Primary Cilia in Cystic Kidney Disease

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Abstract

Primary cilia are small, antenna-like structures that detect mechanical and chemical cues and transduce extracellular signals. While mammalian primary cilia were first reported in the late 1800s, scientific interest in these sensory organelles has burgeoned since the beginning of the twenty-first century with recognition that primary cilia are essential to human health. Among the most common clinical manifestations of ciliary dysfunction are renal cysts. The molecular mechanisms underlying renal cystogenesis are complex, involving multiple aberrant cellular processes and signaling pathways, while initiating molecular events remain undefined. Autosomal Dominant Polycystic Kidney Disease is the most common renal cystic disease, caused by disruption of polycystin-1 and polycystin-2 transmembrane proteins, which evidence suggests must localize to primary cilia for proper function. To understand how the absence of these proteins in primary cilia may be remediated, we review intracellular trafficking of polycystins to the primary cilium. We also examine the controversial mechanisms by which primary cilia transduce flow-mediated mechanical stress into intracellular calcium. Further, to better understand ciliary function in the kidney, we highlight the LKB1/AMPK, Wnt, and Hedgehog developmental signaling pathways mediated by primary cilia and misregulated in renal cystic disease.

11.1 Functions and Features of Primary Cilia

Almost all mammalian cells have an apical protrusion that is used to sense the extracellular environment. This protrusion, the cilium (also known as the flagellum), is an ancient organelle thought to be present on the last eukaryotic common ancestor (Mitchell 2007). Mammalian primary cilia were first reported in 1898 on various iron hematoxylin-stained epithelia, and at that time, primary cilia extending into renal tubules were speculated to

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sense fluid flow (Zimmermann 1898). Following detailed electron microscopic analyses of embryonic and adult cell types, cilia were further proposed to serve as conserved structures that receive and transduce extracellular cues (Poole et al. 1985). Indeed, the primary cilium functions as an antenna to receive light and mechanical and chemical signals. The ciliary membrane, while topologically continuous with the plasma membrane, is distinct in its composition of membrane and protein components. The increase in cell surface area provided by the ciliary membrane protrusion allows for a concentration of receptors that can transduce extracellular signals. Cilia can also shed or lose membrane from their distal tips in order to release materials to the extracellular environment (Hogan et al. 2009; Wood et al. 2013), signal to adjacent organisms (Wang et al. 2014), or turn over its spent protein, including molecular and membrane components (Young 1971; Young and Bok 1969), demonstrating their capacity for two-way communication.

11.1.1 Cilium Assembly

The cilium is composed of a cylindrical arrangement of nine microtubule doublets (A and B tubules) termed the axoneme, which is ensheathed by the cellular plasma membrane (Fig. 11.1). While motile cilia also contain a central pair of microtubules along with accessory structures such as dynein arms and radial spokes for force generation, nonmotile primary cilia lack these structures. Cilia extend and are maintained via intraflagellar transport (IFT), the bidirectional movement of motor and cargo proteins along the microtubular axoneme. IFT was first discovered in the green alga, *Chlamydomonas*, by differential interference contrast (DIC) visualization of particle movement along flagella (Kozminski et al. 1993). Trafficking of proteins from the base to the tip of the cilium in anterograde IFT is powered by motor proteins in the kinesin-2 family (Cole et al. 1998). This anterograde motor sedimented with a large group of proteins could be biochemically purified as two complexes, complex B and complex A (Cole et al. 1998). Complex B proteins are associated with the anterograde kinesin motor and complex A with retrograde transport from the tip to the ciliary base mediated by the cytoplasmic dynein motor (Pazour et al. 1998, 1999; Porter et al. 1999; Signor et al. 1999). Mutations in the complex B IFT proteins or IFT motors result in stunted cilia and flagella, suggesting their requirement for ciliary assembly (Brazelton et al. 2001; Deane et al. 2001; Follit et al. 2006; Fujiwara et al. 1999; Haycraft et al. 2003; Huangfu et al. 2003; Ishikawa et al. 2014; Pazour et al. 2000; Qin et al. 2007; Sun et al. 2004). Accumulation of complex B proteins in swollen ciliary distal tips and impaired retrograde IFT in complex A mutants indicate that complex A proteins are required for retrograde IFT (Blacque et al. 2006; Iomini et al. 2001, 2009; Perkins et al. 1986; Qin et al. 2011; Tran et al. 2008). Additionally, complex A proteins have also been shown to be required for ciliary entry of G-protein coupled receptors in mammalian cells (Mukhopadhyay et al. 2010).

While each type of cilium is specialized to receive appropriate signals within a certain tissue, proteins common to most cilia include tubulins, comprising the structural units of the axoneme, microtubule motors and IFT complexes, the Bardet–Biedl Syndrome-related protein complex (BBsome), which traffics cargo in and out of the cilium, and vesicular trafficking-related small GTPases (ARFs, ARFs, Rabs). As we will discuss in more detail

below, mutant proteins common to several types of cilia result in pleiotropic phenotypes due to ciliary dysfunction in multiple tissues.

11.1.2 Basal Bodies, Early Cilium Formation, and Cilium Disassembly

Cilia are anchored at their base by centriole-derived basal bodies (Fig. 11.1). The basal body is composed of nine microtubule triplets (A, B, and C tubules). The A and B tubules extend from the basal body during cilium formation and become the ciliary axoneme. The basal body is the mother centriole of the pair that forms the centrosome at spindle poles during cell division. During G1 or G0, the basal body acquires accessory structures (distal and subdistal appendages), migrates, and docks to the apical plasma membrane. Docking occurs by attachment of the distal appendages to Golgi-derived vesicles (Sorokin 1962) that fuse to one another and, subsequently, to the apical plasma membrane for cilium extension. This process is mediated by membrane-deforming proteins, EH-domain containing 1 (EHD1) and EHD3 for fusion of distal appendage vesicles into a ciliary vesicle, and by recruitment of GTPase and GTP exchange factors Rab11 and Rabin8 for activation of Rab8 in ciliary vesicle biogenesis (Knodler et al. 2010; Westlake et al. 2011). The basal body then matures through loss of centrosomal protein 110 (CP110) and recruitment of IFT proteins and those of the transition zone (Deane et al. 2001; Lu et al. 2015; Rosenbaum and Witman 2002), a compartment distal to the basal body that is proposed to act as a ciliary gate, regulating ciliary entry of proteins.

Just as there is a mechanism for maturation of centrioles to nucleate cilia, there is also a mechanism to disassemble cilia and release sequestered basal bodies for mitotic progression. Several proteins have been implicated in this process, most notably the Aurora A kinase (Pugacheva et al. 2007), which activates mitotic spindle assembling CDK1-Cyclin-B. Growth factors activate a pathway that stimulates Aurora A kinase and, subsequently, the histone deacetylase, HDAC6, responsible for tubulin deacetylation and cilium destabilization. Another group of kinases, the NIMA-related kinases (NEK), are also involved in ciliary resorption (Hilton et al. 2013; Wloga et al. 2006). Nek2, involved in centrosome separation, is basal body localized and activates a microtubule depolymerizing kinesin, kif24, for ciliary resorption in the G2/M transition (Kim et al. 2015; Spalluto et al. 2012). In *Chlamydomonas*, another microtubule depolymerizing kinesin, kinesin-13, is also required for flagellar disassembly (Piao et al. 2009).

11.1.3 Cilium Length Control

Another fundamental discovery made in *Chlamydomonas* that expanded our understanding of how cilia are maintained was that tubulin continuously turns over at flagellar tips (Marshall and Rosenbaum 2001; Song and Dentler 2001; Stephens 1997). This, along with evidence that flagella exhibit a rapid initial growth that slows as they approach steady state (Rosenbaum and Child 1967) and that flagella disassembly occurs at a constant rate independent of length (Kozminski et al. 1995; Marshall et al. 2005; Marshall and Rosenbaum 2001; Parker and Quarmby 2003), forms the basis of the balance point model of flagellar maintenance (Marshall et al. 2005; Marshall and Rosenbaum 2001). In this model, flagella reach their steady-state length when the assembly and disassembly rates are equal. The length dependence of flagellar assembly is based upon the variable quantity of IFT

material entering the flagellum at different lengths (Craft et al. 2015; Engel et al. 2009; Ludington et al. 2013) as well as the variable quantity of IFT material accumulating at basal bodies prior to trafficking into flagella (Ludington et al. 2013). Cilia in different cell types have a characteristic length, which presumably are well suited for their function. A few examples of characteristic ciliary lengths are shown in Table 11.1.

Chemical and genetic screens (Avasthi et al. 2012; Kim et al. 2010, 2016a) along with single gene perturbation have identified many regulators of ciliary length. In addition to IFT motors, proteins, and cargo that are required for proper cilium assembly, proteins involved in tubulin modification (Pugacheva et al. 2007; Sanchez de Diego et al. 2014) and actin dynamics (Abdul-Majeed et al. 2012; Avasthi et al. 2014; Bershteyn et al. 2010; Kim et al. 2010; Oishi et al. 2006; Sharma et al. 2011) also regulate cilium length. A variety of cell surface receptors have also been identified as modulators of cilium length such as dopamine receptors D1, D5, and other G-protein coupled receptors (Abdul-Majeed et al. 2012; Abdul-Majeed and Nauli 2011; Avasthi et al. 2012). Regulators of protein phosphorylation such as GSK3β, MAP kinases (LF4, Dlk-1, DYF-5), cell cycle-related kinases (Nek1, Nek4, Nek8, Nrk2, Nrk17, Nrk30, Cdc42, Cnk2, LF2, LF5, AurA), and phosphatases (cdc14b) also modulate cilia length (Berman et al. 2003; Burghoorn et al. 2007; Coene et al. 2011; Pugacheva et al. 2007; Tam et al. 2013; Tam et al. 2007; Thiel et al. 2011; van der Vaart et al. 2015; White and Quarmby 2008; Wloga et al. 2006; Zuo et al. 2011). In addition to all of these, cilia respond to second messengers such as calcium, cAMP, inositol 1,4,5-trisphosphate, and associated enzymes protein kinase C, adenylate cyclase III, and inositol polyphosphate 5-phosphatase E, respectively (Abdul-Majeed et al. 2012; Besschetnova et al. 2010; Hatayama et al. 2011; Luo et al. 2012; Ou et al. 2009). Clearly, many different genes are involved in the response of cilia to external signals as well as in coordination with the cell cycle. Additional genes regulating cilium structure and length have been previously reviewed (Avasthi and Marshall 2012; Broekhuis et al. 2013; Keeling et al. 2016), and much is still unknown about how these pathways work together or, indeed, which of them are utilized concurrently. Along with axoneme structure, ciliary membrane must also be regulated for proper assembly, maintenance, and protein composition (Bloodgood 2012). Ciliary formation requires trafficking of Golgi-derived vesicles regulated by small GTPases including Rab8, Rab11, Arf4, and Arl3 (Kim et al. 2014; Knodler et al. 2010; Mazelova et al. 2009; Schwarz et al. 2012; Westlake et al. 2011) as well as membrane regulating proteins EHD1/3 (Lu et al. 2015). In mammalian photoreceptors and *Chlamydomonas* flagella, ciliary membrane is continuously renewed (Dentler 2013; Young 1971; Young and Bok 1969) and membrane/proteins can be shed via ectosome release (Avasthi and Marshall 2013; Hogan et al. 2009; Wang et al. 2014; Wood et al. 2013; Wood and Rosenbaum 2015). While these studies highlight mechanisms of membrane regulation, how ciliary membrane and axoneme dynamics are coordinated remains largely a mystery.

