

The Epithelial Calcium Channel TRPV5 Is Regulated Differentially by Klotho and Sialidase*

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Background: The epithelial Ca²⁺ channel TRPV5 facilitates Ca²⁺ reabsorption in the kidney and is regulated by sialidase and the hormone klotho.

Results: Sialidase stimulates TRPV5 plasma membrane stabilization in a lipid raft-dependent manner, while klotho increased cell surface expression of the channel via its *N*-glycan.

Conclusion: Klotho and sialidase regulate TRPV5 membrane stabilization in a different manner.

Significance: Understanding the regulation of TRPV5 is crucial to treat patients with Ca²⁺-related disorders.

The transient receptor potential vanilloid type 5 (TRPV5) Ca²⁺ channel facilitates transcellular Ca²⁺ transport in the distal convoluted tubule (DCT) of the kidney. The channel is glycosylated with a complex type *N*-glycan and it has been postulated that hydrolysis of the terminal sialic acid(s) stimulate TRPV5 activity. The present study delineates the role of the *N*-glycan in TRPV5 activity using biochemical assays in Human Embryonic Kidney 293 cells expressing TRPV5, isoelectric focusing and total internal reflection fluorescent microscopy. The anti-aging hormone klotho and other glycosidases stimulate TRPV5-dependent Ca²⁺ uptake. Klotho was found to increase the plasma membrane stability of TRPV5, via the TRPV5 *N*-glycan. Sialidase mimicked this stimulatory action. However, this effect was independent of the *N*-glycosylation state of TRPV5, since the *N*-glycosylation mutant (TRPV5^{N358Q}) was activated to the same extent. We showed that the increased TRPV5 activity after sialidase treatment is caused by inhibition of lipid raft-mediated internalization. In addition, sialidase modified the *N*-glycan of transferrin, a model glycoprotein, differently from klotho. Previous studies showed that after klotho treatment, galectin-1 binds the TRPV5 *N*-glycan and thereby increases TRPV5 activity. However, galectin-3, but not galectin-1, was expressed in the DCT. Furthermore, an increase in TRPV5-mediated Ca²⁺ uptake was detected after galectin-3 treatment. In conclusion, two distinct TRPV5 stimulatory mechanisms were demonstrated; a klotho-mediated effect that is dependent on the *N*-glycan of TRPV5 and a sialidase-mediated stimulation that is lipid raft-dependent and independent of the *N*-glycan of TRPV5.

Calcium (Ca²⁺) plays a crucial role in multiple physiological processes such as muscle contraction, neuronal excitability, enzymatic activity, bone formation, cell membrane formation, and exocytosis. It is of physiological importance to tightly regulate intra- and extracellular Ca²⁺ levels. To this end, the Ca²⁺ balance is maintained by the bone, kidney, duodenum and parathyroid glands (1). Secretion of parathyroid hormone (PTH)² by the parathyroid glands results in increased Ca²⁺ re(ab)sorption from both bone and kidney. The rate-limiting step in renal active Ca²⁺ reabsorption is the uptake by the transient receptor potential vanilloid type 5 (TRPV5) channel, which is apically expressed in the distal convoluted tubule (DCT) of the kidney (2–5). The channel is responsible for 8% of the total Ca²⁺ reabsorption (6). TRPV5 is a tetrameric membrane protein containing 6 transmembrane segments, with a cytosolic amino and carboxyl terminus and an extracellular *N*-glycan at asparagine 358. The *N*-glycan is an important post-translational regulatory site, which can affect the plasma membrane retention of the channel (7).

Previous studies have shown that intracellular *N*-glycans contribute to signaling processes, dimer formation, membrane trafficking and polarized sorting of glycoproteins to the apical surface of epithelial cells (8, 9). Extracellular *N*-glycans of plasma membrane glycoproteins are involved in signaling processes, apoptosis and cell-cell contacts. The composition of these glycans and the properties of the glycoproteins can be influenced by the activity of extracellular glycosidases (10–12). For example, previous studies demonstrated that sialidase, a glycosidase, affects caveolar-mediated endocytosis by interacting with glycolipids in the plasma membrane (13). Trafficking of TRPV5 is crucial for controlling its abundance at the plasma membrane and therefore, the Ca²⁺ reabsorption.

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² The abbreviations used are: PTH, parathyroid hormone; CNT, connecting tubule; COPAS, complex object parametric analyzer and sorter; DCT, distal convoluted tubule; EndoF, endoglycosidase-F; IEF, isoelectric focusing; tIEF, isoelectric focusing of transferrin; TIRF-M, total internal reflection fluorescent microscopy; TRPV5, transient receptor potential vanilloid type 5.

A protein that modifies the extracellular *N*-glycan of TRPV5 is the type-1 glycoprotein, klotho (7, 14, 15). Klotho is a glycosidase that participates in the degradation of glycans and thereby affects different glycoproteins (7, 15–17). Other glycosidases, such as β -glucuronidase and sialidase, mimic the plasma membrane stabilization effect of klotho on TRPV5 by modifying its *N*-glycan. It was hypothesized that klotho has β -glucuronidase activity, because this enzyme has homology with β -glucuronidase and is able to hydrolyze glucuronic acids (16). Treatment of Human Embryonic Kidney 293 cells (HEK293) expressing TRPV5 with β -glucuronidase also increased the Ca^{2+} uptake through TRPV5 (7). However, Tohyama *et al.* found that the β -glucuronidase activity of klotho is ~ 26 times lower than that of β -glucuronidase (16). In addition, two highly conserved glutamate residues, essential for the enzymatic activity of β -glucuronidase, are not conserved in klotho. Furthermore, glucuronic acids are uncommon moieties in *N*-glycans of membrane proteins such as TRPV5. Cha *et al.* proposed that klotho operates instead as an exo-sialidase, specifically breaking the α -2,6-linked bonds of the negatively charged sialic acids (14). They postulated that in Chinese Hamster Ovary (CHO) cells, cleavage of sialic acids by klotho exposes the underlying disaccharide galactose- β -(1–4)-*N*-acetylglucosamine, followed by binding of galectins, a group of carbohydrate-binding proteins with a high affinity for the exposed galactose-*N*-acetylglucosamine disaccharides.

One of the galectin family members is galectin-1, a polyvalent protein expressed in various tissues and with a wide range of biological activities. It was suggested that galectin-1 forms a stable complex with TRPV5, resulting in a stimulatory effect on Ca^{2+} reabsorption by TRPV5 retention at the cell surface (14). Galectin-1 is present in both extra- and intracellular compartments, where it binds to carbohydrate and non-carbohydrate molecules, respectively (18). Galectin-1 is found in human tubular epithelial cells and in extensive amounts in the porcine kidney (19).

