

Compartments within a compartment

What *C. elegans* can tell us about ciliary subdomain composition, biogenesis, function, and disease

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The primary cilium has emerged as a hotbed of sensory and developmental signaling, serving as a privileged domain to concentrate the functions of a wide number of channels, receptors and downstream signal transducers. This realization has provided important insight into the pathophysiological mechanisms underlying the ciliopathies, an ever expanding spectrum of multi-symptomatic disorders affecting the development and maintenance of multiple tissues and organs. One emerging research focus is the subcompartmentalised nature of the organelle, consisting of discrete structural and functional subdomains such as the periciliary membrane/basal body compartment, the transition zone, the Inv compartment and the distal segment/ciliary tip region. Numerous ciliopathy, transport-related and signaling molecules localize at these compartments, indicating specific roles at these subciliary sites. Here, by focusing predominantly on research from the genetically tractable nematode *C. elegans*, we review ciliary subcompartments in terms of their structure, function, composition, biogenesis and relationship to human disease.

Overview of Ciliary Compartmentalisation, Transport and Disease

Over the past 15 years more than 30 distinct human diseases, embracing at least 80 genes, have been associated with defects in cilia, which are motile or non-motile microtubule-based extensions protruding from most eukaryotic cell surfaces.^{1,2} These disorders, termed ciliopathies, display a wide range of overlapping pathologies including respiratory dysfunction (e.g., primary ciliary dyskinesia), cystic kidneys (e.g., polycystic kidney disease, nephronophthisis), organ laterality defects (e.g., situs inversus), retinal degeneration (e.g., retinitis pigmentosa), bone abnormalities (e.g., Meckel-Gruber syndrome), obesity (e.g., Bardet-Biedl syndrome) and central and peripheral nervous system defects (e.g., Joubert syndrome).² Collectively, these pleiotropic phenotypes underscore the functional importance of the cilium in a

wide range of cellular processes that includes cell and fluid movement, phototransduction, chemosensation, and mechanosensation, as well as developmental signaling involving sonic hedgehog and possibly Wnt, TGF- β , PDGF α , and Notch ligands.^{3–9}

Conducting these functions are a diverse range of receptors, channels, signaling molecules, transport machineries and ciliopathy proteins that are enriched or almost exclusively found in cilia. While many of these proteins localize along the entire axoneme, others are asymmetrically distributed at distinct ciliary subdomains such as the transition zone and Inv (inversin) compartments, indicating specific roles and disease mechanisms for these subregions.^{10,11} Protein transport and restriction to cilia is driven by various polarized ciliary transport regulators, as well as by cytosolic and membrane diffusion barriers at the ciliary base that restrict access to the organelle and preserve its composition.^{10,12–18} One key pathway is intraflagellar transport (IFT), which operates bi-directionally along ciliary microtubules to deliver and distribute “cargo” proteins required for cilium structure and function.^{19,20} The IFT machinery consists of anterograde (kinesin-2) and retrograde (cytoplasmic dynein) motors, as well as an associated IFT particle comprised of two subcomplexes, A and B. Ciliary protein transport also heavily relies on secretory and endocytic membrane regulators such as coat adaptors, small GTPases and the exocyst complex, all of which facilitate vesicle-derived ciliary membrane (and associated protein) delivery and retrieval.^{21–33} Multiple studies also report that passage of proteins into cilia employs cilium-localized nucleocytoplasmic transport regulators (karyopherins) and nuclear pore complex proteins (nucleoporins),^{17,34–37} although there is some uncertainty about the latter based on recent conflicting evidence.³⁸ For delivery of ciliary proteins, one scenario is vesicle trafficking via secretory pathways to the periciliary membrane, followed by IFT-facilitated entry into cilia. However, this model is overly simplistic because some membrane proteins enter cilia independent of IFT.³⁹ Furthermore, soluble proteins below a certain size can enter the cilium via simple diffusion, indicating IFT may traffic only relatively large proteins (>40–100 kDa).^{17,38} Thus, protein trafficking and sorting to cilia depends on multiple pathways, with no universal mechanism, including the lack of universal ciliary protein sorting motifs.

In this review, we focus on the various subciliary domains, namely the periciliary membrane/basal body, transition zone,

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Inv, and distal segment/tip compartments. Predominantly, we focus on research from the *Caenorhabditis elegans* nematode that has led to a greater understanding of subcompartment composition, biogenesis, function, transport, and relationship to human disease.

Subcompartments of Cilia

All eukaryotic cilia can be subdivided into distinct subcompartments, each with unique ultrastructure and protein compositions (Fig. 1A). The most proximal subcompartment is the mother centriole-derived basal body (BB) and surrounding periciliary membrane, which invaginates in some mammalian cells and Trypanosomes to form a ciliary pocket.²⁷ The BB centriole, normally consisting of 9 triplet microtubules, nucleates ciliary axoneme extension and is plasma membrane-docked via distal appendages, which are also called transition fibers (not to be confused with the Y-links of the adjacent transition zone subcompartment; see below).⁴⁰ These fibers anchor the mother centriole during early ciliogenesis and may form part of a ciliary pore, with septins and possibly nuclear pore complex proteins, regulating protein and membrane transport across the ciliary base.^{10,16,17,40,41} Indeed, in green algae, distal appendages organize the “ciliary bracelet,” a membrane specialization defined by intramembrane particle arrays and absence of certain sterols, which could act as a barrier to lateral membrane transport.^{42,43} Distal appendages also represent a probable site of docking, assembly, and turnaround of IFT trains.⁴⁴ Additional accessory features of the BB are microtubule-associated subdistal appendages, basal feet and ciliary rootlets, all of which are thought to anchor, stabilize and position the centriole and associated axoneme.^{45,46} The periciliary membrane enveloping the BB also serves as a docking site for vesicles carrying cilium-destined cargos, and a zone of endocytosis and membrane/receptor retrieval.^{18,24,27,33}

