Whole-exome sequencing reveals LRP5 mutations and canonical Wnt signaling associated with hepatic cystogenesis

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Polycystic livers are seen in the rare inherited disorder isolated polycystic liver disease (PCLD) and are recognized as the most common extraenal manifestation in autosomal dominant polycystic kidney disease. Hepatic cystogenesis is characterized by progressive proliferation of cholangiocytes, ultimately causing hepatomegaly. Genetically, polycystic liver disease is a heterogeneous disorder with incomplete penetrance and caused by mutations in PRKCSH, SEC63, PKD1, or PKD2. Genome-wide SNP typing and Sanger sequencing revealed no pathogenic variants in hitherto genes in an extended PCLD family. We performed whole-exome sequencing of DNA samples from two members. A heterozygous variant c.3562C > T located at a highly conserved amino acid position (p.R1188W) in the low density lipoprotein receptor-related protein 5 (LRP5) gene segregated with the disease (likelihood of odds score, 4.62) but was not observed in more than 1,000 unaffected individuals. Screening of LRP5 in a PCLD cohort identified three additional mutations in three unrelated families with polycystic livers (p.V454M, p.R1529S, and p.D1551N), again all undetected in controls. All variants were predicted to be damaging with profound structural effects on LRP5 protein domains. Liver cyst tissue and normal hepatic tissue samples from patients and controls showed abundant LRP5 expression by immunohistochemistry. Functional activity analyses indicated that mutant LRP5 led to reduced wingless signal activation. In conclusion, we demonstrate that germ-line LRP5 missense mutations are associated with hepatic cystogenesis. The findings presented in this study link the pathophysiology of PCLD to deregulation of the canonical wingless signaling pathway.

Significance

Polycystic liver disease (PCLD) is an autosomal dominantly inherited disorder characterized by multiple fluid-filled hepatic cysts that may cause an extremely enlarged liver. PCLD is genetically heterogeneous, and mutations in PRKCSH and SEC63 are present in ~25% of PCLD patients. This research identifies four unique LRP5 mutations in four independent families that were all located at highly conserved protein domains. Functional activity analyses suggest that mutant LRP5 reduces wingless (Wnt) signal activation. This study suggests that imbalanced Wnt signaling is related to hepatic cyst formation.

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Results

Extended Polycystic Liver Disease Family. We assessed a 49-y-old female (III/18) by clinical history taking and physical and radiological examination. She presented to us with a severe symptomatic polycystic liver without renal cysts and complained of abdominal distension, pain, dyspea, and pyrosis. There was no history of renal disease, hypertension, or intracranial aneurysms. She used contraceptives for a total of 30 y and had two pregnancies. CT scanning revealed numerous cysts with diameters of 5–18 cm in liver segments I–III and VII. Despite aspiration sclerotherapy and laparoscopic deroofing of the liver cysts, her symptoms persisted, and she enrolled in a clinical trial for long-acting octreotide treatment (22). We expanded our clinical analysis and identified a 40-member Dutch family with three generations of early- and late-onset cystogenesis inherited in an autosomal dominant fashion (PCLD-1). Next, we studied 19 individuals with cystogenesis, of which 16 were affected with a polycystic liver according to the Reynolds criteria (5, 7), and three members were affected with renal cysts that came from the 40-member Dutch family (Fig. L4). The proband (III/18) possessed no pathogenic variants affecting any of the known PCLD or ADPKD genes.

