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Seriously Cilia: A Tiny Organelle Illuminates Evolution, Disease and Intercellular Communication

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Abstract

The borders between cell and developmental biology, always permeable, have largely dissolved. One manifestation is the blossoming of cilia biology, with cell and developmental approaches (increasingly complemented by human genetics, structural insights, and computational analysis) fruitfully advancing understanding of this fascinating, multifunctional organelle. The last eukaryotic common ancestor probably possessed a motile cilium, providing evolution with ample opportunity to adapt cilia to many jobs. Over the last decades, we have learned how nonmotile, primary cilia play important roles in intercellular communication. Reflecting their diverse motility and signaling functions, compromised cilia cause a diverse range of diseases collectively called ciliopathies. In this Review, we highlight how cilia signal, focusing on how second messengers generated in cilia convey distinct information, how cilia are a potential source of signals to other cells, how evolution may have shaped ciliary function, and how cilia research may address thorny outstanding questions.

Introduction

Eukaryotic cells are compartmentalized, tasking organelles with different functions. For example, aerobic respiration occurs in mitochondria and interpretation of genetic information occurs in nuclei. Like mitochondria and nuclei, cilia are organelles that are specialized, but for the tasks of motility and signal transduction. Here, we use the terms cilia and flagella interchangeably, underscoring that our discussion is limited to eukaryotes, with prokaryotic flagella being fundamentally different. Moreover, cilia can be divided into distinct subsets based on structure and function. Some cilia are motile and transport fluid or propel sperm. Other cilia are immotile interpret environmental information or intercellular cues (Figure 1A). These sensory immotile cilia were named primary cilia in reference to their appearance in lung development before (or primary to) motile cilia[1]. This nomenclature extended to encompass all immotile cilia[2]. However, as with most definitions in biology, the

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division of cilia into motile and sensory is not absolute, as motile cilia can have sensory functions.

In this review, we highlight emerging understanding of how, like your radio antenna, primary cilia function in signal transduction. A complication to receiving information that all cells contend with is the lipid bilayer, the scant few nanometers of hydrophobicity that separate the highly regulated intracellular environment from the extracellular world. This delimiting membrane poses a challenge: to respond to the world, intracellular machinery must gain information from the other side of the membrane. Cells have solved this challenge in diverse ways, several of which use cilia. Moreover, cilia share a common structure but can be tuned to receive different types of information in different cell types.

Different modes of ciliary signaling share some requirements (e.g., diverse ciliary signaling pathways rely on a part of the ciliary base called the transition zone to control ciliary protein composition) and have some unique requirements (e.g., different ciliary signals are transduced via distinct ciliary receptors and effectors). Below, we detail how the cilium, an evolutionarily ancient organelle, acts as a dynamic signaling hub for the transduction of both intracellular and extracellular cues. We also reflect on the various roles of cilia in human physiology, and how evolution may have shaped this functional diversity.

Nothing in ciliary biology makes sense except in the light of evolution

Phylogenetics strongly suggests that the last common eukaryotic ancestor (LECA) was a ciliated organism[3–5] (Figure 1B). Because of its ancestral origin, much ciliary biology is shared among currently existing species, and principles discovered in one organism can illuminate biology in others. For example, motor-driven intraflagellar transport (IFT) was discovered in the green alga *Chlamydomonas*[6]. IFT, critical for both ciliogenesis and ciliary maintenance, transports proteins both to the ciliary tip via Kinesin-2 and back to the ciliary base via Dynein-2 (Figure 1C). Identification of the *Chlamydomonas* genes encoding IFT components allowed homologs to be recognized and revealed that the IFT machinery is conserved across ciliated organisms.

Another evolutionarily ancient ciliary machine is the transition zone, a gate at the base of the cilium that helps to regulate ciliary protein composition. Because of the cilium's deep evolutionary conservation, identification of transition zone components in one organism predicts transition zone components of other organisms. For example, CEP290 was discovered to be a component of the *Chlamydomonas* transition zone and subsequently recognized to be a component of the mammalian transition zone[7,8]. Many transition zone components are co-conserved with CEP290 among ciliated phyla, underscoring how, to paraphrase Theodosius Dobzhansky[9], nothing makes sense except in the light of evolution. Humans are no exception to conservation of ciliary components. For example, human mutations in *CEP290* can cause Joubert syndrome, which helped spur the realization that mutations in other transition zone components also cause Joubert syndrome[10–12].

Consistent with the hypothesis that LECA possessed a cilium, many ciliary proteins beyond IFT and transition zone components are conserved across diverse extant ciliated

organisms. Many of these evolutionarily ancient proteins are centriolar components required for ciliogenesis, such as the DISCO complex (including CEP90, OFD1, FOPNL)[13].

Other ancient complexes are essential for ciliary motility. These include the axonemal dyneins, the outer dynein arm-docking complex (ODA-DC), the nexin-dynein regulatory complex (N-DRC) and the radial spokes. In addition, many microtubule inner proteins (MIPs) are widely conserved among existing diverse ciliated organisms. MIPs are required to strengthen the axonemal microtubules and regenerate ATP to fuel the dyneins[14,15].

Additional ancient ciliary proteins include assemblies involved in trafficking proteins to or from cilia. These include lipidated protein intraflagellar targeting (LIFT) involved in trafficking of lipidated proteins to the ciliary membrane, and an octameric adaptor related to Bardet-Biedl syndrome called the BBSome[16].

Not all proteins with conserved roles in ciliary function fulfill their role at the cilium proper. Despite not localizing to centrioles or cilia, cytoplasmic proteins such as the dynein axonemal assembly factors (DNAAFs) co-evolved with cilia and contribute to ciliary motility by assembling the axonemal dyneins before their import into cilia[17].

Thus, extrapolation from extant organisms allows us to partially reconstruct the ancestral cilium of LECA. Most probably, this cilium contained a 9+2 axoneme possessing MIPs for structural reinforcement and generation of ATP from AMP and ADP, as well as radial spokes. These cilia were motile and used DNAAFs to generate axonemal dyneins, some of which docked onto the ODA-DC and were regulated by N-DRC. Within the cilium, IFT along the axonemal proteins was powered by Kinesin-2 and Dynein-2. Select lipidated proteins were transported to these cilia by LIFT, and proteins were transported out of the cilia via the BBSome, both across the transition zone.

While these reconstructions suggest that the LECA had a complex cilium, we should remain aware that living organisms may not fully represent the ancestral ciliary protein complement. For example, an evolutionarily ancient ciliary protein, EVC3, has been lost by extant vertebrates[18].

LECA may have used its cilium both for motility and for sensing extracellular cues. Below, we describe our current understanding of how animal cilia use three distinct signaling systems: Hedgehog, Polycystin and G protein-coupled receptor (GPCR) signaling. These three pathways represent distinct modes of ciliary signaling and are associated with cilia biology in diverse organisms.

Cilia transduce vertebrate Hedgehog signals

Patterning of tissues during embryonic development depends on a surprisingly small number of signaling pathways. One critical means of intercellular communication in developing animals is Hedgehog signaling[19]. Last century witnessed the discovery of Hedgehog proteins as secreted lipoproteins sensed by the receptor Patched (PTCH) to regulate the central transducer, Smoothened (SMO), thereby controlling the activity of the downstream

GLI transcription factor effectors[20,21]. This century has seen a burgeoning understanding of how many animals use primary cilia for Hedgehog signal transduction.

How do cilia transduce Hedgehog signals? Central to many signal transduction pathways are post-translational modifications (e.g., phosphorylation by receptor tyrosine kinases) or changes in protein-protein interactions (e.g., steroid hormone receptor separation from HSP90). Certainly, Hedgehog signaling involves post-translational modifications and changes in protein-protein interactions. Many of these modifications and changes occur in one subcellular locale, the primary cilium, and Hedgehog signaling depends critically on regulated changes in the composition of the primary cilium.

Not only are cilia required for vertebrate Hedgehog signaling, but all of the central components of the Hedgehog signal transduction pathway (PTCH, SMO, PKA, KIF7, SUFU and GLI) spend at least some of their time at the cilium[22–25]. In the absence of Hedgehog stimuli, the cilium possesses PTCH and the GLI proteins are bound to SUFU to prevent transcriptional activity (Figure 2). Some GLI proteins are cleaved to generate a truncated repressor form (GliR) that keeps the Hedgehog transcriptional program off. Hedgehog stimuli induces PTCH ciliary exit and SMO ciliary accumulation, resulting in the formation of GLI activators (GliA). When activated, GLI cleavage is prevented, GLI dissociates from SUFU[26], and GLI turns the Hedgehog transcriptional program on in the nucleus.

How SMO activation leads to GLI activation remains a major gap in understanding. In *Drosophila*, the SMO C-terminus can interact directly with a GLI-containing protein complex[27]. The *Drosophila* and vertebrate SMO C-termini are highly divergent, indicating that vertebrates are likely to activate GLI through a distinct mechanism.

A key intermediary between SMO and GLI is protein kinase A (PKA), which promotes GLI conversion into GliR[28–30]. Vertebrate SMO can bind and inhibit PKA[31,32]. Thus, vertebrate SMO may regulate GLI by blocking PKA-mediated phosphorylation. As Hedgehog signals are morphogens capable of specifying multiple different fates depending on concentration and duration, GLI proteins may be in more states than on and off, with intermediate states transducing the mid-range of the morphogen gradient. How the ciliary machinery parses characteristics of the morphogen gradient to direct differentiation along multiple trajectories remains unclear.

