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REVIEW

Primary cilia proteins: ciliary and extraciliary sites and functions

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Abstract
Primary cilia are immotile organelles known for their roles in development and cell signaling. Defects in primary cilia result in a range of disorders named ciliopathies. Because this organelle can be found singularly on almost all cell types, its importance extends to most organ systems. As such, elucidating the importance of the primary cilium has attracted researchers from all biological disciplines. As the primary cilia field expands, caution is warranted in attributing biological defects solely to the function of this organelle, since many of these "ciliary" proteins are found at other sites in cells and likely have non-ciliary functions. Indeed, many, if not all, cilia proteins have locations and functions outside the primary cilium. Extraciliary functions are known to include cell cycle regulation, cytoskeletal regulation, and trafficking. Cilia proteins have been observed in the nucleus, at the Golgi apparatus, and even in immune synapses of T cells (interestingly, a non-ciliated cell). Given the abundance of extraciliary sites and functions, it can be difficult to definitively attribute an observed phenotype solely to defective cilia rather than to some defective extraciliary function or a combination of both. Thus, extraciliary sites and functions of cilia proteins need to be considered, as well as experimentally determined. Through such consideration, we will understand the true role of the primary cilium in disease as compared to other cellular processes' influences in mediating disease (or through a combination of both). Here, we review a compilation of known extraciliary sites and functions of "cilia" proteins as a means to demonstrate the potential non-ciliary roles for these proteins.

Keywords Primary cilia · Extraciliary · Ciliopathy

Introduction

Cells from all three taxonomic domains of life (archaea, bacteria, and eukaryota) are capable of extending cellular protrusions that provide important functions to the cell. Perhaps the most well-known example is the flagellum, a structure that exists in all three domains, but is structurally distinct in each. Thus, this structure has such evolutionary importance that it evolved three times in early life [1–4]. The eukaryotic flagellum shares many characteristics with the eukaryotic motile cilium [5], with each having an axonemal structure that consists of nine microtubule doublets that surround a central pair of single microtubules, a conformation known as a 9 + 2 axoneme. Both organelles exist only on select cells and have a primary function of motility—a critical function for survival. Spermatocytes, hence reproduction and the survival of sexually reproducing species, depend on the proper motility of flagella [6]. The bronchi of the lungs are lined with motile cilia that participate in mucociliary clearance. Defects in this clearance can result in chronic bouts of inflammation [7]. The ventricles of the brain are lined with ependymal cells expressing motile cilia that are necessary for movement of cerebrospinal fluid. Defective ependymal cilia and improper cerebrospinal fluid flow can lead to hydrocephalus [8]. Given the importance and usefulness of motile cilia in eukaryotes, perhaps it is not surprising that other types of cilia and modified cilia evolved to serve a range of other functions. In recent decades, more attention has been devoted to this diverse range of cilia.

Motile cilia are the most well-known type of cilia, but they are neither the only type of cilia, nor the most abundant type of cilia. Multiple cilia subtypes have been described,

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but there are generally three main groups: motile cilia, primary cilia, and nodal cilia [9, 10]. Motile cilia, as described above, are found on select cells, exist in clusters, and function in motility. Primary cilia can be found on almost all cells, where they usually exist singularly and are important for signaling [11–16]. Unlike motile cilia, primary cilia contain a 9 + 0 axoneme, lacking the central two microtubules found in motile cilia. Primary cilia also lack the dynein arms that make movement possible in motile cilia. However, there are a few exceptions to this categorization, such as olfactory cilia, which are considered primary cilia, but have a 9 + 2 axoneme without dynein arms [17, 18]. Lastly, nodal cilia exist in embryogenesis, and share properties of both motile and primary cilia. Nodal cilia contain a 9 + 0 axoneme like primary cilia, but possess dynein arms like motile cilia. Typically, the outer microtubules of motile cilia are anchored to the central microtubules via radial spokes, allowing for a coordinated whip-like motion. Nodal cilia lack these radial spokes and central microtubules, so instead have a rotary movement that helps create chemical gradients during development that help guide organ growth. In fact, loss of nodal cilia results in situs inversus, a condition where the visceral organs are reversed [19].

History

While motile cilia were described as early as the late seventeenth century by Anton van Leeuwenhoek [20], primary cilia were not described on mammalian cells until 1898 by the Swiss anatomist, KW Zimmerman [21]. Zimmerman drew depictions of singular cilia-like structures anchored by a basal body (an organelle, specifically the mother centriole, that forms the base of a cilium) on epithelial cells protruding into the lumen of the distal convoluted tubules of rabbit kidney [21]. He called these structures "centralgeissel," meaning central flagellum, and speculated that they might serve some sensory function. Later, this organelle was renamed the 'primary cilium' by Sorokin as he observed that this type of cilia developed before motile cilia in the central nervous system [22]. However, primary cilia were largely ignored and thought to be vestigial organelles because they lacked motility, the only known function for cilia at that time. We now know that primary cilia are far from vestigial, and that Zimmerman was correct when he hypothesized that they serve a sensory function. Numerous laboratories have since demonstrated that primary cilia are important for signaling and development [11–16]. Work done in the Rosenbaum laboratory revealed the existence of an intraflagellar transport system in the flagella of *Chlamydomonas reinhardtii* (green algae) [23, 24]. This work led to the discovery that mutations in the gene encoding for the intraflagellar transport 88 (IFT88) protein are causative for polycystic kidney disease and disrupt ciliogenesis [the cell biological process by which the cilium is assembled from the mother centriole of the centrosome (the basal body)] [16]. Thus, polycystic kidney disease became the first disease linked to primary cilia, increasing the relevance of an organelle once thought to be vestigial. But in 2003, the primary cilia field perhaps garnered the most interest when the Anderson laboratory linked the intraflagellar transport system and primary cilia to the sonic hedgehog (Shh) pathway [15]. Shh signaling is a critical developmental pathway that is often upregulated in cancer, and thus this discovery attracted scientists from multiple disciplines to the study of primary cilia.

Ciliopathies

Dysfunctional primary cilia can result in a wide array of developmental diseases that are collectively referred to as ciliopathies [25]. Ciliopathies are difficult to define and categorize, but are increasingly thought to exist as separate syndromes that fall along a ciliopathy spectrum [26, 27]. It is unclear how ciliopathies can be distinct (besides due to differential gene transcription in different tissue types), despite the fact that this almost ubiquitously expressed organelle is defective in some manner in all of them. For example, asphyxiating thoracic dystrophy, also known as Jeune syndrome (JATD) is considered a skeletal ciliopathy as its main features are small limbs and short rib cages [28, 29]. Consequently, JATD affected individuals have abnormally small rib cages, often have underdeveloped lungs, and frequently succumb to respiratory failure at young ages [28]. JATD is joined by Meckel Gruber syndrome (MKS) on the severe end of the ciliopathy spectrum. Individuals with MKS have severe hindbrain defects, renal dysplasia, and multiple organ involvement [30]. MKS is considered one of the most severe ciliopathies as it is usually lethal. On the "milder" end of the ciliopathy spectrum is Bardet–Biedl syndrome (BBS). BBS is usually not lethal, but affected individuals are often obese, have hypogonadism, and progressively lose their eyesight [31].

