SOS2 and *ACP1* Loci Identified through Large-Scale Exome Chip Analysis Regulate Kidney Development and Function

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ABSTRACT

Genome-wide association studies have identified >50 common variants associated with kidney function, but these variants do not fully explain the variation in eGFR. We performed a two-stage meta-analysis of associations between genotypes from the Illumina exome array and eGFR on the basis of serum creatinine (eGFRcrea) among participants of European ancestry from the CKDGen Consortium (n_{Stage1}: 111,666; n_{Stage2}: 48,343). In single-variant analyses, we identified single nucleotide polymorphisms at seven new loci associated with eGFRcrea (*PPM1J*, *EDEM3*, *ACP1*, *SPEG*, *EYA4*, *CYP1A1*, and *ATXN2L*; $P_{Stage1}<3.7\times10^{-7}$), of which most were common and annotated as nonsynonymous variants. Gene-based analysis identified associations of functional rare variants in three genes with eGFRcrea, including a novel association with the SOS Ras/Rho guanine nucleotide exchange factor 2 gene, *SOS2* ($P=5.4\times10^{-8}$ by sequence kernel association test). Experimental follow-up in zebrafish embryos revealed changes in glomerular gene expression and renal tubule morphology in the embryonic kidney of *acp1*- and *sos2*-knockdowns. These developmental abnormalities associated with altered blood clearance rate and heightened prevalence of edema. This study expands the number of loci associated with kidney function and identifies novel genes with potential roles in kidney formation.

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CKD is considered a complex phenotype with a genetic predisposition.[1] Previous genome-wide association studies (GWAS) have successfully identified multiple common genetic risk variants associated with the CKD-defining measures of eGFR and urinary albumin-to-creatinine ratio (UACR).[2–5] Together, these variants explain only a small proportion of the variation in eGFR and UACR.[6] To comprehensively interrogate protein-coding regions and assess the effects of rare variants (minor allele frequency [MAF]<1%), we carried out a two-stage meta-analysis of the association between eGFR on the basis of serum creatinine (eGFRcrea) and variants genotyped on the Illumina HumanExome chip (http://genome.sph.umich.edu/wiki/Exome_Chip_Design) among 111,666 European ancestry (EA) participants from the CKDGen Consortium and assessed the role of genes significantly associated with eGFRcrea in kidney development using embryonic zebrafish models. In secondary analyses, we examined associations with eGFRcrea stratified by diabetes status, and in a smaller subset of EA participants, we also tested eGFR on the basis of cystatin C (eGFRcys) and UACR. An additional 9624 participants of African ancestry (AA) were also used in an independent exome-chip discovery meta-analysis.

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RESULTS

Up to 120,357 participants from 27 studies of EA and up to 11,386 participants from seven studies of AA were included in stage 1 analyses for eGFRcrea, eGFRcys, or UACR. An additional 48,343 participants from 12 studies of EA were included in stage 2 analysis of eGFRcrea. All participants provided informed consent and each of the studies was approved by its governing ethics committee or Institutional Review Board. Sample characteristics and genotyping information for each study are summarized in Supplemental Tables 1 and 2.

In stage 1 single-variant EA analyses, we identified 33 loci associated with eGFRcrea (Supplemental Table 3, Table 1) that met our *a priori* chip-wide significance threshold of $P<3.7\times10^{-7}$(0.05/134299 variants). Of these, eight had not been identified in association with eGFRcrea from previous GWAS analyses; six were missense variants: rs34611728 (*PPM1J*), rs78444298 (*EDEM3*), rs11553746 (*ACP1*), rs2307394 (*ORC4*), rs55760516 (*SPEG*), and rs9493627 (*EYA4*); and two were GWAS tag single nucleotide polymorphisms (SNPs) included on the exome chip due to prior associations in the National Human Genome Research Institute (NHGRI) GWAS Catalog for caffeine/coffee intake:[7,8] rs2472297 (intergenic, near *CYP1A1*) and inflammatory bowel disease[9] rs8049439 (intronic, *ATXN2L*); all were common variants (MAF>1%). A Manhattan plot displaying all 33 chip-wide significant loci is shown in Figure 1. Quantile-quantile plots indicated no inflation of the overall *P* value distribution (Supplemental Figure 1). Regional association plots show the associations of other variants within the 500 Mb region of the index variant of the eight newly identified loci in Supplemental Figure 2.

In stage 2 analyses, we followed up the eight newly identified eGFRcrea loci meeting our significance threshold among an additional 48,343 EA participants from 12 studies (sample characteristics and genotyping information are summarized in Supplemental Tables 2 and 4). All loci but *ORC4* met criteria for replication (a direction of effect consistent with stage 1 analysis; a $P_{1\text{-sided}}<0.05$ from stage 2 analysis; and $P<3.7\times10^{-7}$ from a combined stage 1 and stage 2 analysis; Table 1). Because diabetes mellitus is a major risk factor for CKD, we assessed the genetic associations at these loci in the presence or absence of diabetes to obtain additional insights into potential mechanistic pathways. In total, 11,040 and 94,677 participants were included in the diabetes- and nondiabetes-specific analyses, respectively. In analyses stratified by diabetes status, the β-coefficients were directionally consistent and of similar magnitude between the strata for six out of seven newly identified loci (Supplemental Table 5).

To identify additional novel loci associated with alternative measures of kidney function, we tested the association of single variants with eGFRcys (n=32,861 EA participants) and UACR (n=31,164 EA participants). All observed associations achieving the significance threshold ($P<3.7\times10^{-7}$; Supplemental Table 6) have been previously reported in association with eGFRcrea, eGFRcys, or UACR.[3,4,6]

To further investigate the role of rare variants in kidney function, we performed gene-based tests for 9990 autosomal genes that contained at least two nonsynonymous or splice-site variants with MAF<1%. No evidence of inflation was observed in quantile-quantile plots for gene-based tests (Supplemental Figure 3). Three genes, *SOS2*, *SLC47A1*, and *LRP2*, met the experiment-wide threshold for gene-based significance ($P<2.5\times10^{-6}$; 0.05/19,922 tests [9961 genes × 2 tests]; Table 2; Supplemental Figure 4). The association for *SLC47A1*, a renal solute transporter, was driven by the presence of a single variant, rs111653425, with MAF approximately 1% (Supplemental Table 7). Common variants at *SLC47A1* and *LRP2* have been previously reported in association with eGFRcrea,[4,6] thus implicating both rare and common variants at both loci.[4,6] Conversely, the association with *SOS2* was novel ($P_{SKAT}=5.38\times10^{-8}$; $P_{T1}=3.25\times10^{-6}$). No genes reached the threshold for chip-wide significance in gene-based associations for eGFRcrea stratified by diabetes status, eGFRcys, or UACR.