Emanating from the BB is the transition zone (TZ) compartment, which forms the first 0.5–1.0 μm of the microtubule doublet-containing ciliary axoneme.^{10,47} The TZ is distinguished ultrastructurally by characteristic three dimensional Y-link structures of unknown composition that connect each of the nine doublets with the ciliary membrane (Fig. 1A). Multiple studies implicate the TZ as a ciliary gate, imparting physical and molecular diffusion barriers to cytosolic and membrane protein transport across the ciliary base, thereby serving to separate the cilium from the rest of the cell.^{12–15,48} Indeed, like the BB distal appendages in algae, Y-links in protists and mammals organize a TZ membrane specialization termed the “ciliary necklace,” consisting of concentric rings of intramembrane particles.⁴⁹ Consistent with the TZ being a distinct ciliary subcompartment, numerous proteins implicated in Nephronophthisis, Meckel-Gruber syndrome and Joubert Syndrome specifically localize within this domain and regulate gating, perhaps as structural subunits of the Y-links.^{12–15,48}

The ciliary axoneme distal to the TZ frequently displays distinct proximo-distal patterning at the level of ultrastructure and protein localization, indicative of subcompartmentalisation and

regionalised sensory and signaling functions. For example, variations in microtubule (MT) arrangement define proximal (doublet MTs) and distal (singlet MTs) ciliary segments in nematodes, mating green algae, and various vertebrate cell types (olfactory, pancreatic, renal).^{50–54} Furthermore, mammalian ciliopathy proteins such as Inversin/NPHP2, NPHP3, NPHP9, and ARL13B (in some cell types) localize specifically to a proximal ciliary region (excluding the TZ) termed the Inv compartment.^{11,48,55} Various proteins are also concentrated in distal ciliary regions and at the ciliary tip, including frog olfactory CNG channels and sonic hedgehog signaling proteins such as Gli1/2/3 and Sufu.^{56–58}

C. elegans as a Model for Investigating Cilium Biology and Compartmentalisation

In the *C. elegans* adult hermaphrodite of 959 somatic cells, non-motile primary cilia extend from the distal dendrite tips of 60 sensory neurons (Fig. 1B). Most cilia are part of environmentally exposed pores (sensillae) in the cuticle, serving chemo-, thermo-, and osmolarity-sensing functions.⁵⁰ Importantly, many genes associated with cilium biogenesis, transport and disease are conserved in *C. elegans*. Also, worms possess an extensive experimental toolbox for cilium research such as the availability of mutant alleles, behavioral assays for cilium function, transport assays for IFT and vesicular trafficking, and fluorescence assays for determining precise protein localizations at the sub-ciliary level. Thus, despite lacking most organs associated with mammalian cilia function and disease, *C. elegans* has emerged as a leading metazoan model for investigating basic underpinning cilium biology and pathomechanisms at the cellular level.

Worm research has been instrumental in understanding the molecular basis of IFT, its regulation, and ciliary subtype variation.^{19,59} Furthermore, *C. elegans* research has provided mechanistic insight into ciliopathy protein function such as the role of BBS proteins and Joubert Syndrome-associated ARL-13/ARL13B in IFT regulation, and ciliary gating functions for MKS and NPHP at the TZ.^{15,60–64} Various membrane trafficking regulators have also been linked to *C. elegans* cilium formation and protein transport, including clathrin adaptor (AP1, AP2) subunits, various small GTPases (RAB-5, RAB-8), OSTA-1 (organic solute transporter α -like protein), and SQL-1/GMAP210 (golgi microtubule associated protein).^{24,25,65–67} Finally, nematode forward genetics, transcriptomics and bioinformatics approaches have identified new conserved ciliary proteins, including IFT components DYF-1/IFT70, DYF-2/IFT144, DYF-11/IFT54, DYF-13/TTC26, and IFTA-1/IFT121.^{63,68–72}

C. elegans cilia possess heterogeneous morphologies ranging from rod-shaped to organelles that are forked, branched or with membrane expansions.^{73,74} Most research has focused on the bilateral amphid (head) and phasmid (tail) olfactory sensillae, consisting of 12 and 2 ciliated cells, respectively. In these pores, axonemes are typically rod-shaped, possessing the canonical subcompartments described above, with some notable distinctions^{50,73,74} (Fig. 1A and B). First, the ciliary base lacks a basal body centriole and associated accessory structures.⁷⁴ Until

recently, it was thought that distal appendages are present at the ciliary base; however, a stunning new electron tomography study of 50 3-D reconstructed nematode cilia now shows that the “appendage-type” electron densities are likely to be a flaring out of the ciliary doublet microtubules.⁷³ Thus, it would appear that once the mother centriole has docked to the plasma membrane during cilium initiation, all centriolar and accessory structure is lost. Second, the ciliary base sits at the apex of an $\sim 0.5 \mu\text{m}^2$ asymmetric swelling of the dendrite tip, termed the periciliary membrane compartment (PCMC).²⁴ As will be discussed below, the PCMC is enriched for various ciliary proteins, and serves as a site of homeostatic regulation of ciliary membrane trafficking. Third, amphid and phasmid channel cilia possess bipartite microtubule arrangements that define a proximal or ‘middle segment’ consisting of doublet MTs, and a “distal segment” comprised of singlet MTs due to termination of each doublet B-tubule at the mid-point of the axoneme.⁷⁴ These subcompartmental distinctions, while perhaps unusual, are not specific to *C. elegans*. For example, mature rat sperm flagella lack a basal body centriole and ciliary A-tubule extensions are found in vertebrate cells.^{51,75,76} Also, as will be discussed, the middle segment is analogous to the mammalian Inv compartment. Below follows a detailed description of the nematode ciliary subcompartments, with a focus on their composition, functions, and relationship to disease.

