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Autonomy declared by primary cilia through compartmentalization of membrane phosphoinositides

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The primary cilium is a cell surface projection from plasma membrane which transduces external stimuli to diverse signaling pathways. To function as an independent signaling organelle, the molecular composition of the ciliary membrane has to be distinct from that of the plasma membrane. Here, we review recent findings which have deepened our understanding of the unique yet dynamic phosphoinositide profile found in the primary cilia.

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Introduction

Often described as a hair-like protrusion, the primary cilium resembles cell surface appendages such as the filopodium or the microvillus (Figure 1a–c). Unlike these plasma membrane projections, the primary cilium has been recognized as a cellular organelle based on its ability to establish autonomy on the plasma membrane and accomplish discrete signaling functions. Indeed, genetic and proteomic studies have revealed a multitude of developmental pathways initiated from the primary cilia [1–4]. Mis-localization of ciliary signaling receptors, ion channels or enzymes have been associated with a diverse class of disorders collectively termed ciliopathies [5], supporting the role of primary cilia as an important, independent signaling platform. An outstanding question to date is how the signal transduction mechanism in the primary cilium remains autonomous despite geographic influences from the contiguous plasma membrane, which is a few hundred-folds larger in surface area (Figure 1a). In this mini-review, we will introduce and discuss recent findings on the molecular basis of primary cilia's organellar identity. These studies demonstrate that the compartmentalization of specific membrane lipids underlies primary cilia's capacity to initiate signaling functions independently of the plasma membrane.

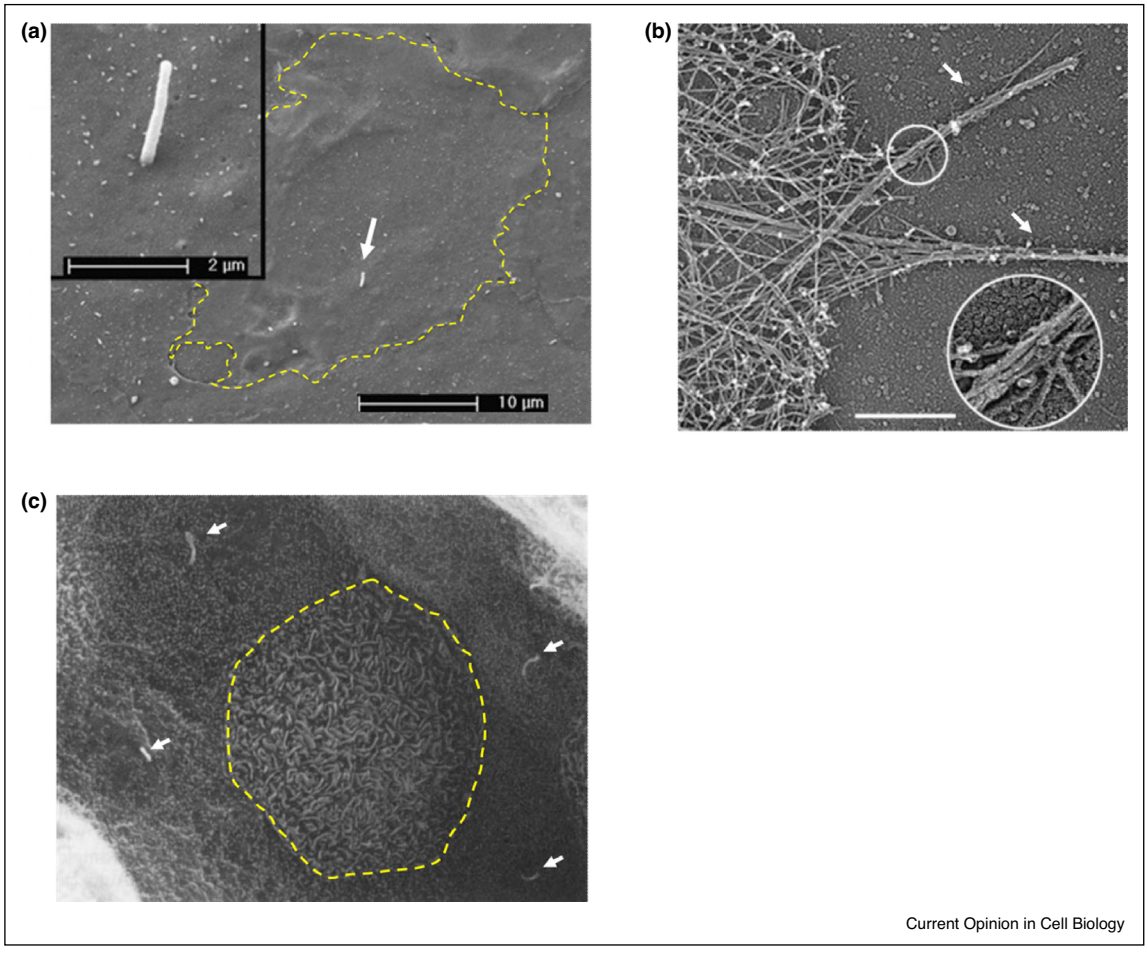
A distinct phosphoinositide profile for two contiguous membranes