Within the kidney, cilium length increases during development, being shortest in renal vesicles and longest in mature fetal nephron segments (Saraga-Babic et al. 2012). Renal ciliary length is also modulated in response to various endogenous and extracellular factors, including hormones and changes in urinary shear stress, which are thought to alter the sensitivity of the ciliated cell to external cues (Besschetnova et al. 2010; Upadhyay et al.

2014). Alterations in length of primary cilia are also observed in response to renal injury and during regeneration/repair processes (Verghese et al. 2008, 2009; Han et al. 2016).

11.2   Ciliopathies: Linking Ciliary Dysfunction to Renal Cystogenesis

The discovery that the Oak Ridge polycystic kidney disease (*orpk*) mouse model resulted from a defect in *Tg737/IFT88* marked a major breakthrough linking dysfunction of primary cilia to human disease (Pazour et al. 2000). Soon afterward, an explosion of discoveries followed demonstrating that mysterious disorders affecting multiple organ systems including the retina, kidney, skeletal system, reproductive systems, and others could be traced to defects in a single ciliary gene. These syndromic diseases, termed ciliopathies, include Meckel–Gruber Syndrome (MKS), Bardet–Biedl Syndrome (BBS), Joubert Syndrome, Jeune Syndrome, Nephronophthisis (NPHP), and others (Table 11.2). These pediatric diseases range in severity, causing death in infancy of individuals with MKS or during late teenage years in some individuals with NPHP, and have overlapping but varying degrees of clinical manifestations, including kidney cysts, intellectual disabilities, craniofacial and skeletal defects, polydactyly, retinal degeneration, hypogonadism, and obesity. Many of these diseases, such as MKS and NPHP, result from disruption of proteins at the transition zone, a proposed hotspot for disease gene networks (Chih et al. 2012; Garcia-Gonzalo et al. 2011; Williams et al. 2011). Disruption of these proteins alters cilia structure, resulting in cilia that are abnormally short, absent, or, less commonly, elongated (Williams et al. 2011). BBS is caused by mutations in one of the 21 genes (Bujakowska et al. 2015; Heon et al. 2016; Lindstrand et al. 2014; Schaefer et al. 2016; reviewed in Khan et al. 2016), most of which encode proteins comprising or facilitating formation or trafficking of the BBSome complex, which has been demonstrated to be a cargo of IFT in *Chlamydomonas* flagella (Lechtreck et al. 2009) and to transport signaling molecules to membrane compartments and within the ciliary membrane (Guo et al. 2016; Guo and Rahmouni 2011). The BBS core is an 8-subunit complex composed of BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9, and BBS18/BBIP1 (Nachury et al. 2007; Scheidecker et al. 2014). Additionally, BBS3/ARL6 and BBS17/LZTFL1 regulate ciliary trafficking of the BBSome (Liew et al. 2014; Marion et al. 2012; Seo et al. 2011), while BBS6, BBS10, and BBS12 form a BBS-chaperonin complex together with BBS7 to promote BBSome assembly (Seo et al. 2010; Zhang et al. 2012). Causative mutations in *IFT* genes have also been identified in BBS patients. IFT27/BBS19 (Aldahmesh et al. 2014) facilitates ciliary exit of the BBSome (Eguether et al. 2014; Liew et al. 2014), and while IFT172/BBS20 (Bujakowska et al. 2015; Schaefer et al. 2016) has been shown to regulate IFT particle turnaround at the ciliary distal tip in *Chlamydomonas* (Pedersen et al. 2005), its connection to the BBSome remains to be explored. While disruption of some BBS proteins impairs ciliogenesis (Marion et al. 2009), mutations of other BBS proteins do not overtly affect cilia structure, but impede ciliary entry or exit of signaling molecules (Berbari et al. 2008; Zhang et al. 2011).

Of the many clinical features of ciliopathies, a major hallmark is renal cystic disease (Quinlan et al. 2008). Renal cystic diseases are mostly inherited and can be classified as Polycystic Kidney Disease (PKD) or as non-PKD, depending on the mutated gene. While the non-PKD diseases comprise the ciliopathies mentioned above, PKD results from mutations in *Polycystic Kidney Disease 1* (*PKD1*), *PKD2, or Polycystic Kidney and Hepatic*

Disease 1 (*PKHD1*) genes. Mutations of *PKD1* or *PKD2* cause approximately 90% of cases of Autosomal Dominant Polycystic Kidney Disease (ADPKD), the most common fatal genetic disease, affecting 1:400–1:1000 adults worldwide. Renal cysts can initiate in the fetus and progressively grow, compressing and compromising surrounding parenchyma and causing end-stage renal disease in the 6th decade of life. Mutations in *PKHD1* cause Autosomal Recessive PKD, which affects 1:20,000 children. The *PKD1*, *PKD2*, and *PKHD1* gene products, polycystin 1 (PC1), PC2, and fibrocystin/polyductin (FPC), localize to various subcellular compartments, including the primary cilium (Barr and Sternberg 1999; Yoder et al. 2002). Ciliary localization of polycystin-GFP fusion proteins in *C. elegans* provided the first demonstration of a link between renal cystic disease and primary cilia (Barr and Sternberg 1999). The ciliary localization of mammalian *PKD* gene products together with evidence that ciliary defects cause renal cysts led to the proposal that ciliary dysfunction may present a unifying etiological mechanism for renal cystic diseases. Recently, mutations in *Glucosidase Alpha; Neutral AB* (*GANAB*), which catalyzes a step in the *N*-glycosylation pathway in the endoplasmic reticulum (ER), have been reported in ADPKD patients, reflecting a critical role for the ER protein maturation process for functional PC1 and its localization to the primary cilium (Porath et al. 2016), discussed in more detail in Sect. 3.

Multiple cellular processes are disrupted in renal cystogenesis that result in cell dedifferentiation, increased cell proliferation, increased fluid secretion of renal epithelial cells, and increased tubular cell apoptosis (reviewed in Calvet and Grantham 2001; Torres and Harris 2006; Zhou 2009). Studies using ADPKD primary renal cells revealed that the combination of high intracellular cAMP levels and low intracellular calcium (Ca^{2+}) drives cell proliferation and fluid secretion of cyst-lining epithelial cells (Calvet 2008; Wallace 2011). In human ADPKD cells, low homeostatic intracellular Ca^{2+} enables aberrant cAMP-mediated activation of Protein Kinase A (PKA) and ERK, which, in turn, activates both cystic fibrosis transmembrane conductance regulator (CFTR) and ERK signaling pathways, causing fluid secretion by epithelial cells into the cyst lumen and increased proliferation of cyst-lining epithelial cells, respectively (Yamaguchi et al. 2004). Addition of a Ca^{2+} ionophore rescued these aberrant responses, and conversely, addition of a Ca^{2+} channel blocker to normal human kidney (NHK) cells caused ADPKD cellular responses, demonstrating the pivotal role of intracellular Ca^{2+} in influencing cellular phenotype (Yamaguchi et al. 2006). Elevated intracellular cAMP is common to cystic kidneys of both orthologous *Pkd* and ciliary mouse models: the former including *Pkd1* (Hopp et al. 2012) and *Pkd2* (Torres et al. 2004) mutant mice and the *Pkhd1*-mutant Polycystic Kidney (PCK) rat (Wang et al. 2008), while the latter group including *pcy* (Yamaguchi et al. 1997), *cpk* (Tao et al. 2015), *jck* (Smith et al. 2006), *Kif3a* (Choi et al. 2011), and *Thm1/Ttc21b* (Tran et al. 2014b). Such observations suggest that increased cAMP may be a universal and fundamental mechanism originating from ciliary dysfunction. Another unifying phenomenon is that loss of PKD or ciliary proteins during kidney development leads to severe, rapidly progressing renal cystic disease, while loss of these proteins after kidney maturation (P12–P14) causes a mild, slowly progressing disease, demonstrating that the developmental state of the kidney strongly influences renal cystogenesis (Piontek et al. 2007). A role for the primary cilium and PC1 in sensing tubular injury and in regulating the

repair–regeneration–redifferentiation response has been proposed, and dysfunction of this adaptive mechanism in mutant kidneys may provide a trigger for cyst formation in the adult kidney (Weimbs 2007).

Yet, differences are evident between PKD and non-PKD renal cystic diseases. In PKD, growth of renal cysts greatly enlarges kidney size, while in non-PKD diseases, such as NPHP, kidney size tends to decrease due to abundant fibrosis. Another contrast between PKD and non-PKD diseases is that while most non-PKD proteins have a fundamental role in ciliogenesis, PC1, PC2, and FPC do not. This is also reflected by the majority of studies that do not report altered ciliary length in *Pkd1/Pkd2* mutants, with the exception of two. In renal epithelia of the PC1$^{RC/RC}$ mouse model, which carries an ADPKD mutation (Hopp et al. 2012) and in cultured *Pkd1*−/− and *Pkd2*−/− derived mouse renal epithelial cells (Jin et al. 2014b), cilia were lengthened. This may relate to the role of PC1/PC2 in modulating intracellular cAMP and Ca^{2+}, since pharmacological manipulation of intracellular cAMP and Ca^{2+} in mouse inner medullary collecting duct (IMCD) cells showed that low Ca^{2+} lengthens cilia, while high Ca^{2+} shortens cilia (Besschetnova et al. 2010).

