Am J Transl Res 2020;12(5):2212-2224
www.ajtr.org /ISSN:1943-8141/AJTR0112204

Original Article

POFUT1 is dispensable for structure, function and survival of mouse podocytes

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Received April 9, 2020; Accepted May 1, 2020; Epub May 15, 2020; Published May 30, 2020

Abstract: *Pofut1* gene encodes a O-fucosyltransferase that adds fucose to the serine/threonine residue in the sequence of $C^2XXXX(S/T)C^3$ of EGF-like domain in a protein. O-fucosylation has been shown to be required for some EGF-like domain-containing proteins to function, e.g., Notch1, and POFUT1 deficiency could affect cellular function and cause diseases. *Pofut1* is ubiquitously expressed, but its essentiality for most cell types is not known. In the present study, we examined the consequence of Pofut1 gene abrogation in mouse podocytes using Cre-loxP system, and found that the conditional knockout mice were indistinguishable from wild-type controls in urinary protein level, glomerular morphology, podocyte foot process ultrastructure, podocyte marker expression and podocyte numbers. These results indicated that POFUT1 is not essential for podocyte structure, function and survival in mice. To understand why POFUT1 is dispensable for podocytes, we searched mouse podocyte essential gene candidates (as determined by single-cell RNA-seq) and found only two POFUT1 substrates, NOTCH2 and tPA. It has been shown that abrogation of these genes does not cause podocyte injury, explaining dispensability of POFUT1 for mouse podocytes and demonstrating a feasibility to predict POFUT1 essentiality for a given cell type. At present, most mouse cell types have been subject to single-cell RNA-seq, making essential gene prediction and thus POFUT1 requirement prediction possible for the cell types.

Keywords: POFUT1, O-fucosylation, podocyte, NOTCH2, tPA

Introduction

O-fucose modification was discovered in many proteins with epithelial growth factor (EGF)-like domains, such as coagulation factors [1-4] and Notch receptors and ligands [5, 6]. Protein O-fucosyltransferase 1 (POFUT1) is the only enzyme responsible for O-fucosylation of the EGF-like domains with the consensus sequence of $C^2XXXX(S/T)C^3$ in these proteins [7, 8]. There are about 100 human proteins that carry the consensus sequence and thus are potentially O-fucosylated by POFUT1 [9].

The importance of POFUT1 and protein O-fucosylation in cells have been demonstrated directly by *Pofut1* gene abrogation in both Drosophila and mouse, which resulted in developmental defects and embryonic lethality [10, 11]. POFUT1 deficiency also causes other abnormalities in both animals and human, including skin and cardiovascular diseases, microcephaly, and muscle aging-related phenotype, etc. [12-15].

Generally, POUFT1 essentiality is implemented by requirement of O-fucosylation for some proteins which play important roles in various cellular processes, such as Notch receptors and ligands [5, 6]. *Pofut1* gene abrogation gives rise to developmental defects that are the same as Notch signaling deficiency [10, 11]. POFUT1 also regulates the development and homeostasis of blood cell lineages through Notch [16-18]. In addition, POFUT1 regulates Notch signaling in lung development [19] and intestinal homeostasis [20], as well as the maintenance of enteric neural crest progenitors [21] and mammary epithelial cell lineages [22]. In human, *POFUT1* gene mutation causes hidradenitis suppurativa-Dowling-Degos disease through impairing Notch signaling [23]. Notch ligands also require O-fucosylation to function [24].

