THE CILIOPATHY GENE NPHP-2 FUNCTIONS IN MULTIPLE GENE NETWORKS AND REGULATES CILIOGENESIS IN C. ELEGANS

By

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ABSTRACT OF THE DISSERTATION

The ciliopathy gene \textit{nphp}-2 functions in multiple gene networks and regulates ciliogenesis in \textit{C. elegans}

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Cilia are hair-like organelles that function as cellular antennae. Cilia are conserved across eukaryotes, and play a vital role in many biological processes including signal transduction, signal cascades, cell-cell signaling, cell orientation, cell-cell adhesion, motility, interorganismal communication, building extracellular matrix, and inducing fluid flow. In humans, cilia are present in a majority of tissue types, and cilia dysfunction can lead to a range of syndromic ciliopathies, including nephronophthisis (NPHP) and Meckel Syndrome (MKS).

Cilia have a microtubule backbone, the axoneme, and are composed of multiple subcompartments, each with a specific function and composition: the transition zone (TZ) anchoring to the axoneme to the membrane, the doublet region extending from the TZ, and in some cilia types, the singlet region extending from the doublet region.

The nematode \textit{C. elegans} is a well-established model of cilia biology, and possesses cilia at the distal end of sensory dendrites. My work focused on exploring the role and function of \textit{nphp}-2, the \textit{C. elegans} ortholog of mammalian \textit{INVS/NPHP2}, and the relationships between \textit{nphp}-2, the TZ, and the doublet region. I found that TZ-associated genes can be grouped into
two redundant genetic modules that interact with *nphp-2* cell-type specific manner to regulate ciliogenesis and cilia placement. I also found that, like its ortholog Inversin (encoded by *INVS*), NPHP-2 localizes to the eponymous Inversin compartment, a subportion of the doublet region. *nphp-2* genetically interacts with other doublet region-associated genes; like TZ-associated genes, these interactions can also be organized into two redundant genetic modules. The doublet region modules regulate many aspects of cilia biology, including ciliogenesis, cilia placement, microtubule patterning, tubulin post-translational modification, and doublet region composition. Additionally, I characterized a positive and a negative regulator of the doublet region modules.

Together, these results help knit together data from mammalian models and *C. elegans*, and build a genetic framework between *nphp-2* and genes from multiple ciliary compartments, pulling together what were disparate threads to lead us to a better understanding of cilia biology.
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Chapter 1: Cilia Biology and the Inversin Compartment

1.1 – Introduction to cilia

1.1.1 – Introduction

On the whole, cilia and flagella are amongst the most remarkable organelles, with wide-ranging structure and function, and define eukaryotes in the same manner as nuclei and mitochondria (Cavalier-Smith, 2002). Members of this class range from the simple thread-like mammalian primary cilia, bent by fluid flow in the renal lumen; to the willow-like chemosensory AWA, used by Caenorhabditis elegans to taste the environment; and to the pair of synchronously beating flagella used by Chlamydomonas to glide through pond water. In humans, cilia are fundamental to life. Their sweeping motion guides eggs through fallopian tubes towards flagella-driven sperm, enabling fertilization. During embryogenesis, cilia drive developmental signaling pathways, break left-right symmetry post-gastrulation, and regulate the development of most organs, including hearts and kidneys. After birth, cilia allow us to see, hear, smell, and taste the world. But for all their variety in role and purpose, a majority of cilia share a similar underlying logic in structure and composition.

1.1.2 – Abstract

Cilia are cellular antennae, a membrane-clad microtubule skeleton protruding from the cell surface. Cilia function in many fundamental processes, including sensation, motility, development, adhesion, and vesicle release. Cilia can be divided into several subcompartments with distinct compositions, structures, and roles. A recently discovered subcompartment is marked by the localization of Inversin, a protein that has been implicated in modulation of Wnt-signaling, cilia placement, and cilia assembly/disassembly. Other proteins have also been
found to localize to this “Inversin compartment”, including Nek8, Nphp3, and Anks6. These Inversin compartment components genetically and physically interact with numerous ciliary and non-ciliary partners; this suggests that the Inversin compartment is intertwined with many aspects of cilia biology. In this chapter, I discuss the biology of the Inversin compartment: its characterization, composition, role in signaling pathways and signal transduction, and its interactions with cilia base components, revealing the many facets of Inversin compartment structure and function. The abundance of new evidence allowed us to explore many questions: How do we define this proximal ciliary compartment? How is it established? What are its structural features? What is its protein and lipid composition? How are these aspects modulated? How are these features conserved across cilia types and organisms? And the $64,000 question: What is the function of the Inversin Compartment?

1.2 – Brief Overview of Cilia Biology

1.2.1 – Cilia structure

Defining the structure of cilia has taken many years and careers, though the task is not yet complete (ultrastructure reviewed in Fisch and Dupuis-Williams, 2011). Mammalian renal tubule primary cilia, a common cilia model, comprise a microtubule backbone clad with membrane, anchored to the cell at the base (Figure 1). At this base lies the centriole-derived basal body, composed of nine microtubule triplets. Nine microtubule doublets extend from these triplets away from the cell, forming the transition zone (TZ). The TZ anchors the ciliary microtubule

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1 NB: Often, when specific ciliary sublocalization of a reporter cannot be determined (usually due to the absence of colabelling with known reporters), nonspecific terms are used. “Proximal cilium” refers to the portion of the cilium nearest the cell body/dendrite, encompassing any or all of the basal body, TZ, InvC, and doublet region; “distal cilium” refers to the portion of the cilium furthest from the cell body, encompassing the singlet region and distal tip.
structure—the axoneme—to the membrane through Y-links composed partly of MKS and NPHP proteins. These membrane anchors pucker the outer ciliary membrane in a distinctive pattern visible by electron microscopy, called the ciliary necklace due to its appearance (Gilula and Satir, 1972). The microtubule doublets of the TZ then extend much further, forming the ciliary shaft. In some cilia types, microtubule singlets, in turn, extend from the doublets. This distal microtubule singlet region can be permanent as in C. elegans amphid channel and phasmid cilia, or transient, as in Chlamydomonas. While the above general arrangement holds true in most cilia, in C. elegans, the basal body degenerates after ciliogenesis, leaving the TZ as the primary anchor (Perkins et al., 1986) (Figure 1). In addition to the structural singlet and doublet microtubules discussed above, there are often a number of microtubule singlets\(^2\) that lie near the center of the cilium and run along its entire length. These are required for motility in flagella and motile cilia, but have an unknown function in sensory cilia.

A word of caution is warranted before moving forward, though. Though almost all literature, including this dissertation, treats cilia as broadly comparable, it is important to remember that there is no such thing as a “standard” cilium. All cilia are specialized for specific purposes, and because of the diversity of these purposes, there are almost no universally common features. Not all cilia have all subcompartments, cilia ultrastructures can be vastly different, many cilia components play different roles in different in different cell types, and many components are not universally conserved in ciliated and flagellated organisms. A brief description of the various ciliary compartments follows (Figure 1).

**Periciliary membrane.** In general, the base of the cilium, including the periciliary membrane,

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\(^2\) These are henceforth referred to as “central singlets” to distinguish them from the nine structural microtubule singlets. In standard cilia and flagella nomenclature, cilia and flagella re categorized as the number of outer microtubules plus the number of central singlets, e.g., 9+2. C. elegans amphid cilia can range from 9+0 to 9+5 (Perkins et al., 1986).
basal body, transition fibers, and transition zone function to regulate ciliary targeting and import of cilia components. The plasma membrane surrounding the cilia base plays a specialized role in cilia biology (Reviewed in Nachury Seeley 2010). This membrane compartment can be five or more times wider than the cilium itself, and is surrounded by a diffusion barrier (Hunnicutt et al., 1990; Vieira et al., 2006). This barrier may be formed by galectin-3 (Clare et al., 2014), Usher Syndrome proteins (Maerker et al., 2008), or Septin 2, the latter of which is required for localization of cilia membrane proteins and cilia function in the Sonic Hedgehog pathway (Hu et al., 2010) (See 1.5.1 – Signal Transduction and Amplification). Membrane composition may be defined solely by lipid rafts, though the actin cytoskeleton underlying the membrane may divide it into “nanocells”, small patches of membrane to which transmembrane proteins are restricted (demonstrated in a series of papers from the Kusumi lab: Fujiwara et al., 2002; Morone et al., 2006; Nakada et al., 2003). Because the cilia and plasma membranes are divided, membrane proteins cannot freely travel between them. The periciliary region acts as a “landing strip” for vesicles from the rest of the cell loaded with ciliary cargo. These vesicles, laden with transmembrane cilia proteins, fuse to the membrane here, allowing the cilia proteins to be trafficked into the cilium through intraciliary trafficking pathways (Pazour and Bloodgood, 2008). The periciliary region is also the likely site of extracellular vesicle release in several ciliated cells, including the CEMs of male C. elegans (Wang et al., 2014). Rab8 and its activator Rabin8, which function together to target endosomes from the cell body to the cilia base, mediate some of these vesicular pathways (Molla-Herman et al., 2010; Moritz et al., 2001). The exocyst (not exorcist!) tethering complex regulates binding of these vesicles to the cilia membrane (modelled in Fogelgren et al., 2011). Both the polarized trafficking factors Rab8/Rabin8 and the exocyst complex are required for ciliogenesis and cilia function, and can lead to disease when disrupted (Fogelgren et al., 2011; Kaplan et al., 2010; Nachury et al., 2007; Zuo et al., 2009).
Distal dendrite. Because the cilia of *C. elegans* are found at the distal end of dendrites, they do not have a circular periciliary membrane patch surrounding them as mammalian cilia do. Instead, the dendritic tip constitutes the periciliary membrane, and can be distinguished from the rest of the dendrite by an enlarged diameter. The volume enclosed within the dendritic tip acts as a delivery and sorting center for ciliary traffic, connecting the ciliary traffic network to the dendritic microtubule traffic network. In addition to the functions described for the periciliary membrane described above, in amphid cilia, the distal dendrite is required for dendritic extension (Heiman and Shaham, 2009).

Ciliary pocket/pore. Much of what is known about the cilia pocket was established in the 1960s, but recent work has linked this region to ciliogenesis, and exocytosis/endocytosis at the cilia base (Sorokin, 1962; Sorokin, 1968; reviewed in Ghossoub et al., 2011). As the primary function of the cillum is as a liaison with the environment, the extracellular environment it lies in is critical to its function; mammalian and *C. elegans* cilia greatly differ in this respect. In mammalian cilia, most of the length of the cillum is directly exposed to the environment. Membrane around the cillum base is invaginated (shaped by the actin cytoskeleton) and the gap between the cillum and the plasma membrane is filled with extracellular matrix (Molla-Herman et al., 2010). The ciliary pocket of mammalian cilia is the site of active endocytosis.

*C. elegans* cilia, on the other hand, are completely buried within the ciliary pores, spaces contiguous with the external environment and wrapped by a sheath and a socket cell (Figure 2). This extracellular lumen is also filled with extracellular matrix (Perkins et al., 1986). Little is known about the composition of this matrix, though matrix proteins are present in ciliary-promoter-derived ciliomes and are upregulated in male ciliated neurons (Wang et al., 2014), and several transmembrane ciliary proteins have interaction domains (including protein-protein and glycosylated protein binding domains) on their external face (Bycroft et al.,
The basal body (BB) forms the “root” of the cilium, anchoring the cilium to the cytoskeleton and connecting the ciliary traffic network to the rest of the cell. The basal body is enriched in gamma-tubulin, suggesting that it is the site of active microtubule nucleation (e.g., Silflow et al., 1999). In mammalian cilia, the basal body is composed of the microtubule triplet centriole and centriolar associated protein (CEP) complex. The CEP is attached to the membrane through nine propeller-like transition fibers. The transition fibers possibly attach to the membrane through Cep164 and ODF2 (Graser et al., 2007; Ishikawa et al., 2005). The transition fibers act as site for docking of intraflagellar transport (IFT) components, in a DYF-19/FBF1 specific manner, bound for the cilium (Wei et al., 2013).

The existence of a basal body in C. elegans cilia is contentious. After ciliogenesis (See 1.2.4 – Ciliogenesis), centrioles undock from the cilia base, leaving a portion of the CEP complex behind (including the ciliopathy protein HYLS-1 [Dammermann et al., 2009]); in daf-19 mutants, which are missing the master ciliogenesis transcription factor, centrioles are found where the cilia base is expected to be (Perkins et al., 1986). This demonstrates that the centriole is present at the cilia base of C. elegans cilia for some portion of time. Some groups maintain that although the mature C. elegans cilia base does not contain a centriole (Perkins et al., 1986), the complex that is left behind constitutes a functionally conserved basal body (Williams et al., 2011). This hypothesis is supported by (1) localization of the centrioles in daf-19 mutants (2) localization of the centriolar protein HYLS-1 to the cilia base in C. elegans, and (3) conservation of ultrastructural features (transition fibers). Both the BB and the C. elegans pseudo-BB function

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3 Transition fibers may not exist in all cilia types. Detailed EM analyses in C. elegans suggest that “transition fibers” may be an EM artifact, and are actually splayed microtubule doublets that are not recognizable as such because the angle between the microtubules and the plane of the EM slices (Doroquez et al., 2014).

4 In this work, we use the term “pseudo-BB” to represent the region of the C. elegans cilium analogous to...
in regulation of traffic into and out of the cilium; IFT components are enriched in this compartment.

**Transition Zone (TZ).** The first compartment of the cilium proper is the transition zone: it is formed of microtubule doublets, and has a complex structure and multiple functions, including anchoring the cilium to the membrane and regulating ciliary traffic. It is described in detail below (and reviewed in Szymanska and Johnson, 2012).

**Doublet region.** The microtubule doublets of the transition zone extend to form the doublet region. In mammalian cilia, the doublet region constitutes the rest of the cilium; in *C. elegans* amphid and phasmid cilia the doublet region constitutes approximately half the length of the cilia shaft, giving the region its alternate name, the middle segment (Perkins et al., 1986). The doublet region has few known specific functions. In mammalian cilia, as it forms the largest ciliary compartment, it is the site of most signal transduction. In *C. elegans*, most signal transduction components localize to both the doublet and singlet regions, such that it is unknown whether the doublet region has specific signal transduction functions. The doublet region does contain passing cargo bound for and returning from the distal singlet region (Ou et al., 2005); this passing traffic can hypothetically be modulated by doublet region components. New evidence also suggests that the doublet region correlates to cilia shape (Malan Silva, Personal Communication).

**Inversin Compartment (InvC).** Unlike the other cilia regions described here, the Inversin compartment is actually a portion of another region of the cilium, the doublet region. This region is defined by the localization of Inversin (Shiba et al., 2009). The Inversin compartment is the main focus of this dissertation; the origin and possible function of the InvC are described in detail below, and in Chapters 3 and 5.
Singlet region. Singlet microtubules extend from the microtubules of the doublet region into the distal region; in some cilia types, only one microtubule from each doublet extends (the B-tubule), whereas in other cilia types both microtubules from each doublet extend (A- and B-tubules) (Perkins et al., 1986), (Malan Silva, Personal Communication). There are exceptions to this: singlets of a few cilia, including CEP and amphid wing cilia (discussed in Chapter 3), are formed de novo (Doroquez et al., 2014; Perkins et al., 1986). The singlet region is often very specialized in shape and defines the function of a given cilium (Doroquez et al., 2014). In C. elegans, it is often the only portion of the cilium that is directly exposed to the environment and so is frequently linked to signal transduction pathways.

Distal tip. The distal tip of the cilium has no microtubules, and functions in vesicle release and turnaround of intraciliary trafficking components. The distal tip may also have a role regulating the length of the cilium.

1.2.3 – The ciliary transition zone

The ciliary transition zone lies at the base of the cilium between the basal body and the cilia shaft, and is so named because it is the transition between the cell and the cilium. The transition zone was first identified in TEM micrographs: it appeared as a circle of microtubule doublets held in place by an interior ring-like structure, linked to the membrane through ‘Y’ shaped protein links (Gibbons and Grimstone, 1960; Ringo, 1967). These Y-links are hypothesized to be composed in part of MKS and NPHP proteins. MKS and NPHP proteins may have other roles in addition to this structural function, although none have any predicted enzymatic domains, and instead contain a large amount of protein-protein, protein-lipid, and calcium binding domains. MKS and NPHP proteins/genes fall into physical and genetic modules (Garcia-Gonzalo et al., 2011; Sang et al., 2011; Warburton-Pitt et al., 2012; Williams et al., 2011). These include, to
date: the MKS module (MKS1, MKS6, B9D1/MKS9, B9D2/MKS10, TCTN1, TCTN2/MKS8), the first NPHP module (NPHP1, NPHP4), and the second NPHP module \(^5\) (NPHP5, NPHP6, Ataxin10). As new TZ genes are identified, they can usually be grouped with one of these modules. While the MKS and first NPHP modules are almost universally conserved, work in \(C.\) \textit{elegans} identifies MKS-5/NPHP-8 as both an MKS and NPHP module component (Williams et al., 2011), whereas work in mammalian cell culture identifies RPGRIP1L/MKS5/NPHP8 as a physical component of the first NPHP module (Sang et al., 2011). In \(C.\) \textit{elegans}, MKS-5 functions to organize the localization of the entire MKS physical module but not components of the NPHP module, which would place it firmly as a member of the MKS module; however, \textit{mks}-5 genetically interacts with both \textit{mks} genes and \textit{nphp}-4, which places outside of both modules (Williams et al., 2011). (For a discussion of the unique genetic interactions between \textit{mks}-5 and \textit{nphp}-2, see Error! Reference source not found.).

The TZ has many proposed functions, but all can be categorized as either gating or anchoring. The first proposed function for the Y-links is anchoring the cilium to the membrane. Y-links physically connect the microtubules of the axoneme to the membrane. Disruption of TZ links in mutants has been linked to misplaced cilia in this and other work (Warburton-Pitt et al., 2014; Warburton-Pitt et al., 2012; Williams et al., 2010). TZ anchoring is required for proper ciliogenesis, as interactions between the TZ, basal body, and membrane are required during the first step of ciliogenesis (See below, 1.2.4 – Ciliogenesis).

The second set of proposed functions relate to a gating function. As the Y-links physically fill the TZ, they act as an occlusion mechanism, preventing large soluble molecules or membrane components from freely diffusing into or out of the cilium (Awata et al., 2014; Cevik et al., 2013; Huang et al., 2011; Kee et al., 2012). Disruption of the TZ leads to leakage of components in both

\(^5\) Or the “JBTS module”, coined by Szymanska and Johnson, 2012.
directions (Cevik et al., 2013; Huang et al., 2011; Williams et al., 2011). Startlingly, 3D reconstructions of TZ EMs reveals that gaps between neighboring Y-links are the exact same shape and size as IFT trains, implying that the TZ plays a critical role in regulation of IFT. The position of the TZ in the cilium necessitates its involvement in other ciliary trafficking pathways, including the nuclear pore-like Ran-importin system (Dishinger et al., 2010; see Error! Reference source not found.) and the Unc119b protein shuttle (Wright et al., 2011; see Chapter 3, and 1.4.4 – Establishing the Inv Compartment), though how these intersect is completely unknown.

1.2.4 – Ciliogenesis

There are two primary methods of ciliogenesis (called here types A and B), which vary depending on cell type (Figure 3) (summarized in Williams et al., 2011).

**Step One (Type A):** The centrosome protein complex is assembled around the centrioles, forming the proto-basal body. This complex then binds the ciliary vesicle, a step that may depend on the ciliopathy gene OFD1/JBTS10 and Talpid3 (Singla et al., 2010; Yin et al., 2009). The microtubules of the basal body then extend and impinge on the vesicle membrane, forming the proto-TZ. (The microtubule growth in this step is likely to be driven by tubulin diffusion, as IFT mutants still have this stunted cilium.) The microtubules of the proto-TZ bind to the vesicle membrane through Y-links. This ciliary vesicle complex then docks and fuses to the plasma membrane.

**Step One (Type B):** After the centriolar protein complex is assembled, it docks directly with the plasma membrane. The microtubules of the basal body then extend, forming the TZ, which further anchors the cilium to the membrane.

**Step Two (Types A and B):** IFT drives further extension of the cilium to its full length, and ciliary subregions are established through microtubule patterning pathways.
An important recent paper clarified several aspects of ciliogenesis in *C. elegans*, and tied them to distinct mechanisms (Williams et al., 2011). The first step is mediated in part by TZ complexes, described in the previous section; disruption of this step in severe TZ mutants may lead to knock-on defects in the second step of ciliogenesis. Targeting of the basal body to the plasma membrane can be controlled by Disheveled, a component of Wnt signaling pathways (Vladar and Axelrod, 2008) (See 1.5.1 – Signal Transduction and Amplification). The second step is driven by IFT and BBS complexes (described in the section below); during this step, the TZ functions primarily as a ciliary gate. The processes of the second step of ciliogenesis continue throughout the lifetime of the cilium, maintaining the cilium. Additional pathways also specifically regulate cilia maintenance, as described below. The division between the two steps of ciliogenesis and the different mechanisms driving each step is highlighted by the facts that defects in IFT mutants appear markedly different from TZ mutants, and that as a whole, TZ mutants tend to have relatively normal IFT rates.

### 1.2.2 – Intraflagellar Transport

Intraflagellar Transport (IFT) is the primary ciliary cargo transport mechanism, consisting of bidirectional movement of IFT particles along the length of the axoneme (Figure 4). Cargo carried by IFT can include receptors and structural components (e.g., tubulins [Bhogaraju et al., 2013; Bhogaraju et al., 2013] and PKD-2 [Bae et al., 2006]). In *C. elegans*, anterograde transport (from the cilia base to the cilia tip) is mediated by two microtubule motors: heterotrimeric kinesin-II (KLP-11, KLP-20, KAP-1) and homodimeric OSM-3. Kinesin-II and OSM-3 function cooperatively in microtubule doublet cilia regions (the basal body, TZ, and doublet region), and OSM-3 alone functions in microtubule singlet regions (Ou et al., 2005). Retrograde transport

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*In the context of this dissertation, cilia and flagella are synonymous terms*
(from the cilia tip to the cilia base) is mediated by IFT dynein, which functions in all cilia regions. The IFT transport complex comprises two subcomplexes, IFT-A and IFT-B, which are linked by the BBSome. In some BBS mutants, the two IFT subcomplexes become separated and travel independently, leading to defects in ciliogenesis. Each of the two anterograde IFT motors is associated with a specific IFT particle: Kinesin-II is bound to IFT-A, and OSM-3 is bound to IFT-B.

Though Kinesin-II and OSM-3 have different intrinsic velocities, because the entire IFT complex is bound together, the complex travels at an intermediate speed. Measurement of IFT particle velocity is often used to characterize trafficking and localization of cilia proteins and diagnosis specific defects in cilia mutants, as different cargo-motor associations or mutant defects yield characteristic velocity profiles (e.g., Table 2). IFT motor function and expression can be modulated in a cell specific manner, either by altering the ratio of motors (Evans et al., 2006) or by unlinking the two IFT subcomplexes (Mukhopadhyay et al., 2007). Secondary motors, such as kinesin KLP-6, can also bind to the IFT complex and modulate its velocity (Morsci and Barr, 2011). IFT is slightly modified in other cilia models: KIF17, the ortholog of OSM-3, functions as the primary IFT motor, whereas Kinesin-II functions to specialize cilia.

Although it was earlier believed that IFT complexes traveled along ciliary microtubules independently (as illustrated in the model in Figure 4), we now know that IFT particles travel along the axoneme in long multi-subunit trains. These trains have dynamic morphology dependent on both the direction of travel (anterograde trains are longer and narrow, while retrograde trains are short and compact [Pigino et al., 2009]) and length of cilium (the longer the axoneme, the shorter the trains, though the number of trains increases [Engel et al., 2009]). IFT trains are tightly associated with the flagellar membrane; this may be mediated via conserved membrane-binding domains in IFT peptides, interactions with ciliary proteins, (e.g., membrane associated ARL-13 and transmembrane MKS-3), or both.
1.3 – Ciliopathies and cystic kidney disease

Cystic kidney diseases are a large group of interrelated disorders, marked by abnormal nephrodevelopment leading to the formation and progressive enlargement of cysts, often comorbid with extrarenal manifestations including retinal degeneration, *situs inversus*, and neurological disorders. This spectrum disorder includes Nephronophthisis (NPHP), Meckel (MKS), Senior-Løken (SLS), and Joubert (JBTS) Syndromes, which in some instances share loci (Reviewed in Baker and Beales, 2009). Initial studies of these disease genes revealed an association between these cystic kidney diseases and the cilium; this class of disease is now also known as the ciliopathies, diseases of the cilium (Figure 5).

Primary renal cilia lie at the root of most cystic kidney diseases. These cilia are thought to act as mechanosensors of urine flow, indicating the onset of glomerular filtration; when development has progressed to this point, the cilia precipitate a shift to a second nephrodevelopmental pathway. Functional (e.g., mutations in mechanoreceptors and ion channels) and morphological (e.g., mutations in ciliogenesis genes, maintenance genes) defects in the cilium break this pathway and subsequently lead to abnormal proliferation, protein sorting, and cyst development (Reviewed in Hildebrandt and Zhou, 2007).

Because a majority of human cell types possess cilia and require them for normal function, ciliopathies have many secondary, extrarenal manifestations. As cilia of different cell types vary in form, function, and basic biology, specific ciliary defects affect these different cell types in different manners. This variety can yield a specific spectrum of symptoms. Clinicians group ciliopathies depending on this set of symptoms: there are over 16 subtypes of Nephronophthisis, 11 subtypes of Meckel Syndrome, and 20 subtypes of Joubert Syndrome (Table 3). As specific subtypes are linked by broader symptoms, causative genes often function
in related pathways (e.g., described above, there is a MKS complex at the TZ composed, in part, of MKS1, MKS3, MKS5, and MKS6). Before the advent of large-scale ciliome screens, mapping mutations in ciliopathy patients was an extremely powerful method of identifying cilia genes and establishing their relationship to one another.

*Caenorhabditis elegans* is a powerful model for investigating these ciliopathies, including autosomal dominant polycystic kidney disease (ADPKD) and NPHP (Barr and Sternberg, 1999; Jauregui and Barr, 2005; Winkelbauer et al., 2005). Though lacking kidneys, *C. elegans* has functionally conserved cilia. Many cystic kidney disease genes are present and perform similar functions to higher fish and mammalian homologs. Novel ciliary genes have been identified in animal models of ciliopathies and subsequently found to be the causative agents in human cystic diseases of previously unknown etiology (e.g., in *C. elegans*, bbs-5 has been linked to Bardet-Biedl Syndrome [Li et al., 2004], and in *Mus musculus*, NEK8 linked to NPHP9 and INVS has been linked to NPHP2 [Otto et al., 2008]), illustrating that animal models provide fertile ground for novel research in this area. Additionally, cilia genes identified and characterized in *C. elegans* have later been found to be causative in newly classified ciliopathies (e.g., mksr-1 in MKS9 and mksr-2 in MKS10 [Dowdle et al., 2011; Hopp et al., 2011; Williams et al., 2008]).

### 1.4 – Characterizing the Inversin Compartment

#### 1.4.1 – Discovering a proximal ciliary compartment

Definitions of the proximal cilium have often been confusing and incompatible. Originally, the term applied primarily to the first handful of microns of a given cilium or flagellum, but without specification to the number of microns or to where to begin measuring this distance. Eventually, it was found that a specialized compartment, with distinct structure, composition, and function
lay in this region. This compartment, the TZ, functions in protocilium-cilia vesicle-cell membrane interactions in early ciliogenesis, and in intraciliary trafficking in the mature organelle (Sorokin, 1962; Williams et al., 2011). The cilia shaft extends from the TZ, and can range in length from one to dozens of microns. However, further evidence emerged that the TZ did not constitute the entire proximal cilium.

In some models, the proximal and distal portions of the cilia shaft can exhibit distinct ultrastructure (Perkins et al., 1986; Reese, 1965). The proximal cilia shaft—the “middle segment” in C. elegans parlance—possesses microtubule doublets arranged about the center of the lumen, similarly to the whole of the flagellar shaft of Chlamydomonas and mammalian primary cilia. The distal portion of the cilia shaft—the “distal segment”, in worm literature—possesses microtubule singlets arranged in a disorganized manner. In the early 1990s, the idea of a distinct post-TZ compartment appeared in Chlamydomonas literature, when it was discovered that the proximal half of Chlamydomonas’ axoneme was associated with different dynein heavy chains than the distal portion (Piperno and Ramanis, 1991), and was specifically glutamylated (Fouquet et al., 1996).

Evidence for a proximal, post-TZ, ciliary compartment has also been found in C. elegans. In work characterizing intraflagellar transport (IFT), a microtubule motor based transport system that builds and maintains cilia and flagella, Jonathan Scholey’s group demonstrated that C. elegans amphid channel cilia were built by two separate, but coordinated, kinesin motors (Snow et al., 2004). They found that while the motor OSM-3 was capable of building the entire cilium (TZ, doublet region, and singlet region), the second IFT motor Kinesin-II was only capable of building the TZ and doublet region (Perkins et al., 1986; Snow et al., 2004). In addition, the localization of Kinesin-II was restricted to this proximal region (Snow et al., 2004). This evidence from Chlamydomonas and C. elegans supported the idea of a distinct conserved, post-TZ ciliary
subcompartment, though the structure and composition appeared to differ between models, and the compartment had no identified function.

The floodgates opened in 2009 when the Yokoyama group published foundational work detailing the ciliary localization of a nephropathy-associated protein, Inversin (Shiba et al., 2009). Using immunogold antibodies, they demonstrated that in mammalian primary cilia Inversin strictly localized to a small ~2µm subcompartment directly distal to the TZ. Further work demonstrated that Inversin was required for the localization of multiple other proteins to this “Inversin compartment” (InvC), including Nphp3, Nek8, and Anks6 (Fukui et al., 2012; Hoff et al., 2013; Shiba et al., 2010). In C. elegans, multiple doublet region-restricted proteins in addition to Kinesin-II components were also found, including ARL-13, ARL-3, HDAC-6, UNC-119, and the Inversin homolog NPHP-2 (Cevik et al., 2010; Li et al., 2010; Warburton-Pitt et al., 2014; Warburton-Pitt et al., 2012); the doublet region was also found to be specifically glutamylated (O’Hagan et al., 2011).

1.4.2 – Defining the Proximal Cilium

The C. elegans doublet region is often understood to be analogous to the mammalian InvC, in part because of the partial-length localization patterns of several ciliary proteins (Blacque and Sanders, 2014; Cevik et al., 2010; Cevik et al., 2013; Warburton-Pitt et al., 2012; Wojtyniak et al., 2013). However, our recent work indicates that C. elegans possesses a conserved InvC that is distinct from the doublet region (Warburton-Pitt et al., 2014). This assessment is strongly supported by evidence demonstrating several C. elegans doublet region proteins, including the Kinesin-II components KLP-11 and KLP-20, the histone deacetylase HDAC-6, and the small GTPase ARL-13, have full length ciliary localization in the primary cilia of multiple mammalian cell types, including HEK293, MEF, IMCD3, RPE1, MDCK, and human fibroblasts (Fan et al., 2004;
Follit et al., 2009; Hori et al., 2008; Larkins et al., 2011; Lee et al., 2012; Pugacheva et al., 2007; Sanchez de Diego et al., 2014; Satish Tammana et al., 2013; Wright et al., 2011; Wu et al., 2006; Zhou et al., 2006). Furthermore, the doublet region-associated protein ARL-13 has been demonstrated to have differing localization patterns in different larval stages (Cevik et al., 2013), but the Inversin ortholog NPHP-2 is restricted to the proximal cilium in all larval stages (Warburton-Pitt et al., 2014). This evidence confirms the hypothesis first proposed by Perkins et al.: the *C. elegans* doublet region is analogous to the whole shaft of the primary cilium, and not the InvC (Perkins et al., 1986). The *C. elegans* microtubule singlet region appears to be a specialized ciliary compartment, not always present, and with widely varying morphology (Doroquez et al., 2014). Other organisms also exhibit a microtubule singlet region, including *Chlamydomonas*, in which a singlet region is present transiently during mating.

### 1.4.3 – The Inversin Complex

Fortuitously, the first InvC component characterized, Inversin, is relatively upstream in the process that establishes the subcompartment—Inversin is strictly required for InvC localization of most known InvC components, and the *C. elegans* ortholog *nphp*-2 is required for localization of TAX-2 and partially required for TAX-4. Inversin localizes to the InvC (Shiba et al., 2009), physically interacts with itself (Wright et al., 2011), and both Inversin and NPHP-2 are possibly membrane associated (Shiba et al., 2009; Warburton-Pitt et al., 2012); Inversin has also been reported to localize to the basal body in addition to the InvC (Otto et al., 2003; Sang et al., 2011). Very little is known about the localization dependencies of Inversin; it has been shown to require the TZ protein B9d2 in zebrafish kinocilia, but these cilia do not possess a proximal InvC (Zhao and Malicki, 2011). The MYND domain encoding *daf*-25 is also required for *nphp*-2 expression or protein stability in *C. elegans* in several cilia types (Wojtyniak et al., 2013). DAF-25,
an ortholog of mammalian Ankmy2, localizes to cilia—though subciliary localization is unclear—and regulates localization of a membrane-associated guanylyl cyclase (Jensen et al., 2010). Inversin requires its ankyrin repeat-containing N-terminal region (Mochizuki et al., 1998; Morgan et al., 1998) for cilia targeting and import, and requires a C-terminal ninein homology domain for restriction to the InvC (Shiba et al., 2009). The Inversin C-terminal region also contains a calmodulin-binding IQ domain that is required for localization and function (Morgan et al., 2002; Otto et al., 2003; Yasuhiko et al., 2001). The calmodulin that physically interacts with Inversin, CALM2, localizes along the entire cilium and at the basal body (Otto et al., 2005; Shiba et al., 2009). The ankyrin repeat region of NPHP-2 also appears to be required for targeting to the cilium in AWB neurons (Wojtyniak et al., 2013), but the C-terminal ninein homology and IQ domains do not appear to be conserved. However, NPHP-2 does encode a predicted Ca\textsuperscript{2+} binding EF-hand which may function analogously to the IQ domain of Inversin (Warburton-Pitt et al., 2012); this EF hand is required for ciliary targeting in amphid channel and phasmid cilia (Warburton-Pitt et al., 2014).

Physical interaction studies have identified a physical complex of Inversin-associated proteins, including Nek8, Nphp3, and Anks6 (Bergmann et al., 2008; Fukui et al., 2012; Hoff et al., 2013; Mahjoub et al., 2005; Shiba et al., 2010). These three proteins require Inversin for restriction to the InvC (Hoff et al., 2013; Shiba et al., 2010), but not for ciliary import (Nakata et al., 2012). Unsurprisingly, the N-terminal portion of Inversin is insufficient for InvC restriction of Nek8 (Shiba et al., 2010). It has been speculated that Nek8 and Anks6 bind Inversin through Inversin’s ankyrin repeat region (Hoff et al., 2013; Shiba et al., 2010). Anks6 stabilizes the InvC complex by enhancing Inversin-Nek8 binding (Hoff et al., 2013). Nek8 and Anks6 themselves physically interact, though they do not require each other for localization; this interaction is mediated by an ankyrin-region hydroxylated arginine of Anks6 and the kinase domain of Nek8 (Hoff et al.,
Like Anks6, Inversin contains a strongly conserved hydroxylation motif in its Ankyrin repeat region (Hoff et al., 2013; Warburton-Pitt et al., 2014), which could plausibly play a similar role in Invesin-Nek8 physical interactions. The hydroxylase that physically interacts with Anks6 and Inversin, HIF1AN (Rual et al., 2005), is not restricted to the InvC (Hoff et al., 2013). Nek8 requires its catalytic kinase domain for InvC localization, but it alone is not sufficient for ciliary targeting. Nek8 with a T162E mutation in the kinase activation loop is hyperactive and seems to lose InvC restriction, but not cilia targeting (Zalli et al., 2012). Nek8 also contains an RCC (remodeling of condensed chromatin) domain that is required for InvC restriction but not ciliary targeting (Sohara et al., 2008). These disparate targeting motifs suggest against a common InvC signal, and the data indicates that Inversin is the earliest known progenitor of the compartment. Further proteins have been identified as physical interactors of the Inv complex, including Ahi1 (Wright et al., 2011), Anks3, Sdha, Nek7, Sptbn1, Eef1d, and Vdac2 (Hoff et al., 2013), though of these, only Ahi1 has a known relevance to cilia biology.

