

# Modelling molecular mechanisms of polycystic liver disease

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Edgar Wills



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# Modelling molecular mechanisms of polycystic liver disease

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# Chapter 1

## *Introduction*

Autosomal dominant polycystic liver disease (ADPLD) is a hereditary disorder causing multiple fluid-filled cysts in the liver, which may cause significant discomfort to affected patients (1) (also see: Lay extra, page 140). A gene defect leads to production of a faulty protein, causing an abnormal cellular response and finally random expansion of parts of the biliary tract (2). In severe cases, the liver becomes deformed, causing a greatly enlarged abdomen. Clinically, the mass effect from this abdominal distension causes pain, nausea, anorexia, shortness of breath and a generally decreased quality of life. The disease is of great biomedical interest for multiple reasons (1, 2): not all genes causing the disease are known, a remarkable mechanism appears to be responsible for the stochasticity of cyst development, and no curative pharmacological treatment has yet been developed. In this chapter I will go into the biomedical background of the disease and will highlight the aims of this thesis.

## *Liver cysts*

### **The human liver**

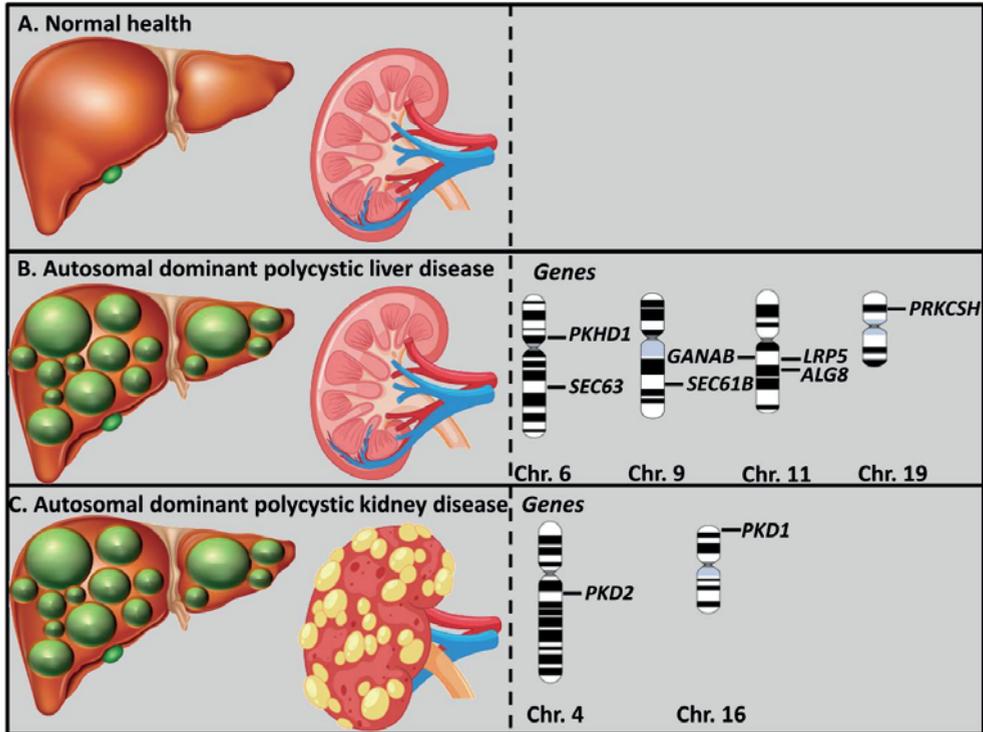
The human liver is the largest internal organ of the human body and the second largest organ after the skin. Present in the right, upper, part of the abdomen, it can weigh up to 1.5kg in healthy adults (3). The liver consists of a right lobe and a left lobe, the first being six times larger than the latter, on average (4). Hepatic functions are critical to support most other organs, and therefore to sustain human life. Most importantly, the liver facilitates detoxification of metabolites ('waste processing'), production of bile ('waste removal'), energy and vitamin storage, and protein synthesis (e.g. blood clotting components). Bile produced by the hepatic cells is excreted into the biliary tract. The main biliary tract starts in the right and left lobe of the liver with two hepatic ducts, which join to form a common hepatic duct. There the cystic duct from the bile-storing gallbladder joins to form a common bile duct, which finally leads into the duodenum.

### **Bile and the biliary tract**

The cells that make up liver parenchyma are hepatocytes and cholangiocytes, both of which are of epithelial origin (4). These cells lie in hexagon-shaped lobules, which consist of hepatocytes and a portal triad. Each portal triad contains a hepatic artery, portal vein and bile duct.

Bile production in the liver starts in tiny canals called canaliculi, lying in between neighboring hepatocytes. The hepatocytes excrete bile salts, e.g. cholic acid and chenodeoxycholic acid, and toxic metabolites into these canaliculi. The bile flows into small bile ducts called ductules, before converging into intralobular ducts and subsequently into the hepatic ducts described previously. Besides the excretion of hepatocytes, cholangiocytes in the ducts also add to the volume, pH and composition of the bile. These cells excrete alkaline fluid to promote micelle formation and to neutralize stomach acid arriving in the duodenum. The fluid consists mostly of  $\text{HCO}_3^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ . Around 250 to 1000ml of bile is produced per day in a healthy adult, the variation being dependent upon the amount of bile salts being excreted from hepatocytes (5).

It is from the intrahepatic part of the biliary tract that cysts, fluid-filled sacs of cells, are thought to form. In PLD, cysts occur separated from the biliary tract. Sporadic cysts are benignly present in 2.5-18% of the general population (6), these cysts represent a frequent, but mostly harmless abnormality. Cysts however may continue to expand to sometimes even more than two liters in volume. In these instances, problems related to abdominal distension may occur, that require clinical intervention.



**Figure 1.** Affected organs and genes in autosomal dominant polycystic liver disease (ADPLD) and autosomal dominant polycystic kidney disease (ADPKD).

**A.)** In normal health, no liver or kidney cysts occur. Only a small percentage of the population may have single, sporadic liver or kidney cysts.

**B.)** *PRKCSH*, *SEC63*, *LRP5* and *GANAB* mutations underlie ADPLD, whereby only the liver develops multiple liver cysts. The kidney is often relatively spared.

**C.)** *PKD1* and *PKD2* mutations underlie >95% of ADPKD cases. Both the liver and kidney develop multiple cysts.

Note: There are indications that *LRP5* and *GANAB* mutations may also cause ADPKD.

## Polycystic liver disease

Polycystic liver disease (PLD) occurs as part of the phenotype of autosomal dominant polycystic liver disease (ADPLD) and autosomal dominant polycystic kidney disease (ADPKD). In dominant autosomally inherited disease, one mutation in any of the two alleles of a gene is sufficient for expression of the disease, thus an affected patient has a 50% chance of producing a child with the disease. ADPLD is rare and occurs in 0.05-0.53% of the population (7). ADPKD occurs more frequently, and is present in 0,1-0,25% of the general population (8).

83-94% of ADPKD patients will develop liver cysts during their life time (9, 10) . PLD is often arbitrarily defined as more than twenty fluid-filled sacs spread out in the liver, but the disease occurs in many shapes and sizes. Polycystic livers from ADPLD and ADPKD are indistinguishable from each other (2), in contrast to those from related disease such as autosomal recessive polycystic kidney disease (ARPKD) and ciliopathies, where hepatic scarring ('fibrosis') and other symptoms (often in the form of a syndrome) may co-occur at a young age. Furthermore, the cysts in ARPKD and ciliopathies are likely in continuity with the biliary tract, whereas those in ADPKD and ADPLD are not. ADPLD and ADPKD patients usually present with complaints from the enlarged liver due to organ displacement ('abdominal distension') as middle-aged adults. Women are earlier, more frequently and more severely affected, likely due to the effect of female hormones such as estrogen (1, 11). Liver sizes of 20 liters ('hepatomegaly') are not unheard of, and symptoms include abdominal distension, early satiety, and limited mobility (12). Biomarkers such as alkaline phosphatase, gamma-glutamyltransferase and Ca19.9 (carbohydrate antigen 19-9) may be used to detect disease occurrence or prognosis (13, 14). Although disease progression is regarded as benign, symptoms often extend to the level that they significantly affect quality of life. Treatment is therefore indicated in symptomatic patients.

## **Treatment**

Several treatment options exist for liver cysts. Conservative, medical management involves administration of somatostatin analogues. This group of therapeutics includes lanreotide, octreotide and pasireotide, which have been used to suppress cyst expansion by their effects on cell growth ('proliferation') and fluid secretion. The somatostatin analogues function through the somatostatin receptors (SSTRs), which affect adenylyl cyclases (AC) to reduce levels of cyclic AMP (cAMP) (15, 16). This molecule was found to be dysregulated in PLD in some studies (17-22), and altered levels cause dysregulation of Ca<sup>2+</sup> signaling. Surgical interventions include aspiration sclerotherapy (AS) (23) and laparoscopic cyst fenestration (15). AS is performed by an intervention radiologist who uses a needle to aspirate fluid from the cyst under ultrasound guidance. The treatment is recommended for simple, sporadic cysts or polycystic liver disease where a single, large or dominant cyst is present. Cyst fluid can be captured and frequently contains cells that have detached from the inner wall ('epithelium') of the cyst. Following fluid withdrawal, a sclerosing agent is injected to destroy the cyst epithelium to prevent further expansion. The most commonly used sclerosant is <150ml 96-100% ethanol (23). Laparoscopic cyst fenestration is a surgical intervention performed through small incisions or keyholes to treat superficial liver cysts. During the procedure, cysts located at the surface of the liver are drained and their domes removed. This allows any secreted fluid to drain into the abdominal cavity where it is taken up by the surrounding cells of the membrane (peritoneum).

### **PRKCSH**

*PRKCSH*, an acronym for protein kinase C substrate 80 K-H, encodes the protein product glucosidase II beta (GIIB). Mutations in this gene are the most frequently occurring cause of isolated polycystic liver disease. Approximately 15% of ADPLD patients possess a mutation in *PRKCSH* (24, 25). The gene is located on chromosome 19 and has a length of 15,712 bases (4). *PRKCSH* isoform 1 has 18 exons with 2,079 bases [NM\_002743.3]. The protein product GIIB consists of 528 amino acids with a molecular weight of 59 kDa (26, 27). GIIB consists of an N-terminal signaling sequence, low-density lipoprotein receptor domain (LDLr), two putative calcium-binding EF-Hand domains, a glutamic acid repeat, a variable protein domain, a mannose-6-phosphate receptor domain and a C-terminal HDEL endoplasmic reticulum (ER)-retention signal. This latter signal is responsible for keeping the glucosidase complex (GIIA and GIIB) in the ER. Glucosidase II functions as an n-linked glycan-processing enzyme, with GIIA as the catalytic component and GIIB as a structural component. It cleaves off two glucose residues in two separate steps, which are tightly linked to protein quality control. Incorrectly folded proteins undergo multiple rounds of glycan processing, and depending on the folding outcome may be processed towards their final location or destroyed in the proteasome following excretion out of the ER.

### **ALG8**

*ALG8*, an abbreviation of *alpha-1,3-Glucosyltransferase*, encodes ALG8. Discovered in 2017 in a large exome-sequencing cohort, it is one of the latest known cyst disease genes. About 3.1% of ADPLD cases are likely caused by the gene (29). It is found on chromosome 11 spanning a length of 38,725 bases. 526 Amino acids are found in isoform 1 [NM\_024079], which weigh approximately 60 kDa. The protein is responsible for adding the second glucose residue to the oligosaccharide precursor group on proteins during N-glycosylation. As this is one of the two glucose residues cleaved by glucosidase II, a role in liver cyst development had been hypothesized for ALG8 even before exome sequencing

### **GANAB**

*Glucosidase II alpha subunit (GANAB)* is one of the most recently found genes in ADPLD and ADPKD (28, 29). Mutations in *GANAB* accounted for 1.9% of ADPLD patients and 0.3% of ADPKD patients. Located on chromosome 11, the gene has a length of 21,901 bases. *GANAB* isoform 3 contains 25 exons across 4,026 bases [NM\_198335.3]. Spanning 944 amino acids, GIIA, *GANAB*'s protein product, weighs 107 kDa. It contains glycoside hydrolase and galactose mutarotase-like domains (26). For the protein function, please refer to the gene card of *PRKCSH*. GIIA, containing the catalytic domain of glucosidase II, joins with GIIB to create an active glucosidase.

### **SEC63**

*SEC63*, short for *SEC63 homologue (S. cerevisiae)*, encodes the SEC63 protein (SEC63p). It was discovered in 2004 as the second gene to cause isolated polycystic liver disease (26, 31). Around 5.7% of ADPLD patients possess *SEC63* mutations (1). The gene is present on chromosome 6 with a length of 90,523 bases. *SEC63* isoform 1 spans 21 exons and contains 6,500 bases [NM\_007214.4]. Although multiple splice variants exist, the main splice variant of SEC63p has 760 amino acids and weighs 88 kDa. SEC63p consists of three transmembrane domains, a luminal DnaJ-like domain (between segment 2-3), and a large cytoplasmic, negatively charged C-terminal domain. It functions with the SEC61 complex, together with SEC62 in as part of the

<p>determined it as a cause (2, 30).</p>	<p>SEC62/SEC63 complex, where it is responsible for protein ER transfer and membrane insertion of transmembrane proteins. The SEC61 complex is also important in protein quality control (2, 31-34). It transfers proteins bound for proteasomal degradation out of the ER and into the proteasome.</p>
<p><b>SEC61B</b>  <i>Sec61 Translocon Beta Subunit (SEC61b)</i>, codes for SEC61B protein. Despite belonging to the molecular complex of SEC63, it was only discovered in 2017 as a cause for ADPLD (29). Approximately 1.3% of cases are likely related to mutations in the gene. This gene is found on chromosome 9 and has a length of 8,556 bases. The mature protein has 4 exons with 545 nucleotides that encode 96 amino acids [NM_006808] weighing 9974 Da. SEC61B forms a heterotrimeric SEC61 complex with SEC61A1 and SEC61G, where it forms the core of the endoplasmic translocon complex. Of the three components, it is the least universally conserved and not essential for the complex's function (29, 35). Its function is poorly understood in comparison to that of its complex partners, but likely includes associations of non-translating ribosomes with the ER to enhance protein translation (29), facilitating cotranslational protein transport and interacting with the signal peptidase during translocation (29).</p>	<p><b>LRP5</b>  <i>LRP5</i>, an acronym for low-density lipoprotein receptor related 5, encodes LRP5. Only published in 2014, it is a recent gene revealed to cause ADPLD. In a cohort of 150 unrelated ADPLD patients without mutation in <i>PRKCSH</i>, <i>SEC63</i> or <i>PKD2</i>, only 3 cases were found. LRP5 is located on chromosome 11 with 150,410 bases, its isoform 1 spans 23 exons for a total length of 5,168 bases [NM_002335.3] (26). The protein size is 1,615 amino acids that weigh 179 kDa. LRP5 is a co-receptor in Wnt signaling. LRP5's extracellular part contains four beta-propeller domains composed of six YWTD repeats, an epidermal growth factor-like domain, and three low-density-lipoprotein receptor-like ligand-binding domains (36). Together with a Frizzled (Fz) receptor, LRP5 binds Wnt ligands (37-40). Through conformational change and phosphorylation of its PPPSP motifs, a docking site for axin1 and the Wnt 'destruction complex' is created. After several more signaling steps this eventually leads to beta-catenin activation and transcription of genes with a TCF/LEF regulatory site. Its role in canonical Wnt signaling is especially well known.</p>
<p><b>PKD1</b>  <i>Polycystic kidney disease 1 (PKD1)</i>, is the first gene proven to cause hepatic and renal cyst development (41). Approximately 85% of all ADPKD patients possess mutations in <i>PKD1</i> (2). The gene is located on chromosome 16 with 47,191 bases. Isoform 1 spans 46 exons for a total of 14,138 bases [NM_001009944.2]. <i>PKD1</i> encodes polycystin-1 (PC1), a very large protein weighing 462.529 kDa, with a size of 4,303 amino acids. PC1 is a membrane glycoprotein with a large N-terminal extracellular region, multiple transmembrane regions and a cytoplasmic C-terminal region. Protein processing by GILB, GIIA and SEC63p are essential for protein</p>	<p><b>PKD2</b>  <i>Polycystic kidney disease 2 (PKD2)</i>, is the second gene discovered to cause hepatic and renal cystogenesis. Mutations in the gene are responsible for disease in approximately 15% of all ADPKD patients (2). Located on chromosome 4, the gene has a length of 70,141 bases. Isoform 1 contains 15 exons spanning 5,089 bases [NM_000297.3]. With 968 amino acids and a weight of 109.691 kDa protein product polycystin-2 (PC-2) is considerably smaller than PC1, with which it forms a heterodimer (48). N-glycosylation is essential to determine the abundance of the protein (49). Similar to PC1, it is a ciliary membrane protein, in PC2's case containing 6 transmembrane domains with</p>

<p>function, and current experimental data favor PC1 as the central, downstream component for all cyst-causing genes except LRP5 (28, 42). Polycystin-2 seems to be crucial for ciliary trafficking of PC1, as well as its proper maturation (42, 43).</p> <p>PC1 contains proline, glutamic acid, serine and threonine amino acids that may facilitate ubiquitin-mediated degradation. Leucine-rich repeats, a C-type lectin domain, 16 immunoglobulin-like repeats and 4 type II fibronectin-related domains implicate the protein in cell-cell or cell-matrix interactions (44). The protein is notably located in the primary cilium, with additional localization on the lateral domain of plasma membranes and to adhesion complexes in polarized epithelial cells (45, 46). At around 200 amino acids, PC1's C-terminal tail contains a G-protein binding domain and a coiled-coil domain (46). It also contains an N-terminal G-protein coupled receptor proteolytic site (GPS), whose cleavage is necessary for its function (47). PC1 was long thought to be a mechanosensitive ciliary calcium ion channel, responsive to urine flow, but recent data suggest that this is incorrect (48). Its exact ciliary function therefore remains elusive.</p>	<p>cytoplasmic N and C termini (44). PC2 functions as a calcium permeable nonselective cation channel, although the previously considered role as a mechanosensitive ion channel currently seems unlikely. Its interaction with PC1 is crucial for ciliary localization of the heterodimeric complex, with the main interaction taking place at their c-terminal cytoplasmic domains (44, 50). The majority of cellular PC2 is located in intracellular compartments other than the primary cilium however, where it likely modulates intracellular calcium stores.</p>
<p><b>PKHD1</b></p> <p><i>Polycystic kidney and hepatic disease 1 (PKHD1)</i>, is a novel gene causing a phenotype similar to ADPLD in mutation carriers. The gene is primarily known in causing autosomal recessive polycystic kidney disease (ARPKD) in humans, yet was found in a cohort of unresolved ADPLD patients (29). 6-11% of ADPLD cases are expected to be caused by the gene based on this study.</p> <p>The gene is 472,941 bases long on chromosome 6. Its 16,282 nucleotides isoform 1 [NM_138694] encode 4,074 amino acids, giving the protein a molecular weight of 446.702 kDa. The fibrocystin protein contains a highly glycosylated N-terminal extracellular domain, a single transmembrane domain, a calcium binding site and multiple plexin-transcription-factor domain (51)s. Although its precise function is unknown, it is another cilium-localized molecule. Like PC1 and PC2, fibrocystin is likely involved in calcium, cAMP, MAPK and EGF(R) signaling (51).</p>	

## **Pathophysiology**

Polycystic livers start out as a simple genetic abnormality in one of two alleles of the fertilized oocyte ('egg'). This single genetic error eventually leads to a faulty protein or protein production, an unknown disturbed pathway, causing a cholangiocyte stem cell with a defective response (possibly through an abnormal primary cilium) and finally a liver cyst. These mechanisms of disease are further elucidated below.

## **Genes and proteins**

A plethora of genes are involved in liver cyst development (also see Gene cards box). Mutations are found in genes *PRKCSH*, *SEC63*, and *LRP5* in up to 35% of ADPLD patients (27, 29, 40), while mutations in *PKD1* and *PKD2* cause ADPKD in virtually all cases (8) (gene cards). A recent study found *GANAB* mutations in a small number of ADPKD patients as well (0.3%) (28). The prevalence of mutations in ADPLD patients is unclear, 2 out of 9 unresolved families in this study had a *GANAB* mutation, and 3 out of 159 ADPLD patients (1.9%) in a later study (29). The gene may cause either severe ADPLD or mild to moderate ADPKD (28). ADPKD, another liver cyst disease, is caused by mutant *PKHD1*. The disease displays a recessive disease pattern. In another recent study, *PKHD1* mutation carriers (i.e. not patients) showed a clinical phenotype similar to that of ADPLD (29). It was estimated that 6-11% of ADPLD cases may be caused by *PKHD1*. The group additionally found two genes on the same biosynthetic route as *PRKCSH* and *SEC63B*; *ALG8* and *SEC61B* respectively. *ALG8* mutations likely occur in 3.1% of patients, while *SEC61B* mutations explain another 1.3%. In total, it is estimated that known genes can explain about 50% of ADPLD cases (29). Ciliopathies with PLD may also occur due to mutations in *IFT88*, *MKS*, *TSC1*, *TSC2*, and *OFD1* (more information below).

The protein products of these genes have several commonalities. *GIIB*, *GIIA*, *ALG8*, *SEC61B* and *SEC63p* function in the endoplasmic reticulum (ER), where they are responsible for processing newly formed proteins. These ER proteins are essential for polycystin (PC) 1 and PC2 processing (29, 42, 49), which are the protein products of *PKD1* and *PKD2* genes. Complete loss of *PRKCSH*, *GANAB*, *ALG8*, *SEC61B* and *SEC63* reduces PC dosage to a critically low level. This is most likely due to a close interplay between *PRKCSH*, *GANAB*, *ALG8*, *SEC61B*, *SEC63*, *PKD1*, and *PKD2*. One feature of ADPLD that remains unexplained is the low penetrance and mild phenotype in some family members with known mutations in genes underlying the disease (28, 52-54). Evidence of familial clustering of severe PLD indicates that genetic modifiers may play a role.

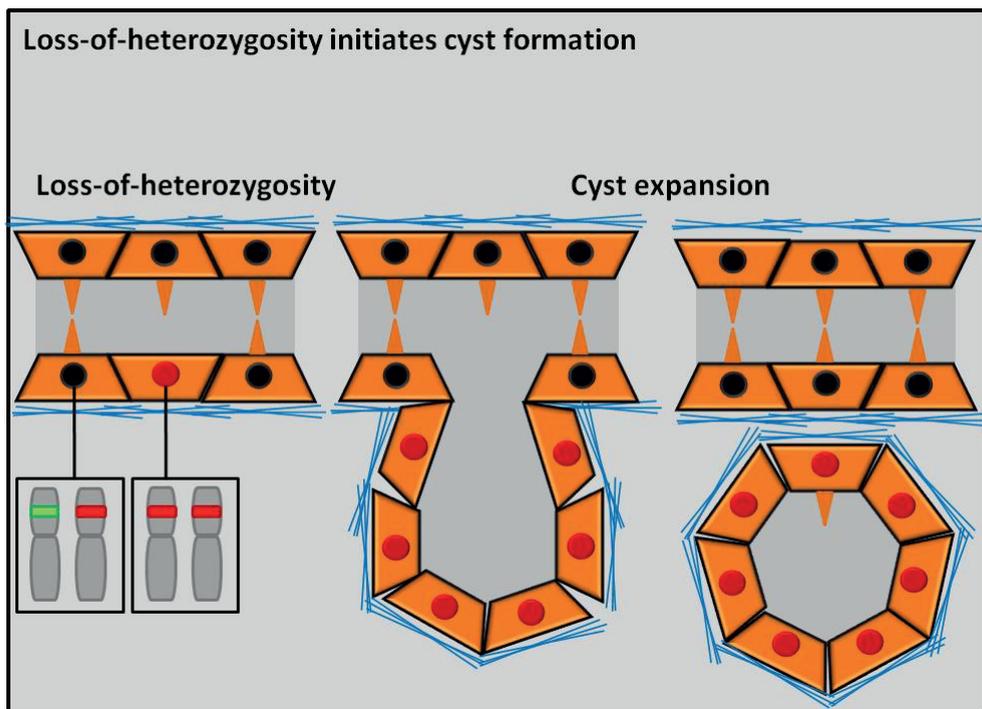
*PC1*, *PC2* and *PKHD1* function in the primary cilium, the cell's antenna. Additionally, *PC1* localizes to the lateral domain of the plasma membrane and to adhesion complexes in polarized epithelial cells (46). The majority of cellular *PC2* is located in intracellular compartments other than the primary cilium, where it likely modulates intracellular calcium stores (44, 50). It remains unclear which processes involving the cilium are dysregulated to cause liver cysts. The finding of *LRP5* mutations in ADPLD (40), suggest that the Wnt signaling pathway is likely implicated.

## **Genes and proteins: loss-of-heterozygosity**

ADPLD is recessive at a tissue level (2, 55-57). A liver cyst occurs when a single cell expands

vigorously due to loss of both normal wild-type alleles. This abnormal stem cell gives rise to a cyst comprised of mutated daughter cells. In PLD the chance of this process occurring is greatly increased, since all cells already have lost the function of their first allele. In the body, somatic second hits occur that then cause loss-of-heterozygosity (LOH) and reduction of the cognate protein activity below the threshold of pathogenicity.

LOH is detectable in ~79% of liver cysts from *PRKCSH* mutation cases, ~7% of cysts of a *SEC63* mutation patient, and in 80% of cysts from a *PKD2* carrier (55-57). Additionally, Janssen and colleagues showed that loss of *PKD2* occurs in sporadic cysts (55). A single, sporadic cyst of a patient had both alleles of *PKD2* inactivated, whereas two normal *PKD2* copies were present in the germline. A strong indication that sporadic cyst formation occurs through the same pathophysiological pathway as polycystic cyst formation, while having to pass through a greater stochastic barrier by having to mutate not one, but two alleles. In kidney cysts, LOH similarly occurs with *PKD1* and *PKD2* (58, 59). No descriptions of LOH for *GANAB*, *ALG8*, *SEC61B*, *PKHD1* or *LRP5* yet exist.



**Figure 2.** Loss-of-heterozygosity initiates cyst formation.

Somatic loss of the wild type allele (green) leads to a cholangiocyte stem cell with two mutated alleles (red). This cholangiocyte displays aberrant growth, leading to cyst formation.

### Pathways

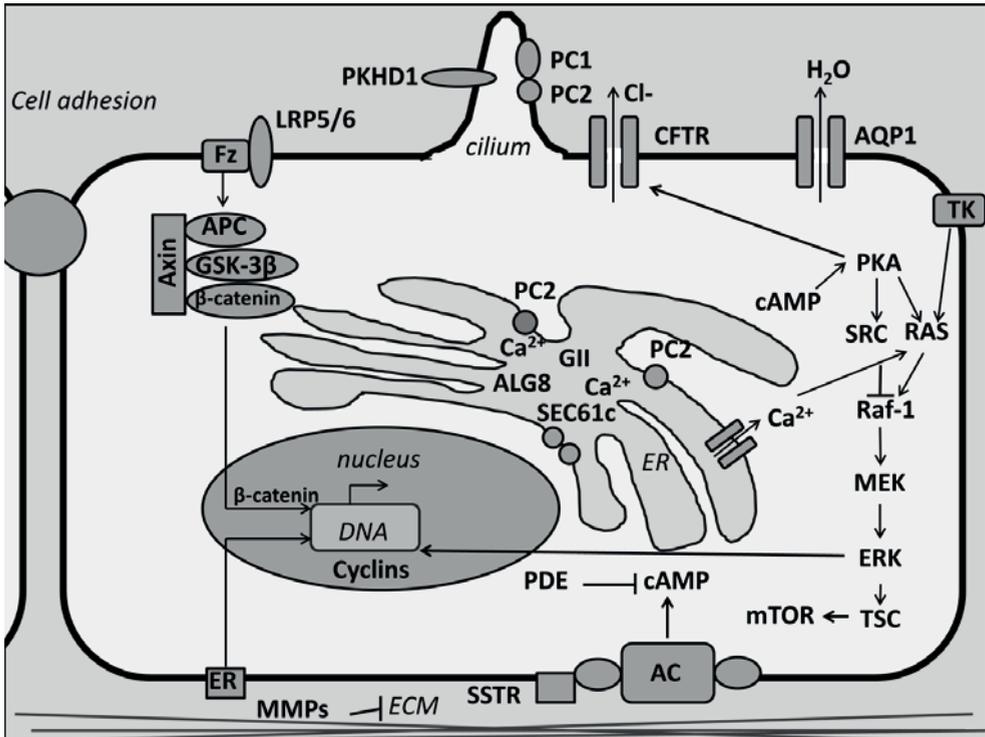
Multiple pathways are implicated in liver cyst development. Suggested pathways and cellular processes include:  $\text{Ca}^{2+}$  (20, 21), cAMP (17-22), PKA/Src/Raf/MEK/ERK1/2 signaling (17, 20, 21, 60, 61), CFTR/AQP1/AE2/fluid secretion (21), pleiotropic growth factors/receptor tyrosine kinases (EGF/EGFR/VEGF/IGF-1/IL-6) (20, 21, 62), mTOR (20, 21, 60), cyclins/CDC25a (21, 63, 64), estrogens/FSH, HDAC6/epigenetics (1, 20), MMPs/ECM remodeling (20, 21), and

cell adhesion (62).

Of foremost importance are calcium and cAMP signaling, which are regulated by the somatostatin analogues that can treat the disease.  $\text{Ca}^{2+}$  signaling is coupled to protein products of multiple cyst related genes. Fibrocystin loss likely disturbs intracellular calcium signaling (51). The polycystins may function as nonselective cation channel. It is currently unclear whether this includes  $\text{Ca}^{2+}$  signaling in the primary cilium (see paragraph 'hepatic cystogenesis: paradigm shift' in the discussion). Other causes for disturbed ciliary signaling may be present. GIB and PKD2 display effects on  $\text{Ca}^{2+}$  signaling from the ER (46, 65-67). The calcium pathway is tightly interrelated with cAMP signaling: increased  $\text{Ca}^{2+}$  decreases cAMP, and  $\text{Ca}^{2+}$  and cAMP influence proliferation and fluid secretion.

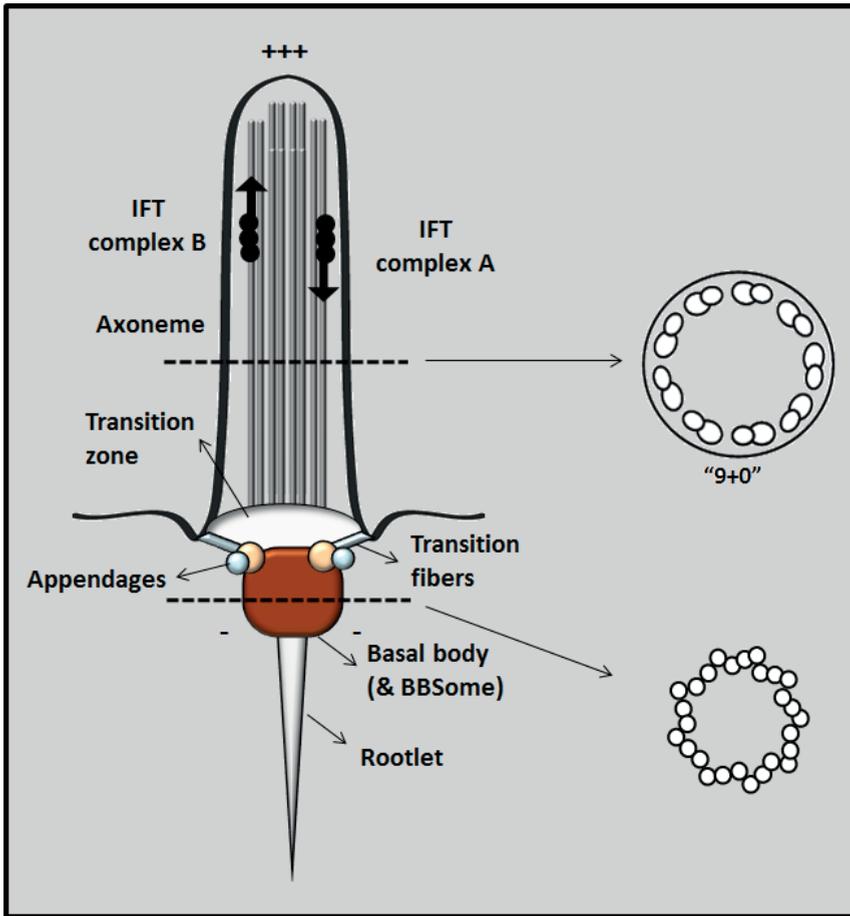
Wnt signaling is a pathway which is implicated in ADPLD by the discovery of disease associated mutations in the *LRP5* gene (40). The protein product of *LRP5* is a receptor that interacts with Frizzled (Fz) receptors, of which 10 subtypes exist in mammals (reviewed in (68)). Similarly, 19 Wnt subtypes are present in mammals. *LRP5* only has one homologue in the form of *LRP6*, with which it shares partially redundant functions. Frizzled and *LRP5* bind Wnt ligand proteins, causing them to activate the Wnt pathway through the Axin2 destruction complex. The final effect of this canonical Wnt signaling pathway is activation of beta-catenin. Beta-catenin then binds TCF/LEF promoters to activate (transcribe) Wnt-sensitive genes (68).

The different Wnt and Fz subtypes are relevant due to their differences in activated pathways. Besides the canonical beta-catenin pathway, Wnt also signals through non-canonical pathways. A particular Wnt-Fz-LRP combination may activate beta-catenin and / or non-canonical pathways (68). The non-canonical pathways are divided into two branches, which are both independent from beta-catenin (69). The first is the planar cell polarity (PCP) pathway, which determines the orientation of the mitotic spindle and thus the direction of the planar cell expansion, as well as the organization and orientation of cellular structures such as cilia. The second is the Wnt/ $\text{Ca}^{2+}$  pathway, which regulates intracellular  $\text{Ca}^{2+}$  release from the endoplasmic reticulum to regulate tissue patterning. Although it is considered a distinct pathway, it shares components with the PCP pathway, and may modulate both canonical and PCP Wnt signaling to induce its effects.



**Figure 3.** Schematic representation of the pathways involved in polycystic liver disease development according to current literature.

ER: Endoplasmic reticulum; ECM: extracellular matrix; DNA: Deoxyribonucleic acid.  
 Fz: Frizzled; LRP5/6: LDL Receptor Related Protein 5/6; APC: adenomatous polyposis coli; GSK-3β: Glycogen synthase kinase 3 beta; SEC61 c.: SEC61 complex; PC1/2: Polycystin-1/2; Cl<sup>-</sup>: Chloride; H<sub>2</sub>O: dihydrogen monoxide; AQP1: Aquaporin-1; TK: Tyrosine Kinase Receptor; PKA: Protein kinase A; SRC: Proto-oncogene tyrosine-protein kinase Src; Raf-1: RAF proto-oncogene serine/threonine-protein kinase; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal-regulated kinases; TSC: Tuberous Sclerosis protein; mTOR; Mammalian target of rapamycin; PDE: Phosphodiesterase; cAMP: Cyclic adenosine monophosphate; Ca<sup>2+</sup>: Calcium; AC: Adenylyl cyclase; SSTR: Somatostatin receptor; MMP: Matrix metalloproteinase; ER: estrogen receptor.



**Figure 4.** Schematic representation of a primary cilium.

Nonmotile primary cilia have a 9+0 microtubule architecture within the ciliary axoneme, which means nine doublet microtubules organized in a circle. These cilia form from a basal body, which is the older of two centrioles. Intraflagellar transport complexes are responsible of transporting molecular cargo along the axonemal microtubule tracks, and thereby promote the unique protein and lipid composition of cilia. At the ciliary base, the BBSome protein complex connects to IFT to actively shuttle proteins and vesicles across the barriers at the ciliary base.

### Primary cilia and cholangiocytes

Outside of PLD, diseases involving liver cysts are mostly caused by genes involving the primary cilium. Normal biliary ducts have solitary, cylindrical primary cilia on virtually all cells, with a length  $>4\mu\text{m}$  (49, 50). Each of these nonmotile cilia has a 9+0 microtubule architecture within the ciliary axoneme (2). That means nine doublet microtubules organized in a circle, without the central singlet microtubule pair and associated dynein-based motility apparatus observed in motile cilia. In quiescent cells, cilia form from a basal body, the older of two centrioles. The link between cilia and the centriole indicates that cilia can only occur during nondividing phases of the cell cycle. The ciliary compartment and membrane have a distinct protein and lipid composition and several barriers that separate this organelle from

the cell's cytoplasm and membrane, respectively (70, 71). Intraflagellar transport (IFT) complexes assemble and maintain a cilium by active, molecular motor-based transport of molecular cargo along the axonemal microtubule tracks. This transport is bidirectional: heterotrimeric kinesin-2 motors power the IFT-B particles to move from the ciliary base towards the tip (anterograde transport), while movement of IFT-A particles in the opposite, retrograde direction is powered by cytoplasmic dynein 1b/2 motors. At the ciliary base, another protein complex called the BBSome connects to IFT to actively shuttle proteins and vesicles across the barriers at the ciliary base (72).

In the liver parenchyma, only cholangiocytes possess primary cilia. Cholangiocyte cilia are thought to be cellular sensors, involved in mechano-, osmo-, and chemo-reception of bile flow (73-76). An old hypothesis states that cilia mechanically bend in response to fluid flow, whereby a mechanosensory calcium channel produces influx of  $Ca^{2+}$  as a second messenger. In recent years, this hypothesis has become more controversial however (see paragraph 'hepatic cystogenesis: paradigm shift' in the discussion).

### **Cholangiociliopathies**

A plethora of crucial ciliary components are affected in different cholangiopathies. To group these diseases together, researchers have aptly coined the term cholangiociliopathies (75). The polycystins are the most important with regard to liver cysts, although their function, and thus, their role in cystogenesis remains poorly understood. In ADPKD, cells lining the medium-sized hepatic cysts (1-3cm diameter) project cilia (into the lumen) that are short ( $1.25 \pm 0.29 \mu\text{m}$ ) and rare (1/200 cells), and the organelles are completely absent from larger cysts (>3cm diameter) (77, 78). Mutations in genes underlying other ciliary components are infrequent and display a milder phenotype. Polycystic livers have also been detected in animals or patients with *IFT88*, *cystin-1*, *Meckel-Gruber syndrome (MKS)*, and *orofacioidigital disease 1 (OFD1)* mutation (2). Most likely, more ciliopathies may develop a phenotype with multiple liver cysts.

The presence of multiple liver cysts is not limited to ADPLD and the cholangiociliopathies, but occurs in a wider spectrum of monogenic diseases. For example, failure to form peroxisomes due to monogenic mutation in *peroxisomal biogenesis factor* genes leads to a syndrome sometimes involving liver cyst formation (79). GIB, SEC63p and SEC61B interact with peroxisomal proteins and are crucial for peroxisome development (80). As such, cyst formation appears to be a complex, heterogeneous process, possibly not relying solely upon disruption of a single pathway. PC1, ciliopathies, Wnt and even peroxisomal biogenesis disorders could represent different routes to a liver cyst.

### **Biliary stem cells and tissue development**

During development of the cholangiocytes in the biliary tract, multiple signaling pathways become active, and their disturbance leads to liver cysts. Most important are Notch, Tumor Growth Factor (TGF)- $\beta$ , and, crucially, Wnt signaling (2, 81-87), all of which are also associated to ciliary signaling (88-91). In embryonic development, and during liver damage, portal veins in the liver secrete Wnt ligands. These induce nearby hepatoblasts, 'bipotent' cells with potential to become hepatocyte or biliary cells, to become prebiliary cells (2). The cells express stem cell marker SOX9, epithelial cell marker KRT18 and biliary cell marker KRT19. The biliary precursors first form a ductal plate surrounding the portal veins, which then doubles to shape two cell layers. Maturation of these layers causes a lumen to form,

generating primitive ductular structures, which after further development become the bile ducts. Not all cells of the ductal plate participate in this process, and those that do not involute or become hepatocytes. Abnormalities in this process of remodeling give rise to ductal plate malformations such as the biliary microhamartomas called von Meyenburg complexes. These abnormal ductal plate remnants might continue growing throughout life, causing cysts to develop (see **chapter 1**). It is thought that the cause for this abnormal behavior of cells is the previously described loss-of-heterozygosity of *PRKCSH*, *SEC63*, *PKD1*, *PKD2*, and possibly *LRP5*, *GANAB*, *ALG8*, *SEC61B* and *PKHD1*.

In adulthood, oval shaped cells within the biliary tract are generally thought to be the local stem cell reservoir. These stem cells are SOX9+, KRT19+ and retain bipotency, allowing them to proliferate and differentiate into hepatocytes and cholangiocytes when required due to circumstances such as biliary damage. The oval cells likely derive from the aforementioned hepatoblasts during embryonic development (92). Upon damage, some of these cholangiocytes also start expressing stem cell marker LGR5+ (93-95), which is known to occur in stem cells of other parts of the intestinal tract (96). LGR5 is part of the Wnt signaling pathway, and actually associates with ADPLD-related protein LRP5 in a complex (97, 98). SOX9+, LGR5+ stem cells may not only contribute to normal tissue homeostasis of the biliary tract, but also to the abnormal expansion of liver cysts upon LOH.

### ***Molecular toolbox to study polycystic liver disease***

Genomic and protein research have made a large leap forward during the past decades, and especially the past years. This progress warrants a few paragraphs dedicated to the new techniques, as it is important to understand the subject by starting from the basics.

#### **Polymerase chain reaction, Sanger sequencing, and SNP arrays**

After discovery of the DNA double helix, enzymes have enabled sequencing and manipulation of DNA (99). Polymerases, ligases, and endo-/exonucleases are at the core of this progress. These enzymes can copy, link together, and cut DNA respectively. Polymerase chain reaction amplifies fragments of DNA or RNA to thousands or millions of copies, allowing easy detection. The nucleotide sequence of these copies can subsequently be determined by a final amplification round with fluorescent nucleotides using Sanger sequencing. The standard deoxynucleotides (dNTPs) are replaced by chain-terminating dideoxynucleotides (ddNTPs). Incorporation of each fluorescent 'A', 'C', 'G', or 'T' nucleotide can be recognized by sensors due to the strong fluorescent signal of many pieces of DNA. These techniques have been applied to broadly scan large segments of DNA in ADPLD families, and study whether any of the large segments, i.e. markers therein, correlate with the phenotype in the family members. Using a method called linkage analysis, the DNA segment with the highest odds to be linked to the phenotype is determined. Within this linked region, all genes are then individually sequenced to look for pathogenic variants (see glossary). In this manner, *PRKCSH* and *SEC63* were found to be a cause for ADPLD in 2003 and 2004 (31, 100, 101).

#### **Next-generation sequencing: exomes, molecular inversion probes, and RNA sequencing**

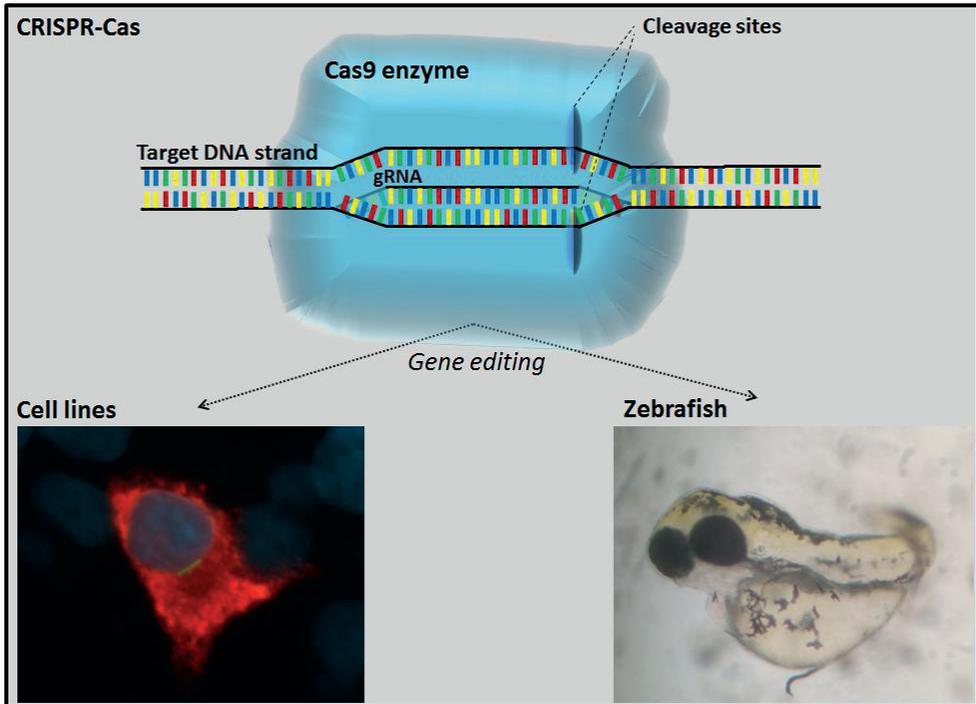
Methods to sequence DNA have kept improving, and 'next-generation' sequencing currently allows cheap and fast determination of the entire gene coding DNA of humans with whole exome sequencing (WES). Instead of sequencing the whole genome of 3 billion bases, only

1% thereof, or 30 million coding bases are sequenced (102). WES is especially effective in rare Mendelian disorders. Since the expected variant is rare, nonpathogenic variants can easily be excluded by sequencing multiple family members. For autosomal dominant disease, two far-removed, disease-affected family members often only share few candidate variants. By specifically looking at the variant – phenotype correlation in additional family members, a candidate variant may be established as the disease cause. The candidate gene can then be validated as a disease cause using cellular and animal models by knocking out the gene and studying the model's phenotype.

Using WES in 2014 (40), Cnossen and colleagues sequenced DNA of two affected pedigrees of an ADPLD family. Amongst several other mutations, they found a mutation in the *LRP5* gene, and subsequently used Sanger sequencing to confirm that the other affected family members shared this abnormality. *GANAB*, *ALG8*, *SEC61B* and *PKHD1* discovered in 2016 and 2017, was discovered by using the same technique (28, 29).

Currently, there is a paucity in finding additional genes by applying WES in affected families. This can be caused by several factors, such as the lack of complete sequence coverage (sensitivity) or the presence of mutations in deep-intronic regulatory sequences that are missed by focusing on the coding sequences in WES. To resolve the sensitivity issue, molecular inversion probe (MIP) sequencing (103, 104) is being considered as an alternative. MIP technology uses small DNA probes with PCR to amplify specific segments of the human genome, which are subsequently circularized. After exonucleases break down all DNA that is not circular, only the circular MIPs remain. In this manner, MIPs allow 30-100 genes to be targeted and sequenced at a lower cost than the 20,000+ genes of the whole genome for hundreds of patients at the same time. This technique will likely be deployed for ADPLD in the near future. Mutations in non-coding deep-intronic regulatory sequences can be detected by sequencing of the entire genome (whole genome sequencing, WGS). This however requires an entirely different bioinformatic analysis pipeline due to the huge expansion of the data load and the search for regulatory patterns. Currently, the cost of this procedure is still often a bottleneck, but it is expected to replace WES in the coming years as the prices go down and the efficiency goes up.

Next-generation sequencing can also be applied to RNA (105). RNA isolated from cells or tissues can be enriched for the messenger (m)RNA. After reverse transcription of the RNA into copy DNA, sequences can be detected as with WES. RNA sequencing not only allows the nucleotide sequence to be determined, but also the amount of mRNA that is expressed per gene. By comparing groups of tissues, e.g. polycystic versus normal cholangiocytes, individual genes or pathways that are differentially expressed are detected.



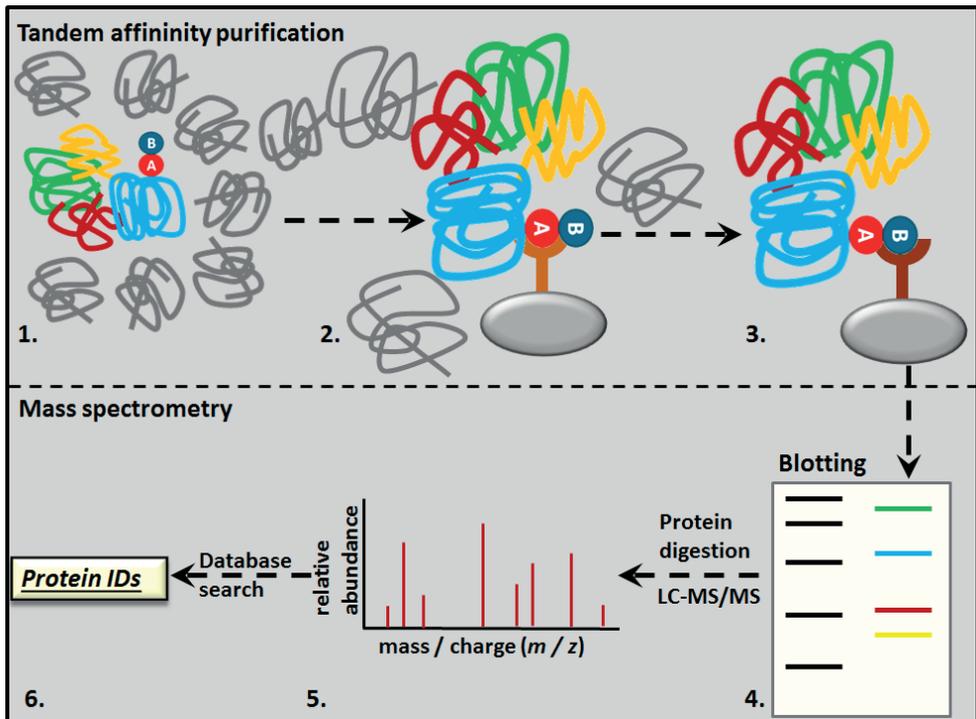
**Figure 5.** CRISPR-Cas9 gene editing.

