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Whole exome resequencing reveals recessive mutations in TRAP1 in individuals with CAKUT and VACTERL association

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Disclosure

The authors report no conflicts of interest.

Web Resources

1000 Genomes Browser, http://browser.1000genomes.org;
Ensembl Genome Browser, http://www.ensembl.org;
Exome Variant Server, http://evs.gs.washington.edu/EVS;
Mutation Taster, http://www.mutationtaster.org;
Gudmap (GenitoUrinary Molecular Anatomy Project), http://www.gudmap.org;
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org;
Polyphen2, http://genetics.bwh.harvard.edu/pph2;
Sorting Intolerant From Tolerant (SIFT), http://sift.bii.a-star.edu.sg;
The Human Protein Atlas, http://www.proteinatlas.org;
UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway.

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Abstract

Congenital abnormalities of the kidney and urinary tract (CAKUT) account for approximately half of children with chronic kidney disease and they are the most frequent cause of end-stage renal disease in children in the US. However, its genetic etiology remains mostly elusive. VACTERL association is a rare disorder that involves congenital abnormalities in multiple organs including the kidney and urinary tract in up to 60% of the cases. By homozygosity mapping and whole exome resequencing combined with high-throughput mutation analysis by array-based multiplex PCR and next-generation sequencing, we identified recessive mutations in the gene *TNF receptor-associated protein 1* (*TRAP1*) in two families with isolated CAKUT and three families with VACTERL association. TRAP1 is a heat shock protein 90-related mitochondrial chaperone possibly involved in antiapoptotic and endoplasmic reticulum-stress signaling. *Trap1* is expressed in renal epithelia of developing mouse kidney E13.5 and in the kidney of adult rats, most prominently in proximal tubules and in thick medullary ascending limbs of Henle's loop. Thus, we identified mutations in *TRAP1* as highly likely causing CAKUT or CAKUT in VACTERL association.

Introduction

Congenital abnormalities of the kidney and urinary tract (CAKUT) occur in 3–6 per 1,000 live births. CAKUT are the most frequent cause for chronic kidney disease in children (~50%)[1, 2] in the US. The acronym "CAKUT" comprises heterogeneous malformations involving the kidney (e.g. renal agenesis, hypodysplasia), and the urinary tract (e.g. vesicoureteral reflux, ureteropelvic junction obstruction)[3]. These congenital anomalies are related because a part of their pathogenesis is an impaired co-development of nephrogenic tissues derived from the metanephric mesenchyme and the ureteric bud[4]. Twenty monogenic causes of isolated CAKUT in humans have been published to date as reviewed recently by Yosypiv[5]. However, they only account for ~10% - 20% of all cases indicating a broad genetic heterogeneity of CAKUT. A recent study on copy number variations (CNVs) in a large cohort of individuals with CAKUT and two publications identifying novel monogenic causes of CAKUT bring further evidence that CAKUT is a condition of extensive genetic heterogeneity[6–8]. CAKUT most frequently occur isolated, but might be associated with extra-renal phenotypes, for instance with VACTERL association (MIM [#192350]). The

acronym “VACTERL” describes the combination of at least three of the following congenital anomalies: vertebral defects (V), anorectal malformations (A), cardiac defects (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R), and limb defects (L). VACTERL association is a rare disease that occurs mostly sporadic in 1/10,000–40,000 live births[9]. Its etiology is enigmatic, although animal models suggest an involvement of Sonic hedgehog signaling[10]. In humans, *ZIC3* mutations are the cause of a closely related non-classic VACTERL condition (VACTERL-X, MIM [#314390])[11, 12]. Additionally, there are six case reports published of individuals with VACTERL association in conjunction with mitochondrial dysfunction as summarized recently by Siebel and Solomon[13]. In order to identify new recessive genes that cause isolated CAKUT or CAKUT in VACTERL association, we performed homozygosity mapping and whole exome resequencing in 24 affected individuals with CAKUT from 16 families, and in 4 individuals with CAKUT in VACTERL.