The localization requirements of Nphp3 have also been clearly characterized. Nphp3 localizes to the InvC in a three step process (Nakata et al., 2012). First, a coiled-coil region targets Nphp3 to the basal body. Second, Nphp3 translocates into the cilium in a manner dependent on its N-terminal myristoyl side chain; deletion of the myristoylatable residue results in cilia base accumulation of Nphp3. Third, residues 202-595 interact with Inversin, which restricts Nphp3 to the InvC (Nakata et al., 2012; Shiba et al., 2010). Similarly to the case of Nek8, the N-terminal portion of Inversin is not sufficient to restrict Nphp3 to the InvC (Shiba et al., 2010).

1.4.4 – Establishing the Inv Compartment

Little is known about how the InvC is initially established in any system, despite repeated attempts. A quick note regarding the interpretation of localization data: it’s difficult. If in a given
gene knockdown an InvC protein is only restricted to the InvC in half of cilia observed, is that gene required for InvC targeting? It clearly is not, because the protein can target and be restricted to the InvC compartment. But on the other hand, it clearly is, as the protein is no longer restricted properly in many cilia. It has been the case where the same data has supported opposite conclusions based on how incomplete penetrance is regarded (Wojtyniak et al., 2013 vs Blacque and Sanders, 2014).

Though IFT is required both to build the unelaborated cilium and to specialize cilia, IFT seems not to play a critical role in establishing the InvC. Neither NPHP-2, UNC-119, nor TAX-2/TAX-4—which colocalize with NPHP-2 in some cell types—have been reported to move along the axoneme in an IFT-associated manner (Warburton-Pitt et al., 2012; Wojtyniak et al., 2013; Warburton-Pitt et al., 2014). The localization of TAX-2/TAX-4 is not restored following photobleaching, also indicating that these proteins are not IFT trafficked (Wojtyniak et al., 2013). Inversin has not been found to directly bind to any IFT polypeptide, though it may interact indirectly (Zhao and Malicki, 2011). In C. elegans, osm-3 mutants can localize TAX-2 in AWB and ASK cilia and TAX-4 in ASK cilia (Wojtyniak et al., 2013). The kinesin-II component klp-11 is also not required for NPHP-2 and UNC-119 proximal cilium targeting in phasmid cilia (Warburton-Pitt et al., 2014), and the Kinesin-II component kap-1 is not required for TAX-2 ciliary targeting in AWB cilia (Wojtyniak et al., 2013). However, Kinesin-II does play some role in localization, if not trafficking, as in Kinesin-II mutants NPHP-2 and UNC-119 both mislocalize to the BB, and TAX-2 expression is reduced (Warburton-Pitt et al., 2014; Wojtyniak et al., 2013). Recent work indicates that an Unc119b-based shuttle system may be responsible for the myristoyl-dependent translocation of Nphp3 into the cilium (Figure 6) (Wright et al., 2011). Briefly, Unc119b binds lauroylated and myristoylated proteins at the BB/TZ ciliary traffic staging area (Zhang et al., 2011), and the complex translocates into the cilium proper. Here, the
GTP-bound form of Arl3 binds Unc119b (Veltel et al., 2008). RP2 binds the Arl3-Unc119b complex and triggers Arl3 to hydrolyze its GTP (Grayson et al., 2002; Veltel et al., 2008), and Arl3 in turn triggers conformational changes in Unc119b that cause the release of acylated cargo into the cilium (Ismail et al., 2012; Wright et al., 2011). Unc119b is then bound by C5orf30—which prevents reassociation of Unc119b and cargo—and then exits the cilium (Wright et al., 2011). One of the cargoes of Unc119b is Nphp3, which binds Unc119b through its myristoyl side chain (Wright et al., 2011). Without Unc119b or the myristoyl side chain, Nphp3 cannot localize to the cilium, and remains at the TZ (Nakata et al., 2012; Wright et al., 2011). Multiple components of the Unc119b shuttle localize to the InvC, including Unc119b itself and C5orf30 (Wright et al., 2011), though other components, including RP2 (Hurd et al., 2010; Hurd et al., 2011) and Arl3 (Wright et al., 2011; Zhou et al., 2006) do not. It remains untested whether Unc119b or C5orf30 require Inversin for localization, or vice versa. The Unc119b shuttle is tightly linked to membrane biology. The myristoylated and palmitoylated RP2 is membrane associated (Chapple et al., 2000; Chapple et al., 2002), Arl3-GTP binds membranes specifically (Ismail et al., 2012; Wright et al., 2011), Unc119b can extract proteins from membranes (Zhang et al., 2011), and known cargoes are all membrane associated (Wright et al., 2011; Zhang et al., 2011). Additionally, casual observation indicates that Inversin/NPHP-2 itself may be membrane associated (Shiba et al., 2009; Warburton-Pitt et al., 2012). The Unc119b shuttle is not sufficient for InvC restriction of shuttled proteins; as described above, Inversin is strictly necessary.

*C. elegans* possesses orthologs of Unc119b (*unc-119*) (Higashide and Inana, 1999), Arl3 (*arl-3*) (Li et al., 2004), RP2 (*rpi-2*) (Williams et al., 2011), PDE6d (*pdl-1*) (Hukema et al., 2006), Arl13b (*arl-13*) (Cevik et al., 2010; Li et al., 2010), and INPP5E (*cil-1*) (Bae et al., 2009), but it is unknown if the shuttle systems themselves are conserved. UNC-119 localizes to the proximal cilium in amphid and phasmid neurons (Warburton-Pitt et al., 2014), and *unc-119* is required for the
localization of the myristoylated receptors ODR-3 and GPA-13 (Zhang et al., 2011). C. elegans UNC-119 is structurally conserved enough to rescue Unc119b/- defects when expressed in mammalian cell culture models (Wright et al., 2011). Unc119b shuttle components also have other roles in C. elegans ciliogenesis: unc-119 is required for singlet region biogenesis (Ou et al., 2007), arl-3 plays a role in IFT regulation and ciliogenesis (Li et al., 2010), and arl-3 depletion suppresses unc-119 ciliary defects (Warburton-Pitt et al., 2014). Additionally, unc-119 appears to be required to traffic NPHP-2, as mutants display an accumulation of dendritic NPHP-2 and a shortened InvC, but nphp-2 is not required for UNC-119 localization (Warburton-Pitt et al., 2014). Curiously, nphp-2 and unc-119 genetically interact in ciliogenesis, resulting in additive defects, suggesting that they function in parallel pathways (Warburton-Pitt et al., 2014).

A large body of work describes amino acid motifs, domains, and post-translational modifications that function to target proteins to the cilium; however, little is known about compartment specific targeting signals, and whether they even exist. Multiple ciliary proteins contain coiled-coils, including the proximally enriched/restricted components Nphp3 (Nakata et al., 2012), Inversin/NPHP-2 (Warburton-Pitt et al., 2012), TAX-4, CNG-3 (Wojtyniak et al., 2013), Evc/Evc2 (Caparros-Martin et al., 2013), LOV-1/PKD-2 (Barr and Sternberg, 1999) and transition zone components Cep290 (Nakata et al., 2012) and Cep164 (Graser et al., 2007), among others. Though Nphp3 requires the coiled-coil domain for TZ/BB targeting, Cep290 does not, indicating that this targeting motif is not universal; it is unknown whether this motif is required for localization of other InvC associated proteins (Nakata et al., 2012). As discussed above, acylation may also function to target proteins to ciliary membranes in a regulatable, reversible, possibly compartment specific manner (Godsel and Engman, 1999; Resh, 1999). In addition to abovementioned Nphp3, RP2, ODR-3, and GPA-13, there are several other myristoylated ciliary proteins, including the Unc119b-interacting cystin (Tao et al., 2009; Wright et al., 2011),
fibrocystin (Follit et al., 2010), Arl6, Tctn2, and the Kinesin-II Kif7 (Nakata et al., 2012), but none of these are restricted to the InvC (Cantagrel et al., 2008; Garcia-Gonzalo et al., 2011; Liem et al., 2009; Ward et al., 2003; Wiens et al., 2010; Yoder et al., 2002). Other ciliary targeting signals, including RVxP and Ax(S/A)xQ, are either not present in known InvC proteins, or not required for targeting (Wojtyniak et al., 2013). InvC restriction is most likely determined through physical interactions with other InvC components and not through a signal sequence or motif.

Finally, other proteins also appear to have proximal ciliary localization in some cilia types, including the Ode1 interactor Lca5 (Coene et al., 2009; den Hollander et al., 2007), the Bug22 ortholog Gtl3 (Ishikawa et al., 2012), and CDKL5 in Chlamydomonas (Tam et al., 2013), though how they functionally, physically, and genetically interact with other InvC components is completely unknown.

1.4.5 – Membrane Dynamics of the Inv Compartment

Differential lipid composition of ciliary membranes has long been linked to specific ciliary localization of membrane-associated proteins. Several InvC-associated proteins possess membrane interaction motifs and localize to the membrane, suggesting the InvC regulates, or is regulated by, membrane interactions. However, little is known regarding characterization and regulation of its membrane biology. Lipid rafts are required for function and localization of CNG channel CNGA1, ortholog of C. elegans InvC component TAX-4, in photoreceptors, though these regions may not correspond to the InvC of other cilia (Brady et al., 2004; Ding et al., 2008). An Inversin physical interactor, Ahi1, is required for ciliary targeting and function of Rab8a, which is required for ciliary membrane trafficking; knockdown of Ahi1 itself leads to membrane and vesicle trafficking associated defects (Hsiao et al., 2009; Sang et al., 2011).

Protein acylation is an important mechanism for both membrane association and InvC targeting,
as seen in the case of the Unc119b shuttle (Nakata et al., 2012; Wright et al., 2011). A newly
described *C. elegans* protein, CIL-7, has been implicated in ciliary membrane and extracellular
vesicle (ECV) trafficking; CIL-7 is intriguing in that it is a myristoylated protein that requires
*unc-119* for proper localization in a myristoyl side chain dependent manner (Julie Maguire,
Personal Communication). The InvC may also be linked to ciliary vesicular dynamics. Inversin
physically interacts with Clathrin, a membrane coat protein (Sang et al., 2011). CIL-7 functions in
regulation of ECV release (Julie Maguire, Personal Communication)

A more heavily characterized protein involved in ciliary membrane trafficking is the prenylated
INPP5E, which is translocated into the cilium by the Unc119b paralog PDE6d in an
Arl13b-specific manner (Figure 6) (Bae et al., 2009; Humbert et al., 2012). This shuttle system is
likely conserved in *C. elegans*, as described above. Interestingly, the *C. elegans* INPP5E homolog
cil-1 may genetically interact with *nphp-2*, indicating a link between the two acyl shuttles; per
contra, *nphp-2* does not appear to be required for PKD-2 trafficking (Error! Reference source
ot found.). However, subciliary localization of CIL-1 is unknown (Bae et al., 2009), and INPP5E
localizes to the entire cilium (Bielas et al., 2009; Humbert et al., 2012; Jacoby et al., 2009),
arguing against an InvC specific role in these cilia types.

The Unc119b shuttle components RP2 and Arl3 appear both to be regulated by membrane
interactions and to link membrane dynamics to the microtubule dynamics. Recent structural
studies suggest that Arl3-GTP has weaker affinity for the cholesterol and sphingolipid-enriched
ciliary membrane than other membranes. This weaker affinity allows Arl3 to dissociate from the
ciliary membrane, revealing physical moieties that mediate Unc119b binding. Arl3 can then
trigger Unc119b cargo release, as described above (Ismail et al., 2012). The Arl3 GAP RP2 is also
membrane-associated (Chapple et al., 2000; Chapple et al., 2002), and requires its myristoyl and
palmitoyl anchors for proper function (Rosenberg et al., 1999). Though both Arl3 and Rp2 are
primarily membrane-associated, both also have established functions in microtubule regulation. Arl3 has been reported to be physically associated with microtubules (Grayson et al., 2002), modulates IFT microtubule motor interactions (Li et al., 2010), and preferentially binds unmyristoylated, non-membrane-associated RP2 (Bartolini et al., 2002). RP2, in addition to functioning as an Arl3 GAP, also functions as a tubulin GAP (Bartolini et al., 2002). These results suggest that a proximal cilium specific membrane compartment is linked to the microtubule dynamics and drives trafficking specificity. However, no known lipid markers reveal such a distinct membrane compartment. A caveat is warranted, though: most cilia lipid markers were observed in *Paramecium* and *Tetrahymena*, which may not have a conserved InvC because they have no identifiable Inversin ortholog.

Several cargos of PDE6d have been identified, including INPP5E, RHEB, Rab28, rhodopsin kinase, and rod phosphodiesterase (Florio et al., 1996; Humbert et al., 2012; Ismail et al., 2011; Thomas et al., 2014; Zhang et al., 2004; Zhang et al., 2007), though these are not known to be InvC restricted.

### 1.4.6 – Ultrastructure and microtubule dynamics

Whereas the transition zone is marked by microtubule doublets attached to the ciliary membrane via MKS-NPHP protein Y-links, there are few ultrastructure details to distinguish the InvC from the rest of the ciliary shaft. The InvC is composed of microtubule doublets, which appear to be spatially associated with the membrane (Shiba et al., 2009); perturbation of this has been associated with ciliary mutants (Cevik et al., 2010). Additionally, Inversin physically interacts with β-tubulin (Otto et al., 2003). However, tubulin post-translational modifications (PTMs) have been linked genetically and physically to the InvC, and Inversin itself associates with microtubules in a polymerization-state dependent manner (Nurnberger et al., 2004). Cilia of
Inv/- mice have superficially wild-type ultrastructure (Shiba et al., 2009); however, nphp-2 worms have disorganized Y-links, overextended B-tubules, and misplaced cilia, which may be due to TZ-anchoring failure (Warburton-Pitt et al., 2014). In worms absent daf-25—which has been linked to nphp-2 expression in some cell types—amphid channel cilia ultrastructure appears normal (Jensen et al., 2010).

PTMs are small side chains added to microtubules after polymerization, altering their affinity to binding partners, which in turn can modulate microtubule dynamics (Reviewed in Verhey and Gaertig, 2007). Many PTMs have been identified to date, including acetylation, polyglutamylation, detyrosination, polyglycylation, O-GlcNAcylation, phosphorylation, palmitoylation, and removal of the two final C-terminal residues. Multiple modifications may be present on the same tubulin subunit, allowing for combinatorial specificity (Redeker, 2010).

Microtubule stability has long been linked to PTMs. Most of these modifications have been studied in the ciliary context, and acetylation and polyglutamylation have specifically linked to InvC biology.

1.4.6.1 – Acetylation. Like other PTMs, acetylation is a marker for stabilized microtubules (Palazzo et al., 2003). Though primary cilia are generally acetylated along their entire length (Piperno et al., 1987), acetylation-associated enzymes, including the histone deacetylase Hdac6 (Hubbert et al., 2002), have been shown to be proximally enriched (Li et al., 2010), and genetically interact with InvC components to control cilia integrity (Mergen et al., 2013; Warburton-Pitt et al., 2014). (Another known tubulin deacetylase, Sirt2, doesn’t seem to function on axonemal microtubules [Li et al., 2010; North et al., 2003]). Acetylation has long been associated primarily with alpha-tubulin residue K40 (L'Hernault and Rosenbaum, 1985). However, the only C. elegans alpha tubulin with this residue, MEC-12, is not expressed in ciliated neurons (Fukushige et al., 1999), anti-acetylated K40 antibodies do not label cilia (O'Hagan et al.,
2011; Warburton-Pitt et al., 2014), and the only known acetyltransferase that acetylates K40, MEC-17, has not been directly linked to cilia function (Akella et al., 2010). Because hdac-6 functions in *C. elegans* ciliary stability, this suggests that *hdac-6* has other possible targets, though none have been identified (Li et al., 2010). Indeed, it has been recently found that β-tubulin also has an acetylatable K252 residue (Chu et al., 2011), and many tubulins are also included in the *Homo sapiens* acetylome (Choudhary et al., 2009), though it remains to be seen whether they have a role in ciliary dynamics.

Acetylation has also been tied to IFT components. Bbip10, a BBSome peptide, is required for both microtubule polymerization and acetylation in zebrafish. Acetylation is restricted to the proximal cilium in some, but not all, cell types. Intriguingly, Bbip10 and Hdac6 physically interact, and Hdac6 antagonizes Bbip10 acetylation function (Loktev et al., 2008). The *C. elegans* Bbip10 homolog is expressed in amphid and phasmid ciliated neurons, though has not been characterized (Kunitomo et al., 2005).

Unfortunately, the primary anti-acetylated tubulin antibody in use, 6-11B-1, is not actually specific to acetylated K40, labeling instead acetylatable tubulin, strongly curtailing the utility of acetylation data in many reports (Soppina et al., 2012).

**1.4.6.2 – Glutamylation.** Microtubule polyglutamylation is required for vertebrate and nematode ciliogenesis and function (O’Hagan et al., 2011; Pathak et al., 2007), and is regulated by TTLL (tubulin tyrosine ligase like) glutamylases and their converse deglutamylases (Reviewed in Verhey and Gaertig, 2007). Unlike acetylation, polyglutamylation has been associated with both an increase and decrease in microtubule stability, suggestive of cell type-specific regulatory activity, or a requirement for balance between stability and instability in normal cilia microtubule dynamics (O’Hagan et al., 2011; Wloga et al., 2010). Ciliary glutamylation is linked to the function of ciliopathy genes, including *nphp-2* in *C. elegans* and CEP41/JBTS15 in
mammalian models (Lee et al., 2012; Warburton-Pitt et al., 2014).

Over the years, polyglutamylated tubulin has been associated with the proximal cilium in many models, including *Chlamydomonas* flagella (Fouquet et al., 1996), zebrafish pronephric cilia (Pathak et al., 2007), *C. elegans* amphid channel and phasmid cilia (O'Hagan et al., 2011; Warburton-Pitt et al., 2014), and human fibroblast cilia (Lee et al., 2012). Ciliary glutamylation has also been associated with modulation of ciliary dynein (which functions in cilia movement), though not IFT dynein, activity (Kubo et al., 2010; Suryavanshi et al., 2010). In a very suggestive finding predating the discovery of the TTLL glutamylase and glycylase family (Janke et al., 2005), Nphp3 was reported to have a TTL domain, though the function of this domain remains completely uncharacterized (Olbrich et al., 2003). Additionally, Inversin physically interacts with zebrafish TTC30B/Fleer (Zhao and Malicki, 2011), a cilia base protein, which—like Nphp3—contains TPR repeats, and is required for tubulin polyglutamylation (Pathak et al., 2007) and proper ciliary localization of Inversin (Zhao and Malicki, 2011). Besides these examples, very few other genes are known to both regulate glutamylation and genetically interact with InvC components. In *C. elegans*, the deglutamylase *ccpp-1* and glutamylase *ttll-4* function to regulate amphid and phasmid ciliary glutamylation, but neither are reported to localize to the proximal cilium (O'Hagan et al., 2011), and neither seem to be strictly required for localization of InvC components, including NPHP-2, UNC-119, and TAX-2 (Warburton-Pitt et al., 2014; Wojtyniak et al., 2013). Similarly, Cep41 is a basal body protein required for ciliary glutamylation; another glutamylase, Ttll6, requires Cep41 for ciliary targeting but, similarly to Cep41, localizes to the basal body (Lee et al., 2012). Recent work by our group has also demonstrated that *nphp-2* mutants exhibit ectopic glutamylation and microtubule overstabilization; other proximal cilium associated mutants, including *hdac-6* and *unc-119*, also have altered glutamylation patterns in amphid and phasmid cilia. The actions of the proximal cilium associated genes appear to lie
upstream of the glutamylation apparatus (Warburton-Pitt et al., 2014).

Overall, studies of microtubule acetylation and glutamylation suggest that the InvC can modulate cilia stability through microtubule acetylation and polyglutamylation, possibly in response in environmental signals. As at least one TTLL protein is predicted to be myristoylated (Tttl11b), and Nphp3 has a TTL domain and is myristoylated (Olbrich et al., 2003), it may be that Unc119b and the InvC function to shuttle and localize the glutamylation apparatus in cilia, and through this pathway, regulate microtubule patterning and ciliogenesis.

1.5 – The Function of the Inversin Compartment

The InvC has been implicated in virtually every cilia-associated pathway in recent years, with the largest body of work exploring signal transduction and amplification. The InvC may also have roles in ciliogenesis, cilia maintenance, cilia placement, cilia specialization, cilia length regulation, IFT regulation, protein trafficking, and cilia motility. As wide ranging as this list is, it neglects downstream and non-ciliary functions of these pathways, including cell cycle regulation (Morgan et al., 2002; Simons and Walz, 2006), cellular motility (Werner et al., 2013), and DNA damage repair (Chaki et al., 2012). I discuss several of these functions in turn.

1.5.1 – Signal Transduction and Amplification

Many signal transduction pathways have been linked to ciliary function, including Wnt, Hedgehog, and Hippo, and signaling components such as CNGs, TRPVs, and Ca\(^{2+}\) channels.

1.5.1.1 – Wnt signaling. Wnt signaling is intimately tied into the function of the InvC. In the canonical Wnt transduction cascade, Wnt ligand binds a Frizzled transmembrane receptor, which then activates intracellular Disheveled. Disheveled then inhibits the activity of an APC/GSK3\(\beta\)/CKI complex, which itself inhibits \(\beta\)-catenin function by targeting it to proteasomal
degradation. When the APC complex is inhibited by Dishevelled, β-catenin can translocate into the nucleus and initiate transcription of Wnt effectors (Reviewed in Huang and He, 2008; MacDonald et al., 2009). The cilium as whole downregulates this pathway (He, 2008); knockout of kinesin Kif3A and Ofd1 results in cells with no cilia (Marszalek et al., 1999) and constitutive activation of Dishevelled (Corbit et al., 2008). Additionally, in ciliated cells, β-catenin accumulates at the cilia base, but in cilia knockdowns, β-catenin accumulates in the nucleus and constitutively activates Wnt-related transcription (Lancaster et al., 2011). This also implies the cilium is not required for canonical signaling, and that cilia retraction may actually potentiate the cell to receive future signals. Conversely, ciliogenesis does not require functional Wnt signaling (Kishimoto et al., 2008).

Inversin, Nphp3, Nek8, Hdac6, and the Inversin physical interactor Ahi1 (Sang et al., 2011), function in regulation of the ciliary Wnt response (Bergmann et al., 2008; Lancaster et al., 2011; Li et al., 2008; Simons et al., 2005). Inversin has been misidentified as a basal body protein in the Wnt literature, which has led to confusion (Mahuzier et al., 2012; Simons et al., 2005). Inversin functions downstream from Frizzled, and is required for the recruitment of Dishevelled to the plasma membrane in response in Frizzled signaling (Lienkamp et al., 2010). Plasma membrane localization of Dishevelled has been associated with upregulation of an alternate Wnt pathway, the planar cell polarity (PCP) pathway (Axelrod et al., 1998). This change of localization is achieved by specific binding of Inversin to cytoplasmic Dishevelled, which is then targeted for degradation. Because this action also downregulates canonical Wnt signaling, Inversin has been called a switch between canonical Wnt and PCP signaling (Simons et al., 2005). In addition to indirectly upregulating PCP, Inversin also physically interacts with the PCP components Prickle and Van Gogh, suggesting that it also plays a direct role in this pathway (Das et al., 2004; Ross et al., 2005; Simons et al., 2005). However, more recent evidence contradicts
these results. In inv-/ mice, no upregulation of canonical Wnt signaling was observed, and β-catenin did not accumulate in the nucleus as found previously (Sugiyama et al., 2011). Additionally, the same group also found that overexpression of Inversin did not downregulate canonical signaling (Sugiyama and Yokoyama, 2006). This discrepancy is suggested to be due to differences between the earlier in vitro methods and the newer in situ data (Sugiyama et al., 2011). Curiously, though Kif3a is best known as a requisite ciliary microtubule motor, it has also been shown to regulate Wnt signaling in a non-ciliary pathway; this further confuses the role of cilia in canonical Wnt signaling (Corbit et al., 2008). Thus, even though Inversin is known to physically and genetically interact with Wnt components in multiple models, the role of Inversin in Wnt signaling is now unclear. Disheveled function has been linked to basal body docking during ciliogenesis; this echoes the role of nphp-2 in cilia anchoring in C. elegans and may reflect a conserved function of Wnt signaling (Vladar and Axelrod, 2008).

Not as much is known about the function of Nphp3 in Wnt signaling. Similarly to Inversin, mutation and KO of Nphp3 both lead to PCP-associated defects in zebrafish development (Bergmann et al., 2008; Zhou et al., 2010). The effects of Inversin and Nphp3 on Wnt signaling are additive, suggesting that they function in parallel pathways (Bergmann et al., 2008). Knockdown of Nek8 also results in situs inversus and other PCP-associated developmental defects. Experimental evidence suggests that Nek8 functions downstream of Inversin, just as Nek8 localization is dependent on Inversin (Fukui et al., 2012). However, such a model is partially contradicted by the fact that knocking down both Inversin and Nek8 leads to an increase in defects over the single knockdowns, which suggests that the genes function in parallel pathways (Fukui et al., 2012).

Ahi1 has been demonstrated to physically interact with Inversin, and Ahi1 knockouts have similar defects in epithelial cell polarization and organization as Nphp3 knockouts (Sang et al.,
Ahi1 has also been shown to positively modulate canonical Wnt signaling in a cilium-specific manner, through regulation of β-catenin nuclear translocation (Lancaster et al., 2009; Lancaster et al., 2011). Disruption of Ahi1 localization in IFT knockout animals also perturbs canonical Wnt signaling (Lancaster et al., 2011). These results suggest that the Ahi1 cell polarization phenotype may be due to unopposed overactivation of the PCP pathway.

There are also several suggestive pieces of data that are currently difficult to fit together. Hdac6 has also been implicated in upregulating Wnt signaling, functioning to deacetylate β-catenin, a step required for nuclear translocation (Li et al., 2008). Though not shown in ciliated cells, yeast experiments indicate that Nphp3 physically interacts with Dysferlin, an Hdac6 inhibitor (Blandin et al., 2013; Di Fulvio et al., 2011); it is unknown whether this interaction functions to regulate Wnt signaling, microtubule acetylation, or other pathways.

1.5.1.2 – Hedgehog signaling. Hedgehog signaling is required for the development and patterning of organs, and unlike Wnt signaling, requires and is promoted by the primary cilium (Corbit et al., 2005; Huangfu et al., 2003). Hedgehog ligand binds to a Patch receptor, which then derepresses Smoothened (Humke et al., 2010; Tukachinsky et al., 2010). Smoothened translocates into the cilium and triggers the dissociation of the Sufu/Gli3 repressor complex; if Sufu/Gli3 cannot localize to the cilium tip, as in Kif3a, Kif7 and Ift88 knockdowns, it cannot be split (Haycraft et al., 2005; Huangfu et al., 2003; Humke et al., 2010; Liem et al., 2009; Tukachinsky et al., 2010). Finally, once separated from Sufu, Gli3 is no longer repressed and can activate transcription. TZ proteins have been shown to modulate Hedgehog signaling, including Rpgrip1l, which alters the ratio of repressed and active Gli3 (Vierkotten et al., 2007). The InvC interactor Arl13b has also been shown to mediate Hedgehog signaling (Caspary et al., 2007). Two physically interacting proteins, Evc and Evc2, localize to the proximal cilium and function in Hedgehog signaling (Caparros-Martin et al., 2013; Dorn et al., 2012). These proteins are required
for the Gli3 repressor complex to translocate to cilia tips—although not for ciliary localization of Smoothened—and without them, Hedgehog signaling cannot be derepressed (Caparros-Martin et al., 2013; Pusapati et al., 2014). Evc2 requires an N-terminal W-peptide for restriction to the proximal cilium and deletion of this domain leads to Hedgehog signaling defects. This region is also required for physical interactions with EFCAB7/IQCE, which anchors Evc/Evc2 to the proximal cilium (Pusapati et al., 2014). EFCAB7 and IQCE both contain many calcium interacting domains, thought the role of calcium in Hedgehog signal transduction and localization of these components is unknown (Pusapati et al., 2014). Evc2 is not required for the localization of Inversin, Arl13b, or IFT component IFT88 (Dorn et al., 2012). Interestingly, the Evc/Evc2 proximal compartment is distinct from both the TZ and InvC, lying between, and partially overlapping, both of them, and marks a novel ciliary subcompartment (Dorn et al., 2012).

1.5.1.3 – Other signaling pathways. Beyond the more general Wnt and Hedgehog signaling pathways, proximal cilium components also function in more specific pathways. Crosstalk has been demonstrated between Hippo and Wnt pathways (Varelas et al., 2010); Wnt components Nek8 and Nphp3 both activate the Hippo intermediate TAZ, inhibiting the Hippo pathway (Frank et al., 2013; Habbig et al., 2012); and Nek8 physically interacts with TGF-β pathway components TGFB and ACVR1 (Barrios-Rodiles et al., 2005). Recent evidence solidifies a link between Hippo signaling and the cilium: the Hippo components MST1/2 and SAV1 antagonize ciliogenesis by downregulating the AuroraA/HDAC6 pathway, associate with TZ proteins, and are required for the ciliary targeting of some cargos (Kim et al., 2014).

In *C. elegans*, activation of the ODR-3 receptor—which requires UNC-119 for ciliary targeting—leads to opening of the TAX-2 and TAX-4 CNG channels (Lans et al., 2004). These channels have been demonstrated to be InvC localized in certain cell types, requiring full length NPHP-2 for restriction (Wojtyniak et al., 2013); this is similar to the manner in which Inversin anchors InvC
components in primary cilia. Additionally, both TAX-2 and NPHP-2 require *daf-25* for expression and localization in AWB cilia (Wojtyniak et al., 2013), though TAX-4 does not (Jensen et al., 2010).

The polycystins have also been linked to the proximal cilium in multiple models. Polycystin-1/PC1 has been reported to be localized to a restricted portion of the cilium, possibly the InvC (Sengupta and Barr, 2014). It has also been suggested that PKD-2, the *C. elegans* Polycystin-2/PC2 homolog, is enriched in the proximal portion of male-specific cilia of *C. elegans* (Bae et al., 2006). PC1 and PC2 have been shown to physically interact (Newby et al., 2002), though they functionally interact in a complex manner (Delmas et al., 2002; Geng et al., 2006; Xu et al., 2003). Both PC1 and PC2 have also been linked to modulation of Wnt signaling components and their downstream phenotypes (Bisgrove et al., 2005; Kim et al., 1999; McGrath et al., 2003; Pennekamp et al., 2002), though some data is contradictory (Le et al., 2004; Miller et al., 2011). In *C. elegans*, PKD-2 trafficking requires CIL-7, a myristoylated protein that interacts with *unc-119* (Julie Maguire, Personal Communication), but does not require *nphp-2* (Error! reference source not found.). In tubular epithelia primary cilia, Nek8 mutants exhibit ciliary accumulation of PC1 and PC2 (Sohara et al., 2008), and PC2 is required for InvC-dependent embryonic nodal signaling (Pennekamp et al., 2002). Other TRP family members have also been associated with the proximal portion of the cilium, including the TRPV channels Nanchung and Inactive in Drosophila (Gong et al., 2004; Lee et al., 2010). A related TRPN, nompC, localizes to cilia tips and functions as a mechanoreceptor (Cheng et al., 2010; Liang et al., 2011); the TRPV channels then amplify the induced current (Lee et al., 2010).

Calcium not only plays an indispensable role in general cilia biology, but may also play a large role in InvC biology. Several of the aforementioned proteins, including Inversin/NPHP-2, CNGA1/TAX-4, CNGB1/TAX-2, EFCAB7, and IQCE have calcium or calmodulin binding domains. In
the case of NPHP-2, the calcium binding EF-hand is required for ciliary targeting/retention (Warburton-Pitt et al., 2014). Additionally, frog olfactory cilia have a proximal, post-TZ enrichment of calcium channels (French et al., 2010). Pkd2, though a nonspecific cation channel, also has an EF-hand, which may function in calcium-based squelching of Pkd2 response or create a positive feedback loop, leading to further Pkd2 channel opening. Calcium signaling also plays a role in nodal cillum-associated left-right pattern formation (Reviewed in Norris, 2012). Because intraciliary calcium levels are very dynamic and are strongly tied to signal transduction and amplification, it is likely that calcium modulates the binding affinities, localizations, and activation status of InvC components. However, this has not been examined in detail in any system.

1.5.2 – Ciliogenesis

InvC components function in ciliogenesis pathways in a very cell type specific manner. Early work in inv/- mice suggested that Inversin was not required for cilia formation in renal epithelial cells (Morgan et al., 2002; Otto et al., 2003; Phillips et al., 2004); however, recent work has both confirmed this earlier data in MEFs (Veland et al., 2013), and linked Inversin/nphp-2 to ciliogenic defects in MDCK and C. elegans amphid channel and phasmid cilia (Mergen et al., 2013; Warburton-Pitt et al., 2012). Cell-specific roles for Inversin are not surprising given the range of functions and localization patterns in different tissues (e.g., Nurnberger et al., 2002; Nurnberger et al., 2004).

1.5.3 – Cilia Cycling

Cilia are dynamic organelles that are retracted and regrown in a cell-cycle dependent manner (reviewed in Quarmby and Parker, 2005). The cell can send disassembly signals to the cilium,
and the cilium may also be able to send signals to the cell to divide. Many InvC-associated proteins have been implicated in cilia cycling, including Nek8, Inversin, and Hdac6 (Morgan et al., 2002; reviewed in Fry et al., 2012).

When cilia disassembly is triggered, the plasma membrane-associated Hef1 translocates to the basal body and phosphorylates Aurora A (reviewed in Plotnikova et al., 2008). Aurora A then phosphorylates Hdac6, which deacetylates the ciliary microtubules, destabilizing them, and leading to their degeneration (Pugacheva et al., 2007). Inversin directly antagonizes this pathway, binding both Aurora A and Hef1, inhibiting both, and reducing their phosphorylation, leading to the stabilization of the ciliary axoneme (Mergen et al., 2013). Loss of Inversin allows the disassembly pathways to become hyperactive, deciliating cells inappropriately; because Inversin functions upstream of Hdac6, Hdac6 inhibitors can block Inversin related ciliation phenotypes (Mergen et al., 2013). In *C. elegans*, the proximally-localized *hdac-6* also seems to function to destabilize amphid channel and phasmid cilia (Li et al., 2010; Warburton-Pitt et al., 2014). Defects in ciliary double mutants—including combinations of *nphp-2, unc-119*, and *arl-13*—can be suppressed by *hdac-6* deletion, indicating this role of the proximal cilium may be conserved (Warburton-Pitt et al., 2014).

Several organisms possess cilia only on terminally differentiated cells, including *C. elegans*. This suggests that any protein or activity required exclusively for cycling would not be conserved in *C. elegans*, allowing for genetic separation of cilia cycling and ciliogenesis. Interestingly, the entire NPHP5/NPHP6 TZ module, which physically interacts with Inversin, is not conserved in *C. elegans* (Sang et al., 2011), suggesting this module may play a role in cilia cycling. However, no studies to date have used this approach to explore cilia cycling.

**1.5.4 – Cilia Length Regulation**
It has been long known that cilia length is strongly tied to proper function (Afzelius et al., 1985), and has been associated with disease (Follit et al., 2008; Mokrzan et al., 2007; Murcia et al., 2000; Sohara et al., 2008; Tammachote et al., 2009). To date, *Chlamydomonas* has long been the primary model used to explore length regulation (Barsel et al., 1988; Jarvik et al., 1984; Kuchka and Jarvik, 1987; Tuxhorn et al., 1998); however, *Chlamydomonas* has no described InvC and no conserved Inv complex components other than a possible Nek8 ortholog. Other data does suggest the presence of an InvC in Chlamydomonas. Lf5p, a serine/threonine kinase which regulates flagellar length, localizes to a 1µm proximal portion of the flagella, but distal to the TZ. Though many flagellar mutants were tested for Lf5p localization, none were found to be necessary (Tam et al., 2013). Lf5p homologs in other models are ambiguous because of the high conservation of the S/T kinase domain, making precise ortholog identifications difficult. No known proteins in any organism share homology with the other non-kinase half of L5fp.