Joubert syndrome (JBTS) is a congenital neurodevelopmental ciliopathy that can occur on a spectrum of ciliopathy phenotypes [32, 33]. JBTS is a relatively rare disorder and is characterized by cerebellar vermis defects, ataxia, irregular eye and tongue movements, abnormal breathing, general hypotonia, and cognitive defects [34]. JBTS can be diagnosed by symptoms, and also by the presence of the "molar tooth sign (MTS)" on axial magnetic resonance imaging (MRI). The MTS is a structural anomaly that occurs due to: (1) elongation of the superior cerebellar peduncles, (2) deepening of the interpeduncular fossa, and (3) cerebellar vermis defects [35]. In its purest form, JBTS is thought to only affect the central nervous system; however, that

categorization is insufficient. JBTS is both a genetically and clinically heterogeneous disorder. Individuals with JBTS can have multiple organ system involvement with a wide range in severity. Interestingly, this rare syndrome has been shown to be caused, so far, by mutations in at least 40 genes [36–38].

We are only just beginning to understand ciliopathies. Multiple genes can be causative for the same ciliopathy, but mutations in the same gene can result in different ciliopathies in different individuals [39–41]. For instance, mutations in *MKS1* are conventionally thought to result in the severe form of MKS. But recent reports have found *MKS1* mutations in individuals with JBTS [42–45], blurring the lines between these two syndromes. Similarly, mutations in *CSPP1* can result in JBTS alone [46, 47] or JBTS accompanied by JATD [48]. Individuals with *CSPP1*-related JBTS can have a wide range of severity and symptoms. To date, attempts to correlate *CSPP1* mutations with phenotype have been unsuccessful [48].

The discrepancy between genes and phenotypes is unresolved in the cilia field despite attempts by multiple laboratories to address this issue. Ben-Omran and colleagues [49] proposed that the heterogeneity in clinical ciliopathy phenotype may be explained by spontaneous events (genetic or epigenetic) that occur during pregnancy. This group's results showed that patients could still have variable phenotypic presentations even if they had the same *CSPP1* mutation and came from the same family. Moreover, these families had a high degree of consanguinity, leading these researchers to infer that these family members had similar genetic backgrounds. Thus, Ben-Omran's group concluded that spontaneous events that occur during pregnancy, and not genetic background, was the more likely culprit for the observed phenotypic heterogeneity of JBTS. However, it is now known that even identical twins are not truly identical, so differences in genetic background cannot be ruled out by this report [50]. Indeed, others have proposed that the range in phenotypes observed in ciliopathies could be due to genetic modifiers unique to each individual [51]. For now, we lack consensus as to why so much phenotypic variance exists within and between ciliopathies, although genetic modifiers are clearly involved. However, we propose that some understanding of the clinical heterogeneity within and between ciliopathies, such as JBTS, can be achieved through analyses of the localizations and functions of cilia proteins outside of the primary cilium. Some of these extraciliary functions have been characterized and others are recognized but not understood. Here, we provide a summary of the many extraciliary sites and functions that have been identified and that may contribute to the variable phenotypes often observed with ciliopathies. Through the elucidation of the extraciliary roles of these cilia proteins, we will gain a better understanding of how primary cilia are implicated in these diseases and how the extraciliary functions of these cilia proteins can possibly contribute to ciliopathy phenotypes.

Extraciliary sites and functions of ciliary proteins

Cell cycle

Cancer

Perhaps, one of the least surprising extraciliary roles for cilia proteins is their participation in cell cycle regulation. At a simplified and structural level, ciliogenesis (construction of a cilium) and mitosis both depend on properly regulated microtubules that protrude from the centrosome. The mother centriole from the mitotic centrosome docks to the plasma membrane and becomes the basal body as the cell enters G0. When a cell begins to divide, the primary cilium is reabsorbed and the centrioles are free to participate in cell cycle division. Thus, both mitosis and ciliogenesis depend on the same organelle, the centrosome [52–54].

At a clinical level, many cancers have upregulated Shh signaling, a pathway that signals through the primary cilium [55]. Defects in primary cilia often affect Shh signaling [15, 56]. Many laboratories have observed cell cycle defects in knockdown and/or overexpression studies of primary cilia proteins. GLOD4, a glyoxalase, and SPATA4, a spermatogenesis associated protein, were identified as critical for both cilia and cell cycle regulation through a whole genome transcription analysis in flagellated and deflagellated *Chlamydomonas* [57]. Knockdown of *GLOD4* in human retinal pigmented epithelial (RPE) cells resulted in delayed cell cycle progression and shorter cilia [57]. Loss of *SPATA4* in RPE cells resulted in cell cycle arrest and a decrease in number of cilia [57].

Primary cilia proteins are sometimes overexpressed in cancers; and overexpressed proteins in cancer are sometimes identified as ciliogenesis proteins. Ciliogenesis proteins are proteins that have been in part associated with the assembly/maintenance of cilia. AHI1 (Abelson helper integration site-1) is a ciliogenesis protein that is implicated in JBTS, and localizes to the basal body/transition zone in arrested cells, and to centrosomes in mitotic cells [58]. In addition, Ahi1 has also been shown to have oncogenic potential in cutaneous T-cell lymphoma (CTCL) [59] and chronic myeloid leukemeia (CML) [60] where it is thought that dysregulation of *AHI1* expression levels lead to these cancers or more aggressive forms of these cancers (reviewed in [61]). In fact, the *AHI1/Ahi1* gene was originally identified by genetic mapping of a retroviral insertion in DNA from leukemia and lymphoma samples with high expression in certain leukemia/lymphoma progenitor cells [62]. CSPP1 (centrosome

and spindle pole associated protein 1) is another ciliogenesis protein that localizes to centrioles and cilia axonemes, and is implicated in ciliopathies and cancer. CSPP1 was first identified by laboratories working in the cancer field. The Aasheim laboratory [63] examined why the follicular subtype of non-Hodgkin's lymphoma sometimes transitions into the large diffuse B-cell subtype. It was during this transition that one isoform of CSPP1 exhibited increased expression and localized to centrosomes and mitotic spindles. Knockdown of *CSPP1* resulted in impaired transition from G1 to S phase [63]. Later, CSPP1 was implicated in cytokinesis by showing that CSPP1 localizes to the cleavage furrow, and that RNAi knockdown of *CSPP1* resulted in metaphase arrest, and regression of the cleavage furrow [64]. We know that CSPP1 is a kinetochore protein important for mitotic spindle dynamics. Moreover, depletion of CSPP1 resulted in increased mitotic spindle velocity and impaired metaphase checkpoint regulation [65]. The Patzke laboratory has shown that CSPP1 is located in the nucleus in some forms of breast cancer [66]. It was not until 2010 [67] that CSPP1 was found to localize to primary cilia, and not until 2014 was CSPP1 implicated in JBTS with or without JATD [46–48]. Recently, histone deacetylase 2 (HDAC2) which is known to be upregulated in cancers [68], has been shown to have a role in cilia disassembly [69]. Pancreatic ductal adenocarcinoma cells have a loss of cilia, but inhibition of HDAC2 rescues primary cilia likely through decreased Aurora A signaling, which is normally associated with cilia disassembly [69]. Thus, cilia proteins and cancer-related proteins are often connected, and even sometimes one and the same.

