Supporting Information

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SI Text

Molecular Biology. GFP-tagged TRPV5 have been described (1). cDNAs for human ST6Gal-1 and ST3Gal-1 (IMAGE Clone; Invitrogen) were cloned into pEF1 expression vector (Invitrogen). Before subcloning, appropriate restriction enzyme sites were added to both ends by using synthetic oligonucleotides and PCR. Point mutations by site-directed mutagenesis were confirmed by sequencing. Sequences for sense and antisense RNAi oligonucleotides for human galectins and sialyltransferases are in SI Table S1.

Preparation of Purified KLe and Klotho-Containing Conditioned Media. Preparation of purified KLe has been described (2). Serum-free conditioned medium containing WT KLe or mutant KLe (mutations of Asp-240, Asn-241, Glu-416, Asn-417, Asn-690, and Glu-691 to Ala) was collected from 293 cells transiently transfected with the expression vector for each protein. The concentration of the proteins in conditioned media was determined by immunoblot analysis using anti-Klotho antibody and the purified KLe of known concentration as standards.

Whole-Cell Patch-Clamp Recording. Cells (HEK or CHO) were cultured and transiently transfected with cDNAs encoding GFP-TRPV5 (0.1 μg DNA per 35-mm dish) with or without additional constructs (1 μg each) as described (1). The total amount of DNA for transfection was balanced by using an empty vector. For knockdown by RNAi, oligonucleotides (200 nM each) were mixed with cDNA for TRPV5 for cotransfection. About 24 h after transfection, cells were incubated with Klotho, bacterial sialidase, and/or β-galactosidase for indicated duration. Forty to 48 h after transfection, whole-cell currents were recorded by using an Axopatch 200B amplifier as described (1). Transfected cells were identified by epifluorescent microscopy. For recording of TRPV5 current, the pipette and bath solution contained 140 mM NaAsp (sodium aspartate), 10 mM NaCl, 10 mM EDTA, and 10 Mm Hepes (pH 7.4) and 140 mM NaAsp, 10 mM NaCl, 1 mM EDTA ,and 10 mM Hepes (pH 7.4), respectively. Capacitance and access resistance were monitored and 75% were compensated. The voltage protocol consisted of 0-mV holding potential and 400-ms steps from −150 to 100 mV in 25-mV increments. Current density was calculated by dividing current at −100 mV (pA; measured at 25°C) by capacitance (pF). Results are shown in mean ± SEM (n = 5–10). Statistical comparison between two groups of data were made by using two-tailed unpaired t test. Multiple comparisons were made by using one-way ANOVA followed by t test.

