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The Emerging Complexity of the Vertebrate Cilium: New Functional Roles for an Ancient Organelle

Review

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Cilia and flagella are found on the surface of a strikingly diverse range of cell types. These intriguing organelles, with their unique and highly adapted protein transport machinery, have been studied extensively in the context of cellular locomotion, sexual reproduction, or fluid propulsion. However, recent studies are beginning to show that in vertebrates particularly, cilia have been recruited to perform additional developmental and homeostatic roles. Here, we review advances in deciphering the functional components of cilia, and we explore emerging trends that implicate ciliary proteins in signal transduction and morphogenetic pathways.

Introduction

The discovery of cilia is almost coincident with the early descriptions of the ultrastructure of the eukaryotic cell (Zimmerman, 1898), and it might even have been part of Leeuwenhook's early forays into microscopy in the 17th century. In the ensuing years, cilia and flagella have been reported in a broad spectrum of cell types (see "Where are Primary Cilia Found?" at http://members.global2000.net/bowser/cilialist.html), and observations have been facilitated by the advent of electron microscopy and immunocytochemistry (Wheatley, 2005). Despite these discoveries, the physiological importance of the cilium had been largely ignored, and some reports even proposed that its existence is nothing more than an evolutionary vestige (Webber and Lee, 1975). The recent association of ciliary proteins with human genetic disorders has highlighted the fact that compromised ciliary function can have profound consequences for the cell and, in some instances, organism viability (Badano et al., 2006b).

Having arisen early in eukaryotic evolution, cilia can be found across a broad phylogenetic spectrum with certain exceptions including *Cyanidioschyzon*, *Arabidopsis*, *Dictyostelium*, and *Saccharomyces* (Cavalier-Smith, 2002). Although cilia are only found in select cell types in invertebrates, such as the dendritic ends of sensory neurons of *Caenorhabditis* or in the sperm and sensory neurons of *Drosophila*, they are present almost ubiquitously in vertebrate cells. Here, we examine some of the newly discovered functions of vertebrate cilia, evaluate their protein content as deduced by an array of studies in diverse ciliated species, and discuss their emerging role as signal transducers in polarized cells.

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A Brief Overview of Ciliary Structure

Cilia are microtubule-based structures, which, tethered to the cell at the basal body, typically protrude from the apical surface of mammalian cells. Although not compartmentalized by a membrane, it is reasonable to consider them as unique organelles. The basal body and its surrounding material serves as the interface between the cilium and the cytoplasm. Basal bodies possess nine radially oriented microtubule triplets composed of A, B, and C fibers, of which A and B extend into the proximal region of the cilium, known as the transition zone. Beyond the transition zone, the axoneme consists of the middle segment, which maintains nine microtubule doublets either with (9+2) or without (9+0) a centrally located pair, followed by the distal segment, a portion of the cilium in which the backbone becomes more variable but is typically composed of nine single microtubule fibers (Dutcher, 2003) (Figure 1).

Because protein synthesis does not occur in cilia, proteins are shuttled to their final ciliary destination by a highly specialized process known as intraflagellar transport (IFT) (Figure 1; for a comprehensive review of ciliary structure and IFT, see Rosenbaum and Witman, 2002). Kinesin molecular motors drive anterograde translocation and, not surprisingly, are necessary for ciliary assembly. In most ciliated organisms, heterotrimeric kinesin-2, composed of motor subunits Kif3A and Kif3B and a nonmotor subunit, kinesin-associated protein (KAP), associates with up to 16 different IFT proteins (Rosenbaum and Witman, 2002). However, in *C. elegans*, heterotrimeric kinesin-2 and homodimeric kinesin-2, osmotic avoidance defective (OSM)-3 motors, cooperate to transfer proteins across the middle segment, whereas only OSM-3 kinesin carries out protein transport across the distal segment to the ciliary tip (Ou et al., 2005; Snow et al., 2004). Recently, Evans et al. (2006) demonstrated that in nematode cilia, modulation of heterotrimeric versus homodimeric kinesin activity influences functional differences in specific sensory neuron types. In vertebrates, evidence suggests that heterotrimeric kinesin-2 is required for IFT, but the level of coordination with other kinesin motors remains unclear. Kif17, the vertebrate ortholog of OSM-3, mediates protein transport in mammalian neuronal dendrites (Setou et al., 2000), and a recent report showed the first direct link between Kif17 and the cilium in mammalian cells in vitro (Jenkins et al., 2006). Retrograde shuttling of proteins, including kinesin-2 and IFT particles, is also required for ciliary maintenance and is mediated by dynein heavy chain (DHC1b, CHE-3, or Dhc2 in *Chlamydomonas*, *C. elegans*, and mammals, respectively) (Rosenbaum and Witman, 2002).

Ciliary structure is strongly conserved from protists to multicellular organisms. The existence of 9+2 organelles across eukaryotes has led to the speculation that this may be the ancestral structure, with 9+0 cilia having arisen later (Cavalier-Smith, 2002; Mitchell, 2004). A traditional view has maintained that cilia with the central pair of microtubules, such as sperm flagella, oviduct, and respiratory tract cilia, are motile, whereas their

