SUPPORTING INFORMATION

The human nephrin Y^{1139}RSL motif is essential for podocyte foot process organization and slit diaphragm formation during glomerular development

Running title: *nephrin endocytosis and glomerular development*

SUPPLEMENTARY FIGURES

Figure S1.

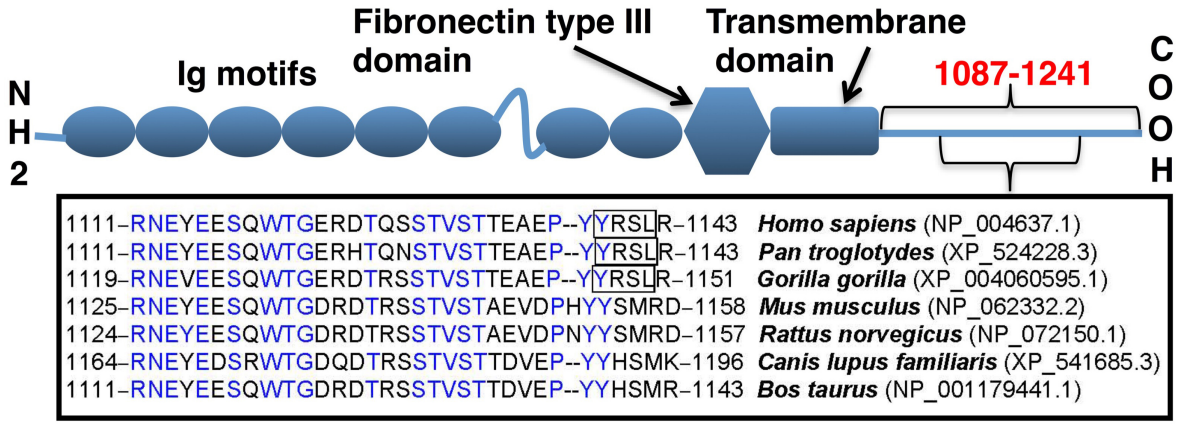

Figure S1. Nephrin domain organization. The cytoplasmic C-terminal tail domain of human nephrin spans amino acid residues 1087 to 1241 (1). The box demonstrates a region of human nephrin sequence spanning the Y^{1139}RSL motif compared to homologous nephrin sequences in other mammalian species. The alignment was generated by MUSCLE (multi sequence alignment with high accuracy and high throughput) version 3.6 (2). Expression of the YRSL sequence is limited to primates' nephrin. The blue font demonstrates residues conserved across mammalian species.

Figure S2.

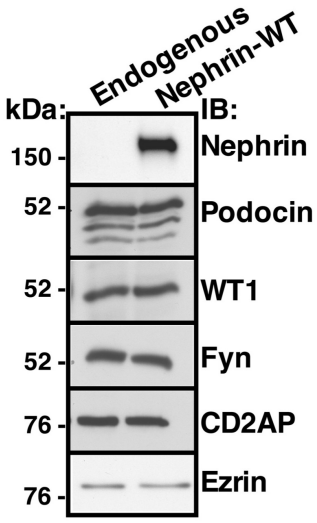

Figure S2. Immunoblots showing expression of podocyte specific proteins and nephrin interacting partners in lysates of T-SV40 immortalized human podocytes. Endogenous nephrin was not detected by western blotting (the left lane: Endogenous). Transfected wild-type (WT) human nephrin without a tag (Nephrin-WT) is shown in the right lane. All other proteins are endogenously expressed. Podocin anchors nephrin at the plasma membrane, the Src family kinase Fyn phosphorylates the intracellular nephrin tyrosine residues, and CD2AP interacts with nephrin and podocin.(3) Nephrin is transcriptionally activated by the Wilms tumor suppressor WT1.(4) Ezrin is a loading control. Expressing human nephrin in podocytes prevented the non-physiologic membrane trafficking itineraries observed in heterologous cell models. (5) Expressing nephrin without a tag prevented non-specific, tag-dependent effects on nephrin trafficking. Experiments were repeated 3 times from different cultures with similar results.

Figure S3.