Although loss of proteins required for ciliary structure usually causes NPHP in the human population, IFT mouse models and the *Nek8* mutant, *jck*, manifest PKD-like disease with enlarged kidneys. Moreover, heterozygosity for both *Pkd1* and the *Nek8$^{jck/+}$* mutation, which in the homozygous form causes longer cilia and an ADPKD phenotype (Smith et al. 2006), caused renal cysts in mice, while single heterozygosity did not, suggesting that molecular mechanisms stemming from deficiency of PC1 and *jck* mutant NEK8 protein may converge (Natoli et al. 2008). Intriguingly, despite that ablation of *Kif3a*, and in turn primary cilia, results in renal cysts, combined loss of primary cilia and PC1/2 ameliorated PC-mediated renal cystogenesis and decreased renal epithelial cell proliferation in mice, suggesting the presence of an as yet unidentified ciliary-mediated signaling pathway that is crucial for PC1/2 cyst development (Ma et al. 2013). Corroborating and extending these data, reduced ciliary length through inhibition of Cyclin Dependent Kinase 5 (CKD5) also ameliorated the renal cystic disease in *jck* mice (Husson et al. 2016). These data underscore the need to define the role of ciliary proteins individually and in PKD to understand mechanisms that exacerbate or attenuate PKD. Disease variability observed among ADPKD patients may be due to mutations/polymorphisms in genes affecting ciliary function.

11.3   Ciliary Localization of Polycystins Is Interdependent and Important for Their Function

Analysis of several ADPKD missense mutations in either *PKD1* or *PKD2* showed that they resulted in the absence of polycystins in cilia (Cai et al. 2014). One of these mutations, PC2^{W414G}, present in the first extracellular loop, retained channel activity, suggesting that functional PC2 in the endoplasmic reticulum (ER) is insufficient to prevent cystic disease and that ciliary localization of polycystins is essential for this function. Thus, understanding ciliary-targeting mechanisms of polycystins is important for understanding PKD biology and finding therapeutic targets.

Several ciliary targeting sites have been uncovered in the PC1 C-terminal cystosolic tail, including a KVHPSST sequence (Ward et al. 2011) and sites within and upstream of the coiled-coiled domain required for interaction with PC2 (Su et al. 2015). An RVxP ciliary targeting sequence in the PC2 N-terminus has also been identified (Geng et al. 2006). Accumulating evidence suggests that ciliary localization of PC1 and PC2 is interdependent (Freedman et al. 2013; Gainullin et al. 2015; Kim et al. 2014; Nauli et al. 2003; Su et al. 2015). In ADPKD-derived, *PKD1*-mutant induced pluripotent stem cells and in *Pkd1*–/– MEFs, PC2 ciliary localization was reduced or undetectable, respectively, while in *Pkd2*–/– MEFs, ciliary PC1 was lacking (Freedman et al. 2013; Gainullin et al. 2015; Kim et al. 2013). Conversely, overexpression of PC2 enhanced both WT and mutant PC1 ciliary targeting, while increasing levels of WT PC1, but not of a PC1 construct lacking its C-terminus, promoted PC2 ciliary localization in mouse IMCD3 cells (Freedman et al. 2013; Su et al. 2015), consistent with a requirement for the PC1 C-terminus in ciliary localization.

To reach the primary cilium, PC1 protein must undergo maturation, which has also been shown to depend on PC2 (Gainullin et al. 2015; Kim et al. 2014). PC1 maturation involves N-linked core glycan addition and cleavage at its G-protein coupled proteolysis site (GPS), which generates extracellular N-terminal (NTF) and membrane-embedded C-terminal (CTF) fragments that remain non-covalently associated, and occurs within the ER prior to subsequent trafficking and N-glycan modification in the Golgi (Wei et al. 2007). *PKD1* missense mutations that interfere with GPS cleavage prevent PC1 maturation and ciliary localization (Cai et al. 2014; Chapin et al. 2010; Kurbegovic et al. 2014). PC1–PC2 association occurs before GPS cleavage of PC1 (Gainullin et al. 2015) and is required for the PC complex to reach the trans-Golgi (Kim et al. 2014). In *Pkd2*–/– mouse embryonic fibroblasts (MEF), cleaved PC1 remained endoH sensitive, suggesting that PC1 must complex with PC2 to traffic through the Golgi. Further, cleavage of PC1 is essential for transport through the Golgi, since a non-cleavable PC1 mutant co-precipitated with PC2 remained endoH sensitive (Kim et al. 2014). *Pkd1^{RC/RC}* hypomorphic mutant mice carrying various *Pkd2*-mutant alleles of differing deficiency revealed that renal cystic disease severity was determined by levels of mature PC1, which in turn correlated with PC2 levels, demonstrating the dependence of PC1 maturation on PC2 in vivo (Gainullin et al. 2015).

11.3.1 Polycystin Transport from the Golgi Apparatus to the Cilium

Golgi-derived vesicles convey proteins to the base of the primary cilium, a process termed polarized vesicle trafficking (reviewed by (Hsiao et al. 2012). This transport is essential for ciliogenesis and is mediated by the Rab and Arf families of small GTPases, which recruit vesicle coating complexes during vesicle budding, docking, and fusion. At the centrosome, Rab11 is essential in vesicular targeting of Rabin8 to recruit and activate Rab8 for ciliary targeting of cargo proteins (Knodler et al. 2010). Two 8-subunit protein complexes, the exocyst and the BBSome, are required for post-Golgi vesicular membrane trafficking and for ciliary entry and exit of signaling proteins, respectively. Within cilia, trafficking of the BBSome and its cargo is mediated by IFT.

Evidence suggests that multi-protein complexes involving the Rab and Arf GTPases, the exocyst, and the BBSome mediate post-Golgi intracellular and ciliary trafficking of

polycystins (Fig. 11.2). In photoreceptors, a protein complex consisting of Arf4 and the ArfGAP with SH3 domain, ankyrin repeat, and PH domain 1 GTPase-activating protein (ASAP1) traffics membrane proteins from the Golgi to the base of the modified cilium (Mazelova et al. 2009). Hypothesizing a role for such a complex in trafficking polycystins, Ward et al. (2011) found that via its KVHPSST ciliary targeting sequence, PC1 interacts with Arf4 and ASAP1 in a complex with Rab6 and Rab11 in the Golgi membranes of primary renal cortical epithelial (RCTE) cells (Ward et al. 2011). The RVxP ciliary targeting sequence of PC2 also allowed binding of PC2 to Arf4, which is localized in the trans-Golgi, suggesting that PC2 also passes through the Golgi network. In another study, a yeast two-hybrid screen identified RAB GTPase Binding Effector Protein 1 (Rabep1) to interact with PC1 in a region containing the coiled-coiled domain within the C-terminal tail (Kim et al. 2014). This interaction was confirmed in primary collecting duct (CD) cells of mouse postnatal kidneys and also occurred in *Pkd2*–/–- MEF. Since this study showed that PC1 exits the ER in a complex with PC2, the presence of PC1-Rabep1 complex in *Pkd2*–/– MEF indicates this interaction occurs in a pre-Golgi compartment. Subsequently, Rabep1 was found to bind to Golgi-localized, gamma adaptin ear-containing, ARF-binding protein 1 (GGA1) in CD cells, an interaction that had been shown previously in trans-Golgi vesicles (Mattera et al. 2003). GGA1 was subsequently found to bind to Arf-like 3 (Arl3), which facilitates ciliary localization of cargo proteins (Wright et al. 2011). Taken together, binding of Rabep1 to PC1 in a pre-Golgi compartment links the PC1/PC2 complex to GGA1 at the trans-Golgi, where GGA1 then interacts with Arl3. In contrast to findings of Ward et al. (2011), PC1 did not co-immunoprecipitate with Arf4 in CD cells (Kim et al. 2014), which may point to differences between cell types and requires further investigation.

In contrast to these two studies, glycosylation analysis of PC2 in whole cell lysates of porcine renal proximal tubular epithelial LLC-PK$_1$ cells, together with live-imaging and electron microscopy, suggested that PC2 destined for primary cilia is trafficked to the cis-Golgi and, subsequently to the cilium, bypassing the mid- and trans-Golgi membranes (Hoffmeister et al. 2011). Supporting this, ciliary protein fractions of RCTE cells contained endoH-resistant PC1 that co-precipitated with endoH-sensitive PC2 (Gainullin et al. 2015). These data imply that PC2 vesicular trafficking is independent of that of PC1. Thus, two possible routes for trafficking of PC2 to the primary cilium are suggested by these studies.

PC1 also interacted with Rab8 (Ward et al. 2011), which is directed to the ciliary base by Abelson Helper Integration Site 1 (AHI1) and targets cargo to the primary cilium. Expression of a dominant-negative Rab8 prevented ciliary localization of PC1 (Ward et al. 2011) and also of PC2 (Hoffmeister et al. 2011). Rab8 is activated by Rab11, which is stimulated by Class II phosphoinositide 3-kinase-C2α (PI3K-C2α), which localizes to pericentriolar recycling endosomes (PRE) near the ciliary base (Franco et al. 2014). shRNA knockdown of PI3K-C2α in IMCD3 cells shortened cilia and resulted in a marked reduction of cells with PC2 ciliary localization, which was rescued upon expression of a constitutively active Rab8 (Franco et al. 2016). *Pi3k-C2α*+/− mice were sensitized to cyst formation on a *Pkd1*+/− or *Pkd2*+/− genetic background or when subjected to ischemia/reperfusion-induced renal injury, substantiating the importance of PC ciliary localization for PC function in vivo.

