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# Post-Translational Loss of Renal TRPV5 Calcium Channel Expression, $Ca^{2+}$ Wasting, and Bone Loss in Experimental Colitis

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## Abstract

**Background & Aims—**Dysregulated $Ca^{2+}$ homeostasis likely contributes to the etiology of IBD-associated loss of bone mineral density (BMD). Experimental colitis leads to decreased expression of Klotho, a protein which supports renal $Ca^{2+}$ reabsorption by stabilizing TRPV5 channel on the apical membrane of distal tubule epithelial cells.

**Methods—**Colitis was induced in mice via administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) or transfer of $CD4^{+}IL10^{-/-}$ and $CD4^{+}$, $CD45RB^{hi}$ T cells. We investigated changes in bone metabolism, renal processing of $Ca^{2+}$, and expression of TRPV5.

**Results—**Mice with colitis had normal serum levels of Ca2+ and parathormone. Computed tomography analysis demonstrated decreased density of cortical and trabecular bone, and there was biochemical evidence for reduced bone formation and increased bone resorption. Increased



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fractional urinary excretion of Ca2+ was accompanied by reduced levels of TRPV5 protein in distal convoluted tubules, with a concomitant increase in TRPV5 sialylation. In mIMCD3 cells transduced with TRPV5 adenovirus, the inflammatory cytokines tumor necrosis factor (TNF), interferon (IFN)γ, and interleukin 1β reduced levels of TRPV5 on the cell surface, leading to its degradation. Cytomix induced interaction between TRPV5 and UBR4, an E3 ubiquitin ligase; knockdown of UBR4 with small interfering RNAs prevented cytomix-induced degradation of TRPV5. The effects of cytokines on TRPV5 were not observed in cells stably transfected with membrane-bound Klotho; TRPV5 expression was preserved when colitis was induced with TNBS in transgenic mice that overexpress Klotho or in mice with T-cell transfer colitis injected with soluble recombinant Klotho.

**Conclusion—**Following induction of colitis in mice via TNBS administration or T-cell transfer, TNF and IFNγ reduce expression and activity of Klotho, which would otherwise protect TRPV5 from hyper-sialylation and cytokine-induced TRPV5 endocytosis, UBR4-dependent ubiquitination, degradation, and urinary wasting of $Ca^{2+}$.

### Keywords

ulcerative colitis; UC; mouse model; osteoporosis

## INTRODUCTION

Loss of BMD is one of the more common extra-intestinal symptoms of Inflammatory Bowel Diseases (IBD). The relative risk of fracture in IBD patients has been estimated to be 40% higher than in general population, with the prevalence of osteopenia and osteoporosis varying significantly depending on the study populations, geographic location and study design, and fall within the range of 22%–77% for osteopenia and 17%–41% for osteoporosis[1]. There are many risk factors predisposing for the loss of BMD in IBD patient population, as we reviewed earlier[2]. One of the potential mechanisms contributing to the IBD-associated bone loss is impaired mineral homeostasis, renal and intestinal $Ca^{2+}$ (re)absorption in particular. Malnutrition together vitamin D insufficiency/deficiency are considered as important factors in the pathogenesis of bone loss in IBD. It is also plausible that decreased expression and activity of key $Ca^{2+}$ transport proteins in the gut and kidney, induced by inflammatory mediators, may be the underlying cause of the reported failures of dietary $Ca^{2+}$ and vitamin D supplementation in IBD[3, 4]. Indeed, in mouse model of Crohn's-like ileitis (TNF-overexpressing TNFΔARE mice), both duodenal and renal $Ca^{2+}$ absorptive epithelia displayed significant down-regulation of TRPV6, calbindin D9K, plasma membrane $Ca^{2+}$ ATPase (PMCA1b), as well as calbindin D28k, and $Na^{+}/Ca^{2+}$ exchanger (NCX1), respectively[5]. In this report, changes in expression of these $Ca^{2+}$ transport genes were accompanied with increased bone resorption, as documented by tomography scanning and increased total deoxypyridinoline in serum of the TNFΔARE mice. Although bone density in IBD patients poorly correlates with cumulative steroid exposure, steroids certainly contribute to the pathogenesis of osteopenia and osteoporosis. One likely mechanism was described by Huybers et al.[6], who demonstrated that in mice oral methylprednisolone significantly decreases duodenal $Ca^{2+}$ absorption. Similar to TNFΔARE, this decrease coincided with decreased TRPV6 and calbindin D9k mRNA and protein expression, with unaltered serum $Ca^{2+}$ or $1,25(OH)2D_3$ levels[6].

In 1970, Joseph Kirsner's group demonstrated elevated urinary $Ca^{2+}$ excretion in patients with ulcerative colitis and with granulomatous regional enteritis[7]. However, this observation or the involved molecular mechanisms were not investigated further in IBD or in animal models. Under physiological conditions, the primary site of active renal $Ca^{2+}$ reabsorption is the epithelium of the distal convoluted tubules, and is mediated via apical selective nonvoltage-gated $Ca^{2+}$ channel, TRPV5. $TRPV5^{-/-}$ mice show robust renal $Ca^{2+}$ wasting

and reduced bone thickness despite the compensatory intestinal $Ca^{2+}$ hyperabsorption accompanied by enhanced duodenal TRPV6 expression[8]. TRPV5 is regulated by transcriptional mechanisms (calciotropic hormones including 1,25(OH)2 vitamin $D_3$, parathormone (PTH), and estrogens), by pH- and $Ca^{2+}$-dependent regulatory mechanisms, trafficking of TRPV5 to the luminal membrane and by physical interactions with associated proteins[9]. It is also controlled by renal Klotho, a multifunctional protein which regulates Pi/$Ca^{2+}$ metabolism as well as aging[10]. Klotho has been directly implicated in the regulation of transepithelial $Ca^{2+}$ transport through regulation of both apical entry as well as basolateral $Ca^{2+}$ exit. Through its glucuronidase activity, Klotho modulates N-glycosylation of TRPV5 at $Asn^{358}$ by removal of the capping sialic acid, which enables physical interaction of galectin 1 with TRPV5 on the cell surface and promotes its apical retention and activity [11, 12]. Indeed Klotho has been demonstrated to prevent renal $Ca^{2+}$ loss[13]. Interestingly, we have demonstrated profound effects of experimental colitis and the associated cytokines (TNF and IFNγ) on renal Klotho expression[14, 15], thus suggesting that this may translate into defective function of TRPV5 and impaired renal $Ca^{2+}$ handling in experimental colitis. We propose a scenario whereby during colitis, TNFα and IFNγ act to decrease Klotho expression and activity, which would otherwise protect TRPV5 from hyper-sialylation, and from cytokine-induced TRPV5 endocytosis, UBR4-dependent ubiquitination, degradation, and urinary $Ca^{2+}$ wasting.

