Ttc30a affects tubulin modifications in a model for ciliary chondrodysplasia with polycystic kidney disease

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Abstract: Skeletal ciliopathies (e.g., Jeune syndrome, short rib polydactyly syndrome, and Sensenbrenner syndrome) are frequently associated with nephronophthisis-like cystic kidney disease and other organ manifestations. Despite recent progress in genetic mapping of causative loci, a common molecular mechanism of cartilage defects and cystic kidneys has remained elusive. Targeting two ciliary chondrodysplasia loci (ift80 and ift172) by CRISPR/Cas9 mutagenesis, we established models for skeletal ciliopathies in Xenopus tropicalis Froglets exhibited severe limb deformities, polydactyly, and cystic kidneys, closely matching the phenotype of affected patients. A data mining-based in silico screen found ttc30a to be related to known skeletal ciliopathy genes. CRISPR/Cas9 targeting replicated limb malformations and renal cysts identical to the models of established disease genes. Loss of Ttc30a impaired embryonic renal excretion and ciliogenesis because of altered posttranslational tubulin acetylation, glycylation, and defective axoneme compartmentalization. Ttc30a/b transcripts are enriched in chondrocytes and osteocytes of single-cell RNA-sequenced embryonic mouse limbs. We identify TTC30A/B as an essential node in the network of ciliary chondrodysplasia and nephronophthisis-like disease proteins and suggest that tubulin modifications and cilia segmentation contribute to skeletal and renal ciliopathy manifestations of ciliopathies in a cell type-specific manner. These findings have implications for potential therapeutic strategies.

DOI: https://doi.org/10.1073/pnas.2106770118

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dysplasia with polycystic kidney disease. Proceedings of the National Academy of Sciences of the United States of America, 118(39):e2106770118.
DOI: https://doi.org/10.1073/pnas.2106770118
Ttc30a affects tubulin modifications in a model for ciliary chondrodysplasia with polycystic kidney disease

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Published September 21, 2021. This article is a PNAS Direct Submission. The authors declare no competing interest.

Skeletal ciliopathies (e.g., Jeune syndrome, short rib polydactyly syndrome, and Sensenbrenner syndrome) are frequently associated with nephronophthisis-like cystic kidney disease and other organ manifestations. Despite recent progress in genetic mapping of causative loci, a common molecular mechanism of cartilage defects and cystic kidneys has remained elusive. Targeting two ciliary chondrodysplasia loci (ift80 and ift172) by CRISPR/Cas9 mutagenesis, we established models for skeletal ciliopathies in *Xenopus tropicalis*. Frotlets exhibited severe limb deformities, polydactyly, and cystic kidneys, closely matching the phenotype of affected patients. A data mining–based in silico screen found ttk30a to be related to known skeletal ciliopathy genes. CRISPR/Cas9 targeting replicated limb malformations and renal cysts identical to the models of established disease genes. Loss of Ttc30a impaired embryonic renal excretion and ciliogenesis because of altered posttranslational tubulin acetylation, glycation, and defective axoneme compartmentalization. Ttc30a/b transcripts are enriched in chondrocytes and osteocytes of single-cell RNA-sequenced embryonic mouse limbs. We identify TTC30A/B as an essential node in the network of ciliary chondrodysplasia and nephronophthisis-like disease proteins and suggest that tubulin modifications and cilia segmentation contribute to skeletal and renal ciliopathy manifestations of ciliopathies in a cell type–specific manner. These findings have implications for potential therapeutic strategies.

Significance

Cilia are tubulin-based cellular appendages, and their dysfunction has been linked to a variety of genetic diseases. Ciliary chondrodysplasia is one such condition that can co-occur with cystic kidney disease and other organ manifestations. We modeled skeletal ciliopathies by mutating two established disease genes in *Xenopus tropicalis* frogs. Bioinformatic analysis identified ttk30a as a ciliopathy network component, and targeting it replicated skeletal malformations and renal cysts as seen in patients and the amphibian models. A loss of Ttc30a affected cilia by altering posttranslational tubulin modifications. Our findings identify TTC30A/B as a component of ciliary segmentation essential for cartilage differentiation and renal tubulogenesis. These findings may lead to novel therapeutic targets in treating ciliary skeletalopathies and cystic kidney disease.

Author contributions: M.G. and S.S.L. designed research; M.G., A.H., P.S., K.G., W.S., R.D., S.S., F. Heeg, K.D., F. Hildebrandt, E.L., and A.K. performed research; M.G., A.H., P.S., K.G., A.K., and S.S.L. analyzed data; and M.G. and S.S.L. wrote the paper.

The authors declare no competing interest.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi/10.1073/pnas.2106770118/-/DCSupplemental.