There are multiple, non-mutually exclusive, suggested mechanisms of length regulation, including diffusion of requisite components (Levy, 1974); free tubulin concentration (Sharma et al., 2011); IFT train size, particle frequency, quantity, and component localization (Engel et al., 2009; Iomini et al., 2001; Marshall and Rosenbaum, 2001; Tam et al., 2003); and alteration of microtubule polymerization kinetics (Marshall and Rosenbaum, 2001), among possibilities. Length regulation has also been tied to several signaling pathways (Abdul-Majeed et al., 2012), including positive control by Notch (Lopes et al., 2010) and non-canonical Wnt signaling (Oishi et al., 2006), and modulation by calcium/cAMP/PKA pathways (Besschetnova et al., 2010).

Cilia length has been linked to several Inv complex components and proximally enriched proteins, including Inversin/*nphp*-2, Nphp3, Nek8, and Pkd1. Nphp3 (Bergmann et al., 2008; Zhou et al., 2010) and Nek8 (Smith et al., 2006) hypomorphs and knockouts exhibit elongated cilia. There is mixed data regarding Inversin/*nphp*-2, with reports suggesting (Mergen et al.,
2013; Warburton-Pitt et al., 2012) or discounting (Phillips et al., 2004; Zhao and Malicki, 2011) a role in length control. These differences are likely due to cell type specific roles of Inversin, similarly to the established tissue-specific elongation phenotypes reported in Pkd1 mice (Hopp et al., 2012). Basal body and TZ proteins also have an established role in length control; both Nde1 and Nphp4/nphp-4 knockouts and mutants exhibit elongated cilia (Burckle et al., 2011; Kim et al., 2011; Warburton-Pitt et al., 2012).

It is more difficult to ascertain stunted cilia phenotypes. For instance, C. elegans requires multiple genes for the formation of a distal microtubule singlet segment (e.g., Hao et al., 2011; Ou et al., 2007; Snow et al., 2004); mutants of these genes have shorter overall cilia, but normal length microtubule doublet segments. Whether these count as “stunted” cilia is a matter of personal preference. In this vein, our recent work shows that multiple InvC-associated genes have elongated or stunted polyglutamylated tubulin compartments; whether this indicates a missized doublet segment, a missized cilium, or ectopic glutamylation is unknown (Warburton-Pitt et al., 2014).

How these different genes regulate cilia length is relatively unknown. nphp-2 genetically interacts with the TZ-associated nphp-4 to regulate IFT velocities (Warburton-Pitt et al., 2012), and both Inversin and Pkd2 interact with cytoskeletal components (Chen et al., 2008; Nurnberger et al., 2004; Rundle et al., 2004).

1.6 – Interactions between the InvC and other ciliary compartments

1.6.1 – Interaction with the Transition Zone and Basal Body

The InvC has long been associated with the TZ and BB; indeed, InvC components are sometimes even mistaken for being TZ/BB components. Many studies have probed the link between the
compartments and found that they are genetically and physically linked, and together have a hand influencing almost every ciliary feature discussed in the above sections.

Physical interactions have been detected between multiple InvC and TZ components. Inversin physically interacts with members of all three TZ genetic modules (the NPHP5/NPHP6/Atxn10 module, the NPHP1/4/8/Plk1 module, and the MKS1/MKS6/Tectonic2 module), interactions that are conserved in many models including mammalian cell lines (Otto et al., 2003; Sang et al., 2011), zebrafish (Zhao and Malicki, 2011), and C. elegans (Andrew Jauregui, PhD thesis). Inversin has also been demonstrated to form a physical complex with B9d2 and TTC30B/Fleer, which regulates IFT, ciliary cargo transport, and non-canonical Wnt PCP signaling (Zhao and Malicki, 2011). Because of these physical interactions, it has been suggested that the InvC may function as a bridge linking the TZ and the cilia shaft (Czarnecki and Shah, 2012). Nphp3, though not as well characterized, has also been shown to interact with TZ components, including Nphp1 and Nphp15/Cep164 (Chaki et al., 2012; Sang et al., 2011).

InvC- and TZ-associated genes have been shown to genetically interact in many, many contexts. Inversin and Nphp4 interact in kidney cyst formation in Zebrafish (Burckle et al., 2011), and the Inversin physical interactor Ahi1 interacts with Nphp1 in opsin localization (Louie et al., 2010). In C. elegans, nphp-4 negatively regulates NPHP-2 expression/stability (Andre Jauregui, PhD thesis), and nphp-2 interacts with nphp-4 and mks-6 to control dendrite and cilia length (Warburton-Pitt et al., 2012).

1.6.1.1 – Ciliogenesis. Interactions between the InvC and the TZ also modulate the two-step process of ciliogenesis, the initiation/docking step driven by TZ and BB components, and the second elongation step driven by IFT. nphp-2 mutant worms have disordered—though not mislocalized—TZ Y-links, which are required for membrane attachment of the axoneme (Warburton-Pitt et al., 2014). Disruption of Y-link organization may be at the root of a cilia
misplacement phenotype seen in npg-2 mutants in which cilia cannot anchor in the correct location (Warburton-Pitt et al., 2014; Warburton-Pitt et al., 2012); this may also disrupt interactions between the cilium and surrounding glial cells, leading to cell non-autonomous defects of fasiculated cilia (Williams et al., 2010; Error! Reference source not found.). These links are in part composed of TZ proteins—including the aforementioned NPHP-4, MKS-5, Cc2d2a, and Ahi1—which may lie at the heart of several observed TZ and InvC ciliogenic phenotypes (Chih et al., 2011; Williams et al., 2011). The elongation step of ciliogenesis has also been linked to Inversin; Nphp1 interacts in Hdad6-Aurora A mediated tubulin stabilization in a manner similar to Inversin (Mergen et al., 2013).

1.6.1.2 – Protein localization. The TZ and BB are also required for proper targeting of InvC-associated proteins. The BB/periciliary membrane associated mks-3 is required to restrict NPHP-2 and ARL-13/Arl13b to the proximal cilium (Cevik et al., 2013; Warburton-Pitt et al., 2014). Multiple TZ-associated genes, are also required for the localization of NPHP-2, ARL-13, TAX-2, and TAX-4 in C. elegans (Cevik et al., 2013; Warburton-Pitt et al., 2014; Wojtyniak et al., 2013). In primary cilia, the TZ has been shown to act as an intermediate step in InvC protein localization, in a manner dependent on coiled-coil motifs (Shiba et al., 2010). The converse dependence does not seem to be true; Inversin is not required for Nphp1/4 TZ localization in primary cilia, and npg-2 is not required for MKS-3, B9 domain proteins, or NPHP-1/-4 TZ targeting (Shiba et al., 2010; Warburton-Pitt et al., 2012).

1.6.1.3 – Signaling pathways. InvC components modulate Wnt signaling pathways in conjunction with cilia base proteins. Both basal body (Gerdes et al., 2007; Kishimoto et al., 2008) and TZ proteins are required for PCP signaling. In addition to the PCP-modulating Inv complex described above, a possible separate PCP-modulating complex containing Inversin, Nphp4, Rpgrip1l, and disheveled also exists (Burckle et al., 2011; Mahuzier et al., 2012). This complex
may be the same as the Inversin-Fleer-B9d2 complex, which has independently been shown to regulate several Wnt related pathways (Zhao and Malicki, 2011). Nphp4 represses canonical signaling in a manner similar to Inversin, by binding disheveled and targeting it for proteasomal degradation (Burckle et al., 2011). Interestingly, Rpgr1p1/Mks5 functions to antagonize disheveled degradation, a function in direct competition with Inversin and Nphp4 (Mahuzier et al., 2012). It is also very intriguing that the three known members of the Inversin/Rpgr1p1/Nphp4 complex have an interesting genetic relationship in *C. elegans*: knocking out any two of these genes results in synthetic ciliogenesis defect (Warburton-Pitt et al., 2012; Williams et al., 2010). Wnt signaling has not been definitively established to function in association with *C. elegans* cilia, though NPHP-2 appears to physically interact with the disheveled DSH-2 (Andrew Jauregui, PhD thesis). Echoing data from mammalian studies, deletion of Wnt components in *C. elegans* does not abrogate cilia formation (Oikonomou et al., 2011).

The TZ also plays a role in other signaling pathways. Like Inversin and Nphp3, Nphp4 inhibits Hippo signaling (Habbig et al., 2011). The Hedgehog regulating Evc compartment appears to partially overlap both the TZ and the InvC, suggesting it requires interactions with both for proper function (Pusapati et al., 2014). As described above, the InvC has also been linked to Ca^{2+} signaling; a very large number of TZ components also have Ca^{2+} binding domains, including the C2 domains of MKS-5, MKS-6 (Williams et al., 2011), Nphp4, Nphp8, Ahi1 (Sang et al., 2011), and Cc2d2a (Gorden et al., 2008; Noor et al., 2008; Tallila et al., 2008), the related B9 domains of MKS-1, MKSR-1, MKSR-2 (Bialas et al., 2009; Williams et al., 2008), and the calmodulin-binding domain of Nphp5, which binds the same calmodulin as Inversin (Otto et al., 2005). C2 domains also function in membrane interactions, an important aspect of TZ function in membrane anchoring. Curiously, while TZ components primarily contain C2-family Ca^{2+} binding domains
excepting IQ domain-containing Nphp5), InvC-associated components tend to possess EF-hand and calmodulin—an EF-hand protein—binding domains. This distinction suggests that the two compartments may have differing functions with respect to calcium. It is tempting to speculate that calcium acts to modulate InvC component function, whereas the TZ acts as a calcium sponge/barrier, preventing the extremely high intraciliary calcium concentration from leaking into the cytoplasm (reviewed in Hu and Nelson, 2011). Signals could then be transmitted out of the cilium by second messengers, and not by direct calcium diffusion, which has been demonstrated to be very slow (Delling et al., 2013).

1.6.1.4 – Other TZ and InvC interactions. IFT is an extraordinarily complex, multi-motor, cell-type specific, ciliary subcompartment regulated, transport system. *C. elegans nphp-2* has been demonstrated to interact with *nphp-4* in regulation of IFT particle velocities (Warburton-Pitt et al., 2012). The *nphp-2* interactor *arl-13* and the InvC-associated *hdac-6* and *arl-3* also all modulate IFT particle composition and velocity (Cevik et al., 2010; Li et al., 2010), and the *nphp-2* genetic interactor *klp-11* (Warburton-Pitt et al., 2014) is an IFT motor itself. The biology underlying these interactions is completely unknown, despite multiple attempts, as the GFP reporters used to characterize IFT sensitize the cilia to defects (Cevik et al., 2010; Jauregui et al., 2008; Li et al., 2010).

1.6.2 – The Inversin Compartment in Disease

Most of the genes discussed throughout this review are linked to a class of syndromes termed the “ciliopathies” because of the cilia dysfunction at their root. Two of the best studied of these ciliopathies are polycystic kidney disease and retinitis pigmentosa, the degeneration of photoreceptors. Mutations in Inversin/Nphp2, Nphp3, Nek8/Nphp9, and Anks6/Nphp16 all lead to nephronophthisis (Hoff et al., 2013; Olbrich et al., 2003; Otto et al., 2003; Otto et al., 2008;
Taskiran et al., 2014). This possibly occurs through dysregulation of Wnt signaling during kidney development when the onset of urine flow should be detected by renal tubule cilia (Simons et al., 2005). A large amount of animal model evidence supports this hypothesis, including work in the inv mouse Inversin model (Morgan et al., 1998), the pcy mouse Nphp3 model (Olbrich et al., 2003), the jck mouse Nek8 model (Liu et al., 2002), and in morpholino-injected Zebrafish (Hoff et al., 2013; Lienkamp et al., 2010; Liu et al., 2002; Simons et al., 2005; Zhou et al., 2010); more generally, overexpression of transgenic constitutively active β-catenin leads to PKD in mice (Saadi-Kheddouci et al., 2001). This research bears out in human NPHP2 patients, who exhibit increased disheveled and β-catenin expression (Bellavia et al., 2010). Mutations found in NPHP patients can prevent proper cilia targeting of the protein product (Smith et al., 2006; Sohara et al., 2008), can alter residues needed for function in Wnt signaling (Simons et al., 2005), or can lead to truncated and nonfunctional proteins that would theoretically prevent localization of downstream effectors (Hoff et al., 2013; Olbrich et al., 2003; Otto et al., 2003). A common disease pathway in the four known InvC nephronophthisis genes is supported by evidence showing that morpholinos against Nek8, Nphp3, and Anks6 yield identical pronephric tubule phenotypes in zebrafish (Hoff et al., 2013). However, it has been shown that the abnormal upregulation of canonical Wnt pathways is not responsible for the growth of kidney cysts, and that the PCP defects that characterize these diseases arise at an earlier stage in development (Sugiyama et al., 2011, reviewed in Wuebken and Schmidt-Ott, 2011).

### 1.6.3 – Conservation of the Inversin Compartment

Though the InvC has only been characterized in a handful of organisms, associated genes are conserved across broad clades. Inversin itself appears to be conserved within the clade Opisthokonta, but Nphp3 appears to be almost universal in eukaryotes, with homologs found
amongst both unikonts and bikonts. Nphp3 is not present in Ecdysozoans—a group containing arthropods and nematodes, among other organisms—suggesting it has been lost. Like Nphp3, Unc119b is also conserved throughout Eukarya, but has not been lost in Ecdysozoans. A significant weakness in the InvC literature is the lack of work examining the conservedness of subciliary localization of InvC components. The localization of both Inversin and Unc119b appears to be conserved, but the localizations of Nphp3, Nek8, TAX-2, and TAX-4 have only been reported in single models.

Not all cell types within organisms with InvC-bearing cilia have a conserved InvC. In C. elegans, NPHP-2 has markedly different localization in different cell types; NPHP-2 appears to be primarily cilia base localized in IL2 and male-specific cilia (Error! Reference source not found.). Moreover, NPHP-2 does not require its EF-hand for localization in IL2 cilia, unlike in other cell types (Warburton-Pitt et al., 2014). Inversin also displays cell type-specific localization patterns; results in IMCD3 cells show that Inversin can localize to a variable length of the proximal cilium (Sang et al., 2011).

These localization differences raise a crucial question: is the InvC determined solely by the localization of Inversin, or can it exist separately of Inversin? Though TAX-2 and NPHP-2 were shown to co-localize in AWB cilia in C. elegans (Wojtyniak et al., 2013), is that enough to claim that TAX-2 is an inversin compartment component, especially since NPHP-2 appears to localize to the cilia base in that cell-type? No experiment has definitively examined the localization of known InvC components in cell types in which Inversin is not normally localized to a classic InvC.

1.7 – Supplemental Information/Material and Methods

This chapter focuses primarily on the compartmentalization of the mammalian renal tubule epithelium primary cilia and C. elegans amphid channel and phasmid cilia, the models in which a
majority of the research has been performed; data derived from other models, including the flagella of *Chlamydomonas*, and the Zebrafish pronephros and Kupffer’s Vesicle cilia, is specifically identified as such.
### 1.7.1 – Abbreviations/Definitions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Inv</td>
<td>Inversin</td>
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<tr>
<td>InvC</td>
<td>Inversin Compartment</td>
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<tr>
<td>BB</td>
<td>Basal Body</td>
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<tr>
<td>pseudo-BB</td>
<td>The basal body region of <em>C. elegans</em>, missing the characteristic centrioles</td>
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<tr>
<td>TZ</td>
<td>Transition Zone</td>
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<tr>
<td>Inv Complex</td>
<td>Physical complex comprising Inversin, Nphp3, Nek8, and Anks6</td>
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<td>IFT</td>
<td>Intraflagellar Transport</td>
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<td>PKD</td>
<td>Polycystic Kidney Disease</td>
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<td>NPHP</td>
<td>Nephronophthisis</td>
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<td>MKS</td>
<td>Meckel Syndrome</td>
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<td>PCP</td>
<td>Planar Cell Polarity</td>
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1.8 – Figures and Tables

![Cartoon of C. elegans and mammalian cilia](image)

**Figure 1. Structures of *C. elegans* and mammalian cilia**

Cartoons of *C. elegans* and mammalian cilia highlighting regions mentioned in the text. Both diagrams show the ciliary and cell membranes and the microtubules of the axoneme (a single line indicates microtubule singlets, and a double line indicates microtubule doublets). The *C. elegans* cilium cartoon illustrates the cilia tip complex, singlet region, the doublet region, the transition zone, the pseudo-basal body, the periciliary membrane, the distal dendrite, and adherens junctions (joining the distal dendrite to neighboring glial cells, not shown). The pseudo-BB complex is represented by a rounded rectangle. Vesicles are shown residing in the cilia base, and docking with the membrane and pseudo-BB. More speculatively, vesicles are also shown in the TZ and the cilia shaft. The mammalian cilium cartoon illustrates the cilia tip complex, the doublet region, the TZ, the basal body, the periciliary membrane, and the ciliary...
pocket. Vesicles are shown residing near the cilia base and docking with the membrane of the cilia pocket. The cilia necklace, not labelled, is the external leaflet of the TZ cilia membrane.

Figure designed based on (Blacque and Sanders, 2014).
Figure 2. *C. elegans* cilia lie in a pore

Schematic of a longitudinal cross-section of amphid cilia in *C. elegans*. Cilia are located at the distal ends of dendrites, and extend into the ciliary pore. The pore is formed from two glial cells: the sheath cell surrounding the proximal cilium, and the socket cell surrounding the distal cilium. The socket cell encircles the cilia bundle tightly, whereas within the sheath cell there is an extracellular space filled with matrix material. Here are tight junctions between the socket and sheath cells, and the distal dendrite and sheath cell. Figure modified from (Perkins et al., 1986).
Figure 3. Ciliogenesis is a two stage process.

Ciliogenesis occurs in two stages: assembly of the centriolar complex, docking to the membrane, and biogenesis of the TZ, followed by extension of the axoneme by IFT. In some cell types, the first step includes docking of the centriolar complex to a ciliary vesicle, which then docks with the membrane; in other cell types, the centriolar complex docks to the plasma membrane directly. Reproduced from (Reiter et al., 2012).
Figure 4. Schematic of cilia structure

A model of the cilium showing the relative structures of the individual ciliary compartments, and IFT motors functioning in them. Basal body is marked with dashed lines to indicate a lack of a centriole. Below are electron micrographs of ciliary cross sections through each of the compartments. Yellow motor is OSM-3, black motor is kinesin-II, and gray motor is dynein.

Figure duplicated from (Williams et al., 2011).
The cystic kidney hypothesis states that the genes mutated in most cystic kidney disease encode proteins that localize to cilia. These genes are conserved across eukaryotes, and while they have a diversity of functions in different organisms, they are still associated with cilia. In humans, a variety of phenotypes are associated with dysfunctional cilia because of the wide tissue-expression of ciliary genes. From (Hildebrandt and Zhou, 2007).
Figure 6. The UNC119B Shuttle

(A) Unc119b and PDE6d are related acyl-binding proteins. Reproduced from (Constantine 2013).

(B) Schematic of the Unc119b shuttle system. The mechanism is described in the text (1.4.4 – Establishing the Inv Compartment). Figure reproduced from (Wright et al., 2011)
1.9 – References


*EMBO J.*


Chapter 2: *nphp-2* interacts with two transition zone modules to regulate ciliogenesis and cilia placement

*Note: This chapter is modified from the following publication:*


2.1 – Abstract

The cystic kidney diseases Nephronophthisis (NPHP), Meckel Gruber Syndrome (MKS), and Joubert Syndrome (JBTS) share an underlying etiology of dysfunctional cilia. Patients diagnosed with NPHP type II have mutations in the gene INVS/NPHP2, which encodes inversin, a cilia localizing protein. Here, we show that the *C. elegans* inversin ortholog, NPHP-2, localizes to the middle segment\(^7\) of sensory cilia, and is partially redundant with *nphp-1* and *nphp-4* (orthologs of human nephrocystin-1 and nephrocystin-4, respectively) for cilia placement within the head and tail sensilla. *nphp-2* also genetically interacts with MKS ciliopathy gene orthologs, including *mks-1*, *mks-3*, *mks-6*, *mksr-1*, and *mksr-2*, in a sensilla-dependent manner to control cilia formation and placement. However, *nphp-2* is not required for correct localization of the NPHP and MKS encoded ciliary transition zone proteins or for intraflagellar transport (IFT). We conclude that INVS/NPHP2 is conserved in *C. elegans*, and that *nphp-2* plays an important role in *C. elegans* cilia acting as a modifier of the previously described NPHP and MKS pathways to control cilia formation and development.

\(^7\) During the time-frame this manuscript was written, the doublet region was known as the “middle segment (MS)”, and the singlet region was known as the "distal region (DR)". These older terms were not originally defined rigorously and were thus used to signify subtly different concepts by different authors.
2.2 – Introduction

2.2.1 – Ciliopathies

Human ciliopathies, including nephronophthisis (NPHP), Meckel Syndrome (MKS), and Joubert Syndrome (JBTS), are a class of genetic disorders defined by defective cilia and cystic kidneys (Badano et al., 2006). The primary nephropathy is often comorbid with retinal degeneration, deafness, polydactyly, obesity and situs inversus (Hildebrandt and Zhou, 2007; Pazour and Rosenbaum, 2002; Wheatley, 1995). Many syndromic ciliopathies share loci; in particular, NPHP, MKS and JBTS have thirteen, ten, and thirteen genetic loci identified, respectively, and exhibit considerable overlap (e.g., NPHP6/MKS4/JBTS5 and NPHP11/MKS3/JBTS6) such that there are currently twenty-five distinct loci in sum (Table 3). Disease class overlap may be due partially to the underlying oligogenic nature of the disorders (Hoefele et al., 2007). Ciliopathy associated genes encode cystoproteins; a majority of these gene products localize to the cilium; the wide array of symptoms associated with ciliopathies is due to the near-ubiquitous presence of cilia on mammalian cells.

2.2.2 – C. elegans as a model to study cilia

Cilia are hair-like microtubule (MT)-based organelles that protrude from cell surfaces. Sensory cilia act to receive and transmit information from the extracellular environment and are integral to many signal transduction pathways (Goetz and Anderson, 2010). Eukaryotic cilia and structurally related eukaryotic flagella are constructed by intraflagellar transport (IFT), a MT motor based transport system. The nematode Caenorhabditis elegans is a powerful system to study fundamental questions in cilia biology and to model human ciliopathies. The primary sensory organs of C. elegans are the amphid and phasmid sensilla in the head and tail (Perkins et
al., 1986). The cilia within these sensilla have a wide range of morphologies and functions, allowing for investigation of ciliary specializations and function (Bae and Barr, 2008; Jauregui et al., 2008). The MKS1-related genes B9D1 and B9D2, first characterized as ciliary proteins in *C. elegans*, are ciliopathic in humans, highlighting the power of this model (Bialas et al., 2009; Dowdle et al., 2011; Williams et al., 2008).

*C. elegans* cilia are located on the distal dendritic endings of sensory neurons, where a centriole-derived complex consisting principally of transition fibers (TF) precedes the microtubule-based axoneme (Dammermann et al., 2009; Perkins et al., 1986). The first segment of the axoneme is the transition zone (TZ), a region where many ciliary proteins congregate, either regulating ciliary traffic, being imported/exported into the cilium, or playing structural/functional roles essential for ciliogenesis and formation of the so-called ‘ciliary gate’ (Rosenbaum and Whitman, 2002). The axoneme extends from the TZ. In *C. elegans* amphid channel cilia, the axoneme comprises a MT doublet middle segment and a MT singlet distal segment (Perkins et al., 1986).

### 2.2.3 – The transition zone ciliary protein complex

In *C. elegans* cilia, most ciliopathy-related proteins are either directly implicated in IFT function (e.g., BBS-1, BBS-7, BBS-8), or localize to the TZ/basal body complex (e.g., NPHP-1, NPHP-4, MKS-1, MKS-3, MKS-6) (Bialas et al., 2009; Blacque et al., 2004; Garcia-Gonzalo et al., 2011; Jauregui and Barr, 2005; Qin et al., 2001; Williams et al., 2008; Winkelbauer et al., 2005). This TZ complex is necessary for early ciliogenesis and the TZ is a region associated with traffic into and out of the cilium (Williams et al., 2011). Single mutations in genes encoding TZ complex members yield mild or nonobservable ciliogenic defects. However in certain pairwise combinations, these mutations cause ciliogenesis defects result that are attributable to
anomalies in centriole/TZ membrane anchoring (Williams et al., 2008; Williams et al., 2010; Williams et al., 2011). Mammalian NPHP1/JBTS4 and NPHP4 physically interact to form a complex (Mollet et al., 2002). In C. elegans, nphp-1 and nphp-4 single and double mutants have superficially normal cilia, though transmission electron microscopy reveals subtle ultrastructural defects (Jauregui and Barr, 2005; Jauregui et al., 2008; Winkelbauer et al., 2005). Neither is required for localization of the ciliary receptors OSM-9 and PKD-2; however, mutations in nphp-4 indirectly affect the velocity of several IFT subcomponents (Jauregui et al., 2008). mks-1 (orthologous to MKS1/BBS13) encodes a B9 domain (a C2-like lipid/calcium binding domain [Zhang and Aravind, 2010]) containing protein in the same family and pathway as mksr-1 and mksr-2 (orthologous to B9D1 and B9D2, respectively); mutations in any or all three of these three genes produce no significant cilia defects (Bialas et al., 2009; Kyttala et al., 2006; Williams et al., 2008). MKS-1, MKSR-1, and MKSR-2 are interdependent for proper localization, and function together with NPHP-1 and NPHP-4 to regulate ciliogenesis (Bialas et al., 2009; Williams et al., 2008). mks-3 (orthologous to MKS3/TMEM67/NPHP11/Meckelin) plays a minor role in sensation mediated behaviors, and genetically interacts with nphp-4 to yield dysfunctional cilia in the double mutant (Williams et al., 2010). Recently, MKS-6 (a C2 domain encoding orthologs of JBTS9/CC2D2A) was shown to function together with NPHP-1 or NPHP-4 to control centriole/TZ anchoring to the membrane, the initial step of ciliogenesis (Williams et al., 2011). These genetic interactions have provided evidence for two functional pathways, or modules: an “MKS pathway” involving MKS-1, MKSR-1, MKSR-2, MKS-3 and MKS-6, and an “NPHP pathway” comprising NPHP-1 and NPHP-4.

Further work in both C. elegans and mammalian systems has reinforced these findings and extended these modules. The Jackson group identified a NPHP5/NPHP6 module in addition to the MKS and NPHP1/NPHP4 modules. These modules are physically linked through mutual
interactions with NPHP2 (Sang et al., 2011). In mice, the MKS module (comprising MKS1, TMEM216, TMEM67, CEP290, B9D1, and CCD2A) is essential for ciliogenesis in some tissues, and is necessary for localization of several membrane-associated ciliary proteins, including Arl13b, Smoothened, and Pkd2 (Garcia-Gonzalo et al., 2011). The MKS module also contains Tectonic1/TCTN1, a hedgehog signaling component, linking ciliogenesis, membrane composition, and signaling (Garcia-Gonzalo et al., 2011). Several of these genetic and functional interactions between TZ-associated genes are conserved in C. elegans, providing a powerful tool to define genetic pathways.

2.2.4 – Inversin in mammalian models

Mutations in INVS/NPHP2 are responsible for NPHP type II, an infantile autosomal recessive disease. The gene product inversin is named for the inversion of visceral asymmetry in the inv mouse, which is normally mediated by nodal monocilia (Morgan et al., 1998; Watanabe et al., 2003). Renal cysts of inv mice resemble those in infantile NPHP2 patients (Phillips et al., 2004). Interestingly, the N-terminal ankyrin repeat containing region of inversin can localize to node cilia and rescue left-right defects, but not renal cysts, indicating that inversin is a multifunctional protein that acts in a cell-type specific manner (Watanabe et al., 2003). In Xenopus and zebrafish, inversin acts as a molecular switch between canonical and noncanonical Wnt signaling by binding cytoplasmic disheveled (Dvl) and targeting it for degradation; disruption of this pathway interchange is concordant with disease expression (Bellavia et al., 2010; Benzing et al., 2007; Simons et al., 2005). In mammals, several isoforms of inversin localize to the proximal segment of sensory cilia, a region termed the “Inv compartment.” The zebrafish ortholog of mksr-2, B9d2, physically interacts with and modulates ciliary localization of inversin, which in turn is necessary for anchoring the NPHP3 and NPHP9 gene products to the same compartment.
(Shiba et al., 2009; Shiba et al., 2010; Zhao and Malicki, 2011).

2.2.5 – Summary of this study

In this study, we show that the *C. elegans* ortholog of INVS/NPHP2, *nphp-2*, is expressed in ciliated sensory neurons and encodes two protein isoforms that localize to the ciliary middle segment or “Inv compartment”. We also demonstrate that *nphp-2* is partially redundant with *nphp-1* and *nphp-4* in regulation of TZ placement, TZ orientation, and IFT particle velocity but not for localization of other TZ proteins. Cilia in the *nphp-2 nphp-4* double mutant are severely compromised, although neither *nphp-2* nor *nphp-4* is necessary for localization of B9 domain proteins, MKS-1, MKSR-1, and MKSR-2 (Williams et al., 2008). Genetic experiments between *nphp-2* and MKS genes encoding TZ-localizing proteins demonstrate a complex interaction network, which varies in a sensillum-dependent manner. We conclude that *nphp-2* plays an important role in *C. elegans* cilia, and that *nphp-2* acts as a sensillum-specific modifier of the previously described NPHP and MKS pathways.
2.3 – Results

2.3.1 – Y32G9A.6 is the *C. elegans* ortholog of INVS/NPHP2

Inversin possess several domains, including protein interacting ankyrin repeats, two nuclear localization signals (NLS), two calmodulin binding IQ domains, a basic residue enriched region, and a C-terminal ninein homology region (Figure 7A) (Morgan et al., 2002). At least two of these regions, the ankyrin repeat and the ninein homology regions, are independently sufficient for ciliary localization of inversin (Shiba et al., 2009; Watanabe et al., 2003). Protein BLAST homology searches against the *C. elegans* genome yielded several candidates, including Y32G9A.6 (See 2.5.3 – *C. elegans* INVS homology search). The Y32G9A.6 promoter contains an X-box motif, found in many ciliary genes, and has been identified in at least two ciliary genomic screens, one of which demonstrated expression in ciliated neurons, and predicted Y32G9A.6 as “inversin-like” (Blacque et al., 2005; Efimenko et al., 2005). Protein primary sequence analysis of Y32G9A.6 predicts a D-box ubiquitination site, a bipartite nuclear localization signal, and an EF-hand (Figure 7A). All domains present in Y32G9A.6, with the exception of the EF hand, are conserved in inversin orthologs across several species (Figure 7A) (Morgan et al., 2002). Rather than having a direct calcium binding EF hand, inversin possesses an IQ domain, which binds the calcium sensor calmodulin. Amino acid sequence comparison of Y32G9A.6 and inversin shows 19.1% identity, and 35.2% similarity, and only marginally less – 16.9% identity and 32.6% similarity – when ankyrin repeats are omitted. This is less than other ciliary proteins, but is still significant (Table 4). Based on BLAST results, presence of an X-box promoter motif, conserved protein motifs, and domain organization similar to inversin, we characterized and showed that Y32G9A.6 is the *C. elegans* ortholog of inversin. This gene was coexpressed and genetically
interacted with the other NPHP genes (see below). We hereafter refer to the sequence Y32G9A.6 as *nphp*-2.

**2.3.2 – *nphp*-2 is expressed in ciliated neurons and encodes two cilium-localized isoforms**

The expression pattern of *nphp*-2 was determined through use of native promoter driven GFP expression. P_{nphp-2}::GFP was expressed in the ciliated sensory nervous system throughout development. In the adult, expression was evident in both hermaphrodite and male ciliated sensory neurons, including amphid, phasmid, and IL2 neurons. *nphp*-2 was also expressed in male specific ciliated sensory nervous system, including the CEM, RnB, and HOB neurons (Figure 7B). Expression in the internal oxygen sensor neuron PQR was visible in the hermaphrodite, but could not be distinguished from the tail sensilla in the male.

RT-PCR with subsequent sequencing was used to confirm the predicted splicing pattern and cDNA sequence, and revealed two distinct isoforms. In addition to the predicted full length transcript (long isoform, NPHP-2L), a more abundant (approx. 10-fold) shorter isoform (NPHP-2S) was detectable. This latter isoform, spliced from within exon 10 to the start of exon 11, lacks a region encoding a twenty-two residue fragment (VLIARKNALFRNYNYHPGTEQ), which does not contain or lie within any identified domain. To ascertain the subcellular localization of each isoform, we used native promoter driven expression of GFP tagged splice form cDNA, *e.g.*, the short form P_{nphp-2}::NPHP-2S::GFP and the long form P_{nphp-2}::NPHP-2L::GFP. Cilia in transgenic NPHP-2S::GFP and NPHP-2L::GFP animals form properly in a wild-type background as determined by dye filling (Figure 13). Both NPHP-2S and NPHP-2L transgenes are functional and rescue dye filling defects in the *nphp*-2 *nphp*-4 double mutant (Figure 14). To determine subciliary localization, NPHP-2S::GFP and NPHP-2L::GFP were coexpressed with
p\textsubscript{\textit{nphp-1}}::NPHP-1::CFP, a transition zone (TZ) marker. NPHP-2S::GFP localized cytoplasmically throughout neurons, including dendrites, cell bodies and axons, and to the middle segment of amphid channel and phasmid cilia (Figure 7C, Figure 15). NPHP-2S::GFP was excluded from the TZ and the nucleus (Figure 7C,D; Figure 15). NPHP-2L::GFP also primarily localized to the middle segments of amphid channel and phasmid cilia; dim signals were observable in cell bodies and dendrites, though not in axons. In addition, several bright NPHP-2L::GFP puncta were observable within the cell body (Figure 7C,D). Both NPHP-2S and NPHP-2L localized to the middle segment at all stages of development, from larval L1 to young adult (Figure 36). The middle segment localization of NPHP-2S and NPHP-2L differs markedly from the TZ localization of most other cystoproteins, including NPHP-1, NPHP-4, MKS-1, and MKS-6 (Bialas et al., 2009; Jauregui and Barr, 2005; Williams et al., 2008; Williams et al., 2011; Winkelbauer et al., 2005). To confirm middle segment localization, the length of the NPHP-2S and NPHP-2L localizations was measured (2.5±0.59 µm) and was found to be similar to the length of the MS as measured in EM micrographs (2-3µm) (David Hall, personal communication). GFP-tagged NPHP-2S and NPHP-2L ciliary motility was undetectable, indicating that NPHP-2 is—like the TZ proteins—unlikely to be a component of the IFT machinery (Figure 36).

2.3.3 \textit{nphp-2} is necessary for proper TZ/cilia placement

The available \textit{nphp-2} mutant \textit{gk653} contains a deletion from upstream of the start codon to within the first intron, removing the start ATG. A hidden Markov model splice site predictor correctly predicts the wild-type splice pattern, but predicts an alternate start site in \textit{gk653}, which yields a transcript with a dysfunctional first exon followed by a continuation of the correct transcript (data not shown). RT-PCR of \textit{nphp-2} cDNA confirms the presence of a transcript in \textit{gk653} mutants (data not shown). We therefore generated (by Mos1 transposon mutagenesis)
and examined two additional nphp-2 alleles, nx101 and nx102, which contain in-frame deletions over ankyrin repeats 5-11 and 5-8, respectively (Figure 7A, Figure 16).

To characterize mutant ciliary phenotypes associated with these deletion alleles, we incubated animals with the lipophilic fluorescent dye Dil, which allows readout of gross cilium structure and dendrite extension (Perkins et al., 1986). The dye is taken up by a subset of amphid and phasmid ciliated neurons that are either directly exposed to the environment (e.g., ASK, PHA) or are embedded in the cuticle (e.g., AWB). Failure of a neuron to take up Dil may be indicative of cilia structural or dendritic extension defects. nphp-2(gk653) mutants had a moderate dye filling defect (Dyf) in phasmid neurons, while amphid neurons appeared normal (Figure 8A).

nphp-2(nx101) and nphp-2(nx102) mutants exhibited phasmid Dyf phenotypes that were weaker than those observed in gk653 animals (0.59 in gk653 vs. 0.83 and 0.85 in nx101 and nx102, respectively). For this reason, we used nphp-2(gk653) for the remainder of experiments.

While no loss of function or null alleles of nphp-2 exist, we predict that these would have stronger Dyf phenotypes than the moderate severity observed in gk653 animals.

