Chapter 5
Functional Study of the Primary Cilia in ADPKD

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Abstract The primary cilium is a microtubule-based organelle that is considered to be a cellular antennae, because proteins related to multiple signaling pathways such as Wnt, PDGFRα, Hh, and mechanosignaling are localized to the membrane of the primary cilium. In the kidney, primary cilia extend from the cell membrane to the lumen of renal tubules to respond to fluidic stress. Recent studies have indicated that the disruption of ciliary proteins including polycystin-1 (PC1), polycystin-2 (PC2), and members of the intraflagellar transport (IFT) family induce the development of polycystic kidney disease (PKD), suggesting that the malformation or absence of primary cilia is a driving force of the onset of PKD. Therefore, in this chapter, the renal cystogenesis mechanism induced by cilia defects and pathogenic ciliary proteins associated with PKD development will be described.

Keywords Cilia • Ciliogenesis • Cystogenesis • Intraflagellar transport

5.1 Primary Cilia

The cilium is a finger-like structure that protrudes from the apical membrane surface of various vertebrate cells. Cilia have been observed to take two forms, motile or immotile, and are conserved in eukaryotes. For a long time, many studies focused on motility of the cilia, because scientists have considered that immotile cilia (now commonly referred to as primary cilia) are evolutionarily degenerated. However, increasing evidence has indicated that primary cilia have a role in regulating various signaling pathways in most mammalian cells and that primary cilia can sense physical and biochemical signals (Singla and Reiter 2006), suggesting that primary cilia may represent a sensory organelle distributed throughout vertebrate cells. Interestingly, immotile cilia are widespread compared to motile cilia in the human

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body. Also, it has been reported that structural or functional defects of the primary cilia are closely related to the onset of various human diseases, including developmental disorders (Satir et al. 2010). For these reasons, it is important to study the function of primary cilia to understand various pathological defects in vertebrates.

What is the difference between motile cilia and primary cilia? Primary cilia differ from motile cilia in their structural aspect. Primary cilia are characterized by an axoneme comprised of nine pairs of outer microtubules (9 + 0 microtubule arrangement). On the other hand, an additional central microtubule pair is observed in motile cilia (Singla and Reiter 2006). Motile cilia consist of an axoneme of nine outer doublet microtubules surrounding a single pair of inner microtubules (9 + 2 microtubule arrangement). Motile and primary cilia are extended from the basal body of differentiated and quiescent cells (Kobayashi and Dynlacht 2011). The basal body is derived from recruitment of the centrosome to the plasma membrane and transition of the mother centriole; thus, cell cycle regulation is a critical cellular event in the assembly or disassembly of cilia (Kobayashi and Dynlacht 2011). Besides these structural differences, the number of primary cilia per cell is distinguished from motile cilia. Primary cilia occur singly on epithelial cells, whereas most cells having motile cilia have one or more motile cilia (Michaud and Yoder 2006).

In vertebrates, most motile cilia are displayed on the ependymal cells of brain ventricles, the epithelial cells of trachea and lung, and the cells of the oviducts (Michaud and Yoder 2006). These motile cilia are involved in the movement of mucus or the circulation of body fluid, whereas primary cilia function in the sensing of extracellular stimulus and signal transduction. In fact, recent studies have revealed that various proteins including ion channels, receptors, and transporters involved in signal transduction are localized to the ciliary membrane and basal body (Satir et al. 2010). Primary cilia are regarded as sensory organelles throughout various tissues due to this special characteristic compared to motile cilia. Examples of primary cilia in mammalian cells include those on the renal tubules, the endothelium covering the cornea, and bile duct epithelial cells in liver (Ibanez-Tallon et al. 2003), suggesting that primary cilia play a role in phototransduction, mechanosignaling, chemosensory, and osmosensory function (Waters and Beales 2011).

5.1.1 Ciliogenesis

Cilia are highly conserved cellular organelles of most mammalian cells. To assemble cilia, several distinct stages collectively known as ciliogenesis are required. Unlike other cellular organelles, primary cilia are a dynamic structure whose assembly depends on cell cycle progression, because the centrioles involved in cell cycle regulation are an essential component in the formation and maturation of basal body of the cilia. For these reasons, proliferating cells must exit the mitotic cycle and enter the G0 stage to free centrioles for axoneme nucleation (Avasthi and Marshall 2012).

