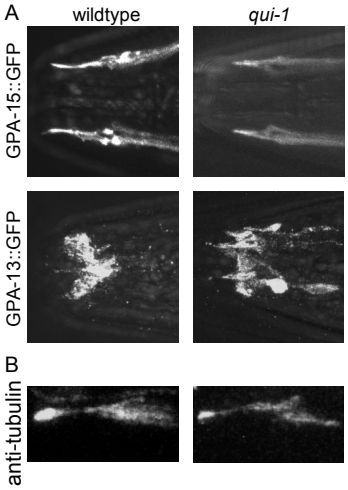

Figure 4. Cilia morphology of *qui-1* animals. **A**. GPA-13::GFP and GPA-15::GFP in cilia of wild type and *qui-1* animals. *qui-1* animals showed staining of GPA-15::GFP only in the cilia middle segments. In wild type animals GPA-13::GFP can be seen in the wing-shaped AWC cilia and, weaker, in the ASH and ADF cilia. In *qui-1* animals GPA-13::GFP accumulates at the transition zone and does not seem to enter the AWC cilia. **B**. Anti tubulin staining of wild type and *qui-1* animals. Both show full-length cilia.

This phenotype was very similar to the phenotype observed in *gpa-3QL* animals. In addition, *qui-1* affects the localization of another Gα subunit, GPA-13::GFP (Figure 4A). However, *qui-1* animals have full-length cilia, since dye-filling and anti-tubulin immunostaining was comparable to wild type animals (Figure 4B, and results not shown). In addition, the IFT dynein subunit XBX-1 and the IFT particle CHE-2 were localized at distal tip of the cilia in *qui-1* animals (data not shown). These data indicate that *qui-1* animals have normal cilia structure and that QUI-1 is involved in the localization of GPA-13 and GPA-15 in the cilia.

Discussion

In this chapter, we describe the identification of several proteins that seem to play a role in the regulation of IFT. We identified four genes, which, when mutated, suppress the dye filling defect of *gpa-3QL* animals and restore the localization of GPA-15::GFP in the distal segments of the sensory cilia of *C. elegans*. In this study, we have merely identified these proteins as components of the machinery that regulates IFT. Extensive functional characterization of each of these proteins will reveal their functions. We expect that these proteins might function in transducing environmental signals to the IFT machinery, the translocation of IFT proteins or cargo from the Golgi or other dendritic compartments to the cilia, gatekeeping at the transition zone or assembly of motor protein – cargo complexes.

Materials and Methods

Strains

All strains were grown at 20°C unless stated otherwise. Wild type animals used in this study were Bristol N2. Strain CB4856 was used for SNP mapping. The following strains were generated in the EMS screen: *sql-1(gj202)III*, *sql-2(gj804)I*, *sql-3(gj823)IV* and *sql-4(gj201)I*. Other strains used: *kin-29(oy38)X, qui-1(gb404) him-8(e1489)IV*, *syIs25[gpa-3QL]X*, *gjIs230[gpa-15::gfp elt-2::gfp]II*, *gjEx244[hsp16-2::gpa-3QL vab-1::rfp]*, *gjIs214[gpa-13::gfp elt-2::gfp]I*. To identify the mutated gene in mutants isolated from the screen, long range PCR fragments were created (Long range PCR kit Qiagen), and injected into the mutants (5 ng), together with *elt-2::gfp* as a marker. If an injected fragment restored the Dyf phenotype of *gpa-3QL,* smaller fragments only containing one gene were created and injected into the mutants.

SNP mapping

gpa-3QL was backcrossed seven times to CB4856 to generate a CB4856 strain with *gpa-3QL* integrated on its X chromosome. This strain was used for SNP mapping of the suppressor mutants of *gpa-3QL* isolated from the EMS screen. We first determined on which chromosomes *sql-1*, *sql-2*, *sql-3* and *sql-4* were located. For more focused mapping the mutants were crossed with the CB4856/*gpa-3QL* strain. Of the F2 progeny 900 singles were assayed for the presence of described SNP marker (Wicks et al. 2001).