Both the exocyst and BBSome appear to mediate ciliary entry of polycystins. Deficiency of Sec10, an exocyst subunit, which localizes to the cilium and controls ciliogenesis (Zuo et al. 2009), resulted in undetectable ciliary localization of PC2 (Fogelgren et al. 2011). Morpholino knockdown of Sec10 in zebrafish phenocopied the PC2 morpholino mutant, resulting in a tail curled upwards, left–right laterality defects, and glomerular abnormalities. Sec10 was also shown to co-precipitate with PC2, IFT88, and IFT52, suggesting that the exocyst together in a complex with IFT proteins mediates ciliary entry of PC2. Using a yeast two-hybrid screen and GST pull-down experiments, BBS1, BBS4, BBS5, and BBS8 were identified as interacting with the PC1 C-terminus, although only loss of BBS1 and expression of a dominant-negative form of BBS3/Arl6 in IMCD cells shortened cilia and prevented PC1 ciliary localization (Su et al. 2014). Since Rab11 interacts with the exocyst and also promotes association of BBS1 with Rabin8 (Knodler et al. 2010), PC-BBSome and PC-exocyst complexes may form at the base of the cilium in connection with Rab11. The BBSome is also implicated in ciliary removal of PC2. In RPE cells deficient for both BBS4 and BBS5, PC2 accumulated in the primary cilium, although overall PC2 protein levels were unaffected, suggesting that BBS4 and BBS5 together mediate removal of PC2 from the primary cilium (Xu et al. 2015). PC2 further undergoes ubiquitination and lysosomal degradation (Hu et al. 2007). Treatment of RPE cells with a lysosomal inhibitor, chloroquine, increased ciliary localization of PC2, similar to the combined deficiency of BBS4 and BBS5 (Xu et al. 2015).

Most of these trafficking studies examined PC1 or PC2 alone. Further examination of whether PC-Rab/Arf, PC-BBSome, or PC-exocyst complexes consist of PC1/PC2 together would help ascertain whether PC1 and PC2 traffic independently or as a complex from the Golgi to the primary cilium. If the polycystins traffic independently, such experiments would further determine at what point of trafficking, e.g., prior to or following ciliary entry, the PC1/PC2 complex is formed.

11.4 Primary Cilium is a Mechanosensory Organelle

After more than 100 years, Zimmermann's hypothesis that primary cilia lining the renal tubules act as flow sensors (Zimmermann 1898) was demonstrated. Fluid flow causes primary cilia of cultured renal epithelial cells to deflect and intracellular Ca^{2+} levels to increase via a mechanism that requires extracellular Ca^{2+} influx and ensuing release of intracellular Ca^{2+} stores (Praetorius and Spring 2001). In cells deciliated by chloral hydrate, Ca^{2+} response was abrogated, demonstrating that primary cilia are essential in flow-mediated elevation of cystosolic Ca^{2+} (Praetorius and Spring 2003). A role for cilia was further made evident by the absence of a luminal flow-induced Ca^{2+} response in microperfused collecting ducts of 2-week-old *orpk* mutant mice, which harbor an *IFT88* hypomorphic mutation causing shortened cilia (Liu et al. 2005b). This abrogated response occurred in tubular segments that were not yet dilated, suggesting defective mechanotransduction might mark tubules sensitized to cystogenesis. In addition, magnitude of the Ca^{2+} response was observed to be twofold higher at P14 than at P7 in wild-type mice, an increase that was not observed in *orpk* mice. This suggests that the cilia-mediated, flow-induced intracellular Ca^{2+} response matures at P14. Interestingly, this time point coincides

with the developmental window that influences renal cystic disease severity (Piontek et al. 2007).

Absence of PC1 or PC2 function in renal cells also obliterated flow-mediated intracellular Ca^{2+} increase (Nauli et al. 2003). This led to the proposal that ciliary PC1 senses flow and activates the Ca^{2+} channel activity of ciliary PC2, causing Ca^{2+} influx into the cilium, which, in turn, results in increased intracellular Ca^{2+}. FPC, TRPV4, and NEK8 also complex with PC2 at the primary cilium, and cells deficient in these proteins have shown impaired mechanotransduction (Kottgen et al. 2008; Manning et al. 2013; Wang et al. 2007). Yet, the variable development of renal tubular cysts among various *Pkhd1* mutant mouse models, with some displaying absence of cysts (Gallagher et al. 2008; Moser et al. 2005) and others presence of cysts (Garcia-Gonzalez et al. 2007; Woollard et al. 2007), the presence of glomerular but not tubular cysts in *Nek8*-null mice, which die at birth (Manning et al. 2012), contrasting with the *Nek8*$^{jck/jck}$ mutant adults, which show a phenotype resembling human ADPKD (Smith et al. 2006), along with the lack of renal cyst formation in *Trpv4*-deficient mice and zebrafish, has questioned the contribution of impaired mechanotransduction to renal cystogenesis (Kottgen et al. 2008). In *Pkhd1*-mutant PCK rats, which model ARPKD and develop renal cysts, TRPV4 mechanosensory function was reduced in cyst-lining epithelial cells and TRPV4 activation attenuated the renal cystic disease (Zaika et al. 2013), demonstrating that impaired fluid flow-induced intracellular Ca^{2+} generation can modulate renal cystic disease, even if it may not be sufficient to initiate renal cystogenesis. Since mutations in *PKHD1* cause ARPKD in humans and rats, but show variable effects in mice, these varying influences of FPC on renal cystogenesis across different species may reflect differential regulation of fluid flow-induced Ca^{2+} responses in mouse, rat, and human kidneys. Likewise, the requirement of TRPV4 in fluid flow-induced Ca^{2+} generation may be cell line or tubule segment specific as illustrated by the lack of involvement of TRPV4 in fluid flow-mediated, PC2- and calcium-dependent induction of *endothelin*-1 expression in IMCD cells (Pandit et al. 2015).

Dysfunctional flow sensing or mechanotransduction may contribute to renal cystogenesis via aberrant calcium-mediated or calcium-independent mechanisms. Collecting duct cells derived from the *orpk* mouse mutant exhibit blunted primary cilia, increased apical calcium entry in response to fluid flow (Siroky et al. 2006) and to epidermal growth factor (EGF) treatment, and an augmented PC2- and TRPV4-dependent proliferative response to EGF (Zhang et al. 2013). PC1 undergoes mechano-regulated cleavages within its C-terminal tail (CTT), generating fragments (CTT, P30, P15) that translocate to the nucleus, interact with various transcription transactivators (CHOP, TCF, STAT3, STAT6), and modulate proliferative, apoptotic, or cytokine-stimulated pathways. Cleavage of the CTT is stimulated by loss of mechanostimulation, as in MDCK cultures under static (no-flow) conditions (Low et al. 2006) or in wild-type mouse kidneys subjected to unilateral obstruction of the ureter, and by loss of mechanosensory function, as in Kif3a-null kidneys which lack primary cilia (Chauvet et al. 2004). PC2 influences cleavage and subsequent stabilization of PC1 CTT via a calcium-independent mechanism. Membrane-localized (non-cleaved) CTT binds and sequesters STAT6 and its coactivator P100 to prevent their nuclear activity (Low et al. 2006), or maintains canonical (JAK-activated, SOCS inhibitable) signaling via STAT1 and STAT3, thought to modulate cytokine-mediated STAT signaling (Talbot et al. 2011, 2014). Cleavage

and nuclear translocation of CTT can be anti-cystogenic, by binding to CHOP, TCF, or β-catenin to prevent interaction with p300, thus inhibiting apoptosis or proliferative Wnt-signaling (Lal et al. 2008; Merrick et al. 2012), or can exacerbate cystic disease via Src-mediated or augmented cytokine-mediated STAT3 activation (Talbot et al. 2011, 2014). Observations of CTT fragments and nuclear staining with antibodies directed to the C-terminus of PC1 in human ADPKD kidneys (Talbot et al. 2011), along with the abilities of leflunomide and pyrimethamine, inhibitors of STAT6 and STAT3, respectively, to ameliorate cystic disease in PKD mouse models (Olsan et al. 2011; Takakura et al. 2011), support the relevance of these CTT-mediated functions in PKD.

For more than a decade, Ca^{2+} influx into the cilium could only be speculated until strategies to visualize Ca^{2+} in the primary cilium were pioneered. Genetically encoded Ca^{2+} indicators were fused to a ciliary targeting sequence of ciliary localized proteins (Delling et al. 2013; Jin et al. 2014a; Su et al. 2013). Jin et al. (2014a) showed that upon mechanical bending of a primary cilium, Ca^{2+} increased in the cilium, which was followed by increased Ca^{2+} in the cytosol, at last providing experimental evidence that primary cilia convert fluid flow shear stress into ciliary Ca^{2+} signals (Fig. 11.3a). Additionally, *Pkd2* siRNA knockdown impeded the increased ciliary Ca^{2+} response, supporting the role of PC2 in mediating flow-induced Ca^{2+} response. This sequence of events has been supported in vivo in the nonmotile cilia of zebrafish Kupffer's vesicle, which is orthologous to the mammalian node. In the node, leftward fluid flow generated by nodal cilia elevates cytosolic calcium in cells on the left side of the node, triggering asymmetric gene expression which is required for left–right asymmetry. In response to leftward fluid flow, Ca^{2+} was shown to increase in nonmotile cilia and, subsequently, in the cytoplasm, supporting that primary cilia transduce mechanical forces into ciliary Ca^{2+} (Yuan et al. 2015). *pkd2* and its putative partner *pkd1-like1* were identified as the molecules required for mechanotransduction in Kupffer's vesicle.

However, by patch clamping the cilium, DeCaen et al. (2013) showed that ciliary ion currents were generated by the gene products of *Pkd1L1* and *Pkd2L1*, while PC1 and PC2 activities were undetectable (DeCaen et al. 2013). In addition, laser disruption of the ciliary tip increased ciliary Ca^{2+}, but caused only small increases in Ca^{2+} at the ciliary base, which did not significantly alter cytosolic Ca^{2+} levels (Delling et al. 2013) (Fig. 11.3b). By developing a transgenic mouse expressing a ratiometric genetically encoded Ca^{2+} indicator in primary cilia and using swept-field confocal microscopy capturing up to 500–1000 frames/s to image cells and tissues derived from this mouse, fluid flow was not observed to induce Ca^{2+} changes in primary cilia of cultured kidney epithelial cells nor in the renal thick ascending limb of loop of Henle, in crown cells of the embryonic node, or in kinocilia of inner ear hair cells (Delling et al. 2016). In incidences when ciliary Ca^{2+} increased, Ca^{2+} was observed first to increase in the cytoplasm and subsequently to diffuse into the cilium. At supraphysiological flow velocities, ciliary tips were observed to rupture, which was followed by extracellular Ca^{2+} entry. Collectively, these results challenge the notions that primary cilia and the polycystins transduce mechanical strain into ciliary Ca^{2+} and, moreover, that ciliary Ca^{2+} regulates intracellular Ca^{2+}.

