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Mutations of the SLIT2-ROBO2 pathway genes *SLIT2* and *SRGAP1* Confer Risk for Congenital Anomalies of the Kidney and Urinary Tract

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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>
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>;
SeattleSeq Annotation 138, <http://snp.gs.washington.edu/SeattleSeqAnnotation138/>;
Sorting Intolerant From Tolerant (SIFT), <http://sift.bii.a-star.edu.sg>;
UCSC Genome Browser, <http://genome.ucsc.edu/cgi-bin/hgGateway>.

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Abstract

Congenital anomalies of the kidney and urinary tract (CAKUT) account for 40–50% of chronic kidney disease that manifests in the first two decades of life. Thus far, 31 monogenic causes of isolated CAKUT have been described, explaining ~12% of cases. To identify additional CAKUT-causing genes, we performed whole exome sequencing followed by a genetic burden analysis in 26 genetically unsolved families with CAKUT. We identified two heterozygous mutations in *SRGAP1* in 2 unrelated families. *SRGAP1* is a small GTPase activating protein in the SLIT2-ROBO2 signaling pathway, which is essential for development of the metanephric kidney. We then examined the pathway-derived candidate gene *SLIT2* for mutations in cohort of 749 individuals with CAKUT and we identified 3 unrelated individuals with heterozygous mutations. The clinical phenotypes of individuals with mutations in *SLIT2* or *SRGAP1* were cystic dysplastic kidneys, unilateral renal agenesis, and duplicated collecting system. We show that *SRGAP1* is expressed in early mouse nephrogenic mesenchyme and that it is coexpressed with *ROBO2* in SIX2-positive nephron progenitor cells of the cap mesenchyme in developing rat kidney. We demonstrate that the newly identified mutations in *SRGAP1* lead to an augmented inhibition of RAC1 in cultured human embryonic kidney cells and that the *SLIT2* mutations compromise the ability of the SLIT2 ligand to inhibit cell migration. Thus, we report on two novel candidate genes for causing monogenic isolated CAKUT in humans.

Keywords

CAKUT; MCDK; multicystic dysplastic kidneys; VUR; vesicoureteral reflux; SLIT2; ROBO2; SRGAP1

INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) account for 40–50% of chronic kidney disease that manifests in the first two decades of life (Brakeman 2008; Smith et al. 2007). The wide range of structural malformations (e.g. unilateral renal agenesis, multicystic dysplastic kidneys, duplex collecting system, vesicoureteral reflux) results from developmental defects of the kidneys and/or the urinary tract (Ichikawa et al. 2002; Vivante et al. 2014). More than 31 different monogenic causes of isolated CAKUT in humans have been described, which account for ~12% of cases (Hwang et al. 2014; Vivante et al. 2014). The pathogenetic basis of CAKUT lies in the disturbance of nephrogenesis due to mutations in genes that play important roles during kidney development. The underlying molecular control of normal nephrogenesis is governed by a large number of genes and signaling pathways that orchestrate these complex events. Perturbation in any of these steps can cause CAKUT, as supported by mouse models and human disease (Rasouly and Lu 2013; Vivante et al. 2014). The metanephric kidney is formed *via* reciprocal interaction between the ureteric bud (UB) and the metanephric mesenchyme (MM), starting at 4 weeks of gestation

in humans or on embryonic day 12 in mice. UB outgrowth is considered the initiating event, which critically depends on tightly regulated GDNF-RET signaling at the interface of the UB and the MM (Costantini and Shakya 2006; Durbec et al. 1996; Jeanpierre et al. 2011; Pepicelli et al. 1997; Sanchez et al. 1996; Tang et al. 1998; Vega et al. 1996). *Slit2* and *Robo2* are expressed as ligand and transmembrane receptor in the UB and the MM respectively. SLIT2-ROBO2 signaling has been shown to play a role in limiting GDNF-RET signaling to the site of the ureteric budding (Grieshammer et al. 2004; Piper et al. 2000). The importance of SLIT2-ROBO2 signaling in metanephric kidney development is underlined by knockout mouse models for *Slit2* and *Robo2*, as well as heterozygous *ROBO2* mutations in humans with CAKUT (Grieshammer et al. 2004; Hwang et al. 2014; Lu et al. 2007).

In humans, most CAKUT-causing genes reported to date exhibit an autosomal dominant mode of inheritance with variable expressivity and incomplete penetrance (Gbadegesin et al. 2013; Hwang et al. 2014; McPherson et al. 1987; Sanna-Cherchi et al. 2013). Nevertheless, mutations in seven recessive CAKUT-causing genes have recently been discovered (Humbert et al. 2014; Kohl et al. 2014; Saisawat et al. 2014).

To identify additional single-gene causes of CAKUT, we conducted whole exome sequencing (WES) followed by a burden analysis of novel heterozygous likely pathogenic variants in 26 unrelated individuals with CAKUT including their parents. Under the assumption that autosomal dominant disease causing genes harbor very few or no pathogenic variants in any healthy control cohort due to purifying natural selection, we hypothesized that likely pathogenic variants in the same gene in two or more unrelated families with CAKUT, with absence of likely pathogenic variants in the same gene from control cohorts (positive genetic burden), may indicate causality. Therefore, we compared the numbers of likely pathogenic variants per gene in our CAKUT cohort to the numbers of different but also likely pathogenic variants in 2,000 Swedish control exomes. Importantly, since all considered variants were novel and, hence, absent from the control cohort, we merely compared the numbers of different, but equally pathogenic variants as previously described by Boyden and colleagues (Boyden et al. 2012).

We identified 29 genes as significantly enriched with potentially pathogenic variants in our group of 26 unsolved CAKUT families. Amongst them, *SRGAP1* was mutated in two families with CAKUT and the only gene functioning in a signaling pathway that previously has been implicated in the pathogenesis of CAKUT.

SRGAP1 is a small GTPase activating protein downstream of the extracellular ligand *SLIT2* and the transmembrane receptor *ROBO2* that was shown to limit the active state of the second messenger *RAC1*, which is important for cell migration (Li et al. 2006; Wong et al. 2001; Yamazaki et al. 2013). We and others recently reported on families with heterozygous mutations in *ROBO2* as a rare monogenic cause of CAKUT (Bertoli-Avella et al. 2008; Dobson et al. 2013; Hwang et al. 2014; Jeanpierre et al. 2011; Lu et al. 2007). To obtain further evidence for heterozygous mutations in *SLIT2-ROBO2* pathway genes on genetic ground, we performed high-throughput exon sequencing in *SRGAP1* and *SLIT2* in a cohort of 749 individuals with isolated CAKUT.