Immunofluorescence and microscopy

Animals were fixed, permeabilized and stained with anti tubulin antibodies as described previously (Finney and Ruvkun 1990). Monoclonal rat antibody against tubulin was purchased from Abcam (ab6160). Secondary antibodies were goat-anti-rat alexa-488-conjugated (Molecular Probes). Dye filling was performed using

DiI (Molecular probes) (Perkins et al. 1986). The localization of fluorescent proteins and cilia morphology was examined using a Zeiss confocal microscope CLSM510.

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Summary and Concluding Remarks

Many environmental signals are detected by specialized sensory neurons, which have cilia extending from the cell surface as long appendices and exposed to the environment. Cilia consist of a microtubular axonemal core surrounded by a membrane and are anchored in the cell by the basal body. The end of the basal body and the beginning of the axoneme are called the transition zone.

Since cilia do not have the capacity to synthesize proteins, all components, both structural and signaling molecules, need to be transported into and out of the cilia. This is probably achieved by a process called intraflagellar transport (IFT) and is driven by three motors in *Caenorhabditis elegans*. Two motors, kinesin-II and OSM-3 kinesin, are used for anterograde transport from the base of the cilium to the distal tip. Both these kinesin motor complexes are involved in the transport in the first 4 μm of the cilium, called the middle segment, whereas only OSM-3 kinesin is required for the transport in the last 2,5 μm of the cilium until the distal tip, called the distal segment. Retrograde transport from the distal tip back to the basal body is dependent on the dynein motor complex. IFT is not only responsible for the transport of structural components but recently three signalling molecules have been described to be transported in the cilia i.e. OSM-9, a transient receptor potential vanilloid channel involved in sensory signal transduction, a PKD associated protein called qilin and Smoothened, a plasma membrane protein involved in hedgehog signaling.

In addition to its function in delivering cargo into the cilia, IFT also plays a role in the regulation of cilia length and might play a role in regulating the localization of signaling molecules. There are several long flagella mutants known in *Chlamydomonas,* including *lf-4* a novel MAP kinase belonging to the male germ cell associated kinase family. The flagella of *lf-4* mutants have 1,5 –2 times the normal length and it has been suggested that this MAP kinase could be involved in regulation of IFT.

Environmental signals can affect cilia structure. Changes in cilia structure are imperative in the dauer response of *C. elegans*, which develops under harsh environmental conditions, such as starvation or overcrowding, into an alternative developmental stage, the dauer larva. The nematode arrests its development prior to reproductive maturity, as a specialized L3 diapause form, also called the dauer larva. Upon improvement of the environmental conditions, the dauer larva progresses to a normal L4 larva and resumes its development into a reproductive animal. The decision between dauer versus growth is controlled by at least two endocrine processes: the DAF-2 (insulin/IGF I receptor) and the DAF-7 (TGFβ) pathway. Under dauer inducing conditions the *daf-7* expression is down-regulated, which is regulated by DAF-11, a guanylyl cyclase.

Not much is known about the regulation of the IFT process and how modulation of this pathway could affect other systems. The aim of the work

described in this thesis was to gain more insight into the regulation of IFT and to determine the involvement of G proteins. Previously, it has been described that a dominant active mutation of the Gα subunit GPA-3 (GPA-3QL) influences the uptake of fluorescent dye by the sensory neurons in *C. elegans*. We hypothesized that this dye filling defect observed in *gpa-3QL* animals could be caused by an IFT defect. In Chapter IV, the careful characterization of the *gpa-3QL* animals is described. *gpa-3QL* mutants revealed a mislocalization of specific signaling molecules in the sensory cilia. We found that GPA-3QL interferes with entry into the distal segments of the cilia of three sensory Gα proteins, GPA-4, GPA-9 and GPA-15. GPA-3QL affects the localization of these Gα proteins most likely via the IFT particle OSM-5, the *C. elegans* homologue of the mouse Polaris/Tg737 IFT protein, which is required for the assembly of motor protein - cargo complexes.