To identify genes that may play a role during kidney development, we used knockdown zebrafish embryos generated by injecting morpholino oligonucleotides (MOs) into single-cell stage embryos. Morpholinos are a commonly used tool in zebrafish screens because they enable an efficient identification of genes that may have a role in developmental processes. The morpholinos targeted genes with nonsynonymous variants (*ppm1j*, *acp1*, *eya4*, *speg*, and *edem3*) and the novel gene-based finding, *sos2* (Supplemental Figure 5A and Supplemental Table 8). General defects in the pronephros or embryonic kidney structure (marked by expanded *pax2a* expression) were observed in *acp1* ATG MO- and *sos2* ATG MO-injected embryos compared with controls (Figure 2, A, E, and I; $P<0.001$ for both). Both *acp1* ATG- and *sos2* ATG-knockdowns showed elongated proximal tubules (increased *slc20a1a* expression) compared with the control group (Figure 2, B, F, and J; mean difference in proximal tubule length: *sos2*=81.7 μm; $P<0.001$; and *acp1*=74.7 μm; $P<0.001$; Figure 2M). The increase in proximal tubule length was not a consequence of increased embryo length, as both *sos2* ATG and *acp1* ATG-morphants had significantly reduced body length relative to controls (Supplemental Figure 5B). Additionally, *acp1* ATG-knockdowns showed shorter distal tubule length (*slc12a3* expression), which may be a consequence of reduced body length (Figure 2, C, G, and K; $P<0.001$). No abnormalities were observed for podocytes (*wt1a* expression) for *sos2* or *acp1* compared with controls (Figure 2, D, H, and L; $P>0.05$).

Because of the potential off-target effects of morpholinos, these developmental findings were validated with secondary splice-site morpholinos designed to target the *sos2* and *acp1* pre-mRNA (Supplemental Figure 5A). The *sos2* and *acp1* splice morpholino-injected embryos had significantly increased proximal tubule length relative to controls (Supplemental Figure 5, C–E). Furthermore, the *acp1* splice-site morpholino-injected embryos had shortened distal tubules (*slc12a3* expression)

consistent with the phenotype induced by the ATG morpholino (Suppemental Figure 5F). The reproducibility of the proximal tubule developmental defects with ATG and splice-site morpholinos adds confidence to the specificity of the morpholino-induced tubule phenotype.

Follow-up *in situ* hybridization experiments to determine expression patterns of *sos2* and *acp1* during zebrafish development did not reveal kidney-specific expression of *sos2* or *acp1;* however, *sos2* and *acp1* were broadly expressed throughout embryogenesis at key stages of kidney development and may be acting to control kidney development (Supplemental Figure 6). In humans, both *sos2* and *acp1* protein are detected in adult renal tubules.[10] No significant developmental abnormalities were observed among MO knockdowns for the remaining genes (Supplemental Figure 6C). These findings suggest that both *SOS2* and *ACP1* may influence embryonic renal development, and that genetic influences on kidney development may contribute to variation in kidney function.

We next sought to determine whether *sos2* and *acp1*-mediated developmental alterations led to abnormalities in kidney function. In zebrafish, edema is a common sign of kidney failure.[11] We first performed an edema prevalence study and identified incidence rates of pericardial and global edema in *sos2* and *acp1* morphant larva. Both *sos2* and *acp1* ATG morpholino-injected embryos had a heightened incidence of pericardial edema beginning at 72 hours post fertilization (hpf) (Figure 3, A–C). The *sos2* morphants developed severe global edema by 120 hpf, whereas the *acp1* morphants presented with only pericardial edema (Figure 3, A–C). Both *sos2* and *acp1* morphants showed indications of embryonic lethality by 120–144 hpf (Figure 3, A–C).

An additional metric for kidney function is the assessment of glomerular filtration and fluid flow by fluorescent dextran clearance.[12] Control, *sos2,* or *acp1* morphant embryos were injected with equal volumes of rhodamine-labeled 70 kDa molecular mass dextran in the cardiac sinus venosus at 72 hpf. Dextran clearance rate was assessed by the quantification of rhodamine fluorescence intensity in a standardized area of the cardiac region at 2, 24, and 48 hours post injection (hpi). *sos2* and *acp1* morphant embryos exhibited decreased dextran clearance at both 24 and 48 hpi relative to controls (Figure 3, D–I, and M). Furthermore, renal tubules marked by fluorescent dextran in *sos2* and *acp1* morphant larva displayed an abnormal morphology (Figure 3, J–L). Specifically, the proximal convoluted tubules were reduced in size and lacked coiling depth. Failed clearance of 70 kDa molecular mass dextran and abnormal tubular structure suggests that morphants may have defects in tubular fluid flow and glomerular filtration.

