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Genome-wide Association Studies Identify Genetic Loci Associated With Albuminuria in Diabetes

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Elevated concentrations of albumin in the urine, albuminuria, are a hallmark of diabetic kidney disease and are associated with an increased risk for end-stage renal disease and cardiovascular events. To gain insight into the pathophysiological mechanisms underlying albuminuria, we conducted meta-analyses of genome-wide association studies and independent replication in up to

5,825 individuals of European ancestry with diabetes and up to 46,061 without diabetes, followed by functional studies. Known associations of variants in *CUBN*, encoding cubilin, with the urinary albumin-to-creatinine ratio (UACR) were confirmed in the overall sample ($P = 2.4 \times 10^{-10}$). Gene-by-diabetes interactions were detected and confirmed for variants in *HS6ST1* and near



RAB38/CTSC. Single nucleotide polymorphisms at these loci demonstrated a genetic effect on UACR in individuals with but not without diabetes. The change in the average UACR per minor allele was 21% for *HS6ST1* ($P = 6.3 \times 10^{-7}$) and 13% for *RAB38/CTSC* ($P = 5.8 \times 10^{-7}$). Experiments using streptozotocin-induced diabetic *Rab38* knockout and control rats showed higher

urinary albumin concentrations and reduced amounts of megalin and cubilin at the proximal tubule cell surface in *Rab38* knockout versus control rats. Relative expression of *RAB38* was higher in tubuli of patients with diabetic kidney disease compared with control subjects. The loci identified here confirm known pathways and highlight novel pathways influencing albuminuria.

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Urinary albumin and serum creatinine are two biomarkers recommended for the routine assessment of chronic kidney disease (CKD) (1). Even at physiological rates of glomerular filtration, small elevations in urinary albumin concentrations are associated with an increased risk for CKD progression, end-stage renal disease (ESRD), cardiovascular events, and cardiovascular and all-cause mortality (2–4). Patients with diabetes are at a particularly high risk for CKD and its sequelae: the prevalence of CKD among individuals with diabetes is >40% compared with ~10% in the general U.S. adult population (5), and the presence of CKD is an important contributor to the excess mortality in diabetes (6). The appearance of significant amounts of albumin in the urine (albuminuria) is a hallmark of diabetic kidney disease (DKD), the incidence of which continues to rise along with type 2 diabetes worldwide (7). Residual diabetes-related microvascular risk represents an important challenge even in treated individuals (8), and DKD remains the leading cause of ESRD. No new effective treatments for DKD have been approved in more than two decades (9), highlighting the importance to better understand its underlying mechanisms.

Using genome-wide association study (GWAS) meta-analysis in general population cohorts, we previously identified a missense single nucleotide polymorphism

(SNP) in the gene encoding cubilin (*CUBN*) in association with the urinary albumin-to-creatinine ratio (UACR) (10). *CUBN* is currently the only genome-wide significant locus for UACR. However, this variant explains only a small fraction of the previously reported heritability of albuminuria, ranging from 0.2 to 0.46 in the general population and in those with diabetes (11–13), suggesting that additional genetic variants remain to be found. Here we report the results of a GWAS meta-analysis of albuminuria traits in the general population performed in almost twice the sample size of our previous study (10), with a special focus on those with diabetes, replication in additional independent individuals, and follow-up investigations in human tissues and a genetically modified animal model of diabetes.

RESEARCH DESIGN AND METHODS

Study Populations

Our study was based on 30 discovery and replication studies mostly from the general population, with the exception of Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) and Genetics of Diabetic Nephropathy (GENDIAN), which enrolled exclusively individuals with type 2 diabetes, totaling 67,452 participants of European

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ancestry across the different analyses (up to 7,787 with diabetes in discovery and replication). The study characteristics, including the distribution of albuminuria and diabetes, are reported in Supplementary Table 1. Study protocols were approved by each local institutional review board or ethics committee, and all human participants gave written informed consent.

Phenotype Definitions and Analytical Strategy

The measurement of urinary albumin and creatinine in each study is reported in Supplementary Table 2. Urinary albumin values below the detection limit of the used assays were set to the lower limit of detection. Rather than using urinary albumin, the UACR was calculated as urinary albumin/urinary creatinine (mg/g) to account for differences in urine concentration. Microalbuminuria (MA) was defined as UACR >25 mg/g in women and >17 mg/g in men (10). Diabetes was defined as fasting glucose \geq 126 mg/dL, nonfasting glucose \geq 200 mg/dL, or treatment for diabetes, or by self-report if this information was not available. Across studies, we evaluated two traits, UACR and MA, and performed four GWAS meta-analyses: MA and UACR in the overall sample, as well as UACR—a continuous trait with higher statistical power—separately among those with and without diabetes. Diabetes-stratified genome-wide association analyses of MA were not performed due to limited sample size. Detailed information on the design, genotyping, imputation, and data management of each study is provided in Supplementary Tables 2 and 3.

Discovery Meta-Analysis, Replication, and Power

Stringent quality control of the genetic data was performed at the individual study level and again at the meta-analysis level using state-of-the-art methods. Missing genotypes were imputed using the HapMap reference panels in 19 studies and the 1000 Genomes reference panels in two studies. Details of genotyping, imputation software, reference panels, and quality filters in each study are reported in Supplementary Table 3.

All GWAS were performed following a standardized analysis protocol. In each study, the natural logarithm of UACR was taken. Subsequently, sex-specific residuals were obtained from linear regression models of \ln (UACR) on age and study-specific covariates, including study center and genetic principal components, to adjust for possible population stratification, if applicable. The continuous sex-specific residuals were then combined and used as the dependent variable that was regressed on imputed allelic dosages for each SNP in the GWAS.

Before the meta-analyses, all study-specific GWAS summary files underwent quality control using the GWAToolbox (14). Genomic-control (GC) (15) correction was applied when the GC factor was >1 . Inverse variance weighted fixed-effects meta-analyses were conducted using METAL (16). The I^2 statistic was used to evaluate between-study heterogeneity (17). All meta-analyses

were performed in duplicate by two independent researchers.