Mechanisms must exist within the ciliary membrane to prevent invasion by molecules governing the structure and function of plasma membrane. A class of lipids known as phosphoinositides uniquely identify subcellular membranes [6]. In the case of plasma membrane, phosphatidylinositol (4,5)-bisphosphate, PI(4,5)P_2, plays a pivotal role in executing molecular signaling, vesicular trafficking and cytoskeletal processes by recruiting diverse proteins equipped with polybasic residues and/or pleckstrin-homology domains. By contrast, the Golgi apparatus utilizes phosphatidylinositol-4-phosphate, PI(4)P, to direct vesicular trafficking with other intracellular membrane compartments [7]. Interestingly, PI(4)P is also present in the plasma membrane, and the ratio between PI(4,5)P_2 and PI(4)P has been proposed to shape the general electrostatic property of the plasma membrane [8] and control different types of morphogenetic processes such as actin comets and ruffling [9]. The lipid composition of primary cilia, however, depicts a different molecular landscape. Recent works have revealed that primary cilia of resting (non-dividing and quiescent) cells actively metabolize PI(4,5)P_2 into PI(4)P through cilia-enriched phosphoinositide-5-phosphatase Inpp5e [10,11,12••,13••]. The consequential absence of PI(4,5)P_2 from the distal cilia coupled with the presence of PI(4)P represents a key characteristic of the ciliary membrane (Figure 2a,b) [12••,13••,14].

PI(4,5)P_2 exclusion frees primary cilia from the reins of actin-driven plasma membrane processes

Assigning a unique molecular identity to the ciliary membrane is crucial in recognizing the primary cilium as a distinct signaling unit from the surrounding plasma membrane. PI(4,5)P_2, together with other phosphoinositides, functionalizes the plasma membrane through the recruitment of various actin regulatory factors which modulate the actin cortex and generate mechanical forces to remodel the plasma membrane into function-specific configurations [15,16]. Actin polymerization at the plasma membrane is required to drive robust, dynamic outward projections such as lamellipodia and filopodia that contribute to cell motility, as well as transient inward