PNAS 2021 Vol. 118 No. 39 e2106770118
https://doi.org/10.1073/pnas.2106770118
ciliary function (20–32). The ciliary Hh pathway is required for chondrocyte proliferation, differentiation, and limb patterning (33–36). Two of the best-characterized SC genes are ift80 (37–39) (mutated in SRPS III and IV and JATD) and ift172 (40–42) (mutated in JATD and MZSDS). Both encode IFT-B subcomplex proteins (43), and although genetic interactions were described, they do not interact physically with each other (40, 43, 44). In model organisms, biallelic loss-of-function mutations result in shortened, missing, or fewer cilia. Morphant and mutant zebrafish larvae have pronephric glomerular cysts, retinal degeneration, pericardial edema, slightly smaller eyes, and a curved tail (39, 40, 45). In addition, ift172 morphants have otolith defects, hydrocephalus, and craniofacial cartilage defects (40). In mice, null alleles of ift80 or ift172 are prenatally lethal (20, 24), while mice with hypomorphic alleles have a low survival rate and suffer from shortening of long bones, a narrow thorax, and polydactyly (24, 46, 47). Chondrocyte-specific loss of function of either gene in conditional knockout mice results in severely shortened limbs (48, 49). Conditional Col2a1; Ift80/fl/fl mice revealed that chondrocytes are disorganized, the growth plate is smaller, and articular cartilage is thickened (48, 50). Loss of ift80 or ift172 reduces the expression of Hedgehog target genes, such as patched1 or gli1 (24, 47), in line with the hypothesis that Ift80 acts downstream of Smoothened as its agnostic SAG, and the second Messenger Gl2 can rescue the loss of Ift80 (48). Embryonic renal dysplasia has been observed in mice with hypomorphic alleles of ift172, but no studies have addressed their role in kidney function.

Mutations in at least 27 known disease genes currently account for most genetic diagnoses in cases of SC (4). However, because SC is a rare genetic disorder, the known causal genes might not cover the complete spectrum of genes relevant to the pathophysiology of SC. A more complete understanding of the molecular network of SC proteins is needed to shed light on its pathogenesis and offer potential therapeutic options.

Here, we describe two models for chondrodysplasia using CRISPR/Cas9–mediated editing of ift80 and ift172 in Xenopus. CRISPR-targeted frogs developed severe limb defects during metamorphosis with shortened limbs due to chondrocyte differentiation defects, syn- and polydactyly, and polycystic kidneys, phenocopying major clinical features of ciliary chondrodysplasia. Impaired ciliogenesis was detected in multiciliated cells (MCCs) and resulted in defective fluid excretion during tadpole stages. Using a data-mining approach, we identified candidate genes with similar molecular properties to the established disease genes. Among these, only ttc30a targeting mimicked the full spectrum of chondrodysplasia phenotypes (i.e., impaired ciliogenesis, fluid retention in tadpoles, polycystic kidneys, and strongly affected limb development, including polydactyly in frogs). Thus, Ttc30a acts in ciliary signaling disrupted in chondrodysplasia and cystic kidney disease. Enhanced expression in chondrocytes and osteocytes of Ttc30a and Ttc30b suggest an evolutionarily conserved role in mammals. Together, our findings identify Ttc30a/B as a critical node in the pathogenesis network of ciliary chondrodysplasia with polycystic kidney disease.

Results

CRISPR/Cas9 Targeting of ift80 and ift172 Leads to Chondrodysplasia and Cystic Kidneys in Xenopus tropicalis. We chose Xenopus to establish a model for ciliary chondrodysplasia because it is an aquatic organism that allows for analysis of limb development, has a high sequence conservation to humans, is an established model in cilia research, and provides good experimental access (50–54). We targeted two well-characterized SC genes (ift80 and ift172) for loss-of-function analysis using CRISPR/Cas9–based genome editing. Indel frequency and knockout scores were used to identify highly effective single guide RNAs (sgRNAs) against both targets in X. tropicalis embryos (SI Appendix, Fig. 1 A–C). Injection of F0 embryos with Cas9/sgRNA ribonucleoprotein (RNP) resulted in mosaic gene disruption and has previously been shown to elicit specific phenotypes at high frequency and penetrance (55–60).

Phenotypic analysis of 3- to 4-day-old embryos (stage 42) injected at the one-cell stage showed prominent generalized edema in both ift80- and ift172-targeted embryos. Importantly, this phenotype was rescued by coinjecting ift80 or ift172 messenger RNA (mRNA), demonstrating the specificity of the sgRNA-mediated loss of function (Fig. 1 A and B). Morpholino oligonucleotide knockdown in X. tropicalis and CRISPR/Cas9 targeting elicited the same phenotype in Xenopus laevis, confirming the specificity of the phenotype in a separate species (SI Appendix, Fig. 1 D and E).

The most prominent phenotypes in chondrodysplasia patients are malformations of the axial skeleton and limbs (i.e., shortened ribs, long bone dysplasia, and polydactyly (4)). Both ift80 and ift172 were expressed in limb buds (SI Appendix, Fig. 1F). Unilateral CRISPR targeting of F0 animals has been shown to be effective to disrupt genes essential for postmetamorphic limb development in Xenopus (61–63). To test if Ift80 and Ift172 act in limb development in Xenopus, we unilaterally injected two-cell-stage embryos, which limits gene editing to only one half of the body while retaining the unaffected half as an internal control. We targeted slc4a5a2 as a negative control, which only results in a loss of pigmentation (59, 64). Apart from the expected loss of skin pigmentation, metamorphic frogs were phenotypically normal, demonstrating that sgRNA/Cas9 injection per se did not have an impact on development (Fig. 1C).