To determine if cilia displacement defects are phasmid sensilla-specific, as dye filling suggests, or general to core ciliated neurons, we directly examined cilia placement in both amphid and phasmid sensilla. To label amphid and phasmid cilia, we coexpressed NPHP-1::CFP, a TZ marker, and CHE-13::YFP, a TF/cilia axonemal marker. In wild-type amphid sensilla, TZs clustered closely together and cilia were tightly grouped (Figure 8C, left). While both NPHP-1::CFP and CHE-13::YFP localized properly in nphp-2 animals, amphid TZs were dispersed over a larger area and their associated cilia were no longer tightly bundled (Figure 8C, right). We quantified TZ spread by measuring the distance between the anterior-most and posterior-most TZs (Williams et al., 2011). We found that nphp-2 TZs in the amphid sensilla were dispersed over a distance three times longer than WT (data not shown). In WT phasmid sensilla, PHA and PHB cilia and TZs
align (Figure 8C, left). In *nphp-2* phasmid sensilla, cilia and TZs were no longer aligned (Figure 8C, right). Use of another TZ marker, NPHP-4::YFP, yielded similar results: correct subcellular localization of the markers, but disorganized TZ regions in *nphp-2*(gk653) animals (Figure 17). In general, amphid sensilla TZ/cilia displacement was less severe than in phasmid sensilla (approx. 1-2 µm vs. 9 µm, respectively), which may explain the lack of an apparent amphid Dyf phenotype.

Next, we explored the possible correlation between the Dyf phenotype and ciliary displacement. *nphp-2* mutants expressing CHE-11::GFP, which marks the ciliary TZ and axoneme (Williams et al., 2011), were incubated with Dil. Displaced cilia that fail to make contact with the phasmid pore are Dyf, whereas properly positioned cilia dye fill. This implies that the phasmid Dyf phenotype likely stems from an anterior shift in phasmid TZ/cilia placement (Figure 8B). As we could not use TZ spread as a measure of cilia displacement in the phasmid sensilla as we did in the amphid cilia, we instead quantified the displacement by measuring the distance from the TZ to the cell body. In *nphp-2* mutants the distance between the cell body and the TZ was significantly shorter than WT (Figure 10), indicating that cilia were displaced towards the cell body. However, *nphp-2* displaced phasmid cilia had a concomitant increase in length, allowing for contact with the phasmid pore. We conclude that *nphp-2* affects TZ/cilia placement in both amphid and phasmid sensilla.

### 2.3.4 – Genetic analysis reveals a complex interaction network between

**C. elegans** ciliopathic orthologs

Given the oligogenic nature of nephronophthisis (Guay-Woodford et al., 2000; Kuida and Beier, 2000; Wolf and Hildebrandt, 2011), we explored genetic interactions of *nphp-2* with ciliopathy disease gene orthologs with TZ-localized products. First, we measured amphid and phasmid dye
filling. Sensilla of the single mutants nphp-1(ok500), nphp-4(tm1925), mks-1(tm2705), mkdr-1(ok2092), mksr-2(tm2452), mks-3(tm2547), and mks-6(gk674) are non-Dyf with the exception of the nphp-2 phasmid Dyf phenotype (Figure 8A) (Williams et al., 2011). However, many double mutant combinations showed a synthetic dye filling defect (SynDyf), exhibiting a wide range of dye filling phenotypes (Table 1 and Figure 18). Double mutants between an MKS gene (mks-1, mks-3, mks-6) and nphp-1 or nphp-4 are severely Dyf in both amphids and phasms, indicating that two parallel redundant pathways, an MKS pathway and a NPHP pathway, that act similarly in amphib and phasm sensilla (Williams et al., 2008; Williams et al., 2011).

Examination of nphp-2 double mutants reveals differences between ciliogenic roles in amphib and sensilla. In phasm sensilla, nphp-2 was SynDyf with nphp-1, nphp-4, mks-3, and mks-6, indicating that nphp-2 acts as a modifier of both the MKS and NPHP pathways (Table 1, bottom). In the context of amphib sensilla, nphp-2 was SynDyf with nphp-1, nphp-4, mksr-1, and mksr-2 and was nonDyf in double mutant combinations with the MKS family members mks-1, mks-3 and mks-6 (Table 1, top).

nphp-2 single mutants showed an increase in amphid TZ spread, a shortening of phasm dendrite length, and an increase in phasm cilia length. Therefore, we measured these features, in addition to amphid cilia length, in several SynDyf double mutants, including nphp-2 nphp-4, mks-6; nphp-2, and mks-6; nphp-4, and in the single mutants nphp-4 and mks-6.

Dendrites, cilia, and TZs were visualized using the IFT-A marker CHE-11::GFP. In the amphib sensilla, single mutants nphp-4 and mks-6 did not exhibit a significant increase in TZ spread. In the double mutants, TZ spread was increased compared to their respective single mutants, indicative of errors in cilia placement (Figure 9A). Phasm dendritic length, an indicator of TZ displacement in phasm sensilla, was significantly decreased in single mutants (73% of the
average length of WT), implying mild TZ displacement. Double mutants had dendritic lengths significantly shorter than any of the single mutants, implying severe TZ displacement (Figure 9B). The dye filling results suggest that nphp-2 has a sensillum-specific role, though nphp-2 interacts with nphp-1 and nphp-4 in both amphid and phasmid sensilla. By contrast, nphp-2 genetically interacts with different members of the MKS family in a sensillum specific manner. The synergistic defects seen in both amphid TZ spread and phasmid dendritic length suggest genetic redundancy between nphp-2 and the NPHP-1/-4 and MKS pathways. Cilia length measurements in both amphid and phasmid neurons also showed synthetic defects: double mutants had significantly shorter cilia than single mutants, with the single exception of the phasmid cilia length comparison between nphp-4 and nphp-2 nphp-4 mutants (Figure 9C). Curiously, the mks-6; nphp-2 double mutant has synthetic defects in both amphid TZ spread and cilia length, whereas mutants are not amphid SynDyf. This may indicate either that the pathway governing dye filling is separate from the pathway governing cilia formation and placement, or that cilia morphology and dye uptake do not always correlate as previously reported, e.g., a retracted cilium may still contact the now elongated amphid pore and Dil solution. Taken with previous work, these data support two primary TZ genetic pathways, an NPHP-1/-4 pathway and an MKS pathway, with a role for nphp-2 as a sensilla-specific modifier.

2.3.5 – nphp-2 and nphp-4 act redundantly to regulate cilia placement and IFT

The genetic interactions between nphp-2 and TZ-associated genes imply redundant roles and functions. We began exploring these by focusing on the association between nphp-2 and nphp-4. In contrast to either single mutant, and unlike most other double mutants, the nphp-2 nphp-4 double mutant was completely Dyf in both amphid and phasmid sensilla (Table 2). We used IFT-B polypeptide OSM-5::GFP and IFT-A polypeptide CHE-11::GFP to visualize ciliary
TF-axonemal structures. In amphid sensilla, wild-type animals exhibit a pyramidal amphid channel ciliary bundle, with all TFs within 5 µm of each other (Snow et al., 2004) (Figure 10a,b). In nphp-2 nphp-4 double mutants, no cohesive bundle was evident, cilia orientation within the bundle was disrupted, and a portion of the amphid channel cilia appeared to be elongated, curly, or stunted (Figure 10e,f). In the amphid sensilla, it was not possible to identify and associate defects with specific cilia. However, in phasmd sensilla, PHA and PHB exhibit both elongated and stunted phenotypes.

Defects in dye filling, cilia length, and cilia placement may be associated with defects in IFT. In amphid channel and phasmd cilia, the IFT machinery is composed of two complexes, IFT-A and IFT-B, which are transported by two anterograde Kinesin-2 motors, slow Kinesin-II and fast OSM-3, towards the cilia tip (Snow et al., 2004). In wild-type amphid channel cilia, the IFT-A associated Kinesin-II heterotrimeric motor traverses only the middle segment, whereas the IFT-B associated OSM-3 homodimeric motor travels along the entire length of the axoneme. As the IFT particles are linked by the BBS protein complex, when a particle is on the middle segment, both motors are engaged and move along at a speed of 0.7-0.8 µm/sec, whereas while on the distal segment, only OSM-3 is engaged, and the particle travels at the speed of 1.0-1.2 µm/sec (Ou et al., 2005; Snow et al., 2004). To probe IFT function, we measured the velocities of IFT-B polypeptide OSM-5::GFP, IFT-A polypeptide CHE-11::GFP, and homodimeric Kinesin-2 OSM-3::GFP. In nphp-2 mutants, GFP-tagged OSM-5, CHE-11, and OSM-3 velocities in middle and distal segments were similar to wild type (Table 2). No abnormal accumulation of IFT reporters was seen at the ciliary base, indicating particles were being loaded onto the axoneme correctly (Figure 10c,d).

Amphid cilia disorganization in the nphp-2 nphp-4 double mutant made it difficult to define the middle and distal ciliary segments. In nphp-2 nphp-4 double mutants, both IFT-A component
CHE-11::GFP (1.02 ± 0.20 µm/s) and IFT-B component OSM-5::GFP (1.03 ± 0.17 µm/s) moved at speeds comparable to the velocity of the IFT particle in the wild-type distal segment (1.13 ± 0.15 µm/s and 1.06 ± 0.16 µm/s, respectively) (Table 2). We also observed that the IFT-B associated motor OSM-3::GFP travelled along the entire cilium at a velocity of 1.12 ± 0.24 µm/s, similar to the distal segment velocity of OSM-3 in WT. The velocities of the IFT particle observed in nphp-2 nphp-4 mutants are similar to IFT-A and IFT-B velocities in Kinesin-II mutants, in which OSM-3 is the only motor associated with the IFT particle (Pan et al., 2006). Previously, it was reported that OSM-3 overexpression in nphp-4 mutants results dendritic accumulation of OSM-3 (Jauregui et al., 2008); we also observe this in nphp-2 nphp-4 animals overexpressing OSM-3. Surprisingly, OSM-3 overexpression suppressed the truncated cilia phenotype in the phasmid sensillum of nphp-2 nphp-4 double mutants (average length 7.40 ± 0.62 µm). This rescue was not concordant with a rescue of the Dyf phenotype (data not shown). We conclude that within amphid cilia, nphp-2 does not modify IFT complex localization, velocity, or loading onto the axoneme. However, in nphp-2 nphp-4 double mutants, OSM-3 is the primary motor driving IFT, perhaps due to the uncoupling of the heterotrimeric Kinesin-II motor from the IFT complex.

2.3.6 – nphp-2 is not required for proper localization of NPHP-1, NPHP-4 or B9 proteins

To explore whether NPHP-2 might influence the localization of TZ proteins, we first tested and observed that nphp-2 is not essential for NPHP-1 or NPHP-4 TZ localization (Figure 8 and Figure 17). To determine whether NPHP-2 is required for the localization of proteins belonging to the second TZ-associated gene pathway, we examined B9 protein localization in nphp-2 mutants. MKS-1::GFP and MKSR-2::GFP localized to the TZ in wild type animals (Figure 11a,g), nphp-2 single mutants (Figure 11b,h), and nphp-2 nphp-4 double mutants (Figure 11c,i), although
several TZs were improperly positioned within the amphid sensillum. In wild-type animals, MKSR-1::GFP localized to both the dendritic tip and the TZ (Bialas et al., 2009; Williams et al., 2008), and is likewise unaffected in nphp-2 single and nphp-2 nphp-4 double mutants (Figure 11d-f). We conclude that nphp-2 is not required for the proper TZ localization of NPHP (NPHP-1/NPHP-4) pathway proteins or B9 proteins of the MKS pathway.
2.4 – Discussion

INVS/NPHP2, mutated in infantile NPHP type II, localizes to the ciliary proximal segment or “Inv compartment” and is a genetic modifier of polycystic kidney disease. Here we show that the C. elegans ortholog of INVS, NPHP-2, localizes to the C. elegans ciliary middle segment, similar to the “Inv compartment” of mammalian cilia, and plays a role in TZ placement. We also find the genetic interactions between nphp-2 and the NPHP1/4 and MKS pathways in C. elegans reflects the physical interaction between NPHP and MKS modules in mammalian systems (Sang et al., 2011).

2.4.1 – The conservation of Inversin and the two C. elegans NPHP-2 isoforms

Use of multiple bioinformatic analyses and examination of expression and localization patterns establishes Y32G9A.6 as the ortholog of INVS (Blacque et al., 2005). INVS encodes an IQ domain, which interacts with calmodulin in a Ca$^{2+}$-dependent manner (Morgan et al., 2002). In contrast to INVS, NPHP-2 is predicted to contain a Ca$^{2+}$ binding EF hand, which may function in a similar manner as the IQ domain. INVS encodes multiple isoforms (Nurnberger et al., 2002; Ward et al., 2004); likewise, we find that C. elegans nphp-2 encodes two isoforms. Both NPHP-2S::GFP and NPHP-2L::GFP localize to the middle segment, are excluded from the TZ, and rescue the nphp-2 nphp-4 SynDyf defect, indicating that both isoforms function in TZ placement. The additional twenty-two amino acid residues in NPHP-2L appear to play a role in restricting NPHP-2L localization to cell-body puncta and the ciliary middle segment, but the functional difference between NPHP-2S and NPHP-2L remains unknown.

2.4.2 – Cause of ciliary defects in nphp-2 animals
The defects present in *nphp-2(gk653)* animals may stem from one of several possibilities, including impingement on dendritic length, TZ placement, or TZ protein localization. First, an alteration of dendritic length could lead to the observed TZ displacement. *nphp-2* mutants have significantly shorter phasmid dendrites than WT animals, as do several TZ mutants. Double mutants have even shorter dendrites, further evidence of synergistic interactions between the genes. Though phasmid ciliogenesis is currently uncharacterized, these ciliary proteins seem to be necessary for phasmid dendritic extension, which may be mediated through ciliary attachment to the sheath and socket cells near the phasmid pore, as suggested by Williams et al (Williams et al., 2010). Second, *nphp-2* may affect TZ placement at the end of the dendrite. This placement may be mediated by the planar cell polarity (PCP) pathway, which, in zebrafish, is upregulated by inversin (Simons et al., 2005). The role of the PCP in the ciliated nervous system of *C. elegans* is unexplored, and is an exciting avenue for future research. Lastly, *nphp-2* mutation may interfere with correct localization of TZ proteins. This does not seem to be the case for localization of NPHP-1, the B9 proteins, the IFT-A polypeptide CHE-11, and the IFT-B polypeptides CHE-13, OSM-3, and OSM-5. However, in mammalian cells inversin is important for localizing NPHP3 and NPHP9. Likewise, NPHP-2 may have a role in the localization of other ciliary proteins in *C. elegans* (Otto et al., 2003; Shiba et al., 2010).

### 2.4.3 – Interactions between middle segment *nphp-2* and the TZ

A crucial question is raised by the middle segment localization of both isoforms of NPHP-2: how does a protein enriched in the middle segment and excluded from the TZ affect TZ placement? The middle segment forms after the TZ is placed at the tip of the dendrite during the first phase of ciliogenesis (Williams et al., 2011). Two distinct possibilities may account for this apparent paradox: first, that NPHP-2 may have a non-middle segment function, and second, that cilia
placement may follow formation of the middle segment. In adult animals, NPHP-2S has a high non-middle segment concentration and is observed in the cell body, dendrite, and dendritic tip; this population of NPHP-2S may modulate cilia placement. Alternatively, NPHP-2 localization could be dynamically regulated during ciliogenesis, with a transient non-middle segment population modulating cilia placement. The genetic interactions between NPHP-2 and TZ proteins in *C. elegans* and physical interactions between NPHP-2 and NPHP and MKS proteins in mammalian systems support this non-middle segment role for NPHP-2. If cilia begin forming before the dendrite has fully extended, the cilium could guide its own placement through interactions with the surrounding cellular matrix. This possibility is supported by evidence that TZ placement defects are linked to defects in attachment of the cilium to the sheath cell (Williams et al., 2010). In addition, SynDyf double mutants had ectopic dye filling of the amphid and phasmid sheath cells, which has previously been linked to malformed cilia (Ohkura and Burglin, 2011).

### 2.4.4 – The Inversin Compartment

A handful of other proteins share an “Inv compartment” middle segment localization pattern with NPHP-2 in *C. elegans*: the membrane associated GTPase ARL-13 (which modulates IFT-A/IFT-B association), the histone/tubulin deacetylase HDAC-6, and the IFT-A associated Kinesin-II motor (Otto et al., 2003; Li et al., 2010; Cevik et al., 2010; Shiba et al., 2010). In zebrafish, inversin ciliary localization is mediated by Fleer (orthologous to *C. elegans dyf-1*) (Zhao and Malicki, 2011), an IFT-B polypeptide and a regulator of tubulin glutamylation (Ou et al., 2005; Pathak et al., 2007). ARL-13, HDAC-6, and Fleer may play a role in IFT regulation via possible tubulin post-translational modifications (Li et al., 2010). In addition, the mutations of the tubulin de glutamylase *ccpp-1* cause ciliary degeneration, as mirrored by a progressive Dyf
phenotype (O’Hagan et al., 2011); OSM-3 appears to be the primary IFT motor functioning in *ccpp-1* mutants. The signaling and tubulin modifying roles of these components suggests that the Inv compartment may link cell signaling, tubulin modifications, and regulation of IFT. Combined, these observations lead to a quandary: does middle segment localization of these components regulate tubulin modification, or vice versa?

### 2.4.5 – Cell-type specificity of genetic networks

We found that amphid and phasmid cilia use modified gene networks (Figure 12) (Perkins et al., 1986). While the evolutionarily conserved IFT machinery is required for cilia formation, ciliary specialization can be shaped by sensillum or tissue specific modifications. Mammalian Tctn1 has tissue specific roles: it is essential for axonemal extension in nodal and neural tube cilia but is only necessary for Arl13b localization in the cilia of the notochord and early gut epithelia (Garcia-Gonzalo et al., 2011). Mammalian Mks1 also acts in a tissue specific manner, essential for formation of nodal and neural tube cilia, but not bile duct and lung cilia (Weatherbee et al., 2009). Sensillum/cell-type specificity is also evident in *C. elegans*: IFT in the cilia of AWB neurons is modified such that the kinesin motor OSM-3 moves independently of Kinesin-II and is not needed to build distal segments (Mukhopadhyay et al., 2007). Additionally, specifically in the cilia of male CEM neurons, the kinesin-3 motor *klp-6* regulates Kinesin-2 driven IFT (Morsci and Barr, 2011). Here, we show that *nphp-2* SynDyf phenotypes vary in a sensillum-specific manner. *nphp-2* interacts with *nphp-1* and *nphp-4* in both amphid and phasmid neurons, but interacts with MKS family members in a sensillum specific manner. We interpret this as evidence for a role for *nphp-2* as a modifier of NPHP-1/-4 and MKS pathways in these sensilla.
2.4.6 – The TZ physical and genetic modules in *C. elegans* and mammalian models

Much recent work has been done to elucidate the physical and genetic interactions between ciliopathy related genes. A mammalian proteomics screen for physical interactors of cystoproteins revealed three distinct physically interacting modules: a NPHP1-4-8 module, a MKS-1-6-TCTN2 module, and a NPHP5-6-ATXN10 module (Sang et al., 2011). The former two modules correspond to the *C. elegans* genetic interaction based NPHP-1/-4 and MKS pathways, though the latter NPHP5-6-ATXN10 module does not appear to be conserved. NPHP-2 acts as a physical nexus between these three modules, which agrees with our findings that nphp-2 may act as a modifier of both the NPHP-1/-4 and MKS pathways (Sang et al., 2011). In addition to the genes examined here, Williams et al. showed that mks-5, a C2 domain encoding gene orthologous to mammalian RPGRIP1L, was SynDyf with nphp-4, mks-6, and mksr-2 in both amphids and phasmids. This led them to link mks-5 to both NPHP-1/-4 and MKS pathways, similarly to the treatment of nphp-2 here (Williams et al., 2011).

In summary, we find that nphp-2 acts as a genetic modifier of the NPHP and MKS pathways in *C. elegans*, and modulates sensillum-specific regulation of ciliary positioning. These results complement and extend our knowledge of the roles of ciliopathy genes in ciliogenesis and cilia placement, and integrate a wide range of ciliopathic genes into a single model.
2.5 – Materials and Methods

2.5.1 – General Molecular Biology Methods

Standard protocols were used for all molecular biology procedures. PCR amplification was used for genotyping and building transgenic constructs using the following templates: *C. elegans* genomic DNA, cDNA, or prebuilt constructs. High fidelity LA Taq (TaKaRa Bio Inc., Otsu, Shiga, Japan) or Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific, Vantaa, Finland) were used for amplification of DNA for constructs. Sequencing reactions were performed on site, and analyzed by SEBS DNA Sequencing Facility (Rutgers University, Piscataway, NJ, USA). PCR primer and construct sequences are available upon request.

2.5.2 – DNA and Protein Sequence Analysis

BLAST (Altschul et al., 1997) was used for identification of gene orthologs in *C. elegans*. Human protein sequence information was provided by NCBI, and *C. elegans* gene and protein sequence information was provided by Wormbase. SAGE data provided by Wormbase (Release WS221). Structural and domain predictions of gene products are by MotifScan (Hau et al., 2007). Percent identity and percent similarity were computed by MatGat 2.02 software (Campanella et al., 2003). Splice predictions in *gk653* deletion mutants were computed using Genemark.hmm (Lomsadze et al., 2005). ApE 1.17 was used for sequence manipulation.

2.5.3 – *C. elegans* INVS homology search

Basic Local Alignment Search Tool (BLAST) homology searches of inversin/nephrocystin-2 led to the identification of five possible orthologs, *unc-44*, *mlt-4*, T28D6.4, F36H1.2, and Y32G9A.6 (in order of decreasing similarity) (Altschul et al., 1997). The *unc-44* promoter does not contain a
palindromic X-box sequence, which is common to many ciliary genes, and the UNC-44 protein contains a DEATH domain and a zona pellucida UNC-5 domain, neither of which are present in Inversin (Blacque et al., 2005; Efimenko et al., 2005). SAGE tagging data also indicates that unc-44 transcripts are not enriched in ciliated neurons (Wormbase Release WS221, Blacque et al., 2005). Likewise, the mlt-4 promoter does not contain an X-box, and the entire length of the MLT-4 peptide encodes only ankyrin repeats. T28D6.4 promoter does contain an X-box, but the gene product is predicted to contain only ankyrin repeats and an SH3 domain, not present in Inversin. F36H1.2 has no X-box, and the predicted gene product has no similarities to Inversin beyond the common ankyrin repeat motif and a possible weak nuclear localization signal. As described in the results, Y32G9A.6 has an X-box, and shares many predicted domains with Inversin, including a bipartite nuclear localization signal and a D-box ubiquitination site, with similar overall domain organization. Performing a reverse protein BLAST homology search on MLT-4 and Y32G9A.6 yielded a match for Inversin, while UNC-44, T28D6.4, or F36H1.2 did not. A similar in depth search was performed for orthologs of NPHP3/MKS7. Only C. elegans klc-2 (kinesin light chain 2) had meaningful homology to NPHP3. klc-2 has a C-terminal domain with medium homology to NPHP-3, has a transcript enriched in ciliated neurons (Blacque et al., 2005), but does not possess an X-box (Efimenko et al., 2005). In addition, NPHP3 does not appear in the top results in a reciprocal BLAST search for KLC-2.

2.5.4 – RT-PCR and transcript sequencing

Total RNA from animals was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse Transcriptase with oligo-dT was used to isolate stable mature mRNA. Transcripts were ligated into pGEM-T-Easy plasmid (Promega, Fitchburg, WI, USA) and transformed into DH10-β competent cells. DNA from transformed colonies was amplified with Phusion, as above, using
the following primer sets: 5’-ATG TCC CAC ACG CTG ATC GAA GCA TTA GAC GAT GAG -3’ with 5’-GTC GAC GGT CTT TGT TTC TTT TCA GCT TTT AAC TC-3’ to amplify the first half of the transcript, and 5’-GTC GAC TAG CGA CGG AAT TGT GGA AAC CGA GA-3’ with 5’- AAG GAA CAG GTG CCT ATG CGT GTC AAG GCA ATC-3’ to amplify the second half of the transcript. These amplicons were then sequenced.

### 2.5.5 – qRT-PCR

Total RNA from animals was isolated using Trizol Reagent. A 7900HT Sequence Detection system (Applied Biosystems, Foster City, CA, USA) was used. Levels of *nphp-2* mRNA were normalized to either actin or to the RNA polymerase large subunit. *nphp-2* mRNA was detected using the following primer sets: 5’-TCG ACT AGC GAC GGA ATT GTG -3’ with 5’-GTT TTG GAG CGT TTG AAT CGG -3’ and 5’-AAA AAG GAG CTG GAG GCA CTG -3’ with 5’-CAA ATC GGA GAT TGG CGG TA-3’. The actin gene, *act-1*, was used as a control, and was detected using the following primer sets: 5’-CCA TTG TCG GAA GAC CAC GTC -3’ with 5’-AGT TGG TGA CGA TAC CGT GCT C-3’. The DNA polymerase gene *ama-1* was also used as a control, and was detected using the following primer sets: 5’-GGA TGG AAT GTG GGT TGA GA -3’ with 5’-CCG AGT AGT TTT TGC GAA GG-3’.

### 2.5.6 – Strains and Maintenance

All strains were cultured at 20°C, unless otherwise noted, under standard conditions (Brenner, 1974). Deletion alleles such as *gk653* were outcrossed to *him-5(e1490)* at least four times. *nphp-2(nx101)* and *nphp-2(nx102)* alleles are deletions of ankyrin repeats 5-11 and 5-8, respectively, generated by imprecise Mos1 excision, and outcrossed once to *him-5(e1490)*. The *mks-6* allele used, *gk674*, deletes portions of a second gene, *xpa-1*, but has no discernible
effects on the function of XPA-1 (Williams et al., 2011). Strains used in this study appear in Table 6, organized by the figure in which they first appear.

2.5.7 – Imaging

Animals were imaged using standard *C. elegans* slide mounts and either a Zeiss Axio Plan-APOCHROMA 63X 1.4 oil DIC or 100X 1.4 oil DIC objectives on a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany) with a Cascade 512B (Photometrics, Tucson, AZ, USA) digital camera, or on a Zeiss Imager.D1M with a Retiga-SRV Fast 1394 digital camera (Q-Imaging, Surrey, BC, Canada). Fluorochromes were either eGFP, VenusYFP, tdTomato, or CFP. IFT motility was recorded and kymographs and particle movement rate were measured using Metamorph (Version 7.6.1.0, MDS Analytical technologies, Sunnyvale, CA, USA) software. Strains were synchronized by picking L4 worms, culturing at 15°C overnight, and imaging within 24 hours. Worms were anesthetized in a drop of M9 containing 50mM muscimol, transferred to an agarose mount slide, and imaged immediately. AutoDeblur (Version 1.4.1, Media Cybernetics, Bethesda, MD, USA) software was used for 3D Blind deconvolution of image stacks. The amphid channel middle segment is the region of the cilium between the transition zone and the point where amphid cilia align; the distal segment is the region of the cilium past this point (Snow et al., 2004). Figures and diagrams were created with Adobe Photoshop CS3 (Version 10.0, Adobe Systems, San Jose, CA, USA) and Adobe Illustrator CS3 (Version 13.0.0, Adobe Systems). Cilia length, dendrite length, and TZ spread measurements were analyzed using ANOVA, followed by Tukey’s post hoc test.

2.5.8 – Dye Filling Assays

For staining of cell bodies and cilia for evaluation of NPHP-2 localization, animals were incubated
with 2.5µg/mL DiO (2.5mg/mL dimethyl formamide stock, diluted 1:1000 in M9) (Invitrogen, Carlsbad, CA, USA) for 30 minutes, rinsed three times, and then allowed to recover on a seeded plate for one hour before imaging.

For scoring dye uptake as an indicator of amphid and phasmid ciliary structural defects, we used a dye filling protocol modified from Tong and Bürglin (2010). Staged one day old, young adult hermaphrodites are washed off plates in M9, and rinsed twice. Worms were then incubated in 40µg/mL DiI (2.5mg/mL dimethyl formamide stock, diluted 1:1000 in M9) (Invitrogen) for one hour in the dark, rinsed twice in M9, and allowed to recover on a seeded plate for one hour in the dark. Animals were anesthetized using 10mM levamisole and mounted on a slide. Worms were scored as fraction of amphid or phasmid cell bodies stained by identifying and scoring individual neurons, and averaging together the twelve amphid neurons (ASK, ADL, ASI, AWB, ASH, and ASJ neurons on both left and right sides) or the four phasmid neurons (PHA and PHB, left and right). These were compared using the non-parametric Kruskal-Wallis test, followed by Dunn’s multiple comparison test. GraphPad Prism (Version 5.01, GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis and bar graph creation.
2.6 – Acknowledgments

We thank the Barr Laboratory for constructive criticism on the manuscript. Some strains were provided by Dr. Bradley Yoder, the *C. elegans* Genetics Center (funded by the National Institutes of Health), the Japanese National BioResources Project, and the *C. elegans* Gene Knockout Consortium.
2.7 – Figures and Legends

Figure 7. *nphp-2* is the ortholog of mammalian inversin, is expressed in ciliated neurons, and encodes two isoforms with slightly different ciliary localization patterns.

(A) The *C. elegans* ortholog of human inversin, NPHP-2, has similar domain structure to human inversin, containing conserved ankyrin repeats, nuclear localization sequences, and destruction box (D-Box). Novel features include a coil and an EF hand. *nphp-2* encodes a full length isoform (NPHP-2L) and a shorter isoform (NPHP-2S) lacking the twenty-two amino acid region indicated by the asterisk (*). *nphp-2(gk653)* removes the start codon but not the upstream X-box RFX transcription factor binding site. *nphp-2(nx101)* and *nphp-2(nx102)* are deletions of ankyrin repeats 5-11 and 5-8, respectively. (B) *nphp-2* is expressed in the ciliated sensory nervous system of both the hermaphrodite and male. (C) The *nphp-2* gene encodes at least two splice forms, one short (NPHP-2S) and one long (NPHP-2L). NPHP-2S::GFP localizes throughout the sensory neuron, in the cell body, axon, dendrite, and cilia, but is excluded from the nucleus and TZ. NPHP-2L::GFP is predominantly localized to middle segments of cilia in the amphid and phasmid sensilla. (D) Co-expression of NPHP-1::CFP and either NPHP-2S::GFP or NPHP-2L::GFP
was used to visualize ciliary TZs in phasmid sensilla. NPHP-2S::GFP localizes to dendrite and middle segments. NPHP-2L::GFP also localizes to the middle segment. The length of NPHP-2S and NPHP-2L middle segment localization is consistent with the length of the total middle segment (Dr. David Hall, personal communication). Arrows indicate a cilia base, the dividing point between the cilium and dendritic tip, and bars indicate the middle segment (MS), transition zone (TZ), or cilium. To the right, subcellular localization of NPHP-2S::GFP and NPHP-2L::GFP (colored in green) and NPHP-1::CFP (colored in blue) are diagrammed. DS, distal segment; TF, transition fibers; D, dendrite. Inversin primary structure in (A) adapted from Otto et al., 2003.
Figure 8. *nphp-2* mutants are moderately dye filling defective and have defects in transition zone positioning.

(A) *nphp-2*(gk653) mutants incubated with Dil show a phasmid but not amphid dye filling defect. ***, p<0.0001, Kruskal-Wallis test with Dunn's post hoc test. Sample size n is the number of cell bodies scored, and is listed above each experimental group. (B) Phasmid ciliary bases and axonemes were labeled with the IFT-A polypeptide marker CHE-11::GFP (a,d) and incubated with Dil (c,f). Cilia that are improperly placed within the animal do not reach the amphid or phasmid channel pore, are not exposed to the environment, and likely lead to dye filling defects (b,e). Arrowheads indicate the base of cilia (transition fibers), corresponding to increased CHE-11::GFP fluorescence. In panel (e), only PHB fills with Dil, presumably because its cilium is correctly positioned. In contrast, the PHA cilium is displaced anteriorly and the PHA neuron does not dye fill. (C) Axonemes of cilia in amphid and phasmid sensilla were labeled with IFT-B
polypeptide CHE-13::CFP (c,f), and amphid and phasmid TZs were labeled with NPHP-1::CFP (a,d). NPHP-1 localized properly in nphp-2(gk653) mutants (d-f), but de-clustering of channel cilia TZs and a separation of phasmid cilia TZ pairs is observed (diagramed, D).
Figure 9. Ciliary single and double mutants exhibit defects in amphid and phasmid cilia length, amphid TZ spread, and phasmid dendritic length.

Cilia, TZs, and phasmid dendrites were labeled using CHE-11::GFP. Data was normalized to N2 WT. Statistical analysis performed was ANOVA followed by Tukey’s post hoc test. Groups of mutants are labeled on the bar graph; two mutants in the same group are not statistically different from one another. Bars represent SEM. (A) TZ spread was measured as the distance from the posteriormost TZ to the anteriormost TZ. npkh-2 was the only single mutant significantly different from WT. All double mutants were significantly different from WT and the single mutants that comprised them. (B) Phasmid dendrite length was measured from the base of the TZ to the cell body. All single mutants had significantly shorter dendrites that WT. All
double mutants had significantly shorter dendrites than the single mutants that comprised them. (C) Cilia length was measured from the base of the TZ to the observable tip of the cilia. In amphid neurons, nphp-4 was the only single mutant significantly different than WT, and all double mutants had significantly shorter cilia than the single mutants that comprised them. In phasmid neurons, nphp-2 had significantly longer cilia than WT and nphp-4 had significantly shorter cilia than WT. mks-6; nphp-2 and mks-6; nphp-4 had significantly shorter cilia than their respective single mutants, whereas nphp-2 nphp-4 did not significantly differ from nphp-4.
Figure 10. *nphp-2* and *nphp-4* are involved in regulating TZ/cilia placement and orientation.

Amphid channel TFs/axonemes were labeled with either IFT-A complex polypeptide CHE-11::GFP (a,c,e) or IFT-B complex polypeptide OSM-5::GFP (b,d,f). Wild-type amphid channel cilia form a tight bundle (a-b); moderate cilia placement defects are seen in *nphp-2* single mutants (c-d). These defects are exacerbated in *nphp-2 nphp-4* double mutants, which also exhibit defects in length and orientation (e-f). Brackets indicate amphid cilia regions, arrows in (c-d) point to the base of cilia placed outside the amphid bundle.
Figure 11. B9 proteins are not mislocalized in nphp-2 single or nphp-2 nphp-4 double mutants.

MKS-1::GFP (a-c), MKSR-1::GFP (d-f) and MKSR-2::GFP (g-i) localize to the TZ of amphid channel cilia in both wild-type animals and nphp-2 nphp-4 mutants (Williams et al., 2008). Additional puncta present using MKS-1::GFP reporter were due to localization of MKS-1::GFP in labial cilia (yellow arrowhead). Brackets indicate spread of amphid channel transition zones, which is greatly expanded in nphp-2 nphp-4 double mutants. Arrows indicate improperly placed TZs in
the *nphp-2* single mutant (b,e,h). Phenotypes are summarized in the cartoon (j).
Figure 12. Schematic of SynDyf genetic interactions between *nphp-2* and TZ associated genes.

Using double mutant analysis, we constructed genetic interaction networks between ciliopathy genes within each sensilla based on their dye filling phenotypes. Genes within a common pathway are not SynDyf. Line weights indicate severity of defects among double mutants, and all shown interactions are significantly different from wild type. Underline indicates single mutant Dyf defect.
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**Fraction of Cyclic Phase-Shifts in Phasemixes**

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**Fraction of Cyclic Phase-Shifts in Amphibics**
Table 1. Quantification of dye filling defects in double mutant amphid and phasmid neurons.

Amphid and phasmid neurons were scored individually, and then averaged with other neurons within the same animal. N is the number of individual neurons scored. The statistical analysis performed was Kruskal-Wallis test followed by Dunn’s post hoc test. * indicates p value < 0.05 versus WT, ** indicates p value < 0.001. In double mutants in nphp-2 background, § indicates p value < 0.05 versus nphp-2 single mutant, §§ indicates p value < 0.001. Scores are plus/minus the standard error of the mean. (From Jauregui et al., 2008). Double mutants that differ significantly are presented in Figure 7.
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**Table 2.** *nphp-2* does not affect IFT velocities in amphid channel cilia.