We also evaluated heart rate in the *sos2* and *acp1* morphants relative to controls because compromised cardiovascular function could contribute to defects in dextran clearance. At 96 hpf (24 hours after dextran injection), the *sos2* (145.5±3.854 bpm; P=0.003) and *acp1* (151.2±2.653 bpm; P<0.001) morphants had an elevated mean heart rate relative to controls (127.8±3.353 bpm) (Supplemental Figure 2G). Although altered

Table 1. Novel variants associated with eGFRcrea in EA participants from single-variant analysis in stage 1 meeting chip-wide significance ($P<3.7\times10^{-7}$) and associations in stage 2 and combined analysis

Locus[a]	dbSNPID	Chr	Position[b]	Variation (Substitution)	Stage 1[c]				Stage 2[d]		Combined		
				A1/A2 (A1 AF)	β (SEM)	*P Value*	I^2	β (SEM)	1-sided *P*	β (SEM)	*P Value*	Prop Var Exp (%)
PPM1J	rs34611728	1	113255456	c.639G>T (L213F)	A/C (0.13)	−0.0103 (0.0013)	1.2E-14	13.2	−0.0059 (0.0023)	4.7E-03	−0.0092 (0.0011)	3.3E-16	0.05
EDEM3	rs78444298	1	184672098	c.2236C>T (P746S)	A/G (0.02)	−0.0183 (0.0034)	5.2E-08	15.3	−0.0225 (0.0055)	1.8E-05	−0.0195 (0.0029)	1.5E-11	0.03
ACP1	rs11553746	2	272203	c.129C>T (T95I)	T/C (0.35)	−0.0049 (0.0009)	2.0E-07	20.7	−0.0032 (0.0016)	2.2E-02	−0.0045 (0.0008)	1.0E-08	0.02
ORC4[e]	rs2307394	2	148716428	c.233A>G (N78S)	C/T (0.32)	−0.0058 (0.0010)	6.8E-09	14.3	−0.0025 (0.0016)	6.0E-02	−0.0049 (0.0009)	8.4E-09	0.03
SPEG	rs55760516	2	220354108	c.8191A>G (R2731G)	G/A (0.33)	0.0059 (0.0009)	4.8E-10	0.5	0.0054 (0.0016)	3.7E-04	0.0058 (0.0008)	1.7E-13	0.04
EYA4	rs9493627	6	133789728	c.829G>A (G223S)	A/G (0.31)	0.0061 (0.0010)	2.3E-10	0.0	0.0049 (0.0016)	1.4E-03	0.0058 (0.0009)	1.4E-11	0.04
CYP1A1	rs2472297	15	75027880	intergenic	T/C (0.24)	0.0057 (0.0010)	7.0E-08	0.0	0.0059 (0.0017)	3.2E-04	0.0058 (0.0009)	3.0E-11	0.03
ATXN2L	rs8049439	16	28837515	intronic	C/T (0.40)	0.0048 (0.0009)	1.3E-07	7.1	0.0045 (0.0016)	1.8E-03	0.0047 (0.0008)	1.2E-09	0.03

A1, effect allele; A2, non-effect allele; A1 AF, effect allele frequency; Chr, chromosome; Prop Var Exp, proportion of variance in ln(eGFRcrea) explained.

[a]Loci are named according to the closest gene on the basis of the position of the lead SNP for new loci.

[b]Position is reported in UCSC Genome Browser build hg19.

[c]Sample size for stage 1 analysis: n=111,666.

[d]Sample size for stage 2 analysis: n=48,343.

[e]This variant reached chip-wide significance ($P<3.7\times10^{-7}$) in the stage 1 samples but did not meet validation criteria in stage 2.

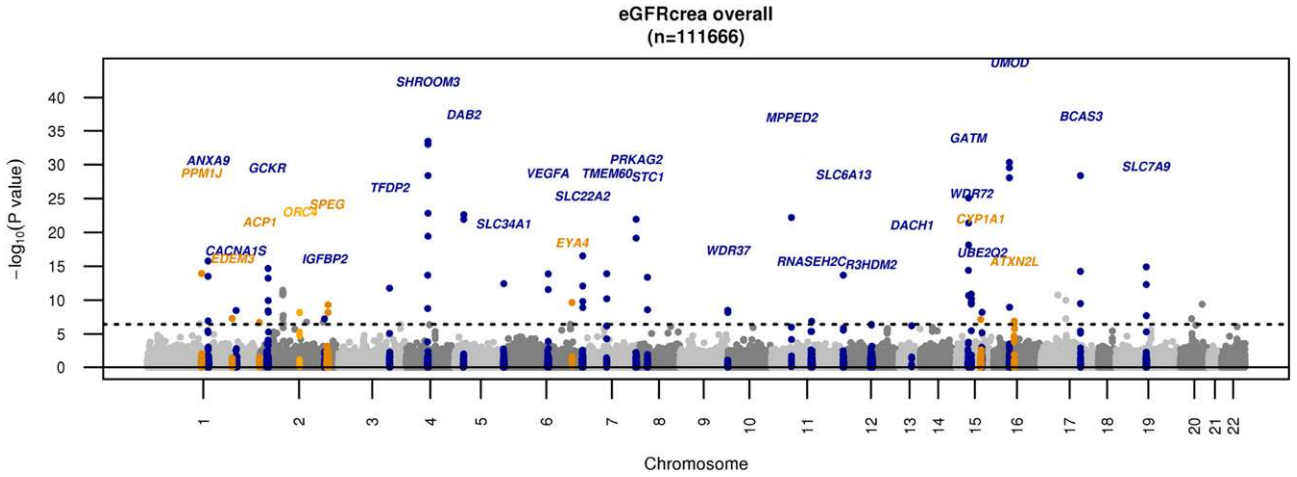


Figure 1. Manhattan plot for single-variant analysis in eGFRcrea among 111,666 EA participants. Newly identified variants are in dark orange. The gene, *ORC4*, not successfully replicated, is in orange. Known loci are in blue.

kidney development, abnormal tubular structure, edema, and decreased dextran clearance all support the conclusion that both *sos2* and *acp1* are regulators of kidney development and function, it remains possible that altered hemodynamics contribute to the edema and dextran clearance phenotypes observed.

In separate single variant analyses among 9624 AA participants, we identified three loci in association with eGFRcrea at chip-wide significance (Supplemental Table 9; $P<3.7\times10^{-7}$). These variants were rare and the limited availability of AA cohorts prevented replication of these findings. The *APOL1* G1 variant, rs73885319, that was a known risk factor for kidney function decline and ESRD in AA populations, was included on the exome array. However, it was not associated with eGFRcrea using an additive genetics model in the AA participants here (P=0.70).