Chapter V describes the identification of a novel IFT regulatory factor *dyf-5*, which encodes a predicted serine threonine kinase, homologous to the mammalian male germ cell associated kinase that belongs to a subfamily of mitogen-activated protein kinases. We found that both gain- and loss-of-function of *dyf-5* affects the dye filling ability of *C. elegans*. We observed excess entry of cargo and IFT proteins into the cilia in loss-of-function *dyf-5* animals, and in contrast reduced entry into the cilia in gain-of-function *dyf-5XS* animals. Moreover, we could not detect motility of IFT proteins in *dyf-5* cilia. These data suggest that DYF-5 functions as a gatekeeper that regulates entry of IFT and cargo proteins into the cilia.

In Chapter VI we address the involvement of GPA-3 and DYF-5 in the dauer formation process. We already found that under dauer inducing conditions, the entry into the cilia of the sensory Gα protein GPA-4 is reduced (Chapter IV), similarly to what we observed in *gpa-3QL* animals under normal conditions. Since *gpa-3QL* animals constitutively form dauer larvae (Daf-c phenotype), we surmised that GPA-3 might be involved in the integration of dauer inducing signals. We found that GPA-3 affects dauer formation by regulating the expression of *daf-7/tgf-β*, by decreasing cGMP levels, most likely by inhibiting the guanylyl cyclase DAF-11. Moreover, we showed that loss-of-function of *gpa-4* results in hypersensitivity to dauer pheromone and reduced *daf-7* expression. Together these data suggest that the withdrawal of GPA-4 from the cilia by GPA-3 will inactivate DAF-11, which is an important step during dauer formation. Since we found that *dyf-5XS* animals have a Daf-c phenotype and reduced expression of *daf-7*, we suggest that also DYF-5 inactivates DAF-11 by withdrawal of sensory molecules such as GPA-4, which subsequently will lead to *daf-7* downregulation and the activation of the dauer formation pathway.

In Chapter VII we performed a forward screen for mutations that suppress the dye filling defect in *gpa-3QL* animals, to look for other factors involved in GPA-3 dependent regulation of IFT. Nine independent suppressor mutants of *gpa-3QL* (*sql*) were isolated and mapping suggested mutations in five loci. Two genes are now identified: C17E4.1, which shows homology to mammalian microtubule-associated protein (MAP1B) and the *C. elegans* homolog for the mammalian Golgi

microtubule-associated protein 210 (GMAP210). From a literature study we could identify two more genes *qui-1* and *kin-29*, which also suppress the non dye filling phenotype of GPA-3QL. QUI-1 is a protein of unknown function and KIN-29 shows homology to the microtubule affinity regulating kinase (MARK).

We have therefore identified a novel mechanism that regulates the localization of signaling proteins in the cilia of *C. elegans*, in response to environmental cues, and we have identified several proteins that play a role in this process (Fig. 1). The change in GPA-4 localization observed in larvae exposed to dauer pheromone requires GPA-3 and the kinesin II anterograde motor complex. We propose that the dauer pheromone can activate GPA-3, resulting in blockage of transport of GPA-4 (and perhaps other signaling molecules) into the distal segments of the cilia. We hypothesize that GPA-3 accomplishes the relocalization of GPA-4 from the distal to the middle segments by affecting the assembly of motor protein - cargo complexes, thereby reducing transport by OSM-3 kinesin and/or stimulating transport by kinesin II (Fig.1). GPA-4 is a positive regulator of the DAF-7/TGFβ-pathway, which inhibits the dauer developmental pathway (Chapter VI). Thus, the altered localization of GPA-4 upon exposure to dauer pheromone might contribute to the dauer response and could partially explain the dauer constitutive phenotype of *gpa-3QL* animals.

