2007

Elucidating the Role of Nephrono Phthisis Proteins Utilizing Caenorhabditis Elegans as a Model

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2007

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ABSTRACT

Numerous disorders are characterized by the presence of cystic lesions within the kidney. The proteins associated with these disorders often localize to cilia, and improper formation or signaling from the cilum has been established as a causative factor leading to cyst formation. In this dissertation my goal was to determine whether the invertebrate nematode *Caenorhabditis elegans* could be utilized as a malleable system to identify pathways involved in human cystic disorders. In particular I wanted to assess the function of two proteins mutated in the human cystic kidney disorder Nephronophthisis, Nephrocystin-1 and Nephrocystin-4. The corresponding genes *nphp1* and *nphp4* were initially selected for study because they both have clear homologs in the nematode, *nph-1* and *nph-4*, respectively. Additionally, both *nph* genes exhibited the presence of X-box sequences in their promoters, suggesting regulation by the X-box binding protein DAF-19, a transcription factor which regulates many cilia specific genes in *C. elegans*. My analysis determined that both *nph-1* and *nph-4* were expressed in ciliated sensory neurons and were indeed regulated by DAF-19. Subsequently, both NPH proteins were shown to co-localize specifically to the transition zone at the base of cilia, a region analogous to the mammalian basal body. Mutants of *nph-1* and *nph-4* were characterized to determine the function they may be playing at this cellular region. Although the cilia in these mutants were formed normally, defects in the cilia-mediated signaling processes of chemotaxis behavior, lifespan regulation, and locomotion in the presence of food were observed. Additionally, the NPH-4 protein was found to be required for NPH-1 protein localization.

Interestingly, a specific Nephrocystin-4 patient mutation was identified that could rescue this localization of NPH-1, but could not rescue the decreased locomotory behavior seen in *nph-4* mutant worms. Locomotory behavior is thought to result from a signal received by the cilia that transmits downstream to EGL-4, the *C. elegans* homolog of cGMP-dependent Protein Kinase G. Analysis of *nph* mutants along with *egl-4* mutants indicated that the EGL-4 protein likely also functions downstream of the NPH proteins. Overall, this dissertation successfully established *C. elegans* as a fruitful model to study Nephronophthisis protein function.

ACKNOWLEDGMENTS

I decided to come to the University of Alabama at Birmingham because I was interested in the research being conducted in the laboratory of Dr. Bradley K. Yoder. Admittedly it was somewhat of a risk to select a graduate program based upon the desire to be part of a lab which I did not know a lot about and had no guarantee that I would be able to join. Fortunately, I was able to become a Yoder lab member and it has been a great experience. I want to thank Brad for all of his help and guidance over the years, he has been a great mentor and I am glad that I selected his laboratory for my graduate studies.

A major reason my experience in the Yoder lab was so enjoyable has been because of my other lab members. I would like to thank the current lab members, Mandy Croyle, Jonathan Lehman, Svetlana Masyukova, Venus Roper, Neeraj Sharma, and Corey Williams and the previous members, Dr. Boglarka Banizs, Dr. Courtney Haycraft, Dr. James Davenport, Dr. Patrick Taulman, Dr. Qihong Zhang, and Kelley Rosborough. Additionally, I must give a special thanks to the previous lab member Dr. Jenny Schafer whose guidance was pivotal to my success as a graduate student. Finally, a note to the "Yoder Lab Clique" (you know who you are), I will miss you all! It just won't be the same playing pass the pigs with anyone else.

Also to my committee members Dr. Kim A. Caldwell, Dr. Creg Darby, Dr. Michael A. Miller, Dr. Erik M. Scwiebert, Dr. Elizabeth S. Sztul, and Dr. Yoder, thank you for all of your helpful suggestions and advice over the years. In the Department of Cell

Biology I would like to thank Rene Eubank, Maxine Rudolph, and Dr. Jim Collawn for their help during my time as a graduate student.

I would like to thank my friends especially, Dr. Kimberly Loesch and Dr. Mita Mookherjee for being my best friends during my time in Birmingham. Thank you to Lindsay Parish for always being available to go catch a movie when I needed to take a break from lab. Thank you to Elisabeth Welty and Becky Smith for listening whenever I needed to vent. Thank you to Molly Moran for her friendship. Thank you to Nich Jones for being a Mappy awesome friend. Also, thank you to my boyfriend Jonathan Hurt for sticking with me during my mood swings while writing this thing.

Finally, I would like to thank my family for their love and support through the years. I especially would like to thank my mom, Mary Bernadette Heekin Winkelbauer, for instilling in me the importance of education which has allowed me to complete my graduate degree.

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LIST OF ABBREVIATIONS

ADPKD	Autosomal Dominant Polycystic Kidney Disease
ARPKD	Autosomal Recessive Polycystic Kidney Disease
BBS	Bardet-Biedl Syndrome
BPK	balb/c polycystic kidney mouse
C. briggsae	*Caenorhabditis briggsae*
cDNA	complementary DNA
C. elegans	*Caenorhabditis elegans*
CI	Chemotaxis Index
CORS	Cerebello-Oculo-Renal Syndrome
CHE	abnormal chemotaxis
CFP	cyan fluorescent protein
DAF	abnormal dauer formation
DNA	deoxyribonucleic acid
DYF	abnormal dye-filling
EGL	abnormal egg laying
EMS	ethyl methanesulfonate
ESRD	end stage renal disease
FPC	Fibrocystin
FUDR	Fluorodeoxyuridine
GFP	green fluorescent protein

GOF	gain of function
IFT	intraflagellar transport
IGF	insulin-like growth factor
INV	inversion of turning
JCPK	juvenile congenital kidney mouse
JS	Joubert Syndrome
LOF	loss of function
LOV	abnormal location of vulva
MDCK	Madin-Darby Canine Kidney
MKS	Meckel-Gruber Syndrome
MTOC	microtubule organizing center
NHD	nephrocystin homology domain
NPH	nephronophthisis
ORPK	Oak Ridge Polycystic Kidney
OFD	Oral-Facial-Digital Syndrome
OSM	osmotic avoidance abnormal
PCK	polycystic kidneys mouse
PCM	pericentriolar material
PCR	polymerase chain reaction
PCY	polycystic kidney disease mouse
PKD	polycystic kidney disease
RFX	regulatory factor binding to the X-box

RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
SH3	src homology 3
TPR	tetratricopeptide
UNC	uncoordinated
YFP	yellow fluorescent protein
WPK	Wistar polycystic kidney rat

INTRODUCTION

Cystic Kidney Diseases

Numerous human disorders are characterized by the formation of cystic lesions within the kidney. These lesions may be acquired throughout the life of the individual or may be the result of a genetic defect. Of particular interest are those diseases which arise via inheritance of mutations, especially Autosomal Dominant Polycystic Kidney Disease (ADPKD), Autosomal Recessive Polycystic Kidney Disease (ARPKD), Bardet-Biedl Syndrome (BBS), Meckel-Gruber Syndrome (MKS), Nephronophthisis (NPH), Oral-Facial-Digital Syndrome (OFD), and Cerebello-Oculo-Renal Syndrome (CORS) (Delous et al., 2007; Guay-Woodford, 2006). Among these disorders there is a high degree of variability relating to mode of inheritance, cyst size, cyst location within the kidney, age of onset, extrarenal manifestations, and the rate of progression leading to onset of End Stage Renal Disease (ESRD) or death resulting from defects in multiple-organ systems. Interestingly, these disorders exhibit allelic or genetic heterogeneity which leads to further phenotypic variation within each disorder.

Autosomal Dominant Polycystic Kidney Disease (ADPKD). ADPKD is inherited in an autosomal dominant manner resulting in a disorder in which symptoms often first appear in adulthood with development of ESRD occurring by the fifth or sixth decade of life or later (Chapman, 2007). The cystic lesions that characterize the disorder are highly variable in size, ranging from millimeters up to 20 centimeters, and are located

throughout the often massively enlarged kidneys (Wilson, 2004). Additionally, a major extrarenal manifestation commonly seen in patients with this disorder is the development of cysts within the liver in 75% of patients by the seventh decade (Rossetti and Harris, 2007). In recent years a great deal has been discovered from research focused on understanding the underlying mechanisms resulting in ADPKD, which is one of the most common human genetic disorders affecting 1 in 400 to 1 in 1000 individuals (Torres et al., 2007). The two defined forms of ADPKD are caused by mutations in the genes *Pkd1* (85% of cases) and *Pkd2* (15% of cases), encoding polycystin-1 and polycystin-2, respectively. It is uncertain whether a 3rd *Pkd* gene may exist that can account for PKD patients in which no mutations in either *Pkd1* or *Pkd2* have been identified.

Patients carrying a mutation in the *Pkd1* gene have a greater degree of disease severity than those exhibiting mutations in *Pkd2*. The *Pkd* gene products Polycystin-1 (PC1) and Polycystin-2 (PC2) are believed to form a complex and function in a common pathway with PC1 acting as a transmembrane receptor interacting with PC2, a calcium permeable ion channel. Interestingly, both of the *Pkd* genes are expressed throughout the renal tubular epithelia and importantly, the corresponding proteins have been localized to cilia as well as to the lateral membranes (Yoder et al., 2002a).

Autosomal Recessive Polycystic Kidney Disease (ARPKD). ARPKD is an autosomal recessive disorder characterized by cystic lesions in the kidney with additional hepatic manifestations. The incidence of this disorder is estimated to be 1 in 20,000 live births (Zerres et al., 1998a; Zerres et al., 1998b). The severity of disease can range from death during the neonatal period to survival into early adulthood. Neonatal death is

mainly associated with respiratory insufficiency due to the presence of enlarged polycystic kidneys and is rarely the result of renal failure. In the event the patient survives infancy, end stage renal disease and liver disease is seen later in childhood.

ARPKD results from mutations in the *PKHD1* gene encoding a large, 4,074 amino acid protein called fibrocystin/polyduction which has been shown to localize to the primary renal cilium with a concentration in the basal body region (Wang et al., 2004). The varying degree of disease severity seen in ARPKD patients is partially explained by allelic heterogeneity. This is largely because there have been over 300 mutations identified in the *PKHD1* gene, which are thought to have variable effects on protein function (Rossetti and Harris, 2007). In addition, the *PKHD1* gene undergoes a very complex splicing pattern that results in numerous different isoforms, which likely also contributes to the variation seen in disease severity (Bergmann et al., 2006).

Bardet-Biedl Syndrome (BBS). BBS is a pleiotropic disorder resulting in a wide variety of phenotypes including, retinal degeneration, obesity, limb abnormalities, learning disabilities, genital abnormalities, and renal dysfunction (Blacque and Leroux, 2006; Katsanis et al., 2001). The renal defects seen in these patients are different from those seen in ARPKD or ADPKD, in that only a small percentage of patients will result in end stage renal disease. The BBS disorder is rare and has been found to occur in only 1:120,000 to 1:160,000 North American and European live births. However, in populations in Newfoundland and Bedouin tribes of Kuwait and Saudi Arabia the incidence is much higher, 1:13,000-1:17,000. Genetic heterogeneity is seen with this disorder resulting from mutations in 12 genes (BBS1 to BBS12), which account for approximately 75%

of BBS cases (Stoetzel et al., 2007). Additional genes are likely to be identified to explain the defects in the remaining 25% of cases. These genes were initially thought to be inherited in an autosomal recessive manner alone. However, recent studies have shown that with regard to some genes, BBS exhibits a complex inheritance pattern in which the inheritance of three mutant alleles at two disease causing loci is required for pathogenicity (Badano et al., 2003). This has been termed triallelic inheritance. Of particular interest, several BBS proteins have been shown to localize to the primary cilium, basal body, or centrosome, indicating a role for these proteins within these structures.

Meckel-Gruber Syndrome (MKS). MKS is an autosomal recessive disorder that is characterized by occipital encepalocele, postaxial polydactyly, ductal plate malformation of the liver, and cystic enlarged kidneys (Alexiev et al., 2006; Consugar et al., 2007). Of the cystic kidney disorders described thus far it is arguably the most severe. Patients with MKS die either in utero or at the latest within a few days after birth as a result of lung and renal failure. As of now three MKS genetic loci have been described, *MKS1* to *MKS3*, that are responsible for this disorder. However, additional loci are expected to be found since these three do not account for all of the known cases of MKS. Recently, the *MKS3* gene was also found to be mutated in patients with Joubert Syndrome type B, which is a cerebellar disorder that is associated with retinal and renal defects (Baala et al., 2007). The gene products of *MKS1* and *MKS3* known as MKS1 and meckelin respectively, localize to the basal bodies and the primary cilium. When these two *MKS* genes are knocked down by RNAi in mammalian cell culture formation of the primary cilium is inhibited (Dawe et al., 2007).

Nephronophthisis (NPH). NPH is an autosomal recessive disorder characterized by a renal histologic triad of tubular basement membrane disintegration, tubular atrophy with cyst development, and interstitial cell infiltration and fibrosis. The cysts formed in this disorder are small and localized to the border between the cortex and the medulla of the kidneys. In contrast to ADPKD, the kidneys in NPH exhibit a normal or slightly reduced size (Hildebrandt and Omram, 2001; Hildebrandt and Otto, 2000). The ensuing disease results in the most frequent genetic cause of ESRD in the first three decades of life. Numerous genetic loci have been identified for NPHP including *NPHP1* encoding Nephrocystin-1 (Otto et al., 2000), *NPHP2* encoding Inversin (Otto et al., 2003), *NPHP3* encoding Nephrocystin-3 (Olbrich et al., 2003), *NPHP4* encoding Nephrocystin-4 (Otto et al., 2002), *NPHP5* encoding Nephrocystin-5 (Otto et al., 2005), and *NPHP6* encoding Nephrocystin-6 (Sayer et al., 2006). Mutations in the *NPHP1* gene are the most prevalent accounting for approximately 25% of patients with NPH. The five remaining NPHP genes each result in mutations in less than 2% of patients indicating that further genetic loci have yet to be discovered (Hildebrandt and Zhou, 2007). In the case of *NPHP1* the most common mutation seen is a large homozygous deletion identified in approximately 80% of patients (Saunier et al., 2000). However, the defects seen in some patients without this deletion have resulted from point mutations causing premature termination or aberrant splicing (Betz et al., 2000; Caridi et al., 2000; Hildebrandt et al., 1997; Saunier et al., 1997). For the other *NPHP* genes, *Inversin* (Otto et al., 2003; Schon et al., 2002), *NPHP3* (Olbrich et al., 2003), *NPHP4* (Hoefele et al., 2005), *NPHP5* (Otto et al., 2005) and *NPHP6* (Sayer et al., 2006) a variety of alleles have been found and include dele-

tions, splice donor site mutations, nonsense mutations, frameshift mutations, and missense mutations.

Each of the six *NPHP* genes corresponds to six different types of NPH, type 1 to 6 respectively. The first three types have been classified in terms of age of onset of ESRD with NPH type 1 being the juvenile form, type 2 being the infantile form, and type 3 being the adolescent form. NPH type 4 is similar to the juvenile form with the causative gene being *NPHP4* instead of *NPHP1*. NPH type 5 and NPH type 6 are merely named as a result of the genes that are mutated in each, *NPHP5* and *NPHP6*. The first four types of NPH have been found to have about 10% of patients presenting with retinitis pigmentosa. However, in NPH type 5 and type 6 all known cases exhibit early-onset of retinitis pigmentosa. This correlation of NPH with retinitis pigmentosa has been termed Senior-Løken Syndrome (SLS). NPH type 1 and NPH type 6 have been shown to be associated with patients having the cerebellar disorder Joubert syndrome type B, similar to what was found for patients with mutations in the Meckel-Gruber gene *MKS3*. Additionally, NPH type 6 has been found to be correlated with the ocular disorder Leber Congenital Amaurosis. The numerous extrarenal manifestations that have been connected to NPH indicate that the *NPHP* gene products are likely to have important functions in multiple organ systems. This is supported by the expression of all of these genes in a variety of tissues in addition to the kidney.

In terms of the NPHP protein localization, initially the protein Nephrocystin-1 was found to be localized to the points of cell-cell and cell-matrix contacts, the adherens junctions and the focal adhesions. More recently the Nephrocystin proteins, with the exception of Nephrocystin-3, have been found to localize to the primary cilium, basal body,

and/or the centrosome. As a result of protein-protein interaction studies it is believed that Nephrocystin-1 through Nephrocystin-4 form a functional complex. Therefore, even though the localization of Nephrocystin-3 has yet to be demonstrated, it is likely that this protein will be found at the same cellular regions where the other Nephrocystins are localized.

Oral-Facial-Digital Syndrome type 1 (OFD1). Oral-facial-digital syndrome is a disorder characterized by defects involving craniofacial regions including, cleft palate, lingual hamartomas, hypodontia, along with various abnormalities of the digits regarding length, positioning, and number. OFD1 belongs to a heterogeneous group of syndromes that is inherited as an autosomal dominantly X-linked disorder that results in lethality in males with an incidence of 1 in 50,000 live births (Thauvin-Robinet et al., 2006). The occurrence of polycystic kidneys is a phenotype commonly seen only in oral-facial-digital syndrome type 1 and not in the other OFD syndromes. Thus far a single genetic locus has been found for the disorder, also termed *Ofd1*. The *Ofd1* gene is expressed ubiquitously in adult tissues and is capable of escaping X-inactivation in humans (de Conciliis et al., 1998). *Ofd1* exhibits allelic heterogeneity in that eighteen mutations have been identified in this gene. Interestingly, in an *Ofd1* knockout mouse heterozygous females were found to develop cystic kidneys and to exhibit defects in cilia formation in these tissues (Ferrante et al., 2006). Also, the OFD1 protein localizes to the centrosome during the cell cycle and to the basal body at the base of cilia in differentiated epithelial cells (Romio et al., 2004).

Cerebello-Oculo-Renal Syndrome (CORS). CORS encompasses a group of disorders that are characterized by defects in the brain, eye, and kidney. Included in this group is the cerebellar disorder Joubert Syndrome (JS) type B. As mentioned earlier, genes that are mutated in both Meckel-Gruber Syndrome and Nephronophthisis have been found to be responsible for the defects seen in JS/CORS patients. Recently, the gene *RPGRIPL1* has been found to be mutated in patients with JS/CORS as well as in MKS patients, indicating that JS/CORS and MKS likely represent a continuum with the same underlying disorder (Delous et al., 2007). Interestingly, the RPGRIPL1 protein has been shown to co-localize with Nephrocystin-4 and Nephrocystin-6 at the cilium basal body and to interact with Nephrocytin-4 (Arts et al., 2007). Taken together these data indicate the likelihood of numerous proteins localized to the cilia basal body including the MKS's, the NPHP's, and possibly the BBS's may be acting together as part of a functional complex. The disruption of this complex is thought to lead to cyst formation within the kidney.

Cilia

Cilia are microtubule based structures comprised of a membrane bound cylinder surrounding a circular arrangement of microtubules which extend out from the basal body (See Figure 1). These organelles can be divided into three different subtypes depending on their microtubule arrangement and their proposed function (Ibanez-Tallon et al., 2003). The most commonly known cilia are the motile cilia which are comprised of a 9+2 microtubule doublet structure with 9 pairs of microtubules surrounding a central pair. This form includes the respiratory cilia in the lungs and those on the ependymal cells lin-

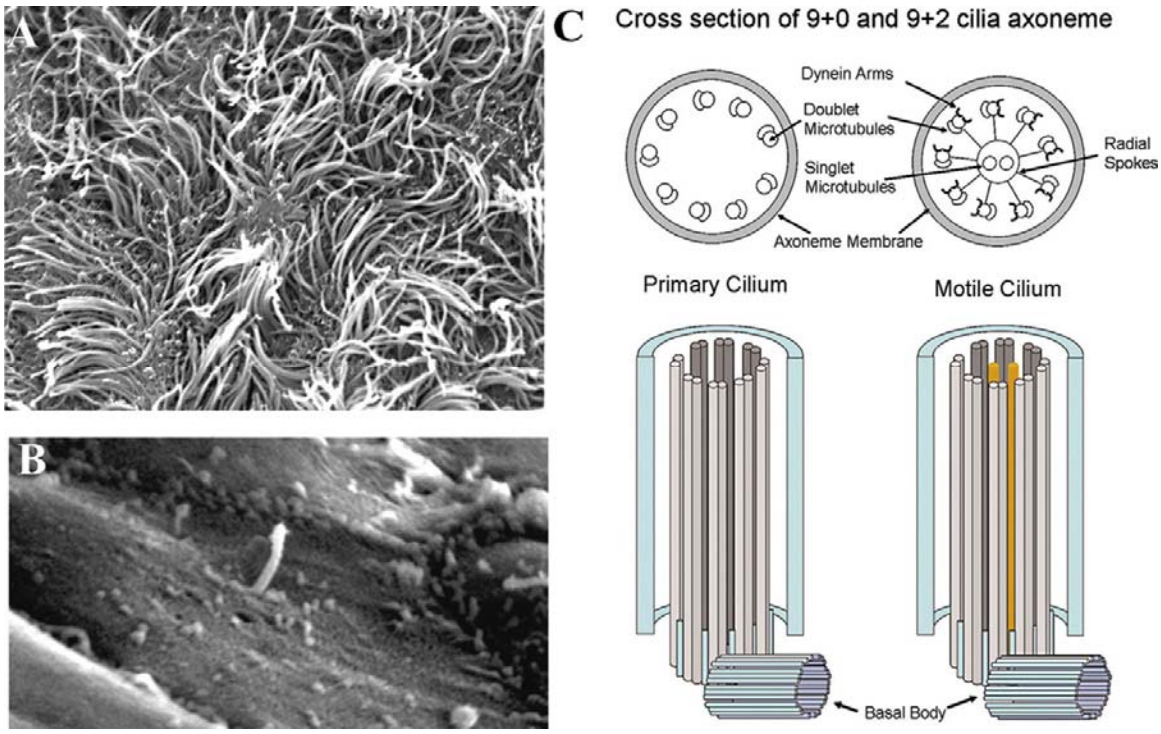

Figure 1. Organization and location of a eukaryotic cilium. (A) Scanning electron microscopic (SEM) image of motile cilia present on wild-type mouse ependymal cells located in the lateral ventricles. (B) SEM image displaying a solitary primary cilium projecting from the surface of an ectodermal cell in the developing limb bud of an embryonic day 10.5 mouse embryo. (C) Structural differences determine the motility of a cilium. Motile cilia (right) consist of nine doublet microtubules surrounding two inner singlet microtubules used to conduct force. Primary cilia (left) are lacking both singlet microtubules and dynein arms.

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ing the ventricles of the brain. The wavelike motility of the 9+2 cilia in these tissues is essential for mucociliary clearance and in cerebrospinal fluid movement. The other two types of cilia are characterized by a 9+0 microtubule arrangement without the central pair of microtubules and are referred to as primary cilia. Cilia of this composition are represented by those on the node, an embryonic signaling center, and those found on most other cells in the mammalian body, including cells lining the nephron. The latter form of primary cilia is thought to be immotile and play a sensory role (Praetorius and Spring, 2001; Praetorius and Spring, 2003). In contrast, the primary node cilia appear to be unique in that while they lack the central pair of microtubules typical of motile cilia, they are capable of a rotational beating pattern. Data indicate that the beating of these cilia play an essential role in specification of the left-right body axis in mice (Nonaka et al., 1998).

