Shroom3 contributes to the maintenance of the glomerular filtration barrier integrity

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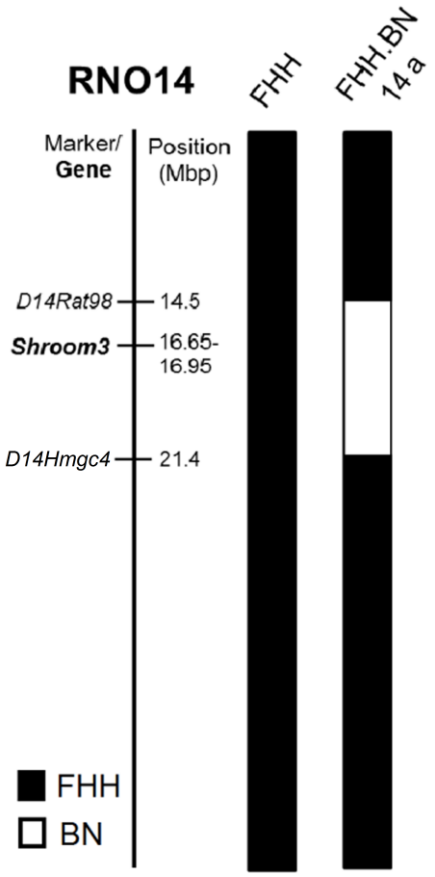
Supplemental materials

Supplemental Table 1. *SHROOM3* variants associated with renal functional traits identified through GWAS. Expression SNP is denoted in bold.

Strongest SNP	Chr:Position	Trait	First Author/Date/ Journal/ Study
rs17319721	4:77587871	eGFRcrea	Kottgen *et al.*/June 2009/*Nature Genet.*/Multiple loci associated with indices of renal function and chronic kidney disease
rs17319721; **rs4256249 (eSNP)**	4:77587871; **4:77677603**	eGFRcrea, eGFRcys**; eGFRcrea**	Kottgen *et al*./May2010/*Nature Genet.*/New loci associated with kidney function and chronic kidney disease
rs9992101	4: 77579455	Serum creatinine	Chambers *et al.*/May2010/ *Nature Genet*/ Genetic loci influencing kidney function and chronic kidney disease
rs13146355	4:77631164	eGFR	Meyer *et al.*/Aug 2010/*PLoS Genet*./ Genome-wide association studies of serum magnesium, potassium, and sodium concentrations identify six Loci influencing serum magnesium levels
rs4859682	4: 77629342	Serum creatinine	Pattaro *et al.*/Mar 2010/ *BMC Med Genet*./ A meta-analysis of genome-wide data from five European isolates reveals an association of COL22A1, SYT1, and GABRR2 with serum creatinine level
rs17319721	4:77587871	Incident CKD	Böger *et al.*/Sep 2011/ *PLoS Genet./* Association of eGFR-Related Loci Identified by GWAS with Incident CKD and ESRD
rs17319721	4:77587871	UACR, albuminuria	Ellis *et al.*/Jul 2012/*Hum Mol Genet.*/ Validated SNPs for eGFR and their associations with albuminuria
rs13146355	4:77631164	Serum creatinine, eGFRcrea	Okada *et al.*/Jul 2012/*Nature Genet.*/ Meta-analysis identifies multiple loci associated with kidney function-related traits in east Asian populations

Abbreviation: Chr: chromosome; SNP: single nucleotide polymorphism; eGFRcrea: glomerular filtration rate, estimated by serum creatinine; eGFRcys: glomerular filtration rate, estimated by cystatin c; CKD: chronic kidney disease; UACR: urinary-to-albumin ratio; eSNP: expression SNP. SNP position is based on NCBI36/hg18 assembly.