In addition to its distinct protein composition, cilia possess a distinct lipid composition[33]. Ciliary lipids may also be critical to Hedgehog signal transduction. One subclass of lipids are sterols. A diverse group of oxidized sterols called oxysterols bind and activate SMO[34–36]. Several SMO-activating oxysterols are enriched in cilia, suggesting that the distinct lipid composition of cilia may be one reason why SMO requires the cilium to activate the downstream pathway signaling[37]. The upstream regulator of SMO, PTCH, can transport sterols across the cell membrane[38–40]. Hedgehog binding to PTCH1 could block this transport, potentially increasing sterol concentration and activating SMO. It will be interesting to ascertain whether endogenous SMO is regulated by oxysterols and/or cholesterol, and whether the regulation of SMO varies by cell type or organism.

Despite the aforementioned conservation of ciliary machinery, exploration of cilia function in diverse organisms has pointed out how evolution has tinkered with ciliary Hedgehog signaling. Whereas vertebrates and sea urchins require cilia for Hedgehog signaling, cilia are dispensable for Hedgehog signaling in many *Drosophila* tissues and in planaria[41–44]. Did the animal ancestor use cilia for Hedgehog signaling? Proteomic profiling of cilia from sea urchins (an early-branching non-chordate deuterostome), sea anemones (non-bilaterians) and choanoflagellates (protists thought to be the closest known relative of animals) revealed that Hedgehog signaling components are associated with cilia in sea anemones, suggesting that the ancestor of bilateria may have employed cilia for Hedgehog signaling[18].

In addition to most known ciliary components, comparing the ciliary proteomes of diverse species identified ciliary proteins of unknown function[18]. For example, this comparison indicated that DYRK2 could be a ciliary kinase involved in Hedgehog signaling, subsequently validated by mammalian genetics[45].

A parsimonious model is that evolution has played with a basal ciliary Hedgehog pathway, either by removing its dependence on cilia in one ecdysozoan (*Drosophila*) or by dispensing with it altogether in another (*C. elegans*). Evolution is most probably continuing to shape ciliary Hedgehog signaling in vertebrates, as alterations in ciliary Hedgehog signaling may underlie relatively recent changes in the eyes of cavefish, the heads of wolves and dogs, and the limbs of flightless birds[46–48].

Future comparisons between cilium-dependent and -independent Hedgehog signaling may help illuminate which attributes are conveyed by the cilium itself. For example, comparing how ciliary and nonciliary Hedgehog signaling differ in kinetics or fidelity may reveal the design principles optimized by both forms of information transmission. One possibility is that relying on protein trafficking to and from cilia may increase signaling fidelity but sacrifice signal transduction speed.

Cilia transduce Polycystin signaling

In addition to their essential role in vertebrate embryonic development, cilia can be important in the homeostasis of adult tissues. *PKD1* and *PKD2* (encoding Polycystin-1 and 2) are justifiably most famous for being the two genes in which mutations cause autosomal dominant polycystic kidney disease (PKD)[49,50]. PKD is a human disease characterized by progressive formation of cysts, leading to kidney failure. Polycystin-1 and 2 are founding members of a subfamily of transient receptor potential (TRP) channels.

The TRP superfamily is comprised of a diverse cohort of tetrameric channel-forming integral membrane proteins. Compared to other members of the TRP superfamily, the Polycystin channel proteins are noncanonical. For example, Polycystin channels are heterotetramers comprised of one Polycystin-1 subunit and three Polycystin-2 subunits[51] (Figure 3A).

In the kidney, most cells of the nephron project a primary cilium into the nephron lumen (Figure 3B). Although Polycystin-1 and 2 localize to several subcellular locations (e.g., sites of membrane protein biosynthesis such as the ER), they prominently localize to cilia[52–54].

Deletion of cilia in the kidney epithelium is sufficient to induce cystogenesis, suggesting that cilia, like Polycystin-1/2 channels, restrain renal epithelial proliferation[55–57].

However, cilia play a complex role in kidney cystogenesis. Cysts resulting from loss of cilia are smaller than the dramatic cysts caused by loss of Polycystin-1/2 function (with cilia present). Moreover, loss of both Polycystin-1/2 function and cilia also results in more mild cystogenesis[58]. One interpretation of these genetic interactions is that cilia act epistatically to PKD1/2 channels and that cilia both restrain and promote renal epithelial proliferation (Figure 3C).

How should we conceptualize these dual functions of cilia in cystogenesis? A perspective comes from understanding of ciliary Hedgehog signaling. Hedgehog signals pattern many tissues, including the neural tube and the limb bud. In the neural tube, Hedgehog signals induce ventral fates. Loss of Hedgehog signaling causes a dorsalization of the neural tube, and gain of Hedgehog signaling causes ventralization of the neural tube. In the limb bud, Hedgehog signals specify the number and identity of the digits. Loss of Hedgehog signaling causes a reduction in digit number and gain of Hedgehog signaling causes a gain in digit number. Unintuitively, loss of cilia causes dorsalization of the neural tube (an apparent loss of Hedgehog signaling) and a gain in digit number (an apparent gain of Hedgehog signaling)[42,59]. This apparent paradox is resolved by recognizing that cilia are essential for both generating the GLI activator and repressor. In the neural tube, Hedgehog patterning is effected by generating GLI activator, whereas in the limb bud, Hedgehog patterning is effected by inhibiting GLI repressor. Thus, loss of cilia removes both GLI activator and repressor in both tissues, but the phenotypic consequences are distinct because of differential reliance on GLI activator or repressor.

As kidney cilia either potentiate or restrain cystogenesis in different genetic contexts, they may function similarly to Hedgehog-transducing cilia: instead of generating both GLI activators and repressors, kidney cilia may generate cyst activators and repressors. In this model, loss of kidney cilia may compromise the formation of a cyst repressor, leading to cystogenesis. In addition, cilia may also generate cyst activators, potentially explaining the aggressive cystogenesis when Polycystin-1/2 channels are absent but cilia are present.

Fascinatingly, restoration of Polycystin-1/2 function in mouse cysts shrinks kidney cysts and reverses associated histological changes such as fibrosis[60]. Thus, if we could discover how Polycystin-1/2 transduces information to control cell proliferation, we might gain insight into pharmacologically tractable approaches to reversing polycystic kidney disease.

A different flavor of Polycystin channel is deployed in the left-right organizer early in development to specify the vertebrate left-right axis. While Polycystin-2 works with Polycystin-1 to limit kidney epithelial cell proliferation, Polycystin-2 collaborates with a paralog of Polycystin-1 called Polycystin-1-like-1 to help the embryo tell left from right[61–63].

Even more than with Hedgehog signaling, deep mysteries about Polycystin remain to be explored. What are the molecular identities of these hypothesized Polycystin-regulated and cilia-dependent cyst activators and repressors? What cations do Polycystin-1/2 flux

to influence kidney epithelial growth and morphology? What cues regulate Polycystin-1/2 activity? In addition to Polycystin-1, Polycystin-1-like-1 and Polycystin-2, we possess three more paralogs of Polycystin-1 and two paralogs of Polycystin-2. If Polycystin-2 paralogs can form functional homomeric channels and productively heterodimerize with all Polycystin-1 paralogs, we may possess 18 different flavors of Polycystin channels. Are each of these possible combinations formed? If so, what do they sense and what do they do? If these other forms of Polycystin channels function in development and physiology, do they all function at cilia and do they function through shared or distinct effectors?

It is likely that answers to these questions will depend on insights into Polycystin signaling derived from studies in diverse organisms. Homologs of Polycystin-2 are present in diverse ciliated organisms, including the green alga *Chlamydomonas*. In *Chlamydomonas*, Polycystin-2 localizes to two rows along the flagella and organizes a ciliary structure required for efficient swimming[64]. Interestingly, *Chlamydomonas* lacks a clear Polycystin-1 homolog, suggesting that Polycystin-2-type proteins can function independently of Polycystin-1 proteins. In *Drosophila*, a homolog of Polycystin-2 localizes to the sperm flagellum and is important for the ability of sperm to navigate the female reproductive tract[65,66]. In *C. elegans*, Polycystin-2 and an ortholog of Polycystin-1 localize to select neuronal cilia and function in male mating[52,67]. Together, these results reveal that Polycystins may have ancient roles in cilia, perhaps dating to the Neoproterozoic Era over 500 million years ago, and that evolution has subsequently deployed Polycystin channels for diverse functions in extant descendants.

Cilia transduce signaling by select GPCRs

Beyond Hedgehog and Polycystin signaling, we have known for decades that select GPCRs sense environmental cues at specialized cilia: the photoreceptor outer segment is a highly modified primary cilium at which Opsins sense light, and olfactory neurons possess atypical cilia adorned by odorant-sensing GPCRs that sense environmental chemicals. The sensitivity of these sensory cilia can be near perfect: amazingly, photoreceptor cells can detect single photons and olfactory neurons can detect single molecules of pheromone[68,69]. Ciliary signaling can also be blazingly fast with photoreceptor peak dim flash response happening at 140ms[70].