## METHODS

### Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Wild type, $Rag1^{-/-}$ and $IL10^{-/-}$ mice (C57BL/6J background) were purchased from the Jackson Laboratory and maintained under speci c-pathogen-free conditions. TRPV5-eGFP transgenic mice, which express eGFP in the epithelium of the late distal convoluted tubules (DCT2), the connecting tubule (CNT), and the initial part of the cortical collecting duct (iCCD)[16], were generously provided by Drs. Jeppe Praetorius and Marlene Vind Hofmeister from the Aarhus University, Denmark. EFmKL46 are transgenic mice overexpressing Klotho under the control of the human elongation factor EF-1α promoter on 129S1/SvImJ background[17].

### Cells, Cytokines, Klotho plasmids, and TRPV5 adenoviral construct

mIMCD3 and MDCK cells, plasmids and adenoviral gene delivery are described in detail in the supplemental Methods section. As cytomix, 100 U/ml of IFNγ, 10 ng/ml of TNFα and 2 ng/ml of IL1β were used throughout the study.

### Experimental colitis

Detailed description of models, including acute TNBS colitis, chronic adoptive T-cell transfer colitis ($CD4^+$ T cells isolated from $IL\text{-}10^{-/-}$ mice transferred into $Rag1^{-/-}$ mice and synchronized with dietary piroxicam) and $CD4CD45RB^{hi}$-T-Cell transfer model (transferred $Rag^{-/-}$ mice with $CD4CD45RB^{hi}$ naïve T cells sorted from WT spleens) is provided in the Supplemental Methods section.

### Real-Time RT-PCR, cytokine and bone turnover analysis

Detailed description of real-time RT-PCR analysis of colonic cytokine expression and the analysis of serum cytokine and bone turnover markers is provided in Supplemental Methods section.

### μCT evaluation of cortical and trabecular bone density

Detailed description of the μCT analysis of mouse femurs is provided in Supplemental Methods section.

### Fractional urinary $Ca^{2+}$ excretion

Plasma (P) and urine (U) creatinine (Cr) and $Ca^{2+}$ were analyzed by commercial assays (Abcam, Cambridge, MA; and BioVision, Milpitas, CA, respectively) from samples harvested at the time of sacrifice. Fractional excretion of $Ca^{2+}$ ($FECa^{2+}$) was calculated according to the following formula:

$$FECa2+=\frac{U[Ca2+]xP[Cr]}{P[Ca2+]xU[Cr]}$$

### In vitro $Ca^{2+}$ uptake

Detailed description of in vitro $Ca^{2+}$ uptake assay[18] in mIMCD3 cells as well as in primary renal tubular cells is provided in Supplemental Methods section.

### Isolation of TRPV5-expressing $GFP^{+}$ renal epithelial cells from TRPV5-eGFP transgenic mice

The use of TRPV5-eGFP transgenic reporter mice[16] for renal tubule isolation is described in more detail in the Supplemental Methods section.

### Cell surface biotinylation, immunoprecipitation, and western blotting

Detailed description of the cell surface biotinylation, immunoprecipitation, and western blot analyses is provided in Supplemental Methods section.

### Immunofluorescence

Detailed description of the protocol used for immunostaining of renal TRPV5 is provided in Supplemental Methods section. Specificity of TRPV5 antibody was verified by immunostaining of adjacent section using mouse monoclonal antibody against distal-convoluted tubule-specific thiazide-sensitive NaCl co-transporter (Millipore, Billerica, MA; data not shown).

### TRPV5 sialylation

Detailed description of the methods used for detection of TRPV5 sialylation in the kidneys of control and colitis mice is provided in Supplemental Methods section. Since colitis results in decreased abundance of total TRPV5, for better visualization, sample loading was adjusted based on TRPV5 densitometric analysis to show equal amount of TRPV5.

### Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) and siRNA knockdown of UBR4

Detailed description the proteomic analysis leading to identification of UBR4 ubiquitin ligase as a TRPV5 binding partner in cymomix-treated distal convoluted tubule cells as well as siRNA-mediated knockdown of UBR4 is provided in Supplemental Methods section.

### Statistical Analysis

Statistical significance was determined by Student t-test or analysis of variance (ANOVA) followed by Fisher PLSD post-hoc test, as appropriate, using SigmaPlot 12 software package (Systat; Chicago, IL). Data are expressed as mean ± standard error of mean (SEM).

# RESULTS

## Development of colitis in TNBS and adoptive T-cell transfer models

TNBS colitis represents a well-characterized model of acute colitis in response to an epithelial injury and a haptenizing agent[19]. Adoptive transfer of $CD4^+$ T cells from IL-10 deficient mice into syngeneic recipients lacking T and B cells and synchronized by dietary exposure to piroxicam induces moderate to severe colitis within 6–8 weeks with symptoms resembling human IBD[20]. The $CD4CD45RB^{hi}$-T-Cell transfer model, is also well-characterized model of chronic colitis and induces both colitis and small bowel inflammation, making this a model similar to Crohn's disease[21].

Development of robust colitis at the end of the study was confirmed by progressive loss of body weight and colonic histology (Fig. 1A, B) as well as colonic expression of TNF, IFNγ, IL17, and IL-1β mRNA (Suppl. Fig. S1).

## Decreased bone mineral density in TNBS and adoptive T-cell transfer colitis

Decreased serum concentration of osteocalcin (more significant in TNBS colitis) and significant increase of serum tDPD suggested a defect in bone remodeling with decreased bone formation, and increased bone resorption, respectively (Fig. 2A, B). Surprisingly, serum levels of RANKL were significantly decreased in both models of colitis (Fig. S2), thus suggesting that in these two models, RANKL does not play a significant role in the pathogenesis of bone loss or that its circulating levels are not reliable markers of bone resorption. Serum levels of TNF were increased in both models, albeit to a greater extent in acute TNBS colitis, while circulating IL-6 was only elevated in TNBS-treated mice (Suppl. Fig. S2). μCT analysis of mouse femurs indicated significant loss of both cortical and trabecular bone in both disease models. This loss of bone mineral density is exemplified by the representative 3D reconstruction images of cortical and trabecular bone in Fig. 2C, D and in the detailed morphometric analyses presented in Suppl. Fig. S3 and S4.

## Impaired renal $Ca^{2+}$ reabsorption and decreased expression of TRPV5 and KLOTHO in renal distal convoluted tubules in experimental colitis

We measured fractional urinary $Ca^{2+}$ excretion ($FECa^{2+}$) based on serum and urine concentration of creatinine and $Ca^{2+}$ at the time of sacrifice. In both models of colitis, $FECa^{2+}$ increased significantly compared to healthy controls (Fig. 3A, B, left panels). We analyzed the expression of cell surface and total TRPV5 and total KLOTHO protein expression in $GFP^+$ renal epithelial cells isolated from control and TNBS-treated TRPV5-eGFP reporter mice. TRPV5 surface and total protein expression was decreased in parallel with decreased KLOTHO (the latter as previously reported[14] (Fig. 3A, middle and right panels). Similar decrease of total TRPV5 and KLOTHO protein expression was observed using total kidney cortex lysate from naïve $Rag1^{-/-}$ mice with T-cell transfer colitis (Fig. 3B, middle and right panels). Immunofluorescence analysis of TRPV5 protein in the distal convoluted tubules in both models of colitis confirmed these results (Fig. 3C).

