

The β -Glucuronidase Klotho Hydrolyzes and Activates the TRPV5 Channel

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Blood calcium concentration is maintained within a narrow range despite large variations in dietary input and body demand. The Transient Receptor Potential ion channel TRPV5 has been implicated in this process. We report here that TRPV5 is stimulated by the mammalian hormone klotho. Klotho, a β -glucuronidase, hydrolyzes extracellular sugar residues on TRPV5, entrapping the channel in the plasma membrane. This maintains durable calcium channel activity and membrane calcium permeability in kidney. Thus, klotho activates a cell surface channel by hydrolysis of its extracellular N-linked oligosaccharides.

Calcium, an essential ion in all organisms, plays a crucial role in processes ranging from formation and maintenance of the skeleton to temporal and spatial regulation of neuronal function. Concentration of blood Ca^{2+} decreases with age in both men and women, and several studies suggest that this is linked to aging-associated disorders. The Ca^{2+} balance is maintained by the concerted action of three organ systems—the gastrointestinal tract, bone, and kidney. Until recently, the mechanism by which Ca^{2+} ions enter the absorptive epithelia was unknown. A major breakthrough came with the identification of an epithelial Ca^{2+} channel family consisting of two members of the Transient Receptor Potential (TRP) superfamily, TRPV5 and TRPV6 (1, 2). TRPV5 is predominantly involved in renal Ca^{2+} handling, whereas TRPV6 is postulated to mediate intestinal Ca^{2+} absorption. TRPV5 and TRPV6 are the most Ca^{2+} -selective channels in the TRP superfamily.

Mice lacking TRPV5 display diminished renal Ca^{2+} reabsorption despite enhanced levels of the calciotropic hormone vitamin D, causing severe hypercalciuria (3). In addition, compensatory hyperabsorption of dietary Ca^{2+} was observed in the intestines of TRPV5 knockout mice. Furthermore, these mice exhibit abnormalities in bone structure, including reduced trabecular and cortical bone thickness (3). In the process of identifying regulatory proteins that maintain the Ca^{2+} balance, we identified klotho as a gene whose expression is decreased in the kidneys of TRPV5 knockout mice. Klotho, a type I membrane glycoprotein of 130 kD (4), is abundantly expressed in mouse kidney as determined by quantitative real-time polymer-

ase chain reaction analysis of mRNA (fig. S1) (4, 5). Klotho mRNA expression was at least 500 times lower in all other analyzed tissues, including brain, lung, muscle, heart, liver, spleen, duodenum, ileum, and bone. Immunoblotting indicated a ~130-kD protein in mouse kidney lysates and urine (Fig. 1B).

Inactivation of the klotho gene in mice (4) resulted in a syndrome resembling human aging, including short life span, bone aberrations, infertility, skin atrophy, and hypercalcemia, as well as an increase in serum vitamin D. Klotho exhibits homology of 20 to 40% at the amino acid level to β -glucosidase enzymes of bacteria, plants, and eukaryotes (4, 6). In general, β -glucosidases participate in the synthesis and degradation of polysaccharides that are involved in processes such as pathogen defense (7), control of signal transduction (8), and modification of hormones (9). In humans, inheritable deficiencies of glycosidases induce a variety of impairments, such as lysosomal storage diseases, Gaucher's and Krabbe's disease, and lactose intolerance (10). In addition to the aging-associated disorders

observed in the klotho-null mice, polymorphisms in the klotho gene have been linked to reduced bone mineral density in humans (11, 12). However, the molecular function of klotho and the downstream targets of this hormone remain to be identified.

Previous studies have indicated that the expression of klotho and TRPV5 is tightly controlled by vitamin D, suggesting a functional link between these proteins in the maintenance of the Ca^{2+} balance (1, 13). On the basis of this correlation and an overlap in the pathophysiology of TRPV5 and klotho knockout mice (i.e., disturbed Ca^{2+} homeostasis and vitamin D metabolism), we investigated whether TRPV5 is a downstream target of klotho. Immunohistochemical analysis of mouse kidney cells revealed colocalization of TRPV5, the vitamin D-sensitive Ca^{2+} -transporting protein calbindin- $\text{D}_{28\text{K}}$, and klotho in the distal convoluted and connecting tubule, which are nephron segments responsible for active trans-epithelial Ca^{2+} reabsorption (Fig. 1A). Although klotho mutant mice show systemic aging phenotypes, only limited organs express the klotho gene (4).