Whole-Exome Sequencing Identifies Pathogenic LRP5 Variant. We performed exome sequencing on genomic DNA in two members (III/18 and II/18) from the PCLD-1 family with an advanced polycystic liver phenotype (Fig. 1B). Exome capture and sequencing were performed using the Agilent SureSelect target enrichment system with SOLiD4 sequencing. We identified 24,178 and 25,332 genetic variants per proband, respectively (Tables S1 and S2). Variants were annotated by a bioinformatics pipeline as described previously (19, 20). Variant follow-up required the presence of at least five unique variant reads (different start sites), and the variant had to be present in at least 20–80% of all reads suggestive of heterozygous changes in a dominant model of disease. Next, we prioritized variants based on predicted amino acid consequences and overlap with common variation (presence in dbSNP v134 and/or an in-house database containing 1,300 analyzed exomes of predominantly European ancestry). We identified 11 unique nonsynonymous variants present in both affected relatives (Table S3). All 11 variants were tested for segregation in both probands and all other family members by Sanger sequencing. The only private nonsynonymous variant that cosegregated completely with the disease was a missense mutation c.3562C > T with evolutionary conserved amino acid change p.R1188W located on chromosome 11q13.2 in the low-density lipoprotein receptor-related protein 5 (LRP5) gene (NM_002335.2; Fig. 1 C and D). We checked sequence coverage and excluded the presence of potential pathogenic variants in the 2 Mb surrounding LRP5. All 19 individuals with cystogenesis possessed LRP5 c.3562C > T (Table S4 and Fig. S1). Analysis of the sequence data revealed linkage at the LRP5 locus with the disease with a significant logarithm of odds (LOD) score of 4.62 (Fig. S2). This LRP5 variant (c.3562C > T) was not detected in genome-wide sequence data from the 1000 Genomes Project (23), 6,500 individuals from the National Heart, Lung, and Blood Institute Exome Sequencing Project (24), or exome data from 1,300 individuals of predominantly European ancestry sequenced in-house (19, 20) and excluded by Sanger sequencing in a control set of 1,000 Dutch DNAs of healthy, unrelated individuals. This LRP5 mutation affected a highly conserved amino acid and was predicted to be damaging by PolyPhen-2, MutPred, and sorting intolerant from tolerant (SIFT) models (Table 1).

LRP5 is a known disease gene causing severe skeletal bone or renal disorders and is associated with metabolic disease (Fig. S3). Therefore, we actively investigated the possible presence of subtle clinical signs in our family by slit-lamp eye examination. These investigations excluded familial exudative vitreoretinopathy (FEVR) in any of the family members (SI Text). We assessed bone density of the lumbar spine and left hip in 13 patients and 9 unaffected relatives. The lumbar T-score was lower in LRP5 mutation carriers but within the normal range, and no member had a bone density disorder. Routine laboratory testing, including renal parameters, did not reveal differences between individuals with and without the LRP5 mutation (Table 2). Therefore, other LRP5-related disorders were ruled out in this index family.

Additional LRP5 Variants in Polycystic Liver Disease. To confirm our results, we sequenced LRP5 in a cohort of 150 unrelated PCLD probands without PRKCSH, SEC63, or PKD2 gene mutations. We identified two additional PCLD families (one Dutch and one Moroccan) and one Dutch PCLD singleton case with private LRP5 missense variants. Mutations c.1360G > A (p.V454M) and c.4587G > C (p.R1529S) segregated with the disease in both families, and unaffected relatives of the singleton case (c.4651G > A; p.D1551N) did not carry the mutation (Fig. S4).

Mutation c.1360G > A (p.V454M) was present in an 86-y-old polycystic liver patient. Her mutation-positive daughter had
several bilateral renal cysts and small hepatic cysts on CT scanning. The daughter died as a result of a metastasized liposarcoma at the age of 49. A 43-y-old Moroccan female had multiple dominant hepatic cysts without renal disease. Her 71-y-old mother had several hepatic cysts without renal cysts. Both carried the c.4587G > C (p.R1529S) mutation in the LRP5 gene. A fourth mutation [c.4651G > A (p.D1551N)] was present in a 65-y-old male with a polycystic liver and three renal cysts. There was no evidence for renal failure. The phenotype of his deceased parents was unknown, and both his healthy children were without hepatic or renal cysts and did not harbor the LRP5 mutation. None of the LRP5 variants were detected in chromosomes from healthy unrelated and ethnically matched controls (Dutch, n = 1,000; Moroccan, n = 525) nor were they present in the in-house or online exome sequencing datasets. In addition, all four LRP5 missense variants affected highly conserved amino acids and again were predicted to be damaging or deleterious by PolyPhen-2, MutPred, and SIFT (Table 1).

