UNDERSTANDING HNF-1β THROUGH IDENTIFICATION OF INTERACTING PROTEINS AND TARGET GENES IN THE KIDNEY

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DEDICATION

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encouraged me to be a serious scientist as well as a better person. Finally, I dedicate my thesis to God who always takes care of me and show me where I am.

UNDERSTANDING OF HNF-1β THROUGH IDENTIFICATION OF INTERACTING PROTEINS AND TARGET GENES IN THE KIDNEY

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Hepatocyte nuclear factor-1β (HNF-1β) is a POU/homeodomain-containing transcription factor that regulates tissue-specific gene expression in the liver, kidney, pancreas, and other epithelial organs. During kidney development, HNF-1β is expressed in renal collecting ducts and all segments of the nephron. Mutation of HNF-1β causes maturity-onset diabetes of the young type 5 (MODY5) and kidney developmental anomalies including renal agenesis, hypoplasia, and cysts. Here, I studied interacting proteins and target genes to understand the function of HNF-1β in the kidney. Yeast two-hybrid screening was performed to identify binding partners of HNF-1β in the kidney. Zyxin and LPP were isolated as putative interacting proteins. The LIM-containing

proteins, Zyxin and LPP, are focal adhesion proteins that shuttle between the cytoplasm and nucleus and play a role in the architectural organization of cells. Both Zyxin and LPP interact with HNF-1β and stimulate the transcriptional activity of HNF-1β in mIMCD3 renal epithelial cells. Epidermal growth factor (EGF), which plays a role in the progression of polycystic kidney disease, induces translocation of zyxin into the nucleus. These studies identify a novel pathway by which signals may be transmitted from the cell surface to regulate the activity of a nuclear transcription factor that is essential for epithelial differentiation in the kidney.

Chromatin immunoprecipitaion and DNA chip analysis (ChIP-chip) were performed to identify direct target genes of HNF-1β in the kidney. Phosphodiesterase 4C (PDE4C) was identified as an HNF-1β target gene. PDE4C belongs to the phosphodiesterase superfamily of enzymes that control the intracellular concentration of cyclic adenosine monophosphate (cAMP) by catalyzing its hydrolysis. cAMP may play a role in cystogenesis by stimulating fluid secretion and cell proliferation. PDE4C is transcriptionally activated by HNF-1β and regulates cAMP levels in mIMCD3 renal epithelial cells. Antibody staining showed that PDE4C is localized in the primary cilium and there interacts with a protein complex containing AKAP150, adenylyl cyclase 5/6 (AC5/6), protein kinase A (PKA), and polycystin-2 (PKD2). These results identify a cAMP-regulating protein complex that is localized in the primary cilium and is disrupted in PKD.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

ABSTRACT vi

PRIOR PUBLICATIONS x

LIST OF FIGURES xi

LIST OF TABLES xiii

LIST OF ABBREVIATIONS xiv

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

RENAL CYSTIC DISEASE 1

HEPATOCYTE NUCLEAR FACTOR-1BETA

PROTEIN STRUCTURE 3

EXPRESSION PATTERN 6

HNF-1BETA IN HUMAN DISEASE 8

THE IN VIVO ROLES OF HNF-1BETA 11

ZYXIN AND LPP

STRUCTURE 13

FUNCTIONS 15

CYCLIC AMP SIGNALING

CYCLIC AMP SIGNALING 17

CYCLIC AMP IN PKD 19

PHOSPHODIESTERASE 4

STRUCTURE 20

CELLULAR FUNCTION AND THERAPEUTIC USE OF PDE INHIBITOR 23

PDE IN POLYCYSTIC KIDNEY DISEASE 24

CHAPTER TWO: ZYXIN AND LPP AS INTERACTING PARTNERS OF HNF-1β

INTRODUCTION 25

RESULTS 27

DISCUSSION 48

MATERIALS AND METHODS 51

CHAPTER THREE: IDENTIFICATION OF A cAMP-REGULATING PROTEIN COMPLEX IN THE PRIMARY CILIUM PROVIDES A COMMON MECHANISM DISRUPTED IN POLYCYSTIC KIDNEY DISEASE

INTRODUCTION 55

RESULTS 58

DISCUSSION 76

MATERIALS AND METHODS 82

CHAPTER FOUR: CONCLUSIONS AND RECOMMENDATIONS

CONCLUSION 88

TRANSCRIPTIONAL ACTIVATION BY ZYXIN FAMILY PROTEINS, ZYXIN AND LPP 88

INVOLVEMENT OF HNF1B IN cAMP SIGNALING COORDINATED BY PRIMARY CILIA 91

BIBLIOGRAPHY 94

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LIST OF FIGURES

FIGURE 1-1 4

FIGURE 1-2 6

FIGURE 1-3 9

FIGURE 1-4 14

FIGURE 1-5 19

FIGURE 1-6 21

FIGURE 1-7 22

FIGURE 2-1 28

FIGURE 2-2 31

FIGURE 2-3 35

FIGURE 2-4 37

FIGURE 2-5 39

FIGURE 2-6 42

FIGURE 2-7 43

FIGURE 2-8 45

FIGURE 2-9 46

FIGURE 3-1 59

FIGURE 3-2 62

FIGURE 3-3 65

FIGURE 3-4 67

FIGURE 3-5 71

FIGURE 3-6 74

FIGURE 3-7 74

FIGURE 3-8 81

LIST OF TABLES

TABLE 1-1 2

TABLE 1-2 10

TABLE 1-3 16

LIST OF ABBREVIATIONS

aa - Amino acid

AC - Adenylyl Cyclase

ADPKD – Autosomal Dominant Polycystic Kidney Disease

AKAP - A Kinase Anchoring Protein

ARPKD - Autosomal Recessive Polycystic Kidney Disease

CAKUT - Congenital Abnormalities of the Kidney and Urinary Tract

cAMP - Cyclic Adenosine Monophosphate

cGMP - Cyclic Guanosine Monophosphate

EGF - Epidermal Growth Factor

EGFR- Epidermal Growth Factor Receptor

Epac - cAMP-dependent guanine nucleotide exchange factor

ESRD – End Stage Renal Disease

HNF - Hepatocyte Nuclear Factor

IHBD - Intrahepatic bile Ducts

LPP - Lipoma Preferred Translocated Partner

MCKD - Medullary Cystic Kidney Disease

MODY - Maturity-Onset Diabetes of the Young

NPHP – Nephronophthisis

PC-1- Polycystin-1

PC-2 - Polycystin-2

PCP - Planar Cell Polarity

PDE - Phosphodiesterase

PKA - Protein Kinase A

PKD - Polycystic Kidney Disease

PKHD - Polycystic Kidney and Hepatic Disease

POU - Pit-1, Oct-1, Oct-2, Unc86 domain

RCAD – Renal Cysts and Diabetes

SH3 - Src-Homology 3

SNP - Single Nucleotide Polymorphisms

TRIP6 - Thyroid receptor Interacting Protein 6

VE - Visceral Endoderm

CHAPTER ONE
Introduction

Renal cystic disease

The kidneys are paired organs that are part of the urinary system and have essential homeostatic functions including the regulation of electrolytes, acid-base balance, and blood pressure. The kidney is also responsible for the reabsorption of glucose and amino acids and is important in the production of hormones including vitamin D, renin, and erythropoietin. ESRD occurs when chronic kidney failure has progressed to the point where kidney function is less than 10% of normal. The kidneys can no longer remove wastes, concentrate urine, and regulate electrolytes. Dialysis and kidney transplantation are only treatments for ESRD.

The kidney is susceptible to cystic disease, a common cause of renal failure. The most clinically significant types of renal cystic disease are hereditary, including autosomal dominant and recessive polycystic kidney disease, nephronophthisis (NPHP), and medullary cystic kidney diseases (MCKD) [1]. Despite many shared pathological features, renal cystic diseases are caused by mutations in different genes (Table 1-1) and show distinct clinical characteristics. Studies of the pathophysiology and genetics of cystic kidney diseases will advance our understanding of fundamental cellular processes and will be essential for the development of new therapeutic approaches.

Table 1-1. Polycystic kidney disease genes and proteins

DISEASE	INHERITANCE	GENE	CHROMOSOME	PROTEIN
ADPKD	AUTOSOMAL DOMINANT	*PKD1* *PKD2*	16p13.3 4q21-23	POLYCYSTIN-1 POLYCYSTIN-2
ARPKD	AUTOSOMAL RECESSIVE	*PKHD1*	6p21-23	FIBROCYSTIN
NPHP	AUTOSOMAL RECESSIVE	*NPHP1* *NPHP2* *NPHP3* *NPHP4* *NPHP5* *NPHP6/* *NPHP7* *NPHP8* *NPHP9*	2q12-13 9q21-31 3q21-22 1p36 3q13.31–3q21.2 12q21.32-q21.33 16p13.3 16q12.2 17q11.1	NEPHROCYSTIN-1 INVERSIN NEPHROCYSTIN-3 NEPHROCYTIN-4 NEPROCYSTIN-5 NEPHROCYSTIN-6/Cep290 GLIS2 NEPHROCYSTIN-8 NEK8 (never in mitosis kinase 8)

MCKD	AUTOSOMAL DOMINANT	*MCKD1* *MCKD2*	1q21 16p12	UROMODULIN

Hepatocyte Nuclear Factor-1beta (HNF-1β)

Protein structure

HNF-1β is a homeodomain-containing transcription factor that is structurally related to HNF-1α and functions as a homodimer or heterodimer with HNF-1α [2-4]. HNF-1α and HNF-1β are encoded by distinct genes on separate chromosomes and are highly conserved in vertebrates [3-7]. Both proteins were first discovered as liver-enriched transcription factors, but their expression is not restricted to hepatocytes [8,9]

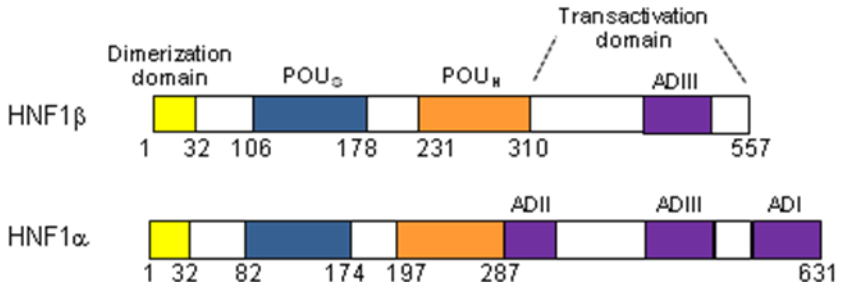


Figure 1-1. Domain structure of HNF1 family members
HNF1 proteins contain an N-terminal dimerization domain (yellow) which is responsible for homo- and heterodimerization. The DNA binding region is composed of a POU specific domain (POU_S, blue) and a divergent POU homeodomain (POU_H, orange). The divergent C-termini (pink) have been shown to mediate transcriptional activation.

Both HNF1 proteins consist of an N-terminal dimerization domain, a bipartite DNA binding region that is composed of a POU-specific domain and a divergent POU homeodomain, and a C-terminal transactivation domain (Fig 1-1) [10-12]. HNF-1α and HNF-1β have similar dimerization domains and DNA-binding properties, but they have distinct activation domains [3,13-15]. Dimerization of HNF-1α and HNF-1β is essential for DNA binding and their association with the transcriptional coactivator, DCoH (dimerization cofactor of HNF1) [16]. Based on the crystal structure of HNF-1α, the dimerization domain forms a unique, antiparallel four- helix bundle that allows the formation of homo- and heterodimers of HNF1 proteins [17-19]. The DNA-binding domain is composed of a POU-specific domain (POU_S) and a divergent POU homeodomain (POU_H). Recent three dimensional structural analysis of HNF-1α shows that the POU_S domain interacts with a 21-amino acid loop of the POU_H domain to create a stable interface that further distinguishes HNF-1α from other flexible POU-homeodomain proteins [20]. HNF-1β contains a 26-amino acid insertion between POU_S and POU_H, depending on the splicing variant [10]. This sequence variability is a key element in differential combinatorial control of gene expression by recruiting different sets of

transcriptional coactivators[21]. In contrast to these rather conserved domains, the C-terminal transactivation domain is the most divergent region between HNF-1α and HNF-1β. The C-terminal domain of HNF-1α contains three distinct activation domains, ADI, rich in serines, ADII, rich in proline, and ADIII, rich in glutamine [12]. However, HNF-1β contains only the glutamine-rich region (ADIII) in the C-terminus. The transactivation domain of HNF-1β was identified using a series of Gal4-fused caboxy-terminal deletion proteins and testing their activity in Gal4-responsive reporter assays. The region between aa 352 and aa 483 is responsible for the transcriptional activity of HNF-1β [22].

Three isoforms of HNF-1β are generated by alternative splicing [23,24]. The first isoform, HNF-1β-A, contains 26 extra aa between POU_S and POU_H, which is excluded in the second isoform, HNF-1β-B. In transient transfection assays, the extra segment of HNF-1β-A enhances transactivation and DNA-binding activity [24]. A third isoform, HNF1β-C, lacks the amino acid sequences located in the C-terminal halves of HNF1β-A and –B. HNF1β-C lacks transcriptional activity in transient transfection assays and inhibits transcriptional activation by HNF-1β-A/B [23].

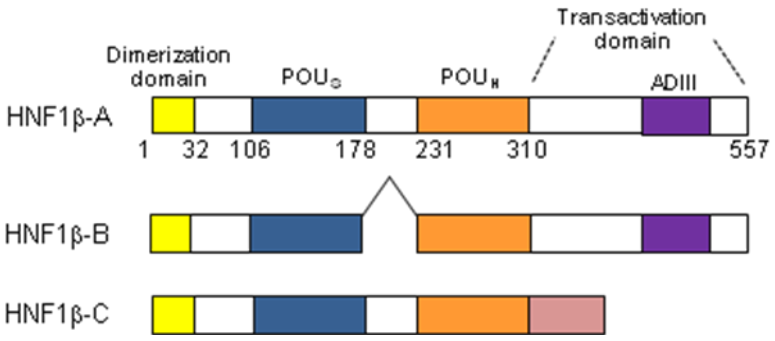


Figure1-2. Schematic diagram of the different isoforms of HNF-1β
HNF-1β-A, -B, and –C proteins contain dimerization domain (yellow) that medicate homo- and heterodimerization among HNF1 family proteins, POU_S (blue) and POU_H (orange) domain which are responsible for DNA binding, and C-terminal transactivation domain.

HNF-1β-A, and –B are expressed at a roughly constant ratio in various organs, however, isoform C is expressed at lower levels [23,24]. All isoforms of HNF-1β differ functionally in their transactivation potential, which provides an additional level of complexity for differential target gene regulation by increasing the number of possible homo- and heterotypic interactions.

Expression pattern

In the adult, HNF-1α and HNF-1β are expressed in similar tissues, including kidney, liver, intestine, and pancreas although at different levels [3,4]. HNF-1β is highly expressed in the kidney and shows low expression in the liver, pancreas, and intestine, whereas HNF-1α is equally expressed in both liver and kidney. Moreover, HNF-1β is present in organs that lack HNF-1α, including the thymus, lung, testis, and ovary [12].

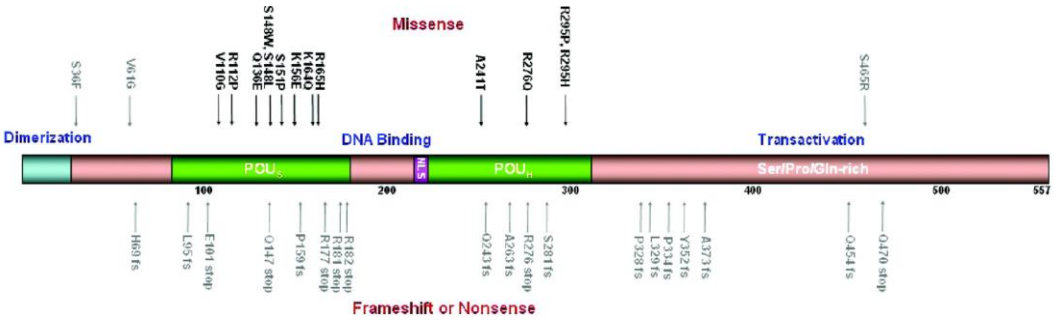
During mammalian development, HNF-1β expression starts at the beginning of gut formation and is detected in epithelium as early as the 2-somite stage [25,26] . According to *in situ* hybridization analysis of mid- to late-embryonic stages, both mRNAs of HNF-1α and HNF-1β are present in polarized epithelia of organs of endodermal and mesodermal origin, including kidney, liver, and the digestive tract [27]. Coffinier *et al* studied the expression pattern of HNF-1β during mouse organogenesis using an NLS-lacZ gene introduced at the HNF-1β locus [26]. X-gal staining is detected in the developing gallbladder and pancreatic buds at E12, and in biliary ducts that develop inside the liver at E14-15. Later and continuing to the adult stage, LacZ expression is strongly detected in the biliary system. During pancreas development, expression is first observed in both the dorsal and the ventral buds. As the buds grow and fuse, HNF-1β/lacZ expression is restricted to the exocrine ducts. In the lung, the cells of bronchi and bronchioles strongly express HNF-1β/lacZ. The kidney is the major site of HNF-1β expression in the adult. In vertebrates, three distinct types of kidneys, the pronephros, mesonephros, and the metanephros, are formed during embryogenesis. HNF-1β/lacZ is expressed at E10 in the epithelium of the developing mesonephros. One day later, the uretic bud emerges from the mesonephric duct and will give rise to the metanephric kidney through the induction of the condensation of the surrounding mesenchyme and its differentiation into tubular epithelium. During kidney morphogenesis, HNF-1β is specifically expressed in comma- and S-shaped bodies, and the epithelia of maturing nephrons. In the mature kidney, HNF-1β expression is detectable throughout the entire nephron, from the proximal tubules to the collecting ducts [26]. In the female, the inner epithelial layer of both the oviduct and the

uterus expresses HNF-1β/lacZ. Epithelial tissues of the male genital organs such as the vas deferens, the epididymis tubules, and prostate, express HNF-1β/lacZ. During development, the expression of HNF-1β is restricted to epithelial cells as they are organized in tubular structures and precedes the expression of HNF-1α, suggesting that HNF-1β participates in early developmental decisions in different cell lineages [12,25-29]. Later, expression of HNF-1α plays a role in the maintenance of epithelial gene expression.

HNF-1β in human disease

The HNF-1β (TCF2) gene is located on chromosome 17q21.3. In human, mutations of HNF-1β have been associated with diabetes, abnormal renal development, and genital tract malformations [30-32]. Some patients with HNF-1β mutations are born with a malformed kidney observed at the end of the first trimester of gestation, indicating that dysfunction of HNF-1β affects kidney formation during early fetal life [32]. Heterozygous mutations in the HNF-1β gene cause maturity-onset diabetes of the young type -5 (MODY5) [31-35]. MODY is a genetically and clinically heterogeneous subtype of type 2 diabetes characterized by early onset (childhood, adolescence, and young adulthood), autosomal dominant mode of inheritance, and insulin secretion defect [36,37]. MODY5 also known as RCAD is frequently associated with congenital abnormalities of the kidney and genitourinary tract [38,39]. To date, more than 50 different heterozygous mutations of HNF-1β have been described including missense, nonsense, framshift, insertion/deletion, and splice site mutations in human with MODY5 [40-42] (Fig 1-3, table 1-2). Most mutations

affect the DNA binding domain [43]. The majority of these mutations are inheried though the germline mutation but several spontaneous mutations and one germline mosaic mutation have been described [32,35,44-46].



Modified from *Lu et al, Biochemistry, 2007*.

Figure 1-3. Disease-causing mutations found in HNF-1β
Most missense mutations are found in the DNA binding domain. Missense mutations are indicated in black and framshift and nonsense mutations are shown in gray.

In addition, mutations of HNF-1β are associated with a variety of renal abnormalities including simple cysts, polycystic kidneys, cystic dysplasia, glomerulocystic kidney disease, familial juvenile hyperuricemic nephropathy (FJHN), and genital abnormalities including Mayer-Rokitansky-Kuster-Hauser syndroms [31,34,35,42,47-50]. Renal cysts and diabetes (RCAD) syndrome is characterized by having features present in two categories, renal cysts and early-onset diabetes and has an

autosomal dominant pattern of inheritance, although spontaneous mutations can produce this syndrome [33,35]. Mutations in HNF-1β result in producing RCAD syndrome [33]. Another syndrome that originates from gene mutations is congenital abnormalities of the kidney and urinary tract (CAKUT). CAKUT is common in humans, occurring at a frequency of approximately 1 in 500 fetal ultrasound examinations [50-52]. HNF-1β has been identified as one of the genes that may cause human CAKUT syndrome [50]. A recent study showed that subjects with sporadic renal hypodysplasia (RHD) may have mutations in HNF-1β [53]. In this study, they found missense variants in HNF-1β and heterozygous deletions of HNF-1β in humans with RHD [53]. Mutations of HNF-1β have been shown to cause pancreatic atropy, exocrine dysfunction, and neonatal diabetes in humans [54]. Furthermore, HNF-1β plays a role as a tumor suppressor in renal, ovarian, and prostate cancer [55-58]. Biallelic HNF-1β inactivation was found in chromophobe renal carcinomas [58,59]. HNF-1β was identified as a target for epigenetic inactivation in ovarian cancer, resulting in dysregulation of target gene expression [56,60]. In prostate cancer, single nucleotide polymorphisms (SNPs) were identified in the HNF-1β gene [57,61,62], providing new insight into a possible genetic link between type 2 diabetes and prostate cancer.