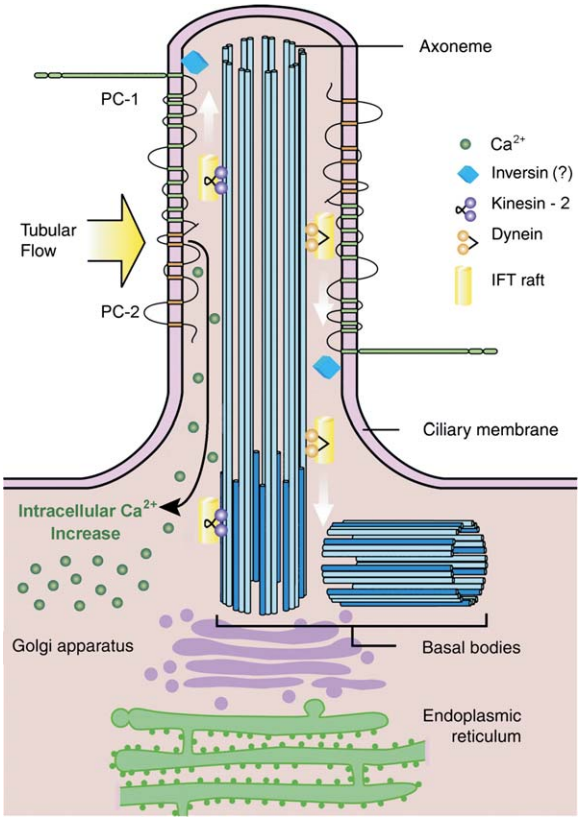

Figure 1. Cilia Mediate Mechanosensation

Cilia are unique cellular structures that typically protrude from the apical surface of mammalian cells. Anchored to the basal body, which consists of nine radially oriented microtubule triplets, the cilium extends from the cell with nine microtubule doublets either without (9+0; shown) or with (9+2; not shown) a central pair of microtubules. Ciliary protein trafficking along the axoneme is facilitated by IFT rafts such as Polaris/IFT88 and is carried out in the anterograde direction by kinesin-2 molecular motors, whereas retrograde shuttling of proteins is conducted by dynein. A mechanosensory role has been demonstrated for cilia; more specifically, extracellular flow causes the cilium to bend and results in an increase of intracellular Ca^{2+}. Evidence suggests that the flow sensation in kidney primary cilia is mediated by PC1 and PC2, transmembrane proteins that colocalize to the ciliary membrane and form a cation-permeable channel. Figure adapted from Figure 1 (Guay-Woodford, 2003) with permission.

absence is indicative of nonmotile sensory cilia, as exemplified by renal, pancreatic, photoreceptor, and olfactory neuron cilia in vertebrates. However, several notable exceptions, such as the 9+0 microtubule structure in diatom gamete flagella (Manton et al., 1970) and eel sperm flagella (Gibbons et al., 1985), suggest that this additional pair of microtubules is not a prerequisite for motility. In the mouse, 9+0 cilia generate fluid flow in the embryonic node (Nonaka et al., 2002), while, in zebrafish, motile 9+0 cilia have been observed in the central canal of the spinal cord (Kramer-Zucker et al., 2005).

Cilia and Motility

Motility is the function most commonly associated with cilia, and in 9+2 cilia, the central microtubule pair appears to be necessary to confer movement via its dynein-mediated link with the surrounding outer doublets (Satir, 1999). The mechanical constraints given by the linkage between dynein arms, microtubule doublets, and radial spokes then convert interdoublet sliding into characteristic ciliary movement. The precise pattern of movement of individual cilia has been studied extensively both on ciliated epithelia and in the flagella of the green alga *Chlamydomonas*. The beat is asymmetric, and it consists of an effective stroke occupying one-quarter of the beat and a recovery stroke. In wild-type flagella, the beat frequency is ~60 Hz, and in flagella lacking outer arm dyneins, the beat form remains nearly normal; however, the frequency decreases to 20–30 Hz (Porter and Sale, 2000). Coordinated beating between adjacent cilia, with each individual cilium having an inherently asymmetric beat, results in directional movement of extracellular fluid overlying ciliated epithelia such as those found in the trachea, choroid plexus, and oviduct. Recently, motile cilia in the ependyma of the brain were shown to be essential for directing long-range directional migration of neuroblasts (Sawamoto et al., 2006).

In addition to the classic functions of ciliary motility, vertebrates are thought to utilize the asymmetry inherent in the structure of the cilium, as well as the resulting asymmetric ciliary movement, as a means to generate handed left-right (LR) asymmetry during development (Tabin and Vogan, 2003). This process depends on a subpopulation of motile cilia not found on ciliated epithelia, specifically the monocilia located on the ventral cells of the mammalian node (organizer) and the epithelium lining Kupffer's vesicle in fish (Amack and Yost, 2004; Essner et al., 2005; Kramer-Zucker et al., 2005; Nonaka et al., 1998). Curiously, several reports show node monocilia that lack the central pair and radial spoke apparatus (Bellomo et al., 1996; Nonaka et al., 1998; Sulik et al., 1994). In addition, the beat pattern of node monocilia may deviate from that observed in *Chlamydomonas* and ciliated epithelia: node cilia have an effective recovery stroke like other motile cilia, but the motion is conical and the directionality of the resulting fluid movement may be, in part, secondary to a posterior tilt of the cilium itself relative to the cell body combined with the inherent asymmetry of the ciliary beat (Nonaka et al., 2005; Okada et al., 2005). Furthermore, these cilia beat in a clockwise direction at frequencies slightly lower than those of ciliated epithelia (Nonaka et al., 1998; Okada et al., 2005; Sulik et al., 1994; Supp et al., 1999). Coordinated beating of >200 (in mouse) monocilia generates leftward directional movement of the extracellular fluid surrounding the node, called nodal flow (Nonaka et al., 1998; Okada et al., 1999).

Directional fluid flow is essential for the development of LR asymmetry; mice with paralyzed node monocilia due to a mutation in the axonemal dyneins *lrd* (Okada et al., 1999; Supp et al., 1999) and *Dnahc5* (Olbrich et al., 2002) and zebrafish with morpholinos targeted against the zebrafish *lrd* ortholog (Essner et al., 2005) have absent nodal flow and randomization of LR asymmetry. The LR defect in $lrd^{-/-}$ mouse embryos is rescued by artificial leftward nodal flow, demonstrating that Lrd functions in LR development via its role in node-cilia motility (Nonaka et al., 2002). The mechanism by which adjacent cells in the node or Kupffer's vesicle coordinate ciliary movement to generate laminar directional fluid flow is poorly understood. One clue comes

from mice with mutations in the ankyrin repeat-containing protein inversin (Inv): these mice have normal movement of the individual node cilium; however, nodal flow is sluggish, due, at least in part, to defective ciliary tilt, and LR development is abnormal (Okada et al., 1999, 2005). These data suggest that Inv may be required to coordinate movement of adjacent cilia.