We have identified four genes that potentially function downstream of *gpa-3* in the regulation IFT, called *sql-4*, *kin-29*, *qui-1* and *sql-1* (Chapter VII). *sql-4* is probably identical to the gene C17E4.1, which codes for a protein that has homology to the mammalian microtubule-associated protein MAP1B. MAPs cover the microtubule and thereby contribute to their stabilization. Moreover, MAPs form a blockage for motors that move along a microtubule. It has been shown that they can even compete with motors for microtubule binding. If SQL-4 would function as a MAP in the sensory cilia of C. elegans one can envisage that loss of SQL-4 could contribute to increased transport into the cilia. KIN-29 encodes a protein kinase with homology to the microtubule affinity regulating kinase (MARK). MARKs can phosphorylate MAPs, resulting in the detachment of MAPs from microtubules. Although the function of KIN-29 in regulating IFT is unclear, it is tempting to speculate that GPA-3 could interfere with the movement of the IFT motor complex along the microtubule, by promoting KIN-29 kinase activity, resulting in removal of MAPs from the microtubular axoneme (Fig. 1).

QUI-1 (Chapter VII) a protein with unknown function was also identified as a possible downstream target of GPA-3. QUI-1 localizes to the transition zone and does not enter the cilia (Carmen Bergamasco and Paolo Bazzicalupo, personal communication). Although these are very preliminary data, we hypothesize that QUI-1 functions at the transition zone by attaching cargo to the IFT complex, or, alternatively, as a cargo adapter for a specific subset of signaling molecules which have to be transported by the IFT complex into the cilia (Fig. 1).

Mutation of *sql-1* (Chapter VII) suppresses the dye filling defect of *gpa-3QL* animals. *sql-1* codes for the *C. elegans* homolog of mammalian GMAP-210 (Golgi microtubule-associated protein 210) . Previous reports suggest that GMAP-210

links the Golgi to the microtubule skeleton and functions in vesicle trafficking between the ER and Golgi. Overexpression of GMAP210 leads to blockage of anterograde and retrograde transport between the ER and the Golgi. It is not clear what function SQL-1/GMAP-210 plays in regulating IFT. It is possible that SQL-1 mediates the translocation of cargo or IFT proteins from the Golgi or the ER to the cilia, and that mutation of *sql-1* reduces transport of proteins downstream of GPA-3 to the cilia, thereby suppressing the effect of *gpa-3QL*. However, more research is required to uncover the link between GPA-3, IFT and SQL-1/GMAP210.

Finally, we have identified the gene mutated in *dyf-5* (Chapter V). DYF-5 encodes a protein kinase homologous to the *Chlamydomonas* LF4 (Berman 2003) and members of the mammalian MAP kinase subfamily of male germ cell associated kinases. Since loss-of-function of *dyf-5* reduces entry of IFT and cargo proteins into the cilia and gain-of-function increases entry of these proteins into the cilia, we propose that DYF-5 functions as a gate keeper that regulates the entry of IFT and cargo proteins into the cilia (Fig. 1). At present, it is unclear if DYF-5 and GPA-3 function in the same or in independent regulatory pathways.

Together, our data suggests that IFT is regulated by at least one, and perhaps two pathways (Fig. 1). These mechanisms render IFT more flexible and provide an additional level of plasticity of the sensory signaling machinery. Further research is necessary to unravel the molecular mechanisms that underlie this regulation and to reveal if similar mechanisms exist in mammals and might contribute to the etiology of cilia related diseases.

