Report

Mutations in *NRIP1* cause dominant urinary tract malformations *via* dysregulation of retinoic acid signaling

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ABSTRACT

Congenital anomalies of the kidneys and urinary tract (CAKUT) are the most common cause of chronic kidney disease in the first three decades of life. Identification of monogenic mutations that cause CAKUT permits insights into related disease mechanisms. However, for most cases of CAKUT the causative mutation remains unknown. We identified a kindred with an autosomal-dominant form of CAKUT. By whole exome sequencing, we identified a heterozygous truncating mutation (c.279delG, p.Trp93fs*) of the *NRIP1* gene in all seven affected members. *NRIP1* encodes a nuclear receptor transcriptional co-factor, which directly interacts with the retinoic acid receptors to modulate retinoic acid transcriptional activity. Functional studies revealed that the NRIP1 altered protein does not translocate to the nucleus, does not interact with retinoic acid receptor alpha (RARα) and fails to inhibit retinoic acid dependent transcriptional activity. In addition, we show that *NRIP1* expression as well as its binding to retinoic acid receptor alpha, is enhanced in the presence of retinoic acid. Expression and knockdown experiments in *Xenopus laevis* further confirmed a role for NRIP1 in renal development. These data indicate that dominant *NRIP1* mutations cause CAKUT by interference with retinoic acid transcriptional signaling, thus shedding light on the well documented association between abnormal vitamin A levels and renal malformations, and suggest a possible gene environment pathomechanism.

INTRODUCTION

Congenital anomalies of the kidneys and urinary tract (CAKUT) constitute the most frequent cause of chronic kidney disease in the first three decades of life, accounting for ~50% of all cases[1; 2]. CAKUT comprises a wide range of structural malformations that result from defects in the morphogenesis of the kidneys and/or the urinary tract[3-6]. The pathologic basis of CAKUT lies in the disturbance of normal kidney development, primarily resulting from mutations in genes that direct this process. Most gene products that cause CAKUT in humans or mice if altered are transcription factors involved in protein-protein interactions and form large transcription complexes[7-13]. These monogenic forms of CAKUT are often inherited in an autosomal dominant manner and exhibit the clinical features of incomplete penetrance and variable expressivity. These features of loose genotype/phenotype correlation, as well as extensive genetic heterogeneity, has rendered gene discovery in CAKUT very difficult. Although ~30 CAKUT genes have been identified[1; 2; 14], approximately 85% of patients with CAKUT do not have mutations in any known genes[15; 16]. Hence, a remaining challenge is to identify the missing components of the pathogenesis of CAKUT, in order to understand how those proteins and transcription factor complexes exert their developmental and tissue specific functions.

In addition to the clear importance of genetic factors on the proper development of the kidney and urinary tract, numerous studies also support the influence of environmental factors on normal and abnormal kidney development [4; 17], suggesting that the pathogenesis of CAKUT can be multifactorial, likely due to a complex interplay between genes and environment. The most prominent example for this is the effect of retinoic acid, the active form of vitamin A, on the kidneys and urinary tract during development. During fetal life, nephrogenesis is influenced by retinoic acid level, such that even moderate maternal vitamin A alterations in either direction can cause CAKUT in humans and rodents[18; 19]. In addition, elegant studies of murine kidney development by Mendelsohn et al. have shown that inactivation of genes in the retinoic acid pathway causes CAKUT in mice [20-22].

To gain further insights into the pathogenesis of CAKUT, we investigated a three generation family with renal malformations by whole exome sequencing (WES). We here identify dominant mutations in the transcription co-factor *NRIP1* gene as causing human autosomal dominant CAKUT by interference with retinoic acid transcriptional signaling, thus shedding light on the well documented association between retinoic acid and renal malformations.

METHODS

Study participants

Following informed consent, we obtained clinical data, pedigree data, and blood samples from individuals with CAKUT from worldwide sources using a standardized questionnaire. Approval for human subjects' research was obtained from the Institutional Review Boards of the University of Michigan, Boston Children's Hospital, Sheba Medical Center, and from other relevant local Ethics Review Boards. Informed consent was obtained from the individuals and/or parents, as appropriate. The diagnosis of CAKUT was made by (pediatric) nephrologists and/or urologists based on relevant imaging.