When TRPV5 was expressed with klotho in human embryonic kidney (HEK293) cells, TRPV5-mediated $^{45}\text{Ca}^{2+}$ influx increased (Fig. 2A). Because klotho is detectable in urine, serum, and cerebrospinal fluid (14), it may operate from the extracellular site to regulate TRPV5 activity. To investigate this mechanism, transfected HEK293 cells expressing TRPV5 (HEK293-TRPV5) were incubated (16 hours) with culture media (supernatant) collected from klotho-expressing cells.

Klotho was detected in supernatant obtained from transfected HEK293 cells expressing klotho (Fig. 2B). TRPV5 activity increased by 178% when cells were treated with supernatant (Fig. 2B). Klotho did not alter TRPV5 expression, suggesting a specific effect on channel kinetics or trafficking

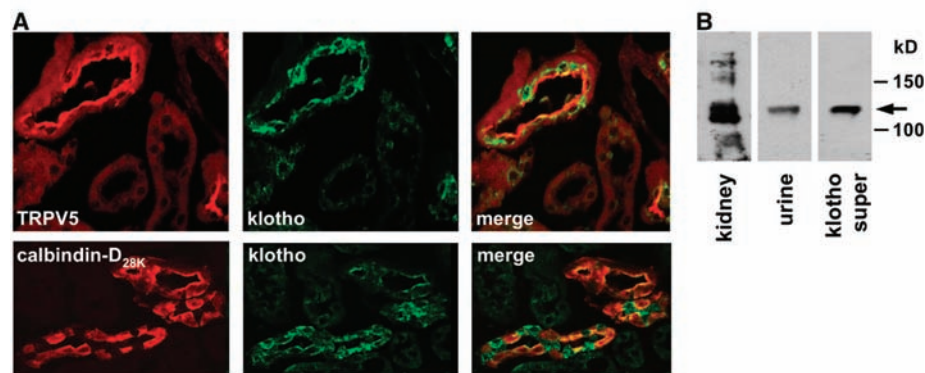


Fig. 1. Localization and expression of klotho. (A) Immunohistochemical analysis of klotho (green), TRPV5 (red), and calbindin- $\text{D}_{28\text{K}}$ (red) in mouse kidney sections. (B) Protein lysates were prepared from mouse kidney and from urine ($n = 3$). Both samples were analyzed for the expression of klotho proteins (indicated by arrow) by immunoblotting with the rat antibody to klotho. Klotho-containing supernatant from transfected HEK293 cells expressing klotho was included as a positive control.