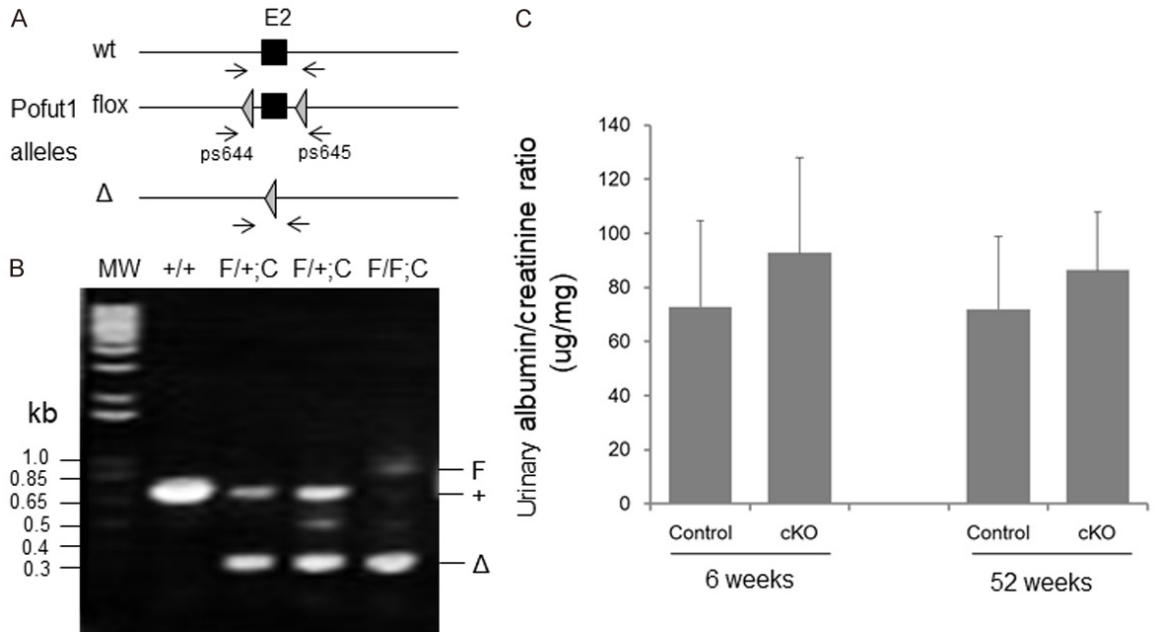

Figure 1. PCR detection of Pofut1 allele with exon 2 deleted in glomeruli isolated from mice and urinary albumin levels of control and Pofut1 cKO mice. A. Schematic of mouse Pofut1 wild-type allele (wt) and the alleles with exon 2 floxed by loxP sites (flox) or deleted (Δ). B. PCR product gel analysis, showing the predicted product from the glomerular samples from mice of various genotypes as indicated. +, wild-type; F: flox; Δ: deletion; C: NPHS2-Cre. C. Urinary albumin/creatinine ratios (ACR) were measured at the age of 6 weeks for control (n=12) and cKO mice (n=11) mice. At the age of 52 weeks, the control (n=10) and cKO (n=10) mice were subject to ACR measurement again. There were no statistically significant difference between the two groups of mice at both 6 and 52 weeks with *p* values of 0.17 and 0.21, respectively, using student's t-test.

POFUT1 is expressed ubiquitously in various mouse tissues [11], suggesting that POFUT1 may be essential for many cell types. As shown in GSE123179 and GSE17142 from the GEO database (http://www.ncbi.nlm.nih.gov/geo/), POFUT1 is also expressed in podocyte, which are part of glomerular filtration barriers. Moreover, Notch components are also expressed in podocytes although at relatively low levels in GSE123179 and GSE17142 from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). It would be interesting to know whether POFUT1 is required for maintenance of podocyte differentiation, structure, function, and survival, particularly through Notch signaling regulation. In the present study we investigated the issues by generation and characterization of mice with POFUT1 gene abrogation specifically in podocytes.

Materials and methods

Generation of mice with podocyte-specific deletion of Pofut1

Pofut1$^{flox/flox}$ mice were generated as described previously [11], and was gift from Dr. Pamela Stanley at Albert Einstein College of Medicine, USA. This mouse line was crossed with podocyte-specific transgenic *NPHS2*-Cre [25] to obtain conditional knockout mice with genotype of *Pofut1*$^{flox/flox}$; *NPHS2*-Cre. PCR genotyping of the *Pofut1* alleles and *NPHS2*-Cre transgene was performed as previously described [11, 25-27]. The primers for Pofut1 alleles are PS644, 5'-GGGTCACCTTCATGTACAAGTGAGTG-3', and PS645, 5'-ACCCACAGGCTGTGCAGTC-TTTG-3', with product sizes indicated in **Figure 1**; *NPHS2*-Cre transgene primers are Cre-F, 5'-GGACATGTTCAGGGATCGCCAGGCG-3', and Cre-R, 5'-GCATAACCAGTGAAACAGCATTGCTG-3', with the product size of 268 bp. The use of mice in the present study conformed to the institutional regulations and requirements concerning the care and use of laboratory animals at Jinling hospital, Nanjing University School of Medicine. The animal ethical committee approval number was 2015NZGKJ-059.

Isolation of glomeruli from mice

Mice were euthanized and perfused with 2.5 mg/ml iron oxide solution in PBS. Kidneys were diced into 1-mm^3 pieces. One hundred microli-

ters of collagenase A (10 mg/ml) and 100 μl of DNase I (1,000 U/ml) were added to the kidney tissues followed by incubation at 37°C for 30 min with rotation. Digested tissue was passed through a 100-μm cell culture strainer and glomeruli were collected by magnetic concentration. Glomeruli were washed with PBS twice. The isolated glomeruli were treated with proteinase K to extract DNA for PCR analysis of allele of deletion.

Urinary albumin to creatinine ratio (ACR) measurement

Urinary albumin and creatinine were measured using mouse albumin specific ELISA and Creatinine Companion Kits (Exocell Laboratories) following the manufacturer's instruction.

Periodic acid-Schiff (PAS) staining of kidney sections

Mice were euthanized and perfused with 4% paraformaldehyde followed by 18% sucrose PBS solution. Kidney tissues were excised and fixed in 10% formalin overnight, dehydrated in graded alcohols and embedded in paraffin. Four micrometer thick sections were cut and stained with PAS reagent following the protocol recommended by the Animal Models of Diabetic Complications Consortium (*http://www.amdcc.org*).

Toluidine blue staining of kidney sections

Five hundred nanometer kidney sections were completely dried. The sections were incubated with staining solution (1% Toluidine Blue and 2% Borate in Distilled Water) until desired staining intensity was achieved. The sections were rinsed with water and air dried and mounted with mounting medium.

Electron microscopy (EM)

Blocks of renal cortex tissue (1 mm^3) were fixed in cold 3.75% glutaraldehyde for 4 h. After washing in 0.1 M phosphate buffer (pH7.5) for 5 times, the renal tissues were post-fixed in 2% osmium tetroxide for 2 h, dehydrated in graded acetones and ethanol and embedded in epoxy resin (SPI Inc., Westchester, PA). Ultrathin sections (80-90 nm) were stained using uranyl acetate and lead citrate, and then examined and photographed using a Hitachi 7500 transmission electron microscope (Hitachi Co., Japan).

Immunofluorescent staining

Kidney cortex tissues were cut and placed in Tissue-Tek OCT, snap-frozen in liquid nitrogen and cut into 5-μm sections using a cryostat (Leica CM 3050S, Germany). The sections were blocked with bovine serum albumin and incubated in primary antibodies against SYNPO (Thermo Fisher Scientific) and laminin α1 (Sigma, St. Louis, MO). Next, the sections were incubated with an FITC-conjugated anti-goat secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 568 goat anti-mouse Ig G1 (both from Invitrogen, Carlsbad, CA).