Hence, we chose to focus on investigating a potential causative role of the newly identified variants in *SRGAP1* and to identify additional mutations in the ligand *SLIT2* in other families with CAKUT.

Here we describe and functionally characterize two novel heterozygous variants in *SRGAP1* and three novel heterozygous variants in *SLIT2*. We demonstrate that *Srgap1* is expressed in early mouse nephrogenic mesenchyme and that *SRGAP1* and *ROBO2* colocalize to the cap mesenchyme in developing rat kidney. Our functional studies further show that the newly identified *SRGAP1* mutations cause augmented inhibition of *RAC1*, whereas the newly identified *SLIT2* mutations diminish its inhibitory effect on neuronal cell migration. We thus present further evidence that heterozygous mutations in *SLIT2*, *ROBO2*, and *SRGAP1* confer risk for developing CAKUT.

MATERIALS AND METHODS

Human subjects

Following informed consent, we obtained clinical data, blood samples, and pedigrees from individuals with CAKUT. The study was approved by the institutional review board (IRB) of the University of Michigan Medical School, Boston Children's Hospital, and local IRBs according to the Declaration of Helsinki. Patients were included in the study if a diagnosis compatible with CAKUT was established by a pediatric nephrologist or (pediatric) urologist. The study comprised 749 individuals from 650 different families with CAKUT from 25 different pediatric nephrology units worldwide. Heterozygous exonic mutations in the following 23 genes that are known to cause isolated CAKUT in humans were excluded prior to this study: *BMP4*, *BMP7*, *CDC5L*, *CHD1L*, *EYA1*, *GATA3*, *HNFB1B*, *PAX2*, *RET*, *ROBO2*, *SALL1*, *SIX1*, *SIX2*, *SIX5*, *SOX17*, *UMOD*, *UPK3A*, *FRAS1*, *FREM2*, *FREM1*, *GRIPI1*, *ITGA8*, and *GREM1*. *HNFB1B*-deletions were excluded by quantitative PCR in individuals with the CAKUT phenotype of renal hypodysplasia.

Whole exome sequencing

For a group of 26 CAKUT families, which remained genetically unsolved in two previous targeted sequencing studies, whole exome sequencing (WES) and a variant burden analysis was performed as described previously (Boyden et al. 2012; Hwang et al. 2014; Kohl et al. 2014). Variants with minor allele frequencies <1% in the Yale (1,972 European subjects), NHLBI GO Exome Sequencing Project (4,300 European and 2,202 African American subjects; last accessed November, 2012), dbSNP (version 135) or 1,000 Genomes (1,094 subjects of various ethnicities; May, 2011 data release) databases were selected and annotated for impact on the encoded protein and for conservation of the reference base and amino acid among orthologs across phylogeny.

For a variant burden analysis likely pathogenic variants detected in the present CAKUT cohort were compared to different, but also likely pathogenic variants of 2,000 Swedish control exomes as previously described (Boyden et al. 2012). A Fisher exact test was used to determine significant enrichment of potentially pathogenic variants ($p < 1.0E-5$) in certain genes in the CAKUT cohort. Variants were considered "potentially pathogenic" if they were

either missense variants affecting amino acid residues evolutionary conserved in vertebrates, splice-site or nonsense variants and absent from large variant databases (EVS, 1000 Genomes, Yale in-house variant database).

Candidate gene mutation analysis

All coding exons of *SRGAP1* and *SLIT2* were sequenced by microfluidic PCR (Fluidigm®) and next-generation sequencing (MiSeq®, Illumina) as described previously by our group (Halbritter et al. 2012; Halbritter et al. 2013). Exon-flanking oligonucleotide sequences for all coding exons of *SRGAP1* and *SLIT2* are available from the authors. Detected variants were filtered against public variant databases (1,000 Genomes and NHLBI GO Exome Sequencing Project) and only novel variants were considered. Following evolutionary conservation analysis, identified mutations were confirmed by Sanger sequencing of genomic DNA.

Immunofluorescence

Immunofluorescence imaging (IF) was performed on rat kidney sections using a Leica SP5X system with an upright DM6000 compound microscope as previously described by the authors (Chaki et al. 2012). Images were processed with the Leica AF software suite. The following primary antibodies were used: SRGAP1 (Santa Cruz Biotechnology, Cat# sc-81939), ROBO2 (Santa Cruz Biotechnology, Cat# sc-31607), SIX2 (abcam, Cat# ab68908), WT1 (Santa Cruz Biotechnology, Cat# sc-192).

RAC1 and CDC42 activity assay

RAC1/CDC42 activity assays were performed according to Pellegrin et al. (Pellegrin and Mellor 2008). Briefly, the GST-PAK1 CRIB domain was purified from the BL21 (DE3) *E. coli*. HEK293T cells were transfected with wild-type or mutant *SRGAP1* using FuGene HD transfection reagent (Promega). Cells were collected 48 hours post-transfection, rinsed with PBS, lysed, and subjected to GST pull-down for 2 hours at 4°C. GST beads were washed 5 times followed by elution with sample buffer. GTP-bound CDC42 and RAC1 were analyzed by Western blotting using anti-CDC42 and anti-RAC1 antibodies (#610929 and #610651, BD Transduction Laboratories). Input controls were analyzed using anti-SRGAP1 (#H00057522-M07, Abnova) and anti- β -actin antibody (ab20272, Santa Cruz Biotechnology).