Cilia as Chemosensory and Mechanosensory Organelles

Cilia as Mechanosensors

The unique geometry of cilia makes them inherently suitable as cellular mechanosensors. The cilium can extend far beyond the cell membrane, and cilia range in length from 3 to 30 μm, with a diameter of only 200–300 nm. The microtubule skeleton of the axoneme connects to the ciliary membrane toward the tip, possibly facilitating direct transduction of forces impinging on the ciliary membrane through the axoneme. The base of the cilium is positioned close to the trans-Golgi apparatus and the nucleus, thus providing immediate access to intracellular signaling machinery (Poole et al., 1997). Along with their "antenna-like" geometry, cilia are flexible and are thus able to respond to fluid shear and forces exerted on the extracellular matrix by bending (Jensen et al., 2004; Schwartz et al., 1997). Although invertebrate cilia have been documented as mechanosensors for some time (reviewed by Wiederhold, 1976), the first clear demonstration of mechanosensation in vertebrate primary cilia came from the observation that isolated bending of the cilium of polarized epithelial MDCK cells caused an intracellular calcium increase. A similar response was generated by applying flow to the cells, and the response was absent in cells treated with chloral hydrate to remove cilia (Praetorius and Spring, 2001, 2003). In vivo, monocilia extend from the epithelium into the lumen of kidney tubules, and, in mouse, multiple mutations affecting ciliary biogenesis are associated with a polycystic kidney phenotype (Badano et al., 2006b). Pazour et al. (2000) demonstrated the first causal link between defects in the IFT protein-encoding gene *Tg737* (*Polaris*) and kidney disease in the mouse. Additional defects in ciliary protein-encoding genes can be subdivided into mutations affecting axonemal motor proteins, such as KIF3A, mutations affecting ciliary receptor function, such as polycystin 1 (PC1) and polycystin 2 (PC2), and those affecting communication of a ciliary signal to downstream pathways, such as mutations affecting Inv. Human polycystic kidney disease (PKD) is an autosomal dominant disease most frequently caused by mutations in *PKD1* and *PKD2*, the genes encoding PC1 and PC2, respectively. Although PC1 and PC2 are thought to have distinct cellular functions, some evidence suggests that, in the kidney, they associate to form a cation-permeable channel (Hanaoka et al., 2000). Notably, PC1 and PC2 can colocalize at the ciliary membrane and the endoplasmic reticulum (Pazour et al., 2002). Flow also induces a calcium response in mouse embryonic kidney cells, albeit with a different time course than that observed in MDCK cells, and cells derived from kidneys of mice with mutations in *Pkd1* fail to generate an increase in calcium in response to flow; furthermore, blocking antibodies directed against either PC1 or PC2 also block the flow-induced calcium response (Nauli et al., 2003). Together, these data suggest that the polycystin complex in kidney cilia functions as a mechanosensor, most likely detecting flow, or possibly directly sensing lumen size (Figure 1).

How does the polycystin complex detect flow at the cilium and then transmit the flow-induced signal? Current evidence supports a bifunctional role for PC1 both as a mechanosensor and as a signal transducer. PC1 has 11 transmembrane domains, a short C-terminal intracellular domain, and a long extracellular region. It binds to the cation channel PC2 via a coiled-coil domain and acts as a receptor, controlling the gating of the PC2 channel (Delmas, 2004). In response to mechanical signals, PC1 undergoes proteolytic cleavage, resulting in translocation of the cytoplasmic C-terminal tail into the nucleus to initiate signaling (Chauvet et al., 2004; Low et al., 2006). However, the features of the polycystin complex that create mechanosensitivity remain elusive. Notably, the flow-induced Ca^{2+} influx is dependent on cilia: cilia are microtubule-based structures, and thus far no direct link of the polycystin complex to microtubules has been identified, suggesting that ciliary bending might propagate a PC1/PC2-mediated influx of intracellular Ca^{2+}.

In addition to kidney defects, mice with mutations in *Pkd2* fail to develop normal LR asymmetry. Specifically, *Pkd2* null embryos retain bilateral symmetry with absent expression of normally left-sided LPM expression of *Nodal* and *Lefty*, randomized heart looping, and right pulmonary isomerism (Pennekamp et al., 2002). At the mouse node, PC2 localizes to monocilia. Analysis of mouse embryos with GFP-tagged LR dynein (lrd) identified two populations of node monocilia: motile cilia containing both PC2 and lrd, and immotile cilia containing PC2 but no lrd. In response to nodal flow generated by motile node monocilia, there is an increase in intracellular calcium in cells at the left margin of the node; this response is absent in *Pkd2* null embryos, suggesting the physical stimulation or a "two cilia" model of leftward nodal flow interpretation (McGrath et al., 2003). Alternatively, the accumulation of morphogens in the node might result in the release of lipoproteins termed "nodal vesicular particles" that accumulate on the left side of the node and promote cilia-mediated signal transduction events that might include hedgehog (Hh) signaling and intracellular Ca^{2+} release (Okada et al., 2005; Tanaka et al., 2005).

The requirement for PC2 in LR development is conserved in zebrafish, as embryos treated with a morpholino directed against *pkd2* undergo abnormal LR development and develop renal cysts (Bisgrove et al., 2005). Further, an asymmetric increase in calcium can be observed at the Kupffer's vesicle (Sarmah et al., 2005). Unlike the kidney, however, ciliary-mediated calcium signaling at the node does not appear to involve PC1, as *Pkd1* knockout mice do not have abnormal LR development (Karcher et al., 2005), and PC1 does not localize to node monocilia. These observations suggest that PC2 may complex with another, yet unidentified partner at the node.

The association between ciliary defects and renal and LR development raises the intriguing possibility that cilia-mediated mechanosensation of fluid flow has a role in the development of other organs with fluid-filled

structures. Some supporting evidence already exists: mice with various mutations that affect cilia develop cysts in the liver and pancreas, which may indicate that cilia sense fluid flow in the developing biliary and pancreatic ducts. Cilia have also been identified in vascular structures, and cardiovascular defects are a prominent feature of most mice with ciliary mutations (Iomini et al., 2004); whether these are secondary to abnormal development of LR asymmetry, or whether they are a manifestation of a direct role for cilia in cardiovascular development, remains unclear.

Cilia as Morphogenetic Mediators

A plethora of studies has firmly established a role for cilia in transduction of extracellular (and extraorganismal) stimuli that include a variety of chemical compounds (such as odorants and alcohols, Dwyer et al., 1998) as well as bioactive molecules, as exemplified by the presence of insulin-like receptors on the ciliary membrane of *Tetrahymena* (Christensen et al., 2003). In vertebrates, these functions appear to have been expanded. Vertebrate Hh, Wnt/Wingless, and platelet-derived growth factor receptor (PDGFR) signaling are three cellular communication pathways pivotal for embryonic growth and development, and recent reports have linked all three pathways to cilia.