## Decreased $Ca^{2+}$ uptake and TRPV5 expression in cytokine-treated renal epithelial cells in vitro

Mouse renal intermedullary collecting duct epithelial cells (mIMCD3), which normally do not express TRPV5 or KLOTHO were stably transfected with an empty vector or with plasmid coding for V5-KLOTHO, followed by transduction with adenovirus encoding human TRPV5 and were treated with cytomix for 48 hours. $^{45}Ca^{2+}$ uptake of was performed with background subtraction from cells transduced with non-coding (empty) adenovirus. Consistent with the fractional excretion data, inflammatory cytokines commonly associated with IBD led to significantly reduced TRPV5-dependent $Ca^{2+}$ uptake in mIMCD3 cells not

expressing KLOTHO (Fig. 4A). However, cytomix was not effective in cells stably transfected with KLOTHO (Fig. 4A, B). Cytomix also significantly inhibited $Ca^{2+}$ uptake in isolated primary renal epithelial cells (Suppl. Fig. S5A). Combinations of TNF+IL1β or IFNγ+IL1β were the most effective in reducing the abundance of cell surface TRPV5 in primary renal epithelia (Suppl. Fig. S5B). Short (4 hr) exposure of isolated $GFP^+$ epithelial cells from TRPV5-eGFP mice to cytomix resulted in decreased expression of TRPV5 at the cell surface but not of the total cellular TRPV5, thus suggesting endocytosis as preceding intracellular degradation in response to inflammatory cytokines (Fig. 4C). Longer exposures of the primary epithelial cells could not be performed due to decreasing cell viability in culture.

## Soluble KLOTHO partially prevents the cytokine-induced loss of TRPV5 in vitro and in vivo

KLOTHO participates in specific removal of α2,6-linked sialic acids and regulates cell surface retention of TRPV5 through this activity by facilitating interaction of desialylated TRPV5 with galectin-1[12]. Since we demonstrated that renal expression of KLOTHO is decreased in colitis (Fig. 3A, B and ref.[14]), we investigated the degree of sialylation of TRPV5 by immunoprecipitating the protein from control and TNBS-treated WT mice, and probing with biotinylated sialic acid-specific SNA lectin. Fig. 5A demonstrates representative image depicting higher degree of sialylation of TRPV5 in colitic mice. Since KLOTHO protein is expressed in both membrane bound and soluble form, we tested if recombinant soluble KLOTHO would be capable of reducing the effects of cytomix on TRPV5 endocytosis *in vitro*. Indeed, such effect could be observed in both MDCK and mIMCD3 cells transduced with ad-*hTRPV5* (Fig. 5B). To further confirm this observation, we induced colitis in WT or EFmKL46, which are transgenic mice overexpressing *Klotho* gene under the control of the human elongation factor EF-1α promoter[17]. EFmKL46 mice overexpress KLOTHO in extrarenal tissues only, therefore any potential effect in the kidney would be the result of soluble KLOTHO. Overexpression of KLOTHO did not affect the development or severity of colitis in EFmKL46 mice (data not shown). However, the reduction in total renal TRPV5 expression was much less pronounced in colitic EFmKL46 mice, thus suggesting at least partial protection of TRPV5 by soluble KLOTHO in experimental colitis (Fig. 5C). In the chronic and progressive model of adoptive transfer of $CD4^+CD45RB^{Hi}$ naïve T-cell into Rag1−/− recipients, mice were 6 weeks post-transfer with PBS or 2μg of recombinant KLOTHO i.p. daily for 7 days. KLOTHO injection did not change the course of disease (data not shown). However, TRPV5 protein level in the kidneys was restored in KLOTHO-injected mice (Fig 5D and Fig 5E). Consistently, we observed normalization of urinary fractional $Ca^{2+}$ excretion in colitic mice administered recombinant soluble KLOTHO (Fig 5F).

## Membrane-bound KLOTHO prevents cytokine-induced ubiquitination, loss of cell-surface and total TRPV5 protein expression

Although ubiquitination of TRPV5 has not been previously described, it is a common phenomenon among internalized membrane proteins targeted for degradation. Such mechanism has been recently demonstrated for a close member of the TRPV family; TRPV6, which is ubiquitinated by an archetypal HECT ubiquitin E3 ligase, Nedd4-2[22]. TRPV5 immunoprecipitated from cytomix-treated mIMCD3 cells showed characteristic laddering pattern when reacted with anti-ubiquitin antibody, which increased in intensity between 4 and 8 hours of treatment (Fig. 6A). This was paralleled by decreased cell surface expression of TRPV5, which preceded decreased total TRPV5 abundance at 8 hours (Fig. 6A). Intriguingly, ubiquitination and loss of TRPV5 protein was not observed in cytomix-treated mKL-mIMCD3 cells, which expressed KLOTHO. Cytomix did not impair physical interaction of KLOTHO with TRPV5 in mKL-mIMCD3 cells (Fig. 6B).

### Ubiquitination by UBR4 E3 ubiquitin ligase leads to proteasomal degradation of TRPV5 in response to inflammatory cytokines

Mass spectrometric analysis of co-immunoprecipitated proteins interacting with HA-tagged TRPV5 in mIMCD3 cells treated with cytomix identified multiple proteins among which only one had known ubiquitin ligase activity; UBR4/p600 a relatively well-characterized ubiquitin E3 ligase[23] (Fig. 7A). Colitis did not affect renal expression of UBR4/p600 mRNA (Suppl. Fig. S6). Transfection of mIMCD3 cells with UBR4 siRNA effectively knocked-down expression of UBR4 mRNA and protein (Fig. 7B). Due to the size of UBR4 (ca. 600kDa) and technical difficulties with PAGE resolution and transfer, dot blot was utilized instead of traditional Western blotting. mIMCD3 cells transfected with non-silencing siRNA or UBR4 siRNA were transduced with Ad-*hTRPV5* and treated with the cytomix for 8 hours. Consistent with data presented in Fig. 6, cytomix treatment led to ubiquitination of TRPV5 in cells transfected with non-silencing siRNA; however, dramatically decreased TRPV5 ubiquitination was observed in cells transfected with UBR4-specific siRNA (Fig. 7C). UBR4 knock-down also led do increased baseline expression of TRPV5 and prevented cytomix-induced loss of TRPV5 protein expression (Fig. 7D). Since adenoviral transduction combined with cytokine and proteasome inhibitor treatment resulted in increased cell death, we used stably transfected and flow-sorted mIMCD3 cells expressing GFP-TRPV5 fusion protein. As expected, cytomix reduced expression of TRPV5, but both proteasome inhibitors (MG-132 and clasto-lactacystin β-lactone) effectively eliminated this response indicating a major role for proteasomal degradation pathway (Fig. 7E). Bafilomycin, an inhibitor of lysosomal degradation, was only partially effective suggesting a contribution of lysosomal degradation in the cytokine-induced loss of TRPV5 (Fig. 7E).