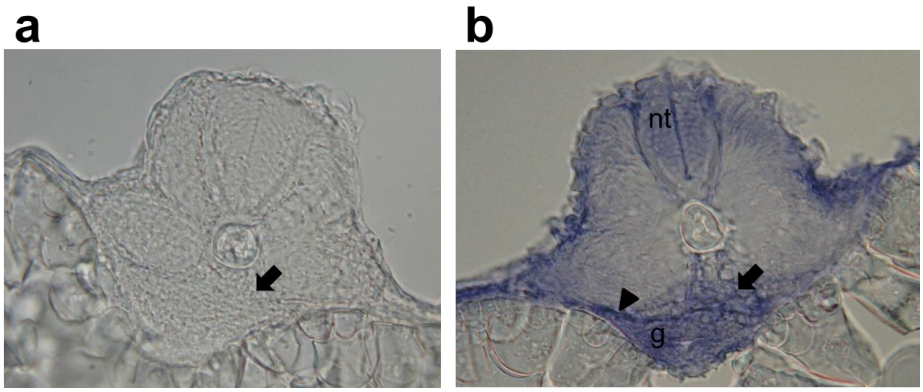


Supplemental Figure 1. Genetic makeup of the parental FHH and FHH.BN14a congenic rat chromosome 14 (RNO14). A 6.1 Mb region (flanking markers*: D14Rat98-D14Hmgc4*) on chromosome 14 containing *Shroom3* gene from the BN rat was introgressed onto the genetic background of the FHH strain.

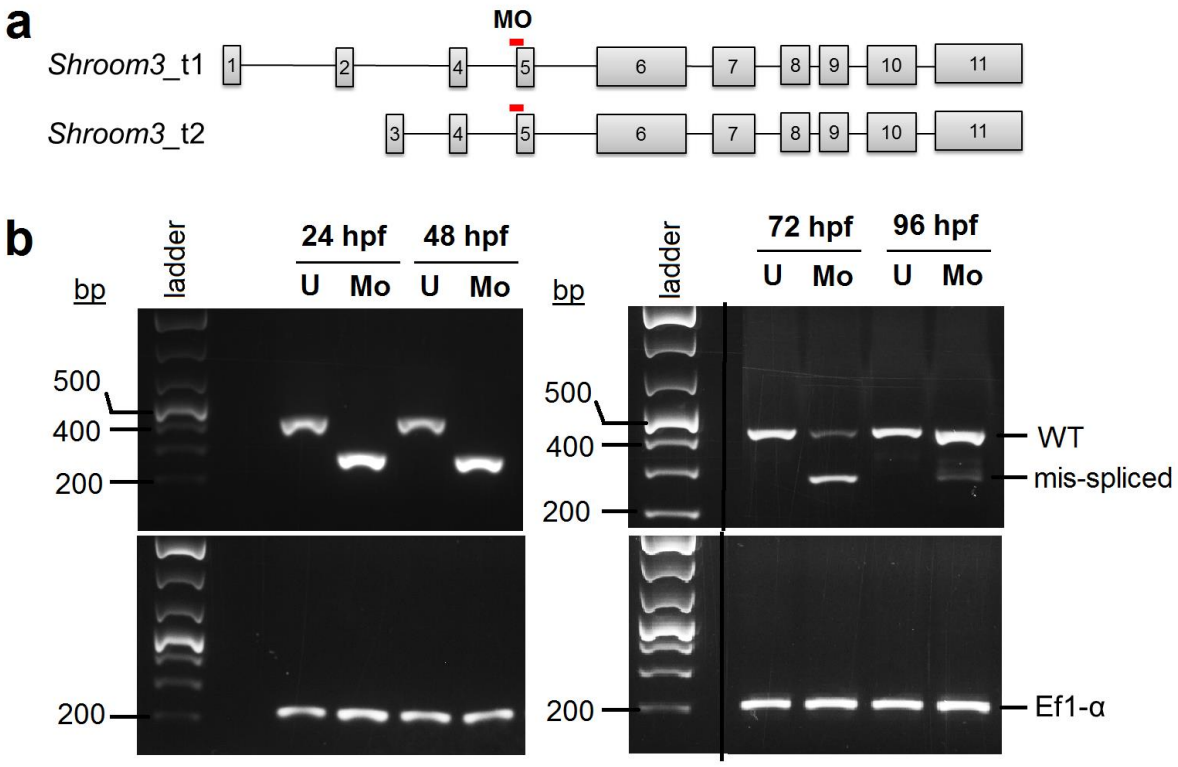
Supplemental Table 2. Non-synonymous variants in the 6.1 Mb congenic region between FHH and FHH.BN14a rats.