Ciliogenesis consists of several ordered steps. First, basal bodies derived from centrosomes are formed and recruited to the apical membrane of the polarized cells, and then docking and fusion with plasma membrane occur to initiate ciliogenesis (Ishikawa and Marshall 2011; Avasthi and Marshall 2012). Finally, the elongation of the ciliary membrane and axoneme continues by the process of intraflagellar transport (IFT) (Avasthi and Marshall 2012). Because all proteins are synthesized in the cytoplasm, systems for the recruitment of proteins related to cilia assembly or functioning at the ciliary tip are required, and this recruitment process is termed IFT (Ishikawa and Marshall 2011).

IFT proteins, which are involved in the bi-directional movement protein complex, are divided into two categories, IFT-A (IFT43, IFT121, IFT122, IFT139, IFT140, IFT144) and IFT-B (IFT20, IFT22, IFT25, IFT27, IFT46, IFT52, IFT56, IFT57, IFT70, IFT74, IFT80, IFT81, IFT88, IFT172), according to their reaction of movement (Taschner et al. 2012). The direction of the transport of IFT-A proteins is from the ciliary tip to the basal body, mediated by the dynein-2 motor protein, so this complex is thought to contribute to retrograde transport, while IFT-B proteins move from the basal body to the ciliary tip, dependent on the action of the kinesin-II motor protein, and this process is called anterograde transport (Cole et al. 1998; Ishikawa and Marshall 2011). Therefore, IFT proteins are recruited to the ciliary tip by kinesin-II and returned to the cell body by dynein-2, leading to the continuous recycling of IFT proteins (Ishikawa and Marshall 2011).

Recent studies demonstrate that the malfunction of most IFT-B complex proteins leads to developmental defects with the shortening or absence of primary cilia in vitro and in vivo (Pazour et al. 2000; Deane et al. 2001; Jonassen et al. 2008; Follit et al. 2006), indicating that these proteins are involved in cilia elongation and the regulation of tissue homeostasis. In contrast, most IFT-A complex proteins do not seem to be essential for cilia assembly (Efimenko et al. 2006; Tran et al. 2008; Tsao and Gorovsky 2008). Although primary cilia are observed in IFT-A complex protein-disrupted models, dysregulated signaling pathways regulated by primary cilia or the mislocalization of ciliary proteins have been observed in these models (Jonassen et al. 2012).

5.1.2 Signaling Pathways Regulated by Primary Cilia

Although primary cilia have no motility, this organelle has essential roles in the developmental stages and genetic diseases in vertebrates (Goetz and Anderson 2010). An accumulation of scientific evidence has demonstrated that signaling molecules related to cell proliferation, differentiation, cell cycle, survival, and autophagy are localized to the membrane of the primary cilium, suggesting that primary cilia are specialized for sensing or transmitting cellular signals. Here, the representative signaling pathways regulated by primary cilia are introduced.