The aim of the present study was to investigate the underlying molecular mechanism of the klotho-mediated TRPV5 regulation and the involvement of galectins. To this end, we delineated the action of different glycosidases, *viz.* klotho and sialidase. Total Internal Reflection Fluorescent Microscopy (TIRF-M) combined with a photo-switchable fluorescent protein enabled us to study the dynamics of TRPV5 proteins on the plasma membrane in HEK293 cells. Finally, isoelectric focusing (IEF), allowed the detection of klotho-mediated sialic acid hydrolysis on a model glycoprotein, transferrin.

EXPERIMENTAL PROCEDURES

DNA Constructs—The pCINeo/IRES-GFP plasmid encoding TRPV5 was generated as described previously (20). TRPV5^{N358Q} was obtained by *in vitro* mutagenesis of TRPV5-pCINeo/IRES-GFP cDNA according to the manufacturer's instructions (Stratagene, La Jolla, CA). The pcDendra-2 (Evrogen, Moscow, Russia) was amplified by PCR and ligated into the pCINeo/IRES-HA TRPV5 construct using the NheI and EcoNI (New England Biolabs, Ipswich, MA) restriction sites. All constructs were verified by DNA sequence analysis.

Immunoblotting and Protein Concentration Determination—TRPV5 protein expression was determined by 8% (w/v) SDS-PAGE and Western blotting using anti-TRPV5 (1:4,000, (21)) with peroxidase-labeled goat anti-mouse IgG (1:10,000, Sigma-Aldrich) antibodies. Protein concentration was measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL), according to the manufacturer's manual.

Electrophysiology—Whole-cell currents were measured with an EPC-10 (HEKA electronic) amplifier using Patchmaster V2.20 software. The borosilicate glass electrode resistance was between 2.5 and 4 $\text{M}\Omega$. The ramp protocol for measuring the current-voltage (*I/V*) relationship of Na^+ consisted of linear voltage ramps from -100 to $+100$ mV within 450 ms repeated every 5 s. The step protocol for measuring the Ca^{2+} current consisted of a 10 s long voltage-step applied from $+70$ to -100 mV. Current traces were sampled at 0.5 ms for the ramp and 2 ms for step protocol. Reported current densities were calculated from the current at -80 mV during the ramp protocol. The standard extracellular solution contained 150 mM NaCl, 6 mM CsCl, 10 mM HEPES (pH 7.4 adjusted with NaOH), 50 μM EDTA, and 10 mM glucose. For Ca^{2+} current measurements NaCl was replaced with equimolar NMDG-Cl and 10 mM CaCl_2 was added. Osmotic differences were adjusted by adding a respective amount of mannitol. The internal (pipette) solution contained 20 mM CsCl, 100 mM Cs-aspartate, 1 mM MgCl_2 , 10 mM BAPTA, 4 mM Na_2ATP , and 10 mM HEPES (pH 7.2 adjusted with CsOH). Data were analyzed using Igor-pro software (WaveMetrics, Oswego, OR).

$^{45}\text{Ca}^{2+}$ Uptake Assay—HEK293 cells were transiently transfected with TRPV5 in pCINeo/IRES-GFP, TRPV5^{N358Q} in pCINeo/IRES-GFP or the empty pCINeo/IRES-GFP vector (mock). One day after transfection, cells were reseeded on poly-L-lysine-coated (0.1 mg/ml) culture dishes and incubated with recombinant klotho (2 $\mu\text{g}/\text{ml}$, R&D systems, Minneapolis, MN), sialidase from *Vibrio cholerae* (27 mU/ml, Sigma-Aldrich), β -glucuronidase from bovine liver (310 units/ml, Sigma-Aldrich), endoF (1 kU/ml, New England), a combination of glycosidases, or human galectin-3 (0.67 $\mu\text{g}/\text{ml}$, Prospec, Ness Ziona, Israel) for 16 h at 37 °C. Radioactive $^{45}\text{Ca}^{2+}$ -uptake in TRPV5-transfected HEK293 cells was determined as described previously (22).

Plasma Membrane Biotinylation and TRPV5 Cell Surface Turnover—HEK293 cells were transiently transfected with TRPV5 in pCINeo/IRES-GFP, TRPV5^{N358Q} in pCINeo/IRES-GFP or the empty pCINeo/IRES-GFP vector (mock). One day after transfection, cells were reseeded on poly-L-lysine-coated (0.1 mg/ml) culture dishes and incubated with recombinant klotho (2 $\mu\text{g}/\text{ml}$, R&D systems), sialidase from *V. cholerae* (27 mU/ml, Sigma-Aldrich), β -glucuronidase from bovine liver (310 units/ml, Sigma-Aldrich), or endoF (1 kU/ml, New England Biolabs) for 16 h at 37 °C. Subsequently, cells were biotinylated as described previously (7). For time point 0 h cells were collected from the plates and disrupted in 1 ml of lysis buffer [1% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5 adjusted with HCl), 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin, 5 $\mu\text{g}/\text{ml}$ proteinase A] immediately after biotinylation. For the other investigated time points, cells were cultured in the absence of glycosidases for an additional 1 or 3 h at

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37 °C, subsequently washed with ice-cold PBS and homogenized in 1 ml of lysis buffer. TRPV5 protein expression at the cell surface and in total cell lysate was investigated as described previously (7).

Total Internal Reflection Microscopy (TIRF-M)—HEK293 cells were transiently transfected with pcDNA3-Dendra2-HA-TRPV5 and pcDNA3-Dendra2-HA-TRPV5^{N358Q} 24 h before the experiment. TIRF-M cells were harvested in 15 ml tubes and resuspended in conditioned medium containing recombinant klotho (2 µg/ml, R&D systems) or sialidase from *V. cholerae* (27 mU/ml, Sigma-Aldrich). The tubes were rotated for 1.5 h at 37 °C to increase the effective reactive surface of the cells. Cells were reseeded on small Petri dishes (WilCo, Amsterdam, The Netherlands) coated with 50 µg/ml fibronectin and cultured for 2–7 h before subjected to TIRF-M. Dishes were washed and placed in a custom-made chamber with Krebs buffer (135 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.5 mM CaCl₂, 20 mM HEPES (pH 7.4 with NaOH), 10 mM D-glucose,) and imaged at room temperature. The Olympus Fluoview FV1000-IX81 confocal microscope, with fully integrated TIRF module, and equipped with a PLAPON60xO/TIRFM-SP-PlanApoChromatic objective 60x oil, NA 1.45 and WD = 0,10 mm lens was used to measure fluorescence. Photoconversion of Dendra2 was carried out using a 405 nm laser for 2 s. TRPV5 membrane trafficking was followed for about 36 min and every 3 min images were acquired of unswitched (488 nm laser, 20 frames/5 s) and switched (559 nm laser, 20 frames/5 s) dendra-TRPV5 proteins. All imaging acquisition was performed with CellM (Tokyo, Japan).