In contrast to control edited at the ift80 or ift172 locus showed severe limb defects. Both fore- and hindlimbs were substantially shorter than in control slc4a5a2-targeted frogs, as quantified in contrast enhanced micro-computed tomography (microCT) scans of mosaic mutant frogs (Fig. 1 C–E, green and red arrowheads). Despite unilateral RNP injections, we observed bilateral limb malformation in some animals, suggesting that in these cases, the first cell cleavage had not fully separated at the time of injection, allowing for gene editing to occur across the midline.

In most animals, the fingers and toes were shortened and less developed, sometimes not even identifiable (Fig. 1C). The number and size of clawed digits of the hindlimbs varied substantially from wild-types, in particular in short, narrow thorax, and polydactyly (20, 24), while mice with hypomorphic alleles of ift172, but no studies have addressed their role in kidney function.

MicroCT images suggested that the shortened, not-yet-ossified limbs consisted mainly of cartilage accumulations (Fig. 1C, green arrowheads), consistent with the phenotype in humans and mouse models (48, 50, 67). Histological sections confirmed that the remaining proliferating zones of cartilage dominated clearly in wild-type and ift80- or ift172-targeted control animals (Fig. 1C). In general, the chondrocytes were less well organized in mosaic mutant froglets (Fig. 1C, 1D), as indicated by the loss of pigmentation (59, 64). Apart from the expected loss of skin pigmentation, metamorphic frogs were phenotypically normal, demonstrating that sgRNA/Cas9 injection per se did not have an impact on development (Fig. 1C).

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In most animals, the fingers and toes were shortened and less developed, sometimes not even identifiable (Fig. 1C). The number and size of clawed digits of the hindlimbs varied substantially from wild-types, in particular in ift80- and ift172-targeted animals (Fig. 1C). X. tropicalis usually possess six digits at the hindlimb, four clawed toes and two other claws (8). Ttc30a/B–targeted frogs, the number of clawed toes were consistently either reduced to less than three (syndactyly) or increased to six or seven (polydactyly) (Fig. 1C). In conclusion, this phenotype is highly reminiscent of the polydactyly phenotype observed in mouse models and human patients (24, 37, 40, 49, 66).

MicroCT images suggested that the shortened, not-yet-ossified limbs consisted mainly of cartilage accumulations (Fig. 1C, green arrowheads), consistent with the phenotype in humans and mouse models (48, 50, 67). Histological sections confirmed that the remaining proliferation zones of cartilage dominated clearly in ift80- and ift172-targeted animals (Fig. 1D). In general, the chondrocytes were less well organized in ift80- and ift172-depleted cartilage than in limbs of controls. However, cartilage differentiation was unaffected in vertebrae, suggesting a limb-specific defect (SI Appendix, Fig. 2A).

Contrast-enhanced microCT was also able to resolve the structure of the mesonephric kidneys in the postmetamorphic frogs at high resolution and revealed polycystic kidneys in most of the mutant animals (Fig. 2 A–C). Quantification and volumetric analysis showed that both ift80 and ift172 disruption resulted in multiple cysts, particularly on the injected side (Fig. 2D). The occurrence of cysts on the uninjected side in ift80 mosaic mutants likely reflects the incomplete segregation of gene editing to one
half, as observed for limb measurements. The total cyst volume was significantly larger on the injected side of ift80-edited froglets (Fig. 2E), while the volume of noncystic renal parenchyma was not significantly different (Fig. 2F).

As ciliopathies can lead to retinal disease, we sectioned both embryo and froglet eyes for histological analysis. Apart from a mildly disorganized outer nuclear layer in ift80-targeted embryos, which may be due to the accompanying edema, we did not observe any obvious photoreceptor defects (SI Appendix, Fig. 2F). Likewise, no laterality defects were found (SI Appendix, Fig. 2C and D).

We conclude that froglets with mutations in ift80 and ift172 displayed shortened extremities with polydactyly and cystic kidney disease, confirming that chondrodysplasia can be modeled in Xenopus. This supplements the available mouse and zebrafish lines and offers additional opportunities to analyze chondrodysplasia genes and their functions (49, 50, 67, 71).

**If80 and Ift172 Targeting Affects MCCs, Resulting in Pronephric Excretion Defects.** Next, we explored the effect of if80 and ift172 disruption on a cellular level. The most prominent phenotype of ift80 and ift172 mutant tadpoles was generalized edema, suggesting a deficiency in water or ion homeostasis due to pronephric, lymphatic, vascular, or cardiac insufficiency. In situ hybridization of established marker genes (aphr, vasculature; nkx2.5, heart; prox1, lymphatic system) did not reveal an obvious developmental disruption of any of these organ systems in Xenopus embryos targeted for if80 and ift172 disruption (SI Appendix, Fig. 3C and D). Given the prominent renal phenotype in froglets, the edema phenotype in embryos, and because Jeune syndrome patients and mouse models frequently suffer from kidney disease, we focused on the pronephros of X. tropicalis embryos (47). We did not find structural tubular defects when staining for the tubular epithelium (SI Appendix, Fig. 3E and F) nor were the expression of segment-specific marker genes (nkce2, distal tubule; slc17a1 proximal tubule) affected (SI Appendix, Fig. 3C and D), suggesting a normal differentiation of tubular tissue.