C. elegans Ciliary Base and Periciliary Membrane Compartments

Despite lacking an anchoring mother centriole and associated appendages,^{73,74} the nematode ciliary base appears to retain the functionality of a basal body. In the first instance, counterparts of many vertebrate basal body-associated proteins are also found at the nematode ciliary base, beneath the transition zone. These include IFT particle proteins, the BBSome, the ciliary rootlet-associated rootletin protein, FBF1/DYF-19 and centriole-associated HYLS-1 (hydrolethalus syndrome 1) (Fig. 2A).^{15,19,29,60,77-79} In *C. elegans*, BBSome proteins control anterograde kinesin-2 motor associations and IFT turnaround, and DYF-19 regulates ciliary entry of assembled IFT machinery.^{64,79} Similar to its mammalian counterpart, *C. elegans* striated rootletin stabilizes ciliary axonemes.^{78,80} Also, non-filamentous rootletin stabilizes nematode cilia lacking rootlets, perhaps by regulating vesicular trafficking in the PCMC.⁷⁸ Thus in worms, a centriole and associated appendages, including distal appendages, are not required for stabilizing ciliary axonemes and maintaining a transport hub at the ciliary base. Instead, these functions may be facilitated by the proximal ends of the nematode ciliary microtubules, which extend proximally into the PCMC.⁷³

As outlined above, the degenerated nematode ciliary base is typically surrounded by a morphologically distinct patch of periciliary membrane, or PCMC, demarcated proximally by a circumferential adherens (belt) junction between the ciliated neuron and supporting glial cell. We consider this swelling of the distal dendrite tip region an adjunct ciliary subcompartment because of its specific enrichment for multiple cilia-related sensory signaling

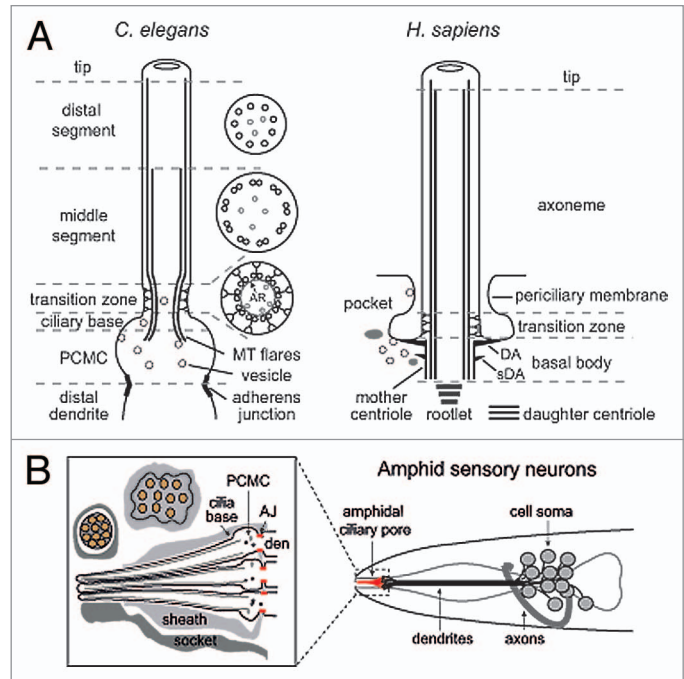


Figure 1. Structural basis of canonical ciliary subcompartments. (A) Left: representation of a canonical *C. elegans* amphid channel sensory neuronal cilium. Ciliary microtubules (MT) extend from a fully degenerated basal body at the ciliary base, with microtubules flaring proximally into the periciliary membrane compartment (PCMC). The PCMC is a swelling of the distal dendrite tip, bounded at its proximal side by a belt-like adherens junction between the sensory neuron and the enveloping sheath support cell (not shown). An $\sim 0.8 \mu\text{m}$ long ciliary transition zone emerges from the ciliary base, consisting of nine closely tethered doublet microtubules (via an internal apical ring (AR); see cross section), with each doublet connected to the ciliary membrane via Y-links. Also present are inner singlet microtubules (gray) extending to varying degrees along the axoneme. The TZ is followed by an $\sim 4 \mu\text{m}$ long middle segment consisting of 9 doublet microtubules, after which the B-tubule of each doublet terminates to establish the nine singlet A-tubule arrangement of the $\sim 3 \mu\text{m}$ long distal segment. Right: representation of a canonical human primary cilium, showing the basal body (and associated distal (DA) and subdistal (sDA) appendages), transition zone and main axonemal compartments. Example also shows the emergence of the cilium from a ciliary pocket, which is an invagination of the periciliary membrane observed for some ciliary subtypes. (B) Schematic of the amphid sensillum—the largest sensory organ in *C. elegans*—in the nematode nose region. Ten ciliary axonemes occupy the environmentally exposed channel (only 3 shown for illustration purposes), created by ciliary axonemes punching through the surrounding sheath (glial) cell. Belt-like adherens junctions (AJ) at the base of each periciliary membrane compartment (PCMC) seal the channel.

and transport proteins. For example, ciliary GPCRs such as ODR-10 (odorant reception), SRBC-66 (dauer signaling), SRG-64, and SRG-36 display prominent localizations at the periciliary membrane relative to all other neuronal plasma membranes.^{24,66} Although the functional significance of these periciliary pools is not understood, they may serve to provide the cilium with rapid access to receptor molecules upon sensory stimulation. In this context, the PCMC would act as a transit station or surplus reservoir for molecules yet to cross into the cilium.