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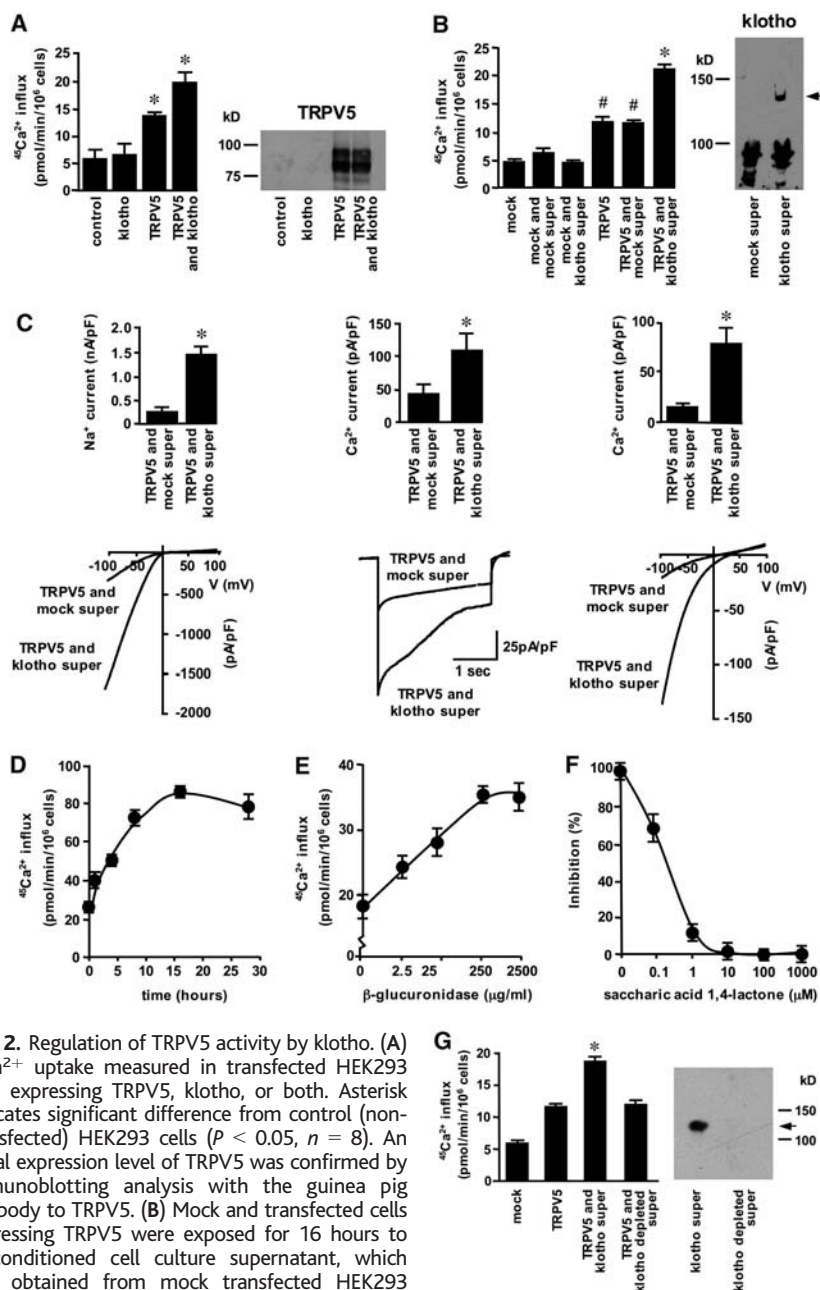


Fig. 2. Regulation of TRPV5 activity by klotho. (A) $^{45}\text{Ca}^{2+}$ uptake measured in transfected HEK293 cells expressing TRPV5, klotho, or both. Asterisk indicates significant difference from control (non-transfected) HEK293 cells ($P < 0.05$, $n = 8$). An equal expression level of TRPV5 was confirmed by immunoblotting analysis with the guinea pig antibody to TRPV5. (B) Mock and transfected cells expressing TRPV5 were exposed for 16 hours to preconditioned cell culture supernatant, which was obtained from mock transfected HEK293 cells (mock super) or transfected HEK293 cells expressing klotho (klotho super) and subsequently analyzed for $^{45}\text{Ca}^{2+}$ influx. Asterisk indicates significant difference from HEK293-TRPV5 cells treated with supernatant from mock-transfected cells. Pound sign (#) indicates significant difference from mock-transfected HEK293 cells ($P < 0.05$, $n = 6$). The supernatants of mock and klotho-transfected HEK293 cells were analyzed by immunoblotting analysis with the use of the mouse antibody to hemagglutinin (HA) to detect the secreted klotho that is epitope tagged with HA (indicated by arrow). (C) HEK293-TRPV5 cells were treated with klotho-containing supernatant or mock supernatant and analyzed by patch-clamp analysis. The cells treated with klotho-containing supernatant displayed significantly increased currents compared with the mock supernatant. Na^+ current at -80 mV: -1526 ± 250 pA/pF versus -299 ± 106 pA/pF ($n = 6$, left); Ca^{2+} peak current: -115 ± 25 versus -45 ± 10 pA/pF ($n = 6$, middle); and Ca^{2+} current at -80 mV: 88 ± 7 versus -14 ± 5 pA/pF ($n = 6$, right). Errors are SEM. Averaged current responses to voltage ramps and hyperpolarizing pulses are depicted below the histograms. (D) HEK293-TRPV5 cells were treated with klotho-containing supernatant for the indicated time and analyzed for $^{45}\text{Ca}^{2+}$ influx ($n = 3$). (E) HEK293-TRPV5 cells were treated for 16 hours with β -glucuronidase at the indicated concentrations and analyzed for $^{45}\text{Ca}^{2+}$ uptake ($n = 3$). (F) HEK293-TRPV5 cells were treated with klotho-containing supernatant in the presence of D-saccharic acid 1,4-lactone, a specific inhibitor of β -glucuronidase (16) ($n = 3$). (G) Klotho was immunoprecipitated from the klotho-containing supernatant and the remaining fluid was analyzed for klotho expression (klotho depleted super). HEK293-TRPV5 cells were treated with depleted and klotho-containing supernatant and analyzed for $^{45}\text{Ca}^{2+}$ influx. Asterisk indicates significant difference from HEK293-TRPV5 cells ($P < 0.05$, $n = 3$). Error bars show means \pm SEM in (A) to (C) and (G); error bars show means \pm SEM in (D) to (F).