Biotinylation of TRPV5—HEK293 cells transiently transfected with TRPV5 in pCINeo/IRES-GFP, TRPV5^{N358Q} in pCINeo/IRES-GFP, or the empty pCINeo/IRES-GFP vector (mock) were reseeded on poly-L-lysine-coated (0.1 mg/ml) culture dishes 1 day after transfection and incubated with sialidase from *V. cholerae* (27 mU/ml, Sigma-Aldrich) or recombinant klotho (2 µg/ml, R&D systems) for 16 h at 37 °C. Subsequently, cells were biotinylated, lysed, and the biotinylated proteins were precipitated from the cell lysate with neutravidin beads (Pierce, Etten-leur, The Netherlands) as described previously (7). For the biotinylation after filipin treatment, the same protocol was used, but the cells were treated with filipin (5 µg/ml, Sigma-Aldrich) for 45 min prior to lysis.

Transferrin Isoelectric Focusing (tIEF)—The IEF assay was carried out as previously described, with the following differences (23). Briefly, plasma samples were incubated for 30 min, with a solution of 6.7 mM ferric citrate and 0.17 M sodium hydrogen carbonate, in a ratio of 1:1 (plasma to solution), to saturate the transferrin with iron. The iron-saturated plasma was treated with 2 units/ml sialidase (Roche Applied Science, Mannheim, Germany), 150 µg/ml recombinant klotho (R&D systems) or left untreated for each of the investigated time points (1, 2, 4, 7 h or overnight). Subsequently, the samples were applied to a hydrated immobiline gel (pH 5–7) on a Phast-System (GE-Healthcare Life Sciences, Piscataway). Transferrin isoforms were detected after immunofixation with rabbit anti-human transferrin antibody (Dako, Glostrup, Denmark) and Coomassie brilliant blue staining.

COPAS Sorting and PCR—Transgenic mice expressing eGFP under the control of the TRPV5 promoter have been described previously (24). Fluorescently labeled DCT/CNT tubules were isolated from transgenic animals, using a Complex Object Parametric Analyzer and Sorter (COPAS) sorter (Union Biometrica, Somerville, MA) as previously described (25, 26). The animal ethics board of the Radboud University Nijmegen approved all of the experimental procedures. Tubular, HEK293 and CHO cell RNA was extracted using TRIzol Total RNA Isolation Reagent (Invitrogen BRL, The Netherlands) and processed into cDNA. The cDNA was mixed with Power SYBR green PCR Mastermix (Applied Biosystems, Foster City, CA) and exon-overlapping primers against TRPV5 and galectin-1 and -3.

Statistical Analysis—In all experiments, the data are expressed as mean ± S.E. Statistical significance ($p < 0.05$) was determined by analysis of variance and a Bonferroni post-hoc test.

RESULTS

Stimulation of TRPV5 Plasma Membrane Retention by Glycosidases—To study the molecular mechanism of the klotho-dependent TRPV5 stimulation, we investigated the effect of various glycosidases on the activity of the channel. HEK293 cells transiently expressing TRPV5 were treated with recombinant mouse klotho (2 µg/ml), sialidase (27 mU/ml), β-glucuronidase (310 units/ml) or endoglycosidase-F (endoF) (1 kU/ml) for 16 h and subjected to patch clamp analysis. HEK293 cells transfected with TRPV5 all showed typical representative inwardly-rectifying I/V curves. Fig. 1A represents cells transiently transfected with TRPV5, untreated or treated with klotho or endoF. The tested glycosidases significantly increased the TRPV5-mediated Na⁺ current density (Fig. 1B), while the current density of mock-transfected cells was not affected (data not shown). The stimulation of TRPV5 channel activity was confirmed by measuring ⁴⁵Ca²⁺ uptake in cells after glycosidase treatment (Fig. 1C). EndoF showed the highest stimulation of TRPV5 (205 ± 6%), but also β-glucuronidase, sialidase, and klotho increased significantly the TRPV5-dependent Ca²⁺ uptake (166 ± 4%, 147 ± 8%, 155 ± 4%, respectively, $p < 0.05$).

Subsequently, we investigated the plasma membrane retention via time-chase analysis. HEK293 cells expressing TRPV5 were treated for 16 h with different glycosidases and subjected to cell-surface biotinylation. To follow the fate of the biotinylated proteins, the cells were incubated for 0, 1, or 3 h without glycosidases. In untreated cells, the amount of biotinylated TRPV5 decreased significantly after 1 and 3 h compared with the initial time point (0 h) (Fig. 1D, upper panels). For klotho-, sialidase-, and endoF-treated cells, only a small decrease of biotinylated TRPV5 channels was detected after 1 and 3 h (Fig. 1D, upper panels). The total expression of TRPV5 was not significantly altered by the glycosidase treatment (Fig. 1D, lower panels).

To further elucidate the mechanism responsible for the increase in TRPV5 activity after glycosidase treatment, the N-glycosylation-deficient mutant TRPV5^{N358Q}, was subjected to time-chase analysis. Compared with wild-type TRPV5 the plasma membrane abundance of the mutant TRPV5^{N358Q}

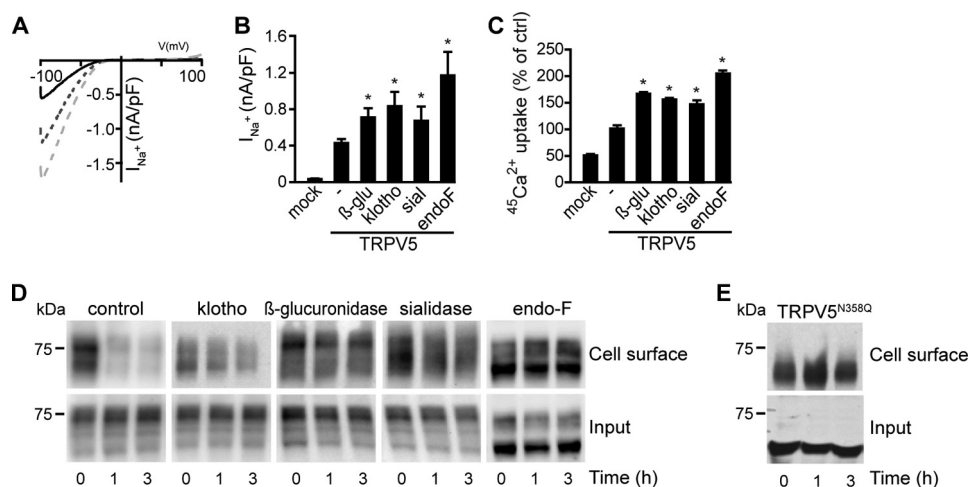


FIGURE 1. Different glycosidases increase the TRPV5-mediated Ca^{2+} uptake via plasma membrane stabilization of the channel. *A*, I/V relationships of untreated, klotho- or endoF-treated HEK293 cells expressing TRPV5 are presented. The *solid line* represents untreated cells, *dashed line* represents klotho-treated cells (*light gray*), and the *dotted line* represents endoF-treated cells (*dark gray*). *B*, TRPV5-mediated Na^+ current in HEK293 cells was measured. Treatment with β -glucuronidase, klotho, sialidase, or endoF all resulted in a significantly increased current ($n = 17-42$). *C*, $^{45}\text{Ca}^{2+}$ uptake assays of HEK293 cells transfected with TRPV5 or the empty vector (mock). These cells were untreated or treated with glycosidase (β -glucuronidase, klotho, sialidase, or endoF); *D*, time-chase assay of cells transfected with TRPV5. The membrane proteins were biotinylated at 0 h. After 3 h incubation without glycosidase, the membrane fraction of TRPV5 was decreased. *E*, time-chase assay of cells transfected with TRPV5^{N358Q}. All data are presented as mean \pm S.E.; $n = 3$; $p < 0.05$, statistically significant.

channel was markedly stable, even after 3 h of incubation (Fig. 1E).