Table 1-2. Mutations in HNF-1β are associated with human diseases
(from *Lu et al, 2007*)

Mutation	Phenotype	Effects
V110G	Glomerulocystic kidney disease	DB/PS

R112P	Diabetes (MODY5)	DB/PS
Q136E	Diabetes (MODY5, cortical renal atrophy)	DB/PS
S148L	Diabetes (MODY5, renal dysplasia)	DB
S148W	Neonatal diabetes, renal cyst	PS
S151P	Renal cysts/genital tract malfunction	DB/PS
K156E	Diabetes, renal cyst	DB/PS
K164Q	Diabetes (MODY5, enlarged glomeruli)	DB/PS
R165H	Diabetes (MODY5, bicornuate uterus)	PS
A241T	Diabetic nephropathy	
R276Q	Diabetes, renal cyst	ID/DB
R295P	Diabetes (MODY5, renal cyst)	DB
R295H	Diabetes (MODY5, glomerular cyst)	DB

DB: DNA binding disruptor, ID: interdomain interaction disruptor, PS: protein stability disruptor

The in vivo roles of HNF-1β

To investigate the in vivo roles of HNF-1β, animal models have been used. HNF-1β homozygous null mice develop normally to the blastocyst stage, start implantation but die later with abnormal or absent extraembryonic region, poorly organized ectoderm and no discernible visceral or parietal endoderm. These results demonstrate that HNF-1β is required for early embryogenesis and plays an important role in visceral endoderm (VE)

specification [29]. Since VE isn't formed in HNF-1β null embryos, not only is its specialized nutritional role impaired but also its post implantation embryonic functions are disrupt [29]. As an animal model of the renal abnormalities in MODY5, transgenic mice expressing a dominant-negative HNF-1β mutant under the control of a kidney-specific promoter have been generated [63]. Transgenic mice are viable at birth but develop renal cysts and renal failure, which is similar to the phenotype of human patients with MODY5. Mice with renal-specific inactivation of HNF-1β develop polycystic kidney disease [64]. Kidney-specific HNF-1β mutant mice show renal cysts and multilayered epithelia, suggesting that inactivation of HNF-1β leads to increased cellular proliferation [64]. In addition, several genes are downregulated in mutant mouse kidneys, including *Umod*, *Pkhd1, Pkd2*, and *Tg737/Polaris,* which are expressed in tubular epithelial cells and are known to be mutated in distinct cystic kidney diseases (table 1-1) [64]. Furthermore, HNF-1β might regulate tubulogenesis by controlling the levels of SOCS-3 expression [65]. In a recent study using tetraploid and diploid embryo complementation, HNF-1β was shown to play an essential role during the earliest stages of urogenital development [66]. In addition, in the lower vertebrate, zebrafish, mutations of HNF-1β affect pronephros development and induce cystic pronephric tubules [67]. HNF-1β also plays a role in specification of the posterior hindbrain during zebrafish development. Zebrafish HNF-1β mutants fail to form a gut of single lumen and instead develop multiple lumens, indicating HNF-1β controls specification of a single lumen [68,69]. Expression of HNF-1β mutants in Xenopus embryos interferes with pronephros development [70]. Different mutations of HNF-1β produce different defects in pronephros development. The

framshift mutation Y352 leads to the reduction or agenesis of pronephric tubules. Other mutants, S151P, E101X, R177X, P159, and Q243, lead to enlargement of the pronephric tubules and the anterior pronephric duct. The different phenotypes produced by different HNF-1β mutants may reflect different subtypes of renal diseases in human [70]. In addition to the effects of HNF-1β in the kidney, liver-specific inactivation of HNF-1β results in severe growth retardation, hypertrophy of the liver, and jaundice caused by abnormalities of the gallbladder and intrahepatic bile ducts (IHBD) [71,72]. In the liver, HNF-1β is essential for normal intrahepatic bile duct differentiation and morphogenesis and lipid metabolism by regulating the expression of hepatocyte-specific genes, *Oatp1* and *Vlcad* [71].

Zyxin and LPP

Zyxin and LPP are members of the zyxin family of proteins that are present at sites of cell-substratum interaction, focal adhesion, and cell-cell adhesion, where they dock with proteins involved in cytoskeleton organization and dynamics [73-76]. The zyxin family consists of zyxin, ajuba, LIMD1, thyroid receptor-interacting protein 6 (TRIP6), WT1-interacting protein (WTIP), and Lipoma preferred translocator partner (LPP).

Protein structures

All zyxin family proteins contain three LIM domains located toward their C-termini and share high sequence similarity within the LIM domain region. LIM domains are cysteine- and histidine-rich domains that form zinc fingers capable of mediating protein-protein interactions. LIM proteins participate in diverse cellular processes through binding to other proteins [77,78]. In addition to LIM domains, zyxin and LPP contain a nuclear export signal that regulates their subcellular distribution between the nucleus and cytoplasm [79-81]. Four proline-rich repeats can interact directly with members of the Ena/VASP family, which are concentrated at focal adhesions, the leading edge of lamellipodia, and filopodial tips [82,83] and are involved in modulation of actin assembly and organization [84-87].

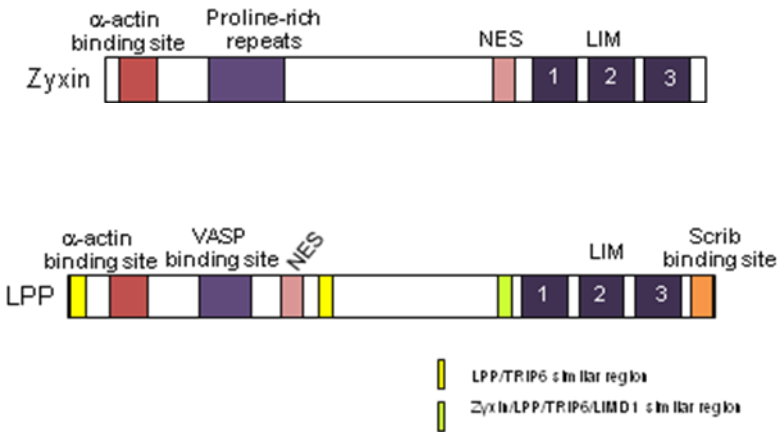


Figure 1-4. Schematic diagram of zyxin and LPP.
In their pre-LIM region, zyxin and LPP harbor a nuclear export signal, NES (pink), proline rich repeat/VASP binding site (light purple), and α-actin binding site (red). Both zyxin and LPP contain three tandem LIM domains (purple).

LPP interacts with the tumor suppressor protein, Scrib, through a Scrib-binding site in the C-terminus and plays a role in Scrib-associated functions [88].

Protein functions

Zyxin is a low abundance phosphoprotein that is localized at sites of cell-substratum interaction in fibroblasts and displays the architectural feature of an intracellular signal transducer [76]. LPP was initially described as the preferred translocation partner in a cytogenetic subgroup of lipomas that is characterized by translocations that mainly involve chromosome 12 [89]. Similar to zyxin, LPP was found to bind to α-actinin in vitro and in vivo [90,91]. However, LPP has a lower affinity for α-actinin than zyxin. Although zyxin and LPP localize at cell adhesions, zyxin is predominantly expressed along stress fibers[92]. Moreover, there is a difference in the relative abundance of these two proteins. In fibroblasts, the expression level of zyxin is five times higher than that of LPP, whereas epithelial cells show similar levels of zyxin and LPP [92].

The regulation of actin dynamics, cell movement, and signal transduction by zyxin and LPP is mediated by their association with a variety of cytoskeletal and signaling proteins (Table 1). Zyxin interacts with h-Warts/LATS1 on the mitotic apparatus, which implicates a significant role for actin regulatory proteins during mitosis [93]. Since zyxin translocates into the nucleus, it is implicated to play a role in gene regulation. Degenhardt *et al* showed that zyxin interacts with the E6 protein from

papillomavirus type 6 which is responsible for hyperproliferation of cutaneous and mucocutaneous epithelial cells, and it functions as a transcriptional activator [94]. LPP also has a functional nuclear export signal by which it can shuttle between the cytoplasm and nucleus [81] and functions as a coactivator of ETS-domain transcription factor, PEA3, as well as additional transcription factors in the nucleus [92,95-97]. The functions of LPP have been revealed by identification of interacting proteins. LPP recruits VASP to specific cellular locations and changes actin dynamics [87]. Palladin has been identified as an interacting partner of LPP, which enhances cell migration and spreading [98]. Another interaction partner of LPP is the tumor suppressor Scrib which is involved in the control of cell adhesion, cell shape, and polarity, and the regulation of planar cell polarity in vertebrates [88,99-101]. Knockdown of LPP in zebrafish results in serious defects in convergent extension, implicating LPP in the noncanonical Wnt/PCP pathway [96].

Table 1-3. Interacting partners of zyxin and LPP.

protein	Cytoskeletal and /or plasma membrane	Signaling molecules	Transcription factors/ nuclear proteins
zyxin	α-actinin	Vav	HPV E6 protein
	Mena/VASP	CasL	SON DNA-binding protein
	H-Warts/LATS1	$P130^{cas}$	
	CRP		
LPP	α-actinin	LASP-1	PEA3

	Mena/VASP	Scrib	ER81
	Palladin		

Dysregulation of renal tubular epithelial cell biology, including cell proliferation, apoptosis, polarity, and extracellular matrix, has been thought to contribute to cystogenesis. Since adhesion-mediated signaling regulates these processes, the integration of signals from the extracellular matrix through the cell surface into the nucleus may be an essential feature to understand the pathophysiology of PKD. The study of shuttle proteins may help to understand the cellular mechanism of PKD.

cAMP signaling

Environmental stimuli relay vital information into the cell by the modulation of intracellular signaling pathways. The diffusible second messenger, cAMP is an important mediator of various signals [102-104]. This ubiquitous second messenger is generated by adenylyl cyclases that are activated by G-protein coupled receptor stimulation [105]. Newly synthesized cAMP diffuses to sites where it can access its effector proteins, including PKA, phosphodiesterases (PDEs), and cAMP-dependent guanine nucleotide exchange factors (Epacs). A principal effector protein is cAMP-dependent protein kinase, PKA, which phosphorylates a myriad of substrates [106]. This process provides a mechanism by

which extracellular stimuli can influence diverse biological processes. Although the diffusion of cAMP within cells is fast, recent studies suggest that enzymes involved in cAMP synthesis and degradation generate boundaries for cAMP diffusion [107], which provide specificity of cAMP signaling to various stimuli within cells. Specificity of cAMP action is facilitated by compartmentalization of signaling proteins within the cells [102,108,109]. A-kinase anchoring proteins (AKAPs) tether effector proteins for cAMP signaling with their downstream targets to facilitate the relay of compartmentalized cAMP signals [110,111]. An important feature of AKAPs is their capacity to interact with other signaling proteins in addition to PKA. The multiprotein complexes composed of AKAPs contain signal termination enzymes, such as phosphodiesterases, and signal transduction enzymes such as kinases, as well as cAMP synthesis enzymes, adenylyl cyclase [107]. cAMP is synthesized by AKAP-adenylyl cyclase (AC) complexes and degraded by AKAP-PDE complex [112]. Distinct tissue distribution, subcellular compartmentalization and differential regulation of these enzymes contribute to the establishment of local cAMP gradients and tight regulation of cAMP signaling.

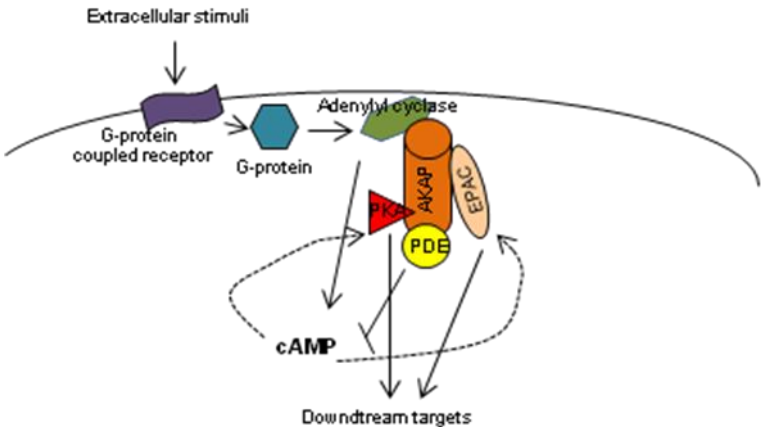


Figure 1-5. Schematic diagram of cAMP synthesis, degradation, and downstream effector activation.
When an extracellular ligand, such as hormone, binds to and activates a G-protein coupled receptor, the signal is passed from the G-protein to adenylyl cyclase (AC). The activated AC converts ATP to cAMP. AKAP protein anchor PKA, effector proteins such as EPAC, and PDE which hydrolyzes cAMP, to specific subcellular locations, which generates cAMP gradients at distinct domains in the cells.

cAMP in PKD

cAMP stimulates epithelial cell proliferation and fluid secretion into cysts of patients with ADPKD [113-115]. Increased levels of cAMP are found in the kidney, liver, and vascular smooth muscle in various models of PKD [116-118], indicating that increased cAMP may accelerate cyst growth and kidney enlargement in patients with PKD. In addition, arginine vasopressin (AVP) V2R antagonists lower the levels of cAMP and inhibit the development and progression of PKD in animal models, such as pck rat (ARPKD disease model), pcy mouse (nephronophthisis), and pkd2$^{-/WS25}$ (ADPKD type 2) [117-119].

Furthermore, clinical trials of AVP V2R antagonists (NCT00413777 and NCT00428948) are currently ongoing for the treatment of PKD. Although the mechanisms responsible for the accumulation of cAMP in PKD are not well understood, recent studies demonstrate that changes in intracellular calcium may be responsible for the elevated cAMP levels in cystic tissues [117,118,120].

Phosphodiesterase 4

Protein structure

To control the levels of cAMP, a signal termination process needs to be located in the right place. A critical regulatory mechanism is the degradation of cAMP. Phosphodiesterases (PDEs) are responsible for catabolism of cyclic nucleotides, cAMP and cGMP [121-123]. In most cells, intracellular cyclic nucleotide levels are largely regulated by PDE activity [123]. The PDE superfamily is large and complex, containing 11 structurally related genes encoding over 60 distinct isoforms [124-126]. Different PDE family members hydrolyze exclusively cAMP (PDE4, PDE7, and PDE8), exclusively cGMP (PDE5, PDE6, and PDE9), or both cAMP and cGMP (PDE1, PDE2, PDE3, PDE10, and PDE11). Each member of the PDE family differs in tissue distribution, inhibitor specificity, and mode of regulation, which contributes to compartmentalize cyclic nucleotide signaling [127,128]. Common features of PDE family members include a highly

conserved catalytic core near the C-terminus, a paired regulatory region, and a unique N-terminal region which confers isoform specificity.

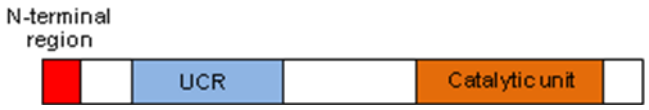


Figure 1-6, Schematic diagram of general structure of phosphodiesterase
PDEs contains a highly conserved catalytic region (orange) near C-termius. Paired regulatory region, upstream conserved region (UCR, light blue), and unique N-terminal region (red).

Among the 11 different PDE family members, PDE4 mainly plays a role in intracellular cAMP degradation [129]. PDE4s play a pivotal role in creating compartmentalized cAMP responses by generating gradients that subsequently regulate distinct cAMP-controlled processes. Since the hydrolytic capacity of PDEs far exceeds the maximum rate of synthesis by adenylyl cyclases, the levels of cAMP are thought to be more sensitive to inhibition of PDEs than to inhibition of ACs. Four genes (A/B/C/D) encode more than 20 different PDE4 isoforms by alternative mRNA splicing coupled to different promoter use [130,131]. Structurally, PDE4 isoforms contain unique N-terminal regions to target their distinct subcellular sites, highly conserved regulatory region1 and 2, and C-terminal catalytic unit. PDE4 isoforms are divided into long, short, super-short, and dead-short forms depending on the absence or presence of upstream conserved

regions (UCRs) [132]. PDE4C is a long PDE4 isoform and is less widely expressed than other PDE4 isoforms [132].

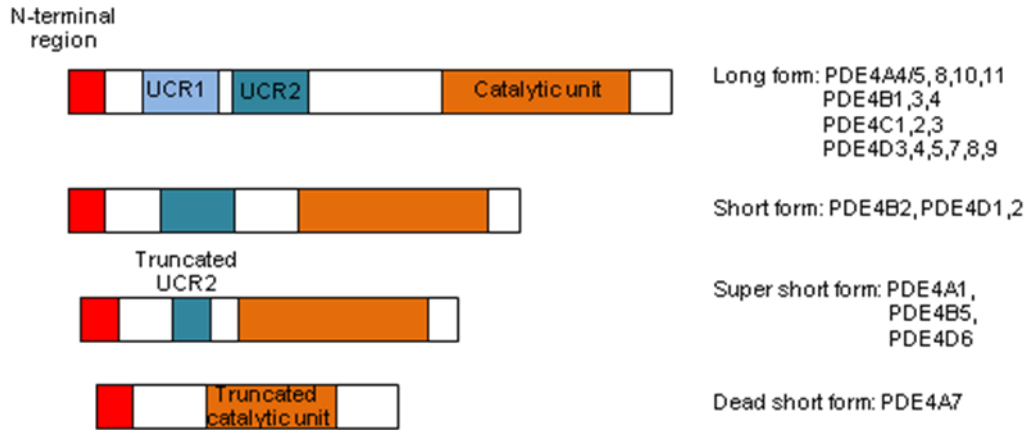


Figure 1-7. Diversity of PDE4 isoforms : long, short, super-short, and dead-short form.
PDE4 isoform is defined by unique N-terminal region. The upstream conserved region (UCR) is a highly conserved regulatory region that has a major functional role in regulating the activity of the PDE4 catalytic unit. Long forms have two UCR domains, short forms lack one UCR1, super-short forms have truncated UCR2 domain, and dead-short forms have inactive catalytic region.

UCR1 and UCR2 seem to interact in PDE4 long forms through electrostatic interactions [133] to form a discrete module that is phosphorylated by PKA and ERK [134,135]. UCR1 is joined to UCR2 by LR1 that is approximately 22-24 amino acids in length and shows heterogeneity between subfamilies. LR2 joins UCR2 to the catalytic unit and shows no similarity between PDE4 subfamilies. The core PDE4 catalytic unit is a

compact structure of 17-helices folded into 3 subdomains which come together to form a deep pocket containing a cAMP binding site. This deep pocket formed by 3 subdomains contains tightly bound Zn^{2+} and loosely bound Mg^{2+}, which are essential for catalytic activity [136,137].

Cellular function and therapeutic use of PDE4 inhibitors

A key feature of individual PDE4 isoforms is their targeting to specific sites/signaling complexes within cells, which leads to compartmentalization of cAMP signaling. The ability of PDE4s to be strategically targeted and anchored in cells may be restricted by association with other proteins. These protein-protein interactions are mediated by their unique N-terminal region. For example, the N-terminus of PDE4A4/5 and PDE4D4 isoforms contain proline-rich sequences which confer interaction with SH3 domain-containing proteins, such as Lyn, Fyn, and Src[138-141]. However, the isoforms interact with different proteins and localize at different subcellular sites. These differences in specificity of interaction arise from different sequences surrounding their distinct proline- and arginine-rich regions.

PDE4s have been implicated in memory and cognition by virtue of the memory-enhancing effects of PDE4 inhibitors. Rolipram, an inhibitor of PDE4, and other inhibitors are involved in the regulation of inflammation, which has led to the development of novel therapies for diseases, such as COPD, asthma, and rheumatoid arthritis [130]. Selective inhibitors of PDE4 have also been studied as therapeutic agents for multiple sclerosis, type 2 diabetes, septic shock, and atopic dermatitis [142-146]. Furthermore,

recent studies have linked PDE4 variants with ischemic stroke and schizophrenia [147-149], which has increased interest in PDE4 as a therapeutic target.

PDE in polycystic kidney disease

Since cAMP plays a key role in cyst progression in PKD, the study of PDEs should have high priority. The levels of cAMP are more sentitive to inhibition of PDE activity because their hydrolytic rate far exceeds the rate of synthesis by adenylyl cyclases [150]. For example, a new class of compounds, the 2-(acylamino)-3-thiophenecarboxylates, is a nonselective PDEs activators and inhibits the growth of Madin-Darby canine kidney cysts [151]. Among many PDE4 isoforms, PDE4C appears to be structurally more distinct from other PDE4 subfamilies, with certain key residues being disordered, suggesting that it may be easier to develop PDE4C-selective inhibitors or activators [152].