To investigate if the newly identified eGFRcrea loci were also associated with diabetes mellitus and arterial hypertension, major risk factors for CKD, we tested for associations between the seven validated eGFRcrea loci with BP and type 2 diabetes (T2D) among EA participants in collaborations with the Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium (CHARGE) Blood Pressure[13] and CHARGE Glycemia-T2D[14] Working Groups. Consistent with prior observations, the majority of variants were not associated with BP traits or T2D (Supplemental Table 10). The exception was rs2472297 at the *CYP1A1* locus, a GWAS tag SNP previously associated with coffee/caffeine intake.[7,8] This SNP was also associated with systolic and diastolic BP (P=7.4×10^{-7}, and 4.6×10^{-11}, respectively).[13]

DISCUSSION

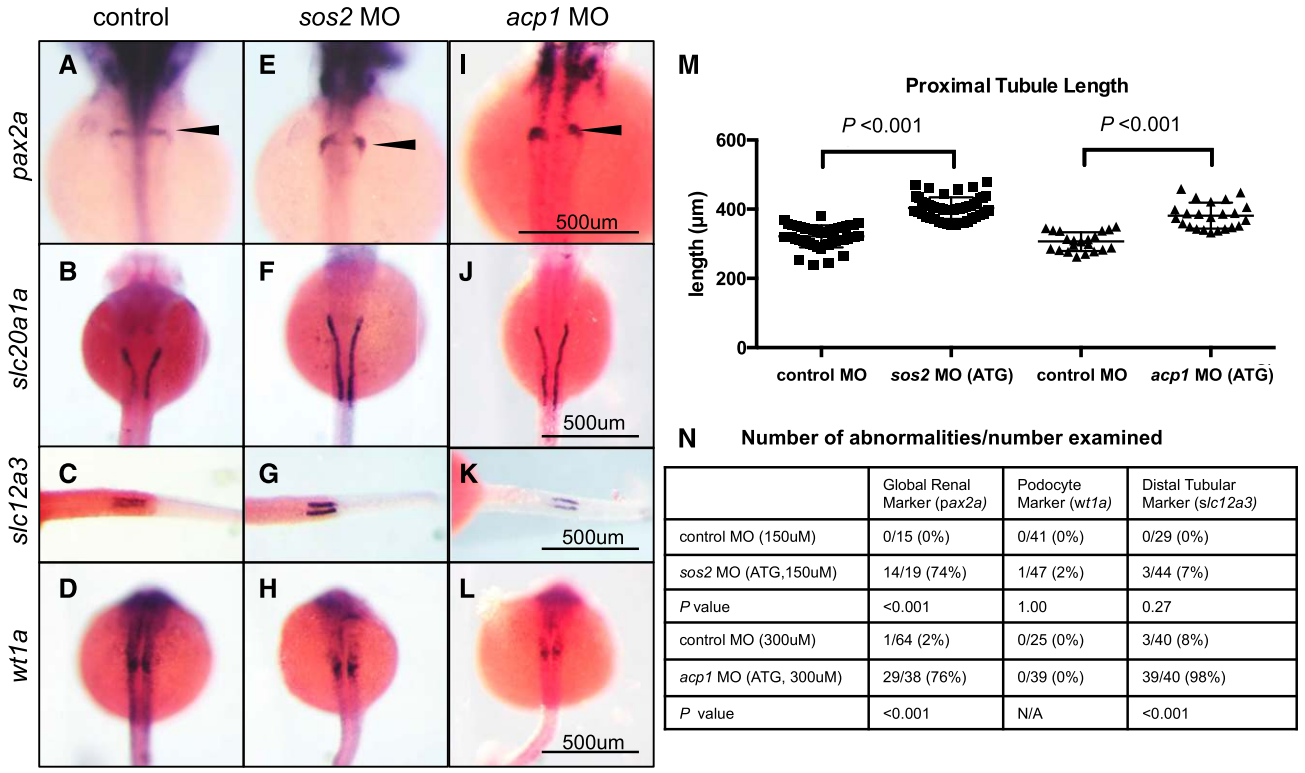
Our main findings are four-fold. First, we identified and validated seven loci associated with eGFRcrea through genotyping on the exome-chip among 160,009 participants of EA in a two-stage study design. Second, the majority of the newly uncovered associations were for common variants with modest effect sizes, which argues against the presence of rare protein-coding variants with large effect sizes represented on the exome chip. Third, we identified one novel association for *SOS2* through gene-based testing. Fourth, we demonstrated altered kidney development and function in zebrafish *sos2* and *acp1* knockdowns.

Our study emphasizes the continued success of efforts combining population-based genetics and model organisms to identify genes underlying kidney function.[4,15] The zebrafish knockdowns provide a systematic model to examine the consequences of gene perturbations in the embryonic renal system of the fish. Our zebrafish morpholino experiments revealed a potential role for *sos2* and *acp1* in kidney development and function. Mutations in the *SOS* gene family (*SOS1* and *SOS2*) lead to Noonan syndrome, a congenital RASopathy (syndromes caused by germline mutations controlling signal transduction pathways) that can feature mild kidney

Table 2. Genes associated with eGFRcrea in EA participants from gene-based analyses meeting chip-wide significance thresholds ($P<2.5\times10^{-6}$)

Gene[a]	Chr	cMAF	*N* Variants[b]	T1[c]			SKAT[d]
				β	SEM	*P* Value	*P* Value
LRP2	2	0.070	38	0.003	0.002	6.7E-02	3.5E-7[e]
SLC47A1[f]	17	0.033	4	−0.033	0.004	7.8E-15[e]	3.4E-12[e]
SOS2[g]	14	0.040	8	0.020	0.004	3.3E-06	5.4E-08[e]

Chr, chromosome; cMAF, cumulative MAF used in analysis; SKAT, sequence kernel association test.
[a]Gene name.
[b]Number of variants used in analysis.
[c]The standard burden test collapses the variants with MAF<1% into a single variable and tests the association between this variable with a phenotype.[30]
[d]The SKAT aggregates individual variant score test statistics.[32]
[e]Meets chip-wide significant threshold, $P<2.5\times10^{-6}$.
[f]Gene-based association results driven by one variant.
[g]Novel gene.