Glycosidase Treatment Retains TRPV5 at the Plasma Membrane—Traditional time-chase experiments are not able to distinguish between delayed recycling of proteins from the plasma membrane or reduced degradation. To overcome this limitation, we established a novel approach by combining live-cell TIRF-M with a photo-convertible (green to red) fluorescent protein, Dendra-2, that was fused to the N terminus of the TRPV5 channel (27). Via this technique an evanescent field at the glass-cell membrane interface permitted specific detection and excitation of the channels close to the plasma membrane ($\sim 70-100$ nm). Proteins in close vicinity to the plasma membrane were photo-switched in TIRF mode using evanescent UV light ($\lambda = 405$ nm). TIRF-M images are shown of the control and treated conditions for Dendra-2-TRPV5 and Dendra-2-TRPV5^{N358Q}, captured at the initial time point after photo-switching and after 30 min (Fig. 2, A and B). The ratio of fluorescent intensity at an initial time point was calculated by $(F_i - F_o)/F_o$, where F_o is fluorescent intensity at an initial time point and F_i = fluorescent intensity at a new time point. To follow the presence of the protein at the plasma membrane in time, the ratio between the switched red fraction (F_{559}) and unswitched green fraction (F_{488}) was calculated $((F_{559}/F_{488})/(F_{559}/F_{488})_{\text{max}})$. After treatment with sialidase ($70 \pm 3\%$) or klotho ($75 \pm 4\%$) the ratio at 30 min was significantly increased compared with control ($47 \pm 8\%$) indicating that TRPV5 is more stable at the plasma membrane (Fig. 2C). To investigate whether there is a glycosidase-dependent effect on the N-glycosylation-deficient mutant, Dendra-2-TRPV5^{N358Q} was subjected to klotho and sialidase treatment. After glycosidase treatment, no significant decrease in the fluorescent ratio of Dendra-2-TRPV5^{N358Q} was found (Fig. 2D).

Klotho and Sialidase Regulate TRPV5 Plasma Membrane Retention Differently—TIRF-M showed that the cells expressing TRPV5^{N358Q} appeared brighter after sialidase treatment,

but not after klotho treatment. The difference between sialidase-treated and klotho-treated HEK293 cells expressing TRPV5^{N358Q} was further investigated. Treatment with klotho of the cells expressing TRPV5^{N358Q} did not alter the plasma membrane availability of this mutant as detected by cell surface biotinylation ($104 \pm 6\%$, $p > 0.2$) (Fig. 3, A and B). For the cells transfected with wild-type TRPV5, the TRPV5 plasma membrane fraction significantly increased after klotho treatment ($140 \pm 9\%$, $p < 0.05$). In contrast, sialidase treatment enhanced the plasma membrane fraction of both TRPV5 and TRPV5^{N358Q} ($151 \pm 8\%$ and $153 \pm 7\%$, respectively, $p < 0.05$) (Fig. 3, C and D).

The distinct actions of klotho and sialidase were further studied by $^{45}\text{Ca}^{2+}$ uptake assays. In line with our biochemical observations, klotho augmented the $^{45}\text{Ca}^{2+}$ influx in HEK293 cells transfected with TRPV5 ($120 \pm 2\%$), but not in cells expressing TRPV5^{N358Q} ($103 \pm 5\%$) (Fig. 3E). In contrast, sialidase increased TRPV5-mediated $^{45}\text{Ca}^{2+}$ uptake in TRPV5-expressing cells ($150 \pm 11\%$) as well as in cells expressing TRPV5^{N358Q} ($149 \pm 10\%$) (Fig. 3E). Co-treatment with sialidase and klotho resulted in an additive increase in TRPV5-mediated Ca^{2+} influx ($170 \pm 8\%$). In contrast, no additional $^{45}\text{Ca}^{2+}$ influx was observed after sialidase and klotho treatment of HEK293 cells over-expressing TRPV5^{N358Q} ($149 \pm 10\%$ and $144 \pm 9\%$, sialidase *versus* sialidase and klotho, respectively, $p > 0.2$). The TRPV5 blocker ruthenium red was applied to determine the magnitude of the TRPV5-mediated $^{45}\text{Ca}^{2+}$ influx (28).

Disruption of Lipid Rafts Increases TRPV5 Plasma Membrane Abundance—Sialidase is known to affect protein trafficking by interacting with glycolipids in lipid rafts (13). To determine whether TRPV5 is present in lipid rafts, lipid rafts in HEK293 cells expressing either TRPV5 or TRPV5^{N358Q} were disrupted using the cholesterol-chelating drug filipin. After filipin treatment, cell surface biotinylation showed a significant increase in TRPV5 and TRPV5^{N358Q} abundance at the plasma membrane (Fig. 4, A–C). Likewise, $^{45}\text{Ca}^{2+}$ uptake assays

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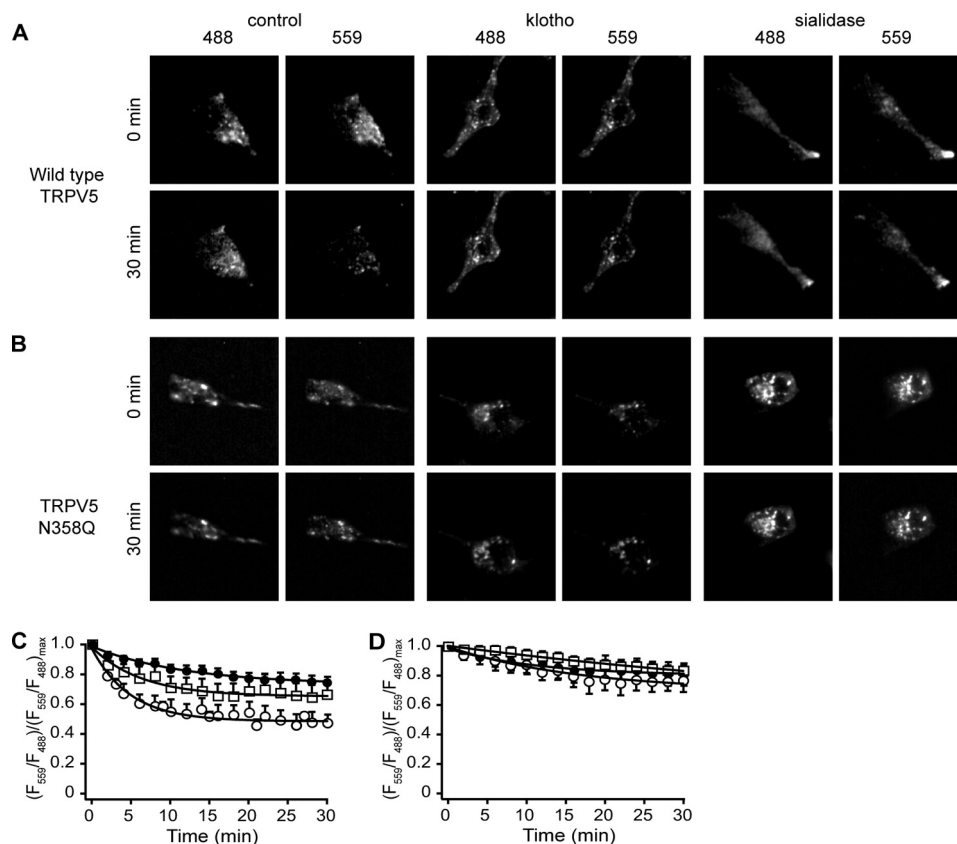