CHAPTER TWO

ZYXIN AND LPP AS INTERACTING PARTNERS OF HNF-1β

Introduction

HNF-1β (hepatocyte nuclear factor-1β) belongs to the POU/homeodomain-containing superfamily of transcription factor that regulate tissue-specific gene expression in the kidney, liver, pancreas, and other epithelial organs [4,27,40,71,153-155]. Heterozygous mutations in HNF-1β are associated with renal cystic diseases and diabetes. [33,156,157]. The various processes controlled by HNF-1β during development and in human diseases could be modulated by interacting partners. For example, binding of coactivator and corepressor, DCoH, CBP, P/CAF, and HDAC1 can regulate the transcription of target genes [22,158-160]. In addition, two novel partners of HNF-1β, E4F1 and ZFP36L, have been identified in the kidney [161]. E4F1 and ZFP36L reduce the transactivation potential of HNF-1β and affect nephrogenesis [161]. ZFP36L is located predominantly in the cytoplasm and shuttles between nucleus and cytoplasm. The nuclear function of ZFP36L was shown to reduce HNF-1β transactivation. Although identifying binding partners of HNF-1β improves our understading of how HNF-1β controls kidney development and diseases, relatively few interacting proteins have been identified.

Zyxin is a low abundant phosphoprotein which is one component of focal adhesions where it regulates integrin-mediated signaling [74,76]. Zyxin has several structural features: an actin-binding region in the N-terminus, a proline-rich region for docking site for Src homology 3 (SH3), and a C-terminal LIM domain involved in protein-protein

interaction [74,76,162-164]. Zyxin also has a functional nuclear export signal (NES) by which zyxin shuttles between the cytoplasm and nucleus [80,165,166]. However, it is unclear how zyxin translocates into the nucleus and what is the function of zyxin in the nucleus. Here, we identify zyxin as a novel HNF-1β interacting protein by yeast two hybrid screening technique. In addition, we identified that the parts of HNF-1β and zyxin that were responsible for this interaction. We also demonstrate that the physical interaction between HNF-1β and zyxin stimulates transcriptional activity of HNF-1β. Furthermore, our results indicate that EGF induces translocation of zyxin into the nucleus via Akt activation and affects the transcriptional activity of HNF-1β.

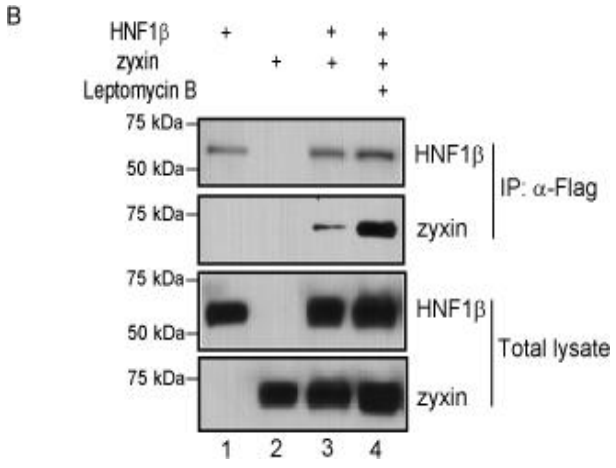
RESULTS

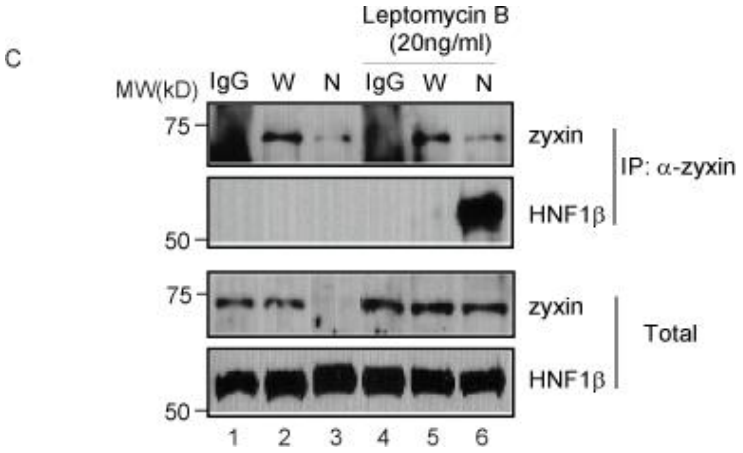
To identify novel HNF-1β interacting proteins, we performed yeast two-hybrid screening. We used full-length HNF-1β as a bait to screen a cDNA library (Matchmaker, Clontech) derived from adult mouse kidney. Twenty-seven positive clones were identified. Sequence analysis and database comparison revealed that one positive clone contained the mRNA coding sequence of zyxin (GenBank™ accession number NM_011777). The specific interaction between HNF-1β and zyxin was verified in yeast two-hybrid assays (Fig. 1A). Yeast were transformed with HNF-1β only, zyxin only, or both HNF-1β and zyxin and then streaked on synthetic drop-out plates. Only yeast transformed with both HNF-1β and zyxin grew on synthetic drop-out plates lacking histidine (Fig. 1A, 3).

To confirm the *in vivo* interaction between HNF-1β and zyxin, expression plasmids encoding HNF-1β-FLAG and zyxin-MYC were cotransfected into HEK293T cells. Cells transfected with zyxin-MYC alone were used as a negative control. Western blot analysis of total cell lysates showed that both FLAG and MYC tagged proteins were detected in cells transfected with HNF-1β-FLAG and zyxin-MYC (Fig 1*B*, lane 3 and 4, lower panel), indicating that both proteins were successfully expressed. After immunoprecipitation with anti-FLAG antibody, zyxin-MYC was dectected in the precipitates (Fig 1*B*, lane 3 and 4, upper panel), indicating co-immunoprecipitation of zyxin and HNF-1β. Treatment with leptomycin B, which blocks CRM-dependent nuclear exportation, resulted in greater immunoprecipitation of zyxin-MYC (Fig 1*B*, lane 4, upper panel), suggesting that zyxin interacts with HNF-1β in the nucleus. To confirm

these results, mouse kidney epithelial cells (mIMCD3 cells) that endogenously express HNF-1β and zyxin, were used for the immunoprecipitation experiments (Fig 1*C*). mIMCD3 cells were incubated in the absence or presence of leptomycin B, separated into cytosolic and nuclear fractions, and subjected to immunoprecipitation. The endogenous HNF-1β and zyxin were detected (Fig 1*C*, lane 1 and 2, lower panel). Zyxin was detected in nuclear fraction derived from leptomycin B-treated cells (Fig 1*C*, lane 3 and 6, lower panel). Immunoprecipitation of endogenous zyxin resulted in co-precipitation of endogenous HNF-1β in leptomycin B-treated cells (Fig 1*C*, lane 6, upper panel), further suggesting an interaction between these two proteins in the nucleus. mIMCD3 cells were transfected with GFP-zyxin and then subjected to immunoflorescence staining with an HNF-1β antibody. HNF-1β and zyxin were co-localized in the nucleus in leptomycin B-treated cells (Fig 1*D*). Taken together, these results suggest a physical interaction between HNF-1β and zyxin in the nucleus.

A


B
HNF1β + + +
zyxin + + +
Leptomycin B +
75 kDa
50 kDa
75 kDa
HNF1β
IP: α-Flag
zyxin
75 kDa
50 kDa
75 kDa
HNF1β
Total lysate
zyxin
1 2 3 4


C
Leptomycin B
(20ng/ml)
MW(kD) IgG W N IgG W N
75
zyxin
IP: α-zyxin
HNF1β
50
75
zyxin
Total
HNF1β
50
1 2 3 4 5 6

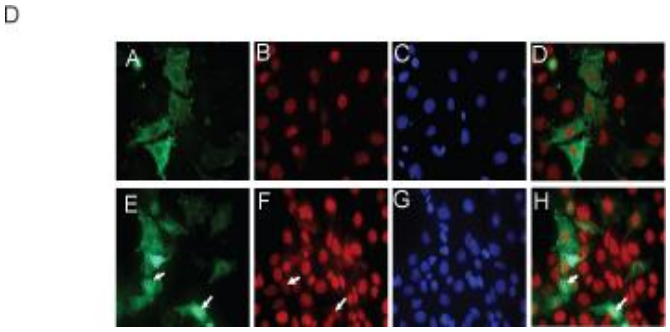
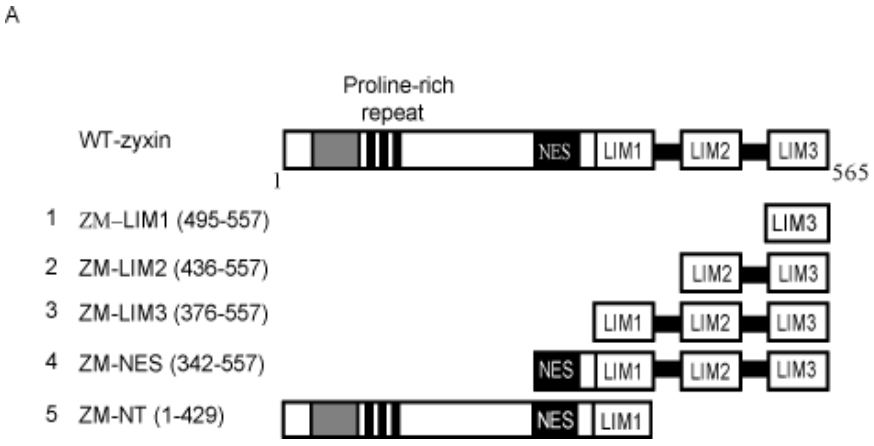


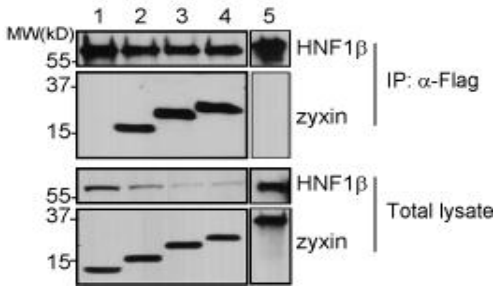
Figure 2-1. Zyxin interacts with HNF-1β.
(A) Results of the yeast two-hybrid screen with HNF-1β as a bait. LexA-HNF-1β fusion vectors were transformed into *S. cerevisiae* (strain L40, genotype *MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::LexA-LacZ*) and screened with a cDNA expression library obtained from adult mouse kidney. Yeast were transformed with LexA-HNF1β only (1), zyxin-AD only (2), or both LexA-HNF-1β and zyxin-AD (3) and then grown on either histidine-contained plate (+His) or medium lacking histidine (-His). (B) HEK293T cells were transiently co-transfected with myc-zyxin and Flag-HNF-1β and incubated in the absence or presence of leptomycin B (5h, 20ng/ml). HNF-1β was immunoprecipitated (anti-Flag) and co-precipitated products were detected by western blotting for zyxin (anti-myc) and HNF-1β (anti-Flag). Input controls are shown on the bottom panels. (C) mIMCD3 cells were incubated in the absence or presence of leptomycin B (20ng/ml) for 5h and separated into nuclear and cytosolic fractions. Endogenous zyxin was immunoprecipitated and coprecipitated HNF1β was detected using anti-HNF1β antibody. W: Whole cell lysate, N: Nuclear extract, IgG: IP with IgG. (D) mIMCD3 cells transfected with GFP-zyxin were treated with (E) or without leptomycin B (20ng/ml) for 2h (A). Cells were then fixed and subjected to immunofluorescence with anti-HNF-1β antibody (B, F). C, G show DAPI and D, H are merged images. Arrows indicate that nuclear zyxin is colocalized with HNF-1β.

To identify the domains of HNF-1β and zyxin that mediate interaction, we generated five MYC-tagged truncated forms of zyxin, ZM-LIM1 (495-557aa), ZM-LIM2 (436-557aa), ZM-LIM3 (376-557aa), ZM-NES (342-557aa), and ZM-NT (1-429aa) (Fig

2*A*). We also generated six flag-tagged deletion mutants of HNF-1β, HM (1-75aa, 1-180aa, 1-290aa, 75-532aa, 180-532aa, and 290-532aa) (Fig 2*C*). The zyxin deletion mutants were individually co-transfected with full-length FLAG-HNF1β into HEK293T cells, and whole cell extracts were immunoprecipitated with anti-FLAG antibody. As shown in Fig 2*B*, all FLAG-tagged HNF-1β proteins were detected in the precipitates (lane 1-5, upper panel). However, only three zyxin mutants, 436-557, 376-557, 342-557, were detected in immunoprecipitates (lane 2-4, upper panel), indicating that the second LIM domain of zyxin is required for the interaction between these two proteins. To identify the zyxin binding domain in HNF-1β, the HNF-1β deletion mutants were co-transfected with wild type MYC-tagged zyxin into HEK293T cells and whole cell lysates were subjected to immunoprecipitation. As shown in Fig 2*D*, five non-overlapping HNF-1β deletion mutants were detected in immunoprecipitates pulled down by anti-MYC antibody (lane 3-7, upper panel), suggesting that the homeodomain of HNF-1β may be essential for the interaction with zyxin.



B


1 2 3 4 5
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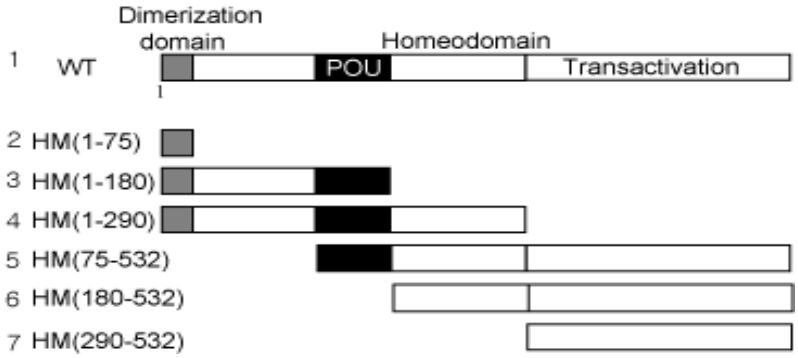
37
15
HNF1β
zyxin
IP: α-Flag
55
37
15
HNF1β
zyxin
Total lysate

C


Dimerization domain
Homeodomain
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POU
Transactivation
1
2 HM(1-75)
3 HM(1-180)
4 HM(1-290)
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7 HM(290-532)

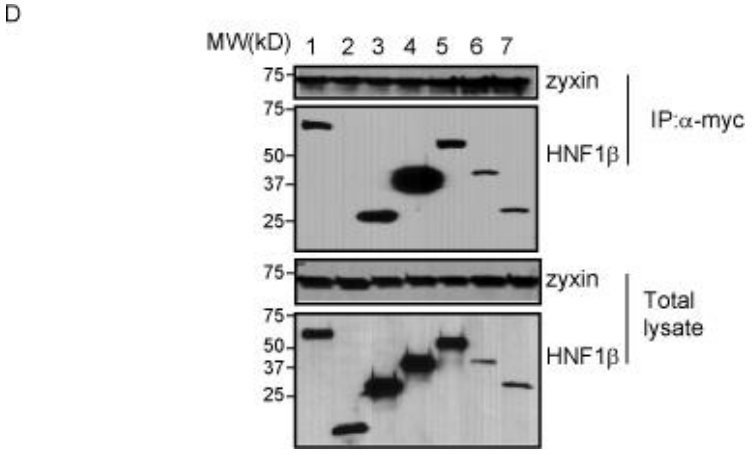
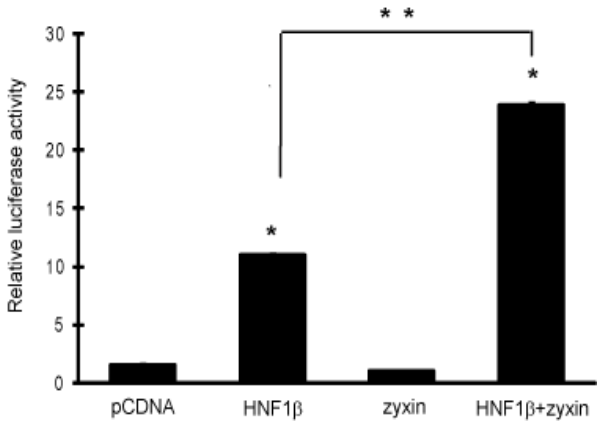


Figure 2-2. Mapping the binding sites of HNF-1β and zyxin.
(A) A diagrammatic representation of full- length zyxin is shown at the top, and truncated mutants are depicted below. (B) HEK293T cells were transfected with pCMV-flag-HNF-1β and additionally received the indicated zyxin deletion constructs. Cell lysates were subjected to co-immunoprecipitation and the zyxin fragments associated with HNF-1β were identified by western blotting with anti-myc antibody. The lower panel shows the expression level of the myc-tagged zyxin fragments and flag-tagged HNF-1β in total cell lysates. (C) Schematic diagram of HNF-1β deletion mutants is shown. (D) HEK293T cells were cotransfected with myc-tagged full length zyxin and the indicated deletion mutant of HNF-1β. zyxin was immunoprecipitated with anti-myc antibody and the HNF-1β fragments bound to myc-zyxin were identified by western blotting with anti-flag antibody. Expression level of zyxin and HNF-1β fragments in total lysates are shown in the lower panel.

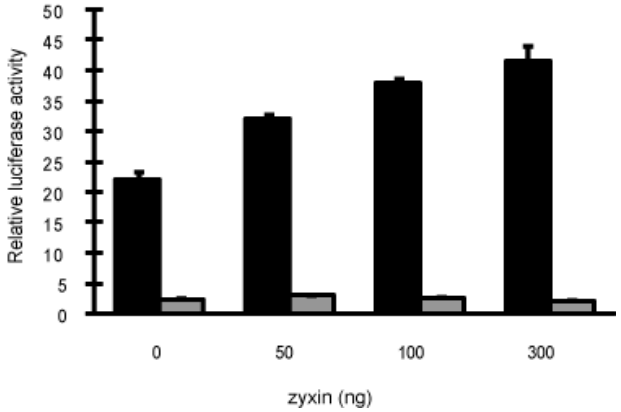
Zyxin has been reported to regulate gene expression by shuttling into the nucleus [165,167]. We studied whether the interaction with zyxin affects the transcriptional activity of HNF-1β. Previously, we reported that HNF-1β regulates *PKHD1* gene expression by directly binding to the promoter region. *PKHD1* is a known target gene of HNF-1β in the

kidney. A reporter plasmid containing 1.9kb of the *PKHD1* promoter driving expression of a luciferase reporter gene was co-transfected with either HNF-1β or zyxin, or both HNF-1β and zyxin, and luciferase activity was measured. As shown in Fig 3*A*, when both HNF-1β and zyxin were co-transfected, *PKHD1* promoter activity was more than in HNF-1β transfected cells. However, when only zyxin was transfected, promoter activity wasn't changed, indicating that zyxin stimulates *PKHD1* promoter activity via HNF-1β. To confirm these results, luciferase reporter gene assays were performed in mIMCD3 cells that endogenously express HNF-1β. As shown in Fig 3*B*, transfection of increasing amounts of zyxin plasmid produced a dose-dependent increase in *PKHD1* promoter activity. However, the activity of a mutant *PKHD1* promoter containing mutations of the HNF-1β-binding site was not increased, indicating that zyxin potentiated the transcriptional activity of HNF-1β. Previous studies showed that the C-terminal transactivation domain of HNF-1β interacted with the coactivator, CBP. Therefore, we examined whether zyxin is contained in a transcriptional complex with CBP and HNF-1β. As shown in Fig 3*C*, the coactivator CBP was detected in immunoprecipitates pulled down by anti-zyxin antibody (lane 2, left panel), indicating that zyxin was present in a transcriptional complex with HNF-1β and CBP in kidney epithelial cells.

A


Relative luciferase activity
30
25
20
15
10
5
0
* *
*
*
pCDNA
HNF1β
zyxin
HNF1β+zyxin

B


Relative luciferase activity
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45
40
35
30
25
20
15
10
5
0
0
50
100
300
zyxin (ng)

C

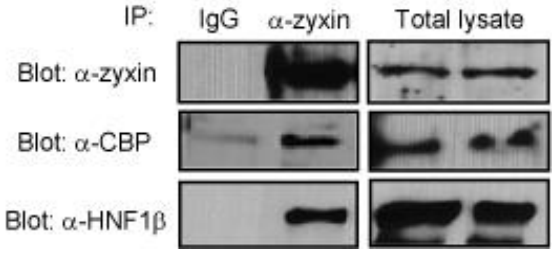
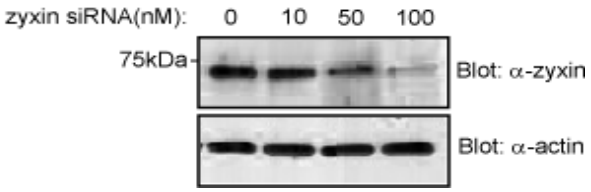

IP:
IgG
α-zyxin
Total lysate
Blot: α-zyxin
Blot: α-CBP
Blot: α-HNF1β

Figure 2-3. Zyxin potentiates the transactivation activity of HNF-1β.
Luciferase reporter gene assays were performed using *PKHD1* promoter-driven luciferase reporter plasmid. (A) HeLa cells were cotransfected with increasing amount of zyxin (0, 50, 100, and 300ng) and a *PKHD1* promoter reporter plasmid. Luciferase activity was increased in cells transfected with both HNF1β and zyxin but not zyxin alone in HeLa cells. (B) mIMCD3 cells were cotransfected with HNF-1β alone, zyxin alone, or both of HNF-1β and zyxin, and a *PKHD1* promoter reporter plasmid. Luciferase activity was measured 48h after transfection. Luciferase activity was increased in mIMCD3 cells transfected with zyxin in a dosage dependent manner in mIMCD3 cells. Results represent three independent experiments. Error bars indicate SD. *, ** indicates $P < 0.05$. (C) zyxin was immunoprecipitated with anti-zyxin antibody and bound products were detected by western blotting with either anti-HNF-1β or anti-CBP antibody in mIMCD3 cells.