Figure 1



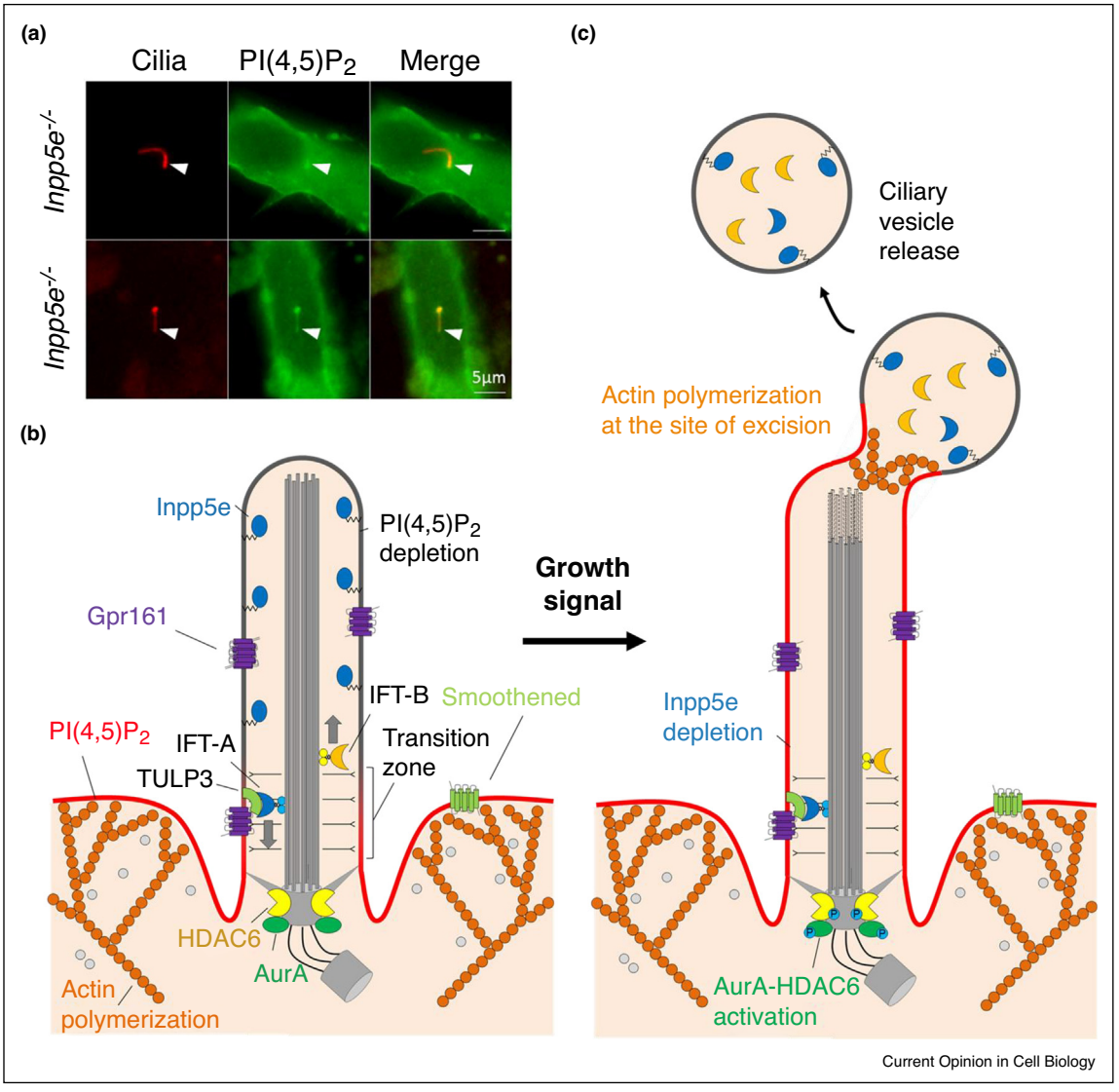
Primary cilium possesses structural similarity to other protrusion structures in plasma membrane. **(a)** An electron microscope image of primary cilium on cultured human umbilical vein endothelial cells. The inset shows the primary cilium indicated by the white arrowhead at higher magnification. The yellow dotted line outlines the cell. Modified from [63]. **(b)** An electron microscope image of filopodium, a cytoskeletal structure protruding from the leading edge of a migrating cell. Filopodium is about 0.2 μm in diameter and 3–10 μm in length [64]. Primary cilium shows similar diameter (0.2 μm) and length (1–10 μm). However, each normal cell has only one primary cilium located above its nucleus, while many filopodia appear at the leading edge during cell migration. The white arrowheads indicate filopodia. Scale bar = 1 μm. The inset shows actin bundle in the filopodium indicated by the circled region at higher magnification. Modified from [65]. **(c)** An electron microscope image of primary cilia, microvilli, and microplicae in kidney collecting duct. Kidney collecting duct consists of two distinct cell populations, principal cells and intercalated cells. Each principal cell exhibits a primary cilium with numerous tiny projections, called microvilli (not so visible in this particular image). Intercalated cells do not possess primary cilium but harbor numerous apical microplicae, which are longer than microvilli but shorter than primary cilia. The yellow dotted line outlines an intercalated cell surrounded by principal cells. The white arrowheads mark primary cilia of principal cells. Modified from [66].

invaginations such as endocytic pits where membrane turnover and signal transduction can take place. On the contrary, actin-dependent membrane functionalization may be incompatible with the function of primary cilia as sensory antennae, which likely necessitates stable structural foundation, and is suppressed by Inpp5e through active PI(4,5)P_2 depletion. Indeed, canonical actin-dependent endocytic and exocytic events could result in acute ciliary membrane turnover that compromises the maintenance of the ciliary domain measuring a mere few square micrometers on the cell surface. Instead, ciliary membrane turnover is re-directed to specialized ciliary pocket structures located around the periphery of the ciliary base [17,18]. The absence of such membrane remodeling processes in the ciliary membrane could further be crucial in preserving the characteristic rod-shaped structure and maintaining a high surface-to-volume ratio, a feature presumably favorable for a signaling antenna [19].