Mitotic figure

Cell division requires both mitosis and cytokinesis, processes that are often impaired in studies examining the function of ciliary proteins. IFT88, the first cilia protein linked to the ciliopathy, polycystic kidney disease [16], has many roles outside of the cilium. One of its many functions is maintaining proper mitotic spindle orientation. *IFT88* mutants have disrupted astral microtubule structure in mitotic figures, misaligned chromosomes, failed localization of various proteins to the mitotic figure, and misoriented mitotic spindles [70]. Interestingly, misorientation of mitotic spindles is not an uncommon abnormality when ciliogenesis proteins are knocked down. For instance, knockdown of *CSPP1* [63, 65], *Tcf2* [71], *Nde1* [72], *Rab11* [73], and *CPAP* and *STIL* [74], all result in defects in ciliogenesis and mitotic spindle orientation.

Even if a cell successfully completes anaphase, it must undergo cytokinesis to establish two separate daughter cells. Interestingly, many proteins that are important for ciliogenesis can be observed at the midbody or cleavage furrow. As previously mentioned, CSPP1 localizes to all these structures and is important for ciliogenesis and cytokinesis. BBS6, a protein implicated in the ciliopathy, Bardet–Biedl syndrome, localizes to the midbody [75]. Knockdown of *BBS6* by siRNA in COS-7 cells results in cells that remain attached to one another by thin cellular bridges, suggesting that *BBS6* knockdown cells fail to undergo cytokinesis [75]. Studies in *Chlamydomonas* have shown that many IFT proteins (Ift27, Ift46, and Ift172) localize to the cleavage furrow [76], suggesting IFT proteins may also have some role in cytokinesis. Recently, an exciting new hypothesis has emerged that the midbody itself may be important for ciliogenesis [77]. After cells undergo cytokinesis, this process leaves behind a midbody remnant (MBR). The MBR can be released into the extracellular space to be degraded or inherited on the surface of one of the daughter cells. The MBR will then migrate along the surface until it is positioned above the centrosome. At this point, the cell absorbs the MBR and ciliogenesis is observed shortly thereafter. Importantly, physical removal of the MBR through glass pipette aspiration results in a dramatic reduction in ciliogenesis compared to control cells [77]. More work will be needed to determine which cilia proteins localize to the midbody and vice versa as the midbody gains significance as an important extraciliary site for cilia proteins.

Cell cycle regulation and ciliogenesis also share, the never-in-mitosis A (NIMA)-related kinases and the Nek kinases [78]. NEK family kinases were initially studied in cell cycle regulation. Some NEK proteins function in establishment of the mitotic spindle, while others are important in the DNA damage response [78, 79], and several NEK proteins have been implicated in ciliogenesis [78]. Nek1 localizes to the basal body and its overexpression results in inhibition of ciliogenesis [80, 81]. NEK8 localizes to the proximal region of primary cilia, though siRNA knockdown of *NEK8* does not appear to impact ciliogenesis [80]. *NEK1* variants have also been implicated in the oral-facial-digital syndrome type II ciliopathy [82], and NEK1 protein has been shown to interact with a causative JBTS protein, CEP104 [83]. The concurrent importance of NEK kinases in mitotic spindle and cilia function, both microtubule-based structures, has led to the hypothesis that NEKs coordinate microtubule-based structures in dividing and non-dividing cells [78].

Cytoskeleton

Ciliogenesis depends on proper microtubule and actin cytoskeleton regulation. Ciliary proteins are increasingly being identified as critical regulators of cytoskeletal dynamics. This does not require that all actin and microtubule proteins be involved in ciliogenesis, but at a minimum suggests that at least some actin and microtubule-guided processes are likely essential for ciliogenesis.

Microtubules

Given that the primary cilium is a microtubule-based organelle, it is not surprising that microtubules are important for ciliogenesis. Drugs that inhibit tubulin polymerization (colchicine and nocodazole) or promote its polymerization (taxol) can disrupt ciliogenesis [84, 85]. Moreover, knockdown of cilia proteins can lead to a disrupted microtubule cytoskeleton [58], and cilia proteins have been shown to bind directly to microtubules [63]. Interestingly, CSPP1 has a microtubule binding domain [86] thought to be important for microtubule regulation so that mitosis can occur.

Perhaps, the most convincing examples that proper microtubule cytoskeletal regulation is important for ciliogenesis are microtubule binding proteins themselves, as many have been shown to affect ciliogenesis. Crescerin, a tubulin-binding microtubule regulating protein, promotes microtubule polymerization. Recently, Crescerin has been shown to localize to centrioles and primary cilia axonemes in inner medullary collecting duct 3 (IMCD3) cells [87]. Deletion of Crescerin in *C. elegans* results in shorter cilia that have an unorganized microtubule axoneme, linking microtubule polymerization to proper cilia architecture [87]. Even microtubule-associated protein (MAP) family proteins, like hSAXO1, have been found to affect ciliogenesis. hSAXO1 localizes to centrioles in RPE cells, and can be seen in the flagella of sperm cells. RNAi knockdown of *hSAXO1* results in shorter cilia, while overexpression leads to longer cilia [88]. hSAXO1 likely affects ciliogenesis by stabilizing microtubules, but microtubules are dynamic structures and proper catalysis of microtubules is also necessary for ciliogenesis.