**LRP5 Expression in Liver Cyst Tissues.** LRP5 has a wide tissue distribution, including liver and kidney, and expression has been reported in Kupffer cells, macrophages, stellate cells, cholangiocytes, and hepatocytes (25). In line with Northern blotting experiments (26), we found abundant LRP5 protein presence in normal liver tissues and relevant for PCLD. In the affected proband (III/18), we observed intense LRP5 staining of cyst-lining epithelium and bile ducts. The intensity of LRP5 expression was comparable in cyst tissue sections derived from a proband (III/18), we observed intense LRP5 staining of cyst-lining epithelium and bile ducts. The intensity of LRP5 expression was comparable in cyst tissue sections derived from a proband (III/18), we observed intense LRP5 staining of cyst-lining epithelium and bile ducts. The intensity of LRP5 expression was comparable in cyst tissue sections derived from a proband (III/18), we observed intense LRP5 staining of cyst-lining epithelium and bile ducts. The intensity of LRP5 expression was comparable in cyst tissue sections derived from a proband (III/18), we observed intense LRP5 staining of cyst-lining epithelium and bile ducts.

For analysis of structural effects of the LRP5 variants, we generated separate models for these domains (Fig. 2 E and F and Table S5) (27). The human LRP5 protein contains 1,615 amino acids and includes a long extracellular region, a single-span transmembrane region, and a relatively short (218 amino acids) intracellular region. The extracellular region consists of four β-propeller domains with subsequent epidermal growth factor (EGF)-like domains. Near the transmembrane region are three LDL receptor class A repeats, whereas on the cytoplasmatic side, five PPPSP motifs are present. The β-propeller domains all consist of six segments of which most carry a characteristic YWTD motif. Arginine 1188 creates hydrogen bonds and ionic interactions in the core of the β-propeller structure, which is predicted to be lost by the tryptophan mutation (p.R1188W). Additionally, the larger tryptophan side-chain will cause steric clashes, which will most likely disturb the whole β-propeller domain. Valine 454 induces several hydrophobic interactions but is also partly exposed to the solvent. Mutant methionine is predicted not to have major structural effects as both amino acids interact similarly (LRP5 D1551N) and one intracellularly located (LRP5 D1551N) mutant, and an empty expression vector as a control. Immunofluorescence imaging in WT and both mutant constructs presented similar localization of LRP5 (Fig. 2 C and D). Western blots of the cell lysates showed comparable protein expression of all constructs (Fig. 2G). Overexpression of the LRP5 constructs in CHO cells increased basal and Wnt3a-induced luciferase activity compared with the empty vector (P < 0.0001). In the presence of Wnt3a, signal

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**Table 1. Summary and in silico analysis of four LRP5 variants in polycystic liver disease**