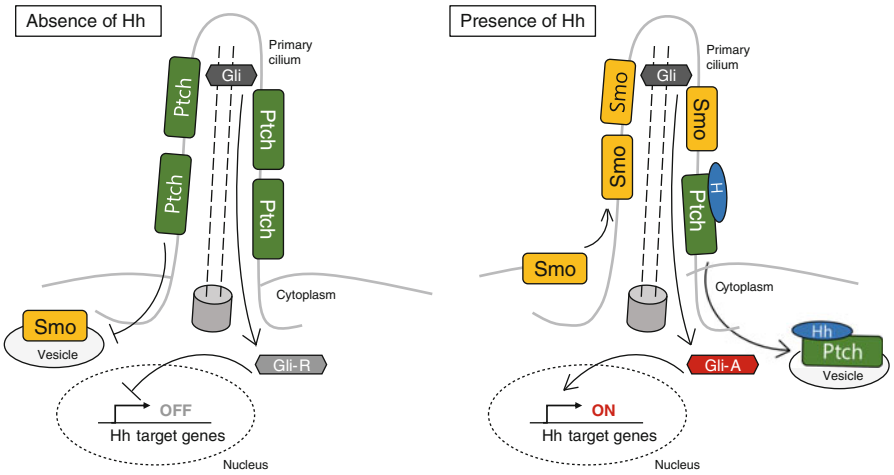

Fig. 5.1 Hh signaling regulated by primary cilia

5.1.2.1 Hedgehog Signaling

Although it has been reported that various signaling pathways are involved in ciliary signaling, the regulatory mechanism of Hedgehog (Hh) signaling in the primary cilia is particularly well understood. Hh signaling is highly conserved in vertebrate development and plays an essential role in regulating tissue patterning and homeostasis (Christensen and Ott 2007; Drummond 2012). Consistent with this, the dysregulation of Hh signaling is observed in models of developmental defects. Recent studies have demonstrated that, in the absence of Hh ligand, Hh ligand binding receptor, Patched (Ptch1), inhibits the recruitment of the Smoothened (Smo) transmembrane protein to the primary cilium (Nozawa et al. 2013) (Fig. 5.1). However, in the presence of Hh ligand, the Hh/Ptch1 protein complex is internalized to the cytosol and Smo proteins accumulate in the ciliary membrane, leading to the expression of Hh target genes via activation of glioma (Gli) transcription factors (Nozawa et al. 2013) (Fig. 5.1).

Several studies have shown that certain ciliary proteins are associated with the localization of Hh signaling molecules. Although mutant mice lacking functional Ift25, which is known as a component of the IFT-B complex, show multiple developmental defects including growth restriction, omphaloceles, and polydactyly, *Ift25* null mice and cells are still ciliated, suggesting that Ift25 is not required for ciliogenesis (Keady et al. 2012). However, interestingly, the same study showed that Hh signaling defects were observed in *Ift25* null mutants and that Ift25 is involved in the regulation of Hh signaling proteins localization on the ciliary membrane (Keady et al. 2012). Other evidence has shown that intestinal cell kinase (Ick), which is known as a ciliary protein, regulates Hh signaling (Moon et al. 2014). *Ick* null mice embryos display an endocrine-cerebro-osteodysplasia (ECO) syndrome-like phenotype with defective Hh signaling (Moon et al. 2014). In addition, this research group

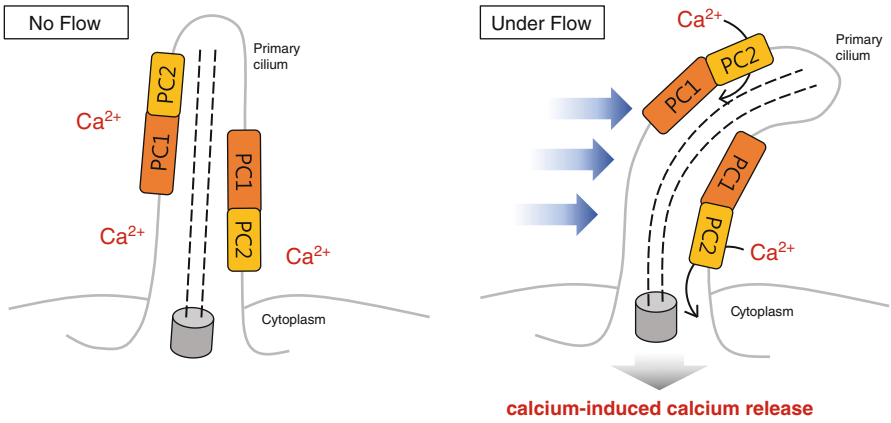

Fig. 5.2 Mechanosignaling regulated by primary cilia

suggested that the knockdown of Ick expression in fibroblasts leads to an increased length of the primary cilia, in which the Smo and Gli2 proteins are abnormally localized (Moon et al. 2014). Taken together, these findings indicate that primary cilia and cilia-related proteins are key regulators of ciliary Hh signaling.

5.1.2.2 Mechanosignaling

Chemical and kinetic stimuli could be detected by the primary cilia, because they are exposed to various hormones, growth factors, chemical substances, fluidic flow, and pressure in the extracellular environment (Basten and Giles 2013). Among the various ciliary responses to extracellular stimuli, in this section, the mechanisms of mechanosignaling by primary cilia are introduced.

The mechanical response of the primary cilia is well understood in renal biology. Representative mechanical stresses include touch, pressure, flow, and vibration, and their detection is collectively referred to as mechanosensation (Basten and Giles 2013). In the renal tubules, which are composed of renal epithelial cells that have primary cilia, the mechanical response to physical phenomena including urine flow occurs through the lumen of the renal tubules. When liquid flow passes through the lumens of the renal tubules, the primary cilia of the renal epithelial cells bend, leading to the initiation of a mechanical response (Fig. 5.2). The deflection of primary cilia in response to luminal flow is recognized by polycystin-1 (PC1) proteins localized to the membrane of primary cilia on the renal epithelial cells. PC1 acts as mechanosensory protein for luminal flow that transmits mechanical stress from the extracellular environment to polycystin-2 (PC2) protein, which is localized to renal primary cilia and associates with PC1 to form a protein complex that functions as a calcium channel (Huang and Lipschutz 2014) (Fig. 5.2). Activated PC2 proteins induce minimal calcium ion influx, leading to massive intracellular calcium release

through calcium-induced calcium release (CICR) (Nauli et al. 2003) (Fig. 5.2). Finally, an increased calcium level of the cytosol regulates various signaling pathways related to proliferation and development.

