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Cilia functions in development

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Abstract

Recent advances in developmental genetics and human disease gene cloning have highlighted the essential roles played by cilia in developmental cell fate decisions, left–right asymmetry, and the pathology of human congenital disorders. Hedgehog signaling in sensory cilia illustrates the importance of trafficking receptors to the cilia membrane (Patched and Smoothed) and the concept of cilia ‘gatekeepers’ that restrict entry and egress of cilia proteins (Suppressor of fused: Gli complexes). Cilia-driven fluid flow in the embryonic node highlights the role of motile cilia in both generation and detection of mechanical signals in development. In this brief review I select examples of recent studies that have clarified and consolidated our understanding of the role of cilia in development.

Introduction

Apical cilia, once ignored as vestigial, are now recognized as essential cellular organelles in the regulation of development [1]. Cilia are microtubule based structures projecting from the apical surface of nearly all cell types [2]. Motile cilia are familiar as the driving force for bronchial mucus clearance and sperm motility while primary cilia are sensory and immotile. Both motile and sensory cilia are assembled and maintained by intra-flagellar transport (IFT), a microtubule motor based delivery of cilia component proteins to the growing tip (anterograde IFT) and retrieval of proteins to the cell body (retrograde IFT) [3]. Embryonic cells employ cilia to anchor membrane receptors and process incoming signals from morphogens (non-motile or sensory primary cilia) and to generate and respond to mechanical signals in the form of fluid flow and shear force in confined spaces (mechanosensory cilia, motile cilia) [1]. Even this simple distinction in cilia subtypes is breaking down since it is now appreciated that motile cilia also have sensory functions [4]. The wide spectrum of human pathologies, collectively termed ciliopathies, associated with mutations in genes required for cilia function includes Meckel-Gruber syndrome (MKS), nephronophthisis (NPHP), Bardet-Biedl syndrome (BBS), Joubert syndrome (JS), Senior-Løken syndrome, Leber congenital amaurosis (LCA), polycystic kidney disease (PKD), and oral–facial–digital syndrome (OFD) [5]. Most recently, Sensenbrenner Syndrome has been added to this growing list [6–8]. The overlap in clinical features of these syndromes, including cystic kidney, laterality defects, nervous system development defects, and retinal degeneration, along with the ever expanding number of gene mutations associated with ciliopathies highlights the complexity of cilia structure and the ubiquity of cilia function [5]. I concentrate here on advances made in the past two years that help clarify how cilia serve as embryonic signaling centers and new insights into the role of cilia generated fluid flow in morphogenesis.

Cilia as developmental signaling centers: recent advances in hedgehog signaling

By anchoring G-protein-coupled receptors in their membranes, primary cilia serve as a cellular ‘antenna’ for extracellular signaling molecules [9,10]. The best studied developmental signal linked to primary cilia is the Hedgehog (Hh) signaling system [11]. Genetic screens in mice lead to the unexpected finding that mutations in proteins required for cilia assembly, IFT proteins, disrupted neural tube patterning mediated by ventral sonic hedgehog signaling [12]. Subsequent studies implicated cilia IFT in Hedgehog signaling in endochondral bone formation [13]. Hedgehog signaling is surprisingly complex [11] and impossible to summarize in a brief review. Instead, I focus here on recent advances in our understanding of components of Hh signaling that highlight the unique functions of cilia as a ‘gated’ signaling center.

In brief, resting cells express the hedgehog receptor and sterol transporter Patched on membrane at the cilia base and axoneme (Figure 1) [14]. Once bound to Hedgehog ligand, Patched is removed from the cilia membrane and inhibition of the seven-transmembrane protein Smoothed (Smo) is relieved, allowing Smo to accumulate in the cilia membrane. Cilia-localized Smo promotes accumulation of Gli transcription factor effectors of hedgehog signaling (Gli2, Gli3) and their binding factor Suppressor of fused (Sufu), specifically at cilia tips [15]. Gli proteins exist in full-length activator forms (GliA) and proteolytically processed repressor forms (GliR) [16]. The output of hedgehog signaling is to shift the balance of Gli repressor to Gli activators and promote transcription of Hedgehog target genes. Cilia tip accumulation of Gli factors correlates with inhibition of Gli proteolytic processing and stabilization of activating forms of Gli proteins [15]. Thus an essential role of cilia is to regulate the ratio of GliA to GliR proteins to control transcription of hedgehog target genes.