Expression analysis of ift80 and ift172 by in situ hybridization revealed both genes to be prominently expressed in MCCs of the pronephros and nephrostomes (Fig. 2G and SI Appendix, Fig. 3A and B). Nephrostomes are the most proximal tubular segments that facilitate uptake of coelomic fluid into the pronephric tubule (Fig. 2H). To test whether impaired fluid uptake into the tubule could explain the observed edema phenotype in if80 and ift172 bilaterally disrupted embryos, we employed a dextran excretion
Fig. 2. Cystic kidney disease and cilia defects in *ift80* and *ift172* CRISPR–targeted *X. tropicalis*. (A–C) CRISPR/Cas9 targeting of *slc45a2*, *ift80*, and *ift172* were performed unilaterally in two-cell–stage embryos. Mesonephros of stage 61 to 63 frogslets were analyzed by microCT scans and kidneys and cysts (red arrowheads in B and C) were segmented for 3D volumetric analysis; red: cysts on the injected side; green: uninjected side. (D) The number of cysts (>0.2mm), (E) the ratio of total cyst volume to kidney volume, and (F) the kidney volume (excluding cysts) was calculated and compared with kidneys of control injected animals. (G) Whole-mount in situ hybridization detects *ift80* and *ift172* in the multiciliated nephrostomes of the pronephros in stage 36 to 38 *X. laevis*. (H) Schematic depiction of the embryonic renal system of Xenopus. (I and J) Excretion assay with fluorescein-dextran at stage 38 to 40. Blue arrowheads point to the proximal part of the pronephros. Yellow arrowheads indicate fluorescence signal in the distal tubule, lacking on the injected side (J). A: anterior, P: posterior; excr: excreting; and emb: embryos. (K) Confocal images of multiciliated epidermal cells (MCCs) stained against acetylated tubulin (cyan). Centrin-RFP fusion protein served as a lineage marker (red arrowheads) and indicates CRISPR-targeted MCCs. Blue arrowheads point to nontargeted (wild type) cells. (L) The ciliated area was determined for each cell. Error bars indicate SEM. *P < 0.05; **P < 0.01; ***P < 0.001. [Scale bars, (A–C) 1 mm, (G and H) 0.5 mm, and (L) 10 μm.]

### Cilia Defects

 assays. Embryos were injected with sgRNA/Cas9 at the two- to four-cell stage, and fluorescently labeled dextran was injected into the coelom at stage 39 to 40 when the pronephros is fully functional. Timelapse movies of *slc45a2* sgRNA-injected (negative control) embryos showed rapid uptake and acceleration of dextran through the pronephric tubule and excretion at the cloaca on both sides in 84.6% of embryos (Fig. 2 I and J). In contrast, entry into the pronephric tubule was observed in only 12.5 and 13.8% of embryos within 3 min on the *ift80*– and *ift172*–targeted side (Fig. 2 I and J), respectively. Dextran uptake was comparable to wild type and much faster on the nontargeted side of unilaterally gene-edited embryos. Normal excretion on the uninjected side could also be indirectly observed in the timelapse movies where dextran entered the medium of *ift80*– and *ift172*–targeted embryos (Movie 1).

Motile cilia in the nephrostomes actively propel fluid into the renal tubules. Thus, structural defects of ciliated cells may explain the observed loss of excretion in CRISPR-targeted tadpoles (45, 71). To analyze the role of *ift80* and *ift172* in motile cilia, we turned to the epidermal MCCs, a well-established and accessible model for ciliogenesis.

**Centrin-red fluorescent protein** mRNA was used as an injection marker to identify and label basal bodies of ciliated cells that had received the CRISPR RNPs, allowing us to analyze CRISPR-targeted and wild-type cells in the same images. Cilia appeared to be fewer and shorter in length, which was confirmed by quantification of cilia in *ift80* and *ift172* knockout cells (Fig. 2 K and L). In contrast, basal body number and apical docking was not altered in crispant MCCs when compared with controls (SI Appendix, Fig. 3G). Thus, the ciliogenesis defect is consistent with findings in other model organisms (45, 71) and a likely cause for impaired tubular uptake.