Anterior subventricular zone neuron migration assay

To make conditioned media of human SLIT2 and its mutants, 80% confluent HEK293T cells were transiently transfected to express SLIT2 using calcium phosphate. 24 hours after transfection, the cell media were replaced with fresh 10% FCS DMEM and incubated in 5% CO₂ incubator at 37°C for three days. SLIT2 conditioned medium was then collected from SLIT2 overexpressing HEK293T cells for functional anterior subventricular zone (SVZa) neuron migration assay. Migrating SVZa cells were dissected from the rostral migratory stream of postnatal rat brains at days 1–5 as described previously. (Ward and Rao 2005) The SVZa cell explants were cultured in a 3:2:1 collagen:matrigel:DMEM mixed gel in the presence of wild-type human SLIT2 or mutant SLIT2 conditioned media for 24 hours. SVZa

cells were fixed in 4% PFA and images were taken under a 10x DIC microscope. DIC images were converted in 8-bit grey-scale images and contrast was increased by 1% (ImageJ software; <http://imagej.nih.gov/ij>). To identify and count migrated cells, matrigel shadows were electronically enlarged by 250% leaving only migrated cells in the periphery visible (Supplementary Figure 6 online). In detail, matrigel shadows were selected using the “Fuzzy select” tool in GIMP2.8 image software (www.gimp.org) with a threshold of 15. The content of the selected area was deleted and filled with black using the “Bucket fill” tool. Subsequently, the black area was enlarged proportionally by increasing length and width by 250% while keeping the center fixed. Following this masking procedure, remaining visible cells were counted using the “cell counter” plugin of ImageJ. Additionally, cell migration distances out of SVZa explants were measured in ImageJ. The length of two migration distances was measured per quadrant. Hence, 24 migration distances were obtained per triplicate. P-values were calculated using the one-tailed Student’s t-test.

RESULTS

Exome sequencing and burden analysis in patients with CAKUT reveals likely pathogenic variants in *SRGAP1*

Since CAKUT are most frequently inherited in an autosomal dominant mode with incomplete penetrance, we hypothesized that different novel heterozygous mutations in the same gene in two or more unrelated individuals with CAKUT could represent monogenic causes for CAKUT. Hence, we performed whole exome sequencing (WES) in 98 individuals from 26 unrelated families with isolated CAKUT. The 26 families included in the present WES study all had an affected child with a diagnosis of the CAKUT spectrum and they all remained genetically unsolved after 2 previous targeted sequencing studies comprising 23 known CAKUT genes (Hwang et al. 2014; Kohl et al. 2014). A burden analysis of novel potentially pathogenic variants in all 26 affected families revealed 29 genes as significantly enriched with novel potentially pathogenic variants as compared to 2,000 Swedish control exomes. Since we only considered novel variants we used a large control cohort regardless of their ethnical background as previously described by one of the co-authors (Boyden et al. 2012). We identified two affected individuals and one affected mother from two unrelated families harboring different heterozygous mutations in *SRGAP1* (Table 1 and Figure 1). In family A4732 of European origin, the index patient A4732-21 and his mother were affected. A4732-21 was diagnosed prenatally with a right multicystic dysplastic kidney (MCDK). At 2.5 years of age there was no renal tissue detectable on renal ultrasound, indicating that the dysplastic kidney remnant had undergone complete involution (Figure 1A). Ultrasound examination of the patient’s asymptomatic mother, A4732-12, demonstrated right non-obstructive duplex kidney (Figure 1B). Both, the index patient and his mother were heterozygous for the *SRGAP1* mutation c.806G>A, leading to the amino acid substitution p.C269Y (Table 1 and Figure 1E–G). The missense mutation p.C269Y affects an amino acid residue conserved in vertebrates and is absent from public variant databases (>17,000 control chromosomes of the NHLBI Exome Sequencing Project and the 1000 Genomes Project) and from 1,972 European subjects in the in-house Yale variant database. In the individual A1041-21 of Arabic/European descent with caudally fused kidneys (horseshoe kidney) including a multicystic dysplastic right upper pole, a

hypodysplastic left upper pole and extra-renal features, we detected the heterozygous *SRGAP1* mutation c.1993C>A, causing an amino acid exchange in a conserved residue, p.P665T (Table 1 and Figure 1). The mutation was inherited from the father, who was clinically not affected. The father was not available for renal ultrasound examination. In order to identify additional families with *SRGAP1* mutations, we screened for mutations in *SRGAP1* in our previously described cohort of 845 affected individuals with CAKUT, which did not reveal additional affected families (Hwang et al. 2014; Sanchez et al. 1996). However, since potentially pathogenic variants in *SRGAP1* were significantly enriched in our CAKUT cohort and since *SRGAP1* is a molecular interactor of the *ROBO2* receptor, a known CAKUT-causing gene, we chose to conduct further functional studies to investigate causality of the newly identified *SRGAP1* mutations (Wong et al. 2001).

***SRGAP1* is expressed in early mouse nephrogenic mesenchyme and colocalizes with ROBO2 to the cap mesenchyme of developing rat kidneys**

To examine the role of *SRGAP1* during normal kidney development, we analyzed a *Srgap1*-lacZ reporter mouse line (Supplementary Figure 1 online). β -galactosidase staining for cells from *Srgap1* promoter-driven lacZ reporter expression at E11.5 detected *Srgap1* in the developing nephrogenic mesenchyme, including the mesonephros and metanephros (Supplementary Figure 1 online). We confirmed expression of *Srgap1* in metanephric mesenchyme at E13.5 and E14.5 by in situ hybridization (Supplementary Figure 2 online). *Srgap1*-expressing cells were absent from the E11.5 mouse nephric duct and the ureteric bud, which is consistent with what is described for *Robo2* during early mouse kidney development.¹⁴ This also is in line with microarray gene expression data in the Gudmap expression database (www.gudmap.org) (Supplementary Figure 3 online). To further relate *SRGAP1* expression pattern to renal developmental phenotypes, we performed immunofluorescence staining of *SRGAP1* in kidneys of embryonic and newborn rats (E16.5 and P0, respectively). We found *Srgap1* to be expressed in rat kidney at E16.5 (Figure 2A +B) and P0 (Figure 2C+D), whereas we did not detect *SRGAP1* in adult rat kidney (data not shown). During rat kidney development, we found *SRGAP1* to be expressed in renal mesenchyme where it colocalizes with *ROBO2* in *SIX2*-positive cap mesenchyme (Figure 2A–D). We detected the strongest overlap of the *SRGAP1* and *ROBO2* signals at the ureteric bud-facing pole of the cap mesenchyme, strongly suggesting active *SLIT2*-*ROBO2*-*SRGAP1* signaling at this site (Figure 2B). These data are highly consistent with the CAKUT phenotype observed in individuals with heterozygous *SRGAP1* mutations. Additionally, *Srgap1* is expressed in developing podocytes, suggesting an additional function here, which is in line with previous work describing podocytes as *Robo2*-expressing cells (Figure 2 A, C, D, and Supplementary Figure 4 online) (Fan et al. 2012; Lindenmeyer et al. 2010).