The Connection Between Cilia and Cystic Kidney Diseases

The study of a variety of genes in numerous organisms for more than a decade has been pivotal in aiding our understanding of the cause of the cystic lesions seen in polycystic kidney diseases. An overwhelming amount of data has been continually pointing to a role for cilium defects in cyst formation. One of the initial breakthroughs in this field came from the analysis of the Oak Ridge Polycystic Kidney (*Tg737*^{ORPK}) mouse. The *Tg737*^{ORPK} mouse was generated as the result of a random insertional mutation in the *Tg737* gene. Analysis of these mice indicated that the *Tg737*^{ORPK} mutation represents a hypomorphic allele. Homozygous *Tg737*^{ORPK} mutants exhibit a complex phenotype which includes cystic lesions in the kidney that resemble those seen in human ARPKD

(Moyer et al., 1994). The renal cilia in *Tg737*^{ORPK} mice were found to be significantly shorter in length than cilia in wild-type mice (Pazour et al., 2000) as well as in *Tg737*^{ORPK} vs. wild type renal epithelial cells in culture (Yoder et al., 2002b). In contrast to the *Tg737*^{ORPK} mutants, mice with complete disruption of the *Tg737* gene (*Tg737*$^{\Delta 2\text{-}3\beta Gal}$) die in mid-gestation. These mutants have severe neural tube and left-right axis patterning defects. The left-right axis abnormalities have been attributed to the loss of cilia on the ventral node (Murcia et al., 2000). Furthermore, it was determined that the loss of *IFT88* and *osm-5*, the *Chlamydomonas* and *C. elegans* homologs of *Tg737*, result in defects in flagella and cilia, respectively (Haycraft et al., 2001; Pazour et al., 2000).

In addition to the *Tg737* ^{ORPK} mouse, there are numerous other mouse and rat models of Polycystic Kidney Disease (PKD) (Guay-Woodford, 2003). The congenital polycystic kidney (*cpk*) mutant mouse resulted from a spontaneous mutation and was the first model of cystic kidney disease to be described. The gene affected in the *cpk* mouse encodes the protein Cystin which co-localizes to cilia along with IFT88/Polaris, the *Tg737* gene product, in a mouse cortical collecting duct cell line (Hou et al., 2002; Yoder et al., 2002a). The Balb/c polycystic kidney (*bpk*) and the juvenile congenital polycystic kidney (*jcpk*) mouse models are phenotypically variable representing ARPKD and ADPKD models, respectively. Interestingly, the defects in these models result from two different alleles within the same gene, bicaudal C (*Bicc1*) (Cogswell et al., 2003). *Bicc1* is expressed early in development and has been detected in the embryonic node with a similar pattern to *Tg737*. It is important to note that in these mutants the cilia develop normally and have not been found to exhibit the cilia defects seen in the *Tg737* ^{ORPK} model.

Of particular interest are mouse models with mutations in the homologs of genes associated with polycystic kidney disease (PKD) in humans. *Pkd1* and *Pkd2* mutant mice have been developed by targeted mutagenesis strategies. The disruption of each of these genes was found to result in a polycystic kidney phenotype due to the loss of the corresponding proteins, Polycystin-1 and Polycystin-2 (Lu et al., 2001; Wu et al., 1998). Both these proteins have also been shown to co-localize to cilia in a mouse cortical collecting duct cell line (Yoder et al., 2002a). The polycystic kidneys (*pck*) rat model arose through a spontaneous recessive mutation and is considered to represent a phenotypic model of ADPKD. However, the defective gene is the homolog of *PKHD1*, the gene responsible for human ARPKD (Ward et al., 2002). *PKHD1* encodes the protein, Fibrocystin (FPC), which also localizes to cilia, as well as at the basal body in kidney sections and kidney cell lines (Wang et al., 2004). Null mouse models of BBS1, BBS2, BBS4, and BBS6 have thus far been generated by targeted deletion of these genes. The resulting mouse strains recapitulate the human syndrome in numerous characteristics including the renal dysfunction (Mykytyn et al., 2004; Nishimura et al., 2004) (Fath et al., 2005; Kulaga et al., 2004; Ross et al., 2005). The cilia in these models are generally formed normally, suggesting a possible role of these proteins in signaling from the cilia. The Wistar polycystic kidneys (*wpk*) rat model arose as a result of a spontaneous mutation in the MKS3 gene (Smith et al., 2006). This model initially was thought to be characteristic of ARPKD because it exhibited cystic dilatation of the collecting ducts which is known to occur in this disorder. However, the liver defects associated with ARPKD were not evident in this model. The inversion of embryonic turning (*inv*) and the polycystic kidney disease (*pcy*) mouse models result from defects in the genes *NPHP2* and *NPHP3*, respec-

tively. *NPHP2* encodes the protein Inversin, which interacts and co-localizes with Nephrocystin in a punctuated pattern in the cilia of MDCK cells (Otto et al., 2003). Nephrocystin-3, the *NPHP3* gene product, interacts with the protein Nephrocystin as determined by co-immunoprecipitation although its localization in the cilia has not yet been described (Olbrich et al., 2003). Finally, the OFD1 mouse model was generated by a targeted deletion of the *Ofd1* gene (Ferrante et al., 2006). Resulting heterozygous females were found to exhibit cystic kidneys in which cilia formation was defective. These data support that there are a wide-variety of animal models of polycystic kidney disease in which the associated defects are related to cilia assembly or signaling activities.

Further evidence for a role of cilia in cystic kidney disease was obtained from analysis of the *C. elegans* homologs of *Pkd1* and *Pkd2*, *lov-1* and *pkd-2* respectively (Barr et al., 2001). Using GFP translational fusion proteins, both LOV-1 and PKD-2 were detected in the cilia of the sensory rays in the male tail, but not in the hermaphrodites. Analyses of worms with mutations in these genes indicated that they were both required for a sensory role in male mating behavior. Interestingly, double mutants of *lov-1* and *pkd-2* show no difference in phenotype than single mutants suggesting both the resulting proteins function in a common pathway, which is known to be true for the human proteins as well. The cilia in *lov-1* and *pkd-2* mutants in *C. elegans* were shown to be properly formed; therefore, the resulting defects were functional in nature and not the result of improper cilia assembly. Intriguingly, the loss of *osm-5*, the *Tg737* homolog, results in a similar male mating defect. In contrast to *lov-1* and *pkd-2* mutants, this phenotype is due to defective cilia assembly.

Finally, some compelling evidence for the cilia/cystic kidney disease connection has come out of studies in the zebrafish model, *Danio rerio*. An insertional mutagenesis screen performed utilizing zebrafish has identified 12 genes that when mutated result in kidney cysts in this organism (Sun et al., 2004). Two genes found from this screen were *vhnf1* and *pkd2*, which are both known genes mutated in cystic kidney disorders in humans. Interestingly, three additional genes, *hi3417*, *hi409*, and *hi2211* were found to be homologous to genes that encode previously identified proteins important for cilia formation, IFT57, IFT81, and IFT172 respectively. This result directly connects cystic kidneys in zebrafish to defects in proteins important for cilia formation.

In summary, the current data support that defects in cilia are a major contributing factor to the formation of cystic lesions in the kidney. These defects can be either structural or functional. Structural abnormalities, such as those observed in the $Tg737^{ORPK}$ mutants, result from defects in proteins required in proper cilia assembly. A functional defect, as seen in the *pkd1* and *pkd2* mutants, results when a protein important for signaling transmission or signal reception is missing from an otherwise properly assembled cilia. In both scenarios, the resulting loss of proteins required for cilia assembly or cilia sensory function results in ciliary defects which in turn are responsible for cyst formation.

Proposed Function of Renal Cilium

The primary cilium extends off the apical surface of the renal epithelium into the lumen of the tubule. As such, it is ideally positioned to function as a sensory organelle to evaluate fluid in the lumen of the nephron. Data from renal cell culture and in perfused tubules supports the idea that one function may be as a mechanosensor that detects fluid

flow. The bending of this organelle in MDCK cells in culture and in perfused tubules results in an increase in intracellular calcium from both extracellular and intracellular stores (Liu et al., 2005; Praetorius and Spring, 2001). Upon deciliation of the MDCK cells or in perfused tubules from *Tg737*^{ORPK} mutants, the flow mediated intracellular calcium increase was abolished (Liu et al., 2005; Praetorius and Spring, 2003). Finally, it was shown in a mouse embryonic kidney cell line that Polycystin-1 and Polycystin-2 function is required for flow mediated mechanosensation and influx of calcium (Nauli et al., 2003). The exact physiological consequence of this calcium increase is uncertain and it will require further investigation to be defined.

Intraflagellar Transport (IFT)

In the previous sections it was described that improper assembly of cilia is a major factor leading to abnormal cell growth and cyst formation in polycystic kidney disorders. Thus, understanding the mechanisms of cilia assembly will likely provide a better understanding of how the loss of this organelle causes such tremendous changes in kidney morphology and physiology. The mechanism by which cilia are assembled is referred to as Intraflagellar Transport (IFT) was first described in the flagella of *Chlamydomonas* and subsequently in *C. elegans* (Scholey, 2003).

IFT is the process by which cilia and flagella are assembled. These organelles project from cells and must be built up starting at the base and adding to the tip. The assembly, maintenance, and proper function of these structures requires a wide variety of proteins including IFT motors, IFT particles, and potential cargo proteins necessary for structure and function, such as tubulin and the polycystins. Since cilia are devoid of pro-

tein synthesis machinery, these proteins must be generated in the cytoplasm and then be transported to the base of the cilia, referred to as the basal body. Once all the necessary machinery is localized around the basal body, a complex is formed consisting of the IFT motors, kinesin and dynein, the IFT particle, and the cargo. This complex moves along the cilium axoneme by utilizing the kinesin motor (kinesin II complex) to move in the anterograde direction and the dynein motor to move in the retrograde direction (see Figure 2). In *Chlamydomonas* (Cole et al., 1998), *C. elegans* (Tabish et al., 1995), and the mouse (Lin et al., 2003) the motor protein kinesin is required for ciliary assembly, suggesting a high degree of conservation of the IFT process throughout evolution. Biochemical analysis of the IFT particle in *Chlamydomonas* indicates that it can be separated by sucrose gradient fractionation, into two distinct components, referred to as complex A and complex B (Cole et al., 1998). Complex A is thought to be associated with retrograde transport while complex B is thought to be associated with anterograde movement. Interestingly, IFT-88 (Polaris/OSM-5) has been identified as a Complex B protein indicating that the cilia defects are due to abnormalities in the process of anterograde movement along the axoneme.

C. elegans as a Model Organism

The nematode *Caenorhabditis elegans* has many characteristics that make it a very useful model system to study cilia assembly (Riddle, 1997). The worm is easy to maintain in an inexpensive manner by growing on a lawn of *E. coli* on agar plates. It has a three day generation time starting at the embryonic stage, going through four larval molts (L1, L2, L3, and L4), and ending with a fertile adult. Also, the nematode predomi-

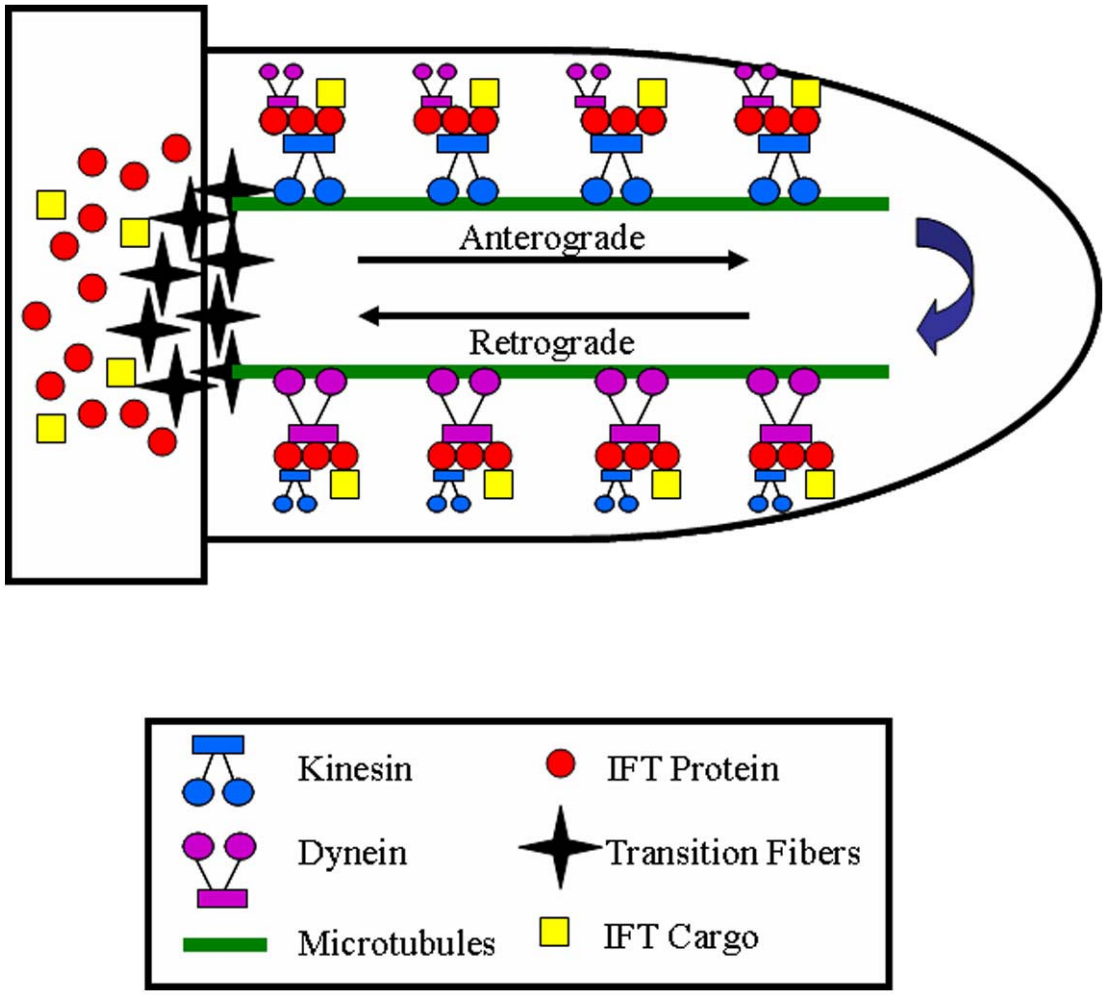

Figure 2. Model of intraflagellar transport (IFT) in a cilium/flagellum. Proteins necessary for cilia formation and maintenance are assembled into the IFT particles (red circles) at the base and transported along microtubules (green lines). The kinesin-II motor complex (blue) transports the IFT particle in the anterograde direction while the dynein motor complex (purple) transports the IFT particle in the retrograde direction. IFT cargo proteins (yellow squares) are carried on the IFT particle. At the tip of the cilium, an uncharacterized switch from kinesin function to dynein function occurs (blue arrow).

Figure and legend used with permission from Proteins involved in cilia formation and function in *Caenorhabditis elegans* University of Alabama at Birmingham Dissertation 2006 Jenny Clayton Schafer

nantly exists as a hermaphrodite that is capable of undergoing self-fertilization producing genetically identical offspring in large quantities. Furthermore, male worms can be generated that allow introduction of new mutations to evaluate potential genetic and epistatic interactions. Another major genetic advantage of the worm is that the genes are compact allowing for the easy identification of promoters. The nematode is transparent and can easily be visualized by light or fluorescence microscopy of live samples immobilized on agar pads or fixed samples. The generation of transgenic strains of *C. elegans* is simply done by the microinjection of the desired construct along with a construct containing a phenotypic marker such as *rol-6* or *unc-122::GFP*. The *rol-6* marker results in a right-handed rolling movement of the transgenic worms that is easily distinguishable from the wild-type sinusoidal movement. When it is necessary to avoid disruption of this normal wild-type movement in the worm a fluorescent marker, such as *unc-122::GFP* can be used. This marker provides bright GFP expression in the coelomocytes without affecting the motility of the worm (Loria et al., 2004). In a transgenic strain the marker and the construct containing the transgene sre maintained as an extrachromosomal array that is passed at variable frequencies to the resulting offspring.

C. elegans, Ciliogenesis, and the Analysis of Cystic Kidney Disease Related Proteins

The nematode *C. elegans* is comprised of just 959 somatic cells, approximately a third of which, 302, make up the nervous system. Of these 302 neurons, 60 exhibit cilia and are collectively referred to as the ciliated sensory neurons. The ciliated sensory neurons are further classified as amphid and labial (inner and outer) neurons found in the head of the nematode as well as the phasmid neurons that are located in the tail (see Fig-

ure 3). At first glance it is not immediately clear the usefulness of studying the roles of cystic kidney disease proteins in *C. elegans*, an organism that does not have a kidney. However, a number of functions of the mammalian kidney are similarly carried out by the ciliated sensory neurons in the worm. One of the major functions of the kidneys is osmoregulation, keeping the solute balance in check. The ciliated sensory neurons of *C. elegans* perform similar functions to sense the external environment and aid the worm to avoid environments that may be detrimental to their survival. Wild-type nematodes in which cilia are properly assembled are able to extend their cilia outward through a pore in the cuticle and examine the external environmental conditions (see Figure 3). The cilia allow the worm to sense the level of food, the osmotic concentrations, the concentrations of chemoattractants and chemorepellants, as well as the presence of a pheromone that is given off in adverse conditions which induces the formation of dauers, an alternative hibernation stage in *C. elegans*. Worms with abnormalities in cilia assembly as well as defects in signaling from the cilium, can be identified by means of numerous phenotypic screens including, dye-filling, osmotic avoidance, dauer formation, chemotaxis, lifespan, male mating behavior, and locomotory behavior.

Dye-filling. In *C. elegans*, the ability of the worm to properly form cilia can be assessed by means of the dye-filling assay. In this simple assay the worms to be tested are rinsed from a plate and are allowed to soak in a solution of a fluorescent dye for 1-2 hours. The worms are then rinsed three times to remove the dye solution and are placed on a plate with a fresh lawn of bacteria. At this point they are observed by fluorescence microscopy for the presence of dye in their sensory neurons. Wild-type worms are able to

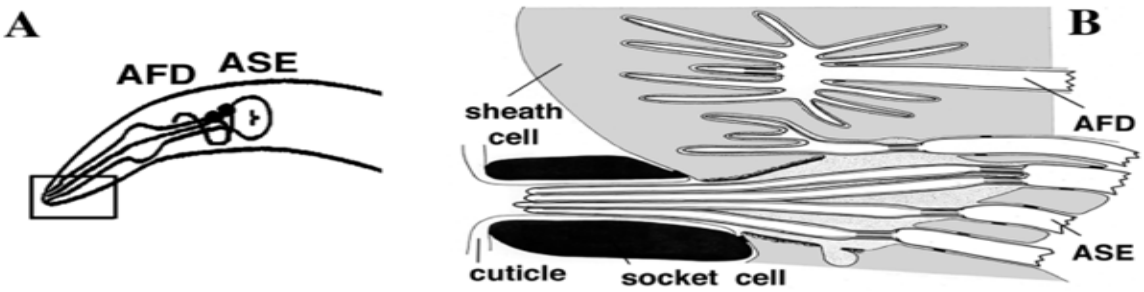

Figure 3. Schematic of amphid ciliated sensory neurons and sensory endings in *C. elegans* (A) The positions in the head of bipolar ciliated sensory neurons ASE and AFD. For the bilateral pairs ASE and AFD of the amphid sensilla, only the left neurons are shown. Cell bodies are dots. The axons of AFD and ASE enter the nerve-ring between the two pharyngeal bulbs, the dendrites extend anteriorly to the tip of the nose. The sensory endings of both neurons are positioned at the tip of the dendrites and are not distinguishable in this drawing. The rectangle depicts the area shown in B. (B) The sensory endings of some amphid sensory neurons (from Perkins et al., 1986). Dendritic tips are shown towards the right. Sensory endings extend from these dendritic tips. The cilium of ASE is exposed to the environment. The microvilli of AFD are encased within the amphid sheath cell. Note the transition zone as the narrowed region at which the dendrite ends and the cilium begins.

Figure and legend used with permission from *Development*. 2001 May;128(9): 1493-505. Haycraft CJ, Swoboda P, Taulman PD, Thomas JH and Yoder BK.

extend their cilia into the external environment allowing them to pull the dye into their neurons. However, cilia mutants are unable to do this and the lack of fluorescent dye in the sensory neurons upon completion of this assay is often a good indication of a defect in proper cilia assembly in *C. elegans*. In a mutagenesis screen performed utilizing this assay 14 mutants were identified and found to have defects in cilia (Perkins et al., 1986).

Osmotic Avoidance. Wild-type *C. elegans* are able to avoid high osmotic concentrations. An assay testing this behavior was developed involving the placement of worms in the center of a ring containing a high osmotic concentration solution such as 4M NaCl (Culotti and Russell, 1978). Worms with properly formed cilia can sense the high osmotic concentration solution and exhibit an avoidance behavior. Cilia formation mutants are unable to sense the high osmolarity and crawl into the solution. Therefore, this assay is also a useful method for identifying mutants with defects in proper cilia formation. Many of the osmotic avoidance defective (*osm*) as well as the dyefilling defective (*dyf*) worms have mutations in proteins required as part of the IFT particle complexes (Cole et al., 1998), including OSM-5 the homolog of IFT88/Polaris.

Dauer Formation. In adverse environmental conditions, such as low food, the worms are able to arrest at the L1 larval stage. Alternatively, they are capable of forming a dauer larva which is arrested between stages L2 and L3. The formation of a dauer occurs due to a decrease in the presence of food, an increase in the presence of dauer pheromone, and an increase in growth temperature (Golden and Riddle, 1984). Dauer larvae are in a state of hibernation with their mouth sealed off from the external environ-

ment and are able to withstand extreme environmental conditions such as treatment with detergent. Upon returning to favorable conditions the dauer larvae resume the lifecycle at the L3 stage. In cilia mutants the ability to properly sense detrimental conditions is diminished and the ability to form dauer larvae is either lost (*daf-d*) or it is constitutively activated (*daf-c*).

Chemotaxis. *C. elegans* exhibit preferences to various chemoattractive stimuli that are sensed through their chemosensory neurons. These attractants can be volatile attractants sensed through the ciliated AWA or AWC neurons such as diacetyl and benzaldehyde respectively (Sengupta et al., 1996; Wes and Bargmann, 2001). To assay chemotaxis behavior in the worm a plate is made in which a chemoattractant is spotted at one end and the control diluent is spotted at the opposite end. A population of worms is placed in the center of the plate precisely between the two spots and the worms are allowed to migrate freely. Over time the percentage of worms capable of reaching the chemoattractant zone (a region within 1.5cm of the attractant) minus the worms found in the control zone, is used to determine a chemotaxis index (CI=the # worms at the chemoattractant zone minus the # of worms at the control zone divided by the total worms in the assay). The CI for wild-type worms is generally over 0.7 with most cilia mutants having chemotaxis indices significantly less than that seen for wild-type worms.

Lifespan. The lifespan of *C. elegans* varies depending on the growth temperature of the worm, in that worms grown at 15°C grow slowly and live longer than worms grown at 25°C. In order to assay lifespan the worms are generally grown at the interme-

diate temperature of 20°C on plates containing fluorodeoxyuridine (FUDR) which allows for the survival of adult worms while inhibiting the survival of their eggs. Worms are observed on a daily basis for survival and are removed from the plate once deceased. In this manner it has been found that many cilia mutants, such as *osm-5*, exhibit increases in lifespan over that seen in wild-type worms (Apfeld and Kenyon, 1999). The signaling pathways that affect lifespan in *C. elegans* involve either signaling through DAF-2, the *C. elegans* homolog of the Insulin/IGF-1 receptor, or through a germ line signaling pathway to a downstream forkhead transcription factor DAF-16. In *daf-2* mutant and germ line ablated worms the lifespan is greatly increased. However, when DAF-16, a transcription factor that is negatively regulated by the DAF-2 signaling pathway, is mutated the life-span is actually shorter than what is normally seen in wild-type worms.

Male Mating Behavior. In *C. elegans* male mating requires functional sensory cilia on the male specific neurons of the tale. To test for defects in this ability a mutant hermaphrodite with motility defects such as an uncoordinated (*unc*) mutant is placed on a lawn of bacteria along with a male being tested for proper mating behavior. The male is observed for 10 minutes for its ability to accomplish the steps involved in mating, 1) Re-sponse to the hermaphrodite, 2) Backing along the hermaphrodite and turning when reaching the head, 3) Location of the Vulva (*lov*), 4) Spicule Insertion, and 5) Sperm Transfer (Liu and Sternberg, 1995). The location of vulva step in this process requires proper signaling from the cilia in the sensory rays of the male tail. As mentioned above in the cilia and cystic kidney disease section, mutants in the *C. elegans* homologs of the *pkd* genes, *lov-1* and *pkd-2* exhibit defects in this process.