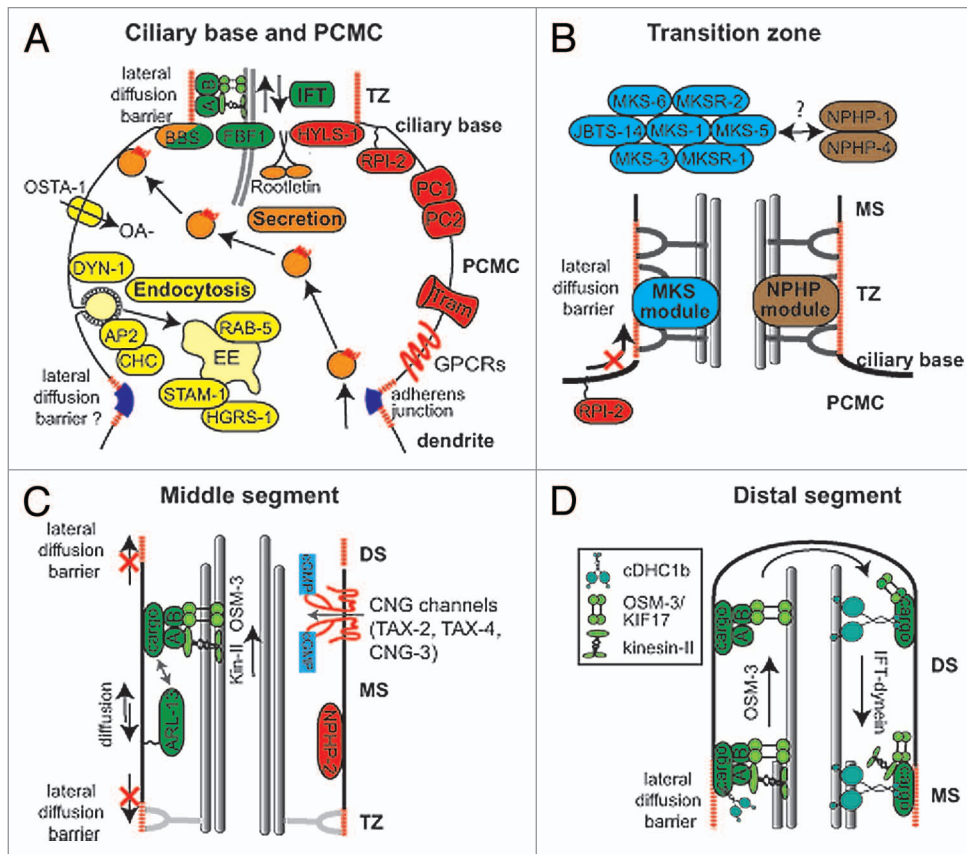


Figure 2. *C. elegans* proteins with distinct roles in specific subcilary compartments. **(A)** Proteins known to be enriched at the periciliary membrane compartment (PCMC) and ciliary base region. These include multiple proteins involved in secretion (orange) and endocytosis (yellow), as well as intraflagellar transport (IFT; green). Other enriched proteins are shown in red. The periciliary membrane is thought to be compartmentalised from the ciliary membrane via a lateral diffusion barrier at the transition zone. Although unproven, the belt-like adherens junction may serve to compartmentalise the PCMC from more proximal dendritic membrane. OSTA-1; organic solute transporter anion-1, DYN-1; dynamin, AP2; clathrin adaptor 2 complex, CHC; clathrin heavy chain, STAM-1; endosomal signal transducing adaptor molecule, HGRS-1; endosomal hepatocyte growth factor-regulated tyrosine kinase substrate, BBS; Bardet-Biedl syndrome complex (BBSome), A; IFT subcomplex A, B; IFT subcomplex B, HYLS-1; Hydrolethalus syndrome protein 1, RPI-2; retinitis pigmentosa gene 2, PC1/2; Polycystin 1/2, Tram; translocating chain-associated membrane protein (translocon), GPCRs; G-protein coupled receptors, OA-; organic anion **(B)** At least 9 proteins linked to ciliopathies are specifically localized in the transition zone (TZ). These are MKS-1/BBS13, MKS-3/TMEM67, MKS-5/RPGRIP1L, MKS-6/Cc2d2a, MKSR-1/B9D1, MKSR-2/B9D2, JBTS-14/TMEM-237, NPHP-1/NPHP1 and NPHP-4/NPHP4. Genetic analyses in *C. elegans* indicate these proteins form distinct, but functionally associated, modules ('MKS' and 'NPHP'), possibly linked via MKS-5. Together, these modules establish TZ connections with the ciliary membrane by regulating the assembly of Y-links. Also, these modules establish the transition zone as a ciliary gate, regulating lateral diffusion of membrane proteins into and out of the cilium (e.g., as shown for RPI-2). MS; middle segment, PCMC; periciliary membrane compartment. **(C)** Middle segment (MS) localization of palmitoylated ARL-13/ARL13B, NPHP-2/Inversin and various cyclic gated nucleotide (CNG) channels. Also shown is an anterograde IFT assembly, which in amphid channel ciliary middle segments is powered by the coordinated (and redundant) activities of two kinesin-2 motors, heterotrimeric kinesin-II (kin-II) and homodimeric OSM-3/KIF17. Lateral diffusion barriers flanking the compartment prevent ARL-13 from diffusing out of the compartment into the transition zone (TZ) and distal segment (DS) compartments. ARL-13 can also associate with IFT trains as potential cargo. **(D)** Assembly of distal segments (DS) via OSM-3 powered anterograde IFT. At tip of middle segment (MS), kinesin-II turns around and is trafficked back to the ciliary base via IFT-dynein (cDHC1b) powered retrograde IFT, leaving OSM-3 to drive anterograde transport of the IFT particle **(A and B)** and associated cargo to the ciliary tip. At tip, OSM-3 becomes retrograde IFT cargo.