***SRGAP1* mutations cause augmented inhibition of RAC1**

SRGAP1 has been shown to act down-stream of *SLIT2*-*ROBO2* and to reduce the GTP-bound (active) state of *RAC1*, which is important for lamellipodial extension and retraction cycles in cell migration (Wong et al. 2001; Yamazaki et al. 2013). We hypothesized that the newly identified mutations in *SRGAP1* in families with CAKUT may have an effect on *RAC1* and *CDC42* activity, which could alter cell migration of the cap mesenchyme during

kidney development. Hence, we studied RAC1 and CDC42 activity using a PAK1-assay in human embryonic kidney cells (HEK293T) transiently overexpressing wild-type SRGAP1 *versus* mutant SRGAP1 proteins (Figure 3). Interestingly, we found that the mutant SRGAP1 proteins p.C269Y and p.P665T shared an increased inhibition on RAC1 activity as compared to wild-type SRGAP1 indicating a gain-of-function effect of the mutations (Figure 3). CDC42 activity was unchanged (Supplementary Figure 5 online).

Novel pathogenic variants in *SLIT2* cause CAKUT in humans

Next, we aimed to identify additional CAKUT-causing genes that are a component in the SLIT2-ROBO2-SRGAP1 signaling pathway. We and others have recently published that heterozygous *ROBO2* mutations cause CAKUT in humans and mice (Hwang et al. 2014; Lu et al. 2007). Intriguingly, mutated *SLIT2* has been reported to cause a CAKUT phenotype in a knockout mouse model making it a promising candidate gene for human CAKUT (Grieshammer et al. 2004). In order to identify *SLIT2* mutations in patients with CAKUT we utilized high-throughput exon sequencing to screen for mutations in *SLIT2* in 749 affected individuals with CAKUT (Hwang et al. 2014; Sanchez et al. 1996). We identified three unrelated individuals with three different heterozygous *SLIT2* missense mutations affecting conserved amino acid residues which were absent from public variant databases (Table 1, Figure 1H–J). Individual A3748-21 with multiple subcortical cysts in both kidneys had the mutation *SLIT2* c.292G>A, p.A98T (Table 1 and Figure 1H–J). Individual A4736-21 with right MCDK was found to have *SLIT2* c.1697G>A, p.S566N (Table 1 and Figure 1H–J). Interestingly, as described in individual A4732-21 with an *SRGAP1* mutation, this individual also showed MCDK and complete involution (Figure 1C–D). The third individual, A3468-21, who was diagnosed with right renal agenesis at 14 years of age, had the mutation *SLIT2* c.2712A>T, p.K904N (Table 1 and Figure 1H–J).

SLIT2 mutations cause reduced inhibition of cell migration

Knockout mice lacking *Slit2* develop supernumerary ureteric buds that remain inappropriately connected to the nephric duct, resulting in CAKUT in the form of multiple ureters and fused dysplastic kidneys (Grieshammer et al. 2004). The SLIT2 protein has a well-studied function as a chemorepellent in neuronal cell migration (Brose et al. 1999; Nguyen Ba-Charvet et al. 1999). Given the CAKUT phenotype of the *Slit2* knockout mouse model, we hypothesized that the newly identified mutations in *SLIT2* would alter its chemorepulsive activity. To test this hypothesis, we studied the function of *SLIT2* mutants on anterior subventricular zone (SVZa) cell migration (Ward and Rao 2005). Neuron precursor cells from the rat olfactory bulb express ROBO2 receptors and are repelled by wild-type SLIT2 (Nguyen-Ba-Charvet et al. 2004). In this model, we overexpressed wild-type SLIT2 and mutants in HEK293T cells and harvested SLIT2-conditioned media 72 hours after transfection. SVZa cell migration in the presence of wild-type SLIT2 or mutant-conditioned media was analyzed after 24 hours by counting migrated cells (Figure 4 and Supplementary Figure 6 and Supplementary Figure 7 online). We observed the expected radial migration of cells in the conditioned media from mock-transfected HEK293T cells. SLIT2 wild-type conditioned media strongly inhibited cell migration (Figure 4 and Supplementary Figure 6 and Supplementary Figure 7 online). However, the *SLIT2* mutations p.A98T, p.S566N, and p.K904N compromised the inhibition of SVZa cell migration,

suggesting a loss-of-function effect (Figure 4 and Supplementary Figure 6 and Supplementary Figure 7 online).

DISCUSSION

In the present study we identified two different heterozygous mutations in *SRGAP1* and three different heterozygous mutations in *SLIT2* in families with isolated CAKUT. We showed that *Srgap1* is specifically expressed in the early nephrogenic mesenchyme in mice and the metanephric mesenchyme in rats where it colocalizes with the transmembrane receptor ROBO2 during metanephric kidney development. We demonstrated that the newly discovered *SRGAP1* mutations have a gain-of-function effect in a RAC1 activity assay and that the mutated *SLIT2* proteins exhibit a loss-of-function effect in repelling migrating *SLIT2*-sensitive cells in a SVZa assay. Although we have not studied the effect of the newly identified mutations in Knock-in murine transgenic models to generate direct evidence for a causative role of the newly described mutations, causality is strongly supported by the aforementioned assays and by genetic evidence on a gene and variant level according to recent guidelines (MacArthur et al. 2014). Hence, we presented evidence that heterozygous mutations in the *SLIT2*-*ROBO2*-*SRGAP1* signaling pathway may confer risk for developing CAKUT in humans.

Our current model of pathogenesis in humans with CAKUT and *SLIT2*-*ROBO2*-*SRGAP1* mutations is based on accumulating evidence that imbalances of a tightly balanced small GTPase network interferes with migrating cells of the ureteric bud and the metanephric mesenchyme during kidney development. The ligand *SLIT2* is expressed in the nephric duct, ureteric bud, and ureteric tips (Supplementary Figure 3 online), whereas its receptor *ROBO2* is expressed in the adjacent metanephric mesenchyme. The present work adds *SRGAP1* as a downstream effector to the established *SLIT2*-*ROBO2* signaling pathway during kidney development. The newly identified deactivating *SLIT2* mutations and activating *SRGAP1* mutations do not allow a harmonic conclusion that either up- or down-regulation of *SLIT2*/*ROBO2*/*SRGAP1* signaling causes CAKUT, because our study showed that gain-of-function mutations in *SRGAP1*, as well as loss-of-function mutations in *SLIT2* may be pathogenic. Instead, we conclude that both up- or down-regulation of a tightly balanced small GTPase network can lead to disease. Following our *in-vitro* functional studies it will be necessary to directly observe the behavior of the mutated proteins during metanephric kidney development, preferably in transgenic knock-in animal models.