To address whether zyxin is required for HNF-1β-mediated gene expression, we used siRNA to ablate the expression of zyxin. Transfection of mIMCD3 cells with siRNA against zyxin caused a substantial decrease in the protein levels of zyxin (Fig 4*A*). The activity of the *PKHD1* promoter in reporter gene assays was then examined. Treatment of siRNA against zyxin reduced *PKHD1* promoter activity (Fig 4*B*). To confirm that zyxin is involved in the regulation of transcription activity of HNF-1β, we tested whether the expression of an HNF-1β target gene, *PKDH1*, is affected by changes in zyxin levels. Control siRNA and zyxin siRNA were transfected into mIMCD3 cells and then mRNA was isolated. mRNA from these cells was reverse-transcribed and then analyzed by quantitative real-time PCR using primers that amplify *PKHD1*. As shown in Fig 4*C*, knockdown of zyxin reduced the expression of the *PKHD1* gene indicating that zyxin is involved in HNF-1β target gene expression. Collectively, these loss-of-function

approaches complement the overexpression studies and demonstrate that zyxin plays a role in HNF-1β-mediated transcriptional activation.

A



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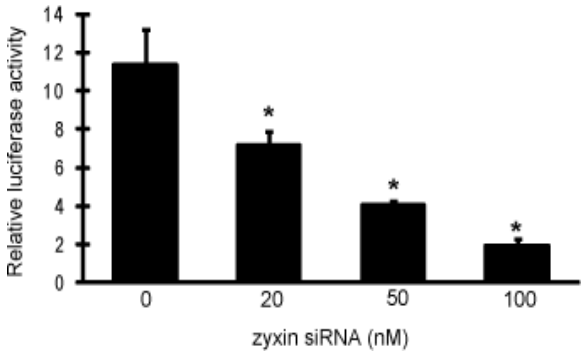

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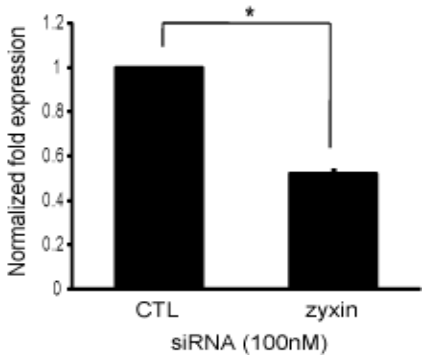
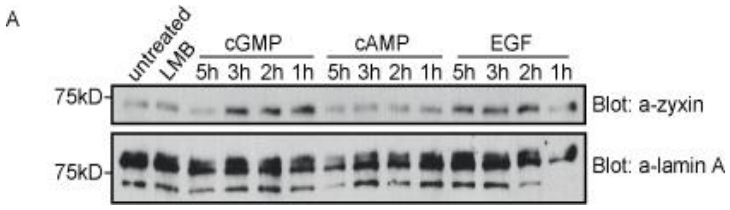


Figure 2-4. siRNA-mediated knockdown of zyxin reduces transcriptional activity of HNF-1β.
mIMCD3 cells were transfected with either control siRNA or zyxin siRNA and cells were subjected to both luciferase assay and western blot analysis. (A) Cell lysates subjected to luciferase assay were analyzed by western blotting with anti-zyxin antibody and anti-α-actin antibody. (B) IMCD3 cells were transfected with siRNA targeting zyxin. After 24h, cells were transfected with *PKHD1* promoter-driven luciferase reporter and then 36h later, luciferase activity was measured. (C) mIMCD3 cells were transfected with control siRNA or zyxin siRNA and 60h later mRNA level of *PKHD1* was quantified by real time RT-PCR. Error bars indicate SD. * indicates $P < 0.05$.

It has been reported that changing the subcellular localization of zyxin might affect cell migration, cell death, proliferation, and gene regulation [80,87,165-172]. Recent studies have shown that the translocation of zyxin into the nucleus is mediated by cGMP, and nuclear accumulation of zyxin was induced by growth factors, NGF and EGF [167,169]. To identify factors that induce the translocation of zyxin into the nucleus in kidney epithelial cells, mIMCD3 cells were treated with cGMP, cAMP, or EGF, and cells were separated into nuclear and cytosolic fractions and subjected to western blot analysis. As shown in Fig 5*A*, zyxin was detected in nuclear extracts derived from cells treated with cGMP and EGF but not cAMP. EGF and EGFR are highly expressed in embryonic kidney and are involved in branching morphogenesis of several tubular organs including kidney. Recent studies also demonstrate their significant role in renal cyst formation in both murine and human ADPKD and ARPKD [173-177]. We examined whether EGF affects zyxin-dependent transcriptional activation. As shown in Fig 5*B*, *PKHD1* promoter activity was increased in EGF-treated cells. Moreover, when cells were transfected with

zyxin, promoter activity was further increased. It has been reported that Akt phosphorylates zyxin, and phosphorylated zyxin is located in the nucleus. Therefore, we examined whether EGF-mediated translocation of zyxin into the nucleus depends on Akt activation. Phosphorylated Akt was detected in cells treated with EGF (Fig 5*C*, lane 2, 3, and 4) and phosphorylation of Akt was blocked in cells pretreated with LY294002, a PI3K inhibitor (Fig 5*C*, lane 6, 7, and 8), indicating that EGF induces phosphorylation of Akt in kidney epithelial cells. To determine whether activation of Akt is important for the translocation of zyxin into the nucleus, mIMCD3 cells and LY294002-pretreated cells were incubated with EGF and cells were separated into nuclear and cytosolic fractions. As shown in Fig 5*D*, zyxin was increased in the nuclear fraction of EGF-treated cells (lane 3 and 4). However, in LY294002-pretreated cells, nuclear zyxin was not increased by EGF treatment (lane 7 and 8). Taken together, these results suggest that EGF induces translocation of zyxin into the nucleus, which is mediated by activation of Akt.



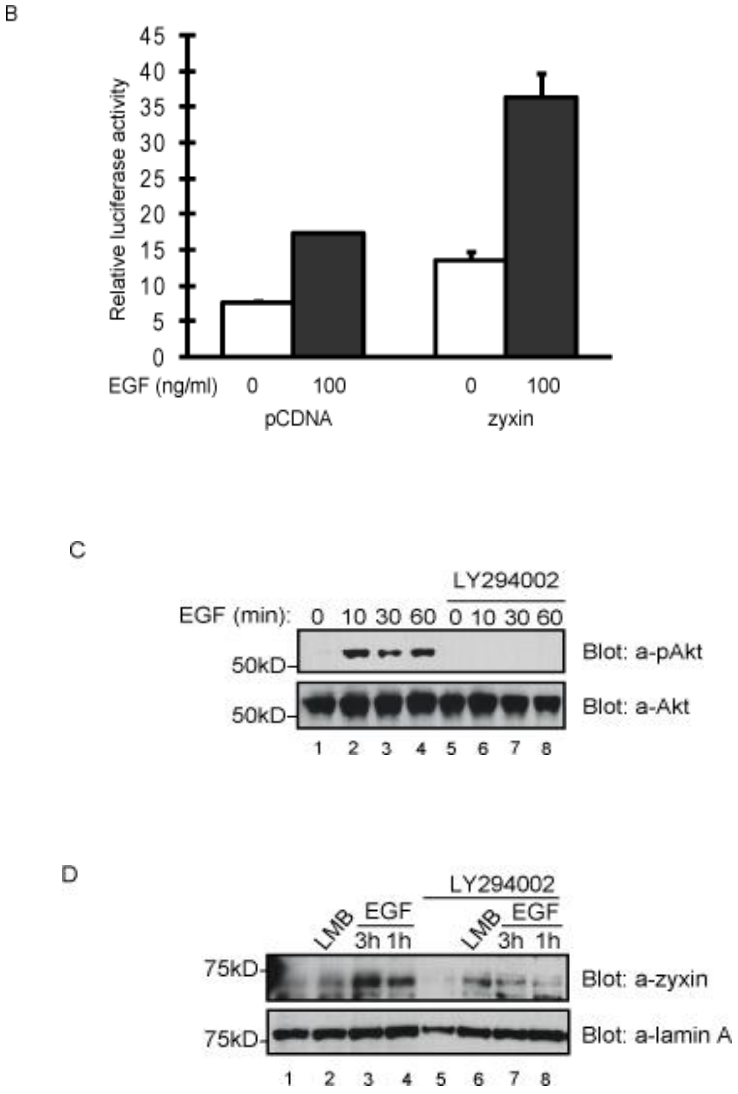


Figure 2-5. Zyxin translocates into the nucleus in response to cGMP and EGF. (A) mIMCD3 cells were treated with cGMP (100nM), dibutylyl-cAMP (100nM), and EGF (100ng/ml) for the indicated time period (1h, 2h, 3h, and 5h). Nuclear extracts were subjected to western blotting with anti-zyxin antibody and anti-laminA antibody. (B) Stably transfected mIMCD3 cells containing a chromosomal integrated *PKHD1*-Gal4-VP16 transgene were transfected with either pcDNA or pCMV-myc-zyxin and Gal4-luciferase reporter plasmid, 24h later cells were treated with EGF (100ng/ml) for 16h,

and luciferase activity was measured 40h after transfection. (C) mIMCD3 cells were incubated in EGF (100ng/ml) for the indicated time period (10min, 30min, and 60min) in the absence or presence of LY294002 (50μM), and cell lysates were subject to western blotting with either anti-Akt or anti-phospho-Akt antibody. (D) mIMCD3 cells were treated with EGF in the absence or presence of LY294002 (50μM). Accumulation of zyxin in nuclear extracts was detected by western blotting with anti-zyxin antibody. Lamin A was used as a nuclear marker protein.

Zyxin knockout mice have no gross abnormality. This result suggests that other zyxin family members, TRIP, LPP, ajuba, and LIMD1, may have overlapping functions that compensate for the absence of zyxin. We also isolated another zyxin family member, called LPP, by yeast two-hybrid screening using HNF-1β as a bait. LPP, Lipoma Preferred translocation Partner, is a member of the zyxin family of LIM domain proteins. LPP contains three LIM domains at its C-terminus, which are preceded by a proline-rich pre-LIM region containing a number of protein interaction domains. LPP has been shown to localize at sites of cell adhesion and shuttle between the nucleus and cytoplasm. Therefore, I examined whether LPP has similar functions to zyxin in regulating HNF-1β function in kidney epithelial cells. I performed co-immunoprecipitation assays to examine the interaction of endogenous proteins. These experiments showed that LPP interacts with HNF-1β in the nucleus of mIMCD3 cells (Fig 2-6).

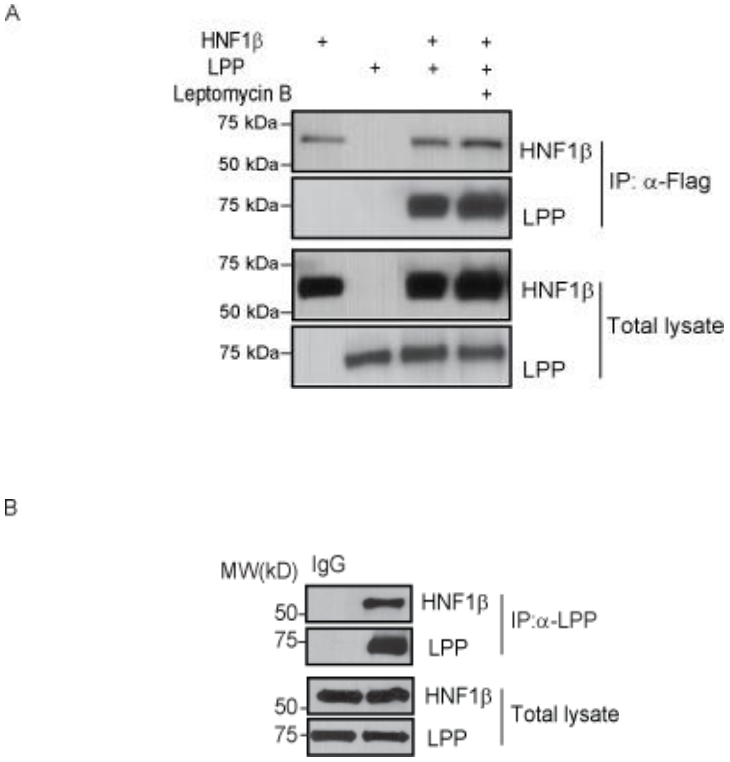
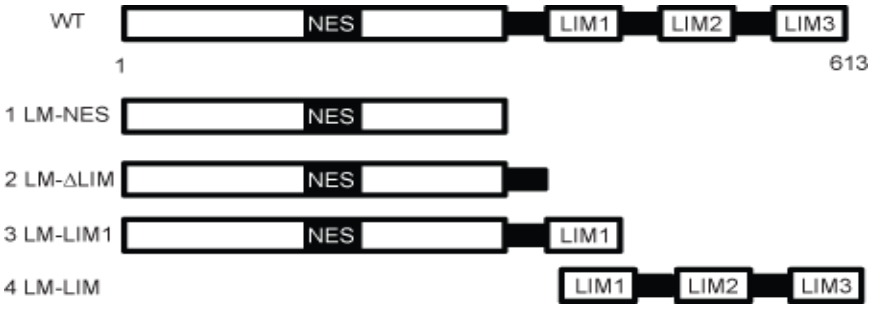


Figure 2-6. LPP interacts with HNF-1β.
(A) HEK293T cells were transiently co-transfected with myc-LPP and Flag-HNF-1β and incubated in the absence or presence of leptomycin B (5h, 20ng/ml). HNF-1β was immunoprecipitated (anti-Flag) and bound products were detected by western blotting for LPP (anti-myc) and HNF-1β (anti-Flag). Input controls are shown on the bottom panels. (B) Endogenous LPP was immunoprecipitated in mIMCD3 cells, and coprecipitated HNF-1β was detected using an anti-HNF-1β antibody.

To identify which domain is important for this interaction, I created deletion mutants of LPP, transfected these with HNF-1β into 293T cells, and performed co-immunoprecipitation assays. These results showed that the LIM domain of LPP is required for the interaction with HNF-1β (Fig 2-7).

A


WT
NES
LIM1
LIM2
LIM3
1
613
1 LM-NES
NES
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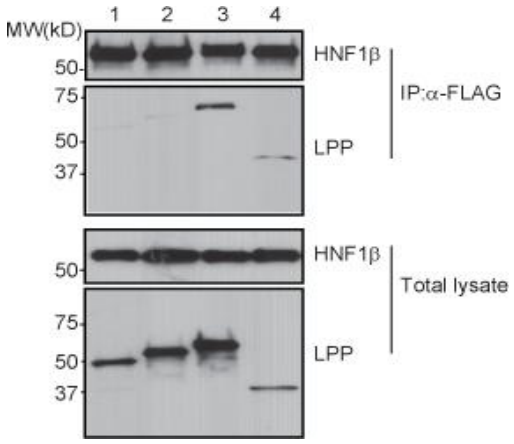
LIM3

B


MW(kD)
1
2
3
4
50
75
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37
HNF1β
IP:α-FLAG
LPP
50
HNF1β
Total lysate
75
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LPP

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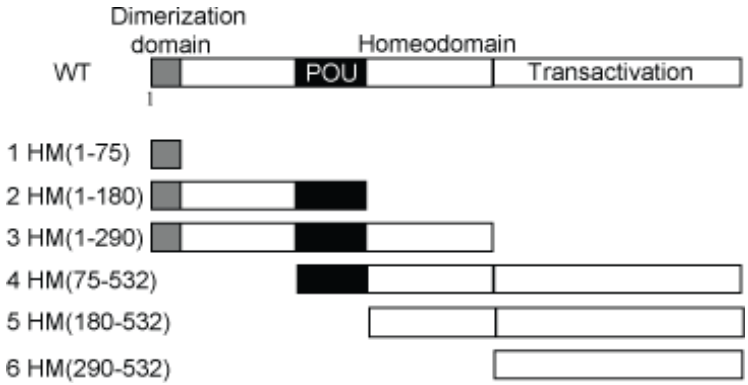

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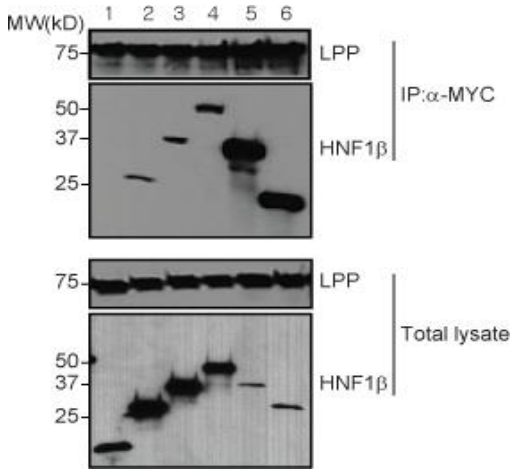


Figure 2-7. Mapping the binding sites of HNF-1β and LPP.
(A) Diagrammatic representation of full- length LPP is shown at the top, and truncated mutants are depicted below. (B) HEK293T cells were co-transfected with pCMV-flag-HNF1β and additionally received the indicated LPP deletion constructs. Cell lysates were subjected to a co-immunoprecipitation assay and the LPP fragments associated with HNF1β were identified by western blotting with anti-myc antibody. The lower panel shows the expression levels of the myc-tagged LPP fragments and flag-tagged HNF1β in total cell lysates. (C) Schematic diagram of HNF-1β deletion mutants. (D) HEK293T

cells were cotransfected with myc-tagged full length LPP and the deletion mutants of HNF-1β. LPP was immunoprecipitated with anti-myc antibody and the HNF-1β fragments bound to myc-LPP were identified by western blotting with anti-flag antibody. Expression levels of LPP and HNF-1β fragments in total lysates are shown in lower panel.

To examine the effect of LPP on the transcriptional activity of HNF-1β, I performed luciferase reporter assays in HeLa cells and mIMCD3 cells. Overexpression of LPP stimulated the activity of the *PKHD1* promoter in both HeLa and mIMCD3 cells, indicating that LPP stimulates the transcriptional activity of HNF-1β (Fig 2-8).

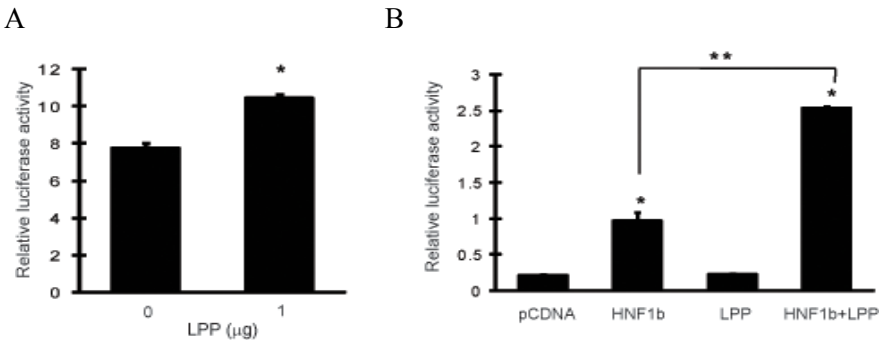
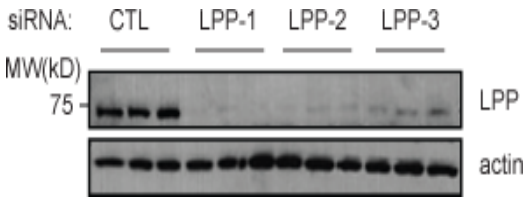


Figure 2-8. LPP potentiates the transcriptional activity of HNF-1β.
Luciferase reporter gene assay was performed using *PKHD1* promoter-driven luciferase reporter. (A) mIMCD3 cells were cotransfected with LPP (1μg) and *PKHD1* promoter plasmid. Luciferase activity was increased in cells transfected with LPP in mIMCD3 cells. (B) HeLa cells were cotransfected with HNF-1β alone, LPP alone, or both HNF-1β and LPP, and the *PKHD1* promoter reporter plasmid. Luciferase activity was measured 48h after transfection. Luciferase activity was increased in cells transfected with both HNF-1β and LPP but not LPP alone. Results represent three independent experiments. Error bars indicate SD. *, ** indicates $P < 0.05$.

I decreased the level of LPP using siRNA and examined the effect on the transcriptional activity of HNF-1β. Luciferase reporter assays showed that the transcriptional activity of HNF-1β was decreased in LPP siRNA-treated cells. I also performed real-time PCR to measure *PKHD1* gene expression in control cells and LPP siRNA-treated cells. *PKDH1* gene expression was reduced in LPP siRNA-treated cells (Fig 2-9). These results show that LPP stimulates the transcriptional activity of HNF-1β.