The katanin family of proteins are enzymes that sever existing microtubules [89] to increase the free microtubule pool necessary for growth. Katanins have been shown to be essential for mitosis, neurogenesis, and now ciliogenesis [90]. Katanin functions as a complex of two proteins, a p60 and a p80 subunit. The p60 subunit is an ATPase that severs microtubules, and the p80 subunit helps localize the katanin complex to centrioles. Overexpression of katanin p60 (Kat1p) leads to the severing of microtubules, and hence, disassembly of cilia [91]. Meanwhile, loss of Kat1p in chick embryos results in the loss of the two central microtubules in motile cilia, resulting in cilia with 9 + 0 axonemes, dynein arms, and radial spokes [91]. In humans, loss of *KATNB1* results in severe microlissencephaly, a condition where the brain is abnormally small and lacks gyri and sulci. In mice, loss of *Katnb1* results in holoprosencephaly, a condition where the brain exists as one mass, instead of having its normal hemispheric divisions. Mice deficient in Katnb1 also have an increased number of centrioles, increased number of cilia, and defective Shh signaling [92]. Thus, katanins may play a role in limiting the number of cilia and centrioles during brain development. The katanin complex of proteins may also be critical for ciliopathies. Primary fibroblasts collected from individuals with JBTS with mutations in *KIAA0556* (JBTS26) have fewer cilia and abnormally long cilia. KIAA0556 binds to microtubules and stabilizes microtubules when overexpressed, but KIAA0556 also interacts with the katanin complex proteins (p60/p80), providing an indirect link between katanin proteins and ciliogenesis [93]. Other katanin-like proteins have been identified and shown to be necessary for ciliogenesis. Recently, missense mutations in the *Katanin p60 subunit A-like 1* (*KATNAL1*) gene were shown to result in neuronal migration defects and defective ependymal cell motile cilia [94]. Katanin-like 2 protein (KATNAL2) has also been implicated in ciliogenesis. KATNAL2 localizes to centrioles, mitotic spindles, midbodies, basal bodies, and axonemes of primary cilia. Knockdown of *KATNAL2* results in decreased ciliation upon serum starvation, inefficient cytokinesis, multipolar mitotic spindles, and an increased number of centrioles [95]. Conversely, overexpression of KATNAL2 often resulted in apoptosis [95]. The involvement of microtubule binding proteins and microtubule regulating proteins in ciliogenesis supports the argument that the integrity of the microtubule cytoskeleton is critical for ciliogenesis, but this one cytoskeletal element alone is not sufficient for cilia assembly.

Actin

While it makes intuitive sense for microtubules to have an essential role in ciliogenesis, the role of actin has until recently been largely ignored. Unlike microtubules, which are found in the cilium (tubulin antibodies are often used as markers for cilia), the presence of actin inside the cilium is debated. However, drugs that affect actin dynamics can alter the process of ciliogenesis. Studies have shown that branched F-actin is inhibitory to ciliogenesis [96], and drugs that inhibit polymerization (cytochalasin D and cytochalasin B) enhance ciliogenesis [97]. Over the years, researchers have noticed that the actin cytoskeleton is disrupted in knockout/knockdown cell lines targeting cilia proteins. For instance, stable *Ahi1* knockdown IMCD3 cells were observed to have a loss of actin stress fibers [58]. *Talpid3* mutants lack cilia, but also develop a variety of actin phenotypes: punctate actin staining in the cytoskeleton, decreased stress fibers, increased filopodia, and stronger actin staining at ruffled membranes [98]. Talpid3 has now been implicated in JBTS [99–101], hydrolethalus, and short rib polydactyly syndrome [102], and even a hybrid ciliopathy with features from both JATD and JBTS [103]. More recently, actin has been directly implicated in ciliogenesis by several important studies. In 2010, a high throughput RNAi genomic screen for modulators of ciliogenesis was performed by the Gleason laboratory, which found that proteins involved in actin

dynamics and endocytosis were important for ciliogenesis [104]. In addition, overexpression of *miR-129-3p*, a microRNA, resulted in the downregulation of several positive regulators of branched F-actin, as well as increases in ciliation and cilia length [105]. Together, it appears that defects in ciliogenesis can be accompanied by alterations in the actin cytoskeleton.

Conversely, loss of cilia proteins often affects the actin cytoskeleton. Retinitis pigmentosa GTPase regulator (RPGR) is implicated in retinal dystrophy, and is localized to primary cilia axonemes where it has been shown to interact with and activate gelsolin (an actin-severing protein) [106]. While RNAi knockdown of *RPGR* in RPE cells resulted in loss of cilia, it also caused increases in the fluorescence intensity of F-actin and dysregulation of several adhesion markers [107]. Recently, the loss of RPGR was found to result in profound changes in gene transcripts implicated in actin–cytoskeletal dynamics that interestingly occur before retinal degeneration [108]. Similarly, knockdown of *NudC*, a chaperone protein, resulted in thicker stress fibers though it increases, rather than decreases, ciliogenesis [109]. Importantly, NudC binds and stabilizes cofilin1, a protein known to be important for actin organization. Mice deficient in *Tg737* (also known as *Ift88*), a model for polycystic kidney disease, have reduced ciliation and decreased stress fibers and focal adhesions [110]. Conversely, mice deficient in polycystin-1 have impaired cilia, but robust stress fibers and focal adhesion. Thus, the relationship between stress fibers and ciliogenesis is not always clear.

We now know that some actin-regulating proteins also affect ciliogenesis. Cordon-blue (Cobl) is known to bind monomeric actin and nucleate it, allowing for the formation of unbranched actin filaments. Cobl also has a polarized apical expression, and morpholino knockdown of *Cobl1* in zebrafish resulted in a loss of apical F-actin and shorter motile cilia [111]. This is important because apical F-actin is thought to be necessary for the mother centriole to dock to the plasma membrane and become the basal body. Several myosin proteins and actin-dependent motor proteins have been shown to be important for ciliogenesis. Multiple laboratories have shown that myosin heavy chain 10 (MYH10) is necessary for ciliogenesis [112, 113], even though MYH10 does not localize to, or around, the primary cilium. Therefore, MYH10 may be affecting ciliogenesis through some role that occurs outside the cilium. Recently, Myosin Va (MyoVa) was shown to be critical for ciliation in RPE cells [114]. Unlike MYH10, MyoVa localizes to the centrosome and interacts with a known causative ciliopathy protein, RPGRIP1L (JBTS7) [114].

The identification of two myosin proteins as ciliogenesis proteins opens up a host of other extraciliary sites to explore. Both MYH10 and MyoVa localize to growth cones and dendritic spines and are known to have functional roles in these two extraciliary sites. Our laboratory has found that Ahi1 positively immunolabels the growth cones in primary mouse hypothalamic neurons [115]. As we await additional studies that show a connection between dendritic spines, growth cones, and cilia, it is important to recognize that these sites may have clinical importance. Decussation defects are often seen in individuals with JBTS [116, 117] and may be a sign of growth cone defects. Similarly, a defect in dendritic spines due to the loss of a cilia protein at this extraciliary site may explain the high occurrence of cognitive defects in ciliopathy-affected individuals.

How actin affects ciliation is unclear, and the answer may be that actin affects ciliation in many different ways. We have already discussed actin nucleating proteins and motor proteins for their ciliogenesis roles, but it is highly likely that more actin-regulating proteins remain to be studied and linked to ciliogenesis. Histone lysine demethylase 3a (KDM3a) negatively regulates ciliation, and Yeyati et al. concluded that KDM3a does so through binding the actin cytoskeleton directly and by regulating actin gene expression [118]. The Marshall laboratory has shown that IFT protein recruitment to basal bodies, a process known to be important for ciliation, is actin dependent [119]. More studies will be necessary to elucidate the many ways actin can affect ciliogenesis.