IFT speed of complex A and B polypeptides in *nphp-2* single and *nphp-2 nphp-4* double mutants was measured by tracing particles on kymographs. The middle segment is the region of the cillum between the TZ and the point where amphid cilia converge (See Fig. 4b). Velocity of IFT-A component CHE-11::GFP, IFT-B component OSM-5::GFP, and Kinesin-2 motor OSM-3::GFP was not significantly altered in middle or distal segments in the *nphp-2* single mutant compared to wild type. OSM-3::GFP velocity was slightly increased in the *nphp-4* single mutant. IFT velocities of all reporters in *nphp-2 nphp-4* double mutants resembled that of IFT velocities in wild-type distal segments, although it was not possible to distinguish between middle and distal segments because of the severe TZ placement and ciliary defects. \(^1\)Data for OSM-3::GFP velocities in *nphp-4* mutants is from Jauregui et al., 2008.
Figure 13. GFP-tagged NPHP-2S and NPHP-2L do not interfere with ciliogenesis.

Wild-type animals expressing native promoter driven NPHP-2S::GFP or NPHP-2L::GFP were incubated with Dil to assess gross cilia morphology. Neurons in both NPHP-2S and NPHP-2L animals dye-filled properly, indicating that overexpression of either NPHP-2 isoform does not lead to ciliogenic defects. The small puncta visible in the panels showing tail dye filling are due to dye filling of the ciliary sheath cells.
Figure 14. GFP-tagged NPHP-2S and NPHP-2L rescue *nphp-2 nphp-4* dye filling defect.

*nphp-2 nphp-4* double mutants expressing native promoter driven (A) NPHP-2S or (B) NPHP-2L were incubated with Dil to assess gross cilia morphology. Both short and long NPHP-2 isoforms rescued the severe *nphp-2 nphp-4* mutant Dyf defect.
Figure 15. NPHP-2 short and long isoforms localize to the middle segment of amphid cilia

Localization of GFP-tagged NPHP-2S and NPHP-2L in the amphid sensillum. Similarly to in the phasmid sensillum, both isoforms of NPHP-2 localize to the middle segment. Panels on the left show both amphid sensilla, and panels on the right show a magnified view of a single amphid channel cilia bundle.
Figure 16. Predicted protein domains encoded by \textit{nphp-2(nx101)} and \textit{nphp-2(nx102)}.

Both \textit{nphp-2(nx101)} and \textit{nphp-2(nx102)} are in-frame deletions, and are predicted to encode truncated NPHP-2 protein. Both are missing a series of ankyrin repeats, \textit{nx101} missing repeats 5-11, and \textit{nx102} missing repeats 5-8.
Figure 17. *nphp-2* exhibits disorganized amphid bundles

Amphid cilia were labeled with CHE-13 fluorescent reporter (c,f), and amphid TZ were labeled with NPHP-4::YFP (a,d). As with the NPHP-1 TZ marker, NPHP-4 localized properly in *nphp-2(gk653)* mutants (d-f), and mutants exhibit the same disorganization of TZ (diagrammed).
Animals were incubated with Dil to assess gross cilia morphology. In wild-type animals, neurons within two amphid bundles and two phasmid pairs were filled with dye. All single mutants except \textit{nphp-2} dye filled normally. Double mutants exhibited varying expressivity of the Dyf phenotype, and were either defective for phasmid dye filling (\textit{mks-1; nphp-2}, \textit{mks-3; nphp-2}, \textit{mks-6; nphp-2}), both amphid and phasmid dye filling (\textit{nphp-2 nphp-4}, \textit{mks-1; nphp-4}, \textit{mks-3; nphp-4}, \textit{nphp-4}, \textit{mks-6; nphp-4}, \textit{nphp-1; nphp-2, nphp-1; mks-1, mks-3 nphp-1}) or were non-Dyf (\textit{mks-1; mks-3, mks-6; mks-1, mks-6; mks-3, nphp-1; nphp-4}). Worms with phasmid Dyf defects were typically missing one or both pairs of phasmid neurons; more rarely, only one neuron of a pair was missing.
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**Table 3. Nephronophthisis (NPHP), Meckel Syndrome (MKS), and Joubert Syndrome (JBTS) share multiple loci.**

Classes of multiple ciliopathies, which are indicated by type number, often share loci. Senior-Løken Syndrome (SLSN) exhibits extensive overlap with NPHP due to its disease classification criteria, nephronophthisis comorbid with retinitis pigmentosa. Loci are also shared with other ciliopathies, including Bardet-Beidel Syndrome (BBS), Renal–Hepatic–Pancreatic Dysplasia (RHPD), Cerebello Oculo Renal Syndrome (CORS) – a subtype of Joubert Syndrome, Oral-Facial-Digital Syndrome (OFD), and hydrolethalus syndrome (HLS). Known *C. elegans* homologs are listed to the right; synonymous names are separated by commas. There are two *C. elegans* orthologs of NEK8, the paralogs nekl-1 and nekl-2, which are separated by an ampersand. References pertain to identification of NPHP, MKS, and JBTS loci, and earliest identification of *C. elegans* homologs. Orthologs found by BLAST are reported only if the highest scoring match has an E value less than $10^{-20}$, and exhibits homology beyond common domains. A question mark (?) indicates that BLAST searching the *C. elegans* protein sequence does not yield the mammalian ortholog as the closest match.
Table 4. *nphp-2* has similar conservation as other *C. elegans* ciliopathic gene orthologs.

Percent identity and percent similarity between full length *NPHP-2* ("full"), NPHP-2 without ankyrin repeats ("no ank"), NPHP-4 and MKS-3 and their mammalian orthologs. Due to slightly lower evolutionary conservation than other ciliary genes, the INVS/NPHP2 ortholog was not immediately identified in *C. elegans*, though percent identity and percent similarity are near those of other genes investigated.

<table>
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<th><em>C. elegans</em></th>
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<th>% Similarity</th>
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<td>NPHP-2 (full)</td>
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<td>19.1</td>
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<td></td>
<td>Fraction Amphid Dyf</td>
<td>N</td>
<td>Fraction Phasmid Dyf</td>
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<td>mks-1</td>
<td>0.95±0.08</td>
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Table 5. *mksr-1; nphp-2* and *mksr-2; nphp-2* mutants are not SynDyf

B9 gene single and double mutants were incubated with Dil to assess cilia morphology. Amphid and phasmid neurons were scored individually, and then averaged with other neurons within the animal. The statistical analysis performed was Kruskal-Wallis test followed by Dunn’s post hoc test. No single mutants were Dyf in either amphids or phasmids. Double mutant phasmid Dyf defects were not significantly different from *nphp-2* single mutant Dyf defects, but double mutant amphid defects were SynDyf in *nphp-2* background. §§§ indicates p < 0.0001 versus *nphp-2* single mutant. Scores are plus/minus the standard error of the mean.
| Figure 7 | PT1995: *pha-1*(e2123); *him-5*(e1490); myEx684[\text{P}_{\text{nphp-2}}::\text{NPHP-2}\text{S}::\text{GFP} + pBX]
PT1994: *pha-1*(e2123); *him-5*(e1490); myEx683[\text{P}_{\text{nphp-2}}::\text{NPHP-2}\text{L}::\text{GFP} + pBX]
PT2354: *nphp-2*(gk653); myEx743[\text{P}_{\text{nphp-2}}::\text{NPHP-2}\text{L}::\text{GFP}, \text{ccRFP}];
yhEx149[\text{NPHP-1}::\text{CFP}, \text{CHE-13}::\text{YFP}, pRF4]
PT2355: *nphp-2*(gk653); myEx744[\text{P}_{\text{nphp-2}}::\text{NPHP-2}\text{S}::\text{GFP}, \text{ccRFP}];
yhEx149[\text{NPHP-1}::\text{CFP}, \text{CHE-13}::\text{YFP}, pRF4]
PT1055: *dpy-5*(e907); *him-5*(e1490); myEx*[\text{dpy-5}(+); \text{P}_{\text{nphp-2}}::\text{GFP}]

| Figure 8 | CB1490: *him-5*(e1490)
PR802: *osm-3*(p802); *him-5*(e1490)
PT2145: *nphp-2*(gk653) *him-5*(e1490)
PT50: *che-11*(e1810); myEx10[\text{P}_{\text{che-11}}::\text{CHE-11}::\text{GFP} + pRF4]
PT1958: *nphp-2*(gk653) *him-5*(e1490); myEx10[\text{P}_{\text{che-11}}::\text{CHE-11}::\text{GFP} + pRF4]
YH237: yhEx149[\text{P}_{\text{nphp-1}}::\text{NPHP-1}::\text{CFP}, \text{P}_{\text{che-13}}::\text{CHE-13}::\text{YFP} + pRF4]
PT1960: *nphp-2*(gk653); yhEx149[\text{P}_{\text{nphp-1}}::\text{NPHP-1}::\text{CFP};
\text{P}_{\text{che-13}}::\text{CHE-13}::\text{YFP} + pRF4]

| Figure 9 | PT1334: *nph-4*(tm925) *him-5*(e1490) V; myEx[\text{CHE-11}::\text{GFP} + pRF4]
MX712: *mks-6*(gk674); nxEx[\text{CHE-11}::\text{GFP} + \text{ROL-6*(su1006)}]
PT2029: *nphp-2*(gk653) *nph-4*(tm925) *him-5*(e1490);
\text{Ex}[\text{P}_{\text{che-11}}::\text{CHE-11}::\text{GFP} + pRF4]
MX: *mks-6*(gk674); *nphp-2*(gk653); nxEx[\text{CHE-11}::\text{GFP} + \text{ROL-6*(su1006)}]
MX716: *mks-6*(gk674); *nph-4*(tm925); nxEx[\text{CHE-11}::\text{GFP} + \text{ROL-6*(su1006)}]

| Figure 10 | PT40: *him-5*(e1490); *osm-5*(sa126); myEx1[\text{P}_{\text{osm-5}}::\text{OSM-5}::\text{GFP} + pRF4]
PT1956: *nphp-2*(gk653); myEx1[\text{P}_{\text{osm-5}}::\text{OSM-5}::\text{GFP} + pRF4]
PT1411: *nph-4*(tm925) *him-5*(e1490) V; myEx1[\text{OSM-5}::\text{GFP} + pRF4]
PT2027: *nphp-2*(gk653) *nph-4*(tm925) *him-5*(e1490);
\text{Ex}[\text{P}_{\text{osm-5}}::\text{OSM-5}::\text{GFP} + pRF4]

| Figure 11 | MX549: *dpy-5*(e907); nxEx[\text{P}_{\text{mks-1b}}::\text{MKS-1b}::\text{GFP} + \text{dpy-5}(+)]
PT2006: *dpy-5*(e907); *nphp-2*(gk653) *him-5*(e1490); \text{Ex}[\text{P}_{\text{mks-1b}}::\text{MKS-1}::\text{GFP}]

+ dpy-5(+)]

PT2026: dpy-5(e907); nphp-2(gk653) nphp-4(tm925) him-5(e1490); 
Ex[P_{mks-1::MKS-1::GFP + dpy-5(+)]

MX349: dpy-5(e907); nxEx[P_{mksr-2::MKSR-1::GFP + dpy-5 (+)]

PT2004: dpy-5(e907); nphp-2(gk653) him-5(e1490); 
Ex[P_{mksr-2::MKSR-1::GFP + dpy-5(+)]

PT2024: dpy-5(e907); nphp-2(gk653) nphp-4(tm925) him-5(e1490); 
Ex[P_{mksr-2::MKSR-1::GFP + dpy-5(+)]

MX342: dpy-5(e907); nxEx[P_{mksr-2::MKSR-2::GFP + dpy-5 (+)]

PT2002: dpy-5(e907); nphp-2(gk653) him-5(e1490); 
Ex[P_{mksr-2::MKSR-2::GFP + dpy-5(+)]

PT2022: dpy-5(e907); nphp-2(gk653) nphp-4(tm925) him-5(e1490); 
Ex[P_{mksr-2::MKSR-2::GFP + dpy-5(+)]

Table 1

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| Table 2 | OSM-3::GFP: Ex [OSM-3::GFP; pRF4]  
|        | PT2483: nphp-2(gk653); Ex[OSM-3::GFP; pRF4]  
|        | PT2484: nphp-2(gk653) nphp-4(tm1925); Ex[OSM-3::GFP; pRF4]  
| Figure 13 | PT2485: nphp-2(gk653); myEx743(P\_nphp-2::NPHP-2::GFP)  
|          | PT2486: nphp-2(gk653); myEx744(P\_nphp-2::NPHP-2L::GFP)  
| Figure 14 | PT: nphp-2(gk653) nphp-4(tm1925); myEx743(P\_nphp-2::NPHP-2::GFP)  
|          | PT: nphp-2(gk653) nphp-4(tm1925); myEx744(P\_nphp-2::NPHP-2L::GFP)  
| Figure 17 | YH224: yhEx142[P\_nphp-4::NPHP-4::YFP; CHE-13::CFP + pRF4]  
|          | PT1962: nphp-2(gk653); yhEx142[P\_nphp-4::NPHP-4::YFP; CHE-13::CFP + pRF4]  

Table 6. List of strains used in this work.

A list of strains used in this work, organized by the figure in which they first appear.
2.8 – References

*Note: These references are formatted to the standards of the Journal of Cell Biology.*


Zhao, C. and Malicki, J. (2011). Nephrocystins and MKS proteins interact with IFT particle and facilitate transport of selected ciliary cargos. *EMBO J.*
Chapter 3: The \textit{nphp-2} and \textit{arl-13} genetic modules interact to regulate ciliogenesis and ciliary microtubule patterning in \textit{C. elegans}

\textit{Note: This chapter is modified from the following publication:}


References in this chapter are formatted in accordance with PLoS Genetics standards.

\section*{3.1 – Abstract}

Cilia are microtubule-based cellular organelles that mediate signal transduction. Cilia are organized into several structurally and functionally distinct compartments: the basal body, the transition zone (TZ), and the cilia shaft. In vertebrates, the cystoprotein Inversin localizes to a portion of the cilia shaft adjacent to the TZ, a region termed the “Inversin compartment” (InvC).

The mechanisms that establish and maintain the InvC are unknown. In the roundworm \textit{C. elegans}, the cilia shafts of amphid channel and phasmid sensory cilia are subdivided into two regions defined by different microtubule ultrastructure: a proximal doublet-based region adjacent to the TZ, and a distal singlet-based region. It has been suggested that \textit{C. elegans} cilia also possess an InvC, similarly to mammalian primary cilia.

Here we explored the biogenesis, structure, and composition of the \textit{C. elegans} ciliary doublet region and InvC. We show that the InvC is conserved and distinct from the doublet region. \textit{nphp-2} (the \textit{C. elegans} Inversin homolog) and the doublet region genes \textit{arl-13}, \textit{klp-11}, and \textit{unc-119} are redundantly required for ciliogenesis. InvC and doublet region genes can be sorted into two modules—\textit{nphp-2+klp-11} and \textit{arl-13+unc-119}—which are both antagonized by the
hdac-6 deacetylase. The genes of this network modulate the sizes of the NPHP-2 InvC and ARL-13 doublet region. Glutamylation, a tubulin post-translational modification, is not required for ciliary targeting of InvC and doublet region components; rather, glutamylation is modulated by nphp-2, arl-13, and unc-119. The ciliary targeting and restricted localization of NPHP-2, ARL-13, and UNC-119 does not require TZ-, doublet region, and InvC-associated genes. NPHP-2 does require its calcium binding EF hand domain for targeting to the InvC. We conclude that the C. elegans InvC is distinct from the doublet region, and that components in these two regions interact to regulate ciliogenesis via cilia placement, ciliary microtubule ultrastructure, and protein localization.

3.2 – Author Summary

Cilia are sensory organelles that are found on most types of human cells and play essential roles in diverse processes ranging from vision and olfaction to embryonic symmetry breaking and kidney development. Individual cilia are divided into multiple functionally and compositionally distinct compartments, including a proximal “Inversin” compartment, which is located near the base of cilia. We used the nematode C. elegans, a well-defined animal model of cilia biology, to characterize the genetics, components, and defining properties of the proximal cilium. The Inversin compartment is conserved in C. elegans, and is established independent of another proximal ciliary region, the microtubule doublet-based region. We showed how components of both the doublet region and the Inversin compartment genetically interact to regulate many pathways linked to core aspects of cilia biology, including ciliogenesis, cilia placement, cilia ultrastructure, microtubule stability, and the protein composition of ciliary compartments. In addition to expanding and clarifying our knowledge of basic cilia biology, these results also have
direct implications for human health research because several of the genes and pathways explored in our work are linked to ciliopathies, a group of diseases caused by dysfunctional cilia.
3.3 – Introduction

Cilia are cellular “antennae” that mediate the transduction of environmental signals into intracellular pathways. Cilia play an integral role in many cellular functions, including developmental signaling, symmetry breaking, cell-cell adhesion, cell-cycle control, stress response, and DNA damage response (e.g., [1-6]). The vast majority of cilia share a set of evolutionarily conserved features: cilia are supported by a microtubule-based backbone, the axoneme; are built by intraflagellar transport (IFT), a microtubule motor driven cargo transport system [7]; and can be divided into structurally and functionally distinct compartments. These compartments include the microtubule triplet basal body which roots the cilium to the cell, the microtubule doublet transition zone (TZ) which anchors the cilium to the membrane, and the microtubule doublet cilia shaft where IFT occurs. The basal body and TZ also act as selective filters for inbound and outbound ciliary cargo, functioning through physical occlusion and cargo-specific recognition mechanisms [7-9]. The cilia shaft has traditionally been treated as an undifferentiated whole [10], though recent evidence has shed light on subdivisions of the cilia shaft [11].

Inversin/Nephrocystin-2 specifically localizes to the Inversin compartment (InvC), a proximal portion of the cilia shaft adjacent to the TZ [12]. This region has been suggested to play a role in signal transduction and amplification [13-15], cilia placement, and ciliogenesis [16, 17]. Products of several genes—including INVS/NPHP2, NPHP3, NEK8/NPHP9, and ANKS6/NPHP16—localize to the InvC. Interactions between InvC genes and other cilia genes have only recently begun to be explored and have not been well-generalized across animal and cell culture models.
The mechanisms that initially establish the InvC are currently unknown, though recruitment pathways for several InvC components are known. Work in vertebrate models has shed light on an InvC-specific physical interaction complex composed of Inversin, Nek8, Nphp3, and Anks6 [18-20]. Nek8, Nphp3, and Anks6 localize to the InvC in an Inversin-dependent manner, but InvC itself localizes independently of the other proteins [18, 19]. Unc119b—a possible, though not proven, InvC component—may mediate an InvC-targeting pathway. In mammalian cells, Unc119b binds myristoylated cargo, including Nphp3, and shuttles it into the cilium. Once the Unc119b-Nphp3 complex has translocated into the cilium, the small GTPase Arl3 triggers Unc119b to release bound cargo [21]. InvC components other than Nphp3 are not known to be myristoylated and shuttled via Unc119b, suggesting additional InvC targeting pathways must exist. Whether Unc119b is required for the localization of InvC and how Unc119b itself is targeted to the proximal cilium is unknown.

The nematode *Caenorhabditis elegans* is a well-studied model of cilia biology [22]. *C. elegans* possesses a ciliated nervous system [23-26] which is primarily used by the roundworm to detect internal and external cues and signals. Amphid channel cilia in the head and phasmid cilia in the tail are exposed to and sense the external environment through cuticular pores [27, 28]. Unlike most mammalian primary cilia, the cilia shafts of *C. elegans* amphid channel and phasmid cilia are divided into two regions: a proximal microtubule doublet-based region attached to the TZ, and a distal microtubule singlet-based region that extends from the doublet region [27, 28]. As both the doublet region of *C. elegans* cilia and the InvC of mammalian primary cilia lie at the proximal end of the cilium, directly adjacent to the TZ at the cilia base, and constitute only a portion of the length of the cilia shaft, previous work has viewed them as compositionally and functionally similar [13, 17, 29]. The relationship between the mammalian InvC and the
C. elegans doublet region of cilia has not been well characterized; Here we present evidence that the InvC and the doublet region are distinct, but overlapping, ciliary regions.

The C. elegans genome encodes orthologs for several of the mammalian InvC-associated proteins, including Inversin itself (NPHP-2), Unc119b (UNC-119), Arl3 (ARL-3), and possibly Nek8 (the uncharacterized paralogous pair NEKL-1 and NEKL-2), but likely not Nphp3 or Anks6. Of these, NPHP-2 and ARL-3 have previously been shown to be doublet region-localizing in C. elegans [17, 30]. C. elegans also possess several doublet region-enriched proteins, which are not InvC restricted in mammalian primary cilia; these include the kinesin-II IFT motor KLP-11 and the membrane-associated small GTPase ARL-13 [30]. The IFT motors Kinesin-II and OSM-3 work cooperatively to carry the IFT assemblies IFT-A and IFT-B and to build the doublet region—OSM-3 alone is sufficient to build the singlet region [31]. ARL-13 likely stabilizes the interaction between IFT-A and IFT-B particles and is required for ultrastructural integrity of the doublet region [30, 32]. arl-13 mutants exhibit multiple ciliary defects, some of which can be suppressed by deletion of histone deacetylase hdac-6, through an unknown mechanism [30]. Mammalian HDAC6 also antagonizes ciliogenesis in mammalian primary cilia: HDAC6 knockouts can suppress ciliogenesis defects arising from INVS/NPHP2 RNAi in MDCK cells [16].

In this work, we aimed to molecularly dissect the proximal cilium of C. elegans and to gain insight into the nature of the InvC and doublet region. We examined interactions between genes associated with the doublet region, determined the territories and localization dependencies of the protein products of these genes, and performed ultrastructural analysis of deletion mutants of these genes. We find that the InvC is conserved in C. elegans, is established early in development, and is distinct from the doublet region. nphp-2 interacts with doublet region genes to regulate cilia placement, microtubule ultrastructural patterning, tubulin glutamylation,
and territory sizes of NPHP-2 and ARL-13. Finally, we show that *nphp-2, arl-13, klp-11*, and *unc-119* fall into two parallel redundant genetic modules, and that interactions between the two modules are modulated by *hdac-6* and *arl-3*. Together, the InvC and the doublet region function in concert to regulate many critical aspects of ciliogenesis and cilia biology.

**3.4 – Results**

**3.4.1 – Genetic interactions between *nphp-2* and *arl-13* are modulated specifically by *hdac-6***

*nphp-2* and *arl-13* single mutants have statistically similar, moderate ciliogenic defects (Figure 19). As *hdac-6* and *arl-3* modulate several *arl-13* phenotypes [30], we sought to determine if *nphp-2* and *arl-13* genetically interact and if *hdac-6* and *arl-3* modulate *nphp-2* phenotypes. We examined double, triple, and quadruple mutant combinations using “dye filling” of ciliated neurons as a gross indicator of ciliogenesis and cilia integrity [27]. Properly formed and placed cilia are environmentally exposed and take up fluorescent Dil dye, whereas stunted or misplaced cilia are not exposed and cannot take up Dil. Unlike the mild dye-filling defects (Dyf) of *nphp-2* and *arl-13* single mutants, *arl-13; nphp-2* double mutants were severely synthetic dye-filling defective (SynDyf) in both the amphids and phasmids (Figure 19). *hdac-6* deletion did not suppress *nphp-2* or *arl-13* Dyf; this is contrary to previously published data indicating that *hdac-6* can partially suppress the weak *arl-13* single mutant Dyf, and may be due to a difference in dye filling or scoring method (cf. [30] and 3.6.7 – Dye-Filling Assays). However, *hdac-6* suppressed the *arl-13; nphp-2* severe SynDyf phenotype to the mild Dyf severity of the single mutants in both amphids and phasmids (Figure 19). *hdac-6* may function by suppressing defects arising from one of the pathways or at a point where the two pathways converge. Combined,
this data indicates that *arl-13* and *nphp-2* act in partially redundant parallel pathways antagonized by *hdac-6*.

*arl-3* has also been implicated as a modulator of the *arl-13* pathway [30]. We found that, unlike the interactions with *hdac-6*, interactions with *arl-3* are cell-type specific (Figure 28). Both *arl-3* single mutants and *arl-3; hdac-6* double mutants were nonDyf in both amphids and phasmids. In amphids, *arl-13; arl-3* was mildly SynDyf, whereas *arl-3; nphp-2* is nonDyf (cf. Figure 19 and Figure 28A). *hdac-6* deletion suppressed the *arl-13; arl-3* phenotype to a severity similar to that of the *arl-13* single mutants. In phasmids, both *arl-13; arl-3* and *arl-3; nphp-2* double mutants exhibited a moderate SynDyf phenotype. *hdac-6* suppressed *arl-13; arl-3* defects, but not *arl-3; nphp-2* defects. Strikingly, in both amphids and phasmids, *arl-3* deletion prevented the *hdac-6* Dyf suppression in the *arl-13; hdac-6; nphp-2* triple mutant described above. The SynDyf phenotype of *arl-13; arl-3* is qualitatively different from the suppression of *arl-13* Dyf defects by *arl-3* found previously [30]. Similarly to the difference in *hdac-6* suppression of *arl-13* defects discussed above, this may be due to different scoring methods or assay conditions. We conclude that *arl-3* functions parallel to both *nphp-2* and *arl-13* pathways, and likely lies in the same regulatory pathway as, but acts antagonistically to, *hdac-6*.

### 3.4.2 – *nphp-2* and *arl-13* genetically interact to regulate amphid cilia ultrastructure

To gain a better understanding of the defects present in *nphp-2* single and *arl-13; nphp-2* double mutants, and to examine the effects of *hdac-6* mediated suppression of the double mutant defects, we used serial-section transmission electron microscopy (TEM) to examine the ultrastructure of amphid channel cilia. Wild-type amphid cilia are divided into three segments based on microtubule ultrastructure: the TZ, the doublet region, and the singlet region (Figure
20A). One of the two microtubules within a doublet, the A-tubule, extends to form the microtubule singlet seen in the distal cilium (Figure 20A1); the second tubule of the doublet, the B-tubule, terminates at the distal end of the doublet region. The doublet microtubules of the TZ and doublet region are arranged in a circular pattern in close proximity to the ciliary membrane (Figure 20A2, A3). Within a particular cilium, microtubule ultrastructural characteristics—the location in the lumen, membrane association, and singlet/doublet architecture—are similar across all nine outer microtubule doublets.

In nphp-2 single mutants, in a given amphid cross-section at a single level, we observe singlet regions of some cilia, doublet regions of other cilia, and TZs of the remaining cilia. This is consistent with amphid cilia that were shifted lengthwise with respect to each other, indicating a potential anchoring defect (Figure 20B3). Additionally, within a given cilium, doublet and singlet microtubule spans were no longer aligned. In sections across the proximal axoneme, this appeared as singlets amongst the expected doublets, and in sections across the distal axoneme, this appeared as doublets interspersed between the expected singlets (Figure 20B1-3). These defects indicate that nphp-2 is required both for microtubule patterning and for cilia anchoring.

While the chemical fixation method utilized here does not preserve Y-link ultrastructure well, nphp-2 mutants exhibited significantly greater Y-link disorder than observed in wild-type animals. The TZ defects seen in nphp-2 animals may be related to both the lengthwise cilia shift and the TZ-placement defect previously reported in nphp-2 mutants [17]. This set of defects has not been reported in any other C. elegans cilia mutant, suggesting that nphp-2 functions in a novel capacity.

arl-13 single mutants also exhibit a range of ultrastructural defects [30, 32]. Doublets are observed in the central lumen of the cilium, which may originate from either displaced outer doublets or mispatterned inner singlets. In both C. elegans arl-13 and hennin/ARL13B mouse
mutants, there is also an increased frequency of early B-tubule detachment from the A-tubule, indicative of microtubule stability or patterning defects [33]. Similar to nphp-2 mutants, ectopic microtubule singlets were sometimes visible in the doublet region of arl-13 worms [30, 32]. arl-13; nphp-2 double mutants exhibited extreme ultrastructural defects, likely causative of the severe Dyf phenotype (Figure 20C1-3). Cilia were almost completely absent from the amphid channel pore. No other cilia were visible even at the distal dendritic level. In one instance, a single cilium was visible in TEM sections. At the TZ level, an incomplete set of doublets was visible within the single cilium (Figure 20C3). Closer to the socket cell/sheath cell transition, a set of singlets was visible (Figure 20C2). At the distal pore, we were unable to resolve any internal structure due to electron dense material filling the cilium (Figure 20C1).

Remarkably, in arl-13; hdac-6; nphp-2 triple mutants almost all defects observed in the double mutant were suppressed (Figure 20D1-3). The only defects remaining were misplaced TZs (Figure 20D3), ectopic singlets in the doublet region (Figure 20D2), and ectopic doublets in the singlet region (Figure 20D1), similar to those present in the nphp-2 single mutant. We did not observe inner doublets as reported in arl-13 single mutants [30, 32]. These results are consistent with the observed hdac-6 suppression of arl-13; nphp-2 Dyf defects.

Because nphp-2 and arl-13 both exhibit ectopic microtubule singlets, nphp-2 mutants exhibit ectopic microtubule doublets, and nphp-2; arl-13 double mutants exhibit severe defects in ciliogenesis, we conclude that nphp-2 and arl-13 function together redundantly in regulation of microtubule patterning and ciliogenesis. Because ciliogenic defects are suppressed by hdac-6, but ectopic doublets and singlets are not, ciliogenesis and microtubule patterning may be independently regulated.
3.4.3 – NPHP-2 and ARL-13 do not require TZ- and doublet region-associated genes for ciliary targeting

Localization of InvC and doublet region components can be broken down into three steps: first the protein is targeted to the cilia base, second, the protein is imported into the cilium, and third, the protein is restricted to a subdomain of the cilium [18]. The factors required for the initial establishment of the InvC and doublet region cilia targeting and localization restriction are unknown. The TZ functions as a regulator of ciliary protein import [Reviewed in [34]], and has been implicated in the import of InvC and doublet region components in mammalian cilia [35, 36]. As both *nphp*-2 and *arl*-13 genetically interact with TZ-associated genes [17, 32], we wanted to determine if NPHP-2 and ARL-13 ciliary targeting and import requires TZ components. In *C. elegans*, TZ genes are organized into two genetic and physical modules—the *mks* module and the *nphp-1+nphp-4* module [8, 17, 20, 37]. We examined the localization of NPHP-2 and ARL-13 in mutants missing a component of each module (Figure 21A,C). In both *nphp-4* and *mks-3* single mutants, NPHP-2::GFP was properly targeted to and imported into the cilium. Mislocalized NPHP-2::GFP puncta in the periciliary region were sometimes visible (Figure 21A). In *mks-3; nphp-4* double mutants, there were severe ciliogenic and dendritic extension errors, as previously reported [17, 37]; in phasmid cilia that were visible and placed properly, NPHP-2::GFP localization appeared as in *mks-3* and *nphp-4* single mutants (Figure 29A). In a wild-type background, ARL-13::GFP localized exclusively to the doublet region in amphid channel and phasmid neurons (Figure 21C). Like NPHP-2::GFP, ARL-13::GFP was targeted to and imported into the cilium properly in *mks-3* and *nphp-4* mutants. Similar to published reports, we also observed ARL-13::GFP mislocalization to the periciliary membrane, as judged by a fluorescent “fringe” surrounding the periciliary region where the membrane lies (Figure 21C, enhanced contrast in Figure 29B), which has been suggested to be due to a failure of the TZ diffusion
barrier [29]. In amphid cilia, like in phasmid cilia, both NPHP-2 and ARL-13 were targeted to the
cilium and imported properly, and both infrequently exhibited mild mislocalization (Figure 30).
We investigated whether doublet region-associated genes were required for doublet region
restriction of NPHP-2::GFP and ARL-13::GFP. hdac-6 had no obvious effect on the localization of
NPHP-2::GFP (Figure 29A). NPHP-2::GFP was targeted and restricted to the proximal cilium in
both klp-11 and arl-13 mutants. In both mutants, periciliary puncta similar to those seen in
TZ-associated mutants were visible (Figure 21B). unc-119 mutants also exhibited proper
NPHP-2::GFP ciliary targeting. In unc-119 mutants, NPHP-2::GFP exhibited a unique distal
dendrite mislocalization pattern, distinct from the periciliary puncta seen in other TZ and
doublet region mutants (Figure 21B).
In klp-11 and nphp-2 mutants, ARL-13::GFP was restricted to a proximal portion of the cilium as
in wild type, but with a periciliary membrane mislocalization pattern similar to TZ mutants.
ARL-13::GFP also did not require unc-119 for ciliary localization. In unc-119 mutants,
ARL-13::GFP and NPHP-2::GFP localized along the distal dendrite in a similar manner (Figure
21D).
In all TZ, doublet region, and InvC mutants examined, NPHP-2::GFP and ARL-13::GFP still
localized to the cilium, suggesting that either unknown factors or redundant pathways are
required for establishing ciliary territories. Periciliary mislocalization was observed for both
reporters across all mutant backgrounds. This suggests either that a delicate, easily perturbed
interaction network is required for NPHP-2 and ARL-13 ciliary import/export, or that the
overexpressed reporter constructs are “leaking” out of the cilium in sensitized mutant
backgrounds, or a combination of both possibilities.
3.4.4 – NP\textsubscript{H}P\textsubscript{2} requires its EF-hand for proper localization and function

We next looked to determine which domains of NP\textsubscript{H}P\textsubscript{2} were required for InvC localization. In mammalian models, several domains in Inversin are required for ciliary targeting and InvC restriction: the ankyrin repeat region, IQ2 domain, and ninein-homologous region (Figure 22A) [12, 38]. The IQ and ninein homology domains are not conserved in \textit{C. elegans} NP\textsubscript{H}P\textsubscript{2}; only the ankyrin repeat region, hydroxylation motif (Figure 35A), and two nuclear localization signals (NLSs) are conserved (Figure 22A) [17]. NLS motifs are hypothesized to play a role in ciliary protein import, and are required for ciliary import of the IFT motor KIF17 [9]. NP\textsubscript{H}P\textsubscript{2} also contains a predicted calcium binding EF-hand which may function in the same calcium detection capacity as the IQ domain of Inversin [17].

We built NP\textsubscript{H}P\textsubscript{2}::GFP constructs missing the EF-hand (NP\textsubscript{H}P\textsubscript{2}-EF\textsubscript{Δ}::GFP, residues 520-533), NLS1 (NP\textsubscript{H}P\textsubscript{2}-NLS1\textsubscript{Δ}::GFP, residues 441-446), or NLS2 (NP\textsubscript{H}P\textsubscript{2}-NLS2\textsubscript{Δ}::GFP, residues 598-603) (Figure 22A). Full-length NP\textsubscript{H}P\textsubscript{2}::GFP localized to a short proximal region of amphid channel and phasmid cilia as well as IL, CEP, OLQ, amphid channel and phasmid cilia, and the AWC wing cilia (Figure 22B). Native promoter driven NP\textsubscript{H}P\textsubscript{2}::GFP was not visible in AWB cilia, as reported previously for AWB-specific promoter driven NP\textsubscript{H}P\textsubscript{2} [13]. NP\textsubscript{H}P\textsubscript{2}-EF\textsubscript{Δ}::GFP was generally faint or absent in amphid channel, AWC, and phasmid cilia, indicating that the EF-hand is strictly required for normal localization of NP\textsubscript{H}P\textsubscript{2} in these cell types. NP\textsubscript{H}P\textsubscript{2}-EF\textsubscript{Δ}::GFP properly localized in IL cilia, indicating that the fluorescent reporter was being synthesized and folded correctly. NP\textsubscript{H}P\textsubscript{2}-EF\textsubscript{Δ}::GFP was additionally present in either CEP or OLQ cilia, though specific identification was difficult due to their close proximity. Deletion of either NLS1 or NLS2 did not perturb NP\textsubscript{H}P\textsubscript{2} localization (Figure 22B).

We next tested whether these domain deletion constructs were functional by attempting to rescue the severe Dyf phenotype of \textit{nphp-2 nphp-4} mutants; \textit{nphp-4} single mutants are nonDyf,
allowing for easy determination of rescue [17]. Rescue of the *nphp-2 nphp-4* SynDyf phenotype by NPHP-2-NLS1Δ::GFP and NPHP-2-NLS2Δ::GFP was comparable to rescue by full length NPHP-2::GFP. NPHP-2-EFΔ::GFP only weakly rescued the SynDyf phenotype, indicating that the EF-hand is critical for both normal function and localization of NPHP-2 (Figure 22C). These results indicate that the calcium-binding EF-hand plays a significant role in both localization and function of NPHP-2 in amphid channel and phasmid cilia, but is dispensable for localization in cilia of CEP, OLQ, and inner labial neurons.