To gain more detailed insights into the molecular signals affected by a loss of *ift80* or *ift172*, we investigated the transcriptional profile of embryos targeted for *ift80* and *ift172*. Because embryos were in vitro fertilized and developed synchronously, stage-dependent variability was minimized between control and CRISPR-targeted embryos. We isolated DNA and confirmed a high indel frequency in both conditions (SI Appendix, Fig. 4 A and B). The top-most down-regulated genes were *ift80* and *ift172*, respectively (SI Appendix, Fig. 4 C–E and Dataset S1), confirming the specificity of our targeting strategy and a direct effect on mRNA levels likely due to nonsense-mediated decay. We found 15 (*ift80*) and 5 (*ift172*) genes to be differentially regulated in either condition (SI Appendix, Fig. 4 D and E) and three genes (abcb1 and two uncharacterized transcripts) to be significantly up-regulated in both conditions. The ATP-dependent multidrug-resistance transporter Abcb1 is transcriptionally
regulated by Gli1 and β-catenin (72, 73). Interestingly, an additional five genes that only reached significance in one condition were also associated with Hh signaling (cng1, uk1, rik3, olf4m, and coll1a1) and four with Wnt signaling (c1ar1, col1a1, olf4m, and uk1) (SI Appendix, Fig. 4F) (72, 74–82). Thus, we detect Hh- and Wnt-dependent gene regulation to be altered upon depletion of ift80 and ift172 in Xenopus embryos. mRNA expression analysis in wild-type embryos of differentially regulated genes identified by RNA-Sequencing (cng1, uk1, and rik3) found an overlapping expression with chondrodysplasia genes in neural tissue and neural crest (SI Appendix, Fig. 4G). However, we did not find a change in the Sonic Hedgehog (Shh) marker nks2.2 after unilateral ift80 or ift172 targeting, suggesting that the differences in mRNA abundance are below the detection level of in situ hybridization (SI Appendix, Fig. 4 H and I).

Data Mining Identifies Skeletal Ciliopathy-Associated Genes. Next, we aimed to identify novel proteins related to the pathogenesis of SC. We reasoned that proteins with similar molecular properties, such as subcellular localization, common protein interactors, or differential regulation in loss-of-function models, could be unrecognized components of the pathogenic molecular mechanism. Therefore, we assembled lists of genes from 90 published genome-wide screens or unbiased analyses (‘resource lists’) (Dataset S1) based on literature curation. We identified 16 resource lists to be significantly enriched for genetically validated chondrodysplasia genes in comparison with 10 million random draws of an equal number of query genes (Fig. 3A). Within these 16 lists, 65 unique genes not yet linked to chondrodysplasia were found to be significantly enriched (Dataset S1). Among these potential candidate genes were three that have been identified as bona fide chondrodysplasia genes (CEP192, Ttc30b, and IFT70) (90, 92). CEP192 (centrosomal protein of 192 kDa) is a number of query genes (Fig. 3A). In conclusion, CRISPR targeting ttc30a in Xenopus replicated all phenotypes observed when targeting the chondrodysplasia genes ift80 and ift172.

To elucidate the expression pattern of mammalian orthologs of ttc30a, we analyzed publicly available single-cell sequencing data of 13.5 mouse limb buds (SI Appendix, Fig. 6 A–E) (93). We found that among the three orthologs, Ttc30b and to a lesser degree Ttc30a1 were most prominently expressed in cartilage, bone, and growth plate clusters (Fig. 5 E–G). The distribution of cell proportions expressing Ttc30b was similar to that of ift80 and ift172 (Fig. 5 G and H). Thus, the expression pattern of Ttc30a and Ttc30a1 suggests a potential role in cartilage formation of mammals. We screened for TTC30A/B mutations in a cohort of 24 unresolved severe ciliary chondrodysplasia cases by exome sequencing, but no likely pathogenic variants were identified. Screening of whole-exome sequencing data in a worldwide cohort of more than 500 patients with nephronophthisis related ciliopathies and cystic kidney disease also did not reveal potentially causative recessive mutations in either TTC30A or TTC30B.

Ciliary Compartmentalization Is Altered in ttc30a- and ift172-Depleted Cilia. Ttc30a mediates tubulin modifications such as glycylation and glutamylination to be deposited within the ciliary axoneme (15, 91, 92). To elucidate the expression pattern of mammalian orthologs of ttc30a, we analyzed publicly available single-cell sequencing data of 13.5 mouse limb buds (SI Appendix, Fig. 6 A–E) (93). We found that among the three orthologs, Ttc30b and to a lesser degree Ttc30a1 were most prominently expressed in cartilage, bone, and growth plate clusters (Fig. 5 E–G). The distribution of cell proportions expressing Ttc30b was similar to that of ift80 and ift172 (Fig. 5 G and H). Thus, the expression pattern of Ttc30a and Ttc30a1 suggests a potential role in cartilage formation of mammals. We screened for TTC30A/B mutations in a cohort of 24 unresolved severe ciliary chondrodysplasia cases by exome sequencing, but no likely pathogenic variants were identified. Screening of whole-exome sequencing data in a worldwide cohort of more than 500 patients with nephronophthisis related ciliopathies and cystic kidney disease also did not reveal potentially causative recessive mutations in either TTC30A or TTC30B.