Lastly, we must consider why both the microtubule and actin cytoskeletons are important for ciliogenesis. Some recent work suggests that coordination between microtubules and actin is important for ciliation. Microtubule actin crosslinking factor 1 (MACF1) coordinates the microtubule and actin cytoskeletons. Deletion of *Macf1* in mice results in loss of polarity in photoreceptors (a modified primary cilium), failed docking of the basal body to ciliary vesicles, a host of microtubule defects, and loss of primary cilia [120]. Another protein that may potentially be important is mouse diaphanous-related formin-1, mDia1. Like MACF1, mDia1 is thought to regulate actin [121, 122] and microtubule cytoskeletons [123, 124]. Curiously, mDia1 has been shown to localize to the mitotic spindle [125, 126], a structure that bares remarkable similarities with primary cilia as previously discussed. Furthermore, mDia1 interacts with the ciliopathy protein, polycystin-2 (PKD2) at mitotic spindles [127]. Given its interaction with a cilia protein, and its localization to an important extraciliary site, mDia1 may be a potential ciliogenesis protein.

Septins

Septins are a class of GTP-binding proteins that have many functions at the cytoskeleton [128]. Septins can act as diffusion barriers and as scaffolds to bind and localize other proteins. Septins can be associated with actin, microtubules, and the cell membrane [128]. Recently, several septins have been

shown to localize within cilia. Septin 2 and septin 9, which are membrane localizing proteins, both can be observed at cilia [129]. Subsequently, RNAi knockdown studies in RPE cells of SEPT2, SEPT7, and SEPT9 demonstrated that these proteins are needed for ciliogenesis and all localize to primary cilia axonemes [130]. Septins 2, 7, 9, and 11 are also important for ciliogenesis of motile cilia [131]. Cytoskeletal proteins are an important extraciliary site for cytoskeletal remodeling, but they are also important for protein trafficking, another important extraciliary function of cilia proteins.

Trafficking

A plethora of trafficking pathways exist to move proteins and G-protein coupled receptors (GPCRs) [132] to the primary cilium for proper signaling and function of this organelle. Furthermore, ciliogenesis requires the proper trafficking of proteins to assemble the cilium. Many trafficking roles have been identified for ciliogenesis proteins, and many excellent reviews have already discussed the many trafficking pathways in and to cilia [133–138]. In this section, we will briefly review some important trafficking studies and discuss their extraciliary implications.

Tubby

Tubby (*TUB*) was initially identified as a gene implicated in obesity [139], but it has since been found that TUB and Tubby-like (TULP) proteins have an important role in trafficking GPCRs to the primary cilium [140, 141], and are thus important for the proper functioning of cilia. Tubby-like protein 3 (TULP3) localizes to the primary cilium axoneme, but its localization is dependent on the proper functioning and expression of various IFT-A complex proteins [142]. IFT-A complexes can carry vesicles containing GPCRs from the cytoplasm toward primary cilia through its interactions with TULP3. Interestingly, TULP3 can selectively bind phosphatidylinositol (4,5)-bisphosphate (PIP2). Since PIP2 preferentially localizes to the transition zone of primary cilia, TULP3 brings IFT-A and its associated GPCRs to the primary cilium [143]. GPCRs are then subsequently released into a PIP2-deficient cilia membrane [144]. The primary cilia membrane has differential expression of phosphoinositides along different sections of the cilium [145], forming a phosphoinositide code [146] that regulates protein trafficking [147]. PIP2 is represented at the transition zone, while the rest of the cilia membrane contains PI4P [146].

The role of phosphoinositides in primary cilium trafficking was expanded through studies examining individuals with oculocerebrorenal syndrome of Lowe (characterized by congenital cataracts and glaucoma, cognitive impairments, hypotonia, and proximal renal tubular dysfunction leading to renal failure). Mutations in the gene *OCRL1*, which encodes for an inositol polyphosphate 5-phosphatase, have been shown to result in Lowe syndrome [148, 149]. More recently, OCRL1 was localized to the primary cilium [150–152] and loss of OCRL1 causes disruptions in the ciliary membrane composition of phosphoinositides, altering proper primary cilium signaling [153].

One of the first genes identified as causative for JBTS [154] was *inositol polyphosphate 5-phosphatase E* (*INPP5E*), whose protein converts PIP2 into PI4P. Knockout of *INPP5E* resulted in the loss of polarized expression of phosphoinositides in the cilium; specifically, loss of INPP5E results in PIP2 being expressed throughout the cilia membrane [155]. The loss of INPP5E resulted in cilia localization of TUB, a tubby family protein which binds to PIP2 that had never been shown to localize to primary cilia axonemes [144]. However, loss of INPP5E resulted in the loss of many GPCRs from the cilia axoneme [144]. Thus, GPCR trafficking into the primary cilium depends on the interaction of IFT-A complexes and TUB family proteins, but also requires a differential expression of PIP2 and PI4P at the cilium.

Although TUB, TULP3, and OCRL1 are important for proper functioning of cilia as signaling centers, these proteins also have functions outside the cilium [156, 157]. Tubby and TULPs are transcription factors [158–160], and thus may have far-reaching effects not localized to cilia. TUB is also a ligand capable of inducing phagocytosis [161]. Likewise, OCRL1 has been implicated in a variety of cellular functions outside of the primary cilium including endocytosis, endosomal trafficking, autophagy, cytokinesis, and actin cytoskeletal dynamics (reviewed in [157]). Further studies are needed to understand how these proteins function inside and outside the cilium, and how these processes either converge on the primary cilium to produce their effects or act independently.

BBSome

Bardet–Biedl syndrome (BBS) is a ciliopathy characterized by obesity, hypogonadism, polydactyly, renal defects, and progressive visual loss [31]. BBS was first identified as a ciliopathy since BBS proteins were exclusively localized to ciliated cells, and because BBS8 was not only shown to localize to centrosomes where it interacted with pericentriolar matrix 1 (PCM1), but also was a ciliogenesis protein [162]. Subsequent work showed that *Bbs4* knockout mice, while still capable of forming primary and motile cilia, failed to form flagella on sperm cells and underwent photoreceptor apoptotic death, both of which are common features seen in ciliopathies [163, 164]. Subsequently, although *Bbs2* and *Bbs4* knockout mice still form primary cilia, these cilia fail to localize the melanin-concentrating hormone receptor 1 (MCHR1) and the somatostatin receptor 3 (SSTR3) to the cilia membrane [165], implicating these BBS proteins in a

trafficking role to primary cilia. It is now well known that BBS proteins can form a trafficking complex of eight proteins called the BBSome that is composed of BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9, and BBS18 [137]. However, BBS proteins are not all alike. While loss of BBS proteins generally is not thought to result in loss of cilia, *Bbs7* knockout mice do have fewer and shorter cilia [166].