Cilia and Hh Signaling

The Hh transduction pathway commands cell fate decisions in a variety of tissues. In vertebrates, Hh ligands (Desert hedgehog [Dhh], Indian hedgehog [Ihh], or Sonic hedgehog [Shh]) bind to their receptor, Patched (Ptch), and derepress the downstream seven-pass membrane protein Smoothened (Smo) and activate the Gli transcription factors (Gli1–3). In the absence of Hh, Gli3 undergoes proteolytic processing and represses the transcriptional activity of Hh target genes. Additionally, Suppressor of fused (Sufu) is a negative regulator of Hh transduction and interfaces with the Gli transcription factors, mediating their nuclear export in the absence of Hh (Ingham and McMahon, 2001).

Hh signaling has been dissected by using several in vitro and in vivo systems over the past decade. Nonetheless, the requirement of cilia for Hh signal transduction was a recent, surprising finding. Huangfu and colleagues (2003) showed that two neural tube mouse mutants, *wimple* (*wim*) and *flexo* (*fxo*), were caused by mutations in the IFT protein-encoding genes, *Ift172* and *Polaris*. Notably, the phenotype of both *wim* and *fxo* bore similarity to Hh mutants, and genetic interaction was demonstrated with *Shh*. Although these data implicated ciliary proteins in this pathway, they did not exclude the possibility that some IFT components might have an alternate role in cytoplasmic transport. Further work associated additional IFT proteins with the same process, not only during neural tube patterning (May et al., 2005), but also during limb development (Haycraft et al., 2005; Liu et al., 2005), by showing that ablation of cilia due to defective IFT results in the loss of Hh signaling.

Additional evidence for the direct involvement of the cilium in Hh transduction was provided by the demonstration that the localization of vertebrate Smo to the cilium is a prerequisite for proper Smo function (Corbit et al., 2005), potentially suggesting that Smo takes on a ciliary localization in an Hh-dependent manner. Still, the precise mechanism by which Smo activates the Hh pathway at the primary cilium remains obscure.

Analysis of other downstream components of the Hh pathway further substantiated the notion that cilia are crucial for normal signal transduction (Figure 2A). Multiple groups showed that IFT proteins interact, directly or indirectly, with the Gli transcription factors. *Ift88* and *Shh* double mutant embryo analysis demonstrated that IFT88 is required for both Gli activator and repressor function, likely because IFT88 is necessary for the structural integrity of the cilium (Liu et al., 2005). Furthermore, Gli3 processing is perturbed in the absence of IFT172/Polaris, Kif3a, and Dnchc2, resulting in abnormally low proportions of Gli3 repressor (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005). Finally, Gli1–3, and the Gli repressor Sufu also localize to the ciliary tip in primary limb cell cultures. Intriguingly, in the absence of cilia, Gli1–3 and Sufu localize to both the nucleus and the microtubule organization center (MTOC), where cilia would have formed (Haycraft et al., 2005), suggesting that Hh might have a role in the organization of the apical microtubule network, a prerequisite for ciliogenesis.

Cilia and Wnt Signaling

Wnt ligands mediate another cellular communication pathway that regulates a diverse spectrum of developmental and regenerative processes. Many proteins have been implicated in Wnt signal transduction (Logan and Nusse, 2004), but the Wnt ligands, the seven-pass transmembrane Frizzled (Fz) receptors, and the downstream effector Disheveled (Dsh), a potential switch between canonical and noncanonical signaling, are key components. In the canonical Wnt signaling pathway, Dsh activation stabilizes cytoplasmic β-catenin; its subsequent trafficking to the nucleus then activates target gene transcription through ternary complex factor (TCF) elements. In the absence of Wnt, β-catenin is degraded by the Adenomatous Polyposis Coli (APC)/Axin/glycogen synthase kinase 3β (GSK3β) complex, and the downstream transcriptional targets remain repressed. Noncanonical Wnt signaling is mechanistically more enigmatic. Probably the best understood noncanonical scheme is the Planar Cell Polarity (PCP) pathway, which requires Fz receptors and the membrane localization of Dsh for signal transmission independent of β-catenin. Although even more obscure, additional noncanonical signaling mechanisms have been proposed, and these include calcium flux, JNK, and Rho pathways (Logan and Nusse, 2004; Veeman et al., 2003).

A growing list of ciliary proteins is being implicated in Wnt signaling. First characterized as a component of the LR pathway (Mochizuki et al., 1998), and subsequently shown to be a cause of nephronophthisis (Otto et al., 2003), Inv has been proposed to be a determinant between canonical and noncanonical Wnt signaling during renal development in vertebrates (Simons et al., 2005). One model suggests that fluid flow over primary cilia results in the increased expression of Inv, which is then somehow linked to cytoplasmic Dsh degradation. According to this scheme, in the absence of flow, Wnt signals through the canonical pathway, whereas the presence of flow over the cilium somehow mediates Dsh localization to the plasma membrane, where it may promote noncanonical Wnt signaling. However, whether