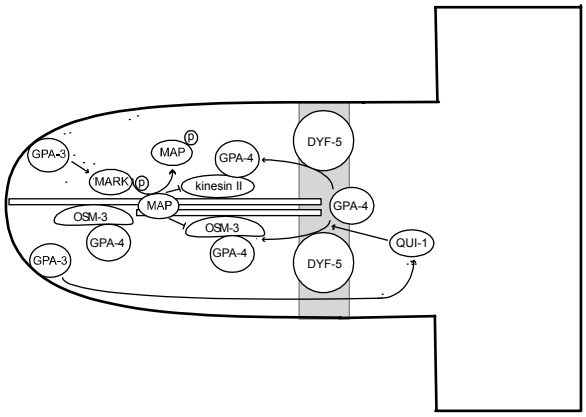

Figure 1. Model for IFT regulation by GPA-3, DYF-5, MARK/KIN-29, MAP/C17E4.1 and QUI-1. GPA-3 influences the entry of GPA-4 into the cilia by affecting QUI-1, which is involved in cargo, such as GPA-4, attachment to OSM-3 kinesin motor. Further regulatory function of GPA-3 is most likely through the MAP/MARK pathway. Unphosphorylated MAPs bind to microtubules, which will form a blockage for IFT motors moving along the microtubules. Upon phosphorylation by MARK, MAP will detach from the microtubules and clear the path for IFT motors. In this way GPA-3 could influence IFT motor motility. DYF-5 functions as a gatekeeper for the entry of IFT particles and cargo into the cilia, independent of GPA-3.

Samenvatting

Cilia zijn kleine uitstulpingen op het celoppervlakte. Ze zijn belangrijk bij de beweging van cellen, zoals bijvoorbeeld bij sperma cellen, maar hebben daarnaast ook een sensorische functie. Wij hebben voor ons cilia onderzoek gekozen voor het model organisme *Caenorhabditis elegans,* aangezien cilia zeer geconserveerd zijn tijdens de evolutie en defecten in cilia niet lethaal zijn in dit organisme in tegenstelling tot vele andere dieren. Alle cilia hebben een vergelijkbare opbouw. Het bestaat uit verschillende buisvormige filamenten omgeven door het celmembraan. Het begin van de cilia wordt de transitie zone genoemd, waarna het eerste deel van de cilia, het middel segment en het uiterste segment volgen. Langs de buisvormige filamenten worden eiwitten vervoerd naar de punt van de cilia via een proces dat intraflagellair transport (IFT) wordt genoemd. Aangezien cilia geen eigen eiwitten kunnen aanmaken, worden structurele, metabolische en signaalverwerkings eiwitten getransporteerd naar de cilia.

C. elegans is een nematode die in de grond leeft en zich voedt met bacteriën en schimmels. Afhankelijk van de temperatuur leeft het ongeveer twee tot drie weken. Een jonge worm gaat door vier larvale fases, L1 tot L4, voordat het volwassen is. Wanneer de omstandigheden niet optimaal zijn door bijvoorbeeld overpopulatie of voedselgebrek, kan *C. elegans* vanuit de L2 fase een alternatieve levensvorm aannemen, genaamd dauer. Een dauer kan vervolgens ongeveer drie maanden overleven. Wanneer de condities verbeteren kan de dauer zich verder ontwikkelen tot een volwassen worm. Een volwassen dier heeft maar 959 cellen, waarvan 60 sensorische zenuwcellen. Dit zijn zenuwcellen die gespecialiseerd zijn in het detecteren van de omgeving. Deze sensorische zenuwcellen hebben cilia aan het uiteinde van hun dendrieten. Deze cilia zijn belangrijk voor het waarnemen van de omgeving. Wanneer de omgeving verandert is het van essentieel belang dat het dier op de juiste manier hierop reageert.

In *C. elegans* wordt IFT aangedreven door drie verschillende motor eiwit complexen, OSM-3 kinesin, Kinesin II en Dynein. Voor het transport van de transitie zone naar het middel segment wordt OSM-3 kinesin en kinesin II gebruikt. Transport van het middel segment naar de punt heeft alleen OSM-3 kinesin nodig. Transport van de punt van de cilia terug naar de transitie zone wordt aangedreven door dynein. Er zijn veel eiwitten bekend die een rol spelen bij IFT. Veelal zijn dit structurele eiwitten. Er zijn diverse aanwijzingen dat IFT gereguleerd wordt. Zo kunnen de lengte van de cilia en de aan- of afwezigheid van bepaalde eiwitten geregeld worden.