Importantly, BBS protein functions are not restricted to the primary cilium, prompting a set of studies seeking to investigate whether BBS is primarily a disorder of defective cilia [167]. The known roles of BBS proteins in cilia were documented in one study, but also the many extraciliary roles of BBS proteins were noted. Briefly, BBS proteins can participate in melanosome transport in zebrafish [168] suggesting these proteins may play a more generalized role in transport/trafficking that is not specific to cilia. Future studies are needed to address whether trafficking or transport of other proteins or organelles are in part mediated by BBS proteins. BBS11, also known as TRIM32, is an ubiquitin ligase that is capable of ubiquitinating and downregulating actin levels [169]. *BBS4*, *BBS6*, and *BBS8* knockout cells have a loss of actin stress fibers and an increase in RhoA-GTP levels [170]. Rho GTPases are important for actin dynamics [171], thus BBS proteins may regulate actin stress fiber levels through control of RhoA-GTP levels. Importantly, decreasing the elevated RhoA-GTP levels in BBS knockout cells was able to rescue defects in cilia length, cilia number, and actin integrity [170]. As there are many other extraciliary functions for BBS proteins, it suggests that BBS proteins may not be entirely cilia specific.

Intraflagellar transport

Another trafficking system important for cilia function is the intraflagellar transport system (IFT). Historically, the IFT system was first described in *Chlamydomonas* [23], and hence its name as the IFT system, and not the intraciliary transport system. Subsequent studies identified *Ift88* as the causative gene in a polycystic kidney mouse mutant [16], thus linking the IFT system and cilia to the first primary cilia ciliopathy. Later, two additional mutant mouse lines were identified in a mutagenesis screen as having mutations in IFT genes that resulted in a Shh phenotype and provided the first indication that primary cilia are critical for proper Shh signaling [15].

The IFT system is a bi-directional system that uses dynein and kinesin proteins for retrograde and anterograde transport, respectively. The anterograde system, IFT-B, helps transport proteins to the tip of the cilium; while the IFT-A complex proteins transport proteins back toward the basal body [172]. We now know that the IFT trafficking system is a general transport system that also functions outside the cilium [142, 173].

While we are unable to cover every IFT study that mentions extraciliary roles, we will discuss a handful of studies to demonstrate the broad extraciliary roles for IFT and its proteins. As mentioned previously, ciliary IFT-A proteins are found in the cytoplasm where they can bind Tubby family proteins to traffic GPCRs to the transition zone of the primary cilium [144]. However, the IFT system can also traffic other cargo. For example, IFT81 and IFT74 directly interact with one another [174], and form the main complex for transporting tubulin monomers to sites where they are necessary [175]. Not surprisingly, defects in IFT can affect the microtubule cytoskeleton. IFT54 interacts with MAP4 and this interaction is important for primary cilia genesis [176]. IFT54 localizes to the base and tip of cilia, and MAP4 usually localizes throughout the cilia axoneme. Mutations in *IFT54* result in decreased MAP4 staining in primary cilia suggesting IFT54 may transport MAP4 to cilia. Mutations in *IFT54* also result in the failure of EB1, a plus-end microtubule protein, to localize to the tips of microtubules [176]. Therefore, it appears that IFT proteins may regulate microtubules in more than one way. Defects in IFT proteins have been implicated in actin defects [177], migration defects [178, 179], and cell cycle defects [180].

It is becoming increasingly clear that at least some IFT proteins participate in endocytic and/or exocytic vesicular trafficking [181]. Rab8, a small GTPase, is important for regulating vesicular transport from the Golgi apparatus [182] and is also critical for ciliogenesis [183]. In fact, a whole conserved system of GTPases are important for ciliogenesis. When bound by GTP, Rab11 increases the guanine nucleotide exchange activity of Rabin 8 to Rab8 [184]. Factors that lead to the failure of Rab8 localization to the basal body result in ciliogenesis defects. IFT121 helps traffic Rab8 vesicles to the cilium [185], and loss of Ahi1 causes Rab8 to fail to localize to the basal body [58]. However, Rab8 is not specific to ciliogenesis. Rab8 functions at many cellular protrusions, and plays a role in migration, polarization, and differentiation [186]. Arl13b (a JBTS gene), although not an IFT protein, is important for regulating endocytic trafficking, and can be seen to colocalize with endocytic markers [187]. Arl13b also plays a role in cell migration [188]. Cilia proteins are increasingly being shown to be important in vesicular trafficking, a function that may implicate cilia proteins in other cell structures.

Golgi apparatus

With cilia proteins being implicated in vesicular trafficking, perhaps it is not surprising that many proteins important for ciliogenesis also affect or localize to the Golgi apparatus, an important organelle for vesicular trafficking. In 1985, Poole observed that the trans-Golgi surface always faces the primary cilium, suggesting there may be some

functional importance to this non-random alignment [189]. Since then, Poole and other laboratories have observed a physical connection between the trans-Golgi and the primary cilium [189–191]. More recently, cilia proteins like IFT20 have been found to localize to the Golgi apparatus and to the cilium [192]. IFT20 is an IFT-B complex protein that is found in both the centrioles and the primary cilium axoneme, and effective knockdown of *IFT20* in RPE cells results in a loss of ciliation [192]. IFT20 is constrained to the Golgi apparatus through binding to Golgin [193], a transmembrane Golgi apparatus protein likely important for many cellular functions [194].

Defects in primary cilia have often been observed to coincide with defects in the Golgi apparatus. Loss of Ahi1 results in the failure of cholera toxin B to transport to the Golgi apparatus, implicating Ahi1 in a vesicular trafficking role [58]. Disorganized Golgi apparatus staining has been observed with a number of mutated or knocked-down cilia proteins: casein kinase 1 delta (CK1-δ) [195], MAP4 [196], TBCCD1 [197], and KIF7 [198]. Importantly, KIF7 (JBTS12) is implicated in JBTS [198]. In a more direct manner, many proteins have now been identified that localize to the Golgi apparatus and to centrioles and/or primary cilia axonemes: IFT20 [192], RC/BTB2 [199], retinitis pigmentosa protein (RP2) [200], HOOK2 [201], CCDC41 [202], and VPS15 [203]. In light of these studies, the Golgi apparatus appears to be an important extraciliary site for cilia proteins. Of note, IFT20 does not immunolabel the Golgi apparatus if cells are fixed with PFA. Instead, IFT20 only immunolabels the Golgi apparatus when cells are fixed with either methanol or PFA prepared in cytoskeletal buffer [204]. Because of the peculiarity of Golgi apparatus fixation, it is possible that many ciliogenesis proteins localize to the Golgi apparatus, but have not yet been shown to localize to the Golgi apparatus because of fixation techniques. As such, the type of fixation utilized needs special consideration in characterizing ciliogenesis protein functions in the Golgi apparatus.