The predominant phenotypes in individuals with heterozygous mutations in either *SLIT2* or *SRGAP1* were multicystic dysplastic kidneys that undergo complete involution during childhood. This fact is suggestive for a pathway-phenotype correlation, although more cases

SUPPLEMENTAL DATA

Supplementary Figure 1. *Srgap1* is expressed in early mouse developing kidney.

Supplementary Figure 2. *Srgap1* is expressed in mouse metanephric mesenchyme and developing glomeruli at E13.5 and E14.5.

Supplementary Figure 3. *Srgap1* and *Robo2* are coexpressed in metanephric mesenchyme, cap mesenchyme, and renal corpuscles in mice at E.11.5 and E15.5

Slit2 is expressed in ureteric bud and ureteric tip at E11.5 and E15.5.

Supplementary Figure 4. *SRGAP1* partially colocalizes with *ROBO2* in developing podocytes.

Supplementary Figure 5. Overexpression of mutant *SRGAP1* does not alter CDC42 activity in cultured HEK293T cells.

Supplementary Figure 6. Mutations in *SLIT2* detected in individuals with CAKUT reduce the chemorepulsive effect of *SLIT2*.

Supplementary Figure 7. Similar amounts of *SLIT2* proteins present in the conditioned media used for the SVZa assay (Figure 4).

and larger pedigrees are needed before we can draw this conclusion and attribute monogenic causality. The presence of unilateral MCDK as a genetic disease seems counterintuitive. However, this is a very common presentation of monogenic CAKUT cases and does not argue against a genetic etiology (Hwang et al. 2014). In family A1041 the affected child inherited the *SRGAP1* mutation from a healthy father with a normal renal ultrasound. This family may represent a case of incomplete penetrance, which is a common finding in CAKUT, and thus does not exclude causality. In this case the treating physician had lost contact with the family and we were not able to obtain renal ultrasounds in other family members.

In summary, by discovery of mutations in *SLIT2* and *SRGAP1* we implicated multiple components of a coherent signaling pathway in the pathogenesis of human CAKUT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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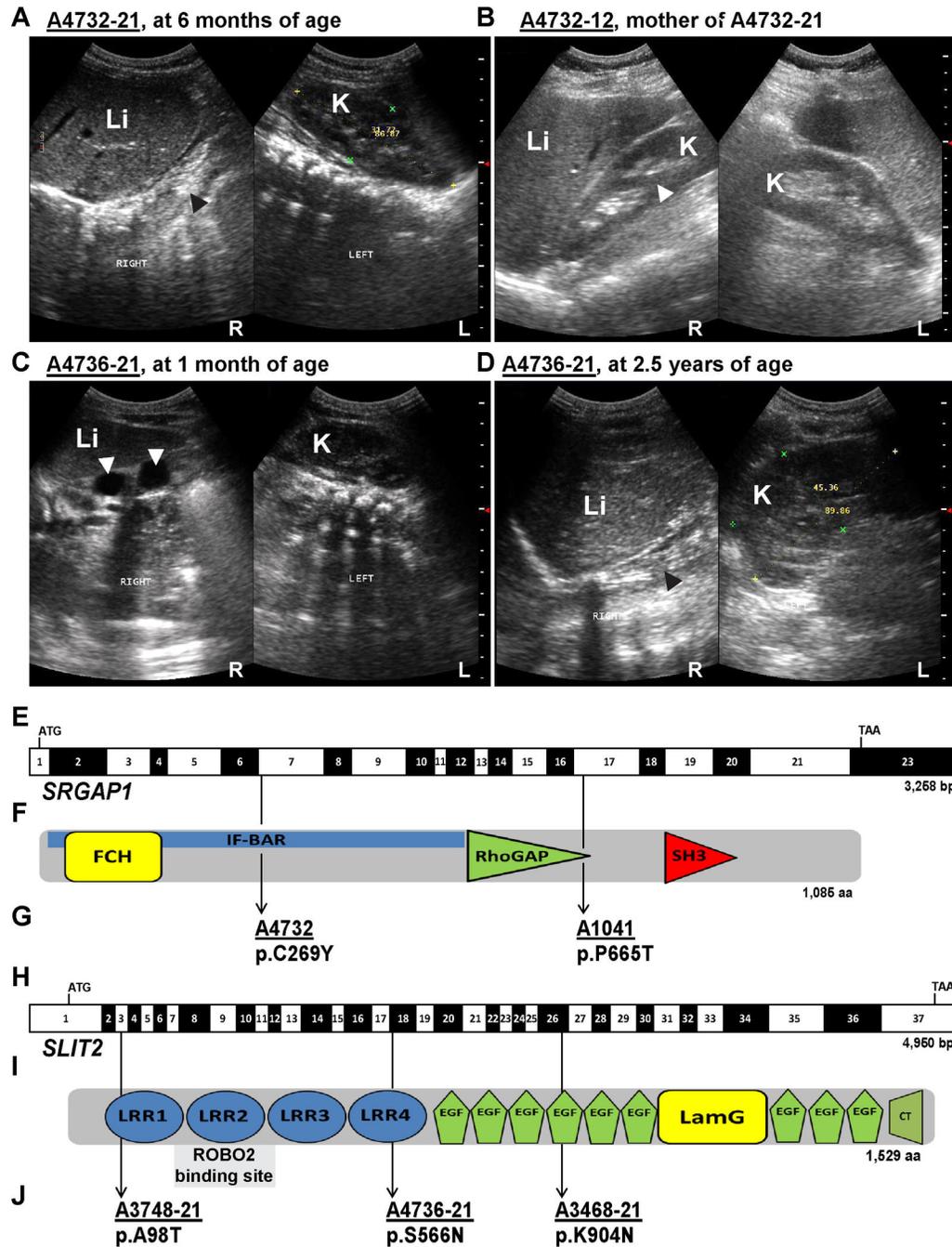


Figure 1. Ultrasonographic findings in two families with CAKUT and localization of newly identified mutations in *SRGAP1* and *SLIT2* cDNAs and proteins
 (A) Renal ultrasonography of individual A4732-21 at age 6 months (*SRGAP1* mutation) showing absence of renal tissue on the right side (black arrow-head) at the expected location adjacent to the liver (Li). The left kidney (K) shows compensatory hypertrophy. Prenatally, right multicystic dysplastic kidney (MCDK) was diagnosed (not shown).