CRISPR/Cas9 Targeting ttc30a Replicates the Phenotype of ift80- and ift172-Targeted Embryos. We designed sgRNAs targeting all Xenopus orthologs of the four candidates for CRISPR/Cas9-mediated loss-of-function experiments. Interestingly, cfap20 and ttc30a crisps formed edema suggestive of embryonic renal excretion defects. This effect was specific, as coinjection with corresponding mRNAs significantly rescued the edema phenotype (Fig. 4 B and C and SI Appendix, Fig. 5 A–E). In order to evaluate a potential effect on limb development, we raised cfap20, ttc30a, c11orf74, and cep192 CRISPR-targeted tadpoles to metamorphosis. Limb formation of cep192, c11orf74, and cfap20 crisps was normal (SI Appendix, Fig. 5 G and H). Froglets with ttc30a mutations, however, developed severe limb defects (Fig. 4D, green arrowheads and SI Appendix, Fig. 5F). Extremities were generally shorter with polydactyly (Fig. 4D and SI Appendix, Fig. 5 F and H). MicroCT scans found large depopulations of cartilage. Histology revealed a chondrocyte differentiation defect, as previously observed for ift80- and ift172-targeted animals. Ttc30a-targeted embryos had reduced fore- and hindlimb length (Fig. 4D), and analysis of the kidneys revealed a significantly higher number of cysts on the injected side of the froglets (Fig. 4E). However, total cyst and kidney volume did not change significantly (Fig. 4E). Excretion assays confirmed that renal tubular uptake of dextran was drastically reduced (Fig. 5 A and B and Movie 2), and epidermal cilia were shorter or absent in embryos injected with sgRNA and RNPs targeting ttc30a (Fig. 5 C and D). In conclusion, CRISPR targeting ttc30a in Xenopus replicated all phenotypes observed when targeting the chondrodysplasia genes ift80 and ift172.

Discussion
SC are caused by pathogenic mutations in genes that encode ciliary proteins. Thus, Mendelian genetics put the spotlight on the primary cilium as a central organelle in the pathophysiology of chondrodysplasias. This led to the insight that several clinically distinct disease entities may have a common molecular origin and are now recognized as a united disease group (4). Today, a
In silico screening for candidates with similar properties to SC genes. (A) Schematic of the in silico screen. Known SC disease genes served as an input list. Resource lists represent datasets from various published screening approaches and were tested for enrichment of genes contained in the input list genes. Genes in significantly enriched resource lists were then scored based on membership or rank. The gene scores were statistically validated by an empirical P value based on 10 million random drawings of input list. (B) Whole-mount in situ hybridizations for four candidate genes (c11orf74, cep192, cfap20, and ttc30a) that were experimentally followed up are shown for tadpoles at stage 31 to 35 (nephrostome expression enlarged) and limb buds (stage 55 to 57). (Scale bars, 0.5 mm.) p, posterior; a, anterior; pr, proximal; and d, distal.

Fig. 3. In silico screening for candidates with similar properties to SC genes. (A) Schematic of the in silico screen. Known SC disease genes served as an input list. Resource lists represent datasets from various published screening approaches and were tested for enrichment of genes contained in the input list genes. Genes in significantly enriched resource lists were then scored based on membership or rank. The gene scores were statistically validated by an empirical P value based on 10 million random drawings of input list. (B) Whole-mount in situ hybridizations for four candidate genes (c11orf74, cep192, cfap20, and ttc30a) that were experimentally followed up are shown for tadpoles at stage 31 to 35 (nephrostome expression enlarged) and limb buds (stage 55 to 57). (Scale bars, 0.5 mm.) p, posterior; a, anterior; pr, proximal; and d, distal.

Validation of Candidates

10 million randomly drawn input lists, empirical p-value for each candidate:

\[ PVaI = P(\sigma_i^{final}(input)) \geq \sigma_i^{final}(resource) \]

A trove of existing datasets, proteomic, and functional screens have contributed to a better understanding of the protein composition of cilia (105, 106). Given that our data-mining approach worked well with chondrodysplasia genes, we used all gene sets annotated in phenotypic series in Online Mendelian Inheritance in Man (OMIM) as input. A significant number of enriched lists could be identified for 13 disease entities, many of which were ciliopathies (e.g., Meckel syndrome, nephronophthisis, and primary ciliary dyskinesia; Dataset S2). Interestingly, Ttc30a was also included in the list of candidates.
candidate-gene list for nephronophthisis. We created a web application to make the resource lists used in our in silico screen accessible and searchable (http://dormantdata.org). Adding further datasets and using novel machine learning algorithms may further enhance the power of a data-driven discovery process. Such refinements could make this approach applicable to other nonciliary diseases in the future or complement prioritization of potential pathogenic loci.