A



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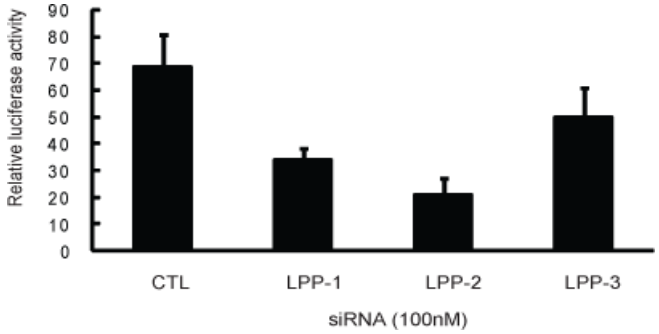

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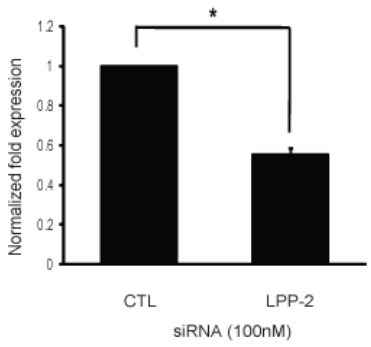


Figure 2-9. siRNA-mediated knockdown of LPP reduces transcriptional activity of HNF-1β.
mIMCD3 cells were transfected with either control siRNA or LPP siRNA and cells were subjected to luciferase assay and western blot analysis. (A) Cell lysates were analyzed by western blotting with anti-LPP antibody and anti-α-actin antibody. (B) IMCD3 cells were transfected with siRNA targeting LPP or random siRNA. After 24h, cells were transfected with *PKHD1* promoter-driven luciferase reporter and 36h later, luciferase activity was measured. (C) mIMCD3 cells were transfected with control siRNA or LPP siRNA and 60h later mRNA level of *PKHD1* was quantified by real time RT-PCR. Error bars indicate SD. * indicates $P < 0.05$.

In summary, another zyxin family protein, LPP, interacts with HNF-1β in the nucleus and has similar functions to zyxin in the regulation of HNF-1β in kidney epithelial cells.

DISCUSSION

Signaling from the cell-matrix interface is important for epithelial polarity, tubular morphogenesis, and directional transport of molecules in kidney tubules [178-182]. Evidence is accumulating that abnormalities of focal adhesion and cell adhesion play important roles in the pathogenesis of polycystic kidney disease. For example, The weakening of focal adhesions caused by the loss of tensin produces renal failure and multiple large cysts in the proximal kidney tubules [178]. Here, we show that focal adhesion proteins, Zyxin and LPP, directly affect gene regulation mediated by HNF-1β in the kidney. We identified focal adhesion components, zyxin and LPP, as novel HNF-1β interacting proteins and found that their second LIM domain was essential for this interaction, which occurred in the nucleus. In addition, we demonstrated that zyxin is present in a transcriptional complex with CBP and stimulates transcriptional activity of HNF-1β. Zyxin shuttles between the cytoplasm and the nucleus where it regulates gene expression [80,167]. Our study shows that zyxin regulates gene expression through physical interaction with the transcription factor HNF-1β in kidney epithelial cells. However, mice that lack zyxin are viable and fertile and do not have any defects in the kidney [183]. Zyxin family members display similar functional domains and subcellular distributions [74,92,183-185]. Among the zyxin family members, two proteins, LPP and TRIP6, are most closely related to zyxin [87,92,183,184], suggesting that they may have functional redundancy.

Both zyxin and LPP were identified as interacting partners of HNF-1β and both stimulate transcriptional activity of HNF-1β in kidney epithelial cells, which sugest that zyxin and LPP may function similarly to regulate gene expression through interactions

with HNF-1β in the kidney. Further studies will be required to determine how these two zyxin family members, zyxin and LPP, regulate the transcriptional activity of HNF-1β in the kidney.

Zyxin acts as a mechanosenor by changing its subcellular distribution, suggesting that zyxin may regulate cell adhesion, spreading, and motility [165,170,171,186,187]. Mechanical force, such as unidirectional cyclic stretch, induces a change in zyxin distribution from focal adhesions to actin filaments [171] or the nucleus [165], suggesting the involvement of zyxin in mechanosensitive gene expression. Although several studies suggested zyxin might be involved in regulating gene expressions in the nucleus, the signals that induce zyxin translocation into the nucleus still remain to be elucidated. It has been reported that Akt-mediated phosphorylation of zyxin forms a complex with acinus to prevent apoptosis, and the complex between zyxin and acinus is induced by growth factors, NGF and EGF [169]. It is possible that EGF may induce the translocation of zyxin into the nucleus. Consistent with this possibility, our results show that EGF induces translocation of zyxin into the nucleus and stimulates the transcriptional activity of HNF-1β. Furthermore, activation of Akt mediated the EGF-induced translocation of zyxin. Epidermal growth factor (EGF) and EGF receptor are highly expressed in the kidney and play a role in branching morphogenesis of tubular organs including lung, mammary gland, and kidney [174]. Furthermore, overactivation of the EGF/EGFR axis contributes to PKD pathophysiology, as EGFR inhibition slows disease progression in ARPKD model. EGF is also increased in the C57BL/6J-cpk mouse, which has an infantile form of polycystic kidney disease (PKD) [175,176,188]. In our study, EGF regulates kidney-specific gene expression controlled by the transcription factor HNF-1β via zyxin translocation. A

previous study showed that HNF-1β regulates tubulogenesis by controlling SOCS-3 gene expression [65]. Our finding identifies a pathway by which HNF-1β regulates tubulogenesis through EGF during kidney development.

In summary, our study identifies zyxin and LPP as HNF-1β binding partners and suggests a novel mechanism for how signals from the cell surface regulate gene expression through a transcription factor that is essential for epithelial differentiation.

MATERIALS AND METHODS

Yeast Transformation and Growth Selection

LexA-HNF-1b fusion vectors were transformed into *S. cerevisiae* (strain L40, genotype *MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::LexA-LacZ*). Yeast were grown in YPDA (2% Difco peptone, 1% yeast extract, 2% glucose, 0.1% adenine hemisulfate) or in synthetic minimal Trp dropout medium (SD-Trp) complemented with 0.1% adenine hemisulfate. For transformation, cultures were grown overnight to an OD_{600} of ~1, centrifuged for 3 min. at 1200× g, washed with 40 mL distilled H_2O, and resuspended in 1 mL H_2O. Aliquots of 50 mL were added to 240 mL PEG 3350 (50% w/v), 50 mL distilled H_2O, 36 mL 1 M LiOAc, 50 mg single-stranded DNA (2.0 mg/ml), and 1 mg plasmid. Proper expression of the bait proteins was confirmed by immunoblotting of total cell lysates with an antibody against LexA. As a control, the absence of autonomous L40 reporter gene activation by the bait proteins was verified.

Library Screening

An adult mouse kidney cDNA library (Matchmaker, Clontech) was used. 100 mL SD-Trp medium was inoculated with *S. cerevisiae*, grown to $OD_{600} > 2$ at 30°C and used to inoculate 1 L YPDA. The culture was further grown to an OD_{600} of 0.7 divided in fourths and pelleted at 4200× g for 15 min at 4°C. The pellets were washed with distilled H20 and transfected with 50 mg of cDNA (1 mg/mL). After incubation at 30°C followed by heat shock at 42 °C for 30 min, cells were harvested by centrifugation at 1900 × g for 3

min, washed with 80 mL distilled H_2O, resuspended in 20 mL distilled H_2O, and plated in 50 14-cm Petri dishes. After 3 days, permeabilized cells were transferred onto Whatman filters and overlaid with 0.2 mg/mL X-gal; 50 mM Tris-HCl pH 7.4;150 mM NaCl; 0.8% agarose. X-gal positive colonies were restreaked in selective medium and rescreened by colony-lift assays. Yeast DNA was isolated from positive clones, and prey plasmids were isolated by transformation into KC8 cells. Prey plasmids were digested with *Bgl*II to determine insert size and sequenced.

DNA Constructs, Antibodies, and Reagents

The full length protein and deletion mutants of zyxin were cloned into pCMV containing MYC tag by PCR. Deletion mutants of HNF1β were produced by PCR amplication from full length HNF1β and cloned into pCMV-Flag vector (Sigma).

Cell Culture, DNA Transfections, and Reporter Gene Assays

HEK293 cells, HeLa cells, and mIMCD3 cells were routinely cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 37 °C incubator with 5.0% CO_2. Transfection was performed in 10% serum conditions using Effectene (Qiagen) following the manufacturer's instructions. Cells were cotransfected with 20 ng pRL plasmid encoding *Renilla* luciferase to control for differences in transfection efficiency. After growth for 48 hours, the cells were lysed in 500 µl passive lysis buffer (Promega Corp., Madison, Wisconsin, USA), freeze-thawed once, and centrifuged. Supernatants (20 µl) were added to 96-well plates, and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega

Corp.), according to the manufacturer's directions. Luciferase Assay Reagent II (100 µl) was added, and light output was measured for 10 seconds using a Wallac VICTOR V multilabel counter (Perkin Elmer, Wellesley, Massachusetts, USA). Firefly luciferase activity was normalized to *Renilla* luciferase activity, which was measured by adding 100 µl of Stop & Glo reagent and measuring light output for 5 seconds.

Immunoprecipitation, Western Blot Analysis, and cell fractionation

Cells were lysed with IP buffer (50M Tris-cl, 150mM NaCl, 5mM EDTA, 0.1% Triton X-100, 0.1% Deoxycholicacid, 100mM PMSF, Protease inhibitor cocktail from Roche). Extracts were clarified by centrifugation 12000rpm for 1 min. For each IP, cell extract proteins were mixed with primary antibody or IgG at 4℃ for overnight, and then incubated with 20 ml protein AG/slurry (1:1, v/v) for 1h with gentle rotation at 4℃. Immunoprecipitates were washed three times with IP buffer and boiled in SDS-loading buffer. After SDS-PAGE, under reducing conditions, proteins were transferred to nitrocellulose ECL membrane and subjected to Western Blot analysis with ECL detection reagent (PIERCE). Primary antibodies used were follows: zyxin, Lamin A, α-Actinin (Santa Cruz), HNF1b( ), FLAG, MYC (Sigma), and Akt, p-Akt (Cell signaling Inc)

siRNA Transfection

Short interfering RNA (siRNA) oligonucleotides against zyxin and control siRNA oligonucleotides were purchased from Santacruz Biotechnology, Inc (Santa Cruz, CA). mIMCD3 cells were transfected with zyxin siRNA (10, 50, and 100 nM) using Lipofectamine 2000 reagent (invitrogen). Endogenous expression level of zyxin was determined by western blotting with anti-zyxin antibody.

RT-PCR

Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen). For RT-PCR, cDNAs were synthesized using the Super ScriptTM first strand synthesis system (Invitrogen) and then amplified by PCR with PKHD1 primers

Real time PCR

Real-time PCR was performed in triplicate using iCycler and SYBR green Supermix reagents (Bio-Rad Laboratories). β-2 microglobulin was used as the control gene for normalization. Collected data were analyzed using IQ5 software (Bio-Rad Laboratories).

Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde and washed with PBS. The cells were incubated with a primary antibody against FLAG (Sigma) and HNF1b (SantaCruz) and then incubated with secondary antibodies. Secondary antibodies were conjugated to Alexa Fluor 594 (Molecular Probes Inc, Eugene, OR) and Alexa Fluor 488. Signals were visualized and captured using a fluorescent microscope.

Statistical Analysis

Statistical analysis was performed by using Student's *t* tests with Dunnett's correction for multiple comparisons where applicable. *P* values <0.05 were considered significant.

CHAPTER THREE

IDENTIFICATION OF A cAMP-REGULATING PROTEIN COMPLEX IN PRIMARY CILIUM REVEALS A COMMON MECHANISM DISRUPTED IN POLYCYSTIC KIDNEY DISEASES

Introduction

Primary cilia are highly structured organelles that are found in many organisms and on most mammalian cells. Cilia are structurally maintained by intraflagellar transport (IFT), which carries structural components from the cell body to the ciliary tip (the anterograde direction) because protein synthesis is not known to occur in the cilium [189]. Anterograde IFT is mediated by the heterotrimeric motor protein, Kinesin-2, which is composed of three subunits, Kif3a, Kif3b, and KAP3[190]. Renal cilia project into the tubule lumen and respond to fluid flow or mechanical bending by stimulating an increase in intracellular Ca^{2+}, suggesting that cilia have a mechanosensory function [191]. PC1 and PC2 proteins mutated in autosomal dominant PKD (ADPKD), are required for cilium-mediated flow sensing [192,193]. In addition to their role in mechanosensation, primary cilia may coordinate various signaling pathways including Wnt, Hh, and mTOR signaling pathways [194-198]. Disruption of cilia function is involved in multiple human syndromes collectively called ciliopathies [189]. In the kidney, structural and functional defects in primary cilia play a major role in pathogenesis of renal cystic diseases. Furthermore, mutations of the ciliary genes, PKD1, PKD2, and PKHD1, have been identified in human PKD. Kidney-specific inactivation of Kif3a results in the absence of primary cilia and

development of renal cysts [199]. Similarly, mutation of a subunit of IFT particles, Tg^{737}/Polaris, produces cysts in the kidney [200,201].

In the kidney, HNF-1β is expressed in epithelial cells composing the renal tubules and collecting ducts [8]. Studies using Xenopus embryos have shown that HNF-1β plays a key role in normal kidney development [40]. Mutations of HNF-1β are the cause of maturity-onset diabetes of the young type 5 (MODY5) which is characterized by early onset diabetes and congenital cystic abnormalities of the kidney including simple cysts, multicystic dysplasia, and glomerulocystic kidney diseases [33,34,202]. Kidney specific HNF-1β inactivation and overexpression of dominant-negative mutant HNF-1β in mice lead to a drastic form of polycystic kidney disease (PKD) [63,64]. Therefore, HNF-1β plays an important role not only in kidney development but also in renal cystic diseases.

The intracellular second messenger, cAMP plays a central role in cystogenesis by stimulating fluid secretion and cell proliferation [113,114,203-205]. Organ culture studies have shown that treatment of cultured kidney, with execess Br-cAMP or the cAMP agonist, forskolin, induces the formation of cysts in proximal tubules and collecting ducts [206]. Furthermore, in the *Pkd1* -/- embryonic kidney, Br-cAMP induces the rapid formation of large cysts in proximal tubules and collecting ducts [206]. cAMP levels are elevated in some animal models of PKD such as pcy mice, Pck rats, and Pkd2 mutant mice [117,118]. Torres *et al* have shown that a vasopressin receptor (V2) antagonist is effective in an animal model of PKD via reduction of renal cAMP levels [118]. In ADPKD and ARPKD cells, an elevation of Ca^{2+} is able to restore the anti-mitogenic response to cAMP [198], supporting the tight link between Ca^{2+} and cAMP in PKD.

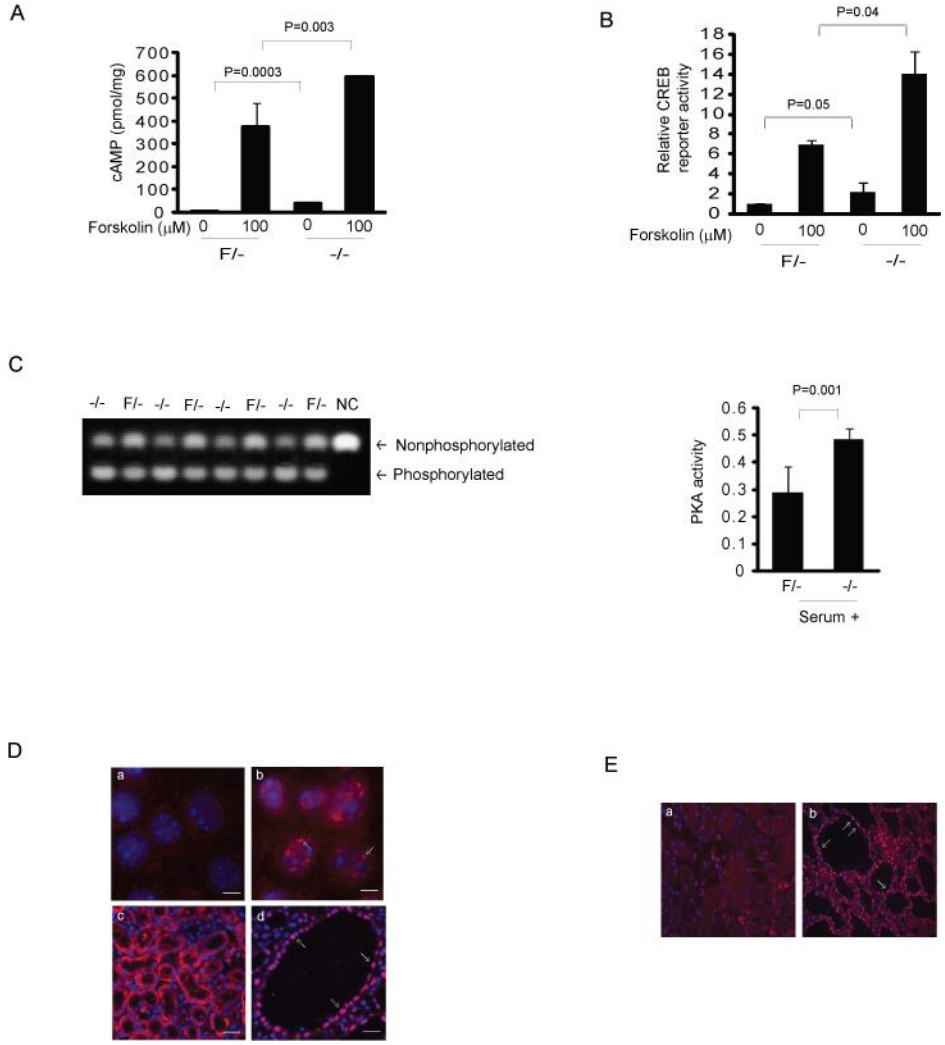
cAMP signals can be compartmentalized in subcellular regions creating microdomains of second messengers within cells [207-209]. The soluble second messenger, cAMP, segregates in cellular microdomains where it locally activates effector proteins such as PKA, phosphodiesterases (PDEs), cAMP-dependent guanine nucleotide exchange factors (Epacs), and adenyly cyclases (ACs) [207,210]. A-kinase anchoring proteins (AKAPs) tether these effector proteins with their downstream targets to facilitate the relay of compartmentalized cAMP signals [110,111]. cAMP is synthesized by AKAP-adenylyl cyclase (AC) complexes and degraded by AKAP-PDE complexes [112]. In the kidney, receptor-mediated agonists that activate adenylyl cyclase and inhibitors of phosphodiesterase accelerate the rate of renal enlargement in PKD [117,211-213].

In this report, we generated Kif3a null cells that lack primary cilia to investigate the mechanism by which primary cilia play a role in cAMP signaling and pathogenesis of PKD. We identified an AKAP150 complex containing AC5/6, PDE4C, and PKA in primary cilia of kidney epithelial cells. PC2 interacts with the ciliary complex, and the function of PC2 as a Ca^{2+} channel is essential for the regulation of cAMP levels.

Results

cAMP signaling is activated in Kif3a null tubular epithelial cells – Previous studies have shown that kidney-specific inactivation of the Kif3a subunit of kinesin II abolishes ciliogenesis and produces polycystic kidney disease [199]. Although loss of primary cilia produces renal cysts, the mechanism by which loss of primary cilia develops cysts is unclear. We generated a Kif3a null renal tubule cell line to study the function of renal primary cilia. cAMP levels are elevated in some animal models of PKD such as pcy mice, Pck rats, and Pkd2 mutant mice [117,118]. Furthermore, excess cAMP causes cyst formation in cultured embryonic kidney [206], indicating that cAMP stimulates cyst formation in the kidney. Therefore, we examined the levels of cAMP in the absence of primary cilia. cAMP levels in kif3a null cells were 10-fold higher than in $Kif3a^{F/-}$ cells (Fig. 1A). Consistent with this result, CREB (cAMP responsible element binding protein) reporter was activity increased in Kif3a null cells compared with in $Kif3a^{F/-}$ cells (Fig. 1B). When cells were treated with forskolin for 30 min, the levels of cAMP and CREB reporter activity were increased more in Kif3a null cells than in $Kif3a^{F/-}$ cells (Fig. 1A, B). PKA activation was measured using the PKA substrate, Kemptide. Phosphorylation of Kemptide was increased in kif3a null cells compared with $kif3a^{F/-}$ cells (Fig. 1C). Phosphorylated PKA substrates accumulated in nuclei of kif3a null cells, whereas they were not observed in nuclei of control cells (Fig. 1D). In the kidney, phosphorylated PKA substrates accumulated in nuclei of $kif3a^{F/-};Cre^{ksp}$ mice. However, they were localized in cytoplasm of $kif3a^{F/-}$ mice (Fig. 1D), suggesting that cAMP-dependent gene expression may be altered in kif3a deficient cells and mice. Increased phospho-CREB positive cells were observed in cystic kidneys from $kif3a^{F/-};Cre^{ksp}$ mice compared to control mice (Fig

1E). Most cystic epithelial cells were phospho-CREB positive (Fig 1Eb), indicating that cAMP signaling is highly activated in *kif3a*$^{F/-}$*;Cre*^{ksp} mice that develop renal cysts. Taken together, these results indicate that kif3a null cells that lack primary cilia contain increased levels of cAMP and show highly activated cAMP signaling, suggesting that cAMP levels may be regulated by primary cilia.