The Cas9 enzyme is guided to a DNA target site by a gRNA, at which location it will induce a double-stranded break in the target DNA. In this thesis this technique has been applied to develop genetic knockouts of ADPLD genes in cell lines and zebrafish.

### Genome editing: CRISPR-Cas

Although genome editing has existed for many decades, efficient, precisely targeted methods have only been around for several years. At the core of this progress lies a surprisingly simple method discovered in 2012 (99, 106). CRISPR-Cas9 has enabled researchers to efficiently and accurately target and edit genomic DNA of almost any eukaryotic cell, including stem cells. The technique makes use of the plasmids and cloning methodology readily available in every biomedical laboratory, which has allowed it to spread quickly. Its medical implications cause difficult bioethical conundrums, as even germline human DNA may be edited.

Cas9 is a targeted endonuclease (see glossary) that cuts both strands of DNA at sites complementary to a guide (g)RNA (figure 5). Bacterial cells use CRISPR, short for Clustered Regularly Interspaced Short Palindromic Repeats, as a gRNA. For laboratory use, artificial gRNAs are used. These gRNAs contain a targeting sequence of 20 nucleic acids in an RNA scaffold, which is recognized by the Cas enzyme. Repair of the double-stranded breaks induced by CRISPR-Cas frequently leads to mutations, and loss of gene function. The ease of use and GPS-like precision of the research tool has led to successful, accurate genetic editing in a variety of fields from agriculture to medicine. We have applied this technique to genes underlying ADPLD in cells and organisms to generate new disease models (**chapters 5 and 7**).



**Figure 6.** Tandem affinity purification combined with mass spectrometry allows identification of protein complexes.

Image inspired by

[http://web.science.uu.nl/developmentalbiology/boxem/interaction\\_mapping.html](http://web.science.uu.nl/developmentalbiology/boxem/interaction_mapping.html).

1. A protein mixture is obtained by lysis of cells, tissues, or organisms.
2. The protein of interest, with associated complex, is purified using a receptor specific for a tag or other part of the protein. This allows enrichment of the protein of interest in the resulting protein mixture. In this thesis, tag A corresponds to a streptavidin tag.
3. A secondary purification step is performed using a receptor specific for another tag or other part of the protein of interest. Further enrichment of the protein of interest occurs. In this thesis, tag B corresponds to a FLAG tag.
4. In preparation of mass spectrometry, components are separated on an SDS-PAGE gel. Protein bands are then cut out and digested by trypsin. Part of the enriched protein mixture can additionally be checked on a blot. For example, western blotting with a silver stain can verify the success of the attempted protein purification.
5. Analysis by LC-MS/MS allows the digested proteins to be recognized by mass, charge and relative abundance.
6. A database search allows identification of known and (before analysis) unknown proteins present in the protein complex, based on the mass and charge found.

### Affinity proteomics and immunofluorescence

Affinity proteomics is a method to study protein complexes. To direct expression of the protein of interest in cells, the coding sequence of the cognate genes (e.g. *PRKCSH*) is cloned as complement DNA (cDNA) into plasmid expression vectors. These vectors enable multiplication of the plasmids in bacteria and translation and production of the protein in laboratory strains of mammalian host cells. To facilitate protein purification and detection,

the proteins are equipped with molecular affinity tags. In this thesis, the use of tandem streptavidin and FLAG tags allow the isolation of highly purified proteins and their associated complexes in a straightforward and quick purification procedure (107), and allows detection with specific antibodies against the epitope tag. Incorporation of a green fluorescent protein (GFP) tag is often selected instead when only the localization of the proteins is required, as GFP, and thus its fused protein of interest, can be readily visualized under fluorescence microscopes without additional immunostaining procedures. Additionally, proteins containing these tags can be recognized by fluorescence-assisted cell sorters (FACS). Following transfection of the plasmids into cells (e.g. transfection of PRKCSH-Strep-FLAG in biliary cells) (107), isolation followed. By fishing out the proteins in two steps (in tandem) with Strep and FLAG tags, a very pure isolation of the protein and interacting proteins can be obtained (tandem affinity purification, TAP). Mass spectrometry can subsequently determine the identity of the interacting proteins. In **chapter 5**, we performed this technique to determine the interacting proteins around PRKCSH, SEC63, and LRP5.

### ***Research models***

Several research models were employed during the course of this thesis. As mentioned, cyst development was studied at multiple levels, and appropriate models were used as required.

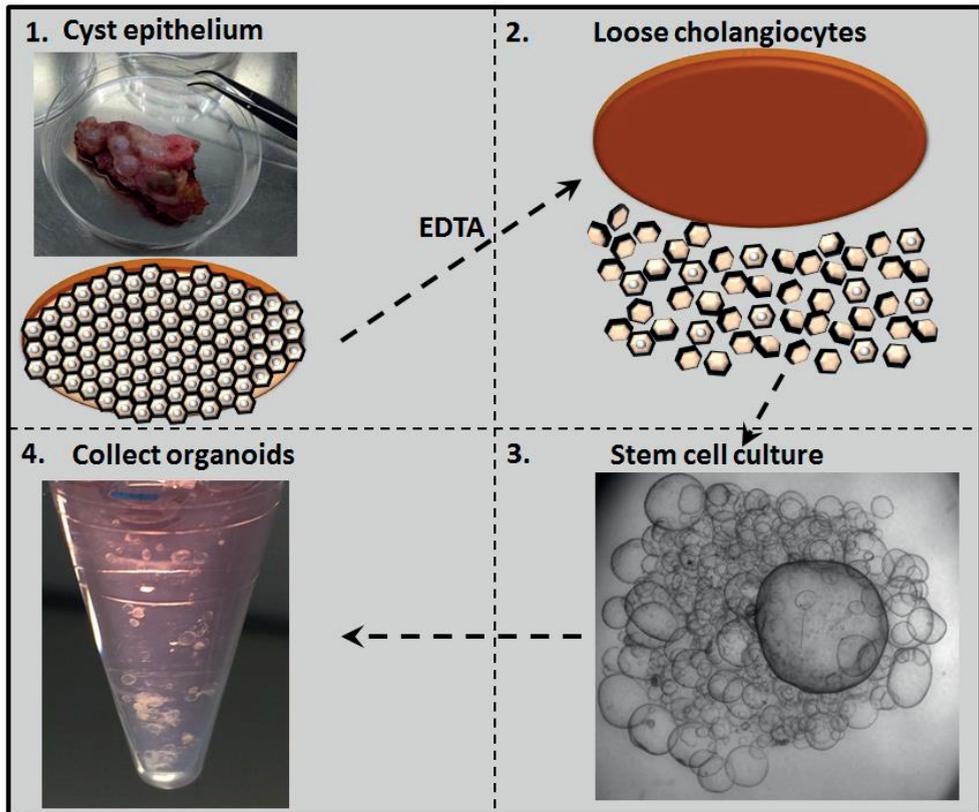
### **Cell lines**

The H69 SV40-virus immortalized cell line is a human cholangiocyte cell line (108), while HEK293T is a well-known model cell line from human embryonic kidney. H69 cholangiocytes develop primary cilia at a high frequency (76). The cell lines have the great advantage that they are easy to work with and can be kept in culture almost endlessly. The disadvantage is that they have lost the genetic signature of healthy primary cells, indicating that the interpretation of test results should proceed with care. The cell lines were applied to the study of the protein interaction network of GLIB, SEC63p and LRP5 in **chapter 5**. Thus far, few human cell lines exist carrying mutations in the PLD genes, and application of CRISPR-Cas9 technology to develop them was one of the research goals of the thesis.

### **Primary cells**

At the start of this PhD-project, primary liver cyst cells could not be kept in culture. From 2008 to 2015, stem cell scientists have discovered methods to isolate and culture human adult stem cells of the intestine, stomach and liver (93-95, 109-111). It became possible to create, maintain and expand adult stem cells, while they retain multi-lineage potential. This has led to the development of so-called organoids, which is defined as a 3D cellular cluster derived from primary tissue. Adult liver stem cells form organoids consisting of hepatocytes and biliary cells.

Aspiration sclerotherapy and cyst fenestration are two interventional treatment options for PLD, whereby parts of cysts are removed. The tissue material from these procedures is valuable as one of the few sources to study human cystogenesis. Cyst epithelium and fluid prove capable of expanding as bipotent cholangiocyte cells, using culture conditions for adult liver stem cells (**chapter 6**). As controls, we cultured cholangiocytes from the healthy liver derived from surgery for liver cancer metastasis. A biobank of cells of different patients was established, which could be characterized by DNA & RNA sequencing, immunohistochemistry and more.



**Figure 7.** Growing organoids from cyst epithelium.

1. Cyst epithelium is collected during surgeries such as laparoscopic cyst deroofing.
2. Cholangiocytes from the epithelium are loosened by EDTA.
3. When placed into circumstances suitable for the growth of liver stem cells, approximately 1% of cholangiocytes will form organoids.
4. Organoids can be collected for further experiments or storage. Examples include DNA, RNA, and protein isolation and follow-up techniques.

### Zebrafish

Zebrafish are a convenient model organism for the study of many diseases. The fish are easy to maintain, readily reproduce, and have short generation times. Development of zebrafish embryos is rapid. Within 5 days organs such as the heart, kidney, and liver grow. As the embryos are translucent, any issue in their development can easily be seen under a microscope.

For ADPLD research, researchers have partially knocked down gene underlying the disease during the embryonic development window of 5 days (112). They observed liver cysts and abnormally curved bodies. This contrasts to the phenotype of fish with a spontaneous *Sec63* mutation described by another study group (113). Fish did not have liver cysts or abnormal curvature, but displayed decreased length, fatty livers, nerve conduction problems, and swim bladder defects. Development of a genetic animal model was attempted by injecting

CRISPR-Cas9 constructs targeting *Prkcsh*, *Sec63*, and *Lrp5* in single-cell zebrafish embryos in **chapter 7** of this thesis.

## **Aim and outline of this thesis**

The aim of this thesis is to gain a better understanding of the pathological mechanisms involved in ADPLD. Clinicians struggle to explain why cysts develop in most of the patient cohort, as the genes underlying >75% of patients are not known. Biomedical scientists remain ambiguous of which pathway gets disrupted following loss of ADPLD protein expression, and how primary cilia are involved. Despite recent progress with somatostatin analogues, there is a struggle to treat patients pharmacologically. Novel disease models are required to unravel the cystogenic pathways, and screens for treatment options. For this reason, novel, suitable cellular and animal models are developed in this thesis, and these models are interrogated at DNA, RNA, protein, organelle, cellular, and organismal level. The research presented here is guided by the following research questions:

## **Research questions**

### **1.) Which underlying germline and somatic genomic abnormalities are present in ADPLD? (chapters 3, 4)**

We isolated DNA from biliary cells obtained from 46 cyst fluid and cyst epithelium samples from 23 patients, and analyzed these using high-density SNP arrays to detect LOH. Since all known cyst genes are recessive on a somatic level, homozygous regions can correspond to regions of interests. We hypothesized that somatic abnormalities, detectable by these SNP arrays, could point the way to novel liver cyst genes. In case of somatic abnormalities, germline DNA was additionally studied. Three types of cyst genes were expected to lie in the abnormal regions. First, genes similar to *PRKCSH* and *PKD1* that lay at the root of disease. Second, in line with the genetic interaction network postulated by Fedeles and colleagues, transheterozygous genes, which are a somatic second-hit in another gene than the germline defect of the patient. Third, modifier genes that influence the rate of cyst development due to germline mutations. By applying these methods, we found that at least half of the patients harbored abnormalities outside of known cyst regions.

### **2.) What are the characteristics of cultured cyst and normal biliary cells as measured by RNA expression? Are any specific pathways dysregulated in liver cyst cells as compared to normal biliary cells? (chapter 6)**

In chapter 5 we use RNA sequencing to determine the gene expression pattern of eight liver cyst cell cultures and compare that with four normal biliary cell cultures. Despite discovery of a genetic interaction network between kidney and liver cyst genes in 2011, it remains unclear which pathway becomes dysregulated upon loss of polycystin-1 expression. The possibility of growing stem cells of liver cysts has opened up a new opportunity to study the mechanism of disease in cystogenesis. We hypothesize that differentially expressed pathways occur between cyst cells and normal biliary cells. We explore whether biliary cells cluster based on genetic background and LOH status, and find evidence in agreement with this hypothesis.

### **3.) What is the ADPLD-associated protein interactome? (chapter 5)**

To discover clues on disrupted pathways in ADPLD, we used tandem affinity purification to isolate ADPLD protein complexes, and analyzed them using mass spectrometry. GIIIB, SEC63p and LRP5 are used as bait proteins to uncover interacting proteins in an unbiased way. We hypothesize that novel interaction partners may point the way towards new ADPLD genes and explain more parts of ADPLD pathophysiology. We explore whether GIIIB, SEC63p and GIIA interact with components of Wnt signaling, cilium formation or other pro-cystogenic pathways. Reversely, the relation of LRP5 to other cyst-related proteins is researched. We detected many known interactors of GIIIB and SEC63p, and several new ones that might point the way towards a cyst interactome.

### **4.) What is the effect of mutation of genes underlying ADPLD on primary cilia and Wnt signaling? (chapter 5)**

In chapter 4 we use CRISPR-Cas9 to induce mutations in the genes underlying ADPLD to study two hypothesized mechanisms of disease. A frequent observance in cyst formation is abnormal or low numbers of primary cilia. LRP5 is the strongest suggestion for an important role of Wnt signaling in cyst development thus far. We hypothesize that loss of *PRKCSH* or *SEC63* leads to reduced ciliogenesis and impaired Wnt signaling. The effect of loss of the genes on LRP5 expression is explored with regard to n-glycosylation and protein maturation. We show here that loss of *PRKCSH* and *SEC63* leads to formation of a lower number of cilia in two cell lines.

### **5.) Do stem cells occur in liver cysts and what are their characteristics? (chapter 6)**

We extracted cells from cyst fluid and epithelium and placed them under conditions suitable for the expansion of adult liver stem cells. Based on data from biliary tracts, we hypothesize that cyst epithelium possesses stem cells capable of *in vitro* proliferation. This fits with the theory of cyst development, which states that a single stem cell acquires a second-hit mutation to cause cyst development. Bipotent cyst stem cells developed, and displayed characteristics of ADPLD, including LOH, expression of ciliary genes, and response to somatostatin analogues.

### **6.) Can zebrafish model polycystic liver disease? (chapter 7)**

CRISPR-Cas9 technology is again applied to induce mutation in the genes underlying ADPLD, but this time in zebrafish. Currently, no accurate models of ADPLD exist, only mice with post-natal disruption of pro-cystogenic genes. As new genes for the disease are discovered, their effects can be validated in test animals. Given the need for new therapeutics, models should be developed that allow drug screens to be conducted without problem. Zebrafish have the advantage of rapid development and translucent embryos. These favorable conditions allow study of the effect of ADPLD mutations on liver development in an organism. We hypothesize that disruption of *PRKCSH*, *SEC63*, *LRP5* or all three genes leads to formation of liver cysts in zebrafish. In **chapter 7**, we compare the phenotype of the mutated fish to those of morpholino knockdown fish (expression of genes is only partially disrupted in morpholino knockdown), and find large discrepancies.

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## Chapter 2

Polycystic liver disease: ductal plate malformation and the primary cilium

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### **Abstract**

Polycystic livers are found in autosomal dominant polycystic kidney disease (ADPKD), caused by *PKD1* and *PKD2* mutations in virtually all cases, and in isolated polycystic liver disease (PCLD), caused by known *PRKCSH* and *SEC63* mutations in only ~20% of cases together. Loss-of-heterozygosity in single hepatoblasts leads to underlying cystogenic ductal plate malformations. Crucially, actual components driving this development remain elusive. Recent advances have unraveled the roles of TGF- $\beta$ , Notch and Wnt signaling, transcriptional regulators like HNF6 and HNF1 $\beta$ , as well as cilium function in hepatobiliary organogenesis. In polycystic liver disease, mutation or defective cotranslational processing of key elements required for primary cilium formation have been implicated. This review recapitulates liver patterning factors in hepatobiliary development and extracts molecular players in hepatic cystogenesis.

### **Lay summary**

Liver cysts, abnormal fluid-filled sacs, are caused by complete loss of genes like *PKD1*, *PKD2*, *PRKCSH* and *SEC63* in the body. This review summarizes the subsequent effects on the molecules responsible for development of the liver and bile ducts, to determine what goes erroneously to cause cysts.

### **Keywords**

polycystic liver disease, loss-of-heterozygosity, hepatobiliary organogenesis, ductal plate malformation, hepatic cystogenesis, primary cilium

## **Brief introduction into polycystic liver disorders**

Liver cysts are a relatively common finding on routine abdominal imaging, being present in approximately 2.5-18.0% of the population [1]. Polycystic livers are defined by the presence of >20 cysts and are estimated to be present in 0.05-0.53% of the population [2]. Polycystic liver disease (PLD) is part of the phenotype of two inherited disorders: autosomal dominant polycystic kidney disease (ADPKD); and polycystic liver disease (PCLD). PCLD is distinguished from ADPKD by the absence of polycystic kidneys. Cystic livers, together with hepatic fibrosis, may also be found in the spectrum of diseases that have dysfunction of the primary cilium (Box 1).

ADPKD, caused by mutations in *polycystic kidney disease (PKD)1* and *PKD2*, is the most common cause for end-stage kidney disease, and is seen in approximately 0.10-0.25% of the population [3]. *PKD1* encodes the transmembrane protein polycystin-1, and is seen in 85% of ADPKD cases. *PKD2* encodes the transmembrane protein polycystin-2, which is responsible for 15% of ADPKD cases. Although polycystic kidneys are the primary presentation, liver cysts occur in 83-94% of patients as an extrarenal manifestation [4, 5]. PCLD is rare and is estimated to be present in about 0.03% of the population [6]. There is considerable genetic heterogeneity as *Protein kinase C substrate 80K-H (PRKCSH)* and *SEC63* mutations have been identified only in about 20% of the cases [7], leaving over 80% of cases currently without a genetic diagnosis.

Cyst development is driven by somatic second hit mutations, which affect wild-type alleles of biliary type cells during early hepatic organogenesis [8, 9]. This process, termed loss-of-heterozygosity (LOH), occurs in most cysts associated with *PRKCSH*, *PKD1* or *PKD2* mutations, and at a lower proportion in *SEC63* mutated cysts [10]. The key processes that lead to development of cysts start early in life. In PCLD it is thought that subsets of cells behave

abnormally during maturation of the ductal plate [11]. Unlike normal biliary plate hepatoblasts, these cyst initiator cells do not undergo regression after disconnection from the biliary tree but continue to proliferate.

The molecular disease mechanism leading from focal LOH to a defective response during ductal plate remodeling represents a key event for cystogenesis, but actual components that drive the development remain elusive. In normal hepatobiliary development, the important roles of Notch, transforming growth factor (TGF)- $\beta$  and Wnt signaling have recently been unraveled and have afforded better insight into transcriptional regulation by the involved transcription factors (i.e. hepatocyte nuclear factor (HNF)6 and HNF1 $\beta$ ). The common denominator in cystogenesis is the mutation of individual genes that are part of these signal transduction routes, and the disruption of many of these encoded proteins affects the primary cilium. This review aims to recapitulate molecular players in cystogenesis by focusing on known liver patterning factors in hepatobiliary development.

### **Box 1. Additional cholangiociliopathies**

Hepatic cysts and fibrosis frequently occur as part of the phenotype of multiple ciliopathies, which have therefore also been termed cholangiociliopathies [37].

#### **Autosomal recessive polycystic kidney disease (ARPKD)**

ARPKD always causes liver disease involving Caroli disease and syndrome, with a prevalence of 0.005% [2]. Caroli disease is defined as ectasia or segmental dilatation of the larger intrahepatic bile ducts (IHBDs), whereas Caroli syndrome is defined as ectasia of small IHBDs and congenital hepatic fibrosis in addition to Caroli disease. It is caused by mutations in *PKHD1*. Common features are enlarged kidneys with cysts, pulmonary hypoplasia, characteristic facies, and contracted limbs with clubfeet.

#### **Meckel–Gruber syndrome (MKS)**

MKS has a prevalence of 1:135 000 and frequently presents with liver cysts [46]. It is a recessive disease caused by mutations in *MKS1*, *MKS2*, and *MKS3*, and in genes also associated with NPHP such as *NPHP3*, *NPHP6*, and *NPHP8*. Renal cystogenesis, polydactyly, and occipital encephalocele are part of the MKS phenotype.

#### **Oral-facial-digital syndrome 1 (OFD1)**

OFD1, an X-linked disease caused by mutations in *OFD1*, presents with fibrocystic liver disease in ~40% of cases [57]. Prevalence estimates vary from 1:50 000 to 1:250 000 [58]. Renal cystogenesis, brain abnormalities, and malformations of teeth, jaw, facial bones, hands, and feet are often seen in OFD1 [36,58].

#### **Tuberous sclerosis (TSc)**

TSc presents with hepatic cysts in ~13% of cases [59] and has a prevalence of 1:10 000 to 1:25 000 [60]. Loss of *TSC1* or *TSC2* leads to hamartomatous lesions in multiple organ systems. Renal cysts and angiomyolipomas are frequently present.

#### **Cranioectodermal dysplasia (CED)**

Three patients with CED, also known as Sensenbrenner syndrome, have been described with liver cysts as part of the phenotype [40,41]. CED is caused by mutations in genes encoding IFT-A proteins *IFT122/CED1*, *IFT121/CED2*, *IFT43/CED3*, and *IFT144/CED4*. Symptoms include kidney cysts, skeletal abnormalities, ectodermal defects, and

characteristic facies.

#### **Von Hippel–Lindau (VHL) disease**

VHL is an autosomal dominant disease, caused by *VHL* mutations [36], which may present with liver cysts [61]. VHL occurs at a frequency of 1:36 000 live births [62]. It is characterized by the development of hemangioblastomas of the central nervous system and retina, neuroendocrine tumors, and renal cysts [36].

#### **Nephronophthisis, Bardet–Biedl, Joubert, and Jeune syndromes**

Ciliopathies NPHP, Bardet–Biedl, Joubert, and Jeune syndromes frequently present with isolated congenital hepatic fibrosis due to ductal plate malformation [37].

### ***Normal hepatobiliary development***

An overview of the process of normal hepatobiliary development and the roles of liver patterning factors therein is critical to understand the molecular mechanisms of hepatic cyst development. Hepatic organogenesis starts at week four (or embryonic day (E) 8.5 in mice [12]), when the liver bud arises from the cephalic part of the primitive foregut near the yolk sac [11]. At that point the pars hepatica, one of two liver bud divisions, initiates liver precursor cells to grow into the mesenchymal separation between the pericardial and peritoneal cavities. These cells start to occupy endothelium-lined spaces of the septum transversum. In this manner, the basic architecture of the liver becomes established, with parenchymal cords and plates alternated by hepatic sinusoids. At week eight (or E10.5 in mice [13]) the first intrahepatic bile duct (IHBD) precursors develop. Cytokeratin (CK)8, CK18 and CK19 positive hepatoblasts start organizing in a ductal plate [11], enveloping the portal mesenchyme. This ductal plate induction is tightly controlled by a signalling environment involving several key signalling pathways [12].

#### **TGF- $\beta$ , Notch and Wnt signalling pathways**

One of the signalling pathways that drives ductal plate development is TGF- $\beta$  [13]. A signalling gradient of increased TGF- $\beta$ /activin near the portal vein is implicated in ductal plate development at mouse E12.5. The TGF- $\beta$  antagonist chordin is present in liver parenchyma at E16.5, while the presence of TGF- $\beta$  transcription factor SMAD5 is limited to portal hepatoblasts [14]. The TGF- $\beta$  gradient occurs in combination with HNF6 induced repression of the TGF- $\beta$  receptor II (TGF-RII) in the liver parenchyma. TGF- $\beta$ RII positive prebiliary cells shape a primary layer near the portal vein, a process that is associated with subsequent loss of TGF- $\beta$ RII expression. Subsequently, a second layer of TGF- $\beta$ RII positive hepatoblasts is created at the parenchymal side of the primary layer [13]. This allows development of a lumen between the asymmetric layers. Maturation of these layers expands in a wave starting at the largest portal veins of the liver hilum propelling towards the periphery, inducing the prebiliary hepatoblasts of the second layer to become cholangiocytes.

Notch signalling is required for the processes of differentiation and tubulogenesis, as well as bile duct density [12, 15]. Notch receptors are activated by ligands such as Jagged1 and Notch2 from the periportal mesenchyme and biliary cells, which signal through (Recombining binding protein suppressor of hairless) Rbpj and hairy and enhancer of split-1

(Hes)/ Hairy/enhancer-of-split related with YRPW motif (Hey) family members. Both TGF- $\beta$  and Notch signalling have consistently been implicated in in vitro murine biliary fate specification of bipotent adult liver stem cells [16].

Similarly, a gradient of canonical Wnt signalling appears to regulate the ductal plate [17]. Effects of Wnt signalling are time- and context-dependent during liver development. In the ductal plate of wild type mice, the Wnt signal transducer  $\beta$ -catenin is strongly present at the membrane, while hepatoblasts elsewhere have a much weaker membranous  $\beta$ -catenin staining. This stabilization of  $\beta$ -catenin is transiently induced at mouse E15.5, reaching a peak membranous intensity on E17.5 up to adulthood. It is therefore hypothesized that Wnt, generated by the portal vein or mesenchyme, may help induce ductal plate formation. This concept is supported by in vitro studies that show that early embryonic liver cultures lack CK19+ biliary cells following  $\beta$ -catenin silencing [18], whereas culture in Wnt3a conditioned media leads to biliary differentiation [19]. Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signalling pathways, together with extracellular matrix (ECM) components complete the extracellular factors involved in specifying biliary cell fate [20].

### **Biliary fate specification depends on a network of transcription factors**

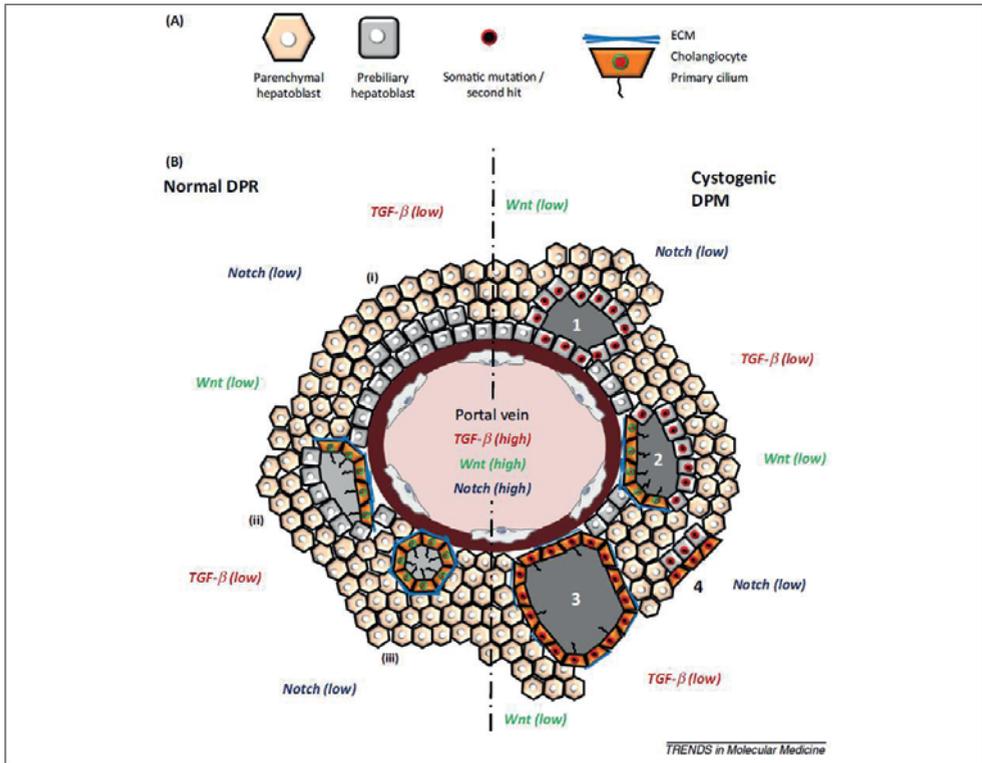
Wnt, TGF- $\beta$  and Notch signalling pathways function in concert with a network of intracellular transcription factors [21]. Biliary fate specification of hepatoblasts relies on hematopoietically expressed homeobox (Hhex), onecut domain family member (OC) 2, HNF6/OC1, SRY-box (Sox)9, and HNF1 $\beta$ . It also depends on absence of hepatocyte fate transcription factors such as T-box transcription factor (TBX)3, HNF1 $\alpha$ , HNF4 $\alpha$ , CCAAT/enhancer binding protein (C/EBP) $\alpha$ , Forkhead box protein A(FoxA)1 and 2. These intracellular transcription factors are not only regulated by the extracellular Wnt, TGF- $\beta$  and Notch signalling pathways, but also function reciprocally. For example, HNF6 and Sox9 are vital to maintaining the TGF- $\beta$  signalling gradient [13, 22]. Modulation of the regulatory factors acts in a timing and context dependent manner, and is required for regulation of hepatobiliary development. The cholangiocyte transcription factor machinery finally leads to expression of specific ciliary genes such as polycystic kidney and hepatic disease 1 (PKHD1) and cystin-1, but biliary differentiation itself may also be regulated by the primary cilium or its components (see below).

### ***Pathophysiology of hepatic cystogenesis***

Hepatic cystogenesis is thought to occur by defective remodeling of the ductal plate, and is considered a process of ductal plate malformation (DPM) [22]. Normally, when primitive ductular structures mature to bile ducts, ductal plate cells not involved in tubulogenesis involute or become periportal hepatocytes [23]. In PCLD, somatic second-hits in wild-type alleles are hypothesized to underlie the failure of single cells to undergo correct remodeling [8]. Studies of *Hnf6*, *Hnf1b* and *cystin-1* mouse knockout models has allowed for a classification of cystogenic remodeling, which is found to occur by at least three distinct origins of DPM (Figure 1) [22], respectively: (i) DPM 1, disturbed differentiation of biliary precursor cells; (ii) DPM 2, impaired maturation of radially asymmetric primitive ductular structures; and (iii) DPM 3, abnormal duct expansion. Additional origins of cystogenic DPM potentially occur, and are described below.

*HNF6* and *HNF1B* have been shown to be downstream regulatory factors that are disrupted in multiple models, and a recurrent phenotype is poorly differentiated or hybrid

hepatobiliary cells with a tendency to develop cysts. These observations have also been made in conditional gene ablation mouse models of hepatocyte transcription factors *C/EBP $\alpha$*  and *Hhex* during early stages of biliary development [24, 25], as well as in a mouse line in which aberrant Wnt signaling is induced by loss of the gene encoding *adenomatosis polyposis coli (APC)* [17]. In the whole liver, disrupted hepatocyte development occurs in addition to cyst development in the three models. Postnatally, embryonic differentiation defects resolve in *Hnf6<sup>-/-</sup>* and *Hnf1b<sup>-/-</sup>* models [22], but DPM and defects of primary cilia persist. In *Hnf1b<sup>-/-</sup>* mice dysplastic ducts additionally persist. *C/EBP $\alpha$ <sup>-/-</sup>* and *Hhex<sup>-/-</sup>* mice may develop adult polycystic liver disease, which has not been investigated for *APC<sup>-/-</sup>* mice. In *Foxa1* and *Foxa2* knockout mice bile duct hyperplasia occurs [26], which is reminiscent of DPM 3. A fourth DPM pattern may be found in models that overexpress the intracellular domain of *Notch2* and knockout of *Wnt5a*, because this gives rise to aberrant remnants of ductal plate cholangiocytes [15, 27]. In these mice, Notch overactivation promotes cholangiocyte survival, biliary differentiation and increased periportal bile duct density. *Xylosyltransferase (Xylt)2<sup>-/-</sup>* mice also develop polycystic liver and kidney disease [28], but *Xylt2* deficiency causes reduced glycosaminoglycan synthesis rather than hepatic morphogen signaling defects. Although increased  $\beta$ -catenin expression is observed and may be a downstream mechanism, *Xylt2<sup>-/-</sup>* mice implicate proteoglycan/ECM abnormalities as an additional mechanism to cystogenesis. Together these data suggest that cystogenic DPM can occur through multiple, distinct mechanisms depending on the genetic background. To date, it is not yet known which of these embryologic mechanisms cause PCLD following somatic loss of the wild-type alleles. Monoclonal proliferation and dilatation of single initiating cells are thought to cause the biliary hamartomas and cysts observed in adult PCLD patients. Recent discoveries in the function of hepatocystin and SEC63p have identified important clues to the cause of DPM in PCLD, and are discussed in the subsequent paragraphs.



**Figure 1.** Schematic representation of ductal plate remodelling and malformation. Ductal plate remodelling (DPR) depends on transforming growth factor (TGF)- $\beta$ , Notch and Wnt signalling gradients, and cellular responsiveness to the respective ligands. Further modulation comes from cell–cell and cell–environment interactions mediated by the extracellular matrix (ECM) and primary cilia. **(A)** Legend. **(B)** Left: normal DPR: (i) prebiliary cells shape a primary layer near the portal vein. (ii) Following maturation of the primary layer, a second layer of prebiliary cells allows a lumen to form. (iii) Biliary tracts are formed when the secondary layer matures to cholangiocytes. Right: ductal plate malformation (DPM) 1, 2, and 3 indicated by white numbers and a biliary hamartoma in the liver parenchyma designated by a black 4. DPM 1, disturbed differentiation of biliary precursor cells; DPM 2, impaired maturation of radially asymmetric primitive ductular structures; and DPM 3, abnormal duct expansion.

### ***PRKCSH, SEC63 loss of heterozygosity, and co-translational protein processing***

*PRKCSH*, located on chromosome 19p13.2, encodes for an 80 kDa protein that has been named hepatocystin [29]. It serves as the noncatalytic  $\beta$ -subunit of glucosidase (G)II, which requires hepatocystin for its endoplasmic reticulum (ER) retention. Cysts of *PRKCSH* mutated patients contain secondary somatic mutations in >76% of epithelia [8], indicating recessivity at the cellular level.

*SEC63*, located on chromosome 6q21, encodes SEC63p, an 88 kDa protein involved in cotranslational protein translocation of secreted or membrane-inserted proteins into the ER. It associates in stoichiometric amounts with Sec61p, where it functions in the Sec61 complex. Together with its luminal counterpart binding immunoglobulin protein, SEC63p is required for translocation and membrane insertion of a subset of ER-translocated proteins.

*SEC63* mutation carriers only display LOH in 1 of 14 cyst epithelia [10]. Cyst development due to *SEC63* variants therefore seems to depend upon another stochastic process.

### Cotranslational protein processing

Protein transport into the ER is dependent upon N-terminal signal peptides, C-terminal tail peptides and signal recognition peptide anchors and occurs co- as well as post-translationally. Signal-peptide containing proteins are transported in three stages: (i) targeting; (ii) membrane insertion at the Sec61 complex; and (iii) completion of membrane translocation. *SEC63p* is especially important for membrane insertion of proteins with multiple transmembrane regions. *SEC63p*-dependent ER transport relies on the sequence of signal peptides [30], and loss of *SEC63p* leads to reduced glycosylation as well as decreased ER membrane insertion of *SEC63p*-necessitating proteins. Interestingly, although loss of plasma membrane protein aquaporin (AQP)-2 is found in both *Sec63*<sup>-/-</sup> and *Prkcsh*<sup>-/-</sup> renal tubular cells, the proteins mediate this effect at different stages of protein biogenesis [30]. Transport of AQP-2 into the ER is unimpaired in *Prkcsh*<sup>-/-</sup> cells, indicating that protein loss occurs at a later stage.

Hepatocystin assists in glycan processing by cleaving two glucose residues in the ER. This allows quality control enzymes calnexin/calreticulin to bind and assist in protein folding. Proteins that are folded correctly undergo cleavage of a final glucose residue by glucosidase II. In case of improper folding, re-glycosylation and additional cycles of glucose cleavage occur, until folding is completed or the protein becomes bound for ER associated degradation (ERAD). ERAD requires retranslocation out of the ER, also involving *SEC63p*. Although additional functions have been suggested (Box 2), hepatocystin and *SEC63p* function in closely related, but distinct stages of protein biogenesis.

**Table 1.** Cystogenic genes, related protein product pathways and DPM malformation<sup>a</sup>

<i>Gene</i>	<i>Pathway</i>	<i>DPM</i>	<i>DPM type</i> <sup>b</sup>	<i>Additional remark</i>	<i>Ref</i>
<i>Apc</i>	Wnt (canonical)	Cystogenic	1	-	[17]
<i>Wnt5a</i>	Wnt (noncanonical)	Bile duct hyperplasia	3	-	[27]
<i>Notch2ICD</i>	Notch	Bile duct hyperplasia, ductal plate remnants	3	Overexpressed	[15]
<i>Hnf6α</i>	Transcription factor	Cystogenic	1	-	[22, 57]
<i>OC-2</i>	Transcription factor	Cystogenic	-	-	[57]
<i>Hnf1B</i>	Transcription factor	Cystogenic, bile duct paucity	2	-	[22]

<b><i>Foxa1 / Foxa2</i></b>	Transcription factor	Bile duct hyperplasia	3	Genes redundant, double knockout	[26]
<b><i>Hhex</i></b>	Transcription factor	Cystogenic	1	-	[25]
<b><i>C/ebpa</i></b>	Transcription factor	Cystogenic	1	-	[24]
<b><i>Xylt2</i></b>	Glycosaminoglycan synthesis	Cystogenic	-	-	[28]
<b><i>ALG8</i></b>	N-glycan processing (CDG-Ih)	Cystogenic, Caroli disease	-	-	[31]
<b><i>PRKCSH</i></b>	N-glycan processing	Cystogenic	3	Somatic LOH	[29]
<b><i>SEC63</i></b>	Protein quality control & membrane insertion	Cystogenic	3	-	[29]
<b><i>PKD1</i></b>	Cilium	Cystogenic	3	Somatic LOH	[9]
<b><i>PKD2</i></b>	Cilium	Cystogenic	3	Somatic LOH	[9]
<b><i>Pkhd1</i></b>	Cilium	Cystogenic	3	-	[56]
<b><i>Cystin-1</i></b>	Cilium	Cystogenic	3	-	[22]
<b><i>IFT88</i></b>	Cilium (IFT-B)	Cystogenic	1/2	-	[45]
<b><i>MKS genes</i></b>	Ciliary axoneme / basal body	Cystogenic, Caroli syndrome	1/2	-	[46]
<b><i>OFD1</i></b>	Cilium / basal body	Cystogenic, Caroli syndrome	-	-	[58]
<b><i>CED genes</i></b>	Cilium (IFT-A)	Cystogenic, Caroli syndrome	-	-	[40, 41]
<b><i>TSC1, TSC2</i></b>	TSc complex / basal body	Cystogenic	-	-	[59]
<b><i>VHL</i></b>	Von Hippel-Lindau / ciliary axoneme	Cystogenic	-	-	[60]

**a** Abbreviations: DPM, ductal plate malformation; LOH, loss of heterozygosity.

**b** DPM 1: differentiation defect described, DPM 2: defect of PDS maturation described, DPM 3: abnormal duct expansion described, -: unknown mechanism; Italics: DPM type not classified by referenced studies.

## ***Effects of defective co-translational protein processing on hepatic signaling***

Around 30% of proteins undergo ER translocation, and are subject to folding and quality control by PRKCSH and SEC63p. Defective processing of a key regulator of biliary cell growth is a likely cause of PCLD [29]. This is thought to be the mechanism that underlies DPM-associated disorders in the context of PRKCSH and SEC63 mutations, as well as those observed in several congenital disorders of glycosylation (CDG) [31]. Patients with type I CDG have defects in synthesis and transfer of glycans, whereas type II CDG patients have defective glycan processing. Many CDG patients have hepatomegaly as part of their clinical phenotypes, and bile duct dilatation has been reported in CDG-Ib, CDG-Id, CDG-Ih and CDG-IIb. Liver cysts have been observed in CDG-Ih. Unsurprisingly, the biochemical defect of at least two of these subtypes (CDG-Ih and CDG-IIb) shows considerable overlap with the protein translocation and folding defects seen in PCLD. Several biliary growth related transmembrane proteins are sensitive to defects in cotranslational processing, and polycystin-1 has recently been strongly implicated in relation to the known PCLD genes [32].

### ***Box 2: Postulated roles of hepatocystin and SEC63p beyond cotranslational processing***

#### **Hepatocystin**

Knockdown of either hepatocystin or glucosidase II induces autophagy, as found by a significant increase in LC3 positive autophagic structures [64]. This did not occur through the unfolded protein response pathway, but rather through inhibition of mammalian target of rapamycin (mTOR) signaling. Although these findings indicate an additional role for hepatocystin in autophagy, they do not preclude an effect of its deficiency on the cilium. Interestingly, a pair of papers recently showed an interdependency of autophagosome induction and cilium formation [43, 65]. Another proposed function of hepatocystin is that of Inositol 1,4,5-Trisphosphate receptor (IP3R) induced regulation of calcium release activity [66].

#### **SEC63p**

SEC63p has been shown to interact with nucleoredoxin (NRX) in a yeast two hybrid assay [67]. This establishes an indirect link of SEC63p to Disheveled 1 (Dvl1), which is also an NRX binding partner. Intriguingly, Dvl1 has been shown to be important in Wnt signaling and planar cell polarity [68].

## **Cotranslational processing and defects in hepatic signaling routes**

*PrkcsH* and *Sec63* function in a genetic interaction network with *Pkd1*, *Pkd2* and *Pkhd1* [32]. Polycystin-1 dosage is the final effector for severity of cyst development within this network. Loss of *PrkcsH* or *Sec63* in combination with *Pkd1* haploinsufficiency significantly worsens cyst development in comparison to loss of individual PLD proteins. Conversely, overexpression of polycystin-1 rescues the phenotype of the PCLD orthologous mice. Importantly, polycystin-1 expression, and that of selected membrane proteins such as Na/K

adenosine 5'-triphosphatase (ATPase), decreases significantly following homozygous deletion of either PCLD gene. A likely cause is the requirement of proteins such as Na/K ATPase  $\beta$ 2-subunit to undergo multiple n-glycosylation dependent courses of folding and quality control [33].

In the study by Fedeles' group, cyst development follows loss of *Prkcsh* or *Sec63* induced at mouse post-natal day 28 [32]. Cysts derived from bile ducts possibly also occur during patients' adult life, but this mechanism does not have to be distinct from embryonic DPM, as bipotent liver progenitors persist in the adult biliary tract [16, 23], and differentiation depends on the same signaling factors found in ductal plate remodeling. Also, abnormal duct expansion of DPM 3 may occur in already differentiated cholangiocytes.

TGF- $\beta$  and Wnt hepatic signaling routes are sensitive to the n-glycosylation state of receptors TGF- $\beta$ RII and low density lipoprotein receptor-related protein (LRP)6 [34, 35]. Membrane expression of TGF- $\beta$ RII decreases following n-glycosylation defects of tunicamycin-treated cells, an effect even stronger for TGF- $\beta$ RII mutants lacking n-glycosylated asparagine sites. N-glycosylation defects of TGF- $\beta$ RII lead to 2.5-5-fold decreases in signaling and phosphorylated SMAD2 levels. Similarly, n-glycosylation of LRP6 is important for maturation, membrane localization and signaling. When n-glycosylation increases due to the loss of Mest protein, levels of active, phosphorylated LRP6 and  $\beta$ -catenin increase [35]. The levels of downstream Wnt-regulated transcription factors such as C/EBP $\alpha$  then decrease, indicating the importance of LRP6 glycosylation.

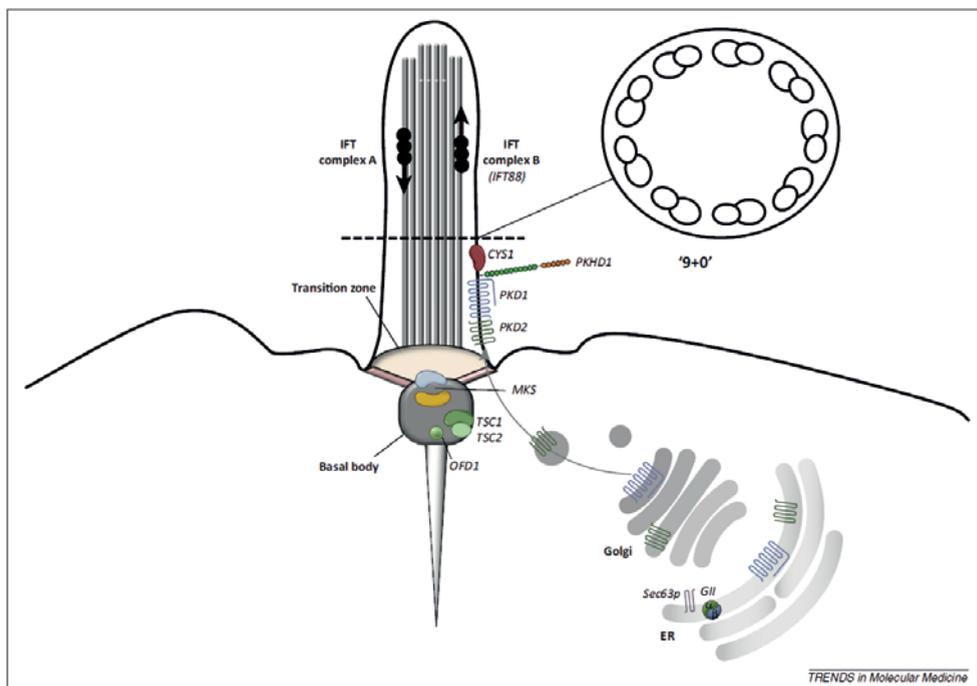
Summarizing these studies, the strongest case has currently been made for the pivotal role of loss of polycystins as a cause for cystogenesis in *PRKCSH* and *SEC63* deficient tissues. Defective Wnt or TGF- $\beta$  signaling may be an alternative route to cystogenesis following impaired n-glycosylation, but other routes have also been postulated, involving primary cilium proteins.

### ***Polycystins and polycystic liver disease as a ciliopathy***

Primary cilia have been identified as key organelles in the pathogenesis of ADPKD and related cystic diseases [36]. In the liver parenchyma, only cholangiocytes possess a primary cilium. Primary cilia are solitary, non-motile organelles on the apical cell membrane surface of most mammalian cells (Figure 2). They are regarded as the cell's antennas, involved in mechano-, osmo- and chemoreception of, for example, bile flow [37-39]. A cilium is assembled from a basal body that itself originates from the older of two centrioles in quiescent cells [36]. Non-motile cilia have a characteristic 9+0 microtubule arrangement. The ciliary compartment and ciliary membrane have a dedicated protein and lipid composition that separate this organelle from rest of the cell. This composition is assembled and maintained by active intraflagellar transport complexes A (IFT-A) and B (IFT-B), along axonemal microtubule tracks, and active protein and vesicle shuttling across different barriers at the ciliary base.

Cilia have been implicated in planar cell polarity, cell cycle control, and numerous essential signalling pathways. Canonical Wnt, mammalian target of rapamycin (mTOR), cyclic adenosine monophosphate (cAMP), G protein-coupled receptors, cystic fibrosis transmembrane receptor, epidermal growth factor receptor, mitogen-activated protein kinase/extracellular signal-regulated kinase and cellular calcium ion (Ca<sup>2+</sup>) signalling all locate to the cilium. The ciliary compartment is also the active site of the protein products of hepatocystic disease genes *PKD1*, *PKD2*, *PKHD1*, *cystin-1*, *intraflagellar transport protein 88 homolog (IFT)-88* and four cranioectodermal dysplasia (CED) genes that are part of IFT-A [36,

40, 41]. Meckel syndrome (MKS)-associated proteins are found in the transition zone, as well as the basal body [42], where tuberous sclerosis (TSC)1 and TSC2 protein products are also located. Oro-facial-digital syndrome 1 protein (OFD)1 is localized at or near the basal body [43], while the *VHL* protein product is associated with the cilium, but not located in it [36]. Noncystic DPM is also frequently observed in ciliopathies such as nephronophthisis (NPHP), Joubert syndrome and Bardet-Biedl syndrome [37]. Consequently, biliary ductal development and cilium expression are intimately linked. Ciliopathies frequently lead to DPM and, vice versa, genes causing DPM often lead to ciliopathy.



**Figure 2.** Localization of cystogenic genes in and around the cilium. The ciliary compartment is the active site of the protein products of hepatocystic disease genes PKD1, PKD2, PKHD1, cystin-1, IFT88, and four CED genes that encode IFT-A proteins. MKS-associated proteins are found in the transition zone, as well as the basal body, where TSC1 and TSC2 protein products are also located. OFD1 is localized at or near the basal body, whereas the *VHL* protein product is associated with the cilium, but not located in it (not shown). In the ER, co-translational processing by hepatocystin and Sec63p is essential for the dosage of polycystin-1 and polycystin-2. Gray arrow: transport from ER through the Golgi to the cilium. Black arrows: intraflagellar transport from ciliary base to ciliary tip and vice versa. Dashed line indicates cross-section of non-motile cilium showing its characteristic '9+0' microtubule arrangement. Abbreviations: GIIb, hepatocystin; Sec63p, Sec63 protein; PKD1, polycystin-1; PKD2, polycystin-2; PKHD1, polycystic kidney and hepatic disease 1; CYS1, cystin-1; IFT88, intraflagellar transport protein 88 homolog; OFD1, oral-facial-digital syndrome 1 protein; MKS, Meckel–Gruber syndrome; TSC1, tuberous sclerosis protein 1; TSC2, tuberous sclerosis protein 2; IFT, intraflagellar transport; ER, endoplasmic reticulum.