FIGURE 2. Klotho and sialidase stabilize TRPV5 on the plasma membrane. *A* and *B*, TIRF-M images of HEK293 cells expressing Dendra-2-TRPV5 or Dendra-2-TRPV5^{N358Q} in both *red* and *green* channels, after 0 and 30 min. *C*, ratio of switched red/switched green fraction of HEK293 cells expressing Dendra-2-TRPV5. These cells were subjected to live-cell TIRF-M. At 0 min Dendra-2 was subjected to UV light ($\lambda = 405$ nm), resulting in a switch from *green* to *red*. *D*, ratio of switched red/switched green fraction of HEK293 cells expressing Dendra-2-TRPV5^{N358Q}. These cells showed no increase in plasma membrane retention after glycosidase treatment. \circ , control; \square , klotho-treated; \bullet , sialidase-treated. All data are expressed as mean \pm S.E.; $n = 3$.

revealed a proportional increase in the functional activity of TRPV5 ($119 \pm 3\%$) and TRPV5^{N358Q} ($122 \pm 4\%$), after filipin treatment *versus* control ($p < 0.05$) (Fig. 4D). A combination of filipin and klotho significantly stimulated TRPV5-mediated $^{45}\text{Ca}^{2+}$ uptake ($100 \pm 6\%$ and $143 \pm 10\%$, control *versus* filipin and klotho, respectively $p < 0.05$). No additional $^{45}\text{Ca}^{2+}$ uptake was observed after combining sialidase with filipin ($100 \pm 6\%$ and $113 \pm 6\%$, control *versus* filipin and sialidase, respectively $p > 0.2$) (Fig. 4E).

Sialidase and Klotho Modify the N-glycan of Transferrin in a Different Manner—The difference between klotho and sialidase was further confirmed by isoelectric focusing (IEF), using human blood transferrin as a model glycoprotein. Via this technique, proteins are separated based on their isoelectric focus point. Since sialic acids are negatively charged, a change in the isoelectric focusing pattern can be detected after sialidase treatment. Plasma transferrin was used as model glycoprotein, since the isoelectric focusing point after removal of the sialic acids has been characterized. The main isoform of human plasma transferrin contains two complex biantennary N-glycans, each terminated by two α -2,6-linked sialic acids (23). The protein was treated with klotho (150 $\mu\text{g}/\text{ml}$) or sialidase (2 units/ml) for different time intervals. Sialidase treatment resulted in a clear shift in the IEF pattern of transferrin, indicating the loss of sialic acids (Fig. 5A). Before treatment, a main isoform with four sialic acids was detected and after 2 h of sialidase treatment all sialic acids were

cleaved. Treatment with klotho did not significantly alter the IEF pattern of transferrin (Fig. 5B).

Galectin-3 Partially Mediates Klotho-dependent TRPV5 Activity—The premise that klotho might not be a specific sialidase, would add new mechanistic data to the hypothesis proposed by Cha *et al.*, who suggested that galectin-1 stabilizes TRPV5 on the plasma membrane after N-glycan modification by klotho-mediated sialidase activity. In CHO cells they showed that the stimulatory klotho effect on TRPV5 was absent after the knock down of galectin-1 (14). However, immunohistochemistry of mouse kidney sections failed to show co-localization between TRPV5 and galectin-1 (Fig. 6A). In addition, galectin-1 was expressed in CHO cells, but not in HEK293 cells, that were used for this study (Fig. 6B). Since the potential ligands for galectin-1 and galectin-3 are similar (29), the expression of galectin-3 in mice sections, HEK293 cells and in Complex Object Parametric Analyzer and Sorter (COPAS)-sorted distal convoluted and connecting tubules (DCT/CNT) was checked. Galectin-3 showed a clear co-localization with TRPV5 in mice kidney sections. Some tubuli were positive for galectin-3, but not for TRPV5, indicating that galectin-3 activity is not restricted to TRPV5-expressing tubuli (Fig. 6C). Galectin-3 was also expressed in HEK293 cells, as was shown by RT-PCR (Fig. 6D). The expression of galectins in the DCT/CNT was further studied after collecting the nephron segments from mouse kidney, by the COPAS large particle flow cytometry (25).