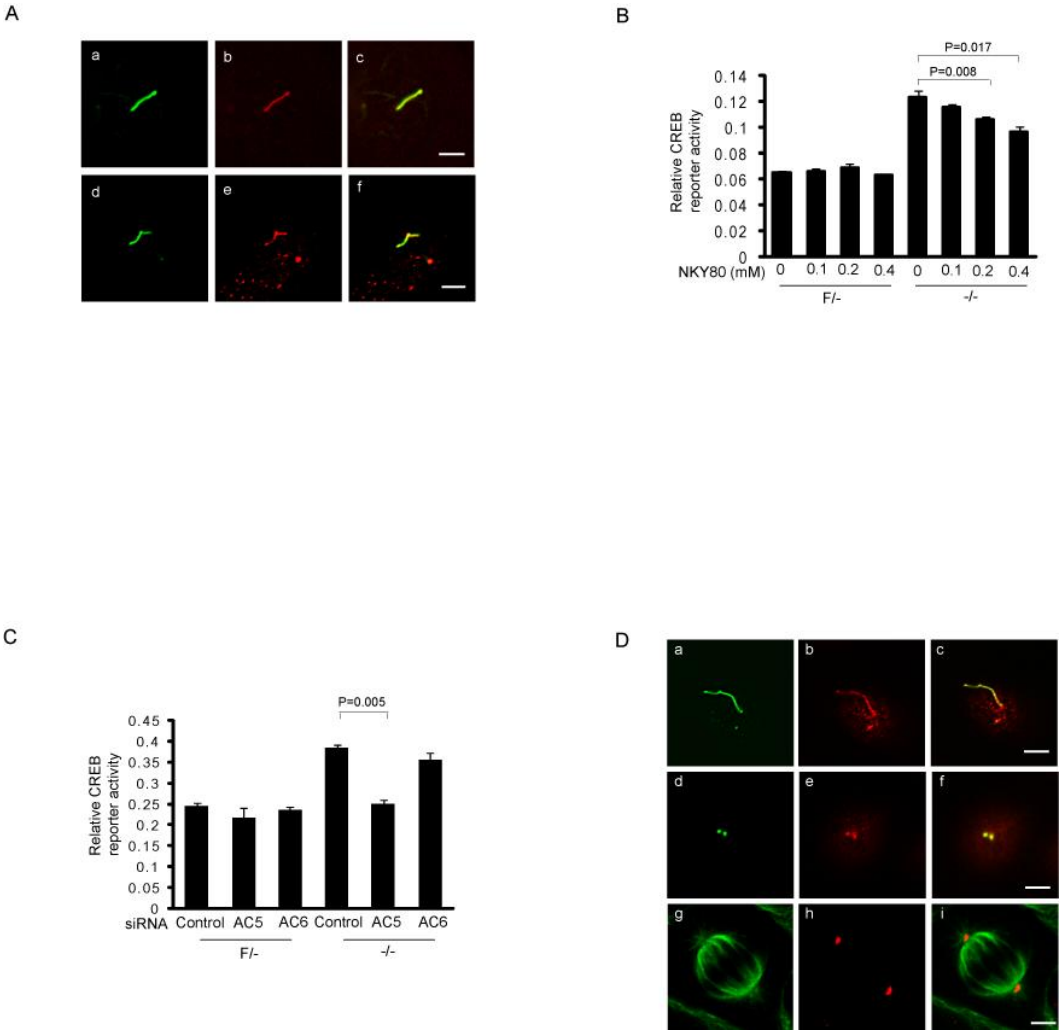
C, D, and E were performed by Akira Suzuki

Figure 3-1. cAMP signaling is activated in Kif3a null cells.
(A) cAMP was measured using an enzyme immunoassay in Kif3a null cells and control cells. Cells were treated with forskolin for 30 min and then cAMP levels were measured. (B) Cells were transfected with a CREB reporter plasmid and pRL-CMV plasmid and then treated with forskolin for 7hrs. CREB reporter activition was measured using dual-luciferase assay system. (C) PKA activity was measured using the fluorescently-labeled PKA substrate, Kemptide. The phosphorylated products were separated from the non-phsophorylated substrate by agarose electrophoresis (left). The phosphorylated bands were excised, and the fluorescence at 570nm was measured (right). Kif3a null cells showed 67% higher PKA activity (n=4). (D) Kif3a F/- and Kif3a -/- cells were stained with anti-PKA substrate antibody (a, b). The phosphorylated PKA substrates shown in red accumulated in nuclei of kif3a null cells (b) but not in control cells (a). *In vivo*, the phosphorylated PKA substrates shown in red also accumulated in nuclei of $Kif3a^{F/-};Cre^{ksp}$ mice (d), but not in $Kif3a^{F/-}$ mice (c). (E) $Kif3a^{f/-};Cre^{ksp}$ kidney and wild type mouse kidney were stained by phospho-CREB antibody. Nuclei were stained by DAPI. Scale bars; 10 μm (a, b) and 20 μm (c, d). Error bars indicate SD. Statistical analysis was done using the Student's t-test.

Adenylyl cyclase 5/6 and AKAP150 are localized in renal primary cilia and AC5/6 is involved in the regulation of cAMP levels in renal epithelial cells - Masyuk *et al*. showed that primary cilia in cholangiocytes contain PC1, PC2 and AC6 [214]. Adenylyl cyclase type 5 and 6 are both Ca^{2+} - and G_i-inhibitable and share most, if not all, biological properties [215]. We examined the subcellular localization of AC 5/6 in $Kif3a^{F/-}$ cells, and Kif3a null cells. In wild-type cells, AC5/6 co-localized with acetylated tubulin which was used as a marker for the primary cilium (Fig. 2A a-f). In Kif3a null cells, cilia and ciliary expression of AC5/6 were absent. AC5/6 was located in the plasma membrane

in Kif3a null cells (data not shown). Next, I generated mIMCD3 kidney epithelial cells that stably expressed either flag-tagged AC5 or flag-tagged AC6. Consistent with the endogenous staining results, flag-tagged AC5 and flag-tagged AC6 were localized in the plasma membrane and primary cilia. To determine whether AC5 or AC6 were responsible for the high levels of cAMP in Kif3a mutant cells, the effects of an AC5 inhibitor, NKY80, were examined. Treatment with NKY80 produced a dose-dependent decrease in CREB reporter activity. In contrast, CREB reporter activity was not affected by treatment with NKY80 in $Kif3a^{F/-}$ cells (Fig. 2B). To confirm these results, expression of AC5 was reduced by siRNA treatment. Knockdown of AC5 decreased CREB reporter activity in Kif3a null cells, but knockdown of AC6 had no effect (Fig 2C). These results indicate that AC5 is involved in increasing cAMP levels in Kif3a null cells.

The specificity of cAMP signaling within cells is accomplished by compartmentalization of the signaling components. AKAPs, A kinase anchoring proteins, form local signal transduction units by tethering different enzymes and adapter molecules [216,217]. AKAP complexes function as molecular relays that generate spatial and temporal cAMP signals [217]. Bauman *et al* reported that AKAP79/150 directly interacts with AC5/6, and knockdown of AKAP79/150 results in higher concentrations of cAMP [218]. Since AC5/6 was localized in primary cilia, the subcellular localization of AKAP150 was examined. We found that AKAP150 was localized in primary cilia (Fig.2Da-c). AKAP150 was also localized in basal bodies (Fig 2Dd-f), mitotic spindle (Fig 2Dg-i), and plasma membrane (data not shown). Kif3a null cells lack expression in primary cilia, but express AKAP150 in basal bodies, spindle poles and plasma membrane (data not shown). These results indicate that the AKAP79/150 complex is located in renal primary cilia.



By Akira Suzuki

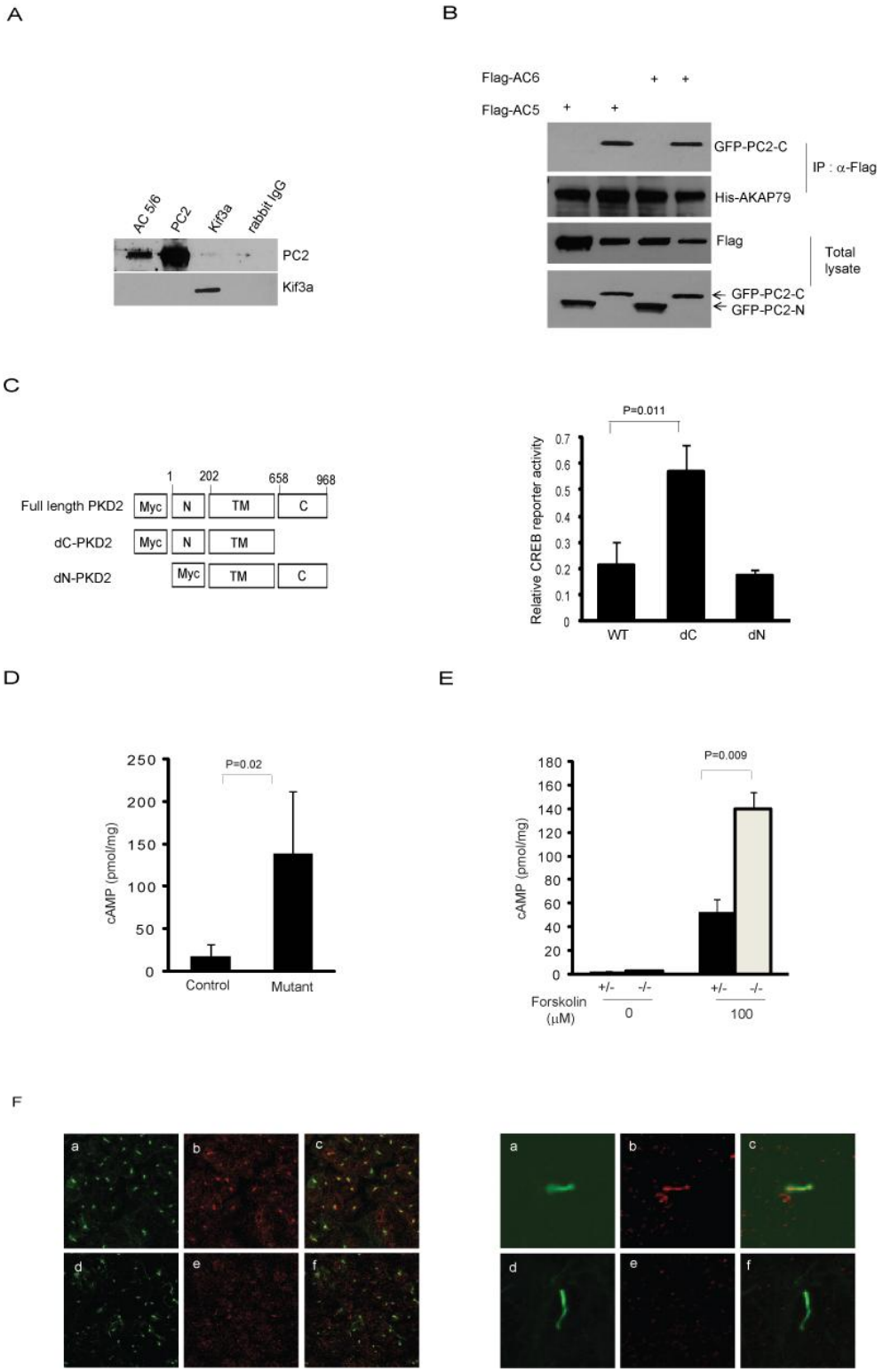
Figure 3-2. Adenyly cyclase 5/6 and AKAP150 are present in primary cilia and AC5 is involved in upregulation of cAMP in kif3a null cells.

(A) The subcellular localization of adenylyl cyclase (AC) 5 and 6 were examined in kif3a null cells and control cells. AC6 (red, b) co-localized with acetylated tubulin (green, a). AC6 co-localized with acetylated tubulin in primary cilia (c) in control cells. AC6 was observed in just plasma membrane in Kif3a null cells (data not shown). An antibody that recognizes both AC5 and AC6 stained in primary cilia (e). AC5/6 was only observed in

plasma membrane in Kif3a null cells (data not shown). Scale bars; 5μm (B) Kif3a F/- and Kif3a -/- cells were co-transfected with CREB-reporter plasmid and pRL-CMV plasmid and then treated with the indicated amount of NKY80. Luciferase activity was measured. (C) Kif3a F/- and Kif3a -/- cells were transfected with siRNA to AC5 and AC6 as well as scrambled siRNA (control). 24hrs after transfection, cells were transfected with CREB reporter plasmid and then 36hrs later, luciferase activity was measured. (D) mIMCD3 cells were co-stained with anti-AKAP150 antibody (red) (b, e, h) and either anti-acetylated tubulin (green) (a), anti-γ-tubulin antibody (green, d), or anti-α-tubulin antibody (green, g). AKAP150 is observed in primary cilia (c), basal bodies (f), and spindle poles (i). Error bars indicate SD. Statistical analysis was performed using ANOVA and Dunnett's Multiple Comparison Test.

Polycystin-2 interacts with the AKAP150 complex and is essential for the regulation of cAMP levels - Since AC5/6 are known to be Ca^{2+}-inhibitable enzymes, we tested the hypothesis that polycystin-2 (PC2/PKD2), a calcium-permeable cation channel, may be involved in the regulation of cAMP through the AKAP150 complex. Indeed, PC2 mutant mice show high cAMP concentrations in the kidney and reduction of cAMP ameliorates the cystic phenotype [118]. The endogeneous interaction among AC5/6, PC2, and Kif3a was examined in mIMCD3 kidney epithelial cells. Cell lysates were immunoprecipitated with anti-AC5/6, PC2, or Kif3a antibody and then immune complexes were blotted with either anti-PC2 or Kif3a antibodies (Fig. 3A). The results indicated that PC2 interacts with AC5/6 but not kif3a in mIMCD3 kidney epithelial cells. To identify the domain of PC2 that interacts with the AKAP79/150 complex, we generated GFP fused C-terminal (GFP-PC2-N) and N-terminal (GFP-PC2-C) fusion proteins of PC2. The fusion proteins were transiently overexpressed with His-tagged AKAP79 together with either flag-tagged AC5 or AC6 in HEK293 cells. Immune complexes precipitated with anti-flag antibody

contained his-tagged AKAP79, flag-tagged AC5/6, and GFP-PC2-C (Fig. 3B). No coprecipitation of GFP-PC2-N was dectected. These results indicate that the C-terminus of PC2 is required for the interaction with the AKAP79/150 complex. To test the functional relevance of the interaction between PC2 and the AKAP150 complex, two deletion mutant PC2s were generated, and CREB reporter activity was measured (Fig. 3C, left panel). The C-terminus-truncated PC2 activated CREB reporter activity, whereas full length and N-terminus-truncated PC2 showed no significant activation (Fig. 3C, right panel). These results suggest that the interaction of PC2 with the AKAP150 complex is critical for the regulation of cAMP levels. cAMP was measured in the absence of PC2 in both kidney cells and tissue. cAMP was increased in the kidney from kidney-specific PC2 knockout mice (P21, n=3) (Fig 3D). The levels of cAMP were increased by forskolin treatment in both PC2 +/- and PC2 -/- cells. However, PC2 -/- cells showed much higher levels of cAMP compared to PC2 +/- cells (Fig. 3E). Taken together, these results demonstrate that PC2 is required to regulate the levels of cAMP in the kidney. PC2 +/- and PC2 -/- cells were immunostained with anti-AC5/6 together with anti-acetylated tubulin antibody. Acetylated tubulin staining shows that both wild-type and mutant cells have primary cilia (Fig. 3Fa, d). AC5/6 were localized in primary cilia in PC2 +/- cells (Fig. 3Fb). However, PC2 null cells did not show AC5/6 in primary cilia (Fig. 3Fe). To determine whether AC5/6 were downregulated or mislocalized in PC2 -/- cells, we measured AC5/6 gene expressions (Fig. 7C). Expression of AC5 was downregulated and AC6 was upregulated in PC2 -/- cells. These results suggest that both gene expression and ciliary localization of AC5 are decreased in the absence of PC2 in kidney epithelial cells.


A
AC 5/6
PC2
Kif3a
rabbit IgG
PC2
Kif3a
B
Flag-AC6
Flag-AC5
GFP-PC2-C
His-AKAP79
IP : α-Flag
Flag
Total lysate
GFP-PC2-C
GFP-PC2-N
C
1
202
658
968
Full length PKD2
dC-PKD2
dN-PKD2
Myc
N
TM
C
P=0.011
Relative CREB reporter activity
WT
dC
dN
D
P=0.02
cAMP (pmol/mg)
Control
Mutant
E
P=0.009
cAMP (pmol/mg)
Forskolin (μM)
0
100
F
a
b
c
d
e
f

Panels A, B, and C were performed by Akira Suzuki

Figure3- 3. C-terminus of Polycystin-2 (PC2) interacts with Adenylyl cyclase 5/6 and regulates cAMP signaling.
(A) Cell lysates were immunoprecipitated with anti-AC5/6, anti-PC2, or anti-Kif3a antibodies. Immune complexes were immunoblotted with anti-PC2 and anti-Kif3a antibodies. (B) pEGFP-PC2-N and pEGFP-PC2-C were co-transfected with either pCMV-flag-AC5 or AC6 together with pCMV-His-AKAP150 in HEK293 cells. 48hrs after transfection, cells were harvested and cell lysates were immunoprecipitated with anti-Flag antibody. (C) Schematic diagram of mutant PC2 lacking C-terminus (dC) or N-terminus (dN) (left panel). mIMCD3 cells were transfected with full length-PC2, dC-PC2, or dN-PC2 together with CREB-reporter and pRL-CMV plasmids. 48hrs after transfection, CREB reporter activity was measured (n=3, right panel). (D) cAMP was measured in kidney tissues from control and PKD2 knockout mice (P21, n=3). (E) PKD2 +/- and PKD2 -/- cells were treated with forskolin (100 μM) for 30 min. cAMP was measured using an enzyme immunoassay. (F) PKD2 +/- and PKD2 -/- cells were co-stained with anti-acetylated tubulin (green) and anti-AC5/6 antibody (red). AC5/6 was not detected in primary cilia of PKD2 null cells. Statistical analysis was performed using Student's t-test.

The Ca^{2+} channel function of PC2 is essential for the regulation of cAMP levels –

PC2 is expressed in the primary cilia of kidney epithelial cells where it has essential roles in mediating Ca^{2+} entry in response to fluid flow and the activation of subsequent signaling pathways [193,219]. Although all adenylyl cyclase isoforms are inhibited by high concentrations of calcium, AC5 and AC6 can be inhibited by sub-micromolar concentrations of calcium and comprise the calcium-inhibitable family of adenylyl cyclase [220-222]. We examined whether the Ca^{2+} channel activity of PC2 plays a role in regulating cAMP signaling. PKD2 -/- cells were transfected with wild type PC2 or a PC2 mutant, D511V, that blocks Ca^{2+} entry [223]. Protein expression of wild type and mutant PC2 was similar (Fig 4, lower panel). Expression of wild-type PC2 reduced cAMP levels

in PKD2 -/- cells, whereas cAMP levels remained elevated following expression of the D511V mutant (Fig. 4, upper panel). These results indicate that the Ca^{2+} channel function of PC2 is important to regulate cAMP signaling.

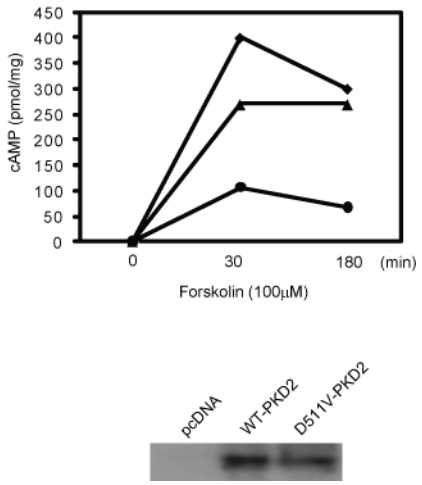


Figure 3-4. The function of PKD2 as a Ca^{2+} channel is important for the regulation of cAMP levels.
PKD2 null cells were stably transfected with wild type PKD2 or D511V mutant PKD2. Cells were treated with forskolin (100μM) for 30 min and 180 min. The levels of cAMP were measured in pCDNA (-■-), WT-PKD2 (-●-), and D511V-PKD2 (-▲-) expressing cells using an enzyme immunoassay (upper panel). Expression of WT-PKD2 and D511V-PKD2 was confirmed by western blotting using an anti-PKD2 antibody (lower panel).