## Cystogenic ciliopathies and ductal plate malformations

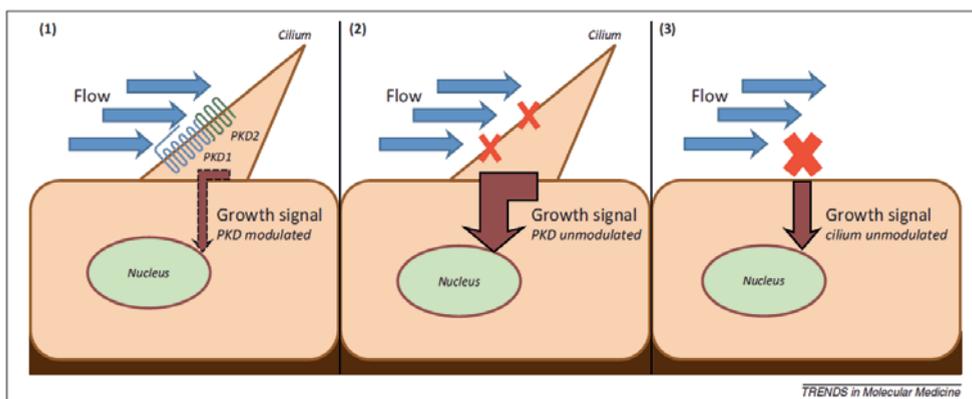
Although ciliopathies are associated with DPM, ciliopathy genes may confer different types of DPM (Table 1). DPM 1 and 2-associated transcription factors *HNF6* and *HNF1B* are main players in the differentiation of biliary hepatoblasts, and loss of these genes leads to ciliary impairment in mice, and paucity of cilia has been observed in human *HNF1B* heterozygotic mutants [22]. Mutant cholangiocytes display reduction of *cystin-1* concurrently with loss of cilia. It is not yet known which of the ciliary genes causes the DPM of *HNF1B* and *HNF6* mutants. The ciliopathy of *cystin-1* mutants is not linked to a cholangiocyte differentiation or duct maturation defect (DPM 1 or 2), but to aberrant bile duct expansion (DPM 3).

At least two ciliopathy associated genes do show differentiation defects. Mutations of *IFT-88* lead to defective ciliary assembly and DPM in mice [44] and man [45]. Cells in the portal area of the mice show differentiation defects as determined by the expression of alpha-fetoprotein and biliary lectins. Similarly, five out of six human Meckel syndrome fetuses possess hybrid cells co-expressing ductal (CK19) and hepatocyte (*HNF4α*, Albumin) markers in DPM-associated cysts [46]. Half of these fetuses have a reduction in, or complete absence of, cilia. The study authors suggest that defective differentiation may have been the initial cause.

Given that ciliopathies cause different DPM categories, dependent on the genetic background, it is not directly clear to which DPM category PCLD and APDKD belong. Cystic expansion of bile ducts occurs following post-natal loss of *Pkd1*, 2, *Prkcsh* or *Sec63* [32] and this implicates DPM 3 as a likely option. Consistent with this hypothesis, cilium-independent cystogenicity of the polycystins has recently been excluded [47].

### Loss of polycystins causes severe cystogenesis through cilia

Polycystin loss without loss of cilia is the likely cause of the severe phenotype of ADPKD livers [47] and likely that of *PRKCSH* and *SEC63* livers as well [32]. Loss of cilia by either a mutation of the gene encoding the kinesin-like protein (*Kif3a*) or by *Ift20* mutation causes milder cystic disease compared to cystogenesis induced by polycystin loss, and polycystin dosage variation following cilium loss does not affect cyst severity. Conversely, if cilia are lost after polycystins, cyst severity increases with time as long as cilia persist, and slows after cilium loss. These findings exclude a cystogenic contribution of polycystins without cilium activity. Polycystins therefore inhibit a cilia-dependent cyst growth signal, which becomes constitutively active upon polycystin loss (Figure 3). Loss of cilia results in slower cyst growth, which is likely regulated by activation of a polycystin-independent basal ciliary cyst growth signal. In summary, the normal role of polycystins appears to be functional ductule adaptation to chemo- or mechanosensation, only allowing derepression of the cilia-dependent growth signal when necessary.



**Figure 3.** Loss of polycystins causes cystogenic growth through cilia.

**(1)** Modulation of a ciliary growth signal by polycystins (PKD) under normal conditions. This probably depends on functional ductule adaptation for chemo- or mechanosensation, only allowing derepression of the cilia-dependent growth signal when necessary (red arrow demarcated by dashed line).

**(2)** Loss of polycystins leads to constitutive activity of a cilia-dependent cyst growth signal (red arrow demarcated by unbroken line).

**(3)** Loss of cilia leads to a polycystin-independent basal ciliary cyst growth signal (red arrow demarcated by unbroken line). Flow: fluid flow such as that induced by bile, depicted by blue arrows. Abbreviations: PKD1, polycystin-1; PKD2, polycystin-2.

### ***Treatment perspectives: cAMP, somatostatin analogues and ciliopathies***

The cAMP signaling pathway is one of many disturbed in PLD, but it is important as the pathway inhibited by somatostatin analogues (SAs) [48, 49]. SAs such as lanreotide, octreotide and pasireotide show limited, but universal effectiveness in curtailing cyst growth [50]. Laboratory work has shown that SAs have beneficial effects on further fibrocystic ciliopathies like PKHD and NPHP [51, 52]. Contrarily, a randomized clinical trial with mTOR inhibitor and SA combination therapy did not show added benefits in comparison to SA monotherapy in PLD [53]. cAMP regulation in cholangiocytes is not only orchestrated by somatostatin receptors (SSTRs), but also by secretin receptors, adenylyl cyclases and phosphodiesterases [51]. In addition, bile components may regulate cAMP levels through cilium-associated chemoreceptors TGR5, a G protein-coupled bile acid receptor, and purinergic G protein-coupled receptor (P2Y)<sub>12</sub> [38, 39]. cAMP levels modulate cholangiocyte proliferation, apoptosis and fluid secretion, and thereby influence hepatic cyst expansion. Another mechanism involving cAMP and cilia has recently been discovered [54], and this mechanism may help explain cAMP abnormalities and SA effectivity in ciliopathies. It has been found that cAMP levels and ciliary length are reciprocally regulated. Following fluid shear-mediated polycystin activation, Ca<sup>2+</sup> signaling decreases cAMP, whose lower level in turn downregulates ciliary length by impairing delivery flux of cilium components. Dynamic regulation of ciliary length may thus be compromised in human cystic diseases involving perturbed polycystin function, while cAMP levels may remain improperly modulated following ciliary disruption.

The *Pkhd1*<sup>-/-</sup> rat model has ciliary disturbances in addition to renal and hepatic cysts [51]. Ca<sup>2+</sup> signaling is curtailed, while cAMP levels are elevated in cystic cholangiocytes. Elevated

cAMP levels are also found cystic cells of multiple polycystic kidney disease animal models, including jck mice ( $Nek8^{jck}$ ), PCK rats ( $Pkhd1^{pck}$ ),  $Pkd2^{WS25/-}$  mice and pcy mice ( $Nphp3^{pcy}$ ) [55]. Cholangiocyte primary cilia of the PKHD $^{-/-}$  rats are at least two times shorter compared to those of control rats. Octreotide can suppress cAMP levels through SSTR 2, 3 and 5 induction in these rats, causing decreased cell proliferation and increased apoptosis. Pasireotide enhances these effects, as the molecule binds more strongly to diverse SSTRs and has a longer half-life [56]. In inner medullary collecting duct cells with NPHP gene knockdown, octreotide prevents defects in spheroid formation [52]. It is therefore plausible that SAs are effective in a larger group of fibrocystic diseases than in PLD.

### **Concluding remarks**

In the past several years, our understanding of the intricacies of signal and transcriptional networks in normal and cystogenic biliary development has expanded tremendously. Initiation of cysts occurs by LOH in single cells, leading to defects in biliary signaling and environmental sensing, with monoclonal proliferation as a consequence. Many studies find ciliary dysfunction precedes cyst development, and this seems to be the common path to hepatic cystic disease. In case of polycystin related PLD, residual ciliary activity is important for the severe cystogenesis phenotype.

Several questions remain, however (see Box 'Outstanding questions'). Most importantly, genetic diagnosis currently eludes up to eighty percent of PCLD cases in the clinical setting. In addition, PLD treatment remains suboptimal. Normal pathways of hepatobiliary development, as well as genes of cyst disease models may yield clues to the actual pathogenesis. A better understanding of cilium-induced growth signals and subsequent DPM in PLD will open up new avenues for therapeutic intervention.

### **Outstanding questions**

- Which are the genes responsible for the 80% of PCLD cases that currently remain unexplained?
- What is the primary pathway that becomes disrupted following loss of polycystins?
- What is the mechanism of DPM following loss of polycystins?
- Is only LOH capable of causing random subsets of biliary cells to develop into cysts following germ line mutations? Or does another chance-based mechanism exist for Sec63 mutations that cause cysts to occur?

## Glossary

**Adenomatosis polyposis coli (APC):** is a member of the destruction complex of the canonical Wnt signaling pathway. Loss of APC leads to constitutive Wnt pathway activation.

**Autophagy/autophagosome:** catabolic mechanism involving breakdown of unnecessary protein components in lysosome organelles of cells, following transport by autophagosomes. Autophagosome induction and cilium formation have recently been found to be interdependent processes.

**Autosomal dominant polycystic kidney disease (ADPKD):** a common genetic nephropathy, in which PLD is found in 83–94% of patients.

**Canonical Wnt signaling:** is involved in cellular differentiation and proliferation in embryonic and adult tissues. Following activation by Wnt ligands, Frizzled and LRP5/6 receptors initiate signaling through the  $\beta$ -catenin transcription factor by induction of a destruction complex.

**Cholangiocytes/biliary cells:** epithelial cells that line the biliary tract.

**Ciliopathy:** genetic disease caused by dysfunction of the cilium or cilium-anchoring structures. Examples include ADPKD, ARPKD, MKS, and, most probably, PCLD. Ciliopathies are as phenotypically diverse as the cells and tissues on which primary cilia are present; cystic kidneys, fibrocystic liver disease, diabetes, retinal degeneration, anosmia, situs inversus, skeletal involvement, mental retardation, obesity, and infertility are all part of the ciliopathy phenotype spectrum.

**Congenital disorders of glycosylation (CDG):** type I CDG has defects in synthesis and transfer of glycans, whereas type II CDG has defective glycan processing. CDG often presents with ductal plate malformation. cyclic AMP (cAMP): a second messenger important in many biological processes. The cAMP signaling pathway is one of many disturbed in PLD but is important as the pathway is inhibited by SAs.

**Cytokeratins (CKs):** are protein markers commonly used to identify cell types. For example, CK19 marks biliary cells.

**Ductal plate:** layer of hepatoblasts enveloping the portal vein/mesenchyme, bound to become biliary tract cholangiocytes and periportal hepatocytes during hepatobiliary development.

**Ductal plate malformation (DPM):** defects in remodeling of the ductal plate during biliary tract development.

**Extracellular matrix (ECM):** includes the interstitial matrix and basement membrane of multicellular tissues. It functions in cell adhesion, cell-to-cell communication, and cellular differentiation. **Hepatoblast:** bipotent cell that may differentiate towards biliary or hepatocyte cells. Normally present during embryology, but bipotent liver progenitors persist in the biliary tract during adulthood.

**Hepatocyte:** main epithelial cell of the liver parenchyma.

**Notch signaling:** notch signaling is involved in cellular differentiation and proliferation in embryonic and adult tissues. Following activation by Notch or Jagged ligands, Notch receptors initiate signaling through Rbpj and Hes/Hey transcription factor families.

**Polycystic kidney disease (PKD):** polycystic kidneys are the primary presentation of PKD, but

polycystic livers are found in most patients. PKD1 and PKD2 are the genes responsible for ADPKD in 85% and 15% of cases, respectively. PKHD1 is the gene responsible for virtually all incidences of ARPKD.

**Polycystic liver disease (PLD):** PLDs are defined as presence of >20 cysts in the liver.

**Polycystic liver disease (PCLD):** a rare form of PLD, which presents without polycystic kidneys. **Primary cilia:** solitary, non-motile organelles on the apical cell membrane surface of most mammalian cells involved in mechano-, osmo-, and chemoreception. In addition, primary cilia are implicated in planar cell polarity, cell cycle control, and numerous essential signaling pathways.

**Protein kinase C substrate 80KH (PRKCSH):** is one of two genes known to cause PCLD, and PRKCSH mutation is found in more than 10% of cases. It encodes the protein hepatocystin that serves as the non-catalytic  $\beta$ -subunit of GII and is involved in n-glycan processing.

**SEC63:** is one of two genes known to cause PCLD. SEC63 mutation is found in more than 5% of cases. It encodes the Sec63p protein, which is part of the Sec61 complex involved in co and posttranslational protein transport.

**Somatostatin analogs (SAs):** include lanreotide, octreotide, and pasireotide. cAMP regulation can be orchestrated by SAs through SSTRs. Lanreotide, octreotide, and pasireotide have different binding affinities for SSTR subtypes as well as different half-lives, which affects clinical effectivity. **Transforming growth factor (TGF)- $\beta$  signaling:** is involved in cellular differentiation and proliferation in embryonic and adult tissues. Following activation by TGF- $\beta$  ligands, TGF- $\beta$  receptors initiate signaling through SMAD complex transcription factors.

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## Chapter 3

Chromosomal abnormalities in hepatic cysts point to novel polycystic liver disease genes

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### **Abstract**

Autosomal dominant polycystic liver disease (ADPLD) is caused by variants in *PRKCSH*, *SEC63*, and *LRP5*, while autosomal dominant polycystic kidney disease (ADPKD) is caused by variants in *PKD1*, and *PKD2*. Liver cyst development in these disorders is explained by somatic loss-of-heterozygosity (LOH) of the wild type allele in the developing cyst. We hypothesize that we can use this mechanism to identify novel disease genes that reside in LOH regions. In this study, we aim to map abnormal genomic regions using high-density SNP microarrays to find novel polycystic liver disease genes.

We collected 46 cysts from 23 patients with polycystic or sporadic hepatic cysts, and analyzed DNA from those cysts using high-resolution microarray (n=24) or Sanger sequencing (n=22). We here focused on regions of homozygosity on the autosomes (>3.0Mb), and large CNVs (>1.0Mb).

We found frequent LOH in *PRKCSH* (22/29), and *PKD1/PKD2* (2/3) cysts of patients with known heterozygous germline variants in the respective genes. In the total cohort, 12/23 patients harbored abnormalities outside of familiar areas. In individual ADPLD cases, we identified germline events: a 2q13 complex rearrangement resulting in *BUB1* haploinsufficiency, a 47XXX karyotype, chromosome 9q copy number loss, and LOH on chromosome 3p. The latter region was overlapping with an LOH region identified in two other cysts.

Unique germline and somatic abnormalities occur frequently in and outside of known genes underlying cysts. Each liver cyst has a unique genetic makeup. LOH driver gene *BUB1* may imply germline causes of genetic instability in PLD.

### **Lay summary**

Liver cysts, abnormal fluid-filled sacs, are caused by complete loss of genes like *PKD1*, *PKD2*, *PRKCSH*, *SEC63* and *LRP5* in the body. The genetic makeup of 46 cysts of 23 patients is studied. Although sharing similarities in loss of known genes, cysts are found to be unique with regard to other genetic abnormalities. Genes outside of known genes are frequently affected, some of which may modify the risk of cyst development.

### **Keywords**

polycystic liver disease, cholangiocytes, loss-of-heterozygosity, microarray, candidate genes

### **Introduction**

Polycystic liver disease (PLD) is part of the phenotype of two inherited disorders; autosomal dominant polycystic liver disease (ADPLD), and autosomal dominant polycystic kidney disease (ADPKD). In 83-94% of ADPKD patients polycystic livers are seen.<sup>1,2</sup> Variants in *Protein Kinase C Substrate 80K-H (PRKCSH)*, *SEC63 homolog (S. Cerevisiae) (SEC63)*, and *Low-density lipoprotein Receptor-related Protein 5 (LRP5)* cause ADPLD, and are present in ~25% of cases,<sup>3,4</sup> while variants in *Polycystic Kidney Disease 1 (PKD1)* and *Polycystic Kidney Disease 2 (PKD2)* are responsible for ADPKD in virtually all cases.<sup>5</sup>

Protein products of genes underlying PLD are located to the endoplasmic reticulum (ER) or primary cilium.<sup>6,7</sup> Experimental data favor a genetic interaction network between ER-localized protein products of *PRKCSH* and *SEC63*, and cilium-localized *PKD1* and *PKD2*.<sup>7,8</sup> The finding of *LRP5* variants in PLD suggests that Wnt signaling may be disrupted downstream of this interaction network. Genes that underlie PLD thus function in distinct organelles and pathways, despite a final common cystogenic effect. Furthermore, ciliopathy-associated

genes act outside of the *PKD1* / *PKD2* genetic interaction network,<sup>7,9</sup> and may also cause liver cysts. The search for new genes should therefore not be limited to currently known genomic sites.

At a tissue level, PLD appears to be a recessive disease. Complete loss of cyst gene expression from diseased epithelium follows loss-of-heterozygosity (LOH),<sup>10-15</sup> which may be related to cyst genetic instability.<sup>16,17</sup> The proportion of somatic variants varies with the gene that is affected in the germline. Recent studies found that second, somatic variants or LOH occurred in 56/71 liver cysts (79%) from patients with *PRKCSH* variants,<sup>11</sup> in 4/5 (80%) *PKD2* variant carriers,<sup>18</sup> but only 1/14 cysts (7%) from a patient with a *SEC63* variant.<sup>10</sup> This indicates LOH incidence depends upon the genetic and phenotypic background.

We hypothesize that a ‘two-hit model’ is a general principle for development of hepatic cysts. Therefore, somatic LOH regions in cyst epithelium may harbor novel candidate PLD causing genes that harbor heterozygous germline variants in the respective cases. Considering the genetic interaction network in PLD,<sup>7,8</sup> digenic or transheterozygous variants at two genetic loci may also play a role. Transheterozygous *PKD1* / *PKD2* variants have been described in renal cysts,<sup>14,15</sup> whereby a variant in one cyst gene is succeeded by a variant in a second cyst gene. Cysts with heterozygous variants in *PRKCSH* and *SEC63* continue to express the relevant proteins.<sup>10,11</sup> It is reasonable to hypothesize that transheterozygosity may be another mechanism in hepatic cyst formation. This study aims to determine novel genetic loci that are involved in cystogenesis both at germline and somatic level. To this end, we followed an unbiased approach and assessed copy-number variations (CNV) and LOH regions in PLD cyst epithelium using a genome-wide high resolution cytogenetic array analysis.

## Methods

### Patient material

We obtained DNA from liver cyst cholangiocytes of 23 newly included patients who underwent either laparoscopic cyst fenestration or aspiration sclerotherapy from 2011-2014 because of large cysts. All patients except one were female, and had single or multiple liver cysts. All patients had severe symptoms and the mean age was 54 (range 42-83) years. Seventeen patients had ADPLD, three had ADPKD, and three had solitary or sporadic cysts. Use of this tissue for research was reviewed and approved by the regional ethics review board “Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen”.

### Cyst work-up

We isolated cholangiocytes by four methods (Figure S1; Table 1). First, as described from 23 previously studied laparoscopy – derived liver cysts (6 patients) obtained from 2010 to 2012,<sup>18</sup> we collected cells from fresh tissue by ethylenediaminetetraacetic acid (EDTA) detachment. Keratin (KRT)-19 staining indicated the purity of each sample. Second, we collected cells from 30 laparoscopy – derived liver cysts of eight patients from 2012 to 2014. These cells expanded into adult liver organoids using conditions suitable for their expansion.<sup>19</sup> Under these conditions, only stem cells with a cholangiocyte-like phenotype expressing KRT19 persisted. DNA from one cyst per patient was studied using high density SNP microarrays (Affymetrix Cytoscan HD). DNA from the remaining 22 cysts was used to assess somatic loss of the wildtype allele of heterozygous *PRKCSH* germline variants by

Sanger sequencing.

Third, symptomatic cyst patients were referred to our hospital for aspiration sclerotherapy. We collected 68 cyst fluid aspirates from 50 patients in 2011 and 2012. We subjected all samples to centrifugation, KRT19 staining and fluorescent-activated cell sorting (FACS) of cholangiocytes (appendix). This yielded eight additional samples for single nucleotide variant (SNP) microarray studies. Fourth, we grew eight cultures from 30 aspiration sclerotherapy fluids collected from 2012 to 2014 using conditions suitable for the expansion of adult liver stem cells.<sup>19</sup> We obtained cyst fluid and epithelium samples and stored them in the course of treatment following the Dutch Code for the proper secondary use of human tissue. Use of this tissue for research was reviewed and approved by the regional ethics review board “Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen”.

### **DNA isolation from cyst cholangiocytes**

We isolated DNA from the cyst cholangiocytes using the QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. For samples with low DNA yields (which included all FACS obtained samples), whole genome amplification (WGA) using the Qiagen REPLI-g Mini kit (Qiagen) was performed.

### **Genetic analysis by microarray and genotyping**

We assessed copy-number variations, LOH regions and regions of homozygosity using genome-wide high resolution cytogenetic array analysis (CytoScan HD, Affymetrix, Santa Clara, CA, USA).

We screened whole blood PLD patient DNA for germline variants in *PKD1* (NG\_008617.1, NM\_001009944.2), *PKD2* (NG\_008604.1, NM\_000297.3), *PRKCSH* (NG\_009300.1, NM\_002743.3), *SEC63* (NG\_008270.1, NM\_007214.4), and *LRP5* (NG\_015835.1, NM\_002335.3) using direct sequencing as described previously.<sup>11</sup> Briefly, we isolated DNA from whole blood using the PureGene DNA isolation kit (Gentra Systems/Qiagen, Minneapolis, Minnesota, USA) or High Pure Polymerase chain reaction (PCR) template preparation kit (Roche, Mannheim, Germany), and stored it at 4°C. PCR amplified *PKD2*, *PRKCSH*, *SEC63*, and *LRP5*, exons and flanking intronic sequences with specific primers (Table S1). Screening for germline variants in *PKD1* (NG\_008617.1, NM\_001009944.2) was done by a method adapted from Tan et al<sup>20</sup>. In short, primer sequences (Table S1) for long range PCR were chosen on specific regions of the *PKD1* gene preventing the amplification of the known duplications of the first 33 exons at the proximal side of the gene. PCR reactions were performed according the manufacturer’s manual using FastStart™ Taq DNA Polymerase System supplemented with GC-RICH solution (Roche) for exon 1 or GeneAmp® High Fidelity PCR System (Life technologies, Carlsbad, USA) supplemented with 5% DMSO for all other fragments. Annealing temperatures during PCR were carefully selected for each amplicon in order to amplify the desired regions. After purification of PCR amplicons from gel using the QIAEXII Gel Extraction Kit (Qiagen) a total of 500 ng of equimolar amounts of the PCR amplicons of each sample were sequenced in a single run using Ion Torrent™ Next-Generation Sequencing (Life Technologies).

All cysts from patients with *PRKCSH* variants were analyzed for LOH using direct sequencing of this variant. We screened the entire coding region of *BUB1 Mitotic Checkpoint Serine/Threonine Kinase (BUB1)* (NG\_012048.1, NM\_004336.4) in a cohort of unrelated

patients with ADPLD (n=100) that met the Reynolds criteria.<sup>21,22</sup> We performed high resolution melting curves (RotorGene-Q; Qiagen) to reveal differences in melting curve shape that correlate to *BUB1* genotype variants and validated these findings by Sanger sequencing. *Adenylate Cyclase 1 (ADCY1)* (NG\_034198.1, NM\_021116.2), *Insulin-Like Growth Factor Binding Protein (IGFBP) 1* (NC\_000007.14, NM\_000596.2), and *IGBPF3* (NG\_011508.1, NM\_001013398.1) were analyzed in a patient with cyst chromosome 7 copy number loss. Exons and flanking intronic sequences were amplified using polymerase chain reaction with specific primers (Table S1). We purified PCR amplicons from gel using the QIAEXII Gel Extraction Kit (Qiagen) and sequenced them with the BigDye terminator kit and ABI3730, ABI310 or ABI3100 Genetic Analyzers (Applied Biosystems, Boston, MA, USA) or from 2014 onwards Ion Torrent sequencing (Life Technologies Carlsbad, CA, USA).

## Data analysis

We considered large regions of homozygosity on the autosomes (>3.0Mb) and large CNVs (>1.0Mb) for further analysis. For samples not derived by FACS/WGA, we placed no limits for size of LOH or copy number variants (CNV) around known cyst genes. We only assessed X chromosomes for whole chromosome abnormalities. For selected cases, we compared the array results derived from cyst cell DNA (somatic) and germline DNA (genomic) to ensure identification of somatic events. We selected cases for germline analysis based on FACS/WGA origin of cyst DNA or presence of non-mosaic >1.0 Mb CNVs. We used individual CytoScan HD data chromosome analysis suite (CHAS, Affymetrix) V2.1. For analysis involving multiple SNP array data, we used Nexus Copy Number (Biodiscovery, El Segundo, CA, USA) V6.0.

### SNP array data

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Wills et al., 2016) and are accessible through GEO Series accession number GSE78808 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=kijpyeauhnbwzqv&acc=GSE78808>). Described variants have also been deposited at NCBI's database of genomic structural variations (Wills et al., 2016) and are accessible through dbVar (<http://www.ncbi.nlm.nih.gov/dbvar>) accession number nstd125.

**Table 1.** Patient characteristics.

Unknown germline variant indicates no variant in PRKCSH (NM\_002743.3), SEC63 (NM\_007214.4), or LRP5 (NM\_002335.3) was found for ADPLD patients, and no variant in PKD1 (NM\_001009944.2) or PKD2 (NM\_000297.3) was found for ADPKD patients.

CNV: copy number variation; chr.: chromosome; CNN: copy number neutral; ROH: region of homozygosity; LOH: loss-of-heterozygosity; Epith.: epithelium; Loss: copy number loss; Gain; copy number gain

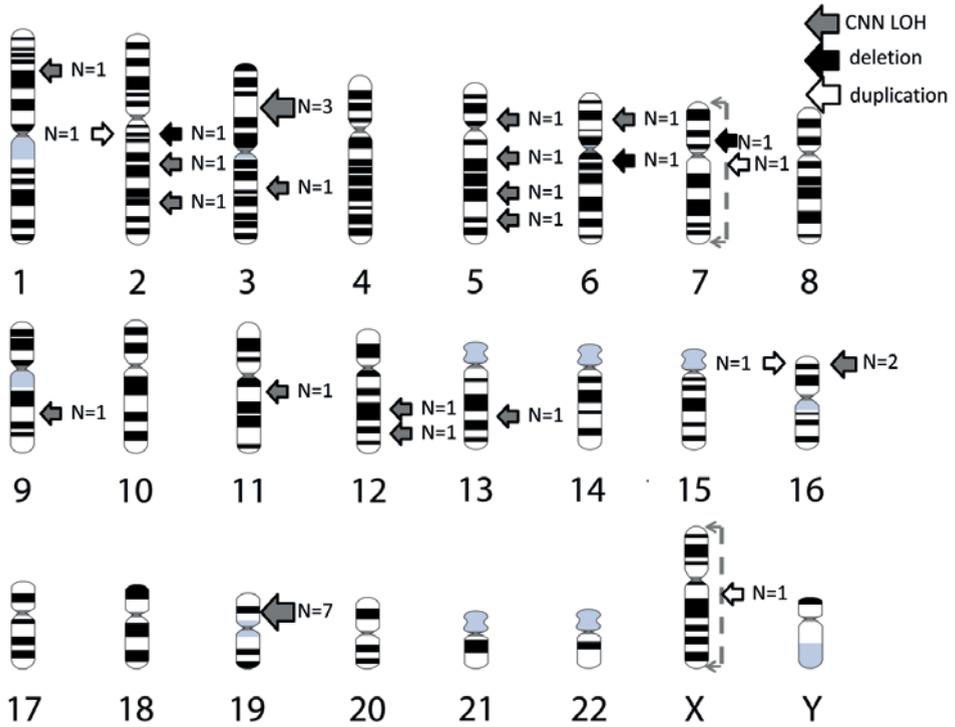
<sup>a</sup> Or abnormality in PKD1, PKD2, PRKCSH, SEC63, or LRP5 in case of sporadic cysts

#	Patient & age	Source	Disease, germline mutation & SNP array CNV	Second hit mutation <sup>a</sup>	Additional events
1	Male, 61 years	Fluid	ADPLD Unknown Loss chr 9q (2.9Mb) CNN ROH chr. 3p (12.7 Mb) CNN ROH chr. 13q (5.4Mb)	None detected	None

2	Female, 58 years	Fluid	ADPLD Unknown	None detected	CNN LOH chr. 5q (3.6Mb)
3	Female, 60 years	Fluid	ADPLD Unknown	None detected	None detected
4	Female, 50 years	Fluid	ADPLD Unknown	None detected	None detected
5	Female, 59 years	Fluid	ADPLD Unknown	None detected	None detected
6	Female, 55 years	Fluid	ADPLD Unknown	None detected	None detected
7	Female, 83 years	Fluid	ADPKD <i>PKD1 hg38</i> <i>chr16:g.2090007T&gt;G</i>	CNN LOH chr. 16p (34.6 Mb)	CNN LOH chr. 1p (6.9Mb) CNN LOH chr. 12q (4.4Mb)
8.1	Female, 44 years	Fluid	ADPLD <i>PRKCSH hg38</i> <i>chr19:g.11437972G&gt;C</i> Gain chr 2q13 (1.3Mb) Loss chr. 2q13 (1.7Mb; including <i>Bub1</i> )	CNN LOH chr. 19p (12.9 Mb)	None detected
8.2	Female, 44 years	Epith.	ADPLD <i>PRKCSH hg38</i> <i>chr19:g.11437972G&gt;C</i> Gain chr 2q13 (1.3Mb) Loss chr 2q13 (1.7Mb)	CNN LOH chr. 19p (1.5Mb)	Loss chr 7p (1.7Mb)
9	Female, 54 years	Epith.	Sporadic cyst -	None	CNN LOH chr. 2q (3.1Mb) CNN LOH chr. 12q (3.7Mb)
10	Female, 57 years	Fluid	Sporadic cyst -	None	None
11	Female, 58 years	Epith.	ADPLD Unknown	CNN LOH chr. 19p (1.7Mb)	Mosaic trisomy chr 7 CNN LOH chr. 12q (3.6Mb)
12	Female, 47 years	Epith.	ADPLD <i>PRKCSH hg38</i> <i>chr19:g.11449074A&gt;G</i>	CNN LOH chr. 19p (20Mb)	CNN LOH chr. 6p (3.3Mb)
13	Female, 70 years	Fluid	ADPLD Unknown	Del chr 4q (39kb)	CNN LOH chr. 5q (5.4 Mb)
14	Female, 52 years	Epith.	ADPLD Unknown	None	None
15	Female, 61 years	Epith.	ADPLD	None	CNN LOH chr. 2q (3.2Mb)

			Unknown		CNN LOH chr. 5p (3.2Mb)
16	Female, 51 years	Fluid	ADPLD <i>SEC63 hg38 chr6:g.107904719del</i>	Del chr 6q (42 Mb)	None
17	Female, 50 years	Epith.	ADPLD <i>PRKCSH hg38 chr19:g.11449074A&gt;G</i>	None	CNN LOH chr. 5q (3.1Mb)
18	Female, 52 years	Fluid	ADPKD <i>PKD1 hg38 chr16 g.[2135508G&gt;A(;);2090517_2090558delinsGGAC], c.[182C&gt;T(;);12171_12212delinsGTCC], p.[Pro61Leu(;);Ser4057Argfs*87]</i>	<i>PKD1 hg38 chr16 g.[2135508G&gt;A(;);2090517_2090558delinsGGAC], c.[182C&gt;T(;);12171_12212delinsGTCC], p.[Pro61Leu(;);Ser4057Argfs*87]</i>	CNN LOH chr. 3p (3.4Mb) CNN LOH chr. 19 (1.2Mb) CNN LOH chr 11 (1.3Mb)
19	Female, 47 years	Epith.	ADPLD <i>PRKCSH hg38 chr19:g.11437972G&gt;C</i> 47XXX	CNN LOH chr 19 (15.9Mb)	Trisomy X (entire) CNN LOH chr. 3p (6.1 Mb)
20	Female, 43 years	Fluid	Sporadic cyst -	Gain CN 2.26 chr. 16p (3.8Mb)	CNN LOH chr. 1p (3.6Mb)
21	Female, 61 years	Fluid	ADPLD <i>PRKCSH hg38 chr19:g.11437972G&gt;C</i>	None	None
22	Female, 42 years	Fluid	ADPLD <i>PRKCSH hg38 chr19:g.11437972G&gt;C</i>	CNN LOH chr 19 (19.6 Mb)	None
23	Female, 51 years	Fluid	ADPKD Unknown	None	None

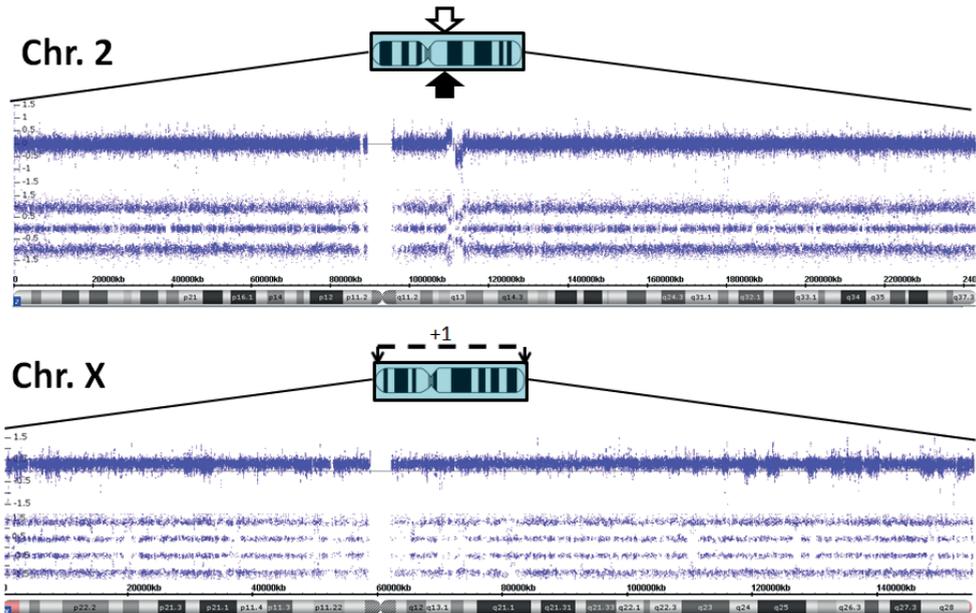
## Results



**Figure 1.** Overview of abnormal regions found in this study by SNP array.

### Patient characteristics

We included DNA from 24 liver cysts from 23 patients for Affymetrix CytoScan HD SNP array analysis in this study (Figure 1, Table 1; Figure S1 for inclusion flow chart), together with 23 cysts from 6 previously described patients (Table S2; Figures S2, S3). In the new cohort, all patients except one were female, and had single or multiple liver cysts. All patients had severe symptoms and the mean age was 54 (range 42-83) years. Seventeen patients had ADPLD, three had ADPKD, and three had solitary or sporadic cysts. Six carried *PRKCSH* variants, one carried a variant in *SEC63* and two had *PKD1* variants.



**Figure 2.** Upper panel: 2q13 complex rearrangement present in genomic blood DNA of PRKCSH variant patient (#8). Lower panel: Presence of three X-chromosomes in genomic blood DNA of blood of another PRKCSH variant patient (#19).

### Germline variants

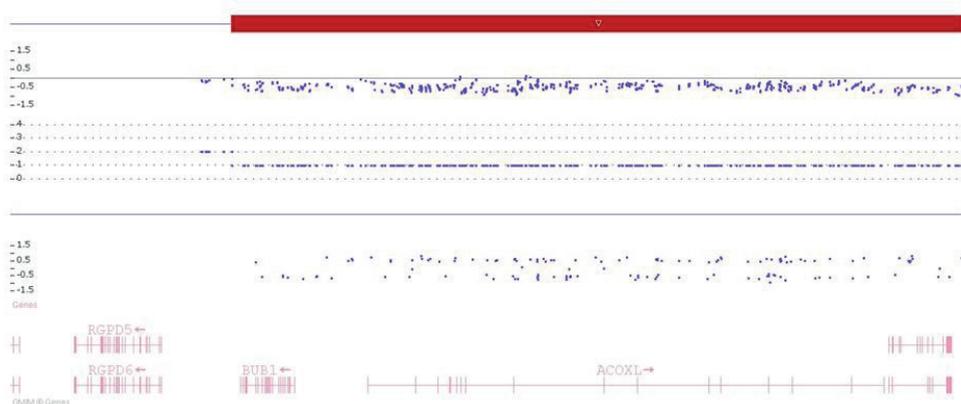
In the total cohort, three patients displayed germline abnormalities detectable by our criteria outside of *PRKCSH*, *SEC63*, *LRP5*, *PKD1*, and *PKD2* genomic regions. We found a heterozygous 3.0 Mb 2q13 complex rearrangement in germline and cyst DNA of a *PRKCSH* variant carrier (patient 8; Figure 2). This rearrangement constituted of a copy number gain as well as a copy number loss, and contained the genes *BUB1*, *ACOXL*, *BCL2L1*, *ANAPC1*, *MERTK*, *TMEM87B*, *FBLN7*, and *ZC3H8\** (Figure 3).

Peripheral blood and cyst DNA of another patient (#1) displayed a large copy number loss (12.7 Mb) of chromosome 3p containing Wnt signaling effector Catenin (Cadherin-Associated Protein), Beta 1 (CTNNB1), amongst others (Figure 4). We additionally identified a large copy number loss (2.9Mb) in patient #1, which contained the genes *SYK*, *NFIL3*, and *ROR2\*\**. This CNV in chromosome 9 occurs with low frequency in a normal, healthy cohort. A third ADPLD patient carried a gain of the entire X-chromosome (Triple X) in her germline and somatic DNA (patient 18; Figure 2).

\* *Acyl-CoA Oxidase-Like (ACOXL)*; *BCL2-Like 1 (BCL2L1)*; *Anaphase Promoting Complex Subunit 1 (ANAPC1)*; *MER Proto-Oncogene, Tyrosine Kinase (MERTK)*; *Transmembrane protein 87B (TMEM87B)*

*Fibulin 7 (FBLN7)*; *Zinc Finger CCCH-Type Containing 8 (ZC3H8)*

\*\* *Spleen Tyrosine Kinase (SYK)*; *Nuclear Factor, Interleukin 3 Regulated (NFIL3)*; *Receptor Tyrosine Kinase-Like Orphan Receptor 2 (ROR2)*



**Figure 3.** BUB1 is present at the centromeric part of the 2q13 loss region.

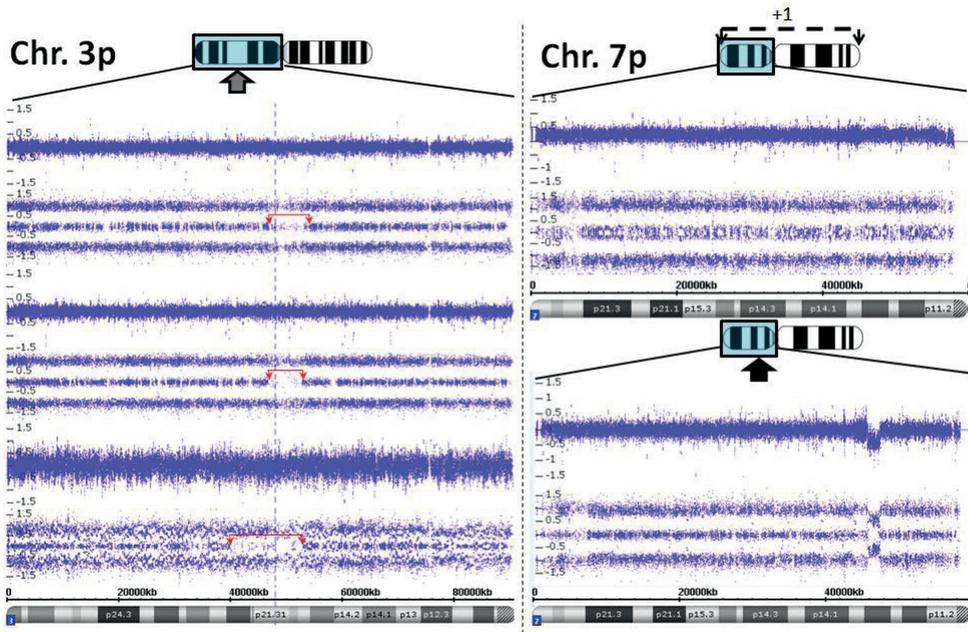
### Germline BUB1 variants

The 2q13 rearrangement pointed to the well-known mitotic checkpoint gene *BUB1*.<sup>23</sup> In order to test whether this gene played a more general role in ADPLD, we studied the gene in genomic DNA from 100 severely affected ADPLD patients. Using high-resolution melt curves (HRM) and subsequent Sanger sequencing on abnormal melting curves, we identified one synonymous SNP (rs370559107: hg38 chr2:g.110672765C>G, silent variant) in these 100 patients. In addition, a single, nonsynonymous SNP (rs61730706; hg38 chr2:g.110667649G>A, c.677C>T, p.(Ala226Val)) was present in the DNA of one patient of this cohort, which occurs with a minor allele frequency of 0.002-0.005 in the general population. Polyphen and SIFT showed conflicting effects (SIFT: 0.28, tolerated; Polyphen (HumDiv): possibly damaging 0.749; Polyphen (HumVar) benign 0.219; Variant taster variant (p=0.617). We did not consider this SNP as causative or disease-related, as it is normally present in the general population.

### LOH surrounding PRKCSH, SEC63, LRP5, PKD1 and PKD2

To validate our approach, the SNP array cohort included seven cysts from six patients with known *PRKCSH* variants. The SNP arrays of five cysts of four patients revealed copy number neutral (CNN) LOH on chromosome 19 at the position where *PRKCSH* is located (Figure S4). Sanger sequencing of additional cyst DNA from patients (#12, 17, and 19) with known *PRKCSH* variants revealed somatic loss of the respective wildtype alleles in 17 out of 22 cysts (Table S3).

DNA of a cyst of patient #7 contained a >30Mb homozygous region on chromosome 16p. Although initially classified as having isolated PLD with a single renal cortical cyst, a germline *PKD1* variant was found (hg38 chr16:g.2090007T>G, c.12632A>C, p.(Glu4211Ala)). No CNN LOH was found in cyst DNA of the other two ADPKD patients by SNP array, and we proceeded by directly sequencing *PKD1* and *PKD2*. Patient #18 had a germline *PKD1* variant (hg38 chr16:g.2135508G>A, c.182C>T, p.(Pro61Leu)).



**Figure 4. Left:** CNN LOH on chromosome 3p of patient #18, 19 and 1, from above to below in that order. **Right upper panel:** Mosaic whole chromosome 7 gain in an ADPLD patient (#11) with unknown variant, chromosome 7p shown. **Right lower panel:** 1.7 Mb loss on chromosome 7p of a *PRKCSH* patient cyst (#8.2).

In the cyst, the second allele was likely affected by a somatic deletion (hg38 chr16 g.[2135508G>A(;);2090517\_2090558delinsGGAC], c.[182C>T(;);12171\_12212delinsGTCC], p.[Pro61Leu(;);Ser4057Argfs\*87]). We detected no variants in germline or somatic DNA of the final ADPKD patient.

LOH across ADPLD genes was a frequent occurrence in somatic DNA of our total cohort, regardless of variant status of germline DNA. Approximately 40% of 34 cysts with high-quality SNP array data displayed LOH or allelic imbalance (AI) across *PRKCSH*, while more than 25% of cysts had *PKD1* LOH or AI. *PKD2*, *SEC63* and *LRP5* both had lower percentages of LOH/AI, ~15%, ~15% and ~10% respectively. Copy number losses and gains in ADPLD occurred less frequently.

### Novel, digenic abnormalities in cysts

As described above, patient #8 presented with two germline events. We identified a *PRKCSH* variant and a complex rearrangement resulting in *BUB1* haploinsufficiency. DNA from her second cyst displayed multiple second hits when compared to peripheral blood. A 1.7 Mb copy number loss region on chromosome 7 was present as well as CNN LOH surrounding *PRKCSH* on chromosome 19 (Figure 4). The loss region includes the genes *ADCY1*, *IGFBP1* and *IGFBP3*, as well as the pseudogene *Septin 7B2* (*SEPT7B2*). It was not detected in the SNP array profile of germline DNA, nor were germline variants detected in any of these genes using Sanger sequencing. Sanger sequencing also did not reveal variants in these genes in the cyst. Another *PRKCSH* mutant cyst displayed mosaic trisomy 7 (#11), although only

present in a subset of cells.

Copy number neutral LOH of chromosome 19 (1.2 Mb), encompassing *PRKCSH*, and chromosome 11 (1.3 Mb), encompassing *LRP5*, was present in cyst DNA of an ADPKD patient (#18, germline *PKD1* variant). In this patient, an additional copy number neutral LOH region was detected on chromosome 3p (hg19 chr3:g.49,735,745\_53,133,526), partially overlapping with a region of a *PRKCSH* variant carrier (#19; hg19 chr3:g.46,715,645\_52,852,488). Notably, patient #1 with a larger copy number neutral LOH on chromosome 3p on germline and cyst DNA also had overlap (hg 19 chr. 3:g.39,874,567\_52,653,645). The minimal region of LOH contained >60 genes, containing multiple tumor suppressor genes (Table S4).

### Sporadic cysts

Two sporadic cysts of the same patient showed novel chromosomal LOH (patient #28; Table S2). These cysts had been previously described without abnormalities encompassing the then known cyst genes *PRKCSH*, *SEC63*, *PKD1*, or *PKD2*. Copy number neutral LOH (2.0 Mb) was found around *LRP5*, a novel cyst gene (Figure S5). Germline DNA revealed no variant in *LRP5*, and the presence of two germline heterozygous *LRP5* SNPs (hg38 chr.11:g.68403545T>C, rs545382; hg38 chr.11:g.68425222G>A, rs556442) indicated that LOH was only present at a somatic level. Unfortunately, no cyst DNA remained, and it could not be confirmed whether this copy number neutral LOH led to loss of wild type alleles by variant.

Finally, the sporadic cyst from patient 20 displayed a 3.8 Mb mosaic copy number gain at the telomeric part of chromosome 16p on cytoscanner. The signal was relatively weak, yet may suggest presence of a cellular subpopulation of this cyst with *PKD1* gain.

### Discussion

Here we show that germline and somatic abnormalities outside of known cyst genes frequently occur in PLD, and many cysts of PLD patients have a unique genetic signature. LOH in known regions was confirmed for *PRKCSH* (22/29), and *PKD1/PKD2* (2/3) variants. In the SNP array cohort of 23 new patients (24 cysts), we detected 12 cysts with sizable copy number losses or LOH outside of earlier identified genomic regions. In 3 patients we found the presence of unique germline aberrations in PLD. Additionally, a patient with a primary liver phenotype had a germline *PKD1* variant. Cyst DNA showed recurrent copy number loss on chromosome 3, with overlap between three patients. On chromosome 7, a 1.7 Mb copy number loss and a mosaic whole-chromosome gain were found. Sporadic cysts displayed LOH around *LRP5* and a mosaic gain surrounding *PKD1* at a somatic level.

Although many genomic abnormalities were present beyond genomic regions known for second hits, most were unique. Chromosomal mapping suggests that each cyst follows an independent genetic pathway, similar to mosaicism observed in tissues of other somatic second hit disorders.<sup>24,25</sup> This likely reflects significant heterogeneity that is at the genomic root of cyst development. It also reflects that general genomic instability presumably precedes cyst development.<sup>16,17</sup> *BUB1* haploinsufficiency is a known driver of chromosomal instability resulting in LOH and tumorigenesis,<sup>23,26</sup> and a 2q13 microdeletion has previously been described.<sup>26,27</sup> While we show that it is unlikely that this specific gene is generally involved in LOH in PLD, other drivers of genetic instability may be present in the disease. Wnt signaling abnormalities appear to be common in PLD. Wnt signaling abnormalities are

associated with tumorigenesis in the liver and kidney, and cause aberrant cyst development in mice.<sup>28,29</sup> *CTNNB1* deletions and *APC* variants are also frequently found in hepatoblastoma.<sup>30,31</sup> Variants in canonical Wnt signaling component *LRP5* causes ADPLD in man,<sup>4</sup> whereas the c-terminal tail of polycystin-1 might interact with beta-catenin.<sup>32</sup> In the same vein, our finding of copy number neutral LOH around *CTNNB1* together with copy number loss of *ROR2*<sup>33</sup> in the germline of an ADPLD patient, together with copy number neutral LOH around *LRP5* in two sporadic cysts of another patient further implicates this pathway as a crucial for cystogenesis.