Position	Gene Symbol	BN nt	FHH nt	AA Change	Polyphen Prediction
16670605	*Shroom3*	T	C	N1704S	Benign
16670607	*Shroom3*	C	G	E1703D	Benign
16670618	*Shroom3*	C	T	V1700M	Possibly damaging
16672808	*Shroom3*	C	T	R1432Q	Possibly damaging
16673036	*Shroom3*	G	A	A1356V	Possibly damaging
16673231	*Shroom3*	T	C	Y1291C	Benign
16682736	*Shroom3*	C	T	G1073S	Possibly damaging
16683924	*Shroom3*	T	C	S677G	Possibly damaging
16684146	*Shroom3*	G	A	L603F	Benign
16684310	*Shroom3*	C	G	R548T	Benign
16684341	*Shroom3*	T	C	M538V	Benign
16684422	*Shroom3*	C	T	A511T	Probably damaging
17032887	*Stbd1*	C	G	S248T	Benign
17033136	*Stbd1*	G	A	P165L	Benign
17033161	*Stbd1*	C	A	A157S	Possibly damaging
17220659	*Art3*	C	G	S109T	Benign
17220661	*Art3*	C	G	Q108H	Possibly damaging
17380227	*Ppef2*	A	G	M396V	Benign
17382773	*Ppef2*	G	A	E454K	Benign
17421453	*Uso1*	A	G	L776S	Benign
18078065	*Parm1*	C	T	A102T	Benign

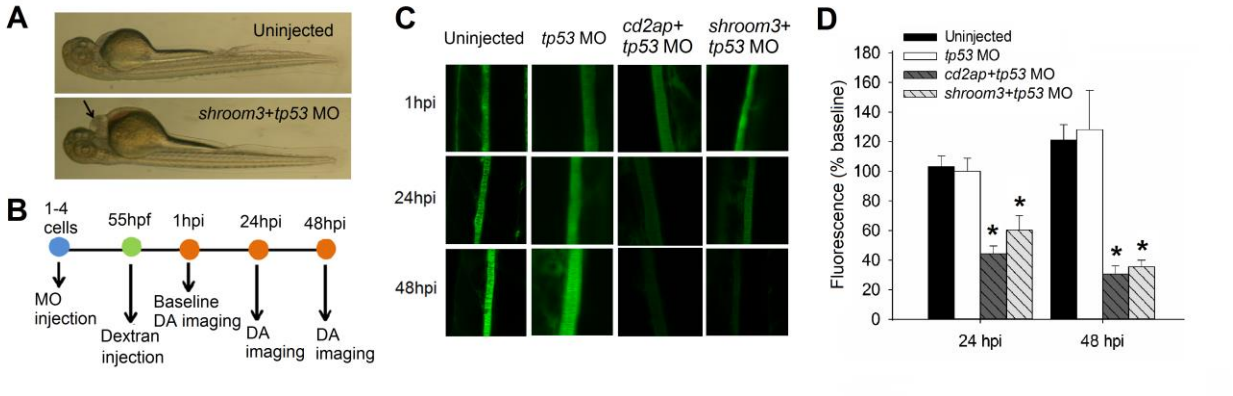
Abbreviation: nt: nucleotide: AA: amino acid. Variant positions and gene symbols are based on rat RGSC3.4 genome assembly and RefSeq database.



Supplemental Figure 2. Zebrafish *shroom3* transcript expression was detected in the pronephros by *in situ* hybridization (ISH). ISH against *shroom3* transcript was performed on 48 hours post fertilization wild-type embryos, using (a) sense-strand probe (negative control) and (b) antisense-strand probe. Transverse cross sections at the embryo midline showed *shroom3* expression in the glomerulus (arrow), pronephric tubule (arrowhead), neural tube (nt), and gut (g).