Whole exome sequencing

To identify a causative mutated gene for CAKUT we investigated family members from a four-generation Yemenite-Jewish family (**Fig. 1**) with an autosomal dominant form of CAKUT characterized predominantly by lower urinary tract involvement, renal hypodysplasia and/or ectopia (**Table 1**). DNA samples from 6 affected individuals (**Fig. 1**) were subjected to whole exome sequencing (WES) as established previously using Agilent SureSelect™ human exome capture arrays (Life Technologies) with next generation sequencing (NGS) on an Illumina™ sequencing platform. Sequence reads were mapped against the human reference genome (NCBI build 37/hg19) using CLC Genomics Workbench (version 6.5.1) software (CLC bio). Mutation calling under an autosomal dominant model was performed by geneticists and cell biologists, who had knowledge regarding clinical phenotypes, pedigree structure, genetic mapping, and WES evaluation (**Table S1**) and in line with proposed guidelines.[23; 24] Sequence variants that remained after the WES evaluation process were examined for segregation.

Whole exome sequencing (WES) analysis

Following WES, genetic variants were first filtered to retain only heterozygous, non-synonymous variants that were shared between the 6 affected relatives of family H subjected to WES. Second, filtering was performed to retain only alleles with a minor allele frequency (MAF) <0.1%, a widely accepted cutoff for autosomal dominant disorders.[25; 26] MAF was estimated using combined datasets incorporating all available data from the 1,000 Genomes Project, the Exome Variant Server (EVS) project, dbSNP138, and the Exome Aggregation Consortium (ExAC). Third, observed sequence variants were analyzed using the UCSC Human Genome Bioinformatics Browser for the presence of paralogous genes, pseudogenes, or misalignments. Fourth, we

scrutinized all variants with MAF<0.1% within the sequence alignments of the CLC Genomic Workbench™ software program for poor sequence quality and for the presence of mismatches that indicate potential false alignments. Fifth, we employed web based programs to assess variants for evolutionary conservation, to predict the impact of disease candidate variants on the encoded protein, and to predict whether these variants represented known disease causing mutations. Finally, Sanger sequencing was performed to confirm the remaining variants in original DNA samples and to test for familial segregation of phenotype with genotype. Variants were also tested for absence from in-house control populations of 200 individuals with nephronophthisis, and 429 individuals with steroid resistant nephrotic syndrome.

Copy number variants analysis

One individual from the index family (Family H individual III8; **Fig. 1A**) was analyzed for copy number variations. Genome-wide genotyping for copy number variant (CNV) analysis was performed on an _____ platform as previously described. Genotype calls and quality-control analyses were performed with Genome Studio v.2010.3 (Illumina) and PLINK software. The CNV calls were determined with generalized genotyping methods implemented in the PennCNV program. CNVs were mapped to the human reference genome hg18 and annotated with UCSC RefGene and RefExon (CNVision program25**).(Hagith Yonath please fill)**

High-Throughput NRIP1 mutation analysis

Massively parallel sequencing of all *NRIP1* exons was performed in ~600 individuals from ~300 different families with CAKUT from different pediatric nephrology centers worldwide using microfluidic PCR (Fluidigm™) and next-generation sequencing (MiSeq™, Illumina) as described previously. Variants were filtered against public variant databases (http://evs.gs.washington.edu/EVS) and only novel heterozygous variants were considered, confirmed by Sanger sequencing, and tested for segregation with the CAKUT phenotype.

cDNA cloning

Full-length human NRIP1 cDNA and retinoic acid receptor alpha (RARα) cDNAs were subcloned by PCR from full-length cDNA (cDNA clone MGC:_____ IMAGE:_____ and cDNA clone MGC:____ IMAGE:_____, respectively). Expression vectors were generated using LR Clonase (Invitrogen) according to the manufacturer's instructions. The following expression vectors were used in this study: pRK5-N-Myc, pcDNA6.2-N-GFP, and p____-FLAG (ASK DANIELA WHAT

FLAG EXPRESSION VECTOR IS). Mutagenesis was performed using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) to generate a clone with the NRIP1 mutation identified in Family H.

Cell culture, transient transfections, and retinoic acid treatment

The experiments described were performed in HEK293 cells purchased from the American Type Culture Collection biological resource center. For transient transfections, HEK293 cells were seeded at 60-70% confluency in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and 1% penicillin/streptomycin and grown overnight. Transfections were carried out using Lipofectamine2000 (Invitrogen) and OptiMEM (????) following the manufacturer's instructions. For experiments involving treatment with retinoic acid (RA), HEK293 cells were treated with 500 nM RA 24 hours after transfection. Experiments were then carried out 24 hours after continuous treatment with RA.