WT1 staining and positive cell counting in glomeruli of mice

OCT-embedded frozen kidneys of 10 Pofut1 cKO mice and wild type mice were cut into 4 μm sections, air-dried and incubated with blocking solution at room temperature for 30 min. Rabbit polyclonal WT1 antibody (C-19; Santa Cruz) and the second antibody goat anti-rabbit IgG (H+L) Alex Fluor 488 (Invitrogen, Carlsbad, CA) were used to stain podocytes at room temperature for 1 h, respectively, followed by DAPI staining and sealing with Fluoromount-G (Southern Biotech). WT1 and DAPI double-positive cells were counted as podocytes. WT1 positive cells in ~20 glomeruli were counted for each mouse, and total number of WT1 positive cells were divided by the number of glomeruli examined, resulting in the average podocyte number per glomerulus of the mouse.

Search for mouse proteins that are predicted to be O-fucosylated by POFUT1

Potential targets of POFUT1 are listed based on a ScanProsite database (https://prosite.expasy.org/scanprosite/) search of all mouse proteins containing EGF like domain (PS50026) that contain the $C^2XXXX(S/T)C^3$ consensus sequence for O-fucosylation cross-referenced with the Uniprot database (https://www.uniprot.org). Splice variants were not considered.

Statistical analysis

SPSS18.0 software was used for the statistical analyses. The experiments described in the

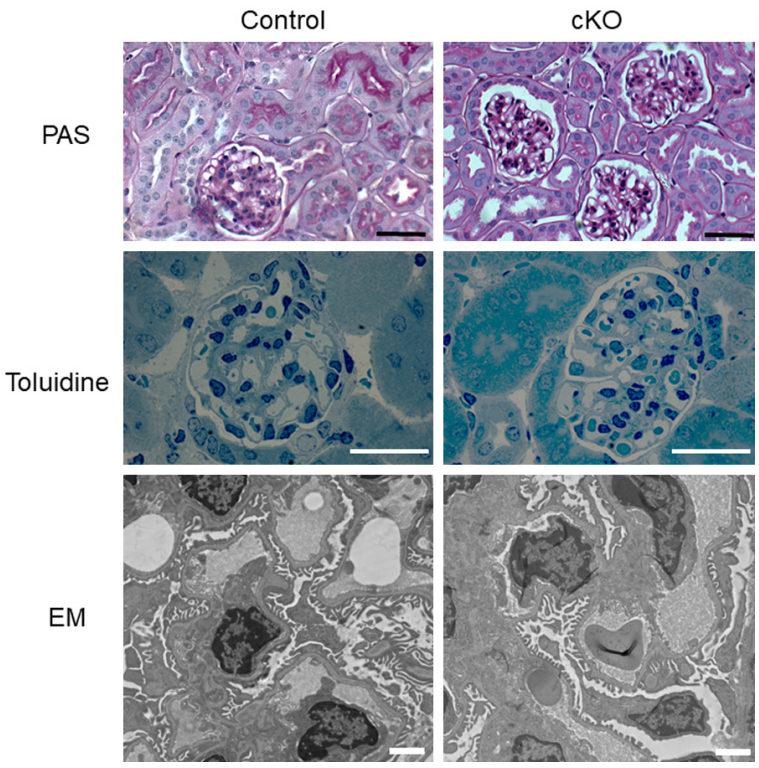

Figure 2. Morphological comparison of glomeruli of control and *Pofut1* cKO mice. PAS and Toluidine staining were performed on kidney sections of the mice, showing indistinguishable glomerular morphology between the two groups of mice. Scale bars: 30 μm. Neither did EM examination show any difference in glomerular ultra-structure between the control and *Pofut1* cKO mice. Scale bars: 2 μm.

present study were performed on 10 mice in each group, and the results were given as mean ± SD. Two-tailed Student's t test was used for comparison between two groups, and $P<0.05$ was considered to be significantly different.

Results

Generation of mice with Pofut1 deletion selectively in podocytes

The mouse line with Pofut1 floxed allele was crossed to podocyte-specific *NPHS2*-Cre transgene line to generate conditional knockout of Pofut1 in mouse podocytes (cKO). PCR genotyping of the Pofut1 alleles and *NPHS2*-Cre transgene were performed as described previously [11, 27].

To confirm that Pofut1 deletion was induced in podocytes, we isolated glomeruli from *Pofut1*$^{f/f}$; NPHS2-Cre cKO and control mice. The genomic DNA was prepared and subject to PCR genotyping to detect the product of deletion. We obtained precise PCR product from the predicted recombination of the floxed *Pofut1* alleles in the mice carrying floxed *Pofut1* alleles and *NPHS2*-Cre transgene (**Figure 1A**, **1B**). The identity of the PCR product was further confirmed by diagnostic digestion using restriction enzyme as described previously [26].

Characterization of POFUT1 cKO mice

To examine the consequence of POFUT1 deficiency to podocytes in mice, we monitored the development of proteinuria (urinary albumin/creatinine ratio, ACR), which is the established marker of podocyte injury, in the cKO mice, and found that their ACRs were all normal at the age of 6 weeks compared with control group. At the age of 52 weeks (~1 year), we measured ACR again and found no difference between the two groups of mice (**Figure 1C**).

PAS staining of kidney was performed for the mice, and the glomeruli of cKO mice were morphologically normal compared with that of controls (**Figure 2**). Toluidine staining also showed normal morphology of glomeruli of cKO mice (**Figure 2**).