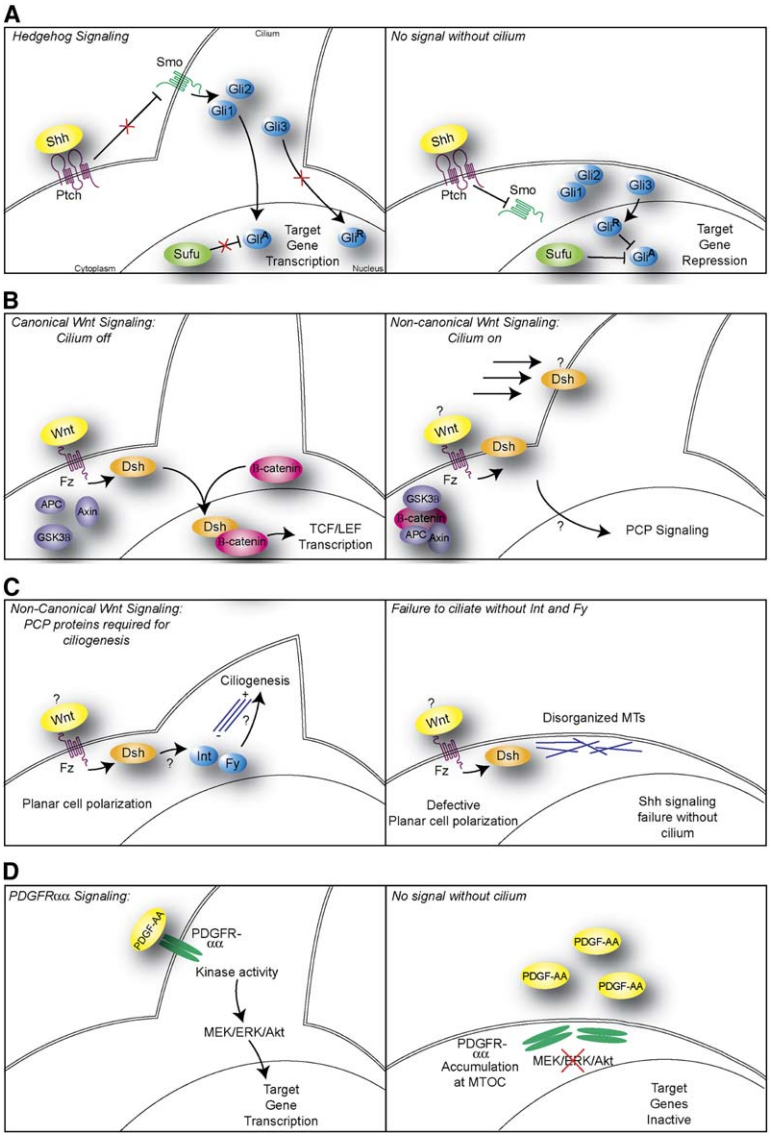

Figure 2. Cilia and Signal Transduction

(A) Cilia are necessary for vertebrate hedgehog (Hh) signaling. Upon Shh ligand binding to Ptch, inhibition of Smo is eliminated, promoting activation of the Gli transcription factors (Gli^A) and their translocation from the cilium to the nucleus. Cells with ciliogenesis defects, such as *Ift172* and *Ift88/Polaris* mutants, cannot localize Smo to cilia (right panel). Subsequently, Gli3 is processed into a repressive variant (Gli^R) and inhibits Gli induction of target gene transcription in conjunction with the transcriptional repressor Sufu.

(B) The cilium acts as a switch between canonical and noncanonical Wnt signaling depending on environmental cues. In a default state (left panel), cells carry out canonical signaling in which Wnt ligand binds to the transmembrane Fz receptor and stimulates the stabilization and translocation of cytoplasmic β-catenin to the nucleus. Changes in ciliary environment, such as fluid flow in the kidney, might result in noncanonical Wnt signaling (right panel), where Dsh localizes to the plasma membrane and β-catenin is unable to escape degradation by the APC/Axin/GSK3β complex.

(C) Noncanonical Wnt components play a role in ciliogenesis. In *Xenopus*, PCP proteins Int and Fy localize to the apical surface of the cell along with Dsh and promote the generation of the cilium. In the absence of Int and Fy (right panel), ciliogenesis fails to occur, perhaps a consequence of disorganized microtubules (MTs) on the apical surface. The lack of a cilium results in Shh signaling failure and tissue patterning defects.

(D) Cilia are required for PDGFRαα signaling in quiescent cells. In postmitotic cells, PDGFRαα localizes to the cilium and confers downstream MEK/ERK/Akt signaling upon binding of the PDGF-AA ligand (left panel). In *Tg737*^{orpk} mutant cells with defective IFT (right panel), the lack of a cilium is coincident with abrogated PDGFRαα signaling, and the receptor accumulates at the microtubule organization center (MTOC).

these in vitro observations (including the induction of flow) are physiologically relevant remains to be demonstrated, and the fact that Inv also localizes to the basolateral membrane of cells further complicated this model, as β-catenin also signals from that site in a Wnt-independent manner (Eley et al., 2004).

The availability of mouse mutants for basal body-specific proteins provided further evidence for a ciliary role for Wnt signal transduction. Two recent reports that focused on genes that cause Bardet-Biedl syndrome (BBS) shed light on this function. In one study, Ross et al. (2005) established that mouse mutants and zebrafish morphants for BBS1, BBS4, and BBS6 interact genetically with the conserved transmembrane PCP protein Van Gogh-like2 (Vangl2), which plays a key role in controlling planar cell polarity and convergent extension movements during development (Torban et al., 2004). Subsequent data also indicated that PCP and convergent extension involvement was not unique to the BBS proteins, but could also be modulated by other components of the basal body, such as the BBS modifier MGC1203 (Badano et al., 2006a), although the question of whether basal body function is necessary and sufficient for Wnt transduction remains unclear.

Cumulatively, these data hint at a tantalizing link between the cilium and Wnt signaling (Figure 2B). Nonetheless, numerous questions remain unanswered, including the temporal relationship between ciliogenesis, Wnt, and Hh. A recent study on two *Xenopus* orthologs of the *Drosophila* PCP genes Inturned (*Xint*) and Fuzzy (*Xfy*) indicated that PCP might lie upstream of ciliogenesis (Figure 2C). Park and colleagues (2006) demonstrated that both *Xint* and *Xfy* morphants not only display convergent extension phenotypes, but that they also display neural defect phenotypes that were due to Shh signaling failure. Functional analyses of Xint and Xfy suggest that they are critical for mandating the proper order of the apical actin cytoskeleton in ciliated cells, which, in turn, is critical for proper polar organization of microtubules and, ultimately, ciliogenesis (Park et al., 2006).

It is quite possible, however, that the cilium maintains one or more Wnt roles subsequent to ciliogenesis. Inv,

which binds directly to Dsh (Simons et al., 2005), is required for the correct angular orientation of nodal monocilia (Okada et al., 2005), and Vangl2 localizes to cilia of both renal and bronchial epithelial cells (Ross et al., 2005). It will be intriguing to decipher these potentially interlinked observations in the context of both development and tissue maintenance.