After meta-analysis, SNPs with average minor allele frequency (MAF) <0.01 were excluded, and another GC correction was applied. There were 2,191,945 SNPs with average MAF >0.05 and present in $>50\%$ of the studies, which were then clustered based on correlation (linkage disequilibrium pruning using $r^2 \leq 0.2$) with the respective index SNP (the SNP with the lowest P value) within windows of ± 1 MB to identify independent SNPs with suggestive association ($P < 10^{-5}$) in one or more of the four analyses.

Replication testing was then performed for signals that were genome-wide significant ($P < 5 \times 10^{-8}$) in any analysis or that showed suggestive association among those with diabetes, motivated by the clinical importance of DKD and the stronger association of the known and validated *CUBN* variant on UACR among those with diabetes (18). Replication was defined as a one-sided P value <0.05 in the meta-analysis of independent replication studies. Five of the nine studies that contributed to replication used imputed dosage, and four studies performed replication genotyping of the index SNPs. A meta-analysis of the replication results was performed. The double GC-corrected results from the discovery meta-analysis and the results of the nine replication studies were meta-analyzed to obtain the overall statistical significance. Unless stated otherwise, all reported P values are two-sided.

Assuming that associated SNPs explain a respective 0.6% and 0.5% of the UACR variance in diabetes (Table 1), there was 95% and 91% power, respectively, to replicate the seven suggestive loci from the discovery stage in an additional 1,800 samples with a one-sided P value of <0.05 .

Additional Analyses to Characterize Novel Loci

Replicated SNPs were further evaluated even in the absence of genome-wide significance because, in addition to the significant replication P value, the low heterogeneity across cohorts and the biological plausibility of the *RAB38* locus further increased confidence in the findings. The SNPs were evaluated in the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) study for association with a primary clinical end point defined as time from the DCCT baseline until time to persistent MA or a secondary end point of time to incident albumin excretion rate >300 mg/24 h or ESRD (10). Time to outcome development or censoring was determined as the number of visit years from the DCCT baseline up to and including the 12th year of EDIC follow-up. Subjects with persistent MA at DCCT baseline and DCCT year 1 were excluded from analyses of that outcome.

Epigenomic map analyses were performed, as described previously (19), by using data from human kidney and kidney proximal tubule epithelial cells that can be accessed at Gene Expression Omnibus (GSE49637).

Table 1—Replicated SNP associations with UACR in individuals with diabetes

	Sample size (n)	Effect on log(UACR) mg/g	SE	P value	r ² %
rs649529, RAB38					
Discovery	5,825	−0.15	0.03	9.3 × 10 ^{−6}	0
Replication	1,962	−0.12	0.05	0.02	0
Combined	7,787	−0.14	0.03	5.8 × 10 ^{−7}	0
rs13427836, HS6ST1					
Discovery	5,509	0.20	0.04	6.1 × 10 ^{−6}	10
Replication	1,890	0.16	0.07	0.03	58
Combined	7,399	0.19	0.04	6.3 × 10 ^{−7}	30

For both variants, the effect of each additional copy of the minor allele (T) on UACR was modeled in an additive fashion. r^2 is provided as a measure of heterogeneity across studies. Imputation quality ranged from 0.41 to 1.0 for rs649529 and from 0.44 to 1.0 for rs13427836. The variants were directly genotyped in four of the replication studies, with a call rate ranging from 0.98 to 1.0 for rs649529 and from 0.99 to 1.0 for rs13427836. The estimated proportion of explained variance in UACR among those with diabetes is 0.6% for rs649529 and 0.5% for rs13427836, using the formula $2 \times \text{MAF} \times (1 - \text{MAF}) \times \text{effect}^2 / \text{var}(\log[\text{UACR}])$, based on the combined effect estimates from Table 1 and the phenotypic variance in the large population-based ARIC Study.

Genetic associations with additional renal function traits, estimated glomerular filtration rate (eGFR), and CKD, were evaluated based on results from GWAS meta-analysis of the corresponding traits within the CKDGen Consortium (C.P., personal communication).

Gene Expression Analyses in Human Tissues

Quantification of transcript abundance in microdissected fractions of human glomeruli and tubuli from surgical nephrectomies, living allograft donors, and portions of diagnostic kidney biopsies (20) was performed using RNA sequencing. Tissue from different renal compartments was separated using microdissection, homogenized, and stored at -80°C . Total RNA of human proximal tubule fractions ($n = 256$) and glomerular cells ($n = 48$) was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quality was assessed with the Agilent Bioanalyzer 2100, and RNA preparations exhibiting RNA integrity number scores >7 were used for cDNA synthesis (library preparation at DNA Sequencing Core at UT Southwestern Medical Center). In short, 1 μg total RNA was used to isolate poly A purified mRNA using the Illumina TruSeq RNA Preparation Kit. Single-end 100 bp sequencing was performed, and the annotated RNA counts (fastq) were calculated by CASAVA 1.8.2 (Illumina). Reads were mapped to the reference genome (National Center for Biotechnology Information build 37, hg19) using Spliced Transcripts Alignment to a Reference (STAR). Reads per kilobase of transcript per million mapped for *HS6ST1* and *RAB38* were compared between glomerular and tubular fractions using a two-sided t test.

Comparison of candidate gene expression between case subjects with DKD proven by biopsy specimen and healthy control subjects was based on publicly available microarray data from human microdissected glomeruli and tubuli (Gene Expression Omnibus, GSE 30122) (20). Raw data were analyzed using the R package "Affy" version 1.44.0, and expression levels were normalized using robust multiarray average. Transcript abundance between

patients and control subjects was compared using two-sided t tests; statistical significance was defined as $P < 8.3 \times 10^{-3}$ ($\alpha = 0.05$ corrected for six comparisons).

Studies of *Rab38* in Rats

To better understand the association of *RAB38* with albuminuria in diabetes, we studied genetically modified rat models of diabetes. Eight *Rab38* knockout (KO) rats on a fawn-hooded hypertensive (FHH) background, seven rats transgenic for the wild-type Brown Norway rat *Rab38* allele, and seven congenic rats were generated and raised as described previously (21–23). *Rab38* KO rats did not express the protein (22). These references also describe the recording of blood pressure and the measurement of glucose and albuminuria. Diabetes was induced by administering streptozotocin (STZ) (STZ; 50 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) to 9-week-old male rats.