We next examined ultrastructure of glomeruli and podocytes of the cKO mice by electron microscopy, but did not find any abnormalities, particularly, foot process effacement, which is the most sensitive marker of podocyte injury (**Figure 2**).

Furthermore, we used immunofluorescence staining to examine the expression of podocyte markers, synaptopodin and basement membrane component, laminin α1. The results showed that the expression of the genes were totally normal in cKO mice compared with that in controls (**Figure 3**).

Finally, we counted podocyte numbers in cKO mice and compared them with that of control

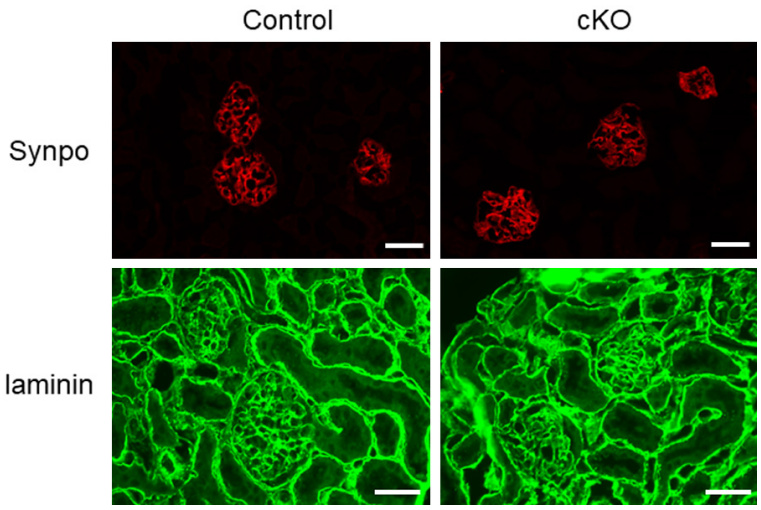

Figure 3. Immunofluorescence staining of podocyte marker, synaptopodin, and basement membrane component, laminin α1 in kidney of control and Pofut1 cKO mice. Scale bars: 40 μm.

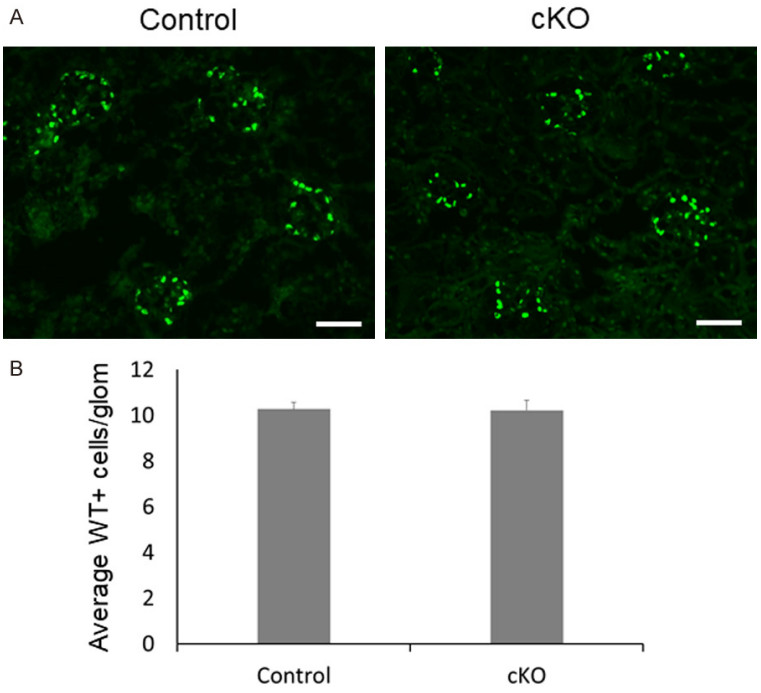

Figure 4. WT1 positive cell counting in glomeruli of mice. A. Representative WT1 fluorescence staining. B. Quantitative results of WT1 positive cells of control (n=10) and Pofut1 cKO (n=10) mice. About 20 glomeruli were examined for WT1 positive cells numbers for each mouse. Scale bars: 50 μm. There was not statistically significant difference between the two groups with a *p* value of 0.66 using student's t-test.

mice to determine whether podocyte numbers were reduced in the cKO mice. WT1 is a marker of podocytes, therefore, WT1-positive cells in glomeruli were counted. The average number of positive cells per glomerulus represented the podocyte number in the mouse. The result showed that there was not any difference in podocyte number between cKO and control groups (**Figure 4**).

Searching mouse proteins potentially O-fucosylated by POFUT1

We next explored why *Pofut1* gene abrogation in mouse podocytes did not affect structure, function, and survival of podocytes. We speculated that mouse podocytes may not express protein substrates for POFUT1 or the substrates are not essential for podocytes, resulting in dispensability of POFUT1 in podocytes.

To prove it, we firstly searched mouse proteins that contain EGF-like repeats with the consensus sequence of $C^2XXXX(S/T)C^3$ in the ScanProsite database following the method described [9]. Seventy-eight mouse genes express proteins that are potentially O-fucosylated by POFUT1 (**Table 1**).