Results

Whole exome resequencing identifies a homozygous mutation in *TRAP1* in CAKUT and in VACTERL association

By homozygosity mapping in a family of two sibs (A3403) with unilateral and bilateral vesicoureteral reflux (VUR) III°, respectively (Figure 1A, B and Table 1), we identified a short 5.2 Mb segment of homozygosity on chromosome 5 (Figure 1C), indicating distant consanguinity of the parents. This finding suggested that in this family CAKUT are most likely caused by a homozygous recessive mutation in an unknown CAKUT gene. We performed whole exome resequencing in individual A3403-21 as described previously by the authors[14, 15]. In order not to miss either a homozygous mutation in a short run of homozygosity or a compound heterozygous mutation (which, as in this case, cannot be excluded *a priori* in families with remote consanguinity[16]), we considered variants not only in the homozygosity peak but within regions of genetic linkage for both sibs (coverage ≥ 4; minor variant frequency, MVF ≥ 0.2). Following variant filtering we retained 38 variants in 13 genes for Sanger confirmation and segregation analysis (Supplementary Table S1 online). Only a single homozygous missense mutation (R469H) in the gene *TRAP1* on chromosome 16p13.3 survived the variant filtering process and segregation analysis (Figure 1D). This homozygous variant in *TRAP1* in A3403-21 and -22 was positioned in a ~1.5 Mb run of apparent homozygosity that was not detected by homozygosity mapping (Figure 1C), because the threshold for detection of “homozygosity peaks” is 2.1 Mb[17].

In family A4252 with CAKUT in VACTERL we performed whole exome resequencing in an affected individual (A4252-21). This girl was born with a right double kidney and duplex ureter, left VUR, esophageal atresia type IIIb, and anal atresia with a vestibular fistula (Figure 1E, F and Table 1). Although there was no consanguinity reported in this family, homozygosity mapping showed unusually broad homozygosity peaks on chromosome 16 on the p-terminus and q-terminus (5.5 and 9.6 Mb, respectively) (Figure 1G). In this case, we hypothesized that CAKUT in VACTERL is caused by a homozygous mutation within these homozygous regions. When evaluating whole exome resequencing data in this individual, the 512,733 variants initially detected (MVF ≥ .55; coverage ≥ 2) were reduced to only 11

variants within the "homozygosity peaks" on chromosome 16 and 18 (Supplementary Table S2 online). The only variant that was confirmed by Sanger sequencing and that altered a conserved amino acid residue was *TRAP1* R469H, the same allele as in family A3403. By comparison of SNPs in the affected girl and her parents, we demonstrated that partial maternal isodisomy of chromosome 16 with two recombinants (one located on the p-arm and one located on the q-arm) was the underlying cause of homozygosity for *TRAP1* R469H (Figure 1G-J).

The *TRAP1* allele c.1406G>A, p.R469H alters an evolutionary highly conserved amino acid residue and it is predicted to be deleterious for protein function by publically available software programs (Table 1 **and** Supplementary Figure S1 online). In the Exome Variant Server (EVS) database, R469H has a minor allele frequency (MAF) of 0.9% in Americans of European descent. In our cohort of 675 individuals with CAKUT, most of them European, the MAF is 1.9%. The three affected individuals from two unrelated families with homozygous *TRAP1* R469H, as well as 6 additional heterozygous carriers share haplotypes at the *TRAP1* locus (Figure S2 online) which speaks for *TRAP1* R469H being a European founder mutation.

Mutation analysis reveals three additional families with *TRAP1* mutations

We subsequently analyzed the coding sequence of *TRAP1* in a cohort of 675 individuals with isolated CAKUT (Supplementary Table S3 online) and 300 individuals with classic VACTERL association (i.e. VACTERL-X and other related disorders have been excluded) using a barcoded multiplex PCR approach and consecutive next generation sequencing as described previously by the authors[18]. As a control group, we included 800 individuals with the distinct renal phenotype of nephronophthisis.

We detected six additional recessive mutations in *TRAP1* in a compound heterozygous state in three additional unrelated families with CAKUT or CAKUT in VACTERL (Table 1, Figure 1K, L, M, Supplementary Figure S1, and S3 online). In individual A3051-21 with a left-sided multicystic dysplatic kidney (MCDK), we found a maternally inherited protein-truncating frame-shift mutation (c.127_137dup, p.R46fs*75). This mutation abrogates the N-terminal mitochondrial targeting sequence of *TRAP1*, which makes this a null allele. The second allele was a missense mutation (c.1324G>A, p.E442K) which segregated from the father.

In individual A4884-21 with CAKUT in VACTERL, including right renal agenesis, vertebral malformations, anal atresia with a rectoperineal fistula, atrial septum defect type II, esophageal atresia, and abnormal position of the thumbs (Table 1 **and** Supplementary Figure S4 online), we detected compound heterozygous missense mutations in *TRAP1* located in the ATPase-domain (c.757A>G, p.I253V) and in the HSP90-domain (c.1573C>T, p.L525F) (Figure 1L).