Additional PCMC enriched proteins (but mostly excluded from cilia) include established and proposed regulators of membrane trafficking. For example, early endosomal proteins STAM-1 (endosomal signal transducing adaptor molecule) and HGRS-1 (endosomal hepatocyte growth factor-regulated tyrosine kinase substrate; Hrs) localize at the PCMC and regulate the ciliary localization of LOV-1 (polycystin-1 ortholog) by preventing its accumulation in the PCMC.⁶⁷ In a recent report, the Sengupta lab described a novel endocytic role for PCMC-localized OSTA-1 (organic solute transporter anion), which genetically interacts

with AP-2 clathrin adaptors, regulates RAB-5 vesicle flux, and facilitates the ciliary localization of various GPCRs.⁶⁶ Also, we and others reported PCMC localizations for RAB-5, DPY-23 (mu2 subunit of AP-2 clathrin adaptor), and DYN-1 (dynamin) and roles for these endocytic regulators in controlling ciliary and PCMC membrane volume in a RAB-8 and BBS-8 dependent manner, as well as the ciliary targeting of sensory signaling and IFT molecules.^{24,67} Indeed, this study allowed us to propose that *C. elegans* PCMC/ciliary membrane homeostasis is regulated by endocytosis-driven membrane retrieval, counterbalanced by

BBS-8 and RAB-8-mediated membrane delivery.²⁴ In agreement with this notion, an exciting recent study from the Barr lab demonstrated that biologically active extracellular vesicles that regulate mating behavior are shed from the periciliary membrane, and that this process is dependent on IFT.⁸¹ Similar findings have been made in *Chlamydomonas*, where bioactive ectosomes regulating daughter cell liberation following mitosis are shed from the flagellum, although in this instance vesicles derive from the tip rather than the base.⁸² Together, the above findings implicate the PCMC as an active zone of endo- and exo-cytosis, similar to what has been described for the ciliary and flagellar pockets of protists and mammalian cells.^{27,33,83}

Other notable *C. elegans* proteins enriched at the PCMC, and specifically the periciliary membrane, include membrane-associated RPI-2 (ortholog of retinitis pigmentosa gene 2; RP2) and the glycoprotein TRAM-1 (ortholog of translocating chain-associated membrane protein; TRAM).^{15,21,69} In Trypanosomes and mammalian cells, RP2 also localizes to the ciliary base where it is reported to link secretory trafficking between the Golgi and the cilium by facilitating docking of Golgi-derived vesicles to the ciliary base.^{84,85} The apparent periciliary membrane localization of the Sec61 ER translocon component TRAM-1 is particularly intriguing, and could suggest the presence of dendritic ER in the PCMC, perhaps in close apposition with the plasma membrane as ER-plasma membrane contact points.⁸⁶⁻⁸⁸ However, conventional chemical fixation techniques have not as yet revealed ER ultrastructure in the nematode PCMC and thus it remains a distinct possibility that the TRAM-1 PCMC signals reflect an atypical translocon functioning at the nematode periciliary membrane.

How various transmembrane (e.g., GPCRs) and membrane-associated (e.g., RPI-2) proteins are enriched at the periciliary membrane remains poorly understood. One possibility is anchoring to the underlying cytoskeleton via retention domains. For example, mammalian podocalyxin is excluded from a ciliary membrane domain by interacting with cortical actin via a PDZ motif.⁸⁹ Another non-mutually exclusive mechanism involves flanking diffusion barriers preventing lateral diffusion out of a periciliary membrane patch. Distally, these barriers likely involve septin rings and transition zone proteins (see next section). Proximally, at least in the case of *C. elegans* sensory neurons, adherens junctions may prevent diffusion of PCMC proteins into the dendritic compartment (Fig. 2A).

***C. elegans* Transition Zone Compartment**

Extending from the cilium base is a ~0.8 μm long transition zone (TZ).⁷⁴ Narrower than the rest of the axoneme, the TZ forms a constricted ring of doublet microtubules, with reduced inter-doublet spacings. Tethered to the inner face of the outer doublet microtubules is a membranous (apical) ring which draws toward it varying numbers of inner singlet microtubules that elongate distally into the axoneme. Characteristic Y-links extend from the junction between the A and B tubules and connect with the TZ membrane. Although vesicles are thought to be largely excluded from cilia, a recent report in *C. elegans* clearly

shows that the vesicles are found in ciliary transition zones.⁷³ With the possible exception of the Y-links (discussed below), little is known regarding the functional relevance of most TZ ultrastructural features.

A primary reason for the recent interest in the TZ is that multiple ciliopathy proteins are specifically localized within this compartment, with some of the first observations made in nematodes. In *C. elegans*, at least 9 ciliopathy proteins are found at the TZ, including NPHP-1, NPHP-4, MKS-1, MKSR-1/B9D1, MKSR-2/B9D2, MKS-3/TMEM67, MKS-5/RPGRIPL, MKS-6/Cc2d2a, and JBTS-14/TMEM237 (Fig. 2B).^{15,90} Functional analyses using single mutant alleles revealed no obvious roles for most of these proteins in cilium structure and TZ integrity, although subtle cilia subtype-specific ultrastructural and IFT defects are observed in *nphp-4* mutants, and *mks-5* mutants possess a reduced dye-filling phenotype indicative of a cilium structure defect.^{15,91-93} However, double mutant worms possessing loss of function alleles in an MKS/MKSR/JBTS-14 gene together with an NPHP gene, but not two genes from the same group, display relatively severe phenotypes, including truncated and misshapen cilia, misplaced axonemes, and chemosensory defects.^{15,90,92} Furthermore, TZ morphogenesis and placement is severely impacted; TZs lack most or all Y-link connectors and are frequently de-anchored from the plasma membrane, extending instead from ectopic proximal positions within the distal dendrite.^{15,90} Also, in phasmid neurons, MKS/NPHP double mutants possess short dendrites, leading to the suggestion that during neuronal differentiation, the membrane-spanning TZ anchors the tip of the nascent dendrite to supporting cells or the extracellular matrix.^{15,92} This is because in *C. elegans*, dendrite elongation occurs via a trailing model, where an anchored dendrite is laid down behind a migrating cell soma.⁹⁴ Thus, in *C. elegans*, MKS and NPHP genes act in a functionally redundant manner to establish physical connections between microtubules, membranes and the ECM, required for ciliary docking and dendrite elongation.