Het doel van het onderzoek dat beschreven wordt in dit proefschrift is om meer inzicht te krijgen in de regulatie van IFT. Veel signalen uit de omgeving van een organisme worden bemerkt met behulp van receptoren. Na binding van een ligand aan een receptor wordt dit signaal in de cel veelal doorgegeven met behulp van een cascade van eiwitten, die geactiveerd worden en vervolgens een ander

eiwit activeren. De eerste stap in de cel wordt vaak gevormd door zogenaamde heterotrimere G eiwitten, die direct geactiveerd kunnen worden door receptoren. G eiwitten bestaan uit drie onderdelen, een α, een β en een γ deel. In inactieve vorm binden de 3 delen elkaar en bindt de α subunit GDP. Na activatie splitst het complex zich in een Gα, nu GTP gebonden, en een $\beta\gamma$ deel. Zowel de Gα als de G$\beta\gamma$ kunnen vervolgens andere eiwitten activeren. Van eerder onderzoek was bekend dat mogelijk G eiwitten een rol spelen bij de ontwikkeling of functie van cilia. Wij hebben dit fenomeen verder onderzocht.

Wormen die een altijd actieve vorm van het Gα eiwit GPA-3 (*gpa-3QL* dieren) tot expressie brengen hebben een defect aan hun cilia. Wild type wormen kunnen door de cilia van hun sensorische neuronen fluorescente kleurstoffen opnemen. *gpa-3QL* dieren kunnen dit echter niet meer. Onze hypothese was dan ook dat GPA-3QL de ontwikkeling en/of de functie van de cilia beïnvloedt. Eerst hebben wij onderzocht wat er precies mis is met de ciia van *gpa-3QL* dieren (hoofdstuk IV). Met behulp van diverse technieken hebben we laten zien dat er structureel eigenlijk weinig mis is met de cilia van *gpa-3QL* dieren. Wij vonden echter dat enkele eiwitten die betrokken zijn bij het doorgeven van signalen in cilia niet juist gelokaliseerd zijn in de cilia: GPA-3QL belemmert de toegang tot de distale delen van de cilia van GPA-4, GPA-9 and GPA-15, 3 andere Gα subunits. Waarschijnlijk wordt dit veroorzaakt door een verandering in het IFT proces in de cilia onder invloed van GPA-3QL. In wild type dieren worden GPA-4 en GPA-15 waarschijnlijk getransporteerd door 2 motor eiwit complexen, OSM-3 en kinesin II. Echter, in *gpa-3QL* dieren worden GPA-4 en GPA-15 voornamelijk getransporteerd door kinesin II en niet met behulp van OSM-3. Hierdoor bereiken GPA-4 en GPA-15 niet de distale segmenten van de cilia.

Onze resultaten hebben een nieuw mechanisme geïdentificeerd waarmee *C. elegans* de lokalisatie van signaal transductie eiwitten in de cilia kan regelen. Zo kunnen wormen zich aanpassen aan veranderende omgevingsomstandigheden. Een belangrijk signaal uit de omgeving is het dauer feromoon. Dit feromoon wordt continu door *C. elegans* uitgescheiden, en wordt waarschijnlijk door de worm gebruikt als een maat voor hoeveel wormen er in zijn directe omgeving aanweizg zijn. Bij hoge feromoon concentraties ontwikkelen *C. elegans* larven zich tot dauer dieren, die langer kunnen overleven en zeer sechte omgevingsomstandigheden kunnen overleven. In dauer larven zijn ook de cilia veranderd. Wij hebben gevonden dat het blootstellen van larven aan dauer feromoon tot gevolg heeft dat GPA-4 en GPA-15 niet meer in de distale delen van de cilia terecht komen, eigenlijk net als we hadden gezien in *gpa-3QL* dieren. Deze verandering van de lokalisatie van GPA-4 en GPA-15 is afhankelijk van de aanwezigheid van GPA-3 en van de motor kinesin II. Deze resultaten suggereren dat omgevingsfactoren, in de cel worden doorgegeven door middel van de Gα eiwit GPA-3, die de lokalisatie van signaal transductie moleculen beïnvloedt door IFT te veranderen.