There is scientific evidence showing that the primary cilia act as mechanosensors via PC1/PC2 protein complex in renal epithelial cells. The research group of Nauli et al. suggested that Pkd1null/null cells and cyst-lining cells derived from human autosomal dominant polycystic kidney disease (ADPKD) kidneys fail to respond to shear stress, although *Pkd1*$^{null/null}$ cells still have primary cilia (Nauli et al. 2006).

These findings suggest that renal primary cilia act as antennae to detect shear stress movement through renal tubules, while the PC1/PC2 protein complex contributes to mechanotransduction signaling mediated by calcium in the renal cilia (Nauli et al. 2003).

5.1.2.3 Mammalian Target of Rapamycin Signaling

The mammalian target of rapamycin (mTOR) signaling pathway plays roles in cell size control, cell growth, and metabolism (Wullschleger et al. 2006). mTOR signaling is driven by two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Wrighton 2010). mTORC1 consists of five protein components: mTOR, Raptor, GβL, PRAS40, and Deptor, while mTORC2 consists of six protein components: mTOR, Rictor, mSIN1, Protor-1, mLST8, and Deptor (Laplante and Sabatini 2009). When mTORC1 is activated by amino acids and growth factors, it phosphorylates p70S6 kinase (S6K) and 4E-BP1 to activate protein synthesis and cell proliferation and regulate cell size (Boehlke et al. 2010). On the other hand, Lkb1 tumor suppressor, which is known as a serine/threonine kinase, inhibits mTORC1-mediated signaling via the cellular energy status sensor, AMP-activated protein kinase (AMPK) (Shaw et al. 2004). mTOR pathways are well studied in cancer biology, while the regulatory mechanism or relationship between mTOR and primary cilia is not completely understood. In addition, although mTOR signaling is highly activated and is thought to be a major disease-causing pathway in PKD, which is considered as a ciliary defect disease (Ibraghimov-Beskrovnaya and Natoli 2011), the precise relationship between primary cilia and mTOR in the kidney has not been fully elucidated.

However, in 2010, the research group of Boehlke and colleagues suggested that the primary cilia regulate cell size through mTOR signaling (Boehlke et al. 2010). According to the findings of this research group, Lkb1 localized to primary cilia regulates flow-dependent mTOR activity and shear stress induces AMPK phosphorylation at the basal body, resulting in the inhibition of mTORC1 activity and a reduction in cell size (Boehlke et al. 2010) (Fig. 5.3). This paper is the first scientific evidence showing that primary cilia act as mechanosensor to modulate mTOR signaling, but further studies are needed to identify the machinery that mediate the recruitment of mTOR pathway regulators to the ciliary or basal body, such as IFT.