## DISCUSSION

Osteopenia and osteoporosis are two of the more common extraintestinal symptoms with a general consensus that IBD patients are at a significantly higher risk of developing metabolic bone disease and low BMD than the healthy subjects. The relative risk of fracture in IBD patients has been estimated to be 40% higher than in general population[1]. While increased osteoclast and decreased osteoblast activity have been implicated in the net bone loss in IBD, the exact molecular basis for reduced BMD has still not been established and is likely multi-factorial. Likely risk factors predisposing to lower BMD in IBD patients include malabsorption of vitamin D, $Ca^{2+}$ and vitamin K, low body mass index, low bone mineral intensity peak in IBD patients with pediatric onset, chronic inflammatory state, type of IBD (CD vs. UC; small intestinal involvement), increasing age, female gender, decreased mobility, and chronic use of corticosteroids. However, recent reports have shown that bone loss occurs even before clinically significant bowel disease has occurred[24] and in pediatric patients, mild cortical bone loss was detected even at the time of diagnosis[25].

Vitamin D insufficiency and deficiency in IBD has received much attention in recent years. However, several studies have shown no relationship between BMD and measured serum $25(OH)D_3$ levels[26,27]. Moreover, studies have indicated that significant proportion of adult and pediatric IBD patients have elevated levels of the bioactive metabolite, $1,25(OH)_2D_3$, which correlates with decreased BMD[28, 29]. Our group recently described that expression of Klotho, a critical regulator of vitamin $D_3$ metabolism and $Ca^{2+}$ homeostasis is decreased in several mouse IBD models[14]. Moreover, we recently demonstrated that high vitamin $D_3$ supplementation during active colitis negatively impacts bone metabolism[30]. These findings indicate a very complex effect of acute and chronic intestinal inflammation on systemic $Ca^{2+}$ homeostasis and bone turnover. In 1970, Breuer et al[7] described elevated urinary $Ca^{2+}$ excretion in patients with ulcerative colitis and granulomatous regional enteritis, an observation confirmed later by Abreu *et al*[28] in CD patients. Huybers *et al*[5] showed that TNFΔARE mice, which develop spontaneous ileitis, have reduced mRNA expression of

duodenal apical TRPV6 $Ca^{2+}$ channel, calbindin-D9k, and basolateral PMCA1b $Ca^{2+}$ pump. The same report highlighted changes in renal $Ca^{2+}$ transporters NCX1 and calbindin-D28k, although with no change in the expression of TRPV5 mRNA. It remained unclear whether the described changes in renal key $Ca^{2+}$ transporters at the transcript levels alone could explain urinary $Ca^{2+}$ losses in IBD patients.

Klotho can directly bind TRPV5 channel at the cell surface and regulate its function by hydrolyzing terminal sialic acid residues of the glycan chains, which could in turn prevent TRPV5 internalization and increase its abundance on the cell surface[12]. This observation suggested that in the face of down-regulated renal Klotho expression in colitis[14], changes in TRPV5 protein rather than its transcript, may be critical in reducing $Ca^{2+}$ reabsorption in the distal convoluted tubules. In the present study, we employed two distinct models of acute and chronic colitis associated with altered bone metabolism and decreased BMD based on μCt analysis. Loss of BMD was paralleled with increased urinary $Ca^{2+}$ excretion which could be reproduced *in vitro* in cells treated with a combination of three prominent mediators of intestinal inflammation. Inflammation-associated loss of TRPV5 protein expression was associated with increased TRPV5 sialylation, which implicated the loss of Klotho expression in this process. Indeed, overexpression of Klotho *in vitro* or in transgenic EFmKL46 mice *in vivo*, prevented the negative effects of inflammatory cytokines on TRPV5 protein and $Ca^{2+}$ absorption. The latter model, in which extrarenal Klotho expression is driven by the human elongation factor EF-1α promoter, suggests that soluble Klotho may be sufficient in preventing the effects of inflammatory cytokines on TRPV5 protein, and opens a potential therapeutic avenue. This notion is further strengthened by our observation that soluble recombinant Klotho prevented cytokine-induced loss of plasma membrane TRPV5 and that physical association of TRPV5 and Klotho was not affected by cytomix treatment *in vitro*.

In the current study, we also describe the fate of TRPV5 protein in response to inflammatory cytokines during Klotho deficiency. Following hyper-sialylation, TRPV5 is internalized and becomes targeted for degradation through the ubiquitin (Ub) system, a mechanism not uncommon in response to cytokine stimulation[31, 32]. Recently, another TRP family member, TRPV6, was shown to be a target of at least one Ub E3 ligase, Nedd4-2[22]. Here, we have identified UBR4, an E3 Ub ligase, as a prominent binding partner of TRPV5 in renal epithelial cells exposed to cytomix. UBR4 has been shown to have E3 ligase activity and it recognizes and binds to proteins bearing specific N-terminal residues that are destabilizing according to the N-end rule. This binding subsequently leads to ubiquitination and degradation of the target protein[23]. A comprehensive study by Nakatani et al.[33] showed that UBR4/p600 localizes in continuous mesh patterns from the nucleus to the cytoplasm and that it perforates the nuclear envelope. Based on their findings, the group also concluded that p600 concentrates at the leading edge of membrane structures. They also described that p600 and clathrin form an integrated structure near the leading edge. Clathrin is known to play an important role in transporting membrane domains between intracellular membrane-bound compartments (including the endosomes) by forming clathrin-coated membrane vesicles[34] and Nakatani et al.[33] postulated that UBR4/p600 is an essential structural component for membrane morphogenesis. Although it is tempting to speculate that UBR4 may be playing a direct role in the internalization of TRPV5 followed by ubiquitination, at present, we don't have the evidence to support this theory. At this point, we hypothesize that the loss of Klotho leads to accumulation of sialylated TRPV5 at the plasma membrane, which creates permissive conditions for the proinflammatory cytokines to trigger endocytosis (or to synergize with intrinsic recycling mechanism) and proteasomal and to a lesser degree lysosomal degradation of the $Ca^{2+}$ channel. Whether UBR4-mediated ubiquitination occurs before, during, or after TRPV5 internalization, or a combination of those, will require additional studies. The end result of the process is decreased renal $Ca^{2+}$

reabsorption and urinary $Ca^{2+}$ wasting. This mechanism, along with the previously implicated changes in intestinal $Ca^{2+}$ absorption, likely contributes to the systemic imbalance of $Ca^{2+}$ homeostasis and to IBD-associated loss of BMD (Fig. 7F). In addition to describing this novel mechanism, our data points to a potential usefulness of recombinant soluble Klotho as adjuvant therapy for osteopenia or osteoporosis in IBD. Hu et al.[35] demonstrated that peripherally administered recombinant soluble Klotho can be identified in the renal tubular lumen by electron immunomicroscopy and can be detected in urine obtained with *in vivo* free-flow micropuncture. The same group demonstrated that in rats injected i.p. with a single dose of soluble recombinant Klotho tagged with an IR dye, the protein persisted in serum for 24 hours and could be detected in urine 20 min after injection and kidneys retained the IR-labeled Klotho for up to two days[36]. Indeed, pre-clinical efficacy of recombinant soluble Klotho has been recently demonstrated by Chen et al.[37] who showed that i.p. injection of soluble Klotho extended the life span of Klotho hypomorphic mice, ameliorated premature aging-related phenotype, attenuated renal fibrosis, and reduced cellular senescence.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **TRPV5** | transient receptor potential vanilloid 5 |
| **DCT** | distal convoluted tubules |
| **PTH** | parathormone |
| **sRANKL** | soluble receptor activator of nuclear factor kappa-B ligand |
| **tDPD** | total deoxypyridinoline crosslinks |