N **Number of abnormalities/number examined**

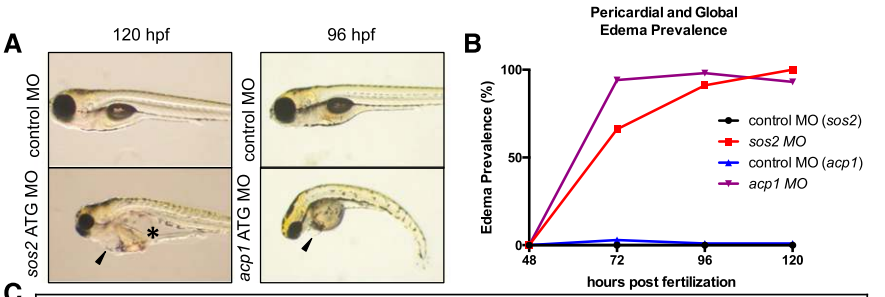
	Global Renal Marker (*pax2a*)	Podocyte Marker (*wt1a*)	Distal Tubular Marker (*slc12a3*)
control MO (150uM)	0/15 (0%)	0/41 (0%)	0/29 (0%)
sos2 MO (ATG,150uM)	14/19 (74%)	1/47 (2%)	3/44 (7%)
P value	<0.001	1.00	0.27
control MO (300uM)	1/64 (2%)	0/25 (0%)	3/40 (8%)
acp1 MO (ATG, 300uM)	29/38 (76%)	0/39 (0%)	39/40 (98%)
P value	<0.001	N/A	<0.001

Figure 2. *sos2* and *acp1* knockdowns result in defective kidney development. (A–D) Whole mount *in situ* hybridization in control embryos demonstrates normal expression of kidney markers, including *pax2a* (global kidney, A), *slc20a1a* (proximal tubules, B), and *slc12a3* (distal tubules, C) at 48 hpf, and *wt1a* (podocytes, D) at 24 hpf. (E–L) *sos2* and *acp1* ATG morpholino (MO) knockdown embryos develop glomerular gene expression defects (E, I, arrowheads) and display elongated proximal tubules (F, J). Knockdown of *acp1* shortened the distal tubules, whereas *sos2* knockdown left distal tubule *slc12a3* expression unaffected (G, K). No abnormalities in podocyte marker *wt1a* were observed for *sos2* ATG- and *acp1* ATG-MOs (H, L). (M) Quantitative assessment of proximal tubule length (*slc20a1a* expression) shows that proximal tubules are elongated in *sos2* ATG- and *acp1* ATG-MO injected embryos. *t* test used to calculate *P* values. (N) Table of observed abnormal embryos and total number examined by kidney markers *pax2a*, *wt1a*, and *slc12a3*, and MO-injected or control status. Fisher exact test used to calculate *P* values.

dysfunction[16] and renal anomalies.[17,18] The *SOS2* protein is expressed in the glomeruli and tubules of kidneys from adult humans.[10] Together, these findings provide further evidence for a potential role for *SOS* proteins in kidney development and function. There are no prior reports of an association between kidney function and *ACP1*, a gene that encodes for an acid phosphatase involved in the immune response and found in erythrocytes.[19] Our observations of kidney abnormalities in *sos2*- and *acp1*-knockdowns provide genes and target tissues for prioritization in future studies of more extensive functional follow-up, diagnostic screening, and potentially drug development.

Although morpholinos are an efficient tool for the rapid evaluation of GWAS hits, they have the potential for off-target effects, and morphant phenotypes are not always recapitulated in genetic mutant models. In this study, we evaluated *sos2* and *acp1* kidney development phenotypes using two independent morpholinos, which we believe adds confidence in the specificity of our phenotypes. Furthermore, we provided evidence that kidney developmental changes correlate with edema and reduced dextran clearance from the blood. We believe that our morpholino screen has allowed us to clarify promising candidates for further study. Simultaneously, we acknowledge that future studies in genetic mutants will enhance and clarify these findings. Because genetic knockout techniques do not necessarily recapitulate exact features of the identified human variants, future experiments are also needed to evaluate the effect of specific variants on kidney development and function.

The majority of identified novel variants were common. The strongest single-variant association with eGFRcrea to date is the common variant rs13329952 at the *UMOD* locus with an effect size of 0.016 ln(ml/min per 1.73 m^2) (MAF=0.19).[15] Given our large discovery sample, our study was adequately powered (>80%) to detect effect sizes of 0.11–0.008 ln(ml/min per 1.73 m^2) for very rare variants to more common variants (0.0005>MAF>0.10) (details of the power calculation can be found in the Supplemental Material). Although the selection of nonsynonymous content was expected to enrich for functional variants with large effect sizes, a possible reason for the lack of these findings might be the design-based,



C

Edema Prevalence						
Control MO				***sos2* ATG MO**		
	pericardial	global	dead	pericardial	global	dead
48 hpf	0/40 (0%)	0/40 (0%)	0/40 (0%)	0/35 (0%)	0/ 35 (0%)	0/35 (0%)
72 hpf	0/40 (0%)	0/40 (0%)	0/40 (0%)	23/35 (66%)***	0/35 (0%)	0/35 (0%)
96 hpf	0/40 (0%)	0/40 (0%)	0/40 (0%)	32/35 (91%)***	0/35 (0%)	0/35 (0%)
120 hpf	0/40 (0%)	0/40 (0%)	0/40 (0%)	7/35 (20%)**	16/35 (46%)***	12/35 (34%)***

Control MO				***acp1* ATG MO**		
	pericardial	global	dead	pericardial	global	dead
48 hpf	0/79 (0%)	0/79 (0%)	0/79 (0%)	0/101 (0%)	0/101 (0%)	0/101 (0%)
72 - 80 hpf	0/79 (0%)	2/79 (3%)	0/79 (0%)	95/101 (94%)***	0/101 (0%)	2/101 (2%)
96 hpf	0/79 (0%)	1/79 (1%)	2/79 (3%)	97/99 (98%)***	0/99 (0%)	1/99 (1%)
120 hpf	0/77 (0%)	1/77 (1%)	0/77 (0%)	91/98 (93%)***	0/98 (0%)	6/98 (6%)*