A more recent surprise of cilia biology has been that select GPCRs also localize to the primary cilia outside of sensory organs. For example, SSTR3, 5HT6, MCHR1, GPR161, GPR88, FFAR4 and MC4R are all GPCRs from different subfamilies that localize to cilia[71–75].

Beyond vision and olfaction, what types of information do ciliary GPCRs communicate? Clues from both mouse and human genetics point to critical roles for primary cilia in energy homeostasis. In mice, deletion of cilia from adult neurons causes obesity[55]. Likewise, in humans, mutations that disrupt ciliary function cause ciliopathies characterized by obesity, including Bardet-Biedl syndrome, Carpenter syndrome, Alström syndrome and MORM[76]. These human diseases are called ciliopathies to denote their common origin in compromised ciliary function[77].

The most common monogenic cause of human obesity are mutations in *MC4R*, encoding the ciliary localized GPCR Melanocortin receptor 4[75,78,79]. Thus, an appealing model is that ciliopathies cause obesity because cilia are fundamentally required for MC4R-mediated control of long-term energy homeostasis (Figure 4A).

Many GPCRs signal through cAMP, and GPCRs can be Gαs-coupled to induce cAMP formation (e.g., MC4R) or Gαi-coupled to inhibit cAMP formation (e.g., SSTR3) via regulation of adenylyl cyclases. Adenylyl cyclase 3 (ADCY3) exquisitely localizes to neuronal cilia and mutations in ADCY3 cause obesity in both mice and humans, suggesting that cAMP generation within cilia is key for energy homeostasis[80–82]. Attenuating cAMP generation specifically in mouse hypothalamic cilia causes hyperphagia and obesity[83], further suggesting that not only does MC4R localize to neuronal cilia, but MC4R regulation of ciliary cAMP controls feeding behavior. Thus, it is likely that feeding behavior is regulated by MC4R activation of ADCY3 to generate cAMP specifically within cilia. If so, MC4R regulation of long-term energy homeostasis can serve as one example of how neuronal cilia control mammalian behavior.

The finding that MC4R requires cilia to signal raises the interesting question of whether other ciliary GPCRs, such as FFAR4, also require cilia to signal. In preadipocytes, FFAR4 localizes to cilia and promotes differentiation[84]. During differentiation, the nascent adipocyte loses its cilium[85]. After differentiation, FFAR4 localizes to the plasma membrane and sensitizes the adipocyte to Insulin[86–88]. These findings raise the intriguing possibility that the same GPCR and ligand may, in one cell state, signal at the cilium with one output and, in another cell state, signal at the plasma membrane with a different output. Thus, by placing a receptor either at the ciliary membrane or at the plasma membrane, cells may respond to the same ligand in two distinct ways, and this choice of placement may be dependent on factors such as stage of differentiation.

Interesting questions about the function of GPCRs at cilia include how ligands are delivered to neuronal cilia and how cells distinguish closely related GPCRs (e.g., SSTR3 and SSTR1) to send one to the cilium and the other to the plasma membrane. For example, if a single cilium contains multiple receptor types, can the cell distinguish their outputs or are they summed? And, importantly, how do cells distinguish second messengers produced at cilia from those produced elsewhere?

The cilium and the cell body can differentially interpret second messengers

The discovery in the 1960s by Sutherland and colleagues that many hormones communicate via the second messenger cAMP was critiqued because it was deemed improbable that different cues could trigger distinct responses through a single chemical entity[89,90]. In the 1980s, Brunton and colleagues proposed that cAMP is compartmentalized within the cell such that different hormones produce distinct pools of cAMP that are interpreted in distinct ways[91,92].

How different cAMP pools communicate different information remains an area of active research. As described above, FFAR4 is an example of a GPCR that may signal sequentially

at the ciliary membrane and the plasma membrane. What happens when distinct GPCRs signal at the ciliary membrane and the plasma membrane simultaneously? One possibility is that physically sequestering signaling pathways to different parts of the cell surface allows parallel information processing to avoid, in the terminology of information theory, intersignal interference. While cells are likely to use many strategies to compartmentalize signaling so as not to confuse the information content, one possible strategy would be to take advantage of the physical differences of the cilium and cell body to distinguish cAMP in each subcellular domain.

To investigate whether the cilium represents a functionally distinct cAMP compartment, we and others have developed tools to control the subcellular production of cAMP. One optogenetic system to exert temporal control of cAMP production uses bacterial photoactivatable adenylyl cyclase (bPAC), which generates cAMP in response to blue light[93]. To distinguish the functions of cilium-generated and cell body-generated cAMP, we generated transgenic zebrafish that express bPAC targeted to cilia (Cilia-bPAC) or cytoplasm (Cyto-bPAC)[94].

Because cAMP and its effector, PKA, negatively regulate the Hedgehog pathway, Hedgehog signal transduction is one potential interpreter of ciliary and cytoplasmic cAMP. Among the many tissues patterned by Hedgehog signals are somites. Surprisingly, generating moderate amounts of cytoplasmic cAMP did not attenuate Hedgehog patterning of somites, but generating the same amount of cAMP within cilia did[94]. Similarly, a ciliary-targeted GPCR that generates cAMP affected Hedgehog signaling, but the same GPCR outside the cilium did not[94]. Perhaps these artificial systems mimic the function of GPR161, a ciliary Gαs-coupled GPCR that inhibits Hedgehog signaling during embryonic development[74]. Thus, cAMP generated in different subcellular sites imparts different information, and the cilium and cell body are functionally distinct cAMP compartments (Figure 4B).

A corollary suggested by the finding that ciliary cAMP inhibits Hedgehog signaling is that a ciliary Gαi-coupled GPCR might be able to lower ciliary cAMP and activate Hedgehog signaling. Indeed, stimulation of the ciliary Gαi-coupled GPCR, SSTR3, with its ligand, somatostatin, can activate the Hedgehog pathway in NIH-3T3 cells[94]. Given the capacity of this ciliary GPCR to activate the Hedgehog signaling pathway when expressed heterologously, it will be interesting to assess whether ciliary GPCRs beyond GPR161 function via GLI modulation.

How might the cilium act as a distinct cAMP compartment? Could there be a barrier to prevent the mixing of ciliary and nonciliary cAMP? Unlike mitochondria and most other organelles, cilia are not entirely membrane bound and are thus contiguous with the cytoplasm. cAMP biosensors revealed that cAMP freely diffuses between the cilium and cytoplasm[94,95], indicating that there was no barrier preventing the mixing of the two pools of cAMP. Similarly, examination of calcium diffusion between the cytoplasm and the cilium revealed no barrier between the two[96]. Thus, two second messengers, cAMP and calcium, freely diffuse between the two compartments, raising the vexing question of how cells distinguish ciliary and cytoplasmic signals.

Computational modeling suggested that the narrow entrance and elongated shape of the cilium imposes different cAMP dynamics in the cytoplasm and cilium: because of the cilium's low volume and high surface-to-volume ratio relative to the cell body, small absolute changes in cAMP within the cilium translate into large increases in ciliary cAMP concentration and negligible changes in cytoplasmic cAMP concentration[94]. Similarly, small absolute increases in ciliary calcium can cause large increases in ciliary calcium concentration without perturbing cytoplasmic calcium concentration[96].

In the sea urchin sperm flagellum, the effectors of cAMP and calcium are probably orders of magnitude more abundant than the second messengers themselves[97]. Thus, as long as effectors have sufficient affinity for their second messengers, activation of effectors within the flagellum happens before the second messenger has a chance to diffuse into the cytoplasm, be exported, or get degraded, a phenomenon termed kinetic compartmentalization[97]. Similar principles may operate within primary cilia to contribute to different effects of ciliary and cytosolic second messengers. Additional plausible parameters may contribute to functional separation of ciliary and cell body signaling (e.g., degradation of cAMP by phosphodiesterases, differential localization of cAMP effectors), but geometry alone may be sufficient to explain how cells distinguish ciliary and cytoplasmic second messengers.

Distinguishing pools of second messengers is likely to be essential for cellular functioning. For example, sperm make use of cAMP and calcium both in the flagellum to control swimming direction and in the head to control acrosomal exocytosis[98]. Mixing the second messengers between the two compartments would disrupt fertilization. If the unique geometry of the cilium is used to distinguish ciliary and plasma membrane signaling, then one might expect that flooding the system with cAMP would erase the ability of the cell to make that distinction. Indeed, the production of super-physiological levels of cAMP using bPAC erases the distinction and allows cytoplasmically produced cAMP to affect Hedgehog signaling[94].

Such experiments raise interesting questions about how the functional compartmentalization between ciliary and cell body signaling is maintained over different signaling regimes. For example, do negative regulators of second messengers (e.g., cAMP phosphodiesterases or calcium buffers) help prevent crosstalk between the two compartments? Additionally, if the second messengers remain restricted to their compartment of origin, the effectors can be in common. However, if signaling within the ciliary compartment must be communicated elsewhere within the cell (as exemplified by Hedgehog signaling, which must ultimately affect nuclear transcription), second messengers must work via effectors specific to their compartment of origin. An ambassador's effectiveness depends on being identified with their country.