In individual EA1717 with CAKUT in VACTERL, including pyelectasis, left VUR, a complex anorectal malformation including anal atresia and persistent cloaca, esophageal atresia, cardiac defects, limb defects and, persistent left vena cava superior (Table 1), we

detected compound heterozygous missense mutations which are both located in the HSP90-domain of *TRAP1* (c.1330T>A, p.Y444N and c.1663G>A, p.V555I).

In order to exclude the presence of recessive mutations in controls, we sequenced the *TRAP1* coding sequence in 800 individuals with the distinct renal phenotype of nephronophthisis (NPHP). We detected the *TRAP1* allele I253V seven times (MAF 0.87%), T444N twice (MAF 0.25%), and R469H twice (MAF 0.025%), all of them as single heterozygous alleles. *TRAP1* R46Sfs*75, E442K, L525F, and V555I were absent from our control cohort. Furthermore, no other possibly deleterious variants were present in a homozygous or compound heterozygous state in 800 individuals with NPHP.

Trap1 is expressed in developing and adult kidney

In order to determine whether *TRAP1* has a function during kidney development, we analyzed *Trap1* expression in developing kidney in mouse embryos 13.5 dpc. *Trap1* expression seemed to be expressed at this stage in renal vesicles according to *Trap1* transcription assays publically available through the Gudmap project. By *in-situ* hybridization in E13.5 mouse embryos, we found *Trap1* to be strongly expressed in kidney, adrenal gland, and gonad. *Trap1* expression specifically localized to renal epithelia (Figure 2).

In order to characterize TRAP1 localization in adult kidney, we performed immunofluorescence stainings in rat using a monoclonal TRAP1 antibody in conjunction with established renal markers (Figure 3). TRAP1 is present most prominently in peanut-lectin-marked proximal tubules in the renal cortex (Figure 3A-B). In renal medulla, we detected TRAP1 in peanut-lectin-negative tubular segments and in NKCC-marked ($Na^+K^+2Cl^-$ co-transporter) thick ascending limbs of Henle's loop (Figure 3C-D). TRAP1 co-localizes with mitochondrial marker MTCO1 in renal cortex and medulla.

Discussion

In the present study, we identified by whole exome resequencing and high-throughput mutation analysis five unrelated families with CAKUT or CAKUT in VACTERL association with recessive mutations in *TRAP1*. Two sibs with CAKUT had a homozygous missense mutation (R469H), which segregated from a common ancestor of their parents. A girl with VACTERL association had the identical homozygous mutation due to maternal isodisomy of chromosome 16 p-ter and q-ter. In a cohort of 675 individuals with CAKUT and 300 individuals with classic VACTERL association we identified 3 additional individuals carrying compound heterozygous mutations in *TRAP1*. Homozygous or compound heterozygous deleterious variants were absent from 800 control individuals. By ISH and IF, we showed that *Trap1* is expressed in early mouse renal epithelia whereas the Trap1 protein is present only in defined segments of developed nephrons in rat.

In 6,500 individuals recorded in the EVS server there are several nonsynonymous variants present in *TRAP1*, including heterozygous truncating variants in 11 individuals. However, deleterious alleles in recessive disease-genes, unlike in dominant disease-genes, do not

underlie direct negative selection through evolution. Consequently the presence of rare deleterious variants in recessive disease genes in a large cohort is an expected finding.

The allele *TRAP1* Y444N, detected as compound heterozygous mutation in an individual with CAKUT in VACTERL, is present homozygously in a single individual of the ESP cohort of 6,500 healthy Americans. However, in the context of CAKUT, this does not necessarily mean the variant is non-pathogenic. CAKUT frequently remain completely asymptomatic. For instance, a double-kidney or unilateral renal agenesis typically are an "accidental finding" in renal ultrasound.

The fact that the homozygous mutation *TRAP1* R469H was found in an individual with CAKUT and an individual with VACTERL association is surprising. However, in CAKUT and in VACTERL association intra-familial phenotypic variability is very common[19–21]. Even in a single individual different CAKUT phenotypes may be present, for instance left renal agenesis and right VUR.

The frequencies of individuals with recessive *TRAP1* mutations in our cohorts (0.15% in CAKUT, 0.6% in CAKUT with VACTERL) suggest that mutations in *TRAP1* are a rare cause of these conditions. Similarly, mutations in two recently identified CAKUT-causing genes, *WNT4* and *DSTYK*, are rare causes of CAKUT[7, 8]. These findings in humans, along with numerous CAKUT-mouse models, indicate that CAKUT are a common clinical phenotype arising from a multitude of different single-gene causes.