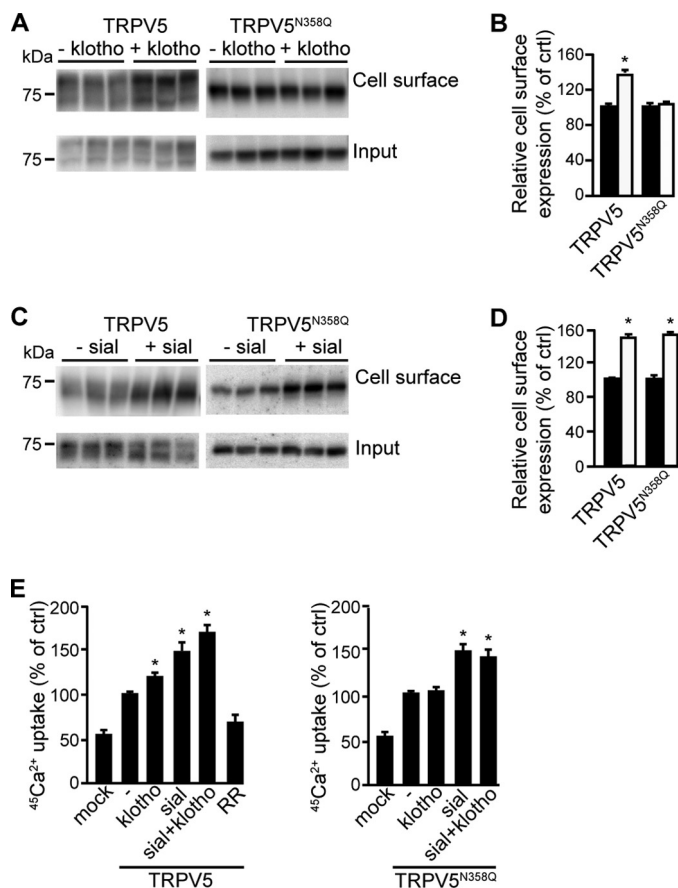


FIGURE 3. TRPV5 stabilization via klotho is N-glycan-dependent. *A*, biotinylation of HEK293 cells expressing TRPV5 or TRPV5^{N358Q} after klotho treatment, a representative blot is depicted; *B*, relative TRPV5 cell surface expression with or without klotho treatment (white and black bar, respectively); *C*, biotinylation of HEK293 cells expressing TRPV5 or TRPV5^{N358Q} after sialidase treatment, a representative blot is depicted; *D*, relative TRPV5 cell surface expression with or without sialidase treatment (white and black bar, respectively); *E*, ⁴⁵Ca²⁺ uptake assay of cells transfected with TRPV5 or TRPV5^{N358Q} after treatment with klotho, sialidase, a combination of these glycosidases, ruthenium red or left untreated. All data are presented as mean ± S.E.; *n* = 3; *, *p* < 0.05, statistically significant.

In isolated DCT/CNT segments only galectin-3 mRNA was detected by RT-PCR, but not galectin-1 (Fig. 6E). In addition, also in mouse urine, only galectin-3 was found, which implies that it has access to the glycan of TRPV5 (Fig. 6F).

The involvement of galectin-3 in the regulation of TRPV5 was further investigated. HEK293 cells, transiently overexpressing TRPV5 were treated with 0.67 μg/ml recombinant human galectin-3. ⁴⁵Ca²⁺ uptake assays revealed an increase in TRPV5 channel activity after galectin-3 treatment (100 ± 2% and 131 ± 6%, control versus galectin-3, respectively, *p* < 0.05) (Fig. 7A), while the activity of the TRPV5^{N358Q} mutant was not affected by galectin-3 treatment (100 ± 2% and 104 ± 4%, control versus galectin-3, respectively, *p* > 0.2) (Fig. 7A). Combining galectin-3 and klotho treatment, or galectin-3 and sialidase treatment, did not result in additional stimulation of channel activity (Fig. 7B).

DISCUSSION

Our study demonstrates that glycosidases augment the plasma membrane retention of TRPV5, where klotho and siali-

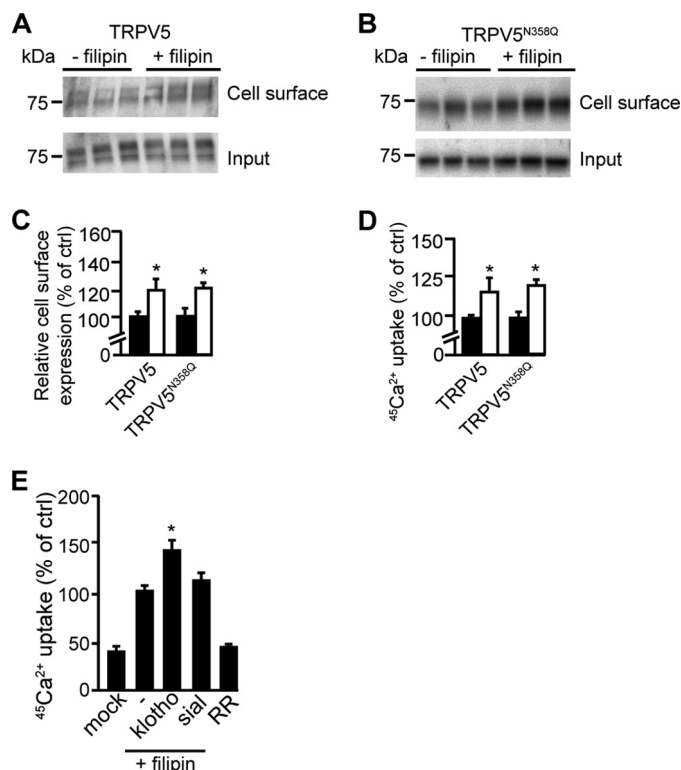


FIGURE 4. TRPV5 plasma membrane retention is regulated via lipid rafts. *A*, biotinylation of HEK293 cells expressing TRPV5 after filipin treatment, a representative blot is depicted. *B*, biotinylation of cells expressing TRPV5^{N358Q} after filipin treatment, a representative blot is depicted. *C*, relative cell surface expression, with or without filipin treatment (white and black bar, respectively) of TRPV5 (*n* = 6) or TRPV5^{N358Q} (*n* = 3) overexpressing cells; *D*, ⁴⁵Ca²⁺ uptake assay in the presence of filipin (*n* = 3). Filipin significantly increases Ca²⁺ uptake independent on the N-glycan of TRPV5 (white bar). *E*, ⁴⁵Ca²⁺ uptake assay in the presence of filipin and klotho or sialidase (*n* = 3). Klotho significantly increased TRPV5-dependent ⁴⁵Ca²⁺-uptake in the presence of filipin, while sialidase treatment gave no additional effect. All data are presented as mean ± S.E.; *, *p* < 0.05, statistically significant.

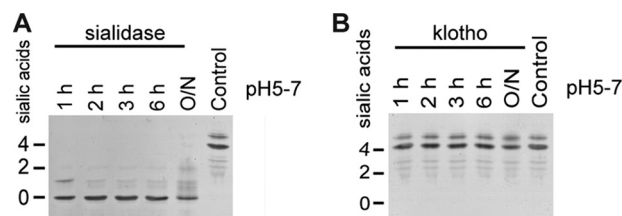


FIGURE 5. The isoelectric focusing pattern of transferrin is not modified by klotho. *A*, tIEF after sialidase treatment. Hydrolysis of sialic acids by sialidase treatment results in a shift of the IEF pattern. *B*, isoelectric focusing of transferrin after klotho treatment. Klotho has no influence on the amount of sialic acids.

dase increase the TRPV5 plasma membrane fraction via distinct mechanisms. The effect of sialidase, in contrast to klotho, is independent of the glycosylation status of TRPV5. Moreover, galectin-3 is a potential mediator for klotho-dependent TRPV5 stabilization. Our conclusions are based on the following observations: (i) biochemical assays showed that klotho, sialidase, β-glucuronidase and endoF increase the TRPV5 channel activity and membrane retention; (ii) the plasma membrane stability of TRPV5^{N358Q} was altered by sialidase, but not by klotho; (iii) IEF demonstrated no modification of the N-glycan sialic acids after klotho treatment, in transferrin; (iv) only galectin-3, but not galectin-1, co-localizes with TRPV5 in DCT/CNT of the