Phosphoinositides delineate cilia-specific signaling networks

While Inpp5e delineates the ciliary membrane from the plasma membrane, the assembly of ciliary signaling

Figure 2



Dynamic membrane lipid reorganization upon growth stimulus. **(a)** Live imaging of ciliary PI(4,5)P_2 in *Inpp5e*$^{+/-}$ and *Inpp5e*$^{-/-}$ mouse embryonic fibroblasts. Primary cilia were visualized by the fluorescent cilia marker 5HT_6-YFP (false-colored red). The PI(4,5)P_2 biosensor $PH^{PLC\delta1}$-mCerulean3 (false-colored green) is confined at the proximal region of cilia in *Inpp5e*$^{+/-}$ cells but localized over the entire length of the primary cilia in *Inpp5e*$^{-/-}$ cells. Modified from [12••]. **(b)** Primary cilium is a microtubule-based organelle on the cell surface which can sense extracellular signals and transmit them to downstream intracellular signaling pathways. To function as a signaling center, primary cilium harbors various proteins including enzymes, ion channels, and receptors such as GPCRs. The trafficking of many of these signaling molecules is regulated by the anterograde IFT-B machinery, which transports signaling molecules into cilia, as well as the retrograde IFT-A machinery, which returns these molecules to the cell body. In resting cells, the ciliary membrane displays a unique lipid composition, in contrast to the PI(4,5)P_2-rich plasma membrane. Such differences in lipid composition is made by ciliary Inpp5e, which dephosphorylates PI(4,5)P_2. In turn, PI(4,5)P_2 depletion represses actin polymerization in primary cilia, and is also involved in the distribution of ciliary proteins (e.g. Gpr161). **(c)** Upon growth stimulation, primary cilium begins to disassemble. Phosphorylated Aurora A kinase by growth stimulation phosphorylates HDAC6, and HDAC6 deacetylates and destabilizes ciliary microtubules for cilia resorption. In addition to this classical pathway, growth signals also decrease ciliary Inpp5e, thereby replenishing PI(4,5)P_2 in primary cilia. This lipid remodeling permits actin polymerization at the tip of cilia, resulting in ciliary vesicle release. Whether the distal end of the axoneme (dotted line) is destabilized at the time and site of the ciliary membrane scission requires further testing. The resultant ciliary vesicles contain a specific set of ciliary proteins including IFT-B and Inpp5e.

functions is generally established by the transition zone composed of a series of Y-shaped structures linking the ciliary membrane and the microtubular axoneme at the proximal cilia, as well as the propeller-like transition fibers connecting the ciliary membrane to the basal body [20,21]. The transition zone and transition fibers assemble a dual-layer gate to retain receptors and ion channels defining the specialized sensory functions of the primary

cilia [22–24]. While ciliary enrichment of Inpp5e relies on the integrity of the transition zone [12••,25], a recent study also suggests Inpp5e regulates the function of the transition zone [26]. Thus, the transition zone and Inpp5e likely cooperate in the determination of ciliary signaling networks. One interesting candidate to discuss would be Hedgehog (Hh) signaling that controls cell proliferation and differentiation during development [27]. Smoothened, a G-protein coupled receptor that traffics in and out of primary cilia through the transition zone to transduce Hedgehog signals, is recently reported to bind to PI(4)P [28•]. The high local concentration of PI(4)P in the ciliary membrane that results from constitutive $PI(4,5)P_2$ de-phosphorylation could potentially generate a hotspot for PI(4)P-regulated Smoothened activity in the cilia. Additionally, $PI(4,5)P_2$ presence in the proximal ciliary membrane could strategically position trafficking regulatory proteins at the ciliary base through lipid binding. One such example is TULP3, which accumulates at the proximal cilia through $PI(4,5)P_2$ binding and facilitates the entry of G-protein coupled receptor Gpr161, a Hh signaling repressor [29,30]. A more recent study has extended this TULP3-dependent trafficking mechanism to additional ciliary receptors and ion channels, thereby highlighting the importance of $PI(4,5)P_2$ compartmentalization in controlling the concentrations of ciliary signaling components [31•]. Taken together, these examples demonstrate that the unique ciliary phosphoinositide profile complements the gatekeeper function of transition zone/fibers in constructing cilia-specific signaling functions.