In conclusion, we propose that recessive mutations in *TRAP1* are a novel rare cause of isolated CAKUT and the first recessive cause of the VACTERL association.

Subjects and Methods

Human subjects

We obtained blood samples and pedigrees following informed consent from individuals with CAKUT and from individuals with VACTERL association. Approval for human subjects research was obtained from the University of Michigan Institutional Review Board and other institutions involved. The diagnosis of CAKUT and VACTERL association was based on published clinical criteria[9].

Homozygosity mapping

We performed homozygosity mapping as described previously[17].

Whole exome resequencing (WER)

Exome library preparation and massively parallel resequencing was conducted using the SeqCap EZ Exome v2 (Nimblegen) and Genome Analyzer II (Illumina). Subsequent variant detection, filtering and analysis have been described previously by the authors[14, 15]. All detected variants were confirmed by Sanger sequencing.

Immunofluorescence microscopy (IF)

IF was performed as previously described by the authors[14] using a Leica SP5X system with an upright DM6000 compound microscope and images were processed with the Leica AF software suite. Antibodies used: TRAP1 (Abcam, [TRAP1-6], Cat# ab2721), MTCO1 (Abcam Cat# ab45918), NKCC2 (LSBio Cat# LS-C150446), NCCT (Millipore Cat# AB3553). Specificity of the anti-TRAP1 antibody for rat TRAP1 was confirmed in immunoblot (Figure S5 online).

In-situ hybridization (ISH)

ISH was conducted on sections of wildtype mouse embryos with an NMRI background at embryonic day 13.5. Mouse embryos were dissected into ice cold phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde/PBS, and then processed into paraffin wax. ISH was performed on paraffin sections (5]m) using antisense probes generated by PCR from an E11.0 total embryo cDNA library, and specific staining was verified using a sense probe. PCR products contained 3' T7 and 5' T3 RNA polymerase binding sites for in vitro transcription and probes were purified using G-50 sephadex columns (GE Healthcare). The 779bp probe for Trap1 spans exons 13–17 (Accession: NM_026508.2).

ISH was performed according to the protocol from (Chotteau-Lelievre et al., 2006) with minor modifications, and detection of AP activity was visualized using BM Purple (Roche Diagnostics). Following staining, slides were quickly dehydrated in 80% and then 100% ethanol, cleared twice for 1 min in xylene (Roth) and coverslips were mounted with Entellan mounting medium (Merck). Photographs were obtained using AxioVision software (Zeiss) with a Zeiss AxioCam and SteREO Discovery.V12 microscope. Three sections from at least 2 different embryos were analyzed.