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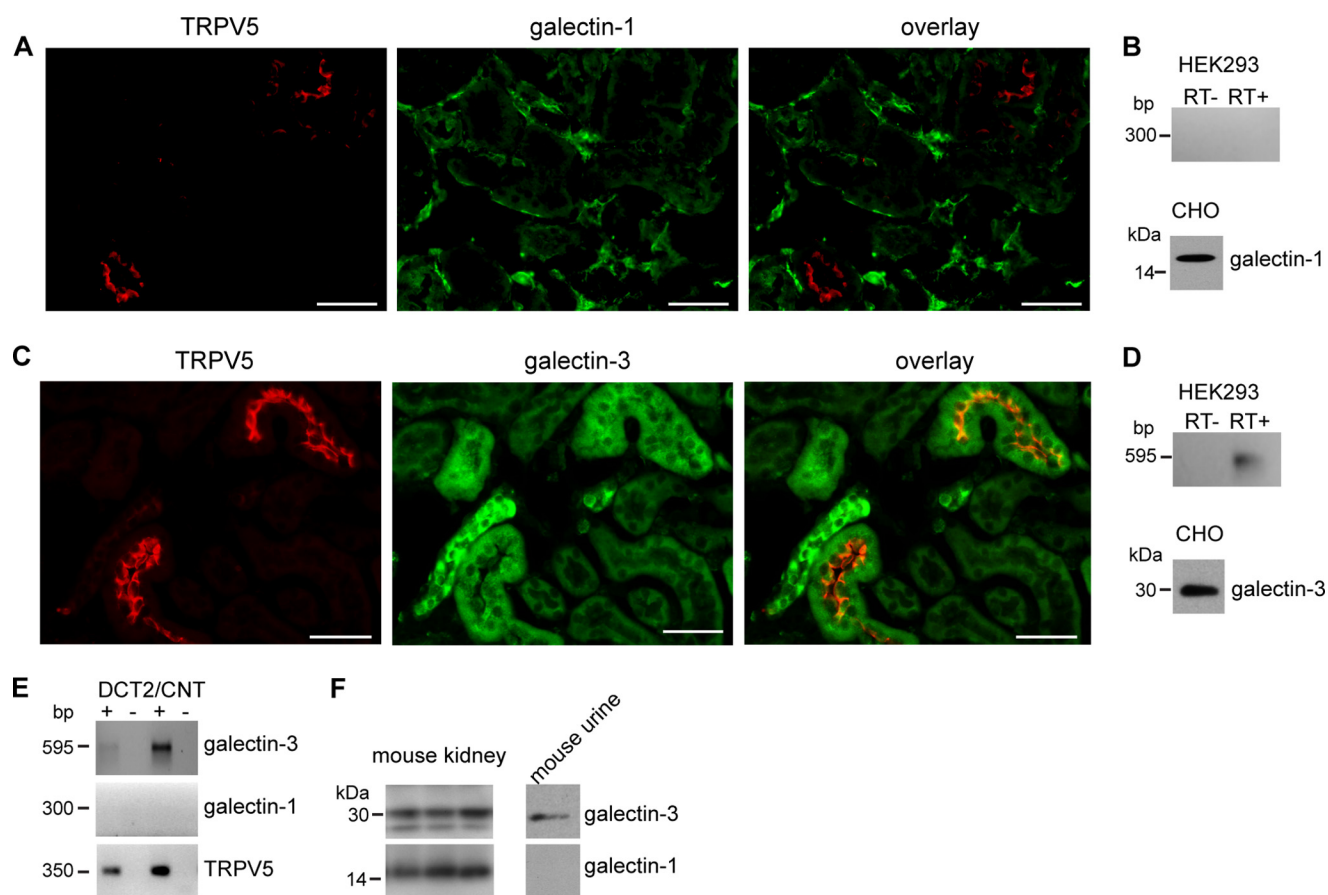


FIGURE 6. Galectin-3 is a candidate mediator of the klotho effect on TRPV5. *A*, immunohistochemistry on DCT/CNT for TRPV5 and galectin-1 shows no co-localization. *B*, immunoblot of CHO cell lysate demonstrated that galectin-1 was present. After RT-PCR on the cDNA of HEK293 cells, no galectin-1 was detected. *C*, immunohistochemistry on DCT/CNT for TRPV5 and galectin-3 demonstrate a co-localization; *D*, immunoblot of CHO cell lysate demonstrated that galectin-3 was expressed. After RT-PCR on the cDNA of HEK293 cells, galectin-3 was detected. *E*, cDNA of the DCT/CNT cells was analyzed by RT-PCR and only galectin-3 was detected. *F*, total kidney lysate of mouse contains both galectin-1 and galectin-3, while mouse urine only contains galectin-3.

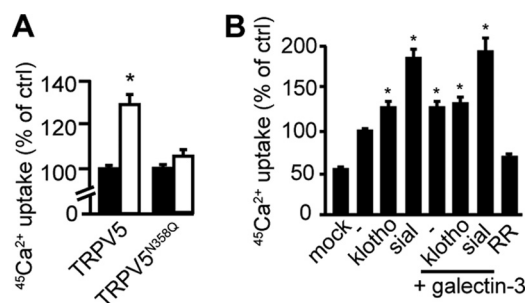


FIGURE 7. TRPV5 channel activity is regulated by extracellular galectin-3. *A*, ⁴⁵Ca²⁺-uptake assay in cells overexpressing TRPV5 ($n = 5$) or TRPV5^{N358Q} ($n = 3$) in the presence and absence of human galectin-3. Galectin-3 significantly increased wild type TRPV5 (white bar) but not TRPV5^{N358Q} channel activity (white bar). All data are presented as mean \pm S.E.; * $p < 0.05$, statistically significant. *B*, ⁴⁵Ca²⁺-uptake assay of cells overexpressing TRPV5 treated with klotho, sialidase, galectin-3, or a combination of these enzymes. No additional stimulation was detected after treatment with klotho and galectin-3. All data are presented as mean \pm S.E.; $n = 5$; * $p < 0.05$, statistically significant.

kidney; (v) galectin-3 increased the TRPV5-mediated Ca²⁺ influx in a *N*-glycan-dependent manner.

It was demonstrated that glycosidase treatment increases the TRPV5 channel activity. This effect was detected by treatment with different types of glycosidase (endoF, sialidase, and β -glucuronidase). TRPV5 channel activity was measured via time-chase experiments in combination with functional analyses (7,

14). A general mechanism or pathway for all the applied glycosidases remains unknown, since their enzymatic effects are distinct: endoF cleaves the entire *N*-glycan (30), sialidase hydrolyzes end-standing sialic acids, while β -glucuronidase cleaves glucuronic acids (31). One hypothesis is that any modification of the *N*-glycan improves the plasma membrane stabilization of TRPV5. Alternatively, since all glycans on the plasma membrane were hydrolyzed, the detected stabilizing effect could be independent of the *N*-glycan of TRPV5. This would mean that the plasma membrane stabilization is due to an indirect effect on TRPV5. However, the stimulatory effect of klotho is dependent on the *N*-glycan of TRPV5.