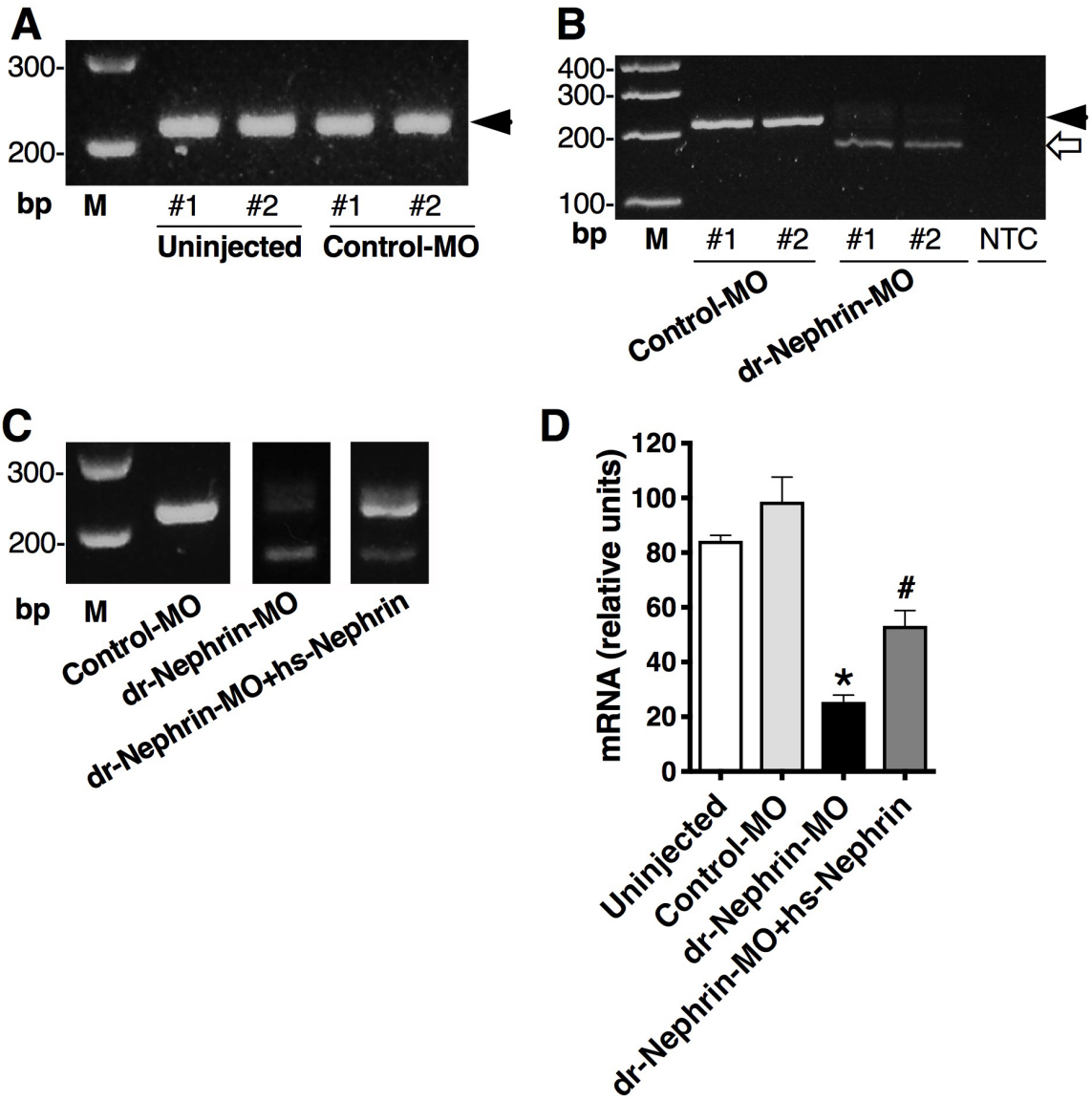

Figure S3. Efficiency of nephrin morpholino (MO) and human nephrin rescue in zebrafish embryos. (A) An image of a 2% gel showing that injection of control MO (Control-MO) did not affect splicing of nephrin mRNA at 4 dpf, compared to uninjected embryos. Shown is zebrafish nephrin mRNA from two embryos (#1 and #2) per condition. The black arrow marks the intact nephrin mRNA. M is molecular ladder. (**B**) Injection of validated nephrin MO (dr-Nephrin-MO) resulted in mis-splicing of nephrin mRNA, as detected by an altered PCR product at 4 dpf. The altered mRNA was ~50 bp smaller (white arrow) than the intact nephrin mRNA (black arrow) detected in the Control-MO-injected morphants. NTC is no template control (primers only). (**C**) Co-injection of the synthetic human (hs)-Nephrin transcripts with the dr-Nephrin-MO resulted in partial rescue of nephrin mRNA expression in morphants. (**D**) Summary of data from A-C. The 4th column represents data pooled from different hs-Nephrin variants. Quantification of the intact PCR product (black arrow) was performed by the ImageJ densitometry using exposures within the linear dynamic range. *P<0.05 *versus* Control-MO and $^{\#}$P<0.05 *versus* dr-Nephrin-MO. 4-6 experiments/group. Error bars, S.E.M.

Figure S4.