The cilium is a source of extracellular signals

Initially observed in *Chlamydomonas*[99,100], a growing body of evidence supports a role for the cilium not just as a receiver and interpreter of extracellular information, but also as a source. In *Chlamydomonas*, flagella are the source of proteolytic enzyme-bearing

extracellular vesicles that degrade the cell wall to liberate daughter cells following mitosis[99]. Activated *Chlamydomonas* gametes shed vesicles that specifically bind the flagellae of other gametes, hinting at signaling functions[101].

Similarly, in *C. elegans*, ciliated sensory neurons release extracellular vesicles containing Polycystins, and purified vesicles trigger mating behaviors in male worms[52,67,102]. In male *C. elegans*, select neurons release ciliary vesicles into the luminal space that surrounds their cilia and then through a pore into the external environment of the animal. Interestingly, *bbs-8* and *rab-28* mutants accumulate extracellular vesicles in the lumen surrounding the cilium, but do not show any change in the release of vesicles into the external environment (Figure 5). Therefore, there may be two functionally distinct steps for ciliary vesicle release in *C. elegans*: ectocytosis from the neuron and then subsequent expulsion into the environment for extracellular communication[103]. In mammals, RAB28 promotes the shedding of the photoreceptor outer segment and *RAB28* mutations cause a form of retinal degeneration, suggesting that RAB28 may have different roles in the regulation of ciliary ectocytosis in nematodes and vertebrates[66,104].

The photoreceptor outer segment is a specialized primary cilium containing stacks of membrane disks concentrated with Opsins, key to phototransduction. Opsins contain 11-cis-retinal which absorbs light to undergo isomerization to all-trans-retinal. In addition to sensing light, these Opsins are damaged by light. Disks containing photooxidized Opsins and all-trans-retinal are shed from the distal outer segment and taken up by the adjoining retinal pigmented epithelium, which rejuvenates the 11-cis-retinal and sends it back to the photoreceptors[105]. Defects in outer segment turnover cause forms of retinal degeneration, highlighting the importance of this specialized form of ciliary ectocytosis for vision maintenance.

Formation of the disks requires branched actin polymerization and a transmembrane protein called Peripherin[106,107]. In the absence of Peripherin, the photoreceptor fails to form internalized disks and, instead, sheds them as extracellular vesicles[108]. These findings suggest that photoreceptor disk formation may be a modification of ciliary ectocytosis, a variant that accumulates the Opsin-bearing vesicles internally, not externally, to enhance light capture. Although photoreceptors are highly specialized, these results raise the question of whether other cell types alter ectocytosis to control ciliary membrane topology and signaling sensitivity.

Ectocytotic control of ciliary composition may occur outside of photoreceptors. In primary ciliated cells, upon SSTR3 stimulation and inhibition of BBSome-mediated retrieval, ciliary ectocytosis increases[109,110]. Similar to photoreceptor disk shedding, ectocytosis requires Actin[109]. The ability of the BBSome to promote GPCR retrieval into the cell body and to attenuate ectocytosis suggests that there are at least two mechanisms for controlling ciliary GPCR content.

Which proteins are shed in ciliary vesicles? Curiously, in the absence of the BBSome, a subset of GPCRs are lost from neuronal cilia[111–113]. Perhaps only certain neuronal cell types

activate ectocytosis in the absence of the BBSome, or perhaps only some types of GPCRs exit cilia via ectocytosis.

Ciliation and deciliation -- the cilium as a transient organelle

With very few exceptions (e.g., metazoan oocytes lack centrosomes, red blood cells lack nuclei), cellular organelles are present across cell types. But unlike the nucleus, ER and Golgi, not all cells have a cilium[114]. For example, blood cells, both red and white, lack cilia.

Ciliation can vary not just by cell type, but also within a cell type. For example, in the skin, many hair follicle cells are ciliated, but few cells in the interfollicular epidermis are ciliated[115] (Figure 6). The dispensability of the cilium for many cellular functions may allow tissues to tune individual cells, even neighboring cells of the same type, to different extracellular cues. For example, the few ciliated cells that line the stomach may respond to different cues than the rest of the gastric epithelium[116].

In addition, some types of cells have cilia only during certain stages of their differentiation. For example, during early stages of neural tube development, the neural epithelium lacks cilia. Soon thereafter, the neural epithelium acquires cilia and uses them for dorsoventral patterning. Conversely, the endodermal epithelium is ciliated early in development but loses cilia as it differentiates into the intestinal epithelium[117].

Control of the timing of ciliation may participate in the timing of responsiveness to extracellular information. A longstanding conundrum has been how cells accurately interpret a morphogen gradient when that morphogen gradient must be generated over time. One possible solution to this conundrum would be to separate generation of the gradient and interpretation of the gradient into different time periods. To pattern the dorsoventral axis of the neural tube, SHH and IHH are expressed at the midline from gastrulation stages. As the neural tube ciliates after gastrulation, temporal control of ciliation may allow neural epithelial cells to ignore Hedgehog cues while the gradient is being created, then, upon ciliating, interpret that mature gradient to accurately read out their dorsoventral position within the neural tube.

Thus, regulation of ciliation may help regulate cellular responses to cues, both spatially and temporally. We do not understand the mechanisms by which cells are ciliated. Some insight may come from the fact that almost all cells retract their cilia before entering mitosis. Cilia extend from a centriole, so deciliation prior to mitosis releases one of the centrioles so that it can become part of the spindle pole. How ciliary disassembly occurs remains poorly understood, although Aurora A kinase is part of the process[118].

Presumably, ciliary disassembly before mitosis frees up the mother centriole to participate in spindle pole formation. Ciliate protists solve the problem of dual roles of centrioles in ciliation and spindle function in a different way: they assemble their mitotic spindles independently of centrioles and thus the basal bodies remain attached to the cell surface throughout the entire lifecycle[119,120].

Do mammalian cells take advantage of the retractability of cilia to shape their responses to non-developmental cues? Multiciliated cells that line the upper airway and clear mucus out of the lung avoid deciliation altogether by being terminally differentiated. However, upon infection by Respiratory syncytial virus (RSV), a serious respiratory pathogen often affecting young children, multiciliated cells in the airway lose their cilia[121,122]. Loss of motile cilia compromises airway clearing, promotes the formation of mucus plugs and predisposes children to opportunistic infections by other respiratory pathogens[123]. While it is likely that the loss of cilia is caused by virally mediated damage, it is possible that aspects of this loss of cilia are adaptive. As respiratory viruses may bud from the ciliary membrane[124], it is possible that retraction of cilia could attenuate viral production. Are there inhibitors of ciliogenesis that cells turn on to negatively regulate ciliogenesis under stressful conditions such as RSV infection? Transcriptomic or proteomic analysis of RSV infected airway epithelial cells may help identify negative regulators of ciliogenesis.

Deciliation is also employed by the devastating frog-killing chytrid *Batrachochytrium dendrobatidis*[125]. *Batrachochytrium* zoospores can swim using a motile cilium[126]. *Batrachochytrium* sense the mucins secreted by frog skin, triggering a transition called encystment during which the cilium is quickly resorbed and the centriole is repurposed for the mitotic spindle during nuclear divisions[127]. Efforts to make chytrid species genetically tractable may shed light on how they resorb their cilia[126,128].

Other unicellular organisms also are flagellated only part time. *Naegleria gruberi* is a free-living protist that can generate two distinct cellular architectures: its feeding, reproducing, crawling amoeba-like form can persist indefinitely without flagella and even without centrioles[129]. However, when stressed, it transforms into a flagellated swimmer to search out better conditions. Astonishingly, this transformation involves de novo assembly of basal bodies and construction of flagella in about an hour. A similar phenomenon occurs in *Tetramitus*, a related free-living amoeba that not only constructs four flagella via de novo basal body assembly but can reproduce in both its flagellated and amoeboid states[130,131].

Not to be outdone by its distant cousin is *Plasmodium*, the protist parasite that causes malaria. *Plasmodium* lacks flagella for most of its lifecycle[132]. In the blood, *Plasmodium* can become a gametocyte which, if swallowed by a feeding mosquito, undergoes three lightning-fast rounds of mitosis and flagellogenesis, resulting in eight flagellated gametes in just 10–15 minutes. These flagella power *Plasmodium* mating in the mosquito's gut. As flagella are essential only for this one stage in its lifecycle, it is possible that a *Plasmodium* mutant lacking, for example, an outer arm dynein (e.g., PF3D7_1213600, PF3D7_1020100 or PF3D7_1114000) could be maintained in its asexual stage in the lab but fail to propagate outside the lab[133].

Similarly, choanoflagellates can retract their flagella to adopt amoeboid forms[134]. Thus, it is possible that the unicellular ancestor of multicellular animals had evolved the ability to adopt either ciliated or unciliated forms, and animals differentially deployed these programs for cell specialization. For example, in sponges, ciliated choanocytes may represent deployment of the ancestral flagellated program and unciliated archeocytes may represent deployment of the ancestral unflagellated program. As noted above, we do not understand

how vertebrate cells decide whether to generate a cilium or not. Part of the decision is probably made at the level of transcriptional control. Two transcriptional regulators of ciliogenesis, RFX and FOXJ1, are providing clues to the evolutionary history of ciliogenesis programs.