Bioinformatics

NGS reads alignment and variant detection was done with Genomics Workbench software (CLC Biotech). Mapping parameter: Global alignment, length fraction = 0.9, and similarity fraction = 0.9. Genetic location is according to the assembly of the Genome Reference Consortium GRCh37.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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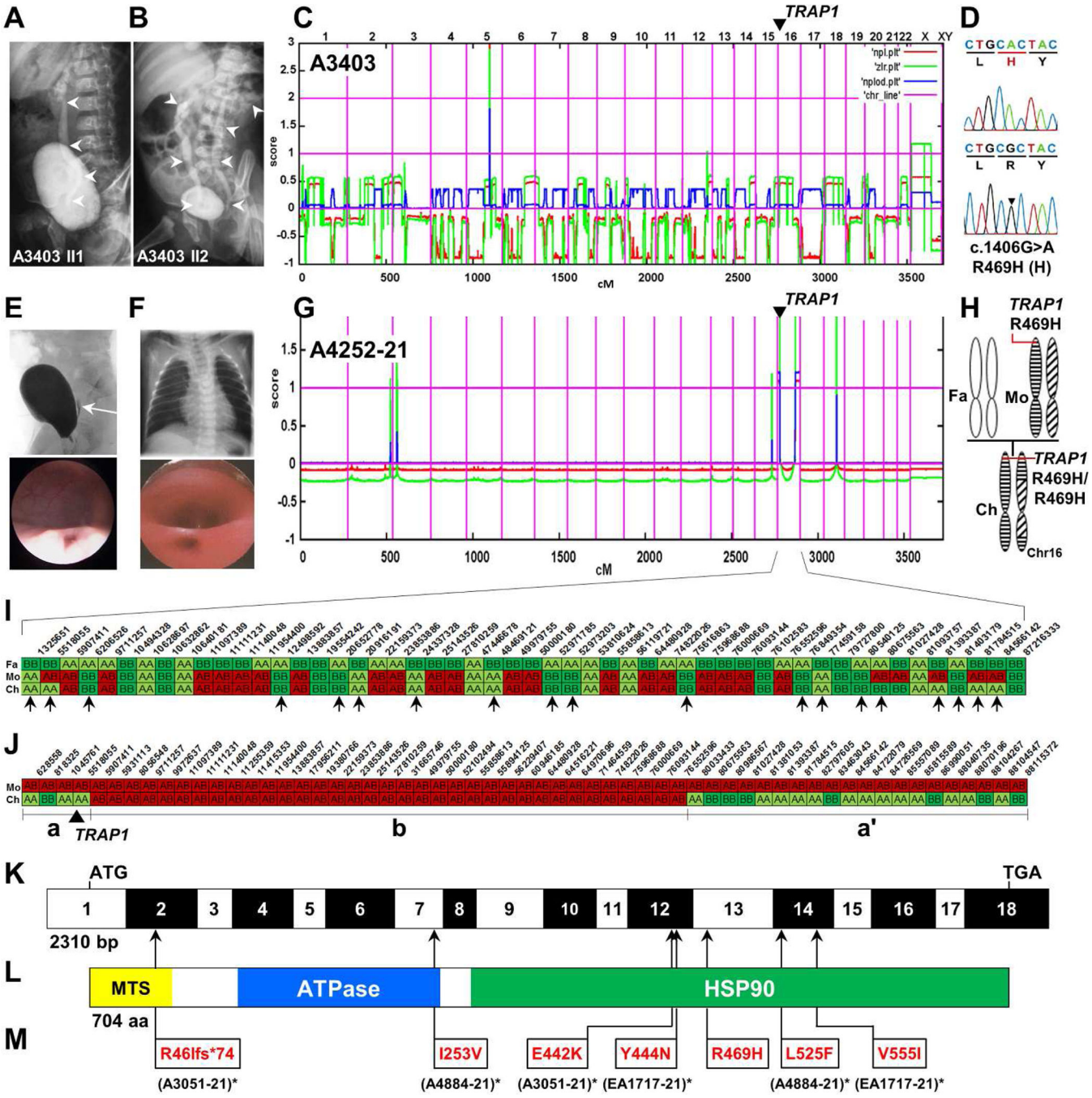

Figure 1. Homozygosity mapping and whole exome resequencing identifies mutations in *TRAP1* as causing CAKUT or VACTERL association

(A, B) Voiding cysturethrograms (VCUG) of CAKUT siblings A3403-21 and -22 showing unilateral vesicoureteral reflux (VUR) grade III and bilateral VUR, respectively (white arrow heads).

(C) Non-parametric LOD (NPL) scores across the human genome in 2 affected sibs. X-axis represents Affymetrix 250k StyI array SNP positions across human chromosomes

concatenated from p-terminal (left) to q-terminal (right). Genetic distance is given in cM. A single peak indicates distantly related parents.

(D) Chromatogram of newly identified homozygous missense mutation (arrow head) in the gene encoding TNF receptor-associated protein 1 (*TRAP1*) over wild type control.

(E) VCUG (upper panel) and cystoscopy (lower panel) demonstrating VUR and a dilated ureteral orifice, respectively.

(F) Chest X-ray (top panel) and esophagoscopy (bottom panel) showing esophageal atresia and esophagotracheal fistula in individual A4252-21 with CAKUT in VACTERL association.

(G) NPL score in an individual A4252-21 with VACTERL association. Two maximum peaks indicate homozygosity at the p-terminus and q-terminus of chromosome 16.

(H) Panel on the right illustrates maternal heterodisomy of chromosome 16 and partial uniparental isodisomy (p-ter and q-ter) of the child (Fa, father; Mo, mother; Ch, child).

(I) Partial haplotypes of selected markers and their physical positions across chromosome 16 in the father (Fa), the mother (Mo), and the affected child (Ch) of CAKUT family A4252. Selected markers (biallelic SNPs; MAF = 0.496 – 0.5) homozygous in the father are shown in green (alleles AA) and light green (alleles BB).

The fact that for 19 of 52 alleles there is paternal non-contribution in the child strongly suggests maternal heterodisomy of chromosome 16. No paternal non-contribution was observed in the child on any other chromosome (data not shown).

(J) Selected markers (biallelic SNPs; MAF = 0.497 – 0.5) heterozygous in the mother (Mo) of family A4252 are shown for alleles coded in red (AB; phase unknown). Note that in the central segment (b), separated by vertical lines, the child's (Ch) haplotype is identical to the mother's. In the p-ter (a) and q-ter (a') segments (a, a') the child is homozygous, indicating maternal isodisomy in these segments.

