Supplemental Figure legends

Supplemental Figure S1. Nephrin colocalizes with podocin, CD2AP and occasionally with clathrin but not with rab5, cathepsin D or p62 in the punctate structures in the ZDF rat glomeruli

(A-F) Double immunofluorescence staining of glomeruli of 34 wks old obese ZDF rats with nephrin and (A) clathrin heavy chain, (B) rab5, (C) cathepsin D, (D) p62, (E) CD2AP or (F) podocin. The antibodies used are listed in Supplemental Table S1. Scale bars: 50 μm.

Supplemental Figure S2. Nephrin and PACSIN2 colocalize at the plasma membrane with caveolin-1 and occasionally with clathrin light chain

(A-D) TIRF microscopy analysis of podocytes overexpressing clathrin light chain-eGFP (A, C) or caveolin-1-DsRedmonomer (B, D), and PACSIN2-mCherry (C) or PACSIN2-eGFP (D). In (A) and (B), podocytes were incubated in the presence of 516-647N for 20 min prior to fixation. TIRF microscopy reveals that nephrin and PACSIN2 colocalize regularly with caveolin-1 and occasionally with clathrin light chain (arrowheads). Mouse PACSIN2-mCherry and caveolin-1-DsRedmonomer were described in (1). Clathrin light chain-eGFP was subcloned into pEF-BOS vector as in (1). Scale bar: 10 μm.

Supplemental Figure S3. PACSIN2 knockdown increases the insertion of nephrin at the plasma membrane. A significant amount of internalized nephrin is recycled back to the plasma.

(A) Western blot showing overexpression of flag-PACSIN2 in podocytes. β-actin is used as a loading control. (B, C) Quantification of Western blots similar as in (A) reveals a 2-fold increase of PACSIN2 (B) without affecting nephrin levels (C). (D) Western blot of mouse podocyte lysates after transfection of scrambled or PACSIN2 siRNAs. (E) Quantification of Western blots similar as in (D) reveals that PACSIN2 siRNA-treatment decreased the level of PACSIN2 by 59%. (F) On-Cell Western analysis shows that knockdown of PACSIN2 increases the amount of nephrin inserted at the plasma membrane (n_{well}=54). Mouse podocytes expressing nephrin were transfected with ON-TARGET plus SMARTpool mouse PACSIN2 (L-045093-01-0005) or siCONTROL Non-Targeting Pool#2 (D-001206-14-05) siRNAs (Dharmacon, Lafayette, CO)

using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 16 h after plating and used for experiments after 48 h. (G, H) To confirm that internalized nephrin is recycled back to the plasma membrane, we performed qualitative immunofluorescence (G) and quantitative On-Cell Western (H) control experiments in which we added the 5-1-6 IgG to the culture medium of podocytes for 30 min, allowing internalization of IgG-labeled nephrin molecules. Thereafter, cells were acid stripped (1,15% acetic acid, 0.5 M NaCl, 4 min on ice) to remove the IgG bound to nephrin molecules still present at the plasma membrane, followed by incubation in culture medium without 5-1-6 IgG for 0-120 min to allow recycling of nephrin. Finally, a fluorescently labeled secondary IgG was applied without permeabilization to stain nephrin that was recycled back to the plasma membrane. With this approach, we observed that a significant amount of labeled, internalized nephrin was recycled back to the plasma membrane, reaching a peak of 15% 30 min after stripping (Supplemental Figure S3G, H). The nuclear marker DRAQ5 (ThermoFisher) was used for normalization in (H). In (H, n_{well}=33-54), * and # indicate that the statistical test was performed against no stripping or 0 min, respectively. Scale bar: 40 µm. Bars show the mean and error bars the standard deviation. The statistical analysis compares the mean of single wells from three experiments combined using Student's t-test. #p<0.05, **/##p<0.01, ****/####p<0.0001

Supplemental Figure S4. Rabenosyn-5-GFP overexpression does not change the expression level of nephrin or PACSIN2

(A) Western blot of rabenosyn-5-GFP overexpressing podocytes. β-actin is included as a loading control. (B-D) Quantification of Western blots similar as in (A) shows a 75% increase of total rabenosyn-5 (B) without affecting nephrin (C) or PACSIN2 (D) levels. Bars show the mean and error bars the standard deviation. Statistical significance is calculated using Mann-Whitney-U test. **p<0.01; ns, nonsignificant

Supplemental Figure S5. High glucose or palmitate do not change the expression levels of PACSIN2 and nephrin after 48 h treatment.

(A-C) Western blot of PACSIN2 and nephrin in podocytes treated with high glucose (A), palmitate (B) or both (C), as well as their respective controls. α-tubulin is included as a loading control. (D-E) Quantification of Western blots similar as in (A-C). Bars show the mean and error

bars the standard deviation. Statistical significance is calculated using Mann-Whitney-U test. ns, nonsignificant

Supplemental Table S1. List of antibodies used in the study.

The 5-1-6 IgG (3) was fluorescently labeled with NHS-ester Attodye 647N (Siegen, Germany), referred to as 516-647N.

Primary IgGs

Antigen	Host	Company/Sup. Reference
alpha-tubulin	mouse	Sigma-Aldrich, St. Louis, MO, USA
beta-actin	mouse	Sigma-Aldrich
cathepsin D	rabbit	Novus Biologicals, Littleton, CO, USA
CD2AP	rabbit	(2)
clathrin heavy chain	mouse	Calbiochem, San Diego, CA, USA
nephrin	guinea pig	PROGEN Biotechnik, Heidelberg, Germany
nephrin "5-1-6"	mouse	(3)
p62	guinea pig	PROGEN
PACSIN2 "P2B"	rabbit	Abgent, San Diego, CA, USA
PACSIN2 "P2P"	rabbit	(4)
podocin	rabbit	Sigma-Aldrich
rab5	rabbit	Cell Signaling Technology,Danvers, MA, USA
rabenosyn-5	rabbit	(5)

Secondary IgGs

Antigen	Host	Company/Sup. Reference
Alexa Fluor 594-anti rabbit	donkey	Molecular Probes, Eugene, OR, USA
Alexa Fluor 594-anti mouse	donkey	Molecular Probes
Alexa Fluor 488-anti mouse	donkey	Molecular Probes
Alexa Fluor 488-anti guinea pig	donkey	Molecular Probes
IRDye 800-anti mouse	goat	LI-COR Lincoln, NE, USA
IRDye 800-anti rabbit	donkey	LI-COR Lincoln
IRDye 680-anti mouse	donkey	LI-COR Lincoln
IRDye 680-anti guinea pig	donkey	LI-COR Lincoln

Supplemental Video S1. Nephrin is internalized in PACSIN2-eGFP positive sites.

Podocytes overexpressing PACSIN2-eGFP (green) and nephrin are incubated in the presence of 516-647N IgG (red). TIRF microscopy is used to observe the endocytosis of nephrin. Time is shown in seconds. Scale bar: 2 μm

Supplemental references

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