By in vivo modeling, we validated the in silico data and identified Ttc30a as a protein involved in chondrocyte differentiation and renal function. It is likely that not all SC-relevant components are known, and potentially deleterious variants may be too rare to be identified by Mendelian genetics because the natural mutation load does not reach saturation for all possible pathogenic mutations to occur or lead to early embryonic lethality. In addition, essential proteins may be protected by genetic compensation of functionally redundant genes (107, 108). The human TTC30A/B locus may represent a critical site that is protected by early and reoccurring duplication events in the tetrapod phylogeny that in-creasingly relied on limbs for locomotion and dexterity (Fig. 4C). Coinjection of ttc30a mRNA partially rescued the phenotype. (D) Unilaterally CRISPR-targeted ttc30a frogslets developed shortened limbs on the injected side (green arrowhead). MicroCT analysis, 3D reconstructions, and histological sections stained with hematoxylin-eosin demonstrated accumulation of car-tilage (green arrowhead). Quantification of limb length reduction of side (green arrowhead). MicroCT analysis, 3D reconstructions, and histological sections stained with hematoxylin-eosin demonstrated accumulation of cartilage (green arrowhead). Quantification of limb length reduction of ttc30a targeted compared with nontargeted side. (E) MicroCT scans show cystic kidneys in ttc30a-targeted animals (red arrowhead). 3D reconstruction of the kidneys and cysts (red) were used for volumetric analysis. Quantifications of cyst number, the ratio of total cyst volume to kidney volume, and total kidney volume (excluding cysts). [Scale bars, (B) 0.5 mm, (D and E, 3D reconstruction) 2 mm, (D, histological section) 200 μm.] R, right; L, left; P, proximal; D, distal. P > 0.05 ns (not significant); *P < 0.05; **P < 0.01; ****P < 0.0001.

Both PTMs stabilize tubulin complexes and are necessary for cilia motility (92). Interestingly, polyglutamylation regulates velocity of the Kinesin-2 complex and therefore the uptake of Hb components (Smo and Gli3) after signaling activation and has recently been found to depend on the Joubert syndrome–associated proteins ARL13B and CEP41 (18, 19, 114). Loss of the Joubert syndrome–associated proteins ARMCO, TOGARAM1, and Kif7 also decreases acetylation (115). Knockout of Kif7 affects PTMs mainly at the tip of the cilia (116, 117). In our model, we find the strongest effect on tubulin glycylcylation and acetylation in the ciliary tip. On one hand, particle turnaround from anterograde to retrograde transport occurs at the tip, and ifi172 is involved in this process (118). Furthermore, the tip is a crucial compartment for Hh-signaling molecule activation, which is disrupted in Kif7 knockout cells that also show decreased acetylation and glutamylation (117).

Our findings suggest that alterations of posttranslational tubulin modifications may contribute to the pathogenesis of ciliopathies.
percentage of cells per cluster expressing clusters. [Scale bars, (A and B) Excretion assay of slc45a2 sgRNA–injected controls and ttc30a-targeted embryos. Blue arrowheads point to the proximal tubule, and yellow arrowheads highlight fluorescent dextran in the distal tubules as a measure of excretion. (C) Confocal images of epidermal MMCs of stage-36 embryos. centrin-RFP (red) marks cells targeted for ttc30a (red arrowheads). Blue arrowheads label wild-type cells. Cilia are stained with acetylated gamma-tubulin (cyan). ([C and D] Detection of Ttc30b expression in respective cell clusters. (E) Clustering of scRNA-Seq data from E15.5 mouse limbs into 10 different cell clusters using typical marker genes. (F) Detection of Ttc30b expression in respective cell clusters. (G) Comparison of percentage of cells per cluster expressing Ttc30a1, Ttc30a2, Ttc30b, Ift80, and Ift172. (H) Expression level of Ttc30b, Ift80, and Ift172 in respective tissue clusters. [Scale bars, (A) 0.5 mm and (C) 10 μm.] GP, growth plate.

Beyond Joubert syndrome, such as chondrodyplasia and nephronophthisis. Intriguingly, the ciliary localization of PKD2 was found to depend on tubulin glutamylation, suggesting a potential role in renal cystogenesis (18, 119).

Like glutamylation, tubulin glycylation appears to be present only in primary cilia of certain cell types. Glycylation was detected in Madin–Darby Canine Kidney (MDCK) cells but absent in mouse inner medullary collecting duct (mIMCD3) and human retinal pigment epithelial (RPE-1) cells (16). Only a subset of cilia in the zebrafish spinal cord and pronephros are glycylated, but none in Kupffer’s vesicle (92). We found that motile cilia in the neural tube and nephrostomes were glycylated but not primary cilia in pronephric tubules. In contrast, both types of cilia are glutamylated. Spinal cord cilia were neither acetylated nor glutamylated but only glycylated. Thus, tubulin amino acid PTMs not only constitute a part of the “tubulin code” that diversifies ciliary functions, but its disruption may contribute to the pleiotropic phenotypes of ciliopathies (119).

TTC30A/B are known ciliary components but have not been associated with skeletal defects. In human tissue, TTC30A and TTC30B are strongly expressed in mammalian ciliated tissues, such as airway epithelia or ductuli efferentes in the epididymis (120). Consistent with our own analysis, single-cell RNA-Sequencing data of embryonic mice revealed an enhanced expression of ttc30a1, ttc30a2, and ttc30b in osteoblasts and chondrocytes (Fig. 5 E–H) (121). A rare missense variant in TTC30B (rc.1157C > T, p.A375V) has recently been described in a large Chinese family with polydactyly, suggesting an autosomal-dominant mode of inheritance (122). However, no other unifying clinical phenotypes were observed in the 27 affected family members. In vitro analysis suggested that down-regulation of TTC30B had an inhibitory effect on Shh-pathway stimulation, consistent with our findings that Ttc30a has a role in limb patterning. Hh signaling does not influence cystogenesis in autosomal polycystic kidney disease, suggesting that other ciliary mechanisms are more likely to be at play (123). Further investigation will need to elucidate which signals are disrupted by loss of Ttc30a in renal cyst formation and which other molecular signals are affected by disrupted posttranslational tubulin modifications and defective ciliary compartmentalization. These insights will be essential to improve the understanding of the pathogenesis of nephronophthisis, chondrodysplasia, and other ciliopathies.