Locomotory Behavior in the Presence of Food. *C. elegans* cilium mutants demonstrate abnormal locomotory behavior in the presence of food compared to that seen in wild-type worms (Fujiwara et al., 2002). The movement of a worm placed on a lawn of bacteria is easily observed via the impression it makes in the lawn it is crawling through. To assay this behavior a single worm which has been grown in the constant presence of food is placed in the center of a lawn of bacteria and is left to move freely overnight. After this time the worm is removed from the plate and the tracks left behind are observed. Wild-type worms exhibit a phenotype where they usually traverse a large portion of the area taken up by the bacterial lawn, this behavior is referred to as roaming. In the case of mutants exhibiting defects in cilia, the area covered by the worm is greatly decreased with the mutants often remaining in the same spot for long periods of time, this behavior is known as dwelling. This dwelling phenotype is seen in the cilia mutant, *che-2* whose gene product functions as part of the intraflagellar transport particle complex B and is required for proper cilia assembly. Interestingly, when this mutant is crossed to a mutant of the *C. elegans* homolog of the cGMP dependent protein Kinase G, EGL-4, this dwelling phenotype is suppressed. In fact, these double mutants take on an excessive roaming phenotype that is seen in *egl-4* single mutants, indicating that EGL-4 acts down-stream of or in parallel to CHE-2. This data lends support to the idea that EGL-4 may play a role in signaling from the cilium.

DAF-19 Regulation of Cilia Genes

DAF-19 is the sole *C. elegans* member of the Regulatory Factor binding to an X-box (RFX) family of transcription factors. This transcription factor binds to a conserved

promoter element referred to as an X-box. The X-box motif in *C. elegans* is approximately 14 base pairs in length and is located within 1000 base pairs upstream of the transcriptional start site of responsive genes. The DAF-19 transcription factor is necessary for the expression of some genes involved in cilia formation and cilia signaling function. Furthermore, *daf-19* mutant worms completely lack cilia as well as the transition zones at the base of cilia, a structure that is analogous to basal bodies in mammalian systems (Perkins et al., 1986). A computer based search of the *C. elegans* genome was conducted to identify potential cilia-associated genes based on the presence of the X-box motif within 1000bp upstream of the ATG (Efimenko et al., 2005; Swoboda et al., 2000). As a result of this search approximately 750 candidate cilia genes were identified. Interestingly, several of the genes identified are homologs of genes that are mutated in cystic kidney diseases including, *bbs-1*, *bbs-2*, *bbs-5*, *bbs-7*, *bbs-8*, *xbx-7* (*mks1*), *F35D2.4* (*mks3*), and *nph-4*.

Nephronophthisis Protein Function Model

The cystic kidney disease, NPH, results from mutations of the genes *NPHP1*, *NPHP2*, *NPHP3*, *NPHP4*, *NPHP5*, and *NPHP6* which encode the proteins Nephrocystin-1, Inversin, Nephrocystin-3, Nephrocystin-4, Nephrocystin-5, and Nephrocystin-6 respectively. The genes *NPHP1* and *NPHP4* are mutated in the juvenile form of Nephronophthisis and are of particular interest being that they are evolutionarily conserved across human, mouse, and *C. elegans* (Otto et al., 2002; Otto et al., 2000). The function of the Nephrocystin-4 protein remains elusive. Furthermore, NPHP4 has no predicted structure elements that provide insight into potential function. In contrast, the

mouse Nephrocystin-1 protein consists of three coiled-coil domains, two E-rich domains, and an SH3 protein interaction domain. In mammalian systems, the Nephrocystin-1 protein is thought to function as a novel docking protein and has been shown to interact with $p130^{CAS}$ (Donaldson et al., 2000), Pyk2, tensin (Benzing et al., 2001), filamin A, filamin B (Donaldson et al., 2002), Inversin (Otto et al., 2003), Nephrocystin-3 (Olbrich et al., 2003), and Nephrocystin-4 (Otto et al., 2002), as well as demonstrating self-association (Donaldson et al., 2002). In addition, NPHP1 co-localizes with $p130^{CAS}$ and E-cadherin at adherens junctions of cell-cell contacts (Donaldson et al., 2000) as well as partially co-localizing with Inversin in a punctuated pattern in the cilia of MDCK cells (Otto et al., 2003). Current data suggest that Nephrocystin-1 organizes a protein complex that regulates the actin cytoskeleton of epithelial cells at adhesion sites such as the focal adhesion or adherens junctions (see Figure 4). In support of the function of Nephrocystin-1 in the focal adhesion signaling complex is the fact that a kidney phenotype resembling Nephronophthisis is observed in tensin (a component of focal adhesions) mutant mice (Lo et al., 1997). Also, the suggestion has been made that Nephrocystin-1 plays a role in establishing cell polarity. The interaction of Nephrocystin-1 with Inversin (NPHP2), Nephrocystin-3 and Nephrocystin-4 as well as the co-localization of this protein with Inversin and Nephrocystin-6 in cilia/basal bodies suggests that these five NPH proteins may function in a common pathway, possibly within cilia. It is important to note however, that the interactions between the NPH proteins were determined by co-immunoprecipitation experiments and thus may not be direct, potentially requiring additional proteins.

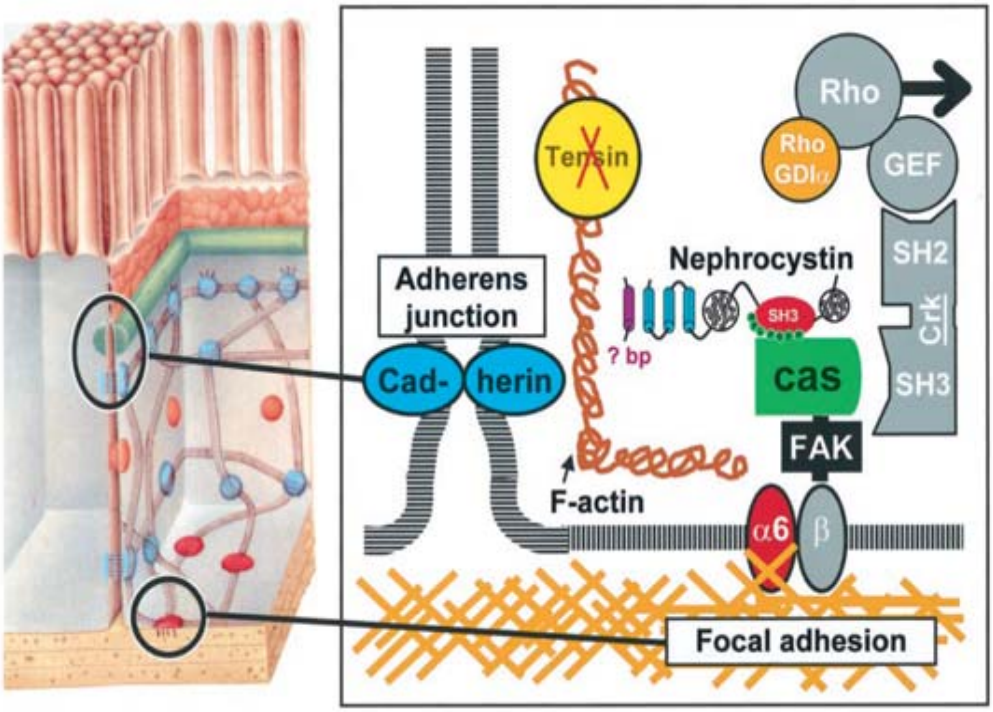

Figure 4. Hypothesis of putative nephrocystin function, stating that nephrocystin might be involved in focal adhesion and/or adherens junction signaling. An illustration of a renal epithelial cell is shown on the left (modified from reference Lodish, 1995); components of adherens junctions and focal adhesions are shown on the right. Adherens junctions of epithelial cells represent E-cadherin-containing cell-cell contacts; focal adhesions represent integrin-containing cell-matrix contacts. Focal adhesions mediate signal transduction from the extracellular matrix to the nucleus. One of the routes is relayed over integrin molecules, focal adhesion kinase (FAK), proteins such as p130cas (cas), adapter proteins such as Crk (containing SH2 and SH3 domains), guanine nucleotide exchange factors (GEF), and small GTPases such as Rho, Rac, or Ras to the nucleus (arrow). Major molecular components of focal adhesion complexes are shaded gray; components with proposed involvement in NPH are shown in color. Nephrocystin might be a component of focal adhesion signaling complexes, because it is a binding partner to and co-localizes with p130cas. It might also be an adherens junction component, because it co-localizes with E-cadherin. The hypothesis, that nephrocystin might play a role in focal adhesions, is emphasized by the fact that the tensin knockout mouse (symbolized by red cross bars over "tensin") exhibits a phenotype very similar to human nephronophthisis, where tensin is an important constituent of focal adhesion signaling complexes.

Figure and portion of legend used with permission from *J Am Soc Nephrol*. 2000 Sep;11(9): 1753-61. Hildebrandt F, Otto E.

Recently, Nephrocystin-1 was shown to localize specifically to the basal body at the base of cilia in respiratory epithelial cells (Schermer et al., 2005). In this study, Nephrocystin-1 was found to be phosphorylated by casein-kinase 2 allowing it to interact with the phoshofurin acidic cluster sorting protein (PACS-1). The binding of Nephrocystin-1 to PACS-1 is thought to be required for the proper localization of Nephrocystin-1 to the base of cilia. The localization of Nephrocystin-1 to the basal body indicates that this region at the base of the cilium may play an important role in cystic kidney disease pathogenesis.

Centrosomes, Basal Bodies, and Transition Zones

Centrosomes act as a microtubule organizing center (MTOC) that consists of two centrioles surrounded by pericentriolar material (PCM). The older "mother" centriole makes up the basal body from which a cilium is formed. As described previously, the proteins required for intraflagellar transport and cilia assembly initially gather around the basal body region. Therefore, the basal body plays an important role in the proper development of cilia. In *C. elegans*, the basal body has been described as somewhat degenerate in nature and has been instead named the transition zone, the region where the distal end of the dendrite transitions into a cilium (See Figure 3). Recent data suggests that defects in this region of the cell in humans may also be linked to cystic kidney disorders. This is evidenced by the fact that several proteins involved in renal cystic disorders associate with either the centrosome or the basal body, including proteins involved in BBS, MKS, NPH, OFD, and CORS. It is tempting to speculate that these proteins may all

function together at this cellular region in common pathways, that when disrupted lead to cyst formation (Guay-Woodford, 2006).

Purpose of Research

At the outset of the studies described in this dissertation we were interested in determining whether *C. elegans* could be developed as a model to study additional proteins which are defective in cystic kidney disorders. Our lab had previously conducted studies characterizing the cilia related functions of *osm-5*, the homolog of *Tg737* which is mutated in the Oak Ridge Polycystic Kidney mouse. The OSM-5 protein was identified as a Complex B component of the IFT particle involved in anterograde transport within the cilium (Haycraft et al., 2001). In addition to these studies our lab characterized another anterograde IFT associated protein, CHE-13, as well as the retrograde IFT associated protein, XBX-1 (Haycraft et al., 2003; Schafer et al., 2003). The study of these proteins along with OSM-5 in *C. elegans* has provided critical information about the wide-ranging effects that structurally defective cilia can cause in this organism. These effects are seen as aberrant phenotypes resulting in defective dye-filling, osmotic avoidance behavior, dauer formation, chemotaxis behavior, lifespan regulation, male mating behavior, and locomotion in the presence of food. In the case of mutants that result in loss of proper cilium structure such as OSM-5, CHE-13, and XBX-1, these phenotypes are often universally affected. Interestingly, a more specific phenotypic effect was identified in the *C. elegans* homologs of the genes mutated in ADPKD, *lov-1* and *pkd-2*. The loss of these genes resulted in male specific defects related to mating behavior (Barr et al., 2001). Importantly, the cilia in these mutants are properly formed and the defective phenotype is

thought to result from the loss of cilia-mediated signaling responses. Due to the wealth of knowledge obtained from studying homologs of cystic disease proteins in this organism we became interested in looking at the Nephronophthisis proteins Nephrocystin-1 and Nephrocystin-4. In this research we found that both of these genes had clear homologs in *C. elegans*, *nph-1* and *nph-4* respectively. Additionally, as a result of a computer based search we determined the *nph-4* gene has an X-box sequence in its promoter. This motif is the binding site for the transcription factor DAF-19 which is known to regulate genes expressed in ciliated sensory neurons. The *nph-1* gene was subsequently found to have a slight variant of the X-box sequence in its promoter as well, hinting at the likelihood that both of the NPH proteins may reside in cilia. Finally, studies in mammalian cell culture indicated that Nephrocystin-1 and Nephrocystin-4 function as part of a common protein complex (Mollet et al., 2002), further adding to our interest in studying these proteins. With these data as an initiating point, I set forth to determine whether *C. elegans* could be developed as a model to study Nephronophthisis protein function. It was hoped that it would provide a tractable system that would provide important insights into the pathogenic mechanisms of this cystic kidney disorder.

THE C. ELEGANS HOMOLOGS OF NEPHROCYSTIN-1 AND NEPHROCYSTIN-4 ARE CILIA TRANSITION ZONE PROTEINS INVOLVED IN CHEMOSENSORY PERCEPTION

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Journal of Cell Science Vol. 118, Issue 23, 5575-5587, December 2005

Format adapted and errata corrected for dissertation

SUMMARY

Nephronophthisis (NPH) is a cystic kidney disorder that causes end-stage renal failure in children. Five nephrocystin (nephrocystin-1 to nephrocystin-5) genes whose function is disrupted in NPH patients have been identified and data indicate they form a complex at cell junctions and focal adhesions. More recently, the nephrocystin proteins have also been identified in cilia, as have multiple other cystic kidney disease related proteins. Significant insights into this cilia and cystic kidney disease connection have come from analyses in simpler eukaryotic organisms such as *C. elegans*. In this regard, we became interested in the *C. elegans* homologs of nephrocystin-1 (*nph-1*) and nephrocystin-4 (*nph-4*) from a database screen to identify genes coordinately regulated by the ciliogenic transcription factor DAF-19. Here we show that expression of *nph-1* and *nph-4* is DAF-19 dependent, that their expression is restricted to ciliated sensory neurons, and that both NPH-1 and NPH-4 concentrate at the transition zones at the base of the cilia, but are not found in the cilium axoneme. In addition, NPH-4 is required for the localization of NPH-1 to this domain. Interestingly, *nph-1* or *nph-4* mutants have no obvious cilia assembly defects; however, they do have abnormalities in cilia mediated sensory functions as evidenced by abnormal chemotaxis and lifespan regulation. Our data suggest that rather than having a ciliogenic role, the NPH proteins play an important function as part of the sensory or signaling machinery of this organelle. These findings suggest that the defects in human NPH patients may not be the result of aberrant ciliogenesis but abnormal cilia-sensory functions.

INTRODUCTION

Nephronophthisis (NPH) is a group of autosomal recessive cystic kidney disorders resulting in chronic renal failure in children. NPH patients share features of renal cyst development at the corticomedullary junction along with irregularities in tubular basement membrane, tubular atrophy, interstitial cell infiltration, and renal fibrosis (Hildebrandt and Omram, 2001; Hildebrandt and Otto, 2000). Mutations in five genes (nephrocystin-1 to 5) have been identified in patients with nephronophthisis (Olbrich et al., 2003; Otto et al., 2002; Otto et al., 2000; Otto et al., 2003) (Otto et al., 2005); however, the function of these proteins and the mechanism by which their disruption leads to the renal pathology remains fundamentally unknown.

The best characterized of the nephrocystin proteins is nephrocystin-1 (Otto et al., 2000). Nephrocystin-1 has several protein interaction modules including N-terminal coiled-coils, a Src homology 3 (SH3) region, and a nephrocystin homology domain (NHD) located in the C-terminal 2/3 of the protein. Data in mammalian systems suggest that nephrocystin-1 functions as a docking protein involved in cytoskeletal organization and cell adhesion regulation at focal adhesions and at cell-to-cell junctions. This is based on binding studies showing that mammalian nephrocystin-1 interacts with adaptor proteins such as proline rich tyrosine kinase 2, p130cas, and actin binding proteins tensin and the filamins (Benzing et al., 2001; Donaldson et al., 2000; Donaldson et al., 2002). Recently, it was demonstrated that mammalian nephrocystin-1 associates with nephrocystin-4 (Mollet et al., 2005). In addition, nephrocystin-2 and nephrocystin-3 are co-immunoprecipitated with nephrocystin-1 antibodies (Olbrich et al., 2003; Otto et al.,

2003), thus, leading to the speculation that these four nephrocystin proteins form a complex and function in the same pathway.

In addition to the localization of nephrocystin-1 to sites of cell contact and the focal adhesions, mammalian nephrocystin-1 and nephrocystin-4 have been detected in primary cilia and basal bodies at the base of cilia. They are thought to bind to β-tubulin, the central component of the cilium axoneme in mammalian cells (Mollet et al., 2005; Otto et al., 2003). In addition, the nephrocystin-4 homolog in *Chlamydomonas* has been shown to be a component of the centriole and the basal body (Keller et al., 2005). Localization to cilia has also been reported for nephrocystin-2 (also known as inversin) and nephrocystin-5 (Otto et al., 2003)(Otto et al., 2005). While nephrocystin-3 has not yet been identified in cilia, the interaction of nephrocystin-3 with the other nephrocystins makes this likely (Olbrich et al., 2003). Thus, the current theory is that the nephrocystins form a multifunctional complex that mediates signaling activity in actin and microtubule based structures; however, the role that this complex plays in these regions of the cell remains unknown.

Cilia are microtubule based organelles that extend off most cells in the mammalian body. They are assembled through an evolutionarily conserved process called intraflagellar transport (IFT) that mediates the anterograde and retrograde movement of protein complexes along the axoneme (Kozminski et al., 1993; Scholey, 2003). Disruption of IFT in mice results in loss of cilia and leads to severe developmental and disease pathologies (Murcia et al., 2000)(Pazour et al., 2000)(Zhang et al., 2004). Intriguingly, many human and murine disorders whose pathology is characterized by renal cyst forma-

tion have now been associated with cilial proteins (Pazour, 2004)(Zhang et al., 2004) thus, highlighting the importance of this organelle in normal renal physiology.

Our understanding of the function of several of the cystic kidney disease related proteins that localize in the cilia has benefited greatly from the analysis of their homologs in simpler eukaryotic organisms such as *Chlamydomonas* and *C. elegans*. For example, the homologs of the cystic kidney disease genes *Tg737* (*osm-5*) (Haycraft et al., 2001), polycystin-1 (*lov-1*), and polycystin-2 (*pkd-2*) (Barr et al., 2001) have been characterized in *C. elegans* and all three of these proteins have been detected in the cilia of sensory neurons. Loss of *lov-1* or *pkd-2* function has no overt effects on the assembly or morphology of the cilia; however, it does impair cilia mediated signaling events required for the male to locate the hermaphrodite vulva resulting in abnormal male mating behavior. In contrast to *lov-1* and *pkd-2*, disruption of *osm-5* results in defects in cilia assembly. Characterization of the OSM-5 protein revealed that it is a key component of the IFT particle (Haycraft et al., 2001; Qin et al., 2001). The OSM-5 protein concentrates at the transition zone at the base of the cilium and in a punctate pattern along the cilium axoneme typical of other IFT proteins. As seen with *C. elegans* lacking *lov-1* or *pkd-2*, *osm-5* mutants exhibit male behavior defects, as do other IFT mutants in *C. elegans*. Thus, the mating defect is likely caused by the loss of cilia on sensory neurons of the male which are required for *lov-1* and *pkd-2* signaling function. In addition to mating abnormalities, *osm-5* and other IFT mutants have defects in chemotaxis, dauer formation, dye-filling, osmotic avoidance, and have extended lifespan, all of which are thought to be caused by loss of cilia and consequently cilia-mediated sensory activity on these neurons (Apfeld and Kenyon, 1999; Starich et al., 1995).

In order to understand the connection between cilia and cystic kidney disease, it is crucial that we identify and characterize additional cystogenic proteins involved in formation and function of cilia. In this regard, we previously described a database genome screen of *C. elegans* and *C. briggsae* to identify components of cilia (Haycraft et al., 2003; Schafer et al., 2003). This search was based on coordinate regulation of many ciliogenic genes by the DAF-19 transcription factor (Efimenko et al., 2005; Swoboda et al., 2000). DAF-19 regulation is mediated through a motif called an X-box that in *C. elegans* is normally located within the first few hundred bases upstream of the start of translation. Among the candidate genes obtained from our search for an X-box in putative promoter regions were many of the previously characterized IFT proteins, and intriguingly the *C. elegans* homologs of nephrocystin-1 (*nph-1*) and nephrocystin-4 (*nph-4)*.

We describe two *C. elegans nph* genes and the NPH proteins and explore their possible function in ciliated sensory neurons. Our results indicate that both *nph-1* and *nph-4* are expressed in ciliated sensory neurons of the male and hermaphrodite in a DAF-19 dependant manner. We show that both NPH-1 and NPH-4 localize to the transition zones at the base of the cilia but not in the cilium axoneme and that NPH-4 is required for correct localization of NPH-1 to this domain. Intriguingly, disruption of either *nph* gene does not affect cilium formation but does result in impaired chemotaxis to volatile attractants and an extended lifespan. These findings along with previous results demonstrating male mating defects in *nph-1;nph-4* double mutants and in animals where *nph-1* or *nph-4* expression was reduced by RNAi suggest a role for the NPH-1 and NPH-4 proteins as part of the general sensory machinery at the transition zone at the base of cilia that is re-

quired for cilia mediated signaling in response to changes in environmental stimuli (Wolf et al., 2005)(Jauregui and Barr, 2005).

MATERIALS AND METHODS

General Molecular Biology Methods

Standard molecular biology procedures were conducted according to Sambrook et al. (Sambrook et al., 1989). *C. elegans* genomic DNA, *C. elegans* cDNA, single worms, and cloned worm DNA were utilized for PCR amplifications, direct sequencing, and sub-cloning as described (Sambrook et al., 1989). PCR conditions and reagents are available on request. DNA sequencing was performed by the UAB Genomics Core Facility of the Heflin Center for Human Genetics.

DNA Sequence Analyses

Genome sequence information was obtained from the National Center for Bio-technology Information (http://www.ncbi.nlm.nih.gov/) or from the Celera Database (http://www.celera.com), Gene sequences were identified using the *C. elegans* database Wormbase and references therein (http://www.wormbase.org). Sequence alignments and conserved motifs were identified using ClustalW (http://www.ebi.ac.uk/clustalw/) and the PileUp, and MotifSearch (MEME) algorithms associated with Wisconsin GCG program (Accelrys,Inc., http://www.accelrys.com/products/gcg_wisconsin_package/program).

A search of the *C. elegans* and the *C. briggsae* genomes was conducted utilizing a computer algorithm to identify potential target genes of the transcription factor DAF-19 as described (Haycraft et al., 2003; Schafer et al., 2003; Swoboda et al., 2000)(Efimenko

et al., 2005). This search involved the analysis of regions upstream of the ATG of predicted genes for the X-box promoter consensus sequence. The search identified a number of previously characterized IFT genes as well as *R13H4.1(nph-4)* in addition to the *C. briggsae* homolog of *M28.7(nph-1)*. Visual inspection of the *C. elegans nph-1* promoter revealed the presence of an X-box sequence that was not identified by the computer based search due to the presence of an extra nucleotide not included in the search algorithm. BLAST and visual inspection were used to identify and compare X-box sequences found in the promoter regions of *C. briggsae* and mouse homologs of *nph-1* and *nph-4*.