(K) Exon structure of human *TRAP1* cDNA. Positions of start codon (ATG) and of stop codon (TGA) are indicated.

(L) Domain structure of the TRAP1 protein. HSP, heat shock protein; MTS, mitochondrial targeting sequence.

(M) Translational changes of detected mutations are shown relative to their positions in *TRAP1* cDNA (see L) and TRAP1 protein (see M) for affected individuals with CAKUT or CAKUT in VACTERL association with recessive *TRAP1* mutations. Family numbers are shown in parenthesis. (* denotes an individual carrying a compound hete)

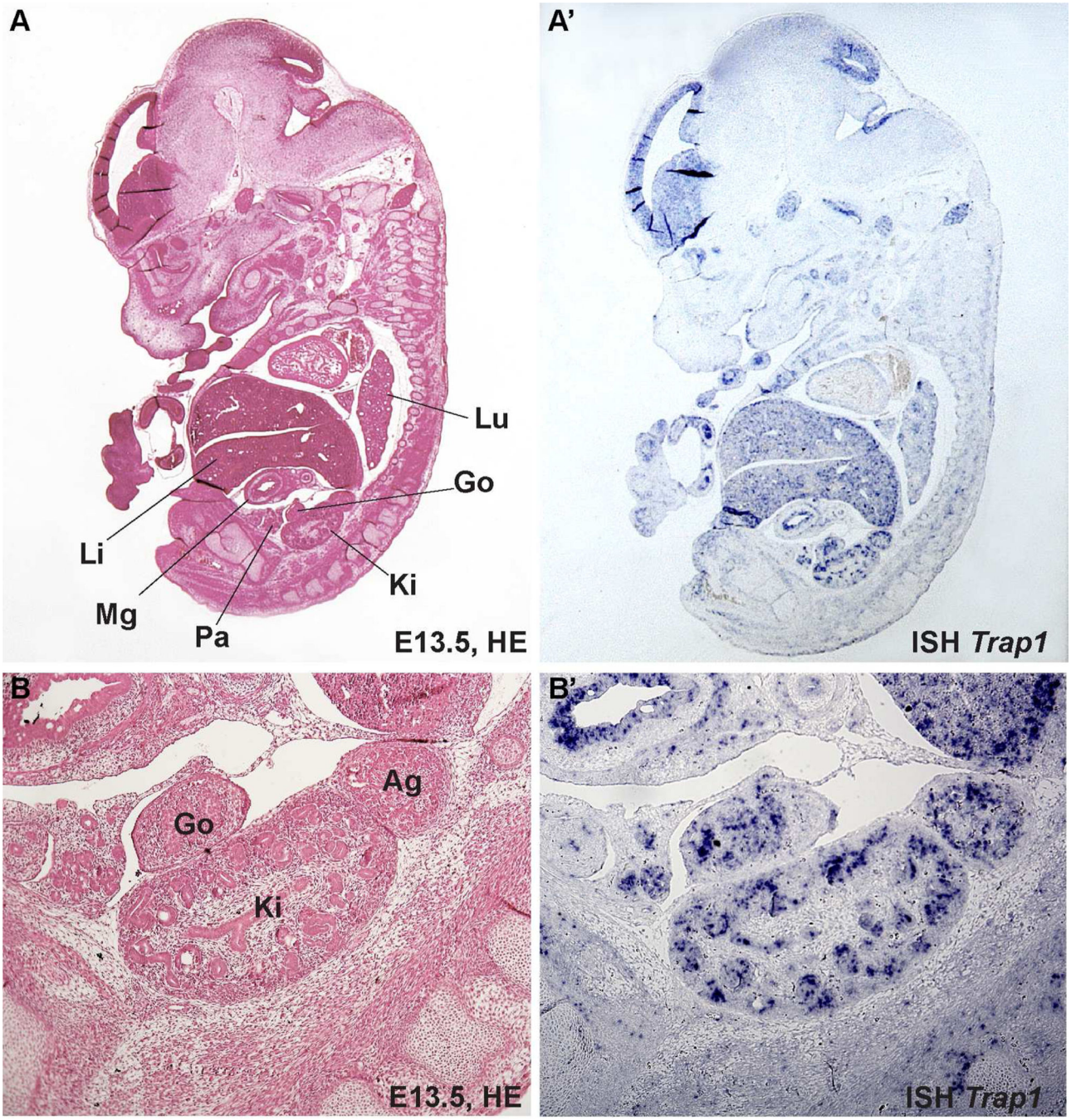

Figure 2. *Trap1* is highly expressed in renal epithelia of E13.5 mouse embryos

The upper panel shows an HE-stained sagittal section **(A)** and a *Trap1*-ISH **(A')** in consecutive sections of a mouse embryo E13.5. Note the prominent *Trap1* expression in the developing kidney (marked "Ki" in the left panel). The lower panel shows higher magnifications of E13.5 mouse kidney. **(B)** HE staining, **(B')** *Trap1*-ISH. The *Trap1*-ISH staining pattern in consistent with *Trap1* being expressed specifically in renal epithelia **(B')**. Ag, adrenal gland; Go, gonad; HE, hematoxylin-eosin; ISH, *in-situ* hybridization; Ki, kidney (i.e. metanephros); Li, liver; Lu, lung; Mg, midgut; Pa, pancreatic primordium.