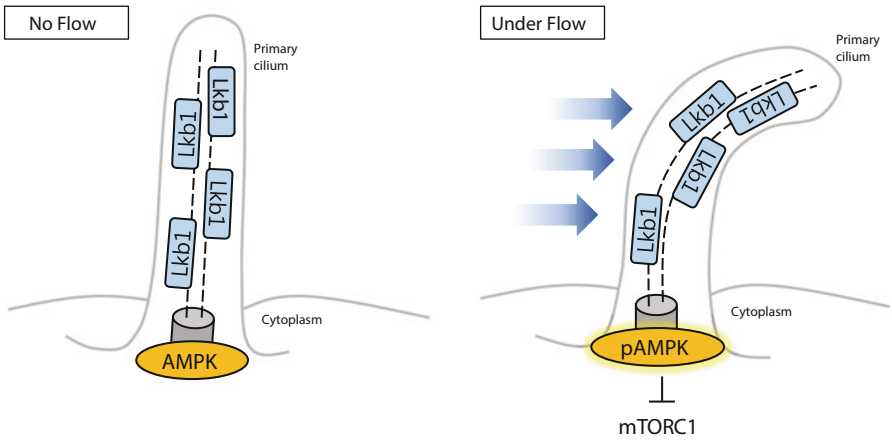

Fig. 5.3 mTOR signaling regulated by primary cilia

5.2 Ciliopathies & PKD

Primary cilia are localized to almost all cells of the human body and function as sensors for the detection of extracellular signals, so it is conceivable that a defective function or structure of the primary cilia is correlated with various human diseases. In fact, defects of the primary cilia are involved in the onset of various diseases, including retinal degeneration, rib/thoracic skeleton defect, pelvic bone defect, polydactyly, liver cysts, hydrocephalus, cardiac defect, mental retardation, airway defect, genital defect, pancreatic cysts, and cystic kidneys (Lee and Gleeson 2011). These multisystemic human diseases are referred to as ciliopathies. Most ciliopathies are caused by mutations of ciliary genes, indicating that the identification of disease-specific functions of mutated ciliary genes is critical for understanding ciliopathies.

Among the many ciliopathies, in this section, we focus on PKD, which is the most common human genetic disorder and is characterized by the formation of fluid-filled cysts in the renal tubules, leading to end-stage renal disease (Patel et al. 2009). Recent studies suggest that PKD is caused by abnormalities of primary cilia, so PKD can be considered as ciliopathy of the kidney. Some studies have demonstrated a relationship between defects of the primary cilia and PKD development. It has been reported that the *PKD1* and *PKD2* genes are mutated in PKD and the proteins encoded by these two genes are localized to the membrane of the renal cilia and form calcium channels, which seem to respond to mechanical stresses including luminal flow through the renal tubules (Chapin and Caplan 2010). Although mutations of these two genes do not affect the ciliogenesis of primary cilia (Ma et al. 2013), a failure to increase calcium influx in response to shear stress is observed in *Pkd1* mutant cells, indicating that defects of polycystin proteins are involved in dysfunctional renal cilia (Nauli et al. 2006; Patel et al. 2009). In addition to these

findings, it has been reported that the inactivation of genes related to ciliogenesis including *Ift20*, *Ift88*, and NIMA (never in mitosis A)-related kinase 8 (*Nek8*) cause a severe PKD phenotype in mice (Jonassen et al. 2008; Jonassen et al. 2012; Lehman et al. 2008; Liu et al. 2002). Interestingly, the loss of genes that are required for cilia assembly such as *Ift20* and *Kif3a* induces a renal cystic phenotype in mice with the absence of renal cilia (Jonassen et al. 2008; Lin et al. 2003), whereas one of the PKD mouse models called juvenile cystic kidney (jck) shows a cystic kidney phenotype with lengthened cilia compared to those of normal kidneys (Smith et al. 2006). Taken together, these findings indicate the dysregulated signaling by which defective primary cilia are associated with PKD development and suggest that functional studies of primary cilia and ciliary proteins might contribute to a better understanding of PKD pathogenesis.

5.3 Disruption of Signaling Pathways in Cilia-Defective PKD Mouse Models

The abnormal regulation of signaling pathways such as proliferation, cell cycle, differentiation, apoptosis, inflammation, and fibrosis has been observed in PKD mouse models. Among these pathways, an increase of proliferation has been considered as a major causative factor in the development of PKD. According to a published paper, inactivation of the *Pkd1* gene in mouse kidney cells induces a severe polycystic kidney phenotype with increased proliferation of the cyst-lining epithelial cells (Shibazaki et al. 2008).

Recent studies show that defective primary cilia or the inactivation of cilia-related genes induces aberrant signaling pathways associated with proliferation, differentiation, and development in various PKD mouse models (Ma et al. 2013; Ibraghimov-Beskrovnaya and Natoli 2011; Jonassen et al. 2008; Eguether et al. 2014; Shibazaki et al. 2008). Consistent with this, it has been reported that various signaling pathways including Wingless (Wnt), planar cell polarity, mTOR, mitogen-activated protein kinase (MAPK), and Hh are dysregulated in various ciliopathy models. The major ciliary pathways related to PKD development are discussed in this section.

5.3.1 Increase of MAPK Signaling in PKD with Ciliary Defects

PC1 and PC2 protein complexes have been shown to localize to the ciliary membrane in the kidney. Renal cilia function as a mechanosensor and cooperate with the PC1-PC2 protein complex in the renal tubules to translate mechanical stress through the lumen into an increase of intracellular calcium ion influx (Bastos and Onuchic

2011). When tubular fluid flow induces bending of cilia, the PC2 calcium channel opens to allow calcium ions to enter into the cilioplasm, resulting in CICR from the endoplasmic reticulum (Jin et al. 2014; Winyard and Jenkins 2011). Indeed, calcium entry in response to shear stress is disrupted in renal epithelial cells that have no cilia or cilia with no PC1. These findings demonstrate that the induction of an increase of intracellular calcium in response to fluid flow requires intact primary cilia with PC1 and PC2 protein complexes.