To determine which of the 78 potential O-fucosylated proteins are expressed in and essential for podocytes, we compared them with mouse podocyte essential genes. Previously, we performed single-cell RNA-seq analysis of mouse podocytes and found a high heterogeneity of gene expression in individual podocytes [28]. We hypothesized that genes expressed in every single podocyte are likely essential for podocytes as a cell type, while the genes expressed only in a portion of podocytes are non-essential. With this notion, we identified 335 podocyte essential gene candidates using 0.5 rpkm as expression cutoff [28]. Here, we lowered the standard by using 0.1 rpkm as the expression

Table 1. Putative mouse genes encoding protein substrates of POFUT1

uniprot ID	gene	name	conserved
Q61549	Adgre1	Adhesion G protein-coupled receptor E1	
A2ASQ1	Agrn	Agrin	√
Q6PGD0	Atraid	All-trans retinoic acid-induced differentiation factor	√
Q61361	Bcan	Brevican core protein.	
O35161	Celsr1	Cadherin EGF LAG seven-pass G-type receptor 1	√
Q9R0M0	Celsr2	Cadherin EGF LAG seven-pass G-type receptor 2	√
Q91ZI0	Celsr3	Cadherin EGF LAG seven-pass G-type receptor 3	√
P97766	Cfc1	Cryptic protein	√
Q8VHS2	Crb1	Protein crumbs homolog 1	√
Q80YA8	Crb2	Protein crumbs homolog 2	√
Q9JLB4	Cubn	Cubilin	√
Q09163	Dlk1	Protein delta homolog 1 (DLK-1)	√
Q8K1E3	Dlk2	Protein delta homolog 2 (DLK-2)	√
Q61483	Dll1	Delta-like protein 1	√
O88516	Dll3	Delta-like protein 3	√
Q9JI71	Dll4	Delta-like protein 4	√
Q8JZM4	Dner	Delta and Notch-like epidermal growth factor-related receptor	√
O35474	Edil3	EGF-like repeat and discoidin I-like domain-containing protein 3	√
Q9QXT5	Egfl7	Epidermal growth factor-like protein 7	√
Q4VBE4	Egflam	Pikachurin	√
Q80YC5	F12	Coagulation factor XII	√
P70375	F7	Coagulation factor VII (EC 3.4.21.21)	√
Q5F226	Fat2	Protocadherin Fat 2	√
Q8BNA6	Fat3	Protocadherin Fat 3	√
Q2PZL6	Fat4	Protocadherin Fat 4	√
Q501P1	Fbln7	Fibulin-7	√
Q61555	Fbn2	Fibrillin-2	√
E9Q7X6	Heg1	Protein HEG homolog 1.	
Q9R098	Hgfac	Hepatocyte growth factor activator	√
Q05793	Hspg2	Basement membrane-specific heparan sulfate proteoglycan core protein	
Q9QXX0	Jag1	Protein jagged-1	√
Q9QYE5	Jag2	Protein jagged-2	√
Q91ZX7	Lrp1	Prolow-density lipoprotein receptor-related protein 1	√
Q9JI18	Lrp1b	Low-density lipoprotein receptor-related protein 1B	√
A2ARV4	Lrp2	Low-density lipoprotein receptor-related protein 2	
O08999	Ltbp2	Latent-transforming growth factor beta-binding protein 2	√
A2AJX4	Malrd1	MAM and LDL-receptor class A domain-containing protein 1.	
Q6DIB5	Megf10	Multiple epidermal growth factor-like domains protein 10	√
Q80T91	Megf11	Multiple epidermal growth factor-like domains protein 11	√
Q80V70	Megf6	Multiple epidermal growth factor-like domains protein 6	√
P60882	Megf8	Multiple epidermal growth factor-like domains protein 8	√
P28825	Mep1a	Meprin A subunit alpha	
P21956	Mfge8	Lactadherin	
B2RPV6	Mmrn1	Multimerin-1.	√
P19467	Muc13	Mucin-13	
P55066	Ncan	Neurocan core protein	√
Q2VWQ2	Nell1	Protein kinase C-binding protein NELL1	√
Q01705	Notch1	Neurogenic locus notch homolog protein 1	√
O35516	Notch2	Neurogenic locus notch homolog protein 2	√
Q61982	Notch3	Neurogenic locus notch homolog protein 3	√
P31695	Notch4	Neurogenic locus notch homolog protein 4	√

Q8R4G0	Ntng1	Netrin-G1	
Q8R4F1	Ntng2	Netrin-G2	
Q8BU25	Pamr1	Inactive serine protease PAMR1	√
Q8VIK5	Pear1	Platelet endothelial aggregation receptor 1	√
P11214	Plat	Tissue-type plasminogen activator	√
P33587	Proc	Vitamin K-dependent protein C	√
Q9CQW3	Proz	Vitamin K-dependent protein Z.	√
Q60841	Reln	Reelin	√
P59222	Scarf2	Scavenger receptor class F member 2	
Q80TR4	Slit1	Slit homolog 1 protein	√
Q9R1B9	Slit2	Slit homolog 2 protein	√
Q9WVB4	Slit3	Slit homolog 3 protein	√
Q70E20	Sned1	Sushi, nidogen and EGF-like domain-containing protein 1	√
Q8R4Y4	Stab1	Stabilin-1	√
Q8R4U0	Stab2	Stabilin-2	√
A2AVA0	Svep1	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	√
Q9WTS4	Tenm1	Teneurin-1	√
Q9WTS5	Tenm2	Teneurin-2	√
Q9WTS6	Tenm3	Teneurin-3	√
Q3UHK6	Tenm4	Teneurin-4	√
Q05895	Thbs3	Thrombospondin-3.	
Q06806	Tie1	Tyrosine-protein kinase receptor Tie-1	
Q91X17	Umod	Uromodulin	√
Q9CZT5	Vasn	Vasorin	√
Q62059	Vcan	Versican core protein	
Q70UZ7	Vwa2	von Willebrand factor A domain-containing protein 2	√
Q9WUA1	Wif1	Wnt inhibitory factor 1	√

cutoff, resulting in 611 podocyte essential gene candidates (**Table 2**).