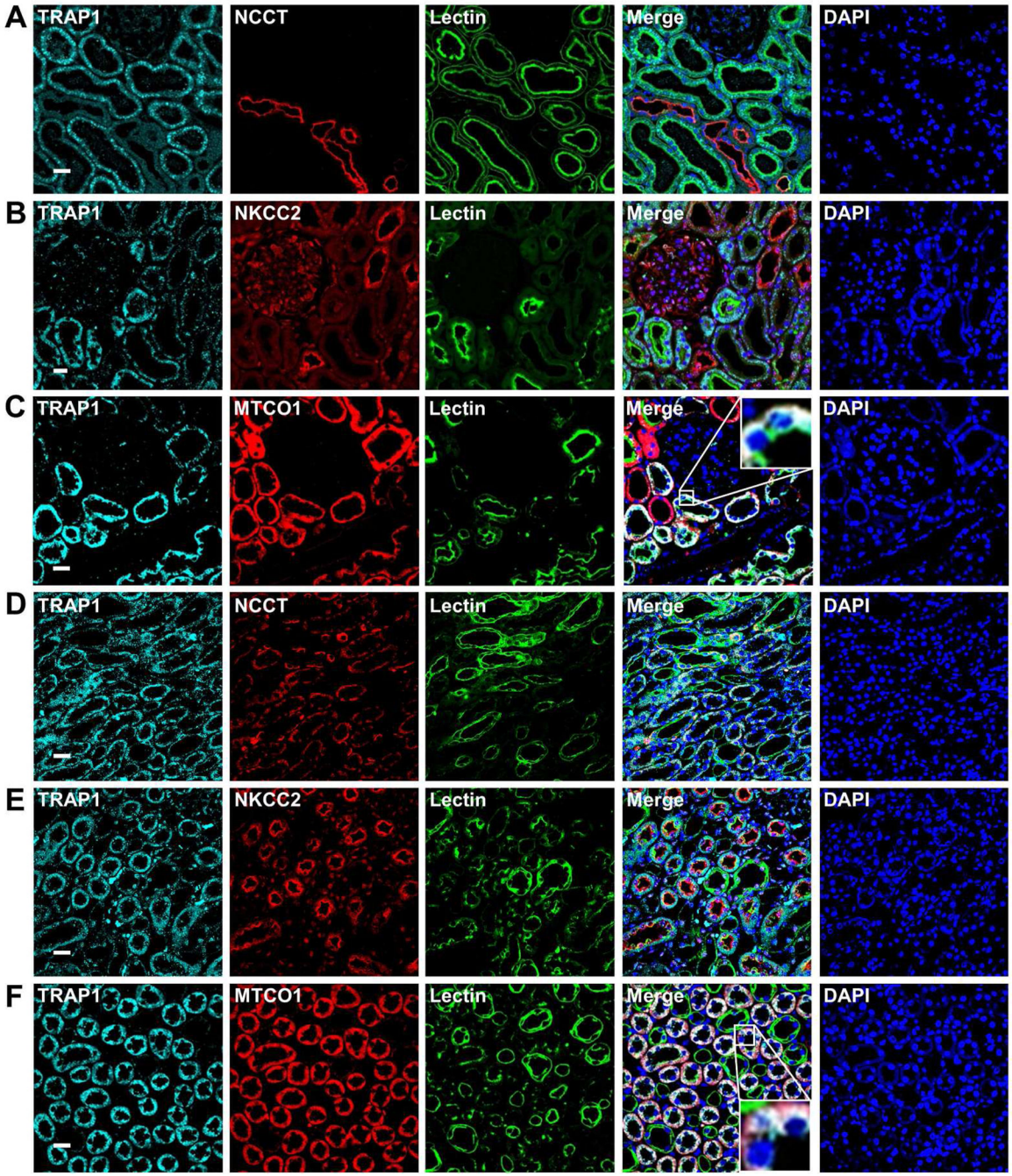

Figure 3. *Trap1* is highly expressed in renal epithelia of E13.5 mouse embryos

The upper panel shows an HE-stained sagittal section **(A)** and a *Trap1*-ISH **(A')** in consecutive sections of a mouse embryo E13.5. Note the prominent *Trap1* expression in the developing kidney (marked "Ki" in the left panel). The lower panel shows higher magnifications of E13.5 mouse kidney. **(B)** HE staining, **(B')** *Trap1*-ISH. The *Trap1*-ISH staining pattern in consistent with *Trap1* being expressed specifically in renal epithelia **(B')**.

Ag, adrenal gland; Go, gonad; HE, hematoxylin-eosin; ISH, *in-situ* hybridization; Ki, kidney (i.e. metanephros); Li, liver; Lu, lung; Mg, midgut; Pa, pancreatic primordium.

Table 1

Mutations of *TRAP1* in 5 families with isolated CAKUT or CAKUT in VACTERL association

Family -Individ. (sex)	Ethnic origin	Nucleotide alteration[a]	Deduced Protein change	Continuous amino acid sequence conservation	MutT[b]	Poly Phen2[c]	SIFT[d]	MAF in EVS[e]	Exon (state; segregation)	Urinary tract phenotypes	Other phenotypes
A3403 −21 (F) −22 (F)	Serbian	c.1406G>A	p.R469H	*E. coli* (*C. elegans* has L)	0.99	0.997	0.00	0.77%	13 (Hom; Fa, Mo)	−21: VUR-III° R −22: VUR-III° R and L	None
A4252 −21 (F)	Central Euro-pean	c.1406G>A	p.R469H	*E. coli* (*C. elegans* has L)	0.99	0.997	0.00	0.77%	13 (Hom; Mo; partial maternal isodisomy)	Double kidney R VUR L	VACTERL association including esophageal atresia IIIb, anal atresia, vestibular fistula
A3051 −21 (M)	Mace-donian	c.127_137dup c.1324G>A	p.R46Sfs*75 p.E442K	N/A *D. rerio*	N/A 0.99	N/A 0.003	N/A 0.3	absent 0.08%	2 (het; Mo) 12 (het; Fa)	MCDK L	None