Clues as to how cilia regulate Gli protein activity have been provided by recent studies of the Gli interacting protein Suppressor of fused. Suppressor of fused (Sufu) is a Gli binding protein that inhibits Gli activity largely by sequestration in the cytoplasm [17–19]. Mutation in mammalian Sufu leads to ligand-independent activation of the Hh pathway, supporting the idea that Sufu acts as a repressor of Hh signaling [20]. The demonstration that deficiency of Sufu can lead to full activation of Hh signaling even in the absence of cilia (Ift88 mutant cells) indicated that a primary function of cilia is to remove Sufu inhibition of Gli activity [21••]. Recent biochemical studies support the idea that in resting cells, Sufu preferentially associates with full-length Gli3 and after proteolytic cleavage, GliR dissociates from the complex to represses Hh target genes [21••]. Activation of Hh signaling promotes dissociation of full-length Gli3 activator from Sufu, allowing subsequent Gli3 phosphorylation and activation of Hh target genes [21••]. Importantly, dissociation of Sufu:Gli3 complexes and Gli3A phosphorylation does not occur in cilia-deficient (Kif3a mutant) cells stimulated with SAG, a small molecule activator of smoothed [21••]. Thus a principal role of cilia in hedgehog signaling is to promote dissociation of inactive Sufu:Gli complexes, allowing the release of Gli activator proteins [23].

While many additional aspects of hedgehog signaling remain to be worked out, an intriguing question is how transit of Sufu:Gli complexes to cilia tips promotes Sufu:Gli dissociation. The cilia tip is the active zone of cilia growth; here, anterograde IFT raft cargo is released and re-assembled with other axonemal proteins to extend the cilium [24]; retrograde IFT then recycles transport proteins to the cilia base [25]. Cilia deficient in retrograde IFT accumulate proteins at the cilia tip resulting in bulbous tip membrane protrusions [26]. It will be interesting to see whether cilia tip associated mechanisms of Sufu:Gli dissociation have co-opted cellular machinery originally evolved for dissociating IFT raft cargo. It will

also be important to resolve the somewhat puzzling role of retrograde IFT in Gli transport and processing since mutation in the retrograde IFTA motor Dync2h1 inhibits Hh signaling [27,28] while mutation in other retrograde IFTA proteins activates Hh signaling [26,29]. Clarification of these results is likely to come from modeling cilia association of Hh signaling proteins as the outcome of shifting the equilibrium toward cilia retention of proteins that are constantly shuttled in and out of the cilium [28], as opposed to a strictly gated process. For instance, the concept of cilia trafficking equilibria has recently been put forward to account for findings that hedgehog signaling deficits caused by the loss of the retrograde IFT A motor Dync2h1 can be compensated by reduction in the levels of anterograde IFT B proteins [30].

Gatekeepers of protein trafficking in the cilium

Control of both initiation of Hedgehog signaling and subsequent processing of Gli proteins is critically dependent on access of signaling receptors (Smo) and downstream effectors (Gli) to the ciliary compartment. For instance, Protein kinase A, a known inhibitor of hedgehog signaling, acts by preventing access of Sufu:Gli complexes to the cilia, thus preventing Sufu:Gli dissociation and Gli activation [23,31,32]. Access to the cilium is likely to be a general principle in cilia function affecting many cell-type-specific cilia signaling systems. The cilia transition zone, a short segment of the cilium just above the basal body, has recently been implicated as an essential ‘gatekeeper’ that limits or controls diffusion of membrane proteins in and out of the cilium (Figure 2) [33•]. This proposed function of the transition zone is correlated with the presence of ‘Y-links’, structures that link cilia microtubule doublets to the cilia membrane in position to restrict membrane protein diffusion or regulate IFT [33•]. The significance of the transition zone is highlighted by recent reports that a complex of proteins encoded by genes found to be mutated in syndromic ciliopathies (MKS/NPHP) function as a multiprotein complex in the cilia transition zone [34••] and act as a barrier to membrane protein diffusion [35••,36••]. Whether MKS/NPHP proteins are part of the Y-link structure or whether they perform a regulatory function in cilia trafficking remains to be determined.