Comparison of the genes encoding the 78 proteins of POFUT1 potential substrates with 611 podocyte essential gene candidates identified two overlapped genes, *Notch2* and *Plat*. However, gene knockout of both Notch2 and Plat is known not to cause any podocyte injury [29, 30], [The International Mouse Phenotyping Consortium (IMPC) (http://www.informatics.jax.org/marker/phenotypes/MGI:97610)]. Therefore, neither Notch2 or Plat is essential for podocytes; alternatively, there may be functional redundancy for them in mouse podocytes, thus explaining why Pofut1 abrogation did not result in podocyte injury in mouse.

POFUT1 expression in individual podocytes

Although *Pofut1* gene expression is detectable in most tissues with mixed population of cells, including mouse podocytes [GEO: GSE123179; GSE17142], it is not known whether *Pofut1* is expressed in every single podocyte, thus being predicted as podocyte essential gene. We examined our data of single-cell RNA-seq of mouse podocytes [28], and found that *Pofut1* was expressed in a portion of podocytes (**Table 3**). This result is supported by the database of the Kidney Interactive Transcriptomics (KIT) (http://humphreyslab.com/SingleCell/) (Data not shown). Thus, *Pofut1* is not a podocyte essential gene.

Discussion

O-fucosylation of EGF-like domain by POFUT1 is required for the function of proteins, e.g., Notch receptors, as shown by *Pofut1* abrogation that causes cellular abnormalities through inhibiting Notch signaling. Notch signaling has been shown to be essential for podocyte formation in development [31, 32] and Notch components are still present in mature podocytes. These studies suggested that POFUT1 might be essential for mouse podocytes. However, we showed that *Pofut1* gene deletion selectively in podocytes did not induce abnormalities in mice, suggesting that POFUT1 activity is not

Table 2. 611 podocyte essential gene candidates predicted by single-cell RNA-seq

9930111J21Rik2	CSDE1	Gm33780	MTMR2	RAB11B	SPTBN1
ACAD9	CSNK1A1	Gm3839	MT-ND1	RAB3B	SQSTM1
ACOT2	CST3	Gm6211	MT-ND2	RAB7A	SRGAP1
ACSL4	CTDSPL	GNB1	MT-ND3	RAC1	SRP14
ACTB	CTNNA1	GNG5	MT-ND4	RACGAP1	SRPK1
ACTN4	CTSV	GOLIM4	MT-ND5	RAD21	SRSF2
ACTR2	CYB5A	GPBP1L1	MT-ND6	RASL11A	Srsf5
AEBP1	CYB5R4	GPC1	MTSS1	Rbm25	SSBP2
AFF4	D330041H03Rik	GPX4	MYCBP2	RBM26	SSR1
AIF1L	DAZAP2	GPX8	MYH9	RBM28	SSR3
AIM1L	DDX5	GRK4	MYL12B	RBM39	ST13
AKR1A1	DDX58	GSK3B	MYL6	RBMS2	SUCLA2
ALCAM	DECR2	GSN	MYLK	RBMS3	SWT1
ALKBH5	DEGS1	GTF2A1	MYO1C	REEP3	SYNJ2BP
ALOX15B	DENND5B	H3F3A/H3F3B	MYO1D	RHEB	SYNPO
ANAPC16	DNAJC11	H3F3A/H3F3B*	MYOM2	RHOA	TAX1BP1
ANXA1	DOCK5	HAUS8	N4BP2L2	RMDN1	TBP
ANXA2	DPP4	HELLS	NAP1L1	ROBO2	TCF21
ANXA4	DPYSL2	HLA-A	NBEAL1	RPL10	TCP1
AOX1	Dst	HLA-A	NCK2	RPL10A	TDRD5
AP1S3	DSTN	HLA-A	NCL	RPL13	TGFBR3
AP2M1	DTNB	HNRNPL	NDUFA1	RPL14	THSD7A
APAF1	DUSP3	HNRNPU	NDUFA13	Rpl14-ps1	TIAL1
APBB2	Dync1i2	HP1BP3	NDUFA3	RPL21	TIMM17B
APBB3	DYNLL1	HSBP1	NDUFA4	RPL23	TIMMDC1
APLP2	DYNLRB1	HSD3B1	NDUFA6	RPL26	TIMP3
APP	DYNLT1	HSP90AB1	NDUFA7	RPL27A	TJP1
ARF3	DYNLT3	HSP90B1	NDUFB8	Rpl32	TLN1
ARGLU1	EEF1A1	HSPB11	Ndufs5	RPL35	TM4SF1
ARHGAP24	EHD2	HTRA1	Neat1	RPL35A	TMBIM1
ARHGAP28	Eif1	HYPK	Nebl	RPL37	TMBIM6
ARHGEF18	EIF3M	IER3IP1	Nedd4	RPL37A	TMCO1
ARPC1A	EIF4A1	IFFO1	Nes	RPL38	TMED7
ARPC2	EIF4A2	IFITM2	NFE2L1	RPL4	TMEM234
ATG16L1	EIF4G2	IFNGR1	NFIA	RPL41	TMEM245
ATP5A1	EMC2	IFT80	NFRKB	RPL7	TMEM30A
ATP5B	ENPEP	IGFBP7	NKTR	RPL8	TMEM50A
ATP5C1	ENSMUSG00000004980	ILDR2	NOA1	RPL9	TMEM59
Atp5e	ENSMUSG00000022820	IMMT	NOP10	RPLP0	TMEM69
ATP5F1	ENSMUSG00000023737	IQGAP1	NOTCH2	Rplp1	TMEM80
ATP5G3	ENSMUSG00000027942	IQGAP2	NPHS1	RPN2	TMOD3
ATP5J	ENSMUSG00000040078	ITCH	NPHS2	RPS11	Tmsb4x
ATP5J2	ENSMUSG00000044285	ITGA3	NPNT	RPS12	Tmsb4x*
ATP5L	ENSMUSG00000057577	ITGAV	NPR3	RPS14	TNFRSF10A
ATP6AP1	ENSMUSG00000061331	ITGB1	NRAS	RPS16	TNS2
ATP6V0E1	ENSMUSG00000064339	ITGB5	NSF	RPS18	TNS3
ATP6V1A	ENSMUSG00000064352	ITM2B	NUPR1	RPS20	TOB1
ATP6V1B2	ENSMUSG00000067344	IVD	OAZ1	RPS23	TOP1

