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**Title:** 1,25(OH)2 vitamin D3 stimulates active phosphate transport but not paracellular phosphate absorption in mouse intestine

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# $1,25(OH)_2$ vitamin $D_3$ stimulates active phosphate transport but not paracellular phosphate absorption in mouse intestine

## Running title: Vitamin $D_3$ and intestinal absorption of phosphate

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## Key points summary

- Intestinal absorption of phosphate proceeds via an active/transcellular route mostly mediated by NaPi-IIb/*Slc34a2* and a poorly characterized passive/paracellular pathway.
- Intestinal phosphate absorption and expression of NaPi-IIb are stimulated by 1,25$(OH)_2$ vitamin $D_3$ but whether NaPi-IIb is the only target under hormonal control remains unknown.
- We report that administration of 1,25$(OH)_2$ vitamin $D_3$ to wild type mice results in the expected increase in active transport of phosphate in jejunum, without changing paracellular fluxes. Consequently, treatment failed to alter phosphate transport in intestinal-depleted *Slc34a2* mice.
- In both genotypes, 1,25$(OH)_2$ vitamin $D_3$ induced similar hyperphosphaturic responses and changes in FGF23 and PTH.
- While urinary phosphate loss induced by administration of 1,25$(OH)_2$ vitamin $D_3$ did not alter plasma phosphate, further studies should investigate whether chronic administration would lead to phosphate imbalance in mice with reduced active intestinal absorption.

## Abstract

Intestinal absorption of phosphate is stimulated by 1,25(OH)$_2$ vitamin D$_3$. At least two distinct mechanisms underlie phosphate absorption in the gut, an active transcellular transport requiring the $Na^+$/phosphate cotransporter NaPi-IIb/Slc34a2, and a poorly characterized paracellular passive pathway. 1,25(OH)$_2$ vitamin D$_3$ stimulates NaPi-IIb expression and function and loss of NaPi-IIb reduces intestinal phosphate absorption. However, it is remains unknown whether NaPi-IIb is the only target for hormonal regulation by 1,25(OH)$_2$ vitamin D$_3$. Here we compared the effects of intraperitoneal administration of 1,25(OH)$_2$ vitamin D$_3$ (2 days, once per day) in wild type and intestinal-specific *Slc34a2* deficient mice, and analyzed trans- vs paracellular routes of phosphate absorption. We found that treatment stimulated active transport of phosphate only in jejunum of wild type mice, though NaPi-IIb protein expression was upregulated in jejunum and ileum . In contrast, 1,25(OH)$_2$ vitamin D$_3$ administration had no effect in *Slc34a2* deficient mice, suggesting that the hormone specifically regulates NaPi-IIb expression. In both groups, 1,25(OH)$_2$ vitamin D$_3$ elicited the expected increase of plasma FGF23 and reduction of PTH. Treatment resulted in hyperphosphaturia (and hypercalciuria) in both genotypes, though mice remained normophosphatemic. While increased intestinal absorption and higher FGF23 can trigger the hyperphosphaturic response in wild types, only the second one can explain the renal response in *Slc34a2* deficient mice. Thus, 1,25(OH)$_2$ vitamin D$_3$ stimulates intestinal phosphate absorption by acting on the active transcellular pathway mostly mediated by NaPi-IIb while the paracellular pathway appears not to be affected.