Paraffin blocks of rat kidney samples were sectioned (6- μm thickness) with a Leica RM2255 rotary microtome (Thermo-Fisher Scientific, Waltham, MA) on Superfrost Plus glass slides (12-550-15, Thermo-Fisher Scientific). Before staining, slides were deparaffinized in changes of CitriSolv (22-143-975, Thermo-Fisher Scientific) and 70% isopropanol. Antigen retrieval was accomplished by incubating in sodium citrate buffer (1.8% 0.1 mol/L citric acid, 8.2% 0.1 mol/L sodium citrate, in distilled water, pH 6.0) in a rice cooker for 30 min. Slides were blocked with PBS blocking buffer (1% BSA, 0.2% nonfat dry milk in PBS) for 30 min and stained with primary antibodies specific for megalin or cubilin diluted in blocking buffer overnight at 4°C . Sheep anti-megalyn and rabbit anti-cubilin were provided by Dr. P. Verroust, INSERM, Paris, France. After two washes in 0.1% Tween 20 (v/v in PBS), slides were incubated with corresponding fluorophore-conjugated secondary antibodies (Invitrogen), diluted in blocking buffer at room temperature for 1 h, and counterstained with 10 $\mu\text{mol/L}$ Hoechst 33342 (Molecular Probes-Invitrogen, H1399). Slides were subsequently mounted in Prolong Gold Antifade reagent (Invitrogen), and images were acquired with a Leica SP5 confocal laser scanning microscope (Center for

Microscopy and Image Analysis, University of Zurich) equipped with a Leica APO $\times 63$ NA 1.4 oil immersion objective.

All experiments were performed in compliance with National Institutes of Health *Guide for Care and Use of Laboratory Animals*, and all used protocols were approved by the local institutional animal care and use committee.

RESULTS

Discovery of Genomic Loci Associated With Albuminuria Traits

The discovery GWAS meta-analyses for the four traits included up to 20 studies and up to 54,450 individuals per trait. The median UACR in the 20 individual studies that contributed to the UACR meta-analysis ranged from 2.5 to 15.6 mg/g. Across all studies, the mean proportion of women was 53%, and the median of average age was 57 years. The prevalence of diabetes in the population-based studies ranged from 1 to 14% (Supplementary Table 1).

There was no evidence of systematic biases influencing the genome-wide association results, as indicated by low genomic control parameters (Supplementary Fig. 1). Only SNPs in the previously identified *CUBN* locus showed genome-wide significant association with both UACR ($P = 2.4 \times 10^{-10}$, Supplementary Table 4 and Supplementary Fig. 2) and MA ($P = 1.3 \times 10^{-10}$, Supplementary Table 5 and Supplementary Fig. 2). The effect of the minor C allele of the index SNP rs10795433 on logarithmic UACR values was fourfold larger among 5,825 individuals with diabetes (0.19 log[mg/g], $P = 2.0 \times 10^{-5}$) compared with 46,061 individuals without diabetes (0.045 log[mg/g], $P = 8.7 \times 10^{-6}$; $P = 8.2 \times 10^{-4}$ for difference). This corresponds, for each additional C allele, to a 5% higher geometric mean of UACR ($\exp[0.045]$) in individuals without diabetes compared with 21% higher average UACR in those with diabetes ($\exp[0.19]$).

Suggestive associations were identified for all four analyses (Supplementary Tables 4–7 and regional association plots in Supplementary Fig. 3). Among the clinically important group of individuals with diabetes, seven genomic loci contained one or more SNPs showing a suggestive association with UACR. These were exclusively identified in the meta-analysis of individuals with diabetes and mapped into or near *HS6ST1*, *CNTN4*, *KBTBD8*, *TFAP2B/PKHD1*, *CHN2*, *WDR11/FGFR2*, and *RAB38/CTSC* (Supplementary Table 7). Following our analytical strategy, we selected the index SNP in each of these seven regions for follow-up among up to 1,962 independent individuals with diabetes.

The Supplementary PDF document contains the QQ and Manhattan plots of all GWAS meta-analyses, the regional association plots, tables with cohort descriptions, and association results of SNPs at $P < 10^{-5}$. Results from discovery GWAS meta-analysis are publicly available at <http://fox.nhlbi.nih.gov/CKDGen/>.

Replication Analyses Implicate *RAB38/CTSC* and *HS6ST1* as Novel Loci for UACR in Diabetes

The replication analyses included nine studies and up to 1,962 individuals with diabetes. The median UACR across

replication studies ranged from 3.8 to 14.5 mg/g. The mean proportion of women was 49%, and the median of average age was 55 years. For the seven SNPs tested for replication (Supplementary Table 8), we assessed whether the one-sided P value was < 0.05 in the combined replication studies (see RESEARCH DESIGN AND METHODS). This was the case for two SNPs: intergenic rs649529 upstream of *RAB38*/downstream of *CTSC* on chromosome 11q14 (Fig. 1A) and the intronic variant rs13427836 in *HS6ST1* on chromosome 2q21 (Fig. 1B). As illustrated in Fig. 1C, each additional copy of the minor T allele of rs649529 at *RAB38/CTSC* was consistently associated with lower UACR among the 5,825 individuals in the discovery and 1,962 in the replication cohorts (combined $P = 5.8 \times 10^{-7}$) (Table 1), with no evidence of heterogeneity across cohorts ($I^2 = 0\%$). This effect corresponded to 13% lower geometric mean of UACR per copy of the T allele. Similarly, rs13427836 in *HS6ST1* showed consistent effects across cohorts (combined $P = 6.3 \times 10^{-7}$) (Table 1), with each copy of the T allele associated with an $\sim 21\%$ higher mean UACR but moderate heterogeneity ($I^2 = 29.9\%$) (Fig. 1D).

The association of rs649529 near *RAB38/CTSC* and rs13427836 in *HS6ST1* was not found in individuals without diabetes ($P = 0.64$ and $P = 0.38$, respectively) (Table 2). Differences in the association with UACR among those with and without diabetes were significant (t test for difference: $P = 6.9 \times 10^{-6}$ for rs649529 and $P = 1.7 \times 10^{-5}$ for rs13427836). Effects for the index variant in *CUBN* are provided for comparison. We also evaluated the association of the replicated SNPs with MA in the setting of diabetes. Information was obtained from a subset of studies with sufficiently high numbers of individuals with diabetes and MA ($n = 2,552$; Atherosclerosis Risk in Communities Study [ARIC], Cardiovascular Health Study [CHS], Cohorte Lausannoise [CoLaus], European Prospective Investigation into Cancer and Nutrition [EPIC], Framingham Heart Study [FHS], Kooperative Gesundheitsforschung in der Region Augsburg [KORA] F3, KORAF4, and Study of Health in Pomerania [SHIP]). Across cohorts, the odds ratio for MA for each copy of the minor allele was 0.84 for rs649529 near *RAB38* ($P = 0.019$) and 1.39 for rs13427836 in *HS6ST1* ($P = 7.8 \times 10^{-4}$), consistent with the direction of the SNP effects on UACR.