toward the plasma membrane. HEK293-TRPV5 cells were treated (16 hours) with klotho-containing supernatant and analyzed by whole-cell patch-clamp analysis. Both the Na^+ and Ca^{2+} currents were stimulated by klotho treatment (Fig. 2C). Measurements in divalent-free solution (EDTA 0.1 mM) revealed increased channel activity (Fig. 2C). Application of a hyperpolarizing voltage step from a holding potential of $+70$ to -100 mV induced an increased Ca^{2+} peak current, whereas no differences in the Ca^{2+} -dependent inactivation behavior of the channel were observed (Fig. 2C). By applying a voltage ramp to cells perfused with a solution containing 10 mM Ca^{2+} , a rise in current amplitude was obtained (Fig. 2C). TRPV5 is a constitutive active Ca^{2+} channel with an open probability of nearly 100% (15). Collectively, these findings imply an increased abundance of TRPV5 at the plasma membrane upon exposure to extracellular klotho. The stimulatory effect of klotho on TRPV5 activity was maximal at 16 hours of incubation, but increased channel activity was apparent after 2 hours (Fig. 2D). Because klotho exhibits β -glucuronidase activity (16), we examined whether purified β -glucuronidase from bovine liver (Enzyme Commission number 3.2.1.31) could mimic the stimulatory effect of klotho on TRPV5 channel activity. HEK293-TRPV5 cells were incubated (16 hours) with increasing concentrations of β -glucuronidase. β -glucuronidase mimicked the effect of the klotho-containing supernatant on TRPV5 channel activity, with a maximal stimulation at 250 $\mu\text{g/ml}$ (Fig. 2E). To further characterize whether the β -glucuronidase activity of klotho is responsible for the stimulatory action, we assessed the effect of D-saccharic acid 1,4-lactone, a klotho inhibitor (16). Klotho-mediated increase of TRPV5 activity was blocked by this inhibitor in a dose-dependent manner, with an apparent median inhibitory concentration (IC_{50}) of 0.5 μM (Fig. 2F). This demonstrates that the stimulatory action of klotho can be attributed to its β -glucuronidase activity. Supernatant that was depleted of klotho by immunoprecipitation failed to increase TRPV5 channel activity (Fig. 2G). Together, these findings demonstrate that the β -glucuronidase activity of klotho activates TRPV5.

The effect of klotho and β -glucuronidase was further evaluated in primary cell cultures from rabbit kidney cells. Connecting tubules and cortical collecting ducts form confluent monolayers that exhibit many characteristics of the original polarized epithelium, including parathyroid hormone and vitamin D-stimulated transepithelial Ca^{2+} transport (17). The addition of klotho-containing supernatant or β -glucuronidase to the apical side of the cell monolayers stimulated transcellular Ca^{2+} transport in these primary kidney cells (Fig. 3).

To elucidate the molecular mechanism underlying the stimulatory effect of extracellular klotho on TRPV5 channel activity, we examined the role of glycosylation in this process. Heterologous expression of TRPV5 in

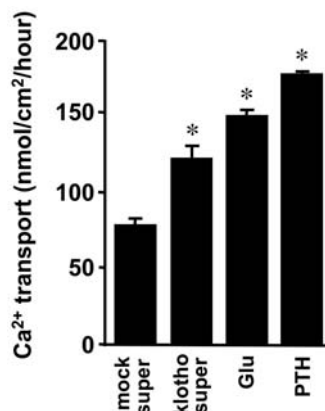


Fig. 3. Transepithelial Ca²⁺ transport is stimulated by klotho. Monolayers of rabbit connecting tubules and cortical collecting ducts primary cell cultures were apically treated with the supernatant from klotho-expressing cells or mock-transfected cells for 16 hours. Net apical to basolateral Ca²⁺ transport was measured in the presence of mock supernatant (apical), klotho-containing supernatant (apical), 250 μg/ml β-glucuronidase (Glu, apical), or 10 nM parathyroid hormone (PTH, basolateral). Asterisk indicates significant difference from cell cultures treated with mock supernatant (*P* < 0.05, *n* = 6). Error bars show means + SEM.