### 3.4.5 UNC-119 is associated with the doublet region

Mammalian Unc119b localizes to the proximal cilium and is required for the targeting of several proteins to the InvC [21], and *C. elegans unc-119* is required for singlet region biogenesis in amphid cilia [39], suggesting a compartment-specific role. We therefore examined the localization of *posm-6::GFP::UNC-119*. In phasmid cilia, GFP::UNC-119 localization was similar to NPHP-2::GFP and ARL-13::GFP: its localization was restricted only to a small proximal portion of the cilium and was excluded from the TZ. Similar to localization in phasmid cilia, GFP::UNC-119 likely localized to the doublet region and not the entire cilium of amphid cilia (Figure 23A). GFP::UNC-119 was not motile in cilia.

We also examined the dependence of UNC-119 localization on doublet region-associated genes. Specific amphid mislocalization was difficult to determine, and we therefore focused on phasmid cilia (Figure 31). In phasmid cilia, GFP::UNC-119 did not require *nphp-2, arl-13, hdac-6*, or *klp-11* for ciliary targeting or restriction to the proximal cilium (Figure 23B).
3.4.6 – IFT motors and unc-119 genetically interact with doublet region-associated genes

We examined the genetic interactions between the doublet region-associated genes nphp-2, arl-13, and their modulators hdac-6 and arl-3 with the two IFT motors, osm-3 and klp-11. In both amphids and phasmids, arl-13; klp-11 was SynDyf [32]. arl-13; klp-11 dye-filling defects were slightly suppressed by deletion of hdac-6 (Figure 24A, Figure 32A). klp-11; arl-3 double mutants yielded a very mild SynDyf phenotype (Figure 28B). osm-3 single mutants are missing a singlet region and are completely Dyf, which precludes searching for synthetic interactors of osm-3. Instead, we assayed osm-3 double mutants for suppression of the severe dye-filling defect; in no mutant background examined was the defect suppressed (Figure 24B, Figure 32B).

We next examined genetic interactions between unc-119 and other doublet region-associated genes. unc-119 single mutants were severely Dyf, with the phasmid phenotype being less severe than the amphids (Figure 24B, Figure 32A) [39]. In phasmids, both nphp-2 and klp-11 were SynDyf with unc-119. Surprisingly, we found that hdac-6 suppressed Dyf defects in the unc-119; nphp-2 double mutant to the level of the nphp-2 single mutant (Figure 24B). arl-3 deletion was also able to significantly suppress the unc-119 Dyf phenotype in both amphids and phasmids (Figure 28B).

Genetic interactions between nphp-2, arl-13, klp-11, unc-119, hdac-6, and arl-3 are summarized in Figure 24C. Doublet region-associated genes fall into two redundant pathways or modules: nphp-2+klp-11 and arl-13+unc-119. Deletion of two genes within a module does not result in an increase in Dyf severity over that present in single mutants, but deletion of any two genes from different modules yields a SynDyf phenotype. Interactions between these modules are regulated by hdac-6 and arl-3.

Double mutants with a deletion in a single TZ gene and a single doublet region gene are SynDyf.
To determine whether *hdac-6* mediated suppression of SynDyf defects extended to these cross-compartmental genetic interactions, we assayed for suppression of SynDyf defects in *nphp-2 nphp-4* mutants by *hdac-6* and *arl-3*. We found that neither *hdac-6* nor *arl-3* suppressed the severe *nphp-2 nphp-4* SynDyf defect (Figure 32D). This suggests that *hdac-6* functions specifically in doublet region pathways.

**3.4.7 – Axonemal glutamylation is downstream of the action of *nphp-2*, *arl-13*, *unc-119*, and *hdac-6***

Post-translational glutamylation predominantly occurs on the C-terminal tails of α- and β-tubulin of axonemal B-tubules [40-44], and regulates microtubule stability and IFT motor function [45]. Glutamylation is specifically associated with the doublet region, as B-tubules define and are only present in the doublet region. Additionally, in *Chlamydomonas* and *Paramecium*, TZ microtubules are not glutamylated [40, 46]. In *C. elegans* and vertebrates, mutants with defects in tubulin glutamylation or *arl-13* exhibit B-tubule degeneration [33, 45, 47]. We therefore determined whether doublet region associated genes regulated tubulin glutamylation, or whether tubulin glutamylation specified the localization of doublet region proteins.

In wild-type animals, the anti-glutamylated tubulin antibody GT335 labeled the doublet region of amphid channel and phasmid cilia (Figure 25A) [47]. *nphp-2* mutants exhibited characteristic cilia displacement in the amphids, but no qualitative changes in head cilia glutamylation. The glutamylation signal in *nphp-2* phasmid cilia ranged from wild-type-like to extremely elongated, which is consistent with the TEM observation of B-tubules extending into the distal axoneme (Figure 20B). *arl-13* mutants exhibited elongated staining in amphid channel cilia. Amphid staining in *unc-119* mutants was extremely shortened and cilia were angled inwards. *hdac-6* mutants appeared to have shortened GT335 staining of amphid channel cilia. We also observed
significant differences in the length of the phasmid GT335 ciliary signal. Both \textit{arl-13} (3.95±0.25µm) and \textit{nphp-2} (4.30±0.32µm) mutants had phasmid staining significantly longer than in wild type (2.79±0.07µm), while \textit{unc-119} (2.40±0.05µm) mutants had staining significantly shorter (Figure 34E). The length of GT335 staining in amphid cilia was not quantified due to the difficulty of unbiased measurement of a single cilium within the amphid bundle.

To determine if doublet region-associated protein localization was dependent on tubulin glutamylation status, we examined the localization of NPHP-2::GFP and GFP::UNC-119 in \textit{ccpp-1} and \textit{ttll-4} mutants. Mutants of \textit{ccpp-1}, which encodes a tubulin de glutamylase, display degenerating amphid channel and phasmid cilia with a concomitant dye-filling defect; this is suppressed by deletion of the opposing glutamylase, encoded by the tubulin tyrosine ligase-like gene \textit{ttll-4} [45]. In each mutant, NPHP-2::GFP and GFP::UNC-119 reporters were targeted to cilia and were restricted to the proximal cilium similarly to wild-type (Figure 25B,C). This was surprising in the case of \textit{ccpp-1} mutants, as cilia degenerate as the worm ages (Figure 25B,C). These reporters were still doublet region-associated in earlier larval stages of \textit{ccpp-1} mutants when ciliary degeneration was not as severe (Figure 36C,D). Combined, these results indicate that \textit{nphp-2, arl-13, unc-119}, and \textit{hdac-6} lie upstream in regulation of tubulin glutamylation pathways, and the localization patterns of their protein products are not defined by tubulin glutamylation.

### 3.4.8 – Doublet region protein territories are genetically regulated

To allow for a more direct comparison of subciliary localization, we stained transgenic NPHP-2::GFP, ARL-13::GFP, and GFP::UNC-119 strains with GT335 (Figure 26). NPHP-2::GFP did not fully overlap with GT335, only colabelling the proximal portion of the doublet region of amphid and phasmid cilia. However, ARL-13::GFP colabelled with a greater portion of the GT335
doublet region signal in amphid cilia than did NPHP-2::GFP, and completely colabelled with GT335 in phasmid cilia (Figure 26, Figure 33A); this suggests that ARL-13 is associated with the microtubule doublets that define the doublet region. GFP::UNC-119 colabelled with either a significant fraction of the length of the GT335 signal. Additionally, GFP::UNC-119 did not extend beyond the GT335 labelled doublet region, indicating that GFP::UNC-119 is excluded from the TZ, which is not labelled by GT335.

We also examined the territory length of each of the fluorescent reporters as a fraction of the total cilia length. Transgenic animals were incubated with Dil to label the length of the cilium. NPHP-2::GFP marked a significantly shorter fraction of the length of the cilium than did ARL-13::GFP or GFP::UNC-119 (Figure 33C).

To understand how the localization of doublet region-associated proteins is genetically regulated, we measured the length of the cilium marked by NPHP-2::GFP, ARL-13::GFP, GFP::UNC-119, and KAP-1::GFP—a component of Kinesin-II—in different mutant backgrounds (Figure 34). ARL-13::GFP and NPHP-2::GFP have interdependent localizations: in arl-13 mutants, the NPHP-2::GFP territory was extended along the cilium, and in nphp-2 mutants, the ARL-13::GFP territory was extended. unc-119 mutants exhibited shortened ARL-13::GFP and NPHP-2::GFP territories (Figure 34A,B). Additionally, klp-11 mutants had a shorter NPHP-2::GFP, but not ARL-13::GFP, localization signal (Figure 34A). No significant differences were found in the territory lengths of GFP::UNC-119 and KAP-1::GFP in any of the mutant backgrounds (Figure 34C,D). The length of the tubulin glutamylation signal is also genetically controlled; the GT335 signal is shortened in unc-119 mutants, and elongated in nphp-2 and arl-13 mutants (Figure 34E).

In sum, the NPHP-2::GFP territory size is shorter than the territories of doublet region components ARL-13 and Kinesin-II, marks a shorter length of the cilium than ARL-13 and
UNC-119, is shorter than the doublet region-linked glutamylated tubulin signal, and colabels only a portion of both amphid channel and phasmid GT335 staining. We conclude that NPHP-2 marks a region of the cilium distinct from the doublet region, and propose that this region is analogous to the InvC of mammalian cilia.

3.5 – Discussion

In this study, we present evidence that (1) *C. elegans* possesses a conserved InvC compartment, (2) interactions between *nphp-2* and *arl-13* regulate microtubule ultrastructural patterning, (3) InvC and doublet region sizes are distinct and genetically regulated, (4) *hdac-6* and *arl-3* modulate interactions between *nphp-2*, *arl-13*, *klp-11*, and *unc-119*, (5) and that microtubule glutamylation is downstream of the action of InvC and doublet region genes. Additionally, we found that genes associated with the proximal cilium (TZ, InvC, and doublet region) can be grouped into parallel genetic modules, which interact to drive ciliary anchoring and proper ciliogenesis. Finally, we addressed several possible mechanisms for the ciliary targeting and InvC restriction of NPHP-2.

3.5.2 – The nature of the doublet region

There has been confusion regarding the relationship between the mammalian InvC and the *C. elegans* doublet region. Because amphid channel and phasmid cilia have elongated microtubule singlets, doublet region proteins appear to be proximally restricted. This was also taken in reverse: proteins localized to the proximal cilium were considered de facto doublet region components. As the mammalian InvC is similarly characterized by proximally restricted proteins, and both the mammalian InvC and *C. elegans* doublet region had an underlying ultrastructure of microtubule doublets, the *C. elegans* doublet region and the mammalian
proximal InvC were considered analogous [19, 20, 35, 235, 236]. In *C. elegans*, a number of factors have been associated with the doublet region, including NPHP-2, ARL-13, UNC-119, ARL-3, HDAC-6, the Kinesin-II components KAP-1/KLP-11/KLP-20 [9, 235, 237], and glutamylated tubulin [18].

However, mammalian orthologs of many *C. elegans* doublet region proteins localize along the entire cilium [22-32]; this casts doubt on the equivalence between the *C. elegans* doublet region and the mammalian InvC. In mammalian primary cilia, the majority of the axoneme is composed of microtubule doublets; it is likely that the *C. elegans* ciliary doublet region is analogous to the entire mammalian ciliary doublet-based cilia shaft. To better clarify the associations between protein localizations and ciliary anatomy, in this work we refer to regions of the cilium by referencing the underlying ultrastructure. This clarifies localization confusion between systems: “ARL-13/Arl13b localizes to the doublet region of cilia” is true in both *C. elegans* amphid channel cilia and mammalian primary cilia.

However, several factors associated with the *C. elegans* doublet region do have proximally restricted localization in mammalian primary cilia, including NPHP-2 and UNC-119. Additionally, each doublet region-associated protein likely has different localization requirements: Kinesin-II is known to be doublet associated and is not influenced by glutamylation [9, 18], NPHP-2 localizes to a restricted subregion of the doublet region, and ARL-13 is membrane associated and its localization has been proposed to be regulated by membrane composition [33]. Because of this, we conclude that in *C. elegans*, what has been known as the proximal cilium (or “middle segment”) is not a distinct region of the cilium patterned by underlying microtubule doublets, but is rather a set of overlapping but distinct protein territories (modelled in Figure 27).
Calcium signaling plays a crucial role in signal transduction and ciliary function [58, 59]; cilia have high intraciliary calcium concentrations, and many TZ proteins possess calcium binding domains [60]. The NPHP-2 vertebrate homolog, Inversin, has two identified calmodulin-binding IQ domains [61, 62], one of which is required for proper localization [12]. Calmodulin detects intracellular calcium concentrations through a calcium-binding EF-hand. Though *C. elegans* NPHP-2 does not encode a predicted IQ domain, it does possess an EF-hand. This EF-hand is required for the localization and function of NPHP-2 in amphid and phasmid cilia, similarly to the IQ2 domain of Inversin. A significant difference exists between the EF-hand of NPHP-2 and the IQ2 domain of Inversin: deletion of the EF-hand of NPHP-2 results in a complete lack of ciliary localization, whereas deletion of the IQ2 domain of Inversin results in a mislocalization of Inversin along the entire cilium. In both systems, Inversin/NPHP-2 no longer localizes to the InvC. This suggests that Ca$^{2+}$ detection/binding by and the subsequent hypothetical modulation of the activity of Inversin/NPHP-2 is a critical, conserved feature of the protein. Two possibilities arise for the function of these domains: Ca$^{2+}$ specifies the localization of NPHP-2, modulates the activity of the protein, or both. In the first case, intraciliary calcium might bind to NPHP-2, and change binding affinities and physical properties of the protein. This may prevent NPHP-2 from exiting the cilium, and possibly restricting it to the InvC. In the second case, intraciliary calcium concentrations can be modulated by the opening and closing of ciliary calcium channels in response to stimuli; NPHP-2 would then act as a component in signal transduction pathways linking calcium flux to downstream effects (e.g., modulation of glutamylation, localization of other proteins, Wnt signaling pathways, ciliary maintenance pathways). If calcium does specify InvC restriction, then in *C. elegans*, non-InvC targeted NPHP-2 may be cleared actively or passively out of the cilium through a nonconserved means. It is also possible that the
calcium-dependency of Inversin and NPHP-2 has diverged.

### 3.5.4 – UNC-119 is a proximal ciliary protein

We found that UNC-119 localizes to the proximal cilium and is excluded from the distal region. GFP::UNC-119 and GT335 colabel, indicating that GFP::UNC-119 is excluded from the TZ, as TZ microtubules are not glutamylated (See Figure 26, in which GT335 colabels with TZ-excluded ARL-13::GFP and NPHP-2::GFP). In *C. elegans*, UNC-119 labels a shorter portion of the cilium than ARL-13 or GT335, markers associated with the doublet region. Additionally, mammalian Unc119b physically interacts with the InvC component Nphp3 and is proximally restricted in cilia of RPE cells, suggesting that Unc119b is associated with the InvC [21]. However, GFP::UNC-119 marked a larger portion of the cilium than did NPHP-2::GFP.

The *C. elegans* genome encodes homologs of many of many Unc119b shuttle proteins, including Unc119b, Arl3, and RP2, and two myristoylated ciliary proteins which require unc-119 for ciliary localization [63]. *arl-3* genetically interacts with unc-119 and nphp-2, suggesting that in *C. elegans* the components of the shuttle are in place. These shuttle components do not appear to be required for the localization of NPHP-2, as NPHP-2 is imported into the cilium in unc-119 and arl-3 mutants. In unc-119 mutants, NPHP-2 exhibited a unique distal dendritic localization pattern that cannot be attributable to TZ leakage; it is unknown whether this population represents NPHP-2 that has not been properly imported into the cilium or is mistargeted NPHP-2.

### 3.5.5 – The function of *hdac-6*

In both mammalian systems and *C. elegans*, Hdac6/*hdac-6* functions as an antagonist of ciliogenesis and cilia stability [16, 48]. In mammalian primary cilia, Hdac6 functions as an
α-tubulin K40 deacetylase regulates microtubule function and stability [48, 64-67]. The *C. elegans* genome encodes a single α-tubulin with the acetylatable residue K40, MEC-12. However, there is no direct evidence for mec-12 expression in amphid and phasmid neurons, and the anti-α-tubulin-K40 antibody 6-11b-1 does not label amphid and phasmid cilia in WT animals or *hdac-6* mutants (Figure 37) [68]. Alternative tubulin acetylation sites may exist, including on β-tubulin [69]; *hdac-6* could deacetylate these secondary sites. NPHP-2 contains a predicted N-terminal acetylation site (at 2S) which may be deacetylated by HDAC-6; this may modulate binding between NPHP-2 and its targets [70]. Additionally, HDAC-6 may have other unidentified ciliary targets [30]. Determining the mechanism by which HDAC-6 acts as a genetic modifier of InvC and doublet region gene defects is an important future direction.

### 3.5.6 – Doublet region and InvC components modulate tubulin post-translational modification

Tubulin glutamylation is associated with the proximal portions of microtubule B-tubules in *C. elegans* cilia, mouse spermatozoa flagella, and *Chlamydomonas* flagella [42, 45, 71]. In *C. elegans*, microtubule glutamylation is linked to microtubule ultrastructure, stability, and maintenance [45, 72]. Multiple doublet region genes regulate microtubule glutamylation, and we observed a correlation between ectopic glutamylation and ectopic microtubule doublets. Additionally, ciliary targeting of doublet region proteins is not dependent on glutamylation status. Therefore, *nphp-2, arl-13, hdac-6*, and *unc-119* function upstream of microtubule glutamylation, which may enable them to exert an influence on microtubule patterning, IFT, ciliogenesis, and, in the case of *unc-119*, singlet region biogenesis. These pathways may be conserved: in Arl13b/*hennin* mutant mice, ciliary microtubule glutamylation intensity is reduced, and microtubule B-tubules have ultrastructural defects [33, 50].
Doublet region- and InvC-associated genes form genetic modules

In *C. elegans*, TZ-associated genes can be grouped into two distinct, partially redundant genetic and physical modules [8, 17, 37]. Doublet region- and InvC-associated genes may be grouped in a similar manner into a *nphp-2+klp-11* module and an *arl-13+unc-119* module. *hdac-6* appears to function outside the two modules, negatively regulating both: deletion of *hdac-6* in SynDyf double mutants suppresses the SynDyf phenotype. In mammalian primary cilia, Hdac6 also plays an antagonistic role, destabilizing cilia through deacetylation of tubulin, a pathway suppressed by Inversin [16]. *arl-3* may function outside of the two modules in a cell-type specific manner; in phasmids it genetically interacts with components from both modules, but in amphids *arl-3* only genetically interacts with only the *arl-13+unc-119* module and not the *nphp-2+klp-11* module. In amphid cilia, *nphp-2* also does not interact with the TZ SynDyf network.

Curiously, a further two genetic module organization exists between TZ genes and the InvC/doublet region genes. *nphp-2 nphp-4, mks-3; nphp-2, and arl-13; nphp-4* double mutants exhibit severe ciliogenic defects [17]. Deletion of one TZ gene from either TZ module and one doublet region gene from either doublet region module yields a SynDyf phenotype, though not all combinations have been tested. The TZ SynDyf network and the doublet region SynDyf network can be thought of as two genetically interacting “super-modules”, each consisting of two to three sub-modules described in this and previous work [8, 17, 20, 37].

Origin of the Inversin Compartment

The localization requirements for multiple InvC localizing components have been previously determined, but how the InvC is initially established is not known (See 5.2.1 – InvC Biogenesis).
In *C. elegans*, the InvC is likely established early in cilia development, as NPHP-2 is proximally restricted in phasmid cilia as early as the first larval L1 stage immediately following hatching (Figure 36A), and is not motile (Figure 36B), unlike the larval stage-dependent dynamic localization of ARL-13 [29]. We have eliminated several mechanisms for the establishment of the InvC. Ciliary ultrastructure does not seem to play a role, as in both mammals and *C. elegans*, the localization of Inversin/NPHP-2 is associated with only a sub-portion of the doublet region where there are no identifiable ultrastructural features [12]. Tubulin post-translational modifications also do not appear to specify the InvC, as *nphp-2* (and genetically interacting doublet region components) lies upstream of glutamylation pathways. The TZ does not appear to play a major role in specifying the InvC. NPHP-2 still localizes and is restricted to the proximal cilium in TZ single and double mutants. IFT is another candidate mechanism, but we found that although IFT components genetically interact with InvC and doublet region associated genes, Kinesin-II is not required for NPHP-2 localization. In mammalian cilia, the Unc119b shuttle is required for the ciliary import of Nphp3; this activity is upstream of the action of Inversin in Nphp3 localization. In *C. elegans* phasmid cilia, NPHP-2 does not require either *unc-119* or its effector *arl-3* for ciliary import or InvC restriction (Figure 29A).

Several mechanisms for establishing the InvC remain. First, calcium may play a role. Both Inversin and NPHP-2 require a calcium binding domain for InvC localization [12]; the origin and nature of the calcium signal these domains detect is unknown. Second, the InvC may also be initially established by a diffusion of factors from the cilia base. A third possibility is that cilia membrane composition may help define the InvC. The cilium has a distinct membrane composition from the plasma membrane, and the different ciliary subregions may also have differential membrane composition.
3.5.9 – Final Summary

We propose that the logic underlying the establishment of the NPHP-2/Inversin compartment may be similar in *C. elegans* and mammals, in a manner independent of microtubule ultrastructure. We have shown that doublet region- and InvC-associated genes interact to guide ciliogenesis, cilia placement, cilia ultrastructure, protein composition, and tubulin post-translational modification. The next challenge is to determine what initially patterns the doublet region and InvC, and to understand the function of these cilia regions.
3.6 – Methods and Materials

3.6.1 – General Molecular Biology

Standard protocols were followed for all molecular biological procedures. PCR amplification using Taq polymerase (New England BioLabs, Ipswich, MA, USA) was used for genotyping deletion alleles, and was followed by restriction digest for SNP diagnosis. PCR amplification for construction of transgenic constructs was performed with Phusion High fidelity DNA polymerase (New England BioLabs), templated off *C. elegans* genomic DNA. Sequencing was performed offsite (GeneWiz, South Plainfield, NJ, USA). PCR primer and construct sequences are available upon request.

3.6.2 – Bioinformatics and Computer Tools

Protein BLAST was used to find sequence orthologs [78]. All protein sequence information other than that of *C. elegans* was provided by NCBI, and all *C. elegans* nucleotide and protein sequences were provided by WormBase (Releases WS229 and WS234). Structural motif and domain predictions were generated by MotifScan [79]. Acetylation motifs were identified using NetAcet 1.0 [70]. Coiled-coil regions were identified with COILS [80]. ApE 2.0.36 was used for sequence manipulation, annotation, and restriction site identification.

3.6.3 – Strains and Maintenance

All strains were cultured at room temperature, unless otherwise noted, under standard conditions [81]. Transgenic strains using *pha-1* selection were grown at 25°C, and *pha-1(e2123)* mutants were grown at 15°C. Deletion alleles were outcrossed to *him-5* at least four times.

Strains used in this study are listed in Table 8A. Alleles used were as follows: *nphp-2(gk653),*
Primers used for diagnosis are listed in Table 8B. All transgenic strains used in Figure 19 to Figure 26 were tested using dye-filling for dominant negative defects in ciliogenesis. We observed no adverse effects in NPHP-2::GFP and GFP::UNC-119 transgenic strains but did find dominant negative defects in ARL-13::GFP transgenic strains (Table 7).

The full length isoform of NPHP-2 was used in all NPHP-2 reporter constructs. The full length isoform, NPHP-2L, differs from the shorter isoform, NPHP-2S, in that the shorter isoform is missing 22 non-conserved amino acids of unknown function. Both isoforms have similar subciliary localization.

### 3.6.4 – Electron Microscopy

*nphp-2* and *arl-13; hdac-6; nphp-2* young adult animals were fixed using 3.5% glutaraldehyde + 1% PFA in 0.1M HEPES and then in 1% OsO4 + 1.25% K4Fe(CN)4 in 0.1M HEPES. Samples were infiltrated and embedded in Embed-812 plastic resin. *arl-13; nphp-2* young adult animals were fixed using high-pressure freeze fixation and freeze substitution in 2% OsO4 + 2% water in acetone as the primary fixative [82]. Samples were slowly freeze substituted in an RMC freeze substitution device, before infiltration with Embed-812 plastic resin. Images for wild-type animals fixed by a comparable immersion fixation method (cf. [27]) are now curated by the Hall lab at Einstein courtesy of E. Hedgecock. These wild type images are also available online at www.wormimage.org.

For TEM, serial sections (70 nm thickness) of fixed animals were collected on copper slot grids coated with formvar and evaporated carbon and stained with 4% uranyl acetate in 70% methanol, followed by washing and incubating with aqueous lead citrate. Images were captured
on a Philips CM10 transmission electron microscope at 80kV with a Morada 11 megapixel TEM CCD camera driven by iTEM software (Olympus Soft Imaging Solutions).

For each strain, we imaged three individuals that were fixed chemically. Additionally, we were concerned that the severe defects seen in the *arl-13; nphp-2* double mutant were partially due to the harsh chemical fixation method. We fixed a fourth double mutant using high pressure freeze (HPF) fixation, which introduced fewer artifacts to confirm the validity of the chemical fixation data. We used the same strain in the construction of the double *arl-13; nphp-2* and triple *arl-13; hdac-6; nphp-2* mutants as was used in the previously published EM of the *arl-13* single mutant.

### 3.6.5 – Imaging

Animals were mounted on 5% Noble agar pads on standard microscope slides, and immobilized with a five minute incubation in 10mM sodium azide. Worms were imaged using a Zeiss Plan-AXIOCHROMA 100X 1.4NA oil objective on a Zeiss Axio Imager.D1M (Zeiss, Oberkochen, Germany) with a Retiga-SRV Fast 1394 digital camera (Q-Imaging, Surrey, BC, Canada). Exposure time for antibodies was 100ms, and exposure time for GFP fluorophores was 250ms. Images were captured and manipulated using Metamorph software (Version 7.6.1.0, MDS Analytical technologies, Sunnyvale, CA, USA). Image stacks were 3D deconvolved using Auto Deblur software (Version 1.4.1, Media Cybernetics, Bethesda, MD, USA). Figures and diagrams were created with Adobe Photoshop CS3 (Version 10.0, Adobe Systems, San Jose, CA, USA) and Adobe Illustrator (Version 13.0.0, Adobe Systems). Image brightness and contrast were modified uniformly across an image, but gamma was not adjusted from 1.00. Brightness manipulations are similar, but not identical, across panels and figures. Significant variations in absolute intensity are noted where appropriate. For all strains, unless noted, worms were picked at L4.
stage 24 hours before imaging.

3.6.6 – Statistical Analysis

All statistical analysis was performed with a combination of GraphPad Prism (Version 5.01, GraphPad Software, La Jolla, CA, USA) and Microsoft Excel (Version 14.0.7106, 32-Bit, Microsoft Corporation, Seattle, WA, USA). Sample size (n) for all figures is listed in Table 9. Minimum p value for significance was set at 0.01 for all analyses unless otherwise specified. All parametric and continuous data types were analyzed using unpaired t-tests with Welch’s correction to avoid assumption of equal variance. When multiple t-tests were performed on related data sets presented together, the Holm-Bonferroni multiple comparison adjustment was used to ensure the total alpha for the analysis did not exceed 0.01. All nonparametric and discontinuous data types were analyzed using Mann-Whitney U-test. Similar to the analysis of continuous data types, the Holm-Bonferroni multiple comparison adjustment was employed to ensure total alpha for all related comparisons did not exceed 0.01. Specific pairwise comparisons made are described in figure legends. Letters on graphs indicate statistically distinct groups, e.g., all groups marked ‘A’ are significantly different from all groups marked ‘B’.

3.6.7 – Dye-Filling Assays

Staged young adult hermaphrodites were washed of plates with M9, and then rinsed three further times in M9, using gentle centrifugation to pellet the worms between rinses. Worms were then incubated in 40µg/mL Dil (2.5mg/mL dimethyl formamide stock, diluted 1:1000 in M9) (Invitrogen) for 30 minutes in the dark. Worms were then rinsed three times in M9 as before, and were then placed on a seeded plate for a further 30-60 minutes to recover and flush dye from the digestive tract. Animals were anesthetized with 10mM sodium azide and then
immediately scored on a compound microscope (see Imaging section) using for dye-filling by manual counting of filled cell bodies. Cell body counts within the amphid or phasmid organs were averaged together to yield the average number of cells filling per organ per worm, and subjected to statistical analysis. Ectopically dye-filled neurons (e.g., IL2s) were not included in the total count.

3.6.8 – Antibody Staining

Antibody staining was performed under the standard Ruvkun-Finney protocol (Anatomical Methods, [83]) using GT335, an antibody against branch point single and polyglutamylated tubulin. Both primary (GT335, mouse, 1:100, Enzo Life Sciences) and secondary antibody (Alexa Flour 568 goat anti-mouse, 1:2000 dilution, Life Technologies) washes were performed at 4°C overnight. Young adult hermaphrodites were selected for imaging using the number of eggs in the animal—between one and ten—as a proxy for age.
3.7 – Acknowledgements

We thank L. Gunther and G. Perumal for help with high pressure freezing procedures. We thank E. Hedgecock for contribution of archival images to the Hall lab collection. We thank members of the Barr lab, including Robert O’Hagan, for critical reading of the manuscript and assistance with reagents and procedures. We also thank Jinghua Hu, Oliver Blacque, and the *C. elegans* Genetics Center for strains and reagents, the Japanese National BioResource Project and the *C. elegans* Gene Knockout Consortium for deletion mutants, Wormbase for sequence information. We are also grateful to the Rutgers *C. elegans* community, because you can't start a fire without a spark.
### 3.8 – Abbreviations List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Dyf</td>
<td>Dye Filling defective</td>
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<tr>
<td>GT335</td>
<td>Anti-glutamylated tubulin antibody</td>
</tr>
<tr>
<td>IFT</td>
<td>Intraflagellar Transport</td>
</tr>
<tr>
<td>InvC</td>
<td>Inversin Compartment</td>
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<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney cells</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>RPE</td>
<td>Retinal Pigment Epithelial cells</td>
</tr>
<tr>
<td>SynDyf</td>
<td>Synthetically Dye filling defective</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TZ</td>
<td>Transition Zone</td>
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3.9 – Figures and Legends

Figure 19. The synthetic dye-filling defective phenotype of *arl-13; nphp-2* mutants is modulated by *hdac-6*

*hdac-6* deletion suppresses *arl-13; nphp-2* double mutant phenotypes. In phasmids, *nphp-2* and *arl-13* single mutants are mildly Dyf, which is not suppressed by *hdac-6* deletion. In both amphids and phasmids, *arl-13; nphp-2* is severely SynDyf and was suppressed by *hdac-6*. Data in both panels was analyzed with pairwise Mann-Whitney U-test between all groups, followed by the Holm-Bonferroni multiple comparison adjustment with a total alpha of 0.01. Groups from either panel sharing a capital letter are not significantly different, whereas groups from either panel with different capital letters do differ significantly.
**Figure 20. nphp-2 single, arl-13; nphp-2 double, and arl-13; hdac-6; nphp-2 triple mutants exhibit defects in ciliary ultrastructure**

Horizontal panels indicated by 1, 2, and 3 correspond to singlet region, doublet region-to-singlet region transition, and TZ levels, respectively, and are comparable across the genotypes. The insets show an enlarged view of the region within the white box, and are diagrammed in the accompanying cartoon. All scale bars are 250nm. (A1-3) In wild-type amphids, all doublet B-tubules within a cilium have similar spans. (A1) Distal microtubule singlets are devoid of B-tubules. B-tubule containing microtubule doublets are present in (A2) the doublet region and (A3) the TZ. (B1-3) In nphp-2 animals, amphid channel cilia are shifted lengthwise with respect to each other (cf. Figure 6A). Within a cilium, spans of microtubule doublet B-tubules are asynchronous; arrows in insets indicate these microtubules. TZ Y-links are disorganized (C1-3) In arl-13; nphp-2 animals, most amphid channel cilia are absent. (C1) The distal end is filled with electron dense material with unresolvable microtubules. (C2) A single, stub-like cilium is visible, consisting of only microtubule singlets. (C3) TZ microtubules are abnormal with some missing microtubule doublets. We also observe vesicle-like structures at this level that are indicated by arrows. (D1-3) In arl-13; hdac-6; nphp-2 animals, most amphid cilia are visible. Ectopic singlets and doublets are still present. Cilia are shifted posteriorly towards the tail. (D1-2) Insets show asynchronous microtubules within cilia.
Figure 21. NPHP-2 and ARL-13 do not require TZ-, doublet region-, and InvC-associated genes for ciliary targeting

(A) In WT, NPHP-2 is restricted to the proximal cilium. In both *nphp-4* and *mks-3* mutants, NPHP-2::GFP was targeted to the cilium and restricted to the post-TZ proximal cilium. Several NPHP-2::GFP puncta were visible in the periciliary compartment. (B) In doublet region and InvC mutants, NPHP-2::GFP was targeted to the cilium and restricted to the post-TZ proximal cilium in *klp-11*, *arl-13*, and *unc-119* mutants. *arl-13* and *klp-11* mutants exhibited periciliary NPHP-2::GFP puncta, and *unc-119* mutants exhibited distal dendritic accumulation of NPHP-2::GFP. (C) In WT, ARL-13::GFP localizes to the proximal cilium. In both *nphp-4* and *mks-3* mutants, ARL-13::GFP was targeted to the cilium and restricted to the post-TZ proximal cilium. ARL-13::GFP also mislocalized to the periciliary membrane compartment in TZ mutants. (D) In *nphp-2* and *klp-11* mutants, ARL-13::GFP was targeted to the cilium and restricted to the post-TZ proximal cilium. In these mutants, ARL-13::GFP also mislocalized to the periciliary membrane compartment, and in *unc-119* mutants, ARL-13::GFP mislocalized to the distal dendrite.
Periciliary puncta – arrowheads, periciliary membrane – white arc, distal dendrite/periciliary accumulation – white bar. Periciliary membrane localization was judged by a visible enrichment of ARL-13::GFP on the edges of the periciliary compartment without a concomitant enrichment in the interior lumen of the periciliary region.
Figure 22. The EF hand is necessary for proper NPHP-2 localization and function

(A) Diagram of the domains present in Inversin, NPHP-2, and each of the NPHP-2 domain deletion constructs. (B) Localization of GFP-tagged NPHP-2 domain deletion constructs. In the head, full length NPHP-2::GFP localizes to the InvC of amphid channel cilia, to the base of IL2 cilia, and to the base of either OLQ or CEP cilia. In the tail (outlined by dashes), NPHP-2::GFP localizes to the InvC of phasmid cilia. Localization of both NPHP-2-NLS1Δ::GFP and NPHP-2-NLS2Δ::GFP appeared roughly wild-type in all cell types, though both constructs appeared enriched in AWC wing cilia. NPHP-2-EFΔ::GFP is present in IL2 and CEP cilia but fails to localize to amphid channel and phasmid cilia. (C) NPHP-2-NLS1Δ::GFP, NPHP-2-NLS2Δ::GFP, and NPHP-2-EFΔ::GFP can rescue nphp-2 nphp-4 amphid and phasmid dye-filling defects, but NPHP-2-EFΔ::GFP rescue is significantly worse than full length NPHP-2::GFP in both amphid and phasmid neurons. Yellow arrowheads – IL2 cilia, red arrowheads – OLQ, blue arrowheads – CEP cilia, white bar – amphid/phasmid bundle. Letters indicate statistically distinct groups. Data was analyzed with pairwise Mann-Whitney U-test against both positive and negative controls, followed by the Holm-Bonferroni multiple comparison adjustment with a total alpha of 0.01. Non-transgene expressing siblings were used as negative controls in rescue experiments.
Figure 23. UNC-119 localizes to the proximal cilium in phasmids and does not require DR and InvC genes to target the cilium

(A) N-terminal GFP tagged UNC-119 localizes to dendrites and cilia of amphid channel and phasmid neurons. The TZ gap (arrow) can be discerned in the cilia of amphid channel neurons. In cilia of the CEP and OLQ neurons, GFP::UNC-119 fluorescence was faint and infrequently visible. In phasmid cilia, GFP::UNC-119 localizes to the doublet region, and is excluded from the TZ (arrow) and singlet region. (B) GFP::UNC-119 localization in doublet region- and InvC-associated mutants. GFP::UNC-119 localizes to the proximal cilium in all mutant backgrounds examined.
Figure 24. klp-11 and unc-119 genetically interact with arl-13 and nphp-2 in an hdac-6 dependent manner.