Characterization of Genetic Effects by Markers of Kidney Function and Diabetes

We next investigated whether the gene-by-environment interaction was also observed for the eGFR, another measure of kidney function, and/or diabetes or glycemic traits. No statistically significant associations were found between rs649529, rs13427836, rs10795433, and eGFR in those with or without diabetes (Table 2) and no associations were found with CKD. There were also no statistically significant associations of these variants with type 2 diabetes, fasting blood glucose, or plasma hemoglobin

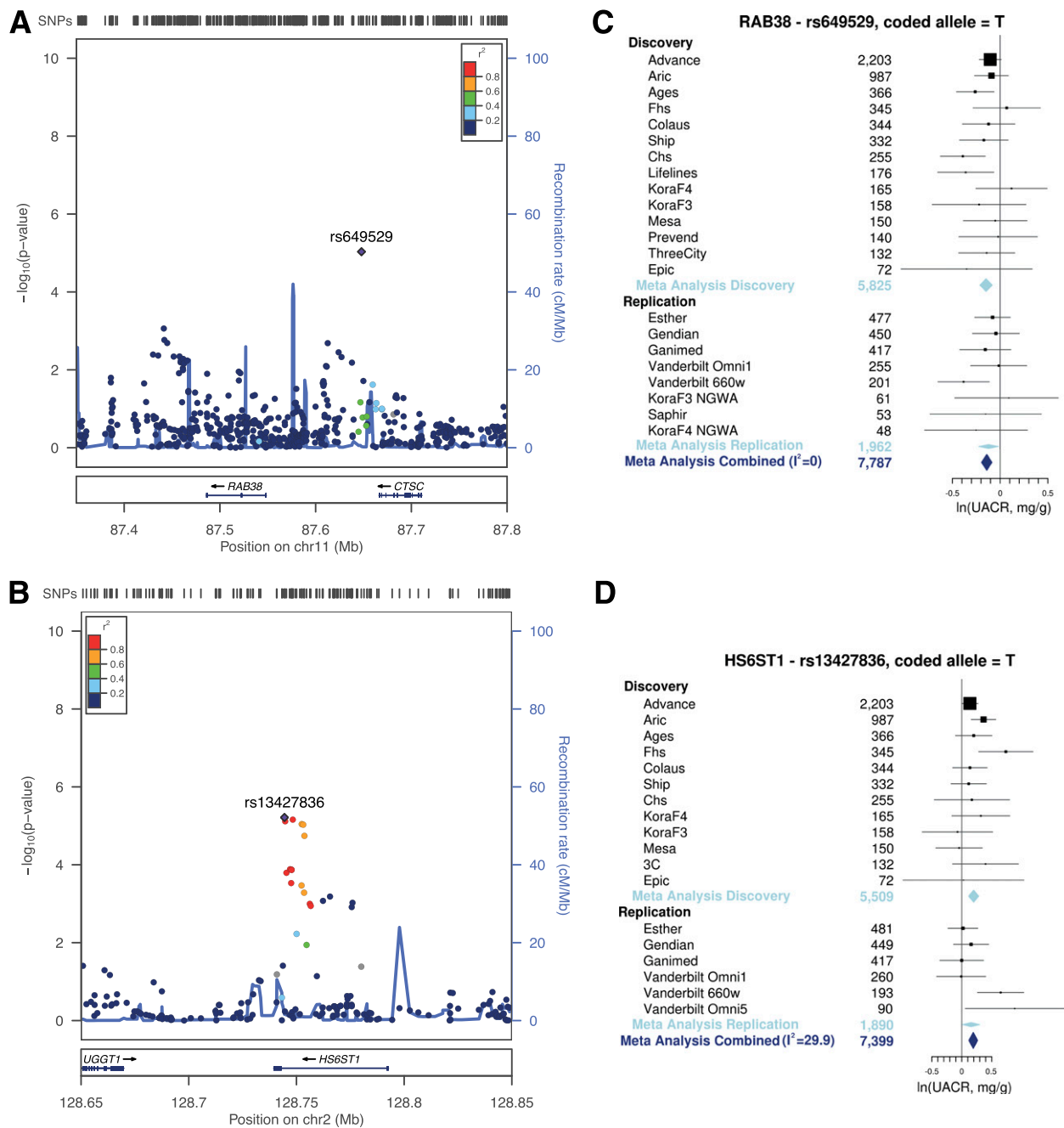


Figure 1—Overview of associated genomic loci at *RAB38/CTSC* and *HS6ST1* and consistent association with albuminuria in diabetes across the contributing studies. *A*: Regional association plot of the *RAB38/CTSC* locus on chromosome 11. *B*: The T allele at rs649529 is associated with lower UACR across discovery and replication studies. *C*: Regional association plot of the *HS6ST1* locus on chromosome 10. *D*: The T allele of intronic rs13427836 is associated with higher UACR across discovery and replication studies. The solid squares indicate the mean difference and are proportional to the weights used in the meta-analysis. The solid vertical line indicates no effect. The diamond indicates the weighted mean difference, and the lateral tips of the diamond indicate the associated CIs. The horizontal lines represent the 95% CI.

A_{1c} concentrations (Table 2), indicating that the observed associations pertain to albuminuria in the setting of diabetes rather than to diabetes or impaired glucose metabolism per se. A comprehensive search in the National Human Genome Research Institute GWAS Catalog (24) did not reveal any significant associations between the two validated SNPs or their proxies with other diseases or traits.