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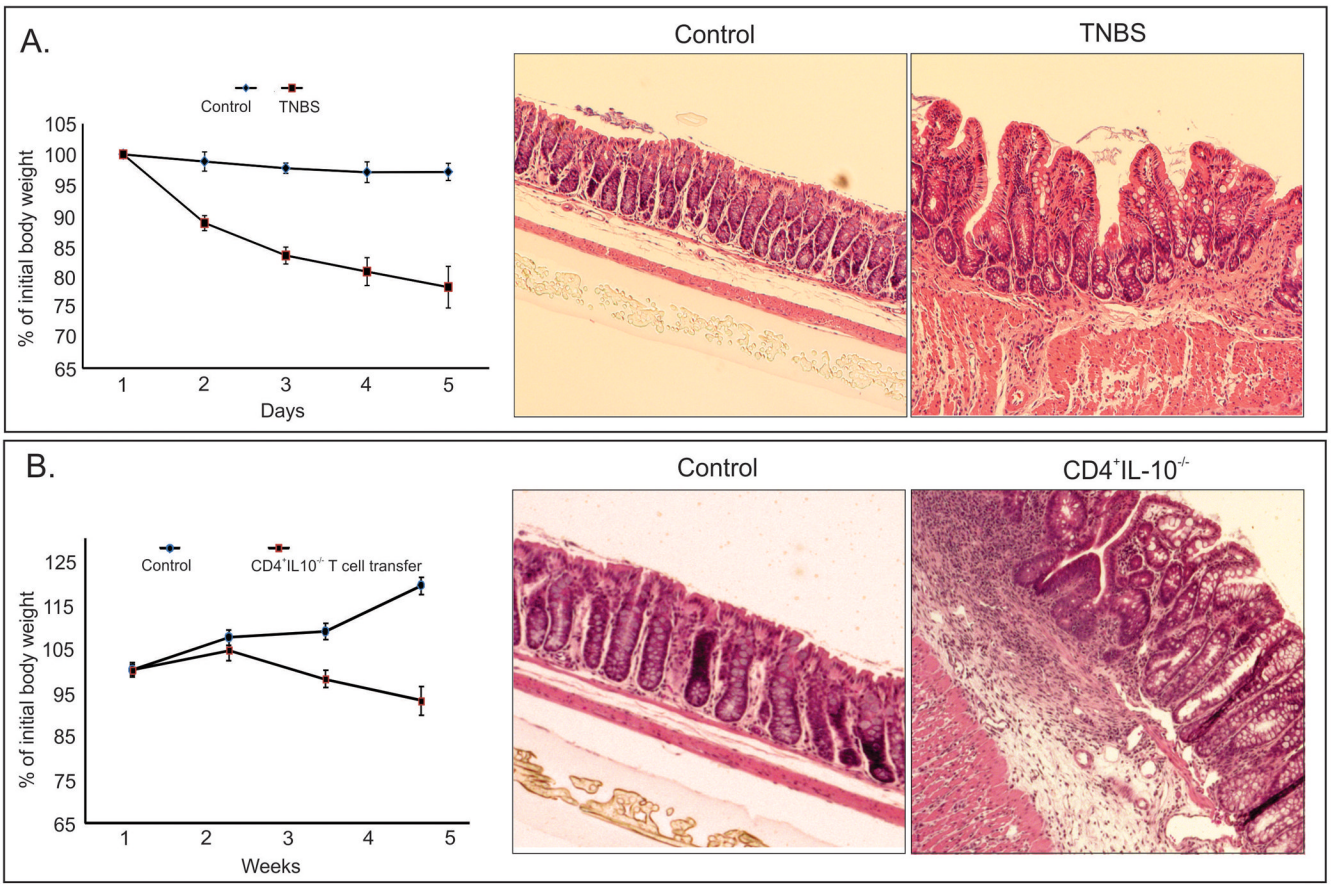


**Fig. 1. Development of colitis in (A) TNBS and (B) $CD4^{+}IL^{-/-}$ adoptive T-cell transfer models**

Body weight changes are shown as percentages of the baseline value and are means ± SEM. (A) Five days after TNBS treatment, haematoxylin and eosin (H&E)-stained colon tissue sections were examined. (B) For T-cell transfer colitis, colon tissues were examined after 6 weeks post T-cell transfer. Data are representative of at least three independent experiments.