Trisomy X, the final germline abnormality we found, is unlikely to be involved in cystogenesis. Although female gender is a risk factor for cyst development and complications,<sup>34</sup> this is most likely due to sex hormones.<sup>35,36</sup> Furthermore, gene expression of triple-X cells is largely limited to one copy by X-inactivation, excluding 5-10% of genes on the X-chromosome located in pseudoautosomal regions.<sup>37</sup> We see this as a chance finding, considering its prevalence of 1 in 1000 females.

On a somatic level, possible digenic or transheterozygous variants and a *PKD1* gain were present on cysts. The 1.7 Mb chromosome 7 copy number loss of a *PRKCSH* variant carrier might point towards a novel transheterozygous modifier region. Haploinsufficiency of genes such as *IGFBP1*, *IGFBP3* and *ADCY1* might be relevant in cyst development, as these genes are related to the IGF and adenylyl cyclase pathway that the anti-cystogenic somatostatin analogues are involved in.<sup>38-41</sup> Surprisingly, another cyst displayed a mosaic gain over the whole of chromosome 7, indicating that overexpression of these or other genes may also be relevant for cyst development. More difficult is the recurrent, overlapping LOH region on chromosome 3 (49,735,745-52,653,645). Although two cysts display clear LOH, the third may have had a partially normal cellular subpopulation. Over 60 genes are present at this location, none of which are known cyst-related genes. This 3p21.3 region does contain a cluster of tumor suppressor genes,<sup>42</sup> such as tumor susceptibility gene *Rassf1a*.<sup>43</sup> The cluster frequently undergoes LOH in early formation of different tumors,<sup>42</sup> including hepatocarcinomas and cholangiocarcinomas.<sup>44</sup> The mosaic gain of *PKD1* in one cyst may be consistent with overexpression of *Pkd1* in mice leading to cystogenesis.<sup>45</sup> The mosaicism of this region was relatively low however, and may represent an artefact. Given the substantial investment that went into genotyping this cohort, we express our disappointment that we found no more specific leads to clarify the origination of multiple cysts in the liver.

Concluding, our chromosomal mapping indicates significant genetic heterogeneity outside of known second-hit regions of liver cysts. We identified unique cystogenic regions, as well as characteristics of general genomic instability in hepatic cyst DNA. These findings may explain the large number of ADPLD cases without a known variant, as well as phenotypic dissimilarities between similar cyst germline variants.

## Abbreviations

ADPKD	autosomal dominant polycystic kidney disease
ADPLD	autosomal dominant polycystic liver disease
AI	Allelic imbalance
CNN	copy number neutral
CNV	copy number variation
Del	deletion
ER	endoplasmic reticulum
FACS	fluorescent-activated cell sorting
LRP5	low density lipoprotein receptor-related protein 5
LOH	loss of heterozygosity
PCR	polymerase chain reaction
PLD	polycystic liver diseases (ADPLD and ADPKD)
PKD1	polycystic kidney disease 1 protein (polycystin-1)
PKD2	polycystic kidney disease 2 protein (polycystin-2)
PRKCSH	protein kinase C substrate 80K-H (80 kDa protein, heavy chain)
SEC63	Saccharomyces cerevisiae homolog 63
SNP	single nucleotide variant
Wnt	wingless-type MMTV integration site family member
WT	wildtype

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## Conflict of Interest

None.

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## Chapter 4

### Liver cyst gene knockout in cholangiocytes inhibits cilium formation and Wnt signaling

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### **Abstract**

Mutations in the *PRKCSH*, *SEC63*, and *LRP5* genes cause autosomal dominant polycystic liver disease (ADPLD). The proteins products of *PRKCSH* (alias *GIIB*) and *SEC63* function in protein quality control and processing in the endoplasmic reticulum (ER), while *LRP5* is implicated in Wnt/ $\beta$ -catenin signaling. To identify common denominators in the PLD pathogenesis, we mapped the PLD interactome by affinity proteomics, employing both HEK293T cells and H69 cholangiocytes. Identification of known complex members, such as glucosidase IIA (GIIA) for *PRKCSH*, and *SEC61A1* and *SEC61B* for *SEC63*, confirmed the specificity of the analysis. *GANAB*, encoding *GIIB*, was very recently identified as an ADPLD gene. The presence of *GIIB* in the *LRP5* complex pinpoints a potential functional connection with *PRKCSH*. Interestingly, all three PLD-associated protein complexes included filamin A (FLNA), a multifunctional protein described to play a role in ciliogenesis as well as canonical Wnt signaling. As ciliary dysfunction may also contribute to hereditary liver cyst formation, we evaluated the requirement of *PRKCSH* and *SEC63* for ciliogenesis and Wnt signaling. By CRISPR-Cas9 induced knockdown of both ADPLD genes in HEK293T cells and H69 cholangiocytes, we identified that their depletion results in defective ciliogenesis. However, only H69 knockouts displayed reduced Wnt3a activation. Our results suggest that loss of *PRKCSH* and *SEC63* leads to general defects in ciliogenesis, while quenching of the Wnt signaling cascade is cholangiocyte-restricted. Interactions of all three PLD-associated protein complexes with FLNA may mark a common link between the ADPLD proteins and the cystogenic processes driving this disease.

### **Lay summary**

Isolated liver cysts, fluid-filled sacs in the liver, are caused by loss of *PRKCSH*, *SEC63*, and *LRP5* genes. Primary cilia, the cell's antennas, are disturbed due to this loss. Wnt signaling, important in development of tissues and organs, is similarly impaired. Evidence of a connection between *PRKCSH*, *SEC63*, *LRP5* and another protein, filamin A, may be the cause for these disturbances of cilia and Wnt signaling.

### **Keywords**

polycystic liver disease, primary cilia, Wnt signaling, protein interactome, CRISPR-Cas9

### **Introduction**

Polycystic liver disease (PLD) is part of the phenotype of two disorders; autosomal dominant polycystic liver disease (ADPLD), and autosomal dominant polycystic kidney disease (ADPKD). ADPLD is caused by mutations in *PRKCSH*, *SEC63*, and *LRP5* in up to 25% of cases (1, 2), and *PKD1* and *PKD2* mutations cause ADPKD in virtually all cases (3). Recently, mutations in *GANAB*, encoding the glucosidase IIa (GIIA) subunit, were also found to cause ADPKD and ADPLD in a very small percentage of cases (4). *GANAB*, together with *ALG8*, encoding  $\alpha$ -1,3-glucosyltransferase, and *SEC61B* were identified very recently as ADPLD genes in a large exome sequencing effort (5). Cysts frequently display loss-of-heterozygosity (LOH) (6-11). Wild-type alleles are completely lost by this process, which appears to be crucial for cyst development.

*PRKCSH*, *SEC63* and *GIIB* proteins are located in the endoplasmic reticulum (ER), *LRP5* on plasma membranes, and polycystin-1 (PC1) and polycystin-2 (PC2), encoded by *PKD1* and

PKD2 respectively, on the primary cilium (2, 12, 13). A study on a genetic interaction network between *PRKCSH*, *SEC63*, *PKD1*, and *PKD2* implicated *PKD1* as the rate limiting component of cyst formation (12-14). *PRKCSH*, also known as glucosidase IIb (GIIB), functions in n-glycan processing through its interaction with GIIA. *PRKCSH* maintains the complex in the ER through a localization signal (15). *SEC63* is present on the ER membrane in the *SEC61* protein translocon, where it facilitates protein translocation and transmembrane protein insertion. Both ER proteins are part of post-translational quality control and processing of PC1 and PC2 (12-14), while GIIA was shown to be required for maturation of PC1, and subsequently for correct surface and ciliary localization of both polycystins (4). Although direct evidence of the suggested function of the polycystins in ciliary calcium trafficking has remained elusive (16, 17), much credence has been given to the hypothesis that they are important for ciliary function (18-20). Intact cilia are important drivers of cyst development (21). Primary cilia in biliary ducts act as mechano-, osmo-, and chemosensors (22), and structural ciliary abnormalities may be present in cysts (23, 24). Although it has become clear that *PRKCSH*/GIIB (and recently GIIA), and *SEC63* protein loss leads to defective PC1 processing, resulting in a cilia-dependent cyst formation (4, 14), it remains unclear whether other aspects of cilium formation, cilium structure, or cilium-directed pathways are also affected.

The polycystin complex has been implicated in MAPK/ERK, mTOR, and cAMP pathways (21), yet disturbance of these pathways is not sufficient to cause cilia-dependent cyst growth. Wnt/ $\beta$ -catenin signaling is another pathway essentially linked to primary cilia function. *LRP5*, the most recent identified ADPLD gene (2), implicates Wnt signaling in cystogenesis. Wnt signaling through *LRP6*, a close homologue of *LRP5*, is dependent upon n-glycosylation (25). Similarly, several experimental data link *SEC63* to Wnt signaling by binding to nucleoredoxin (26), a protein that binds to Disheveled (27) and determines its levels (28), while the c-terminal tail of polycystin-1 directly associates with beta-catenin, thus regulating canonical Wnt signaling (29). Finally, primary cilia may restrict the activity of the canonical Wnt pathway by spatial sequestering of beta-catenin to the cilium (30, 31). It remains to be identified if *PRKCSH* and *SEC63* directly interact with Wnt signaling components, and/or if mutations directly affect the Wnt pathway.

In this study we first aimed to unveil the associating functional protein modules and pinpoint the candidate common pathogenic denominators using affinity proteomics. Our results suggest that none of the three PLD-associated proteins (*LRP5*, *PRKCSH* and *SEC63*) plays a direct role in either Wnt signaling or cilium function, as none of the canonical players in these processes were found to interact. However, *FLNA*, a signaling scaffold protein previously shown to bind to polycystin-2 (PC-2), and to be involved in ciliogenesis as well as canonical Wnt signaling, was present in all three PLD-associated protein complexes. By CRISPR-Cas9 mediated knockdown of *LRP5*, *PRKCSH* and *SEC63* in HEK293T cells and H69 cholangiocytes, as well as evaluation of patient-derived cells, we determined the importance of these genes for correct ciliogenesis and Wnt signaling.

## Materials and Methods

### CRISPR-Cas9 gene knockout

For generation of *PRKCSH*, *SEC63* and *LRP5* mutant cell lines, we developed CRISPR-Cas9 constructs based on the pSpCas9(BB)-2A-GFP plasmid. pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene, Cambridge, USA; plasmid # 48138) (52). This plasmid contains a mammalian-codon optimized Cas9 with nuclear targeting sequences, a gRNA backbone, and enhanced green fluorescent protein (eGFP) for selection. We designed oligo pairs of 20-nt on "http://tools.genome-engineering.org" (table 2), which provides a score from 0-100 based on the specificity of target sites. We annealed oligos and ligated them into PX458 plasmid, and subsequently transformed them into competent *E. coli*. We screened plasmids from two *E. coli* clones per construct using Sanger sequencing. Verified constructs were transfected into HEK293T and H69 cells in 6-wells plate using FuGene HD (Promega, Madison, USA).

**Table 1.** Target sites and oligos used for CRISPR-Cas gene knockout.

Scores indicate target site specificity on a scale from 1-100, higher scores are more specific.

\*Target site found in sequence following ATG/start codon

Gene	Exon	Target site	Oligo 1 (5'→3')	Oligo 2 (5'→3')	Score
<i>PRKCSH</i>	2*	ACCCATGTGCTGGGCCGTGGAGG	CACCgACCCATGTGCTGGGCCGTGG	AAACCCACGGCCAGCAGCATGGGTC	67
<i>PRKCSH</i>	3	GGATGGTGGCCGAACCGTCCAGG	CACCgGGATGGTGGCCGAACCGTCC	AAACGGACGGTTCGGCCACCATCCc	94
<i>SEC63</i>	1*	TCCCGCCGACATACTACCTCTGG	CACCgTCCCGCCGACATACTACCTC	AAACGAGGTAGTATGTCCCGGGAc	94
<i>SEC63</i>	2	GGTGTATGGTATCGTTTACGG	CACCgGGTGTATGGTATCGTTTA	AAACTAAACGATACCACATACCCc	88
<i>LRP5</i>	1*	GCTGGCGCTGTGCGGCTGCCCGG	CACCgGCTGGCGCTGTGCGGCTGCC	AAACGGCAGCCGCACAGCCAGCCc	60
<i>LRP5</i>	2	CAACCCCGGGACGTACGGCTGG	CACCgCAACCCGGGGACGTACGGC	AAACGCCGTACGTCCCGCCGTTGc	99

### Selection of knockout cells

We sorted single GFP+ cells into a 96 well's plate using FACS on an Aria SORP sorter (BD Biosciences, San Jose, CA, USA). Remaining H69 cells were additionally seeded to a 6-well's plate, and we used 50% filtered, conditioned medium from 70% confluent H69 cells to improve their growth rate. From the 6-well's plate, we transferred clones individually onto a 96-well's plate after 2-3 weeks. We conducted Sanger sequencing for targeted regions, as well as western blot of protein products to confirm knockouts.

For both cell lines, we screened 15 clones per construct for mutations at a DNA and protein level. For experiments, we selected two *PRKCSH*<sup>-/-</sup> or *SEC63*<sup>-/-</sup> clones per construct, and validated loss of protein expression on western blot in duplo.

### DNA isolation and genotyping

We screened clones for mutations in *PRKCSH*, *SEC63*, and *LRP5* using direct sequencing as described previously (7). Briefly, we isolated DNA from trypsin-detached cells using the PureGene DNA isolation kit (Gentra Systems/Qiagen, Minneapolis, USA) or High Pure PCR template preparation kit (Roche, Mannheim, Germany), and stored it at 4°C. Polymerase chain reaction (PCR) amplified *PRKCSH*, *SEC63*, and *LRP5* exons with specific primers (table S3).

## DNA constructs

We generated entry clones encoding full-length human *PRKCSH*, *SEC63*, and *LRP5* from cDNA from liver tissue; 528, 760 and 1615 amino acids respectively [NCBI Reference Sequence: NM\_002743.3 (*PRKCSH*); NP\_001001329.1 (*PRKCSH*), NM\_007214.4 (*SEC63*), NP\_009145.1 (*SEC63*); NM\_002335.3 (*LRP5*); NP\_001278831.1 (*LRP5*)]. We isolated total RNA from liver tissue using TRIzol Reagent (Life Technologies, Carlsbad, USA), and produced oligo dT cDNA by RT Transcriptor First Strand cDNA synthesis kit (Roche Applied Sciences, Mannheim, Germany). Full-length PCR fragments were obtained using the FastStart High Fidelity PCR System (Roche) using primers from table S4. Fragments were cloned into the Gateway entry vector pDONR201 (Life Technologies). LRP5 intracellular (ICD) fragment constructs, encoding the C-terminal amino acids 1409–1615 were generated using the LRP5 full-length entry clone as a template. LRP5ICD was added to the experiments to improve protein complex capture. Removing the transmembrane portion of proteins generally increases (water-soluble) protein yield. We made expression constructs from all entry clones using Gateway cloning procedures (Life Technologies) according to the manufacturer's protocol in N- and C-terminal TAP expression vectors. LRP5 mutant constructs were previously generated using the Quick Change-II-XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, USA) and a pcDNA3.1V5His WT-LRP5 as a template (table S2) (2). Sequences were confirmed by Sanger sequencing.

## Immunostaining and western blotting

We transfected plasmids expressing cTAP-PRKCSH, nTAP-SEC63, nTAP-LRP5, and nTAP-LRP5ICD in H69 and HEK293T cells with FuGene HD (Promega). We lysed cells using lysis buffer (50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% NP-40) on ice with complete protease inhibitor cocktail (Roche) 48 hours after transfection. Cells were spun down after 10 minutes incubation on ice and supernatant was collected. Sample Buffer (4x NuPAGE) was added to the supernatant and heated for 10 min at 70°C. Samples were run on NuPAGE Novex 4–12% Bis-Tris SDS-PAGE gels. We performed immunostains with anti-Flag, anti-PRKCSH, or anti-SEC63 as well as with anti-alpha tubulin as primary antibodies (conditions shown in Table S5), and goat anti-rabbit IRDye800 and goat anti-mouse IRDye680c as a secondary antibodies (1 mg/ml, Molecular Probes, Waltham, USA). Washing steps used PBS-Tween (PBS with 0.2% Tween 20). We kept western blots in the dark and scanned them on an Odyssey infrared imaging system (Li-Cor, Lincoln, USA). Bands were detected and processed by ODYSSEY application software (Version 1.2, Li-Cor).

## Cell culture and DNA transfection

HEK293T were cultured in high glucose DMEM AQmedia (Sigma Aldrich, St. Louis, USA), supplemented with 10% FCS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 10mM HEPES. H69 cells were cultured in DMEM/F12, supplemented with 10% FCS, 1% penicillin/streptomycin, and 10mM HEPES. For DNA transfections, HEK293T and H69 cells were seeded, grown overnight, and then transfected using FuGene HD (Promega).

## Tandem affinity proteomics and mass spectrometry

Cells transiently expressing the Streptavidin-FLAG (SF-TAP)-tagged PRKCSH (cTAP), SEC63 (nTAP), and LRP5 (nTAP, ICD) fusion proteins were lysed on ice with complete protease inhibitor cocktail (Roche Diagnostics) in lysis buffer (50 mM Tris-HCl (pH7.5), 150 mM NaCl,

1% NP-40). The Streptavidin- and FLAG based tandem affinity purification steps were performed as previously described (53, 54). 5% of the final eluate was evaluated by SDS-PAGE followed by silver staining, according to standard protocols, while the remaining 95% was subjected to protein precipitation with chloroform and methanol. Protein precipitates were subsequently subjected to mass spectrometry analysis and peptide identification as previously described (55). Proteins identified in SF-TAP control experiments (empty SF-TAP vector), or identified in more than 15% of the experiments from our in-house TAP-proteome data collection (33), were removed as nonspecific complex members. Protein interaction networks were visualized by Cytoscape software (56).

### **Immunofluorescence and microscopy**

We cultured HEK293T and H69 cells on poly-L-lysine coated glass slides under standard cell culture conditions in Dulbecco's modified Eagle's medium (DMEM) or DMEM/Ham's F12 with 10% fetal calf serum (FCS). Serum-starved medium (H69: Opti-MEM1; HEK293T: DMEM/PBS 1:1) induced cilia for 72hr prior to staining. Subsequently, we fixated cells in 3.8% paraformaldehyde in phosphate buffered saline (PBS) and permeabilized with 1% Triton-X-100 in PBS. We blocked cells with freshly made 2% bovine serum albumin (BSA) in PBS for 1 hour, followed by 1 hour incubation with primary antibodies (in 2% BSA in PBS) at room temperature. We washed cells with PBS, and added secondary antibodies at room temperature for 30 min. We used the following primary antibodies: anti-PRKCSH, anti-SEC63, anti-PDI, anti-ARL13B, anti-FLAG, anti-peroxisomal biogenesis factor 14, anti-alpha-tubulin, and anti-beta-actin (table S5). Secondary antibodies (all from Life Technologies) were: anti-rabbit IgG Alexa Fluor 488, and anti-mouse IgG Alexa Fluor 568 (Table S6). We protected glass slides with stained cells with Vectashield (Vector Laboratories, Burlingame, CA) and placed them on a microscopic glass slide. We performed microscopy on an Axio Imager ZI fluorescence microscope (Zeiss, Sliedrecht, The Netherlands) with an ApoTome slider. We processed images using AxioVision (Zeiss) software, and we assessed three images per experiment. Percentages of ciliated cells are depicted as mean  $\pm$  S.D.

### **Cell cycle analysis**

Cell cycle analysis was performed with HEK293T wild type cells, 2 PRKCSH knock-out cell lines and a SEC63 knock-out cell line in log phase. Approximately  $1 \times 10^6$  cells were collected using trypsin and the cells were washed with PBS. The cells were fixed by adding 70% ethanol to the cell pellet while mixing and incubated for 1 hour at 4°C. The cells were then washed with PBS at  $850 \times g$  and 1 ml of Propidium Iodide working solution (40  $\mu\text{g}/\text{mL}$  PI and 100  $\mu\text{g}/\text{mL}$  RNase A in PBS) was added to the cells. After an incubation of 30 minutes at 37°C cells were measured with the FC500 flowcytometer (Beckman-Coulter, Indianapolis, IN) with excitation at 488nm (15mW) and emission at 610nm longpass filter. The first peak represents the G1 phase, the second peak the G2 phase and in between the S phase.

### **(De)glycosylation assay**

To check whether PRKCSH and SEC63 can influence the maturation and expression of LRP5, HEK293T wild type cells, 2 PRKCSH knock-out cell lines and a SEC63 knock-out cell line were transiently transfected with the pcDNA3.1V5His WT-LRP5 construct because of the low endogenous LRP5 expression in HEK293T cells. After 48 hours cells were lysed with lysis buffer (50 mm Tris-HCl (pH7.5), 150 mm NaCl, 1% NP-40, 0.1% SDS) on ice with complete

protease inhibitor cocktail (Roche). Total protein was subjected to Endoglycosidase H or N-Glycosidase F (Roche) treatment according to the manufacturer's manual. 20 Micrograms of treated and non-treated total protein samples were subsequently analyzed using SDS-PAGE followed by western blotting. LRP5 was visualized by the Clarity ECL-kit (Bio-Rad Laboratories, Hercules, CA) using the primary V5-antibody (Life Technologies) and the secondary rabbit anti-mouse-HRP antibody (Dako; Glostrup, Denmark).

mRNA levels of the cell lines were analyzed by isolating RNA using TRIZOL (Life Technologies) according to the manufacturer's manual. cDNA was produced using iScript cDNA Synthesis Kit (Bio-Rad). Real time PCR was performed in triplicate with SybrGreen using primers LRP5 forward (5'- GAGATCCTCCGTAGGTCCGT-3') and reverse (5'- CCAAGCGAGCCTTCTACAC-3') or primers housekeeping gene B2M forward (5'- ATGAGTATGCCTGCCGTGTG-3') and reverse (5'- CCAAATGCGGCATCTTCAAAC-3'). The protein and mRNA measurements were performed in duplicate.

### **Wnt Luciferase Activity Assays**

We performed Wnt luciferase assays as described previously (2). For the activity assay, we seeded  $5.0 \times 10^3$  HEK293T cells, or  $5.0 \times 10^3$  human cholangiocyte 69 (H69) cells per well in a 96-well plate in triplicate. After 24 h, we transiently transfected cells using FuGene HD (Promega) with 100 ng LRP5 construct or empty vector and 100 ng of TCF/LEF1 Reporter or 100 ng negative control according to the manufacturer's instructions (Signal TCF/LEF1 Reporter Assay Kit; Qiagen). Twenty-four hours after transfection, medium was replaced by medium with or without 50% Wnt3a conditioned medium to initiate Wnt signaling. We cultured cells for another 24 h, and detected luciferase activity using the Dual-Glo Luciferase Assay System (Promega) in an InfiniteM200-Pro plate reader (Tecan, Männedorf, Switzerland). Firefly luciferase activity was normalized to Renilla luciferase activity to correct for variations in transfection efficiencies. Values are reported as means  $\pm$  S.E.M. Each experiment was conducted in triplicate.

### **RNA isolation and RT-qPCR**

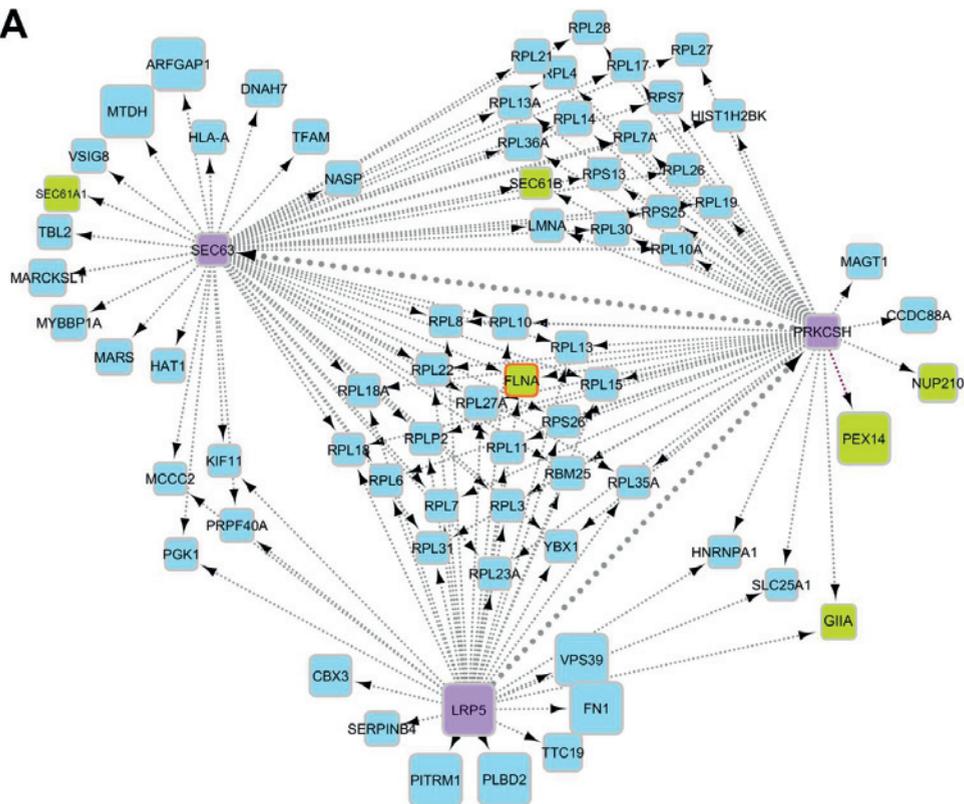
Total RNAs were isolated using TRIzol according to the manufacturer's protocol. We reverse transcribed RNA into complementary DNA (cDNA) using a iScript cDNA synthesis kit according to the protocol (Bio-Rad, Hercules, USA). 1  $\mu$ l of resulting cDNA was used for RT-qPCR. Briefly, the RT-qPCR was carried out on a thermal cycler (CFX96, Bio-Rad), using the 2- $\Delta\Delta$ Ct SYBR green protocol (57). We amplified axin 2 and beta-2-microglobulin (reference) in 40 cycles. RT-qPCR data were analyzed using the CFX-Manager software, which validates primer quality by analyzing melting curves. All the RT-qPCRs were repeated three times with triplicates for each treatment. For RT-PCR, all values were represented as mean  $\pm$  S.D.

### **Statistical analysis**

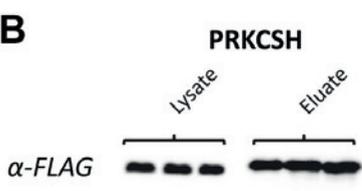
For cilia measurements, luciferase assays, and RT-qPCR we used Mann-Whitney test to test for significance. A two-tailed P-value of  $<0.05$  was considered significant.

## Results

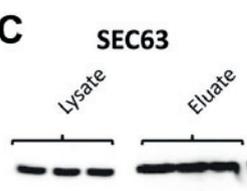
### A



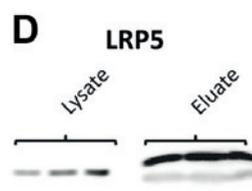
### B



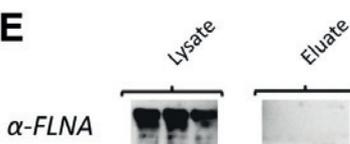
### C



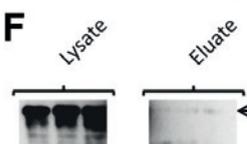
### D



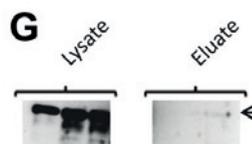
### E



### F



### G



**Figure 1.** PLD-associated protein interaction network. **(A)** The network is constructed using mass spectrometry data of the indicated tandem affinity purified PLD proteins PRKCSH, SEC63, LRP5 or the intracellular domain of LRP5 (in purple) as bait in HEK293T and H69 cells. Except for GANAB/GIIA and SEC61A1, proteins shown are either selected for their uniqueness in this dataset (the size of the node is proportional to the uniqueness) compared to a recently published large protein interaction dataset ((32), bait count  $\leq 10$ ) or are identified in the affinity purification data of at least two out the three different TAP-tagged bait proteins (LRP5, PRKCSH, SEC63), and identified in replicate experiments. Proteins highlighted in green are discussed in the results. *(continued on next page)*

(continued from last page) FLNA is a SYSCILIA gold standard protein ((56) (red border). The network was visualized using Cytoscape software (54). **(B-G)** SEC63 and LRP5 associate with FLNA in vitro. **(B-D)** Western blot analysis of HEK293T cell co-immunoprecipitation containing either PRKCSH, SEC63 or LRP5 as bait protein. An antibody against FLAG showed the input of bait proteins PRKCSH, SEC63 or LRP5 respectively. **(E)** Following co-immunoprecipitation with PRKCSH as bait, an antibody against FLNA detected no co-precipitating protein at 280 kDa, corresponding to the expected size of FLNA on western blot. **(F, G)** Following co-immunoprecipitation with SEC63 **(F)** or LRP5 **(G)** as bait, an antibody against FLNA detected a specific co-precipitating protein of 280 kDa, corresponding to the expected size of FLNA on western blot. Arrow: Expected size of FLNA.

**Table 2A:** Selected proteins captured with PRKCSH as bait

Protein	# out of 19 TAPs found	Protein coverage (average)
PRKCSH	19	51%
$\alpha$ -subunit glucosidase (GIIA)	19	57%
Peroxisomal biogenesis factor 14 (PEX14)	5	19%
nucleoporin 210kDa (NUP210)	5	13%
Filamin A (FLNA)	3	5%
Myosin IB (MYO1B)	3	7%

**Table 2B:** Selected proteins captured with SEC63 as bait

Protein	# out of 4 TAPs found	Protein coverage (average)
SEC63	4	57%
SEC61B	4	38%
SEC61A1	4	17%
transducin (beta)-like 2 (TBL2)	4	45%
Thymopoietin (TMPO)	4	35%
ADP-ribosylation factor GTPase activating protein 1 (ARFGAP1)	4	33%
Methionyl-tRNA synthetase (MRS)	4	13%
Dynein, axonemal, heavy chain 7 (DNAH7)	4	2%
Metadherin (MTDH)	3	8%
Filamin A (FLNA)	2	12%
Peroxisomal biogenesis factor 19 (PEX19)	1	10%

### Tandem affinity proteomics determines the PLD-associated interactome

We performed affinity proteomics to identify putative physical connections between the molecular machinery of PRKCSH, SEC63, and LRP5, and to find molecular clues for the observed abnormalities (Fig. 1). Tandem affinity purification followed by mass spectrometry was performed both in H69 cholangiocytes as well as in HEK293T cells. Recently, we and others demonstrated that HEK293T cells are very efficient in assessment of interacting protein complexes and networks, both of the entire cell (32) as well as of a specific organelle such as the cilium (33). In total, we performed 5 experiments with the full length LRP5 protein, 6 experiments with the LRP5 intracellular domain (ICD), 7 experiments with SEC63, and 19 experiments with PRKCSH, resulting in 2357 TAP-MS identifications (Table S1, Fig. 1).

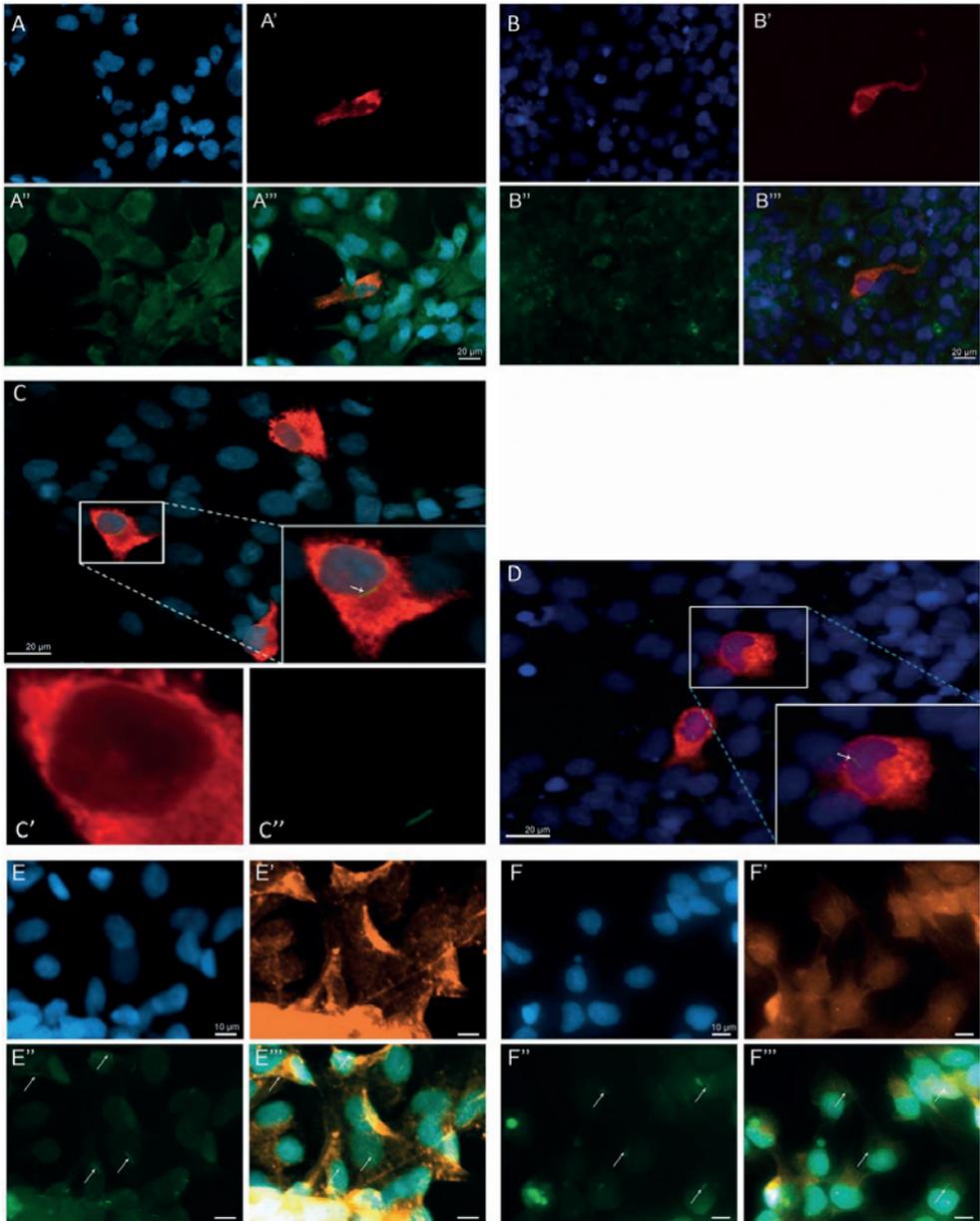
PRKCSH TAPs captured 233 proteins, and 94 remained after filtering. SEC63 bait caught 353 prey proteins, 155 of which remained after filtering. LRP5 TAPs captured 371 proteins, and filtering left 119 proteins. Glucosidase IIA (GIIA), the main binding partner of PRKCSH/GIIB, was present in all 19 PRKCSH TAP experiments (Fig. 1). Five PRKCSH TAPs captured nucleoporin 210kDa and another five caught peroxisomal biogenesis factor (PEX) 14. SEC63 captured interaction partners SEC61B and SEC61A1 in four of seven experiments, and one SEC63 TAP captured another peroxisomal biogenesis factor, PEX19. Since peroxisomes require the ER for their formation (34), we assessed peroxisome formation in our knockout cells. We found that peroxisome formation was uninhibited as determined by immunofluorescence for PEX14 (Fig. S1). LRP5 full-length protein did not localize to the primary cilium (Fig. S2). GIIA was identified to associate to the LRP5 ICD complex in two out of six experiments, without PRKCSH/GIIB being detected. Interestingly, Filamin A (FLNA) was found to interact with all three bait proteins (detected in 9/37 TAP experiments). Although immunofluorescence did not indicate co-localization of FLNA with the bait proteins (data not shown), co-immunoprecipitation confirmed the presence of small amounts of FLNA with SEC63 and LRP5ICD (Fig. 1B). GIIA was also present with co-immunoprecipitation. These results indicate that LRP5 protein might interact with GIIA, and that all proteins underlying ADPLD might have an interaction with FLNA in common.

**Table 2C:** Selected proteins captured with LRP5 full-length protein as bait

Protein	# out of 4 TAPs found	Protein coverage (average)
LRP5	4	22%
kinesin family member 11 (KIF11)	3	7%
proteasome 26S subunit, non-ATPase 12 (PSMD12)	3	8%
Filamin A (FLNA)	2	10%

**Table 2D:** Selected proteins captured with LRP5-ICD as bait

Protein	# out of 6 TAPs found	Protein coverage (average)
LRP5	6	9%
protein phosphatase 2, catalytic subunit, beta isozyme (PPP2CB)	6	17%
protein phosphatase 2, regulatory subunit B, alpha (PPP2CA)	6	30%
chromobox homolog 3 (CBX3)	5	18%
phospholipase B domain containing 2 (PLBD2)	5	6%
carboxypeptidase, vitellogenic-like (CPVL)	3	10%
Filamin A (FLNA)	2	2%
$\alpha$ -subunit glucosidase (GIIA)	2	2%

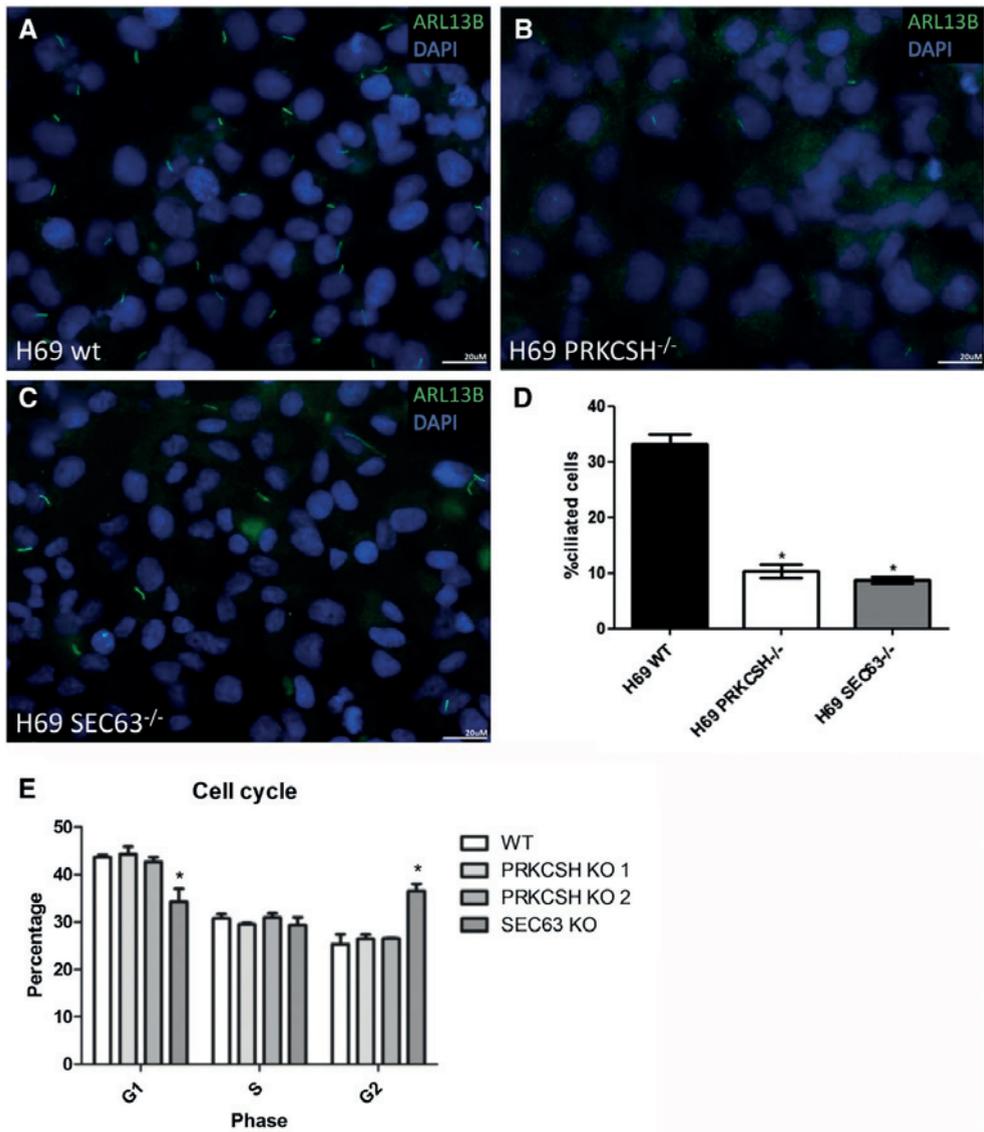


**Figure 2.** PRKCSH and SEC63 localize to the ER of H69 cholangiocytes, without any presence in primary cilia. **(A)** PRKCSH (red) co-localizes with ER-marker PDI (green). **(B)** SEC63 (red) co-localizes with ER-marker PDI (green). **(C)** PRKCSH (red) does not co-localize with cilia-marker ARL13B (green). **(D)** SEC63 (red) does not co-localize with cilia-marker ARL13B. **(A–D)** DAPI (blue) stains the nuclei; size bars correspond to 20  $\mu\text{m}$ . **(E, F)** PRKCSH and SEC63 localize to the ER of ciliated H69 cholangiocytes, without any presence in primary cilia. **(E)** PRKCSH (red) does not co-localize with cilia-marker ARL13B (green). Arrows indicate cilia. **(F)** SEC63 (red) does not co-localize with cilia-marker ARL13B. Arrows indicate cilia. **(E, F)** DAPI (blue) stains the nuclei; size bars correspond to 10  $\mu\text{m}$ .

PRKCSH and SEC63 TAPs captured mainly ER localized proteins, most of which are implicated in ER protein synthesis, including many ribosomal proteins (Table 1; Table S1). Immunofluorescence staining co-localized PRKCSH and SEC63 protein products with protein disulfide isomerase (PDI), a resident enzyme of the ER, without any detectable signal at primary cilia (Fig. 2), which was in line with our affinity proteomics results.

### **CRISPR-Cas9 induced knockdown of *PRKCSH* and *SEC63* disrupts ciliogenesis**

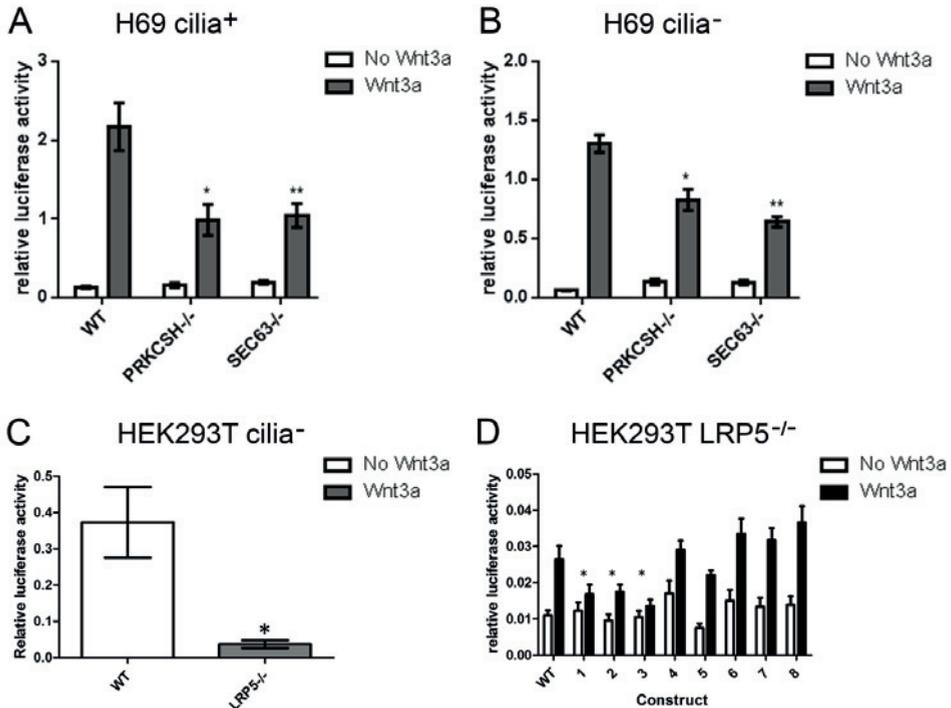
In order to study the effect of *PRKCSH*, *SEC63*, and *LRP5* mutations, the genes were knocked out in H69 cholangiocyte and HEK293T cells using CRISPR-Cas9 technology to introduce deletions resulting in loss of protein. All constructs were effective in inducing mutations, albeit with different efficiencies (Fig. S4 and S5). We subsequently assessed *PRKCSH* and *SEC63* clones expected to have bi-allelic deletions for loss of protein on western blots (Fig. S6). Two knockout clones per gene were selected for HEK293T cells and H69 cholangiocytes, which all completely disrupted the cognate protein production. Cilium formation of *PRKCSH* and *SEC63* knockout cells was clearly diminished when compared to controls, for both H69 and HEK293T cells (Fig. 3A-D; Fig. S7). Both stress-free proliferative as well as serum-starved ciliogenic conditions displayed decreased ciliogenesis of knockout cells. Wildtype (WT) H69 cholangiocytes had cilia on  $33.1\% \pm 5.7\%$  of cells, *PRKCSH*<sup>-/-</sup> H69 on  $10.3\% \pm 3.43\%$  ( $p < 0.0001$ ) of cells and *SEC63*<sup>-/-</sup> H69 on  $8.7\% \pm 1.9\%$  ( $p < 0.0001$ ) of cells. WT HEK293T showed cilia on  $9.0\% \pm 3.7\%$  of cells, whereas *PRKCSH*<sup>-/-</sup> and *SEC63*<sup>-/-</sup> HEK293T displayed cilia on  $2.3\% \pm 1.3\%$  ( $p = 0.0002$ ), and  $4.2\% \pm 1.9\%$  ( $p = 0.004$ ) of cells respectively. Evaluation of the cell cycle progression by flow cytometry did not reveal major differences (Fig. 3E, Fig. S8), although a small shift could be observed of the population of cells in G1 (slightly decreased) to G2 (slightly increased) upon SEC63 knockout, while the percentage of cells in S phase is the same as in WT cells (Fig. 3E). No significant differences in any cell cycle phase could be observed upon PRKCSH depletion. These data indicate that mutations in the genes underlying ADPLD significantly reduce ciliogenesis.



**Figure 3.** Depletion of PRKCSH or SEC63 in cholangiocytes disrupts ciliogenesis. (A) H69 cholangiocytes form cilia upon serum depletion. Cilia are marked by the ciliary GTPase ARL13B (green). (B) CRISPR-Cas9 mediated knock-down of *PRKCSH* significantly reduces the frequency of cilia. (C) CRISPR-Cas9 mediated depletion of *SEC63* renders a similar reduction of ciliogenesis. (A–C) DAPI (blue) stains the nuclei; size bars correspond to 20 μm. (D) Graphical representation of the significant reduction of the number of cells that carry cilia (shown in (A–C)) in cells depleted of *PRKCSH* (69% reduction) or *SEC63* (74% reduction) compared to wildtype cells in three independent experiments. \*:  $p < 0.05$  (E) Graphical representation of cell cycle distribution in WT versus *PRKCSH* or *SEC63* KO HEK293 cells, determined by flow cytometric analysis of cell cycle with propidium iodide DNA staining (plots included in online Supplementary Material, Fig. S7). Results are expressed as % of counted cells in each phase of the cell cycle. Two independent experiments; means  $\pm$  standard deviations are presented. \*:  $p = 0.0005$ .

## Knockdown of *PRKCSH* and *SEC63* reduce Wnt signaling in H69 cholangiocytes

Since Wnt signaling is disrupted by *LRP5* mutations of ADPLD, and can be affected by mutation of ciliary genes, we assessed Wnt signaling in *PRKCSH* and *SEC63* knockouts using a TCF/LEF1 luciferase reporter assay. Wnt signaling was significantly decreased due to loss of *PRKCSH* and *SEC63* in H69 cholangiocytes in three independent experiments (Fig. 4). Wnt3a-treated WT H69 cells showed a significantly higher luciferase activity ( $2,2 \pm 0,1$ ;  $1,3 \pm 0,02$ ) in both ciliated and non-ciliated conditions in comparison to *PRKCSH*<sup>-/-</sup> ( $1,0 \pm 0,07$ ,  $p=0,0056$ ;  $0,83 \pm 0,03$ ,  $p=0,0019$ ) and *SEC63*<sup>-/-</sup> cells ( $1,0 \pm 0,05$ ,  $p=0,0019$ ;  $0,64 \pm 0,01$ ,  $p<0,0001$ ).