Strains

Worm Strains were obtained from the *Caenorhabditis* Genetics Center, *C. elegans* Knock-Out Consortium, and the National BioResource Project in Japan. The strains were grown using standard *C. elegans* growth methods (Brenner, 1974) at 20°C unless otherwise stated. The wild-type stain was N2 Bristol. The following strains were used: RB743 *nph-1(ok500)II*, CB3970 *unc-4(e120)II*; *bli-1(e769)II*, JT6924 *daf-19(m86)II*; *daf-12(sa204)X*, JT204 *daf-12(sa204)X*, DR550 *osm-5(m184)X*, FX925 *nph-4(tm925)V*, and YH278 *nph-1(ok500)II*; *nph-4(tm925)V*. YH192 yhEx119 (*tx: nph-1::CFP*) in N2, YH269 yhEx168 (*tx: nph-4::DsRed2*) in N2, YH238 yhEx150 (*tx: nph-1::CFP*;*tx: che-13::DsRed2*) in JT6924, and YH240 yhEx150 (*tx: nph-1::CFP*;*tx: che-13::DsRed2*) in JT204 were utilized for *nph-1* and *nph-4* expression analyses. YH220 yhEx138 (*tl: nph-1::CFP*;*tl: nph-4::YFP*), YH224 yhEx142 (*tl: nph-4::YFP*;*tl: che-13::CFP*) in N2, YH237 yhEx149 (*tl: nph-1::CFP*;*tl: che-13::YFP*) in N2, YH230 yhEx145 (*tl: nph-4::YFP*) in RB743, YH309 yhEx149 (*tl: nph-1::CFP;tl: che-13 YFP*) in FX925 were

utilized for NPH-1 and NPH-4 protein localization analyses. YH307 yhEx185 (*tl: che-13::YFP*) in YH278 was utilized to measure cilia length in the double *nph* mutant background. YH317 yhEX193 (tl: *nph-1::GFP*) in RB743 and YH331 yhEx202 (tl: *nph-4::YFP*) in FX925 were utilized for rescue experiments. These rescue lines were generated using UNC-122::GFP (Loria et al., 2004) as a marker instead of *rol-6(su1006)* to examine chemotaxis rescue.

The RB743 strain was outcrossed 3 X utilizing the closely linked CB3970 strain. The mutation in the resulting strain was confirmed by PCR. The YH278 double mutant strain was generated by crossing RB743 male worms with FX925 hermaphrodites. The resulting F2 offspring obtained from self fertilization were screened by PCR to identify strains containing both mutations.

Generation of Constructs and Strains

The vectors used for generating the promoter and genomic fusion constructs were modified from pPD95.81 (gift of A. Fire). The pCJF6 vector was created by removing the GFP from pPD95.81 and replacing it with the CFP from pPD134.96 (Haycraft et al., 2003). Similarly, the pCJF7 vector was created by removing GFP from pPD95.81 and replacing it with YFP from pPD132.102. The pCJ102 vector was created by replacing the GFP from pPD95.81 with dsRed2 from pDsRed2 (Clontech, Palo Alto, CA). The transcriptional *nph-1::CFP* vector (pCJ114) and the translational NPH-1::CFP vector (pCJ148) were created by inserting a 2 kb and a 3.6 kb fragment respectively into the pCJF6 vector, each containing 300 bp of the *nph-1* promoter amplified from N2 genomic DNA. A fragment containing 1 kb of the promoter of *nph-4* was generated by PCR from

N2 genomic DNA and was cloned into vector pCJ102 to generate the transcriptional *nph-4::DsRed2* vector (pCJ162). In addition the promoter and the entire *nph-4* gene were cloned into vector pCJF7 to generate the translational NPH-4::YFP fusion construct pCJ146. Owing to the presence of two predicted genes contained in two introns of *nph-4*, the 5' end of *nph-4* including the predicted promoter was amplified from N2 genomic DNA and the 3' end of *nph-4* was amplified from *C. elegans* cDNA. These two fragments were ligated together at the *ClaI* site located at 1729 bp in the cDNA sequence and subsequently were cloned into pCJF7. Additional expression constructs used were the pCJ49.2 transcriptional *che-13::DsRed2* which was previously described (Haycraft et al., 2003), pCJ36.1 translational CHE-13::CFP, and pCJ37.3 translational CHE-13::YFP. The transgenic strains above were generated as described (Mello et al., 1991). All PCR was performed using AccuTaq-LA DNA Polymerase (Sigma, St. Louis, MO) according to manufacturer's instructions.

Imaging

Worms were anesthetized using 10 mM Levamisole and were immobilized on a 2% agar pad for imaging. Imaging was performed using a Nikon Eclipse TE200 inverted microscope and captured with a CoolSnap HQ camera (Photometrics, Tucson, AZ). Shutters and filters were computer driven. Images were processed using Metamorph software (Universal Imaging, Downingtown, PA). Confocal analysis was performed on a Leica DMIRBE inverted epifluorescence / Nomarski microscope outfitted with Leica TCS NT Laser Confocal optics and software (Leica. Inc.; Exton, PA). Optical sections through the Z axis were generated using a stage galvonometer or step motor. Further

processing of images was done using Photoshop 7.0 (Adobe Systems, Inc., San Jose CA).

DAF-19 Regulation

RNA was isolated as described previously (Haycraft et al., 2003) from JT204 and JT6924 mutants. Reverse transcribed RNA(cDNA) was generated using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. PCR was performed using primers that result in amplification of fragments of 250 bp *(nph-1)*, 300 bp *(nph-4)*, and 750bp *(snt-1)*. *snt-1* served as an internal non-DAF-19 regulated control.

To test DAF-19 regulation in vivo, the transgenic line YH238 was generated by injection of *tx: nph-1::CFP* and *tx: che-13::DsRed2* into the JT6924 *daf-19(m86);daf-12(sa204)* mutant background. The YH238 strain was then crossed to the JT204 *daf-12(sa204)* strain resulting in the same extrachromosomal array being present in the *daf-19* (+) background. The strains used contain a mutation in *daf-12(sa204)X* to suppress the Daf-c phenotype of *daf-19(m86)II*.

The X-box sequence of the *nph-4* gene was mutated in the pCJ162 construct by performing site-directed mutagenesis, mutating the first three, the middle two, and the last three bases, generating construct p255. This resulted in the X-box sequence, TAATCC TC GACTTG, mutated from the original X-box sequence, ATTTCC AT GACAAC. This construct consisting of *tx: nph-4(Mut X-box)::DsRed2* was co-injected into N2 worms along with *tx: osm-5::CFP* which contains a functional X-box. Expression was examined in 18 individual F1 Rol offspring.

Assays

Dye-filling using DiI (Molecular Probes, Carlsbad, CA) and osmotic avoidance assays were performed as described previously (Starich et al., 1995).

The ability of *C. elegans* strains to form dauer stages was tested as described previously (Starich et al., 1995). Briefly, mutant and wild-type 'starved' worms were collected and resuspended in 1 ml of 1% SDS solution. The worms were incubated with rocking for 1 hour and washed three times in sterile distilled water. Surviving worms were examined immediately after plating on bacterially seeded plates.

The lifespan assay used was adapted from Dorman et al. (Dorman et al., 1995; Gandhi et al., 1980). Worms were bleached to synchronize the population and the resulting eggs were seeded to fresh NGM plates and incubated at 20°C. The day the eggs were seeded was considered the day of hatching d=0. Upon reaching the L4 stage, the animals were picked to new plates containing 5-fluoro-2'-deoxyuridine (FUDR) (Sigma-Aldrich, St. Louis, MO) to inhibit growth of progeny. The animals were observed daily for survival and dead worms were removed.

Chemotaxis assays to volatile attractants were performed essentially as described previously (Blacque et al., 2004). Briefly, 10 cm chemotaxis plates were made as described (Matsuura et al., 2004). A spot was marked at the center of each plate as well as at opposite sides, 0.5 cm from the edge. A zone was drawn at each side 1.5 cm from each of these spots representing the chemoattractant zone and the control zone. To the spots in each zone 1 µl of 1M sodium azide was added as an anesthetic. In the chemoattractant zone, 1 µl of chemoattractant (diluted 1:100 in 95% ethanol) was added and at the opposite control zone 1ul of 95% ethanol alone was added. Young adult worms (50-150) were

deposited in the center of the plate and were counted at 15 minute intervals over the course of 90 minutes. The efficiency of chemotaxis at each time point was calculated as the chemotaxis index, the number of worms at the chemoattractant zone minus the number of worms at the control zone divided by the total number of worms on the plate.

RT-PCR of *nph* gene expression in *nph-1(ok500)* and *nph-4(tm925)*.

RNA was isolated as described previously (Haycraft et al., 2003) from *nph-1(ok500)* and *nph-4(tm925)* mutants. Reverse transcribed RNA was generated using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. PCR was performed using primers that result in amplification of fragments crossing over the deleted region for each respective mutation and these fragments were sequenced. The results from the RT-PCR analyses of expression from the *nph-1* or *nph-4* mutant loci are included in supplementary data (Fig S2).

RESULTS

***nph-1* and *nph-4* are expressed in ciliated sensory neurons.**

Previously, we described a genome sequence-based search to identify genes involved in ciliogenesis and cilia function in *C. elegans* (Haycraft et al., 2003; Schafer et al., 2003; Swoboda et al., 2000) (Efimenko et al., 2005). Among the candidate genes obtained from this search were several homologs of mammalian genes known to be associated with cystic kidney disorders such as Bardet-Biedl Syndrome (BBS) (Blacque et al., 2004) and *nph-4* that is responsible for the renal pathology in juvenile nephronophthisis patients. The X-box motif in *nph-4* is located at position -168 relative to the translational

start site and is near a consensus sequence with functional X-boxes identified in other genes (Table 1).

In addition to searching the *C. elegans* genome for X-box sequences this search was also conducted in the closely related worm *C. briggsae*. The promoter region of the *C. briggsae* homolog of the *nph-1* gene also contains a strong X-box sequence. Visual inspection of the promoter region of the *C. elegans nph-1* homolog revealed a close match with the consensus X-box located at position -77 in *C. briggsae* relative to the translational start site (Table 1). The putative X-box in the *nph-1* promoter was not identified in the *C. elegans* screen due to the presence of an additional nucleotide in the spacer region between the left and right half-sites of the X-box motif that was not permitted by the parameters of our search algorithm. While less common, a three nucleotide spacer between the two X-box half-sites has been reported in several mammalian genes known to be regulated by the homologs of DAF-19 (Emery et al., 1996).

We also searched the *C. elegans* genome for the homologs of nephrocystin-2, nephrocystin-3, and nephrocystin-5. However, our analysis of the candidates indicated that any homology between *C. elegans* proteins and mammalian nephrocystin-2, nephrocystin-3, and nephrocystin-5 was restricted only to the ankyrin, TPR, or IQ domains respectively. Thus, we do not believe that the *C. elegans* genome encodes homologs of mammalian nephrocystin-2, nephrocystin-3, or nephrocystin-5.

The presence of putative X-box motifs in the promoters of *nph-1* and *nph-4* suggested that they would be expressed in ciliated sensory neurons of *C. elegans* and that further characterization of these genes would provide important insights into the mechanism by which cilia defects result in renal cystic diseases. In contrast to the ubiquitous

Table 1

Species	Gene	Location[a]	X-box Sequence[b]		
C. elegans	*nph-1*(M28.7)	-77 to -63	**GTT**G**CC**	**A**GG	**GG**C**AAC**
C. briggsae	*nph-1*(CBG03043)	-68 to -55	**GTT**G**CC**	**AT**	**GG**T**C**C
Mouse	*nephrocystin-1*	-14 to -1	**GTTTCC**	C**T**	**GA**C**AAC**
C. elegans	*nph-1*(R13h4.1)	-168 to -155	A**TTT**C**C**	**AT**	**GA**C**AAC**
C. briggsae	nph-4(CBG23249)	-186 to -173	A**TTT**C**C**	**AT**	**GG**C**AAC**
Mouse	nephrocystin-4	-231 to -218	**GTC**T**CC**	TA	**GG**T**AAC**
Consensus X-box		(*C. elegans*)	**GTHNYY**	**AT**	**RR**N**AAC**

[a]For C. elegans and C. briggsae these positions are relative to the translational start site. The mouse positions are relative to the start of transcription.

[b]R=G/A; Y=C/T; H=A/T.C; N=G/A/T/C; Bold denotes a match to the C. elegans X-box consensus sequence.

nature of cilia in the mammalian body, cilia are found on only a subset of neurons (60 out of 302) in the head (amphid and labial) and tail (phasmid) of the *C. elegans* hermaphrodite, with the male containing an additional 52 ciliated neurons (Ward et al., 1975; Ware R. et al., 1975; White et al., 1986). To determine the expression patterns of *nph-1* and *nph-4*, we generated transcriptional fusion constructs consisting of the promoter region (300 bp up from the ATG) of *nph-1* fused with CFP (*nph-1::CFP*) or the promoter (1000 bp up from the ATG) of *nph-4* fused with DsRed2 (*nph-4::DsRed2*). Transgenic lines expressing these constructs were generated and the resulting expression patterns were compared to those of two known IFT genes (*osm-5* and *che-13*) (Haycraft et al., 2003; Haycraft et al., 2001). In agreement with the presence of an X-box motif, *nph-1* and *nph-4* expression was detected in most ciliated sensory neurons in the hermaphrodite (Fig. 1 and 2). There was no expression of *nph-1* or *nph-4* evident in non-ciliated cell types. Overall, the expression pattern of *nph-1* was similar to that of the IFT genes (Fig. 2C). In the case of *nph-4*, we were able to generate only a single stable transgenic line. Analysis of this line shows *nph-4* expression in a subset of ciliated sensory neurons in the head of the worm as well as in most of the phasmid neurons in the tail. We do not believe that these data reflect the expression of the endogenous *nph-4* gene. This is based on the analysis of NPH-4 protein localization showing that it is expressed in a much broader spectrum of the ciliated neurons than seen using the transcriptional fusions and on the recently published data by Wolf et al. indicating *nph-4* expression in most of the amphid and labial neurons (Wolf et al., 2005). The reason for this difference in expression and the difficulties in generating these stable lines is not known.

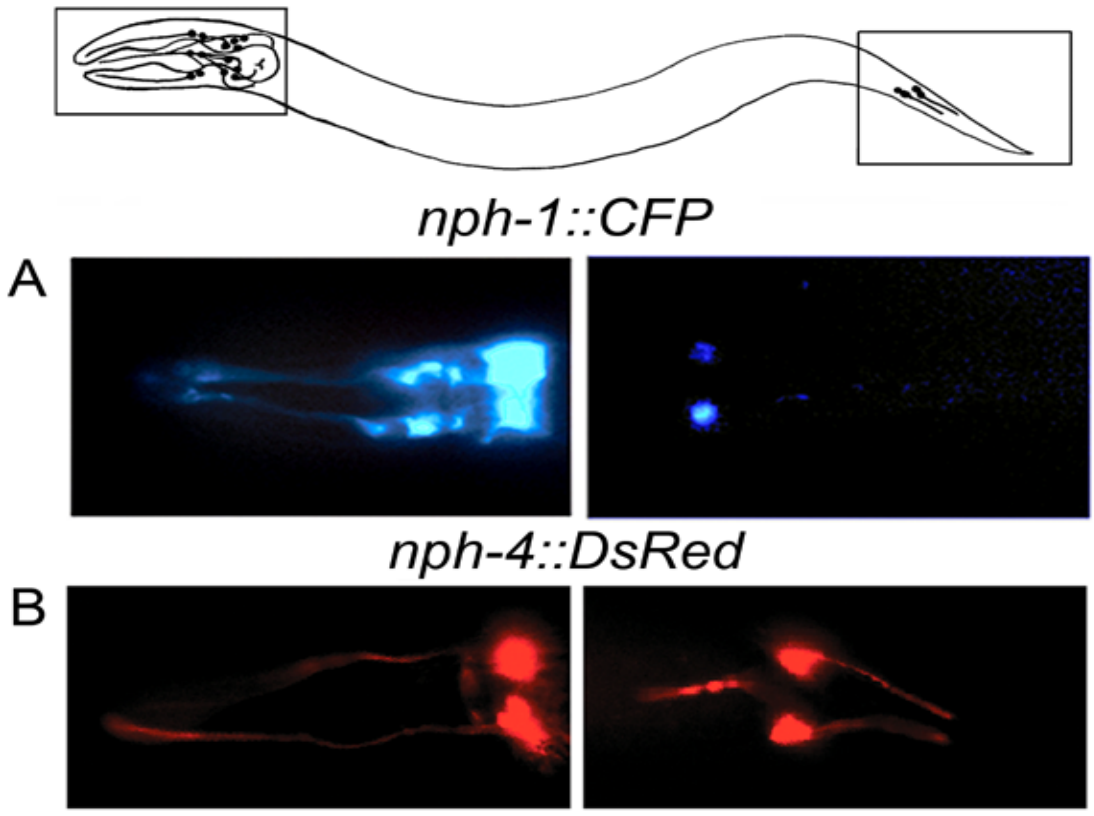


Figure 1. Expression of *nph-1* and *nph-4* in *C. elegans*.
(Top) Diagram showing the position of the neurons examined in A and B. (A) *nph-1::CFP* (L1 Stage) and (B) *nph-4::DsRed2* (adult) are expressed in the ciliated sensory neurons of the worm including the amphid and labial neurons in the head (left panels) as well as the phasmid neurons in the tail (right panels). In this figure and all following figures, anterior is toward the left.

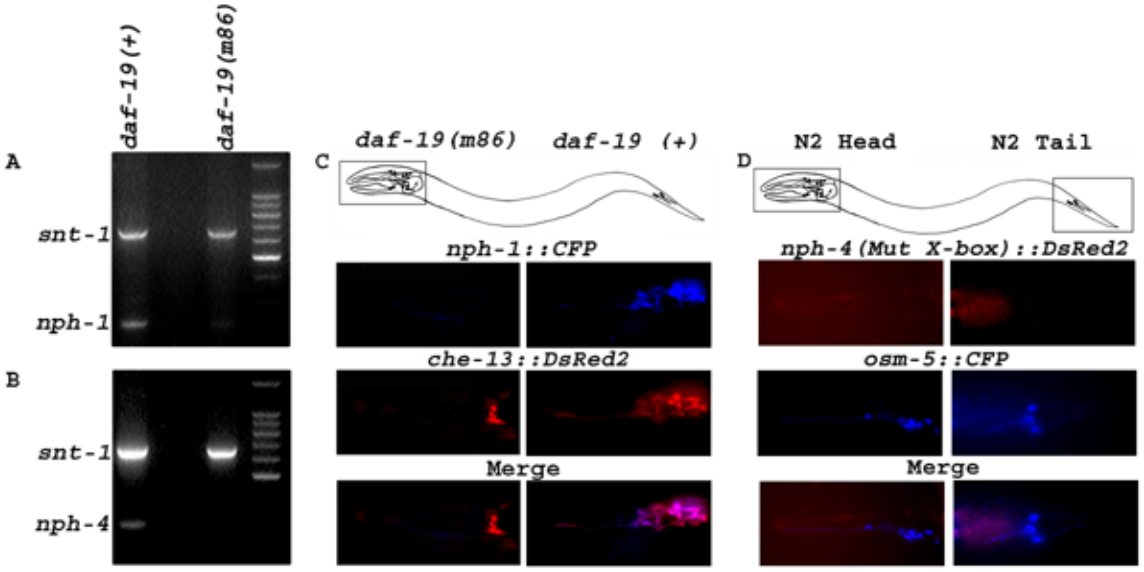


Figure 2. DAF-19 regulation of *nph-1* and *nph-4*.
RT-PCR analysis of (A) *nph-1* and (B) *nph-4* showed a marked decrease in expression of both genes in the *daf-19(m86)* mutant background compared to that seen in the *daf-19(+)* background. Synaptotagmin (*snt-1*) was used as a control for a neuronal gene whose expression is DAF-19 independent. (C) *In vivo* expression analysis of *nph-1::CFP* in *daf-19(m86)* mutant (left panels) versus *daf-19(+)* (right panels) backgrounds. *nph-1::CFP* expression was greatly diminished in the *daf-19(m86)* background compared to *daf-19(+)*. A similar reduction in expression is seen for *che-13::DsRed2*, a gene encoding an IFT protein and is known to be regulated by DAF-19. The merge of *nph-1::CFP* and *che-13::DsRed2* expression in the *daf-19 (+)* background shows that both of these genes are expressed in the same cells. (D) *In vivo* expression analysis in wild type worms using the *nph-4(mut)::DsRed* transgene in which the X-box has been mutated. Mutation of the X-box results in the loss of *nph-4* expression in the ciliated sensory neurons relative to the same *nph-4* transgene with a wild type X box (see Fig. 1B) or the IFT gene *osm-5::CFP* with wild-type X-box.

DAF-19 regulates expression of *nph-1* and *nph-4*

To assess the importance of the X-box sequences in the *nph-1* and *nph-4* promoters, we evaluated whether a similar motif was present in the homologs of these genes in other ciliated organisms. In *C. briggsae* a consensus X-box was present in the putative promoter regions of both *nph-1* and *nph-4*. The X-box motifs were located in nearly the identical positions relative to the start of translation of these genes in both organisms (Table 1). Comparison of the promoter regions outside of the X-box sequence failed to show a high degree of conservation. Similarly, in the promoter regions of the mouse *nph-1* and *nph-4* homologs, a near consensus X-box sequence was identified located at position -14 for nephrocystin-1 and -231 for nephrocystin-4 relative to the start of transcription. Together these data suggest an evolutionarily conserved transcriptional regulatory mechanism controlling the expression of genes involved in cilia assembly and function that includes many of the IFT genes, several BBS genes, and now two genes involved in NPH (Efimenko et al., 2005).

To explore the potential importance of the X-box, we compared *nph-1* and *nph-4* expression levels in *daf-19(m86)* mutant and *daf-19*(+) backgrounds. This analysis was conducted using transgenic lines expressing the transcriptional fusion constructs and by semi-quantitative RT-PCR to determine the effect on expression of the endogenous genes. RT-PCR analyses showed a significant decrease, but not complete abolition, of *nph-1* and *nph-4* expression in *daf-19(m86)* mutant versus *daf-19(+)* backgrounds (Fig. 2A,B) while the expression level of the non-DAF-19 regulated neuronal gene synaptotagmin (*snt-1*) remained unchanged. Similarly, we detected a marked reduction in the expression of *nph-1::CFP* due to the loss of DAF-19 in transgenic worms (Fig. 2C). These

results paralleled that of *che-13*, a known target for DAF-19 regulation (Haycraft et al., 2003). Finally, we generated transgenic worms that carry a mixed extrachromosomal array consisting of a *nph-4::DsRed2* transgene with a mutated X-box sequence and an *osm-5::CFP* transgene with a wild type X-box. In contrast to the *osm-5::CFP* which is expressed in ciliated sensory neurons, expression from the *nph-4(mut)::DsRed2* construct with the mutated X-box was not evident in any of the independent worms analyzed (n=18, Fig. 2D).

NPH-1 and NPH-4 localize to the base of cilia.