<table>
<thead>
<tr>
<th>Family ethnicity</th>
<th>Mutation (c.DNA)</th>
<th>NM_002335.2</th>
<th>Predicted effect on protein</th>
<th>PolyPhen2</th>
<th>MutPred</th>
<th>SIFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCLD-1 Caucasian (Dutch)</td>
<td>c.3562C &gt; T</td>
<td>p.R1188W</td>
<td>Probably damaging 1.00</td>
<td>0.552</td>
<td>Deleterious 0.00</td>
<td></td>
</tr>
<tr>
<td>PCLD-2 Caucasian (Dutch)</td>
<td>c.1360G &gt; A</td>
<td>p.V454M</td>
<td>Possibly damaging 0.872</td>
<td>0.520</td>
<td>Deleterious 0.01</td>
<td></td>
</tr>
<tr>
<td>PCLD-3 Moroccan</td>
<td>c.4587G &gt; C</td>
<td>p.R1529S</td>
<td>Possibly damaging 0.610</td>
<td>0.288</td>
<td>Deleterious 0.00</td>
<td></td>
</tr>
<tr>
<td>PCLD-4 Caucasian (Dutch)</td>
<td>c.4651G &gt; A</td>
<td>p.D1551N</td>
<td>Probably damaging 0.999</td>
<td>0.235</td>
<td>Deleterious 0.00</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 2. Baseline characteristics of PCLD-1 family**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No hepatic cystogenesis and no LRP5 mutation (n = 9)</th>
<th>Hepatic cystogenesis and LRP5 mutation (n = 13)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>55 ± 12</td>
<td>56 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>Female sex, no. (%)</td>
<td>6 (67)</td>
<td>9 (69)</td>
<td>NS</td>
</tr>
<tr>
<td>Bone density: Hip</td>
<td>T-score: −0.19 ± 0.78*</td>
<td>−0.08 ± 0.93</td>
<td>0.042</td>
</tr>
<tr>
<td>Z-score: 0.69 ± 0.68*</td>
<td>0.95 ± 0.94</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Bone density: L1-L2-L3-L4</td>
<td>T-score: 1.18 ± 0.95*</td>
<td>0.19 ± 0.88</td>
<td>NS</td>
</tr>
<tr>
<td>Z-score: 1.81 ± 0.89*</td>
<td>1.18 ± 1.03</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>69 ± 14</td>
<td>73 ± 20</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.39 ± 0.71</td>
<td>5.44 ± 1.01</td>
<td>NS</td>
</tr>
<tr>
<td>GFR (MDRD) (ml/min/1.73 m²)</td>
<td>87 ± 13</td>
<td>85 ± 25</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7 ± 2.5</td>
<td>26.4 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.35 ± 0.71</td>
<td>1.80 ± 0.93</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.34 ± 0.40</td>
<td>1.38 ± 0.32</td>
<td>NS</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.44 ± 0.82</td>
<td>3.25 ± 0.78</td>
<td>NS</td>
</tr>
<tr>
<td>Non HDL (mmol/L)</td>
<td>4.06 ± 0.93</td>
<td>4.07 ± 0.93</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>5.5 ± 0.6</td>
<td>5.7 ± 0.8</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1C (mmol/mol)</td>
<td>37 ± 6</td>
<td>39 ± 9</td>
<td>NS</td>
</tr>
</tbody>
</table>

*NS, not significant.

*There was one missing value because of degenerative abnormalities of the lumbar spine in an 85-y-old woman.
activation was significantly down-regulated by 30% and 45%, taking into account the basal activity in both LRPS mutants compared with the LRPSWT. We also detected a significant decreased (P < 0.001) activity of LRPS mutant p.D1551N without the presence of Wnt3a (Fig. 2H). Luciferase activity assays were repeated in HEK293 and human liver-derived H69 cells, where comparable significant results were obtained (Fig. S5).

Altered Expression Levels of Wnt Target Genes. Subsequently, we conducted quantitative PCR (qPCR) experiments of transducers and transcription factors associated with the canonical Wnt signaling. Our analyses show that HEK293 cells transfected with mutant LRPS led to altered expression levels of target genes compared with LRPSWT (Fig. S6). There was a significant increased gene expression of transducers adenomatous polyposis coli, glycogen synthase kinase 3β (GSK3β), and leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) and transcription factor v-my avian myelocytomatosis viral oncogene homolog (c-Myc) in LRPSR1188W or LRPSD1551N compared with LRPSWT. Similarly, axis inhibitor-1 (AXIN-1), axis inhibitor-2 (AXIN-2), lymphoid enhancer-binding factor 1 (LEFI), SRY-box 9 (SOX9), fibroblast growth factor 18 (FGF18), and cyclin D1 (CCND1) were also up-regulated. In the presence of the extracellular regulator Wnt3a, expression levels of several Wnt signaling components changed (Fig. S7). Decreased expression levels were found for GSK3β, AXIN-1, AXIN-2, LGR5, c-Myc, CCND1, and LEFI compared with LRPSWT in both mutated LRPS constructs. These results are in line with the functional consequences of these LRPS mutations as shown with luciferase activity assays (Figs. S8 and S9).

Discussion

This study identifies LRPS as a novel gene associated with hepatic cystogenesis in patients clinically diagnosed with PCLD. The initial discovery was made in two affected relatives from an extended Dutch family by exome sequencing (21). A private missense mutation c.3562C>T (p.R1188W) in the LRPS gene segregated with 18 affected relatives (>40 y), with a significant LOD score of 4.62. These findings are corroborated by the presence of three additional private missense LRPS variants in two PCLD families and one PCLD singleton. All unique variants identified in LRPS affected highly conserved amino acids and were predicted to be damaging or deleterious. The identification of LRPS as a causative gene follows that of PRKCSH (15%) and SEC63 (6%) for isolated polycystic liver disease in a PCLD cohort (18).