Supplemental Figure 3. Morpholino (MO) injections affect splicing of *shroom3* transcript in zebrafish. (a) The *shroom3* MO was designed to target the intron splice-acceptor site upstream of *shroom3* exon 5. Red horizontal line indicates the binding-site of *shroom3* MO on the two *shroom3* transcript splice-variants, t1 (accession number: JX455752) and t2 (accession number: JX455753) (zebrafish *shroom3* sequence was published previously by Ernst et al. 2012). (b) PCR analysis indicated that the *shroom3* MO disrupted proper splicing of the zebrafish *shroom3* transcript. The effect of MO lasted for at least 72 hours post fertilization (hpf). Partial recovery of wild-type *shroom3* mRNA by 96 hours post fertilization (hpf) may have underestimated the effect of *shroom3*-deficiency. We therefore engineered continuous expression of a *shroom3* dominant negative as a mean to validate the effect of *shroom3* on glomerular filtration barrier function. (bp= base pair, U= Uninjected control, Mo= Morphant)



Supplemental Figure 4. Knockdown of *shroom3* by morpholino (MO) causes cardiac edema and increased glomerular permeability. (A) At 3 days post fertilization (dpf), uninjected control zebrafish developed normally. Injection of *shroom3*+*tp53* MO resulted in cardiac edema (indicated by arrow). (B) Shown is the protocol for the 70-kDa FITC-labeled dextran clearance assay. (C) Representative fluorescence images of individual dorsal aorta at 1, 24, and 48 hours post injection (hpi) for each group are shown. (D) FITC intensity was measured in relation to the intensity at 1 hpi. Both uninjected and *tp53* MO injected zebrafish had preserved FITC intensity at 24 and 48 hpi. Injection of *cd2ap*+*tp53* MO or *shroom3*+*tp53* MO caused significantly decreased FITC signal, suggesting that knockdown of *cd2ap or shroom3* disrupts glomerular filtration barrier size selectivity, therefore increasing the rate of dextran clearance. *p<0.05 vs. uninjected and *tp53* MO-injected zebrafish

Supplemental Table 3. Clinical characteristics of subjects used in targeted genotyping of *SHROOM3*.

	Controls	Non-diabetic ESKD
N	760	1705
Age (years)	48.4 ± 12.7	54.6 ± 14.6
Female (%)	57.9	43.7
Body mass index (kg/m^2)	29.2 ± 7.4	27.2 ± 6.98
Fasting serum glucose (mg/dL)	89.2 ± 13.6	88.6 ± 8.66
Duration of ESKD (years)	-	2.2 ± 1.64
Blood urea nitrogen (mg/dL)	13.3 ± 4.5	-
Serum creatinine (mg/dL)	1.03 ±0.46	-

Abbreviation: ESKD: End-stage kidney disease

Supplemental Table 4. Association results of all genotyped variants in *SHROOM3* with non-diabetic end-stage kidney disease in African Americans. (Statistically significant SNPs are denoted in bold)