## Introduction

Homeostasis of inorganic phosphate (Pi) is a critical task faced by all vertebrates, since derangements of plasma phosphate, both as excess and deficiency, result in pathological systemic alterations. Among them, rickets/osteomalacia and skeletal muscle myopathy are consequences of chronic hypophosphatemia, whereas ectopic mineralization including nephrocalcinosis and vascular calcifications develops in chronic hyperphosphatemia (for review see (Wagner *et al.*, 2019; Koumakis *et al.*, 2020)). Dietary phosphate, consisting of both inorganic and organic compounds, is digested and absorbed mainly by the epithelial cells of the small intestine, although the large intestine may also have some contribution (for review see (Hernando & Wagner, 2018)). Absorption depends on two distinct mechanisms: an active component mediated to a major extent by the *Slc34a2* $Na^+$/phosphate cotransporter NaPi-IIb (Hilfiker *et al.*, 1998; Sabbagh *et al.*, 2009; Hernando *et al.*, 2015; Ikuta *et al.*, 2018), though the presence of alternative transporters has been suggested (Candeal *et al.*, 2017), and a passive component predicted to proceed paracellularly but the identity of which remains unknown (Knoepfel *et al.*, 2019). Once in the circulation and according to cellular needs, Pi can be either transported to bones, skeletal muscles and soft tissues, or filtered and be excreted by the kidney. Renal reabsorption by the proximal tubule epithelia is a highly regulated process that ensures that the amount of Pi excreted with urine is in balance with systemic requirements. Renal reabsorption is mediated by the *Slc34a1*/NaPi-IIa (Magagnin *et al.*, 1993) and *Slc34a3*/NaPi-IIc (Segawa *et al.*, 2002) cotransporters, whereas the role of the recently described renal Slc34a2/NaPi-IIb cotransporter remains unclear (Motta *et al.*, 2020). The quantitative role of the *Slc20* family in the intestinal and/or renal transcellular transport of phosphate is probably minor, at least in mice (Pastor-Arroyo, Acta Physiologica; *in press*).

Whilst not studied to the same detail as renal handling, intestinal absorption of dietary Pi is also subjected to regulation, with low dietary Pi and $1,25(OH)_2$ vitamin $D_3$ as main physiological stimuli (for review see (Hernando & Wagner, 2018)). $1,25(OH)_2$ vitamin $D_3$ is a steroid hormone primarily involved in mineral homeostasis. Its deficiency results in rickets, though its role in extraskeletal processes such as regulation of immune system, cancer progression and cardiovascular or autoimmune diseases is being debated (for review see (Christakos *et al.*, 2016)). The final step of its bioactivation from 7-dehydrocholesterol-derived inactive precursors takes place in the epithelial cells of renal proximal tubules, where the precursor 25(OH) vitamin $D_3$ is hydroxylated at position C-1, thus generating the hormonally active $1,25(OH)_2$ vitamin $D_3$ . This step is catalysed by the mitochondrial CYP27B1/1α-hydroxylase (Takeyama *et al.*, 1997), the deficiency of which is responsible for vitamin D-dependent rickets type I (StArnaud *et al.*, 1997). Also in the kidney, $1,25(OH)_2$ vitamin $D_3$ (as well as 25(OH) vitamin $D_3$) is further hydroxylated at position C-24 by the

mitochondrial CYP24A1/24-hydroxylase, generating 24-hydroxylated metabolites which are hormonally inactive (Beckman *et al.*, 1996). *Cyp24a1* ablation in mice leads to impaired bone mineralization (St-Arnaud *et al.*, 2000), and inactivating mutations in humans are one of the causes of idiopathic infantile hypercalcemia (IIH) (Schlingmann *et al.*, 2011). Expression of renal hydroxylases, and therefore plasma levels of the hormone, is under stringent control. $1,25(OH)_2$ vitamin $D_3$ represses the transcription of the anabolic *CYP27B1* (Murayama *et al.*, 1999), whereas it stimulates the expression of the catabolic *CYP24A1* (Roy & Tenenhouse, 1996), thus suppressing its own production. A similar effect is elicited by the fibroblast growth factor 23 (FGF23), a phosphaturic hormone released by osteocytes and osteoblast (Shimada *et al.*, 2004). In contrast, parathyroid hormone (PTH), also a phosphaturic hormone and a main regulator of Pi and $Ca^{2+}$ homeostasis, promotes the renal transcription of *CYP27B1* via the nuclear receptor NURR1, whereas it represses *CYP24A1*, thus stimulating renal synthesis of $1,25(OH)_2$ vitamin $D_3$ (Shigematsu *et al.*, 1986; Brenza *et al.*, 1998; Murayama *et al.*, 1999). In addition, $1,25(OH)_2$ vitamin $D_3$ stimulates the production of FGF23 (Kolek *et al.*, 2005; Saito *et al.*, 2005), whereas it inhibits PTH synthesis (Silver *et al.*, 1985; Demay *et al.*, 1992a).