LRPS is a single-span transmembrane protein that acts as a coreceptor with Frizzled protein family members for transducing signals by Wnt proteins. Wnt signaling directs a number of fundamental physiological mechanisms such as cell proliferation, cell polarity, and cell fate determination during embryonic development (28). Until now, LRPS variants were linked to a spectrum of Mendelian genetic diseases. LRPS-related disorders include autosomal dominant conditions with abnormal bone density, such as endosteal hyperostosis and osteosclerosis (29–32), but also eye disorders such as recessive and dominant forms of FEVR (29, 33, 34). Our findings expand the clinical spectrum of LRPS-associated phenotypes because there were no extrahepatic features in patients with LRPS mutations in our studies. Specifically metabolic disorders, diabetes mellitus, skeletal, and retinal diseases were absent in the index family, and these were not reported for the other three families. We performed specific clinical investigations in extended family 1 and excluded FEVR in individuals with LRPS germ-line mutations by slit-lamp eye examination. Bone density measurements ruled out bone diseases in individuals with and without PCLD. Hepatic or renal cystogenesis has not been observed in association with FEVR or with bone diseases.

Why different LRPS mutations can result in such a wide spectrum of complex diseases that targets different tissues remains to be determined. LRPS is detected by Northern blot analysis, immunohistochemistry, and in situ hybridization studies in several tissues including the liver and kidney (25, 26). Until now, LRPS mutations were linked to pathological retina or bone development. In the extended PCLD-1 family, we identified the LRPSD1551N mutation in 22 individuals, all identified in the hepatic and/or renal cystogenesis. Two members were too young (<40 y) to develop hepatic cysts, and one individual is an example of incomplete penetrance. Indeed, there was considerable clinical heterogeneity in affected patients without complaints or patients with severe abdominal discomfort in the PCLD-1 family. This clinical heterogeneity has been described in families with
and SEC63-associated PCLD (14, 18), and penetrance of PRKCSH-associated PCLD is estimated at ~80%. Intrafamilial phenotypic variability suggests that modifier genes and/or environmental factors play a major role in PCLD disease expression. Similarly, the clinical expression of LRP5-associated bone and eye diseases is highly variable (27).

There have been efforts to recapitulate the involvement of LRP5 mutations in a number of bone and eye disorders in mice. A targeted KO Lrp5<sup>sm1Jasak/Lrp5<sup>sm1Jasak</sup> mouse suffers from unspecific hepatobiliary abnormalities that might implicate phenotypic overlap with our observed human phenotypes (35). Human and mouse LRP5 share a high degree of amino acid identity to corepressor low density lipoprotein receptor-related protein 6 (LRP6) and have a similar domain structure (36). Furthermore, there has been speculation about functional redundancy between both transmembrane coreceptors of the canonical Wnt signaling pathway (37). The homozygous KO Lrp6 mouse is embryonic lethal and possesses polycystic kidneys (37) compatible with the ADPKD phenotype. There is further experimental data to suggest that renal cystogenesis in ADPKD is linked to defective canonical Wnt signal transduction. Perturbations of polycystins cause inappropriate levels of β-catenin, and activation and inactivation of the Wnt signaling pathway are reported in different polycystic kidney disease mice models during embryonic or postnatal development (38–42). Our findings provide a direct link between canonical Wnt signaling and polycystic liver disease as Wnt signaling is reduced in mutant LRP5 compared with WT LRP5. A moderate reduction of signaling activity is in line with known LRP5 missense mutations in retina and bone disorders (43, 44). These observations support a role of an imbalanced canonical and noncanonical Wnt signal transduction in the pathogenesis of LRP5-associated polycystic diseases. We speculate that LRP5, along with other genes implicated in hepatic cystogenesis, PKD1, PKD2, PRKCSH, and SEC63, is part of the functional genetic network. Experimental studies have observed that reduced dosage of these gene products, and in particular polycystin-1, is required to cause cyst formation (45, 46).