(B) Renal ultrasonography of the patient's mother (*SRGAP1* mutation) showing separation of the central echogenic complex in the right kidney (white arrow-head) indicating a non-obstructive duplex kidney. The left kidney is normal.

(C) MCDK on the right side in individual A4736-21 (*SLIT2* mutation) at age 3 months (white arrow-heads denote two large cysts).

(D) In the same individual, at age 2.5 years, no renal tissue was detectable on ultrasound on the right (black arrow-head), indicating complete involution of the MCDK. The left kidney (K) shows compensatory hypertrophy.

(E) Exon structure of human *SRGAP1* cDNA. The position of the start codon (ATG) and stop codon (TGA) are indicated.

(F) Domain structure of the *SRGAP1* protein.

(G) Mutations detected in *SRGAP1* in two families with CAKUT (family identifiers are underlined) are depicted as black arrows indicating their positions in relation to exons and protein domains (see also Table 1).

(H) Exon structure of human *SLIT2* cDNA.

(I) Domain structure of *SLIT2*.

(J) Mutations of *SLIT2* detected in three unrelated individuals with CAKUT (see also Table 1).

aa, amino acids; bp, base pairs; CT, C-terminal cysteine knot domain; EGF, epidermal growth factor-like domain; FCH, Fes/CIP4 homology domain; IF-BAR, inverse F-BAR domain; K, kidney; LamG, laminin G domain; L, left; Li, liver; LRR, leucine-rich repeat domain; R, right; RhoGAP, GTPase-activator protein for Rho-like GTPases domain; SH3, Src homology 3 domain.

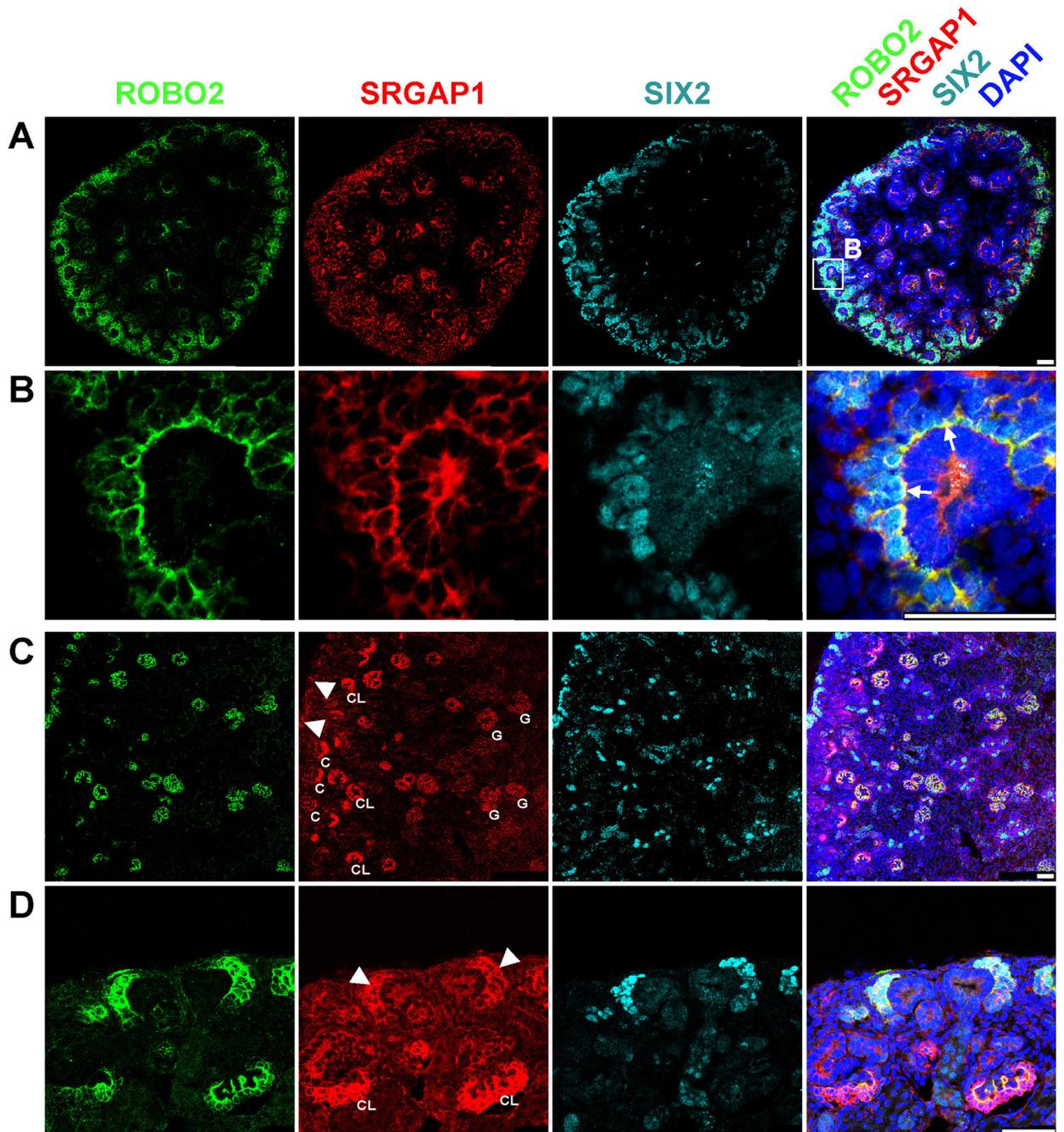


Figure 2. *SRGAP1* is coexpressed with *ROBO2* in cap mesenchyme in rat kidney at E16.5 and P0 Immunofluorescence microscopy of sections of rat kidneys at E16.5 (A and B) and at P0 (C and D).

(A–B) *ROBO2* (green) is expressed in *SIX2*-positive (blue-green) cap mesenchyme and developing glomeruli. *SRGAP1* (red) colocalizes with *ROBO2* in cap mesenchyme.

(B) Enlargement from (A). Note that the overlap of *ROBO2* and *SRGAP1* (yellow) in cap mesenchyme is most prominent at the ureteric tip-facing pole of the cap mesenchyme (arrows) indicating active *ROBO2*-*SRGAP1* signaling at this site.

(C–D) ROBO2 is expressed in SIX2-positive cap mesenchyme (arrow-heads) and in different stages of development of glomerular podocytes (C, comma-shaped body; CL, capillary loop stage glomerulus; G, mature-appearing glomerulus).