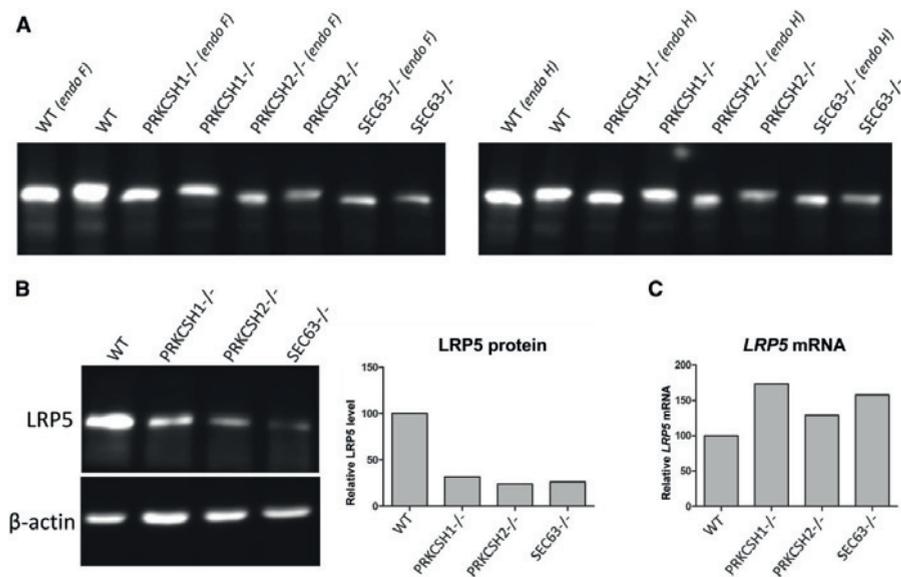


**Figure 4.** Depletion of *PRKCSH* and *SEC63* in cholangiocytes and loss of *LRP5* impairs Wnt signaling. **(A)** TCF/LEF1 luciferase assays to measure levels of activated  $\beta$ -catenin, and hence canonical Wnt signaling following transfection of indicated cell line by reporter constructs. Knockout H69 cholangiocytes with cilia induced by serum starvation ('cilia<sup>+</sup>') display significantly decreased Wnt signaling in comparison to controls. **(B)** TCF/LEF1 luciferase assays as in (A). Knockout H69 cholangiocytes in normal conditions that induce no extra cilia ('cilia<sup>-</sup>') also display significantly decreased Wnt signaling in comparison to controls. **(C)** TCF/LEF1 luciferase assays as in (A). Depletion of Wnt co-receptor *LRP5* in HEK293T cells leads to reduced Wnt signaling under conditions as in (B). **(D)** Co-transfection of luciferase assay as in (A) together with *LRP5* constructs as indicated. We used *LRP5* mutant constructs that contained recently identified ADPLD mutations (2), which demonstrated decreased Wnt signaling. Three out of eight mutant *LRP5* constructs showed impaired Wnt signaling. Constructs are numbered as in table S2 (1. *LRP5* c.1360G>A, p.Val454Met, 2. *LRP5* c.1680G>T, p.Trp560Cys, 3. *LRP5* c.3562C>T, p.Arg1188Trp, 4. *LRP5* c.4587G>C, p.Arg1529Ser, 5. *LRP5* c.4651G>A, p.Asp1551Asn, 6. *LRP5* c.3468G>C, p.Gln1156His, 7. *LRP5* c.3403C>T, p.Arg1135Cys, 8. *LRP5* c.3107G>A, p.Arg1036Gln.). **(A-D)**: \*:  $p<0,01$ , Mann-Whitney *U*-test.

Additional testing confirmed this pattern. Two different H69 knockout clones with knockout of *PRKCSH* or *SEC63* displayed similarly decreased Wnt signaling (Fig. S9A), while tests using axin2 mRNA expression as Wnt readout showed consistent results (Fig. S9B). In contrast, in HEK293T cells Wnt signaling was not affected by loss of *PRKCSH* and inconsistently affected by loss of *SEC63* (Fig. S9C, D). For *SEC63*<sup>-/-</sup> HEK293T cells effects on Wnt signaling appeared to be clone rather than mutation specific.

### LRP5 glycosylation is not dependent on PRKCSH and SEC63

As a disrupted function of the glucosidase PRKCSH/GIIB (and also GANAB/GIIB that was identified in our TAP dataset) is expected to induce N-glycosylation defects, we evaluated if LRP5 was a substrate affected by CRISPR-Cas9 mediated PRKCSH knockout, and if possibly SEC63 also plays a role in this. Our glycosylation assay showed that LRP5 is similarly glycosylated between WT, *PRKCSH*<sup>-/-</sup> and *SEC63*<sup>-/-</sup> HEK293T cells (Fig. 5A). Endo F and Endo H treatment similarly removed N-glycosylation of LRP5 between the types of cells, as indicated by the reduced molecular weight of the protein on western blot (Fig. 5A). Interestingly, the LRP5 signal intensities seemed to be decreased in the knockout cells. This was confirmed by evaluation of LRP5 protein expression levels in relation to β-actin (Fig. 5B). To evaluate if the reduced levels were caused by a transcriptional downregulation, we assessed the LRP5 mRNA levels in the knockout cells, which were not reduced and even slightly increased (Fig. 5C).



**Figure 5.** ADPLD knockouts show reduced expression of LRP5, without an abnormal protein glycosylation pattern. (A) Both WT and ADPLD knockout cell lines only display glycosylated LRP5. N-glycosylation can be removed by Endo F and Endo H treatment, resulting in a smaller protein and molecular weight. (B) LRP5 expression is reduced in ADPLD knockout cells in comparison to wildtype cells, without a comparative loss in β-actin expression, indicating that the reduction is induced by loss of PRKCSH or SEC63. (C) Relative LRP5 mRNA expression is slightly increased in *PRKCSH* and *SEC63* knockout cells in comparison to WT cells. *PRKCSH1*<sup>-/-</sup> = *PRKCSH*<sup>-/-</sup> clone 1; *PRKCSH2*<sup>-/-</sup> = *PRKCSH*<sup>-/-</sup> clone 2.

### Effects of patient *LRP5* mutations on Wnt signaling in *LRP5*<sup>-/-</sup> HEK293T

HEK293T cells without *LRP5* displayed significantly impaired Wnt signaling in comparison to wild-type cells (Fig. 4C). Transfection of these cells allowed assessment of Wnt signaling abnormalities without presence of endogenous *LRP5*. We used *LRP5* mutant constructs that contained recently identified ADPLD mutations (2) that demonstrated decreased Wnt signaling (Table S2). Three out of eight mutant *LRP5* constructs showed impaired Wnt signaling (Fig. 4D). Specifically, construct 1 (c.1360G>A, p.Val454Met), construct 2 (c.1680G>T, p.Trp560Cys), and construct 3 (c.3562C>T, p.Arg1188Trp) significantly decreased Wnt signaling, while construct 5 (c.4651G>A, p.Asp1551Asn) decreased it all three times without reaching significance. The remaining mutant constructs were either similar to or higher than the wild-type construct in luciferase intensity.

### Discussion

Here we show that cyst genes and proteins involved in liver cyst development play an important role in Wnt signaling and cilium formation. CRISPR-Cas9 induced knockout of *PRKCSH* and *SEC63* in HEK293T and H69 cells leads to a significant decrease in cilium formation, without affecting cell proliferation. Independent of cilium number, Wnt signaling is decreased in H69 *PRKCSH*<sup>-/-</sup> and *SEC63*<sup>-/-</sup> cells. By contrast, HEK293T KO cells show clone-dependent effects on Wnt signaling. Finally, patient's *LRP5* mutant constructs show reduced Wnt signaling compared to wildtype *LRP5* in *LRP5*<sup>-/-</sup> HEK293T cells.

Affinity purification experiments confirm known interactor GIIA for *PRKCSH*, and SEC61 complex proteins for *SEC63*, validating the current approach. It shows that *PRKCSH* and *SEC63* interact with *PEX14* and *PEX19* respectively. The identification of these peroxisomal proteins confirms previous studies. Peroxisomes form in the ER, where they bud off in foci to form mature peroxisomes (34). *PEX14* is localized in the ER near *SEC63* when newly formed (35), and combined *SEC62/SEC63* loss impairs peroxisome biogenesis. Although liver cysts may be found in peroxisome biogenesis disorders (36), our findings indicate no further link to ADPLD development. Single loss of *PRKCSH* or *SEC63* did not affect *PEX14* presence or localization in our cell lines.

We were able to discover novel *PRKCSH* interacting partners, such as *NUP210*, *FLNA*, and *MYO1B*, while *SEC63* complex interactors include *TBL2*, *TMPO*, *ARFGAP1*, *MRS*, *DNAH7*, *MTDH*, and *FLNA*. No Wnt signaling interactors are present for *LRP5* full-length protein or intracellular domain, but *LRP5* was found in a complex with *KIF11*, *PSMD12*, *FLNA*, *PPP2CA*, *PPP2CB*, *CBX3*, *PLBD2*, *CPVL*, and *GIIA*.

Several of our findings do further implicate Wnt signaling in hepatic cystogenesis, which pathway has previously been found to cause cyst development (2, 29, 37, 38). First, the interaction of *LRP5*-ICD with *GIIA* points towards n-glycan processing of this part of *LRP5*. Second, loss of ADPLD genes *PRKCSH* and *SEC63* in H69 cells leads to a small decrease in Wnt signaling, and, third, this small Wnt signaling decrease is similar to that observed for *LRP5* mutations present in ADPLD families ((2) and this study). An explanation may be that n-glycosylation of *LRP6* is necessary for its maturation and plasma membrane localization (25). *LRP5* processing may be similarly n-glycosylation dependent, which protein displays approximately 71% homology (39) and contains at least four predicted n-glycosylation sites ((40-42), (<http://www.cbs.dtu.dk/services/NetNGlyc/>)). It is unknown whether *LRP5* maturation and membrane translocation also requires *SEC63* and the *SEC61* complex, like many transmembrane proteins (43, 44), but *SEC63* has been linked previously to Wnt

signaling through nucleoredoxin (26). Interestingly, GANAB, the gene encoding GIIA, is the most recently identified ADPKD and ADPLD gene (4), and required for maturation of PC1. The N-glycosylation of LRP5 however, that we could observe in HEK293T cells (Fig. 5) was not diminished by depletion of either PRKCSH/GIIB or SEC63, which suggests that LRP5 is not a substrate of glucosidase II and SEC63 is not involved in the regulation of LRP5 N-glycosylation. Our findings together strengthen the case for Wnt signaling abnormalities as a common contributing factor in hepatic liver cyst development. If this is unrelated to maturation and trafficking of polycystin-1 remains to be identified. The fact that we determined that knock out of either PRKCSH or SEC63 led to reduced levels of LRP5, while the transcript levels were slightly elevated, suggests connectivity of the ADPLD-associated pathways downstream of gene transcription.

The cell-line dependent effect of PRKCSH and SEC63 loss on Wnt signaling is a surprising finding. This is either a limitation to the applicability of our findings, especially regarding the use of HEK293T cells to evaluate this as in contrast to the H69 cholangiocytes, this cell line shows variability between different knock out clones. However, it may also point towards a tissue-specific effect of loss of ADPLD genes. In man, *PRKCSH* and *SEC63* mutation only leads to significant cyst development in the liver, and little to no cyst development in the kidney (45). Another limitation is that ciliary formation did not affect Wnt signaling impairment. Wnt signaling is putatively inhibited by primary cilia (30, 31), but Wnt signaling was impaired in both proliferative (low cilia formation) and serum-starved (high cilia formation) conditions. Cilium-independent mechanisms, such as those described above, are therefore most likely the cause of the observed Wnt defect.

Filamin A may provide a link between the PLD proteins, ciliogenesis and Wnt signaling. Although with the available reagents no co-localization could be identified with PRKCSH, SEC63 and LRP5, co-immunoprecipitation indicates the protein might link to SEC63 and LRP5. Filamin A is a molecular scaffold protein involved in cell motility and signaling, and interacts with a large number of proteins (46). The protein is part of the SYSCILIA gold standard protein list of known ciliary components (47). Filamins crosslink cortical actin filaments into a dynamic 3D structure, and are thought to function as a signaling scaffold for a variety of cellular processes. Filamin A has been shown to be important for stability of meckelin and polycystin-2 (48, 49), and *FLNA* loss leads to basal body positioning and ciliogenesis defects (48). The meckelin-filamin A interaction may additionally be required to maintain correct levels of Wnt signaling.

Ciliogenesis is also defective in PLD in comparison to healthy tissues. Normal biliary ducts have solitary, cylindrical primary cilia on virtually all cells, with a length  $>4\mu\text{m}$ . In medium-sized hepatic cysts (1-3cm diameter) cilia are short ( $1.25 \pm 0.29 \mu\text{m}$ ) and rare (1/200 cells), and the organelles are absent from larger cysts ( $>3\text{cm}$  diameter) (50, 51). Our findings indicate that loss of *PRKCSH* and *SEC63* may lie at the root of these ciliary abnormalities. Both H69 and HEK293T cells with loss of these genes display a significant decrease in cilium formation under proliferative and serum-starvation conditions. This implicates structural ciliary abnormality in addition to previously found functional abnormality (12-14) in ADPLD cells.

In conclusion, our results suggest that loss of PRKCSH and SEC63 leads to general defects in ciliogenesis, while quenching of the Wnt signaling cascade is cholangiocyte-restricted.

Interactions of all three PLD-associated protein complexes with FLNA may mark a common link between the ADPLD proteins and the cystogenic processes driving this disease.

## Abbreviations

**CRISPR:** Clustered regularly interspaced short palindromic repeats

**MS:** Mass spectrometry

**TAP:** Tandem affinity proteomics

**LOH:** Loss-of-heterozygosity

**WT:** Wild-type

**ADPLD:** Autosomal Dominant Polycystic Liver Disease

**ADPKD:** Autosomal Dominant Polycystic Kidney Disease

**PLD:** Polycystic Liver Diseases (ADPLD and ADPKD)

**PKD1:** Polycystic Kidney Disease 1 protein (Polycystin-1)

**PKD2:** Polycystic Kidney Disease 2 protein (Polycystin-2)

**PRKCSH:** Protein Kinase C Substrate 80K-H (80 kDa protein, Heavy chain)

**SEC63:** SEC63 homolog (*S. cerevisiae*)

**LRP5:** Low density lipoprotein Receptor-related Protein 5

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## Chapter 5

Bipotent adult stem cells derived from cyst epithelium expand into organoids retaining key characteristics of polycystic liver disease

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## **Abstract**

**Backgrounds & aims:** Polycystic liver disease (PLD) is an autosomal dominantly inherited disorder caused by mutations in genes such as *PRKCSH* and *SEC63*. It has been thought that cysts develop from biliary progenitor cells due to loss-of-heterozygosity (LOH), leading to aberrant proliferation or defects in differentiation. Cyst expansion can be suppressed by somatostatin analogues such as lanreotide. There is no human *in vitro* model available that truly recapitulates polycystic liver disease. We hypothesize that PLD progenitors can form bipotent liver organoids that carry key features of cyst development.

**Methods:** Cells from normal biliary duct (n=6), cyst biliary epithelium (n=60) and cyst fluid (n=31) were isolated and placed under conditions suitable for expansion of human adult liver stem cells. We analyzed genetic LOH, gene expression, differentiation capacity, response to lanreotide and cilium formation of these organoids.

**Results:** Cholangiocytes from cyst biliary epithelium (47/60) and cyst fluid (9/31) proved capable of expanding as bipotent liver organoids. Multiple cyst organoids displayed LOH surrounding *PRKCSH* or *SEC63* regions. Organoids formed cilia when proliferation was inhibited. Neither expansion nor differentiation of PLD organoids was impaired. RNAseq revealed differential gene expression depended on organoid genetic background. Lanreotide significantly decreased expansion of liver organoids in comparison to negative control (197% ± 46% versus 547% ± 28%; p: 0.038).

**Conclusions:** Biliary progenitor cells from patient cyst epithelium and fluid expand into liver organoids. They recapitulate key characteristics of PLD, and are a promising human *in vitro* model for research, diagnostics and treatment of polycystic liver diseases and cholangiociliopathies.

## **Lay summary**

Self-renewing cells of liver cysts, abnormal fluid-filled sacs, can be grown outside of the body. Grown in this manner, they are a promising development for research, diagnosis and treatment of various diseases of the liver.

## **Keywords**

Liver disease, Cholangiocytes, Disease model, RNAseq, Drug test

## **Introduction**

Polycystic liver disease (PLD) is a phenotypical expression of isolated polycystic liver disease (ADPLD), and of autosomal dominant polycystic kidney disease (ADPKD)(1). PLD is caused by germline mutations in *PRKCSH*, *SEC63*, *LRP5*, *PKD1*, and *PKD2* (1, 2). ADPKD is the most prevalent (~1:1,000) (genetic) cause of end-stage renal disease. By contrast, ADPLD is infrequent and seen in ~1/158,000 of the general population. Patients with ADPKD have an increased total liver volume, and a recent study found that 81% of female ADPKD patients have severe hepatomegaly (3). An important feature of PLD is increased cholangiocyte proliferation and fluid secretion, causing symptoms related to abdominal distension. This can be suppressed by somatostatin analogues such as lanreotide (4-6).

A common event that underlies these autosomal dominant disorders is complete loss of cyst gene expression from diseased epithelium (7-12). This is the result of the presence of a germline mutation that is accompanied by somatic inactivation of the second allele of a PLD

gene (second hit). The target cell of this genetic process is likely to be the cholangiocyte that carries stem-cell like capacities. Although this is an attractive concept, experimental data that demonstrated expansion from mutated cholangiocytes into a cyst are lacking. Cyst development is thought to be dependent upon ductal plate malformation (DPM) (1, 13) caused by disturbed differentiation of biliary precursors, impaired maturation of bile ducts, or abnormal duct expansion. Experiments in animal models have shown that the specific mechanism is dependent on the genetic background (1, 13). Progress in studies on the mechanism of cystogenesis in presence of PLD mutations has been hampered because models that rely on biliary differentiation of human induced pluripotent stem, embryonic, or HepaRG cells are incomplete. These cells are derived from non-cystic tissues that do not have the same genetic make-up, including second hit mutations, as human cysts (14, 15). Thus far, no human 3D cyst model that possesses second hits, and that is capable of differentiation has been available.

Recent studies have indicated that there are novel treatment options for PLD (16, 17), but at the same time highlight limitations of current models. There is poor access to primary human biliary tissue, which limits the scale of experiments. This illustrates the need for a more reliable human *in vitro* cyst model allowing large-scale pharmacological screens. The discovery of a method that allows unlimited expansion of liver derived bipotent stem cells opens avenues for development of a novel model (18-20). We hypothesize that PLD cholangiocyte-like stem cells can develop into bipotent liver organoids with cyst like features. Here, we describe the isolation of cholangiocytes from human cyst biliary epithelium and cyst fluid, their expansion into bipotent liver organoids, and their stem cell, cholangiocyte and PLD-associated phenotype. We compare PLD-associated and normal stem cells by gene expression profiling, and assess their capacity to differentiate into hepatocytes and biliary cells. Finally, we demonstrate the translational value of liver organoids by evaluating the response to lanreotide on their expansion, and the presence of the molecular machinery required to respond to somatostatin analogues.

## **Methods**

### **Patient material**

We obtained cyst epithelial cells from 40 patients with 91 PLD or with sporadic cysts. From this input material, 56 cysts from 17 patients could be included for this study. All included patients were female and had a median age of 54 years (43-70). Written informed consent was obtained for the use of secondary tissue, and samples were collected under appropriate ethics approval (“Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen”).

### **Cholangiocyte isolation**

Primary human cyst epithelial cells were derived from surgical biopsies obtained through laparoscopic cyst fenestration, or isolated from cyst fluid that was acquired during aspiration sclerotherapy. Whole blood was collected from these patients. We obtained biopsies from healthy liver from clean resection margins of colorectal cancer liver metastasis surgery. Cholangiocytes were isolated from normal biliary ducts as described earlier (21), without enrichment for liver progenitors. Briefly, 0.5-1cm<sup>3</sup> tissue was rinsed with DMEM (GIBCO by Invitrogen, Carlsbad, CA, USA) with penicillin/streptomycin, and minced using preparative scissors. Minced tissue was then incubated for 1 hour at 37 degrees C in digestion solution

(2.5 mg/ml collagenase B (Roche, Mannheim, Germany), 0.1mg/ml DNase I (Sigma, St. Louis, MO, USA) in HBSS containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (GIBCO)) while slowly mixing on a magnetic stirrer (MR2002; Heidolph, Schwabach Germany). Digestion was stopped by adding DMEM with 1% FCS (GIBCO).

Cells were isolated from cyst epithelium by incubation of the luminal side of epithelia on 500  $\mu\text{L}$  50mM EDTA for 30 minutes at room temperature. Remaining tissue was further prepared as tissue from normal biliary ducts, with elongation of the collagenase digestion step to 2-3 hours. Isolated cells from this second isolation were frozen in stocks. Organoid cultures were preferentially started from the EDTA isolation to maximize presence of cyst lumen derived cells. If this was unsuccessful, frozen stocks of collagenase isolated cells were used.

Cells from cyst fluid of aspiration sclerotherapies were spun down by centrifugation. To maximize live cell numbers, 10  $\mu\text{L}$  of spun-down cells were seeded along with 25  $\mu\text{L}$  Matrigel. Any remaining cells were frozen as stocks. In case of organoid growth, cells were passaged until a stable culture was established. If growth-arrest occurred, additional cells from the frozen stock were subsequently seeded and passaged until a culture was founded.

### **Human adult bipotent liver stem cell expansion medium**

Expansion medium (EM) consisted of AdDMEM/F12 (Invitrogen) with 1% N2, B27 (minus vitamin A) supplements (both from GIBCO), 1,25mM N-Acetylcysteine (Sigma), 10mM nicotinamide (Sigma), 50ng/ml EGF (Peprotech), 1 $\mu\text{g}$ /ml Rspo1 (Peprotech), 10nM gastrin (Sigma), 25 ng/ml HGF (Peprotech, Offenbach, Germany), 100ng/ml FGF-10 (Peprotech), 5  $\mu\text{M}$  A83.01 TGF- $\beta$  inhibitor (Tocris, Ellisville, MO, USA), and 10  $\mu\text{M}$  forskolin (Tocris). The first 3-4 days after seeding cells, 50% Wnt CM (homemade prepared as described in (22, 23), and 10  $\mu\text{M}$  Y27632 Rho-kinase inhibitor (Sigma) were added. Cells (~10,000) were seeded in 25  $\mu\text{L}$  growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) in non-attaching 48-wells plates (BD Biosciences). EM was replaced every 2-4 days. After 10-14 days, organoids were removed from Matrigel, mechanically dissociated and seeded in fresh Matrigel. Cells were subsequently passaged every 7-14 days with a 1:3 to 1:8 ratio, depending on the number of organoids.

### **Long-term organoid storage**

Frozen stocks were prepared from dissociated organoids mixed with AdDMEM/F12 (Invitrogen) with 10% DMSO, then placed overnight in -80 degrees C, followed by -180 degrees C nitrogen storage.

### **Hepatocyte and biliary differentiation medium**

For hepatocyte differentiation, 25ng/ml BMP7 (Peprotech) was added to the EM 7-10 days prior to splitting. Following 2-4 days of culture with EM + BMP7, hepatocyte differentiation medium (hDM) was added. hDM consisted of AdDMEM/F12 with 1% N2, B27 (minus vitamin A) supplements, 50ng/ml EGF, 10nM gastrin, 25ng/ml HGF, 500nM A83.01 TGF- $\beta$  inhibitor, as well as 10 $\mu\text{M}$  DAPT (Abcam, Cambridge, MA, USA), 30 $\mu\text{M}$  dexamethasone (Sigma) and 100ng/ml FGF19 (Peprotech). Biliary differentiation medium consisted of EM without Rspo1, nicotinamide, A83.01 TGF- $\beta$  inhibitor, and forskolin. Organoids were cultured in biliary differentiation medium (bDM) for seven days before experiments.

For induction of cilia, cells were cultured for seven days in normal expansion medium, and 100nM nocodazole (Sigma) was added 2 hours before fixation (24).

## **DNA isolation**

Organoids were removed from medium and mechanically dissociated before DNA isolation. Only organoids with less than 5 passages were used for DNA isolation. DNA from organoids was isolated using the QIAamp DNA Micro kit (Qiagen) according to the manufacturer's protocol, and stored at 4°C. For samples with low DNA yields, whole genome amplification using the Qiagen REPLI-g Mini kit (Qiagen) was performed. DNA was isolated from whole blood using the high pure PCR template preparation kit (Roche) according to the manufacturer's protocol, and stored at 4°C.

## **RNA isolation from organoids**

Organoids were removed from medium, mechanically dissociated before RNA isolation. For gene expression tests, RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. For RNA sequencing, RNA from organoids was isolated using the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Organoids used in RNA sequencing had been passaged less than 10 times, and had been cultured under the same circumstances for two weeks prior to RNA isolation.

## **Genotyping**

Whole blood patient DNA was screened for germline mutations in PRKCSH, SEC63, and LRP5 using direct sequencing as described previously (8). Exons and flanking intronic sequences were amplified using polymerase chain reaction with specific primers (Supplementary Table 1). The amplified fragments were purified (QIAEXII Gel Extraction Kit; Qiagen, Hilden, Germany) and sequenced with the BigDye Terminator Kit and ABI3730 capillary sequencer (Perkin-Elmer Applied Biosystems, Boston, MA).

## **Cytogenic array analysis**

Copy-number variations and loss of heterozygosity (LOH) regions were assessed using genome-wide high resolution cytogenetic array analysis (CytoScan HD, Affymetrix, Santa Clara, CA, USA). For analysis of individual cytoscan HD data chromosome analysis suite (ChAS, Affymetrix) V2.1 was used.

## **(reverse-transcriptase) Polymerase chain reaction**

Isolated RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands). Targets were amplified for 40 cycles using gene-specific primers and Sybr-Green (Life Technologies) in a thermal cycler (C1000 / CFX96, Bio-Rad). Primers used are listed in supplementary table 2. For RT-PCR, all values were represented as mean  $\pm$  S.D. Mann-Whitney nonparametric test was used for statistical analysis,  $p < 0.05$  was considered statistically significant.

## **Immunofluorescence**

Organoids were washed once and fixed whole-mount for 20 minutes using paraformaldehyde (PFA; 3.8%) at room temperature. Samples were subsequently permeabilized with PBS 0.5% Triton-x100 with 1% bovine serum albumin, and incubated overnight with primary antibodies. Following three washes with PBS 0.3% Triton-x100, samples were incubated 2 hours with secondary antibodies. Images were obtained using confocal microscopy (Olympus FV1000, Shinjuku, Tokyo, Japan). Antibodies used are listed in supplementary table 3 and 4.

## **Scanning electron microscopy**

For cilium expression, organoids were grown in regular expansion medium on 8-well chambered cover slips (Ibidi, Martinsried, Germany). Matrigel containing organoids was cut open using a razor. Cells were washed once with PBS and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h. After washing cells twice with sodium cacodylate buffer, cells were stored in this buffer at 4 °C until further fixation with 1% OsO<sub>4</sub> (osmium tetroxide) for 30 min. Coverslips were then washed with 0.1 M sodium cacodylate buffer and dehydrated in a graded series of alcohol washes for 10 min each. Finally, coverslips were critical point dried, mounted on stubs, sputter coated with gold and viewed in a JEOL SEM6340F field emission scanning electron microscope.

## **Library preparation**

RNA-seq libraries were prepared with the Illumina TruSeq mRNA Sample Preparation kit (Illumina Inc.; San Diego, CA, USA) according to the manufacturer's protocol. Libraries were validated on an Agilent Bioanalyzer 2100. The standard protocol from Illumina for the preparation of RNA-SEQ libraries from total RNA includes a step in which polyA mRNA selection results in the removal of the bulk rRNA from the sample. This results in the removal of background signal. Synthesis of first and second strand cDNA was followed by end repair and addition of "A" to the ends and ligations of the adapters for sample multiplexing. Enrichment of the samples was carried out by PCR. The amplified libraries were size selected and libraries were quantified by PicoGreen assay (Life Technologies). We used TruSeq<sup>TM</sup> SR Cluster Kits v3 (Illumina) for cluster generation in an Illumina cBOT instrument following the manufacturer's protocol. Six indexed libraries were loaded into each lane of flow cells. Sequencing was performed on an Illumina HiSeq 2500 instrument (Illumina) by the manufacturer's protocol. Multiplexed two lanes of single-read runs were carried out with a total of 150 cycles per run (including 7 cycles for the index sequences). Custom programs were used to demultiplex individual samples using their unique index. Each sample had 25 million reads on average. We used CLC Genomics Workbench 7 for RNAseq alignment and statistical analysis of the data. Human HG19 was used as reference sequence.

## **Transcript Profiling**

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Wills et al., 2015) and are accessible through GEO Series accession number GSE73579 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73579>).

## Gene expression analysis

To calculate gene expression intensity, read counts were normalized to reads. Methods for data normalization and analysis are based on the use of “internal standards” (25) that characterize some aspects of the system’s behavior, such as technical variability, as presented elsewhere (26, 27). The comparison of these methods with some other normalization and analysis procedures was presented in (28). Created initially for the analysis of microarray data they were slightly modified to the needs of RNAseq data analysis. The two-step normalization procedure and the Associative analysis functions are implemented in MatLab (Mathworks; MA, USA) and available from authors upon request. These algorithms are also obtainable from an R package *diffGeneAnalysis*, available as a part of Bioconductor packages (<http://www.bioconductor.org/packages/2.5/bioc/html/diffGeneAnalysis.html>). Heatmaps were generated with the Spotfire Decision Site 9 (TIBCO; Palo Alto, CA, USA) with gene subsets created from the list of significant genes. Functional analysis of identified genes was performed with Ingenuity Pathway Analysis (Ingenuity® Systems; Redwood City, CA, USA, <http://www.ingenuity.com>). Principal components analysis (PCA) was performed on log<sub>2</sub>-transformed normalized data to visualize variations between the samples of the different groups using R (version 3.1.11).

## Lanreotide and expansion experiments

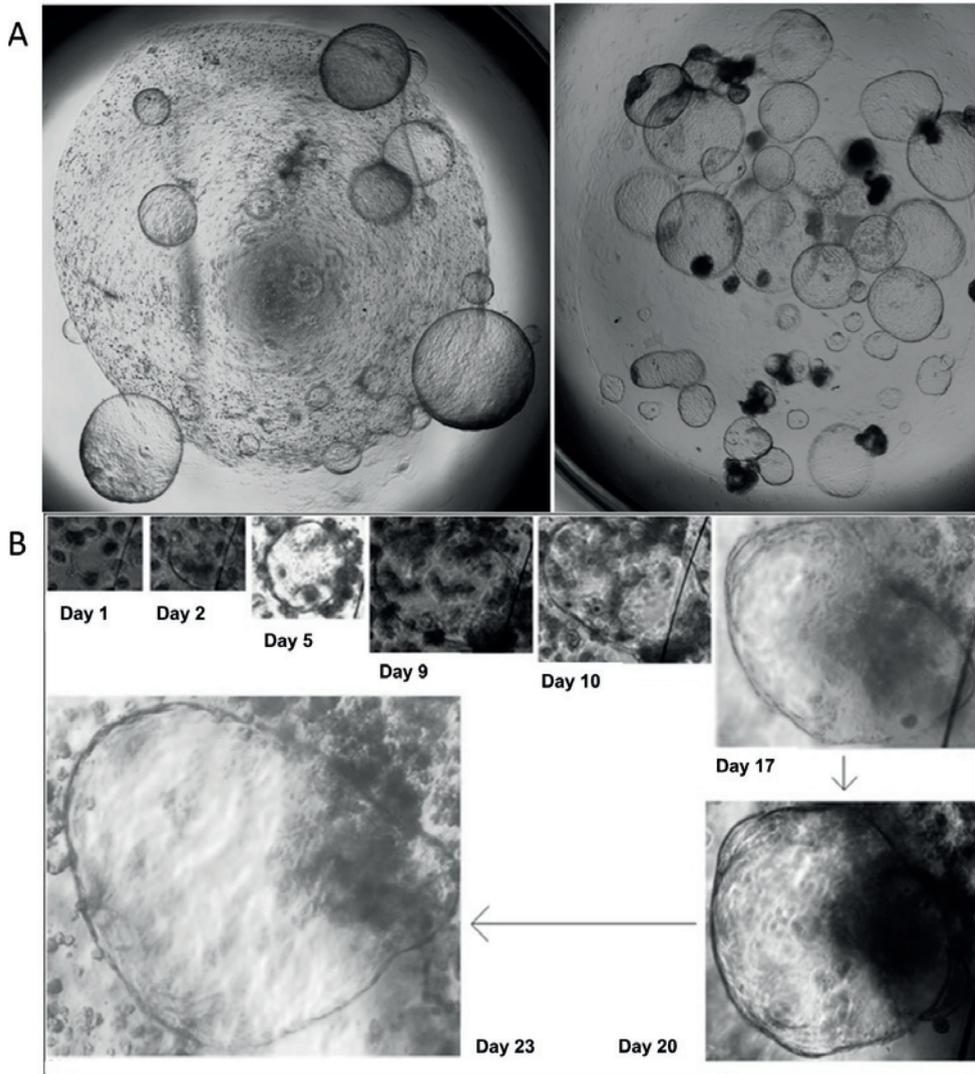
In three independent experiments, control (0.1 M acetic acid) or lanreotide ( $10^{-7}$ M in 0.1M acetic acid) was added twice a day, at 12-hour intervals for seven days. Organoid development was followed by light microscopy and circumferential areas were quantified by Image J software (NIH). Data were expressed by percentage change in circumferential area (mean  $\pm$  SEM) on day 7 in comparison to day 1. Statistical analysis was performed by Student’s t-test.

**Table 1.** List of organoids and origin used in experiments. All cyst epithelium came from laparoscopic cyst fenestration, and all cyst fluids came from aspiration sclerotherapy.

\*: Limited to experiments shown in this article. We used only 1 line per experiment when multiple cyst lines from the same patient were available. p: passage

Name	Patient	Origin	Disease	Mutation	Passage time	Organoid studies*
PLD1	Female, 45 years	Epithelium	ADPLD	<i>PRKCSH</i> 292+1G>C	1 week	-
PLD2	Female, 54 years	Epithelium	Solitary cyst	-	1 week	IF, gene expression & differentiation, RNAseq (p6)
PLD4	Female, 55 years	Fluid (300mL; turbid, orange)	ADPKD	Undetermined	2 weeks	-
PLD5	Female, 55 years	Fluid (2,5L; clear, yellow)	Solitary cyst	-	2 weeks	-
PLD7	Male, 59 years	Fluid (300mL; clear, yellow)	ADPLD	Unknown	2 weeks	-
PLD8.1-8.10	Female, 56 years	10 Epithelia	ADPLD	Unknown	1 week	IF, gene expression & differentiation, RNAseq (p6)
PLD9.1-9.13	Female, 47 years	13 Epithelia	ADPLD	<i>PRKCSH</i> 1362-2A>G	1 week	Cytoscan, IF, RNAseq (p6)
PLD10	Female, 70 years	Fluid (500mL; clear, yellow)	ADPLD	Unknown	2 weeks	gene expression & differentiation, RNAseq (p4)
PLD11	Female, 52 years	Epithelium	ADPLD	Unknown	1 week	-
PLD12.1-12.4	Female, 61 years	4 Epithelia	ADPLD	Unknown	1 week	RNAseq (p5)
PLD13	Female, 51 years	Fluid (970mL; clear, yellow)	ADPLD	<i>SEC63</i> c.964del	1 week	Cytoscan, IF
PLD14.1-14.16	Female, 50 years	16 Epithelia	ADPLD	<i>PRKCSH</i> 1362-2A>G	2 weeks	-
PLD15	Female, 52 years	Fluid (100mL; clear, yellow)	ADPKD	<i>PKD1</i> c.182C>T	1 week	RNAseq (p6)
PLD16.1-16.14	Female, 47 years	14 Epithelia	ADPLD	<i>PRKCSH</i> c.292+1G>C	1 week	Cytoscan, RNAseq (p6)
PLD17	Female, 43 years	Fluid (200mL; clear, yellow)	Solitary cyst	-	1 week	RNAseq (p6)
PLD18	Female, 55 years	Fluid (1L; clear, yellow)	ADPLD	Unknown	2 weeks	-
PLD19	Female, 61 years	Fluid (400ml; clear, yellow)	ADPLD	<i>PRKCSH</i> 292+1G>C	1 week	-

## Results



**Figure 1.** Organoids grown from cyst-fluid derived cells.

**A:** Organoids derived from cyst cells. Left: PLD10, 2 week culture, no passage; right: PLD4, 5 week culture, passage 3. Images of a well, digitally reconstituted from multiple partial images.

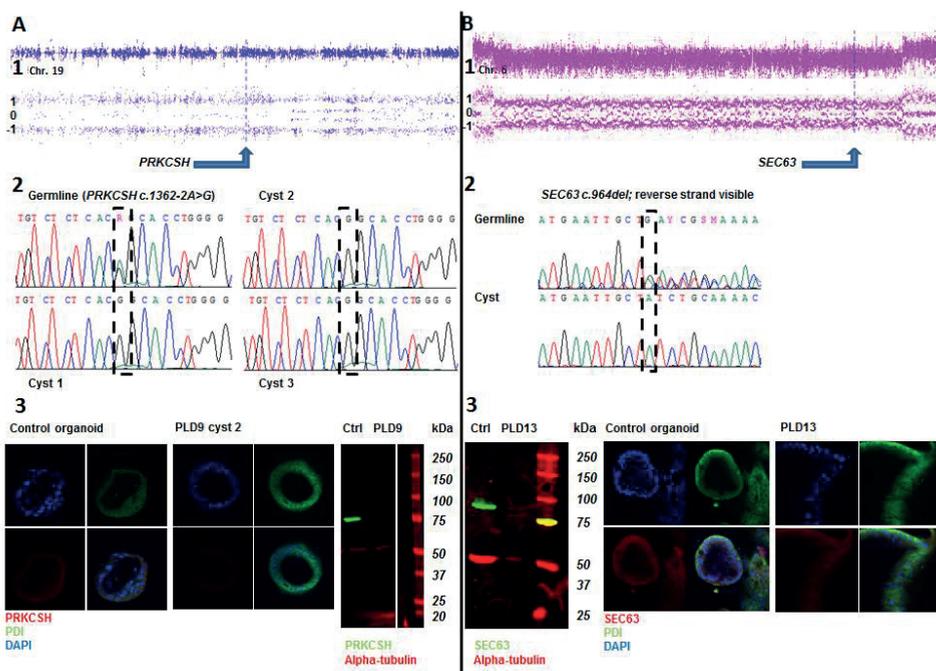
**B:** A single organoid from a cyst fluid cell over the course of 23 days, same magnification.

### **Biliary progenitors form organoids from cyst epithelium and fluid**

We were able to expand and maintain cholangiocytes derived from epithelium of liver cysts (figure 1, table 1). This is consistent with the presence of a pool of biliary progenitor cells or precursors thereof in cyst epithelium. Under these conditions, we maintained cultures of organoids deriving for >4 months, or >20 passages. Cultures expanded from virtually all cyst epithelia obtained by laparoscopic fenestration. In total, 47 of 60 cyst epithelia from 8

patients successfully grew organoids (table 1).

Since cyst fluid is routinely available from aspiration sclerotherapy of dominant liver cysts, we aimed to isolate and expand biliary progenitors from this fluid. By placing cyst fluid derived cells in conditions suitable for human adult liver stem cell expansion, we could often observe growth. Up to thirty organoids from individual stem cells grew from all cells isolated from cyst fluid. 9 out of 31 seeded cyst fluids established long-term cultures. Clear, yellow cyst fluids had the highest efficiency for culture establishment (~36% / 8 of 21), while darker, more turbid fluids yielded less long-term cultures (~10% / 1 of 10). Many cyst fluid derived organoids could be passaged like epithelium derived organoids (every 7 days), but some fluid derived cultures survived only with 14-day passaging intervals. We presume this difference may be related to the smaller original population of fluid-derived cells, in which more quiescent stem cells occur. No differences in gene expression were observed (see below). We could freeze and thaw all cultures according to the described protocol.



**Figure 2.** Loss-of-heterozygosity in cyst – derived organoids. Comparison to control organoid derived from healthy liver from clean resection margins of colorectal cancer liver metastasis surgery.

**A:** Loss of PRKCSH protein expression induced by loss-of-heterozygosity in multiple cyst – epithelium derived organoids from a 47 year old ADPLD patient with a PRKCSH c.1362-2A>G mutation.

1. Cytoscan HD displaying copy number neutral loss-of-heterozygosity surrounding PRKCSH on chromosome 19 (i.e. loss/sparsity of blue dots at 0 (heterozygous state) in comparison to blue dots at -1 and 1 (homozygous states)). Location of PRKCSH on chromosome 19 indicated by dotted line.

2. Chromatograms depicting the PRKCSH c.1362-2A>G mutation in germline and cyst – derived organoid DNA (mutation located in black dotted box). All three cysts display loss-of-heterozygosity of the PRKCSH c.1362-2A>G mutation. (continued on next page)

(continued from last page)

**3.** Immunofluorescence indicated loss of PRKCSH protein (red) expression had occurred, immunoblot confirmed this loss of PRKCSH protein (green) in comparison to alpha-tubulin control protein (red). Immunofluorescent image: PDI (green): ER marker; DAPI (blue): nuclei marker.

**B:** Loss of SEC63 protein expression induced by loss-of-heterozygosity in a single cyst – fluid derived organoid from a 51 year old ADPLD patient with a SEC63 c.964del mutation. Comparison to control organoid derived from healthy liver from clean resection margins of colorectal cancer liver metastasis surgery.

**1.** Cytoscan HD displaying loss-of-heterozygosity induced by allelic loss surrounding SEC63 on chromosome 6 (i.e. loss/sparsity of purple dots at 0 (heterozygous state) in comparison to purple dots at -1 and 1 (homozygous states), inward shift of -1 and 1 channels in combination with downward shift of most upper channel indicates allelic loss). Location of SEC63 on chromosome 6 indicated by dotted line.

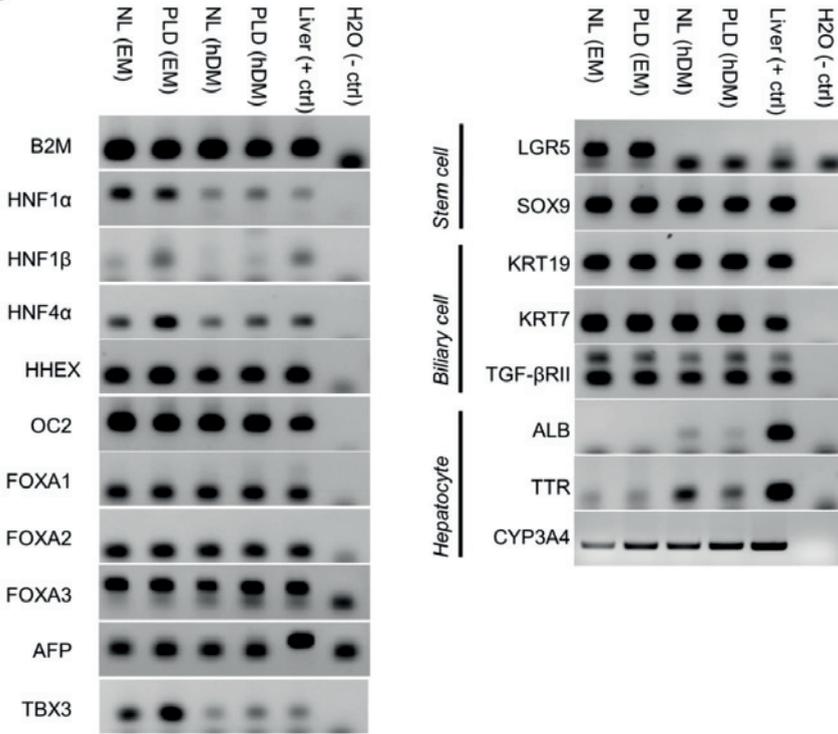
**2.** Chromatograms depicting the SEC63 c.964del mutation in germline and cyst – derived organoid DNA (mutation located in black dotted box). The cyst displays loss-of-heterozygosity of the SEC63 c.964del mutation.

**3.** Immunofluorescence indicated loss of SEC63 protein (red) expression had occurred, immunoblot confirmed this loss of SEC63 protein (green) in comparison to alpha-tubulin control protein (red). Immunofluorescent image: PDI (green): ER marker; DAPI (blue): nuclei marker. PDI (green): ER marker; DAPI (blue): nuclei marker. Organoids recapitulate cyst epithelium genetic background

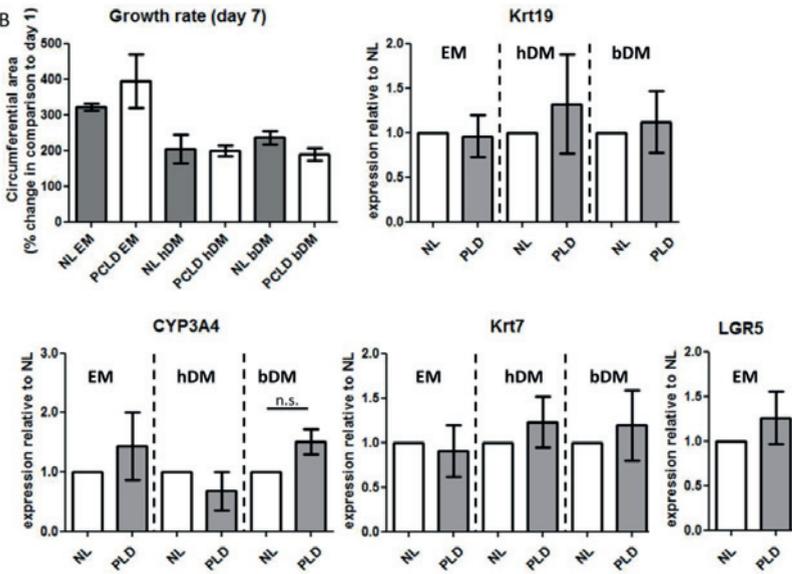
In contrast to the majority of PLD patient cells from healthy tissue, cysts are believed to develop due to monoclonal expansion of cells with somatic second hits or LOH. To confirm the presence of LOH, as well as recapitulation of patient germline abnormalities, we employed genome-wide high resolution cytogenetic array analysis on DNA derived from cyst organoids.

Organoids possessed germline mutations, and also harbored additional mutations consistent with a second-hit hypothesis of cystogenesis (figure 2). In a cyst organoid line of a *PRKCSH* patient, chromosome analysis found a 13 megabase CNN LOH region on chromosome 19. Mutation analysis of multiple cyst epithelium organoid lines of this patient led to identification of recurrent *PRKCSH* LOH (c.1362-2A>G). Immunofluorescence of one of these lines indicated loss of PRKCSH protein expression. Genetic analysis of cyst fluid derived organoids led to identification of a 42 megabase chromosome 6 deletion encompassing *SEC63*. Subsequent analysis of germline DNA revealed a *SEC63* mutation (c.964del), which led to a frameshift (p.Gln322Serfs\*5). There was complete loss of wildtype *SEC63* in cyst organoid DNA. Immunofluorescence indicated lower fluorescent signal for cyst organoids in the SEC63 band. On western blot, no PRKCSH or SEC63 protein was present in mutated organoids (supplementary figure 1).

**A**



**B**



(Legenda on next page)

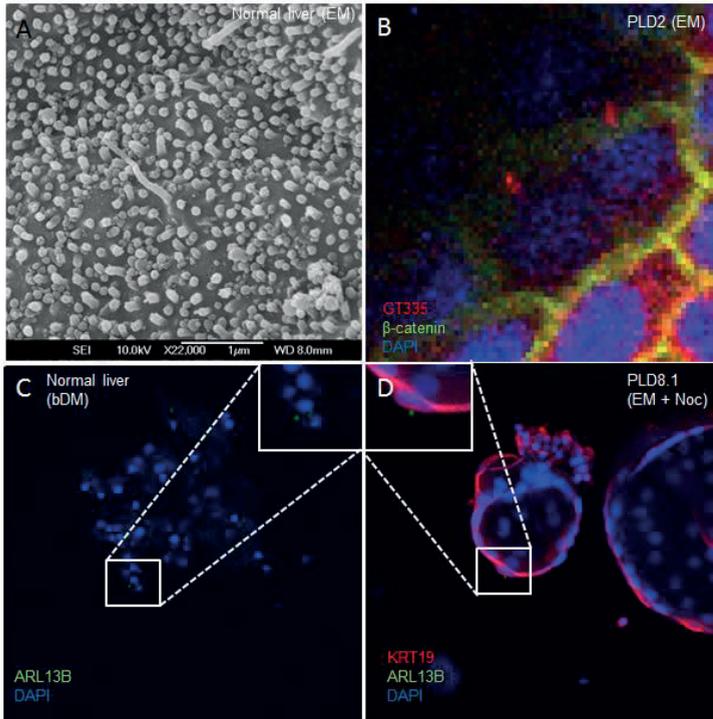
**Figure 3.** Proliferation and differentiation of cyst and normal liver organoids (also see 20 for further characterization of normal liver organoids).

**A:** Expression of a panel of hepatobiliary genes expressed in normal (NL) and cyst (PLD) organoids in comparison to normal liver tissue. Stem cell (LGR5, SOX), cholangiocyte (KRT7, KRT19, TGF- $\beta$ 2) as indicated. For AFP, and LGR5 cDNA from fetal instead of adult liver was used as a positive control. In EM, both organoid types expressed stem cell markers SOX9 and LGR5, and expressed cholangiocyte markers KRT7, KRT19, and TGF- $\beta$ 2. LGR5 expression was lost with hepatocyte differentiation. Transcription factors FOXA1, FOXA2, FOXA3, OC2, HHEX, HNF1 $\alpha$ , HNF1 $\beta$ , HNF4 $\alpha$ , and TBX3 were expressed. Albumin and CYP3A4 expression increased in both normal and cyst organoids following hepatocyte differentiation. Expression of HHEX, HNF1 $\alpha$ , HNF1 $\beta$ , FOXA3, HNF4 $\alpha$ , and TBX3 decreased under hDM conditions. No AFP expression was present under any condition.