Immune synapse

When T cells come into contact with a target cell, the two cells form a point of contact known as the immune synapse. The immune synapse is thought to be important for activation of T cells and for directed contact and secretion events [205]. The establishment of the immune synapse requires cytoskeletal rearrangements and activation of different pathways that have remarkable similarities to those present during ciliogenesis [206]. Centrosomes must first polarize to the immune synapse [207–209] during targeted killing. Next, the mother centriole docks to the plasma membrane at the immune synapse using distal appendages similar to those used in ciliogenesis [210]. The immune synapse also requires polarized trafficking of different components, a process carried out by the intraflagellar transport system [211]. Notably, IFT20, which was previously mentioned as a ciliogenesis protein localizing to the Golgi apparatus, is similarly important for vesicular recycling at the immune synapse [173]. Similar to cilia, the regulated trafficking of vesicles is likewise important for the establishment of the immune synapse [212]. Vesicular trafficking at both the primary cilium and the immune synapse involve the small GTPases, Rab29 [213] and Rab8 [214]. Perhaps, what is most striking is that T cells do not have primary cilia, yet they contain and utilize many proteins important for ciliogenesis to construct this completely separate structure, the immune synapse. This example indicates that labeling a protein a 'cilia protein' may not be appropriate in many instances.

Miscellaneous

Cellular inclusions

Cilia proteins have been occasionally observed in various cellular inclusions. Ahi1 is found in donut-like structures called stigmoid bodies in hypothalamic mouse neurons [115]. Little is known about stigmoid bodies, but several proteins and receptors localize at this structure including: estrogen receptors [215], aromatase [216], HAP1 [217], 5-HT_7 receptors [218], and androgen receptors [219]. ANO1, a Ca^{2+} activated Cl^- channel, localizes to the primary cilia axoneme, and either drug inhibition or shRNAi knockdown of ANO1 results in fewer and shorter cilia [220]. Curiously, ANO1 also localizes to a donut-shaped inclusion called a "nimbus". Again, little is known about the nimbus other than it forms before the mother centrioles dock to the plasma membrane and may function as a scaffold for the assembly of cilia components [220]. Cilia proteins have been observed in another donut-shaped inclusion called the loukoumasome. This intracellular tubulin-based ring contains its own intracellular primary cilium that immunolabels positively for adenylyl cyclase 3 (AC3); a protein localized to primary cilia in some cell types. This structure also contains two other proteins important for ciliogenesis, gamma-tubulin and MYH10 [221].

Although we have limited knowledge regarding these inclusions, the presence of cilia proteins in all three suggests that the presence of these proteins may not be coincidental. These proteins may have a functional role at these sites or these inclusions may provide a method of sequestering cilia proteins. Much more information is needed to better understand these inclusions and their potential ciliary and extraciliary roles.

Fixation methods

A potential source of variation for the reported information on cilia and cilia proteins may be due to variances across different laboratories, in particular, differences in fixation methods. Cilia proteins at ciliary and extraciliary sites show variable immunolabeling that is dependent on the fixation method used; therefore, it is possible that many cilia proteins are insufficiently characterized because they have not been studied with amenable fixation methods [204].

Different types of cilia may be affected

Finally, another potential source of variance in disease manifestation may lie with the type of cilium or combinations of cilia that are affected. Currently, the motile cilia field, nodal cilia field, and primary cilia field exist as fairly separate research areas. This is unfortunate as all three types of cilia share similarities in structure and some overlap in protein composition. Some cilia proteins are known to localize to more than one type of cilia. Centrosome protein 290 (CEP290) can be found at the centrosomes of both motile cilia and primary cilia, and loss of CEP290 results in loss of both types of cilia [222]. Similarly, RPGR, a primary cilia protein, has been observed at the transitional zone of airway motile cilia in mice [223]. RPGR mutations are associated with primary cilia dyskinesia with retinitis pigmentosa, a motile ciliopathy [224]. Furthermore, individuals with RPGR mutations demonstrate decreased motile ciliary beat coordination, and a disturbed orientation of their motile cilia [225]. BBS proteins appear to be important at both motile cilia and primary cilia. BBS proteins are known to localize to the base of motile cilia [226] and are important for trafficking to primary cilia [165]. More recently, BBS mutant mice have been found to have defects in motile cilia morphology and have decreased cilia beat frequency [226]. Importantly, individuals with BBS mutations have decreased number of respiratory cilia, and have an increased prevalence of a variety of pulmonary issues, including asthma and neonatal respiratory distress [227]. As the cilia fields progress, more examples of cilia proteins localizing to different cilia types are likely to be found. The possibility exists that different cilia proteins localize to different combinations of cilia types, and thus manifest more often as one ciliopathy over another. It is also conceivable that cilia proteins function differently or have different levels of importance within the three cilia subtypes.

Ciliopathies: a disease of the organelle or the proteins?

Ciliopathies are currently defined as diseases in which the primary defect is in the primary cilium; however, accounting for the heterogeneity of this spectrum of disorders has been hampered. To date, there are at least a dozen ciliopathies with many more suspected that may be identified in the future. These ciliopathies are all thought to result from defects in one organelle that exists on almost all cells in the body, but can present with a different set of symptoms, defects, and severities. Some deficiencies such as defective kidneys are common across all ciliopathies, but why the ciliopathies present with different severities of kidney defects is unclear. Primary cilia can be found on almost all human cells, and thus should be expected to affect all organ systems; but this ubiquity also presents a problem because it fails to explain why all organ systems are not affected in the same way if the same causal organelle is defective (besides differences in gene expression in various tissues).

JBTS is known to result from mutations in at least 40 different genes that commonly result in defective cilia. Given this convergence upon the cilium, it is easy, and maybe even accurate, to draw the conclusion that defective cilia are the source of ciliopathies. However, if defective cilia result in skeletal defects in Jeune syndrome, then the question remains why JBTS produces defective cilia, but not the same skeletal defects.

Could mutations in different cilia genes cause loss of cilia in some organs while preserving cilia functionality in other organ systems, thus explaining why some organ systems are affected and others are not? Our laboratory has shown that mutations in *CSPP1* can result in JBTS with or without Jeune syndrome [48]. Thus, it is possible for defective cilia to be concurrently causative for both a JBTS phenotype and a Jeune syndrome phenotype, but curiously, not in every case. Currently, it is not known why *CSPP1* mutations can result in two ciliopathies in one individual, and result in only one ciliopathy in another, but this is an important question for the cilia field to address in the future. This lack of understanding leaves open the possibility that other explanations, even those that are equally confusing, may exist.