The identification of LRP5 associated with hepatic cystogenesis affords a better understanding of the pathophysiology of PCLD. Positivity for immunoreactive LRP5 in cyst epithelium suggests that the protein is structurally intact. Functional assays in three different cell systems indicate that the mutation renders the protein to be less functional and that leads to inhibition of the canonical Wnt signaling (41, 42).

In conclusion, PCLD is a genetically heterogeneous disorder that may be caused by LRP5 gene mutations. Our results link hepatic cystogenesis to dysregulated canonical Wnt signaling and are in line with data that were generated from ADPKD models (41, 42). Polycystins modulate Wnt signaling during organ development and contribute to hepatic and renal cystogenesis (42, 47, 48). Our study provides evidence that mutations in LRP5 are related to hepatic cyst development and fuels the hypothesis that canonical Wnt signal transduction is important in polycystic diseases. It is possible that disruption of other (downstream) members of the Wnt signaling pathway may be associated with initiation of hepatic and renal cyst formation.

**Materials and Methods**

**Human Subjects.** Ultrasound images of liver and kidneys were acquired using a 3.6-MHz general purpose clinical echo system (Acuson x150; SiemensAG) equipped with a linear array linear transducer. Blood samples were collected from all 40 subjects, and DNA was extracted from blood leukocytes using the HP-PCR Template Preparation kit (Roche Applied Science). Formalin-fixed paraffin-embedded liver cyst tissue specimens were available from proband III/18 after laparoscopic cyst fenestration. DNA samples from 1,000 Dutch and 525 Moroccan healthy unrelated individuals were used as controls. All subjects provided written informed consent for all investigations. This study was approved by the Medical Ethics Committee of the Radboud university medical center, The Netherlands.

**Sanger Sequencing.** PCR products of proband III/18 for all exons of the PRKCSH, SEC63, PKD1, and PKD2 genes were analyzed by traditional Sanger sequencing on ABI310 or ABI310 Genetic Analyzers (Applied Biosystems). Complete sequence analysis for PKD1 variants was conducted by an experienced Center (Bioscientia). Exon and exon-intron boundaries of LRP5 were determined using the Genome Bioinformatics Group of University of California, Santa Cruz (UCSC) Genome Browser, and unique primers were designed (Primer3). Heterozygous changes in LRP5 were detected by high-resolution melting curves (RotorGene-Q; Qiagen) and validated by Sanger sequencing.

**Whole-Exome Sequencing.** Exome enrichment was performed using the SureSelect Human All Exon 50-Mb Kit (Agilent), covering ~23,000 genes. Emulsion PCR and bead preparation were made by using the EZbead system according to the manufacturer’s instructions followed by SOLiD sequencing (Life Technologies). Reads were mapped to the hg19 reference genome using SOLiD Bioscope software v.1.3 and annotated as described previously (19, 20). All nonsynonymous variants shared in both affected individuals, absent or with very low frequency in dbSNP, were tested and validated in healthy and affected members of PCLD-1 family for cosegregation by high-resolution melting curve analysis and Sanger sequencing.

**Linkage Analysis.** We calculated the two-point LOD score for linkage in the extended family using the SuperLink-v1.6 program in EasyLinkage-v5.08 software package. To determine the actual two-point LOD score for the LRP5 mutation that was detected in this family, the mutation was considered to be a microsatellite marker in close proximity of LRP5 (D15S4117). An autosomal dominant mode of inheritance was assumed with a penetrance of ~80%, and the disease allele frequency was estimated at 0.0001 (Fig. S2 and SI Text).

**Clinical Investigations.** Because LRP5 mutations are associated with bone density disorder, metabolic, and ocular developmental diseases, we performed additional clinical investigations of members from the PCLD-1 family with a clear genotype-phenotype relation. An ophthalmologist (C.E.N.) performed an eye examination by a slit lamp to exclude retinal disorders such as FEVR. At the same time, we analyzed metabolic and renal parameters in blood from these 22 individuals of the PCLD-1 family. We assessed bone density of the lumbar spine and left hip by dual energy X-ray absorptiometry (DXA scan; Hologic Discovery A). Results are reported as T- and Z-scores, which reflect the number of SDs by which a patient’s value differs from the mean of a group of young or age-matched normal controls, respectively.