Loss of Inpp5e and end of primary cilia

The presence of primary cilia is, nevertheless, not perennial but is instead dynamically regulated by the cell cycle state; while cilia assemble during the G_0/G_1 phases, they disassemble upon mitotic entry [32]. The accelerated cilia disassembly caused by genetic ablation of Inpp5e demonstrates Inpp5e control in cilia stability [10,11]. Under physiological conditions, cell cycle re-entry in quiescent cells is accompanied by the disappearance of Inpp5e from primary cilia, which results in an accumulation of $PI(4,5)P_2$ prior to cilia disassembly (Figure 2c) [33••]. Hence, the diminution of the cilia-specific $PI(4,5)P_2$-depleted membrane domain on the cell surface is associated with the loss of primary cilia [33••]. Although little is known about the molecular mechanism of Inpp5e loss in the cilia, we speculate that re-localization and/or degradation is responsible. As one of the classical cell cycle regulators, Aurora A kinase (AurA) serves as a key controller of cilia stability by disassembling axonemal microtubules through activation of histone deacetylase 6 (HDAC6) [32]. AurA can also regulate Inpp5e via direct phosphorylation [34•], and pharmacological inhibition of AurA preserves Inpp5e in cilia of growth-stimulated cells [33••]. Independently, Inpp5e is reported to bind to cargo proteins of cilia trafficking such as ARL13B [35,36], PDE6D [35,37,38] and CEP164 [35]. These results suggest functional and/or biochemical interactions between the two molecules, and it is of great interest to understanding if and how they regulate one another for their functionality.

$PI(4,5)P_2$ accumulation generates a 'cut here' signal in primary cilia read and executed by actin

Intriguingly, ciliary $PI(4,5)P_2$ accumulation triggers the scission of distal cilia which consequently get released as vesicles into the external environment (Figure 2c) [33••]. Emerging evidence indicates that cilia could be invaded by polymerizing actin upon specific signaling induction. In parallel, Nager *et al.* reported that certain cilia-residing G protein-coupled receptors (GPCRs) may be removed via signal-dependent ciliary vesicle release, and this process was revealed by pharmacological inhibition to depend on actin and its regulators, such as myosin 6, drebrin, Arp2/3 and α-actinin 4 [39••]. However, approaches based on an endpoint evaluation of pharmacological inhibitors could not readily inform when and where F-actin plays a role in ciliary vesicle release. To circumvent this challenge, methods for visualizing and perturbing actin polymerization inside primary cilia of live cells are required. Phua *et al.* [33••] performed time-lapse, live-cell imaging with simultaneous visualization of F-actin during growth-induced ciliary vesicle release and discovered acute and transient F-actin assembly at the site of ciliary membrane scission. A combination of live-cell imaging and *in situ* perturbation indicated that actin polymerization inside primary cilia is indeed responsible for growth-induced ciliary vesicle release. Importantly, inhibition of the release of ciliary vesicles also impaired cilia disassembly, revealing a mechanism of how the loss of $PI(4,5)P_2$ compartmentalization could lead to cilia instability. This model is supported by the detection of $PI(4,5)P_2$-binding actin regulators in cilia including cofilin-1, fascin, Kras and Sorting nexin 9 (SNX9) with elevated $PI(4,5)P_2$ [33••]. While a recent ciliary proteomic study by Kohli *et al.* has also detected actin regulators in primary cilia, whether these proteins depend on $PI(4,5)P_2$ for cilia localization and their role in primary cilia requires further investigation [40•]. A detailed molecular mechanism of the vesicle shedding process is thus anticipated. Since the structure of primary cilia is supported by a microtubule-based axoneme whose rigidity may counteract the scission, it would be relevant to dissect whether microtubule depolymerizing pathways such as AurA-HDAC6 and PLK1-KIF2A could spatially degrade the axoneme at the cilia scission site in concert with actin polymerization [32,41].