Materials and Methods

Animal Maintenance and Handling. Adult animals were kept according to the German and Swiss law for care and handling of research animals. Husbandry and treatment were approved by the local authorities (Regierungspräsidium Freiburg and Veterinäramt Zürich).

Microinjection. Embryos were obtained by in vitro fertilization (X. laevis and X. tropicalis) or natural mating (X. tropicalis). For injections, embryos were
Fig. 6. Ttc30a affects posttranslational tubulin modifications and ciliary compartmentalization. (A) Glycylation (cyan) in combination with acetylation (red) and (B) glutamylation (cyan) of pronephric and neural tube cilia were detected by immunostaining in wild-type embryos at stage 39. Cells of the pronephros and neural tube were visualized by Lectin staining (white/gray). White arrowhead points to ventral cilia of the neural tube, and red arrowhead to primary cilia of the pronephric tubular system. Stainings of the nephrostomes and tubular system are also shown as single channels. L, lateral; M, medial; D, dorsal; V, ventral; gly, glycylation; lec, lectin; glu, glutamylation; and acet, acetylation. Influence of targeting ift80, ift172, and ttc30a by CRISPR/Cas9 on glycylation (C) and glutamylation (D) was analyzed by immunostainings of epidermal cilia at stage 36 (glutamylation/glycylation, cyan; acetylation, red). (E and F) A schematic model for the role of ttc30a during bone and kidney development. (E) Ttc30a interacts with Kif17 in the cilium and is crucial for tubulin modifications (blue, glycylation; red, glutamylation; and yellow, acetylation) (15, 92, 112). Mutations of ttc30a in Xenopus reduce acetylation and glycylation, negatively impact cartilage differentiation, and result in kidney cysts (F). Green, intraflagellar transport (IFT)-B proteins; red circled, known SC genes; gray, ciliary tubulin. Error bars indicate SEM. *P < 0.05; **P < 0.01; and ***P < 0.001. (Scale bars, 10 μm.)
Analysis of CRISPR/Cas9 Frogslets. Frogslet for limb analysis were euthanized and fixed in MOPS pH 7.4, 2 mM EDTA, 1 mM MgSO4, 3.7% formaldehyde (MEMFA). Iodine contrast enhancement was done by washing frogslet in 1x phosphate-buffered saline for 2 d, followed by dehydration to ethanol, and staining in 1% iodine solution (Sigma-Aldrich, 20772) in ethanol. After washing with 100% ethanol, the animals were scanned with a Caliper Quantum Fx from PerkinElmer. Imaris 9.5.1 was used to determine the volume of kidneys and kidney cysts. The length of limbs was measured with Fiji software (unranked and critical parameters used as a basis for the statistical analysis by multiple testing by the Benjamini–Hochberg procedure (false discovery rate < 0.05).

Data Availability. All study data are included in the article and/or supporting information. The raw data associated with SI Appendix, Fig. 4 C–E was deposited at the Sequence Read Archive (SRA) with the BioProject accession number PRJNA670560 (125). The code used in the in silico screen is publicly available at GitHub, https://github.com/genespi-freiburg/Geneset (126).

ACKNOWLEDGMENTS. We are grateful for technical assistance from Alena Sammarco, Jessica Kleindienst, Marko Vujanovic, Claudia Meyer, the staff of the Life Imaging Center Freiburg at the Center for Biological Systems Analysis of the University of Freiburg, the Center for Microscopy and Image Analysis, and the Zurich Integrative Rodent Physiology of the University of Zurich. This study was supported by the German Research Foundation to S.S.L. (Emmy Noether Program Li-18172/1-P, P.S. (Project 192904750-CRC 992 Medical Epigenetics), A.K.O (KO 35985-1), Project-ID 431984000-SFB 1453 Nephrogenetics and Centre for Integrative Biological Signalling CBIBS–EXC–2189–Project ID 390039984), and E.L. (Project-ID 431984000-SFB1453 Nephrogenetics), the European Union’s Horizon 2020 research and innovation programme to E.L. (MCDS-Therapy Grant Agreement No. 754825) and S.S.L. (DIRECT Grant Agreement No. 804474), the EQUIP Program for Medical Scientists, Faculty of Medicine, University of Freiburg to P.S., the NIH (No. R01DK68306) to F. Hildebrandt, and the Swiss National Science Foundation to S.S.L. (National Centre of Competence in Research NCCR Kidney.CH) and (Project ID 310030_189102/1).

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