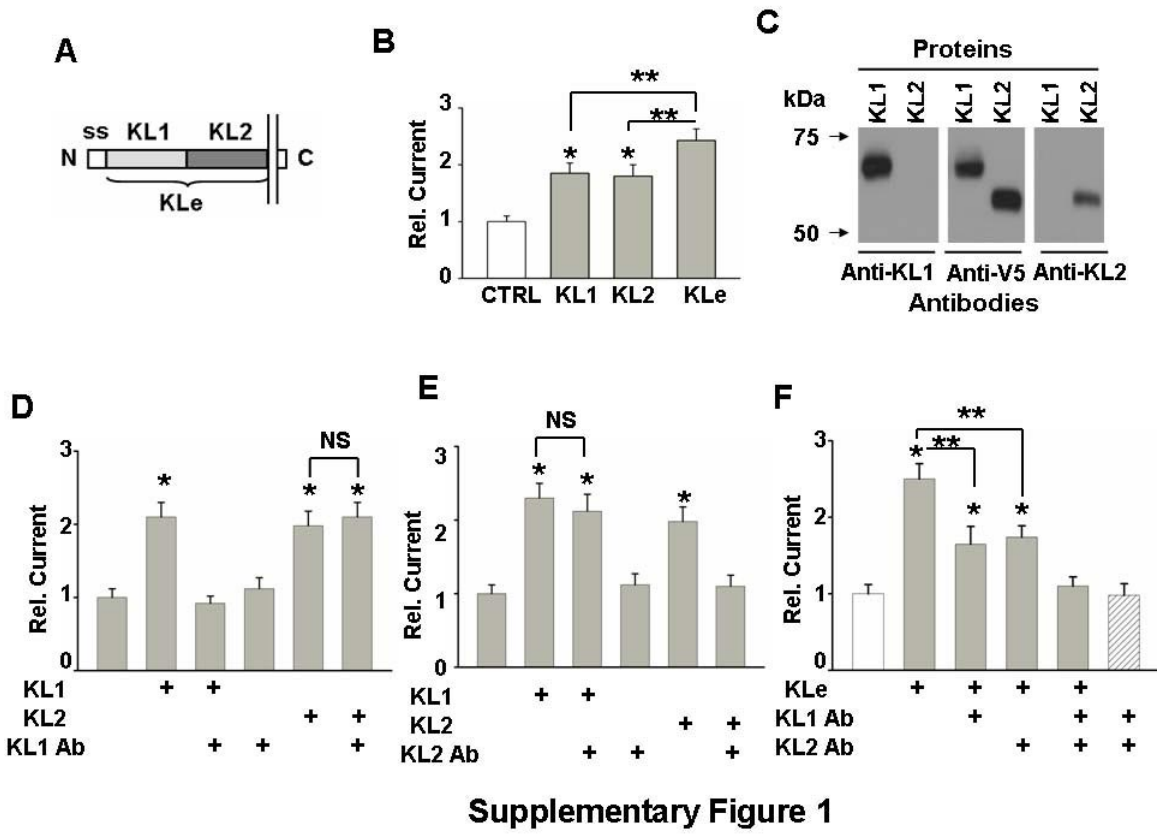
Surface Biotinylation Assay. Cells were washed twice with ice-cold PBS before incubation with 0.75 ml of PBS containing 1 mg/ml EZ-link-NHS-SS-Biotin (Pierce) for 30 min at 4°C. After quenching by TBS [140 mM NaCl, 10 mM Tris·HCl (pH 7.4), 5 mM KCl], cells were lysed in RIPA buffer [150 mM NaCl, 50 mM Tris·HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS] containing protease inhibitor mixture (Roche). Biotinylated proteins were precipitated by streptavidin agarose beads (Pierce), heated at 50°C for 3 min, and separated by SDS/PAGE for Western blot analysis. TRPV5 proteins were detected by a mAb against GFP. Each experiment shown was performed at least three times with similar results.

Coimmunoprecipitation of TRPV5 with Galectin-1. Cells transfected by WT GFP-TRPV5 or N358Q mutant were treated with or without Klotho in the culture medium overnight. Cell surface proteins were cross-linked by using a membrane-impermeable dithio-bis-suffosuccinimydyl propionate (DTSSP) (Pierce) for 1 h. After quenching by TBS, cells were lysed in a RIPA buffer supplemented with 100 mM lactose and centrifuged. Supernatants were immunoprecipitated by a monoclonal anti-GFP antibody (1:200 dilution; Clontech). Immunoprecipitates were reduced by DTT, separated by SDS/PAGE, and analyzed by Western blot. Galectin-1 was detected by using a polyclonal anti-galectin-1 antibody (Cell Sciences).

Staining by SNA and MAA. CHO cells were fixed by 4% paraformaldehyde (in PBS) for 20 min at room temperature, followed by incubation with biotinylated SNA or MAA (60 μg/ml; Vector Laboratories) for 30 min at 37°C and incubation with Alexa 594-conjugated streptavidin (1:500 dilution; Molecular Probes) for an additional 30 min at 37°C. Fluorescent images were obtained by using a Nikon Eclipse TE2000-U fluorescent microscope and overlaid with differential interference contrast (DIC) images as described (3). In each experiment, gain for fluorescence detection was adjusted equal by using untransfected cells as a control.

KLe. The KLe is composed of two internal repeats, KL1 and KL2, each sharing amino acid sequence homology to family 1 glycosidases (2, 4, 5) (Fig. S1*A*). Despite differences in amino acid sequence in the putative active site, the KL1 and KL2 domains of Klotho each independently increased TRPV5 current density (Fig. S1*B*). The effect of each individual domain on TRPV5 current was slightly less than that of the full extracellular region (KLe). The effects of KL1 and KL2 were prevented by DANA (data not shown). An antibody against the KL1 domain (4) neutralized the effect of KL1 but not of KL2 on TRPV5 (Fig. S1*D*). Conversely, an antibody against the KL2 domain (6) neutralized the effect caused by KL2 but not by KL1 (Fig. S1*E*). Each domain-specific antibody partially neutralized the effects of KLe (Fig. S1*F*). Effects of antibodies against KL1 and against KL2 were additive (Fig. S1*F*).