Another barrier or ‘gate’ controlling access to cilia is the so-called ‘ciliary necklace’, a ring of intramembrane proteins at the junction of the apical plasma membrane and the cilia base adjacent to the transition zone (Figure 2). Recent reports establish an unexpected function for components of the nuclear pore complex in transit of proteins past the ciliary necklace [37•,38]. The nuclear pore complex regulates traffic in and out of the nucleus [39]. Importin b and the small GTPase ran are key elements of this transport system that have now been shown to also function in regulating protein transit to the cilium via the ciliary necklace [37•,38]. Septin 2, a highly conserved protein responsible for maintaining distinct membrane domains in budding yeast, is also expressed in the ciliary necklace region and functions there as a barrier to membrane protein diffusion [40]. These observations indicate that multiple cilia membrane-associated barriers regulate trafficking of specific proteins to and from the cilium. In addition, specific apical membrane retention signals can restrict diffusion of proteins into the cilium [41]. Elucidation of the molecular nature of the transition zone and protein sorting signals should yield new insights into how cilia membrane composition is regulated and ultimately how cilia signaling is controlled.

Cilia membrane protein transport is mediated by cilia targeted coatamer-like proteins (the ‘BBSome’ [42]) and by the activity of multiple small GTPases including rab8, rab11, and rab23 [43,44]. The temporal sequence of rab8, rabin8, a rab8 guanine nucleotide exchange factor, and rab11 activities in ciliogenesis has recently been elegantly demonstrated by live cell imaging [45]. Further studies of other cilia-associated small GTPases including arf4, arl13b, arl2, arl3, arl6, rabl4/IFT27, and rabl5/IFT25 [46] are likely to identify molecular

switches that regulate cilia gatekeeper functions, cilia membrane composition, and cilia structure [47,48].

Cilia, fluid flow, and development

Another major function of cilia in development is to generate fluid movement in confined embryonic spaces and to sense and interpret fluid shear force (mechanotransduction) as a developmental signal. The best studied example of this occurs in the embryonic node, or Kupffer's vesicle (KV) in fish, where leftward fluid flow is harnessed as a developmental cue for breaking left-right symmetry. Seminal findings of left-right asymmetry disorders in human patients with immotile cilia syndromes [49] ultimately lead to the discovery of motile cilia and fluid flow in the mouse node [50], a transient embryonic midline structure. In the node, anterior-posterior polarity is transformed into left-right polarity by the action of planar polarity signaling on cilia basal body position and orientation (reviewed in [51]). Tipping the basal body to the posterior results in an asymmetric cilia beat stroke and a strong leftward flow and a weaker rightward return flow [52]. Asymmetric left-sided expression of Nodal is observed after the initiation of flow and artificial reversal of flow is sufficient to reverse situs [53]. So how might fluid flow be transduced into a morphogenetic signal? One view is the flow delivers lipid-encapsulated morphogens preferentially to the left side of the embryo [54]. However for this to work, released morphogens would have to be avidly removed from circulation on the left side since return rightward flow would randomize their distribution in the node. As an alternative, a compelling case for left-sided mechanotransduction by cilia can be made. This idea posits that both motile and sensory cilia exist in the node, with sensory cilia positioned at the edges of the node to transduce flow into an increase in intracellular calcium, ultimately signaling left-sided Nodal expression [55]. This model is supported by findings that calcium is elevated on the left side of the node [55] and knockdown or mutations in the cilia-associated calcium channel Polycystin2 (PKD2) result in randomized L/R asymmetry [56,57]. PKD2 is one of two genes mutated in Autosomal dominant polycystic kidney disease [58]. The other, PKD1, interacts with PKD2 to form active ion channels [59]. A somewhat puzzling finding was that PKD1 is not expressed in the node; so how do PKD2 proteins form an active channel? The answer recently reported by two groups is that a PKD1 ortholog, PKD1L1, is expressed in its place in the node and mutations in PKD1L1 lead to left-right asymmetry defects [60, 61].