ATP6V1G1	ENSMUSG00000071107	JAK1	OGT	RPS24	TOP2A
ATRX	ENSMUSG00000072692	JUP	ORC3	RPS25	TPM3
B2M	ENSMUSG00000081471	KANK1	OSBPL9	Rps27/Rps27rt	TPT1
BBX	ENSMUSG00000081552	KDELR2	P3H2	Rps27/Rps27rt*	TRAM1
BCAT2	ENSMUSG00000083563	KHSRP	PABPC1	RPS27L	TRIB2
BIRC6	ENSMUSG00000083594	KIAA1107	PAIP1	RPS29	TSC22D1
C19orf53	ENSMUSG00000085279	KIAA1109	PAK1	Rps3a1	TSPAN13
C6orf47	ENSMUSG00000085328	KIF1B	PAM	RPS5	TSPAN15
C920009B18Rik	ENSMUSG00000085334	KIF5B	PAN3	RPS7	TSPAN3
Calm1	ENSMUSG00000085586	KLHL9	PARVA	RPS8	TTC3
Calm1*	ENSMUSG00000085950	KRCC1	PBRM1	RSRP1	TWF1
CALR	ENSMUSG00000086967	LACTB2	PCMTD1	S1PR4	Ubb
CANX	ENSMUSG00000089828	LAPTM4A	PCNP	SAP18	UBL5
CAPS2	ENSMUSG00000089940	LCP1	PDCD4	SBDS	UBN2
CBX1	ENSMUSG00000090262	LGR4	PDIA3	Scd2	UCP2
CBX3	ENSMUSG00000090286	LIN7C	PDIA4	SCHIP1	UNC13D
CCNT1	ENSMUSG00000090353	S100a11	PDIA6	SCP2	UQCR11
CD2AP	ENSMUSG00000092400	LOC102640619	PDLIM2	SDC4	UQCRB
CD59	ENSMUSG00000093760	LPIN2	PDXDC1	SEC22B	USP9X
Cd59a	ENSMUSG00000094030	LPL	PFN1	SELK	VDAC1
CD81	ENSMUSG00000094472	LRRC58	PHYKPL	SEMA3E	VEGFA
CD9	ENSMUSG00000096808	LRRC8A	PIAS4	SEMA3G	VEPH1
CDC26	ENSMUSG00000097287	LRRFIP1	PIGV	SENP1	VIM
Cdc42	ENSMUSG00000097695	LUC7L3	PKIB	SEP15	Vmn1r63
CDC42BPA	ENSMUSG00000097815	LYPLA1	PLAT	SEPP1	Vmn2r55
CDC42SE2	ENSMUSG00000097911	LYRM9	PLCE1	SEPT10	VPS53
CDC7	ENSMUSG00000098178	MAFB	PLOD2	SEPT11	WAPAL
CDK14	ENSMUSG00000098183	MAGI2	PLS3	SEPT2	WDR1
Cdkn1c	EPB41L5	MALAT1	Plscr2	SEPT7	WT1
CERS6	ERMP1	MAP1LC3B	PNISR	SEPW1	WTAP
CHCHD2	EZR	MAPT	Podxl	SERBP1	YARS
CHMP2A	FAM81A	MATR3	POLDIP3	SERINC3	YBX1
CHMP5	FGD4	MERTK	POMP	SET	YIPF1
CHPT1	FGFR1	MIER1	PPIA	SGIP1	YME1L1
CLASP2	FKBP1A	MKLN1	PPP1CB	SH2D4A	YWHAE
CLIC3	FKBP8	MMP12	PRDX1	SHISA3	YWHAQ
CLIC5	Fnbp1l	MOCS2	PRDX3	SIK2	YWHAZ
CLK1	Foxd2os	MORF4L1	PRMT1	SKP1	ZAK
CLTC	Foxn3	MPC2	PRRC2C	SLC18B1	ZBTB20
CMPK1	FTH1	MPP5	Psg16	SLC25A3	ZBTB8OS
CNBP	FTL	MRFAP1	PSMA3	SLC25A43	ZDHHC21
COL4A3	FUBP3	MRPL20	PSMC2	SLC39A1	Zfp60
COL4A4	FYCO1	MRPL27	PTBP3	SMCO1	Zfp940
CORO2B	GADD45A	MRPL48	PTGES3	SMDT1	ZFR
COX4I1	GAS5	MRPL51	PTH1R	SMG1	ZKSCAN3
COX6A1	GAS7	MRPS14	Ptma	SMIM10L1	ZMAT1
COX6B1	GATAD1	MSI2	PTP4A1	SMIM14	ZNF207
Cox6c	GLRX2	MT-ATP6	PTP4A2	SNU13	ZNF253
COX7B	Gm11783	mt-Atp8	Ptprd	SNX5	ZNF277

COX8A	Gm16222	MTCH1	PTPRO	SOD1	ZNF3
CPA6	Gm16702	MT-CO1	PTRF	SON	ZNF488
CPNE3	Gm21596/Hmgb1	MT-CO2	PURA	SPARC	ZNHIT3
CROT	Gm26782	MT-CO3	QKI	SPCS1	ZSCAN26
CRYAB	Gm33780	MT-CYB	R3HDM4	SPOP	

Note: a gene marked by * is a distinct isoform of the gene with the same name.