Variant Evaluation

Using publicly available data of genetic effects on gene expression (25), we found an association in *cis* between rs649529 and transcript levels of *RAB38* ($P = 5.4 \times 10^{-6}$) and the neighboring *CTSC* ($P = 7.6 \times 10^{-7}$), consistent with a regulatory effect of this variant in whole blood. Corresponding data for kidney-specific tissues are currently not available, but we used epigenetic maps generated

Table 2—Replicated SNP associations with additional kidney function and diabetes-related traits

Trait	<i>n</i>	Effect (OR)	SE	<i>P</i> value	<i>P</i> difference*
<i>rs649529, RAB38/CTSC</i>					
UACR, diabetes; log(mg/g)	7,787	−0.14	0.03	5.8×10^{-7}	6.9×10^{-6}
UACR, no diabetes; log(mg/g)	45,094	−0.004	0.008	0.64	
eGFR _{crea} , diabetes; log(mL/min/1.73 m ²)	11,527	0.003	0.004	0.46	2.8×10^{-1}
eGFR _{crea} , no diabetes; log(mL/min/1.73 m ²)	118,427	−0.001	0.001	0.59	
CKD (eGFR <60 mL/min/1.73 m ²)	118,114	(1.01)	0.02	0.57	
Type 2 diabetes	63,390	(1.02)	0.02	0.32	
Fasting glucose (mmol/L)	46,186	0.003	0.006	0.65	
HbA _{1c} (%)	46,368	0.004	0.004	0.31	
<i>rs13427836, HS6ST1</i>					
UACR, diabetes; log(mg/g)	7,399	0.19	0.04	6.3×10^{-7}	1.7×10^{-5}
UACR, no diabetes; log(mg/g)	34,830	0.010	0.012	0.38	
eGFR _{crea} , diabetes; log(mL/min/1.73 m ²)	11,092	0.008	0.006	0.13	1.3×10^{-1}
eGFR _{crea} , no diabetes; log(mL/min/1.73 m ²)	114,247	0.000	0.001	0.94	
CKD (eGFR <60 mL/min/1.73 m ²)	113,612	(0.97)	0.02	0.23	
Type 2 diabetes	63,390	(1.00)	0.03	0.94	
Fasting glucose (mmol/L)	46,186	−0.005	0.004	0.22	
HbA _{1c} (%)	46,368	0.003	0.005	0.61	
<i>rs10795433, CUBN†</i>					
UACR, diabetes; log(mg/g)	5,825	0.19	0.04	2.0×10^{-5}	8.2×10^{-4}
UACR, no diabetes; log(mg/g)	46,061	0.045	0.01	8.7×10^{-6}	
eGFR _{crea} , diabetes; log(mL/min/1.73 m ²)	11,522	0.007	0.005	0.18	1.9×10^{-1}
eGFR _{crea} , no diabetes; log(mL/min/1.73 m ²)	118,299	0.0007	0.001	0.61	
CKD (eGFR <60 mL/min/1.73 m ²)	118,121	(1.04)	0.02	0.08	
Type 2 diabetes	63,390	(1.00)	0.03	0.88	
Fasting glucose (mmol/L)	46,186	−0.003	0.005	0.52	
HbA _{1c} (%)	46,368	−0.002	0.005	0.73	

DM, diabetes mellitus; eGFR_{crea}, GFR estimated by serum creatinine levels; OR, odds ratio. Effects represent the change in trait associated with each additional copy of the minor allele for each of the SNPs. For continuous traits, units are provided; the effect for binary outcomes, shown in parentheses, represents the OR. Except for UACR in diabetes, estimates refer to the discovery samples of the respective trait and to the published resources for the glycemic traits. Fasting glucose and HbA_{1c} were evaluated among individuals free of diabetes. For the kidney traits, *P* values and SEs are corrected using genomic control. **P* value for difference from a two-sample *t* test: $t = (\text{effect}_{\text{DM}} - \text{effect}_{\text{nonDM}}) / (\text{SE}_{\text{DM}}^2 + \text{SE}_{\text{nonDM}}^2)^{0.5}$ which, for large sample sizes is distributed as a normal (0,1). The correlation between $\text{effect}_{\text{DM}}$ and $\text{effect}_{\text{nonDM}}$ is assumed to be 0. Associations with type 2 diabetes were tested using the publicly available summary statistics dataset from the DIAGRAM (DIAbetes Genetics Replication And Meta-analysis) Consortium (12,171 case subjects and 56,862 control subjects) (40). Associations with fasting glucose and plasma hemoglobin A_{1c} concentrations were evaluated using the publicly available results from the MAGIC Consortium (www.magicinvestigators.org) (41,42). †UACR effect estimates for UACR in diabetes for *CUBN* are provided from the discovery stage.

from human adult kidney tissue (19) (see RESEARCH DESIGN AND METHODS) to further examine the regulatory potential of index SNPs. The intronic index SNP in *HS6ST1* and several proxies mapped into enhancer regions. Similarly, the *CUBN* index variant rs10795433 mapped into an intronic enhancer region. The region in which the index variant at *RAB38/CTSC* is located was annotated as not mapped/repressed in these cells, thus preventing further examination. All proxies in strong linkage disequilibrium with these three index SNPs ($r^2 > 0.6$, 1000G v5 reference panel) (26) were intronic (*CUBN* and *HS6ST1*) or intergenic (*RAB38/CTSC*).

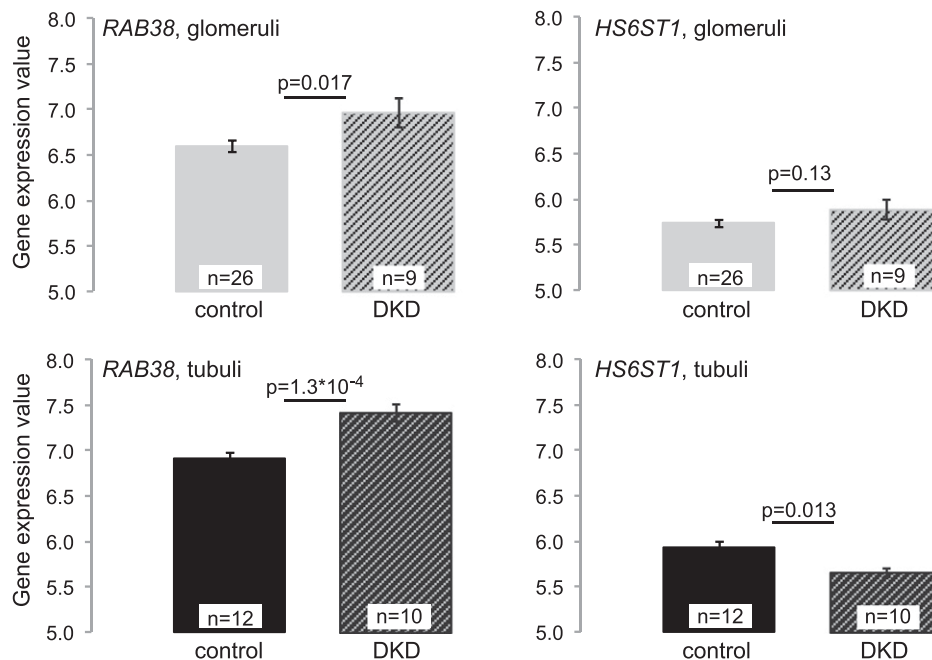
Clinical Characterization Including Gene Expression of Replicated Loci