HEK293 cells and subsequent immunoblot analysis of cell lysates revealed TRPV5 with a molecular size ranging from 75 to between 90 and 100 kD (Fig. 4A). These protein forms were not detected in control cells. The protein of 75 kD reflects the core TRPV5 protein, whereas the protein of 90 to 100 kD represents the complex glycosylated form (18). Sequence analysis predicts an N-glycosylation site at an asparagine residue (N³⁵⁸) positioned between transmembrane segments 1 and 2. The functional role of glycosylation in klotho-mediated TRPV5 stimulation was investigated by mutating this asparagine residue to glutamine (TRPV5-N358Q). This prevented TRPV5 glycosylation when expressed in HEK293 cells, but the cells showed normal Ca²⁺ influx that was not different from cells expressing wild-type TRPV5 channels (Fig. 4A). The stimulatory effect of klotho or β-glucuronidase on TRPV5 was abolished in the HEK293 cells expressing the TRPV5-N358Q mutant, whereas HEK293-TRPV5 cells showed an increased Ca²⁺ influx activity (Fig. 4A). These results indicated that abolishment of glycosylation prevents accumulation of TRPV5 in the plasma membrane. This suggests that the klotho-mediated TRPV5 accumulation is due to the specific hydrolysis of extracellular sugar residues from the channel protein. Because the TRPV5-N358Q mutant did not accumulate in the plasma membrane upon klotho treatment, the klotho-modified TRPV5 glycan is required for trapping the wild-type channel in the plas-

ma membrane. Possibly, the klotho-mediated hydrolysis of TRPV5 exposes a new sugar residue that is a candidate of a plasma membrane retention mechanism. Klotho also increased the activity of the highly homologous TRPV6 channel, whereas a glycosylation-deficient TRPV6 (N357Q) mutant failed to respond to klotho or to the β-glucuronidase (fig. S2). Thus, klotho stimulates TRPV channel activity by sugar hydrolysis at the conserved glycosylation site between transmembrane segments 1 and 2.

To investigate klotho-mediated hydrolysis of the glycosylated TRPV5 protein, HEK293 cells transiently expressing the channel were labeled for 48 hours with D-[U-¹⁴C]glucose. After 32 hours of labeling, the cells were incubated for 16 hours with klotho-containing supernatant, mock supernatant, or β-glucuronidase. TRPV5 was subsequently immunoprecipitated. Incubation of the HEK293-TRPV5 cells with klotho-containing supernatant or β-glucuronidase caused a decrease in D-[U-¹⁴C]glucose-labeled TRPV5, indicating cleavage of extracellular ¹⁴C-sugars (Fig. 4B). This indicates that klotho exhibits β-glucuronidase activity by hydrolyzing sugars from glycosylated TRPV5 channels.

To determine the functional consequences of the klotho action on TRPV5, the plasma membrane expression of TRPV5 was examined in klotho- and mock-treated HEK293-TRPV5 cells. Klotho-containing supernatant increased the plasma membrane TRPV5 expression, as determined by membrane surface