**B:** No significant differences in proliferative or differentiation capacity could be observed between 3 normal (NL) and 3 liver cyst (PLD) organoid lines in three independent experiments, as determined by Mann-Whitney U non parametric testing. Proliferation measured by change in organoid circumferential area on day 7 in comparison to day 1. Differentiation capacity measured by KRT19, KRT7, CYP3A4 and LGR5 expression after 7 days in indicated condition (i.e. EM, hDM, bDM). Gene expression in proliferating and differentiating conditions.

LGR5+ liver organoids are normally constituted of bipotent biliary stem cells (20), and it has been hypothesized that cysts might develop due to abnormal differentiation of biliary precursors (1, 13). We set out to characterize and compare normal liver and cyst organoid gene expression in expanding and differentiating conditions (figure 3). In EM, both organoid types expressed stem cell markers SOX9 and LGR5, and expressed cholangiocyte markers KRT7, KRT19, and TGF- $\beta$ 2. Transcription factors FOXA1, FOXA2, FOXA3, OC2, HHEX, HNF1 $\alpha$ , HNF1 $\beta$ , HNF4 $\alpha$ , and TBX3 were expressed.

The hDM condition allows differentiation to both hepatocyte and cholangiocyte lineages with concomitant loss of progenitor marker LGR5 (20). As bipotency was extensively tested in this previous study, we only set out to confirm this in our organoids by RT-PCR. Albumin and TTR expression was exclusive to the hDM condition in both normal and cyst organoids, while CYP3A4 expression increased in comparison to EM. LGR5 expression was lost with differentiation. Expression of HHEX, HNF1 $\alpha$ , HNF1 $\beta$ , FOXA3, HNF4 $\alpha$ , and TBX3 decreased under hDM conditions. No AFP expression was present under any condition. Expression of hepatocyte and cholangiocyte markers in the hDM condition confirmed the presence of both these cell types in the organoids.



**Figure 4.** Primary cilia are sporadically present on liver organoids.

**A:** Scanning electron microscopy image of liver organoids in EM. A putative primary cilium of  $\sim 2\mu\text{m}$  is visible on a cell.

**B:** Polycystic liver organoids express few, but clear primary cilia when grown in medium aimed at stem cell proliferation. Shown is PLD organoid line 2 from solitary liver cyst epithelium of a 54-year old female patient with (also see table 1).

DAPI: blue;  $\beta$ -catenin: green; GT335: red.

**C:** Polycystic liver organoids express primary cilia when grown in conditions suitable for biliary differentiation. Shown is a normal liver organoid derived from healthy liver from clean resection margins of colorectal cancer liver metastasis surgery.

DAPI: blue; ARL13B: green; KRT19: red.

**D:** Polycystic liver organoids express primary cilia when treated with nocodazole, a pharmacological stimulant of cilia, 2 hours before fixation. Shown is PLD organoid line 8.1 from cyst epithelium of a 56-year old female ADPLD patient (also see table 1).

DAPI: blue; ARL13B: green; KRT19: red.

### Organoid expansion and differentiation capacity

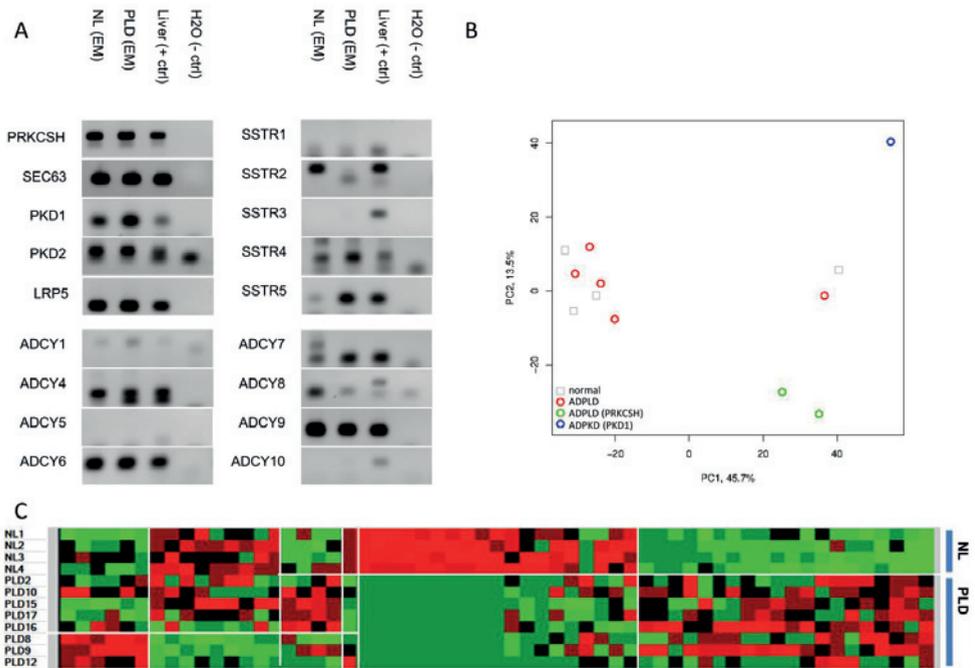
Using EM, bDM and hDM conditions, we compared expansion and differentiation capacity of cyst bipotent biliary progenitors ( $n=3$ ) to that of normal bipotent biliary progenitors ( $n=3$ ) in 3 independent experiments (figure 3). Gene expression was similar among both types of organoids. All in all, we could exclude a differentiation defect.

Since *LRP5* mutations have recently implicated abnormal Wnt signaling in cystogenesis, we additionally cultured 3 normal and 3 cyst liver organoids without Wnt agonist Rspo1. Both culture types simultaneously arrested growth within two weeks of culture in three

experiments (data not shown). This indicates that Wnt/Rspo1 signaling is not disturbed in cyst organoids.

### Organoid primary cilia and polarization

Besides differentiation problems, primary cilia dysfunction is crucial to cyst development, and we assessed their presence on organoids. Under EM conditions, primary cilia were present, but on relatively few cells (figure 4). On whole mounts stains with DAPI and GT335 or ARL13B, we observed a small number of cells with cilia (figure 4; supplementary figure 2). Cells cultured without proliferative growth factors Rspo1, nicotinamide, A83.01 TGF- $\beta$  inhibitor, and forskolin (bDM) for 7 days expressed more cilia, as did those cultured with 100nM nocodazole 2 hours prior to fixation. Cells lining organoids with lumens had predominantly basolateral  $\beta$ -catenin, and only rarely were cilia observed on the external surface of organoids (depicted with PLD8.1).



**Figure 5.** Gene expression of organoids.

**A:** Expression of a panel of cyst-related genes expressed in organoids. PRKCSH, SEC63, LRP5, PKD1, PKD2, SSTR1-5, and ADCY1-10 as indicated. For ADCY8 cDNA from fetal instead of adult liver was used as a positive control.

**B:** Principal component analysis of indicated liver organoid samples.

**C:** Heatmap of differentially expressed genes. NL: Normal liver organoids; PLD: ADPLD/ADPKD organoids. Red: high expression; black: average expression; green: low expression.

## RNA sequencing and pathway analysis

We aimed to determine whether specific genes or pathways were present and / or disturbed in bipotent progenitors passaged 4-6 times (table 1;

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73579>). RNA sequencing of normal liver organoids (n=4) and PLD organoids (n=8, including three *PRKCSH* mutants) was performed, which yielded ~25 million reads per sample. Organoids expressed cyst genes *PRKCSH*, *SEC63*, *LRP5*, *PKD1*, and *PKD2* (figure 5). Many components of nonmotile primary cilia were clearly expressed in all organoids (supplementary table 5), as determined by the SYSCILIA gold standard list (syscilia.org). In addition, ciliopathy genes(1) involved in liver cyst development such as *PKHD1*, *MKS1*, *OFD1*, *IFT88*, and *TSC1* were also expressed. Cellular components related to the lanreotide response were expressed such as somatostatin receptors 1, 2, 4, and 5, while *SSTR3* was not. *SSTR2* was expressed in only some lines of normal and liver cyst organoids (data not shown). Adenylyl cyclases 1, 4, 6, 7, and 9 were expressed, *ADCY5*, 8 and 10 were not expressed.

Following normalization, principal component analysis, F-means clustering, and heat maps revealed different clustering of normal and PLD organoids (figure 5). Clustering was dependent upon mutation status. Two organoids with known *PRKCSH* mutations with LOH on chromosome 19 (PLD9 and PLD16) clustered together on principal component analysis (figure 5), but separately from other organoids. PLD15, from an ADPKD patient with a *PKD1* mutation background, also clustered separately from other organoids. F-means clustering revealed that these samples displayed significant co-expression of genes (supplementary figure 3). PLD9, PLD15, and PLD16 shared expression of a large set of genes, separating them from the other samples. As mentioned above, these organoids had a background of *PRKCSH* and *PKD1* germline mutation and somatic LOH. Another set of genes was shared between these samples and PLD17, with an unknown mutation. PLD9 and PLD16 (both *PRKCSH* mutants) shared expression of a smaller subset of genes. PLD15, the only sample grown from a liver cyst of an ADPKD patient with *PKD1* mutation, displayed a distinct expression pattern when compared to other PLD samples. We found no relation with culture origin (epithelium versus fluid) with PCA clustering, removing this as a source of bias. These data indicate that the mutational background of cysts are an important cause of differential gene expression.

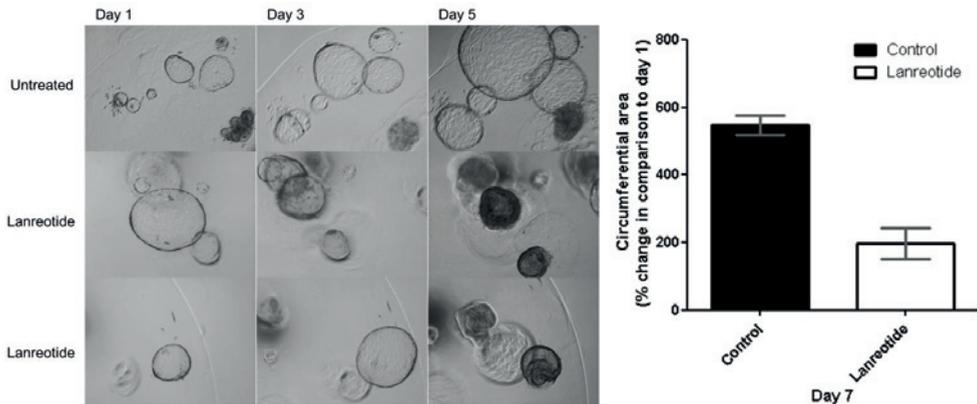
Genes such as *KIF5C*, *PXDN*, *SEPT5*, *GBA3*, *PSCA*, and *FOLR1* were expressed many fold higher in the PLD group compared with the control (NL) group (supplementary table 6).

Three out of four normal liver organoids shared a large set of genes (supplementary figure 4). A small set of genes clustered differently between control and PLD organoids. PLD organoids displayed a heterogenous expression pattern within their group. Despite this heterogeneity, cyclic (c)AMP responsive element binding protein 1 (*CREB1*) was found to control many of the genes that were differentially expressed between normal and PLD liver organoids. No pathways were significantly dysregulated in the PLD organoid group.

### Lanreotide response

Cholangiocytes from polycystic livers are known to respond to lanreotide by decreased growth, and we aimed to determine whether liver organoids would also be inhibited. Using normal EM in combination with 100nM lanreotide every 12 hours, no response could be observed (data not shown). We figured this may have had to do with competition by factors such as EGF and forskolin, which are known to have effects on cAMP signaling. We opted to

reduce all growth factors in the EM to 10%, except B27 and N2 supplements, and to add 100nM lanreotide every 12 hours to the medium. Subsequently, a significant effect of lanreotide was observed in three independent experiments (figure 6). Lanreotide significantly decreased expansion of liver organoids in comparison to control ( $197\% \pm 46\%$  versus  $547\% \pm 28\%$ ;  $p: 0.038$ ).



**Figure 6.** Liver organoids respond to lanreotide by decreased growth. Lanreotide significantly decreased expansion of liver organoids in comparison to negative control ( $197\% \pm 46\%$  versus  $547\% \pm 28\%$ ;  $p: 0.038$ ).

## Discussion

Here we describe the methods to obtain patient-derived 3D cyst organoid lines from cyst epithelium and fluid, which recapitulate key features of polycystic liver disease. Crucial to polycystic liver development are presence of somatic second hits (e.g. in *PRKCSH* and *SEC63*), which we show here to be present in cyst derived organoids. Analysis of expression of *LRP5*, *PKD1*, and *PKD2* cyst genes indicates that loss of these wild type alleles occurs in liver organoids. We found that *SOX9*, *LGR5*, *KRT7*, *KRT19*, and *TGF-βRII* expression is present in expanding organoids, indicating that biliary progenitors are responsible for proliferation. Differentiation capacity of cyst organoids was intact, as measured by hepatocyte marker *CYP3A4*, and cholangiocyte markers *KRT19* and *KRT7*. Using RNAseq we were able to show that clustering of organoids depended on their genetic background, as *PRKCSH* and *PKD1* mutant organoids shared gene expression. No significantly different pathways occurred in our PLD organoids, likely related to the heterogeneity of the organoids' genetic background. Lastly, the response of the organoids to lanreotide indicates their translational value.

An important finding is that bipotent cyst progenitors confirm the long-standing hypothesis that cysts develop from and are maintained by biliary stem cells (8). We found LOH in cyst derived organoids which is consistent with previous reports (7-12). For example, we discovered *SEC63* LOH in our cell culture from a *SEC63* mutant PLD patient. To our knowledge, this is only the second finding of a somatic second hit in *SEC63* (7). Since neither expansion nor hepatoblast differentiation was dysregulated, we may exclude them as the mechanism of cyst formation under the tested circumstances (1, 13). In addition, human organoids expressed somatostatin receptors (including *SSTR2* and 5) and adenylyl cyclases involved in lanreotide response and cyclic AMP signalling. These findings, along with

organoids' response to the somatostatin analogue are in line with *in vitro* findings in 3D hepatic cysts from polycystic kidney disease rats (4, 6), and iPS-derived cholangiocytes (15). Interestingly, *RSPO1*, *LGR5*, and *LRP5* cooperate, amongst others, in generating the Wnt signalling crucial to stem cell development and maintenance (29). Although beyond the scope of this study, this may implicate that *LRP5* mutations found in PLD(2) may affect cystic stem cells.

Our model has several advantages over previous models. PLD organoids could be grown from cells present in cyst fluid expanded in 3D, while retaining somatic second hits (which germline cells do not have (7-12)). This shows its advantages over use of cholangiocytes derived from HepaRG, embryonic or induced pluripotent stem cells (14, 15). Additionally, cyst fluid – derived liver organoids denote bodily fluids as a novel source of primary bipotent liver cells. Other fluid-derived cells, such as metastasizing stem cells from hepatocellular carcinomas or cholangiocarcinomas in blood or bile fluid may also be cultured using these methods. RNAseq showed no separate clustering of organoids on the basis of epithelial or fluid origin, removing this as a source of differences between cultures. The response of the liver organoids to lanreotide shows their potential for use in (personalized) drug screens or other molecule screens. Similar screens have already been conducted with colorectal and prostate organoids (30, 31). As in these studies, further experiments may generate dose-response curves for different organoid lines to exploit their specific sensitivities.

Even though this study shows promising results, it has some limitations. First, some derived organoid lines may not reflect the whole cyst cell population, when cyst fluid organoids originated from few stem cells. Contaminating cells may also occur, such as normal biliary progenitors from the healthy side of cyst epithelium. It is therefore important to assess cell origins by assessing LOH and / or mosaicism regions. Second, under the tested conditions, relatively few cells expressed primary cilia. More cilia may be found in better differentiated biliary cells (14, 15). Conditions for biliary differentiation are expected to become more optimized, given extensive experiments. Third, as these reflect our first attempts at isolating and expanding cyst fluid progenitors, we anticipate that methods can be further improved. Efficient extraction of viable, biliary cells, e.g. through sorting EPCAM+ cells(21), or seeding all unsorted cells of larger cyst volumes might enhance culture establishment efficiency. A larger pool of initiating stem cells will improve the chances of obtaining stem cells with normal proliferation speed. Finally, we believe the heterogeneous gene expression of the organoids included for RNA sequencing to be a reflection of their heterogeneous genetic background, rather than a limitation of the technique. All organoids were cultured in the same conditions for at least two weeks, and variability of our treatment of the cells was at a minimum. Moreover, *PRKCSH* mutant organoids showed similar clustering on PCA, while *PRKCSH* and *PKD1* mutant organoids shared a large set of genes that are differentially expressed from remaining samples. We believe this indicates the heterogeneity observed with RNAseq is dependent upon the genetic background of each organoid, rather than the culturing technique.

In conclusion, we believe that the development of cyst-derived bipotent liver progenitor lines is an important innovation for research, diagnostics, and treatment of various polycystic liver diseases as well as cholangiociliopathies.

## **Abbreviations**

**ADPKD:** autosomal dominant polycystic kidney disease

**ADPLD:** autosomal dominant polycystic liver disease

**bDM:** biliary differentiation medium

**EM:** expansion medium

**DPM:** ductal plate malformation

**hDM;** hepatocyte differentiation medium

**LOH:** loss of heterozygosity

**PLD:** polycystic liver disease

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## **Conflicts of interest**

The authors disclose no conflicts.

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## Chapter 6

Efficient genome editing for genes underlying autosomal dominant polycystic liver disease indicates noncystic phenotype in individual mutagenized zebrafish larvae

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## **Abstract**

**Introduction:** Autosomal dominant polycystic liver disease (ADPLD) is caused by PRKCSH, SEC63, and LRP5 mutations. Thus far, few animal models exist mimicking the human phenotype. Zebrafish can be used as a stable, scalable animal model, but the phenotype of most genetic knockouts for ADPLD is unknown. We aimed to establish zebrafish with induced mutations as an ADPLD model.

**Methods:** We developed gRNAs targeting three genes underlying ADPLD. CRISPR-Cas9 constructs were injected into embryos, and the embryonic phenotype was studied.

**Results:** gRNAs effectively targeted the genes, with high mutagenesis efficiencies (>75%) for Prkcsh and Sec63. Efficient bigenic editing was possible for these two genes. Any mutation in Prkcsh and Sec63 prevented larvae from reaching adulthood. Crispants did not display liver cysts. Only crispants with in-frame Lrp5 mutations matured, which did not have obvious phenotypic abnormalities.

Prkcsh- and Sec63-crispant presented with growth retardation. Sec63 and Prkcsh-/Sec63-double mutant larvae additionally failed to inflate swim bladders.

**Conclusion & discussion:** Fully penetrant CRISPR-Cas9-based ADPLD gene knockdown in zebrafish causes death after the larval stage. Crispants do consistently show a phenotype of retarded growth and failure to inflate their swim bladder. These abnormalities may be used for future studies of underlying pathways or drug screens. Our described gRNAs might form a first step in developing liver-specific knockout fish to increase the cystogenic timeframe.

## **Lay summary**

Genes involved in liver cyst development in humans, fluid-filled sacs in the liver, can be disrupted in zebrafish. In zebrafish larvae, loss of these genes do not cause cysts, but cause a failure to thrive and grow. For the *Sec63* cyst gene, this could be associated with failure to inflate swim bladders, an organ important for zebrafish movement in the water.

## **Keywords**

polycystic liver disease, zebrafish, CRISPr-Cas9, disease model

## **Introduction**

Liver cysts occur in the context of autosomal dominant polycystic liver disease (ADPLD), autosomal dominant polycystic kidney disease (ADPKD), and autosomal recessive polycystic kidney disease (ARPKD). Renal cysts are the predominant feature of the latter two diseases. ADPLD has a prevalence of 1:158,000<sup>1</sup>, and is caused by mutations in the *PRKCSH*, *SEC63*, and *LRP5* genes<sup>2-5</sup>. ADPKD is caused by mutations in the polycystin genes *PKD1* and *PKD2*, which encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively. Recently, a large whole exome sequencing effort added four causative genes to this list: *ALG8*, *GANAB*, *SEC61B*, and *PKHD1*, together with the previously known explaining about 50% of the ADPLD cases<sup>6</sup>. Liver cysts are the result of loss-of-heterozygosity (LOH)<sup>7-12</sup>, in which process wild-type alleles are completely lost from diseased cells. It is experimentally shown that loss of ER-localized PRKCSH or SEC63p leads to impaired processing of crucial ciliary components, most prominently PC1<sup>21</sup>. This was bolstered by the identification of *ALG8*, *GANAB*, and *SEC61B* that act in the same ER-based protein processing pathway as PRKCSH and SEC63, and showed reduced trafficking and maturation of PC1<sup>6</sup>. However, as the function of PC1 is unknown, the relationship to cyst formation remains elusive. Mutations in *LRP5* cause

ADPLD, which gene encodes a Wnt-signaling receptor. This mutation might implicate Wnt-signaling as a process disrupted following loss of PC1 in the cystogenic process<sup>13,14</sup>. Cyst growth can be curtailed, to some extent, by somatostatin analogues such as pasireotide<sup>15-20</sup>. Recapitulating this pathological process in animal models has proven to be difficult. *PrkcsH*<sup>-/-</sup> and *Sec63*<sup>-/-</sup> mice are embryonically lethal<sup>21</sup>. Liver-specific conditional *PrkcsH*<sup>-/-</sup> and *Sec63*<sup>-/-</sup> mice do allow post-natal cyst formation. Polycystic kidney (PCK) rats and *Pkd2*<sup>WS25/-</sup> mice exist, but these are models for ARPKD and ADPKD respectively<sup>16,22</sup>, rather than for ADPLD. *In vitro* models include human polycystic cholangiocytes and 3D polycystic kidney (PCK) rat cholangiocytes<sup>16,17</sup>, but there is poor access to primary human biliary tissue and maintaining cells through passages is difficult<sup>23</sup>. For modeling purposes, animal models are still superior to *in vitro* cultures in many aspects.

New ADPLD models are urgently required to gain more insight in the pathophysiology, validate pathogenicity of new candidate genes and variants, and develop new therapeutic approaches. Zebrafish have surfaced as valuable animal models for many diseases, especially for those where mice models proved to be inadequate<sup>24-26</sup>. In an attempt to fulfil the need for new ADPLD models, zebrafish morpholino (MO) knockdown was previously performed for *sec63*, *prkcsH* and *pkd1a*<sup>27</sup>. Zebrafish morphants showed abnormal body curvature, liver cysts, and somatostatin analogue pasireotide inhibited cyst growth. Another study described *sec63*<sup>St67</sup> mutant larvae, which displayed increased hepatic ER stress and liver steatosis, and failed to survive past 14dpf<sup>28</sup>. In contrast with the *sec63* MO study, *sec63* mutants do not display body axis curvatures and do not develop liver cysts. Although this does not resolve the discrepancies between the studies, the study authors suggested that the observed pathologies of *sec63*<sup>St67</sup> mutants precede cyst formation<sup>28</sup>. Another cause might be aspecific toxic side effects of morpholinos.

Here, we employed CRISPR-Cas9 to induce mutations in the ADPLD genes of zebrafish larvae. We aimed to generate mono- and oligogenic mutations of *prkcsH*, *sec63*, and *lrp5* in zebrafish using CRISPR-Cas9 mutagenesis, to establish new models for ADPLD and study liver cyst development.

## Methods

### Fish keeping and husbandry

We confirm that the experiments were carried out in accordance with European and institutional guidelines on animal welfare (2010/63/EU). We bred Tupfel long fin (TLF) zebrafish and raised them under standard conditions<sup>40</sup>. Adult zebrafish of the TLF strain were used to obtain embryos that were raised in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, and 0.33 mM MgSO<sub>4</sub>) supplemented with 0.1% (w/v) methylene blue.

### CRISPR-Cas9

We designed two gRNAs targeting different exons of each gene using ZiFiT (table 1; <http://zifit.partners.org/ZiFiT/>), and cloned these into pDR274 expression vector (Addgene #42250)<sup>30</sup>. gRNA constructs in pDR274 were linearized using Dral restriction enzyme, and RNA was synthesized using MAXIscript® T7 *in vitro* Transcription kit (Ambion; cat#AM1312M) with the turboDNase step according to protocol. We synthesized capped Cas9n mRNA from XbaI-linearized pT3TS-nCas9n (Addgene #46757) with T3 polymerase using mMACHINE mMESSAGE Kit (Ambion; cat#AM1344) according to the manufacturer's

protocol<sup>31</sup>. This Cas9 nickase is a zebrafish codon optimized protein with a SV40 nuclear localization signal. The injection mixture contained 25ng/μL gRNA, 150ng/μL Cas9n mRNA, 0.2M KCl, 0.05% phenol red diluted in twice demineralized water. We injected a volume of 1 nL into the cell of 1-cell stage embryos with a Pneumatic PicoPump pv280 (World Precision Instruments), which was later raised to 5 nL for *lrp5* exon 2 gRNA. To generate double and triple mutants, we mixed injection mixtures of *prkcsh* exon 4, *sec63* exon 7, and *lrp5* exon 2 in a 1:1:2 ratio, and used 2 nL injection volumes. A minimum sample size of 50 larvae was used in each injection experiment.

**Table 1.** CRISPR-Cas9 target sites and oligos used for gRNA cloning.

Gene	Exon	Target site	Oligo 1	Oligo 2
<i>prkcsh</i>	Exon3 (R)	GGCTTGTTCTCCTCATAAAA	TAGGCTTGTTCTCCTCATAAAA	AAACTTTTATGAGGAGAACAAG
<i>prkcsh</i>	Exon4 (R)	GGTGGGTTTGTAAACCAGCAT	TAGGTGGGTTTGTAAACCAGCAT	AAACATGCTGGTTACAAACCCA
<i>sec63</i>	Exon7	GGTCTATGGGTTGGCATTTA	TAGGTCTATGGGTTGGCATTTA	AAACTAAATGCCAACCCATAGA
<i>sec63</i>	Exon9 (R)	GGAACCTCAATGTTGTCTGT	TAGGAACCTCAATGTTGTCTGT	AAACACAGACAACATTGAAGTT
<i>lrp5</i>	Exon2	GGTCAGTGATCTGGAGGACG	TAGGTCAGTGATCTGGAGGACG	AAACCGTCTCCAGATCACTGA
<i>lrp5</i>	Exon3	GGAGACAAACCGCATCGAAG	TAGGAGACAAACCGCATCGAAG	AAACCTTCGATGCGGTTTGTCT

## Genotyping

We isolated DNA from embryos and larvae using a custom-made genotyping buffer (40mM NaOH, 0.2mM EDTA in sterilized water). For genotype-phenotype comparisons, we separated tail from head segments, taking care to avoid affecting the liver and abdominal organs. We placed single embryos, larvae or larval tails in an appropriate volume of genotyping buffer and heated it to 95°C for 20 minutes. Suspensions were vortexed until homogenous and diluted 1:10 as DNA input for PCR. For assessing the effect of CRISPR-Cas9 injections, we amplified targeting sites using exon-specific primers (table 2). Mutagenesis was confirmed by screening for multiple sized products on a 4% agarose gel, followed by Sanger sequencing when determining gross morphology and liver phenotype.

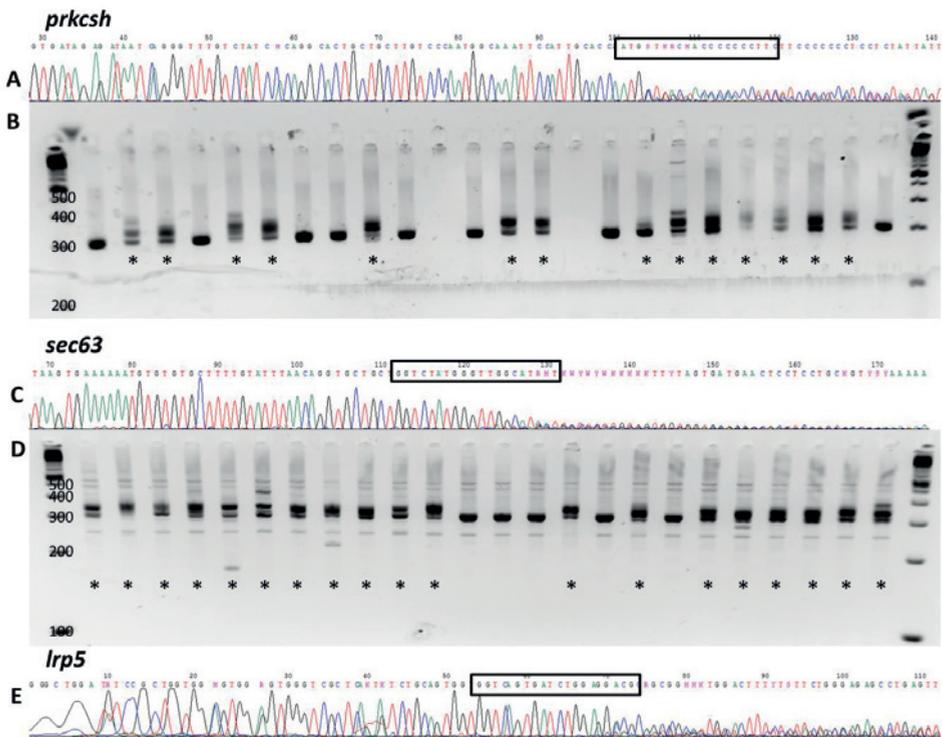
**Table 2.** *Prkcsh*, *Sec63*, *Lrp5* sequencing primers. Nt: nucleotides

Gene	Exon	Forward primer	Reverse primer	Product size (nt)
<i>prkcsh</i>	3	ACCACGAGGAGTACCACTGT	TCGTCTGAACCGTCTTTGCA	230
<i>prkcsh</i>	4	TCTAGAATCTTCGCCAAGA	CACATTTGGCTCCAAGTGTGT	308
<i>sec63</i>	7	GCACCGTTTTGCCTGTGATT	ACATAAGCAGCAATAGTGAGC T	304
<i>sec63</i>	9	TCCTGATCAACACAACACAGC T	TTCCAGCTCCGAATCAAC	380
<i>lrp5</i>	2	CCTTTGCTGCTTTTCGCCAA	TCTGTTTGATGGCCTCTCG	172
<i>lrp5</i>	3	TTGCAGCTATGAAGGAGGCC	AGGGTTTAGTGCAATCGCT	220

## Macroscopic phenotype, liver and pronephron histological analysis

At desired stages, we made photographs of gross morphology of individual, live larvae under a Leica MZFLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany). After tail removal for genotyping, we fixed larvae overnight in 4% formalin. We subsequently placed 10-40 larvae or larval head regions on a 1% agarose mold intended for paraffin embedding<sup>41</sup>. Sizes of wells in the agarose mold were adapted to 2.5 mm for larval head regions. Fish were dehydrated in a series of ethanol steps (50%, 75%, 85%, and 95%) and xylene and embedded in paraffin. Following paraffin embedding, we made sections and stained them using H&E. Slides were scanned using a Panoramic scanner 250 (3D Histech, Budapest, Hungary) and independently assessed by the study authors ESW and SABH.

## Results



**Figure 1.** Effective CRISPR-Cas9-based genome editing of ADPLD genes in zebrafish.

**A:** Sequence traces of *prkcsH* surrounding the gRNA target site of DNA from a pool of embryos, mutagenized by a CRISPR-Cas9 construct targeting *prkcsH* exon 4. The chromatogram indicates different mutations occurring within the target site.

Black circle: gRNA target site on forward Sanger sequence.

**B:** Assessment of genome editing by *prkcsH* exon 4 gRNA in individual embryos as determined by heteroduplex analysis of PCR amplified DNA on electrophoresis gels. In case of a successful amplification, a high frequency of heteroduplex occurrence is seen (indicating successful mutagenesis).

\*: Incorporated mutation as detected by double/multiple bands upon agarose gel electrophoresis.

**C:** Sanger sequences of gRNA target site of DNA from pool of embryos, mutagenized by CRISPR-Cas9 construct targeting *sec63* exon 7. The chromatogram indicates different mutations occurring within the target site.

Black circle: gRNA target site on forward Sanger sequence.

**D:** Assessment of genome editing by *sec63* exon 7 gRNA in individual embryos as determined by heteroduplex analysis of PCR amplified DNA on electrophoresis gels. In case of a successful amplification, a high frequency of heteroduplex occurrence is seen (indicating successful mutagenesis).

\*: Incorporated mutation as detected by double/multiple bands upon agarose gel electrophoresis.

**E:** Sanger sequencing confirmed only infrequent mutations in *lrp5* exon 2 in DNA from a pool of embryos injected with a high volume (5nl) of injection mixture.

### **Efficient mutagenesis of ADPLD genes leads to lethality of zebrafish larvae**

To induce mutations in the genes underlying ADPLD, we set out to generate zebrafish mutants for *prkcsh*, *sec63* and *lrp5*. We injected *Cas9* mRNA along with gene specific gRNAs into 1-cell stage embryos. We designed two gRNAs per target gene, of which only the gRNAs targeting *prkcsh* exon 4 and *sec63* exon 7 were able to generate indels with a high frequency (>75%), as determined by heteroduplex analysis of PCR amplified DNA on electrophoresis gels (Figure 1, Figure S1). For *lrp5*, we could not induce any mutations under normal injection conditions. As we initially set out to establish stable mutant lines, we increased the dose of CRISPR-Cas9 mixture from 1 nL to 5 nL, in an attempt to obtain larvae carrying *lrp5* mutations. This indeed resulted in larvae carrying mutation, but only sporadically.

Only ~20% of *prkcsh* gRNA, ~23% of *sec63* gRNA and ~12% *lrp5* gRNA injected fish hatched independently, in comparison to ~53% for (uninjected) wildtype fish on average. Only few of the *prkcsh* and *sec63* gRNA injected larvae (total injected n=50-100 in two biological replicates) grew to adulthood. While mutations were frequently found in injected larvae, genetic analysis of DNA from tail fins did not reveal mutations in any of the surviving adults. Likely, mutations in these genes are not compatible with complete development. Indeed, mutations were commonly found in those larvae that died during development. For *lrp5*, only fish with mosaic in frame indels hatched and grew to maturity. We observed up to three different mutant alleles in the offspring of a single couple/fish, underlining the mosaic state of the injected generation. These recurrent in-frame mutations lead to replacement of an important peptide sequence (figure 2), but left the remaining protein intact. The region is conserved across species, indicating abnormalities are likely to have deleterious effects (figure 2).

Remaining gRNA constructs proved ineffective in inducing mutations (Figure S1). For *lrp5* exon 3 gRNA, an unexpected SNP was present at the target site (c.403A>G) that was not present in the zebrafish reference DNA, likely preventing mutagenesis. Mutagenesis was therefore possible with 3 out of 6 gRNAs, and 2 of these had high efficiency in biallelic disruption.

### Wildtype sequence

-S- -D- -L- -E- -D- -A- -A-  
CAG GAT CTG GAG GAC GCG GCC

### Mutant allele 1

-S- -D- --- -H- --- -A- -A-  
CAG GAT C-- --- -AC GCG GCC

### Mutant allele 2

-S- -D- -L- --- -E- --- -A-  
CAG GAT CTG GA- --- --G GCC

### Mutant allele 3

-S- -D- -L- -E- -W- -S- -V- -I- -H- -A- -A-  
CAG GAT CTG GAG TGG TCA GTG ATC CAC GCG GCC

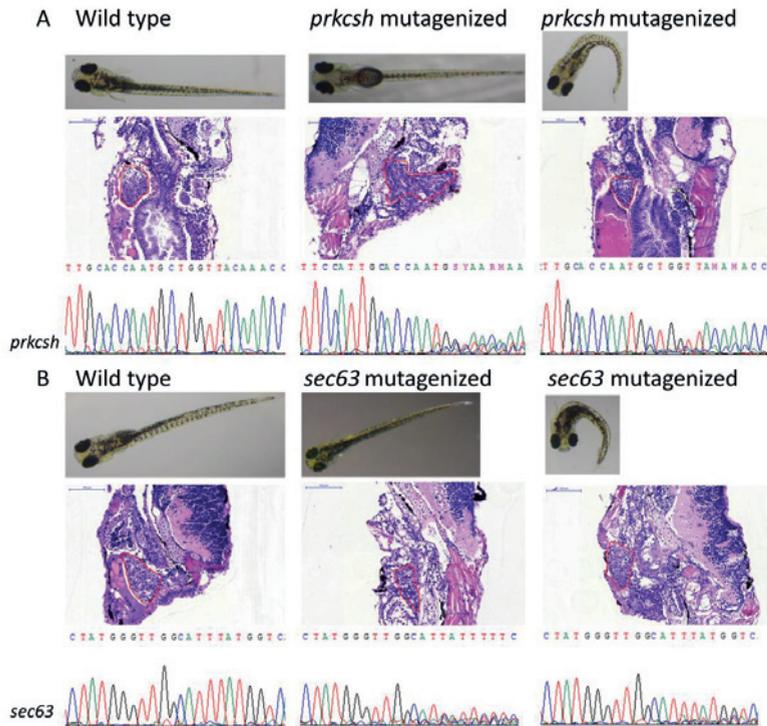
Human	S	G	L	E	D	A	A
Chimp	S	G	L	E	D	A	A
Olive baboon	S	G	L	E	D	A	A
Rat	S	G	L	E	D	A	A
Mouse	S	G	L	E	D	A	A
Dog	S	G	L	E	D	A	A
Horse	S	G	L	E	D	A	A
Chicken	S	G	L	E	D	A	A
Frog	S	G	L	E	D	A	A
Tetraodon	S	D	L	V	D	A	A
Zebrafish	S	D	L	E	D	A	A
Fruitfly	R	D	L	A	E	A	M

**Figure 2.** Three in frame deletions/insertions observed after injection of a Cas9-RNP mixture targeting *lrp5* exon 2.

inset: The in frame deletions/insertions lead to replacement of one, two or three amino acids in a region that is conserved across species. Red boxes indicate the deleted regions.

Only ~20% of *Prkcsh* gRNA, ~23% of *Sec63* gRNA and ~12% *Lrp5* gRNA fish hatched, in comparison to ~53% for wildtype fish on average. Homozygous mutations in all three genes affected early development. Of 50-100 *Prkcsh* and *Sec63* gRNA injected larvae in two experiments each, only fish without mutations grew to maturity. Mutations were only present in larvae that died before 14 days. For *Lrp5* injections, only fish with mosaic in frame insertions/deletions hatched and grew to maturity to pass along germline mutagenized alleles. These recurrent in frame mutations lead to replacement of an important peptide sequence (figure 2), but left the remaining protein intact. The region is conserved across species, indicating abnormalities are likely to have deleterious effects (figure 2).

The *Lrp5* mutant fish were capable of germline transmission of affected alleles. By outcrossing, we observed both WT and heterozygous F1 fish as offspring. Offspring from a single couple inherited wild type alleles, and up to three different mutated alleles, which further indicated the mosaic state of mutant parents. Concluding, *Prkcsh* and *Sec63* mutations lead to larval lethality, and only in frame *Lrp5* mutations are compatible with reaching adulthood.



**Figure 3.** Liver histology of CRISPR-Cas9 mutagenized zebrafish larvae. Depicted for each larva are a microscope image, a histologic section of the liver (outlined in red), and the chromatogram of the relevant target sequence. Depicted WT control larvae coincidentally had not yet inflated swim bladders.

**A:** Mutations in *prkcsh* exon 4 did not cause liver cysts as determined by histological slides of Crispants. Both the wildtype larvae as well as the *prkcsh* crispants showed identical liver histology. **B:** Mutations in *sec63* exon 7 did not cause liver cysts as determined by histological slides of Crispants. Both the wildtype larvae as well as the *prkcsh* crispants showed identical liver histology.

### Cas9-RNP injected somatic mutant larvae do not develop liver cysts

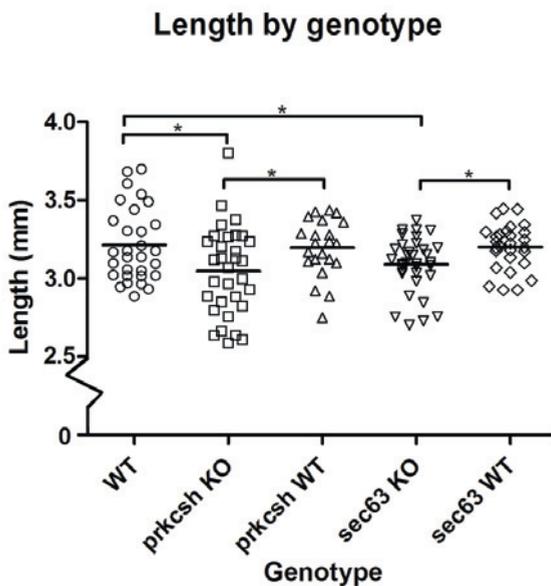
Despite the embryonic lethality of single mutants, we investigated whether Cas9-RNP-injected, somatic mutant larvae (Crispant) are valuable as a short-lived animal model. To assess the effect of mutations on the gross morphology and liver phenotype of these larvae (figure 3), we took microscopic images of whole, live larvae, and carefully removed tails for genotyping. Remaining upper body regions were embedded in paraffin for histology. As such, we could reliably determine both the genotype and phenotype of individual Cas9-RNP injected larvae. Injected larvae devoid of mutations in the tail fin served as controls.

For *prkcsh* crispants, we observed no relation of somatic mutations with fish body curvature (figure 3; figure S2). We furthermore observed major organs such as the eyes, swim bladder and fins for abnormalities, but none displayed a phenotype correlating with the presence of somatic mutations. None of the *prkcsh* crispants showed liver cysts upon histological

investigation.

Like the *prkcsh* crispants, *sec63* crispants did not display defects in the body axis. There were also no gross morphological abnormalities in any of the other major organs. However, mutations of *sec63* almost completely correlated with failure to inflate swim bladders (figure S3), indicating high penetrance of this phenotype as assessed by microscopy. Histological investigation of the *sec63* crispant showed that, like in *prkcsh* crispants, liver cysts were not present.

The size of crispants was significantly decreased in comparison to wildtype fish and injected fish without detectable somatic mutations (figure 4). We found a mean length of 3.046mm (95% c.i. 2.944-3.148mm) for *prkcsh* knockouts, 3.089mm (95% c.i. 3.028-3.150mm) for *sec63* knockouts, in comparison to 3.212mm (95% c.i. 3.124-3.300mm) for wildtypes. Mean size of *lrp5* exon 2 heterozygous mutant fish was not significantly different from WT fish from the same batch (figure S4).



**Figure 4.** Length of zebrafish is decreased due to mutations in *prkcsh* and *sec63*. *Prkcsh* and *sec63* crispants showed decreased length in comparison to wildtype fish and unsuccessfully mutagenized fish injected by a Cas9-RNP mixture.

Only straight fish were included for analysis.

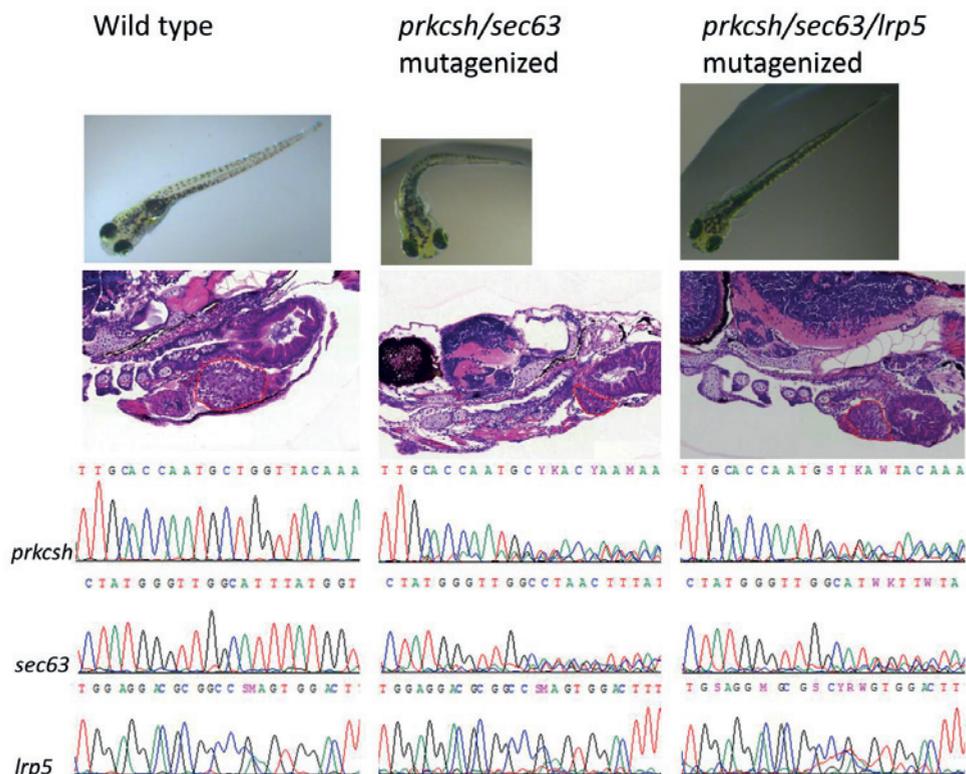
WT: uninjected WT fish; *Prkcsh* KO: crispants injected by *prkcsh* Cas9-RNP mixture with *prkcsh* mutations; *prkcsh* WT: larvae injected by *prkcsh* Cas9-RNP mixture without *prkcsh* mutations; *Sec63* KO: crispants injected by *sec63* Cas9-RNP mixture with *sec63* mutations; *sec63* WT: larvae injected by *sec63* Cas9-RNP mixture without *sec63* mutations. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .

### Cas9-RNP injected somatic multiplex mutant larvae do not develop liver cysts

To try to optimize conditions for liver cyst development we aimed to generate multiplex mutations and knock out two or more genes at the same time to develop a strong cystogenic genotype<sup>30,31</sup>. Previous experiments in mice showed a genetic interaction network exists

between the known ADPLD genes. Mutations or haploinsufficiency in more than one ADPLD gene will worsen the associated cystic phenotype<sup>21</sup>.

To this end, we injected 150 embryos with a Cas9-RNP mixture containing the gRNAs for *prkcsh*, *sec63* and *lrp5* gRNAs. The majority of the injected larvae (132) did not survive past 5dpf. However, 14 out of the 18 surviving larvae were found to carry mutations in both *prkcsh* exon 4, and *sec63* exon 7 (figure S5). Moreover, one single larva of those 14 contained mutations in all three targeted genes. This larva showed a mosaic 6-nucleotide *lrp5* deletion in the region described above. In this multiplex genome editing experiment, we never found larvae with mutations in only one of the three target genes.



**Figure 5.** Liver histology of CRISPR-Cas9 mutagenized zebrafish larvae. Depicted for each indicated larva are a microscope image, a histologic section of the liver (outlined in red), and the chromatogram of the relevant target sequence. Combined mutations in *prkcsh* exon 4, *sec63* exon 7 (and *Lrp5* exon 2) did not cause liver cysts.

Since we have shown that multiplex genome editing is possible for the genes underlying ADPLD, we continued our investigations of the phenotypes of multiplex ADPLD crispants. The phenotype of *prkcsh/sec63* double mutant fish was also normal with regard to body curvature. Similarly to *sec63* single mutations, *prkcsh/sec63* double mutations almost completely correlated with failure to inflate swim bladders (figure 5; figure S5). The double crispants did not display additional gross morphological abnormalities. Even the larva in which all three genes were successfully targeted presented no abnormal features other than

an uninflated swim bladder. Summarizing, introduction of multiplex somatic mutations in ADPLD genes did not result in the development of liver cysts.

## Discussion

We set out to develop zebrafish models for ADPLD. Here we show introduction of *prkcsh*, *sec63*, and *lrp5* mutations by CRISPR-Cas9 in zebrafish, and their associated phenotypes. Unfortunately, genetic knockout consistently caused death in our Cas9-RNP injected larvae in (late) larval stages (up to 14dpf). Here we show introduction of somatic *prkcsh*, *sec63*, and *lrp5* mutations by Cas9-RNP injection in zebrafish embryos, and their resulting phenotypes. While mutagenesis of the first two genes was very efficient, and led to somatic mutations in the majority of injected embryos, *lrp5* mutagenesis proved to be very inefficient for the two selected targets. Furthermore, *prkcsh/sec63* double crispants could easily be generated within one generation. Of the three ADPLD genes investigated here, only larvae with in-frame *Lrp5* mutations developed into adults. Homozygous loss-of-function mutations in *prkcsh*, *sec63* and *lrp5* are apparently lethal during larval development. The *prkcsh* fish did not reach maturity due to unknown reasons. As for the *sec63* Cas9-RNP injected larvae, the failure to inflate their swim bladders is at least one major reason why these fish did not survive to adulthood. As *lrp5* mutations in zebrafish were previously shown to result in severe developmental defects<sup>32,33</sup>, the in-frame *Lrp5* mutations identified in adult Cas9-RNP inject fish likely do not result in complete loss of function. Alternatively, the mosaic nature of the introduced somatic mutations could also leave sufficient cells with intact *Lrp5* for normal development. Both *prkcsh* and *sec63* fish displayed a failure to thrive as judged by total body length at 5dpf. Due to the aforementioned developmental problems of mutants, we adapted our approach to a gene-targeting assay, in which we directly analyzed the phenotype of larvae injected with highly efficient Cas9-RNP complexes targeting *sec63* and *prkcsh*. As mentioned before, these Cas9-RNP-injected, somatic mutant larvae are also known as crispants. Several studies have previously shown that this approach can result in sufficient cells with somatic mutations to mimic the effect of homozygous mutations<sup>34-37</sup>. For this purpose, we established a reliable method to concurrently determine both the genotype and phenotype of individual crispants.