Many of the genes whose expression is regulated by DAF-19 have been found to encode proteins that localize to cilia and are often involved in IFT and/or ciliogenesis. To determine if the *C. elegans* nephrocystins are cilia associated proteins, we generated transgenic lines that co-express NPH-1 and NPH-4 as translational fusions with CFP and YFP respectively. Analysis of multiple independent lines showed that NPH-1 and NPH-4 colocalize at the distal end of the dendrites of most, if not all, amphid, labial, and phasmid neurons of the hermaphrodite and the sensory rays of the male tail (Fig. 3A and data not shown). This localization corresponds to the transition zone (analogous to the mammalian basal body) at the base of cilia as revealed by the analysis of transgenic worms coexpressing the NPH proteins along with the IFT protein CHE-13 (Fig. 3B, C, and supplementary material, Fig. S1) (Haycraft et al., 2003). The transition zone is thought to be a site where cilia proteins concentrate and assemble into complexes prior to being transported into the cilium axoneme (Scholey, 2003). However, in contrast to the

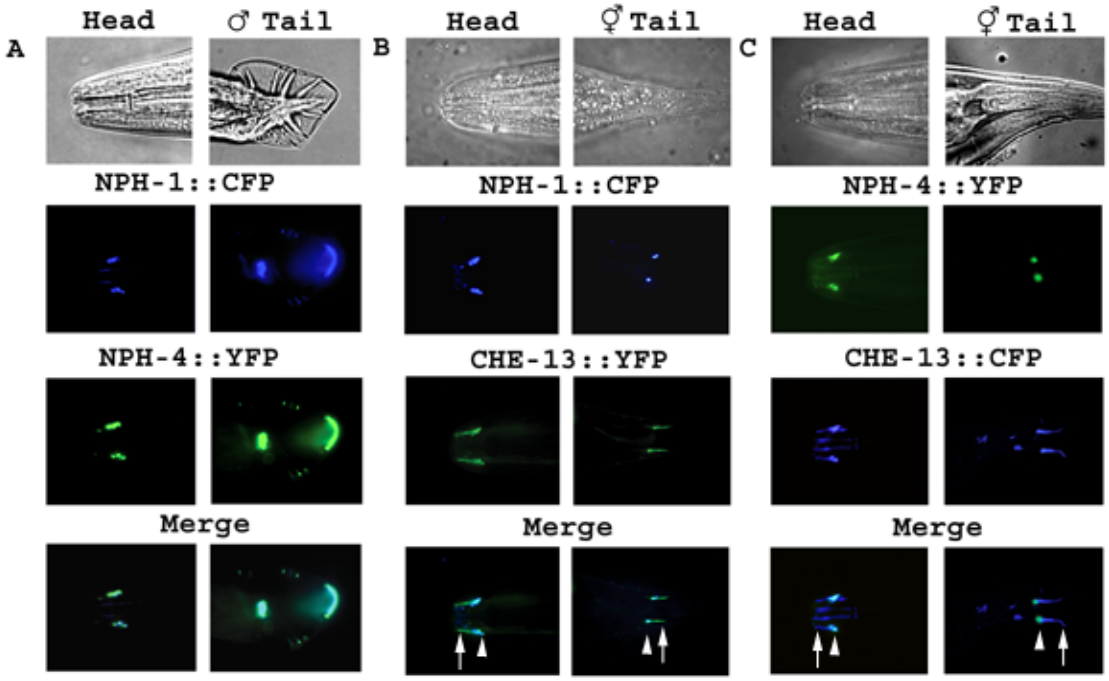
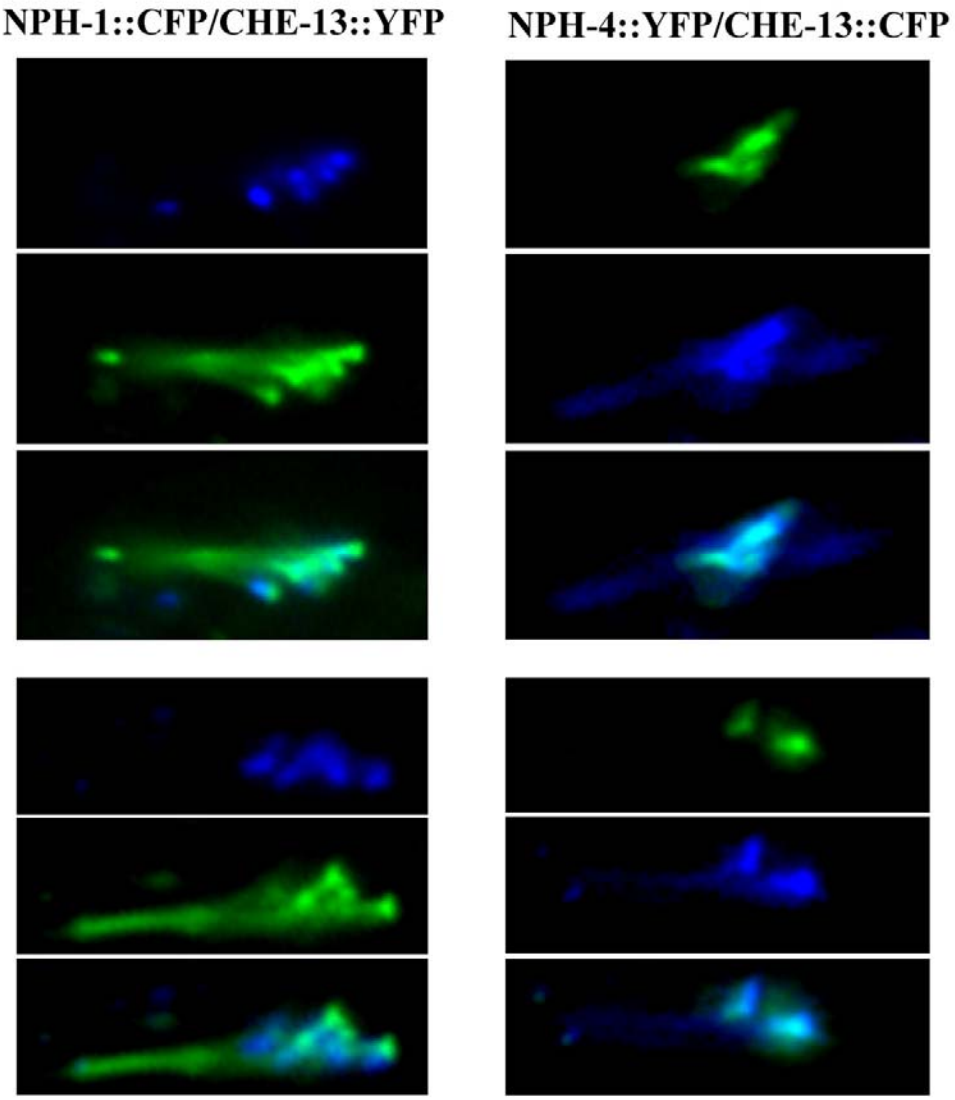


Figure 3. Colocalization of the NPH-1 and NPH-4 proteins to the transition zone at the base of cilia in *C. elegans*.

(A) Transgenic lines were generated that express NPH-1::CFP and NPH-4::YFP under control of their endogenous promoters. NPH-1 and NPH-4 co-localized to the distal end of the dendrites of the ciliated sensory amphid and labial neurons in the head of the worm (left panels) and the sensory rays of the male tail (right panels). (B,C) The localization of NPH-1 (B) and NPH-4 (C) was further evaluated by generating transgenic lines that co-express the IFT protein, CHE-13. CHE-13 was detected at the transition zones (arrow-heads) and in the cilium axoneme (arrows); however, both NPH-1 and NPH-4 were re-stricted to the transition zone. There was no detectable signal for NPH-1 or NPH-4 in the axoneme. For enlarged images of the cilium region see Fig. S1 in supplementary mate-rial.



Supplemental Figure 1:
Enlarged images of the amphid cilia region demonstrating the localization of (left) NPH-1::CFP and (right) NPH-4::YFP to the transition zones of the amphid cilia in relation to the localization pattern of the IFT protein CHE-13.

characterized IFT and BBS proteins, analysis of NPH-4::YFP and NPH-1::CFP failed to show any localization of the fusion proteins within the cilium axoneme even with increased exposure during image acquisition.

Characterization of *nph-1* and *nph-4* mutant alleles.

To begin analyzing the function of the NPH proteins at the transition zone, we obtained RB743 *nph-1*(*ok500*) and FX925 *nph-4*(*tm925*) mutant strains from the *C. elegans* Knockout Consortium and the National BioResource Project (Japan), respectively. Sequence analysis of the *nph-1* genomic region isolated from *nph-1(ok500)* mutants indicates a deletion that begins in intron 5 and extends into the terminal exon, thus deleting exon 6 through the beginning of exon 9. If expressed the mutant protein would lack C-terminal amino acids after residue 358 including much of the nephrocystin homology domain, but would retain the SH3 domain in the N-terminus (Fig. 4). Importantly, this mutation would delete much of the region that in mammalian nephrocystin-1 is thought to directly interact with the nephrocystin-4 protein (Mollet et al., 2005).

The *nph-4*(*tm925*) mutation is an intragenic deletion that begins in intron 2 and extends into intron 6 deleting amino acids 86 to 264 out of 1305 total (Fig. 4). While NPH-4 has no well-characterized motifs, this mutation does delete a highly conserved sequence found in human, mouse, *C. elegans, C. briggsae*, and *Xenopus tropicalis* homologs suggesting its functional importance. In addition, the mutation would remove a majority of the region thought to mediate the interaction of nephrocystin-4 with nephrocystin-1 (Mollet et al., 2005).

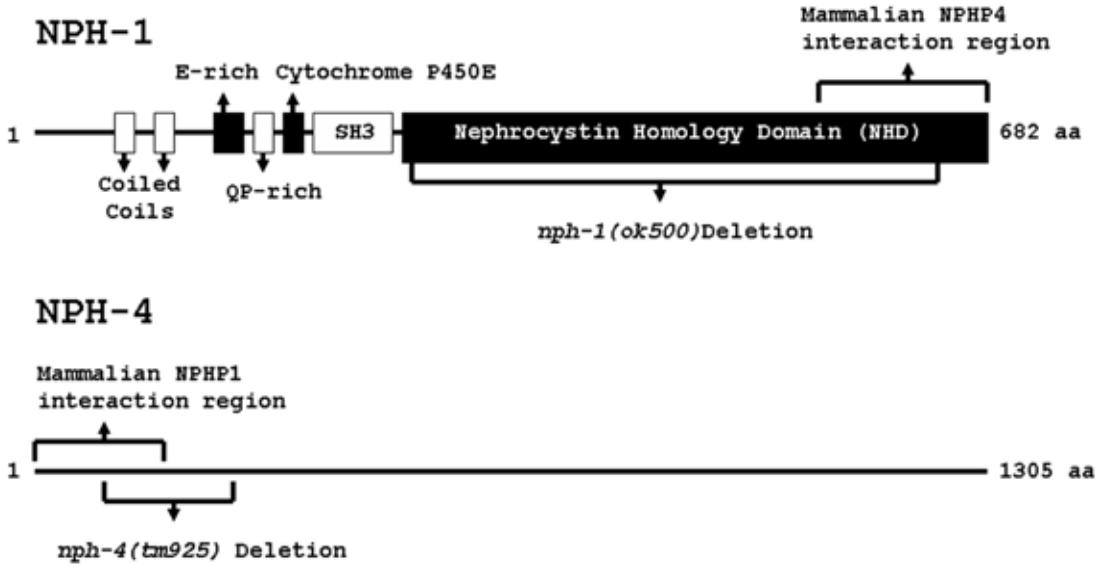
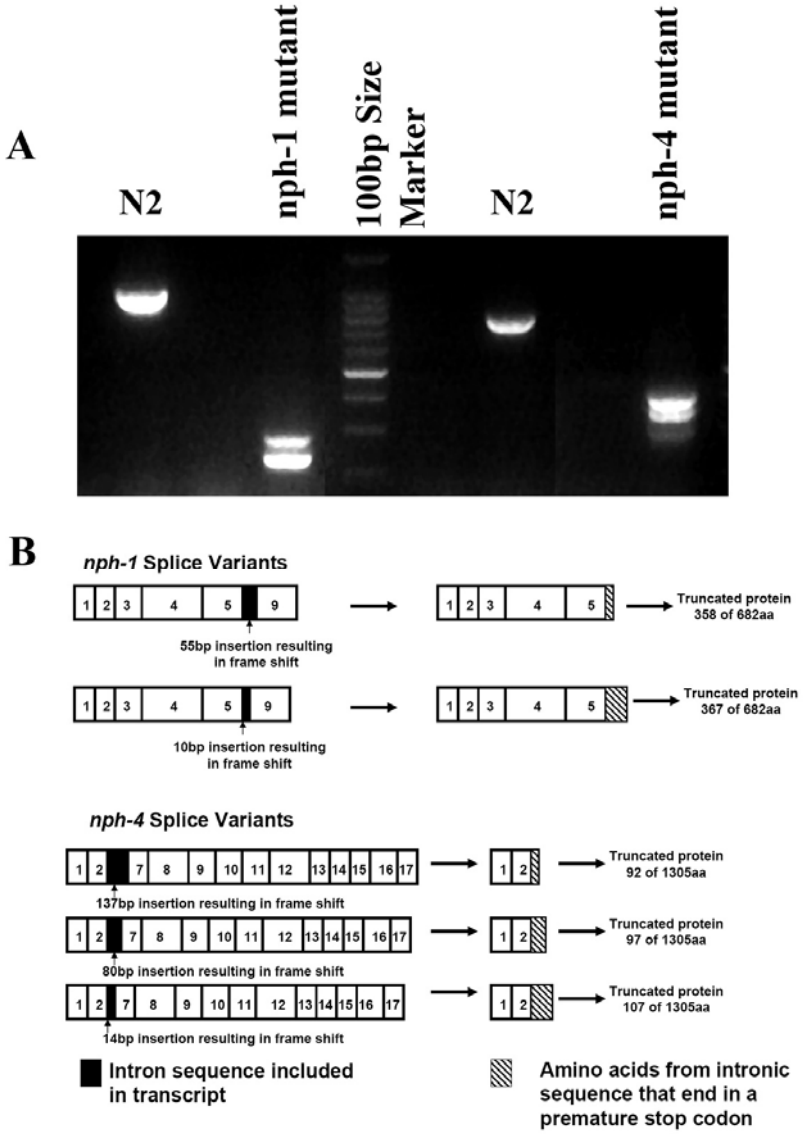


Figure 4. *C. elegans* NPH-1 and NPH-4 protein domain structures.
(A) The *C. elegans* NPH-1 protein consists of two coiled-coil domains, an E-rich domain, a QP-rich domain (glutamate-proline rich domain), Cytochrome P450E, an SH3 domain, and the nephrocystin homology domain (NHD). The deleted region (amino acids 281-641) in the *nph-1(ok500)* mutant is indicated as well as the nephrocystin-4 mammalian interaction region (C-terminal 131 amino acids). The protein sequence is based on the mRNA sequence derived from NM_063897. (B) The NPH-4 protein in *C. elegans* does not exhibit any well-characterized domains. The deletion which spans amino acids 86-264 in *nph-4(tm925)* mutants and the region involved in the interaction with mammalian nephrocystin-1 (N-terminal 176 amino acids) is indicated. The protein sequence is based on the mRNA sequence from AY959881.

To better characterize these mutations, we analyzed the expression of *nph-1* and *nph-4* in the corresponding mutant by RT-PCR. The data indicate that in both mutants there is abnormal splicing of the mutated transcripts that results in two variants in *nph-1(ok500)* mutants and three variants in *nph-4(tm925)* mutants (see supplementary material, Fig. S2). Sequence analyses of the products in the *nph-1(ok500)* mutants indicate that both variants delete most of the nephrocystin homology domain (NHD) and result in reading frame shifts and with early truncation of the protein. The abnormal splice variants in *nph-4(tm925)* mutants all cause changes in the reading frame and result in early termination of the protein with the largest protein being 107 amino acids out of 1,305. Therefore it is likely that these mutations represent null alleles. Additionally in both mutants, the deletions affect regions important for formation of the NPH complex (Mollet et al., 2005).

Cilia formation is normal in *nph* mutants.

To begin characterizing the phenotypes associated with mutations in the *nph* genes, we assessed whether the *nph-1*, *nph-4*, or *nph-1*; *nph-4* double mutants exhibited any abnormalities associated with ciliogenesis or cilia morphology. In *C. elegans*, the amphid and phasmid neurons, which express *nph-1* and *nph-4*, extend cilia through the cuticle where they can absorb fluorescent hydrophobic dye in the medium (Starich et al., 1995). In contrast, *C. elegans* with cilia assembly defects (such as the IFT mutants) are unable to absorb dye (Dyf phenotype). Our results from the dye-filling assay indicate that there are no major morphological abnormalities in the cilia as seen in *osm-5* or *che-13* IFT mutants since both the single and the double *nph* mutants absorb fluorescent dye



Supplemental Figure 2:
Analysis of *nph-1(ok500)* and *nph-4(tm925)* expression and splicing. A) Gel analysis of the RT-PCR products indicate that two and three abnormal products are generated from the *nph-1* and *nph-4* mutants respectively compared to the N2 controls. B) Schematic representation of the protein products formed from the abnormally spiced transcripts.

identical to that seen in the N2 wild-type controls (Fig. 5A)(Haycraft et al., 2003; Haycraft et al., 2001). However, it should be noted that there are IFT mutants (i.e. several of the complex A mutants or *klp-11* and *kap-1*) that do not or only partially dye-fill (Starich et al., 1995)(Perkins et al., 1986)(Snow et al., 2004). Thus, to further evaluate possible defects in cilia structure, we generated *nph-1*/*nph-4* double mutants expressing the cilia marker CHE-13 fused to YFP and measured the length of the cilia on the amphid and phasmid neurons. The data indicate that there are no overt differences in cilia length in the double mutant relative to wild type controls (Fig. 5B,C).

nph mutants exhibit defects in chemotaxis and lifespan regulation.

In *C. elegans*, cilia on the amphid and phasmid neurons play important roles in sensory perception that allow the worm to evaluate its surroundings. Despite normal cilia, the *nph-1; nph4* double mutant males have been shown to be defective in mating response and Wolf et al show pronounced defects in mating efficiency in both single mutants using RNAi knockdown approaches (Wolf et al., 2005)(Jauregui and Barr, 2005). To further assess whether the *nph* mutant hermaphrodites have abnormalities in cilia mediated sensory funcitons, we assayed whether the mutants have defects in dauer formation, osmotic avoidance, lifespan regulation, and chemotaxis.

The dauer larva is a protective stage of the *C. elegans* life cycle brought on by stressful conditions (Golden and Riddle, 1984; Starich et al., 1995). It can be induced by pheromones released in response to lack of food or overcrowding which are thought to be perceived by the ciliated sensory neurons. To evaluate the effect of *nph* mutations on

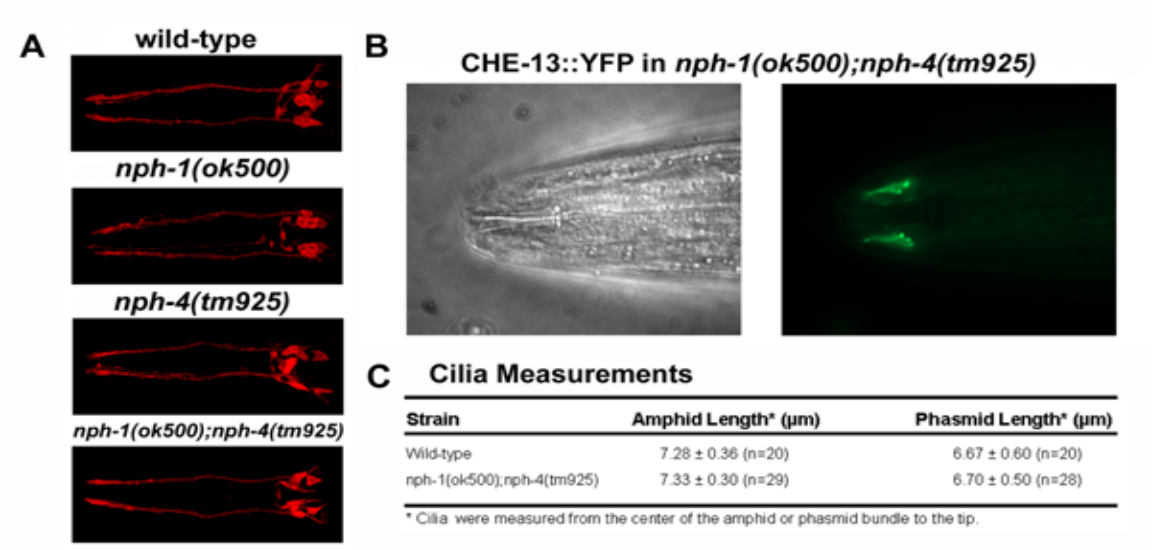


Figure 5. Cilium structure analysis of *nph-1; nph-4* double mutants.
(A) Cilium structure was analyzed by evaluating the ability of *nph-1*, *nph-4*, and double *nph-1; nph-4* mutants to absorb DiI (dye-filling assay). There were no overt differences detected between any of the mutant strains when compared to that of wild type controls. (B) Cilium structure was further analyzed in *nph-1; nph-4* double mutants using the CHE-13::YFP fusion protein. Cilium structure was indistinguishable from wild type controls and CHE-13::YFP was properly localized throughout the cilium. (C) To evaluate possible effects of the *nph* mutants on cilium structure, the length of cilia was determined in *nph-1; nph-4* double mutants expressing CHE-13::YFP. There were no statistically significant differences detected in the cilia of wild type and double mutant lines. Identical results were obtained with *nph-4* single mutants (data not shown).

dauer formation, wild-type worms, *nph-1*, *nph-4* single mutants, *nph-1*;*nph-4* double mutants, and *osm-5* mutants were starved to induce dauer formation and then treated with 1% SDS solution. Dauers are able to survive SDS treatment and can re-enter the life cycle when plated on bacterial lawns. As shown previously, the *osm-5* mutants are unable to form dauer larvae in response to starvation and no viable animals were obtained after SDS treatment. In contrast, wild-type *C. elegans* and *nph* mutants under the same conditions produced numerous dauer stage larvae indicating that *nph-1* and *nph-4* are not required for dauer formation (data not shown).

The cilia on sensory neurons that extend through the cuticle in *C. elegans* are also important in sensing osmotic concentrations. Wild-type N2 worms are able to sense regions of high osmolarity and exhibit an avoidance behavior, while IFT mutants are defective in osmotic avoidance (Osm phenotype). To determine if the loss of the NPH proteins results in an Osm phenotype, we placed a ring of 4M NaCl or 8M glycerol on plates and mutant or wild-type worms were placed in the center of the ring. Worms that were unable to sense the osmotic concentration, such as the *osm-5* or *che-13* IFT mutants, migrated freely into and across the ring. In contrast, the *nph* mutant worms avoided the high osmotic zones similar to the wild-type controls, suggesting that *nph-1* and *nph-4* are not required for osmotic avoidance (data not shown).

Cilia mediated sensory reception is also thought to influence lifespan in *C. elegans* (Apfeld and Kenyon, 1999). This is supported by the fact that many of the IFT mutants, such as *osm-5*, have a marked increase in lifespan. While the role that cilia play in this process remains elusive, it has been proposed that lifespan in *C. elegans* is regulated in part by receptors located in the cilia that receive environmental signals. To determine

whether the NPH proteins play a role in this process, *nph-1*, *nph-4*, *nph-1*;*nph-4*, and *osm-5* mutants and wild-type N2 control worms were synchronized and their survival was followed daily. The single *nph-1* and *nph-1;nph-4* double mutant exhibited an extension in lifespan compared to the wild-type controls (Fig. 6); however, the extension was not as dramatic as seen in the *osm-5* IFT mutant. Interestingly, *nph-4* single mutants have a lifespan similar to that of the *osm-5* mutants. In all three independent lifespan assays conducted, the *nph-4* mutation had a more severe effect on lifespan than did the double *nph-1; nph-4* mutations. The reason for the more severe phenotype in *nph-4* mutants is uncertain; however, we suspect that it indicates that NPH-4 has a role in regulating NPH-1 function. In the absence of NPH-4, NPH-1 would exhibit aberrant activity that could lead to the more severe phenotype. This aberrant NPH-1 activity would then be removed in the *nph-1; nph-4* double mutants resulting in a less severe phenotype. We are currently exploring this possibility.