1. Yeh BI, Kim YK, Jabbar W, Huang CL (2005) Conformational changes of pore helix coupled to gating of TRPV5 by protons. *EMBO J* 24:3224–3234.
2. Kuro-o M, *et al.* (1997) Mutation of the mouse klotho gene leads to a syndrome resembling aging. *Nature* 390:45–51.
3. Zeng WZ, *et al.* (2002) Evidence for endocytosis of ROMK potassium channels via clathrin-coated vesicles. *Am J Physiol* 283:F630–F639.
4. Ito S, Fujimori T, Hayashizaki Y, Nabeshima Y (2002) Identification of a novel mouse membrane-bound family 1 glycosidase-like protein, which carries an atypical active site structure. *Biochim Biophys Acta* 1576:341–345.
5. Rye CS, Withers SG (2000) Glycosidase mechanisms. *Curr Opin Chem Biol* 4:573–580.
6. Kato Y, *et al.* (2000) Establishment of the anti-Klotho monoclonal antibodies and detection of Klotho protein in kidneys. *Biochem Biophys Res Commun* 267:597–602.



Supplementary Figure 1

Fig. S1. Anti-Klotho antibodies abolish the effects of Klotho on TRPV5. (*A*) Membrane topology of mouse Klotho (not drawn in scale). N, amino terminus; C, carboxyl terminus; ss, signal sequence; KL1, KL1 repeat; KL2, KL2 repeat; KLe, extracellular domain of Klotho. (*B*) Effects of KL1 repeat, KL2 repeat, and KLe (100 pM each) on TRPV5. *, $P < 0.05$ vs. vehicle control (CTRL; open bar). **, $P < 0.05$ between indicated groups. (*C*) Specificity of anti-KL1 and anti-KL2 antibodies. Purified recombinant KL1 domain and KL2 domain proteins (V5-epitope tagged) were blotted with KM2076 (anti- KL1), KM2119 (anti-KL2), or an anti-V5 antibody (Invitrogen). (*D*) Effects of anti-KL1 antibody. *, $P < 0.05$ vs. before addition of KL1, KL2, or KL1 antibody (Ab). (*E*) Effects of anti-KL2 antibody. *, $P < 0.05$ vs. before addition of KL1, KL2, or KL2 Ab. (*F*) Effects of anti-KL1 and/or anti-KL2 antibodies. The concentration of anti-KL1 and anti-KL2 antibody was 2 nM when used alone and 1 nM when used in combination. *, $P < 0.05$ vs. before addition of KLe, KL1 Ab, or KL2 Ab. **, $P < 0.05$ between the indicated groups.