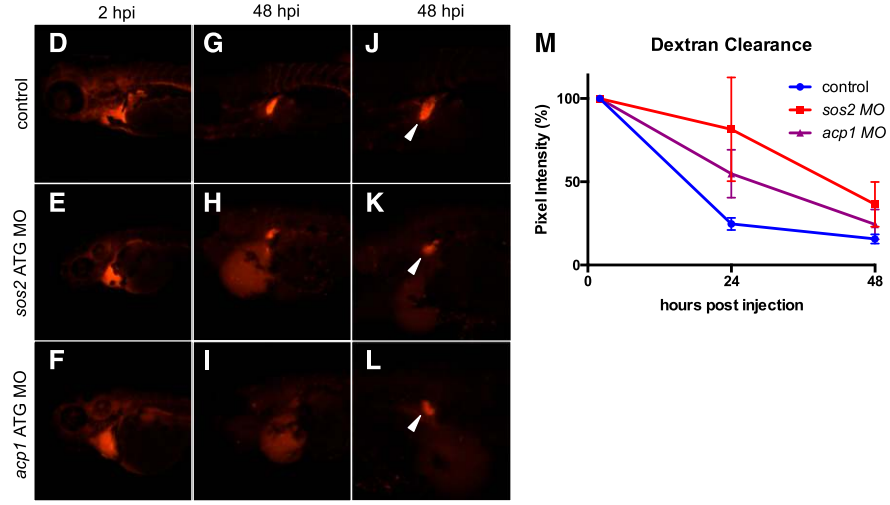


Figure 3. *sos2* and *acp1* knockdowns result in altered kidney function. (A) *sos2* and *acp1* morphants develop edema, which is a sign of kidney failure in zebrafish. *sos2* morphants display severe global edema at 120 hpf, with fluid accumulation in the pericardium (black arrow) and intestinal tract (black star). At 96 hpf, *acp1* morphants have severe pericardial edema (black arrow). (B–C) Incidence of edema and embryonic lethality in *acp1* and *sos2* morphants with Fisher exact test. *Indicates *P* value <0.05; **indicates *P* value <0.01; ***indicates *P* value <0.001. (D–I) Embryos were injected with control, *sos2*, or *acp1* morpholino at the single cell stage and subsequently injected with 70 kDa molecular mass fluorescent rhodamine dextran at 72 hpf. Dextran fluorescence intensity was measured over 48 hpi. Dextran-injected embryos show equal loading at 2 hpi. Compared with control embryos, *sos2* and *acp1* MO injected embryos have reduced dextran clearance in the cardiovascular region over time. (J–L) Morphant embryos have altered convoluted tubule morphology at 120 hpf (48 hpi) (white arrows). (M) Dextran fluorescence intensity over time as normalized to starting fluorescence intensity.

limited coverage of rare variants on the exome chip. The exome chip was primarily designed to assess nonsynonymous rare variants that had been observed among approximately 12,000 sequenced participants that were not selected on the basis of kidney disease (http://genome.sph.umich.edu/wiki/Exome_Chip_Design), and thus, we would not expect kidney disease–causing mutations to be well represented on the exome chip. Although the convenience and low cost of chip-based genotyping arrays focused on exonic variants were influential in facilitating a large number of cohorts to participate, as is necessary for a well powered study, the limited coverage of the exome affected the ability of both single-variant and gene-based tests to assess all exonic variation in association with kidney function. Thus, we cannot rule out *bona fide* rare variant associations with eGFRcrea. Large-scale whole exome or whole genome sequencing will be able to adequately address the unresolved issue of the contribution of rare variants to the variation of kidney function in the general population. Because of limited

sample size in the diabetes stratum compared with the nondiabetes stratum, we did not have adequate power to assess unique genetic associations in the diabetes stratified analysis. We did not implement a random effects meta-analysis as an alternative screening procedures for novel loci because the between-study heterogeneity was small. The analysis module that we used cannot correctly account for the different allele dosage between women and men and therefore we were unable to implement association analysis for chromosome X variants. Although our study supports the observation that gene-based analyses aggregating rare nonsynonymous variants are able to identify new loci in association with common complex phenotypes[14,20–22] and can additionally uncover new rare missense variants within known loci, the number of loci identified through gene-based methods remains a small minority of the findings compared with single-variant results. Finally, the zebrafish knockdowns helped us to screen novel loci that appear to have a role in kidney development or function. However, extrapolation of the relationship between the novel loci among patients with more advanced CKD remains to be determined; follow up studies are needed to assess the association of these loci with ESRD and incident CKD.

In summary, we identified eight novel loci (seven common single variants and one gene with multiple rare coding variants) associated with kidney function. Functional experiments in zebrafish highlighted potential roles for *SOS2* and *ACP1* in embryonic kidney development. Future whole exome and whole genome sequencing studies will be needed to assess the full spectrum of rare genetic variants on kidney function.

CONCISE METHODS

Study Participants

Across all traits analyzed, a total of 120,357 participants from 27 studies of EA were included in stage 1 of this study. An additional 48,343 participants from 12 studies of EA were included in stage 2. A total of 11,386 participants from seven studies of AA were also included in separate analyses. Study-specific characteristics are summarized in Supplemental Tables 1, 2, and 4. All participants provided informed consent and each study was approved by its governing ethics committee or Institutional Review Board.