NRIP1 reporter gene assays

For reporter assays (Dual luciferase reporter assay system, Promega) HEK293 cells were seeded in 24-well dishes and transfected with constant amounts of reporter plasmids (kind gift from Li-Na Wei) and 100 ng of pRL-TK Renilla Luciferase™ for normalization. The total amount of expression plasmid was kept constant by adding empty pcDNA6.2-N-GFP. Per transfection, 500 ng of **pGL3.RARE ()-luciferase reporter plasmid** (which harbors four retinoic acid response elements: _____) and 500 ng of pcDNA6.2-N-GFP.NRIP1 expression plasmid were used. For competition experiments, the amount of pcDNA6.2-N-GFP.NRIP1_WT was held constant and increasing amounts of pcDNA6.2-N-GFP.NRIP1_p.Trp93fs* was added. Firefly luciferase™ and Renilla luciferase™ activities were measured 24 hours after transfection. All transfections were performed in triplicate, and individual experiments were repeated at least three times. After normalization, the mean luciferase activities and standard deviations were plotted as "fold activation" when compared with the empty expression plasmid. p values were determined using the Student's t-test.

Immunofluorescence and confocal microscopy in cell lines

For immunostaining, HEK293 cells were seeded on Fibronectin-coated coverslips in 6-well plates. After 16-24 hours, cells were transiently transfected using Lipofectamine2000 (Invitrogen) following the manufacturer's instructions. Experiments were performed 24–48 hours after transfection. Cells

were fixed for 15 min using 4% paraformaldehyde and permeabilized for 10 min using 0.1 **(? Double check this)** % Triton X-100. After blocking with 10% donkey serum + BSA, cells were incubated with primary antibody overnight at 4°C. The following day, cells were incubated in secondary antibody for 60 min at room temperature, and subsequently stained for 5 min with 1× DAPI in PBS. Confocal imaging was performed using the Leica SP5X system with an upright DM6000 microscope, and images were processed with the Leica AF software suite. Immunofluorescence experiments were repeated at least two times in independent experiments. The following antibodies were used for immunofluorescence experiments: mouse anti-Myc (sc-40, Santa Cruz Biotechnology) and rabbit anti-NRIP1 (ab42125, Abcam), both diluted 1:100. Donkey anti-mouse secondary antibodies conjugated to Alexa Fluor 488 or 594 and donkey anti-rabbit secondary antibody conjugated to Alexa Fluor 594 were purchased from Invitrogen.

Co-immunoprecipitation assays

Co-immunoprecipitation experiments were performed using protein lysates from transfected HEK293 cells. Cell lysates were precleared with Protein G (? Or A) Sepharose beads (GE Healthcare -- CONFIRM) at 4°C overnight in IP lysis buffer (_____) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and EDTA (_____). Co-immunoprecipitation of Myc-tagged proteins was performed using Myc agarose beads (Invitrogen?). The beads were washed five times with lysis buffer and proteins were eluted from the beads by incubating in loading buffer for 30min at 30°C (DOUBLE CHECK TIME AND TEMP). Samples were analyzed by Western blot with anti-Myc (sc-40, Santa Cruz Biotechnology) and anti-FLAG (____, Sigma) antibodies at 1:1000 dilution. Horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Santa Cruz Biotechnology. 10% of the input was loaded as a control. Experiments were repeated at least three times in independent experiments.

Mice methods (*in situ* and RA dependency in explant cultures.)

Dear Andreas, please add methods text in relation to Fig 3C and Fig 4. Thanks!

Methods : Xenopus laevis experiments SOEREN – please add text. Thanks!