The synthetic genetic associations observed in *C. elegans* implicate a modular organization of MKS, MKSR, and NPHP proteins, with NPHP-1/4 comprising an 'NPHP' module, and MKS-1/3/6 and MKSR-1/2 proteins comprising an 'MKS' module.¹⁵ Consistent with this idea, NPHP-4 is required for TZ localization of NPHP-1, and MKS/MKSR proteins display interdependent TZ localizations; also, proteins of one module are not required for the TZ localization of proteins from the other module, indicating distinct pathways for assembly at the TZ.^{15,92,95} The one exception is MKS-5, required for the TZ assembly of all examined NPHP and MKS/MKSR proteins; thus, MKS-5 is proposed to be a central component in docking MKS and NPHP modules at the TZ, perhaps as a linker between the two modules.¹⁵

MKS and NPHP module genes also facilitate a bidirectional membrane diffusion barrier at the TZ that prevents leakage of periciliary membrane proteins (RPI-2; TRAM-1) into cilia, and leakage of ciliary membrane-associated ARL-13 out of cilia.^{15,48} These phenotypes were observed in single MKS and NPHP gene mutants, indicating barrier defects despite grossly normal TZ

ultrastructure.¹⁵ Interestingly, using quantitative imaging and an in vivo FRAP assay to assess flux kinetics across the TZ, we found that ARL-13 leakage out of cilia is enhanced in double mutants lacking Y-links, compared with the corresponding single mutants.⁴⁸ Thus, similar to what is found for the structural integrity of the TZ, MKS and NPHP genes function in a non-redundant and synthetic fashion to establish a TZ membrane diffusion barrier. Interestingly, TZ formation and barrier functions generally do not require IFT genes,¹⁵ although the TZ barrier to ARL-13 diffusion partially depends on the putative IFT-B component, DYF-13/TTC26.⁴⁸ These nematode data are consistent with mammalian and *Chlamydomonas* studies showing that TZ-localized proteins comprise a diffusion barrier that controls ciliary and flagellar membrane protein composition.¹²⁻¹⁴

Biochemical interaction data from mammalian cells also implicate TZ components as part of distinct MKS/JBTS and NPHP modules. The MKS/JBTS module comprises at least 12 TZ-localized components that form a dense protein interaction network.⁹⁶ This module includes essentially all the proteins genetically associated with the *C. elegans* MKS module, as well as tectonic (TCTN1/2/3), TMEM (TMEM17/216/231), and AHI1 proteins. The NPHP module includes a TZ-localized subcomplex of three interconnected proteins, NPHP1, NPHP4, and NPHP8/MKS5.⁹⁷ Consistent with a distinct functional nature, there are few biochemical associations between NPHP1/4/8 and components of the MKS/JBTS module.⁹⁶ Interestingly, the mammalian NPHP module is also thought to include two additional subcomplexes, NPHP2/3/9 and ATXN10/NPHP5/NPHP6, which localize predominantly to the Inv and basal body compartments, respectively.^{55,97} Thus, these subcomplexes expand NPHP module functions beyond the TZ into neighboring ciliary compartments.

Although it is not known how NPHP/MKS/JBTS modules facilitate the ciliogenic and membrane diffusion barrier functions of the TZ, one model is regulation of TZ membrane properties such as lipid composition, fluidity and steric restriction. It has also been suggested that TZ modules are structural components of the microtubule-anchored Y-links which may directly organize biochemical and biophysical features of the TZ membrane.⁹⁶ As structural subunits, NPHP proteins could anchor Y-links to the axoneme via their reported ability to interact with microtubules, whereas the C2/B9 lipid binding domains of MKS-1/2/5/6 and MKSR-1/2, together with various TMEM proteins, could tether Y-links to the TZ membrane.⁹⁶ Furthermore, the coiled-coiled domains of CEP290/NPHP6, MKS-6/Cc2d2a, and MKS5 could form the Y-link stalk.⁹⁶ Consistent with these data, *Chlamydomonas* CEP290 localizes at the Y-links,¹³ and efforts are on-going using immunogold labeling and super-resolution imaging to reveal the precise ultrastructural localizations of other TZ proteins. Also, the further dissection of the TZ membrane and protein environment, its fluidity and diffusive properties is being made possible by strategies employing lipid reporters and FRAP assays, and at least in *Chlamydomonas*, it seems TZ membranes can be isolated and biochemically analyzed.

Middle Segment/Inv Compartment

Extending from the TZ of most amphid and phasmid cilia is the middle segment compartment, defined ultrastructurally by a canonical arrangement of nine outer doublet microtubules.⁷⁴ At the distal end of middle segments, the B-tubule of each doublet terminates, thus signaling the transition to the microtubule singlet (A-tubule)-containing distal segment, discussed in the next section. Most amphid/phasmid ciliary middle segments are ~4 μm long, although that of the amphid winged cilium (AWB), which lies adjacent to the amphid channel, is shorter at ~2 μm .⁹⁸ Notably, 38 of the 60 ciliated neurons lack a middle/distal segment distinction; instead, doublet microtubules extend to the ciliary tips, either in canonical (ring of 9 doublets) or atypical arrangements.⁵⁰ In amphid and phasmid neurons, two kinesin-2 motors, canonical heterotrimeric kinesin-II and an accessory OSM-3-kinesin (KIF17 ortholog), cooperate to drive middle segment anterograde IFT and function in a fully redundant manner to build this subdomain.⁹⁹

The middle segment appears analogous to the vertebrate Inv compartment, initially described in mouse renal epithelial cells based on specific localization of the NPHP2/INV ciliopathy protein to a proximal ciliary segment, excluding the TZ.¹¹ Since then, several other ciliopathy-associated Inv compartment proteins have been identified including NPHP3, NPHP9/NEK8, ANKS6, and ARL13B,^{48,55,100} and some of these proteins (Arl13b, NPHP2) dynamically exchange at the compartment's membrane.^{11,101} Consistent with the middle segment being an Inv-like compartment, *C. elegans* NPHP2 and ARL13B orthologs also localize specifically at the middle segment and nematode ARL13B (ARL-13) exchanges at the membrane (Fig. 2C).^{48,61,62,102} Middle segment restriction is also observed for various cyclic nucleotide-gated (CNG) cation channel subunits TAX-2, TAX-4, and CNG-3.^{103,104}