Phenotype Definitions

Serum creatinine was measured in each study as described in the Study-Specific Methods section in the Supplemental Material, and calibrated to the National Health and Nutrition Examination Study data to account for between-laboratory variation.[23,24] eGFRcrea was estimated using the four-variable Modification of Diet in Renal Disease (MDRD) Study Equation.[25] Cystatin C, an alternative biomarker of kidney function, was measured in a sub-set of participating studies. eGFRcys was estimated as $76.7\times$(serum cystatin C).[25] All eGFRcrea and eGFRcys values <15 ml/min per $1.73m^2$ were set to 15, and those >200 ml/min per $1.73m^2$ were set to 200 to avoid undue influence from outliers. UACR was defined as urinary albumin (mg/L)/urinary creatinine (mg/dl)*100. All analyzed traits (eGFRcrea, eGFRcys, and UACR) were natural log (ln)-transformed. Diabetes was defined as fasting glucose $\geq$126 mg/dl, pharmacologic treatment for diabetes, or by self-report. Hypertension was defined as systolic BP $\geq$140 mmHg, diastolic BP $\geq$90 mmHg, or pharmacologic treatment for hypertension.[15]

Genotypes

Genotyping was conducted in each study using the Illumina Human Exome BeadChip (http://genome.sph.umich.edu/wiki/Exome_Chip_Design). This genotyping array containing 247,870 markers focuses on exonic variants discovered through exome sequencing of approximately 12,000 individuals. Illumina's GenTrain version 2.0 clustering algorithm in GenomeStudio, zCall,[26] or a combination of both procedures were used to call genotypes. To improve genotype calling of low frequency and rare variants, genotypes from eight of the contributing cohorts (Atherosclerosis Risk in Communities [ARIC], Age, Gene/Environment Susceptibility-Reykjavik Study [AGES], Cardiovascular Health Study [CHS], Framingham Heart Study [FHS], Rotterdam Study [RS], Health, Aging and Body Composition Study [Health ABC], Family Heart Study [FamHS], and Jackson Heat Study [JHS]) were jointly clustered and called *via* the CHARGE Consortium algorithm.[27] Other participating cohorts were called individually. Among them, CROATIA-Korcula and Generation Scotland (GS) applied the cluster file from the CHARGE Consortium for genotype calling. Details regarding genotyping and quality control within each study are summarized in Supplemental Table 2.

Statistical Methods for Stage 1

By following a centralized analysis plan, each study performed two sets of analyses: single-variant analysis and gene-based analysis. The primary meta-analyses were focused on the EA population and a secondary set of meta-analyses were focused on the AA population due to a substantially smaller sample size. Where not specified otherwise, R software was used for data management, statistical analyses, and graphing.[28]

Single-Variant Analysis

Each study performed association analyses of the following phenotypes and models: (*1*) ln-transformed eGFRcrea, (*2*) ln-transformed eGFRcys, (*3*) ln-transformed UACR, and (*4*) ln-transformed eGFRcrea stratified by diabetes status. These association analyses were based on linear regression models adjusting for age, sex, study site (if applicable), family structure (if applicable), and the first ten principal components to control for population stratification. All analyses were performed assuming an additive genetic effect and all analyses were stratified by ancestry.

For single-variant meta-analysis, study-specific results were combined for each trait in a fixed-effects model using METAL.[29] We restricted single-variant meta-analyses to (*1*) autosomal variants, (*2*) polymorphic variants, (*3*) variants existing in the joint calling effort within the CHARGE consortium[27], and (*4*) with minor allele count $\geq$20 across all cohorts in each ancestry group. Bonferroni correction for the number of variants tested within each ancestry was used to set the significance threshold for each analysis (chip-wide significance), corresponding to $P<3.7\times10^{-7}$ (0.05/134299 variants) for EA analysis and $P<5.5\times10^{-7}$ (0.05/91187 variants) for AA analysis.

Gene-Based Analysis

Single-variant analysis methods have limited power to detect association for rare variants. Recently developed gene-based methods, where defined variants contained in a gene region are collapsed into one unit for analysis, provide additional statistical power[30] to investigate the role of rare variants on kidney function traits.

For gene-based meta-analysis, study-specific results were combined using the seqMeta package for R.[31] The same phenotypes and covariates were used as for single-variant testing and analyses were again stratified by ancestry. We used two gene-based tests for aggregated analysis of rare single nucleotide variants (SNVs): (*1*) T1,[30] which is more powerful when all variants within the gene region affect the phenotype in the same direction; and (*2*) the sequence kernel association test,[32] which allows for bidirectional effects and is more powerful when there are both protective and deleterious variants within the same gene. Both gene-based tests were restricted to variants with MAF<1% and variants likely to exert a major effect on the gene product (stop gain/loss, nonsynonymous, or splice-site variants on the basis of annotation with dbNSFP [v.2.0]).[33] Genes containing at least two variants with a cumulative minor allele count ≥20[20] were included in the analysis. In total, we tested 9990 autosomal genes meeting our established thresholds and filters. Bonferroni correction for the number of genes and tests performed was used to set the significance threshold for the gene-based analysis corresponding to $P<2.5\times10^{-6}$ (0.05/19,922 tests [9961 genes × 2 tests]) in EA analysis and $P<2\times10^{-6}$ (25,378 tests [12,689 genes × 2 tests]) in AA analysis.

Statistical Methods for Stage 2

Chip-wide significant results from single-variant meta-analyses were brought forward for testing in stage 2 where each new study followed the same methodology as stage 1. Details regarding the genotyping and population characteristics of each cohort can be found in Supplemental Tables 2 and 4.

Study-specific results from stage 2 cohorts were combined using the same meta-analysis approach and software as in stage 1 (fixed-effects model in METAL[29]). Criteria for validation were: (*1*) a direction of effect consistent with stage 1 analysis, (*2*) a one-sided *P* value <0.05 from stage 2 analysis, and (*3*) $P<3.7\times10^{-7}$ from combined stage 1 and stage 2 analysis.

The percentage of phenotypic variance explained by each novel locus was estimated as $R^2=\beta^2 var(SNP)/var(ln[eGFRcrea])$, where β is the estimated effect of the SNP on ln(eGFRcrea), and $var(SNP)=2*MAF_{SNP}*(1-MAF_{SNP})$. Var(ln[eGFRcrea]) was estimated in the ARIC study. All loci were assumed to have independent effects on the phenotype.

Associations with Other Traits

To examine potential associations of the novel eGFR-associated variants with other correlated traits and conditions, we performed external look ups for systolic and diastolic BP in collaboration with the CHARGE Blood Pressure Working Group (*n*=145,872)[13] and for T2D in collaboration with the CHARGE Glycemia-T2D Working Group (*n*=10,240 T2D cases and 63,105 controls)[20] among EA participants.