In *C. elegans*, an RFX transcription factor is essential for ciliogenesis[135]. Homologs of RFX are required for ciliogenesis in specific cell types in mice: RFX3 is required for motile ciliogenesis, RFX4 is required for ciliogenesis in parts of the brain, and RFX2 is required for sperm flagellogenesis[136–138]. In a choanoflagellate, an RFX transcription factor promotes ciliogenesis and is necessary for expression of another transcription factor, FOXJ1[139]. In mammals, FOXJ1 regulates genes involved in ciliary motility[140,141]. Thus, RFX and FOXJ1 are likely part of an ancient transcriptional network employed by animal ancestors to build cilia and subsequently modified in animal lineages[142,143].

A more even sampling of the eukaryotic tree will yield a more complete understanding of how cilia are built by extant descendants of LECA. For example, are there additional conserved nontranscriptional regulators of ciliation? And what additional factors does RFX need to induce ciliogenesis in unciliated cells?

Human genetics and disease inform ciliary biology

Among the diverse organisms with conserved ciliary biology are humans. Consequently, ciliary dysfunction in people causes disease. Mutations in more than 200 genes cause 35 distinct ciliopathies, each with partially overlapping phenotypic manifestations. Perhaps because clinicians can get to know their patients extremely well and follow them for years, clinicians provide perceptive insights into ciliary functions.

Clinicians have often collaborated closely with biologists to uncover how cilia participate in human physiology. Perhaps the most famous of these interactions were those that led to the first recognized ciliopathy, Primary Ciliary Dyskinesia (PCD). In the early 1970s, Bjorn Afzelius was working at the University of Stockholm as a PhD electron microscopist. A physician, Rune Eliasson, solicited Dr. Afzelius’s help in analyzing the ultrastructure of sperm of infertile men[144].

Electron microscopy of these sperm revealed the absence of dynein arms[145]. In addition to infertility, these men had the hallmarks of PCD (then called Kartagener syndrome): bronchiectasis, chronic sinusitis and, sometimes, situs inversus. Association of these ciliary defects with low or no mucociliary transport in the nose led to the realization that PCD is caused by defective ciliary motility[146]. Thus, cutting-edge experimental approaches and clinical insights were complementary in discovering that ciliary motility is essential for lung health and left-right axis patterning.

Since the discovery of ciliary motility defects as the cause of PCD, mutations resulting in ciliary dysfunction have been uncovered as causes of other human ciliopathies. One example is Bardet-Biedl syndrome (BBS), characterized by retinal degeneration, polydactyly, kidney cysts, anosmia, cognitive impairment, and obesity. Many of the genes mutated in BBS affect the BBSome, involved in exporting membrane-associated proteins from the cilium[147,148].

The ongoing revolution in the cell biological understanding of disease allows for a shift in classification, from causative tissue to causative subcellular process. The reclassification of diverse inherited diseases as ciliopathies highlights the commonalities between them and may serve to point out shared biology and shared therapeutic approaches going forward. Similar reclassification of other types of non-ciliary diseases by which subcellular process underlies them may help other fields recognize therapeutically exploitable commonalities for diseases previously regarded as unrelated. One example may be emerging recognition of the role of the integrated stress response (ISR) in a range of neurological diseases for which ISR inhibitor ISRIB may be useful[149].

Beyond inherited ciliopathies, Hedgehog-associated cancers such as basal cell carcinoma and medulloblastoma highlight how cilia can participate in acquired diseases. These cancer cells are ciliated and require their cilia to transduce oncogenic Smoothened signaling[115,150]. Another example of how cilia may participate in acquired disease may be some forms of Parkinson's disease, characterized by loss of dopaminergic neurons in the brain. Gain-of-function mutations in Leucine Rich Repeat Kinase 2 (LRRK2) are a rare cause of Parkinson's disease[151]. Increased LRRK2 activity inhibits ciliogenesis[152–155]. Correspondingly, mice bearing Parkinson's disease-causing mutations in LRRK2 or lacking the counteracting PPM1H phosphatase exhibit a disruption of cilia in about half of striatal cholinergic neurons and astrocytes[153,156]. Normally, these cholinergic neurons respond to Hedgehog signals to support the survival of dopaminergic neurons[157]. Thus, an intriguing model of how mutations in LRRK2 cause Parkinson's disease is that they attenuate striatal ciliogenesis, limiting Hedgehog signaling, thereby leading to loss of dopaminergic neurons (Figure 7). Perhaps because altered ciliary function is limited to a fraction of striatal cholinergic neurons and astrocytes, mutant LRRK2 does not cause widespread ciliary dysfunction. It will be interesting to discover whether cilia play a role in more common forms of Parkinson's disease, how germline mutations result in cell type-specific ciliary disruptions, and what other acquired diseases alter ciliary function.

This discovery of an organelle at the origin of disparate diseases and the coining of the term ciliopathy indicates how nosology, the classification of disease, may be changing. Nosology reflects progress in our understanding of biology: for example, classical Greek physicians recognizing that diabetics produce excess urine, focused their attention on the kidney. Not until the eighteenth century did a more sensitive test, tasting urine, allow for subclassification of diabetes into two forms: mellitus ("from honey") and insipidus ("without taste"). This modest diagnostic advance helped to move physicians' focus from the kidney to the causative organs, reclassifying diabetes as a pancreatic disease (mellitus) and a pituitary disease (insipidus). Similarly, a diverse list of diseases grouped based on their affected tissues are now being reclassified based on which organelle underlies their etiology, with ciliopathies being one key example.

Conclusion

Cilia are ancient, complex machines comprised of several hundred different components that have been evolving for hundreds of millions of years to move and interpret diverse environmental and intercellular signals. Investigating cilia will continue to illuminate

general principles of subcellular organization and information transmission. For example, understanding how cilia are compartmentalized will elucidate how transduction machinery is organized within a subcellular space to interpret extracellular cues. Understanding how ciliary lipids control signaling will reveal how organelle-specific lipids regulate protein function. Understanding how cells distinguish ciliary and plasma membrane signals will uncover how the cell holds multiple conversations simultaneously without confusing the information. Thus, investigating cilia will help reveal fundamental ways in which cells use specialized subcellular compartments to interpret their environments. Studying how cilia are constructed and signal will continue to identify human mutations that cause ciliopathies, how those mutations cause disease and, perhaps someday, how we can cure those diseases.

Beyond the fascinating field of cilia biology, there has been concern lately about the current and future status of the field of developmental biology[158–160]. Part of the concern stems from a perceived reduction in the importance of developmental biology research. Perhaps this perception stems from a constricted definition of what developmental biology entails. While the traditional domain of “good old God-fearing developmental biology”[161] contains many important, unanswered questions, a more expansive definition of developmental biology might be the study of all biological processes requiring more than one cell and relevant to embryogenesis. Under such a definition, stem cell biology, organoids, embryoids, regeneration and computational approaches applied to cell populations (e.g., pseudotime analysis) are all subdomains or tools within developmental biology. When considered from this perspective, developmental biology is experiencing explosive growth.

The recent exciting discoveries in cilia biology described above underscore how cell biological tools can help drive discovery of important and general principles in developmental biology. Of course, apart from cilia, there are many instances of how cell biological approaches have led to fascinating developmental biology discoveries. For example, investigating how the actin cytoskeleton is spatially regulated has helped reveal how convergent extension drives neural tube closure[162] . And investigating how cadherins control cell-cell adhesion has shed light on how mechanical forces sculpt cell shape[163].

Just as biochemistry and genetics are simultaneously fields in their own rights and the source of experimental approaches used by developmental biologists, it is likely that cell biology will both remain its own domain and continue to provide insights, approaches and tools that inform developmental biology. Blurring of the borders between previously distinct fiefdoms of biology can be disorienting as it disrupts long-standing definitions of fields. Still, the borders between biochemistry, genetics, cell biology and developmental biology, always imprecise, are only becoming more so, with cilia biology being a prime example of how synergistic cell and developmental biological perspectives drive discovery[164].

The advances detailed in this Review underscore the importance of cilia in evolutionary, developmental, cell, and human biology. Evolutionarily conserved machinery hints at ancestral ciliary function and continues to inspire avenues of inquiry into ciliary functions in animals living today. Hedgehog, Polycystin and GPCR signaling are examples of how ciliary signaling shapes embryonic development, maintains adult tissue populations, and provides a platform for reception of extracellular sensory cues. The cilium, despite being incompletely

membrane bound, also functions as a distinct compartment that responds to local signaling molecule concentrations. Beyond its role as a signaling receiver, there is evidence that cilia can produce signals to be received by other cells. Additionally, there is great diversity in the spatiotemporal dynamics of ciliation amongst species and even amongst tissue types within one organism. This variety and complexity can not only be appreciated at the cellular level but also at the level of human disease, as ciliary defects cause a wide variety of diseases depending on the tissue location and type of the ciliary dysfunction. Ciliary biology lies at the boundary between biological disciplines, lending itself beautifully to a diverse range of tools, analyses, and inquiries that can be rigorously applied to understanding this complex organelle.