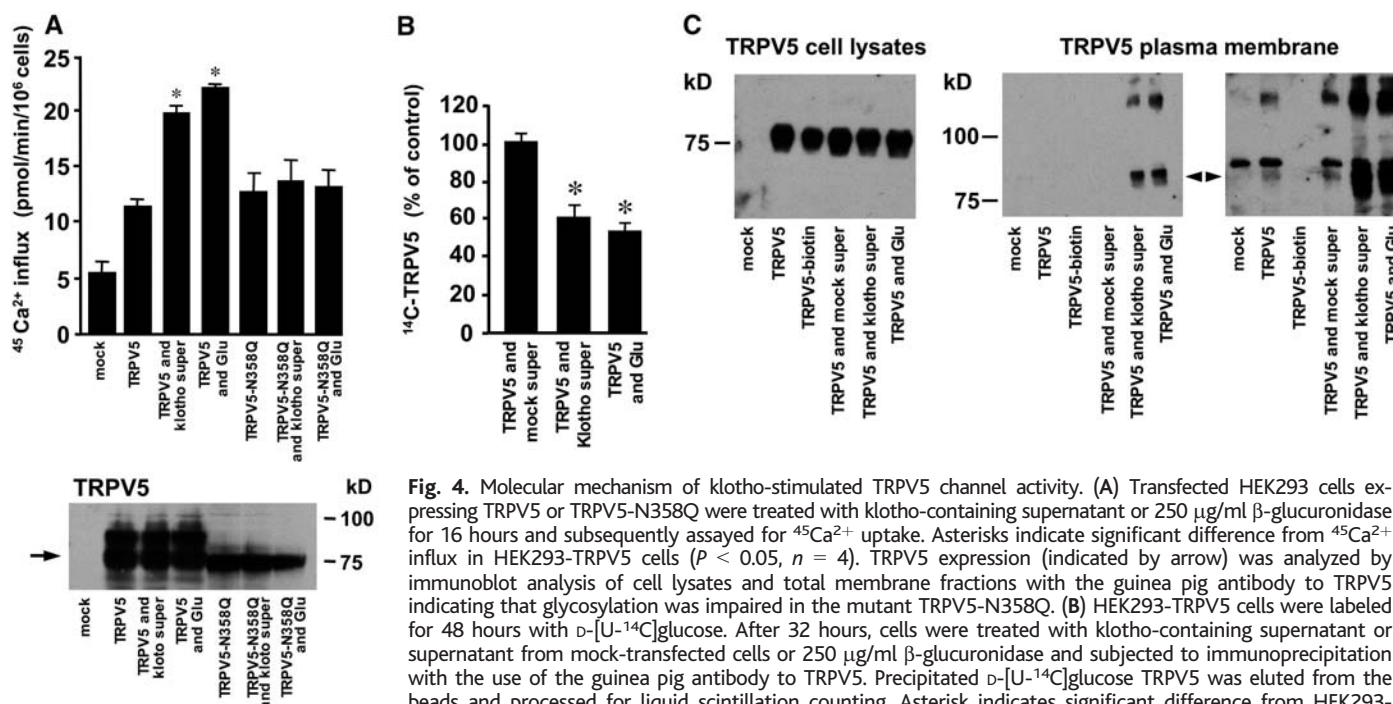


Fig. 4. Molecular mechanism of klotho-stimulated TRPV5 channel activity. (A) Transfected HEK293 cells expressing TRPV5 or TRPV5-N358Q were treated with klotho-containing supernatant or 250 μg/ml β-glucuronidase for 16 hours and subsequently assayed for ⁴⁵Ca²⁺ uptake. Asterisks indicate significant difference from ⁴⁵Ca²⁺ influx in HEK293-TRPV5 cells (*P* < 0.05, *n* = 4). TRPV5 expression (indicated by arrow) was analyzed by immunoblot analysis of cell lysates and total membrane fractions with the guinea pig antibody to TRPV5 indicating that glycosylation was impaired in the mutant TRPV5-N358Q. (B) HEK293-TRPV5 cells were labeled for 48 hours with D-[U-¹⁴C]glucose. After 32 hours, cells were treated with klotho-containing supernatant or supernatant from mock-transfected cells or 250 μg/ml β-glucuronidase and subjected to immunoprecipitation with the use of the guinea pig antibody to TRPV5. Precipitated D-[U-¹⁴C]glucose TRPV5 was eluted from the beads and processed for liquid scintillation counting. Asterisk indicates significant difference from HEK293-TRPV5 cells treated with mock supernatant (*P* < 0.05, *n* = 6). Error bars show means + SEM. (C) HEK293 cells

were transfected with TRPV5 and treated for 16 hours with klotho-containing supernatant, mock supernatant, or 250 μg/ml β-glucuronidase. TRPV5 expression was determined by immunoblotting in total cell lysates to demonstrate equal expression (left). Cell surface proteins were biotinylated extracellularly, precipitated with streptavidin-agarose beads and immunoblotted for TRPV5 (middle, low exposure; right, high exposure). As a negative control, biotin was omitted in the procedure.

protein biotinylation, explaining the observed increase in channel activity (Fig. 4C). Treatment of cells with mock supernatant had no effect. Total expression of TRPV5 in cell lysates was equivalent in all conditions (Fig. 4C). The klotho-mediated increase in channel abundance was mimicked by the addition of 250 μ M β -glucuronidase, indicating that extracellular hydrolysis of TRPV5-associated carbohydrates and consequent plasma membrane accumulation depends on the glucuronidase activity of the klotho protein.