RESULTS

NRIP1 mutations cause CAKUT

To identify a causative mutated gene for CAKUT we investigated a four-generation Yemenite Jewish family with seven individuals who have congenital anomalies of the kidneys and urinary tract (CAKUT). Renal dysplasia was the predominant phenotype (5 individuals) together with Vesicoureteral reflux (VUR) (4 individuals) and/or ectopia (2 individuals) (**Fig. 1A & B, Table 1 and Supplementary Fig. 1**). The age at diagnosis ranged from the prenatal period to late adulthood (**Table 1**). Two of the seven affected individuals underwent surgical intervention including unilateral nephrectomy for their malformations. None of the affected individuals had extra-renal malformations or syndromic features. The pedigree structure was compatible with an autosomal dominant mode of inheritance with variable expressivity and incomplete penetrance (**Fig. 1A**). Individual II:1 had renal US showing bilateral small renal cyst (**Supplemental Fig. 1**), which can be a common finding in his age group and is not necessarily related to the CAKUT phenotype in this family. Therefore, for the initial WES analysis, which considered affected only members, individual II:1 was not included. Under the hypothesis that mutation of an autosomal dominant gene causes CAKUT in this family, we selected six affected family members (individuals III:3, III:4, III:5, IV:7, IV:8 and IV:9) for whole exome sequencing (WES). Given the pedigree structure (**Figure 1A**), the six affected individuals are expected to share about 3.125% of all alleles by descent, allowing us to reduce 32-fold the thousands of variants from normal reference sequence that are expected to result from WES (**Table S1**). We identified a heterozygous truncating mutation (c.279delG; p.Trp93fs*) in the gene encoding the *Nuclear Receptor Interacting Protein 1* (*NRIP1*) [MIM 602490, RefSeq accession number NM_003489.3] (**Table 1 and Fig. 1C-D**). Segregation analysis revealed that this mutation was shared by all seven available affected family members as well as by individual II:1, who as expected from the pedigree structure, is an obligatory mutation carrier given that his brother (indevidual II:8) was found to have CAKUT on renal ultrasound (**Fig. 1A and Table 1**) and to harbor the *NRIP1* mutation (**Fig. 1A**). In addition, the mutation was absent from all 7 unaffected family members available for study, from all available databases of healthy controls (**Table 1**), as well as from two in-house control cohorts of 200 individuals with nephronophthisis and 429 individuals with nephrotic syndrome. Finally, we also showed by CNV analysis that the affected index patient (IV:8) lacked heterozygous deletions that have previously been associated with CAKUT in the *HNF1B* locus and the DiGeorge/velocardiofacial syndrome locus.[27]

The NRIP1 mutation interferes with nuclear translocation, transcriptional repression, and* NRIP1 *interaction with RARα

NRIP1 is a nuclear receptor transcriptional co-factor [28]. Previous biochemical analyses showed that the murine NRIP1 protein harbors two putative nuclear localization signals (NLS) and four repression domains (RD) that mediate its transcriptional repression (ref) (**Fig. 1C**). In addition, it has been shown to directly interact with the retinoic acid receptors (RAR and RXR) and to suppress their retinoic acid mediated transcriptional activity (Farooqui, 2003; ref). The NRIP1 protein contains ten nuclear receptor interacting motifs (LxxLL) spread throughout the molecule (**Fig. 1D**) (Augereau, 2006). Interestingly, the binding of NRIP1 to retinoic acid receptors (RAR) and retinoic X receptors (RXR) was suggested to also require a slightly different sequence, an LxxML motif, located at the protein's C-terminal end (Chen et al 2002).

To examine how the c.279delG (p.Trp93*) mutation, which segregated with the CAKUT phenotype in the large family H conveys *NRIP1* loss of function, we evaluated *NRIP1* for its known functional features. These include: i) nuclear localization, ii) transcriptional repression, and iii) interaction with retinoic acid receptor alpha (*RARα).* Transfection of expression constructs in HEK293 cells revealed that, whereas the wild-type NRIP1 protein translocated into the nucleus – the NRIP1 altered protein (p.Trp93fs*) predominantly remained localized in the cytoplasm (**Fig. 2A, 2B**). In target cells, retinoic acid (RA), the active form of vitamin A, acts as a ligand for nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The complex binds to a regulatory DNA segment, the RA response element (RARE), to control transcription of RA target genes (16. Cunningham, 2015). Accordingly, we next determined whether the *NRIP1* mutation affects NRIP1 retinoic acid dependent transcriptional activity using NRIP1 expression plasmids and a reporter plasmid harboring retinoic acid response elements (RARE) (ref). While the wild-type NRIP1 expression construct acts as transciptional repressor, and completely supressed retinoic acid mediated transcriptional activity, the mutant construct (p.Trp93fs*) showed lack of transcriptional repressor activity (**Fig. 2C**).