To evaluate target tissues within the kidney, we characterized the identified loci using data for tissue-specific

gene expression. Clinical characterization was conducted using data from patients with DKD and healthy control subjects (27) and a prospective study of individuals with type 1 diabetes (28).

We used publicly available data (27) to compare relative expression of *RAB38*, *CTSC*, and *HS6ST1* between patients with DKD confirmed by biopsy specimen and healthy control subjects (see RESEARCH DESIGN AND METHODS). After multiple testing correction, only *RAB38* expression levels were significantly different, with higher expression in tubuli of DKD patients compared with control subjects ($P = 1.3 \times 10^{-4}$) (Fig. 2A). We also used RNA sequencing data from microdissected human kidney samples to quantify *RAB38* and *HS6ST1* expression in human glomeruli and tubuli. *HS6ST1* showed higher expression levels than *RAB38*, and both genes showed

A Comparison of Relative Gene Expression, DKD Patients and Controls



B Comparison of Gene Expression (RNAseq) Across Tissues

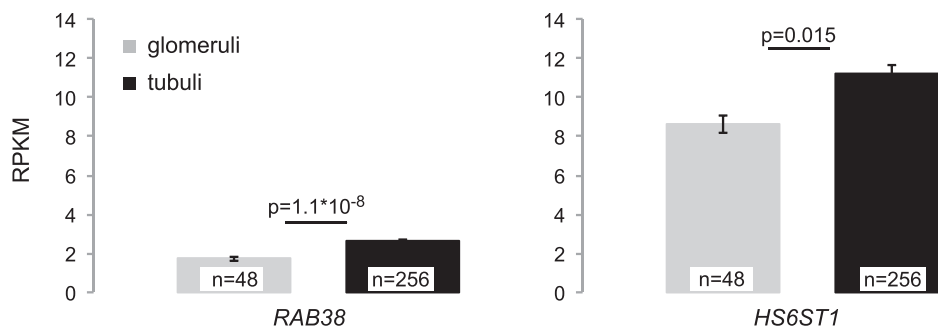


Figure 2—*RAB38* and *HS6ST1* expression across kidney tissues. **A:** Comparison of *RAB38* and *HS6ST1* expression (microarray) in tubuli and glomeruli of patients with DKD and control subjects shows significantly higher *RAB38* expression in tubuli of DKD patients than in tubuli of control subjects (significance threshold $0.05/6 = 8.3 \times 10^{-3}$ for investigating *RAB38*, *CTSC*, and *HS6ST1* in tubuli and glomeruli). *CTSC* expression was not significantly different between DKD case subjects and control subjects in tubuli ($P = 0.11$) or glomeruli ($P = 0.03$). Expression levels are shown as robust multiarray average–processed gene intensity values. **B:** *RAB38* and *HS6ST1* transcript abundance quantified from RNA sequencing (RNAseq) is detected at high levels in human tubuli but also in glomerular cells. Transcripts were quantified by reads per kilobase of transcript per million mapped (RPKM). The error bars in **A** and **B** correspond to the SEM.

higher expression in tubuli than in glomeruli (Fig. 2B). The difference between tubular and glomerular expression was more pronounced for *RAB38* ($P = 1.1 \times 10^{-8}$) than for *HS6ST1* ($P = 0.015$).

To investigate whether the effect of the replicated SNPs extended to kidney disease progression in the setting of type 1 diabetes, the SNPs were tested for association with incident MA (268 case subjects, primary end point) and a combined end point of time to macroalbuminuria or ESRD (133 case subjects, secondary end point) among up to 1,304 participants with type 1 diabetes in the DCCT/EDIC study (28). Neither SNP showed a significant association (Supplementary Table 9).

Diabetic *Rab38* KO Rats Show Increased Urinary Albumin Excretion

We aimed to further substantiate our findings by obtaining experimental support. We focused on the examination of *RAB38* because it was the gene implicated by higher gene expression in tubuli of DKD patients compared with control subjects and because previous studies of *Rab38* KO and transgenic rats have confirmed its role in albuminuria in FHH rats and highlighted a role in tubular albumin reuptake (21,22). We thus examined these animals in the setting of diabetes as outlined in Fig. 3A. Injection of STZ in 9-week-old rats successfully induced diabetes in all strains (Fig. 3B). Blood glucose rose from normal values before injection of STZ (congenic, 205 ± 3 mg/dL; transgenic,