A surprising outcome of these studies is that PKD1L1 is expressed, at least in Medaka fish, on all KV cilia, including motile cilia [61]. Can cilia be both motile and sensory? Precedent for this is seen in human airway cilia where bitter taste receptors are present on motile cilia and can signal changes in cilia beat rate [62]. Lung and oviduct motile cilia also respond to mechanical resistance and the viscosity of the medium by altering their beat rate, a homeostatic response that ensures continued medium propulsion (reviewed in [4]). In the node, motile cilia may encounter resistance to beating in areas of increased local flow and relay this mechanical signal by elevating cytoplasmic calcium. One further level of complexity in cilia mechanotransduction is that the calcium wave induced in ciliated epithelial cells by fluid flow is not directly propagated by cilium bending but rather by ATP secretion and stimulation of purinergic receptors on neighboring cells [63–65]. Since purinergic receptors are ubiquitous in embryos and signal via calcium release [66], it will be interesting to see whether they play a role in propagating node calcium signaling and establishment of L/R asymmetry.

In addition to signaling in the embryonic node, cilia-driven fluid flow impacts morphogenesis of the larval zebrafish kidney as well as development of the mammalian brain. In the zebrafish pronephros, motile cilia-driven fluid flow signals collective migration

of epithelial tubule cells, resulting in convolution of the nephron and functional association with the vasculature [67]. In the brain, cerebrospinal fluid (CSF) is circulated by motile cilia and cilia defects cause fluid back pressure leading to hydrocephalus or swelling of the brain ventricles [68]. In addition, directional migration of neuroblasts from the subventricular zone to the olfactory bulb occurs in response to CSF flow and establishment of a concentration gradient of guidance molecules [69]. Also, in the developing central nervous system, spontaneous rhythmic firing of neurons is required to establish proper neuronal connectivity. A recent report presents provocative findings that rhythmic neuronal activity is dependent on fluid flow, in this case most likely providing a fluid shear mechanical signal that stimulates neural activity [70].

Conclusions

Cilia serve multiple roles in development and function in highly context dependent ways. Sensory cilia display a unique spectrum of receptors depending on the cell type and can impact development in diverse ways. Motile cilia exert long range, non-cell autonomous effects via generating fluid flow, and thereby guiding tissue morphogenesis under the control of mechanical signals. That cilia can be both motile and sensory offers new possibilities for interpreting cilia function. Now that proteomic analysis of cilia has identified over 1,000 cilia-associated proteins, we can expect many new insights on cilia gene function in the next few years. The field has moved beyond simple association of cilia gene mutations with pathology; our current and future goals should be to determine how different complexes of proteins function together to carry out specific cilia functions or build cilia substructures. To this end, biochemical protein interaction studies, analysis of compound genetic mutants, and high resolution electron microscopic structural analysis will all play essential roles in determining how cilia work. Finally, cilia are not static structures; they adapt to signals in their environment and alter their structure or activity in response [71,72]. Future studies promise to integrate subcellular structure of the cilium with cell physiology, developmental patterning, and morphogenesis while revealing new aspects of the dynamic nature of these organelles.