We previously showed that monogenic CAKUT can be secondary to a dominant negative pathomechanism. To test this possibility, using a luciferase reporter assay we co-transfected NRIP1 wild-type construct with increasing amounts of the mutant NRIP1. This did not yield a dose-dependent lack of repression, thereby not supporting a dominant negative effect of the mutant protein on transcriptional activity of the wild type protein (**Supplemental Fig. 2**). Furthermore, since proteins that hetero/homo dimerize are particularly prone, when altered, to exert dominant-negative effects by sequestering functioning molecules into inactive dimers, we tested the

interaction between the altered NRIP1 protein and retinoic acid receptor alpha. Notably, the altered NRIP1 protein still contains one intact interaction motif (**Fig. 1C**), which, according to previously solved ligand binding domains crystalized structures (ref), may theoretically be sufficient to interact with the nuclear receptor ligand binding domain of the RARα (ref). We performed overexpression experiments of RARα with either wild-type or mutant *NRIP1*. Interestingly, the wild-type NRIP1 protein co-localizes with RARα in a speckled nuclear pattern, while, when over-expressed with the altered NRIP1 protein, RARα localized diffusely in the nucleus while the mutant NRIP1 remained in the cytoplasm (**Fig. 2D**), making a dominant negative pathomechanism unlikely. Furthermore, by co-immunoprecipitation experiments we show that the *NRIP1* mutation abrogates NRIP1-RARα interaction (**Fig. 3A-B**), again arguing against a dominant negative pathomechanism.

Retinoic acid increases NRIP1 binding to RARα in vitro and increases NRIP1 mRNA expression in mouse explant organ cultures

Formation of the ureter and kidney begins with ureteric budding when this epithelial tube sprouts from the base of the Wolffian ducts just above the urogenital sinus - an embryonic structure which will eventually give rise to the urinary bladder. Distally the ureteric bud invades the renal metanephric mesenchyme and, following successive branching, gives rise to the renal collecting duct system. The ureteric bud stalk differentiates into the ureter, and its proximal part is transposed to the primitive bladder via the common nephric duct. Elegant studies have shown that the common nephric duct undergoes apoptosis thereby severing connections with the Wolffian duct as the ureter orifice is transposed to its final insertion site in the bladder ureter (Batourina, 2005). This crucial step is controlled by retinoic acid induced signals which govern ureter maturation and formation of proper connections between the bladder and ureter (Batourina, 2005).

In order to further characterize the role of NRIP1 in retinoic acid signaling, we first aimed to determine whether *NRIP1* is expressed in retinoic acid dependent tissue in the development of the murine urogenital system. We performed RNA *in situ* hybridization on whole-mount preparations of urogenital systems of E11.5 to E18.5 mouse embryos and on sections of the urogenital system at E11.5 to E18.5. Strikingly, *Nrip1* enhanced expression was found in the nephric duct, ureter and collecting duct at very early developmental stages (**Fig. 4**). Next, we tested for NRIP1 retinoic acid dependency with respect to *NRIP1* expression and interaction with retinoic acid receptor alpha. Our results suggest that in *Nrip1* expression is enhanced by retinoic acid (**Fig. 3C)**, thereby identifying it as a novel retinoic acid-responsive gene (**Fig. 3C**). In addition, the binding of NRIP1

with retinoic acid receptor α, was enhanced in the presence of retinoic acid treatment done on transfected HEK293 cells (**Fig. 3A2**).

Functional *in vivo* studies in *Xenopus laevis* show a major role for NRIP1 during renal development which is abolished with the mutant NRIP1

Expression and knockdown experiments in Xenopus *laevis* further confirmed a role for NRIP1 in renal development. Since unilateral injections allow a tissue restricted knockdown and analysis of organ specific phenotypes, we turned to the Xenopus model to analyze the developmental events in renal formation in further detail. Nrip1 is expressed during Xenopus development, and was enriched within the renal tubules (**Fig. 5**). Knock down with a translation blocking nrip1 MO inhibited pronephros development and resulted in distorted structure of the pronephrones (h/k; p<0,001). This could partially be rescued with NRIP1 wt (i/k; p=0,007) but not with NRIP1 G279 del (j/k; p=0,429) (**Fig. 6**). These data support the role of *Nrip1* during early tubular morphogenesis, and is consistent with the human CAKUT phenotype.