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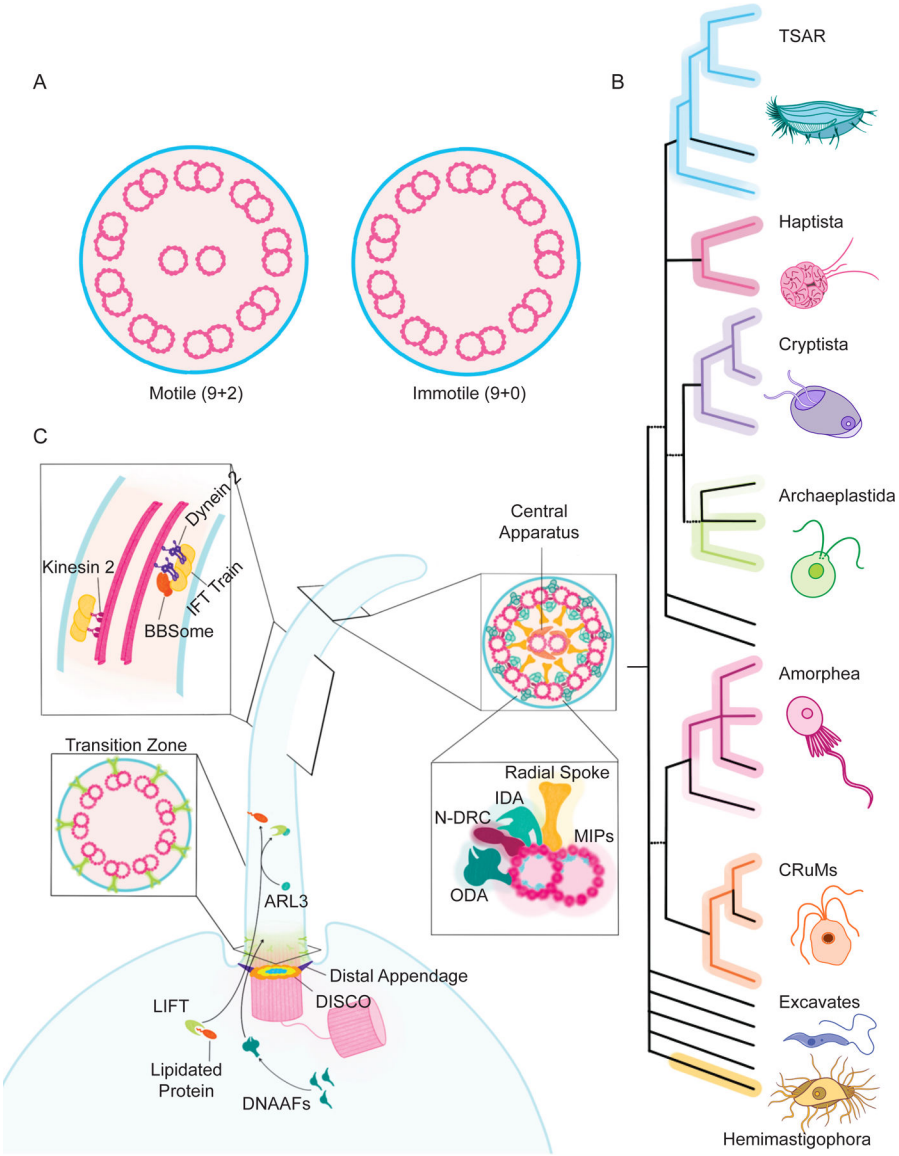

Figure 1 - Motile and immotile cilia differ structurally and LECA possessed a sophisticated cilium

A) Most motile cilia (left) have an axoneme comprised of nine circumferential doublets and a central pair. Most primary cilia (right) have an axoneme lacking the central pair. **B)** A formulation of the eukaryotic tree of life, depicting major clades. Ciliated lineages are depicted with colored lines, unciliated groups are in black and lineages with both are in both. While some groups lack cilia (e.g., Rhodophyta), and some groups contain both ciliated and unciliated members (e.g., Chloroplastida), ciliated organisms exist in all extant clades. Thus, the last eukaryotic common ancestor was probably a ciliated protist. Figure modified from reference 164. **C)** Ciliated lineages share genes encoding ciliary proteins, including components of the LIFT system for transporting lipidated proteins. In the cilium, ARL3 triggers LIFT to deposit cargoes such as ARL13B and INPP5E into the ciliary membrane. Other evolutionarily ancient protein complexes involved in ciliary function include DISCO, a distal centriolar complex involved in forming distal appendages, DNAAFs involved in

assembling dyneins, the transition zone controlling the protein composition of the ciliary membrane, IFT driving transport along the ciliary axoneme, the BBSome helping export select proteins from the cilium, the MIPs reinforcing the ciliary microtubules, and the central pair apparatus, radial spokes, and N-DRC that, together, coordinate the IDAs and ODAs that beat the cilia.

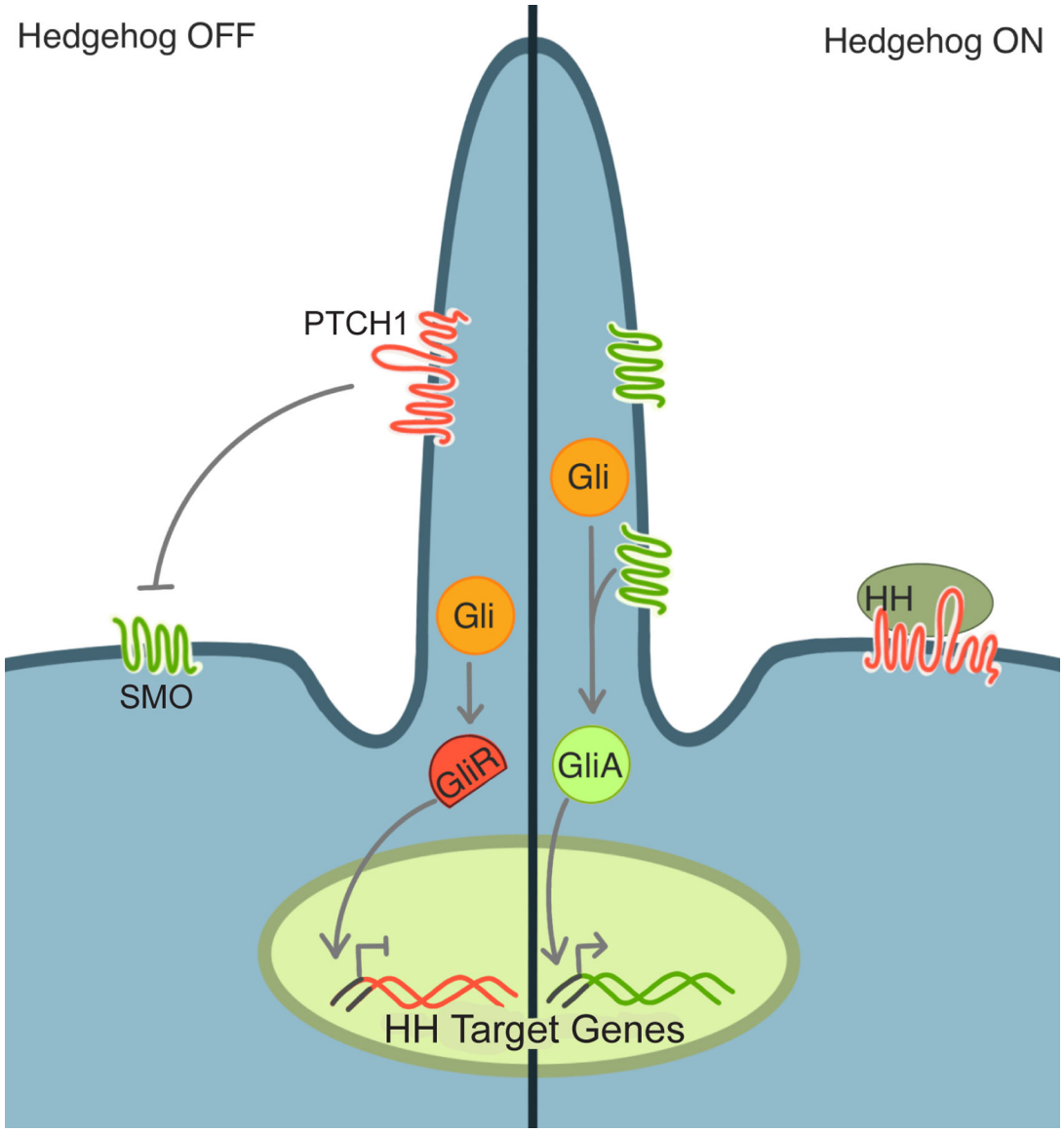

Figure 2 –. Ciliary Hedgehog signaling translates different ciliary protein composition into different transcriptional outputs.

In the absence of Hedgehog (HH) signals (left), PTCH is present in the cilium and keeps SMO out and inactive. Without active SMO in the cilium, GLI transcription factors are converted into a repressive form (GliR), translocate to the nucleus and keep the Hedgehog transcription program off. In the presence of HH signals (right), SMO accumulates in the cilium and converts GLI into an activator form (GliA) which induces the Hedgehog transcriptional program.