A4884 −21 (F)	Dutch	c.757A>G c.1573C>T	p.I253V p.L525F	*E. coli* (*X. tropicalis* has V, *S. cerevisiae* has L) *E. coli*	0.99 0.99	0.433 0.942	0.00 0.00	0.91% absent	7 (het; Mo) 14 (het; Fa)	Renal agenesis R	VACTERL association including cervical/thoracic hemivertebrae, 5 dysplastic short ribs R, anal atresia with rectoperineal fistula, ASD type II, esophageal atresia, abnormal position of thumbs
EA1717 −21 (F)	Dutch	c.1330T>A c.1663G>A	p.Y444N p.V555I	*C. elegans* *C. intestinalis*	0.99 0.99	0.985 0.115	0.03 0.39	0.91%[f] absent	12 (het; Fa) 14 (het; Mo)	Pyelectasis and VUR L	VACTERL association including anal atresia, esophageal atresia, ASD, VSD, hypoplastic/absent humerus, persistent L vena cava superior, cloaca

[a] *TRAP1* cDNA mutations are numbered according to human cDNA reference sequence NM_016292.2, where +1 corresponds to the A of ATG start translation codon.

[b] MutationTaster score. Range: 0 – 1.0, 1.0 being most deleterious.

[c] PolyPhen2 (HumVar) score. Range: 0 – 1.0, 1.0 being most deleterious.

[d] SIFT score. Range: 0 – 1.0, 0 being most deleterious.

[e] Minor allele frequency in 8,600 alleles of Americans of European descent.

[f] One individual is homozygous for this allele.

The following abbreviations are used: ASD, atrial septum defect; CAKUT, congenital abnormalities of the kidney and urinary tract; F, female; Fa, mutation segregating from the father; L, left; N/A, not applicable; M, male; MCDK, multicystic dysplastic kidney; Mo, mutation segregating from the mother; MutT, MutationTaster; ND, no data; R, right; VSD, ventricular septum defect; VUR-III°, vesicoureteral reflux 3rd degree.