DISCUSSION

We here identify a truncating mutation of *NRIP1* as a novel autosomal dominant cause of human CAKUT. By studing NRIP1 cellular localization, luciferase assay, and protein-protein interaction, we demonstrate loss of function for truncating *NRIP1* mutation that segregated in a large CAKUT pedegree. We generated additional data that suggest that dysregulation of *NRIP1* dependent retinoic acid signaling during kidney and ureter morphogenesis causes CAKUT. Furthermore, by expression and knockdown experiments in *Xenopus laevis we* further confirmed a role for NRIP1 in renal development and the role of NRIP mutation as a novel cause of CAKUT in humans.

Nuclear receptor-interacting protein 1 (NRIP1), also known as receptor-interacting protein 140 (RIP140) (ref), is a transcriptional co-regulator, which has been shown to play a role in fine tuning of the activity of large number of transcription factors during development (ref). NRIP1 was mainly implicated as a corepressor of gene expression by interacting with different nuclear receptors, including the retinoic acid receptors (ref, ref). Nonetheless, the role of *NRIP1* during kidney and ureter development as well as its relation to retinoic acid (the active form of vitamin A) in this context has been largely unknown. In the current study, our finding of a germline *NRIP1* mutation as a novel cause of human CAKUT provides a link between the well documented association, of vitamin A/retinoic acid and renal malformations (Wilson, 1948; Batourina, 2005 ref).

Retinoic acid is a signaling molucule crucial for the embryonic formation of many organs (Duester G 2008, Cell). Whithin human (ref) as well as rodent (ref) embryos, axcess or deficiency of retinoic acid, cause CAKUT. Classic mouse models studies have highlighted the importance of retinoic acid signaling for proper ureter maturation and ureter-bladder connection (ref ref ref). Perturbation of this process in mice can result in hypo/dysplastic kidneys as well as in backflow of urine to the ureters, clinically known as vesicoureteral reflux (VUR). Interestingly, renal dysplasia and VUR were both the predominant phenotypes in the kindred we studied here (**Fig. 1., Table 1**). Our results suggest that NRIP1 inhibits retinoic acid-induced transcription. In turn, NRIP1, a target gene of retinoic acid, serves as a feedback inhibitor for this pathway. We postulate that loss of this feedback inhibition may mimic retinoic acid excess as described in the CAKUT mice models (ref), thereby leading to renal malformations.

Our study highlights several insights with respect to the complex genetic basis of CAKUT (ref,Knores). In addition to genetic heterogeneity, low penetrance mutations, and a variable

expressivity already described in monogenic CAKUT, our results emphasize possible gene-environment interactions as an additional level of complexity in human CAKUT.

NRIP1 mutations have never been implicated before in a human disease. Furthermore, early *NRIP1* truncating mutations are absent from all available WES databases, supporting intolerance for this gene for loss of function (see Exac: pLI score of 0.99). This is consistent with our finding that the early truncating *NRIP1* mutation which we identified results in a haploinsufficiency pathomechanism rather than a dominant negative effect. The fact that we did not find a second CAKUT family with *NRIP1* mutations supports the general notion that CAKUT is probably caused by very large number of different and extremely rare disease-causing mutations in large number of different genes (vivante rev).

Supplemental Data

Supplemental Data include 2 figures and 1 table.

Acknowledgments

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Web Resources

http://www.renalgenes.org

UCSC Genome Browser, http://genome.ucsc.edu/ cgi-bin/hgGateway

1000 Genomes Browser, http://browser.1000genomes.org.

Ensembl Genome Browser, http://www.ensembl.org.

Exome Variant Server, http://evs.gs.washington.edu/ EVS.

Polyphen2, http://genetics.bwh.harvard.edu/pph2. SeattleSeq

Sorting Intolerant From Tolerant (SIFT), http://sift.bii.astar.edu.sg.

Online Mendelian Inheritance in Man (OMIM), http://www. omim.org/

ExAC Browser Beta, http://exac.broadinstitute.org

Gudmap (GenitoUrinary Molecular Anatomy Project), http://www.gudmap.org

http://www.mutationtaster.org/

FIGURE LEGENDS

Figure 1. Identification of *NRIP1* mutation in index Family H with CAKUT.