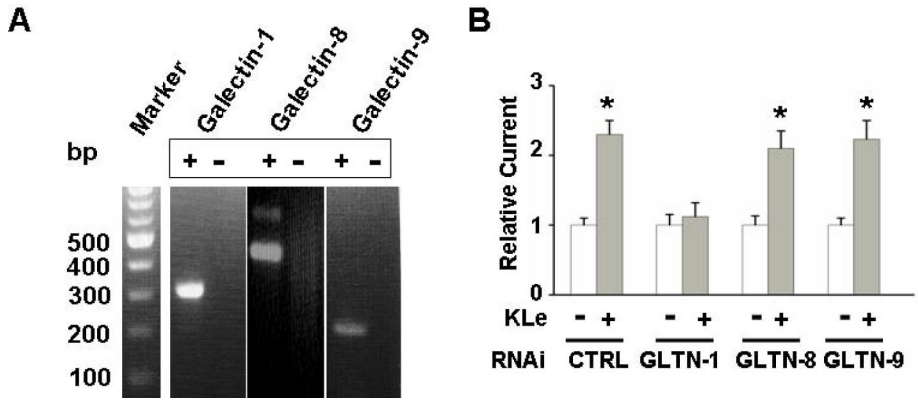


Fig. S2. Effects of knockdown of galectins on Klotho-mediated increase of TRPV5 currents. (*A*) Expression of galectin-1, -8, and -9 in HEK cells analyzed by RT-PCR. + and − are with or without reverse transcriptase, respectively. Forward and reverse primers for galectin-1, galectin-8, and galectin-9 were 5′-gaacctgggcaaagacag-3′ and 5′-ggctggctgatttcagtc-3′, 5′-gtacccaagcatctagtctg-3′ and 5′-taccagctccttacttccag-3′, 5′-gaactttcagactggcttcagtgg-3′ and 5′-accttgaaatctgagctctgcacc-3′, respectively. Galectin-3, galectin-4, galectin-7, and galectin-10 were also detected (data not shown). Galectin-2, galectin-12, galectin-13, and galectin-14 were not detected. (*B*) Knockdown of galectin-1, but not galectin-8 and galectin-9, prevents Klotho-mediated increase of TRPV5 currents. Cells were cotransfected with GFP-TRPV5 mixed with control oligonucleotide or RNAi oligonucleotides for the indicated galectin. Because knockdown of galectins may affect forward trafficking of membrane proteins (1), we analyzed the effects of RNAi experiments by comparing the relative currents. *, $P < 0.05$ vs. before KLe (open bar).

1. Delacour D, *et al.* (2006) Requirement for galectin-3 in apical protein sorting. *Curr Biol* 16:408–414.

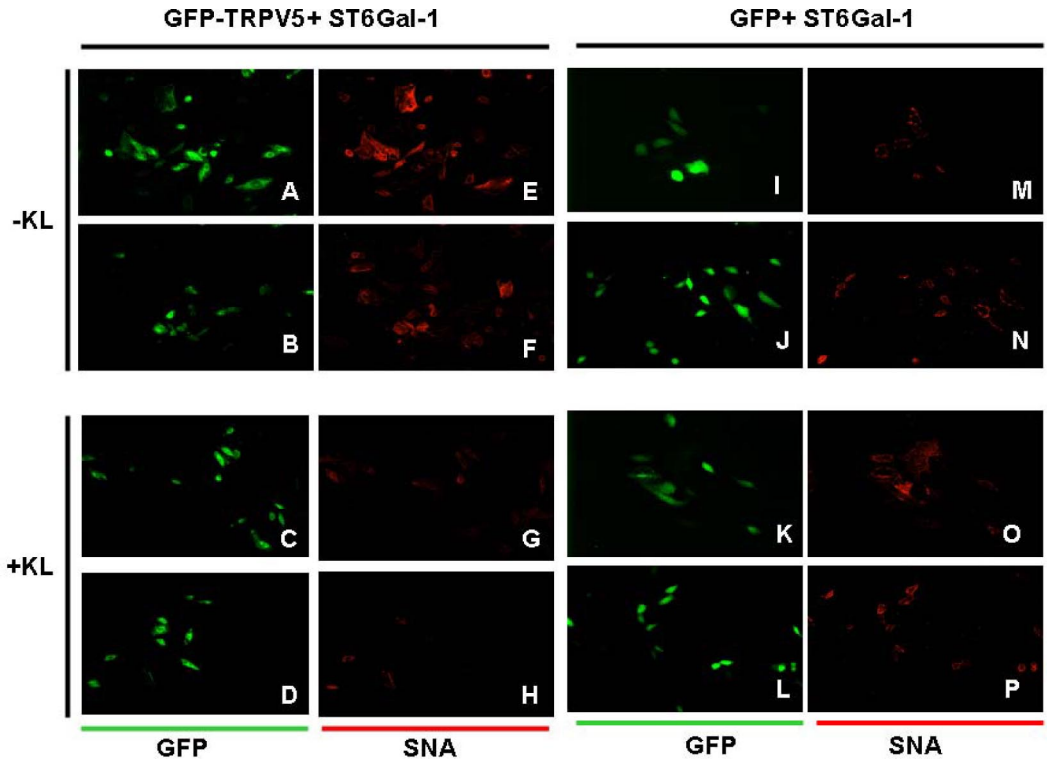


Fig. S3. Treatment by Klotho prevents binding of SNA to TRPV5. CHO cells were transfected with GFP-TRPV5 and ST6Gal-1 (*A–H*) or GFP and ST6Gal-1 (*I–P*). Cells were treated with or without Klotho (indicated by +KL and −KL, respectively) for 1 h before staining for SNA by incubating with biotinylated SNA (as indicated), followed by Alexa 594-labeled streptavidin. In each experimental condition, fluorescent images were acquired by gating for GFP fluorescence or Alexa-594 fluorescence (as indicated by GFP and SNA, respectively) to detect transfected cells and labeling by SNA, respectively.