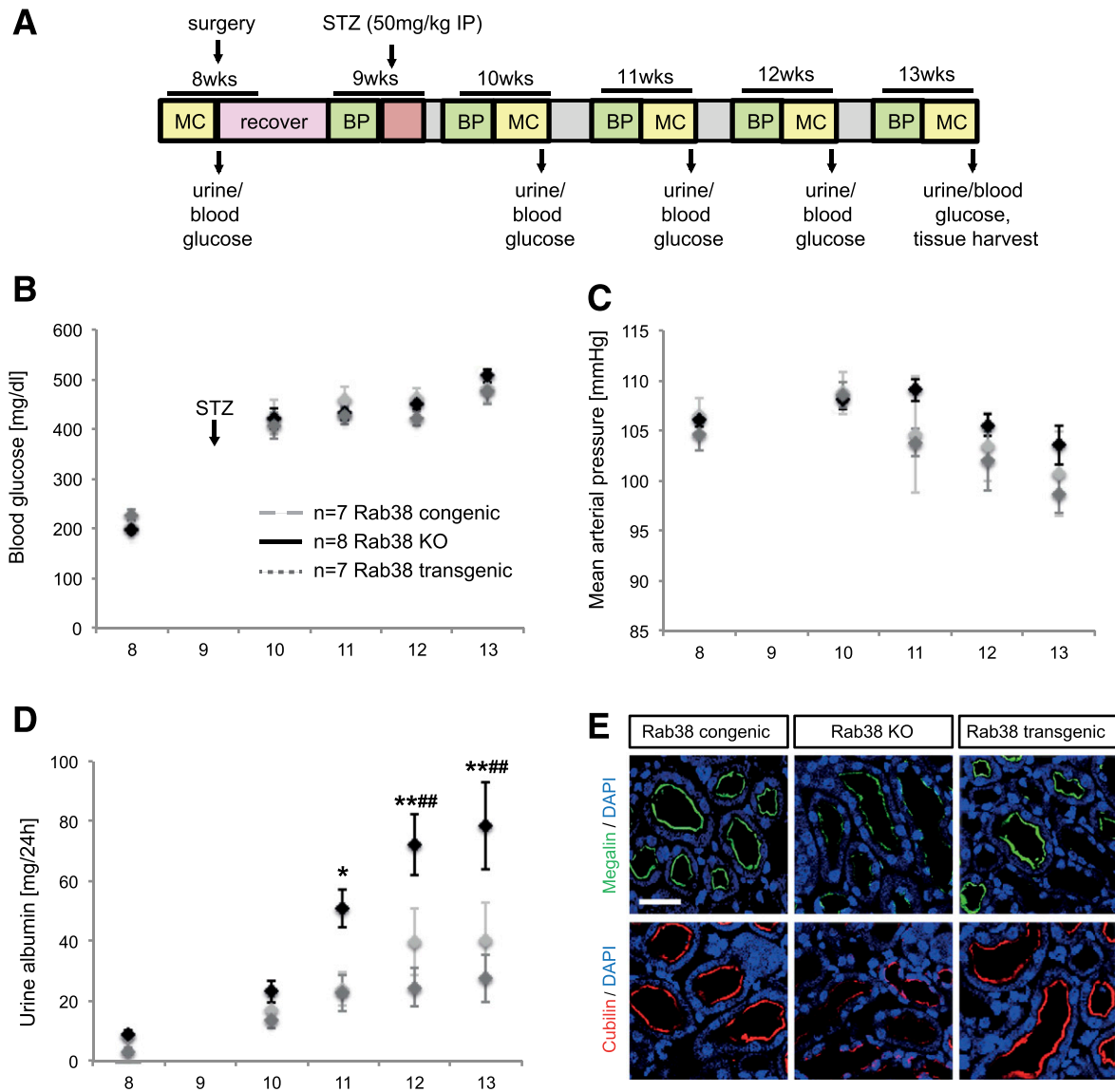


Figure 3—Comparison of *Rab38* congenic, transgenic, and KO rats after induction of diabetes. **A**: Experimental setup and timeline. BP, blood pressure measurement via radio telemetry; MC, metabolic cage. **B**: Comparison of blood glucose concentrations. **C**: Comparison of mean arterial pressure. **D**: Comparison of urinary albumin concentrations. * $P < 0.05$, ** $P < 0.01$ KO vs. transgenic, ### $P < 0.01$ KO vs. congenic. **E**: Expression of endocytic markers. Immunofluorescence staining for megalin (green, top panel) and cubilin (red, bottom panel) in kidneys from all three rat strains. Nuclei counterstained with DAPI (blue). Scale bar, 50 μm . Data are presented as mean \pm SEM. The results for blood pressure measurement, urinary albumin excretion, and blood glucose were analyzed by two-way ANOVA, followed by the Tukey post hoc test.

227 \pm 11 mg/dL; KO, 198 \pm 7 mg/dL) to high values that indicate severe hyperglycemia 1 week after STZ (congenic, 422 \pm 35 mg/dL; transgenic, 406 \pm 27 mg/dL; KO, 420 \pm 21 mg/dL). Blood glucose levels remained high at age 11, 12, and 13 weeks and showed no significant differences among strains (Fig. 3B). There were no significant differences in mean arterial blood pressure among congenic, transgenic, and KO animals freely moving around the cage. All animal strains showed a tendency toward decreased blood pressure 3–4 weeks after injection of STZ (Fig. 3C).

As illustrated in Fig. 3D, *Rab38* KO animals showed a progressive increase in urinary albumin excretion that

became statistically significant 2 weeks after injection of STZ. At 4 weeks after injection, *Rab38* KO animals had an albumin excretion of 79 \pm 14 mg/day, whereas albumin excretion was only 28 \pm 8 mg/day in transgenic ($P < 0.01$) and 41 \pm 13 mg/day in congenic animals ($P < 0.01$). These data indicate that diabetic rats without *Rab38* are more susceptible to the development of albuminuria than congenic and transgenic animals with functional *Rab38* despite a similar degree of hyperglycemia in all animals. Kidney sections obtained from a subset of animals showed a higher average glomerulosclerosis score (2.9 \pm 0.3) compared with congenic (2.2 \pm 0.1) and

transgenic (2.2 ± 0.1) rats ($P < 0.05$) (Supplementary Fig. 4), but differences were subtler than those observed for urinary albumin excretion.

To further clarify how loss of *Rab38* may lead to albuminuria, we performed immunohistochemistry staining of megalin and cubilin, known to mediate albumin reuptake in the proximal tubulus, in kidney sections of all three animal strains. Cubilin and megalin were markedly reduced at the luminal membrane of proximal tubular cells in *Rab38* KO rats compared with congenic and transgenic control animals (Fig. 3E), consistent with a role of *Rab38* in regulating the abundance of cubilin and megalin at the cell surface. In contrast, there was no significant difference in the number of structures positive for the lysosomal marker LAMP1 among the three strains.