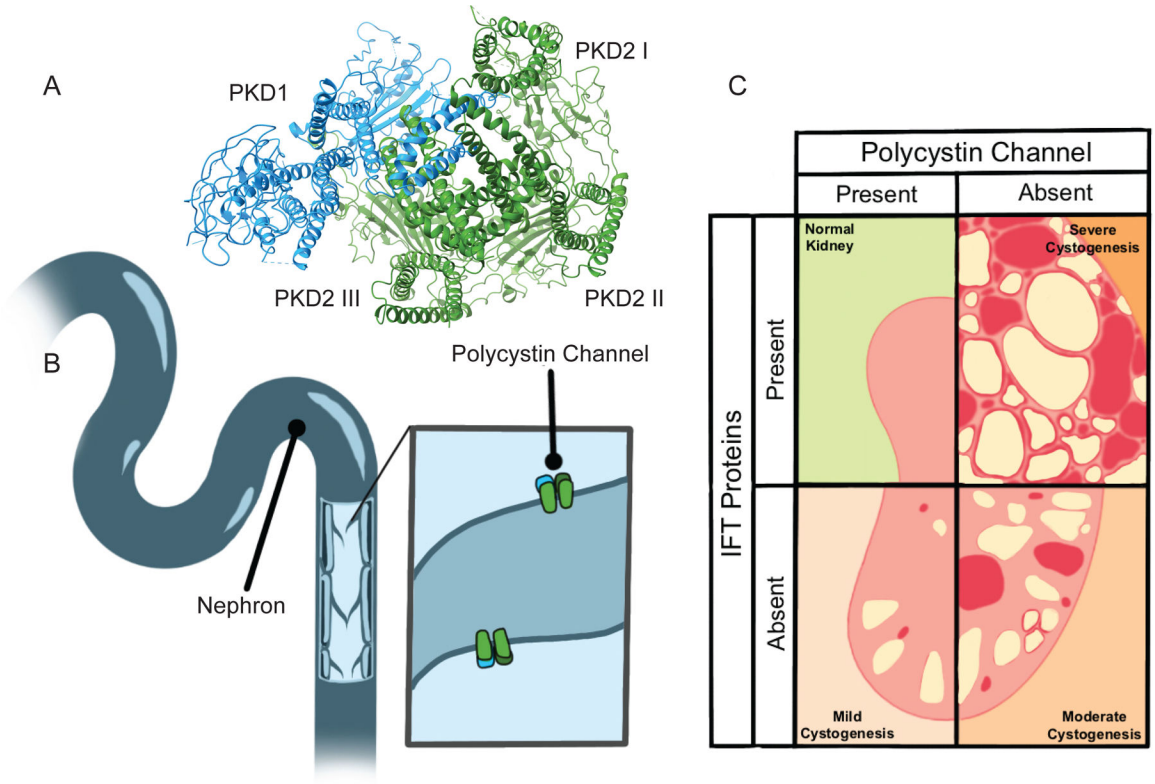

Figure 3 –. Polycystin channels protect the kidney from cystogenesis.

A) Polycystin channels are heterotetramers each comprised of one PKD1 subunit and three PKD2 subunits. Image of PDB 6A70 human PKD1/PKD2 complex (Su et al., 2018) created with UCSF ChimeraX. **B)** Almost all nephron epithelial cells project a Polycystin channel-bearing cilium into the lumen through which filtrate flows. **C)** Removing cilia from nephron epithelial cells causes mild cystogenesis. Removing Polycystin function by abrogating either PKD1 or PKD2 causes severe cystogenesis. Removing both cilia and Polycystin function causes moderate cystogenesis, revealing cilia-dependent cyst activation (CDCA) (Ma et al., 2013).

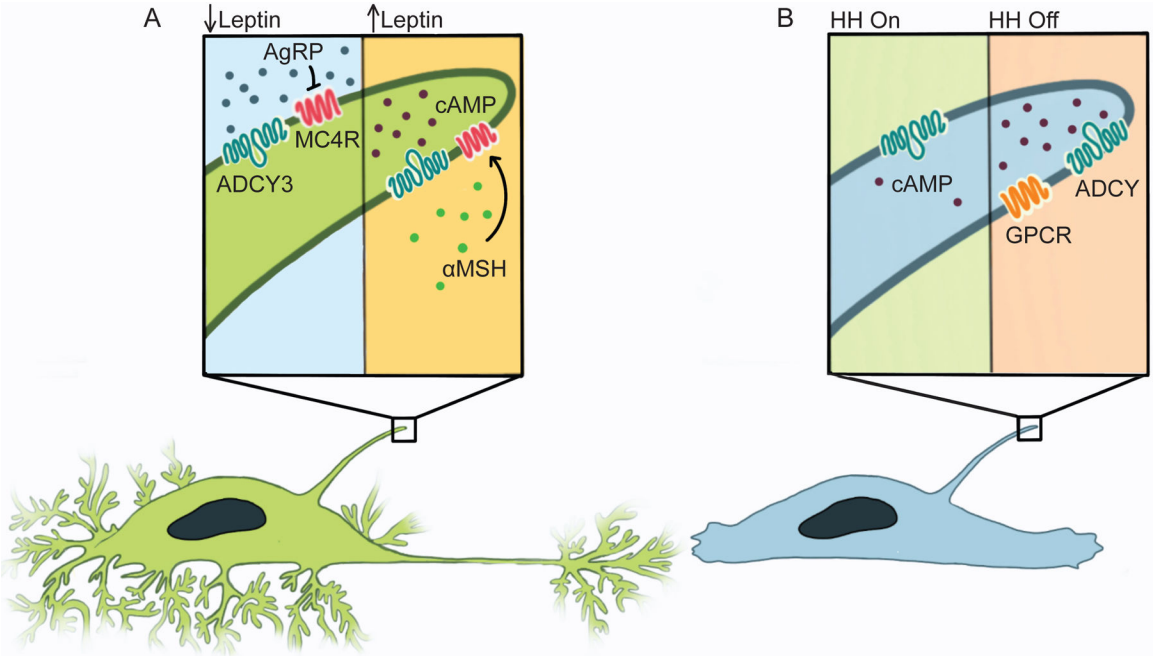

Figure 4 –. Different ciliary environments facilitate specific signaling cascades

A) Left: When peripheral energy stores are depleted, Leptin levels fall, leading Leptin-responsive neurons in the hypothalamus to make AgRP, an inverse agonist of MC4R. In the absence of MC4R activity, ADCY3 in neuronal cilia does not generate cAMP, spurring feeding. Right: When peripheral energy stores are repleted, Leptin triggers hypothalamic neurons to make αMSH, an MC4R agonist (green). Stimulating MC4R induces ADCY3 to generate ciliary cAMP, suppressing feeding. **B)** cAMP generated in the cilium activates a ciliary pool of PKA to turn off Hedgehog signal transduction.