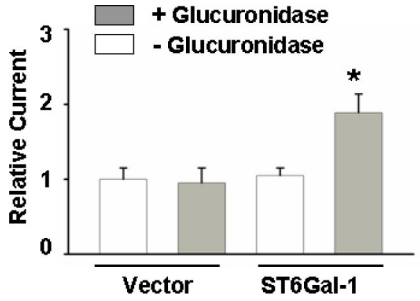


Fig. S4. Effect of β-glucuronidase on TRPV5 in CHO cells. Cells were cotransfected with GFP-TRPV5 plus ST6Gal-1 or an empty vector and incubated with bovine liver β-glucuronidase (0.1 μM) for 16 h. Similar results were observed by using 1 μM bovine liver β-glucuronidase (data not shown). *, $P < 0.05$ vs. vehicle (−glucuronidase; open bar).

Table S1. Sequences for RNAi oligonucleotides for human galectins and sialyltransferases

Target gene	Sense	Antisense
Galectin-1	CCAACACCAUCGUGUGCAAUU	UUGCACACGAUGGUGUUGGUU
Galectin-8	GAACUUUGAUUGUGAUAUGUU	CAUAUCACAAUCAAAGUUCUU
Galectin-9	GGACAGAUGUUCUCUACUCUU	GAGUAGAGAACAUCUGUCCUU
ST3Gal-1	CGGCCUGGUUCGAUGAGAGGUUCAA	UUGAACCUCUCAUCGAACCAGGCCG
ST3Gal-2	CCGACUGGUUUGACAGCCACUUUGA	UCAAAGUGGCUGUCAAACCAGUCGG
ST3Gal-3	CCUGCUGAAUUAGCACCAAGUACG	CGUACUUGGUGGCUAAUUCAGCAGG
ST3Gal-4	CCAAGACCACCAUGCGUCUCUUCUA	UAGAAGAGACGCAUGGUGGUCUUGG
ST3Gal-5	GCGCACCACUGUCUGACCUUGAAUA	UAUUCAAGGUCAGACAGUGGUGCGC
ST3Gal-6	GAAUGUCUAUUGGGUGGCACCUGUG	CACAGGUGCCACCCAAUAGACAUUC
ST6Gal-1	GCUGCGUCCUGGUCUUUCUUCUGUU	AACAGAAGAAAGACCAGGACGCAGC
ST6Gal-2	UCUGCAGGCGCAAUCCUCAACUCUU	AAGAGUUGAGGAUUGCGCCUGCAGA
ST6GalNAc-1	GCACAAUCAUGGAAGAGCCAGGACA	UGUCCUGGCUCUUCCAUGAUUGUGC
ST6GalNAc-2	CCACAAGGACAGGACCUGCAGUAUA	UAUACUGCAGGUCCUGUCCUUGUGG
ST6GalNAc-4*	GAUAUAGCAGUGUGCCAGAUGGGAA	UUCCCAUCUGGCACACUGCUAUAUC
ST8Sia-1	CCCUGCCCAUCUCUUUGCUAUGACU	AGUCAUAGCAAAGAGAUGGGCAGGG
ST8Sia-2	GGGAACCCUGAAGCCUGGAGAUAUU	AAUAUCUCCAGGCUUCAGGGUUCCC
ST8Sia-3	GGGCCUUCUUUGGAUCCCUGCAUU	AAUGCAGGGAUCCAAAGAAUGGCCC
ST8Sia-4	GCUGGCUCUUCAAUCUUCCAGCACA	UGUGCUGGAAGAUUGAAGAGCCAGC
ST8Sia-5	GCCUGGAGCUGAGGCACGAAAUAUU	AAUAUUUCGUGCCUCAGCUCCAGGC

*ST6GalNAc-3 is not present in humans.