Unlike JBTS, which has been linked to genes with a wide range of functions, Jeune syndrome's causative genes tend to be centered around protein transport, specifically, the IFT system. The IFT system can function both inside and outside of the cilium so the possibility remains that mutations in this system can be causative for Jeune syndrome because of: (1) defective cilia, (2) defective extraciliary functions of the IFT system, or (3) defective IFT function both inside and outside the cilium. Given the uniqueness of skeletal defects to a few ciliopathies where the main defects are in the IFT system, it is possible that these skeletal ciliopathies are unlike the other ciliopathies because the extraciliary roles of IFT proteins may contribute more to the skeletal phenotype than primary cilia. Rather, the extraciliary functions of IFT proteins may play a bigger role in Jeune syndrome. However, this explanation is not fully satisfactory since mutations in *IFT88* are implicated in polycystic kidney disease, a ciliopathy not

known to have overt skeletal defects. Thus, there is currently no clear explanation for why ciliopathies are so heterogeneous despite the convergence of studies from multiple laboratories implicating this single organelle. Future studies comparing and contrasting IFT proteins in bone cells and non-bone cells may help to shed light on this heterogeneity.

Lastly, it is possible that defective cilia may be an indirect outcome of some as yet unidentified causative defect in ciliopathies. Cilia are known to be reactive to their environment, and this presents a confounding variable in cilia studies. For instance, lesions of the medial forebrain bundle in genetically wild-type rats create a hemiparkinsonian model that was sufficient to cause lengthening of cilia in the dopamine-deficient hemisphere [228]. Thus, changes in cilia could easily be mistaken as a false positive and might be explained by non-genetic means. In fact, the culture media in which cells are grown has a significant impact on cilia number and length [229]. Most cilia laboratories serum deprive their cells to induce cilia formation for study, but the opposite is also true in that adding serum to media induces cilia regression. Since primary cilia are now thought to participate in secretory signaling [230], it becomes important for cilia laboratories to determine whether their observed cilia defects are due to structural defects of ciliogenesis or an impairment of the extracellular environment. One way to control for this may be to compare the media of control and experimental cells to see if there are any significant changes that may contribute to ciliogenesis or cilia function. Additionally, frequent renewal of media may also serve as a control. To more directly test a role, conditioned media from cells with defective cilia (due to genetic mutation or RNAi knockdown) could be used to grow wild-type cells in an attempt to observe whether the media (and what is released into that media) affects cilia or whether the effect of the genetic mutation affects ciliary function. Studying normal cilia dynamics and how they respond to their environment will be important when attributing cilia changes to a mutation or its environment. However, at present, the source of commonality between the ciliopathies and the cilia proteins that is directly causative for the observed phenotypes remains unknown.

We propose that a fuller understanding of extraciliary sites and functions of "cilia" proteins will aid in our search for a common causality for ciliopathies. This common factor is likely to be (1) a pathway or process that is dependent on all the extraciliary functions of "cilia" proteins, and/or (2) an impairment of the pathway or process might result in defective cilia. Finding such a common factor will help explain why defective cilia do not seem to contribute to some phenotypes observed on the ciliopathy spectrum. There are currently many in vitro experiments that implicate genes and proteins in ciliogenesis, but these genes/proteins have not yet been linked to disease. This could be because no one has yet studied the gene in terms of disease or it may be that some genes will cause defective cilia in vitro, but are so detrimental that they result in embryonic lethality. Finally, not all defective cilia will necessarily result in disease. Ciliopathy research often involves identifying a genetic mutation, and seeking out ciliopathy patients with such mutations. This method may introduce confirmation bias. In fact, O'Connor and colleagues identified a patient with congenital myasthenic syndrome with a *CSPP1* mutation that was previously reported [48], but this patient lacked an MTS and did not have JBTS. This discrepancy is interesting because the O'Connor group's patient also had *MYO9A* mutations in addition to the *CSPP1* mutation. This suggests that the *MYO9A* mutation may be a genetic modifier, but could also suggest that not all patient's with *CSPP1* mutations will develop JBTS. It will be interesting to see how many patients have mutations in "cilia" genes, but do not have ciliopathies. This work will be important to rule out such potential confirmation biases. Moving forward, it is important to identify the extraciliary functions of "cilia" proteins, to try to understand how the extraciliary functions may work together, and to determine how each contribute to ciliogenesis.

Conclusion

It has become increasingly clear that ciliogenesis is one of many functions that cilia proteins are capable of performing. Extraciliary sites and functions have been identified for many cilia proteins, making one question whether it is even possible to have a protein that works solely at the cilium. The presence of extraciliary sites complicates much of cilia research. If we knockdown a protein via shRNAi, we knockdown that protein's ciliary and extraciliary sites, making it difficult to tease out whether an observed phenotype is due to a defective cilium, a defective extraciliary function, or both.

Distinguishing between extraciliary functions and ciliary functions is difficult and often not considered. An ideal approach for determining which functions are directly attributable to the primary cilium alone would be to remove the cilium without disrupting any other organelle or cellular process. Unfortunately, no satisfactory method currently exists to accomplish this. For now, the best approach may be to thoroughly study one protein at a time and then compare results from different laboratories studying different proteins to establish which functions/symptoms are due to loss of cilia versus loss of a specific cilia protein. Only by identifying, understanding, and ruling out the contributions of extraciliary sites and functions, can we finally attribute phenotypes to the primary cilium, and have confidence in calling such diseases as "ciliopathies."

Given that all JBTS and BBS genes identified to date converge on the cilium, one might ask how we are able to reconcile this fact with our premise that extraciliary functions are implicated in and are important factors for ciliopathies. To understand this argument, we need to first address the issue of convergence. It is certainly plausible that the convergence on the primary cilium can be direct (i.e., all "cilia" proteins converge first at the primary cilium) or it could be indirect (i.e., the cilia proteins are all part of a process that precedes and is necessary for ciliogenesis). The indirect pathway is in part supported by data demonstrating that some proteins important for primary cilium formation are not even localized at the primary cilium (e.g., MYH10), but instead function at the level of cytoskeletal dynamics. Therefore, if this indirect pathway is critical, then one would predict that the loss of proteins implicated in a process that precedes and is necessary for ciliogenesis would then result in the loss of both the preceding process and the downstream process (ciliogenesis). In the indirect pathway model, these proteins may not be "cilia" proteins, but instead function primarily at a different process that precedes ciliogenesis. A potential example of a necessary and preceding process for ciliogenesis may be polarity. If polarity is necessary for ciliogenesis, then loss of proteins important for the establishment of polarity would result in a defect in both polarity and ciliogenesis. As such, it is important to consider whether an observed phenotype is due to loss of a "cilia" protein or loss of a protein that indirectly results in defective ciliogenesis. It will be important for future work to determine whether primary cilia defects are due to a direct cilia pathway alteration, implicating primary cilia directly in disease, or whether critical cell biological pathways are defective with the end result concluding in a primary cilia defect. Thus, it was our intent in this review to highlight the potential extraciliary roles of cilia proteins in possibly mediating the defects observed in the ciliopathies, which may actually converge on other indirect cellular pathways such as polarity.

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