With this strategy, *prkcsh*, *sec63* and double crispants did not show polycystic livers on histologic sections. Likely, as larvae developed from Cas9-RNP-injected eggs, neither *Prkcsh* nor *Sec63* protein seems to severely influence embryonic development. The proteins appear to have a detrimental effect on larval development (in case of *sec63* crispants: such as swim-bladder inflation).

Our data are consistent with three of the four previously described experiments for ADPLD genes in zebrafish, as described below. The phenotype of our *sec63* crispant larvae is in line with that previously reported for homozygous *sec63<sup>st67/st67</sup>* mutation fish<sup>28</sup>. *St67* fish fail to inflate swim bladders and show decreased total body length, which we confirmed in our experiments. The *st67* allele is the result of chemical mutagenesis. As such, *sec63<sup>st67/st67</sup>* fish may carry additional mutations in the genome. The fact that we find a similar phenotype in *sec63* crispants larvae indicates that the failure to inflate swim bladders is indeed the result of loss of *Sec63* function. It furthermore shows that our method of transient gene-targeting with CRISPR-Cas9 injection and subsequent phenotypic analysis of individual fish shortly after injection is reliable. Similar to the *sec63<sup>st67/st67</sup>* larvae, our crispants did not show liver cysts during early larval development as a phenotypic consequence of the mutations. Our results are also consistent with experimental data that find MOs against *lrp5* paralogue

*lrp6* are lethal when knockdown is complete<sup>32</sup>. Only careful titration of the MO to lead to incomplete knockdown resulted in live fish.

Similarly, transient CRISPR-Cas9 mediated gene editing of *lrp5* in zebrafish was also previously described to result in developmental defects of skeletal and neural tissue<sup>33</sup>. The study authors did not determine mutagenesis frequency, type of mutations, or the presence of mutations in the fish whose phenotype they described. They also did not describe the hepatic phenotype. Our approach is more reliable since we, in contrast to the aforementioned study, determined the presence of somatic mutations in individual larvae prior to phenotypic analysis.

The lack of liver cysts in our experiments conflict with a morpholino (MO) knockdown study for the ADPLD genes *prkcsH*, *sec63*, and *pkd1*<sup>27</sup>. This study finds abnormal body curvature and liver cysts in the morphants. Discrepancies between phenotypes of zebrafish knock-outs and MO knockdowns of the same gene could be the result of off-target effects and MO toxicity, but also from the knockdown of maternally contributed mRNA with translation blocking MOs. Several phenotypes, such as body axis curvature, pericardial edema and renal cyst formation can also result from off-target effects or MO toxicity. Although liver cysts have not been described before to result from off-target effects or MO toxicity, it has to the best of our knowledge never been investigated, as liver cyst formation can only be determined with histological analysis. As proper controls to establish knockdown specificity were not described in the aforementioned ADPLD study, it cannot be excluded that the liver cysts result from unwanted MO effects. Their finding that liver cyst formation can be attenuated by treatment with pasireotide; a drug prescribed to curtail cyst growth does support their description of liver cysts in these morphants however. Although differential effects of MOs and CRISPR-Cas9 knockdown due to different compensatory mechanisms cannot be excluded, it is likely that limitations in the experimental design underlie the observed differences in phenotype.

It was previously shown in mouse models that knockout of multiple polycystic liver genes speeds up the development of liver cysts<sup>21</sup>. In an attempt to create a genetic environment in favor of early liver cyst development, we performed multiplex genome editing, targeting *prkcsH*, *sec63* and *lrp5*. The genetic results were similar to those previously described for multiplex genome editing<sup>31</sup>. In the cited study, zebrafish with mutations in up to five genetic loci were developed. The generation of double mutants and even a triple mutant in our study further confirms their reported results.

We considered the possibility that the genetic mosaicism observed in the crispants could confound results, yet found this to be unlikely due to the similarity of the *sec63*<sup>st67/st67</sup> mutant to our *sec63* crispants<sup>28</sup>. Additionally, several recent studies found a consistent phenotype of its crispants with data from other sources like morphants and disease-specific human cells<sup>34-37</sup>.

It remains difficult to explain the lack of cyst formation in zebrafish after mutagenesis of the genes underlying human liver cyst development. One probable cause might be that the cysts need more time to develop, as these fish all died before reaching adulthood. Another possible cause are potential differences underlying biliary tract development in humans and zebrafish<sup>38</sup>, for example in ciliogenesis. This might need to be addressed in future studies.

We suspect that more time is needed for cyst development in zebrafish with mutations in ADPLD genes. However, mutations in these genes effect survival of the fish, making it

impossible to study their effects on liver morphology and function. Therefore, we suggest liver-specific and / or conditional knockout fish should be the next step in this line of research. This approach has already been taken to develop liver cysts by conditional liver-specific *Prkcsh* and *Sec63* knockout in mice<sup>21</sup>. The gRNAs for *sec63* and *prkcsh*, described in this paper, could be expressed in concordance with Cas9 under transcriptional control of a liver-specific promotor, such as *fabp10*<sup>39</sup>. With this method, we can abolish the lethal effects that loss of function of the ADPLD genes has on other tissues than the liver. Such an elegant model can make a valuable contribution to our understanding of ADPLD pathophysiology, and might be relevant to more dominant disorders with loss-of-heterozygosity as a mechanism of disease.

Concluding, Cas9-RNP mediated mutagenesis of *prkcsh*, *sec63* and *lrp5* in zebrafish has a significant effect on zebrafish development. As a result, adult fish carrying nonsense mutations in these genes could not be obtained. Analyses of the Cas9-RNP injected larvae with somatic mutations in these genes revealed consistent absence of liver cysts. It appears that the genetic mechanism of ADPLD, loss of heterozygosity (LOH), cannot be modeled in zebrafish by means of conventional mutants, or the Cas9-RNP injected somatic mutants called crispants. Therefore, we recommend a more elegant approach, using e.g. tissue specific knockouts, for all genetic diseases that are the result LOH.

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#### **Competing Financial Interests**

The authors report no conflicts of interest.

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## Chapter 7

Building pancreatic organoids to aid drug development

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comment on:

gutjnl-2016-312423 "Human pluripotent stem cell-derived acinar/ductal organoids generate human pancreas upon orthotopic transplantation and allow disease modelling"

Much of our understanding of human diseases comes from the study of model systems such as cell lines. Cell lines derive from cells that have obtained the property to proliferate indefinitely, often by immortalization or isolation from cancerous tissue. They have the great advantage that they are easy to work with and can be kept in culture almost endlessly. The disadvantage is that they have lost the genetic signature of healthy primary cells, thwarting the interpretation of test results. This has led to the search for human cell systems that accurately recapitulate healthy or disease human primary tissues. Advances in stem cell technology have made it possible to create, maintain and expand induced or adult stem cells while they retain multi-lineage potential. This has led to the development of so-called organoids, which is defined as a 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells or induced pluripotent stem cells (iPSCs). Organoids are capable of self-renewal and self-organization into multiple types of differentiated cells, while maintaining the phenotype of the original tissue. In view of these advantageous properties organoids have been generated for many of the gastrointestinal tissues, particularly colon and liver but also stomach [1, 2]. Pancreatic organoids have gone through a long developmental phase, and have recently matured to their first practical application. Starting with the discovery that pinpointed the lack of insulin as a cause for diabetes in 1920s, scientists attempted to use artificial pancreatic tissue as a path towards a cure. As of 2005, scientists turned to iPSCs to develop *in vitro* pancreatic cells. Taking a long route from definite endoderm specification [3], towards foregut endoderm and pancreatic endoderm specification [4], and finally ending with development of pancreatic  $\beta$ -cells [5, 6], we can model pancreatic development from start to finish. One man's particular crusade against the type I diabetes of his children, culminated in the production of artificial human glucose-sensitive, insulin-secreting beta cells in a petri dish [6]. Testament to this scientific tour-de-force is the addition of alpha and delta cells to the system. Despite this remarkable progress, development of iPSC-derived pancreatic cells of acinar and ductal lineage have long remained elusive. In 2015, scientists from the group of Hans Clevers in the Netherlands established conditions for the culture of human adult pancreatic stem cells [7], following their culturing recipe of human adult intestinal stem cells [1]. Subsequent experimentation led to the mastering of the conditions for 3D culture of pancreatic ductal and acinar cells from iPSCs, opening up a wealth of possibilities to model hereditary diseases of the (exocrine) pancreas [8]. One of the most common inherited diseases of the pancreas is cystic fibrosis (CF). An important phenotypical expression of CF is exocrine pancreatic failure which follows the more common pulmonary symptoms. CF is an autosomal recessive disease caused by mutations in the *cystic fibrosis transmembrane conductance regulator (CFTR)*. This gene encodes a chloride channel that is vital for electrolyte and fluid secretion of bronchiolar and ductal epithelia. Defective CFTR results in decreased secretion of chloride and increased reabsorption of sodium and water across epithelial cells. This increases viscosity and reduces clearance of secretions from the respiratory tract, pancreas, GI tract, and sweat glands. The central pathology of CF is CFTR dysfunction and fortunately there are many CFTR modifiers in clinical development. These agents activate or correct CFTR channel function. In view of the large number and variable effects of underlying CFTR mutations, the prediction of a clinical response to new therapeutics in a particular patient represents a significant hurdle. This is where organoids can really make the difference. Organoids recapitulate the genotype and phenotype to the level that they are reminiscent of the tissue under study in both architecture and composition. Indeed, primary intestinal organoids from CF patients show strongly decreased swelling in comparison to those of healthy subjects when induced by forskolin. CFTR

chemical corrector VX-809 (lumacaftor) and potentiator VX-770 (ivacaftor) have been shown to restore function of these CF organoids [9]. On subsequent testing of rectum-derived organoids, *in vitro* drug responses did correlate with outcome data from clinical trials of these two drugs [10]. In this issue of Gut, Hohwieler and colleagues further advance the technology and now report a method to produce pancreatic exocrine cells from human pluripotent stem cells using a (mostly) small-molecule based protocol [11]. They use iPSC cells generated from cystic fibrosis patients to model a CFTR-like phenotype in organoids. This approach has the advantage that it better mirrors the phenotypical, ultrastructural and functional features of mature pancreatic tissue.

The authors started out by achieving high yields of PDX1-positive pancreatic endoderm through stimulation of their set of small molecules. Several iPSC lines were then coaxed into PDX1/NKX6.1-positive pancreatic progenitors (PPs) using growth factors leading to sustained exocrine and ductal marker expression. Pancreatic maturation into cyst-like pancreatic organoids (POs) was aided by placement of these PPs in Matrigel facilitating 3-dimensional development. These POs expressed specific exocrine markers such as amylase, chymotrypsin C, SOX9 and keratin-19. In addition, organoids displayed carbonic anhydrase activity and CFTR expression, while ultrastructural analysis indicated presence of microvilli, tight junctions and secretory granula characteristic of the exocrine pancreas. The authors then moved on to disease modeling. By reprogramming keratinocytes derived from hair of two CF patients, the authors developed clonal iPSC lines carrying bonafide CF mutations that lead to loss of CFTR function. They convincingly show that POs with mutant CFTR developed similarly to those with wild type CFTR. Upon transplantation into mice, both type of POs developed into cells bearing markers of ductal, acinar and beta cells. The CF phenotype became apparent because CFTR channel activators forskolin and IBMX elicited luminal swelling of POs with wild-type CFTR but not with mutant CFTR channels. These iPSC-derived POs respond to forskolin and CFTR-correctors similarly to the rectal organoids developed by Clevers. CFTR gene supplementation through chemically modified RNA also led to phenotypic rescue.

The apparent difference in organoid swelling may serve as a fine readout for the identification of CFTR enhancers. Indeed, this model can act as a humanized platform for (organ- and patient-specific) drug-screening and allow testing of a wealth of human therapeutic options, including gene therapy. As such it embodies true precision medicine, as we may expect that results from drug interventions from a patient-specific PO can be translated faithfully in that patient. More importantly, their model is a significant step closer to the relevant target tissue, while their source materials, keratinocytes, have the advantage that they can be obtained non-invasively. All in all this study adds a new model for the study of diseases of the exocrine pancreas, and provides clinicians with a non-invasively derived tool for individually predicting patient clinical response to new CF pharmaceuticals. The organoid revolution has expanded to areas outside the laboratory space and has begun to enter the arena of clinical gastroenterology. The shrewd use of organoids by Hohwieler et al. to study pancreatic disease is just one example of the wealth of possibilities that are just around the corner. It is easy to predict that organoids will help us to navigate our way through drug development in a time efficient and cost-effective way.

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# Chapter 8

## Chapter discussion

ADPLD is a disease with major parts of its pathophysiology unexplained, and its mechanisms of disease are currently under considerable scrutiny. For up to 80% of patients no genetic basis for the disease was previously found (1-3). Even with the most recently discovered genes, only 50% have a genetically pinpointed cause (4). Within families with similar mutations, large discrepancies in phenotype cannot be explained (3, 5-7). It remains unclear which pathway becomes disrupted after loss of central cystogenic component polycystin-1 (8), although the cyst gene *LRP5* might point towards Wnt signaling (2). Even where a consensus exists on primary cilia as the organelle causative of cyst development, this view is under debate. No animal model currently exists that accurately recapitulates ADPLD. Somatostatin analogues remain expensive and with only limited effects in curtailing cyst growth. Unsurprisingly, no pharmacological cure currently exists.

In this chapter, the results of this thesis' multipronged approach to tackle these issues by answering key research questions using studies at a genetic, RNA, protein, pathway, organelle, stem cell and organismal level, are recapitulated and critically discussed in view of the current knowledge of the field. In addition, an outlook to and perspectives for future research are proposed. Finally, an important overview is presented coining the opportunities for therapy, both available and under development.

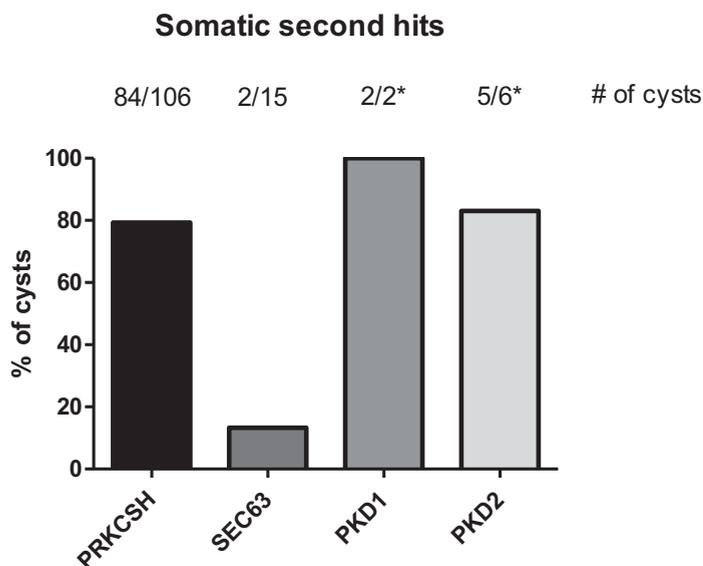
### **1.) Which underlying germline and somatic genomic abnormalities are present in ADPLD? (chapters 3, 4)**

We isolated DNA from biliary cells obtained from 46 cyst fluid and cyst epithelium samples from 23 patients, and analyzed these using high-density SNP arrays to detect LOH encompassing novel genes. In accordance with previous results, we found that in 76% (22/29) and 67% (2/3) of liver cysts of *PRKCSH* and *PKD1* mutation patients respectively, LOH is present. We additionally found LOH in one cyst of a *SEC63* mutation carrier. Abnormalities in novel regions outside of known regions underlying cyst formation were present in 12 of 23 patients. Germline DNA of three individual patients contained aberrations at four regions. One patient with a known *PRKCSH* mutation harbored a complex rearrangement at chromosome 2q13, and this led to haploinsufficiency of the *BUB1* gene. In the germline of another *PRKCSH* mutation patient trisomy X (47XXX) was present. The final germline abnormality was present in the DNA of a patient without germline mutations in any of the known genes underlying cyst development. Her DNA harbored copy number loss at chromosome 9q and an LOH region of chromosome 3p. The last 3p region overlapped with somatic LOH of another two patients.

### **Literature comparison**

Our findings are consistent with previous studies by Janssen and colleagues that show somatic second hits leading to LOH as the main genetic mechanism in cystogenesis (9-11). When combining our data with these studies, we find that somatic second hits are present in 79%, 13%, 100%, and 83% of liver cysts of patients with mutations in *PRKCSH*, *SEC63*, *PKD1*, and *PKD2*, respectively (figure 1). A small side-note should be made with regard to the occurrence of LOH in *SEC63* mutant cysts; although Janssen's study included 14 cysts, these came from only one patient. A second patient was included in our data, indicating that LOH was actually present in both analyzed patients that carry a *SEC63* germline mutation.

Moreover, the predominant mechanism of LOH consistently appears to be telomeric CNN LOH in case of *PRKCSH*. Contrarily, *PKD1*, *PKD2* and *SEC63* LOH appears to occur by interstitial deletions.



**Figure 1.** Somatic second hits are present in *PRKCSH*, *SEC63*, *PKD1*, and *PKD2* mutation cysts. Somatic second hits are defined as LOH or mutation of wild-type alleles. The numbers are calculated from data presented in chapter 3 Wills et al, Wills, Crossen et al 2016, and historic data from Janssen et al, 2011; Janssen et al, 2012; Janssen et al 2015.

\*1 ADPKD patient with unknown germline mutation excluded

One feature of ADPLD that remains unexplained is the low penetrance and mild phenotype in some family members with known mutations in genes underlying the disease (3, 5-7). Evidence of familial clustering of severe PLD suggests that genetic modifiers may play a role. An example is the hypothesis that incompletely penetrant heterozygous variants in *LRP5* may play a role in the penetrance of the PCLD phenotype of *PKHD1* carriers, as the authors noted co-occurrence of variants in both genes (4). Secondary germline abnormalities found in *PRKCSH* mutation patients may provide further insight into the cause. Our data may provide such an example. The complex aberration on chromosome 2q13 leads to haploinsufficiency of the *BUB1* gene. The protein product of this gene plays a crucial role in mitotic spindle assembly checkpoint and thereby chromosome congression (the process whereby chromosomes are properly aligned along the mitotic spindle) (12). Loss of the protein leads to aneuploidy and chromosomal instability. Loss of function or reduced expression of *BUB1* is present in human tumors such as colon, esophageal, gastric, breast cancer and melanoma (13, 14). Consistently, mice with haploinsufficiency of *BUB1* together with heterozygous mutations in genes such as *APC* and *P53* present with increased colon tumorigenesis and thymic lymphomagenesis (15). Although dependent on the genetic background, these data show that *BUB1* haploinsufficiency can drive tumor formation through tumor suppressor gene LOH. Indeed, in cysts from the patient we found CNN LOH encompassing *PRKCSH* on chromosome 19, and an interstitial chromosome 7p deletion.

Recurrent CNN LOH on chromosome 3p21.3 in germline (n=1) and somatic (n=2) DNA from ADPLD patients, may have implicated another tumor suppressor region in disease pathophysiology. The region contains a cluster of tumor suppressor genes. It is involved in early formation of tumors, including hepatocarcinomas and cholangiocarcinomas (16-18). Both *BUB1* and the 3p21.3 tumorigenesis related regions may represent genetic modifiers involved in causing differences in disease penetrance in ADPLD families.

### Strengths and limitations

A strong point of our approach is that our high-density SNP array can in principle detect larger LOH regions. However, although it did detect abnormalities for known genes *PRKCSH* and *SEC63*, novel regions were not definitively identified. Only recurrent LOH on chromosome 3p was found, the meaning of which is not directly clear.

The main limitation of our approach to study germline and somatic genomic abnormalities lies in our methodology. Whereas medium to large chromosomal abnormalities are easily detected by our employed high-density SNP array, smaller mutations are likely missed. As we cross-sectionally linked genetic regions to disease, we cannot confer causality of the mutations we found to the phenotype we are studying.

### Outlook

The new data is valuable, yet requires validation through additional studies as described hereafter. Causality may be proven by mutating the affected regions in animal models and determining the phenotypic outcome. This includes *BUB1* and recurrent LOH on chromosome 3p. Adding *BUB1* haploinsufficiency to a genetically susceptible animal model (e.g. *Pkd2*(*WS25*<sup>-/-</sup>), *Prkcsh*(+/-) or *Sec63*(+/-) mice) could elucidate whether cystogenesis occurs earlier, more frequently, or more severely. The LOH region on chromosome 3p could be studied in a similar manner.

Another main purpose of future genetic studies is to determine the genetic defects responsible for the remaining >50% of genetically unexplained APLD cases. Since family-based linkage studies and SNP array LOH region studies have yielded no new genes in the past years, several alternative methods might be applied. In ADPLD cohorts, a large panel of hypothesized candidate-genes can be screened by molecular inversion probe analysis (19, 20). Any commonly affected gene or mutation can be selected for further evaluation of its pathogenicity. Given the strongly reduced cost of whole-exome sequencing, the remaining cohort could be genetically mapped by this technique. This may unveil non-biased common genetic abnormalities that pinpoint a new disease gene. Whole-genome sequencing may next provide a final strategy to uncover new disease causing variants that were not identified by the other techniques. Non-coding regions of the genome have hardly been studied in ADPLD, and may contain explanative abnormalities.

### **2.) What are the characteristics of cultured cyst and normal biliary cells as measured by RNA expression? Are any specific pathways dysregulated in liver cyst cells as compared to normal biliary cells? (chapter 6)**

In chapter 6 we used RNA sequencing to determine the gene expression pattern of eight liver cyst cell cultures and compared that with four normal biliary cell cultures. The cell cultures expressed all genes underlying cyst formation when mutated, and many genes considered important in the process. They expressed ciliary genes and genes of the cellular machinery involved in the somatostatin response (i.e. somatostatin receptors and adenylyl

cyclases). Although gene expression profiles were heterogeneous, with overlap between diseased and normal tissue, this appeared dependent on the genetic background. We found that two *PRKCSH* mutants clustered separately from the other normal and polycystic cultures on a principal components analysis (PCA), and that a *PKD1* mutant also separates by PCA. No pathways were found to be regulated significantly different between liver cyst and control samples with gene set enrichment analysis.

### Literature comparison

The heterogeneous expression pattern appears to indicate that variability of gene expression between humans exceeds any homogeneity induced by cyst gene mutations. It also fits genetic data that ADPLD is a heterogeneous disease, wherein >50% of cases go unexplained on a genetic basis (1, 2, 4). Two cysts with a similar *PRKCSH* LOH genetic background did cluster together, indicating that the pathways disturbed in these cell lines are similar. More cysts with a similar genetic background are required for further analysis. Pathways such as Wnt, Notch, BMP, pleiotropic growth factor/receptor tyrosine kinase may reveal relevant differential expression, as has been shown in ADPKD (21, 22). Downregulation of cilium-associated genes such as HNF1B, PKHD1, IFT88 and CYS1 may be present in ADPKD (22), which is relevant to the current discussion on the role of primary cilia in cyst development. These genes were indeed expressed in the cultured liver stem cells, indicating the value of the cultures for further studies.

Our findings highlight the importance of unraveling the consequences of loss of specific genes underlying liver cysts, rather than taking the cohort as a whole. The development of these liver cyst organoids allows this. As we obtain organoids from more patients and cysts, larger cohorts with specific genetic mutations might be compared to obtain gene- or patient-specific abnormal expression patterns.

### Strengths and limitations

RNAseq allows detection of a large number of RNA transcripts at a high coverage depth to unravel disturbed gene expression pathways. Several large disadvantages exist however. The genetic heterogeneity of PLD confounded detection of a specific dysregulated pathway. We found the clearest results by comparing the *PRKCSH* mutant samples to the remaining samples, which indicated disturbance of ER related pathways. Our samples did not include biological replicates. Although we attempted to apply a workaround by comparing the PLD group to normal liver cell group, a subsequent attempt might better use biological replicates, as few dysregulated pathways were present. This might yield more robust data for the analysis of each sample. Another confounder may be the culture conditions we applied. Under these circumstances, few to none differentially regulated pathways existed in the data. Although this could be related to genetic heterogeneity, this may also indicate that both normal and PLD cells adopt a similar expression pattern in our culture media.

Alternatively, one normal liver sample displayed different gene expression in comparison to the other normal samples. Skewing by this sample of the gene expression of the normal sample group may be the cause of the low number of differentially expressed pathways between the normal and PLD samples groups.

Summarizing, although relevant expression data was generated, subsequent studies may benefit from comparing larger groups with similar genetics, while preferably using biological replicates.

## Outlook

It proved very difficult to extract pathways involved in cystogenesis due to technical issues. Three methodological adjustments may resolve the underlying technical issues. First, studying the gene expression of many organoids with a similar genetic background may yield a novel disturbed pathway. The similar genetic background is essential, in our study only these cultures clustered together. This can be done in patient cells with the frequent *PRKCSH* mutation, but can also be performed in CRISPR-Cas9 generated knockout cell lines for rarer mutations.

A second method entails growing different normal and patient stem cell cultures, and removing (or reducing) single growth factors. As the serum-less culture method contains only defined biological agents, removing each will likely uncover a pathway that is overactivated in cyst cultures in comparison to normal cells. Depending on the pathway, this may take some time; growth factors like EGF can be removed for many passages without effect on normal cell cultures. In this manner, researchers uncovered specific overactivated pathways like Wnt/APC in colon cell carcinomas (23). Later they experimentally confirmed the genetic basis of how these stem cells grow without their usual growth factors (24). Third, individual stem cell cultures can be screened with libraries of small molecules with a broad spectrum of pharmaceutical action (25, 26). Specific vulnerabilities or abnormal pathways may be uncovered and disrupted in this manner. Combining these approaches may increase the likelihood that one or several abnormal pathways in genetic subgroups of ADPLD will be uncovered.

### **3.) What is the ADPLD-associated protein interactome? (chapter 5)**

To obtain clues about any disrupted pathways in ADPLD, we used a ‘*guilt-by-association*’ approach, employing tandem affinity purification (TAP) to isolate ADPLD protein complexes, and subsequently use mass spectrometry (MS) to identify their protein composition. We elucidated an interactome surrounding the known proteins underlying hepatic cystogenesis. The *PRKCSH* protein, also known as glucosidase 2 beta (GII B), connected to glucosidase 2 alpha (GII A, protein product of *GANAB*) in all 19 TAP-MS repeats. SEC63p captured SEC61A1 and SEC61B in 4 successful experiments. As these are all known interactions in the endoplasmic reticulum, this validated the specificity of our approach. Although the functional relationship with ADPLD requires further investigation, a number of exciting interactome members was unveiled in our experiments. The discovery of *SEC61B* as an ADPLD-causing gene validates the ‘*guilt-by-association*’ approach method as a gene-discovery tool for hepatic cyst development. *SEC61B* was not known to cause ADPLD at the start of the study. We considered nucleoporin 210kDa (5/19 GII B TAP-MS), peroxisomal biogenesis factor 14 (PEX14; 5/19 GII B TAP-MS), and PEX19 (1/4 SEC63p TAP-MS) frequent interactors. We additionally found five more interacting proteins that were present in all SEC63p TAP-MS repeats: transducin (beta)-like 2 (TBL2), thymopoietin (TMPO), ADP-ribosylation factor GTPase activating protein 1 (ARFGAP1), methionyl-tRNA synthetase (MRS), heavy chain 7 of axonemal dynein (DNAH7).

### **Literature comparison**

The interactions with PEX14 and PEX19 confirmed the ER biogenesis of peroxisomal proteins (27), where especially the SEC61/SEC62/SEC63 complex appears to play a crucial role for the formation of peroxisomes. Even more interesting were interacting proteins that all three proteins underlying ADPLD had in common, potentially pointing towards the common

mechanism driving hepatic cystogenesis. Particularly frequent was filamin A, a scaffold protein that is important in cellular signaling. FLNA is a molecular scaffold involved in cell motility and signaling (28-30), and its loss leads to basal body positioning and ciliogenesis defects. We thus propose that the link of FLNA to the ADPLD proteins may (partially) explain impaired ciliogenesis observed in ADPLD gene knockouts. In summary, this interactome underlying cyst formation may provide a framework to explain direct abnormalities following loss of protein expression or disrupted protein function due to genetic mutation.

### Strengths and limitations

The tandem affinity proteomics used in our study is a powerful technique (31, 32), and essential to the success of our *guilt-by-association* approach. Indicative of this is that the gene encoding GIIA, the protein most prominently associating to GIIIB in our dataset, was recently indeed found to be mutated in ADPLD (3). Similarly, the gene encoding SEC61B, the protein associated to SEC63p in our dataset, was recently also found to be mutated in ADPLD (4). Similar to GIIIB and SEC63p defects, loss of GIIA and SEC61B leads to reduced maturation and ciliary localization of PC1 and PC2. Loss of these latter proteins putatively leads to cyst development. Further extrapolating from the data, it is possible that SEC61A1 is also involved in cystogenesis. In comparison to SEC61B, this gene is more conserved and more essential to SEC61 complex function however (4, 33). These results indicate that at least a subset of the proteins in our ADPLD-associated interactome are encoded by candidate genes for the disease, and can be included in targeted gene sets for ADPLD diagnostics, e.g. employing MIPs.

The main surprise of our affinity proteomics study to us is that it failed to detect any Wnt-signaling related partners for LRP5, or its intracellular domain. There are multiple possible explanations. First, the cell lines studies may not be suitable to detect Wnt interaction partners due to lack of expression. This seems unlikely however; as Wnt has previously been studied in HEK293T cells and assays indicated clear Wnt activity (34). Second, our method may not be sufficiently suitable to detect Wnt signaling interactors, for example due to the transient nature or low affinity of interactions between Wnt signaling components.

### Outlook

In order to optimize the dissection of the LRP5-associated interactome to include the expected Wnt signaling components, two routes may be explored. First, a chemical cross-linking step may be included in the affinity proteomics procedure to “freeze” the transient or weak interactions (35, 36). This will also allow a more structural investigation of the interaction interfaces of the identified (sub) complexes. Second, an alternative approach to the proteomics can be employed, such as yeast-two-hybrid screen of a relevant cDNA library (e.g. human cholangiocyte derived) to identify binary interaction partners for LRP5. Nonetheless, the novel interaction partner FLNA is a potentially central component of the PLD interactome, even for LRP5. One way to validate our data might be to further study the interactions by co-immunoprecipitation and co-localization on immunofluorescence. Interacting protein motifs may then be determined, mutated or removed to further assess the molecular interactions or effects of gene mutations.

#### **4.) What is the effect of mutation of genes underlying ADPLD on primary cilia and Wnt signaling? (chapter 5)**

In chapter 4 we use CRISPR-Cas9 to induce mutations in the genes underlying ADPLD. We found that we can efficiently knock out *PRKCSH*, *SEC63*, and *LRP5* in H69 cholangiocytes and HEK293T cells. In both of these cell lines, formation of primary cilia was significantly reduced by loss of *PRKCSH* or *SEC63*. In H69 cholangiocytes, Wnt signaling is additionally disrupted significantly as measured by luciferase assay and axin 2 mRNA expression. These findings indicate cilium formation requires normal *PRKCSH* and *SEC63* expression, and that Wnt signaling may similarly be affected depending on the cell type.

#### **Literature comparison**

The reduced ciliation of *PRKCSH* and *SEC63* knockout cells and the cell type-specific effects on Wnt signaling have several implications. Normal biliary ducts have cilia on virtually all cells, which organelles almost disappear in large sized liver cysts (1/200 cells) (37, 38). Functional ciliary abnormalities by loss of polycystins are therefore not the only consequence of *PRKCSH* and *SEC63* loss. Whether the structural ciliary abnormalities follow functional ciliary abnormalities, or are a direct result of losing genes involved in cystogenesis remains unclear.  $\text{Ca}^{2+}$  and cAMP signaling may influence ciliary length (39), and polycystins influence these pathways. Diminished growth of primary cilia could be related to these processes.

Reduced Wnt signaling following *PRKCSH* and *SEC63* mutation in H69 cholangiocytes is similar to that observed with *LRP5* mutations from patients (2). Surprisingly, out of the many functions contributed to GIIIB and SEC63p, this is one of the first to be cell-type specific. HEK293T cells only showed inconsistent effects of loss of the genes. It might be a bridge too far to claim this underlies the tissue-specific outcomes of loss-of-heterozygosity, but it may point in that direction.

The cause of the Wnt impairment is a second issue. Our data indicate GIIA as an interactor of the intracellular domain of LRP5. LRP5 contains at least 4 n-glycosylation sites that are known to be processed by glucosidase II. For LRP6, a homologue of LRP5, the n-glycosylation sites are necessary for normal maturation, plasma membrane localization and Wnt signaling (40). We believe that Wnt impairment following loss of *PRKCSH* may be LRP5-dependent. Whether *SEC63* is important for LRP5 membrane localization is not known, but the protein may link to Wnt signaling through nucleoredoxin according to literature (41).

The N-glycosylation of LRP5 however, that we could observe in HEK293T cells was not diminished by depletion of either *PRKCSH*/GIIIB or *SEC63*, which suggests that LRP5 is not a substrate of glucosidase II and SEC63p is not involved in the regulation of LRP5 N-glycosylation. Our findings together still strengthen the case for Wnt signaling abnormalities as a common contributing factor in hepatic liver cyst development. If this is unrelated to maturation and trafficking of polycystin-1 remains to be determined. It has been suggested that PC1 is a coreceptor for noncanonical Wnt ( $\text{Ca}^{2+}$ ) signaling, which may imply an indirect relationship between LRP5 and PC1 (4).

The fact that we determined that knock out of either *PRKCSH* or *SEC63* led to reduced levels of LRP5, while the transcript levels were slightly elevated, suggest connectivity of the ADPLD-associated pathways downstream of gene transcription.

## Strengths and limitations

We managed to study primary cilia and Wnt signaling in ADPLD gene knockouts as expected. One problem of CRISPR-Cas9 may be aspecificity of induced double-stranded breaks. Obtained results may then be related to off-target mutations. Similarly, when growing out clones from single cells, a confounder effect may be clone-specific, rather than mutation-specific. By using multiple clones for each experiment we likely circumvented this problem. Furthermore, effects on cilia and Wnt were not only observed in multiple clones, but also in both (*PRKCSH* and *SEC63*) genetic knockouts. This strengthens the validity of our observations and conclusions.

## Outlook

Further studies on the effects of *GANAB*, *ALG8*, and *SEC61B* knockout on ciliogenesis and Wnt signaling are warranted, as are the studies on the effect of *PRKCSH* and *SEC63* knockout. Some researchers suggest ciliary abnormalities are a bystander effect, rather than a causative effect in cyst development ((42, 43), see below). A primary effect of *PRKCSH* and *SEC63* knockout is reduced maturation and localization of PC1 and PC2 due to defective n-glycosylation and quality control (8). This reduced polycystin expression is likely causative for cyst development, as increasing polycystin expression in *PRKCSH* and *SEC63* knockouts could partially rescue the cyst phenotype. Reduced ciliogenesis could be a secondary effect of polycystin loss. Ciliary length depends on ciliary signaling routes such as cAMP/Ca<sup>2+</sup> (39). These signaling pathways are affected by polycystins, although possibly not the polycystins themselves, but by polycystin-like proteins in the primary cilia (42). Future research should address the effect of *PRKCSH* and *SEC63* mutation on *PKD1* and *PKD2*, as well as look at primary cilia in *PKD1* and *PKD2* knockouts. Even before we understand the precise pathogenetic basis of polycystic liver disease, it may be valuable to use pharmacological screens to detect improved ciliogenesis in PLD gene knockout cells. The impaired ciliogenesis represents a first, easily detectable abnormal readout of PLD cells.

### **5.) Do stem cells occur in liver cysts and what are their characteristics? (chapter 6)**

In chapter 6 we apply conditions for adult liver stem cell culture on cells deriving from cyst epithelium and fluid, and find development of liver cyst stem cells. To our knowledge, we were the first to culture cyst cholangiocytes from different patients efficiently and reliably. Characteristics of the cultured cholangiocytes were consistent with cyst epithelial origin. We found presence of *PRKCSH*, *SEC63*, and *PKD1* LOH in cultures from respective mutation carriers. The cyst stem cells were bipotent, and could differentiate into biliary cells and hepatocytes. In multiple cultures, expression of biliary marker keratin-19 together with primary cilia marker ARL13B was present. Culture with somatostatin analogue lanreotide reduced expansion of the cyst cultures.

Summarizing, we believe cyst stem cell culture represents a significant step forward for the ADPLD research field and may in the future be used to unravel pro-cystogenic pathways and drug modifiers.

## Literature comparison

The cultured liver cyst stem cells signify a novel opportunity for research of ADPLD. These cells possess the exact genetic background responsible for cyst development. This contrasts with previous attempts with cholangiocytes derived from induced pluripotent stem cells or

cell lines (44, 45). LOH, one of the hallmarks of cyst development (9-11, 46), is present only in these adult liver stem cells. This disease-specific genetic background increases the likelihood of detecting relevant abnormal pathways of ADPLD.

Through genome-wide RNA sequencing it became clear that gene expression depends on the genetic background. Principal component analysis indicated that *PRKCSH* and *PKD1* mutation cells cluster separately from remaining cells. A mutation-specific approach to elucidating disturbed pathways therefore may have a higher chance of yielding novel insights into ADPLD. Moreover, these cells allow nonbiased screens of small molecules or therapeutic agents (e.g. off-patent drugs) to develop new hypotheses or therapies. Especially when new therapeutics are available, patient-specific cyst cell cultures may be exploited for personalized medicine. Indeed, this has already been taken advantage of for colon carcinoma cells (25), whose organoid cultures are considered as an ex-vivo platform to personalize anti-disease treatment.

A small, but significant additional development is the use of cyst fluid cells. Stem cells that derive from free-floating cholangiocytes in fluid may grow. Fluid is routinely obtained by aspiration sclerotherapy, and other sources of floating cholangiocytes may also exist. Although cholangiocarcinomas can be notoriously difficult to detect, blood-borne metastases may be cultured using the techniques described in Chapter 6. Especially later stage cholangiocarcinomas could be targets for this, and screening any obtained cultures for drug sensitivities may benefit patient treatment. As far-fetched as this may appear, exactly this has been done already with prostate cancer metastasizing in blood (47).

### Strengths and limitations

One of the main limitations of our study described in Chapter 6 is that we did not show the presence of LGR5+ cells in biliary tissue or epithelia of liver cysts. However, we clearly show that in isolating cholangiocyte-like cells and placing them in conditions suitable for the expansion of adult liver stem cells, an LGR5+ population exists or can be induced. Their genetic background confirms the LOH hypothesis of liver cysts, and indicates a population of cyst stem cells that lose heterozygosity. It remains unclear whether bipotent LGR5+ stem cells contribute to cyst expansion, or that they are only a side phenomenon that happens to be exploitable for *in vitro* culture. Future studies may look at the presence and role of LGR5+ cyst stem cells in *in vivo* liver cysts.

### Outlook

For the cyst-derived liver stem cells there are many additional directions for future research. First and foremost is to use the new cell lines for drug screens. Multiple off-patent drug libraries (e.g. Prestwick FDA-approved drug library (48)) exist, that allow for rapid screening for effective compounds with a short time to clinical application.

A second direction is to produce primary cilia on the organoids. For this, better biliary differentiation is most likely required. The current strategy to induce differentiation into biliary cells involves removing all proliferation-stimulating factors. Although this allows for some differentiation to occur, the cells enter a quiescent state in which they degenerate within days. A differentiation growth factor cocktail is currently in development at the Hubrecht institute that allows the cells to differentiate and survive better. When this protocol is published, it will be interesting to study cilia formation in normal and cyst-derived organoids.

A third aim is using the stem cells to better compare germline and somatic mutations. A

large biobank of different cyst stem cells is under development. When sufficient budget is available, or techniques have advanced to become less costly, WES or WGS may be applied to these cyst stem cells and their corresponding germlines. A first step has been undertaken by applying MIP analysis on these stem cells.

### **6.) Can zebrafish model polycystic liver disease? (chapter 7)**

We attempted to develop polycystic liver disease zebrafish by knocking out the genes underlying ADPLD. *Prkcsh* and *Sec63* mutagenized efficiently by CRISPR-Cas9, while *Lrp5* mutants developed only infrequently with higher injection doses. We successfully generated fish with mutations in both *Prkcsh* and *Sec63*. Although larvae developed, mutants died before reaching maturity. We found that larvae with *Prkcsh* and *Sec63* mutation had a reduced length, and that *Sec63* mutants did not inflate swim bladders. Larvae developed with in-frame *Lrp5* deletions or insertions, and these grew to maturity. Compound heterozygotes for these in frame abnormalities did not display a phenotype with regard to gross morphology or length. None of the three mutants developed liver cysts, nor did *Prkcsh/Sec63* double knockouts.

### **Literature comparison**

The most significant conclusion of our zebrafish study is how discordant the phenotype of our fish is compared to a study describing morpholino knockdown fish (49). Whereas polycystic livers, curved body axis (a feature characteristic of fish with ciliopathies), and a response to pasireotide occurred in the knockdowns, none of our knockout fish presented with these abnormalities. A key oversight of this morpholino article was their description of a phenotype with a morpholino that did not actually target the *PKD1*. The larvae displayed polycystic livers, when in fact the morpholino targeted the reverse mRNA transcript of a random ion channel instead of *PKD1*.

Not all was bad when comparing to previous literature. Our *SEC63* knockout larvae displayed an absence of swim bladders, and decreased fish length. Both features are present in *SEC63* fish with homozygous *Stm67* mutation (50). Our *PRKCSH* mutants similarly displayed a decreased total body length, but without a further phenotype. Importantly, larvae co-injected with the CRISPR-Cas9 construct, but without mutation, did not display any phenotype.

In doing this, we have not only established the phenotype of ADPLD gene knockout larvae, but also a new technique for quick genotype-phenotype screens in zebrafish. Our embryos were injected at the single-cell stage, and harvested at 5dpf. The larvae generally had different mutations present in a mosaic pattern of the body, following CRISPR-Cas9 injection. Nonetheless, if mutations occurred, the phenotype was consistent with that described in an established knockout line. We believe our technique may be a valuable addition to short-term screening methods in zebrafish.

### **Strengths and limitations**

The absence of liver cysts in our zebrafish may be caused by limitations in our study design, or by differences in the physiology of *Danio rerio* in comparison to *Homo sapiens*. Due to embryonic lethality and failure to thrive of our mutagenized zebrafish larvae, we could not evaluate adult livers of knockout fish. Although generally assumed to develop during embryology, the absence of liver cysts in zebrafish during this stage could mean two things. One, zebrafish do not develop liver cysts due to known ADPLD mutations, or two, zebrafish

develop liver cysts due to known ADPLD mutations under different conditions. Precursor cells may simply require more time to develop into cysts. A crucial difference exists between our fish and the situation in patients with ADPLD. LOH is unlikely to play a role in our knockout fish, which already frequently develop compound heterozygotic mutations. If zebrafish cannot grow liver cysts, a different cholangiocyte physiology is a possible explanation. It seems that, in the current body of zebrafish literature, no cilia have ever been described on bile ducts. One review even noted this absence of evidence for the cellular structures (51). If one assumes that primary cilia play an important role in the development of cysts, although currently a disputed view, complete, physiological lack of cilia implicates lack of cysts. There can be no zebrafish cholangiociliopathy if no cilia on cholangiocytes occur in normal, healthy *Danio rerio*.

## Outlook

Although zebrafish larvae did not develop hepatic cysts within the experimental setting we applied, future studies may work around the problems observed in post-natal development of ADPLD-relevant mutants. One way to do this is by limiting the presence of mutations to the liver. Ablain and colleagues have recently made available the tools and methodology for tissue-specific CRISPR-Cas9 mutagenesis (52). The system works by cloning a plasmid with a tissue-specific promoter, a codon-optimized Cas9 enzyme, one or multiple gRNAs of choice, GFP and Tol2 gene transfer vector. For the zebrafish liver, a frequently used promoter is the FABP10 gene promoter (53). gRNAs targeting zebrafish *PRKCSH*, *SEC63* and *LRP5* are described in chapter 7, while the remaining components are available from commercial vendors. It is possible that zebrafish with this transgene in their germline may develop to maturity, and that their adult liver or mesonephron displays cysts. If not, one of the previously described hypotheses may be a cause for the lack of a phenotype.

## Future prospects

### *Hepatic cystogenesis: paradigm shift*

In late years, a shift in thinking has occurred on the relation of liver cyst development and primary cilia. This shift has major implications for the pathophysiology of the disease, which were unforeseen at the start of this thesis. Although no components of the thesis are invalidated, acquired data will have to be appreciated from a different vantage point. Analogies may be drawn to medicine, in which different fields have seen radical changes in treatment approach as research progresses. Examples include late versus early oral nutrition in acute pancreatitis patients shortly after admission (54, 55), fixed versus age-dependent reduction of FEV1/FVC < 70% as a criterion for chronic obstructive pulmonary disease (56), and step-up/pyramid approach versus a top-down/early aggressive treatment of rheumatoid arthritis (57). In each of these instances, the former approach replaced the latter, with great, irreversible changes in patient outcomes as a consequence (57). This progressive understanding is often a difficult topic. Our view of the optimal evidence-based model can shift significantly.

A similar process is occurring in the field of cystic diseases. Whereas researchers for years, up to and including the start of this thesis, had considered cysts to be caused primarily by ciliary dysfunction, the tables are turning. More researchers have been calling to consider ciliopathy a side-effect of the primary cause of cyst development, rather than the primary cause itself. A major paradigm shift in the field of liver cysts, and progressive understanding indeed, considering the many studies published from the initial view. In the next paragraph the pros and cons of the “ciliopathy first” approach are reviewed in light of current evidence.

### **Hepatic cystogenesis: primarily primary cilia?**

Important questions regarding polycystins remain unanswered to date: what function leading to cystogenesis is lost in PKD, and, despite their undisputed ciliary localization, is the cilium also the primary cellular locale of polycystin function (table 1)? Some studies suggest that the polycystins perform their primary function in the plasma membrane or ER (58-60), although this is hard to prove in absence of more functional insights.

Whichever function they perform, their relation to ADPLD proteins G1IB and SEC63p seems clear following a publication by Fedeles and Somlo in 2011 (8, 61). G1IB and SEC63p are important for biogenesis of both polycystins, and the amount of lost polycystin-1 leads to a dose-dependent effect on cyst formation.

It is clear that primary cilia are affected by loss of polycystins (37, 38), but unclear how this relates to cystogenesis. Somlo’s research group found that knocking out crucial ciliary components IFT20 and KIF3A in combination with PKD1 or PKD2 leads to decreased cystogenesis in a mouse model of ADPKD (62). These results suggest that the presence of primary cilia in absence of polycystins is worse than the absence of primary cilia altogether with regard to cyst development. Removing primary cilia actually ameliorates the polycystin-absence-induced cystogenesis.

Somlo continued to ascribe an important role for primary cilia in cyst development. In a subsequent paper he provided five points of evidence for his view (63): First, both polycystins are expressed in the ciliary membrane; Second, the majority of gene products associated with fibrocystic disease are expressed in the cilia-basal body complex; Third, a forward genetic screen in zebrafish based on cystic pronephron phenotype identified a

number of cilia-related proteins, including PC2; Fourth, disruption of cilia alone is sufficient to develop cysts in prospective testing; and Fifth, generally considered the strongest evidence, fibrocystic disease-associated gene products, including PC2, are associated with left-right axis defects in animal models. To these points, it may be added that there are suggestions that the polycystins function as ciliary sensors of cell injury (64), inducing proliferation upon damage.

Opponents also present a large body of evidence in their favor. Most notably, there is no direct evidence for the exact mechanism of polycystin action in the cilium leading to cystogenesis (42). Despite the many gene products associated with fibrocystic disease in the cilia-basal body complex, this may be related to effects outside of the primary cilium. For example, IFT mutants appear to also affect the microtubule cytoskeleton outside of primary cilia (65). Left-right axis defects may also occur in the context of non-ciliary disorders due to aberrant FGF or Notch signaling (66, 67) (although downstream these signaling abnormalities may still cause ciliopathy). Additionally, a paper by Clapham and colleagues in *Nature* described no clear effect on calcium signaling by the regular polycystins in the primary cilium (42). Instead, PKD1L1 and PKD1L2, 2 PKD-like proteins, form ciliary ion channels responsible for calcium signaling in the primary cilium. The PKD1L1-PKD2L1 complex controls ciliary calcium concentration and modifies cilia-dependent signaling pathways such as SMO-activated GLI2 translocation and GLI1 expression (42). Further upsetting the classic view on primary cilia, in March 2016 Clapham and colleagues published a follow-up paper in *Nature* that concludes that primary cilia are not calcium-responsive mechanosensors following extensive testing (43). Not even nodal primary cilia responsible for left-right axis determination showed mechanosensitive calcium responses. Any changes in calcium signaling always started from cytoplasmic sources during their high-speed imaging tests used to detect calcium flow. Clapham and colleagues conclude that situs inversus and cystic disease caused by loss of polycystins are not due to loss of mechanically induced cilia-initiated calcium signaling.

In light of all these data, it remains possible that the effect of polycystin loss on primary cilia is a bystander effect rather than a primary or causative effect of cystogenesis.