To further explore the possible connection between the NPH proteins and sensory perception, we tested whether loss of the NPH proteins altered the response of mutant worms to the volatile chemoattractants benzaldehyde and diacetyl that are perceived by two separate classes of neurons, the AWC and AWA neurons, respectively (Sengupta et al., 1996; Wes and Bargmann, 2001). Using standard chemotaxis assays (Bargmann et al., 1993; Blacque et al., 2004), we found that the majority of the wild-type N2 worms chemotax into the attractant zone within 30 minutes of placing the worms on the chemotaxis plates. In addition, the path by which the N2 worms migrate is relatively unidirectional toward the source of the attractant. In contrast to the N2 controls, *nph-1*, *nph-4*, and the *nph-1; nhp-4* double mutants exhibited a significant delay in their chemotaxis

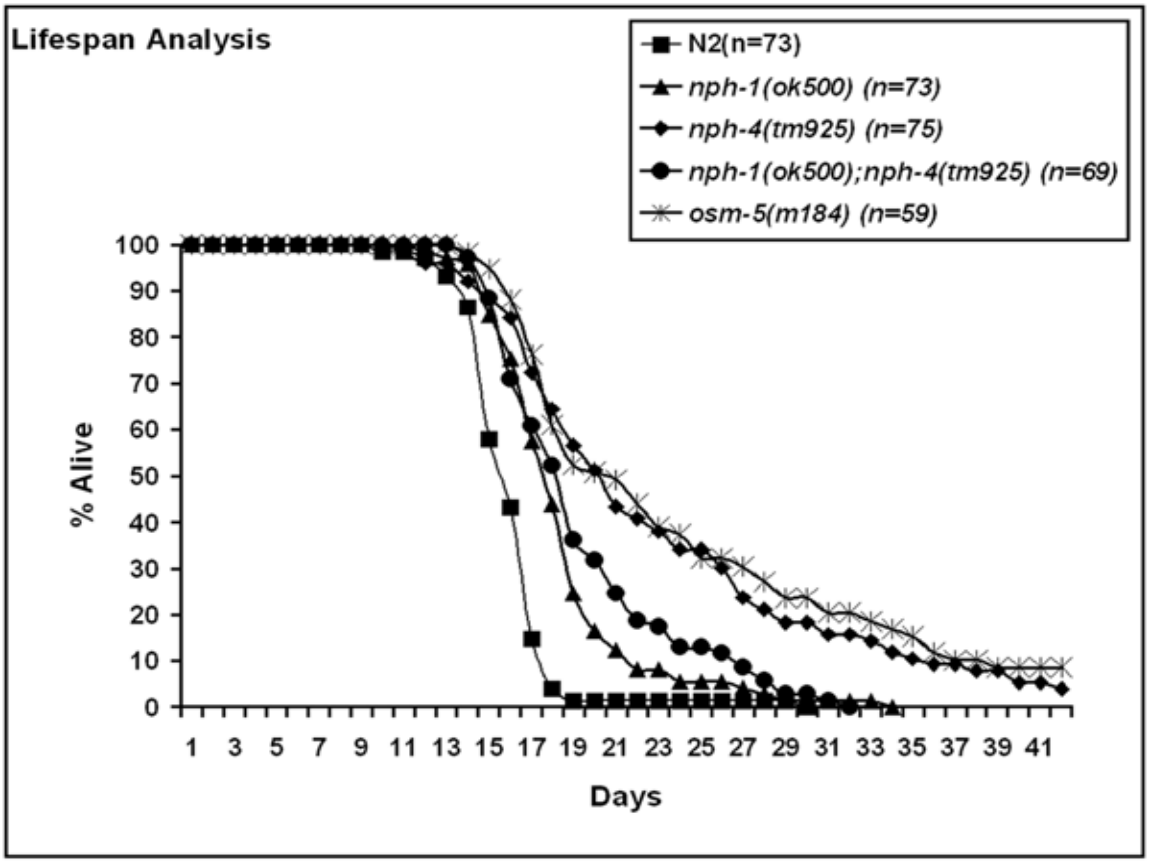


Figure 6. Lifespan analysis of *nph-1* and *nph-4* single and double mutants in *C. elegans*.

The lifespan of *nph-1*, *nph-4*, and *nph-1; nph-4* double mutants was compared to N2 wild-type controls and the long lived *osm-5* cilia mutants. The *nph-1(ok500)*, *nph-4(tm925)*, and *nph-1(ok500);nph-4(tm925)* double mutant strains had a significant expansion in lifespan compared to the N2 worms. While the *nph-1(ok500)* and *nph-1(ok500);nph-4(tm925)* double mutants did not have as severe of an increase in lifespan as *osm-5* mutant worms, single *nph-4(tm925)* mutants were indistinguishable from *osm-5* mutant worms. Similar data were obtained on three independent lifespan experiments (average of 75 total worms in each genetic category) conducted for this analysis.

response toward both attractants (Fig. 7A,B). While many of the mutant worms do eventually reach their destination at the attractant, the migration path was less unidirectional than that of the N2 control worms and was typical of mutants with sensory defects associated with loss of cilia function (Starich et al., 1995). Importantly, in both mutant lines the chemotaxis defects were corrected by expression of the respective wild-type *nph* gene (Fig. 7A,B).

NPH-4 is required for NPH-1 localization to the transition zone.

The mammalian nephrocystin proteins are thought to function in the same pathway and there is data to suggest a physical interaction between several of the NPH proteins (Mollet et al., 2002; Mollet et al., 2005). The specific chemotaxis and lifespan defects in the absence of cilia structure abnormalities in the *nph-1* and *nph-4* mutants also support this conclusion in *C. elegans*. To obtain further insight into this possibility, we analyzed the effect of each *nph* mutation on the localization of the reciprocal NPH protein. In *nph-1* mutants, NPH-4 localization at the transition zones was unchanged from that in wild type controls (Fig. 8A). In contrast, NPH-1 was not localized to the transition zone in the *nph-4* mutants (Fig. 8B). Passing the NPH-1::CFP extrachromosomal array from the *nph-4* mutants onto a wild type background restored NPH-1 localization to the transition zone (Fig. 8C). In addition, *nph-1* expression is maintained in the *nph-4* mutants (data not shown), thus indicating that NPH-4 function is required for normal localization of NPH-1 to the transition zone and supporting the concept that the NPH proteins function as part of the same complex.

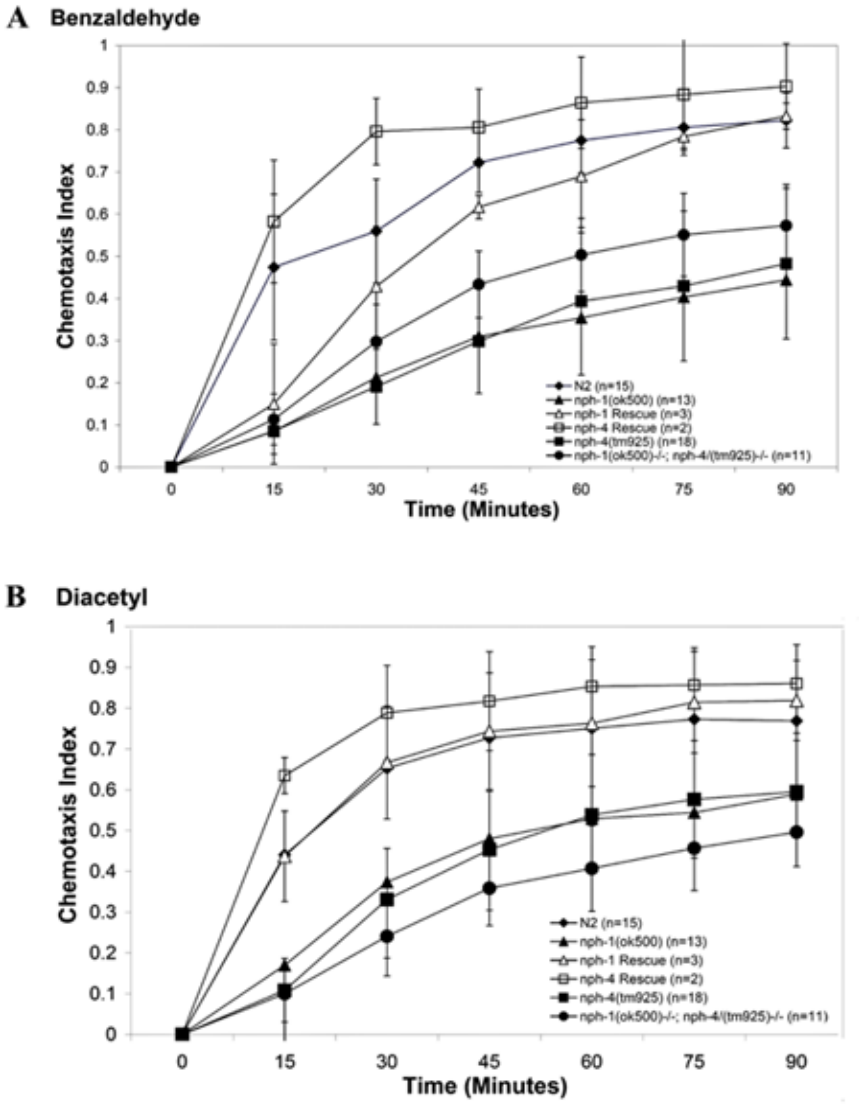


Figure 7. The *nph-1* and *nph-4* single and *nph-1;nph-4* double mutants exhibit defects in chemotaxis toward volatile attractants

Comparison of chemotaxis indices for the wild-type N2 and the *nph* mutants in response to (A) benzaldehyde and (B) diacetyl indicate that both single mutants and the double mutant exhibit abnormal response to these attractants. To demonstrate that the phenotypes were due to mutation of the *nph* genes, the chemotaxis defect in *nph-1* and *nph-4* mutants was rescued by expression of *nph-1::CFP* and *nph-4::YFP* respectively. Both the *nph-1* and *nph-4* rescued lines exhibited a strong chemotactic response to (A) benzaldehyde or (B) diacetyl similar to that obtained for wild type controls. Error bars represent the standard deviation and n refers to the number of independent experiments with an average of 100 worms on each plate being evaluated.

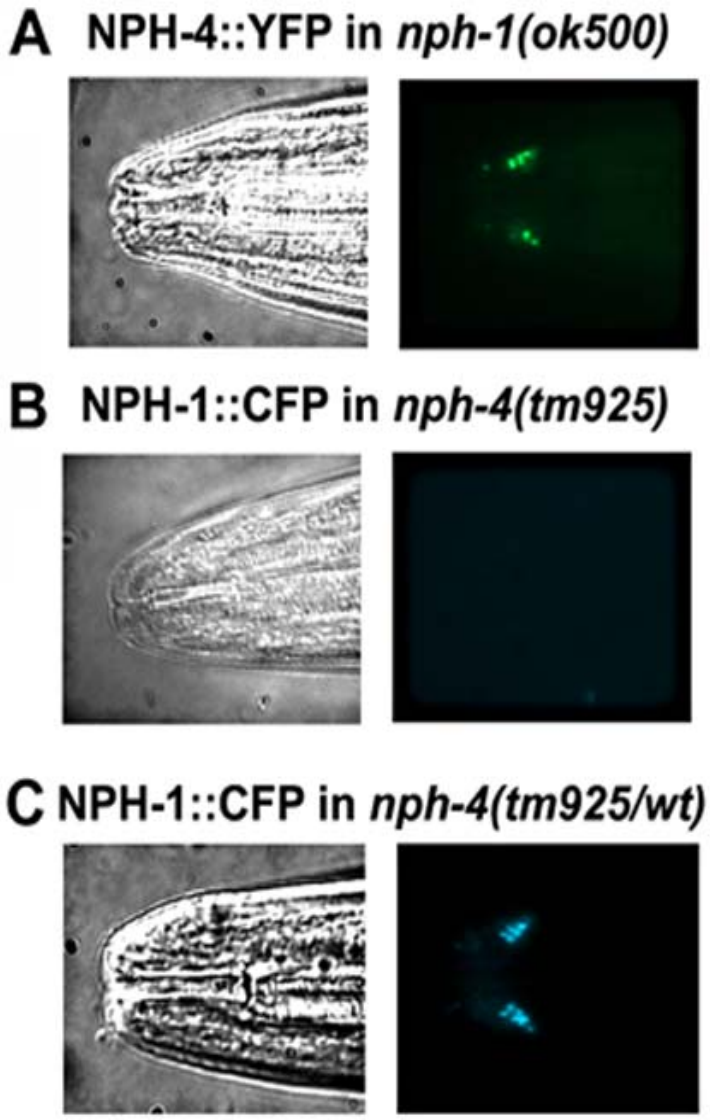


Figure 8. The NPH-4 protein is required for proper localization of NPH-1 to the transition zone.
To explore the possibility that the NPH proteins function as part of the same complex, we analyzed the localization of the NPH proteins in the reciprocal mutant background. (A) Compared to wild type controls, NPH-4::YFP localization was not altered by loss of *nph-1*. (B) In contrast, NPH-1::CFP was not detected at the transition zone in *nph-4* mutants indicating that NPH-4 is required for NPH-1 localization. (C) The localization of NPH-1::CFP to the transition zone was restored by crossing the strain back to wild type worms.

DISCUSSION

The development of renal cysts is common to a number of human and murine disorders including autosomal recessive (ARPKD), autosomal dominant polycystic kidney disease (ADPKD), nephronophthisis (NPH), Bardet-Biedl Syndrome (BBS), and oralfacial digital syndrome (OFD) (Ansley et al., 2003; Romio et al., 2004; Wilson, 2004). The identification of several genes associated with the pathologies in these disorders has revealed that a unifying cause may be an association with defects in cilia assembly or cilia mediated functions. However, the role of most of these "ciliocystic" proteins and the spectrum of functions of renal cilia remains largely unsolved. Currently it is believed that renal cilia function as mechanosensors that evaluate changes in fluid flow through the lumen (Praetorius and Spring, 2001; Praetorius and Spring, 2003). Deflection of the cilium axoneme initiates an increase in intracellular calcium which requires the polycystins, the proteins which are disrupted in human ADPKD (Nauli et al., 2003).

To further understand the connection between cilia and renal cyst development, we have utilized *C. elegans* as a malleable system to facilitate functional analysis of ciliocystic proteins. As part of these analyses, we conducted a nematode genome sequence based screen to identify genes expressed in the sensory cilia. The basis of the screen was the co-regulation of several cilia genes by DAF-19 (Efimenko et al., 2005; Swoboda et al., 2000). Among the candidate genes were several IFT proteins, homologs of genes involved in human Bardet-Biedl Syndrome, as well as the homologs of nephrocystin-1 and nephrocystin-4. Thus, we speculated that the *nph* genes would encode cilia associated proteins and that NPH would represent another model system to evaluate cilia dysfunction and renal cystic disease.

Our initial characterization of *nph-1* and *nph-4* confirmed that both genes are regulated by DAF-19 in agreement with the presence of an X-box in the promoter region and that they are expressed in ciliated sensory neurons of both the male and hermaphrodite. The presence of an X-box sequence in the promoters of these genes in the related nematode *C. briggsae* and in the putative promoter regions of nephrocystin-1 and nephrocystin-4 in the mouse argues that this motif is functional in these genes. We confirmed this in the case of *nph-4* by mutating the X-box sequence and demonstrating a marked reduction in expression from the transgene. In addition, the expression pattern of *nph-1* and *nph-4* shown here and previously by Wolf et al and Jauregui and Barr is similar to that seen for DAF-19 regulated IFT genes such as *osm-5* or *che-13* (Haycraft et al., 2003; Haycraft et al., 2001) and the recently described *bbs-1*, *bbs-2*, *bbs-5*, *bbs-7*, and *bbs-8* (Blacque et al., 2004)(Li et al., 2004)(Efimenko et al., 2005). However, in contrast to OSM-5, CHE-13, BBS-7 and BBS-8, the NPH proteins were not detectable in the cilium axoneme at this level of analysis. Rather, NPH-1 and NPH-4 remained at the transition zones at the base of the cilia. We know that the extrachromosomal arrays used to conduct these localization studies encode functional proteins since they are able to rescue the mutant chemotaxis phenotypes. In addition, our data indicate that *nph-1* and *nph-4*, are distinct from the IFT proteins and most of the BBS proteins in that the *nph* mutants show no gross cilia morphology abnormalities. Thus, from these data it seems unlikely that the *C. elegans* NPH proteins have a role in cilia assembly as proposed recently by Wolf et al. (Wolf et al., 2005).

Characterization of the first four mammalian nephrocystins has suggested that they form a complex and function in a common signaling pathway (Mollet et al., 2005;

Olbrich et al., 2003; Otto et al., 2003). Despite the lack of clear homologs to nephrocystin-2 and nephrocystin-3 in *C. elegans*, our data further support this conclusion based on the similar sensory defects observed with the single and double *nph* mutants in *C. elegans* and from the mislocalization of NPH-1 protein in *nph-4* mutants. The latter data also suggests that one function of NPH-4 is to anchor NPH-1 at the transition zones. This is supported further by recent data in *Chlamydomonas* identifying the NPH-4 protein, but not NPH-1, as a central component of the centriole that forms the basal body from which the flagella emerges(Keller et al., 2005). Since homologs of the additional nephrocystins do not appear to be in the *C. elegans* genome, we are unable to test the effects of *nph-4* mutation on their localization; however we are utilizing the power of the *C. elegans* model system to screen for additional proteins that may function as part of the NPH complex and may provide novel insights into the function of the mammalian proteins.

Despite the lack of any morphological defect in the cilia, our analyses of the *nph* mutants reveal that they exhibit phenotypes typical of mutants with defects in cilia mediated signaling. While osmotic avoidance and dauer formation were overtly normal, the *nph* mutants did exhibit an increase in lifespan as well as abnormalities in chemotaxis toward two volatile chemoattractants. The effects in the *nph-1* and *nph-1; nph-4* double mutants were not as pronounced as those reported for a typical cilia assembly mutant, such as *osm-5*; but were significantly different from wild-type controls. In contrast, mutation of *nph-4* was found to have a more severe effect on lifespan. Although we do not know the reason for the more severe phenotype in the *nph-4* mutant than in the *nph-1; nph-4* double mutants, a possibility is that the NPH proteins have a role in regulating each others' functions. This is supported by the dependence of NPH-1 on NPH-4 for localiza-

tion to the transition zone. Thus, in the *nph-4* mutants, NPH-1 may be free from regulatory influences normally provided by NPH-4 and lead to a more severe phenotype, which is then removed in the case of the double mutants. Additionally, NPH-1 could act genetically downstream of *nph-4* in the *nph* pathway and suppress aspects of the *nph-4* mutant phenotype in the *nph-1; nph-4* double mutant worms. Studies to address these issues are currently underway.

In contrast to the effect on lifespan, we detected no consistent differences between the single and double mutants with regards to their chemotaxis response toward volatile attractants. The reason for this differential effect on the lifespan versus chemotaxis phenotypes is unknown but may indicate that these proteins have slightly different roles in these sensory responses or in different neurons involved in chemotaxis and lifespan regulation.

It has recently been demonstrated that RNAi mediated knockdown of *nph-1* or *nph-4* results in a male mating defect reminiscent of *lov-1* and *pkd-2* mutants (Wolf et al., 2005). Additionally, Jauregui and Barr have shown that the *nph-1; nph-4* double mutants, but not single mutants, have defects in the response step of male mating (Jauregui and Barr, 2005). The reason for the discrepancy in the mating phenotype between the genetic mutants and the knockdowns is unknown. It is surprising that the phenotype is more severe in the knockdown mutants since RNAi approaches are notoriously ineffective in neurons in *C. elegans*. The male mating defects along with our data indicating that *nph* mutants have abnormalities in chemotaxis and lifespan support a role for NPH proteins as regulators of signaling activity mediated by cilia on the sensory neurons.

Since the cilia in *nph* mutants appear normal, it will be interesting to determine whether disruption of the NPH proteins affects localization or transport of chemoreceptors or channels within the cilia such as *odr-10*, which mediates responses to diacetyl (Sengupta et al., 1996), or *pkd-2* which is involved in male mating. Alternatively, loss of NPH proteins may disrupt events downstream from these receptors or channels and may be involved in transmission of the cilia mediated signaling to the cell body. In support of these possibilities, it has recently been found that X-box containing genes can be divided into two separate groups depending upon their X-box sequence relationship to the consensus and to the distance of the X-box relative to the ATG. Based on these criteria, the *nph* genes can be classified as group 2 X-box genes which are thought to perform more specialized sensory functions in contrast to group 1 genes which are hypothesized to be involved in cilia assembly (Efimenko et al., 2005). While the exact role of the NPH proteins in cilia remains elusive, *C. elegans* and the powerful genetic approaches afforded by this system will provide an important research tool to begin evaluating these possibilities and lead to a better understanding of the functions of renal cilia and how defects in this organelle lead to the formation of cysts in the kidney.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. L. Guay-Woodford for critical reading of this manuscript. We thank Dr. A. Fire for the gifts of *C. elegans* expression vectors. We thank Dr. S. Clark for the UNC-122::GFP construct. The *C. elegans* Genome Sequencing Consortium provided sequence information and the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health, provided some of the *C. elegans* strains used in this

study. We thank the *C. elegans* Knockout Consortium and the National BioResource Project in Japan for providing the *nph-1* and *nph-4* deletion mutants. This work was supported by grants to B.K.Y. from the National Institute of Diabetes and Digestive and Kidney Diseases Grants DK65655 and DK62758 and by a grant to P.S. from the Swedish Foundation for Strategic Research (SSF) in Stockholm, Sweden. Additional support was provided by NIH training grants DK07545 (CJH) and 5 T32 GM008111(JCS).

FOOTNOTES

Article published online ahead of print JCS 10.1242/jcs.02665. Article and publication date are at http://jcs.biologists.org/cgi/content/full/118/23/5575.

Supplementary material available online at

http://jcs.biologists.org/cgi/content/full/118/23/5575/DC1

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PROTEIN KINASE G ACTS DOWNSTREAM OF NEPHRONOPHTHISIS PROTEINS DURING LOCOMOTORY BEHAVIOR IN CAENORHABDITIS ELEGANS.

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In preparation for *Journal of the American Society of Nephrology*

Format adapted for dissertation

ABSTRACT

Nephronophthisis (NPH) is an autosomal recessive disorder characterized by cyst formation within the kidney. The nematode *Caenorhabditis elegans* has been established as an advantageous model in which NPH protein function can be analyzed. Two of the genes mutated in NPH, Nephrocystin-1 and Nephrocystin-4 have homologs in *C. elegans*, *nph-1* and *nph-4* respectively. The gene products NPH-1 and NPH-4 localize to the transition zone at the base of cilia in the ciliated sensory neurons. As in mammalian systems, the NPH proteins function as part of a complex based on the observation that localization of NPH-1 to the transition zone requires the presence of NPH-4. Although *C. elegans* hermaphrodites with mutations in either of these genes exhibit normal cilia morphology, they have defects in cilia-mediated signaling activities associated with chemotaxis responses and lifespan regulation. Here we demonstrate that *nph-1* and *nph-4* single and double mutants display an additional phenotype in which they undergo diminished locomotion in the presence of food. Based on this phenotype, we utilized *C. elegans* to assess the functional significance of a *nph-4* point mutation observed in human nephronophthisis patients. Although the construct containing this mutation was able to restore NPH-1 localization to the transition zone, it was not sufficient to rescue the locomotion defect. Similar abnormalities in locomotory behavior are observed in mutants lacking cilia as well as in *C. elegans* with a gain of function mutation in the cGMP-dependant protein kinase EGL-4. In contrast, loss of function mutants in *egl-4* cause an excessive roaming phenotype. To assess whether the NPH proteins function in a similar pathway as EGL-4, we generated double mutant strains with *nph-1* or *nph-4* and the *egl-4* loss or gain of function mutants. Interestingly, the phenotypes of the *egl-4* mutation were displayed

over that of the *nph* mutants. These data raise the possibility that a function of the NPH complex may be to impede EGL-4 signaling activity and that in the absence of the NPH proteins EGL-4 signaling is hyperactivated causing the impaired locomotory activity.