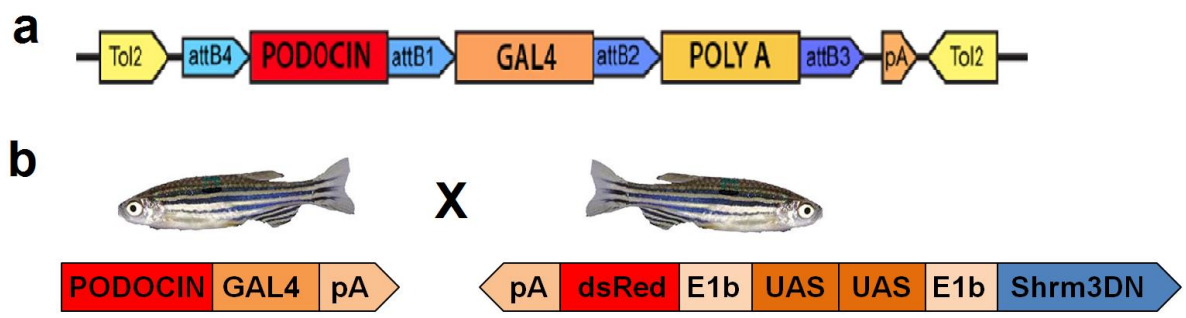
A. Coding and splice-site variants

SNP	Position (HG19)	N (Case/Contro)	MAF (Case/Control)	P	OR	95% CI
rs191518122	77550139	1446/1087	0.0038/0.0023	0.47	1.53	0.48-4.88
rs141460234	77631216	1455/1095	0/0.0005	-	-	---
rs139587539	77659907	1457/1105	0/0	-	-	---
rs146652221	77660381	1455/1091	0.001/0.0018	0.82	1.19	0.25-5.6
4:77660512	77660512	1457/1110	0/0	-	-	---
4:77660518	77660518	1474/1057	0.0003/0	-	-	---
rs344141	77660731	1475/1055	0.4217/0.42	0.2	1.13	0.94-1.36
rs144001652	77660860	1446/1089	0.0014/0.0028	0.15	0.29	0.05-1.59
rs76656494	77661017	1455/1098	0.0017/0.0032	0.79	0.85	0.24-2.94
rs140229037	77661127	1474/1048	0/0.0014	-	-	---
rs61741135	77661704	1454/1104	0.0079/0.0072	0.91	1.04	0.51-2.13
4:77661996	77661996	1456/1112	0/0	-	-	---
rs113001394	77662014	1472/1055	0.0003/0.0005	0.47	2.96	0.15-57.03
rs371443861	77662106	1431/1100	0.0003/0.0014	0.86	0.8	0.07-9.18
rs145035894	77662157	1470/1041	1/1	2	2	2-2
rs147732653	77662361	1489/1144	0.0007/0.0026	0.29	0.41	0.08-2.13
COSM1431111	77662378	1455/1112	0/0	-	-	---
4:77662502	77662502	1462/1154	0/0	-	-	---
rs181194611	**77663057**	**1463/1081**	**0.0062/0.0009**	**0.014**	**7.95**	**1.53-41.46**
rs141613065	77663058	1480/1045	0.0034/0.0024	0.41	0.57	0.15-2.17

B. Non-coding variants

SNP	Position (HG19)	N (Case/Control)	MAF (Case/Control)	P	OR	95% CI
rs13136800	77364746	1456/1105	0.091/0.078	0.045	1.28	1.01-1.63
rs12640246	77368470	1449/1104	0.23/0.22	0.61	1.05	0.87-1.26
rs4859678	77386372	1476/1034	0.1829/0.16	0.51	1.07	0.88-1.29
rs1511817	77386678	1452/1091	0.41/0.41	0.77	1.03	0.85-1.24
rs11721509	77433888	1451/1085	0.17/0.16	0.82	0.98	0.81-1.19
rs4859688	77439979	1325/686	0.43/0.41	0.4 [a]	1.06	0.92-1.22
rs17002091	**77500947**	**1449/1102**	**0.13/0.15**	**0.037** [a,b]	**0.83**	**0.7-0.99**
rs72868158	**77528915**	**1290/670**	**0.11/0.12**	**0.016**	**0.73**	**0.57-0.94**
rs6838801	77549559	1327/682	0.26/0.26	0.61 [a]	1.04	0.89-1.22
rs6532535	77562538	1318/685	0.47/0.47	0.59 [a]	1.04	0.9-1.2
rs4859456	77562958	1335/684	0.47/0.47	0.52 [a]	1.05	0.91-1.21
rs13114029	77575268	1457/1102	0.019/0.02	0.8	0.94	0.59-1.51
rs13108785	77576070	1329/685	0.34/0.32	0.063 [a]	1.15	0.99-1.34
rs151021596	77638937	1450/1098	0.011/0.011	0.72	0.9	0.49-1.64
rs11735292	77653298	1475/1053	0.0319/0.039	0.26	0.81	0.57-1.16
rs344137	77654812	1325/678	0.16/0.17	0.43 [a]	0.93	0.76-1.12
rs17002201	**77681958**	**1448/1089**	**0.17/0.19**	**0.015** [a,b]	**0.83**	0.71-0.96
4:7700260	77700260	1475/1050	0.0003/0.001	0.56	0.48	0.04-5.4
rs72663250 (proxy to rs55650799)	**77708325**	**1326/684**	**0.13/0.16**	**0.037** [a]	**0.81**	0.67-0.99

Association values are adjusted for admixture, gender, and *APOL1* genotype, using either dominant model or addictive model (indicated by superscript 'a'). Values adjusted for admixture and *APOL1* genotypes were indicated by superscript 'b'. Abbreviations: MAF= minor allele frequency; OR= odds ratio; 95% CI= 95% confidence interval.



Supplemental Figure 5. Generation of podocyte-specific *shroom3* dominant negative (shrm3DN) mutant zebrafish. (a) Schematic of the final expression construct, pDestTOl2CG2;podocin:Gal4VP16:polyA, is provided. (b) To generate podocyte-specific shrm3DN mutant, we crossed the *podocin*:GAL4 transgenic fish with a transgenic line that had constitutive expression of a UAS linked to shrm3DN and dsRed mCherry allele. dsRed mCherry expression in podocytes was used as a marker to select offspring with *podocin*:Gal4;*UAS*:shrm3DN expression.