PDE4C is a novel HNF-1β target gene that interacts with the AKAP150 complex in primary cilia - To identify direct target genes of HNF-1β in the kidney, we used ChIP-on-chip combined with cDNA microarray analysis [65]. Using this approach, we identified PDE4C as a novel target gene of HNF-1β. In the ChIP-chip assay, the enrichment of hybridization signals on the PDE4C promoter indicated that HNF-1β binds the promoter (Ma et al, 2007). Analysis of other PDE family members indicated that PDE4C is the only target gene of HNF-1β. Next, we examined the subcellular localization of PDE4C. PDE4C was co-localized with acetylated tubulin in primary cilia of mIMCD3 kidney epithelial cells (Fig. 5Ab). To determine whether PDE4C interacts with the AKAP150 complex, mIMCD3 cells were transfected with pCMV-flag-PDE4C and subjected to immunoprecipitation 48hrs after transfection. The protein complex precipitated by anti-flag antibody contained AKAP150, AC5/6, and PKA-RIIa as well as PDE4C (Fig. 5C). These results suggest that AKAP150 and PDE4C are located in renal primary cilia and form a protein complex to regulate cAMP levels in the kidney. The PDE4C promoter contains two candidate binding sites for HNF-1β located 870-580 bp upstream from the translation start site (Fig. 5D). To confirm that PDE4C is a direct target gene of HNF-1β, we performed ChIP assays using chromatin from mIMCD3 cells. We isolated DNA fragments bound by HNF-1β by immunoprecipitation with anti-HNF-1β antibody and then measured the presence of the PDE4C promoter region by PCR using primers flanking the HNF-1β-binding site (Fig 5H). The PDE4C promoter sequence was enriched by immunoprecipitation with anti-HNF-1β antibody compared with isotype IgG, indicating that HNF-1β binds to the PDE4C promoter region in mIMCD3 cells. We

confirmed this binding *in vivo* by ChIP assay using chromatin from mouse kidney tissue. Consistent with the results obtained in mIMCD3 cells, the PDE4C promoter was enriched by immunoprecipitation with anti-HNF-1β antibody (Fig 5H). These results confirmed that HNF-1β was associated with the PDE4C promoter both *in vitro* and *in vivo*. To determine whether HNF-1β regulates PDE4C expression, we measured the level of PDE4C mRNA in 53A cells that express dominant-negative HNF-1β mutant (HNF-1βΔC) following treatment with mifepristone [22]. Quantitative RT-PCR analysis showed that the expression of the HNF-1βΔC mutant reduced the level of PDE4C mRNA (Fig 5B). Anti-PDE4C antibody staining in DN-HNF1β induced cells showed reduced fluorescence intensity compared to control cells (Fig 5Ab, e, h). Taken together, these results indicate that expression of PDE4C is downregulated in DN-HNF-1β expressing kidney epithelial cells, which suggests that HNF-1β plays a role as a transcriptional activator of PDE4C. To test this possibility, we cloned the mouse PDE4C promoter into the pGL3-Basic plasmid (Fig 5D) and performed reporter gene assays. HeLa cells were transfected with the PDE4C promoter-reporter plasmid and either HNF-1β or control plasmid, and luciferase activity was measured. Luciferase activity was increased in HNF-1β expressing cells (Fig 5E). Next, mIMCD3 cells were co-transfected with the PDE4C promoter reporter plasmid and dominant negative HNF-1β (DN-HNF-1β) mutant plasmid. Luciferase activity was decreased by the HNF-1βΔC mutant (DN-HNF-1β) (Fig 5F). To verify whether the HNF-1β binding sites are functionally important, we mutated each of the consensus binding sites by site directed mutagenesis. Mutation of each site reduced PDE4C promoter activity by approximately 70%, and mutation of both sites

mutation further reduced promoter activity (Fig 5G). Taken together, these results demonstrate that HNF-1β functions as a transcriptional activator of the PDE4C gene by directly binding to the PDE4C promoter.

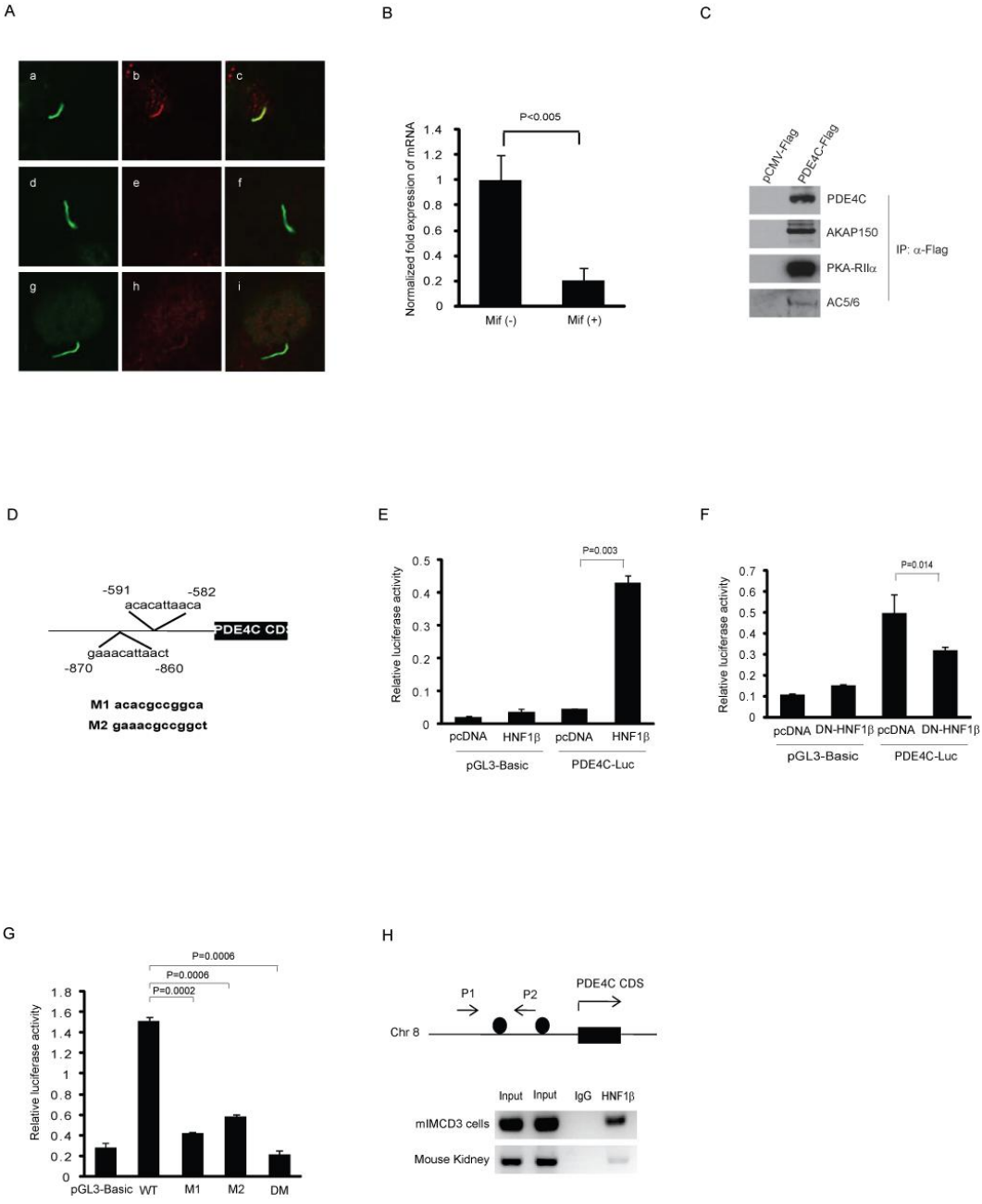


Figure 3-5. PDE4C is directly regulated by HNF-1β, localized in primary cilia, and interacts with the AKAP150 complex.

(A) 53A cells were incubated in the absence or presence of mifepristone for 48hrs and immunostained with antibodies against acetylated tubulin (green, a,d,g) and PDE4C (red, b, e, h). (B) PDE4C mRNA levels were analyzed by quantitative real time PCR. (C) mIMCD3 cells were transfected with pCMV-Flag-PDE4C plasmid. 48 hrs after transfection, cells were harvested and subjected to immunoprecipitation with an anti-flag antibody. Western blotting was performed with anti-PDE4C, AKAP150, PKA-RIIα, and AC5/6 antibodies. (D) There are two possible HNF-1β binding sites in the PDE4C promoter. The PDE4C promoter region was cloned into the pGL3-basic vector. Two sites were mutated (M1, M2) by site directed mutagenesis and mutant promoters were cloned. (E) HeLa cells were cotransfected with HNF-1β and PDE4C promoter-reporter gene. Luciferase activity was measured 48hrs after transfection. (F) mIMCD3 cells were cotransfected with dominant-negative mutant HNF-1β (DN-HNF-1β) and PDE4C promoter-reporter, and luciferase activity was measured. (G) mIMCD3 cells were transfected with reporter plasmids containing wild type, mutant (M1 and M2), and double mutant (DM) PDE4C promoter, and subjected to luciferase assay. (H) Schematic diagram of the mouse PDE4C promoter. Arrows indicate primers that were used for ChIP assays (upper panel). Occupany of the PDE4C promoter by endogenous HNF-1β in chromatin from mIMCD3 cells and mouse kidney was verified by ChIP assay (lower panel). Statistical analysis was performed using Student's t-test.

cAMP is increased in HNF-1βΔC expressing cells, HNF-1β knockout kidney, and PDE4C knockdown cells – Kidney-specific HNF-1β knockout mice develop polycystic kidney disease, and HNF-1β mutant mice show increased expression of cAMP-regulated genes, Aquaporin2 (*Aqp2*) and arginine-vasopressin-receptor type 2 (*Avpr2*) [64], which suggests that HNF1β might regulate cAMP signaling in the kidney. The levels of cAMP were measured using both enzyme immunoassays and CREB reporter assays in 53A cells. Intracellular cAMP was increased by forskolin treatment in uninduced cells (Fig 6B, C). In HNF-1βΔC expressing cells, the levels of cAMP were much higher than in uninduced cells (Fig 6B, C). *In vivo*, cAMP was elevated in HNF-1β knockout kidneys

compared to wild-type kidneys (Fig 6A). These results suggest that HNF-1β might regulate cAMP signaling pathway in the kidney. There are four distinct PDE4 genes, PDE4A, B, C, and D, which are expressed in most cells and play roles in many cellular processes [224]. To investigate whether PDE4C is important for the regulation of cAMP level in kidney epithelial cells, we reduced the expression of PDE4C using four different PDE4C siRNAs and then measured cAMP levels. Expression levels of PDE4C were examined by quantitative RT-PCR (Fig 7A, right panel). Cells were treated with siRNAs and transfected with a CREB reporter gene 24hrs after siRNA treatment. CREB reporter activity was increased in PDE4C knockdown cells, and the magnitude of the increase correlated with the degree of PDE4C knockdown using different siRNAs (Fig 7A, left panel). These results indicate that PDE4C plays a role in the regulation of cAMP level in kidney cells. One possible mechanism explaining how HNF-1β regulates cAMP signaling in the kidney may be though regulation of PDE4C gene expression. Next, we examined PDE4C in PKD2 null cells. Cells were immunostained with anti-PDE4C antibody along with anti-acetylated tubulin antibody. PDE4C was localized in primary cilia in PKD +/- cells (Fig 7Bb, c), but no ciliary staining was seen in PKD2 -/- cells (Fig 7Be, f). Expression of PDE4C mRNA was not different between PKD2 +/- and PKD2 -/- cells (Fig 6C), indicating that PDE4C is mislocalized in the absence of PC2. Taken together, these results indicate a connection between the transcription factor, HNF-1β, the PKD gene (PKD2), and a cAMP-regulating protein complex located in primary cilia.

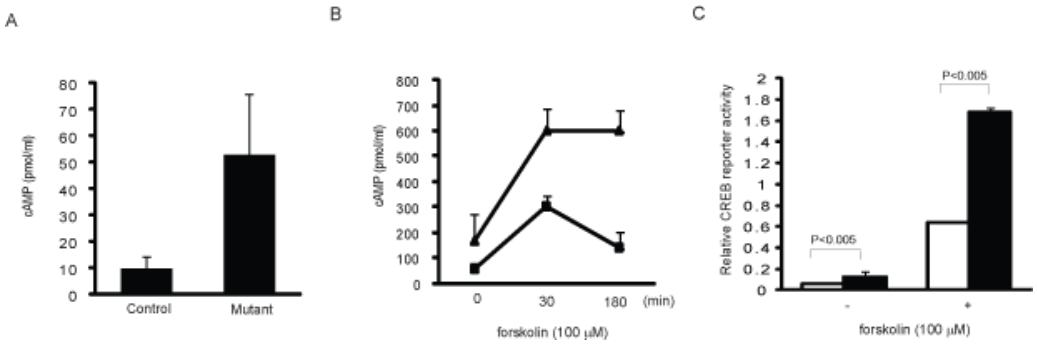
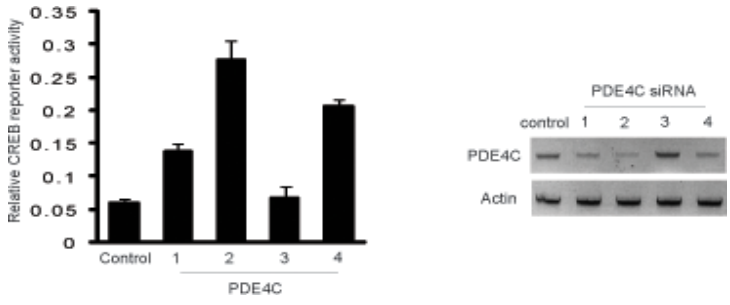


Figure 3-6. cAMP is elevated in HNF-1β mutant cells and kidney
(A) cAMP was measured in HNF-1β knockout kidney (P7, n=2) using an enzyme immunoassay. (B) 53A cells were incubated in the absence (■) or presence (▲) of mifepristone for 48hrs and cAMP was measured by enzyme immunoassay after treatment with forskolin for indicated time. (C) 53A cells were incubated in the absence (open bar) or presence (solid bar) of mifepristone for 48hrs. Cells were transfected with CREB-reporter plasmid and treated with forskolin for 7hrs. Luciferase activity was measured. Statistical analysis was performed using Student's t-test.

A



B C

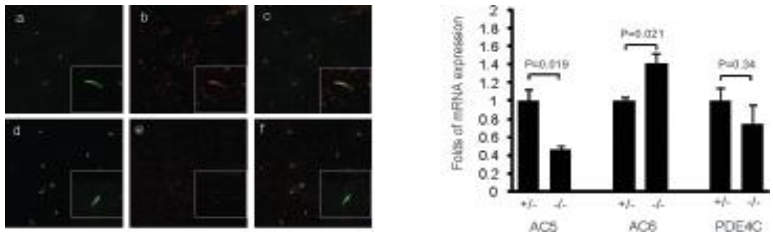


Figure 3-7. cAMP is increased in PDE4C knockdown cells and PC2 is required for the ciliary localization of PDE4C.

(A) mIMCD3 cells were transfected with either control siRNA or four different PDE4C siRNAs and cells were subjected to both luciferase assay (left) and quantitative RT-PCR (right). (B) PKD2 -/- cells were co-stainined with anti-acetylated tubulin (green, a, d) and anti-PDE4C antibody (red, b, e). PDE4C was not localized in primary cilia in PKD2 null cells (e, f). (C) mRNA expression of PDE4C, AC5, and AC6 was analyzed by quantitative real time PCR. Statistical analysis was performed using Student's t-test.

Discussion

In the present study, we show that an AKAP150 complex that contains adenylyl cyclase 5/6, phosphodiesterase 4C, polycystin-2 and PKA-regulatory subunit IIα is localized in renal primary cilia and is responsible for the regulation of cAMP signaling in renal epithelial cells. Our previous study showed that Kif3a is required for the maintenance of primary cilia, and the loss of cilia produces renal cysts with characteristics of human PKD [199]. The proteins defective in human and murine PKD localize to primary cilia and the basal body [225], indicating that ciliary defects are tightly linked to renal cyst formation. Nevertheless, the mechanism by which ciliopathies produce renal cysts is still unclear. Our findings provide a possible mechanism which is coordinated by primary cilia in the kidney.

cAMP is synthesized in response to various extracellular stimuli in the cells. Kif3a null cells that lack primary cilia showed increased cAMP levels after forskolin treatment as well as higher basal levels of cAMP (Fig 1 A, B), suggesting that primary cilia may be involved in regulating the levels of cAMP by regulating synthesis and degradation. Elevated cAMP stimulates cAMP dependent protein kinase (PKA), which phosphorylates a number of target protein and activates effector signaling pathways [226]. In the kidney, the cAMP-PKA pathway phosphorylates epithelial ion channels including aquaporin-2 (AQP2), and CFTR, K+ channels including RomK, and Na2+ channels [227-230], which are involved in water and electrolyte transport [211,231]. Normally, cAMP functions as an anti-mitogenic factor in kidney epithelial cells. However, it activates B-Raf and Erk and stimulates cell proliferation in PKD epithelial cells [115]. These results support the role of cAMP in renal cyst formation. In both Kif3a null cells and Kif3a null

cystic kidneys, PKA substrates accumulated in the nucleus (Fig 2E), which suggested that elevated cAMP may alter gene expression by phosphorylation of proteins involved in gene transcription. In Kif3a null kidney, β-catenin accumulates in the nucleus [199] and superTOPFlash assays show increased Wnt responsiveness in Kif3a null renal epithelial cells (unpublished data). Canonical Wnt signaling has been implicated in promoting epithelial cell proliferation in the kidney [232]. Loss of primary cilia produced by the absence of Kif3a increases Wnt signaling [233], suggesting that ciliary defects cause cyst formation through increased Wnt signaling. We observed that TOPFlash reporter activity was increased by forskolin treatment in Kif3a null cells, and overexpression of mutant β-catenin which is mutated at PKA-phophorylation sites decreases reporter activity in Kif3a null cells (unpublished data), suggesting that β-catenin may be one of the activated PKA substrates that accumulates in the nucleus and stimulates cell proliferation.

Masyuk *et al*. showed that primary cilia in cholangiocytes express PC1, PC2 and AC6 [214]. They also showed that calcium influx suppressed the forskolin-stimulated cAMP increase. A recent study showed that type 2 vasopressin receptors were functionally coupled with AC5/6 in primary cilia and regulated local production of cAMP in LLC-PK_1 renal epithelial cells [234], supporting the ciliary localization of AC5/6. We show that AC5/6, Ca^{2+}-inhibitable enzymes, are localized in primary cilia of kidney epithelial cells and play a role in the regulation of cAMP signaling. In Kif3a null cells, elevated cAMP levels were decreased by NKY80, an AC5 inhibitor, and AC5 knockdown with siRNA. The ciliary localization of AC5/6 is necessary for the regulation of cAMP levels in the kidney and loss of cilia results in increased AC5-dependent cAMP levels. AC5 and AC6 interact with other important signaling molecules such as capacitative calcium entry

(CCE) channels [235], nitric oxide signaling protein [236], phosphodiesterases [237], and Na^+/H^+ exchanger [238]. Here, we found that AC5/6 interact with PC2 (Fig. 5A). The functional interaction between AC5/6 complex and PC2 suggests a mechanism for interaction of cAMP-mediated signaling and Ca^{2+} signaling in renal primary cilia.

The specificity of cAMP signaling is accomplished by compartmentalization of the signaling components. AKAPs are scaffold proteins that tether signaling proteins in subcellular microdomains [216]. Bauman *et al.* showed that AKAP150 and its human ortholog AKAP79 directly interact with AC5/6, and AKAP79/150 anchoring PKA modulates AC activity in a negative feedback manner [218]. They also showed that gene silencing of AKAP79/150 results in disruption of the cAMP gradient in the plasma membrane of HEK293 cells. AKAPs also cluster PKA with PDEs to terminate cAMP signals [210,239,240]. For example, AKAP250 clusters PKA with PDE4 isoforms close to the plasma membrane [241]. We found that AKAP150 and PDE4C are localized in primary cilia and form a complex with AC5/6 as well as PKA. We identified PDE4C as a direct target gene of HNF-1β by ChIP-chip analysis. In a previous study, high expression of genes regulated by cAMP has been found in kidney-specific HNF-1β knockout mice [64], which suggests that HNF-1β affects cAMP signaling in the kidney. Here, we showed that dominant negative mutant HNF-1β elevated cAMP in kidney epithelial cells, indicating that HNF-1β plays a role in the regulation of cAMP signaling. Although ACs are maximally activated by forskolin, cAMP was elevated much higher in DN-HNF-1β expressing cells, which suggests that the increase may be due to the difference in cAMP degradation. Hydrolysis of cAMP by phosphodiesterases controls both the magnitude

and the duration of cAMP signaling [210,239]. These results suggest that the role of PDE4C to degrade cAMP may be disrupted in PKD. The AKAP protein complex plays a role in controlling cAMP-PKA signaling pathway in primary cilia by balancing synthesis and degradation of cAMP in the kidney epithelial cells. Disruption of this complex contributes to enlargement of kidneys in PKD.

Bending of renal primary cilia elevates intracellular Ca^{2+} [191]. PC1 and PC2 are colocalized in primary cilia and are required for cilium-mediated flow-sensing in renal epithelial cells [193]. Renal cystic tissues have elevated intracellular cAMP, which stimulates fluid secretion and cell proliferation [117,118]. Stimulation of cell proliferation by elevated cAMP can be induced by reducing basal Ca^{2+} concentration [115]. Moreover, increasing Ca^{2+} levels restores normal anti-mitogenic response to cAMP in PKD cells [198]. Another study showed that *Pkd2*$^{+/-}$ vascular smooth muscle cells (VSMCs) have defective intracellular Ca^{2+} ($[Ca^{2+}]_i$) regulation and high levels of cAMP, which leads to an abnormal phenotype [242]. Accumulated evidence supports the tight link between reduced Ca^{2+} and increased cAMP in PKD. We showed that PC2 interacts with the AKAP150 complex through its C-terminus. Furthermore, a C-terminal deletion mutant of PC2 induces high levels of cAMP. The PC2 mutant, D511V, blocks Ca^{2+} entry and disrupts intracellular Ca^{2+} concentration [223,243]. The high levels of cAMP in PKD2 null cells were rescued by wild-type PC2 not by the D511V mutant that lacks Ca^{2+} channel activity. These results suggest that PC2 regulates cAMP signaling not only by physical interaction with AKAP150 complex but by modulating Ca^{2+} in kidney epithelial cells. Ayda *et al*. showed that PC2 plays a role in the delivery of protein to membrane by regulation of protein trafficking in Schizosaccharomyces pombe [244]. Mislocalization of

AC5/6 and PDE4 in PC2 -/- cells suggests a possible role of PC2 as a scaffold protein or ciliary trafficking protein.