Most of the $1,25(OH)_2$ vitamin $D_3$ effects are mediated by the VDR (Haussler *et al.*, 1995). In humans, mutations in the *VDR* result in vitamin D resistant rickets type II (Hughes *et al.*, 1988). Among the best characterized biological activities of $1,25(OH)_2$ vitamin $D_3$ are the stimulation of intestinal absorption and renal reabsorption of $Ca^{2+}$. These effects are mostly due to transcriptional upregulation of genes involved in uptake of $Ca^{2+}$ across the epithelial apical membrane (Trpv6 and Trpv5 transient receptor potential vanillin channels), as well as of intracellular $Ca^{2+}$-binding proteins (Calbindin-$D_{9k}$ and Calbindin-$D_{28k}$) and of basolateral transporters (plasma membrane ATPase Pmca-1b and $Na^+/Ca^{2+}$ exchanger Ncx-1) involved in efflux (for review see (de Barboza *et al.*, 2015; Christakos *et al.*, 2016). Some of these changes can also be attributed to the higher intracellular $Ca^{2+}$ levels associated to $1,25(OH)_2$ vitamin $D_3$ treatment (Hoenderop *et al.*, 2003; Renkema *et al.*, 2005). In addition, $1,25(OH)_2$ vitamin $D_3$ stimulates also intestinal Pi absorption. Pi transport in everted intestinal loops from $1,25(OH)_2$ vitamin $D_3$-treated rats is higher than control animals (Harrison & Harrison, 1961). Moreover, intestinal absorption of Pi is reduced in conditions associated with low $1,25(OH)_2$ vitamin $D_3$, including high dietary phosphate, thyroparathyroidectomy or treatment with high dosages of disodium ethane-1-hydroxy-1,1-diphosphonate, a pyrophosphate analogue that reduces active vitamin $D_3$ synthesis (Hill *et al.*, 1973; Rizzoli *et al.*, 1977). In all these instances, intestinal Pi absorption could be stimulated by administration of $1,25(OH)_2$ vitamin $D_3$, an effect that was proposed to be mediated by an hormonally-induced increase in the number of Pi carriers expressed in the intestinal mucosa (Danisi *et al.*, 1980). Indeed, the expression of NaPi-IIb in the gut is under the control of the steroid hormone (Hattenhauer *et al.*, 1999), and its mRNA and/or protein

levels are accordingly reduced in *Vdr* deficient mice (Segawa *et al.*, 2004; Kaneko *et al.*, 2011). Both in mice and rats, short administration of 1,25$(OH)_2$ vitamin $D_3$ was reported to specifically increase the uptake of Pi and the expression of NaPi-IIb in jejunum, without significantly changing these parameters in duodenum and ileum (Marks *et al.*, 2006). In addition to regulating transcellular transport processes, 1,25$(OH)_2$ vitamin $D_3$ also modulates the expression of several claudins responsible for intestinal and/or renal paracellular transport of water (claudin-2; (Zhang *et al.*, 2015)), $Ca^{2+}$ (claudin-2, and 12: (Fujita *et al.*, 2008; Dimke *et al.*, 2013)) and $Mg^{2+}$ (claudin-16: (Kladnitsky *et al.*, 2015)). Recently, the increase in intestinal absorption of Pi (and $Ca^{2+}$) induced by lithocholic acid was reported to require the Vdr but to be independent of NaPi-IIb (Hashimoto *et al.*, 2020). Therefore, the aim of this study was to investigate whether NaPi-IIb is solely responsible for the increased intestinal absorption of Pi induced by 1,25$(OH)_2$ vitamin $D_3$ or whether paracellular Pi absorption is also regulated.