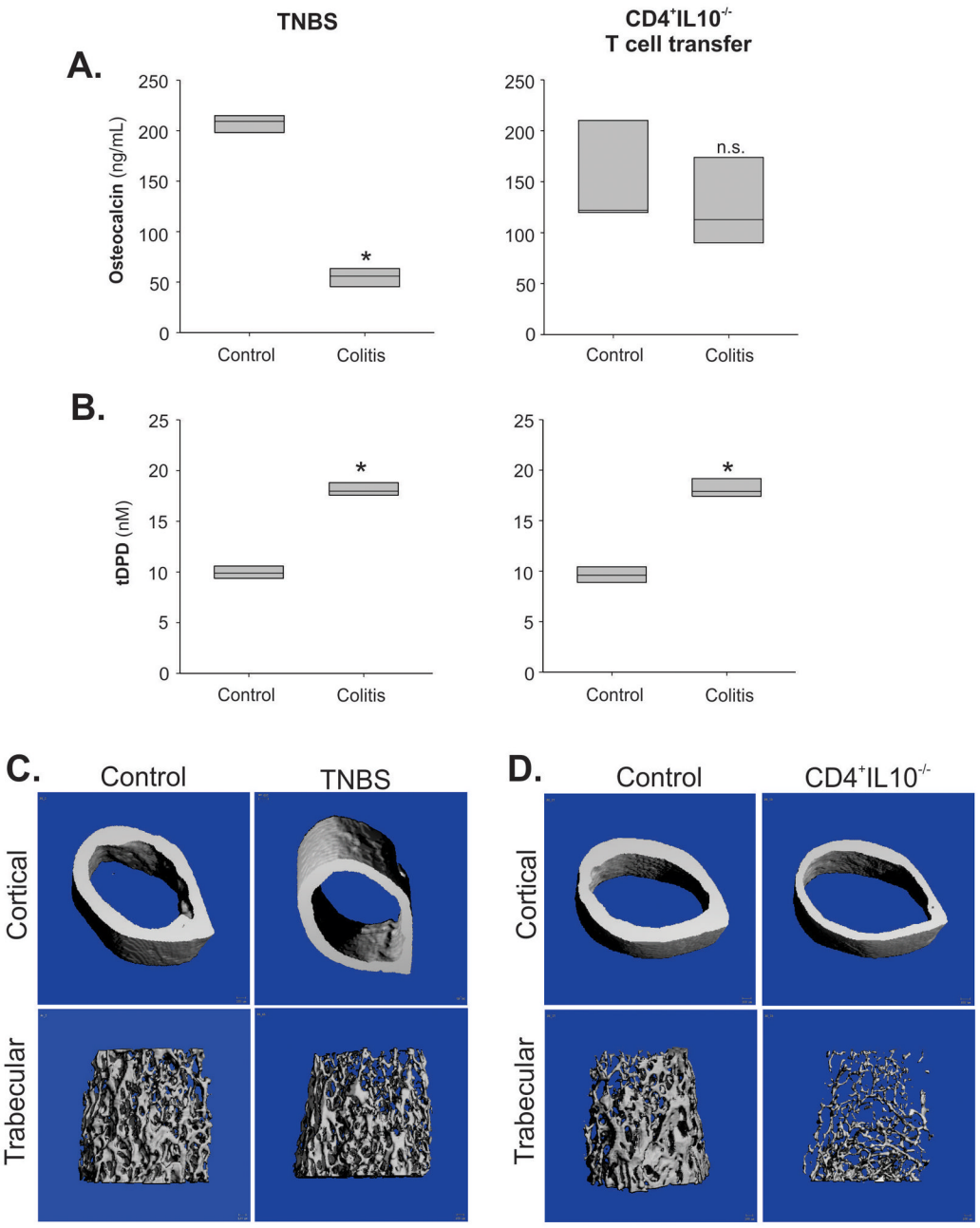


**Fig. 2. Altered bone metabolism and osteopenia in TNBS and CD4$^{+}$IL$^{-/-}$ adoptive T-cell transfer models**

(A) Serum concentration of osteocalcin (bone formation marker) and (B) tDPD (bone resorption marker); * $p<0.05$; t-test. (C and D) Representative μCT 3D-reconstruction of the femoral midshaft, cortical and distal femoral metaphyseal trabecular bone of control and colitic mice.

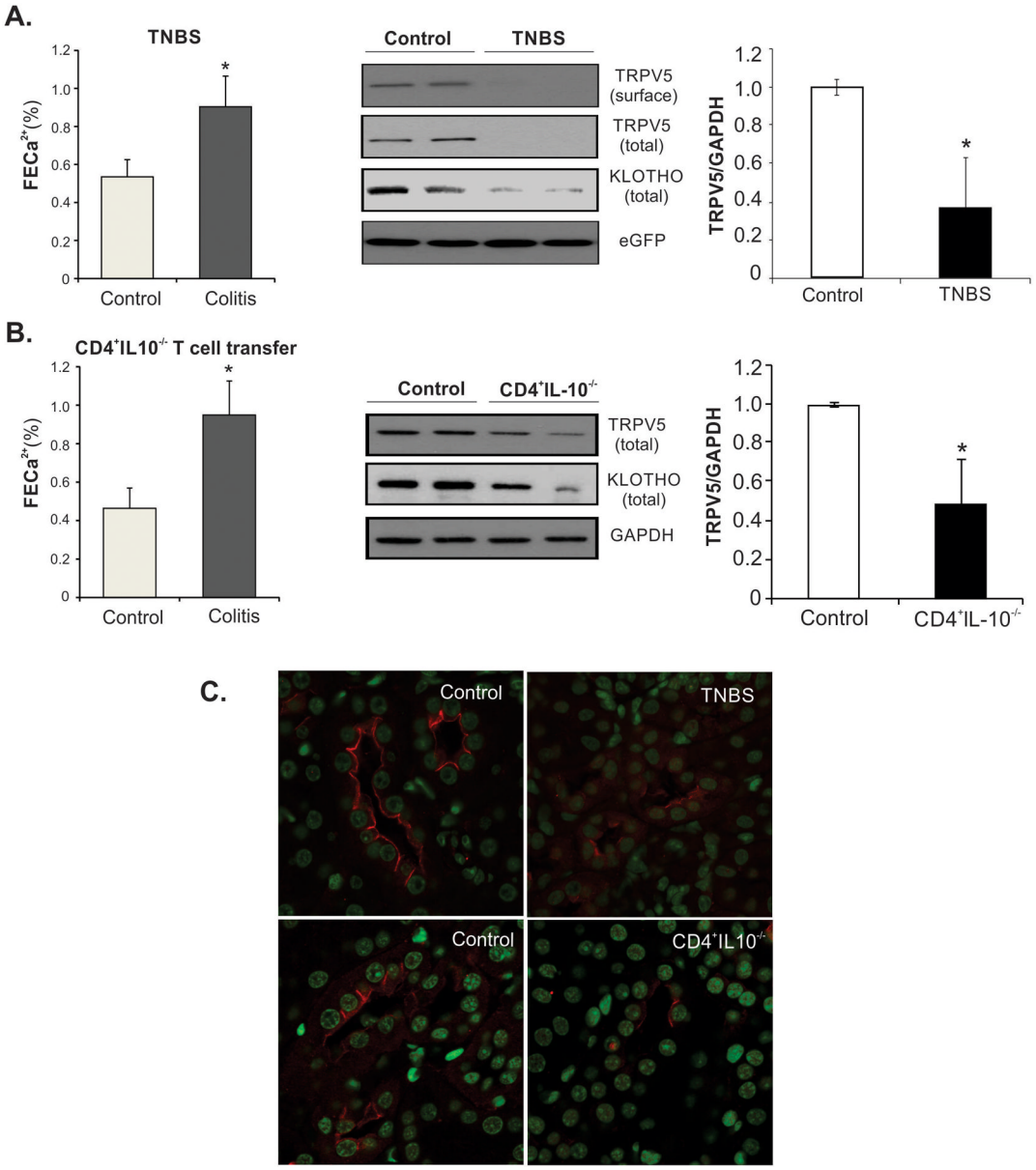


**Fig. 3. Decreased renal $Ca^{2+}$ reabsorption in experimental colitis**

(**A**) TNBS colitis; fractional urinary $Ca^{2+}$ excretion ($FECa^{2+}$) (left panel); cell surface and total TRPV5, and KLOTHO expression in cortical epithelial cells of TNBS-treated TRPV5-eGFP reporter mice (middle panel; eGFP used as a leading control demonstrating equal contribution of TRPV5-expressing epithelial cells of DCT and connecting ducts); summary of Western blot analysis of total TRPV5 protein expression in the renal cortex in TNBS colitis (right panel; mean ± SD, *$p<0.05$; Student t-test). (**B**) $CD4^{+}IL10^{-/-}$ adoptive T-cell transfer model; fractional urinary $Ca^{2+}$ excretion ($FECa^{2+}$) (left panel); total expression of TRPV5 and KLOTHO in the renal cortex (middle panel); summary of Western blot analysis of total TRPV5 protein expression in the renal cortex in T-cell transfer colitis (right panel; mean ± SD, *$p<0.05$; Student t-test). (**C**) Immunofluorescent staining of TRPV5 (red) of the two models of colitis (nuclei counterstained in green).