Cilia and PDGF Signaling

A third significant signal transduction pathway is mediated by PDGFR, a prototypical receptor tyrosine kinase. The PDGF family of proteins consists of three receptors and five ligands, which contribute to cell migration and proliferation by activation of several downstream signaling events, including Mek/Erk, Akt, PI3K, and Ras/Raf/MAPK (Tallquist and Kazlauskas, 2004).

In addition to the Hh and Wnt pathways, PDGFRα-mediated cellular signaling has been associated with the cilium (Schneider et al., 2005) (Figure 2D) via a demonstration that PDGFRα expression was upregulated in quiescent fibroblasts and localized to the cilium, extending from the base to the tip in a time-dependent manner. Moreover, stimulation with PDGF-AA ligand resulted in the activation of downstream signaling pathways (Mek1/2-Erk1/2 and Akt). Finally, in *Tg737*^{orpk} mutant cells, PDGFRα localized to where cilia should have been, but it was unable to confer signaling during growth arrest. Although in vivo work will be required to decipher the physiological relevance of these findings, the emerging image is of a central role for the ciliary structure in diverse signaling pathways.

The Functional Components of Cilia

Cumulatively, the above-described observations hint at a broad role for cilia across phyla. This appears to be especially pervasive in vertebrates and is coincident with the expansion of ciliated cells compared to the presence of a few discrete ciliated cell types in *Drosophila* and *C. elegans*. A major challenge is therefore to identify the functions of cilia and to discriminate panorganismal functions from vertebrate-specific functions, since those might underlie profound evolutionary shifts. Toward that goal, a number of studies have sought to catalog the total complement of proteins required for ciliary function. Many of these have focused on analyses in invertebrates, but they have provided data that can be integrated with information from vertebrate studies to provide a developing picture of the components of cilia in vertebrate cells.

The initial observation of aberrant flagellar phenotypes in *Chlamydomonas* inspired early investigations into the structural and functional elements of the flagellum. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) not only facilitated the first estimates on the diversity and size of the ciliary proteome, but it also helped to define proteins pertinent to the assembly and function of the organelle. Some initial calculations placed the count at ~150 polypeptides at the basal body and as many as 250 proteins in the axoneme (Dutcher, 1995a, 1995b). Still, the procurement of these estimates was limited by insufficient protein resolution, which was not addressed until the advent of optimized mass spectrometry (MS) coupled to whole-genome sequence data (Aebersold and Mann, 2003).

Characterization of the Basal Body and Ciliary Proteome

One of the first efforts to deduce the human ciliary proteome involved the characterization of proteins in purified ciliary axonemes from a human bronchial epithelial cell line (Ostrowski et al., 2002). Their MS analyses yielded >200 putative axonemal proteins, and although this study was hampered by the fact that a high proportion of proteins identified were represented by only one peptide, this data set still provided a glimpse of the complexity of the cilium. The human centrosomal proteome was reported subsequently and further demonstrated the utility of MS for proteome analysis (Andersen et al., 2003).

On the other end of the evolutionary spectrum, an MS-based approach focused on the centriole of *Chlamydomonas* identified 45 candidates, which were then subdivided into 2 categories (Keller et al., 2005). One class represented genes upregulated upon deflagellation, and the other contained conserved components of the centriole. Perhaps the most complete MS analysis of the eukaryotic ciliary proteome to date is the description of the *C. reinhardtii* flagellar protein repertoire (Pazour et al., 2005). In a study with heavy emphasis on organellar purity, more than 600 flagellar proteins were identified, and over half of these were reported with high confidence and many were validated subsequently by deflagellation expression studies. In a subsequent study, Broadhead and colleagues (2006) employed MS on the *Trypanosoma* flagellum and identified 331 putative flagellar proteins, which they validated further in silico and by RNAi.

An improved understanding of the inductive process of ciliogenesis (and flagellogenesis) and the transcriptional regulation of loci pertinent to ciliary biology has also been used to identify ciliary proteins. The specific induction of flagella-related genes that ensues ~30 min after flagellar amputation in *Chlamydomonas* permitted Stolc and colleagues (2005) to perform whole-genome DNA microarray analysis and to identify >200 genes that were transcriptionally upregulated during flagellar regeneration. Not only were these analyses indicative of proteins potentially required for flagellogenesis, but they also revealed both upstream and downstream transcriptional targets. In a parallel approach, two groups carried out a genome-wide search for genes possessing the X-box promoter domain, the *DAF-19* transcription binding factor motif that typically regulates the expression of ciliary genes in *C. elegans* (Blacque et al., 2005; Efimenko et al., 2005). One group reported some 150 X-box-containing genes (Efimenko et al., 2005), whereas the second study supplemented X-box data with serial analysis of gene expression (SAGE) sequences to screen for cilia-specific genes and reported some 50 strong candidates meeting both selection criteria (Blacque et al., 2005).

The availability of genomic sequence information from multiple species has provided a third independent avenue for filtering the ciliary component of proteomes. Ancient eukaryotes evolved into separate lineages in which the cilium was either retained or lost, and two independent groups exploited this key difference to define the proteomic intricacy of the basal body and cilium (Avidor-Reiss et al., 2004; Li et al., 2004). In one study,

the authors compared the nonciliated *Arabidopsis*, *Dictyostelium*, and *Saccharomyces* genomes with those of five ciliated eukaryotes, by using *Drosophila* as the anchor species. This subtraction yielded ~200 genes that were conserved across ciliated organisms but absent in nonciliated species. In parallel, Li et al. (2004) presented a multifaceted comparative genomics analysis, which also capitalized on the genomic differences between ciliated versus nonciliated organisms. The subtraction of common eukaryotic genes shared between two ciliated species (*H. sapiens* and *C. reinhardtii*) but absent from the nonciliated plant *A. thaliana* resulted in 688 genes, termed the Flagellar Apparatus-Basal Body (FABB) proteome. Additional functional and genetic tests validated the enrichment of their data set for ciliary proteins, including the determination of flagellar phenotypes and the use of this resource to identify genes that cause human ciliopathies.