The pathogenesis and progression of inherited forms of PKD appear to be significantly dependent on intracellular Ca^{2+}, cAMP, and defects in primary cilia [120]. Here, we identified an AKAP150 protein complex containing AC5/6, PDE4C, PKA, and PC2 in primary cilia of kidney epithelial cells (Fig. 8), which is disrupted in different forms of PKD, including HNF-1β mutant cells, Kif3a null cells, and PKD2 null cells. In our study, HNF-1β may be involved in regulating cAMP signaling in kidney epithelial cells through controlling the expression of two genes, PDE4C and PC2 (Fig. 8). This study provides a common mechanism coordinated by primary cilia, which regulates cAMP in the kidney and is disrupted in PKD.

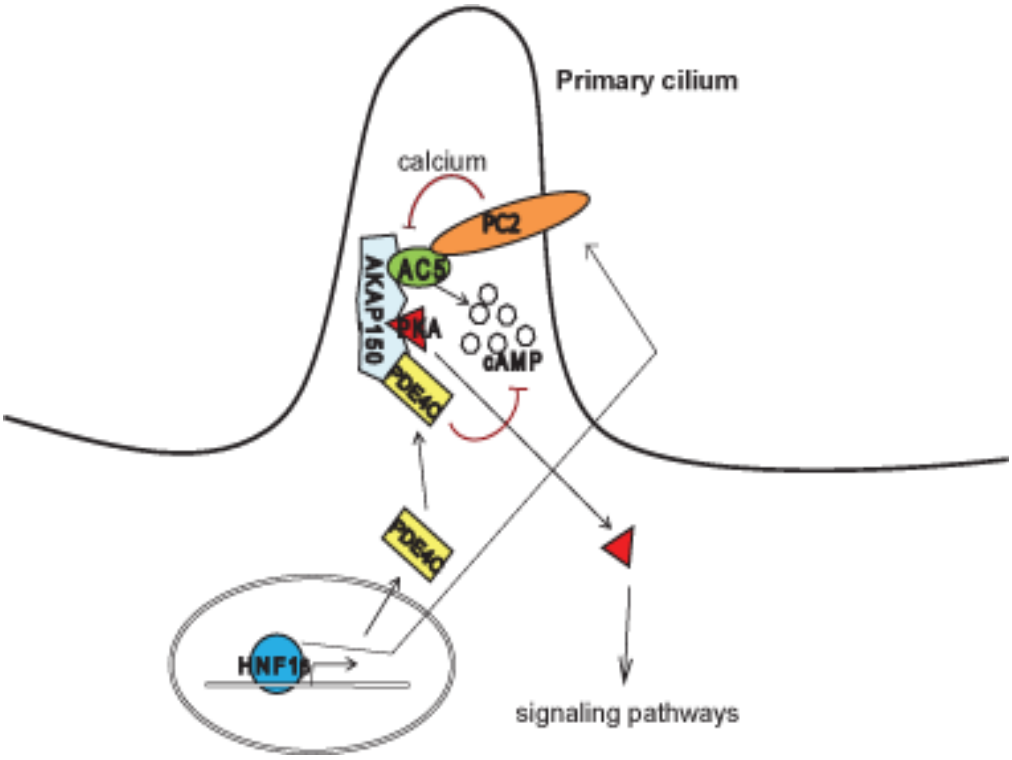


Figure 3-8. Protein complex in primary cilia regulates cAMP signaling and is disrupted in PKD.

A protein complex containing AKAP150, AC5/6, PKA, PDE4C, and PC2 is localized in primary cilia. This AKAP150 complex regulates cAMP levels via AC5/6 and PDE4C. PDE4C and PKD2 (PC2) are transcriptionally regulated by HNF-1β. The interaction of PC2 with the complex through its C-terminus is required for the regulation of cAMP levels. PC2 functioning as a Ca^{2+} channel might inhibit AC5/6, which is Ca^{2+}-inhibitable. PC2 is also required for the ciliary localization of AC5/6 and PDE4C. Disruption of the complex due to mutation of HNF-1β, loss of primary cilia, or mutation of PC2 leads to dysregulation of cAMP signaling.

Materials and Methods

Animals

cre^{Ksp} transgenic mice[245], *Cre*^{pkhd1} mice (William et al., 2008), *Kif3a*$^{fl/+}$ and *Kif3a*$^{+/-}$ [246], *PKD2*$^{fl/fl}$ (Dr. Somlo, Yale University), temperature-sensitive SV40 large T antigen transgenic mice, *H-2K*^{b}*-tsA58* (Charles River) were used in this study.

Cell Culture and DNA Transfections

HeLa cells and mIMCD3 cells were routinely cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 37 °C incubator with 5.0% CO_2. Transfection was performed in 10% serum conditions using Effectene (Qiagen) following the manufacturer's instructions.

Antibodies and reagents

The following antibodies and reagents were used. kif3a (Sigma), acetylated tubulin (Sigma), α-tubulin (Sigma), γ-tubulin (Sigma), pPKA Substrate (Cell Signaling), AC6 (FabGennix), AC5/6 and PDE4C (Santa Cruz), AKAP150 (Upstate), anti-HNF1β (Santa Cruz Biotechnology), anti-antin (Sigma-Aldrich Inc), PC2 (Dr. Somlo, Yale University) GFP (Covance), FLAG-M2 (Sigma). Forskolin was purchased from Sigma (Saint Louis, MO). NKY80 was purchased from Calbiochem. Full-length human PKD2 was provided from Dr. Somlo (Yale University). FLAG tagged-AC5 and AC6 were from Dr. Taussig (UT Southwestern). His-tagged AKAP79 was from Dr. Scott (Howard Hughes Medical Institute, Oregon Health Science University).

Generation of Kif3a-/- cell lines

Cell lines were established from medulla of kidney from *Kif3a*$^{fl/-}$*; H-2K*^{b}*-tsA58* mice as described by Sweeney et al [247]. Briefly, kidneys were perfused with ice-cold PBS, removed, minced, and digested with 0.5% collagenase type XI (Sigma) in DMEM/F-12 for 30 min at 37°C. The digest was passed though 0.4μm cell strainer (BD Falcon) then centrifuged (800 g for 5 min), and the cell pellet was washed in cold DMEM/F-12, centrifuged again, and resuspended in epithelial cell medium (DMEM/F-12 with 1.3μg/ml of sodium selenite (Sigma), 5μg/ml of insulin (Sigma), 5μg/ml of transferring (Sigma), 1μg/ml of 3,3,5'-triiodo-L-thyronine (Sigma)) supplemented with 2% fetal bovine serum (FBS). The cell suspension was plated onto tissue culture dishes. Cells were maintained for at least 2 passages with epithelial medium with 2%FBS and 0.1unit/ml of IFN-γ (Sigma) (growth medium). Then, single cell suspension was prepared to establish clonal cell line. Once colonies were established, cultures were expanded at the 33°C permissive temperature with growth medium. The established cell lines were checked the expression of ksp-cadherin by RT-PCR to confirm their origin. Prior to study, the cells were switched to medium lacking IFN-γ and cultured at 37°C for 3 days to down-regulate SV40 large T antigen.

ChIP-chip and Quantitative ChIP Assays

mIMCD3 cells and mouse kidney tissue fragments were cross-linked with 1% formaldehyde at room temperature. Chromatin was extracted from the nuclei, sonicated, and immunoprecipitated with anti–HNF1β antibody. We amplified immunoprecipitated DNA using promoter-specific primers and quantified it using real-time PCR. For ChIP-chip analysis, we amplified the immunoprecipitated DNA by LM-PCR as described

previously [65,248]. LM-PCR products were fluorescently labeled and hybridized to promoter tiling arrays that covered 1.5 kb of the promoter regions of 26,842 mouse genes (NimbleGen Systems). We analyzed the scanned images of the hybridized arrays using NimbleScan 2.0 (NimbleGen Systems). We converted the raw microarray data into scaled log ratios and visualized them using SignalMap software (NimbleGen Systems). We performed data analysis and target identification as described previously [248]. To locate HNF-1β binding sites, we used Peak Finding software (Ren Lab, UCSD). We used a significance threshold of $P \leq 0.2$ as the cutoff for defining binding sites. The ChIP-chip data are available on the web site of the UT Southwestern O'Brien Kidney Research Core Center (http://www.utsouthwestern.edu/nephrology/obrien/researchdatarepository.html).

Quantitative Real Time PCR and RT-PCR

Total RNA was extracted using Trizol (Invitrogen). Real time PCR was performed in triplicate using iCycler and SYBR green Supermix reagents (Bio-Rad Laboratories). β2-Microglobulin was used as the control gene for normalization. Data were analyzed by using IQ software (Bio-Rad Laboratories). Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen). For RT-PCR, cDNAs were synthesized using the Super Script™ first strand synthesis system (Invitrogen) and then amplified by PCR

siRNA Transfection

Four different short interfering RNA (siRNA) oligonucleotides against PDE4C and control siRNA oligonucleotides were purchased from Dharmacon. mIMCD3 cells were transfected with four different PDE4C siRNAs (#1, 2, 3, and 4) using Lipofectamine 2000 reagent (invitrogen).

Retroviral infection

MMPCreGFP [249] plasmid was provided by Dr. Silver. Retroviral packaging, infection and transfection were performed as described previously [249]. Briefly, MMPCreGFP plasmid was transfected into 293T cells with packaging components using FuGene6 (Roche). Supernatants were harvested at 48 and 78 h after transfection.

Indirect Immunofluorescence Microscopy

The cells were grown on round coverslips and fixed with 4% PFA in PBS or ice-cold methanol, then permeabilize in 0.2% Triton-X in PBS. The kidney from *Kif3a*$^{fl/-}$*; cre*^{Ksp} mice and *Kif3a*$^{fl/+}$*;cre*^{Ksp} were fixed with 4% PFA then subjected to cryosections. The coverslips or sections were stained described previously [199].

Cloning and Reporter gene assay

PDE4C promoter was amplified by using long-range PCR kit (Roche) and subcloned into pGL3-Basic vector (Promega). Integrity of promoter sequence was confirmed by sequencing analysis. Site directed mutagenesis was performed by using QuikChange kit (Stratagene). Sequencing analysis was performed to confirm the presence of desired mutations. Cells were cotransfected with 20 ng pRL plasmid encoding *Renilla* luciferase to control for differences in transfection efficiency. After growth for 48 hours, the cells were lysed in 500 µl passive lysis buffer (Promega), freeze-thawed once, and centrifuged. Supernatants (20 µl) were added to 96-well plates, and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's directions. Luciferase Assay Reagent II (100 µl) was added, and light output was measured for 10 seconds using a Wallac VICTOR V multilabel counter (Perkin Elmer). Firefly luciferase activity was normalized to *Renilla*

luciferase activity, which was measured by adding 100 µl of Stop & Glo reagent and measuring light output for 5 seconds. pCDNA3-PKD2-D511V plasmid was provided by Dr. Tsiokas in University of Oklahoma. Following PKD2 constructs were made using PCR; full-length PKD2 (1-968aa), N-truncated PKD2 (212-968aa), C-truncated PKD2 (1-657aa), full-length PKD2 with D511V mutation. They were ligated into pCMV-Myc (BD bioscience).

Immunoblotting

Cells were lysed with lysis buffer (50M Tris-cl, 150mM NaCl, 5mM EDTA, 0.1% Triton X-100, 0.1% Deoxycholicacid, 100mM PMSF, Protease inhibitor cocktail from Roche). Extracts were clarified by centrifugation 12000rpm for 1 min and boiled in SDS-sample buffer. After SDS-PAGE, under reducing conditions, proteins were transferred to nitrocellulose ECL membrane and subjected to Western Blot analysis with ECL detection reagent (PIERCE).

Intracellular cAMP assay

cAMP levels were measured by enzyme immunoassay (Assay Designs). Protein concentration was determined by Coomassie Plus Bradford assay (Pierce)

Non-radioactive in vitro PKA assay

The cells grown in 10 cm dish were lysed in 0.5 ml lysis buffer containing 25 mM Tris-HCl (pH 7.5), 0.5mM EDTA, 0.5mM EGTA, 10mM 2-mercaptoethanol, 0.5 mM PMSF, and Complete mini (Roche), then homogenized using a Dounce homogenizer. The lysates were cleared and determined the protein concentration. Lysates containing 5µg protein were subjected to a kinase reaction with the fluorescence-labeled PKA substrate, Kemptide (Promega). The phosphorylated Kemptide was sparated by 0.8% agarose gel

electrophoresis, and the fluorescence of the negatively charged, phosphorylated Kemptide was visualized by a Luminescent Image Analyzer. The negatively charged bands were excised and dissolved in Gel Solubilization Solution (Promega), then the absorbance of 570 nm of each sample were read using ELX808 plate reader (BIO-TEK Instrument).

Co-immunoprecipitation

Cell extracts were prepared in IP buffer (10mM Tris(pH7.4), 150mM NaCl, 1% TritonX, 5mM EDTA, protease inhibitor cocktail). After centrifugation at 10,000g for 15 min, the samples were incubated with anti rabbit IgG beads (eBioscience) for 30 min, and then supernatants were incubated with 2μg of antibody overnight, and then with anti rabbit IgG beads for 1 hour. Samples were washed 5 times with IP buffer. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane. The transferred proteins were visualized using ECL (Pierce) and captured on film (Denville). For tagged protein, the extracts were incubated with anti FLAG beads (Sigma) for 30min after centrifugation at 20,000g for 15min.

Statistical analysis.

Statistical tests were done using two-tailed unpaired Student's t-test with Exel or ANOVA and Dunnet's Multiple Comparison Test with GraphPad Prism.

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Dysregulation of transcription of many different genes has been linked to human diseases. Altered gene expression is often caused by dysfunction of transcriptional activators or repressors. HNF-1β, a transcription factor expressed in the kidney, is known to control the expression of cystic disease genes, including *Pkhd1*, *Umod*, and *Pkd2,* and mutation of HNF-1β produces cystic kidney disease in humans and mice. Functional studies of a PKD-specific transcription factor, HNF-1β, may give insights into designing therapeutic targets for PKD. Two approaches were used to study the molecular function of HNF-1β: an unbiased proteomic approach, Yeast two-hybrid screening, and a combinatorial genomics approach, ChIP-chip. The studies described here demonstrate that extracellular stimuli regulate transcriptional activity of HNF-1β through interacting proteins located in the cytoplasm and HNF-1β controls the cAMP signaling pathway through its target gene, PDE4C.

Transcriptional activation by zyxin family proteins, zyxin and LPP

Integration of signals from the cell surface to the nucleus is an essential feature of unicellular and multicellular organisms. Some signal transducers known as shuttle proteins have been identified that act as both cytoskeletal and signaling proteins. For example, cell adhesion receptors and their cytoskeletal binding partners regulate

trafficking of signaling proteins between the nucleus and cytoplasm and thereby influence gene expression [81,92,250]. Here, the zyxin family members, zyxin and LPP, were identified as interacting partners of HNF-1β that stimulate transcriptional activity of HNF-1β in the kidney. Zyxin and LPP recruit VASP, vasodilator-stimulated phosphoprotein, to a specific cellular location and there change actin dynamics [87]. Actin rearrangements occur during epithelial-mesenchymal transitions (EMT), which plays a fundamental role in tissue homeostasis, embryonic development, and disease progression [251]. A functional analysis of zyxin in EMT reveals a role for the protein in mediating actin-membrane linkages at cell-cell junctions [252]. In endocardiac cells, zyxin is essential for cell migration and actin fiber reorganization in TGF-1β-induced EMT and contributes to endocardiac morphogenesis [253]. In this study, a potent EMT inducer, Twist1, regulated expression of zyxin in TGF-1β-EMT, and depletion of zyxin abrogated either Twist1-dependent or TGF-1β-dependent EMT, which demonstrates that the signaling pathway, TGF-1β-Twist1-zyxin plays a role in valvulogenesis during the development of the heart [253]. During kidney development, the kidney epithelium originates from cells that undergo MET and the reverse process, EMT, is a significant mechanism of progressive tissue fibrosis of kidney, liver, and lung [251]. During the MET, Snail, a transcription factor best known for triggering EMT, is downregulated. Snail suppresses Cadherin-16 expression through direct repression of HNF-1β during renal development [254]. Tissue-specific inactivation of HNF-1β results in pancreatic agenesis and biliary dysgenesis during embryonic development [71,255]. A common feature of the mutant phenotype is a defect in epithelial tubulogenesis. Our previous study showed that HNF-1β regulates HGF-

mediated tubulogenesis by controlling expression of SOCS-3 [65], which provides one possible molecular mechanism by which HNF-1β regulates tubulogenesis in the kidney. Reduced expression of zyxin or expression of a zyxin mutant result in alteration of cell scattering behavior during HGF-mediated EMT in MCDK cells [252]. The study described in chapter 2 suggests that zyxin may play a role in regulating the transcriptional activity of HNF-1β during HGF-mediated tubulogenesis.

Planar cell polarity (PCP) is a form of spatial organization of cells in tissue that was first described in *Drosophila melanogaster*. Accumulating evidence implicates PCP in kidney development and suggest that the loss of oriented cell division and convergent extension downstream of PCP signaling leads to cystic kidney disease [256-258]. Renal specific-knockout of HNF-1β results in distortion of mitotic alignment which disrupts tubular architecture (tubular dilation and cyst formation) [256]. This result shows a clear correlation between PKD and the disruption of PCP. Although the loss of PCP contributes to PKD, the molecular function of HNF-1β in PCP remains unclear. A previous study showed that HNF-1β regulates kinesin family member 12 (Kif12), which participates in orienting cell division [259]. This study suggests that HNF-1β may be involved in PCP through controlling Kif12 expression. LPP interacts with the tumor suppressor, Scrib which plays a role in the regulation of cellular adhesion, cell shape, and polarity [260,261]. This interaction suggests that Scrib may mediate communication between cell-cell contacts and the nucleus through LPP.

Recent evidence shows that a noncanonical Wnt pathway is involved in PCP in vertebrates[262]. Wnt4-deficient mice show renal agenesis because they cannot undergo

MET, which demonstrates the essential role of Wnt during renal development [263]. Another study showed that Wnt 9b is essential for the early inductive response of the metanephric mesenchyme (MM) to cause condensation of mesenchymal cells [264]. In addition, Wnt signaling is associated with the pathogenesis of PKD. For example, the kidneys of transgenic mice expressing constitutively active β-catenin show cysts in all segment of the nephron [232] and Kif3a-deficient tubular epithelial cells have abnormal β-catenin levels [265]. Recently, Vervenne *et al* showed that LPP is involved in wnt/PCP signaling by cooperating with Scrib, and that gene expression of LPP is controlled by noncanonical Wnt signaling in *zebrafish* [96]. In this study, morpholino knockdown of LPP resulted in defects in convergent extension, a phenotype of noncanonical Wnt signaling mutants. As described earlier, Scrib is implicated in the regulation of PCP in vertebrates and interacts with LPP, which suggests that Scrib-mediated signaling pathways may transduce into the nucleus through the shuttling protein LPP. Based on recent evidence, the study herein suggests one possible mechanism by which HNF-1β is involved in the regulation of Wnt and PCP signaling in the kidney.

Involvement of HNF-1β in cAMP signaling coordinated by primary cilia

Small molecules such as cAMP are rapidly formed and diffuse into the cytosol to activate downstream effector proteins. This process provides a mechanism by which extracellular stimuli can influence diverse cellular processes. PDEs provide the sole means of inactivating cyclic nucleotides and thus play important regulatory roles in various signaling pathways mediated by cyclic nucleotides [224]. As described above, abnormal cAMP metabolism plays a significant role in the pathogenesis of PKD. PDE

isozyme specific inhibitors are useful for studying cyclic nucleotide signaling, and pharmacological agents suited for "signaling transduction pharmacotherapy" [266]. In several areas of medicine such as cardiovascular, pulmonary, and endocrine disease, the study of PDE isozymes has been applied to develop therapeutic agents. Recently, several studies with experimental disease models suggest the involvement of PDE isozymes in the pathophysiology of renal tubular dysfunction [267,268]. For example, in microdissected inner medullary collecting duct (IMCD) from adrenalectomized rats with a urinary concentrating defect, cAMP-PDE activity was increased and AVP-stimulated cAMP accumulation was decreased, whereas adenylyl cyclase activity was not changed [269]. The study described in chapter 3 shows that a specific isoform of PDE4, a target gene of HNF-1β, is essential for the regulation of cAMP signaling governed by primary cilia. In the kidney, HNF-1β controls the expression of cystic genes including *Pkhd1*, *Pkd2*, and *Umod* [64], which indicates that HNF-1β regulates the function of renal primary cilia through controlling gene expression. The study described in chapter 3 provides further evidence showing how HNF-1β regulates ciliary functions in the kidney. Kidney-specific HNF-1β knockout mice showed increased expression of genes that are regulated by cAMP, suggesting that HNF-1β is involved in cAMP signaling. Identification of PDE4C, as an HNF-1β target gene supports a connection between HNF-1β and cAMP signaling that is coordinated by primary cilia. The central importance of the PDE protein family is underscored by the plethora of PDE subfamilies and isoforms derived from splice varients that share the same catalytic function, whereas they are expressed in specific tissues and in specific subcellular areas and there control compartmentalized cAMP

gradients. These studies lead to more effective therapeutic treatments for PKD by demonstrating the involvement of specific PDE4 isoform in regulating cAMP signaling within renal primary cilia. The regulation of cAMP signaling by AKAP150 protein complex in primary cilia provides a common mechanism that is dysregulated in different PKD models, revealing a novel potential therapeutic target for PKD treatment.

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