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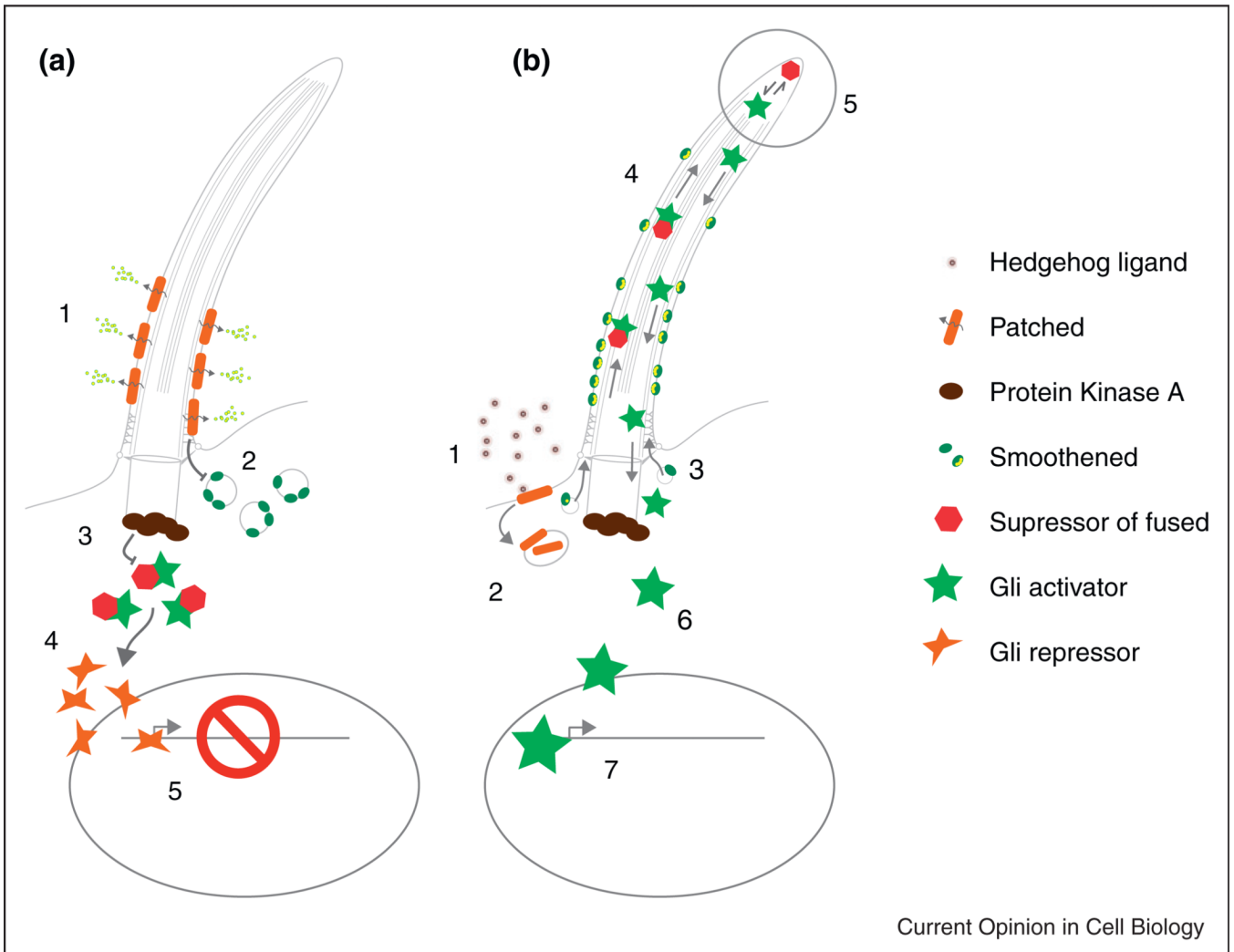
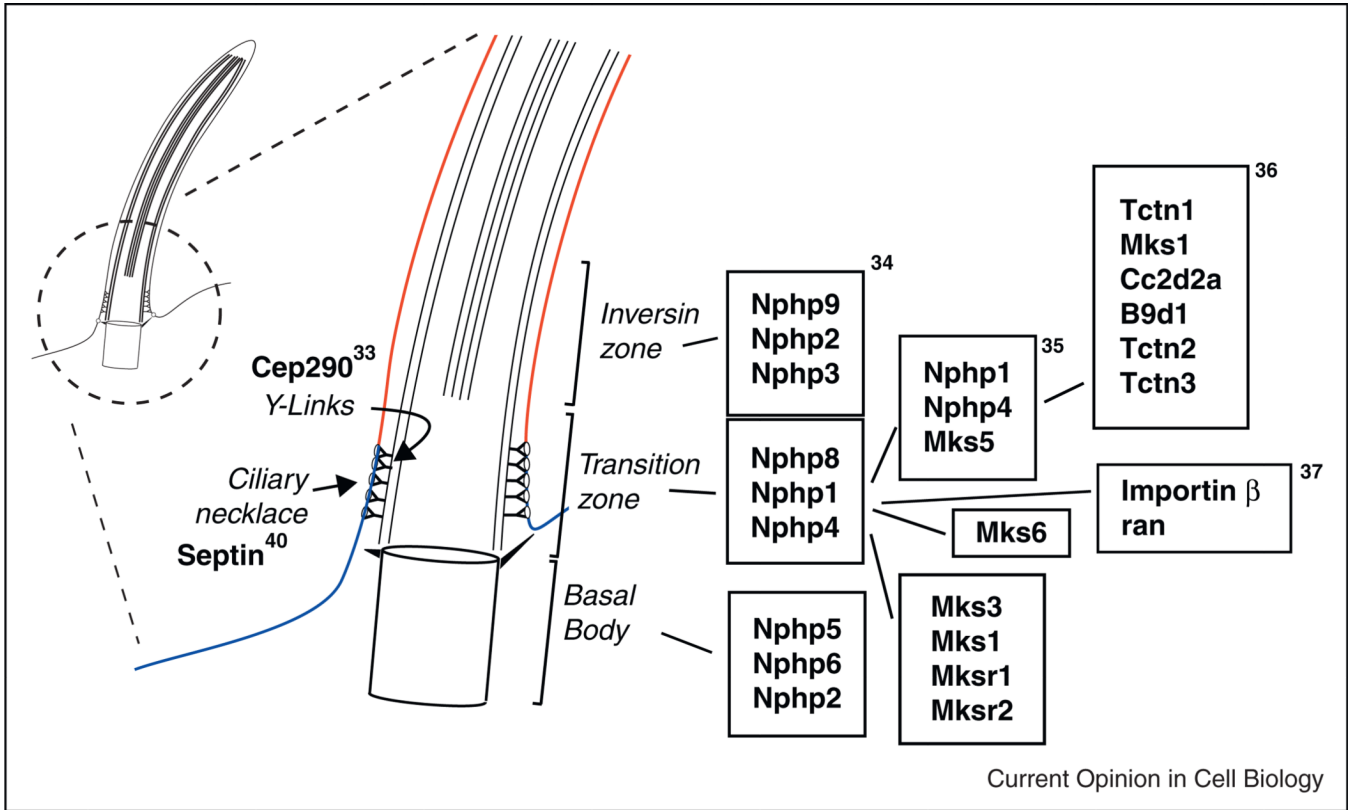