Mathematical modeling of ciliary deflection and cellular response has provided additional perspectives (Fig. 11.3c). By integrating data from measurements of shear stress-induced

intracellular Ca^{2+} generation in microperfused and split-open rabbit cortical collecting ducts with theoretical modeling, Liu et al. (2003) proposed that activation of increased intracellular Ca^{2+} initiates at the base of the cilium where the cilium transfers mechanical forces to the cytoskeleton (Liu et al. 2003). Consistent with the ciliary base having an important role in mechanotransduction, another mathematical model suggested that ciliary deflection causes strain to build up at the base of the primary cilium and in the surrounding cell membrane (Rydholm et al. 2010). This strain buildup occurred approximately 30 s following induction of fluid flow coinciding with the 30 s time delay observed to generate a flow-induced intracellular Ca^{2+} response in MDCK cells (Praetorius and Spring 2001). In addition to strain accumulating at the ciliary base, results of another computational study showed that deflection of primary cilia causes strain to accumulate also at the nucleus and Golgi apparatus (Khayyeri et al. 2015). Thus, analysis of transcriptional or posttranslational changes upon fluid flow may merit investigation.

Taken together, the data suggest that the intermediary steps between ciliary deflection and increased intracellular Ca^{2+} require further study. Differences in cell lines, imaging techniques, use of cell monolayers versus perfused or split-open tubules, and species may contribute to the discrepancies. The debate on the role of the primary cilium as a mechanotransducer of ciliary Ca^{2+} continues (Tran and Lechtreck 2016). Super-resolution imaging and additional in vivo studies may be important in defining the mechanosensory role of primary cilia.

11.5 Primary Cilium is a Signaling Organelle

Multiple signaling pathways are disrupted in ADPKD (reviewed in Song et al. 2009; Torres and Harris 2006), and accumulating evidence suggests that a number of these are mediated by primary cilia. We discuss Ca^{2+}/cAMP, LKB1/AMPK, and Wnt signaling, which play important roles in PKD. In addition, we review Hedgehog (Hh) signaling, whose regulation at the primary cilium is the most characterized, and thus, ciliary mechanisms learned from this pathway may enable ideas that can be applied to other signaling molecules relevant to PKD.

11.5.1 Ca^{2+}/cAMP

Ca^{2+} levels are fivefold higher in the cilium than in the cytosol demonstrating that the cilium is a separate Ca^{2+} compartment (Delling et al. 2013). Additionally, studies by Choi et al. (2011) indicate that PC2 is integral to the formation of a complex that regulates cAMP and Ca^{2+} at the primary cilium. The PC2 C-terminus interacts with A-kinase anchoring protein 150 (AKAP150), which creates a scaffold for binding of protein kinase A (PKA); adenylyl cyclases 5 and 6 (AC5/6), which synthesize cAMP; and phosphodiesterase 4C (PDE4C), which catabolizes cAMP (Choi et al. 2011). Loss of PC2 in *Pkd2*–/– derived renal epithelial cells resulted in the absence of ciliary AC5/6 and PDE4C and elevated intracellular cAMP levels. Expression of wild-type PC2, but not of a PC2 mutant lacking Ca^{2+} channel activity, decreased cAMP levels, suggesting that the PC2 Ca^{2+} channel activity specifically regulates cAMP levels. Using proximity labeling, Mick et al. (2015) also detected the ciliary presence of PKA and AC5/6 in IMCD3 cells (Mick et al. 2015). Collectively, these data suggest that

cilia are distinct Ca^{2+}/cAMP signaling organelles. The influence of ciliary Ca^{2+} in modulating ADPKD has been demonstrated genetically in mice (Jin et al. 2014b). The voltage-dependent L-type calcium channel, CaV1.2, localizes to primary cilia, but is absent from cilia in *Pkd1*–/– and Pkd2–/– derived renal epithelial cells. Deletion of *CaV1.2* in *Pkd1* heterozygous mice caused the growth of few but extremely large renal cysts, while cysts did not occur in single *Pkd1* heterozygotes.

Although mechanisms linking ciliary Ca^{2+}/cAMP to cytosolic pools require further investigation, targeting cAMP formation in preclinical models of ADPKD has proven extremely effective (Gattone et al. 2003; Torres et al. 2004; Wang et al. 2005, 2008). Intracellular cAMP formation can be attenuated by inhibiting arginine vasopressin receptor 2 (AVPR2), which stimulates AC6, or activating the somatostatin receptor, which inhibits AC6. Administration of Tolvaptan, an inhibitor of AVPR2, together with pasireotide, an activator of somatostatin receptors in the $Pkd1^{RC/RC}$ mouse, resulted in lower renal cystic and fibrotic volumes than treatment with either Tolvaptan or pasireotide alone, demonstrating that targeting both receptors simultaneously is more effective than targeting one alone (Hopp et al. 2015). Knockout of *Pde1a*, *Pde1c*, or *Pde3a*, which catabolize cAMP, in a $Pkd2^{-/WS25}$ mouse model exacerbated renal cystogenesis, suggesting that activating these enzymes may also be beneficial (Ye et al. 2015).

The Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and its Outcomes (TEMPO) 3:4 study was a 3-year phase III clinical trial, in which early-stage ADPKD patients anticipated to have rapid renal cyst growth were randomly assigned to either a Tolvaptan or a placebo treatment group (Torres et al. 2011). ADPKD patients administered Tolvaptan showed reduced kidney volume and reduced symptoms of kidney malfunction, including flank pain, hypertension, and albuminuria, compared to patients administered a placebo (Torres et al. 2012). Tolvaptan has been approved for ADPKD patients in Japan, Canada, and Europe. In the USA, the Food and Drug Administration considered the side effects, such as polyuria, excessive thirst, and risk of liver injury, to outweigh the benefits. However, an open-label extension study (TEMPO 4:4) is ongoing (https://clinicaltrials.gov/ct2/show/record/NCT01214421).

11.5.2 LKB1/AMPK

The mammalian target of rapamycin (mTOR) pathway is aberrantly activated in cyst-lining epithelial cells of human ADPKD tissue (Shillingford et al. 2006), and administration of mTOR inhibitor, rapamycin, has attenuated renal cystogenesis in most PKD rodent models tested (Shillingford et al. 2006; Tao et al. 2005; Wahl et al. 2006; reviewed in Ibraghimov-Beskrovnaya and Natoli 2011). mTOR signaling is essential for cell metabolism and growth and is mediated by two multi-protein complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 phosphorylates and activates p706S kinase (S6K) and inhibits 4E-BP1 to stimulate protein synthesis, enabling cell growth. While nutrient and amino acid availability stimulates mTORC1, energy stress and the "energy sensor" molecule, AMP-activated protein kinase (AMPK) inhibits mTORC1 in a ciliary-dependent manner (Boehlke et al. 2010).

Upon fluid flow, the ciliary-localized liver kinase B1 (LKB1) tumor suppressor kinase phosphorylates AMPK, which accumulates at the ciliary base and suppresses mTORC1 and, in turn, cell growth (Boehlke et al. 2010). *Kif3a* cko mice were shown to have larger collecting duct cells, demonstrating the ciliary role in regulating mTOR signaling and cell growth. Further, MDCK cells deficient for Kif3a or Ift88 showed larger cell size only when subjected to fluid flow, demonstrating the importance of fluid shear stress as a stimulus. In LKB1-deficient cells, fluid flow also resulted in larger cells, and while P-AMPK levels of whole cell lysates were not altered, P-AMPK specifically at the basal body was reduced. *Pkd2* kd MDCK cells subjected to fluid flow did not show larger cell size nor elevated levels of P-S6K, suggesting that fluid flow stimulation of LKB1/AMPK signaling is independent of flow-mediated intracellular Ca^{2+} generation.

In addition to flow-mediated suppression of mTOR signaling, other regulators of mTOR signaling have been identified. Lack of ubiquitination of the hepatocyte growth factor (HGF) receptor, c-Met, also activates mTOR signaling in a PKD mouse model, and administration of a c-Met inhibitor attenuated renal cystogenesis in *Pkd1*-null mutant embryos (Qin et al. 2010). Additionally, treatment of MDCK cells with metformin, which activates AMPK, suppressed mTOR signaling and CFTR activity, and administration of metformin to *Pkd1* mouse mutants either prior to or during renal cyst formation attenuated renal cystic disease (Takiar et al. 2011).

Despite the success of mTor inhibitors in attenuating cystic disease in most preclinical models of PKD, clinical trials of sirolimus (rapamycin) and its derivative, everolimus, in ADPKD patients have not shown the same efficacy (Ruggenenti et al. 2016; Serra et al. 2010; Walz et al. 2010). In an 18-month study, no difference in total kidney volume was observed in patients treated with sirolimus compared to patients receiving standard care (Serra et al. 2010). In a 24-month study, ADPKD patients receiving everolimus showed a lower total kidney volume increase, but also a lower parenchymal volume increase, and kidney function was not better than those of patients receiving a placebo (Walz et al. 2010). In another study, ADPKD patients at stages 3b and 4 of chronic kidney disease received sirolimus for 1 year but showed greater decline in glomerular filtration rate (GFR), increased proteinuria, and higher total kidney volume than patients receiving conventional care (Ruggenenti et al. 2016). The study concluded that sirolimus was unsafe and ineffective in ADPKD patients. Another study is ongoing to determine if pulsed administration of sirolimus will be more effective (https://clinicaltrials.gov/ct2/show/NCT02055079). Rapamycin acts mostly on mTORC1, and feedback mechanisms may result in elevated mTOR signaling via mTORC2. A new mTOR inhibitor that inhibits both complexes has shown efficacy in a rat model of PKD (Ravichandran et al. 2015). Alternatively, targeting the pathway upstream of mTORC1/2 at the level of AMPK or cMet might also be more effective. Additionally, administration of lower doses of mTOR inhibitors as part of a combination therapy may also yield more beneficial results. Finally, targeting drug delivery to the kidney may be most effective at reducing toxicity and increasing therapeutic efficacy (Shillingford et al. 2012). Folate receptors are expressed at high levels in the kidney, and thus, folate receptor-mediated endocytosis may be exploited to target compounds to the kidney. Indeed, administration of folate-conjugated rapamycin (FC-rapamycin) to adult mice reduced mTOR signaling in the kidney and not in the spleen. FC-rapamycin also attenuated

PKD in *bpk* (*BALB/c polycystic kidneys*) mice, mutant for *Bicaudal C Family RNA-binding protein 1* (*Bicc1*) (Cogswell et al. 2003), which regulates *Pkd2* via microRNA miR-17 (Tran et al. 2010).