Om uit te zoeken hoe GPA-3QL de lokalisatie van G eiwitten in de cilia beïnvloedt hebben we een genetische screen uitgevoerd. Hierbij hebben we een nieuw eiwit gevonden dat een rol speelt bij de regulatie van IFT, DYF-5 (hoofdstuk

V). Echter, DYF-5 lijkt geen rol te spelen bij het proces dat geregeld wordt door GPA-3QL. DYF-5 codeert voor een serine threonine kinase. Dit kinase is aanwezig in vele organismen die cilia hebben, onder andere in *Chlamydomonas* en in zoogdieren. Mutante *C. elegans* die geen functioneel *dyf-5* gen meer hebben (*dyf-5* dieren) en dieren die veel extra kopiën van het *dyf-5* gen hebben (*dyf-5XS* dieren) hebben problemen met het opnemen van kleurstoffen in de sensorische zenuwcellen. Zowel IFT eiwitten als signaal transductie moleculen hopen zich op in de cilia van *dyf-5* dieren, terwijl we deze eiwitten juist veel minder terugvinden in de cilia van *dyf-5XS* dieren. Deze resultaten suggereren dat DYF-5 een functie heeft bij het reguleren van de toegang van IFT en vracht eiwitten tot de cilia.

Vervolgens hebben we de betrokkenheid van GPA-3 en DYF-5 bij het proces van dauer formatie onderzocht (hoofdstuk VI). Wanneer *C. elegans* wordt blootgesteld aan dauer feromoon, verandert de lokalisatie van GPA-4 en komt het niet meer in de distale delen van de cilia. Dit is vergelijkbaar met wat we in *gpa-3QL* dieren onder normale niet-dauer omstandigheden zien. De resultaten beschreven in hoofdstuk VI suggereren dat GPA-3 GPA-4 onttrekt uit de cilia, waardoor de normale signaal transductie cascade die dauer vorming remt niet meer wordt geactiveerd. Hierdoor wordt de produktie van een groeifactor *daf-7* onderdrukt, en dauer vorming gestimuleerd. Aangezien *dyf-5XS* dieren problemen hebben met het dauer formatie proces en een verlaagde *daf-7* productie hebben, denken we dat ook DYF-5 betrokken is in dit proces.

Tenslotte wordt in hoofdstuk VII beschreven hoe we vier nieuwe genen hebben geïdentificeerd die betrokken zijn bij de regulatie van IFT onder invloed van GPA-3 Mutaties in deze genen herstellen de opname van fluorescente stoffen in de sensorische zenuwcellen van *gpa-3QL* dieren. Deze genen zijn *sql-4*, *sql-1*, *kin-29* en *qui-1*. SQL-4 codeert voor een eiwit dat homologie vertoont met een zoogdieren eiwit dat microtubuli kan binden, MAP1B. SQL-1 is de *C. elegans* homoloog van het zoogdieren eiwit GMAP-210 dat een rol speelt bij transport van het endoplasmatisch reticulum naar het Golgi. KIN-29 is een kinase dat lijkt op kinases die de binding van eiwitten aan microtubuli regelen. De functie van QUI-1 is nog niet bekend. Verder onderzoek is nodig om uit te zoeken wat precies de functie van deze eiwitten is bij de regulatie van IFT.

Curriculum vitae

naam:	Jan Adrianus Burghoorn
geboren:	1 oktober 1971 te Wirdum
1984-1988	M.A.V.O. nylan te Leeuwarden
1988-1990	H.A.V.O. Troelstra scholengemeenschap
1990-1992	V.W.O. Troelstra scholengemeenschap
1992-1998	Biologie, Rijks Universiteit Groningen stages: Moleculaire Genetica, Rijks Universiteit Groningen Groep van Prof.dr D. Dubnau, Public Health Research Institute, te New York (USA).
1998-2000	Medewerker bij Voortplanting en Ontwikkeling, ErasmusMC
2000-2006	Promotieonderzoek, afdeling Celbiologie en Genetica, Erasmus Universiteit Rotterdam. promotor: Prof.dr F.G. Grosveld copromotor: Dr. G. Jansen
2006	Postdoc afdeling Biosciences, Karolinska Institute, te Stockholm Onder supervisie van Dr. P. Swoboda

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