Panel A shows the pedigree of index family H. Squares represent males, circles females, black symbols affected persons, white symbols unaffected persons and white symbol with black dot denotes obligatory mutation carrier. Pedigree is compatible with an autosomal dominant mode of inheritance with incomplete penetrance and variable expressivity. Roman numerals denote generations. Individuals are numbered with Arabic numerals if DNA was available for study. The arrow points to the proband IV:8. WT denotes the wild-type allele. p.Trp93* indicates the mutation c.279 deletion of G in *NRIP1*, leading to a frame shift mutation and introducing a premature stop codon. The mutation fully segregated heterozygously (WT / p.Trp93*) across all seven affected individuals examined and was absent from seven unaffected family members available for study (WT / WT). Individual II:1, is an obligatory mutation carrier given the pedigree structure and segregation analysis. Red circles indicate the persons selected for whole exome sequencing analysis.

Panel B shows voiding cystourethrogram (VCUG) (left panel) and renal ultrasound (right panel) of the index individual IV:8, revealing severe grade 5 left vesicoureteral reflux and hydroureter (white arrows). The index case presented during infancy following screening and is managed expectantly. He had no extra renal manifestations.

Panel C shows exon structure of human NRIP1 cDNA and domain structure of the nuclear receptor interacting protein 1 (NRIP1) protein. NRIP1 contains two putative nuclear localization signals (blue); four transcriptional repression domains (RD, yellow), and 10 interaction motifs LxxLL/LxxML (red & orange). Start codon (ATG) and stop codon (TAA) are indicated.

Panel D shows chromatograms of the heterozygous mutation detected in *NRIP1* (in relation to exons and protein domains) in the index family (red) with CAKUT. The index family's heterozygous mutation c.279delG leads to a frameshift and premature stop codon resulting in p.Trp93fs*.

Figure 2. NRIP1 p.Trp93fs* fails to translocate to the nucleus, does not suppress RA activation and fails to interact with retinoic acid a receptor alpha (RARα).

Panel A: Immunofluorescence staining of HEK293 cells transfected with Myc-tagged wild-type (WT) NRIP1 and Myc-tagged NRIP1 p.Trp93fs*. WT NRIP1 localizes to the nucleus, while the altered NRIP1 protein remains predominantly localized in the cytoplasm.

Panel B: Western blot of cytoplasmic (C) and nuclear (N) extracts from transfected HEK293 cells showing localization of WT NRIP1 predominantly in the nucleus (Anti-Sp1) and NRIP1 p.Trp93fs* in the cytoplasm (Anti-GAPDH).

Panel C: Luciferase assay of HEK293 cell transfected with Mock GFP, GFP-tagged WT NRIP1, and GFP-tagged NRIP1 p.Trp93fs* mutant and subsequently treated with retinoic acid. WT NRIP1 suppresses retinoic acid induced transcriptional activity while the mutant form fails to do so.

Panel D: Immunofluorescence staining of HEK293 cells co-transfected with GFP-tagged RARα and either Mock Myc, Myc-tagged WT NRIP1, or Myc-tagged NRIP1 Trp93fs*. RARα and WT NRIP1 co-localize in the nucleus (speckled pattern), while the Trp93fs* mutant protein remains in the cytoplasm. ** $p<0.01$; NS, not significant.

Figure 3. Retinoic acid increases NRIP1 binding to RARα *in vitro* and increases *NRIP1* mRNA expression in mouse kidney explant organ cultures.

Panel A: Co-immunoprecipitation of protein lysates of WT NRIP1 and RARα overexpressed in HEK293 cells. Treatment with RA increases WT NRIP1 binding to RARα (orange boxes). The Trp93fs* mutation abrogates binding to RARα.

Panel B: Quantification of data shown in Panel A. RARα co-immunoprecipitation band intensity was normalized to WT NRIP1 immunoprecipitation band intensity in the absence and presence of retinoic acid. * $p<0.05$, average of 3 separate experiments.

Panel C: NRIP1 mRNA is downregulated after treatment with the RA antagonist, BMS493, and upregulated following treatment with retinoic acid (Andreas please give concentrations).