DISCUSSION

In this GWAS discovery meta-analysis of 2,191,945 SNPs in up to 54,450 participants of 20 studies, we replicated the association of the previously identified *CUBN* locus and UACR as well as MA at genome-wide significance and identified several suggestive signals among individuals with diabetes. Two of these loci, *RAB38/CTSC* and *HS6ST1*, showed evidence of independent replication, and *RAB38* was further supported by functional studies in a rat model. Our findings point to mechanisms in renal handling of albumin that associate with albuminuria in humans in the setting of diabetes. They thus represent examples of gene-by-diabetes interactions resulting in a complex trait that manifests when environmental exposure and genetic susceptibility variants occur together (29).

Not all individuals with diabetes develop DKD, suggesting that neither the presence of hyperglycemia nor genetic variants alone are sufficient to elicit the renal damage that typically manifests itself as albuminuria in diabetes. Our observations therefore raise the question of how the diabetic environment may result in the manifestation of genetic effects on albuminuria. The lack of association between both genetic variants and type 2 diabetes or specific glycemic measures in humans indicates that their effects occur without influencing diabetes per se. This notion is further substantiated by the fact that diabetic *Rab38* KO rats showed higher urinary albumin concentrations compared with controls despite the presence of similar blood glucose concentrations.

A difference between our observations in humans and rats is that the effect of genetic variation near *RAB38* on albuminuria was only found in humans with but not without diabetes, whereas *Rab38* KO rats without diabetes also progress to albuminuria (22). A potential explanation is that KO rats represent a null mutation, allowing for the genetic component to take full effect without needing further aggravation by environmental factors. Conversely, many human susceptibility variants of complex traits do not result in a complete loss of function but instead are of a regulatory nature. The effect of such variants may become apparent only upon an environmental challenge,

such that genetically determined alterations in renal albumin handling could manifest themselves in the setting of hyperglycemia and/or diabetes due to a number of mechanisms that secondarily affect albumin reabsorption, including an increased load of filtered albumin due to hyperfiltration or impairment of the glomerular filter. Along these lines, our observation of significantly higher *RAB38* transcript abundance in tubuli of DKD patients than control subjects may indicate an adaptation of the tubular machinery for albumin reabsorption in this setting. Moreover, genetics effects of the index SNP at the *CUBN* locus on albuminuria were four times as large in individuals with diabetes compared with those without diabetes, supporting alterations of tubular albumin handling in the setting of diabetes.

RAB38 encodes a member of the small Rab GTPase protein family that regulates intracellular vesicle trafficking between organelles and is important in exo-, endo-, and transcytosis (30). Expression of *Rab38* at the mRNA and protein level was observed in proximal tubule cells of wild-type rats (31). FHH rats, a natural *Rab38*-null mutation, show increased urinary albumin excretion without changes in glomerular permeability (21). In these animals, the expression of a Brown Norway *Rab38* transgene led to phenotypic rescue, and knockdown of *Rab38* in a proximal tubule cell system significantly decreased albumin endocytosis (22). Together, these observations support an important role for the small Rab GTPase *RAB38* in the reabsorption of filtered albumin.

Impaired *RAB38* function may lead to increased albumin excretion via different mechanisms: altered intracellular vesicle transport may affect albumin reabsorption or recycling of reabsorbed albumin back to the plasma membrane (32). Alternatively, altered *RAB38* function may affect the delivery of proteins required, such as cubilin or megalin, for albumin endocytosis, the mechanism underlying albuminuria in Dent disease (33). Our experimental data showing reduced abundance of cubilin and megalin in *Rab38* KO but not in control rats are consistent with the latter hypothesis. Finally, that impaired *RAB38* function may directly cause glomerular damage, in turn leading to increased concentrations of urinary albumin, is also conceivable.

Although the combined evidence from *Rab38* KO rats along with the gene expression and GWAS data strongly implicate *RAB38* as the gene underlying albuminuria in humans, the intergenic index SNP mapped upstream of *RAB38* and downstream of *CTSC* and was associated with transcript levels of both genes in whole blood. We can therefore not exclude the possibility that *CTSC* may be the causal gene underlying the observed associations or that it contributes to the phenotype in addition to *RAB38*. *CTSC* encodes for a lysosomal cysteine protease. Rare mutations in the gene cause autosomal-recessive Papillon-Lefèvre syndrome. No renal abnormalities have been reported in affected patients (34), *Ctsc* KO mice do not show kidney abnormalities (35), and the gene has not been linked to albuminuria or kidney disease.

The other genomic locus associated with albuminuria in diabetes contains *HS6ST1*, encoding the enzyme heparan sulfate (HS) 6-O-sulfotransferase that catalyzes the 6-O-sulfation of HS and heparin (36,37). HS are anionic side chains of HS proteoglycans, which are components of basement membranes, extracellular matrix, and cell surfaces. Several studies have reported that inactivation or removal of HS leads to proteinuria, and biopsy specimens from patients with diabetes revealed changes in HS sulfation patterns compared with control subjects (38). Thus, a genetic variant altering the activity or abundance of the enzyme may lead to altered albuminuria. The underlying mechanisms could be manifold, because HS has been reported to affect not only glomerular filtration but also growth factor signaling, composition, and functions of the glomerular basement membrane as well as functions at the endothelial surface layer (38) and the proximal tubule (39).

Strengths of our study include its large sample size, specific examination of individuals with diabetes, and consistent effects across a variety of studies underscoring the relevance of our findings at the general population level. In addition, we performed careful characterization of a replicated finding through in vivo experiments in *RAB38* KO and control rats that cannot, however, elucidate the exact mechanism by which genetic variation at this locus influences albuminuria in humans.

Limitations include the fact that the replicated SNPs did not achieve genome-wide significance, necessitating future confirmation in even larger studies, and that we could not assess allele-specific gene expression in human kidney tissues. We focused on European ancestry study participants and mostly on individuals with type 2 diabetes. Future studies should therefore examine these associations among individuals of additional ancestries and in well-powered studies of patients with type 1 diabetes. Although results were combined after study-specific analyses, biological variation in UACR and different urine collection and storage methods may have resulted in increased variation and thus reduced statistical power to reveal significant associations.

Additional studies are required to determine the causal variants and the exact underlying molecular mechanism by which genetic variation at *RAB38/CTSC* and *HS6ST1* associates with albuminuria in humans. An elucidation of the underlying mechanisms and the contributions and differences of albuminuria of glomerular and tubular origin may improve our understanding of proteinuric kidney diseases in general but may be especially relevant to DKD, the most common cause of ESRD.

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