11.5.3 Wnt

Wnt signaling is activated upon binding of Wnt ligand to a Frizzled (Fz) receptor. Downstream of this, Wnt signaling partitions into canonical and noncanonical pathways, determined by the presence of co-receptors. While canonical Wnt signaling regulates cell proliferation, differentiation, and cell fate, noncanonical signaling directs tissue organization and morphogenesis and causes transient increases in intracellular Ca^{2+}. For the purpose of this review, we will focus predominantly on the canonical pathway. For a review on noncanonical signaling and ADPKD, see Tran et al. (2014a).

In the canonical pathway, in the absence of ligand, a destruction complex comprised of scaffolding protein, Axin2, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β) phosphorylates and targets β-catenin for degradation. In the presence of ligand, binding of Wnt to Fz recruits the low-density lipoprotein (LDL)-related protein 6 (LRP6) co-receptor, cytoplasmic Dishevelled (Dvl), Axin2, and GSK3β concomitantly disassembling the destruction complex. Stabilized β-catenin translocates to the nucleus and dimerizes with the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to activate target genes, such as *Lef1, Axin2,* and *c-myc,* promoting cell proliferation (reviewed in Oh and Katsanis 2013).

Two studies show that fluid flow can regulate Wnt signaling. Fluid flow across IMCD cells increased the expression of *inversin*, which is mutated in NPHP2, and downregulated β-catenin (Simons et al. 2005). While experiments in *Xenopus laevis* embryos and mammalian renal epithelial cells suggested that *inversin* antagonizes canonical Wnt signaling while promoting the noncanonical Wnt/Planar Cell Polarity pathway (Simons et al. 2005), kidneys of *inversin*-null mouse mutants showed normal regulation of canonical Wnt signaling (Sugiyama et al. 2011), suggesting regulation of the balance between mammalian canonical and noncanonical Wnt branches may be more intricate in vivo. Fluid flow in the mouse embryonic node has also been shown to regulate Wnt signaling via feedback loops with *Cerl2* (Nakamura et al. 2012). An early event in establishing left–right asymmetry is the decay of *Cerl2* mRNA on the left side of crown cells in the mouse node induced by fluid flow. Wnt3 promotes *Wnt3* expression and further *Cerl2* mRNA degradation, and conversely, Cerl2 promotes Wnt3 degradation. These data suggest that flow-mediated decay of *Cerl2* enables asymmetric Wnt signaling around the node. This contrasts with the effect of fluid flow over IMCD cells, which leads to downregulated β-catenin.

Pathway components, such as β-catenin, APC, and Dishevelled 3, localize to the ciliary axoneme, while P-β-catenin, which is targeted for degradation, is present at the basal body (Corbit et al. 2008; Mick et al. 2015). In *Kif3a*–/– embryos and in Wnt3a ligand-stimulated cells derived from *Kif3a*–/–, *IFT88^{orpk/orpk}*, and *Ofd1*–/– mice harboring the Batgal Wnt reporter, reporter activity increased, suggesting that cilia act as a "brake" in canonical Wnt signal transduction (Corbit et al. 2008). In vitro studies suggest that Jouberin (Jbn), encoded by *AHI1*, which is mutated in Joubert Syndrome, facilitates nuclear translocation of β-

catenin and that the primary cilium acts to sequester Jouberin and β-catenin away from the nucleus (Lancaster et al. 2011).

Yet, the functionality of primary cilia in mediating Wnt signaling has been unclear. IFT mutant embryos show Hedgehog (Hh) mutant phenotypes and not phenotypes characteristic of misregulated Wnt signaling (reviewed in Eggenschwiler and Anderson 2007). Additionally, although *Kif3a*–/–; *Batgal* and *Ift88*–/–;*Batgal* embryos showed increased Wnt reporter activity (Corbit et al. 2008), *Thm1^{aln/aln}*; *Batgal* and *Ahi*–/–;*Batgal* embryos, which have shortened and absent cilia, respectively, showed normal reporter activity (Lancaster et al. 2011; Stottmann et al. 2009). Interestingly, *Dnchc2*–/–;*Batgal* embryos showed decreased reporter activity in the kidney but increased activity in the midbrain (Lancaster et al. 2011). This varying effect of *Dnchc2* deficiency was demonstrated in vitro. Moderate inhibition of *Dnchc2*, which did not affect cilia structure, dampened Wnt response, while greater inhibition of *Dnchc2*, which shortened cilia, increased Wnt response. Thus, ciliary regulation of Wnt signaling is context dependent; some organs, such as the brain, are more sensitive to dosage of a particular ciliary gene. These data also suggest that deficiency of different ciliary genes differentially influences Wnt signaling output.

The correct balance of Wnt signaling is critical for maintaining renal tubular integrity, and both inappropriate activation and inhibition of the pathway can lead to renal cysts. Renal-specific overexpression of active β-catenin or loss of APC, which negatively regulates β-catenin, caused renal cysts in mice (Qian et al. 2005; Saadi-Kheddouci et al. 2001). Additionally, enhancing canonical Wnt signaling by ablating *Aquaporin 1* (*Aqp1*) in a mouse model of PKD exacerbated renal cystogenesis (Wang et al. 2015). AQP1 interacts with β-catenin, GSK3β, LRP6, and Axin1, and deficiency of AQP1 increased the levels of stabilized β-catenin. Finally, in an *Mks1* knockout mouse model of Meckel Syndrome with shortened cilia, canonical Wnt signaling was upregulated prior to cysts forming in embryonic mutant kidneys (Wheway et al. 2013). Conversely, in *Ahi*–/– mice, which model NPHP, canonical Wnt signaling was reduced at 5 months of age, and renal microcysts and tubular dilations ensued at 12 months of age (Lancaster et al. 2009).

However, misregulated Wnt signaling does not precede cystogenesis in all mouse models of cystic kidney disease. In mice with renal-specific deletion of *Kif3a, Ift20, Ift140,* or *Tmem67*, upregulation of canonical Wnt signaling was detected after renal cysts were formed, suggesting that other mechanisms underlie initiation of renal cystogenesis (Jonassen et al. 2008, 2012; Leightner et al. 2013; Lin et al. 2003). A direct role for loss of polycystins on Wnt signaling in vivo is also controversial. *Pkd1*–/–; *TCF-lacZ* embryonic kidneys and *Pkd2/WS25; TCF-lacZ* adult kidneys showed normal reporter activity in cyst-lining epithelia and normal levels of β-catenin in whole kidney lysates, even after cysts had formed (Miller et al. 2011). In direct contrast, canonical Wnt signaling was upregulated in cystlining epithelial cells of E17.5 *Pkd1*–/–; *TCF-lacZ* kidneys, and levels of total and active β-catenin of whole kidney lysates were also elevated (Qin et al. 2012). Varying expression levels and patterns by different Wnt reporter lines and the absence of Wnt reporter activity in sites of known canonical Wnt signaling have been reported. Different background strains may also possibly account for the discrepancies observed in reporter activity as well as β-catenin levels.

Recently, PC1 has been shown to bind various Wnt ligands. Binding of WNT9B resulted in increased intracellular Ca^{2+} via Ca^{2+} influx, and WNT9B and WNT3A induced whole-cell currents in WT MEFs but not in *Pkd2*–/– MEFs (Kim et al. 2016b). Combined morpholino knockdowns of dvl2 and pkd1 or of wnt9a and pkd1 in *Xenopus* showed synergistic effects on inducing a pronephric cystic phenotype, indicating these molecules function in the same pathway. These data are the first to identify ligands of PC1 and indicate that misregulated Wnt/Ca^{2+} signaling is causative in ADPKD.

11.5.4 Hedgehog

The first signaling pathway discovered to occur at the primary cilium was the mammalian Hh pathway. The first major discovery was made when ethylnitrosourea (ENU)-mutagenized mouse embryos with Hh signaling phenotypes, such as neural tube patterning defects and polydactyly, were found to have mutations in *Ift* genes (Huangfu et al. 2003). Hh signaling is essential for patterning and development of most vertebrate organs and is also critical for tissue homeostasis (reviewed in Ingham et al. 2011). Hh ligands initiate signaling by binding the transmembrane receptor, Patched (PTCH1), at the cilium. Once bound, PTCH1 diminishes from the cilium, and concomitantly, the transmembrane signal transducer, Smoothened (SMO), is enriched in the cilium and activated (Corbit et al. 2005; Rohatgi et al. 2007). A series of dephosphorylation and phosphorylation events activate full-length Glioblastoma transcription factors, GLI2 and GLI3 (Niewiadomski et al. 2014), which accumulate at the ciliary distal tip (Haycraft et al. 2005). In the absence of ligand, full-length GLI3 protein is processed into GLI3 repressor, which also requires the primary cilium. GLI transcription factors translocate to the nucleus, and the balance of GLI activators (GLIA) to GLI3 repressor determines the level of Hh signaling output (Christensen and Ott 2007; Eggenschwiler and Anderson 2007).