Plasma membrane retention can occur via two distinct routes; a delayed retrieval from the plasma membrane and subsequent degradation of the channel, or via an accelerated/increased insertion at the plasma membrane. It is difficult to distinguish between both routes based on time-chase experiments. To pinpoint the fate of glycosidase-treated TRPV5, we developed a novel assay, combining the live-cell imaging technique, TIRF-M, with photoactivatable fluorophores. Important advantages of TIRF-M over confocal microscopy are the higher Z resolution, high signal to noise ratio, increased contrast in fluorescence and reduced bleaching. The ease in combining kinetic studies with local information in living samples results in fast data acquisition (32). We showed that TRPV5 has a reduced mobile fraction

after sialidase or klotho treatment, indicating that these glycosidases enable TRPV5 to reside longer on the plasma membrane. Our control, TRPV5^{N358Q} lacks *N*-glycosylation and is devoid of any glycosylation-dependent regulation. This mutant demonstrated no decrease in the mobile fraction after glycosidase treatment. Overall this mutant had a smaller mobile fraction compared with TRPV5.

While conducting the TIRF-M based live-cell imaging it was observed that after sialidase treatment, the cells expressing TRPV5^{N358Q} appeared brighter. This effect was not detected after klotho treatment. This prompted us to investigate the effect of sialidase on TRPV5^{N358Q}. An increased plasma membrane abundance of TRPV5^{N358Q} was detected via biotinylation, after sialidase treatment. It was noticed that biotinylated TRPV5 often runs as a smear band on SDS-PAGE. The reason for this effect remains unclear, however, it could indicate that plasma membrane TRPV5 is more heterogeneous compared with intracellular TRPV5.

The effect of sialidase treatment on the TRPV5^{N358Q} mutant was confirmed using ⁴⁵Ca²⁺ uptake experiments. TRPV5-mediated ⁴⁵Ca²⁺ uptake was increased after treatment with both sialidase and klotho in an additive manner, indicating that klotho and sialidase act via different pathways. Interestingly, Cha *et al.* postulated that sialidase did not increase the plasma membrane abundance of TRPV5^{N358Q} (14). However, in their study CHO cells were used as a model system, while in the present study HEK293 cells were employed.

We proved that filipin, an inhibitor of microdomain clustering, increases the cell surface expression of TRPV5/TRPV5^{N358Q} and ⁴⁵Ca²⁺ uptake through TRPV5 and TRPV5^{N358Q}. This finding indicated that TRPV5 and TRPV5^{N358Q} channels are present and internalized in clustered microdomains. Recently, it has been suggested that sialidase prevents microdomain clustering and lipid raft formation by hydrolyzing sialic acids from glycosphingolipids (13). Microdomain clustering is known to stimulate caveolae-mediated endocytosis and since sialidase treatment inhibits this microdomain clustering, the endocytosis is reduced (13). Because TRPV5 is present in these microdomains, the channel is most likely stabilized on the plasma membrane by sialidase via reduced lipid raft formation and thereby a decreased caveolae-mediated endocytosis. In agreement with this hypothesis, sialidase treatment did not result in additional stimulation of ⁴⁵Ca²⁺ uptake after disrupting the lipid rafts by filipin. This finding, together with the sialidase-mediated stimulation of Ca²⁺ uptake via TRPV5^{N358Q}, and increased wild type TRPV5 activation after klotho treatment in the presence of filipin, suggested that klotho and sialidase act via two different pathways to modulate channel activity. Sialidase, in contrast to klotho, stimulates TRPV5 via a mechanism that is independent of the *N*-glycan of TRPV5.

To further confirm this hypothesis IEF was employed using the well-characterized transferrin as a model protein. Sialidase altered the IEF pattern of transferrin, a substrate exposing terminal sialic acids, including α 2,6-linked sialic acids (33, 34). Klotho treatment did not result in any modification, suggesting a lack of sialidase activity of klotho. Alternatively, it is possible that sialidase activity of klotho is tightly structure-related, cleaving only one sialic acid that is presented in a specific sugar

signature, which may be absent in transferrin. The difference between sialidase and klotho prompted us to investigate the following step in the proposed mechanism. Cha *et al.* suggested that galectin-1 is a key player in the regulation of TRPV5. Interestingly, immunohistochemistry on renal mouse cortex sections showed no co-localization of galectin-1 with TRPV5. Galectin-1 is not expressed in HEK293 cells and therefore the *in vitro* measured effect of klotho is likely to be galectin-1 independent. Since the potential ligands for galectin-1 and galectin-3 are highly similar, we investigated the role of galectin-3 in this process. Galectin-3 is a carbohydrate-binding protein that is highly abundant in type A intercalated cells of the cortical collecting duct and the distal tubules (35, 36). An important difference between galectin-1 and galectin-3 is that the dimer galectin-1 requires a terminal galactose for binding, while the oligomer galectin-3 binds to repeating [-3Gal- β -(1-4)-GlcNAc- β -1-]_n or poly-*N*-acetylglucosamine sequences that not necessarily contain a terminal β -galactoside residue (37). Galectin-3 co-localized with TRPV5, although the presence of galectin-3 is not restricted to TRPV5-expressing tubuli. In addition, COPAS sorted DCT/CNT cells were enriched with galectin-3 mRNA, while galectin-1 mRNA was not detected, indicating that galectin-3 may be involved in TRPV5 regulation. In contrast to the TRPV5^{N358Q} mutant, wild type TRPV5 channel activity was increased upon treatment with galectin-3. This suggests that galectin-3-mediated channel activity is dependent on the *N*-glycan of TRPV5. Interestingly, co-treatment with galectin-3 and klotho did not additively stimulate TRPV5 activity implying that klotho and galectin-3 stimulate TRPV5 activity in a similar manner. An interesting observation, as detected with TIRF-M, biotinylation, ⁴⁵Ca²⁺ uptake experiments and electrophysiology was the stable expression of TRPV5^{N358Q} mutant on the plasma membrane without any treatment. It was proposed that galectin stabilizes TRPV5 on the plasma membrane. However, due to the absence of an *N*-glycan on the TRPV5^{N358Q} mutant, it is striking that this mutant retains on the plasma membrane. Since the TRPV5^{N358Q} mutant was transiently overexpressed, one could hypothesize that some glycosylation-deficient channels are forced to the plasma membrane. Since the entire *N*-glycan is absent, the protein cannot be appropriately regulated by the cell and will remain more stable on the plasma membrane.

Likewise, Kohno *et al.* demonstrated a significant difference between the internalization rates of the wild type endothelial differentiation gene-1 product (Edg-1, a G-coupled receptor) and the nonglycosylated N30D-Edg-1 mutant (39). The wild type G-coupled receptor was internalized at higher rates compared with the nonglycosylated N30D-Edg-1 receptor. Another explanation could be that the channel is destabilized due to steric hindrance in the presence of the *N*-glycan, resulting in a reduced amount of TRPV5 on the cell surface. Consequently, any reduction of the *N*-glycan of TRPV5 results in an increase of plasma membrane stability and, therefore, channel activity.

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