N-glycosylation of proteins is highly conserved from yeast to human and has a stabilizing effect on protein structure that facilitates proper folding and trafficking of membrane proteins. Protein glycosylation has been implicated in cellular functions such as modulating protein structure and localization, cell-cell recognition, and signaling in multicellular systems. Protein glycosylation can be modulated by adaptations in the biosynthetic pathway. For instance, dietary variations and hormones such as estradiol can affect the display of cell-surface carbohydrate epitopes

(19). Our study shows that a glycosylated cell surface protein, TRPV5, can be controlled by the extracellular glucuronidase klotho to regulate its biological function. Because klotho and TRPV5 colocalize in the distal part of the nephron, klotho could exert its stimulatory effect by an auto- and/or paracrine mechanism. Furthermore, TRPV5 and klotho are both positively regulated by the biosynthetic vitamin D pathway. This concomitant control will ensure high Ca^{2+} transport capacity and reduce Ca^{2+} excretion to preserve normal blood Ca^{2+} levels during periods of dietary Ca^{2+} insufficiency.

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Supporting Online Material

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Materials and Methods
References

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Reciprocal Interference Between Specific CJD and Scrapie Agents in Neural Cell Cultures

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Infection of mice with an attenuated Creutzfeldt-Jakob disease agent (SY-CJD) interferes with superinfection by a more virulent human-derived CJD agent (FU-CJD) and does not require pathological prion protein (PrPres). Using a rapid coculture system, we found that a neural cell line free of immune system cells similarly supported substantial CJD agent interference without PrPres. In addition, SY-CJD prevented superinfection by sheep-derived Chandler (Ch) and 22L scrapie agents. However, only 22L and not Ch prevented FU-CJD infection, even though both scrapie strains provoked abundant PrPres. This relationship between particular strains of sheep- and human-derived agents is likely to affect their prevalence and epidemic spread.

In transmissible spongiform encephalopathies (TSEs) such as human CJD, sheep scrapie, and bovine spongiform encephalopathy (BSE), B and T cell adaptive immune responses to a foreign infectious agent have not been detected (1). Nonetheless, an attenuated CJD agent, designated SY, was able to prevent superinfection by the more virulent and rapidly lethal FU-CJD agent (2). These experiments exploited two human CJD agents that, when passaged in mice,

were readily distinguished by profound differences in the incubation time to disease and the distribution of brain lesions. The attenuated "slow" SY produced only small medial thalamic lesions typical of sporadic CJD in mice, whereas the virulent "fast" FU strain, isolated only in Japan, caused widespread severe lesions with many amyloid deposits (Table 1). Clear protective effects of SY-CJD against superinfection by FU-CJD were demonstrable with both intracerebral and peripheral challenges, and SY-protected mice could live free of disease for >600 days, a typical mouse life span (3, 4). By comparison, there was minimal interference between scrapie strains 22C and 22A (5). This raised the possibility that protective effects might be restricted to particular strains of

CJD or to unusual agent strain combinations. We sought ways to evaluate interference between different combinations of TSE agents, and to determine whether apparently unrelated agents—such as those propagated from human CJD and from sheep scrapie cases—could be antagonistic.

Mice can respond to CJD agents through a variety of myeloid cell and innate defense mechanisms (6–8). Thus, it was relevant to determine whether different agent strains could prevent superinfection in simplified cell cultures that lack B, T, and myeloid cells. Neural cells, which can be susceptible to TSE agents, would be incapable of producing many of the myeloid cell cytokines that can participate in strain interference in vivo. If interference could be demonstrated in neural cells, it would show that more universal cellular pathways are sufficient for protection. These culture models also might be used to identify crucial, and possibly novel, molecular pathways of innate immunity to TSE agents.

We developed a rapid, simple, and flexible test of interference in GT1-7 cells (hereafter called GT cells), a murine hypothalamic cell line. We previously found that these cells support the replication of a variety of mouse-passaged CJD and scrapie agents (Table 1) (9, 10). A neomycin-resistant plasmid was introduced into the target GT cells (GTneo) to allow their selection by G418 antibiotic treatment (11) (Fig. 1). Infected GT challenge cells were killed by adding G418 to cocultures, and the pure GTneo target cells were then passaged and assayed for PrPres, a surrogate marker for infection in GT cells. Although PrPres does not quantitatively correlate with infectious

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