(A) klp-11 single mutants are not Dyf. klp-11 is SynDyf with arl-13, which is partially suppressed by deletion of hdac-6. (B) unc-119 single mutants are moderately Dyf. unc-119 is SynDyf with both klp-11 and nphp-2. unc-119; hdac-6; nphp-2 triple mutants exhibit suppression of the unc-119 Dyf phenotype. (C) Diagram of interactions between klp-11, arl-13, nphp-2, unc-119, and hdac-6, and between DR and TZ genes, based on SynDyf phenotypes presented in panels A
and B and in Figures 1 and S5. The T-bar indicates *hdac-6* mediated suppression of SynDyf phenotypes. Data was analyzed with pairwise Mann-Whitney U-test between wild type, double mutants, and their respective single mutants, followed by the Holm-Bonferroni multiple comparison adjustment. *, significant versus single mutants at a total alpha of 0.05. **, significant versus single mutants at a total alpha of 0.01. *nphp-2, arl-13, hdac-6*, and *arl-13; hdac-6* Dyf data is presented in Figure 1.
Figure 25. nphp-2, arl-13, and hdac-6 regulate glutamylation in head and tail cilia

(A) Worms stained with GT335 anti-glutamylated tubulin antibody. In WT background, GT335 labels amphid and phasmid doublet region microtubules, and CEP doublet region and singlet region microtubules consistently, and OLQ cilia inconsistently. CEP distal microtubule singlets were also labelled by GT335. Inner labial cilia were also glutamylated, but specific IL1 and IL2
Identification was not possible. *nphp-2* mutants showed characteristic posterior shifted cilia, and glutamylolation in the head looked similar to WT, with infrequent weak inner labial cilia staining. Phasmids exhibited an elongated glutamylolation signal. *arl-13* mutants exhibited elongated staining of amphid bundle, CEP, and OLQ cilia. Amphid staining in *unc-119* mutants was extremely shortened and cilia were angled inwards. *unc-119* mutants exhibited almost no staining of CEP, IL, and OLQ cilia. *hdac-6* mutants displayed shorter amphid bundle staining, and reduced IL and OLQ staining. (B) In *ttll-4* mutants, NPHP-2::GFP and GFP::UNC-119 localize similarly to WT. (C) In *ccpp-1* mutants, both NPHP-2::GFP and GFP::UNC-119 localize similarly to in WT, and GFP::UNC-119 is present in the TZ and accumulates at the distal dendrite. Yellow arrowheads – IL2 cilia, red arrowheads – OLQ cilia, blue arrowheads – CEP cilia, white bar – amphid/phasmid bundle.
Figure 26. NPHP-2::GFP, ARL-13::GFP, and GFP::UNC-119 colabel with GT335 staining

In amphid channel and phasmid cilia, NPHP-2::GFP, ARL-13::GFP, and GFP::UNC-119 signals overlap with GT335 staining. ARL-13::GFP and GT335 staining overlap completely. NPHP-2::GFP does not overlap completely with GT335. TZ staining by GT335 is frequently visible, as in the NPHP-2::GFP overlay. Though the signal of GFP::UNC-119 is dim due to the staining procedure, GFP::UNC-119 appears not to overlap completely with GT335. Data is quantified in Figure S6A.
Figure 27. Model of the composition of the proximal cilium

ARL-13 is depicted as membrane associated based on published characterizations [32]. NPHP-2 is depicted as membrane associated based on the membrane association of Inversin in mammalian primary cilia, and because in *C. elegans* NPHP-2 reporters appear membrane associated by casual observation [12, 17]. Kinesin-II is microtubule associated, and UNC-119 is depicted nonspecifically because of the diffuse localization of GFP::UNC-119. Poly-glutamylated tubulin is depicted as a modification of the B-tubule, as reported [40].
Dye filling was used to test for synthetic interactions between InvC and doublet region genes. (A) arl-3 interacts with nphp-2 and arl-13 in a sensillum-specific manner. arl-3 single mutants and arl-3; hdac-6 double mutants are nonDyf in amphids and phasmids. In both amphids and phasmids, arl-13; arl-3 double mutants are mildly SynDyf, which is suppressed by hdac-6 deletion. arl-3 deletion did not suppress arl-13; nphp-2 defects. hdac-6 mediated suppression of arl-13; nphp-2 defects requires arl-3. In phasmids, but not amphids, nphp-2 genetically interacted with arl-3, but this was not suppressed by hdac-6 deletion. (B) arl-3 genetically
interacts with *unc-119* and *klp-11* in amphids and phasmids. *arl-3* deletion moderately suppressed *unc-119* single mutant Dyf. *klp-11; arl-3* was slightly SynDyf. Data in panel A was statistically analyzed in conjunction with data from Figure 1 because of the overlap in genotypes examined. Both panel A and Figure 1 were analyzed with pairwise Mann-Whitney U-test between all groups, followed by the Holm-Bonferroni multiple comparison adjustment with a total alpha of 0.01. Genotypes from either panel A of this figure or Figure 1 sharing a capital letter are not significantly different, whereas groups from either panel with different capital letters do differ significantly. Data panel B was analyzed using pairwise Mann-Whitney U-test between double mutants, their respective single mutants, and wild type followed by the Holm-Bonferroni multiple comparison adjustment with a total alpha of 0.01. **, double mutant phenotype is significantly different from both respective single mutants.
Figure 29. Localization requirements of NPHP-2::GFP and ARL-13::GFP in phasmid cilia

(A) NPHP-2::GFP does not require mks-3; nphp-4, hdac-6, or arl-13; hdac-6 for ciliary targeting or restriction to the proximal cilium. (B) Contrast enhanced version of Figure 3C,D. ARL-13::GFP mislocalizes to the periciliary compartment in TZ and doublet region mutants.
Figure 30. NPHP-2 and ARL-13 do not require TZ-, doublet region-, and InvC-associated genes for ciliary targeting in amphids

(A) Localization of NPHP-2::GFP is subtly disrupted in mks-3, nphp-4 and mks; nphp-4 mutants. Periciliary puncta in amphid channel cilia and altered staining in IL2, CEP, and OLQ cilia are visible. (B) The NPHP-2::GFP amphid bundle is shortened in klp-11 and unc-119 mutants, and elongated in arl-13 mutants. Periciliary puncta are visible in klp-11 and arl-13 mutants. hdac-6 deletion does not suppress the arl-13 phenotype. (C) ARL-13::GFP localizes primarily to the doublet region of amphid channel cilia, and to a nonspecific proximal region of IL2 cilia. In mks-3 and nphp-4 mutants, ARL-13::GFP localization in amphid channel cilia looks grossly wild-type. CEP and OLQ cilia show increased ARL-13::GFP localization. (D) unc-119 mutants exhibit distal dendritic accumulation of ARL-13::GFP. klp-11 and nphp-2 mutants exhibit increased CEP and OLQ ARL-13::GFP localization. Arrowheads indicate periciliary puncta. Bar indicates distal dendritic localization.
GFP::UNC-119 localization in InvC and doublet region mutants

Figure 31. Amphid UNC-119 localization in InvC and doublet region mutants

GFP::UNC-119 appears similar in wild-type (Figure 5A), *nphp-2*, and *hdac-6* amphid channel cilia.

*klp-11* and *arl-13* exhibit an accumulation of GFP::UNC-119 in amphid channel, OLQ, CEP, and inner labial cilia. *hdac-6* deletion does not suppress GFP::UNC-119 mislocalization in *arl-13* mutants. Green arrow – TZ gap, yellow arrowheads – IL2 cilia, red arrowheads – OLQ cilia, blue arrowheads – CEP cilia, white bar – amphid bundle.
Figure 32. Amphid dye-filling of IFT and unc-119 mutants

(A) klp-11 single mutants are not Dyf. klp-11 is SynDyf with arl-13, which is suppressed by deletion of hdac-6. (B) unc-119 single mutants are severely Dyf. hdac-6; nphp-2 suppresses the unc-119 Dyf phenotype to a small degree. (C) osm-3 single mutants are both severely amphid and phasmid Dyf. In no double mutant was this suppressed. (D) hdac-6; nphp-2 nphp-4 and
*arl-3; nphp-2 nphp-4* worms were assayed for suppression of SynDyf defects. Neither strain exhibited significant suppression as compared to the *nphp-2 nphp-4* double mutant. Data was analyzed with pairwise Mann-Whitney U-test between wild type, double mutants, triple mutants and their respective single mutants, followed by the Holm-Bonferroni multiple comparison adjustment. **, significant versus single mutants at a total alpha of 0.01.
Figure 33. NPHP-2::GFP marks a significantly smaller region of the cilium than ARL-13::GFP and GFP::UNC-119.

(A) Worms expressing NPHP-2::GFP, ARL-13::GFP, and GFP::UNC-119 reporters were stained with GT335. The ratio of the length of each reporter localization pattern to the length of the entire cilium, minus the TZ was computed per-cilium. NPHP-2::GFP marks a significantly shorter proportion of the cilium than either ARL-13::GFP or GFP::UNC-119. (B) Absolute lengths of data presented in panel A. (C) Worms expressing NPHP-2::GFP, ARL-13::GFP, and GFP::UNC-119
reporters were incubated with Dil to label cilia. The ratio of the length of each reporter localization pattern to the length of the entire cilium, minus the TZ was computed per-cilium. NPHP-2::GFP marks a significantly shorter proportion of the cilium than either ARL-13::GFP or GFP::UNC-119. (D) Absolute lengths of data presented in panel C. Phasmid cilia lengths in transgenic strains as measured using Dil staining, and reporter localization size. Data in each panel was analyzed with pairwise t-tests with Welch’s Correction, followed by the Holm-Bonferroni multiple comparison adjustment for a total alpha of 0.01.
Each data point represents the averaged lengths of the GFP signal or immunofluorescence in all visible and distinct phasmid cilia within a single animal. (A) NPHP-2::GFP localization length is significantly decreased in klp-11 and unc-119 mutants, and significantly increased in arl-13 mutants. hdac-6 deletion partially suppresses the arl-13 phenotype. (B) ARL-13::GFP localization length is increased in nphp-2 and decreased in unc-119 mutants. (C) GFP::UNC-119 localization length does not change significantly in any strain. (D) KAP-1 localization length measurements were more variable than the other reporters due to a faint KAP-1::GFP signal, but were not significantly different from wild type in any strain. Localization length was significantly altered in nphp-2 mutants. (E) nphp-2 and arl-13 had significantly longer GT335 signals than in wild type. nphp-2 mutants have increased variability in GT335 signal lengths compared to WT. (F) Diagram representing genetic control of doublet region protein localization in phasmid cilia. An arrow between a gene and a protein/GT335 indicates that the gene is required for the protein/GT335 to label its entire territory. A T-bar between a gene and a protein/GT335 indicates that the gene restricts the size of the protein territory/GT335 signal length. Data was analyzed with an unpaired t-test with Welch’s Correction against wild type, followed by the Holm-Bonferroni multiple comparison adjustment for all comparisons in a given panel. **p<0.01.
Figure 35. NPHP-2 contains a strongly conserved hydroxylation motif and a predicted acetylation site

(A) Hydroxylation motif is highlighted in green. Hydroxylated asparagine is highlighted in blue. ANKS6 and INVS alignment are from [19]. (B) NPHP-2 domain model. NPHP-2 contains a predicted acetylation site at 2S, and two predicted SUMOylation sites. Green letters indicate modified residues. Acetylation and SUMOylation motifs do not appear to be conserved.
Figure 36. Localization of NPHP-2 and UNC-119 in L1 stage worms

(A) NPHP-2::GFP localizes to the proximal portion of the cilium in L1 animals. (B) Kymograph of NPHP-2::GFP in L1 phasmid cilia. No movement of NPHP-2::GFP was detectable. Total duration of recording was 40 seconds. (C) NPHP-2::GFP localizes to amphid and phasmid cilia in L1 and L4 stage ccpp-1 mutants. (D) GFP::UNC-119 localizes to amphid and phasmid cilia in L1 and L4 stage ccpp-1 mutants. White bars indicate ciliary GFP::UNC-119 localization. White arrow indicates abnormal cilia base accumulation of GFP::UNC-119.
Figure 37. The anti-acetylated tubulin antibody 6-11b-1 does not label amphid channel and phasmid cilia in either WT animals or *hdac-6* mutants.

6-11b-1 antibody labels only dendrites of the touch neurons. *hdac-6* deletion does not increase the amount of acetylation detected in touch neurons. Non-specific labelling is seen in the buccal cavity, and not in amphid cilia, as determined by analysis of 3D image stacks.
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**Table 7. Transgenic strains used in this study have mild to no ciliogenic defects**

Worms from each transgenic strain imaged or otherwise quantified in Figures 1-9—other than strains with a Dyf or SynDyf background—were assayed for dye-filling defects to determine if there were any gross defects in ciliogenesis or cilia placement. Scores reported are average fraction of cell bodies taking up dye, plus/minus std. err. Yellow cells mark possible dominant negative defects. Transgenic strains expressing NPHP-2::GFP and GFP::UNC-119 did not exhibit defects, but strains expressing ARL-13::GFP all exhibited mild defects.
### Strain List

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**Transgenic Strains**

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PT2806: nphp-2(gk653) him-5(e1490) V; jhuEx352(parl-13::ARL-13::GFP + pBx)
PT2807: klp-11(tm324) IV; him-5(e1490) V; jhuEx352(parl-13::ARL-13::GFP + pBx)
PT2808: unc-119(ed3) III; him-5(e1490) V; jhuEx352(parl-13::ARL-13::GFP + pBx)
PT2809: him-5(e1490) V; myEx848(posm-6::GFP::UNC-119 + ccRFP)
PT2641: nphp-2(gk653) him-5(e1490) V; myEx848(posm-6::GFP::UNC-119 + ccRFP)
PT2640: arl-13(gk513) I; him-5(e1490) V; myEx848(posm-6::GFP::UNC-119 + ccRFP)
PT2642: klp-11(tm324) IV; him-5(e1490) V; myEx848(posm-6::GFP::UNC-119 + ccRFP)
PT2638: hdac-6(ok3203) IV; him-5(e1490) V; myEx848(posm-6::GFP::UNC-119 + ccRFP)
PT2639: arl-13(gk513) I; hdac-6(ok3203) IV; him-5(e1490) V; myEx848(posm-6::GFP::UNC-119 + ccRFP)
PT2847: ttll-4(tm3310) III; him-5(e1490) V; myEx743(pnphp-2::NPHP-2L::GFP + ccRFP::GFP)
PT2848: ccpp-1(ok1821) I; him-5(e1490) V; myEx743(pnphp-2::NPHP-2L::GFP + ccRFP::GFP)
PT2852: ttll-4(tm3310) III; him-5(e1490) V; myEx848(posm-6::GFP::UNC-119 + ccRFP)
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Table 8. List of strains and PCR deletion diagnosis primers used in this work

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<tr>
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<th>Figure 1</th>
<th>Figure 4C</th>
<th>Figure 6A</th>
<th>Figure 6B</th>
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**Figure 4C**

- **WT**
  - 99
- **nphp-2 nphp-4**
  - 88
- **myEx743(pnphp-2::NPHP-2L::GFP + ccRFP)**
  - 11
- **nphp-2 nphp-4; myEx849(pnphp-2::NPHP-2L-EFdel::GFP + ccRFP)**
  - 47
- **nphp-2 nphp-4; myEx850(pnphp-2::NPHP-2L-NLS1del::GFP + ccRFP)**
  - 27
- **nphp-2 nphp-4; myEx851(pnphp-2::NPHP-2L-NLS2del::GFP + ccRFP)**
  - 20

**Figure 6A**

- **WT**
  - 60
- **klp-11**
  - 40
- **klp-11; nphp-2**
  - 120
- **arl-13; klp-11**
  - 100
- **hdac-6 klp-11**
  - 40
- **klp-11; arl-13; hdac-6**
  - 53

**Figure 6B**

- **unc-119**
  - 59
- **unc-119; klp-11**
  - 40
- **unc-119; nphp-2**
  - 82
- **arl-13; unc-119**
  - 38
- **unc-119; hdac-6**
  - 42
- **unc-119; hdac-6; nphp-2**
  - 50
### Figure 8C

Ratio of fluorescent reporter length to GT335 length

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<td>ARL-13::GFP and GT335 colabel</td>
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Length fluorescent reporter length

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Length of GT335

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*In some animals, GFP was too dim to measure, leading to a difference between GFP and GT335 Ns. These unmatched lengths have no effect on ratio data, as all ratios were computed per-cilium.*

### Supplemental Figure 1

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*In some ARL-13::GFP animals, Dil was too dim to measure, leading to a difference between GFP and Dil Ns. These unmatched lengths have no effect on ratio data, as all ratios were computed per-cilium.

### Supplemental Figure 7A

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### Supplemental Figure 7B

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### Supplemental Figure 7C

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### Supplemental Figure 7D

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### Table 9. Experimental sample sizes for figures with statistical analysis.

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</tbody>
</table>

*Each worm score is the average of 2-4 cilia*
3.9 – References

Note: These references are formatted to the standards of PLOS Genetics.


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Chapter 4: Conclusion

4.1 Key Findings

This work supports and extends our understanding of the TZ, InvC, and doublet region in significant ways. I established that both Inversin and the Inversin compartment are conserved in *C. elegans*, and that the worm is a viable model to study this gene.

In my first paper (Warburton-Pitt et al., 2012), I characterized *nphp-2* and found that it is required for proper TZ placement. I also demonstrated that TZ genes are organized into two distinct genetic modules, and that *nphp-2* interacts with these in a cell-type specific manner. Other work in *C. elegans* and mammalian systems has corroborated this finding. I found that *nphp-2* is not required for the localization of TZ components; as ciliary anchoring is a function of the TZ, this suggests that anchoring defects in *nphp-2* mutants are not due to TZ assembly failure. I also found that *nphp-2* and the TZ gene *nphp-4* interact strongly to guide multiple aspects of cilia biology, including cilia placement and IFT.

In my second paper (Warburton-Pitt et al., 2014), I explored the relationship between *nphp-2* and doublet region associated genes. I found that these genes are also organized into two parallel genetic modules, interactions between which are modulated by *hdac-6* and *arl-3*. Additionally, I found that contrary to previous reports, UNC-119 localizes to the proximal cilium and not the TZ. I also demonstrated that ciliary glutamylation lies downstream of the action of doublet region-associated genes, and that glutamylation does not specify the localization of doublet region proteins. How the InvC and doublet region are initially established is unknown in any system. Our data rules out multiple possible mechanisms, including IFT, the UNC-119 shuttle, TZ, tubulin glutamylation, and microtubule ultrastructure.

Chapter 1 of this thesis is the first comprehensive review of the InvC to date,
summarizing over five years of work in the field. I introduced several hypotheses, including the hypothesis that the *C. elegans* ciliary “middle segment”/doublet region is analogous to the mammalian doublet region and not to the mammalian InvC. I also proposed and explored many possible functions for the InvC. As a whole, this chapter will help synthesize results from across multiple organisms to give a more complete and easy to understand overview of the field than was previously available.

### 4.2 Future Directions

With the advancement of the field, and the work presented herein, many, many unanswered questions and unexplored paths remain. Here, I discuss several immediate questions and possible experimental approaches. The function of the InvC is omitted, as it is thoroughly discussed in Chapter 1 (1.5 – The Function of the Inversin Compartment).

#### 4.2.1 – InvC biogenesis

The single biggest mystery regarding the InvC is how it is initially established. While many possible mechanisms have been put forward, tested, and ruled out, I have no definitive answer to date. Currently, the only positive data that I have is from mammalian and *C. elegans* domain deletion experiments, which found that the calmodulin binding IQ and ninein homology domains of Inversin, and the EF-hand of NPHP-2 are required for ciliary targeting and InvC restriction (Shiba et al., 2009; Warburton-Pitt et al., 2014). Chapter 3 explores and discusses several other mechanisms in *C. elegans*, including IFT, TZ scaffolding/restriction, tubulin glutamylation, and microtubule ultrastructure. Many possible mechanisms remain unexplored, and further possible mechanisms remain unimagined. Here, I discuss several possibilities and experimental treatments in turn. An in-depth discussion of some of these is presented in
Chapters 1 and 3 (1.4.4 – Establishing the Inv Compartment and 3.5.8 – Origin of the Inversin Compartment).

4.2.1.1 – Calcium signaling. The high intraciliary calcium concentration belies the importance of calcium signaling in cilia biology (Reviewed in Masyuk 2014). Calcium can function signal transduction and/or signaling cascades. As the earliest known determinant of the InvC, Inversin, requires its calmodulin-binding IQ2 domain for restriction to the InvC, calcium signaling likely plays a role in specifying the InvC (Shiba et al., 2009). NPHP-2 also requires its calcium binding EF-hand for InvC localization (Warburton-Pitt et al., 2014). A significant difference exists between the EF-hand of NPHP-2 and the IQ2 domain of Inversin: deletion of the EF-hand of NPHP-2 results in a complete lack of ciliary localization, whereas deletion of the IQ2 domain of Inversin results in a mislocalization of Inversin along the entire cilium. In both systems, Inversin/NPHP-2 no longer localizes to the InvC. If calcium has a conserved role in the specification of InvC restriction, then in C. elegans, non-InvC targeted NPHP-2 may be cleared actively or passively out of the cilium through a non-conserved means. It is also possible that the calcium-dependency of Inversin and NPHP-2 has diverged.

However, knowing that calcium-binding is required for localization does not allow us to determine exactly how this is achieved. Inversin/NPHP-2 may localize based on pre-existing calcium concentration gradients, or calcium may stabilize already InvC localized Inversin/NPHP-2. In mammalian systems, calmodulin localizes throughout the cilium and is not enriched in the InvC (Shiba et al., 2009); whether this population of calmodulin is all bound to calcium, or if only small amounts of the calmodulin are bound to calcium, is unknown.

Inversin/NPHP-2 may also act as a calcium “sponge” at the base of the cilium, functioning to maintain high intraciliary calcium and reducing leakage into the cytoplasm.

To clarify the complex link between calcium and the InvC, an experimental approach can be
taken. A calcium indicator can be used to determine if NPHP-2 is required to maintain ciliary
calcium levels or if a ciliary calcium micro-domain exists by comparing WT animals, nphp-2
mutants, and worms expressing NPHP-2-ΔEF. As the EF-hand is not required for IL localization of
NPHP-2, these new experiments should be performed across cell types to explore how cell
specific localization may be achieved. A calcium chelator can be used to determine if calcium is
required for the maintenance of the InvC (Mank and Griesbeck, 2008). It is difficult to determine
the role of calcium in establishment of the InvC as calcium cannot be completely removed
during development. Partial depletion of calcium in sensory neurons could be used instead using
a calcium reporter (Miyawaki et al., 1997). To avoid the problem of non-cilium-specific calcium
depletion, a cilia-specific depletion method should be used. Perforating the ciliary membrane
using OSM-9, a calcium TRP channel, may allow the excess calcium in the cilium to dissipate out
of the cell (Robert O’Hagan, Personal Communication). Additionally, if a ciliary targeting signal
could be identified, it could be fused to a calcium chelating protein, restricting the chelator to
the cilium. If no such targeting signal exists, then a portion of a cilia-targeted protein could be
substituted; for example, minimal targeting regions have been determined for ARL-13 (Cevik et
al., 2013).

4.2.1.2 – Membrane composition. Most cellular compartments have membranes with distinct
composition, suited for their specific task. The cilium possibly has several membrane
microdomains, including the periciliary membrane surrounding the cilium at the dendritic tip,
and the membrane of the cilium proper (e.g., Bae et al., 2009; Chih et al., 2011; Kaplan et al.,
2012). Multiple doublet region/InvC components are associated with membrane biology. ARL-13
is inserted into the membrane through a palmitoyl group (Cevik et al., 2010), NPHP-2 appears to
be enriched near the membrane (Warburton-Pitt et al., 2012), and HDAC-6 has a predicted FYVE
phosphatidylinositol 3-phostphate (PI3P) binding domain (Maureen Barr, Personal
Communication). The palmitoyl group of ARL-13 is required for doublet region restriction but not ciliary targeting, strong evidence that the membrane plays a role in localizing at least some InvC and doublet region components (Cevik et al., 2010). It is still possible that the membrane does not specify ARL-13 restriction, and that other membrane-associated factors are required. It is possible that specific membrane composition drives localization of InvC and doublet region components through these physical interactions. (It is also possible that these components regulate membrane composition; for a discussion of this, see 1.4.5 – Membrane Dynamics of the Inv Compartment.) The localization of NPHP-2 and UNC-119 have been examined in several combinations with PIP-associated gene mutants, including age-1 (a PIP 3-kinase), daf-18 (a PI3P 3-phosphatase), and cil-1 (a Pi(3,5)P 5-phosphatase), but these genes do not appear to be required (Error! Reference source not found.). The length of the UNC-119 territory was also measured in these mutants, but was not significantly altered compared to wild-type. However, this data does not rule out a role for membrane composition in InvC biogenesis, and does not touch upon membrane trafficking pathways.

As specific relevant phospholipids are not known, a broad approach needs to be taken. First, the localization of ARL-13, NPHP-2, UNC-119, and HDAC-6 should be determined in all mutants of PIP kinase and phosphatase genes expressed in ciliated neurons. As HDAC-6 may bind PI(3)P, one might predict that it HDAC-6 will mislocalize in age-1 mutants if the membrane determines localization.

Second, fluorescent markers can be used to determine ciliary membrane composition and the role of the membrane in determining ciliary microdomains. Specific PIPs can be fluorescently labelled, including by 2XFYVE (labelling PI(3)P), AKT[PH domain][labelling PI(3,4,5)P 3 and PI(3,4)P 2], and PLCΔ[PH domain][labelling PI(4,5)P 2] (Bae et al., 2009). Sphingolipids rafts can be detected using BODIPY, which exhibits changes in emission wavelength depending on
concentration (Pagano and Chen, 1998). In addition to BODIPY tagging of cholesterol (Holtta-Vuori et al., 2008), the cholesterol composition of the cilium can be determined using dehydroergosterol, a fluorescent analog of cholesterol which functionally mimics cholesterol in living cells (Mukherjee et al., 1998). An alternative is using a fluorescent protein reporter such as Perfringolysin-O-domain D4::GFP, which preferentially labels cholesterol in lipid rafts (Johnson et al., 2012; Ohno-Iwashita et al., 2004). A non-specific fluorescent marker, Laurdan/C-Laurdan, changes its excitation wavelength depending on the fluidity of the surrounding membrane (Kim et al., 2007; Parasassi et al., 1997). (C-Laurdan is more photostable and water soluble than Laurdan.)

If any of these membrane markers reveals a proximal cilium cilia membrane microdomain, that marker could be colabelled with NPHP-2::GFP, ARL-13::GFP, GFP::UNC-119, and GT335 (anti-glutamylated tubulin antibody which marks doublet region microtubule doublets) to determine the specific subciliary localization of the marker. This information could then be used to select membrane pathway mutants to assay for localization of doublet region and InvC components. In the case of cholesterol, a faster, though less definitive alternative to fluorescent labelling would be to raise worms on cholesterol-deficient media, and then determine localization of InvC and doublet region markers. Cholesterol depletion has previously been used to explore the link between membrane biology, the transmembrane ciliopathy ortholog MKS-3, and IFT (Juan Wang, personal communication). Analysis of other membrane components in such a manner is not possible, as the other components are produced endogenously.

4.2.1.3 – The EVC Compartment.

As described in Chapter 1 (1.4.4 – Establishing the Inv Compartment), in mammalian cilia, there exist two proteins, Ecv and Evc2, that localize to a region called the Evc compartment (EvcC), laying in-between (or partially overlapping both) the TZ and the InvC (Dorn et al., 2012; Pusapati...
et al., 2014). Evc and Evc2 physically interact, require each other for proper localization, and positively regulate ciliary hedgehog signaling. Evc2 possesses a motif termed the W-peptide that is required for restriction to the Evc compartment; the W-peptide binds to the calcium and calmodulin binding complex EFCAB7-IQCE. When the interaction between Evc/Evc2 and EFCAB7-IQCE is disrupted, Evc and Evc2 localize along the entire cilium. This is similar to the calcium-dependence of Inversin/NPHP-2, which require calcium and calmodulin domains to be restricted to the InvC (Shiba et al., 2009; Warburton-Pitt et al., 2014). Several questions arise: Is the Evc/Evc2 complex required for InvC biogenesis or maintenance? Do InvC or TZ components specify the EvcC? Evidence suggests that the Evc/Evc2 complex is not required for the localization of Inversin or Arl13b, though the reverse has not been examined (Pusapati et al., 2014); it maybe that EvcC components interact with both TZ and InvC proteins, restricting the EvcC to the margin between these two larger compartments. Is the EFCAB7-IQCE complex required for InvC biogenesis and does it play the same function in organizing both the InvC and the EvcC? That both InvC and EvcC biogenesis requires proteins sharing domains is intriguing, but this relationship has not been examined to date. Is the EvcC conserved in C. elegans, and can C. elegans be used to study this novel compartment? A cursory search indicates that only EFCAB7 may be conserved in C. elegans (T02G5.3), which possesses an X-box, the general cilia gene promoter. The other EvcC components (IQCE, Evc, and Evc2) do not yield strong BLAST matches. This suggests that while the EvcC itself is likely not conserved in C. elegans, the mechanism that links a subciliary localization pattern to calcium may be present. Regardless of whether the EvcC is present in C. elegans or is linked to calcium signaling, it is of note that the cilium possess two distinct microcompartments, both adjacent to the TZ, both regulated by calcium, and both functioning as signaling components (Wnt in the case of the InvC and Hedgehog in the case of the EvcC). Further work in mammalian models is needed to explore
the relationship between these compartments.

4.2.1.4 – The Transition Zone.
The TZ and InvC are functionally linked, as demonstrated by SynDyf data presented in Chapters 2 and 3 (Figure 12, Figure 24). Several TZ and InvC components also physically interact, including NPHP-2 and NPHP-4 in C. elegans (Andrew Jauregui, PhD Thesis), and Inversin and Nphp1, Nphp5, and Ahi1 in mammalian systems (Sang et al., 2011). As the TZ is assembled early in ciliogenesis, before the docking of the ciliary vesicle to the membrane, and the TZ acts as a gate regulating ciliary traffic, it is possible that the TZ also functions to organize ciliary compartmentalization (Williams et al., 2011). Indeed, in mammalian models, the coiled-coil domain of Nphp3 is required for physical interaction with and targeting to the TZ prior to ciliary import (Nakata et al., 2012).

Evidence in C. elegans, however, indicates that a role for the TZ in organizing the InvC is unlikely. In TZ mutants, both NPHP-2 and ARL-13 were targeted and restricted to their respective territories, indicating that the TZ is not even required for ciliary targeting of these proteins (Figure 21, Figure 29, Figure 30). Since the TZ complex is formed of two redundant modules, it may be possible that in TZ single mutants the second, unaffected module is sufficient for ciliary targeting and territory restriction of NPHP-2 and ARL-13; however, in the mks-3; nphp-4 double mutant (in which both TZ modules are knocked out), NPHP-2 is still localizes to the InvC even in cilia with severe placement defects (Figure 30). It was only possible to determine in the TZ was strictly required for InvC localization of NPHP-2, and not whether the TZ played any role at all; periciliary mislocalization of NPHP-2 may be due to either TZ defects or transgene overexpression defects (}
3.4.3 – NPHP-2 and ARL-13 do not require TZ- and doublet region-associated genes for ciliary targeting).

Because of the genetic redundancy of TZ, and the different roles each TZ protein plays in the complex, it might be that the mks-3; nphp-4 double mutant does not represent a full TZ knockdown. mks-3, a transmembrane protein, is the furthest downstream gene in the MKS module (Williams et al., 2011); examining localization of NPHP-2 in an mks-5; nphp-4 double mutant may be more informative because mks-5 is the furthest known upstream MKS module gene. Additionally, the TZ may be required for NPHP-2 localization in a cell-specific manner: no cell-type specific localization analysis was undertaken in the mks-3; nphp-4 mutants. This would not be surprising, as NPHP-2 appears TZ localized in IL2 cilia, and does not require the calcium binding EF-hand for proper localization in IL2 cilia (Warburton-Pitt et al., 2014).

4.2.1.5 – Glutamylation, acetylation and the target of hdac-6.

Post-translational modification of tubulin and other proteins may also play a large role in establishing the InvC. Microtubules can be labeled for specific purposes by the addition of several moieties, via polyglutamylation and acetylation, among others (Verhey and Gaertig, 2007). Though the doublet region of amphid channel and phasmid cilia is polyglutamylated, experiments in Chapter 3 demonstrate that this polyglutamylation is likely to be downstream of the action of the InvC and doublet region (O’Hagan et al., 2011; Warburton-Pitt et al., 2014). However, I only examined the roles of ccpp-1 (a deglutamylase) and ttl-4 (a glutamylase); five other ttl genes are conserved in C. elegans (Robert O’Hagan, Personal Communication). ttl-4 was chosen for the initial analysis because it can suppress ccpp-1 ciliary degeneration in amphid channel and phasmid cilia, indicating that it is the reverse enzyme of ccpp-1 in that cell type (O’Hagan et al., 2011). ccpp-1 has other reverse enzymes in other cell types; whether these
regulate the localization of NPHP-2 in those cell-types is wholly unknown.

The genetic interactions between the doublet region SynDyf network and *hdac-6* strongly, though not strictly, suggest that acetylation plays a major role in the function of the InvC and doublet region. Because the targets of *hdac-6* are unknown, analysis is difficult. As *hdac-6* is a general antagonist to ciliogenesis, it is not surprising that NPHP-2, ARL-13, and UNC-119 all localize properly in *hdac-6* mutants. If acetylation of some target is required for proper InvC biogenesis, then localization defects would be evident in two conditions. First, overexpression of HDAC-6 should lead to a reduction in total acetylation levels of its target, possibly leading to InvC and doublet region localization defects. Second, once the target of HDAC-6 is found, then deleting it should also lead to localization defects.

Because trying to identify *hdac-6* targets has been fruitless due to the paucity of relevant data regarding acetylated ciliary proteins (Li et al., 2010), an unbiased approach must be taken. Initially, it should be established whether or not *hdac-6* is functioning as a deacetylase in cilia. This can be achieved by mutating a residue in the two active sites of HDAC-6, and then determining if inactive HDAC-6 can prevent suppression of Dyf defects. If HDAC-6 is not functioning as a deacetylase, its catalytic domains may only be functioning in binding of acetylated targets; this role can be tested by replacement of a residue in the active site to a bulky residue, preventing bending. Regardless of whether the deacetylase function of *hdac-6* is required for its ciliary activity, a screen for physical interactors of HDAC-6 can be performed. A physical interaction screen would provide valuable data in both *C. elegans* and mammalian studies; an alternative screen, a screen for suppression of *hdac-6* mediated suppression, would not be possible because of the number of unrelated Dyf genes that would be found (*hdac-6* suppression is specific to only the doublet region SynDyf network). A third, slightly biased, alternative would be to look for overlaps in the mammalian acetylome and genes possessing an
X-box or having higher expression in ciliated neurons (Blacque et al., 2005; Efimenko et al., 2005). Candidates could then be tested for ciliary localization of the protein, and Dyf defects and NPHP-2 mislocalization in the gene mutant.

### 4.2.1.6 – Extracellular/environmental interactions.

All of the previous discussions presuppose that the factors required for InvC organization all lay within the cilium itself. In many mammalian cells, the cilia base is placed several microns within the cell, and the membrane forms a circular invagination, called the ciliary pocket, around the cilia base (Molla-Herman et al., 2010). This cilia pocket may mediate the span of the InvC through interactions between the cilium and either the extracellular matrix filling the pocket, or the plasma membrane forming the other side of the pocket. In *C. elegans* amphid neurons, amphid cilia lie within the amphid pore, which is formed from the amphid sheath and socket cells (Perkins et al., 1986). In the distal portion of the pore, cilia are bundled tightly and are in close contact with the socket cell. The level of the transition between the socket and sheath cells marks the transition between the doublet and singlet regions of the cilium; at this level, cilia are still tightly bundled and closely associated with the glial cells. Further proximal, cilia are spread out and are dissociated from the glial cells; here, they are primarily surrounded by extracellular matrix filling the amphid pore. In both *C. elegans* and mammalian models, there may be interactions between cilia and either the ECM or neighboring membrane. This is supported by evidence in IMCD3 cells, which have cilia but no ciliary pocket, and have no strictly defined InvC (Sang et al., 2011). In addition to the possibility of ECM-mediated ciliary compartmentalization, in *C. elegans*, either sheath membrane or the membrane of nearby cilia may mediate the size of the InvC; contact with other cilia may exclude InvC components such that NPHP-2 localizes only in the proximal portion of the cilium where they are not bundled. If extracellular interactions regulate ciliary compartmentalization, I predict that cilia placement
would correlate with both the length of the InvC (possibly patterned by ECM/sheath/other cilia) and the length of the doublet region (possibly patterned by sheath-socket transition).