Figure 1.

Recent advances in cilia-mediated Hedgehog signaling. For a complete overview of Hh signaling see Ref. [11]. **(a)** Resting cell state with Hh signaling inhibited. The Hedgehog receptor Patched acts as a sterol efflux transporter on the cilia membrane, restricting the amount of Smoothened seven transmembrane protein present on the cilium (2). Protein kinase A activity prevents access of Sufu:Gli complexes (3) to the cilium. Cytoplasmic processing of Gli to the Gli repressor form (4) and transit to the nucleus maintains Hh target genes in a repressed state (5). **(b)** Activation of Hh signaling in cilia by Hh ligand binding. (1) Hedgehog ligand binds Patched (1), promoting removal from the membrane and blocking its sterol efflux function (2). Sterol bound Smoothened accumulates in the cilia membrane (3), abrogates PKA inhibition of Sufu:Gli complex entry into the cilium, allowing transport of Sufu:Gli complexes to the cilia tip (4). Cilia tip accumulation of Sufu:Gli promotes dissociation of full-length activator Gli proteins by undefined mechanisms (5), allowing Gli activator egress from the cilia (6), transit to the nucleus (7) and activation of Hh target genes.



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Figure 2. The ciliary gate. The ciliary neck and transition zone lie just above the basal body and play important roles in limiting diffusion of cilia membrane proteins and regulating cilia protein traffic. Septin expression in the ciliary neck demarcates the apical cell membrane (blue) from the cilia membrane (red). Other protein complexes contribute to Y-link structures (Cep290) and to regulated trafficking or retention of cilia proteins (Importin β/ran and complexes of NPHP/MKS proteins). Boxed proteins represent biochemically defined complexes identified in the noted references.