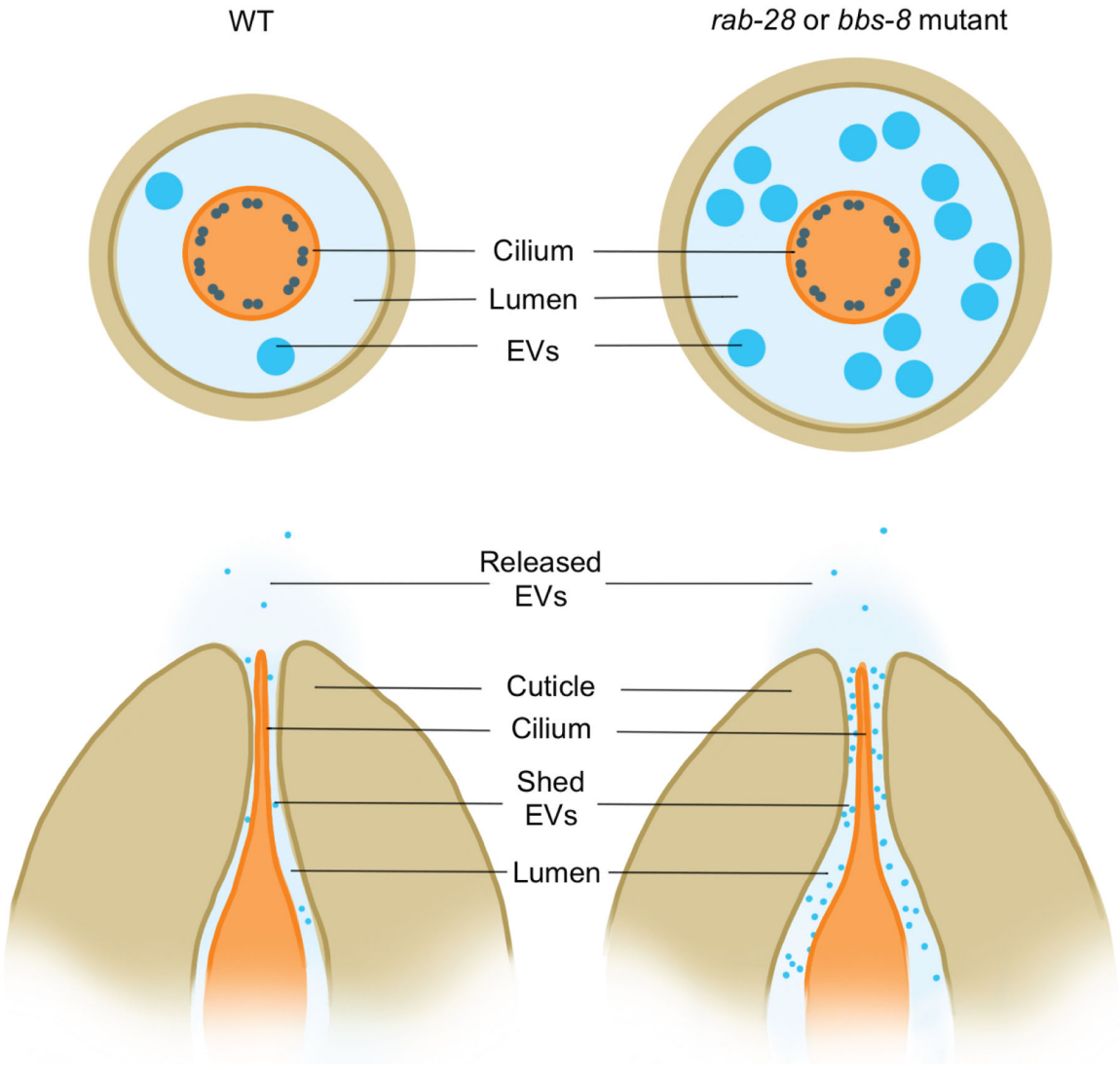

Figure 5 - Some C. *elegans* cephalic male neuronal cilia release extracellular vesicles.
Mutations in *rab-28* or *bbs-8* increase ectocytosis of extracellular vesicles into the lumen surrounding the cilia.

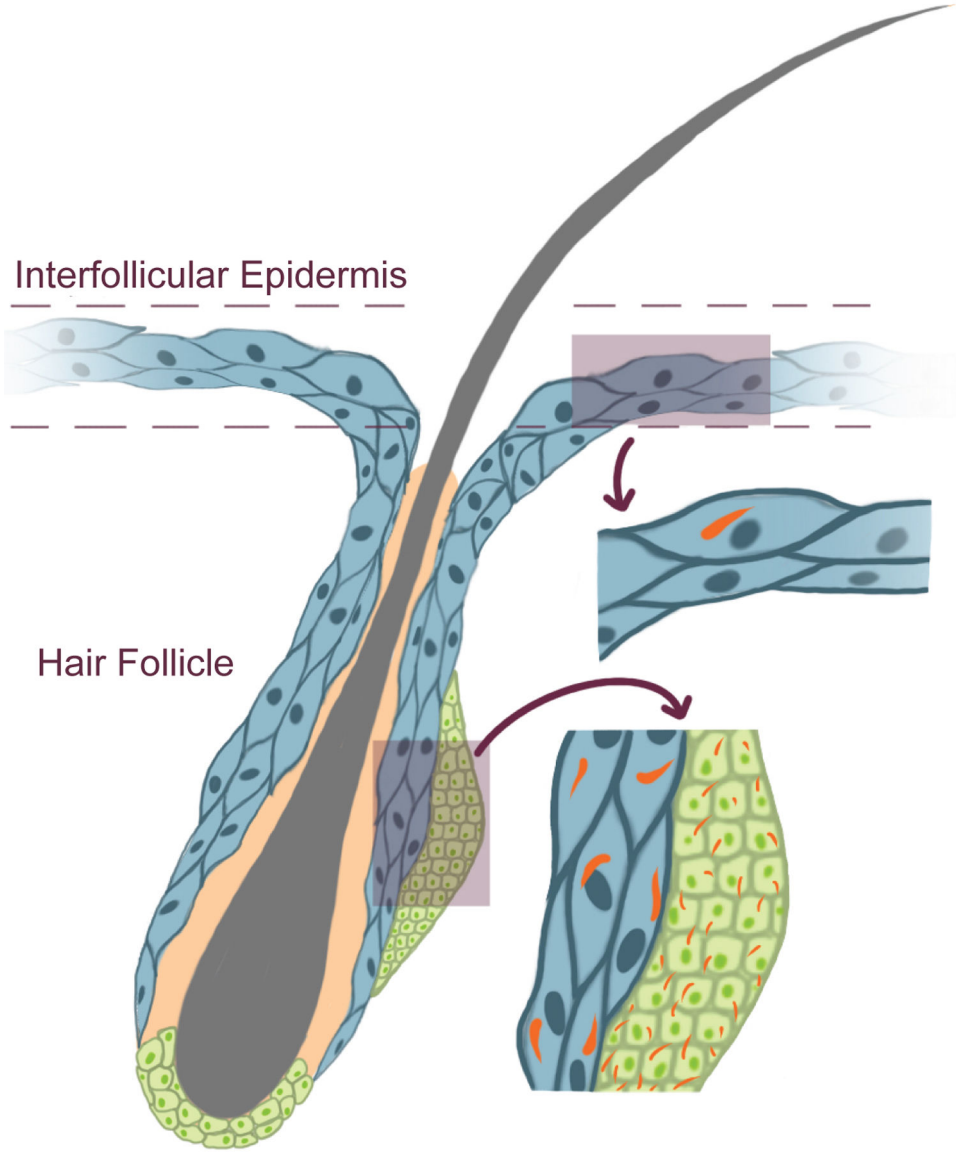

Figure 6 –. Not all animal cells possess cilia.

For example, the skin is comprised of hair follicles and the interfollicular epidermis. Most cells making up the interfollicular epidermis are unciliated. In contrast, most cells of the hair follicle are ciliated. What controls which cells are ciliated and which are not remains unknown.

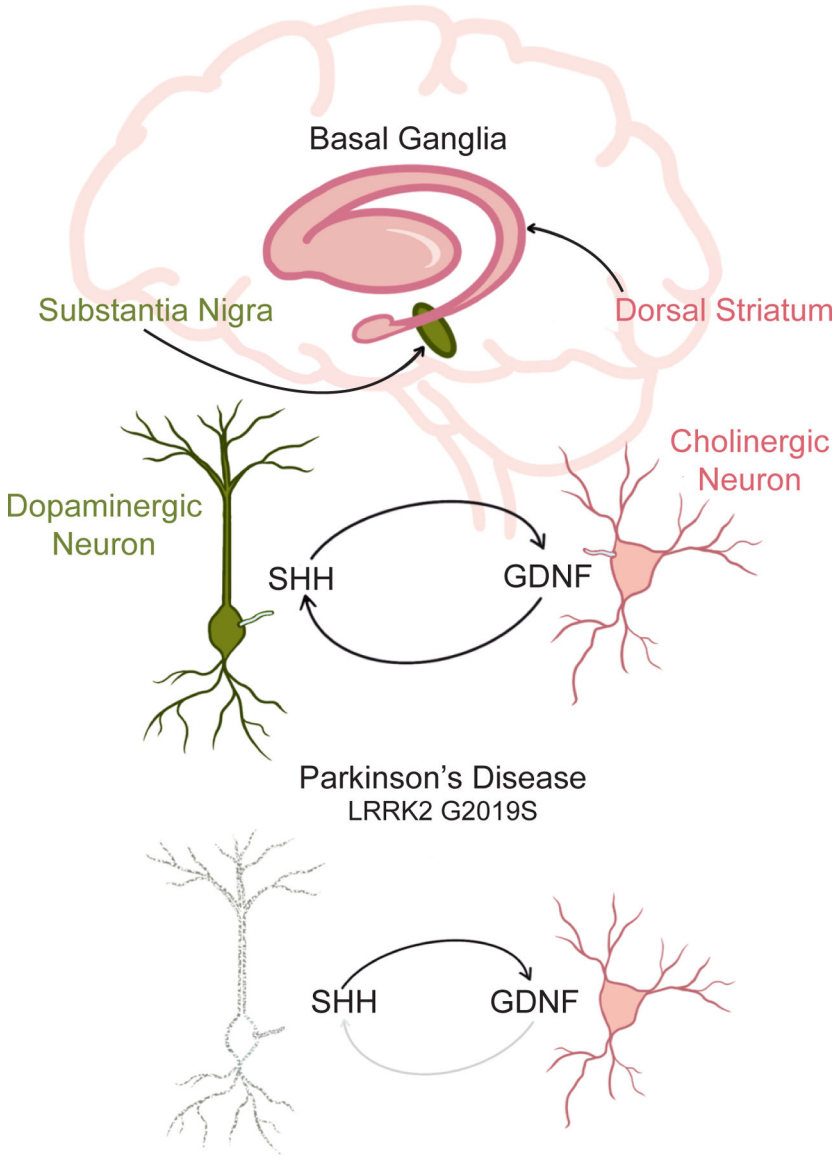

Figure 7 - A model of ciliary involvement in neurological disease.

In Parkinson's disease, dopaminergic neurons in the substantial nigra of the basal ganglia are lost. These neurons are supported by GDNF produced by cholinergic neurons in the striatum. A rare form of Parkinson's disease is caused by gain-of-function mutations (e.g., G2019S) in a kinase, LRRK2, that disrupts cilia in the striatum. With compromised cilia, the cholinergic neurons do not respond to the Hedgehog signals produced by the dopaminergic neurons and, consequently, fail to produce GDNF, perhaps leading to the death of the dopaminergic neurons.