4.2.2 – The role of Wnt signaling in C. elegans ciliated neurons

Mammalian Inversin has few established functions, including a well-characterized role in the regulation of Wnt signaling (Simons et al., 2005) (Discussed in 1.5.1 – Signal Transduction and Amplification). Canonical Wnt signal functions by derepressing β-catenin activity (Reviewed in Huang and He, 2008; MacDonald et al., 2009). An extracellular soluble Wnt ligand binds to the transmembrane receptor Frizzled. Frizzled then binds to and activates disheveled, which then recruits Axin and APC, which were bound to β-catenin; this, in turn, prevents them from binding GSK3β which phosphorylates beta-catenin; phosphorylated β-catenin is ubiquitinated and degraded. When Wnt ligand is present and the Axin/APC/GSK3β complex is dissociated, beta-catenin enters the nucleus, binds to TCF/LEF transcription factors, and induces transcription. Inversin interferes with this process by binding disheveled and targeting it for degradation; this prevents the activation of disheveled and then subsequent inactivation of the Axin/APC/GSK3β destruction complex. As a consequence of the downregulation of this pathway, a second Wnt induced pathway, the planar cell polarity pathway, is induced; as suggested by the name, the PCP functions in orienting cells that are part of two dimensional surfaces, including the orientation of cilia on these surfaces.

I did not explore this possible function of nphp-2 in this work for two reasons. First, though the entire Wnt signaling pathway is present and does function in C. elegans, Wnt signaling has not been shown to function in ciliated neurons; establishing C. elegans as a viable model for ciliary Wnt signaling was beyond the scope of this project. Second, most of the mammalian work establishing Inversin as a Wnt modulator (Benzing et al., 2007; Lienkamp et al., 2010; Simons et
al., 2005) was completed before the localization of Inversin was characterized in detail (Hoff et al., 2013; Nakata et al., 2012; Shiba et al., 2009; Shiba et al., 2010); thus, the models presented in these earlier works are not compatible with the newer InvC complex model, as they draw assumptions based on the previously believed cytosolic/cilia base localization of Inversin.

However, this avenue of research should continue to be explored in light of newer advancements. NPHP-2 may physically interact with DSH-2 (shown in an uncontrolled yeast two-hybrid experiment), tantalizing evidence that this pathway is conserved and functional (Andrew Jauregui, PhD Thesis). It is difficult to analyze this pathway in *C. elegans* however, as all three *dsh* genes (*dsh-1*, *dsh-2*, and *mig-5*) are required for viability, functioning in early embryogenesis (Hawkins et al., 2005). *dsh-1* is expressed in unspecified neurons (McKay et al., 2003), and has a second role in targeting ACR-16 to the post-synaptic region, which is required for synaptic plasticity (Jensen et al., 2012). *dsh-2* is also reported to be expressed in unspecified neurons, though no neuronal function has been reported (McKay et al., 2003). Both *mig-5* and the *mom-5* Frizzled homolog are also reported to be expressed in the nerve ring, though colabelling was not used (Hao et al., 2006; Wu and Herman, 2006).

An important future direction is to establish whether Wnt signaling is a downstream effect of cilia functioning in *C. elegans*. To achieve this, it must be shown that in ciliated neurons, Wnt signaling components are expressed, Wnt pathways are functional, Wnt pathway output can be modulated by the cilium, and that Wnt components change localization and stability in response to ciliary pathways. (1) To demonstrate that Wnt genes are expressed in ciliated neurons by examining, in strains coexpressing a *daf-19* transcriptional reporter, transcriptional reporters of all three Disheveled genes, all four frizzled genes, the Axin homologs *axl-1* and *pry-1*, the APC homolog *apc-1*, the TCF homolog *pop-1*, and the β-catenin homolog *bar-1* (Harris et al., 2010). (2) It should also be demonstrated that the Wnt pathway is active in ciliated neurons; this can
be achieved with use of a modified POPTOP system (Green et al., 2008). The POPTOP system functions as readout of β-catenin activity. This system is viable in *C. elegans* and has been used to study cell polarity (Green et al., 2008). (3) If the canonical Wnt pathway is functioning in ciliated neurons, output of that pathway should be examined in various ciliary mutants, such as *nphp-2*, TZ mutants, doublet region mutants, and mutants of the modulators *hdac-6* and *arl-3*. Wnt output should also be examined in double mutants, as these processes may be regulated by redundant pathways. In addition to Inversin, Nphp4 also has a demonstrated role in Wnt modulation (Burckle et al., 2011); as *nphp-2* and *nphp-4* are redundant in ciliogenesis they may also be redundant in Wnt modulation (though these pathways could be one and the same). Additionally, to further refine the interaction between ciliary Wnt and ciliary pathways, Wnt activation should be examined in worms expressing modified versions of NPHP-2, including the N-terminal half, the C-terminal half, and full length missing the EF hand, D-box, and SUMOylation signals; the D-box and SUMOylation signals in particular may play a role in Wnt modulation as they be required for the targeted degradation of disheveled. (4) A final step would be to examine the subcellular localization and intensity of Wnt components in different ciliary mutants, which may reflect changing activation of the pathway by the cilium. (5) It may also be desirable to examine physical interactions between Wnt components and ciliary proteins. Though Inversin is known to physically interact with disheveled, other proteins may be involved (Simons et al., 2005). Like NPHP-2, ARL-13 possesses a SUMOylation motif (which is required for its function) (Li et al., 2012); additionally, PCP upregulation requires membrane accumulation of disheveled (as opposed to the cytoplasmic disheveled targeted by Inversin). This suggests that membrane associated-proteins may also modulate Wnt signaling. Establishing *C. elegans* as a ciliary Wnt signalling model would provide valuable insight into control and modulation of Wnt pathways.
4.2.3 – Regulating cilia placement

One of the earlier npih-2 phenotypes discovered was the misplacement of cilia (Warburton-Pitt et al., 2012). Using TZ fluorescent markers, it appeared that the bases of cilia were mispositioned, which prevented cilia from extending into the cilia pore and being exposed to the external environment. There were three possible defects that could lead to such a phenotype: dendritic extension defects, where the cilium is misplaced because of the failure of dendritic pathfinding, targeting, or tip anchoring pathways; ciliary anchoring defects, where the dendrite extends but the cilium cannot seat itself properly via interactions with neighboring cells or extracellular scaffolding; or glial cell defects, in which the cilium is anchored to glia or associated extracellular matrix, but the glia themselves are misplaced. These three possibilities are not mutually exclusive, and defects seen in different mutants may reflect these different underlying causes; this may also reflect the genetic redundancy which characterizes cilia genetics.

4.2.3.1 – Dendritic extension defects. Only the birth of amphid ciliated neurons is known in detail (Heiman and Shaham, 2009). After the final cell division and migration, the amphid cell body attaches to a site at the nose of the worm; this interaction is mediated by DEX-1, present on the neuronal membrane, and DYF-7, an ECM component. The anchoring site on the membrane is thought to be in association with the proto-cilium. Subsequently, the cell body migrates away from the nose, towards its final location within the nerve ring. As the cell body migrates, the physical attachment between the neuron and the ECM causes the membrane to stretch like taffy, elongating as the cell moves and forming the dendrite. Defects in the DEX-1/DYF-7 anchoring result in a cell body that has correctly migrated, but with a very short dendrite that does not extend more than a micron or two beyond the nerve ring. Amphid cilia of npih-2 single and npih-2 npih-4 double mutants only exhibit a slight
misplacement (3-5 microns) of cilia bases, which does not appear superficially similar to reported amphid Dex mutants (Figure 10). This suggests that nphp-2 and TZ genes are not required for dendritic anchoring either singly or in combination. The mild misplacement defect may arise from alternative mechanisms; it may also represent defects in a hypothetical post-anchoring fine-tuning of cilia placement.

It is completely unknown whether phasmid dendrites are formed similarly. If they are not, the cell body may be placed prior to neurite outgrowth—cilia defects may perturb this outgrowth. Several ciliary genes that are part of the TZ and doublet region SynDyf networks, including unc-119, have been linked to neurite branching in C. elegans, and other SynDyf genes can be predicted to function in neurite pathfinding based on biochemical functions of their mammalian orthologs, including possible Wnt regulation by nphp-2 (Ackley, 2014; Knobel et al., 2001; Zou, 2004).

Two striking pieces of evidence suggest both that phasmid dendrites form similarly to amphid dendrites and that ciliary components play a role in this process. First, overexpression of ARL-13 in mks-5 mutants leads to a highly penetrant Dex phenotype in both amphids and phasmids (Error! Reference source not found.), similar in appearance to the reported Dex phenotype in ex-1 and dyf-7 mutants. These defects are not seen when ARL-13 is overexpressed in other mks mutant backgrounds; that this phenotype is only evident in mks-5 mutants is unsurprising because of the role of mks-5 as the master-patterning component of the MKS physical TZ module. Secondly, when determining the localization of NPHP-2 in an mks-3; nphp-4 background, many animals exhibited a phasmid Dex phenotype, also similar to reported Dex phenotypes (Figure 30). In mks-3; nphp-4 animals expressing CHE-13::YFP, cilia placement appears intermediate between a full Dex phenotype and the misplacement seen in nphp-2 single mutants; this may suggest either overexpression of NPHP-2 as causing the Dex defect, or,
less likely, that overexpression of CHE-13 suppresses the defect. Taken together, this data is suggestive of a link between dendritic extension and cilia genes, but is currently too insubstantial to draw firm conclusions.

4.2.3.2 – Ciliary anchoring defects. The subtle misplacement of cilia in cilia mutants may be due to failure of the cilium to position itself properly in relation to its external environs. Two regions of ciliated neurons are closely associated with anchoring: the TZ of the cilium, and the dendritic tip. I consider these in turn.

4.2.3.2.1 – TZ anchoring: Within a cilium at the TZ, the axoneme is linked to the membrane through a large complex composed in part of MKS and NPHP proteins (termed a “Y-Link” due to its appearance in early EM micrographs) (Perkins et al., 1986). The Y-links may interact with transmembrane proteins and phospholipids, which in turn can bind the ECM and neighboring cells; this direct attachment between the axoneme and its external milieu is what may determine cilia positioning. Components of other cilia compartments, including the basal body region, the periciliary membrane, and the InvC may also mediate physical interactions with the environment, though nothing is known of this. Additionally, a physical link between cilia and their environment is supported by recent evidence in male specific cilia indicates that intraciliary trafficking may be regulated by external ECM components, including mec-5 and mec-9 (Deanna DeVore, Personal Communication). Y-link organization is disrupted in many TZ mutants and in nphp-2 mutants; this provides a plausible mechanism for cilia misplacement in these mutants (Warburton-Pitt et al., 2014). EM micrographs do not support or disprove this hypothesis, as a non-descript extracellular matrix surrounds the TZ region of the amphid bundle (no good quality phasmid EMs exist), in contrast with the aqueous, empty pore that surrounds the doublet and singlet regions (Perkins et al., 1986).
4.2.3.2.2 – Dendritic Tip Anchoring: Anchoring between the dendritic tip and the environment appears to be primarily mediated by tight junctions (Perkins et al., 1986). Both Nphp4 and Inversin localize to tight junctions in some mammalian tissue types, though these tight junctions are not in association with cilia (Delous et al., 2009; Nurnberger et al., 2002). In C. elegans, the orthologs NPHP-4 and NPHP-2 do not appear to localize to this region of the cell in most cell types, though a cursory examination shows that NPHP-2 may localize to this region in the ray cilia of male worms. Ciliary proteins may function in this region transiently during early stages of ciliogenesis, as this period of development has not been well characterized. At least one ciliary protein, ARL-13, has already been demonstrated to have a developmentally dynamic localization pattern, though in its case, localization still does not extend out of the cilium (Cevik et al., 2013). On the other hand, EMs of nphp-2 mutants indicate that tight junctions are intact, and disruption of tight junctions has not been reported in any cilia mutant (Warburton-Pitt et al., 2014). Taken together, the weak evidence in support and the moderate evidence against this proposition suggest that dendritic tip anchoring is not an important mechanism of ciliary anchoring. To test this suggestion, (1) tight junction components should be identified in the various ciliomes (2) and mutants of these components tested for cilia placement defects, (3) EM micrographs should be obtained from a number of cilia mutants and carefully analyzed, (4) and tight junction markers should be examined in cilia mutants.

4.2.3.2.3 – InvC anchoring? A more speculative possibility is that the InvC itself mediates anchoring between the cilium and the environment. Though Inversin is an intracellular, soluble protein, it does localize to adherens junctions in some cell types, indicating that Inversin can mediate cell-cell interactions (Nurnberger et al., 2002). This hypothesis has not previously been suggested because the InvC is not yet formed at the time of cilia docking during early
ciliogenesis (Williams et al., 2011). However, the ciliary vesicle docking and cilia anchoring steps of ciliogenesis may be in fact two separate steps. If anchoring occurs after elongation of the axoneme begins (and thus after InvC biogenesis), it is possible the InvC can regulate final cilia placement; deletion of \textit{nphp-2} would thus lead to mispositioning defects.

An alternative is that TZ anchoring is not initially static; recent evidence in \textit{Drosophila} shows that the TZ is “pushed” or “extruded” into the cytoplasm after initial ciliogenesis by axonemal extension (Basiri et al., 2014). If such TZ mobility also exists in \textit{C. elegans}, it may be that the InvC is providing the primary ciliary anchoring after the TZ begins to move. The mild displacement defects seen in \textit{C. elegans} TZ and \textit{nphp-2} mutants is on the same order of magnitude as would be expected if there were defects in this “TX extrusion” pathway; this is significantly different from the more severe dendritic extension defects on cilia placement.

\textbf{4.2.3.3 — Glial cell defects.} Two types of glial cells support amphid channel and phasmid cilia (among other cilia types): the socket cell, which surrounds the distal end of the cilia bundle, and the sheath cell, which surrounds the proximal portion of the bundle and the majority of the length of the dendrite (Figure 2) (Perkins et al., 1986). These glial cells have very different morphologies: the socket cell wraps around the bundle of cilia and forms tight junctions with itself, and is covered by a thick layer of ECM continuous with the cuticle, while the sheath cell surrounds all ciliated dendrites individually, as they are embedded in and form tight junctions with it, forming a non-simply connected topology. Additional tight junctions connect the sheath and socket cells for a given cilia bundle together. The junction between the sheath and socket cells lies in the same plane as the doublet to singlet transition within cilia, providing the subtle suggestion that these cells may influence (or be influenced by) cilia compartmentalization.

Strong evidence links cilia placement to these glial cells. \textit{mks-3; nphp-4} double mutants exhibit
misplaced cilia (as described above); visualizing the sheath cell with F16F9.3 reveals that the sheath cell is also misplaced, but is still attached correctly to the cilium. Even more remarkably, visualizing the socket cell with MAGI-1 in the same mks-3; nphp-4 double mutant reveals that the socket cell is properly placed at the ciliary pore adjacent to the cuticle (Williams et al., 2010).

As neither mks-3 nor nphp-4 are expressed in any cells other than ciliated neurons, cilia positioning may actually dictate sheath placement, instead of the supposed other way round. mks-3 and nphp-4 may convey signals (possibly from signals transduced by the cilium itself) into the sheath cell that instruct the sheath where to position itself. The dissociation of the socket and sheath cells may arise from four distinct possibilities. (1) mks-3 and nphp-4 may actually be expressed in sheath and socket cells at levels that are not detectable using standard techniques. (2) mks-3 and nphp-4 may function in cilia, transmitting signals to the sheath cell that it requires to properly coordinate its interaction with the socket cell. (3) The sheath cell is born alongside the ciliated neuron, binds the dendritic tip of the ciliated neuron, and travels with the dendrite as it makes its way to the cilia pore; if the cilia are misplaced, then the sheath cell never comes into contact with the socket cell and no junctions can form. (4) The tight junction between the socket and sheath cells is weak and is simply too physically weak to hold the cells together when any amount of tensile force is applied between them (the tight junction acting as a permeability barrier instead of a structural role). Of these possibilities, all four are possible in the phasmid organ, but only 1, 2, and 4 are possible in the amphid organ.

mks-3; nphp-4 is the only genotype that has been examined for ciliary defects; future work should focus on examining the roles of the other TZ and doublet region genes in this phenotype. Placement defects in doublet region mutants may arise from their defects in TZ organization, which subsequently interfere with ciliated neuron-glia interactions.
4.2.4 – Are NPHP-2 cell body accumulations linked to membrane trafficking pathways?

Above (4.2.1.2 – Membrane composition) I discussed the possible roles of membrane trafficking pathways in the origin of the InvC.

In addition to InvC localization, NPHP-2L also localizes to puncta within the cell body (Warburton-Pitt et al., 2012). It was originally hypothesized that these puncta represented centriolar localization, as a large number of ciliary genes have alternate roles in cell cycle regulation (Andrew Jauregui, PhD Thesis). However, I saw that there were infrequently two to three NPHP-2L puncta in amphid and phasmid cilia, and upwards of 6-8 puncta in male specific neurons; this eliminates the possibility of centriolar localization. Instead, these accumulations are reminiscent of membrane trafficking microcompartments seen with Rab8 pathway markers (Kaplan et al., 2010). NPHP-2, the InvC and ciliary compartmentalization have all been associated with membrane biology (See 1.4.5 – Membrane Dynamics of the Inv Compartment).

Rab8 is a small GTPase that functions in ciliogenesis and regulation of traffic between endosomes and the periciliary membrane (Bae et al., 2006; Moritz et al., 2001 Nachury et al., 2007; Omori et al., 2008). Mammalian Rab8 has been physically and genetically linked to many ciliary components, including CEP290 (MKS-4), a predicted lipid binding protein that lies downstream of Rab8 function (Tsang et al., 2008); CC2D2A (MKS-6), which mediates vesicle trafficking (Tallila et al., 2008); and in worms, the BBSome, which links IFT-A and IFT-B and regulates multiple aspects of IFT machinery assembly and function (both CEP290/MKS4 and CCD2A/MKS6 are components of the TZ MKS module). RAB-8 is targeted to the basal body, and then binds to the BBSome, which transports it into the cilium (Nachury et al., 2007).

While rab-8 mutants do not have any overt ciliogenic defects in C. elegans cilia, overexpression leads to amphid and phasmid dye-filling defects, suggesting that RAB-8 may act as an antagonist
of ciliogenesis (Kaplan et al., 2010). Additionally, RAB-8 overexpression leads to ODR-10 (a ciliary receptor) trafficking defects in AWB cilia. UNC-101/AP-1, a clathrin coat component, is also required for proper ODR-10 targeting and amphid/phasmid ciliogenesis. As in the cilia of unc-119 mutants, cilia of unc-101 mutants do not have singlet regions, and like nphp-2 mutants, unc-101 mutant cilia have microtubule patterning defects and alterations in ciliary glutamylation. RAB-8 overexpression phenocopies these aspects of unc-101 mutants (Kaplan et al., 2010; Ou et al., 2007).

In addition to their ciliary localization, RAB-8 and UNC-101 also localize to puncta in the cell body in both mammalian cell models and C. elegans ciliated neurons (Kaplan et al., 2010). In C. elegans, colabelling reveals that these puncta do not overlap, but instead directly abut, marking two separate regions that function in subsequent steps in the same membrane trafficking pathway: UNC-101 functions in vesicle budding from the trans-golgi network, and then rab-8 functions in endosomal sorting. (This can be seen in the case of ODR-10, which colocalizes with RAB-8, indicating that it accumulates here after leaving the TGN but before being trafficked to the cilium). Whether NPHP-2L puncta localize to one of these compartments is unknown. Such a role for NPHP-2/Inversin would not be surprising: like Inversin, Ahi1/JBTS3 acts as a nexus physically linking multiple TZ modules (Sang et al., 2011); Ahi1 also binds Rab8, and may play a role in membrane trafficking (Hsiao et al., 2009).

Separate (or forked) cilia membrane trafficking pathways may exist; these pathways function in parallel to IFT trafficking pathways. The Arl13b/INPP5E complex and IFT are required for PC2/PKD-2 trafficking, but Rab8, NPHP-2, and Arl3 are not (Bae et al., 2009; Cevik et al., 2010; Kaplan et al., 2010; Zhang et al., 2013); rab-8 and unc-101 are required for ODR-10 trafficking, but IFT is not (Kaplan et al., 2010). ARL-13 and IFT do not function in NPHP-2 trafficking; whether rab-8 and unc-101 do is unknown (Warburton-Pitt et al., 2014). nphp-2 and arl-13 genetically
interact in *C. elegans*, which suggests that these genes have multiple functions (and function in separate pathways) or that they function in the same pathway before (*arl-13*) and after (*nphp-2*) it forks. Additionally, *rab-8* is not required for TAX-2 and TAX-4 trafficking in ASK or AWB cilia, whereas *nphp-2* is required; *rab-8* deletion can suppress *tax-4* cilia membrane extension defects however (Wojtyniak et al., 2013).

Membrane trafficking is a hugely complex field; the above discussion scratches it only with a microtome. The focus of the following proposed set of experiments is to determine the role of NPHP-2 in cilia membrane trafficking pathways. The above data suggest that NPHP2 may function in *rab-8*, but not *cil-1*/INPP5E membrane pathways. (1) First, double labeling between NPHP-2L, RAB-8, and UNC-101 will reveal if NPHP-2 localizes to one of the *rab-8* pathway puncta. This will determine if NPHP-2L functions in, or is shuttled by, this system. NPHP-2L may not overlap with the other markers; whether this NPHP-2L punctum lies in close association with the RAB-8 and UNC-101 compartments will help determine if NPHP-2 actually marks a separate step in the process. (2) Second, NPHP-2 localization should be examined in *rab-8* and *unc-101* single and double mutants. This will determine if NPHP-2 is trafficked by this pathway. (3) Third, the localization of known *rab-8* pathway cargo (including ODR-10) should be examined in *nphp-2* single mutants, and *nphp-2, rab-8, unc-101* double and triple mutants. (4) Fourth, as membrane composition has a critical role in trafficking, membrane composition in these mutants should be determined, using the markers proposed above (4.2.1.2 – Membrane composition). (5) Fifth, *unc-119* functions in trafficking of membrane-associated proteins (and can insert and remove them from the membrane), genetically interacts with *nphp-2* and the membrane-associated *cil-1* and *arl-3* (the former, but not the latter, required for PKD-2 trafficking), and phenocopies *unc-101* ciliogenesis defects. Whether *unc-119* functions in membrane trafficking has not been thoroughly explored; if *unc-119* has a role, the localization of *rab-8* pathway cargos and *cil-1*
pathway cargoes should be determined in *unc-119* mutants, and the localization of UNC-119 should be determined in *rab-8* and *unc-101* mutants.

Together, this set of experiments will greatly expand our understanding of ciliary membrane trafficking and targeting, linking them to other aspects of cilia biology, and possibly assigning function to *nphp-2* and *unc-119*.

### 4.2.5 – Ciliary protein shuttles (non-IFT methods of active transport)

As discussed in Chapter 1 (1.4.4 – Establishing the Inv Compartment), a set of three protein trafficking shuttles is associated with the localization of ciliary proteins: the Unc119b, the Pde6d, and the BART shuttles. There is strong evidence that these shuttles play key roles in cilia import and dynamics of the ciliary membrane, though the mechanisms and genetics are largely unknown.

The Unc119b shuttle has been thoroughly characterized (Wright et al., 2011). Briefly, (1) Unc119b binds myristoylated cargo through a conserved hydrophobic binding pocket, (2) the Unc119b-cargo complex is translocated into the cilium, (3) the GTPase Arl3-GTP binds to the complex, (4) Rp2 binds Arl3-GTP and causes Arl3-GTP to hydrolyze its GTP, (5) Arl3-GDP induces conformational changes in the binding pocket of Unc119b that cause it to release its cargo, (6) and then C5orf30 binds empty Unc119b and escorts it out of the cilium (Figure 6). This overview reveals the paucity of details needed to fully understand the Unc119b shuttle: What are the cargoes of Unc119b? How/where does Unc119b bind its cargoes? Unc119b can remove acylated proteins from the membrane; what sort of membrane can it remove them from (e.g., intracellular vesicles, MVBs, plasma membrane)? How is the complex translocated into the cilium? How does Arl3-GDP revert to Arl3-GTP? What triggers Rp2? How is the Unc119b-C5orf30 complex exported from the cilium? Can Unc119b target its cargoes to specific subdomains of the
cilium? Can membrane composition alter the function of Unc119b?

Many components of the Unc119b shuttle are conserved in *C. elegans* and localize to the cilium, suggesting the worm may be a valid model to examine this shuttle system. *UNC119B/unc-119*, *ARL3/arl-3*, and *RP2/rpi-2* are conserved (Harris et al., 2010); C5orf30 and the only known Unc119b cargo (InvC restricted Nphp3) are not. However, two other cargoes of UNC-119 have been identified (GPA-13 and ODR-3, both myristoylated G-protein subunits), *arl-3* deletion suppresses defects in *unc-119* mutants, and the InvC progenitor *nphp-2* genetically interacts with *unc-119* (Warburton-Pitt et al., 2014; Zhang et al., 2011). Additionally, mutations in *cil-7*, which encodes a myristoylated protein, lead to a reduction in total neuronal UNC-119 fluorescence; this suggests that CIL-7 may not be a cargo but be a regulator of UNC-119 (Julie Maguire, Personal Communication).

Several experiments are possible: (1) Screen the *C. elegans* genome for predicted N-terminal myristoylated proteins, and cross-reference this list with genes containing an X-box. This list would constitute possible UNC-119 binding partners, and should not be longer than a few dozen genes. (2) Examine localization of these predicted binding partners in *unc-119* mutants. (3) Once two or three proteins have been identified that require *unc-119* for proper localization, then they can be used to dissect the *unc-119* shuttle; two or three proteins are required so that conclusions regarding the shuttle can be generalizable. Questions that can explored using UNC-119 cargo reporters include the roles of *rpi-2, arl-3*, and *nphp-2* in ciliary restriction and release of cargoes from UNC-119, the role of the TZ in ciliary translocation, and whether IFT is required. This experiment will hopefully resolve the conundrum of why *arl-3* deletion suppresses *unc-119* defects. The current model of Unc119b shuttle function predicts that *arl-3* defects should phenocopy *unc-119*; it may be that the shuttle and ciliogenesis roles of *unc-119* are separate. (4) The role of *unc-119* in singlet region biogenesis can also be explored: *unc-119*
mutants have defects in biogenesis or singlet region protein localization. *mec-12*, which encodes
an alpha-tubulin, is a likely candidate for this, as its mammalian ortholog is myristoylated.

*C. elegans* may also be a useful model to study the Pde6d protein shuttle. Pde6d is closely
related to Unc119b, and functions similarly: it binds farnesylated proteins through a
hydrophobic pocket, the shuttle-cargo complex is shuttle into the cilium and is bound by
Arl2/3/13, of which hydrolysis of Arl3-GTP triggers the release of the cargo (Humbert et al.,
2012). However, these details are not certain and I have drawn them largely through inference
and comparison with the Unc119b shuttle. Many of these components too are conserved in
*C. elegans*, including all three *arl* genes, *PDE6D/pdl-1*, and a possible cargo, *INPP5E/cil-1* (Bae et
al., 2009; Harris et al., 2010). Curiously, many of these components are specifically linked to
PKD-2 trafficking in male ciliated neurons, suggesting that this shuttle may function specifically
in these cell types. Initial proposed experiments regarding this shuttle serve to establish that it
does indeed exist, as opposed to the Unc119b experiments, which serve to clarify the
mechanism and targets. First, the epistatic interactions between these shuttle components
should be established by examining localization of components in mutant animals missing other
components. Second, the cargo binding pocket of PDL-1 should be altered such that it can no
longer bind farnesy1 groups; the localization of this mutant PDL-1 should be examined to
determine whether PDL-1 functions as a cargo shuttle or whether the binding pocket regulates
the function and localization of PDL-1. Third, the relationship between the various *arl*s that bind
PDL-1 is muddled; this model can be used to test the interactions and requirements of these *arl*s
by examining CIL-1 localization in the *arl* mutants (or PKD-2::GFP, as CIL-1::GFP is difficult to
construct), and animals with ARL constitutively bound to GTP or GDP. Fourth, bioinformatic
databases should be used to try to identify other pathway components, both upstream and
downstream from *pdl-1*. Large physical and genetic databases exist for both mammalian models
and *C. elegans*. Finally, as PKD-2 localization defects have been demonstrated to be largely due to defects in extracellular vesicle pathways, and the biogenesis of these vesicles is intimately tied to membrane biology, it would be unsurprising if the PDL-1 shuttle functioned in this pathway, perhaps by loading membrane-associated proteins onto vesicle membranes.

The BART shuttle, also related to the Unc119b shuttle is almost completely uncharacterized (Bailey et al., 2009; Sharer et al., 2002). BART (Binder of Arl2) is not conserved in *C. elegans*, so it is unlikely that *C. elegans* would be a viable model to characterize this pathway. Additionally, BART has not been associated with cilia and has only been associated with mitochondrial transport and centrosomal processes.

### 4.2.6 – The TZ and doublet region may be dynamic

Several pieces of evidence suggest that ciliary protein localization and compartmental composition are dynamic depending on developmental stage or environmental conditions, including physical interactions between InvC and TZ proteins (Sang et al., 2011), localization of ARL-13 in L1 stage worms (Cevik et al., 2013), and the role of nphp-2 in cilia positioning (Warburton-Pitt et al., 2014; Warburton-Pitt et al., 2012). Though this assessment appears superficially facile, it is not necessarily obvious in *C. elegans*, as cilia form only on terminally differentiated neurons, and likely do not go through the cycle of retraction and growth as do cilia of other organisms (Perkins et al., 1986). Of note is the fact that ciliogenesis is plastic: in *che*-2 mutants, which have no cilia, CHE-2 expression *at any time* in the animal’s life, including the adult stage, can rescue *che*-2 mutant defects, allowing a functional cilium to grow (Fujiwara et al., 1999). (See also 4.2.3 – Regulating cilia placement – InvC anchoring).

#### 4.2.6.1 – Evidence 1: TZ localization of InvC and doublet region components.

The first suggestion that cilia are not static arose from physical interaction experiments. Work in
mammalian systems revealed that NPHP-2/Inversin and Nphp3 physically interact with components of the transition zone (Sang et al., 2011; Andrew Jauregui, PhD Thesis). This is somewhat peculiar as the localization of NPHP-2/Inversin and Nphp3 do not overlap with the TZ; this may be explained by (1) transient localization, (2) low quantities of TZ localized protein, or (3) developmental stage specific localization. Addressing the first possibility, it was found that Nphp3 is targeted to the TZ before it is imported into the cilium; this was only observable when certain domains of Nphp3 were deleted, causing it to accumulate at the TZ—Nphp3 TZ localization was too transient to be visualized otherwise (Nakata et al., 2012). It is still unknown when and why NPHP-2/Inversin interacts with TZ components, though it may also be for ciliary targeting. NPHP-4 physically interacts with the N-term ankyrin repeats of NPHP-2 (Andrew Jauregui, PhD thesis); an important experiment would be to determine the localization of the N-terminal portion of NPHP-2 (this has only been done in specialized AWB cilia, where the ankyrin repeat portion was targeted to the cilium but localized along all the AFD branches [Wojtyniak et al., 2013]). The second possibility, that the quantities on InvC and doublet region proteins residing at the TZ are too small to visualize, is not easily addressed. Several methods to increase a possible dim signal include use of a sensitive confocal microscope, use of tandem GFP tagged protein (e.g., NPHP-2::GFP::GFP::GFP), or use of anti-GFP antibodies (as multiple antibodies can bind to a single target, whether the target be the protein of interest or the primary antibody).

4.2.6.2 – Evidence 2: Doublet region protein localization is developmentally dynamic. A surprising recent discovery by the Blacque Lab was that ARL-13 localization is not static through development, and that during L1, ARL-13 localized along the entire phasmid cilium and undergoes IFT (Cevik et al., 2013). It is unknown whether this represents an earlier stage of
ciliogenesis, or whether it reflects a different function of the cilium during that life stage. Little
time has been spent characterizing cilia in these early stage larvae; most features of the cilium,
including ultrastructure, ciliary protein and membrane composition, and IFT composition and
velocities are unknown, so interpretation of the ARL-13 result is difficult. I examined the
localization of NPHP-2 in L1 animals, and found that it was still restricted to the proximal cilium
(Warburton-Pitt et al., 2014). As ARL-13 is generally associated with microtubule doublets, it is
tempting to speculate that at this stage the entire cilia shaft is doublets, and that because the
InvC is not organized by ultrastructure, it appears similar to in adults. This question is rich with
possibility, and entails many further experiments and questions. In addition to the unknowns
outlined (ultrastructure and IFT) above, other salient aspects to investigate include
characterizing glutamylation, looking at localizations of TZ and doublet region proteins,
examining localization in conjunction in *dex-1* and *dyf-7* mutants (Heiman and Shaham, 2009),
looking at localization during embryogenesis, capturing a virtual time-lapse from L1 to L4 (made
by following an individual worm for only a few hours and then combining data from multiple
different staged worms together), and so on.

4.2.6.3 – Evidence 3: Ciliary anchoring is mediated by *nphp-2*. (See also 4.2.3 – Regulating cilia
placement) *nphp-2* mutants exhibit misplaced cilia; because the InvC is not formed prior to
ciliary docking, InvC components cannot localize to the InvC and their localization may vary
depending on developmental stage. Ciliogenesis is a two-step process that begins with the
formation of a pericentriolar complex, which is followed by the binding of the ciliary vesicle, and
then extension of the TZ (Williams et al., 2011). At this point, the vesicle docks with the plasma
membrane, and then IFT begins and fully elongates the cilium. This model leads to several
questions: How does the ciliary vesicle identify the patch of membrane to which to will dock?
Are docking and anchoring synonymous or are they separate, temporally distinct steps? How does an InvC component modulate cilium position when the InvC does exist prior to docking?
The second question suggests that Inversin may function in cilia anchoring after the formation of the InvC, and does not entail dynamic, stage dependent localization. The third question, which supposes that docking and anchoring are the same step, suggests that Inversin must localize to a different part of the cilium during this early phase.

4.3 – Concluding Remarks

The primary cilium was once thought of as a functionless vestigial organelle. Further work continually reveals hidden complexity in ciliary structure, composition, and function, yielding an organelle that some have termed “the brain of the cell,” an organelle that modulates processes ranging from cell-cell adhesion to DNA damage response, from signal transduction to ECV release, and from cell motility to mitosis.
Recent work has dissected TZ composition and layout using super-resolution microscopy, revealing that the once thought to be homogenous TZ is in fact an intricately laid-out assembly. Data suggests that this may be true for the InvC as well, possessing an Evc/Evc2 “micro-compartment”, and membrane-associated and axoneme-associated complexes. A radical interpretation may be that there is no distinct TZ and InvC, that they are one and the same, and that the proximal ciliary localizations I observe are actually a gradient of continually varying protein localizations, a striped compartment composed of many overlapping localization patterns. The proteinaceous TZ Y-links have traditionally been used to delineate this compartment, though whether all Y-links have identical composition, and whether Y-link components can participate in other assemblies elsewhere is unknown.
The body of work characterizing the InvC fills in crucial holes in the grand picture, but as is
(happily) expected, has led to even more questions. How does it regulate aspects of ciliogenesis when it has yet to form at that stage? What sets up the InvC in the first place? Does it regulate biogenesis of the singlet region? How does it regulate IFT? How does it integrate all of the different functions ascribed to it? Are there even more ciliary micro-compartments that are still hidden to us? And finally, what is the relationship between the separate, yet inseparable, InvC and TZ?
4.4 – References