Table 3. Pofut1 rpkm in 20 single mouse podocytes

Cell	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Ave
RPKM	0	4.15	0	38.35	0.07	0	0	0.59	0	0	0	0.68	0	0	0	0.31	0	0	0	0	2.21

required for podocyte structure, function and survival. We further explored the reason underlying the dispensability of POFUT1 in mouse podocytes and found none of the predicted POFUT1 substrates is essential for podocytes according to literature and the databases generated by single-cell RNA-seq.

We carefully examined the cKO mice with *Pofut1* knockout in podocytes by multiple experimental analyses, including urinary albumin level, morphology, ultrastructure, marker gene expression and podocyte number. There was no difference in these parameters between cKO and control mice, clearly indicating that POFUT1 is dispensable for mouse podocytes.

To explore why POFUT1 is dispensable for mouse podocytes, we identified 78 proteins that are predicted to be O-fucosylated by POFUT1 in the mouse genome by searching Scanprosite. Among the 78 genes, only *Notch2* and *Plat* are in the 611 mouse podocyte essential gene candidates. Since *Pofut1* abrogation in mouse podocytes did not cause phenotypes, it is either O-fucosylation of the two proteins is not required for their function in podocytes, or these two proteins are not essential for mouse podocytes. Notch signaling, particularly Notch2 signaling, is required for podocyte development [31, 32]. However, Notch2 appeared not to be required in mature podocytes as podocyte-specific knockout of Notch2 in podocytes did not show any phenotypes [29]. *Plat* knockout also showed no phenotypes of podocytes, indicating that *Plat* is dispensable for podocytes [30], [The International Mouse Phenotyping Consortium (IMPC) (http://www.informatics.jax.org/marker/phenotypes/MGI:97610)]. The dispensability of *Notch2* and *Plat* in mouse podocytes explains why POFUT1 is not required for podocytes. It should be noted that Pofut1 is the only gene encoding O-fucosyltransferase that adds fucose to Notch2 and tPA, therefore we conclude that POFUT1 and its O-fucosylation activity are not required for podocytes in mice.

POFUT1 dispensability may also be reflected by its expression in single podocytes [28]. We found that Pofut1 mRNA was detected in small portion of cells analyzed (**Table 3**). This result was consistent with that from the database KIT, and together suggested that POFUT1 is not essential for podocytes as a cell type. However, POFUT1 may be actually expressed in every single podocyte, but it was not detected in every single podocyte due to technical variations in the sequencing process. Nevertheless, *Pofut1* knockout in podocytes did not cause podocyte injury, demonstrating that POFUT1 is not essential for podocyte structure, function and survival under physiological condition.

In the present study, we retrieved mouse genes that encode proteins potentially O-fucosylated by POFUT1 at genome-wide level, and found 78 such genes. We compared list of the 78 proteins with that of human proteins, and found most of the proteins are common in the two species (**Table 1**), suggesting a high conservation in evolution, and thus potential function of O-fucosylation for these proteins. Although O-fucosylation of these proteins appears not to be important in podocytes, it could be essential for other cell types in the body. It is interesting to know which tissues or cell types express the O-fucosylated proteins and whether these proteins are essential for the cell types. This can be achieved by taking the approach described in the present study, i.e., comparing the 78 mouse genes encoding potentially O-fucosylated proteins with the essential gene candidates of the cell type of interest. At present, single-cell RNA-seq data are available for most

cell types in the databases, e.g., GEO, and their essential gene candidates can be inferred by the method as we described previously [28]. On the other hand, the O-fucose may not be necessarily essential for the proteins as the case of Cripto whose O-fucose is clearly dispensable for its function in Nodal signaling [33].

POFUT1 has an O-fucosyltransferase independent function in cells. It can serve as a chaperone that assists Notch proteins to fold and traffic properly in endoplasmic reticulum [34, 35]. At present, NOTCH1 is the only protein that has been reported to require POFUT1 protein as chaperone. Since *Notch1* is essentially not expressed in normal podocytes and *Notch1* knockout in podocytes does not affect podocytes [29], the observation that POFUT1 deficiency in podocytes does not cause phenotypes may further suggest that chaperone activity of POFUT1 is very limited.

Finally, it is well established that Notch1 is *de novo* expressed or upregulated in podocytes in glomerular injury mouse models and human glomerular diseases, and that Notch1 activation mediates podocyte injury as shown by the observation that Notch1 gene deletion specifically in mouse podocyte alleviates podocyte injury in the mouse models [36]. Since Notch1 is a substrate of POFUT1 and O-fucose is required for Notch1 to function [5, 10, 11], we expect that *Pofut1* gene abrogation in podocytes would inactivate de novo expressed NOTCH1 protein thereby protecting podocytes from injury. It is interesting to test this speculation.

Acknowledgements

This work was supported by the grants from the National Natural Science Foundation of China (81670653 and 81970619 to SS), the National Key Research and Development Program of China (2016YFC0904103 to ZL) and Planned Projects for Postdoctoral Research Funds (2019 K181 to SZ). We thank for Dr. Pamela Stanley and Dr. Erwin Bottinger for reagents and supports.

Disclosure of conflict of interest

None.

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