The middle segment displays cilium subtype and developmental stage heterogeneity. For example, in AWB cilia, ARL-13, TAX-4, and CNG-3 localize to middle and distal segments, although middle segment enrichment is still observed.^{61,104} Similarly, in mouse cells, despite proximal ciliary restriction of Arl13b in tracheal and oviduct cilia,⁴⁸ this protein decorates the entire ciliary axoneme of other cell types.^{14,105,106} Similar observations have been made for mammalian Inversin/NPHP2.⁹⁷ Furthermore, the middle segment is developmentally regulated because in *C. elegans* phasmid neurons, ARL-13 localization extends to the tips of newly formed cilia, before quickly restricting to a proximal subdomain as the worm ages.⁴⁸

How proteins are targeted to and restricted at the Inv compartment remains unclear. One obvious targeting mechanism is IFT, which in *C. elegans* prevents accumulation of ARL-13 and TAX-2/4 at the periciliary membrane.^{48,104} Consistent with an active transport model, ARL-13 can undergo IFT-like movement in developing cilia and human ARL13B associates with IFT complex B via IFT46/74 interactions.⁴⁸ However, because IFT operates along the entire cilium length, it remains unclear how IFT delivers proteins to the middle segment. One possible solution is that middle segment proteins dock with the kinesin-II

motor, which operates only to the middle segment tip before turning around. In support of this notion, TAX-2/4 targeting to the middle segment of AWB cilia depends on kinesin-II, but not OSM-3-kinesin.¹⁰⁴ However, this is not a universal mechanism because middle segment targeting of TAX-2 in ASK cilia is independent of kinesin-II and OSM-3-kinesin.¹⁰⁴ Thus, either the two motors are functionally redundant or TAX-2 targeting to ASK cilia employs another as yet unidentified IFT motor, or is IFT independent.

Targeting and restriction of proteins to the middle segment also involves membrane diffusion barriers that block lateral diffusion out of the compartment. As outlined above, MKS and NPHP proteins regulate a TZ barrier to ARL-13 diffusion and may represent a general strategy for proximal gating of the middle segment domain.⁴⁸ However, little is known about presumed barriers at the distal side, which lack all defining ultrastructural features of the TZ. Like the TZ, a distal barrier may possess distinct lipid compositions or steric properties that prevent middle segment protein associations. Interestingly, in Trypanosomes, detergent-resistant membrane (DRM) patches are enriched in the distal flagellum vs. the proximal/mid flagellum,¹⁰⁷ thus potentially allowing the distal ciliary association or exclusion of particular membrane proteins.

Aside from establishing flanking barriers, distinct lipid compositions in the Inv compartment itself may retain proteins with lipid-anchoring post-translational acylation modifications such as myristoylation and palmitoylation. Such modifications are already known to facilitate ciliary targeting of mammalian NPHP3, a Trypanosome calcium-binding protein, and *C. elegans* and mammalian Arl13b/ARL-13.^{61,62,108,109} In the case of mammalian NPHP3, its ciliary localization depends on a GTPase activation cycle involving ARL3 and its cognate activator, RP2, which regulates release of myristoylated NPHP3 from an inhibitory interaction with UNC-119, thus allowing association with the compartment's membrane.¹¹⁰ This mechanism probably also involves NPHP2, which was shown to act as an intraciliary molecular anchor to stabilize the Inv compartment localization of NPHP3 (and NEK8).⁵⁵ Such stabilizations are likely to involve interactions with the cytoskeleton and indeed an early report showed enrichment of actin in the proximal region of quail oviduct cilia.¹¹¹ Finally, it is interesting to note that N-terminal palmitoylation of *C. elegans* ARL-13, and possibly mammalian Arl13b, prevents nuclear targeting, which suggests this reversible modification regulates shuttling between the nucleus and the Inv domain. Indeed, mammalian Arl13b possesses a candidate nuclear targeting sequence and there is evidence of nuclear Arl13b signals.¹⁰¹ Functional connections between the Inv domain and the nucleus are also suggested by localization of NPHP9/Nek8 and Inversin to both compartments.¹¹²⁻¹¹⁴

The sequestration of multiple ciliopathy proteins and channels/receptors within the Inv domain implicates important ciliogenic, sensory, signaling and transport-related roles for this compartment. For example, *C. elegans* ARL-13 regulates cilium length and morphology, chemo- and osmosensation, middle segment association of OSM-3 and kinesin-II motors, and the ciliary levels of PKD-2 and the TRPV channel,

OSM-9.^{61,62} Similarly, mammalian Arl13b controls the distribution and exchange dynamics of Shh proteins and regulates neuronal migration and radial glial scaffold formation.^{101,105,115,116} In *C. elegans*, NPHP-2 regulates the ciliary localization (AWB) of at least one sensory signaling molecule, TAX-2, and genetically interacts with TZ-localized MKS and NPHP components to control cilium formation, TZ placement and dendrite length, thus establishing functional relationships between middle segment and TZ compartments.^{102,104} Similar domain interactions have been described in mammals, with NPHP2 biochemically interacting with TZ-localized proteins B9D2, RPKG1L, and NPHP4, as well as the MKS1/MKS6/TECT2 submodule.^{97,117,118} Indeed, a number of important signaling and cellular functions have been described of mammalian Inv compartment proteins, with NPHP2 linked to planar cell polarity (PCP) signaling and NPHP9 to the DNA damage response.¹¹⁹⁻¹²² However, it seems unlikely that all such functions are solely orchestrated from within the Inv compartment because as outlined above, Inv proteins localize along the entire ciliary axoneme of certain cells types.⁹⁷ Also, in mitotic cells, Inv domain proteins localize at the centrosome (NPHP9, NPHP2), spindle poles (NPHP2), and the mid-body (NPHP2).^{113,114,123}

Distal Segment Compartment

Extending from the middle segment of amphid and phasmid channel cilia is a ~3 μm long distal segment, comprised of 9 outer singlet MTs (A-tubules) due to B-tubule termination at the middle segment tip. In the branched AWB cilium, the distal segment is longer (~5 μm) and contains relatively few singlet A-tubules, and terminates with a membrane expansion (fans).⁵⁰ Microtubule singlet-containing distal segments are also found at the flagellar tips of mating green algae, and in various vertebrate olfactory, pancreatic, and renal cell cilia.⁵¹⁻⁵⁴ Thus, distal segments may be a common feature of many eukaryotic cilia.