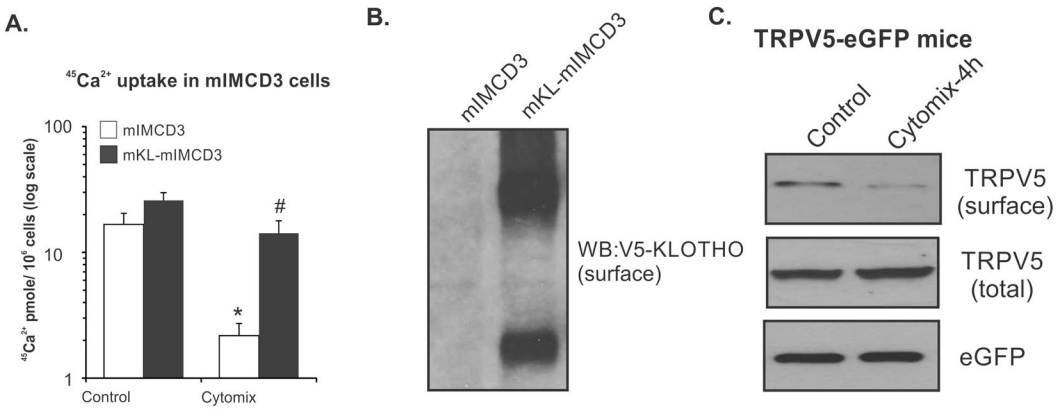


**Fig. 4. Decreased $Ca^{2+}$ uptake and TRPV5 expression in cytokine-treated renal epithelial cells in vitro**

**(A)** TRPV5-mediated $^{45}Ca^{2+}$ uptake in cells stably transfected with empty vector (mIMCD3) or membrane-bound Klotho (mKL-mIMCD3), transduced with Ad-HA-TRPV5 and treated with control medium or cytomix for 48 hours. * $p<0.05$ control mIMCD3 vs. cytomix-treated mIMCD3, # $p<0.05$ cytomix-treated mIMCD3 vs. mKL-mIMCD3; (**B**) Verification of KLOTHO expression in mIMDC3 cells stably transfected with pcDNA3.1/ V5-His-human KL plasmid which codes for full-length human Klotho cDNA. KLOTHO V5 epitope tag was detected by Western blotting. (**C**) Loss of cell surface TRPV5 after short (4-hour) exposure of renal cortical epithelial cells from TRPV5-eGFP Tg mice to cytomix.

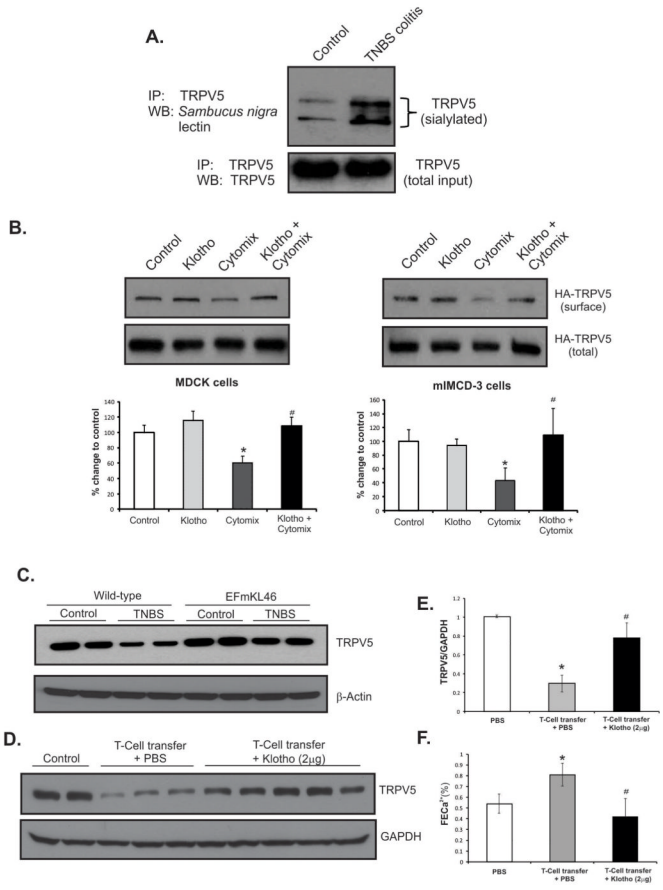


**Fig. 5. (A) Hypersialylation of renal TRPV5 in experimental colitis**

TRPV5 was immunoprecipitated from control or TNBS-treated mice, and probed with biotinylated lectin from *Sambucus nigra*. Detection with streptavidin-HRP conjugate (image representative of n=3). ***(B) Recombinant soluble KLOTHO (20ng/ml) prevents TRPV5 loss of surface expression in MDCK and mIMCD3 cells transduced with Ad-HA-TRPV5 and treated with cytomix*** (images representative of three independent experiments). The bottom panels depict densitometric quantification of TRPV5 protein levels. Data are expressed as % change ± SD. *$p < 0.05$, compared to untreated cells. #p<0.05 compared to cytomix treated. ***(C) TRPV5 expression in control and TNBS-treated wild-type and EFmKL46 transgenic mice.*** Total TRPV5 expression in renal cortex with β-actin as loading control. ***(D) Renal TRPV5 protein expression in $CD4^{+}CD45RB^{hi}$-T-Cell transfer model.*** Control (PBS-injected) and T-cell transferred mice were injected i.p. with PBS or recombinant Klotho (2μg/mouse) daily for 7 days starting 6-weeks post-transfer. ***(E) Densitometric quantification of TRPV5 protein in $CD4^{+}CD45RB^{hi}$-T-Cell transfer model.*** Data normalized to GAPDH. ***(F) The effects of recombinant Klotho injection on the fractional urinary $Ca^{2+}$ excretion ($FECa^{2+}$) in $CD4^{+}CD45RB^{hi}$-T-Cell transfer model***. Mean ± SD, *$p < 0.05$, when compared to PBS injected, #p<0.05 compared to Klotho injected mice.

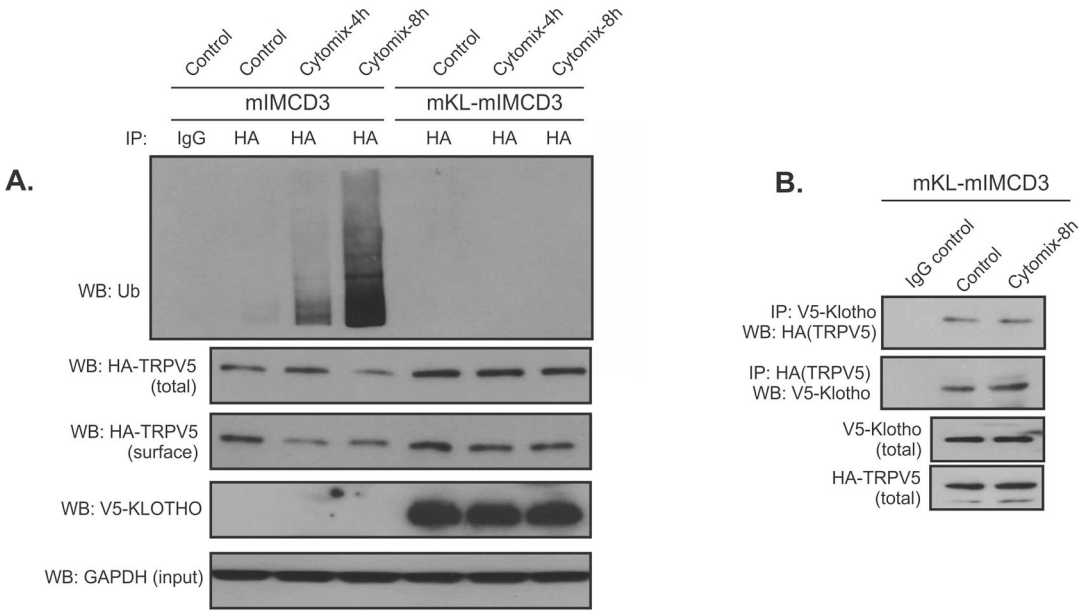


**Fig. 6. (A) Ubiquitination, cell surface expression, and degradation of TRPV5 in response to cytomix in mIMCD3 and mKL-mIMCD3 cells**