(C) Overview of renal cortex showing different stages of embryonic nephron development. The nephrogenic zone is marked by SIX2-positive cap mesenchyme directly underneath the renal capsule (arrow-heads). Different stages of glomerular development on a gradient from early (C, CL) to late (G) express ROBO2 and SRGAP1.

(D) SRGAP1 (red) is coexpressed with ROBO2 (green) in cap mesenchyme (arrow-heads) and highly expressed in developing podocytes in capillary loop stages glomeruli (arrows). Scale bar: 50 μ m.

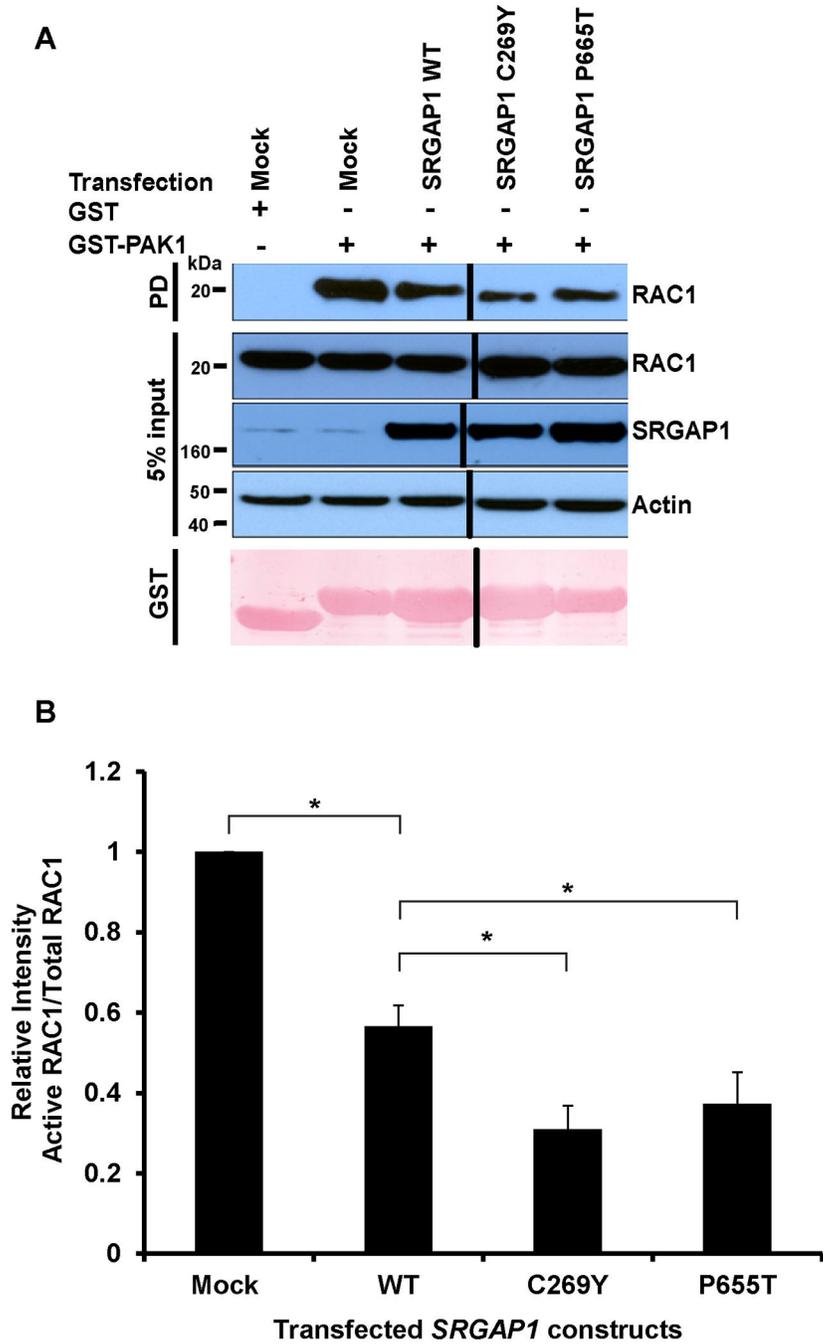


Figure 3. RAC1 activity is reduced upon overexpression of SRGAP1 mutants in cultured HEK293T cells

(A) Active GTP-bound RAC1 precipitated by GST-PAK1 pulldown (PD) from HEK293T cells transfected with wild-type (WT) versus mutant SRGAP1 constructs. Note that HEK293T cells transfected with human SRGAP1 mutants C269Y and P665T exhibit an enhanced decrease in RAC1 activity compared to wild-type SRGAP1. The efficiency of SRGAP1 transfections and 5% input control were confirmed by immunoblotting with anti-SRGAP1 and anti-actin antibodies, respectively.

(B) Relative RAC1 activities (PAK1-bound RAC1/total RAC1) based on anti-RAC1 immunoblot signal intensities in **(A)**. Ratios are normalized to mock transfection. A and B represent 5 experiments each.

*, $P < 0.05$ (Student's t-test); Error bars indicate one standard deviation (n=3).