How cutting the tip modulates primary cilia

The excision and release of cilia tips in mammalian cells are proposed as a mechanism to facilitate cilia disassembly and to progress the cell cycle [33••], a finding

consistent with the tightly-meshed relationship between cilia life cycle and the cell cycle [42–44]. Understanding the molecular significance of these cellular events has just begun, but we have several clues. A proteomic analysis of shed ciliary vesicles has revealed that the vesicles preferentially contain anterograde motor-associated IFT-B over retrograde motor-associated IFT-A components [33••]. One might then speculate that cilia disassembly would be induced because cilia dispose of IFT-B components to quickly and efficiently lose the power to supply materials required for cilia maintenance, thereby compromising the stability of ciliary structure. Alternatively, it could be that cell cycle is advanced due to the implication of ciliary vesicle release in Smo-independent Hedgehog activation [33••]. While a direct disposal of established Hh signaling effectors such as Gli3 and Sufu in ciliary vesicles could potentially underlie this effect, it could also be relevant to explore roles of ciliary vesicle release in modulating Hedgehog signaling through additional non-canonical routes [45], including MAPK, TGF-β, PKC, PI3K/Akt and mTOR, molecules that are not entirely unfamiliar to primary cilia [46–48]. Thus, the release of vesicles from cilia containing signaling components could also be viewed as a way to alter the signaling state of cilia. Supporting this idea, ciliary vesicle release has been proposed as a mechanism to remove activated GPCRs to acutely terminate ciliary signaling [39••].

A potentially non-autonomous role of cilia vesicles

If the extrusion of ciliary material via extracellular vesicle release may be perceived as a mechanism to dilute the structural and/or functional identity of primary cilia, the ciliary vesicles may be transporting a piece of cilia identity in the extracellular environment. Interestingly, ciliogenesis has been shown to display dependence on 'past memory' — Paridean *et al.* demonstrated that an intracellular remnant of the ciliary membrane which remains attached to the mother centriole during cell division promotes faster cilia assembly in the daughter cell inheriting the mother centriole [49]. Similarly, daughter inheritance of the cytokinetic midbody remnant in polarized epithelial cells has also been shown to promote ciliogenesis [50•]. Thus, the possibility of ciliary vesicles regulating the re-establishment of ciliary spatial domain on the cell surface post-mitosis should be pursued. Finally, ciliary vesicle release is also observed in various organisms and under different biological contexts [51]. Ciliated sensory neurons of *C. elegans* release bioactive ciliary vesicles [52], while *Chlamydomonas* shed vesicles containing sexual agglutinin SAG1 from the flagella into the extracellular environment [53]. Back to mammals, instances of ciliary vesicle release have been detected *in vivo* [54,55], and promoted by genetic mutations of additional ciliary components [56]. Dissecting the cilia-associated vesicles in each scenario could lead to universal principles underlying the autonomous and non-autonomous functions of these unique ciliary outputs.

Conclusion and outlook

Recent studies featured in this review have revealed the unique membrane composition of the primary cilia. We also discussed how the specific lipids and phosphatases differentially galvanize ciliary functions under quiescence and growth conditions, with a particular emphasis on cilia-derived vesicle formation. We end this review by raising three questions. First, do phosphoinositide enzymes besides Inpp5e play any role in the regulation of ciliary membrane composition? There are reports of phosphatases and kinases such as OCRL [57], INPP5B [58] and PI4P5KIγ [59] located at or near the primary cilia, and revealing how these enzymes coordinate with one another in regulation of ciliary lipid profiles would provide a more comprehensive view. Second, are there any lipids other than phosphoinositides contributing to the ciliary functions? The ciliary membrane could possess a unique sterol composition [60] that may give rise to characteristic biophysical properties [61], or chemosensory functions [62]. Lastly, can we use the accumulated knowledge to deduce a new treatment strategy for ciliopathies? Because dysregulation and mislocalization of ciliary phosphoinositide enzymes lead to ciliopathies, rectifying these malfunctioning enzymes and abnormal phosphoinositide profiles may in the future inform drug designs that can restore ciliary functions.

At all levels in biology, form and function are closely related. In retrospect, the miniscule size and characteristic shape of the solitary primary cilium could be pertinent to its role as a signaling antenna of the cell. To further deepen our understanding on the form-function interplay at primary cilia, a technique that can directly de-form primary cilia may become handy for assessment of its functional consequence. With the exciting questions to be addressed, and new techniques to be developed, this tiny organelle will continue to guarantee the attention from a diverse scientific audience.

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