## Material and Methods

**Ethical approval.** Animal breeding and experimental handling complied with the Swiss Animal Welfare laws and had been previously approved by the local veterinary authority (Kantonales Veterinäramt Zürich; license number 156/2016). Experiments performed in UK (intestinal absorption *in vivo*) complied with the UK Animals (Scientific Procedures) Act, 1986, Amendment Regulations 2012 (license number P7D597F7F), and were approved by the University College London (Royal Free Campus) Comparative Biology Unit Animal Welfare and Ethical Review Body Committee. Methods conform to the principles and regulations as described in the Editorial by Grundy (Grundy, 2015).

**Animal handling and collection of samples.** Experiments were performed with wild type and intestinal-specific *Slc34a2* deficient male mice about 3-4 months old. Animals received standard diet (KLIBA, SA) containing 0.8% Pi, 1% $Ca^{2+}$ and 800 u/Kg vitamin $D_3$. Mice were housed individually in metabolic cages (Tecniplast) and were injected intraperitoneally once a day during two days with either 4 μg/Kg BW of 1,25$(OH)_2$ vitamin $D_3$ or vehicle (peanut oil/ethanol/PBS). Stool and urine (under mineral oil) were collected during the last 24 hours. At termination (48 hours after the first injection), mice were anaesthetized with isoflurane prior to the collection of blood, mucosa from intestinal segments, kidneys and bones; mice died of exsanguination. Aliquots of urine and plasma as well as tissue samples were snap frozen in liquid nitrogen and stored at -80°C until further use.

**Quantification of Pi and $Ca^{2+}$ in stool, urine and plasma.** The concentration of Pi in stool, urine and plasma was measured according to the Fiske Subarow method (Sigma Diagnostics), whereas the QuantiChrom Calcium assay kit (Bio-Assay Systems) was used to quantify the concentration of total $Ca^{2+}$. Prior to determinations, stool samples were processed as described (Sabbagh *et al.*, 2009). Faecal excretions were normalized to food intake, whereas urinary outputs were normalized to the concentration of creatinine (quantified according to the Jaffe method, Wako Chemicals).

**Quantification of FGF23 and PTH in plasma.** Plasma levels of intact FGF23, the C-terminal FGF23 fragment and PTH were quantified by Elisa (Immunotopics) following the manufacturer's protocols.

**Quantification of mRNA expression.** Total RNA was extracted from mucosa of intestinal segments, kidneys and femurs (RNAeasy Minikit, Qiagen) and was reverse transcribed to cDNA (TaqMan Reverse Transcription Kit, Applied Biosystems). The cDNA was used as template for real time PCR using a commercial PCR Master Mix (TaqMan) and gene specific pairs of forward (Fw) and reverse (Rv) primers together with FAM/TAMRA-labelled probes (Pb). All sequences are shown in Table 1. The expressions of the genes of interest were normalized to the expression of 18S according to the

formula $R = 2^{[Ct(control\ gene)-Ct(test\ gene)]}$, where R is the relative ratio and Ct indicates the cycle number at a given threshold.

**Determination of intestinal $^{32}$P fluxes.** $^{32}$P fluxes across jejunum and ileum were measured in Ussing chambers as described (Knoepfel *et al.*, 2019). In brief, tissue samples inserted in a circular adaptor (3.2 mm diameter) separated apical and basolateral chambers filled with constantly oxygenated Ringer solutions buffered at pH 6.0 (adjusted with 10 mM MES/NaOH), that were kept at 37°C with a water jacket. Both chambers were initially filled with a Ringer containing (in mM): 145 NaCl, 3.5 KCl, 3.6 $NaHCO_3$, 1 $MgCl_2$, 1.3 $Ca^{2+}$-gluconate and 5 glucose. After 30 minutes equilibration, an apical-to-basolateral Pi gradient of either 100 µM or 70 mM was established by adding on the apical side $NaH_2PO_4$ to a final concentration of either 100 µM or 70 mM while leaving the basolateral side with 0 Pi. Aliquots from the basolateral side (400 µl) were taken immediately before as well as 60, 90 and 240 minutes upon addition of $^{32}$P to the apical chamber. At each time point, apical aliquots were also extracted to avoid changes in hydrostatic pressure. Basolateral $^{32}$P was quantified using a β-counter (Packard BioScience).