Figure 4. ***Nrip1*** **shows specific early expression in the Wolffian (nephric) duct, ureter, and collecting duct epithelium of the developing urogenital system of the mouse.**

Comparative *in-situ* hybridization analysis of expression of *Nrip1* in whole urogenital systems and on sections of E11.5, E12.5, E14.5, and E18.5 wild-type urogenital systems. At least three independent specimens were analyzed each.

bl, bladder; cd, collecting duct; cl, cloacae; k, kidney; t, testicular duct; u, ureter (stalk);um, ureteric mesenchyme; ue, ureteric epithelium; ut, ureteric tip; wd, Wolffian duct.

Figure 5: Nrip1 deficiency affects pronephros development in Xenopus.

(a-c) Whole mount in situ hybridization of nrip1 in X. laevis shows its occurrence in the pronephros during development. Expression starts in the pronephric anlage at stage 24 (a, a') and is present in the tubules of stage 33 tadpoles (c, c').

(d-k) Functional analysis of nrip1 in X. laevis. Embryos were unilaterally injected with nrip1 MO and/ or NRIP1 encoding mRNA at the 4-cell stage. Stage 39 tadpoles were stained with fluorescein conjugated lectin to visualize the pronephros (d-j). The size of the pronephros was measured and the proportion between the injected (right) and uninjected (left) side calculated (k). The structure of the pronephros was not changed by microinjections of the NRIP1 wt (e/ k) mRNA or its truncated variant (NRIP1 G279 del; f) compared to the controls (d'/k). Knock down with a translation blocking nrip1 MO inhibited pronephros development (h/k; $p<0,001$) and could partially be rescued with NRIP1 wt (i/k; $p=0,007$) but not with NRIP1 G279 del (j/k; $p=0,429$). Error bars represent SEM. ** $p<0.01$, ***$p<0.001$ (MWU-test)

Table 1. Dominant *NRIP1* mutations detected in individuals with congenital anomalies of the kidneys and urinary tract.[a]

Family - individual	Ancestry	Nucleotide alteration[a]	Exon (zygosity)	Alteration in coding sequence[a]	Allelic loss of function (nuclear localization/ luciferase/ Co-IP with RARα) [a,c]	Presenting symptoms or diagnostic test (at age in years)	Kidney phenotype	Treatment	Serum creatinine [mg/dL] (at age in years)
H	Yemeni Jews		7 (het)						
II:8		c.279delG		p.Trp93fs*	(+/+/+)	Renal US (58)	R small pelvic kidney with hydronephrosis	Conservative	normal
III:3		c.279delG		p.Trp93fs*	(+/+/+)	Abdominal mass (newborn)	L MCDK; R VUR, dilated ureter and dysplasia	L nephrectomy R nephrostomy	1.5 (35)
III:4		c.279delG		p.Trp93fs*	(+/+/+)	Abdominal pain (7)	L dysplastic kidney and VUR	L nephrectomy	0.75 (27)
III:5		c.279delG		p.Trp93fs*	(+/+/+)	Renal US (3)	Bilateral grade 2 VUR	Conservative	0.64 (28)
IV:7		c.279delG		p.Trp93fs*	(+/+/+)	Renal US (2)	Small right kidney	Conservative	normal
IV:8		c.279delG		p.Trp93fs*	(+/+/+)	Hydronephrosis on renal US (prenatal)	L VUR grade 5 and dysplasia; R VUR grade 2	Conservative	normal
IV:9		c.279delG		p.Trp93fs*	(+/+/+)	Renal US (prenatal)	L ectopic dysplastic Kidney	Conservative	normal

[a]cDNA mutations are numbered according to human cDNA reference sequence NM_003489.3, isoform (NRIP1), where +1 corresponds to the A of ATG start translation.
[b]Mutation was absent from 200 control individuals with renal ciliopathies, 429 individuals with steroid-resistant nephrotic syndrome, from ~13,000 healthy control alleles of the EVS (exome variant server http://evs.gs.washington.edu/EVS), from and 2,577 control individuals of the "1,000 human genomes project" (http://www.1000genomes.org), and from ~112,000 control chromosomes of the ExAC server (http://exac.broadinstitute.org).
[c] "+" indicates a defect of the allele in one of the 3 assays of NRIP1 function: Nuclear localization (**Fig. 2A**), luciferase assay (**Fig. 2C**) or Co-IP with RARα (**Fig. 3A**).

Co-IP, co immunoprecipitation; het, heterozygous;L, left; MCDK, multicystic dysplastic kidney; NA, not applicable; ND, no data; R, right; US, ultrasound; VUR, vesicoureteral reflux.

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