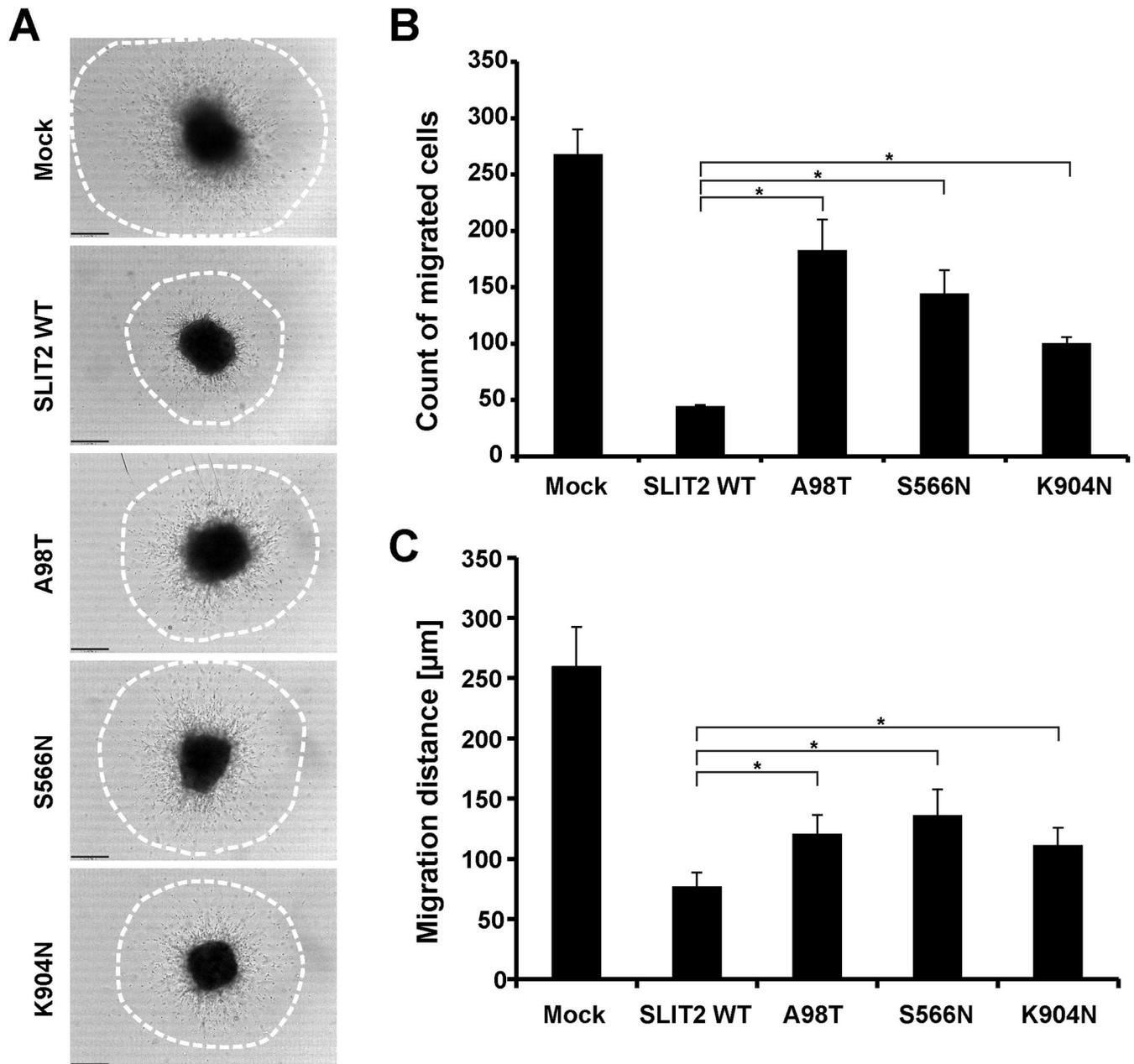


Figure 4. *SLIT2* mutations identified in CAKUT patients compromise its inhibition of SVZa neuronal migration

(A) Representative DIC images show SVZa neuronal migration after 24 hours in the presence of *SLIT2* wild-type (WT) or *SLIT2* mutants (A98T, S566N, K904N) conditioned media. Media from mock transfected HEK293T cells were used as a negative control. Note that wild-type *SLIT2* inhibits neuronal migration whereas the *SLIT2* mutants show a diminished inhibition. Dot circles in processed image column represent the areas where the number of migrated cells out of SVZa explants and migration distance were quantified (see also Supplementary Figure 6 online).

(B) Quantification of migrated SVZa cell counts in (A). Note wild-type SLIT2 has less migrated cells out of SVZa explants. SLIT2 mutants A98T, S566N, and K904N show a diminished inhibitory effect on migrated cell counts.

(C) Quantification of average SVZa cell migration distances in (A). Note wild-type SLIT2 inhibits migration of SVZa cells. SLIT2 mutants A98T, S566N, and K904N show a reduced inhibitory effect on migration indicating partial loss-of-function.

Scale bar: 100µm.

*, $P < 0.05$ (Student's t-test); Error bars indicate one standard deviation (n=3).

Table 1

Clinical phenotypes and mutations detected in *SRGAP1* and *SLIT2* in individuals with CAKUT from 5 families.

Family	Individual	Sex	Origin	Phenotype	Segregation	Nucleotide Change	Amino Acid Change	Conser vation ^a	SIFT	PP2 HumVar	GERP	PhyloP	EVS
<u>Mutations in <i>SRGAP1</i></u>													
A4732	-21	M	MAC	R MCDK R ureteroceles	Ma	c.806G>A	p.C269Y	<i>D.r.</i>	DEL	0.84	4.78	0.982	0/13,006
	-12 (mother)	F		R duplicated Kidney									
A1041	-21	F	ARAB/EU	Horseshoe kidney, R MCDK L RHD, cleft palate, mental retardation	Pa	c.1993C>A	p.P665T	<i>C.i.</i>	DEL	0.309	5.05	0.589	0/13,006
	-11 (father)	M	ARAB	unknown									
<u>Mutations in <i>SLIT2</i></u>													
A3748	-21	M	USA	B subcortical Cysts	no parental DNA	c.292G>A	p.A98T	<i>D.r.</i>	TOL	0.938	6.06	0.992	0/13,000
A4736	-21	M	MAC	R MCKD	Ma ^b	c.1697G>A	p.S566N	<i>C.i.</i>	TOL	0.908	5.5	0.997	0/13,006
A3468	-21	M	MAC	R renal agenesis	no parental DNA	c.2712A>T	p.K904N	<i>D.r.</i>	TOL	0.341	1.51	0.738	0/13,006

^aContinuous evolutionary conservation of the amino acid residue considering the following species: *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis*, *Danio rerio*, *Ciona intestinalis*.

^bThe mother has no signs of CAKUT in renal ultrasonography.

ARAB, Arab countries; B, bilateral; *C.i.*, *Ciona intestinalis*; DEL, deleterious; *D.r.*, *Danio rerio*; EVS, exome variant server (<http://evs.gs.washington.edu/EVS/>); EU, Europe; GERP, GERP score (<http://mendel.stanford.edu/SidowLab/downloads/gerp/>); L, left; MAC, Macedonia; MCDK, multicystic dysplastic kidney; PhyloP, PhyloP score; PP2 HumVar, PolyPhen2 humvar score (<http://genetics.bwh.harvard.edu/pph2/>); R, right; RHD, renal hypodysplasia; SIFT, sorting intolerant from tolerant score (<http://sift.jcvi.org/>); TOL, tolerated.

Nucleotide change numbering refers to the cDNA position of the following transcripts: NM_020762.2 (*SRGAP1*) and NM_004787.1 (*SLIT2*) where +1 corresponds to the A of ATG translation start codon.