**In Silico Analysis and Homology Modeling.** We used three computational tools for the prediction of the functional effect of mutational variants (PolyPhen-2, Mutpred, and SIFT). LRP5 protein structure was created by using an LRP6 template as a start homology model and reconstruction by the YASARA& WHAT-IF Twinset. PDB files were available as templates for homology modeling of WD40 domains (β-propeller subdomains) to incorporate the identified extracellularly located LRP5 mutations. Separate models were visualized for analysis of structural effects by YASARA.

**Immunohistochemistry Studies.** Formalin-fixed paraffin-embedded liver cyst tissue from proband III/18, four unrelated PCLD patients with PRKCSH <i>c.1341–2A>G</i> mutations, and normal human liver tissue were available for immunohistochemistry studies. Staining intensity for the presence of LRP5 was compared between LRP5 and PRKCSH mutants.

**Expression Constructs.** Total RNA was isolated from liver tissue using TRizol Reagent (Invitrogen), and oligo(dT) DNA was obtained by RT Transcriptor First Strand cDNA synthesis kit (Roche Applied Sciences). Full-length WT LRP5 was obtained using the FastStart High Fidelity PCR System (Roche). LRP5 was cloned into the mammalian expression vector pcDNA3.1<sup>V5</sup>_His TOPO-TOPO (Invitrogen) and checked by Sanger sequencing. An extracellularly <i>c.3562C>T</i> (p.R1188W) and an intracellularly located <i>c.4651G>A</i> (p.D1551N) LRP5 constructs were generated by mutating the pCDNA.LRPS.WT vector using the Quick Change-II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

**Luciferase Activity Assays.** We used three cell lines to assess the functional effects of WT and mutant LRP5. For the activity assay, 5.0 × 10<sup>4</sup> CHO cells, 5.0 × 10<sup>4</sup>
HEK293 cells, and 5.0 × 10^4 human cholangiocyte 69 (H69) cells per well were seeded in a 96-well plate in triplicate. After 24 h, cells were transiently transfected using X-tremeGene HD (Roche) with 100 ng LRP5 construct (LRP5wt; LRP5R1184W; LRP5D1515A) or empty vector and 100 ng of TCF/LEF1 Reporter or 100 ng negative control according to the manufacturers’ instructions (Cignal TCF/LEF1 Reporter Assay Kit; Qiagen). Sixteen hours after transfection, medium was replaced by medium with or without 250 ng/mL human Wnt3a (5036-WN; R&D Systems) to initiate Wnt signaling. Cells were cultured for another 24 h, and luciferase activity was detected using the Dual-Glo Luciferase Assay System (Cat. No. E2920; Promega) in an InfiniteM1000-Pro plate reader (Tecan). Firefly luciferase activity was normalized to Renilla luciferase activity for variations in transfection efficiencies. Values are reported as means ± SD. These experiments were conducted in triplicate and performed three times. Western blotting was also performed to analyze possible differences in expression levels between the WT and mutant LRP5.

qPCR. We conducted transient transfections of HEK293 cells with LRP5 constructs as previously described. The signaling was activated by addition of Wnt3a for 24 h. Total RNA was extracted with TRIzol (Invitrogen). Template cDNA was obtained using the iScript cDNA synthesis kit (Biorad). Expression levels of Wnt target genes were assessed twice by qPCR experiments (in triplicate) using the CFX96 real-time detection system (Biorad).

Intracellular Wnt signaling was assessed by qPCR using the Cignal TCF/LEF1 Reporter Assay Kit (Qiagen). The expression of Wnt target genes was assessed twice by qPCR experiments (in triplicate) using the CFX96 real-time detection system (Biorad).

Immunofluorescence Imaging. A total of 4.0 × 10^4 CHO cells per well were seeded on poly-lysine-coated cover glasses on a 24-well plate and transiently transfected with 100 ng of LRP5wt or mutant LRP5 construct. After 24 h, medium was refreshed, and cells were cultured for another 24 h followed by paraformaldehyde fixation and immunofluorescence staining.

Statistics. Groups were compared using descriptive statistics and compared by the Student t test. Both groups were compared by using the nonparametric Mann–Whitney U test. P < 0.05 was considered statistically significant. All analyses were calculated by SPSS software v18.0.

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