mIMCD3 or mKL-mIMCD3 cells were transduced with Ad-HA-TRPV5- and then treated with control medium or medium supplemented with cytomix for 4 or 8 hours. Cell surface proteins were biotinylated and pulled down with streptavidin beads, while TRPV5 was immunoprecipitated with anti-HA antibody. Immunoprecipitates were probed with antibodies against ubiquitin (Ub). Surface and total expression of TRPV5, total expression of KLOTHO, and GAPDH (loading control) were analyzed by western blotting. ***(B) Cytomix does not affect physical interactions of KLOTHO and TRPV5 in mKL-mIMCD3 cells***. mKL-mIMCD3 cells were transduced with Ad-HA-TRPV5- and treated with control medium or cytomix for 8 hours. TRPV5 or KLOTHO were immunoprecipitated with respective epitope tag antibodies and the interacting counterpart detected by Western blotting (image representative of three independent experiments).

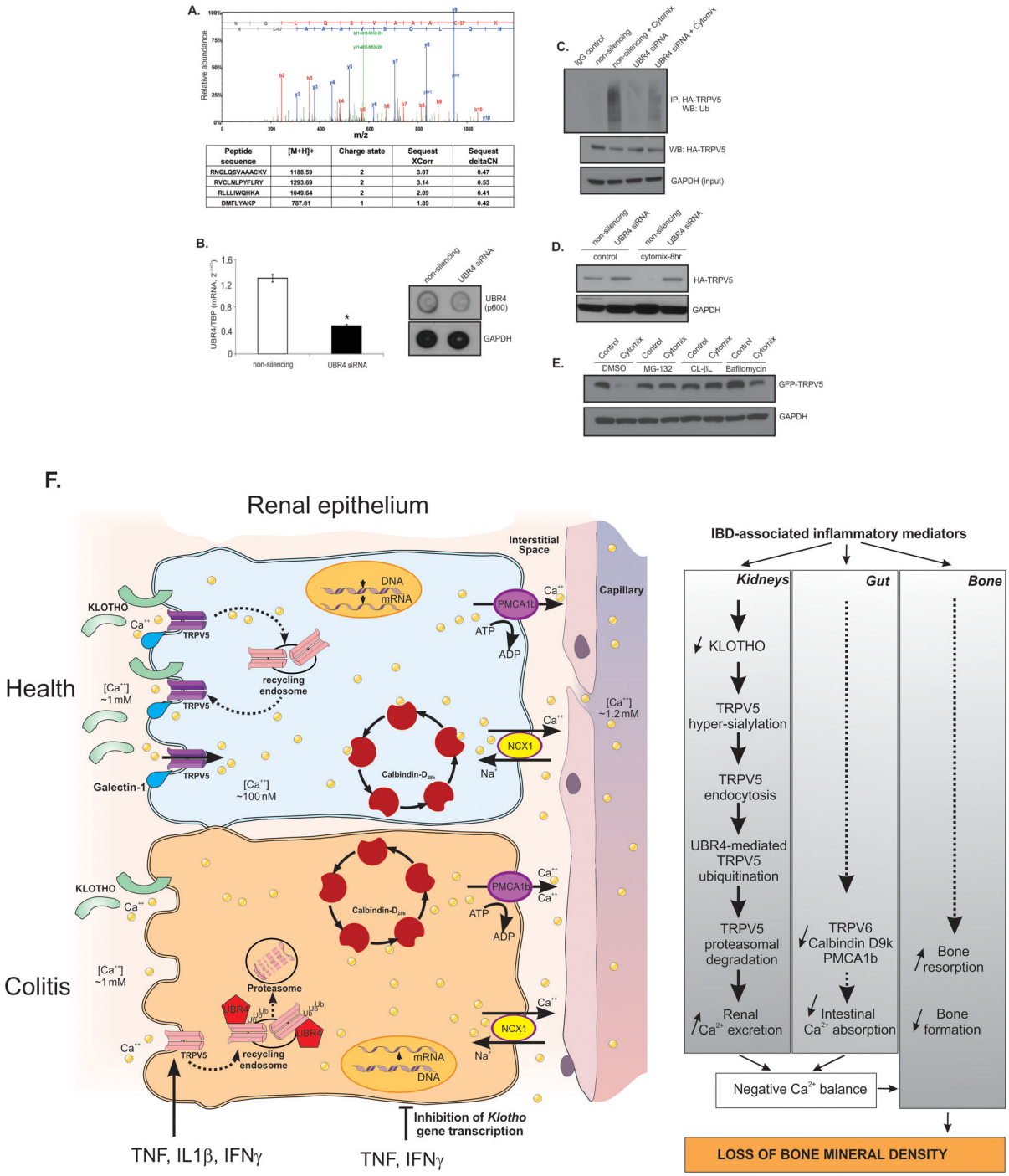


**Fig. 7. UBR4/p600 ubiquitin ligase is responsible for TRPV5 ubiquitination and proteasomal degradation in response to cytomix**

(**A**) A typical MS/MS spectrum for one of the peptides identified from UBR4 protein is shown with its corresponding b/y fragment ions used in determining the sequence. The high-scoring peptides as described in Materials and Methods are listed in the table below. (**B**) Decreased expression of UBR4 mRNA by qRT-PCR(left) and UBR4 protein (by dot blot; right) in mIMCD3 cells transfected with UBR4 siRNA. (**C**) mIMCD3 cells were transduced with Ad-HA-TRPV5, transfected with non-silencing or UBR4 siRNA, and treated with

control or cytomix medium. HA-TRPV5 was immunoprecipitated and probed for ubiquitin (Ub) and pulled-down TRPV5; GAPDH was detected in total cell lysate as input control. (**D**) Expression of total TRPV5 and GAPDH in Ad-HA-TRPV5-transduced mIMCD3 cells transfected with respective siRNA and treated with control medium or cytomix. (**E**) mIMCD3 cells stably transfected with GFP-TRPV5 were treated with control or cytomix medium supplemented with DMSO or with proteosomal or lysosomal inhibitors, MG-132, clasto-lactacystin β-lactone (CL-βL), and bafilomycin. Expression of total TRPV5 and GAPDH was analyzed by Western blotting (all images representative of at least three independent experiments). (**F**) Summary cartoon of the proposed mechanism responsible for increased urinary $Ca^{2+}$ loss during colitis.