IFT, the BBSome, ciliary Ca^{2+}, and the phospholipid content of the ciliary membrane tune this developmental pathway by ultimately regulating ciliary localization of Hh signaling components. In mice, loss of all IFT proteins misregulates Hh signaling (Nozawa et al. 2013). Loss of most complex B proteins, which mediate anterograde IFT, causes the absence of cilia and inability to transduce the Hh signal (Huangfu and Anderson 2005; Huangfu et al. 2003; Liu et al. 2005a). In contrast, loss of IFT complex A proteins, THM1 and IFT122, which largely mediate retrograde IFT, sequesters proteins in bulb-like structures at the distal tip of shortened cilia and causes inappropriate activation of the Hh pathway due to enhanced GLI2A and GLI3A activities (Qin et al. 2011; Tran et al. 2008). In addition to cilia compartmentalizing the Hh signaling cascade, IFT is integral to Hh transduction by trafficking signaling components within cilia. Unlike most complex B proteins, IFT25 and IFT27 are not essential for ciliogenesis but form a subcomplex that is critical for ciliary import of GLI2 and ciliary export of the BBSome and its cargoes, PTCH1, SMO, and Gpr161, a negative regulator of the pathway (Eguether et al. 2014; Keady et al. 2012; Liew et al. 2014). As a result, loss of IFT25 or IFT27 does not alter cilia structure but disrupts activation of the pathway. Ca^{2+} modulates IFT (Collingridge et al. 2013), and treatment of MEF with Smoothened Agonist (SAG) resulted in elevated ciliary Ca^{2+} after 24 h (Delling et al. 2013). Delling et al. (2013) propose that rather than SAG directly activating Ca^{2+} channels, SAG recruits PC2-L1 to primary cilia to "tune" Ca^{2+} levels for proper function of

IFT proteins, such as IFT25, which has a Ca^{2+} binding site. Delling et al. (2013) further propose that other Hh signaling molecules be examined for Ca^{2+} dependence. More recently, the phospholipid content of the ciliary membrane has also been shown to be a critical regulator of the pathway. Phophoinositide phosphatase, *INPP5E*, in which mutations cause ciliopathies (Jacoby et al. 2009), creates a distinct phosphoinositide distribution in the ciliary membrane, whereby phosphoinositide 4-phosphate (PI(4)P) is present in the membrane that ensheathes the axoneme and phosphoinositide 4,5-bisphosphate (PI(4,5) P2) comprises the membrane at the ciliary base (Chavez et al. 2015; Garcia-Gonzalo et al. 2015). Loss of INPP5E caused mislocalization of PI(4,5)P2 to the ciliary membrane enclosing the axoneme, which increased ciliary localization of Hh negative modulators, PI(4,5)P2-binding Tulp3, Gpr161, and IFT140, resulting in inactivation of the pathway.

Like the Wnt pathway, regulation of Hh signaling has been studied mostly in cells that do not undergo shear stress. However, Hh signaling also has a role in chondrocytes, which do undergo mechanical strain. In embryonic chondrocytes, Hh signaling is essential for proliferation and differentiation in the growth plate, while in adult chondrocytes, aberrant activation of Hh signaling leads to degeneration of chondrocytes in osteoarthritis. Chondrocytes subjected to cyclic tensile strain (CTS) showed increased *Ihh* transcription and increased Hh activity as assessed by levels of *Gli1* transcripts (Thompson et al. 2014). In *Ift88^orpk/orpk* cells subjected to CTS, *Ihh* transcription increased but *Gli1* transcription did not, demonstrating that the primary cilium is not required for *Ihh* transcription but for transduction of the pathway. Thus, in chondrocytes, Hh signaling is regulated in a mechanosensitive manner (Shao et al. 2012; Thompson et al. 2014).

Evidence that Hh signaling is increased in renal cystogenesis is emerging. In a genome-wide transcriptome analysis of human ADPKD renal cystic tissue, expression of Hh signaling components, including *Gli2*, was upregulated (Song et al. 2009). Additionally, several mouse models of renal cystic disease show increased Hh signaling. Mutation of the *Glis2* transcription factor, a member of the Kruppel-like C2H2 zinc finger protein subfamily, including the GLI proteins, causes NPHP in humans and mice, and transcriptome analysis of *Glis2*−/− kidneys revealed an upregulation of *Gli1*, a direct target of the Hh pathway (Attanasio et al. 2007). In vitro, Glis2 repressed the Hh pathway and *Glis2* knockdown resulted in transformation of renal epithelial cells to a fibroblast appearance, suggesting that reduced levels of Hh signaling may maintain renal tubular epithelial cells in a differentiated state (Li et al. 2011). Conditional deletion of IFT140, a complex A protein, resulted in cystic kidney disease and increased expression of *Gli* transcripts in cystic kidneys, suggesting increased Hh signaling (Jonassen et al. 2012). Similarly, perinatal deletion of *Thm1*, which also encodes a complex A protein (Tran et al. 2008) and is mutated in patients with ciliopathies (Davis et al. 2011), also caused cystic kidney disease in mice and increased expression of *Gli* transcripts in cystic kidneys (Tran et al. 2014b). Further, *Gli* transcripts were also increased in *jck* and *Pkd1* cko cystic kidneys.

In a study examining the effects of corticosteroid overexposure on kidney development, addition of Hh inhibitor, cyclopamine, reduced hydrocortisone-induced cysts without affecting organ growth, implicating increased Hh signaling in this mechanism of cystogenesis (Chan et al. 2010). Hh inhibitors also prevented cyst formation in cultured

embryonic kidneys of *Thm1, jck,* and *Pkd1* mutant mice (Tran et al. 2014b). Downregulating Hh signaling in orthologous PKD mouse models in vivo will help determine a functional role for increased Hh signaling in PKD-mediated renal cystogenesis.

11.6 Perspectives

While aberrant regulation of multiple signaling pathways has been implicated in ADPKD, this complexity is not unique to ADPKD but occurs also in cancer and correlates with more advanced disease. This emphasizes the need to understand the molecular mechanisms of early disease and, ultimately, those that initiate disease processes. In ADPKD-derived, *PKD1*-mutant iPS cells, only the absence of ciliary PC1 and PC2, and not abnormalities in cell proliferation, apoptosis, or ciliogenesis, was observed, suggesting that ciliary mislocalization of proteins may be early events (Freedman et al. 2013). Thus, determining the proteins resident in cilia in healthy and disease states may help reveal initiating mechanisms. Advances in proteomics and imaging technologies may facilitate such investigations. Recently, proximity labeling of primary cilia using APEX technology was demonstrated to successfully uncover novel proteins in wild-type and *Ift27*–/– primary cilia (Mick et al. 2015). APEX technology provides remarkable sensitivity to allow temporal "snapshots" of both transient and stable residents of the dynamic ciliary proteome. Performing similar experiments in PC1/PC2-deficient cells, which also have primary cilia, seems feasible. Proximity labeling may also be applied to examining proteins at the ciliary base, the ER, and Golgi apparatus in response to fluid flow and in *PKD1/2* mutant cells to further analyze the cellular events important in PKD biology.

Super-resolution imaging has refined the localization of proteins at the ciliary base. Combining stimulated emission depletion (STED) super-resolution imaging with transmission electron microscopy has provided a nanometer-scale view of the spatial distribution of seven proteins at the transition zone and distal appendices in mammalian cells (Yang et al. 2015). This level of resolution can be applied to proteins uncovered using proteomics technology in the axoneme, ciliary base, ER, and Golgi apparatus. Live imaging of renal proximal tubules of Cilia[GFP] mice that express ciliary-localized somatostatin receptor 3 (Sstr3)::GFP shows that fluid flow causes cilia to bend and lie almost parallel to the apical cell surface (O'Connor et al. 2013). These experiments can be extended to analyzing cilia behavior at various stages of renal cystogenesis and correlating with fluid flow-induced intracellular Ca^{2+} generation. The achievement of imaging ciliary Ca^{2+} in vivo can also be applied to possibly other organelles and possibly other pathways.

Thus far, targeting cAMP levels by administration of Tolvaptan has proven most effective in treating patients with ADPKD. Still this therapy can be ameliorated to improve efficacy and reduce side effects. Targeting other pathways in combination with Tolvaptan and directing drug delivery to the kidney may potentiate therapeutic benefits. Alternatively, targeting the PC protein defect and ciliary mislocalization has been proposed as a more proximal therapy (Cai et al. 2014; Trudel et al. 2016). Approximately, 30% of mutations are missense mutations for which the protein defect may be potentially targeted. Since ciliary localization of Hh signaling components is regulated by phospholipid content of ciliary membrane, investigations into the role of the ciliary membrane lipid content on PC ciliary localization

may also be warranted. Glycosphingolipid metabolism modulates ADPKD in mouse models (Natoli et al. 2010) and is involved in the formation of apical membrane and primary cilia of differentiated MDCK cells (Pescio et al. 2012). Thus, the future of ADPKD research promises exciting discoveries toward finding effective therapies and ultimately a cure.

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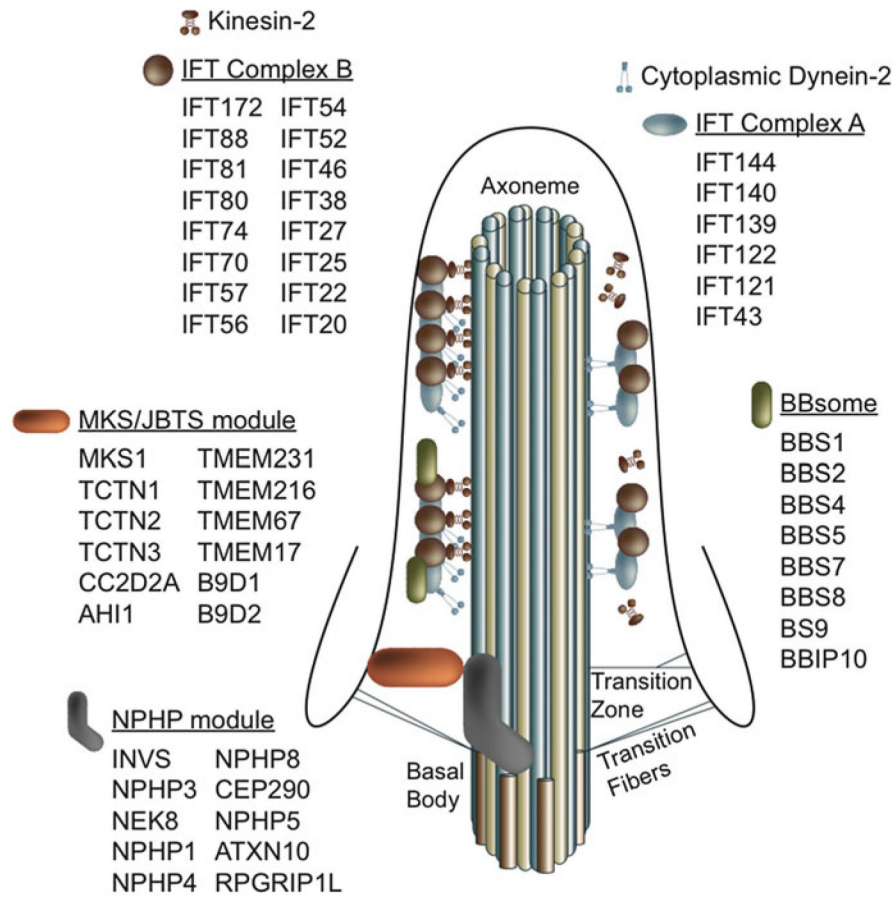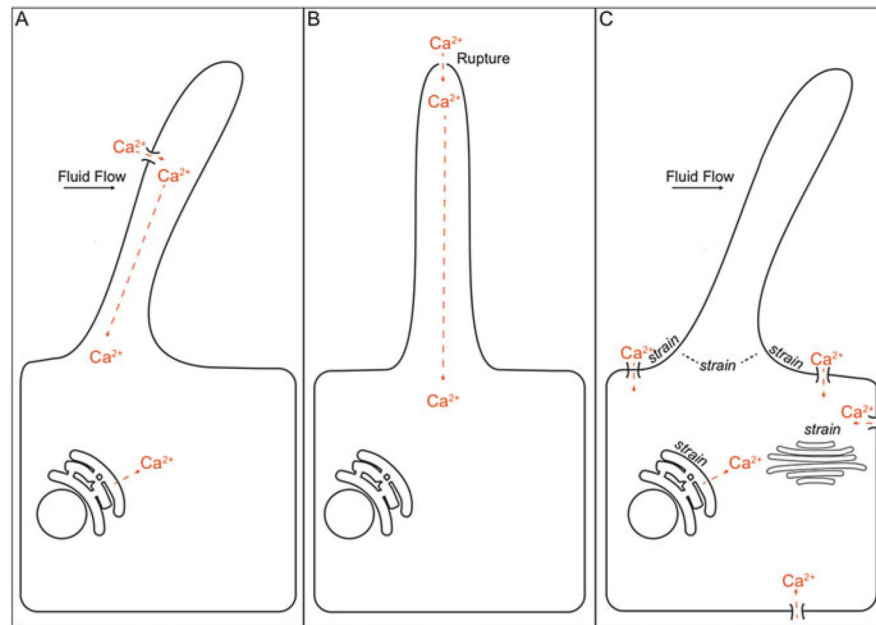
Fig. 11.1.