Supplemental Table 5. FHH rat harbors non-synonymous variants in several genes that have been identified by genome-wide association studies for chronic kidney disease.

Gene	Chromosome	Genomic Position	BN	FHH
Umod	1	177,741,029	C	G
Umod	1	177,741,245	T	C
Dab2	2	55,727,505	A	G
Dab2	2	55,727,598	G	C
Dab2	2	55,727,668	G	C
Mecom	2	114,844,878	C	T
Ino80	3	105,941,054	A	G
Ino80	3	105,979,571	G	T
Ino80	3	105,979,617	G	C
Gnas	3	165,214,394	T	G
Alms1	4	119,859,053	C	G
Alms1	4	119,867,722	T	C
Alms1	4	119,877,408	G	C
Alms1	4	119,879,053	A	G
Alms1	4	119,879,086	T	C
Alms1	4	119,885,426	C	T
Alms1	4	119,885,860	C	A
Prkag2	4	5,659,266	A	G
Casp9	5	160,711,084	T	C
Tfdp2	8	101,256,460	A	G
Tfdp2	8	101,390,941	G	A
Aldh2	12	36,109,675	C	T
Aldh2	12	36,110,126	G	A
Aldh2	12	36,110,183	T	C
Aldh2	12	36,112,403	C	T
Aldh2	12	36,114,800	T	C

Variant position and gene symbol are based on rat RGSC3.4 genome assembly, and sequence of FHH/EurMcwi (MCW) was compared to the BN reference genome. Genetic variants can be downloaded at the Rat Genome Database Variant Visualizer (http://rgd.mcw.edu/rgdweb/front/select.html).

Supplemental Table 6. Morpholino and PCR primer sequences used in the study

Morpholino (MO) antisense oligonucleotide sequence

MO-targeted gene	MO sequence (5' to 3')
shroom3	AGGCTCACTCCGTCTGTAAAAGAGA
cd2ap	CATACTCCACCACCACCTCAACCAT
tp53	GCGCCATTGCTTTGCAAGAATTG

Zebrafish sequence primer used to assess morpholino-induced splicing defects

Target (F: forward, R: reverse)	Primer sequence (5' to 3')
shroom3_1F	CGGACACTTTTGGTTTTTGG
shroom3_1R	CTGTGGTCAAGGCTTTCCAT
ef1-α_F	TCTCTCAATCTTGAAACTTATCAATCA
ef1-α_R	AACACCCAGGCGTACTTGAA

Zebrafish sequence primer used to generate *shroom3* in situ hybridization probe

Target (F:forward, R: reverse)	Primer sequence (5' to 3')
shroom3_2F	GGCCCTCAAGACTCAGACAG
shroom3_2R	GCCCCCTTTAGGTTCTCAAA

Rat sequence primer used to amplify full-length *Shroom3* cDNA

Target (F:forward, R:reverse)	Primer sequence (5' to 3')
Shroom3_1F	ATGAGGACCCCCGAGAACTTG
Shroom3_1R	TCAAGCTGTGAGCGGTAACC
Shroom3_2F	GGTTACCGCTCACAGCTTGA
Shroom3_2R	GGTTAAAGAGGAGAGGTCAACG

Primers used for site-directed mutagenesis

Target (F:forward, R:reverse)	Primer sequence (5’ to 3’)
ΔG1073S_F	GCTCCACGCTCAGCCTGTCTGGCC
ΔG1073S_R	AGGCCTTGCTGTCGCGCTCGAA
ΔY1291C_F	CTCTCAGCTGCTCGGACAGAGGCCA
ΔY1291C _R	GCCTAGATCCAGGGCCAGCCAA
ΔA1356V_F	AGATGCCAGAGTCTCCTCCGTG
ΔA1356V_R	GGGAAGTGGCCGTGCATTCCTG
ΔP1244L_F	CGCCAGGATGTGCTTTTGGGGCAAGACAG
ΔP1244L_R	CTTGTCTGCGGTGGCGAGAGATGACCT