The application of these diverse approaches, each of which carries its own strengths and limitations, might potentially provide the opportunity to enrich heavily for the ciliary proteome by combining all available data. To explore this possibility, we have recently integrated all existing basal body and ciliary proteomics data into a freely available resource: the ciliary proteome database (http://www.ciliaproteome.org; A. Gherman et al., submitted) This provides an interface for the community to explore numerous aspects of the ciliary proteome, including detailed gene and protein information across multiple species. Currently, this database contains ~1000 nonredundant human putative ciliary and basal body proteins, and among those, some 40% of proteins have been identified by at least two independent studies.

Clearly, the combined proteome will overcome some of the limitations of the various approaches, since the identification of the same protein by two unrelated studies will enrich for true positives. At the same time, there is good reason to believe that noise will not be eliminated until all putative candidates are validated experimentally, and that some bona fide ciliary proteins will be missed.

Despite the power of MS, a major limitation stems from the risk of false positives due to impure protein preparations, coupled with the potential to miss low-abundance proteins in the analysis. Furthermore, since MS only provides a snapshot of an organelle's protein makeup, the basal body or ciliary proteome may vary depending on the cellular context such as cell type, point in the cell cycle, or developmental stage at the time of protein harvest. It is notable that although Pazour et al. (2005) identified a likely ortholog of PC2, a five to one enrichment of Ca^{2+} channel proteins was predicted from the comparative genomics analyses (Avidor-Reiss et al., 2004; Li et al., 2004) in comparison to MS (Broadhead et al., 2006; Ostrowski et al., 2002; Pazour et al., 2005). This might reflect a low abundance in the cell that did not exceed the threshold for detection, or that the cellular context at the time of harvest did not involve ciliary mechanosensory action or Ca^{2+} signaling.

Other approaches are equally susceptible to other impurities. For example, comparative genomics probably introduces a significant fraction of false positives or negatives since (1) for any set of species selected, the presence of cilia is not necessarily the sole discriminating factor; (2) ciliary proteins might have been recruited to nonciliary roles; and (3) close protein family members, some, but not all, of which are relevant to ciliary biology, might mask out some relevant components. For example, heat shock protein 90 and α-tubulin represent two proteins clearly associated with the cilium or basal body, as evidenced by their identification in at least five different proteomics reports (Andersen et al., 2003; Broadhead et al., 2006; Keller et al., 2005; Ostrowski et al., 2002; Pazour et al., 2005; Stolc et al., 2005), but they were missed by comparative genomics (Avidor-Reiss et al., 2004; Li et al., 2004). Moreover, comparative genomics depends heavily on species conservation and carries the risk of not detecting rapidly evolving centrosomal or ciliary proteins, such as BBS10, a novel type II chaperonin associated with the centrosome that has recently been demonstrated to be a major contributor to BBS (Stoetzel et al., 2006).

Limitations aside, reconciliation between the various proteomics approaches and their resulting data will provide a useful starting point both for the characterization of additional ciliary components (Figure 3), the elucidation of novel functions of the cilium, and the genetic dissection of human ciliopathies.

The Challenges Ahead

An understanding of the vertebrate cilium will have a profound impact across several fields of study. A number of seemingly diverse genetic disorders are proving to be associated with ciliary and/or basal body dysfunction, putting forth the notion of the ciliopathy, a broader entity that encompasses discrete clinical subphenotypes (Badano et al., 2006b). At the same time, a renewed appreciation for the complexity of the organelle is raising a number of key questions. What is the precise connection between ciliary proteins, cell migration, and cell shape? Likewise, the determinants of mammalian ciliogenesis are largely unclear. Finally, there have been some tantalizing links between the cilium and the regulation of the cell cycle (Quarmby and Parker, 2005), but that relationship and its potential ramifications to mammalian homeostasis remain to be solved.

A central issue is whether there is an emerging trend in the observed association between the cilia and morphogenetic signal transduction. First, the causal relationship between cilia and developmental signaling pathways is unclear. With respect to Wnt, some data argue that the signal is required for ciliogenesis (Park et al., 2006); however, the evidence pointing to a requirement of cilia for Wnt transmission is also compelling (Ross et al., 2005; Simons et al., 2005), perhaps indicating multiple functions or a looping signal. In addition, the localization of Hh and PCP effectors on the cilium might imply that Hh and Wnt receptors might also localize to the ciliary membrane, but this has not yet been demonstrated for Ptch or Fz, perhaps due to a localization requirement mandating that these receptors be in discretely different cellular compartments from their respective effectors. This might, in turn, either suggest that there is a relationship between the apical cellular membrane and the contents of the cilium, or that novel Hh, Wnt, or other receptors with specific ciliary functions are yet to be identified. In that regard, it is notable that the combined