Unlike middle segment assembly, which employs two coordinated and functionally redundant kinesin-2 motors (kinesin-II, OSM-3), only OSM-3 kinesin enters the distal segment to drive anterograde IFT (Fig. 2D).⁹⁹ In *osm-3* mutants, cilia terminate at the middle segment tip and these worms are osmo- and chemosensory defective, highlighting the functional importance of the distal segment compartment.⁷⁴ Additional nematode genes required for distal segment assembly include various IFT genes (*dyyf-1/IFT70*, *dyyf-6/IFT46*, *dyyf-13/TTC26*, *ift-74*, *ift-81*), a MAP kinase (*dyyf-5*), α and β tubulin isoforms (*tba-5*, *tbb-4*) and the AP-1mu1 clathrin adaptor subunit (*unc-101*).^{63,124-126} Many of these genes regulate OSM-3 functions: *dyyf-1*, *dyyf-6*, *ift-74*, and *ift-81* activate OSM-3 motor activities, and *dyyf-5* regulates OSM-3 speeds; also, IFT-74 biochemically associates with OSM-3.^{63,124,125} In contrast, TBB-4 and distal segment-enriched TBA-5 proteins do not regulate OSM-3 motor speeds but instead stabilize distal segment singlet microtubules.¹²⁵

Despite a dedicated assembly pathway, the sensory and signaling proteins specifically functioning in the nematode distal segment remain mostly unknown. Indeed, unlike the other ciliary

subcompartments, to our knowledge very few, if any, proteins have been found to be enriched in the nematode ciliary distal segments. However, in other organisms, distal cilium/tip enrichment has been observed for a number of proteins. In rats, odour-detecting CNG channels preferably cluster within the microtubule singlet-containing distal segments of olfactory neuronal cilia.⁵⁸ Also, the *Xenopus laevis* calponin homology and microtubule-associated protein (CLAMP) of unknown function is enriched in the distal cilium of multi-ciliated epidermal cells, although it remains unclear if this axonemal region is comprised of doublet or singlet microtubules.¹²⁷ Numerous proteins associate with the ciliary tip region, a site of microtubule assembly/disassembly, IFT turnaround and signaling.¹²⁸ These include the mammalian microtubule stabilizing and length control protein EB3,¹²⁹ *Chlamydomonas* EB1,¹³⁰ mammalian sonic hedgehog signaling mediators GLI2, GLI3, and SUFU,^{56,131} and a mechanosensory *Drosophila* TRPN channel, NOMPC.¹³² Very few studies have addressed how proteins are targeted to and restricted at distal ciliary regions, although in *Xenopus*, the planar cell polarity effector protein Fuz regulates proximo-distal localization patterning of MAP7 (microtubule-associated protein 7) and CLAMP, which are enriched in proximal and distal ciliary regions, respectively.¹³³

One important aspect of the distal ciliary axoneme, and in particular the ciliary tip, is that it is an incorporation site for structural subunits of the axoneme (e.g., tubulin, radial spokes) and is where IFT motor-particle assemblies are remodelled and cargo-unloaded as they switch from anterograde to retrograde transport phases.¹³⁴⁻¹³⁶ Although little is known about how these events are regulated, multiple distal segment/tip localized proteins are likely involved. For example, in *Chlamydomonas*, tip turnaround of IFT employs an IFT172 mediated interaction with tip-localized EB1.¹³⁷ In *C. elegans*, IFT particle turnaround at the ciliary base and tip is dependent on the BBSome at these locations, via a mechanism involving DYF-2/IFT144-mediated interactions.⁶⁴

Finally, the ciliary tip is a site of membrane shedding. In *Chlamydomonas*, biologically active ectosomes are released from the flagellar tip,⁸² and in photoreceptors, spent membranous disks are shed from the outer segment tip for engulfment and degradation by neighboring retinal pigment epithelial cells.¹³⁸ Such findings suggest that the tip may serve as a local region of ciliary membrane remodeling and turnover, as well as a site for release of biologically active vesicles involved in extracellular processes, which could possibly include cell-cell communication.

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Concluding Remarks

The specific localization of multiple ciliopathy proteins and signaling molecules to distinct regions of the cilium has ignited efforts to investigate the subcompartmentalised nature of the organelle. Using multiple model systems including *C. elegans*, our understanding of subcompartment composition and related transport is advancing. For example, the ciliopathy protein-enriched transition zone appears to regulate the cilium's signaling capacity by serving as a ciliary gate to regulate transport into and out of the organelle. However, many questions remain. First, what is the functional basis of asymmetric ciliopathy and signaling protein distributions along the ciliary axoneme (e.g., Inv domain vs. distal cilium), and why do some subcompartments display cilium subtype as well as developmental stage heterogeneity? Presumably, such distributions allow the formation of signaling complexes at distinct regions of the ciliary membrane in a cilium subtype and age-dependent manner, but the reasons for this are unclear. Second, what are the mechanisms that separate one compartment from another: how are lateral membrane diffusion barriers defined and what are the anchoring and transport mechanisms that restrict proteins to a particular compartment? Third, what are the functional relationships between ciliopathy proteins and modules that localize in distinct compartments? We know that these relationships exist from biochemical and genetics experiments, but the signaling output from such interactions and their relationship to the spectrum of ciliopathy phenotypes remains poorly understood. Fourthly, as a final thought, it is clear that the subcompartmentalised nature of the cilium provides a new paradigm for understanding transport and signaling mechanisms operating at highly regionalised sites of an organelle; thus, the resultant findings should provide broader insight into the more general question of cellular compartmentalisation and the organization of signaling activity at distinct patches of plasma membrane.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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