NHGRI GWAS Catalog and PubMed Queries

For all newly identified and validated variants, we interrogated the NHGRI GWAS Catalog (https://www.ebi.ac.uk/gwas/;10/12/15) for the lead SNP at each locus and for SNPs in linkage disequilibrium with the lead SNP (within 1 Mbp and $r^2>0.5$ from 1000 Genomes Pilot in CEU; http://www.broadinstitute.org/mpg/snap/) to assess association with other traits (see Supplemental Table 11 for full listing of GWAS Catalog associations). Additionally, for each locus we searched PubMed (http://www.ncbi.nlm.nih.gov/pubmed;10/12/15) for publications on kidney/renal function or CKD.

Functional Studies in Zebrafish

To investigate a potential role of the newly identified genes during kidney development, we assessed the functional consequences of gene knockdown in zebrafish embryos. We used antisense MO technology to knock down genes identified on the basis of validated associations for nonsynonymous variants and for novel gene-based loci. Two independent MO probes were used for each gene. Zebrafish were maintained in accordance with established Institutional Animal Care and Use Committee protocols.

Morpholinos (Gene Tools) were designed against zebrafish target genes. Morpholino sequences are as follows: *sos2* (5′ GCACCGGGAACAACCACACAACTTT 3′), *sos2* (exon 2) (5′ CCTGCACCTATAAACACAGAATAGA 3′), *ppm1j* (5′ AATTTGTGACATCAGCGGCACGGTA 3′), *acp1* ATG (5′ TCCGCTGGAAGCCGCCATATTGGTC 3′), *acp1* (exon 1) (5′ TATAGCATTTCTTACCCAAGCACAC 3′), *speg* ATG (5′ TCTTCTCTTCAGTAACTTTTCTCAT 3′), *edem3* (exon 1) (5′ AGTCCTCACACAGACACATACCTCA 3′), and *eya4* ATG (5′ CAGATCCTGTGTATTCTCCATCAGT 3′). Zebrafish embryos were injected with various concentrations of MO (*sos2* ATG–150 μM, *sos2* (exon 2)– 400 μM, *ppm1j* ATG, *acp1* ATG, and *acp1* (exon1)– 400 μM, *edem3* ATG– 300 μM, *eya4* ATG– 400 μM, *speg* ATG– 100 μM) at the one-cell stage. We fixed embryos in 4% paraformaldehyde at relevant developmental stages for analysis by *in situ* hybridization (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). Distinct pronephros (embryonic kidney) structures were visualized using a series of established markers: *pax2a* (global), *wt1a* (podocyte), *slc20a1a* (proximal tubule), and *slc12a3* (distal tubule). *acp1* probe was generated from zebrafish cDNA using the following primers: 5′ TGGAGAATAGACAGTGCCGC 3′ (forward 1) and 5′ TTTTCACGCTGCTTGCCTTC 3′ (reverse 1), and 5′ GTGGAGAATAGACAGTGCCG 3′ (forward 2) and 5′ CAGGAAGGCTTTGCATC 3′ (reverse 2). *sos2* probe was generated from zebrafish cDNA using the following primers: 5′ GTGTTCGAGGAAGGAGCACA 3′ (forward) and 5′ TGATGTTCCACCCACTGACG 3′ (reverse).

Abnormal gene expression patterns were identified by direct comparison to control embryos that were injected with a standard control MO designed by GeneTools (SynGene, Cambridge, UK). Developmental phenotypes were scored by two independent researchers. Fisher exact tests were used to test for normal and abnormal embryonic phenotypes for the *pax2a*, *wt1a*, and *slc12a3* markers and *t* test was used to test for differences in proximal tubule length for the *slc20a1a* marker; *P*<0.05 was set as a threshold for statistical significance. To evaluate proximal tubule length, the distance between the most anterior and the most posterior tip of the right proximal tubule (from standardized dorsal-view images) was measured using the imageJ measurement tool. Fisher exact test was used to test for differences in edema prevalence; *P*<0.05 was set as a threshold for statistical significance.

Dextran clearance experiments were performed following a previously described protocol.[12] Seventy-two hours after morpholino

injection, embryos were anesthetized in a 1:20 dilution of 4 mg/ml Tricane in embryo water and placed on a 2% agarose injection mold. An equal volume of tetramethylrhodamine dextran (70 kDa molecular mass; Invitrogen, Carlsbad, CA) was injected into the cardiac sinus venosus of each embryo, and individual embryos were sorted into designated wells for timelapse imaging. Fluorescent microscopy images were taken at 2 hpi (74 hpf), 24 hpi (96 hpf), and 48 hpi (120 hpf) for each sorted embryo to assess loading fluorescence and the dextran clearance over time. Fluorescence intensity in the cardiac region was measured as the mean grayscale value using Image J as previously described.[34] Remaining fluorescence intensity at each time point was normalized to the starting intensity and plotted as a percentage of the initial fluorescence intensity. To evaluate edema, morpholinos were injected into the single-cell stage embryo and embryos were examined every 24 hours for evidence of edema. The number of affected embryos was recorded as a fraction of total number of injected embryos.

Power Calculation

Power for association was evaluated for eGFRcrea assuming a mean of 4.5 ln(ml/min per 1.73 m^2) with standard deviation of 0.2 ln(ml/min per 1.73 m^2), estimates from the EA samples in the ARIC study, using QUANTO power calculator, version 1.2.4 (http://biostats.usc.edu/Quanto.html) at the significance level of 3.7×10^{-7} for a variant with MAF of 0.1, 0.05, 0.03, 0.01, 0.005, 0.001, or 0.0005. In stage 1 with sample size n=111,666, there was at least 80% power to detect effect sizes of 0.008, 0.011, 0.015, 0.026, 0.036, 0.08, or 0.11 ln(ml/min/1.73m^2), respectively. For stage 2, using one-sided tests, there was 92% power to detect an effect size of 0.018 for a variant with MAF of 0.02 (minimum MAF among all eight SNPs tested) on the basis of 48,343 samples in stage 2 and correcting for the eight SNPs tested.

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DISCLOSURES

Caroline S. Fox and Audrey Y. Chu are employed by Merck Research Laboratories as of December 14, 2015 and July 18, 2016, respectively.

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