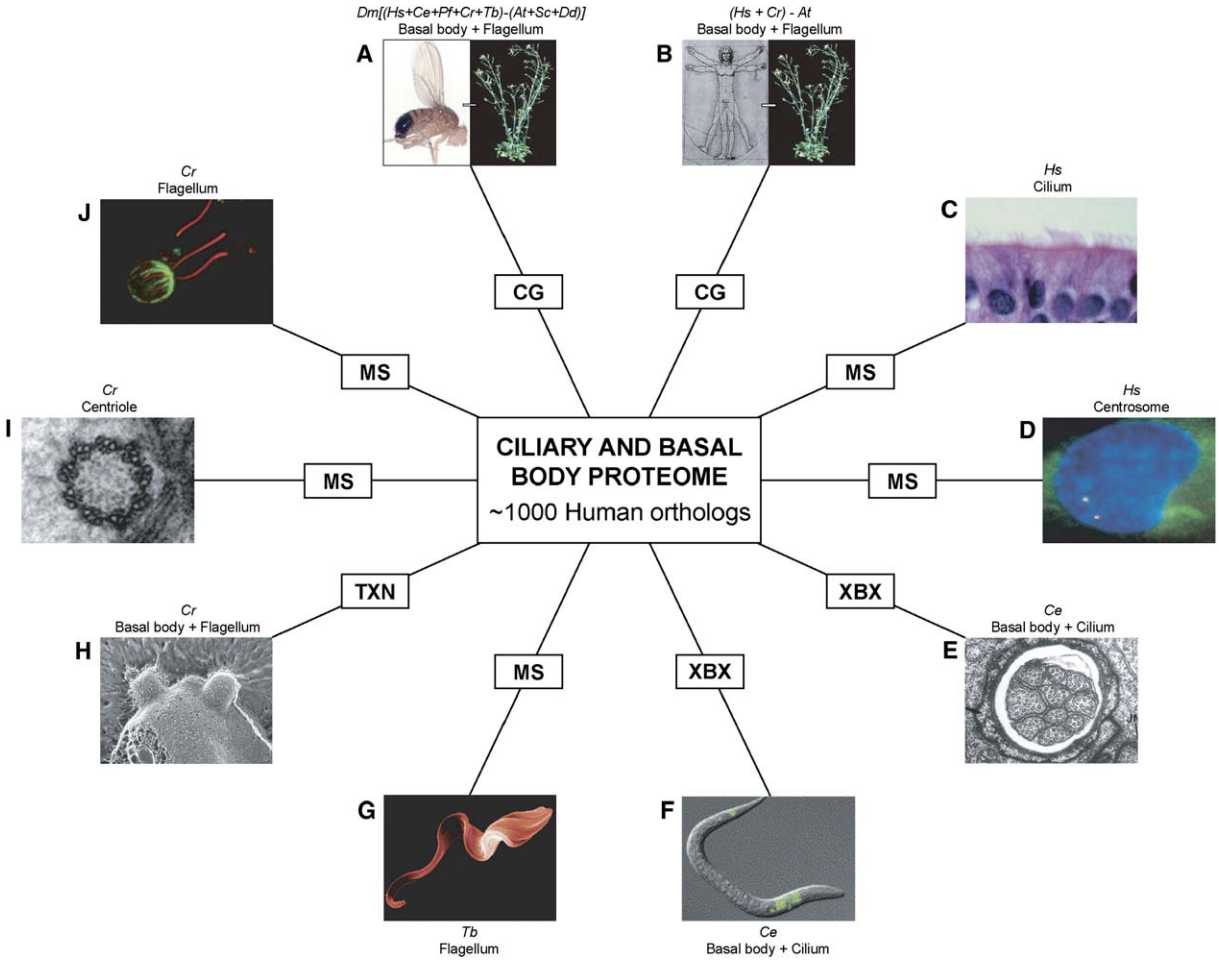

Figure 3. The Ciliary and Basal Body Proteome

The combination of multiple proteomics reports yields a repertoire of ~1000 nonredundant ciliary and basal body proteins.
(A and B) Whole-genome subtraction of ciliated from nonciliated organisms was reported by (A) Avidor-Reiss et al. (2004) and (B) Li et al. (2004).
(C) Human bronchial epithelial cilia were analyzed by MS. Image cropped from Figure 1A, Ostrowski et al. (2002), with permission.
(D) Novel human centrosomal proteins were validated by expressing green fluorescent protein (GFP) fusion proteins in U2OS cells. The merged GFP (green) with γ-tubulin centrosomal marker (red) indicates centrosomal localization. Image reproduced from Figure 2A, and reprinted from Andersen et al. (2003) by permission from Macmillan Publishers Ltd.
(E and F) *C. elegans* genes responsible for ciliogenesis were elucidated by a genome-wide search for genes with an X-box upstream regulatory motif (Efimenko et al., 2005; Blacque et al., 2005; respectively). Cross-section of amphid sensory cilia (E) reprinted from Figure 3a (Perkins et al., 1986), with permission from Elsevier. (F) Cilia-related candidate genes were experimentally verified with GFP tags in vivo. Image reproduced from Figure 1, (Blacque et al., 2005), with permission from Elsevier.
(G) Trypanosome flagellar proteins were analyzed by MS. Image adapted from Figure 1A, (Landfear, 2006) and reprinted by permission from Macmillan Publishers Ltd.
(H) Genes related to ciliary generation are upregulated upon flagellar amputation of *Chlamydomonas* and were catalogued by Stolc et al. (2005). Electron micrograph of deflagellated *C. reinhardtii* by Ursula Goodenough (Washington University, St. Louis, MO), reprinted from Figure 2 (Quarmby, 2004), with permission from Elsevier.
(I and J) MS was employed to elucidate the *Chlamydomonas* centriole (I) (Keller et al., 2005) and flagellar (J) (Pazour et al., 2005) proteomes, respectively. Scanning micrograph of a *C. reinhardtii* centriole (I) cropped from Figure 2H (Fromherz et al., 2004), with permission from the Company of Biologists. Ciliated species are denoted as: *C. elegans* (*Ce*), *C. reinhardtii* (*Cr*), *D. melanogaster* (*Dm*), *H. sapiens* (*Hs*), *P. falciparum* (*Pf*), *T. brucei* (*Tb*), and nonciliated species: *A. thaliana* (*At*), *D. discoideum* (Dd), *S. cerevisiae* (Sc). Proteomics method is indicated by: comparative genomics (CG), mass spectrometry (MS), transcriptional profiling during flagellar regeneration (TXN), analysis of genes containing an X-box (XBX).

ciliary proteome contains numerous transmembrane proteins (Tms), some of which have topological similarities to known receptors. For example, MKS3, a probable ciliary protein that causes exencephaly in humans, is a Tm with some similarities to Fz (Katsanis, 2006; Smith et al., 2006). Other receptors, such as the Somatostatin receptor (Handel et al., 1999) and the Serotonin 5-HT(6) receptor (Brailov et al., 2000), have also been localized to cilia, but their role in the context of this organelle remains unclear. It is intriguing to consider the notion that cilia have acquired some signaling roles specific to cell fate determination and homeostasis during evolution. This hypothesis is supported by the observation that although both *Drosophila* and mice have cilia, only the latter species requires cilia for Shh signaling (Huangfu and Anderson, 2006).

There is little doubt that our appreciation of the role of the cilium in mammalian biology will undergo profound changes. At the same time, the recognition of novel functions might temper our ability to study some aspects of the organelle in traditional models, such as *C. elegans* and *C. reinhardtii*. As such, a new toolkit must be developed that will include siRNAs and constructs for ciliary proteins, as well as conditional mammalian ciliation and deciliation models. At the same time, a new opportunity presents itself to apply established tools and reagents, such as in vitro and in vivo signal transduction pathway reporters, to the study of the new and exciting questions concerning the complex role of the cilium.

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