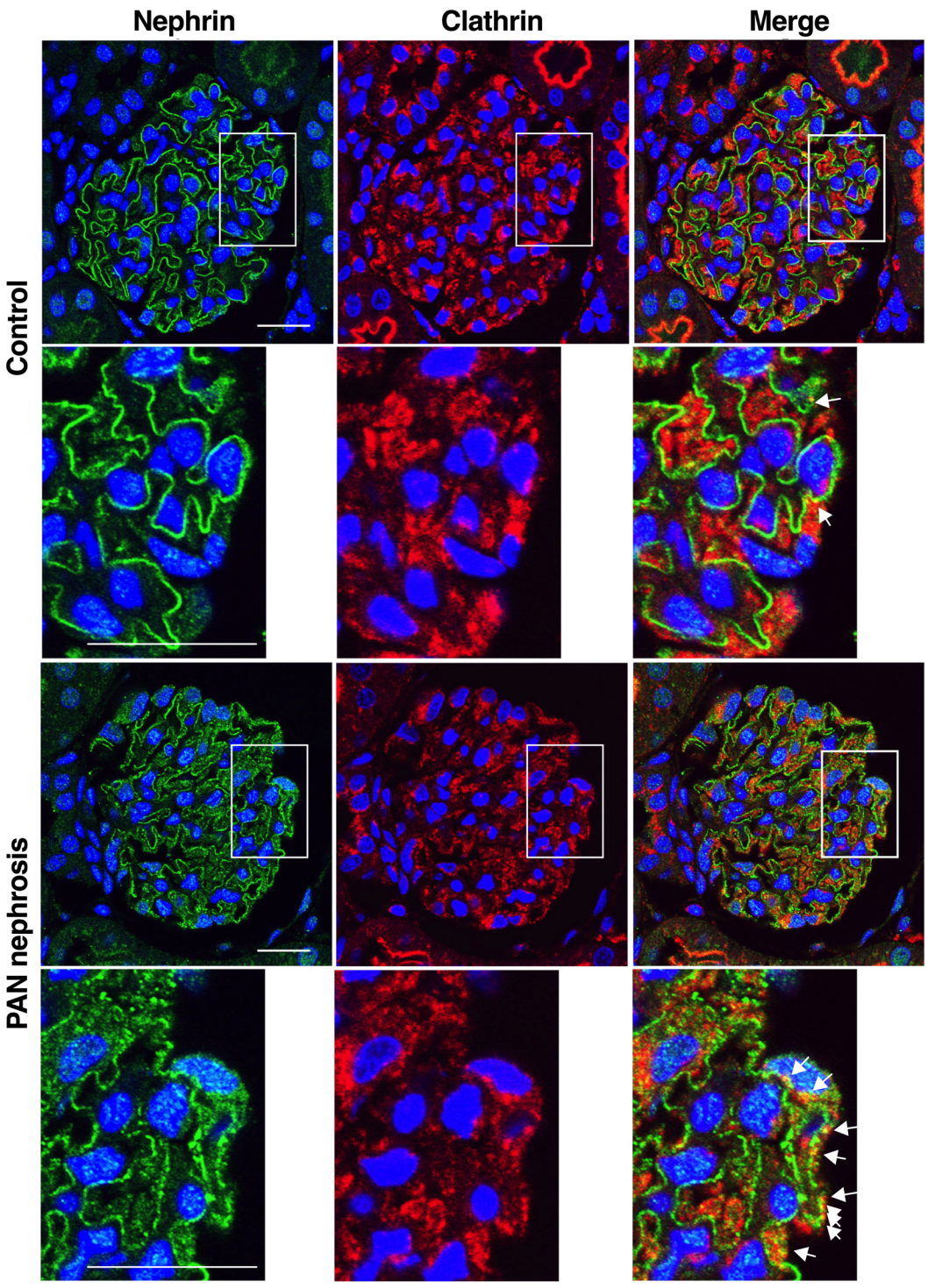

Figure S4. Rat nephrin is internalized via clathrin-dependent endocytosis. To examine whether nephrin can be internalized by clathrin-dependent mechanism in the absence of YRSL, we examined co-localization between clathrin and nephrin in rat glomeruli from the PAN nephrosis model. Immunofluorescent staining and confocal microscopy images of rat glomeruli demonstrating that the co-localization between nephrin (green) and clathrin (red) was increased in nephrotic animals, compared to controls (arrows). The experimental protocol was approved by the Animal Care Committee of Kyorin University School of Medicine, Tokyo, Japan (6). Male Sprague–Dawley rats that weighed 150–200g were from Saitama Experimental Animal Supply (Sugito, Saitama, Japan). PAN nephrosis was induced by a single intravenous injection of PAN (120 mg/kg body weight) or saline (control), and the rats were euthanized on the 3rd day, as previously described (6). Kidney cortex samples were fixed in 4% paraformaldehyde for 2h at 4 ºC and processed (6). For immunofluorescence staining, reactions of the primary antibodies, mouse monoclonal anti-clathrin heavy chain and rabbit polyclonal anti-nephrin antibody pAB2 (5μg/ml both) occurred overnight at 4 ºC, and the immune-complexes were visualized using Alexa Fluor 555-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:800 dilution), respectively. Slides were examined under a confocal laser scanning microscope that was equipped with a Krypton/Argon laser (MRC1024; Bio-Rad, Hercules, CA, USA). Scale bar = 10μm, each. Experiments were repeated in 3 animals/group with similar results.

REFERENCES

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