Signaling related to calcium ions is important for maintaining the homeostasis of renal epithelial cells, because most of the calcium-related signaling pathways are associated with cell proliferation in the kidney. Indeed, a lower intracellular calcium level is observed in the renal epithelial cells of PKD (Mangolini et al. 2016; Yamaguchi et al. 2006). This calcium restriction in renal epithelial cells allows the cAMP-dependent activation of MAPK signaling, leading to an increase of cell proliferation, which is a typical hallmark of renal cyst expansion during PKD development (Mangolini et al. 2016).

5.3.2 Increase of mTOR Signaling in PKD with Ciliary Defects

Another aberrant signaling pathway linked to defective primary cilia in PKD is mTOR. Inappropriate activation of mTOR signaling is common feature of PKD and is caused by the inactivation of ciliary genes including *Pkd1* and *Ift88*, suggesting that defects of ciliary function are related to aberrant mTOR signaling (Mostov 2006). Indeed, as described above, it has been revealed that Lkb1, which is known as a negative regulator of mTOR, is localized to primary cilia and inhibits mTORC1 activity under conditions of fluid flow (Boehlke et al. 2010). Consistent with this, the activation of mTOR signaling and an increase of renal epithelial cell size are observed in the kidney of kinesin family member 3A (*Kif3a*)-defective mice models that show a cystic kidney phenotype with a complete loss of renal cilia (Boehlke et al. 2010). Taken together, these findings indicate that primary cilia in the kidney play a role in regulating cell proliferation via the activation of mTOR signaling in response to luminal flow.

5.3.3 Cilia-Dependent Cyst Activating Mechanism in PKD

Primary cilia of the renal epithelial cells seem to function as negative regulators of cyst expansion. As described above, this function is supported by studies on PKD mice models generated by the inactivation of various ciliary genes, indicating that the loss of primary cilia promotes renal cyst formation in vivo. However, in 2013, a newly discovered mechanism referred to as the cilia-dependent cyst activating (CDCA) pathway was proposed. Interestingly, it was suggested that the loss of renal cilia inhibits cyst growth following the loss of polycystins in vivo (Ma et al. 2013).

In addition, it was shown that the size and severity of renal cysts are associated with the length of the time interval between the early loss of polycystins and the subsequent ablation of cilia (Ma et al. 2013). While the loss of cilia or polycystins alone results in the development and progression of renal cysts, renal cilia involution reduces the progression of cyst growth induced by the inactivation of polycystins (Ma et al. 2013; Lee and Somlo 2014). This evidence strongly suggests that the progression of cysts in PKD is regulated by the duration of the interval time between the initial loss of polycystins and the subsequent disappearance of renal cilia (Ma et al. 2013; Lee and Somlo 2014). This is a novel molecular mechanism that explains the relationship between polycystins and primary cilia in the kidney, but the signaling molecules related to this pathway have not yet been elucidated. Therefore, it is critical to define the components related to CDCA to propose answers to the unresolved questions in PKD pathogenesis.

5.4 Concluding Remarks

Primary cilia are thought to be cellular antennae that regulate diverse signaling pathways in mammalian cells. Indeed, various signaling components related to cell proliferation, differentiation, and development are localized to cilia. It has been reported that the dysfunction of renal cilia induced by the inactivation of various ciliary genes is associated with the development of PKD. Emerging evidence has suggested the existence of a primary cilia-independent role of ciliary genes. Recent studies indicated that the protein Ift88, which is known as a critical component of cilia assembly, can also induce cell migration (Boehlke et al. 2015) and is required for the G1/S transition in non-ciliated cells (Robert et al. 2007). These findings indicate that ciliary proteins might have not only cilia-dependent but also cilia-independent roles. In addition, it has been reported that ciliary defect phenotypes differ between tissues, although the same ciliary protein was inactivated in mice (Moon et al. 2014; Chaya et al. 2014), demonstrating that ciliary proteins might have tissue-specific functions. Therefore, defining the cilia-dependent or cilia-independent roles of ciliary proteins and the tissue specificity of ciliary function will lead to a better understanding of the molecular mechanisms of various ciliopathies including PKD.

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