**Table 1.** Arguments favoring and discrediting the role of primary cilia in cystogenesis

<b>Pro</b>	<b>Contra</b>
PC1 and PC2 localized in the primary cilium	PC1 and PC2 also localized in the plasma membrane and ER membrane
Disruption of cilia alone is sufficient to develop cysts in prospective testing	No direct evidence for polycystin related ciliary effect leading to cystogenesis
Majority of gene products associated with fibrocystic disease are expressed in the cilia-basal body complex	Many cilia-related gene products may have a function outside of the primary cilium
Ciliopathy, including PC2 mutation, leads to left-right axis defects	Left-right axis defects can also occur in the context of non-cilia disorders due to aberrant FGF or Notch signaling
Ciliary polycystins putatively function as sensors of cell injury	Ciliary calcium signaling is not caused by the polycystin complex ion channel
	Cilia-initiated calcium signaling does not occur

### ***Curing polycystic liver disease: state-of-the-art and future perspectives***

Due to the rapid development of new techniques, a small section of this thesis should be devoted to thoughts on how to cure this disease. Surprisingly, many of the tools to cure or eradicate the disease may already be available. Although some are not ready (yet?), because they are in the early stages of development, but with time and refinement, it is likely that application in clinical practice will become possible. This chapter will keep to the theme of going from bench to bedside and will start at the genetic level, going up to the organism.

#### **Genetic selection or editing**

It really is a brave new world with regard to genome editing techniques, such as pre-implantation genetic diagnostics (PGD) or genome editing through CRISPR-Cas9 or related methods. PGD is a technique which selects embryos following genetic analysis on one of eight blastomere cells on day 3 of *in vitro* fertilized eggs (single blastomere technique) (68, 69). Alternatively, a blastocyst biopsy targeting the trophectoderm on day 5 of development can yield more DNA without sacrificing any part of the embryo. In combination with cryopreservation, this allows more time for genetic testing. In other words, PGD can prevent hereditary disease (see table 2). PGD can cost between \$11,500 and 18,500 (70), while some technical challenges exist in the form of IVF side effects (multiple pregnancy risk, ovarian hyperstimulation syndrome) and risks of misdiagnosis. CRISPR-Cas9, has already been used to experimentally treat beta-thalassemia in non-viable human embryos (71). It will become possible to choose or edit genes in the germline, but we run a great risk of losing (parts of) our humanity, or coming down a slippery slope. With suitable knowledge and adequate refinement, off-target or unintended harmful effects will most likely eventually be excluded. The question then is which conditions to treat. Thus far, only severe hereditary diseases qualify for PGD, whereas less severe genetic diseases are initially excluded. PGD has potential social benefits in reducing the overall burden of disease, as well preventing a lifetime cost of chronic disease. Once the technique has been established and becomes more acceptable, it is likely that less severe disease will qualify for treatment. This represents a slippery slope as we need to start to define which inherited diseases are

acceptably prevented and which need to be avoided. This is an ethical tight rope. Besides this point, embryo selection may devalue certain lives. Clearly, great care is warranted when choosing which hereditary afflictions we intend to treat.

On the Dutch website for pre-implantation genetic diagnostics couples with a greatly increased risk of having a child with a severe genetic condition, or increased risk of loss of pregnancy due to a chromosomal abnormality can apply (72). A list for previously treated diseases exists:

**Table 2.** Diseases treated by PGD in the Netherlands, based on data from <http://www.pgdnederland.nl/>

Disease	Primary consequence
Huntington’s disease	Neurodegeneration: mental decline, behavioral symptoms, muscle discoordination
Hereditary breast-ovarian cancer	Hereditary breast-ovarian cancer
Myotonic dystrophy type I	Muscle wasting, cataracts, myotonia
Familial adenomatous polyposis coli (FAP)	Numerous adenomatous polyps, increased (colon)cancer risk
Marfan’s syndrome	Connective tissue disorder: cardiac complications
Neurofibromatosis I	Neural tumors
Cystic fibrosis	Pulmonary failure due to repeated infections
Spinal muscular atrophias	Muscle wasting
Fragile X syndrome	Autism, intellectual disability
Hemophilia A/B	Impaired hemostasis
Duchenne muscular dystrophy	Muscle wasting
Chromosomal abnormalities	Increased risk of pregnancy loss

Some of these diseases (i.e. hereditary breast-ovarian cancer, FAP, cystic fibrosis) have much in common with ADPKD. For example, they are frequently (autosomal dominant) inherited disease, causing loss of function of specific organs, which severely impairs health at a relatively young age. In the case of ADPKD, renal failure may occur at a relatively early age, while glomerular filtration rates progressively decline even earlier. Besides other symptoms due to cyst mass effects, this by itself may be enough to warrant PGD, especially in families with a generally severe phenotype. Families occur with an early-manifesting phenotype (73), and the total number of early-onset ADPKD patients might equal the number of ARPKD patients in pediatric nephrology (74). Indeed, the first healthy twins born from pre-implantation genetic diagnosis for ADPKD has been described in 2004 (75), and the KDIGO group (Kidney disease: improving global outcomes) has placed PGD on the research agenda (76). They recognize a need to examine the performance of the blastomere and trophectoderm biopsy techniques. Nonetheless, the advice from KDIGO is to discuss PGD as a reproductive choice with patients with ADPKD.

ADPLD should be treated as a different entity. While severe ADPLD can have a large impact on quality of life, liver function does not get threatened, nor is the disease assumed to progress to cancer. PGD should be considered on a case-by-case basis, if at all. For example, if multiple members within a family require surgical intervention such as liver transplantation, an assessment of the ethicality of PGD may be wise. It is possible that with time ADPLD will become a suitable indication for genomic editing and PGD.

Concluding, even though genomic editing of the human germline may still lie in the future, PGD is currently available and applied to severe hereditary conditions. ADPKD is a suitable candidate condition for the treatment, while ADPLD may apply on a case-by-case basis. Meanwhile, it may be sensible to develop medical options for treatment.

### **Pharmacological treatment**

Multiple routes can lead to new pharmacological treatment choices for ADPLD. First, large groups of off-patent, generic drugs can be tested in repurposing screens. Second, small molecules with different pharmacological properties can be evaluated for effects on cyst cells. Third, drugs with known effects may be chosen based on properties expected to be effective in inhibiting cysts. Fourth, drugs may be rationally designed to obtain wanted effect. Or fifth, a combination of these options. Of course, somatostatin analogues have a proven effect on curtailing cyst growth, but even these are not ideal due to cost (\$7000-11000/month (77), and currently ~€1000/month in the Netherlands) and limited benefits. There is an unmet need for new drugs. In this chapter, we evaluate which drugs can be rationally picked or may be designed based on literature to inhibit cyst growth (table 3). A relevant screening platform will be required.

### **Pharmacological treatment: RNA level**

Mutations in *PRKCSH*, the gene most frequently mutated in ADPLD, are often related to splice-site mutations. For example, in the Netherlands, the *PRKCSH* c.292+1G>C has a high prevalence (1). Targeting incorrect splicing in ADPLD appears to be a fitting strategy. Recently, scientists have developed splice-switching oligonucleotides (SSOs). These chemically modified antisense oligonucleotides are capable of redirecting splice site selection, and have done so since first attempted to restore correct splicing in beta-thalassemia (78). Researchers published one of the early successes of the technique in 2007, when exon-skipping by SSO was successful for restoring dystrophin to 3-12% of normal levels in a clinical trial for Duchenne's muscular dystrophy (79). In contrast to other antisense oligonucleotides, the SSOs are modified in a manner that causes them to not be degraded by RNase H. Chemical modifications allow SSO with activity localized to specific organs such as the liver (80). Although a mutation such as *PRKCSH* c.292+1G>C cannot be removed, the splice site at the end of its affected exon can be skipped. This would cause a truncated GIB protein to occur, which has potentially less harmful effects than a very early stop codon. A strategy that is worth exploring.

### **Pharmacological treatment: targeting translation**

Although less frequent than splice-site mutations in ADPLD, nonsense mutations also occur within the spectrum of mutational causes. Recently, scientists have developed stopcodon read-through drugs. These drugs suppress translation termination at premature stopcodons by favoring codon binding by aminoacyl-tRNA incorporation/elongation over binding by termination factors (81). Aminoglycosides such as G418 (geneticin) and gentamicin have been studied since the late 90s for use as stopcodon read-through drugs. Historically, their main application was as antibiotic agents, where they prevent incorporation of amino acids in bacterial translation by binding the 30s ribosomal subunit. Although the aminoglycosides bind far more weakly to eukaryote ribosomes, read-through effects clearly occur in multiple studies (reviewed in (82)). Unfortunately, due to side effects such as nephro- and ototoxicity,

aminoglycosides are not viable long-term treatment options.

A recently developed read-through drug by the name of ataluren has proven effectivity with a benign safety profile in clinical testing, which was subsequently launched in Europe for the treatment of cystic fibrosis (83). The drug is an oxadiazole with few off-target side effects and oral bioavailability. Its effectivity as a read-through drug is unsure in view of the data of several studies however (81), but a larger body of evidence has shown effectivity, including aforementioned clinical trials. Testing read-through drugs that affect the nonsense mutations in ADPLD may be an effective strategy.

### **Pharmacological treatment: targeting posttranslational quality control**

A prime function of GIB and SEC63p is their function in quality control. When the underlying genes are mutated, proteins undergo less rigorous quality control. Protein misfolding, followed by degradation in the proteasome is a consequence. Fedeles and colleagues proposed making use of this property of mutated cyst cells by applying proteasome inhibitors (8). These drugs can increase the quantity of misfolded proteins that are not degraded, which leads to an increased cellular unfolded protein burden to toxic levels. In animal models this increased the rate of cyst cell apoptosis. Secondly, they observed an increase in the steady-state levels of Pkd1 by MG132 treatment, which may (putatively) prevent or ameliorate cellular dysfunction due to lack of Pkd1. In *PrkcsH<sup>flox/flox</sup>; Ksp-Cre* mice, i.v. carfilzomib (5mg/kg) with weekly carfilzomib for 6 weeks had a profound effect on cyst progression, without adverse effects. An independent study observed favorable effects of proteasome inhibitor MG115 in another disease model of ADPLD (64). It may be valuable to screen proteasome inhibitor effects on human cell lines, with apoptosis, PKD1 level and ciliogenesis as outcome measures.

### **Pharmacological treatment: additional pathways**

A large number of drugs exist that have known or putative effects in preventing cyst progression, many of which are reviewed in (84). These will each be briefly highlighted, with a special focus on those published in higher impact factor journals and those not described in this review article. The most well-known drugs, somatostatin analogues, do not fall within the scope of this paragraph about future therapeutics, since their effects are already well-studied in clinical trials for ADPLD. Similarly, mTOR inhibitors, are well-studied, but are generally considered to have inadequate effects in halting cyst progress (84-89). This drug class is unlikely to yield promising effects in further studies.

### **Cyclin inhibitors**

Vitamin K3 is the first relevant drug, as it showed some promising effects in animal studies (90). Masyuk and colleagues found that cell division cycle 25 homolog A (Cdc25a) was overexpressed in cystogenesis, causing cell cycle deregulation. By inhibiting Cdc25a using vitamin K3 or PM-20, hepato-renal cystic areas decreased in PCK rats and *Pkd2<sup>ws25/-</sup>* mice. In another study, the cyclin-dependent kinase inhibitors r-roscovitine and S-CR8 appeared to block hepatic and renal cystogenesis (91). These two independent studies indicate that cyclin inhibition may be a valid therapeutic approach in ADPLD.

## **2-deoxyglucose**

2-deoxyglucose causes metabolic reprogramming through inhibition of glucose metabolism (92). A beautiful paper published in *Nature Medicine*, human and murine cyst cells of ADPKD display increased aerobic glycolysis through a mechanism known as the Warburg effect. Named after Otto Warburg, this scientist showed that cancer cells generate energy through glycolysis with reduction of pyruvate through lactate even in circumstances of sufficient oxygen. The effect may sustain tumor cell proliferation through altering biosynthetic pathways. 2-deoxyglucose, by preventing this tumor effect, caused a decrease in kidney weight, volume and cystic index in two mouse models of ADPKD. The drug, or derivatives thereof, may prove a potent therapeutic strategy against cyst development.

## **Metalloproteinase inhibitors**

Another newcomer on the field is marimastat. This pharmaceutical is part of the metalloproteinase inhibitor drug class, which prevent proteins involved in breaking down extracellular matrix (ECM) from functioning. In one study, Banales et al discovered an inhibiting effect on cyst growth (93). Theoretically, this occurs because cysts are no longer capable of growing through the now-undegradable ECM. Unfortunately, marimastat has not yet passed clinical trials aimed at human use of the drug. Trials for ADPLD patients may be considered if a clinical drug is developed with a tolerable safety profile.

## **Ursodeoxycholic acid**

The same group studied ursodeoxycholic acid (UDCA) in experimental models of PLD, and found it inhibited cystogenesis (94). Supposedly, besides normalizing bile acid flow through cystic livers, the drug halts hyperproliferation via a PI3K/AKT/MEK/ERK1/2-dependent mechanism. In a recent clinical trial by d'Agnolo and colleagues (95), the drug failed to live up to expectations. UDCA when administrated for 24 weeks did not reduce total liver volume in advanced PLD. In a post-hoc analysis it did decrease liver cyst volume in ADPKD patients. As study authors mention however, the study was not powered for subgroup analyses of ADPKD and ADPLD patients. Further study is required.

## **Wnt inhibition**

One final pathway worth modifying is that of canonical Wnt signaling, which recently proved to be involved in polycystic liver disease. Scientists are developing drugs that may modify the pathway in a clinical setting. An example of this is LGK974 (96), a porcupine inhibitor that blocks Wnt secretion, currently in clinical testing. It has thus far proven effective against multiple tumor types in animal models, and may be worth screening in PLD drug tests.

## **Calcium agonist and remaining drugs**

One of the putative primary pathogenic pathways involved in cyst development is decreased calcium signaling. Researchers studying this zoomed in on the Trpv4 calcium-entry channel in PCK rats, and found that activating the channel could inhibit cell proliferation and *in vitro* cyst growth (97). Although promising, the authors of the study conclude there are currently no drugs available to test the effect in clinical practice.

Some smaller studies suggest drugs like celecoxib (through VEGF/Raf/MAPK/ERK signaling pathway inhibition) (98), VEGF inhibitors (99), HDAC inhibitors (through improved cell cycle

regulation and ciliogenesis) (100, 101), isoliquiritigenin and thiophenecarboxylates (through inhibition of fluid secretion) (102, 103), and narengenin (104) might inhibit cystogenesis. PPAR-gamma inhibitors also inhibit cyst formation in study published in PLoS one (105, 106). Surprisingly, telmisartan, generally used as an angiotensin-2 receptor antagonist is used to block PPAR-gamma. Although far from conclusive data, it may be valuable to study these compounds in PLD drug screens. The step to animal experiments is long however, and the issue with mTOR inhibitors (successful in experimental models, but unable to alter the natural course of ADPKD) suggest that way to translation is difficult.

**Table 3.** Rationale for drugs in ADPLD based on mechanism of action, many of which are reviewed in (84).

<b>Drug class</b>	<b>Examples</b>	<b>Mechanism of action</b>
Splice-Switching Oligonucleotides (78-80)	-	Correct aberrant splicing, induce expression of a therapeutic splice variant, or induce expression of a novel therapeutic splice variant
Read-through drugs (81-83)	Aminoglycosides (e.g. Gentamicin, G418), negamycin, ataluren	Causing ribosomes to ignore premature stop codons / nonsense mutations
Proteasome-inhibitors (8, 64)	MG132, MG115, carfilzomib	Preventing proteasome degradation of poor quality proteins
CDC25a inhibitors (90)	Vitamin K3, PM-20	Cell cycle regulation through inhibition of overexpressed cyclin-related proteins
2-deoxyglucose (92)	-	Metabolic reprogramming, inhibition of glucose metabolism
CDK inhibitors (91)	R-roscovitine, S-CR8	Cell cycle regulation through cyclin-dependent kinase inhibition
Metalloproteinase inhibitors (93)	marimastat	Metalloproteinase inhibition to prevent tissue expansion
mTOR inhibitors (84-89)	Sirolimus, tacrolimus, everolimus	mTOR pathway inhibition to prevent cell proliferation
PPAR-gamma inhibitors (105, 106)	Pioglitazone, telmisartan	Inhibition of PPAR-gamma pathway to prevent cell proliferation
Ursodeoxycholic acid (94, 95)	-	Prevention of biliary cell proliferation
Estrogen receptor inhibitors (38)	Tamoxifen	Estrogen inhibition to prevent cell proliferation
VEGF receptor inhibition (99)	SU-5416	VEGF pathway inhibition to prevent cell proliferation
Calcium stimulators (97)	?	Intracellular calcium enhancement to prevent cell proliferation
Wnt signaling inhibitors (96)	LGK974	Canonical Wnt signaling inhibition
Fluid secretion inhibition (102, 103)	Isoliquiritigenin, Thiophenecarboxylates	Fluid secretion inhibition (CFTR- or cAMP-mediated)
HDAC inhibitors (100, 101)	ACY-1215	various
NSAID (98)	Celoxib	VEGF/Raf/MAPK/ERK signaling pathway inhibition
naringenin (104)	-	-

## Surgical treatment

One final option for treatment of severe, refractory polycystic liver disease is currently liver transplantation. Liver transplantation represents a formidable procedure and the patient will be on lifelong immunosuppressive therapy to prevent rejection of the foreign liver tissue. A marriage between adult liver stem cell culture and CRISPR-Cas9 might remove this latter disadvantage, while remaining ethically sound. Scientists can isolate adult liver stem cells from the patient before transplantation, and may subsequently remove disease-causing mutations using CRISPR-Cas9 (107). Following suitable testing to prevent unwanted off-target effects, liver cells can be cultured to sufficient quantities for retransplantation. Although still far off, this strategy will allow autologous liver stem cell transplantation. Testing is currently underway for copper accumulation disease in a dog model of Wilson's disease (108), in which disease CRISPR-Cas9 repaired cells will have a survival advantage. If successful, the next step will be generating more complex liver tissue for transplantation.

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# Chapter 9

## Summary

Autosomal dominant polycystic liver disease is a hereditary disorder causing multiple fluid-filled cysts in the liver, which may cause significant discomfort to patients suffering from it. A gene defect in *PRKCSH*, *SEC63*, *LRP5*, *GANAB*, *ALG8* or *SEC61B* leads to production of a faulty protein, causing an abnormal cellular response and finally random expansion of parts of the biliary tract (**Chapters 1 and 2**). Similar effects are seen with *PKD1*, *PKD2* and *PKHD1* mutations, which cause autosomal dominant and recessive polycystic kidney disease (ADPKD & ARPKD). *PKHD1* was recently found to cause liver cysts in an autosomal dominant fashion in mutation carriers.

In the body, somatic second hits occur that then cause loss-of-heterozygosity (LOH) and reduction of the cognate protein activity below the threshold of pathogenicity. In this thesis, we studied cyst development at multiple biological levels.

## DNA

We isolated and analyzed DNA from biliary cells obtained from 46 cyst fluid and cyst epithelium samples from 23 patients (**Chapter 3**). In 76% (22/29) and 67% (2/3) of liver cysts of *PRKCSH* and *PKD1* mutation patients LOH is present respectively. We additionally found LOH in one cyst of a *SEC63* mutation carrier. Abnormalities in novel regions outside of known regions underlying cyst formation were present in 12 of 23 patients. A *BUB1* mutation and recurring 3p mutation indicate that risk factors for ADPLD may modify disease course, and germline modifier genes may be present.

## RNA

We used RNA sequencing to determine the gene expression pattern of eight liver cyst cell cultures and compared that with four normal biliary cell cultures (**Chapter 5**). The cell cultures expressed many genes important in cystogenesis, including all known genes underlying cyst formation and ciliogenesis. Although no significantly different pathways between normal and cyst cultures were discovered, on principal component analysis plots *PRKCSH* and *PKD1* mutation organoids clustered separately from other PLD organoids. Including a larger number of organoids with known mutations will likely yield more information with regard to clustering and mutation-specific disrupted pathways.

## Protein

We used tandem affinity purification (TAP) to isolate ADPLD associated protein complexes, and analyzed them using mass spectrometry (MS) (**Chapter 4**). Known interactors were present. *GIIB* interacted with *GIIA*, and *SEC63p* with *SEC61A1* and *SEC61B*. Interactions of *GIIB* and *SEC63p* with *PEX* proteins confirmed the ER origin of peroxisomes, and a possible relation to cyst development. Finally, *GIIB*, *SEC63p* and *LRP5* shared a common interaction with filamin A (*FLNA*). *FLNA* is a scaffold important in diverse cellular signaling pathways, which include Wnt signaling and ciliogenesis.

## Organelle/cilium

After knocking out *PRKCSH* or *SEC63* in H69 and HEK293T cells, we studied ciliogenesis and Wnt signaling (**Chapter 4**). In both of these cell lines, formation of primary cilia was significantly reduced by loss of *PRKCSH* or *SEC63*. In H69 cholangiocytes, Wnt signaling is additionally disrupted significantly as measured by luciferase assay and axin 2 mRNA expression. These findings indicate cilium formation requires normal *PRKCSH* and *SEC63* expression, and that Wnt signaling may be disrupted due to their mutation depending on the cell type.

## Cell

We used two separate techniques to create cell lines modelling ADPLD. First, as mentioned above, H69 and HEK293T were modified using CRISPr-Cas9 to lose *PRKCSH* or *SEC63* expression. Second, cholangiocytes from cyst epithelium and fluid were placed into conditions suitable for the expansion of liver stem cells (**Chapter 5**). These cells developed into liver organoids, which carried the genetic background of hepatic cysts. Both of these models are valuable for screening for critical components of ADPLD pathophysiology that might be exploited for pharmaceutical treatment.

## Organism

*Prkcsch*, *Sec63* and *Lrp5* in 1-cell stage zebrafish embryos were disrupted by CRISPR-Cas9 technology (**Chapter 6**). Larvae with single, double and triple mutations in the genes underlying cystogenesis developed. Both *Prkcsch* and *Sec63* mutation larvae displayed decreased length, and a failure to reach adulthood. In case of *Sec63* mutations, swim bladders did not inflate. Only larvae with in-frame *Lrp5* mutations developed, and these could reach adulthood. No differences in fish length were observed. In none of the fish, any liver cysts developed. Even without liver cysts, the knockout zebrafish might be valuable for screening purposes, to evaluate the effects of potential pharmacological agents on surrogate parameters like fish length and swim bladder inflation. The developed gRNA sequences may be used to develop zebrafish with liver-specific knockout of cyst genes.

## Concluding remarks

Concluding, this thesis has broadened the view of the genetic basis of ADPLD, as well as pointing towards new mechanisms of disease and avenues for further research. The research indicates that researchers should exploit mutation-specific rather than disease-specific models to determine crucial components of ADPLD pathophysiology and therapeutical options.

### ***Lay extra: From gene to organism.***

Genes are at the basis of life, generating organisms through multiple steps; genes in DNA specify RNA, which codes proteins, cells, tissue, organ and organism growth. Like a stack of dominos, if one falls down, it will have consequences for the next biological process. It is a small miracle that despite development being a game of statistics, humans generally grow up healthy and fit. Below the normal process of development is outlined in simple terms.

#### **DNA**

At the basis of this complexity lies the hereditary or genetic data present in the DNA. This molecule stores all the information required to create everything from a simple gene, to a multicellular organism. Due to its extraordinary stability, evolution 'chose' DNA for long-term storage of genetic information. In human beings, every gene is present in two copies ('alleles') on two long strands called chromosomes. This is fortunate, as when one copy is broken ('mutated'), the other copy can often serve as a backup. DNA is copied by enzymes called polymerases, of which multiple variants exist. For example, DNA polymerases are the enzymes that generate new copies of DNA. In the human body, most DNA polymerases have proof-reading capabilities. This means that after they copy DNA, they check (or proofread) whether the DNA was copied correctly, and correct their mistakes if necessary.

In this manner, human DNA polymerases are highly accurate (=high fidelity), and make mistakes in as little as 1 in 100 million nucleotides (the letters of DNA) (1). Repair enzymes fix 99% of these errors, leaving an error rate of 1 in 10 billion nucleotides. All human genes (also known as the human genome) are 3.2 billion nucleotides x 2 (two copies/alleles), leaving us with 0.64 copy mistakes every time the DNA is copied before a cell divides. There are 37,2 trillion cells in the human body (2), and 240 billion cells in the human liver. This indicates there is a lot of chance for each gene to obtain a mutation (=DNA error) simply based on DNA replication during cell division. Other mechanisms of mutations also exist, such as UV-light or toxin induced DNA damage. Luckily however, humans possess most genes in pairs (known as alleles).

#### **RNA and proteins**

RNA polymerase generates RNA molecules from DNA. Like DNA, RNA consists of nucleotides, but with slightly different chemical components. As such, RNA is more unstable and fleeting, but has the additional capability to perform functions influencing its environment. RNA can store information and affect / process molecules at the same time. It can be copied from DNA with only slight modifications, and some of these RNA copies can subsequently perform the functions stored in the genes. For example, the enzymatic function of ribosomal RNA allows proteins to be formed. More important are the messenger RNAs, which work as instructions to build the proteins of the body.

Genes on DNA usually encode such proteins. Although proteins cannot store genetic information, they are the most important component to create the cells of our bodies. Proteins have a myriad of functions. They are enzymes, building blocks, cellular signals and more. Enzymatic proteins can create, or catalyze, production of a variety of molecules. Proteins are also often a scaffold for other molecules to bind on. And importantly, proteins are responsible for action and reaction of cells in response to their environment.

## Organelles and cells

Proteins perform their function in different organelles of the cell. An example includes the endoplasmic reticulum, where many newly created proteins are processed by other, older proteins to function correctly. Another is the primary cilium, which is only present on some cells. This organelle functions as a cellular antenna, where proteins such as polycystin-1 and polycystin-2 are thought to respond to external stimuli to produce a response within the cell. This is the smallest living unit of most organisms, and virtually all cells are capable of the processes described above. From the instructions contained on DNA, RNA and proteins cause cells to develop. Different cell types exist. For example, the most important cell types of the liver are the hepatocytes and biliary cells. Hepatocytes are responsible for enzymatic processing of nutrients, vitamins, and waste, while biliary are responsible for removal of this processed waste through bile fluids.

Each cell knows how to develop due to its environment, and other cells surrounding it. Crucial for these interactions are signaling proteins. Cell-cell and cell-environment interactions thus shape the tissues of the human body.

## Tissues, organs and organisms

The development of the biliary tract in human embryos is a prime example of tissue formation. Cells from the so-called portal veins of the embryonic liver secrete proteins that induce adjacent cells to become biliary cells. These proteins belong to a class called 'Wnt' proteins. The Wnt proteins simply diffuse between the cells, and as such, the farther away the cell, the lower the amount of Wnt it receives. Whereas high-dose Wnt induces biliary cells, lower-dose Wnt induces periportal hepatocytes, liver cells surrounding incoming veins from the intestines, to develop. Absent Wnt causes centrovenular hepatocytes, liver cells surrounding veins towards the heart, to grow (3). Each of these cell-types has a slightly different function, based on the requirements from its environment. Biliary cells develop the primary cilia, which cellular antennae can sense bile flow and composition. Although the single Wnt gradient is a simplified view, it can be imagined that multiple signaling gradients occurring in different locations and times. Together they cause all tissues to develop.

Organ development is the end result of this complex interaction between genes, proteins, cells, and tissues. The arteries, veins, hepatic parenchyma, biliary tracts, and immune system all come together to form the liver. The correct location and size of each of the tissues is essential for normal organ development, and for development of the human organism as a whole.

## Concluding remarks

Only some of the genes that are responsible for liver cyst development are known, and it is unclear which processes they end up affecting to cause the disease. At each of the described levels problems leading to disease may occur. The tools and knowledge to interrogate each of the above processes in great detail now exist, and many of them were applied in this thesis. How and why is described in this thesis starting in **Chapter 1**. As a lay reader, either the summary, Dutch 'samenvatting' and Dutch 'eenvoudig gezegd' following directly hereafter may be more appropriate reading material however.

As a small extra, the number of liver cells with a mutation in paragraph 'DNA' corresponds to

the number of liver cells with a mutation in the second copy of *PRKCSH*. This gene is 15712 nucleotides in length. Although other mechanisms are also involved, e.g. a mechanism named interstitial deletion; this is at least a part of a statistical calculation that is the basis of random expansion of part of the liver into cysts.

### Glossary

**Gene:** Most basic coding unit of DNA. Sequence of nucleotides encoding a protein, rRNA, tRNA, or other type of functioning RNA.

**Allele:** One of a pair of genes (but may contain minor differences in nucleotide sequence).

**Chromosome:** One of 23 strands of DNA in humans, two copies of each chromosome exist in most cells ('diploid state').

**Biliary cell:** Cell of the biliary tract.

**Cholangiocyte:** Identical to biliary cell.

**Hepatocyte:** Main cell type of the liver.

**Portal vein:** Blood vessel carrying blood from the intestines and spleen to the liver.

**Wnt signaling:** Signaling pathway involved in development of tissue, cell division/proliferation and differentiation (the process of become a mature cell type like a biliary cell or hepatocyte).

**Primary cilium:** The cell's antenna. An organelle (part of a cell/cell structure) found on many cells in the human body.

**Endoplasmic reticulum:** Network of tubular membranes within the cell, responsible for functions such as protein processing/maturation.

## Samenvatting

Autosomaal dominante polycysteuze leverziekte (ADPLD) is een erfelijke aandoening, die meerdere met vocht gevulde cysten in de lever veroorzaakt. Deze cysten kunnen toch flinke klachten bij patiënten leiden. Een genetisch defect in *PRKCSH*, *SEC63*, *LRP5*, *GANAB*, *ALG8* of *SEC61B* leidt tot productie van een foutief eiwit, dat leidt tot een abnormale cellulaire reactie en uiteindelijk willekeurige groei van delen van de galwegen (**Hoofdstuk 1 en 2**). Gelijke gevolgen worden met *PKD1*, *PKD2* en *PKHD1* mutaties gezien, welke autosomaal dominante en recessieve nierziekte (ADPKD & ARPKD) veroorzaken. In het lichaam treden somatische tweede abnormaliteiten op, die verlies-van-heterozygositeit (LOH) onderliggen. LOH veroorzaakt een vermindering van de relevante eiwit producten veroorzaakt, tot onder de drempel van pathogeniciteit. In deze thesis, hebben we cyste ontwikkeling op meerdere biologische niveaus bestudeerd.

### DNA

We isoleerden en analyseerden DNA van biliaire cellen verkregen van 46 cystevocht en cyste-epitheel monsters van 23 patiënten (**Hoofdstuk 3**). In respectievelijk, 76% (22/29) en 67% (2/3) van de levercysten van *PRKCSH* en *PKD1* mutatie patiënten is LOH aanwezig. Bovendien vonden we LOH in een cyste van een *SEC63* mutatie patiënt. Abnormaliteiten in nieuwe regio's buiten de bekende regio's betrokken bij cyste-ontwikkeling waren in 12 van 23 patiënten aanwezig. Een *BUB1* mutatie en herhaaldelijk voorkomende 3p mutaties wijzen erop dat risico factoren van ADPLD ziekteprogressie kunnen beïnvloeden. Ze wijzen er ook op dat kiembaan genen naast de cystegen het ziektebeeld zouden kunnen beïnvloeden.

### RNA

We gebruikten RNA sequencing om het gene expressie patroon van acht lever cyste culturen te vergelijken met dat van vier normale biliaire cel culturen (**Hoofdstuk 5**). De cel culturen brachten veel genen tot expressie die belangrijk zijn in cyste vorming; alle genen die cystogenese onderliggen, evenals genen betrokken bij ciliogenese. Alhoewel geen significant tussen cyste en normale culturen verschillende celsignaleringsroute werd ontdekt, toonde "principal component analyse" aan dat *PRKCSH* en *PKD1* mutatie organoïden separaat groepeerden ten opzichte van normale organoïden. Door inclusie van een groter aantal organoïden met een bekende mutatie kan in een vervolgonderzoek waarschijnlijk meer informatie vergaard worden over groepering van de culturen en mutatie-specifieke verstoorde celsignaleringsroutes.

### Eiwit

We hebben tandem affiniteit purificatie (TAP) gebruikt om ADPLD geassocieerde eiwit complexen op te zuiveren, en hebben deze geanalyseerd met massa spectrometrie (MS) (**Hoofdstuk 4**). Bekende directe interacties werden geïdentificeerd, zoals de associatie van Glucosidase II b (GIIB) met GIIA, en SEC63p met SE61A1 en SEC61B, wat de gebruikte methode valideerde. Interacties van GIIB en SEC63p met perixosomale biogenese eiwitten bevestigden de herkomst van perixosomen uit het endoplasmatisch reticulum. Tegelijk duidde de interactie op een mogelijke relatie van de organellen met cyste vorming. Tenslotte deelden GIIB, SEC63p en LRP5 een gemeenschappelijke interactie met filamine A (FLNA).

FLNA is een zgn. “steigerewit”, waar meerdere cellulare signaleringsroutes mee associëren, waaronder componenten van de Wnt signalering en ciliogenese.

### Organel/cilium

Na uitschakeling van *PRKCSH* of *SEC63* in H69 en HEK293T cellen, bestudeerden wij de ciliogenese en Wnt signalering (**Hoofdstuk 4**). In beide cellijnen was ciliogenese significant verstoord door verlies van de genen. In H69 cholangiocyten werd Wnt signalering tevens verstoord, zoals gemeten door luciferase assay en axine 2 mRNA expressie. Deze vondsten wijzen erop dat ciliumvorming normale *PRKCSH* en *SEC63* expressie vereisen, evenals dat Wnt-signalering verstoord kan worden door mutatie, afhankelijk van het celtype.

### Cel

We gebruikten twee verschillende technieken om cellijnen te creëren die ADPLD modelleren. Als eerste, zoals boven gezegd, werden H69 en HEK293T genetisch gemodificeerd door CRISPR-Cas9. Als tweede werden cholangiocyten uit cyste epitheel en vocht in condities geschikt voor de groei van leverstamcellen geplaatst (**Hoofdstuk 5**). Deze cellen ontwikkelden tot lever organoïden, welke de genetische achtergrond van levercysten bezitten. Beide modellen zijn waardevol voor screening van essentiële onderdelen van ADPLD pathofysiologie, die kunnen worden gebruikt voor medicatie-ontwikkeling.

### Organisme

*Prkcsh*, *Sec63* en *Lrp5* werden in 1-cellige zebrawis embryo's verstoord door CRISPR-Cas9 (**Hoofdstuk 6**). Larven met enkele, dubbele en driedubbele mutaties in de genen verantwoordelijk voor cyste-vorming ontwikkelden. Zowel *Prkcsh* als *Sec63* mutant larven vertoonden een verminderde lengte, als ook niet-gedijen, waarbij de volwassenheid niet werd gehaald. In geval van *Sec63* mutatie vulden zwemblazen zich niet. Alleen larven met in-frame *Lrp5* mutaties ontwikkelden en konden de volwassenheid bereiken. Er werden geen verschillen in vislengte gevonden bij deze mutanten. In geen van de vissen werden levercysten gevonden. Zelfs zonder levercysten zouden deze zebrawissen echter waardevol kunnen zijn voor het screenen van effecten van mogelijke farmacologische agentia. Surrogaat parameters als vislengte en het opblazen van zwemblazen kunnen hier dan mogelijk voor worden gebruikt. Het uitschakelen van de cyste-vorming veroorzakende genen alleen in de lever met de ontwikkelde gRNAs is ook een mogelijke optie.

Concluderend heeft deze thesis de visie op de genetische basis van ADPLD verbreed, als ook nieuwe ziekte-mechanismen en routes voor vervolgonderzoek aangewezen. Het onderzoek wijst erop dat onderzoekers mutatie-specifieke in plaats van ziekte-specifieke modellen moeten gebruiken om essentiële componenten van ADPLD pathofysiologie en therapeutische opties te ontdekken.

## Eenvoudig gezegd

### Het ontstaan van levercysten

Autosomaal dominante polycysteuze leverziekte (ADPLD) is een erfelijke ziekte, die meerdere met vocht gevulde blazen, ofwel cysten, in de lever veroorzaakt. Kinderen van een ADPLD-patiënt hebben een kans van 50% (1 op 2) om het ziektebeeld te krijgen. Groei van deze cysten veroorzaakt klachten door druk in de buik ontstaan. Er zijn momenteel zeven genen bekend die levercysten zonder andere lichamelijke afwijkingen veroorzaken. Dit zijn de *PRKCSH*, *SEC63*, *LRP5*, *GANAB*, *ALG8*, *PKHD1* en *SEC61B* genen (bij conventie worden genen schuin geschreven). *PKHD1* kan in sommige gevallen ook tot cysten in de nieren leiden, zoals ook de daarnaast bekende *PKD1* en *PKD2* genen.

### De fout in het tweede genkopie

Een patiënt heeft een fout in een van de twee kopieën van een gen die op het DNA bestaan. De meeste cellen heb dus gelukkig nog een goede kopie. Als door pech in de lever een fout ontstaat in het tweede kopie, ontstaat pas een levercyste.

### Gevolgen van verlies van beide genkopiën

Het is onduidelijk wat precies met de galwegcel gebeurt na verlies van beide genkopiën. Elk fout in deze genen leidt tot een fout in een corresponderend RNA en eiwit, dat vervolgens leidt tot problemen in de cellen en organen van het lichaam.

Eén van de meest opvallendste vondsten is het verlies of afwijkend raken van de antenne van de cel (het zogenaamde primaire cilium). Een theorie is dat door deze kapotte antenne een verwarde cel ontstaat, die te veel delingen doorgaat en te veel vocht produceert, en zodoende een cyste in de lever maakt.

### Wat we niet weten

Op meerdere niveaus is het ontstaan van levercysten niet compleet duidelijk:

1. Op DNA gebied zorgen de zeven bekende 'ADPLD' genen slechts voor maximaal de helft van de patiëntgevallen.
2. Op RNA en eiwit niveau is het ontstaan van levercysten onduidelijk. Welke deel of eiwitstelsel van de cel verstoord raakt na de fout in het tweede genkopie is nooit overtuigend bewezen.
3. Ook hoe de verstoring van de antennes van cellen, gerelateerd is aan cyste-groei vereist nader onderzoek.
4. Als laatste zou een nieuwe, effectieve medicatie-optie buitengewoon nuttig zijn om ziekte vooruitgang bij patiënten te voorkomen. Een accuraat en makkelijk toe te passen model van het ziektebeeld zou daarbij goed van pas komen.

In deze thesis werd onderzoek gedaan naar levercyste-vorming op meerdere biologische niveaus.

### DNA

We isoleerden en analyseerden DNA van galwegcellen van 46 cysten van de 23 patiënten (**Hoofdstuk 3**). In ongeveer driekwart van de door *PRKCSH* en *PKD1* ontstane levercysten

bevestigden we een fout in het tweede genkopie van de cellen. Bovendien vonden we een foutief tweede genkopie in een cyste van een *SEC63* mutatie patiënt. Ook in nieuwe gebieden vonden we bij 12 van 23 patiënten fouten op het DNA.

## RNA

We vergeleken RNA van acht levercyste kweken met dat van vier normale galwegcel kweken (**Hoofdstuk 5**). De celkweken hadden RNA van genen die belangrijk zijn in cyste vorming; genen die vorming van levercysten veroorzaken (*PRKCSH*, *SEC63*, *LRP5*, *GANAB*, *PKD1* en *PKD2*), evenals genen betrokken bij de antennes van cellen. Een specifieke eiwitgroep als oorzaak voor de levercysten werd niet gevonden, waarschijnlijk is daarvoor bij een vervolgonderzoek een grotere groep vereist.

## Eiwit

We onderzochten welke eiwitten aanwezig zijn rondom *PRKCSH*, *SEC63* en *LRP5* in de cel (**Hoofdstuk 4**). Een aantal bekende partnereiwitten werden gevonden. Dit waren het *GANAB* eiwit (glucosidase IIA) voor *PRKCSH*, en *SEC61A1* en *SEC61B* voor *SEC63*. *GANAB* en *SEC61B* werden later onafhankelijk ontdekt als oorzaken voor levercysten.

Interessanter was dat alle drie de eiwitten een verbinding hadden met het filamine A eiwit (*FLNA*). *FLNA* is een steigereiwit, een eiwit waarop andere eiwitten een groter celonderdeel bouwen. Meerdere celonderdelen zijn verbonden met *FLNA*, waaronder de Wnt eiwitfamilie waartoe *LRP5* hoort, als ook de vorming van de antennes van cellen.

## Verlies van de antenne van de cel

Na uitschakeling van *PRKCSH* of *SEC63* in celkweken, bestudeerden wij de vorming van celantennes en de activiteit van de Wnt eiwitten (**Hoofdstuk 4**). Door verlies van *PRKCSH* of *SEC63* vormden cellen minder celantennes. Ook werd in onderzochte galwegcellen de Wnt activiteit verstoord. Deze vonden wijzen erop dat de celantennes en Wnt activiteit normale *PRKCSH* en *SEC63* genen vereisen.

## Cel

In deze thesis hebben we gepoogd nieuwe modellen voor het ziektebeeld te maken. Daarvoor maakten we zelf foutjes in de genen *PRKCSH*, *SEC63* en *LRP5*. Als tweede namen we cellen van de levercysten en plaatsten die in condities geschikt voor de groei van leverstamcellen (**Hoofdstuk 5**). Deze cellen ontwikkelden tot leverkweken, welke het precieze DNA van levercysten bezitten. Beide modellen zijn waardevol voor onderzoek van essentiële onderdelen van het biologisch mechanisme achter het ontstaan van levercystenvorming, die kunnen worden gebruikt voor de ontwikkeling van medicijnen.

## Organisme

De *Prkcsh*, *Sec63* and *Lrp5* genen werden in zebrafisjetjes uitgeschakeld (**Hoofdstuk 6**). Nieuwgeboren zebrafisjes met verlies van de genen ontwikkelden, afhankelijk van het succes met verlies van 1 tot alle drie deze genen. Zowel *Prkcsh* als *Sec63* mutant larven waren korter. De visjes konden niet opgroeien tot volwassenen. In geval van *Sec63* mutatie vulden zwemblazen zich niet. De zwemblazen zijn een orgaan van vissen verantwoordelijk voor de verplaatsing van vissen in de diepte en hoogte.

Alleen visjes met milde fouten in het *Lrp5* gen ontwikkelden zich tot een volwassen vis. In geen van de vissen werden levercysten gevonden. Zelfs zonder levercysten zijn deze zebravissen mogelijk waardevol voor het testen van nieuwe medicijnen. Er kan dan worden gekeken naar verbetering van de lengte van de vissen en het opblazen van zwemblazen onder invloed van deze medicijnen. Mogelijk kan ook geprobeerd worden om de genen achter de cystevorming alleen in de lever uit te schakelen. Hierdoor hebben de vissen een grotere kans om volwassen te worden, met een langere tijd voor cyste-ontwikkeling.

Concluderend heeft deze thesis de visie op de genetische basis van ADPLD verbreed, als ook nieuwe ziekte-mechanismen en routes voor vervolgonderzoek aangewezen. Het onderzoek wijst erop dat onderzoekers gen-specifieke in plaats van ziekte-specifieke modellen moeten gebruiken om belangrijke onderdelen van het ontstaan van de levercysten en medicijnen te ontdekken.

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## Research data management

Appropriate research data management is important for safeguarding scientific integrity, open science, safekeeping of valuable datasets and the reuse of data. The FAIR (Findable, Accessible, Interoperable and Reusable) principles are a recently published method for proper management of data (1). The data obtained during my PhD at the Radboud university medical center (Radboudumc) are archived in general accordance to these principles. Electronic data are stored on a secure server of the Radboudumc for the Gastroenterology department. The data is accessible by the head of the laboratory, as well as the junior researchers studying relevant topics. The Information and Communications Technology (ICT) of Radboudumc supports the server. Primary and secondary data of all projects that were stored in the local server were additionally backed-up to the university servers on a weekly basis. The animal study in **Chapter 6** was carried out in accordance with European and institutional guidelines on animal welfare (2010/63/EU). Published articles contain part of the data generated and analyzed during this thesis, the additional files being available upon request from the corresponding author.

1. Wilkinson MD, Dumontier M, Aalbersberg IJ, Appleton G, Axton M, Baak A, Blomberg N, Boiten J-W, da Silva Santos LB, Bourne PE, Bouwman J, Brookes AJ, Clark T, Crosas M, Dilloi, Dumon O, Edmunds S, Evelo CT, Finkers R, Gonzalez-Beltran A, Gray AJG, Groth P, Goble C, Grethe JS, Heringa J, ESH ESC Task Force for the Management of Arterial Hypertension, Hooft R, Kuhn T, Kok R, Kok J, Lusher SJ, Martone ME, Mons A, Packer AL, Persson B, Rocca-Serra P, Roos M, van Schaik R, Sansone S-A, Schultes E, Sengstag T, Slater T, Strawn G, Swertz MA, Thompson M, van der Lei J, van Mulligen E, Velterop J, Waagmeester A, Wittenburg P, Wolstencroft K, Zhao J, Mons B. The FAIR Guiding Principles for scientific data management and stewardship. *Sci Data* 3: 160018, 2016.

## List of publications

1. Wills ES, te Morsche RHM, van Reeuwijk J, Horn N, Geomini I, van de Laarschot LFM, Mans DA, Ueffing M, Boldt K, Drenth JPH, Roepman R. *Liver cyst gene knockout in cholangiocytes inhibits cilium formation and Wnt signaling*. **Human Molecular Genetics**. 2017 Nov 1;26(21):4190-4202. [IF 5.689]
2. Wills ES, Drenth JP. *Building pancreatic organoids to aid drug development*. **Gut**. 2017 Mar;66(3):393-394. doi: 10.1136/gutjnl-2016-312865. Epub 2016 Sep 21. [IF 16.658]
3. Wills ES, Cnossen WR, Veltman JA, Woestenenk R, Steehouwer M, Salomon J, Te Morsche RH, Huch M, Hehir-Kwa JY, Banning MJ, Pfundt R, Roepman R, Hoischen A, Drenth JP. *Chromosomal abnormalities in hepatic cysts point to novel polycystic liver disease genes*. **Eur J Hum Genet**. 2016 Dec;24(12):1707-1714. doi: 10.1038/ejhg.2016.97. Epub 2016 Aug 24. [IF 4.287]
4. Wills ES, Roepman R, Drenth JP. *Polycystic liver disease: ductal plate malformation and the primary cilium*. **Trends Mol Med**. 2014 May;20(5):261-70. doi: 10.1016/j.molmed.2014.01.003. Epub 2014 Feb 5. Review. [IF 10.11]
5. Wills ES, Jonkers DM, Savelkoul PH, Masclee AA, Pierik MJ, Penders J. *Fecal microbial composition of ulcerative colitis and Crohn's disease patients in remission and subsequent exacerbation*. **PLoS One**. 2014 Mar 7;9(3):e90981. doi: 10.1371/journal.pone.0090981. eCollection 2014 [IF 3.534]



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(Portugees)

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Graphpad prism

HTML

SPSS

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Patiëntinclusie,  
NGS, SNP micro-

array, adult stem  
cells, CRISPr-Cas

### Cursussen

EHBO, BLS, AED.

VMT,

11/2012 – 04/2018 **Promotie afdeling Maag-, Darm-, en Leverziekten (PhD)**  
RadboudUMC Nijmegen  
“*Modelling molecular mechanisms of polycystic liver disease.*”

12/2016 – heden **Arts-Assistent Interne Geneeskunde**  
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Master- / Combistage MDL / Medische Microbiologie

09/2007 – 8/2012 **Biology of Disease (Master of Science)**  
Universiteit Utrecht

09/2004 – 8/2007 **Biomedische Wetenschappen (Bachelor of Science)**  
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09/1998 – 7/2004 **Gymnasium (Natuur & Gezondheid, Latijn, Economie 1,2)**  
Gymnasium Haganum (Den Haag)

## Beurzen

### 2017 – Gastrostart Beurs

E. Wills, R. Roepman, J. Drenth. *The role of primary cilia in gastric endocrinology.* [€8.000]

### 2015 – RIMLS PhD grant round

E. Wills, R. Roepman, J. Drenth. *A comprehensive, molecular route to a cure for PCLD.* [~€200.000]

### 2014 – IGMD PhD grant round.

W. Cnossen, E. Wills, R. Roepman, J. Drenth. *Wnt signalling in hepatic cystogenesis.* [~€200.000]

## Prijzen

### 2014 – Oral free paper prize UEGW.

*Bipotent adult liver stem cells as a model to study polycystic liver disease.* Session viral hepatitis, cytokines and liver regeneration.

2014 – Travel grant UEGW. Top abstracts basic science.

## Overige studie-onderdelen

4/2010 – 6/2010 **Electieve stage**

Wageningen Universiteit  
*Plants and Health*

9/2008 – 5/2009 **Literatuuronderzoek**  
Academisch Ziekenhuis Maastricht  
*“Löfgren's syndrome and vitamin d insufficiency”*

9/2007 – 6/2008 **Mastercursussen**  
Universiteit Utrecht  
*Mechanisms of disease, immunity and infection, biomolecular and cellular cardiology, vascular biology and immunity, essentials of neuroscience, pathology*

#### **Extracurriculaire activiteiten tijdens studie**

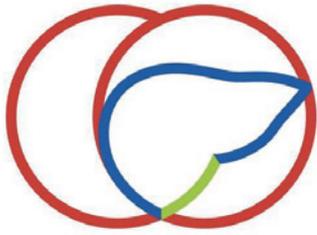
1/2011 – 5/2012 **Rosalind Franklin Contest (RFC)**  
Nationale Wedstrijd voor Geneeskundestudenten  
*Verantwoordelijke Acquisitie*

10/2008 – 7/2009 **Clinical Investigator Science Symposium (CISS)**  
Symposium opleiding arts-klinisch onderzoeker  
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#### **Overige Werkervaring**

2/2008 – 6/2008 **Casca Dura Capoeira**  
Basisschoolleraar Capoeira  
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2005 – 2007 **Studentalent Uitzendbureau**  
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NVH

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