INTRODUCTION

Recent data have revealed that many proteins associated with cystic kidney diseases localize to the cilia or the basal body at the base of the cilium. This suggests that, defects in the formation of or the signaling from these structures is likely part of a common mechanism by which cysts form. Therefore, further understanding of the functions of proteins that localize to these organelles may lead to a better appreciation of the mechanisms by which these pathologies arise.

Nephronophthisis (NPH) is an autosomal recessive disorder that is characterized in part by the formation of cysts at the corticomedullary border of the kidney.[1] Thus far mutations in six genes (*NPHP1* through *NPHP6*) responsible for NPH have been identified. These genes encode the proteins Nephrocystin-1 through Nephrocystin-6.[2-7] Two of these genes *NPHP1* and *NPHP4* are evolutionarily conserved in the nematode *Caenorhabditis elegans*. Analyses of NPH patients have identified several missense mutations in the *NPHP4* gene, many of which are located in regions of high conservation between the homologs in *C. elegans*, *C. briggsae*, mouse, and human.[8] The significance of these missense mutations with regards to localization or function is unknown.

Previously, we characterized the *C. elegans* homologs of *NPHP1* and *NPHP4*, *nph-1* and *nph-4* respectively.[9] We found the corresponding proteins NPH-1 and NPH-4 to specifically localize to the transition zone at the base of cilia without entering the cil-

ium axoneme in the sensory neurons. This localization has since been confirmed in mammalian cell culture in respiratory and renal cell lines for NPHP1,[10, 11] whereas NPHP4 has been shown to be localized to the centrosome, which lies adjacent to the transition zone, of COS-1 cells.[12] Our previous studies also determined that *nph-1* and *nph-4* mutants undergo normal cilia formation but, have defects in cilia mediated signaling activities that result in chemotaxis abnormalities and an extension in lifespan. In addition, we found that the NPH-4 protein was required for the proper localization of the NPH-1 protein to the transition zone.

C. elegans mutants with defects in proteins required for Intraflagellar Transport (IFT), the process by which cilia are assembled, have abnormal chemotaxis responses, increased life span, and a smaller body size compared to wild-type worms. In addition, the IFT mutants exhibit reduced locomotory behavior (dwelling phenotype) when placed individually on a lawn of bacteria, compared to that seen for wild-type animals.[13, 14] This phenotype is also seen in *C. elegans* with a gain of function mutation in the *egl-4* gene, the *C. elegans* homolog of the cGMP dependent Protein Kinase G (PKG).[15] In contrast, the loss of function *egl-4* mutations cause excess roaming in the presence of food and an enlarged body size. This loss of function mutation is able to suppress the small body size and the dwelling behavior seen in the IFT mutant *che-2* suggesting that EGL-4 functions downstream of signals initiated in the cilium.

Here we show that *nph-1* and *nph-4* single and double mutants in *C. elegans* also exhibit a dwelling phenotype when placed on a lawn of bacteria. In *nph-4* mutants, the introduction of the wild-type NPH-4::YFP transgene was able to correct the dwelling phenotype and also restore NPH-1 localization at the transition zone. In contrast, expres-

sion of *nph-4* containing a missense mutation observed in human NPH patients failed to correct the dwelling phenotype although NPH-1 localization was again detected at the transition zone. These data indicate this mutation results in a NPH-4 protein which undergoes proper trafficking to the transition zone and is able to establish the proper interactions for the formation of the NPH complex, however, it is nonfunctional with regards to cilia mediated signaling activities that regulate locomotory behavior. In this study we also assess the genetic connection between the *nph* genes and *egl-4* by analyzing the phenotype of *C. elegans* with double mutations in these genes. The data from the double mutants indicate that the phenotype of the *egl-4* mutation was exhibited. This suggests that the EGL-4 protein likely acts downstream of both of the NPH proteins and that a function of the NPH complex may be to impede EGL-4 activity.

RESULTS

nph-1 and *nph-4* mutants exhibit diminished locomotory behavior.

Previously, we described the characterization of the *C. elegans* mutants *nph-1(ok500)* and *nph-4(tm925)* with regard to chemotaxis and lifespan.[9] Here we further analyzed these two mutants along with an additional *nph-1* allele, *nph-1(tm1701)*. The *nph-1(tm1701)* mutant has a deletion knocking out amino acids 120-280 which includes several protein interaction domains including the SH3 domain (Fig. 1A). To further characterize this mutation we analyzed the expression of the mutant allele by RT-PCR (data not shown). We found that this mutation results in an in-frame deletion leaving the remainder of the protein including the Nephrocystin Homology Domain in tact, suggesting that this mutation may not represent a null allele.

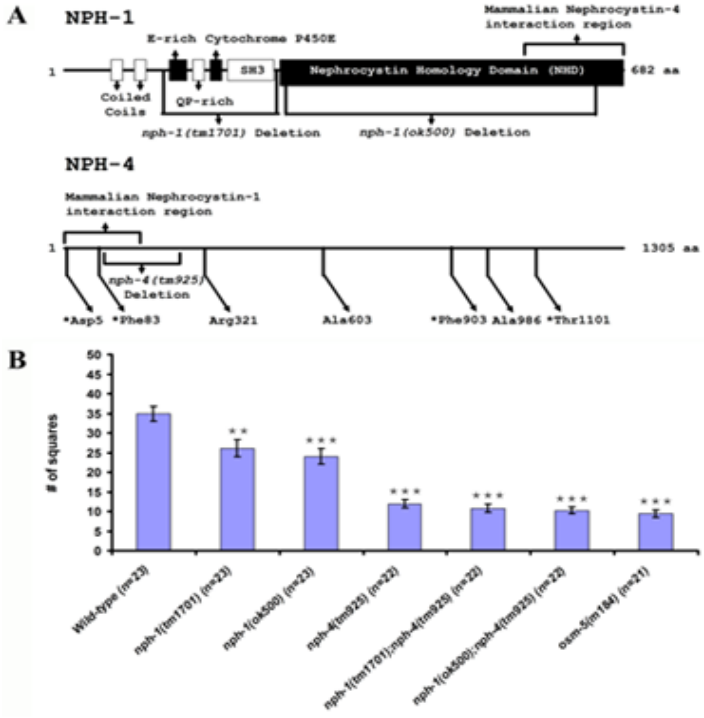

Figure 1. Locomotory behavior analysis of *C. elegans nph* single and double mutants.

(A, top) The *C. elegans* NPH-1 protein consists of two coiled-coil domains, an E-rich domain, a QP-rich domain (glutamate-proline rich domain), Cytochrome P450E, an SH3 domain, and the nephrocystin homology domain (NHD). The two alleles of *nph-1* used in this study are shown. *nph-1(tm1701)* deleted amino acids 120-280 and *nph-1(ok500)* lacks amino acids 281-641. The protein sequence is based on the mRNA sequence derived from NM_063897. (A, bottom) The NPH-4 protein in *C. elegans* does not exhibit any well-characterized domains. The deletion which spans amino acids 86-264 in *nph-4(tm925)* mutants is shown. Also, shown are all residues of known human patient mutations which are conserved between human and *C. elegans* with an asterisk beside those conserved in all of *C. elegans*, *C. briggsae*, mouse, and human. The protein sequence is based on the mRNA sequence from AY959881. (B) The *nph-1* and *nph-4* single and double mutants exhibit a significant decrease in locomotory behavior indicated by the number of squares entered for each strain compared to wild-type worms. The *osm-5(m184)* strain represents a cilia mutant known to exhibit a dwelling phenotype. For this and subsequent figures, error bars correspond to the standard error of the mean (sem) and n refers to the number of independent experiments. The cut-off for statistical significance is represented as *P<0.05, **P<0.005, ***P<0.0005 and was compared to wild type control. There was no statistical difference between the two alleles of *nph-1*.

Mutations that disrupt cilia formation in *C. elegans* result in defects in chemotaxis responses, dauer formation, lifespan regulation, male mating behavior, and cause abnormal locomotory activity in the presence of food.[13, 14, 16-19] To assess whether the *nph* mutants display this additional cilia-mediated signaling phenotype, we analyzed the locomotory behavior of *nph-1* and *nph-4* mutants on food by counting the number of squares on a grid the worm enters after being allowed to roam freely over the course of 18 hours. Wild-type worms on average entered 34.9 squares whereas the IFT mutant *osm-5(m184)* which lacks cilia only entered 9.4 squares. Although cilia appear morphologically normal in the *nph* mutant hermaphrodites, both the *nph-1(tm1701)* and *nph-1(ok500)* mutants exhibited a significant reduction in activity entering 26.1 and 24 squares, respectively (Fig. 1B). Interestingly, a much more dramatic decrease is seen in the *nph-4(tm925)* mutant that entered an average of 12 squares, a similar amount to that seen in *osm-5(m194)*. Additionally, we analyzed *nph-1 (tm1701);nph-4(tm925)* and *nph-1(ok500);nph-4(tm925)* double mutants which entered 10.8 and 10.3 squares respectively, a comparable amount to that seen in the *nph-4(tm925)* mutant alone. This result would be predicted based on our previous data showing that the NPH-4 protein is required for proper localization of NPH-1 to the transition zone.

Transgenic *nph-4(tm925)* mutants expressing NPH-4(Phe83Leu)::YFP incompletely rescue the mutant phenotype.

The analyses of human NPH patients have identified several mutations in the *NPHP4* gene including premature terminations, deletions, and several missense mutations.[5, 8, 20] Although NPH-4 lacks predicted protein domains, there are multiple regions

of high sequence conservation shared among eukaryotic species. Interestingly, several of the missense mutations identified in human NPH patients occur in these highly conserved regions (Fig. 1A). Therefore, analysis of these missense mutations may be informative with regards to identifying regions within this protein which are important for various functions including trafficking, protein-protein interactions, and signaling activities. Here we analyze one of the human mutations *NPHP4* Phe91Leu that corresponds to *C. elegans nph-4* phenylalanine 83 (Fig.1A). This phenylalanine residue is located at the beginning of a region of high sequence conservation, suggesting that it may be part of a functionally important domain (Fig. 2A).

In order to determine the effect that this mutation has on NPH-4 function we performed site directed mutagenesis to convert phenylalanine 83 to leucine in the wild-type NPH-4::YFP construct, recapitulating the mutation seen in human patients. The mutagenized construct (NPH-4::YFP(Phe83Leu)) was injected into *nph-4(tm925)* mutant worms along with the full length construct of NPH-1::CFP. The resulting transgenic worms were analyzed for localization of each of the NPH proteins and for their ability to rescue the locomotory behavior defects seen in the *nph-4(tm925)* mutant worms. As seen with the wild type NPH-4::YFP protein, the NPH-4::YFP(Phe83Leu) protein was also able to localize correctly to the transition zone (Fig. 2B). Furthermore, both the wild type and mutant NPH-4::YFP proteins were able to restore NPH-1::CFP localization at the transition zone at the base of cilia on both the amphid and phasmid neurons. Therefore, the Phe83Leu amino acid change does not disrupt targeting of the NPH-4 protein to the transition zone nor does it prevent assembly of the NPH complex.

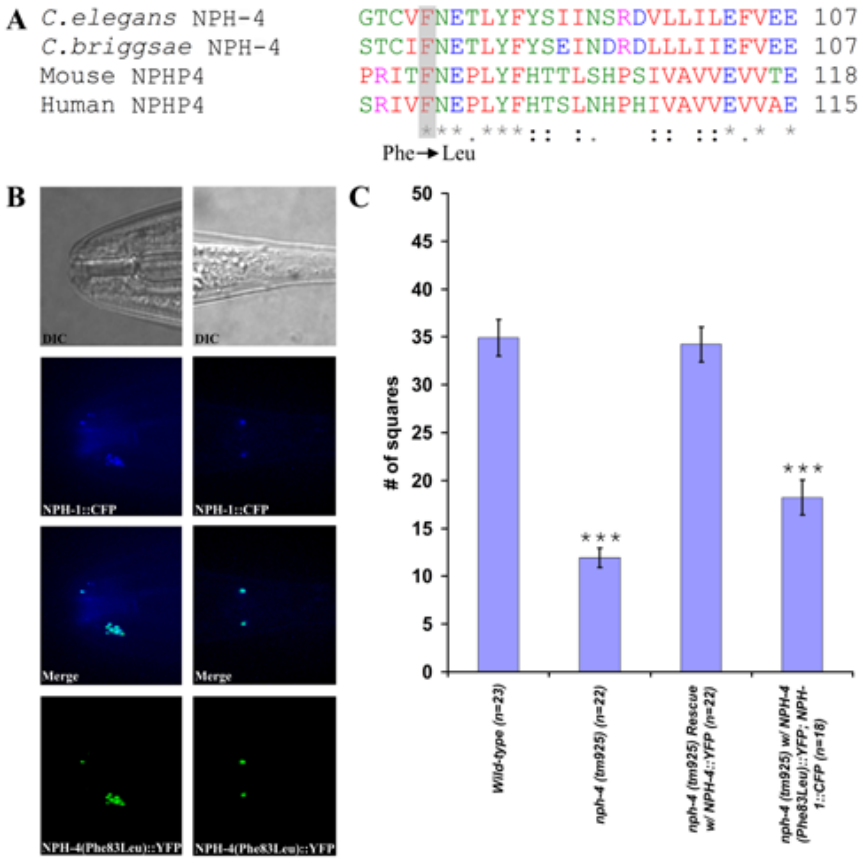

Figure 2. Localization and locomotory behavior in transgenic worms expressing NPH-4(Phe83Leu)::YFP.

The protein sequences of NPH-4 from *C. elegans*, *C. briggsae*, mouse, and human were aligned. The human residue Phenylalanine 91 was conserved across these four organisms (highlighted). The corresponding *C. elegans* residue Phenylalanine 83 was converted to a Leucine via site-directed mutagenesis. (B) In transgenic *nph-4(tm925)* mutants, NPH-4(Phe83Leu)::YFP localized normally to the transition zone at the base of cilia. The NPH-1::CFP protein localization was restored to the transition zone and co-localized with NPH-4(Phe83Leu)::YFP as seen in the merged image, in the amphid neurons in the head(left panels) and the phasmid neurons in the tail(right panels). (C) *nph-4(tm925)* mutants display a significant decrease in locomotory behavior that can be rescued back to wild-type levels by introduction of the wild-type NPH-4::YFP transgene (Rescue). Although transgenic *nph-4(tm925)* mutant worms expressing the NPH-4(Phe83Leu)::YFP construct had improved locomotory activity compared to the *nph-4(tm925)* mutant alone it was markedly different than wild-type controls or the rescued lines.

Knowing that the NPH-4(Phe83Leu)::YFP protein was targeted correctly to the base of the cilium, we next assessed whether this protein would be able to rescue the defect in locomotory behavior seen in *nph-4(tm925)* mutant worms. Transgenic *nph-4(tm925)* worms containing the wild-type NPH-4::YFP rescue construct exhibit tracking behavior similar to that seen in wild-type worms entering on average 34.2 squares (Fig. 2C). In contrast, transgenic *nph-4(tm925)* mutants expressing the NPH-4(Phe83Leu)::YFP construct exhibit a dwelling phenotype, with these worms entering an average of 18.2 squares. Although an improvement over the *nph-4(tm925)* mutants it is significantly different than that seen in wild-type worms or in transgenic worms expressing the NPH-4::YFP rescue construct.

Analysis of genetic interactions between *nph* and *egl-4*

egl-4 encodes the *C. elegans* homolog of the cGMP dependent Protein Kinase G (PKG). Loss of function mutations of *egl-4* cause excess locomotory activity while gain of function mutations cause a dwelling phenotype similar to that seen in the *nph-4(tm925)* mutants. Furthermore, an *egl-4(n478)* loss of function mutation was identified as a suppressor of the dwelling phenotype exhibited by *C. elegans* with mutations in the IFT gene *che-2*.[13] Together these data suggest that EGL-4 functions downstream of the cilia initiated signaling events that regulate locomotory behavior. Based on these observations we wanted to assess whether the NPH proteins also function as part of the *egl-4* pathway.

To accomplish this we generated *C. elegans* strains with mutations in *nph-4* or *nph-1* along with an *egl-4(n478)* loss of function or *egl-4(ad450)* gain of function muta-

tion and analyzed the resulting phenotypes. In agreement with previously reported data, loss of function mutations in *egl-4(n478)* caused a significant increase in roaming activity (47.7 squares) while the *egl-4(ad450)* gain of function mutants exhibited a severe dwelling phenotype (8.7 squares, Fig. 3). The *nph1/4, egl-4(n478)* double mutants exhibited a roaming phenotype that was not statistically different than the the *egl-4(n478)* strain alone (Fig. 3; *nph-1(tm1701);egl-4(n478)* 45.1 squares; *nph-1(ok500);egl-4(n478)*, 46.7 squares; and *nph-4(tm925);egl-4(n478)*, 41.7 squares). In addition, we also analyzed the phenotype of the *nph*-1 mutants containing the gain of function mutation which revealed a more severe dwelling phenotype resembling the *egl-4(ad450)* mutants alone (*nph-1(tm1701);egl-4(n478)* 9 squares; *nph-1(ok500);egl-4(n478)* 9.7 squares). Similar analysis with the *nph-4* worms and the *egl-4(ad450)* gain of function mutants is not feasible due to the similarities in phenotypes of each of the single mutants. Together these data indicate that *egl-4* mutations are able to modify the severity of the phenotype seen in the *nph* mutants.

DISCUSSION

The formation of cystic lesions in the kidney is a feature shared by a large number of human disorders. Among them are Autosomal Dominant Polycystic Kidney Disease (ADPKD), Autosomal Recessive Polycystic Kidney Disease (ADPKD), Bardet-Biedl Syndrome (BBS), Cerebello-Oculo-Renal Syndrome (CORS), Meckel-Gruber Syndrome (MKS), Nephronophthisis (NPH), Joubert Syndrome (JBS), and Oral-Facial-Digital Syndrome (OFD).[1, 21-26] A unifying feature seen in most of these disorders is that the associated genes encode proteins that localize to the cilia or basal body.

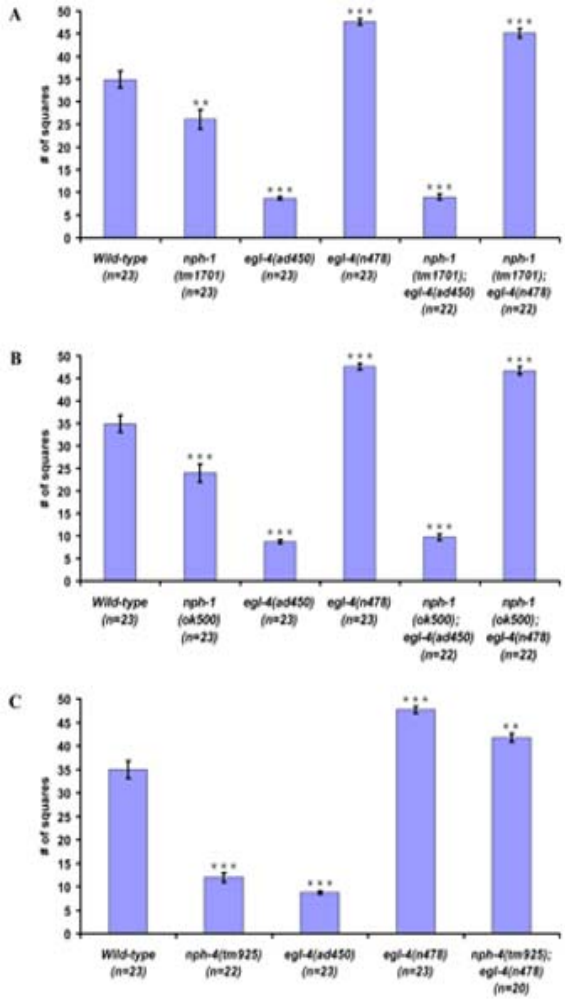

Figure 3. Analysis of genetic interactions between *nph* and *egl-4* mutants.
Locomotory behavior of *nph* mutants and *egl-4(ad450)* gain-of-function mutants is significantly decreased compared to that seen in wild-type worms. In *egl-4(n478)* loss-of-function single mutant worms the locomotory behavior is significantly increased over that seen in wild-type worms. (A) Double mutant *nph-1(tm1701);egl-4(n478)* and *nph-1(tm1701);egl-4(ad450)* worms display the roaming or dwelling behavior seen in the *egl-4* single mutants. (B) *nph-1(ok500);egl-4(n478)* and *nph-1(ok500);egl-4(ad450)* double mutants behaved in this same way as well. (C) *nph-4(tm925);egl-4(n478)* double mutants result in the excessive roaming behavior. These data indicate that the *egl-4* mutations are able to suppress the dwelling phenotypes seen in the *nph* single mutants.

The NPH proteins reside in the basal body region in mammalian cells as well as at the cilium transition zone in the nematode *C. elegans*. Studies in *C. elegans* have revealed that the NPH proteins likely function as part of a complex although the pathway in which they function is still unknown. Mutations in the NPH proteins in *C. elegans* indicate that these genes are not required for cilia formation; however, they are needed for proper cilia-mediated signaling functions regarding chemotaxis behavior and lifespan. Here we demonstrate that *nph* mutants also exhibit a locomotory phenotype that is associated with IFT mutants lacking cilia.[13, 14] This phenotype was much more severe in the *nph-4* mutant than in *nph-1* mutants, in agreement with our previous data indicating that the NPH-4 protein is required for normal localization of NPH-1 at the transition zone. We also confirm that the phenotype in the *nph-4* mutants is due to loss of the NPH-4 protein by expressing the wild type *nph-4* gene in the mutants. Interestingly, while a mutant form of NPH-4 that recapitulates a pathologic missense mutation detected in human NPH patients was able to restore the localization of NPH-1 to the transition zone, it was not sufficient to fully rescue the defect in locomotory behavior. These results indicate that this domain is not required for targeting, trafficking, or retention of NPH-4 to the transition zone or for recruitment of NPH-1 into the NPH complex; however, it does appear that this mutation impedes the normal signaling function of the NPH complex. The mild rescue of the *nph-4(tm925)* mutants expressing NPH-4(Phe83Leu)::YFP with regards to the locomotory defect could be due to a number of factors. In the transgenic *nph-4* mutants with the NPH-4 Phe83Leu mutation, the protein is still made and localizes normally to the transition zone. Therefore, even with this mutation present it is possible that this mutant version of NPH-4 retains some low level of protein function with regards to lo-

comotory behavior. Alternatively, the *nph-4* mutant worms analyzed for rescue with the NPH-4(Phe83Leu)::YFP also contained NPH-1::CFP as shown previously. It is possible that since these mutants have endogenous NPH-1 present that this over-expression of NPH-1 may result in the minimal increase in locomotory behavior. Regardless, of the slightly higher locomotion seen in these worms it is still apparent that the level of locomotion is significantly less than that seen in wild-type and *nph-4* rescue strains.

The NPH-4(Phe83Leu) mutation occurs in a region of highly conserved sequence shared in both worms and humans. In addition to this mutation, there are several other missense mutations that have been identified in the human NPH-4 protein that occur in conserved regions. Based on the current data, we believe *C. elegans* will be a very useful tool for identifying functional domains in NPH-4 and for further dissection of the mechanism leading to cystic disease in NPH patients. Overall, these data establish that both NPH proteins are required for a signaling pathway regulating normal locomotory behavior as previously observed with the IFT mutants which lack cilia. However, it is evident that NPH-4 plays a more significant role in this pathway functioning downstream of the NPH-1 protein.