Structure of primary cilium. Multi-protein complexes at the transition zone, basal body, and along the axoneme are required for building and maintaining the primary cilium
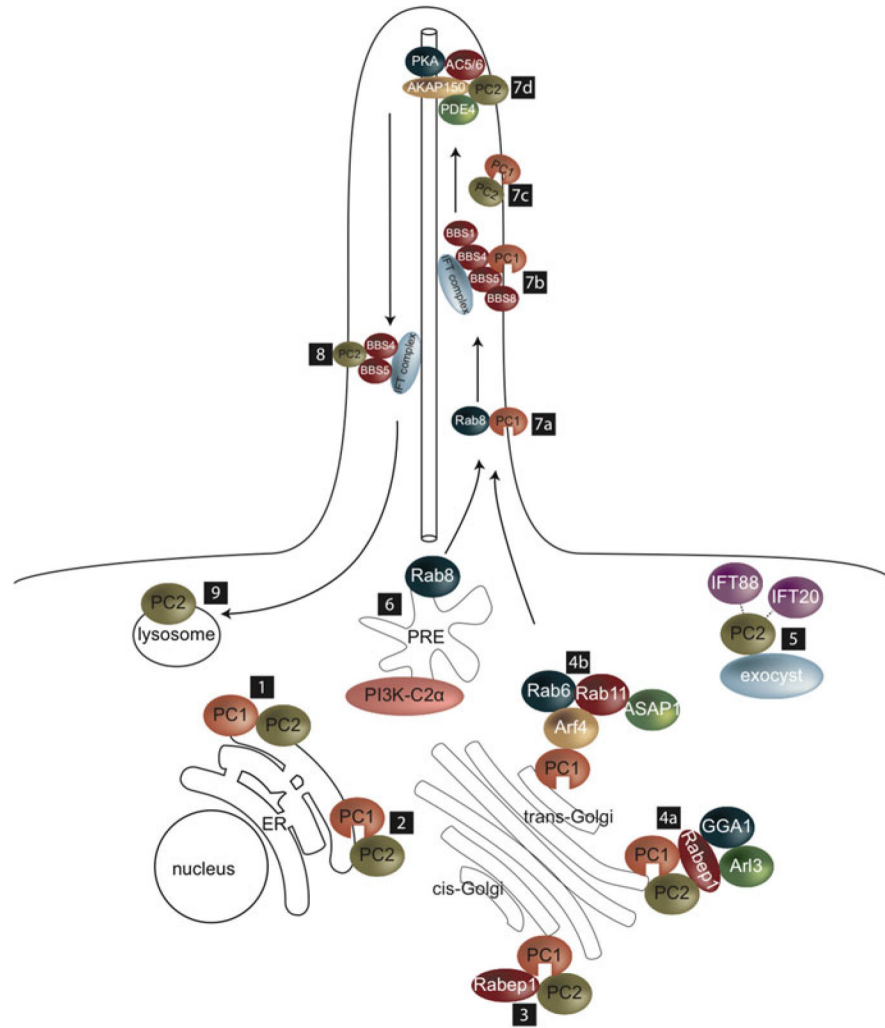
Fig. 11.2.
Intracellular trafficking of polycystins to primary cilium. Intracellular and ciliary trafficking
of polycystins involves the following steps: (1) PC1 and PC2 interact in the ER (Kim et al.
2014; Gainullin et al. 2015); (2) PC1 is cleaved (Kim et al. 2014; Gainullin et al. 2015); (3)
PC1/PC2 complex interacts with Rabep1 in pre-Golgi complex (Kim et al. 2014); (4a)
Rabep1 bridges PC1/PC2 complex to GGA1, which binds to Arl3 at the trans-Golgi (Kim et
al. 2014); (4b) PC1 in a complex with Arf4, ASAP1, Rab6, and Rab11 in Golgi (Ward et al.
2011); (5) PC2 interacts with exocyst, in a complex with IFT88 and IFT20, which facilitates
ciliary entry of PC2 (Fogelgren et al. 2011); (6) PI3K-C2α in the pericentriolar recycling
endosome (PRE) activates Rab8 (Franco et al. 2014), which interacts with PC1 and also PC2
and (7a) facilitates ciliary entry of PC1 and also of PC2 (Ward et al. 2011; Hoffmeister et al.
2011; Franco et al. 2016); (7b) Binding of PC1 to BBS1 and the BBSome facilitates ciliary
entry of PC1 (Su et al. 2014); (7c) PC1 and PC2 complex in the cilium (Nauli et al. 2003);
(7d) PC2 is in a ciliary complex with AKAP150, AC5/6, PDE4, and PKA (Choi et al. 2011);
(8) BBS4 and BBS5 mediate ciliary removal of PC2 (Xu et al. 2015); (9) PC2 is degraded in
the lysosome (Hu et al. 2007; Xu et al. 2015)

Fig. 11.3.

Models of fluid flow shear stress-induced intracellular Ca^{2+} generation. (**a**) Fluid flow induces ciliary Ca^{2+} influx, which releases intracellular Ca^{2+} stores (Jin et al. 2014a; Yuan et al. 2015). (**b**) Ciliary Ca^{2+} influx induced by ciliary membrane rupture increases Ca^{2+} at ciliary base but does not release intracellular Ca^{2+} stores (Delling et al. 2013). (**c**) Bending of primary cilium induced by fluid flow causes strain at base of cilium, which induces Ca^{2+} influx via apical and basolateral membranes and, subsequently, release of intracellular Ca^{+2} stores (Liu et al. 2003; Rydholm et al. 2010; Khayyeri et al. 2015)

Table 11.1

Mammalian ciliary lengths

Cell/tissue type	Cilium length (μm)	Citation
Embryonic neural tube	0.97 ± 0.17	He et al. (2014)
Rat tail tendon fascicles	1.35 ± 0.11	Lavagnino et al. (2013)
Mouse embryonic node	2–2.5	Alten et al. (2012)
Mouse olfactory bulb	22	Ying et al. (2014)
Rat cholangiocytes (large bile ducts)	7.35±1.32	Masyuk et al. (2008)
Rat cholangiocytes (small bile ducts)	3.58±1.12	Masyuk et al. (2008)
Pancreatic duct	2	Cano et al. (2004)
Pancreatic islet	2.5	Ait-Lounis et al. (2007)
Kidney	3–3.5	Pazour et al. (2000)
N1 hypothalamic neurons	2–2.5	Han et al. (2014)
Hypothalamic arcuate nucleus	5.5 ± 0.44	Han et al. (2014)
Ventromedial hypothalamus	5.3 ± 0.39	Han et al. (2014)

Table 11.2

Ciliopathies

Ciliopathy	Organ systems affected	References
Alström Syndrome	Visual, auditory, cardiovascular, endocrine, hepatic, renal, skeletal, neural	Hearn et al. (2005)
Bardet–Biedl Syndrome	Skeletal, neural, visual, renal, reproductive	Ansley et al. (2003)
Birt–Hogg–Dubé Syndrome	Renal (carcinoma and cysts), lung, integumentary	Luijten et al. (2013)
Ellis van Creveld Syndrome	Skeletal, cardiac	Ruiz-Perez and Goodship (2009)
Jeune Syndrome	Skeletal, renal	Beales et al. (2007)
Joubert Syndrome	Renal, visual, neural	Louie and Gleeson (2005)
Juvenile cystic kidney disease	Renal	Smith et al. (2006)
Kartagener Syndrome/Primary ciliary dyskinesia	Respiratory, neural, laterality, reproductive	Afzelius (1976), Camner et al. (1975)
Leber congenital amaurosis	Visual	den Hollander et al. (2007)
Meckel–Gruber Syndrome	Renal, neural, hepatic	Dawe et al. (2007)
Nephronophthisis	Renal	Otto et al. (2003)
Orofaciodigital Syndrome 1	Skeletal	Romio et al. (2004)
Polycystic kidney disease	Renal, hepatic, cardiovascular	Pazour et al. (2000)
Retinitis pigmentosa	Visual	Hong et al. (2001)
Senior–Loken Syndrome	Renal, visual	Otto et al. (2005)
Sensenbrenner Syndrome	Skeletal	Walczak-Sztulpa et al. (2010)
Tuberous sclerosis	Neural, integumentary, cardiovascular, renal, respiratory	Hartman et al. (2009)
Usher Syndrome	Visual, auditory	Bonneau et al. (1993)
Von Hippel–Lindau	Tumors in blood vessels and adrenal glands, renal (carcinoma and cysts)	Esteban et al. (2006)