There is currently very little known about the pathway involved in regulating locomotory behavior. Overall the pathway appears to be initiated by the reception of a signal that requires the cilia on the sensory neurons. Based on genetic data, the model predicts that this signal is transmitted from the cilium to regulate EGL-4 activity that balances the normal level of roaming and dwelling behavior seen in wild-type worms (Fig. 4 left). Our data indicate that the NPH complex may function as part of this signaling pathway. This is based on the genetic data indicating *egl-4* loss and gain of function mu-

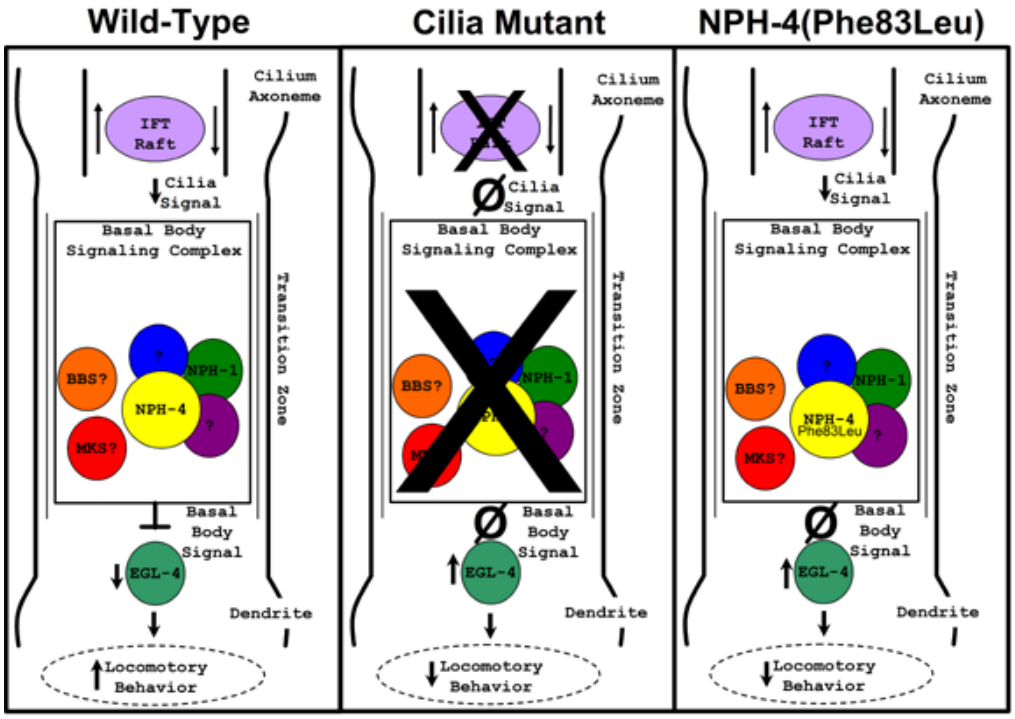

Figure 4. Model

In wild-type worms the cilia are properly formed and able to sense the external environment. Signals initiated in the cilia are transmitted through the basal body signaling complex which in turn can induce effects on downstream proteins, such as EGL-4. In wild-type worms the NPH complex at the base of the cilium functions to regulate EGL-4 activity in response to cilia initiated signals leading to a normal roaming behavior. In cilia mutant worms lacking components of IFT such as CHE-2 or OSM-5, or signaling components such as NPH-1 and NPH-4, the inhibition of the EGL-4 protein is lost resulting in an increase of EGL-4 activity. This would lead to a dwelling phenotype seen in these mutants. In worms where the NPH-4 protein has a Phe83Leu missense mutation, the NPH complex is still able to form but fails to efficiently regulate EGL-4 activity resulting in diminished locomotory behaviors.

tations are able to influence the severity of the dwelling phenotype exhibited by the *nph* mutants. Therefore, the EGL-4 protein appears to function downstream of the signal from the cilium as well as the transition zone proteins NPH-1 and, NPH-4. Interestingly a similar pathway was recently described for the EGL-4 homolog in *Chlamydomonas* (CrPKG), where its activity was found to regulate flagellar adhesion that occurs during fertilization.[27] Thus, our data support a model where signals from the cilium and the NPH proteins located at the transition zone function to impede EGL-4 activity. The loss of the cilium (IFT mutants) or the NPH proteins would lead to diminished locomotory behavior seen in the *nph* and cilia mutants (Fig. 4 center). This would explain why *egl-4* gain-of-function mutants have a similar dwelling phenotype to that seen in these cilia mutants and *nph* mutants.

CONCISE METHODS

General Molecular Biology Methods

General molecular biology was conducted following standard procedures as described. [28] *C. elegans* genomic DNA, *C. elegans* cDNA, single worms, and cloned worm DNA were utilized for PCR amplifications, direct sequencing, and subcloning as described. [28] PCR conditions and reagents are available on request. DNA sequencing was performed by the UAB Genomics Core Facility of the Heflin Center for Human Genetics.

DNA Sequence Analyses

Genome sequence information was obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Gene sequences were identified

using the *Caenorhabditis elegans* database Wormbase and references therein (http://www.wormbase.org). Sequence alignments and conserved motifs were identified using ClustalW (http://www.ebi.ac.uk/clustalw/).

Strains

Worm Strains were obtained from *Caenorhabditis* Genetics Center, *C. elegans* Knock-Out Consortium, and the National BioResource Project in Japan. The strains were grown using standard *C. elegans* growth methods [29] at 20°C unless otherwise stated. The wild-type stain was N2 Bristol. The following strains were used: RB743 *nph-1(ok500)II*, FX1701 *nph-1(tm1701)II*, FX925 *nph-4(tm925)V*, MT1073 *egl-4(n478)IV*, DA521 *egl-4(ad450)IV*, DR550 *osm-5(m184)X*, YH476 *nph-1(tm1701)II; nph-4(tm925)V*, YH502 *nph-1(ok500)II*; *nph-4(tm925)V*, YH501 *nph-1(tm1701)II; egl-4(ad450)IV*, YH477 *nph-1(tm1701)II; egl-4(n478)IV*, YH528 *nph-1(ok500)II; egl-4(ad450)IV*, YH478 *nph-1(ok500)II; egl-4(n478)IV*, YH480 *nph-4(tm925)V*; *egl-4(n478)IV*. YH452 yhIS19 (tl: *nph-4::YFP*) in FX925 was used for rescue experiments. YH457 yhEx277 (tl: *nph-1::CFP*; tl: *nph-4(Phe83Leu)::YFP*) in FX925 was utilized to determine the effect of this conserved patient mutation on protein localization and function. The rescue and site directed mutagenesis lines were generated using UNC-122::GFP[30] as a marker instead of *rol-6(su1006)* to need to examine locomotory behavior rescue.

The *nph:egl* double mutant strains were generated by crossing *nph* male worms with *egl-4(n478)* hermaphrodites. The resulting F2 offspring obtained from self fertilization were screened by PCR to identify strains containing *nph* mutations. The presence of the *egl-4(n478)* mutation was confirmed by PCR amplifying the regions containing the

point mutation, sending the PCR fragment to be sequenced, and observing the sequence for the presence of the point mutation.

Generation of Constructs and Strains

The translational NPH-1::CFP (pCJ148) and NPH-4::YFP (pCJ146) vectors have been described previously.[9] The pCJ146 vector was modified by the addition of the conserved patient mutation of T to C at 247bp into the cDNA, in the *nph-4* gene by site-directed mutagenesis as follows. The primers were designed using the Primer X website: http://bioinformatics.com/primerx/. PCR was performed on the pCJ146 construct using these primers to add the desired mutation. The resulting reaction was digested with *DpnI* to remove the parental vector and was subsequently transformed into NovaBlue singles, Novagen (San Diego, CA). DNA was isolated form the transformants and the presence of the desired mutation was confirmed by sequencing. The resulting construct p279 - NPH-4::YFP (Phe83Leu) was coinjected with the pCJ148 vector into the FX925 strain. The transgenic strains above were generated as described [31]. All PCR was performed using AccuTaq-LA DNA Polymerase (Sigma, St. Louis, MO) according to manufacturer's instructions.

Imaging

Worms were anesthetized using 10 mM Levamisole and were immobilized on a 2% agar pad for imaging. Imaging was performed using a Nikon Eclipse TE200 inverted microscope and captured with a CoolSnap HQ camera (Photometrics, Tucson, AZ). Shutters and filters were computer driven. Image acquisition was done using Metamorph

software (Universal Imaging, Downingtown, PA). Further processing of images was done using Photoshop 7.0 (Adobe Systems, Inc., San Jose CA).

Assays

The locomotory behavior assay was performed as described.[13] Briefly, a single L4 worm was placed in the center of a uniformly sized lawn of bacteria on a 6cm plate and was allowed to move freely for 18 hours. After this time the worm was removed from the plate by aspiration. The tracks were quantitated by counting the number of squares on a grid (each square 3mm X 3mm) the worm tracks entered.

Statistics

Statistics were performed utilizing a 2 tailed independent Student's T-test for comparisons of mutant strains to wild-type. Error bars represent standard error of the mean (sem).

ACKNOWLEDGMENTS

We thank Dr. A. Fire for the gifts of *C. elegans* expression vectors. We thank Dr. S. Clark for the UNC-122::GFP construct. The *C. elegans* Genome Sequencing Consortium provided sequence information and the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health, provided some of the *C. elegans* strains used in this study. We thank the *C. elegans* Knockout Consortium and the National BioResource Project in Japan for providing the *nph-1* and *nph-4* deletion mutants. This work was supported by a NIH grant to B.K.Y. (RO1DK065655)

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SUMMARY

Establishment of C. elegans as a Model of Nephronophthisis Protein Function

The study of the *C. elegans* homologs of the gene mutated in the Oak Ridge Polycystic Kidney mouse as well as the genes mutated in human ADPKD has added to our overall knowledge of the mechanism responsible for cystic kidney diseases and to the functions of the affected proteins, OSM-5, and LOV-1 and PKD-2 respectively. Loss of the OSM-5 protein in *C. elegans* results in structurally stunted cilia, which in turn causes these mutants to inadequately sense the external environment (Haycraft et al., 2001). In the case of LOV-1 and PKD-2 mutants the cilia are properly formed, but these worms still exhibit sensory defects with regards to male mating behavior (Barr et al., 2001). The purpose of this dissertation was to establish *C. elegans* as a model system to assess the functions of the proteins NPH-1 and NPH-4, whose human homologs are mutated in the cystic kidney disease Nephronophthisis.

The genes that are mutated in cystic kidney disorders often encode proteins that associate with cilia. Therefore, my initial hypothesis was that NPH-1 and NPH-4 would localize to cilia in the sensory neurons of the worm. We had reason to believe this hypothesis may be correct since we identified an X-box motif in the promoter region of both *nph* genes indicating that they may be regulated by the transcription factor DAF-19, that regulates many cilia associated genes. The first step taken was to determine whether the genes *nph-1* and *nph-4* were expressed in the ciliated sensory neurons in the worm, since these are the only cells in this organism that contain cilia. The generation and ob-

servation of transgenic worms containing transcriptional fusion constructs of both genes confirmed that these genes were expressed in the amphid, labial, and phasmid neurons in the worm, as was expected. Subsequently, the regulation of these genes by DAF-19 was confirmed *in vitro* by RT-PCR and *in vivo* by expression analyses in *daf-19* mutant compared to *daf-19* +/+ worms.

With this evidence that the *nph* genes were expressed in the ciliated sensory neurons, the possibility that the corresponding proteins would be found to localize to cilia seemed increasingly likely. At this point the localization of NPH-1 and NPH-4 was observed in transgenic worms co-expressing translational fusion constructs for both of these proteins. Both proteins co-localized to the same region at the distal end of the dendrite where the cilia reside, but the fluorescence remained in spots at what looked to be the base of the cilia also known as the transition zone. The specific localization of the NPH proteins to the transition zone, but not within the cilium axoneme, was confirmed by observing transgenic worms co-expressing full length constructs of each NPH protein along with the cilia marker CHE-13. This result established the NPH proteins as being among the first cystic kidney disease related proteins to localize solely to the base of cilia at the transition zone, which is analogous to the mammalian basal body, but not in the cilia axoneme. These data implicate basal body dysfunction as a cause of the defects seen in human Nephronophthisis and possibly other cystic kidney disease patients.

To gain further knowledge as to the role the NPH proteins might be playing at the transition zone, mutants of *nph-1* and *nph-4* were obtained and characterized. Interestingly, the cilia in these mutants formed normally explaining the lack of defects with regards to dye-filling and osmotic avoidance in these worms. These mutants also had nor-

mal regulation of dauer formation at 20°C and 25°C indicating a lack of the Daf-d phenotype that is seen in IFT mutants without cilia.

Analysis of lifespan in both *nph-1* and *nph-4* single and double mutants indicated an extension over that in wild-type worms. However, the *nph-1* single and the *nph-1;nph-4* double mutants exhibited a milder increase in lifespan in comparison to that seen in *nph-4* single mutants. This was an unexpected result and the reason behind it is not entirely clear. It is possible that in terms of lifespan signaling the NPH-4 protein prevents the NPH-1 protein from performing an additional function that allows for a further increase in lifespan when NPH-4 is not present, with this effect being lost in the double mutants that are missing both proteins.

In addition to the extension of lifespan, the *nph* mutants also displayed defects in the ability to undergo chemotaxis toward the chemoattractants, diacetyl and benzaldehyde. However, unlike the unusual results observed for lifespan, the chemotaxis behavior was diminished to comparable levels in both the single and the double mutants. The implications of this data will be discussed below.

The final phenotype that was assayed in the *nph* mutant worms for this thesis was that of locomotion in the presence of food. In this case, the *nph-4* mutants exhibited a severe decrease in locomotion compared to wild-type worms. Again, as was seen for the lifespan analysis the *nph-1* mutant phenotype was less severe than the *nph-4* mutant. However, unlike in the lifespan assay the *nph-1;nph-4* double mutants also took on the severe phenotype seen in the *nph-4* single mutants. An additional piece of data that likely explains these results is the fact that the NPH-1 protein is unable to localize to the transition zone in the *nph-4* mutant background. Therefore, a *nph-4* single mutant should re-

sult in a similar phenotype to that seen in a *nph-1;nph-4* double mutant assuming that the transition zone localization of NPH-1 is required for the phenotype in question.

With this in mind the following conclusions can be drawn as a result of these phenotypic assays. The lifespan is most dramatically extended in the *nph-4* mutant background that is unable to properly localize NPH-1 to the transition zone. Therefore, it seems likely that the previously proposed additional function of NPH-1 in the *nph-4* mutants must be occurring elsewhere in the sensory neurons. This is interesting considering that there is evidence to support a role for Nephrocystin-1 outside of the cilia at cell junctions in mammalian systems (Donaldson et al., 2000). In contrast, the similar defects in chemotaxis in both the *nph* single and double mutants could be simply explained by the loss of NPH-1 protein at the transition zone alone. The *nph-1*, *nph-4*, and the *nph-1;nph-4* mutants are all effectively defective in NPH-1 localization to the transition zone. This is either due to lack of the gene or in the case of the *nph-4* mutants the loss of proper localization. Therefore, removing NPH-1 from the transition zone is enough to cause the chemotaxis defects alone because the loss of *nph-4* appears to have no further effect. Next, the similar severe decrease in locomotion seen in the *nph-4* single and *nph-1;nph-4* double mutants, that is diminished in comparison to that of the *nph-1* single mutants is easily explained. Assuming the transition zone localization of NPH-1 is required for its ability to undergo proper locomotion, both the *nph-4* and the *nph-1;nph-4* mutants should behave essentially the same as is seen here. Interestingly, since the *nph-1* mutants have a smaller effect on the locomotory behavior phenotype compared to *nph-4* mutants it suggests that NPH-4 functions downstream of NPH-1, which is also supported by the localization data. Finally, work from Maureen Barr's lab has indicated that *nph-1;nph-4* dou-

ble mutants, but not single mutants, display a diminished ability to respond to a hermaphrodite during mating (Jauregui and Barr, 2005). This is interesting considering the fact that *nph-4* single mutants should behave similarly to the *nph-1;nph-4* double mutants, if the transition zone localization of NPH-1 is required for the behavior being assayed. Therefore, the most likely explanation for the mating response being only reduced in the double mutants is a result of an additive effect of the loss of NPH-1 function outside of the transition zone as well as the loss of NPH-4 function.

Overall, it seems evident that NPH-1 and NPH-4 function as part of multiple cilia-mediated signaling pathways leading to subtle, but important variations in phenotypic outcomes. Utilization of the nematode *C. elegans* has allowed us to appreciate these subtleties that would have been difficult to separate in higher organisms. These studies have managed to establish the usefulness of examining Nephronophthisis proteins in *C. elegans*.

Relevance of C. elegans Studies to Human Disease

The relevance of conducting studies in *C. elegans* is often brought into question, due to the vast differences between this invertebrate nematode and human beings. For example, the research described in this dissertation involves the analyses of two cystic kidney disease associated proteins in an organism that does not even have a kidney. However, in terms of the cystic kidney disease field a great deal has been learned from studies of proteins affected in these diseases in the *C. elegans* sensory cilia. Additionally, the analysis of the proteins involved in intraflagellar transport has been greatly aided by the study of these proteins in lower organisms such as *Chlamydomonas* and *C. elegans*.

One major reason these organisms have been so useful in these studies is that in contrast to mammalian systems, cilia are not required for viability.

This dissertation has been able to provide further direct evidence for the relevance of analyzing cystic kidney disease proteins in the nematode. The translational fusion construct of NPH-4 that was utilized to determine the localization of this protein to the transition zone, was mutagenized at the highly conserved residue Phenylalanine 83. The mutagenesis resulted in a conversion of this residue to a Leucine, recapitulating a missense mutation identified in human NPH patients. Transgenic *nph-4* mutants were generated with this NPH-4(Phe83Leu)::YFP and NPH-1::CFP. The observation of these worms indicated that the mutagenized NPH-4 was able to localize normally to the transition zone and it was also able to rescue the *nph-4* mutant's inability to properly localize NPH-1. However, these same transgenic worms were unable to completely rescue the severe dwelling phenotype seen in the *nph-4* mutants.

The inability of the NPH-4(Phe83Leu) construct to rescue the decreased locomotion seen in the *nph-4* mutants lends further support to the significance of utilizing *C. elegans* to study human disease associated proteins. In this case the NPH-4(Phe83Leu) protein retains the ability to undergo proper targeting and trafficking to the transition zone. Further, it is also able to restore normal trafficking or retention of NPH-1. However, the single amino acid change in this protein greatly diminishes the protein's ability to rescue the defect in locomotory activity. This information furthers our knowledge about the potential role a single amino acid appears to be playing in *C. elegans* NPH-4 and possibly human Nephrocytin-4 function. There are several other mutations identified in human NPH-4 that occur in equally conserved domains. Thus analysis of these NPH-4 proteins

in C. elegans that recapitulate the mutations identified in human NPH patients will provide important insights into the molecular basis of the disease.

Protein kinase G and the Basal Body Signaling Complex

The most dramatic phenotype identified to date involving the *nph* mutants in *C. elegans* is the severely diminished locomotion in the presence of food. This is particularly evident in the *nph-4* and *nph-1;nph-4* mutants. This same phenotype is seen in cilia IFT mutants as well as in gain of function (gof) mutants of *egl-4*, the *C. elegans* homolog of the cGMP dependent Protein Kinase G (PKG). In order to determine where the NPH proteins function in this locomotory behavior signaling pathway we analyzed double mutants of each of the *nph* genes along with loss of function (lof) and gain of function (gof) mutants of *egl-4*. As a result of this analysis we found that *nph1/4;egl-4* (lof) mutants exhibit a suppression of the diminished locomotory behavior seen in the *nph* single mutants, overall taking on the *egl-4* (lof) excessive roaming phenotype. A similar suppression is seen in double mutants of the cilia IFT gene *che-2* along with this *egl-4* (lof) mutant as well. Additionally, *nph-1;egl-4* (gof) mutants also took on the *egl-4* (gof) phenotype. Mutants of *nph-4;egl-4* (gof) could not be sufficiently analyzed in this way because both of the mutations result in a similar dwelling phenotypes that would be difficult to distinguish between.

The model that I have proposed describing the relationship between cilia, basal body, and PKG signaling in *C. elegans* is as follows. In wild-type worms a signal is received from the cilia and is transmitted to the basal body. The basal body region consists of a congregation of proteins that is believed to form a signaling complex, including

NPH-1 and NPH-4. This basal body signaling complex in turn functions to negatively regulate EGL-4 signaling by an unknown mechanism. Two possible ways EGL-4 signaling could be diminished is by decreasing protein abundance or by more directly affecting the protein activity. I favor the latter alternative and envision a scenario where the basal body signaling complex holds EGL-4 in an inactive state or prevents interactions with other factors required for inducing EGL-4 activity. Therefore, when the signal from the cilia is lost or the basal body signaling complex is disrupted, EGL-4 is activated resulting in the diminished locomotory behavior seen in these animals.

It seems likely that the basal body signaling complex is composed of numerous proteins that may also have connections to other non-NPH forms of cystic kidney disease. An excellent candidate for a protein that may function as part of this signaling complex is RPGRIPL1. This protein which is associated with Cerebello-Oculo Renal Syndrome (CORS) patients localizes to the basal body and interacts with Nephrocystin-4 in mammalian systems (Arts et al., 2007). Interestingly, it has recently been hypothesized that CORS, Joubert Syndrome (JS), and Meckel-Gruber Syndrome (MKS) may actually represent the same underlying disorder (Delous et al., 2007). Therefore the genes mutated in JS and MKS are also potentially important components of the basal body signaling complex. This theory is further supported by the localization of MKS1 to the basal body.

Conclusions and Future Directions

The work in this dissertation has served to firmly establish *C. elegans* as a relevant model for studying Nephronophthisis protein function. Here I have shown that the proteins NPH-1 and NPH-4 localize to the transition zone at the base of cilia and loss of

these proteins result in cilia-mediated signaling defects in the worm. In terms of future directions, there are numerous experiments that could be conducted that would further broaden our knowledge as to the role the NPH proteins are playing.

First, the studies in this dissertation have established that the EGL-4 protein functions downstream of the NPH proteins at the basal body, in addition to its role downstream of cilia. The gene *egl-4* is ubiquitously expressed in the worm and has multiple isoforms. Mutations in *egl-4* have wide-ranging consequences beyond the effects on locomotion that have been characterized here. Most of these defects are not seen in the *nph* mutants suggesting they arise due to affects of *egl-4* on other pathways. In relation to the cilia, *egl-4* is expressed in the ciliated neurons of the head, but the protein localization has yet to be determined in these cells (Hirose et al., 2003). However, it seems likely that this protein functions within the cilia because its homolog in *Chlamydomonas* has been localized to flagella and was identified to be important for the flagella-mediated process of fertilization in this organism (Wang et al., 2006). Determining the protein localization of EGL-4 and the effect of the *nph* mutations have on this localization will lead to a better understanding of the connections between cilia, basal body, and PKG signaling.

Additionally, studies need to be conducted to identify additional components of the proposed basal body signaling complex. One means of accomplishing this is based on a candidate gene approach in which genes such as the *C. elegans* homolog of RPGRIPL1 are characterized based on their established connection to the NPH proteins. However, an approach that identifies novel components of the NPH pathway would also be extremely beneficial in delineating the functions of these proteins. In this regard, we are optimizing an EMS mutagenisis screen to identify secondary mutations that are capa-

ble of suppressing the attenuated ability of the *nph-4* mutant worms to undergo chemotaxis and locomotion. As a result of a small pilot study we were able to identify a mutagenized *nph-4* worm that exhibited a normal chemotaxis response and had significantly suppressed the dwelling phenotype. In fact these *nph-4* suppressor mutants have an excessive roaming phenotype (58.9 squares) even over what is seen in *egl-4* loss of function mutants (47.7 squares). We believe that this mutagenesis strategy will likely provide us with further mutants that function in the NPH signaling pathway. Additionally, the one mutation that we identified is being mapped to identify the gene involved.

The studies described in this dissertation have laid the groundwork for more specifically determining the roles of the NPH proteins. We are hopeful that the mutagenesis screen described above will result in the identification of additional proteins that have connections to cystic kidney disease related dysfunction. Overall, a large amount of information has been gained from the study of cystic disease proteins in *C. elegans*, but there is still much left to do.

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