**Determination of intestinal dilution potential**. Dilution potentials to calculate the apical to basolateral and basolateral to apical permeabilities for $Na^+$ ($P_{Na}$), $Cl^-$ ($P_{Cl}$) and Pi ($P_{Pi}$) were measured in Ussing chambers as described recently (Knoepfel *et al.*, 2019). Briefly, samples from jejunum and ileum were inserted into circular adaptors and let to equilibrate for 30 minutes which the apical and basolateral compartments perfused with a standard Ringer solution containing (in mM) 145 NaCl, 3.6 KCl, 3.6 $NaH_2PO_4$, 5 glucose, 1 $MgCl_2$ and 1.3 $Ca^{2+}$-gluconate, pH 6. Both compartments were perfused independently at a flow rate of 5-10 ml/min. Upon equilibration, transepithelial voltage ($V_{te}$) was recorded in the sequential presence of 145 mM NaCl (standard Ringer), 30 mM NaCl (osmolarity maintained with mannitol pH 6) and 140 mM $NaH_2PO_4$ at pH 6 (NaCl partially replaced by $NaH_2PO_4$). Solutions (oxygenated and prewarmed at 37°C) were first changed at the apical and then at the basolateral sides (whereas the opposite side was always bathed with standard Ringer), and differences in $V_{te}$ induced by these changes were measured under open-circuit conditions. Absolute permeabilities for $Na^+$, $Cl^-$ and phosphate were obtained by the Goldman-Hodgkin-Katz equation (Kimizuka & Koketsu, 1964) and $R_{te}$ according to Ohm's law (Rte=ΔVte/I).

**Intestinal Pi absorption *in vivo.*** *In vivo* absorption of Pi in the ileum was quantified by the *in situ* ligated intestinal loop technique as described (Marks *et al.*, 2006). Briefly, mice were anesthetized with an intraperitoneal injection of 60 mg $kg^{-1}$ pentobarbitone sodium (Pentoject, Animalcare Ltd) and 10 cm of distal ileum cannulated. Upon flushing first with 0.9% saline and then air, the ileum was instilled with 200 µl of uptake buffer containing (in mM) 16 Na-HEPES, 140 NaCl, 3.5 KCl and either 100 µM or 70 mM $KH_2PO_4$ plus $^{33}$P and tied off. Blood samples were collected via cardiac puncture

after 10 minutes incubation with the uptake buffer. Plasma $^{33}P$ was quantified using a liquid scintillation counter (Perkin Elmer).

**Western blots.** Samples of BBM (20 μg) or homogenates (40 μg) were separated on SDS/PAGE gels and transferred to PVDF membranes (Millipore) by standard procedures. Upon blocking with 5% milk powder in TBS (30 minutes/room temperature), membranes were first incubated (overnight/4°C) with primary antibodies against NaPi-IIa (1: 3000; (Custer *et al.*, 1994), NaPi-IIb (1: 3000; (Hilfiker *et al.*, 1998), NaPi-IIc (1: 3000; (Nowik *et al.*, 2008), Cyp24a1 (1: 1000; Proteintech: ≠21582-1-AP), VDR (1: 500; Santa Cruz: ≠sc-13133), β-actin (1: 10000; Sigma: ≠ A5316) and β-tubulin (1: 20000; Sigma ≠T4026) and then with the appropriate HRP-conjugated secondary antibody (GE Healthcare) (2 hours/room temperature). Upon exposure to HRP substrate (Millipore), chemiluminescence signals were detected with the LAS-4000 luminescent image analyser (Fujifilm) and further quantified (ImageJ). Quantifications are shown as ratios to either actin or tubulin.

**Statistical analysis.** Differences between groups were analysed by t-test (2 groups) or Anova/ Bonferroni's test (multiple groups), as indicated. $P< 0.05$ was considered significant**.** All data are shown as individual values together with mean ± SD.

## Results

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ regulates the mRNA expression of renal Cyp27b1 and Cyp24a1 in wild type and in intestinal-specific *Slc34a2* deficient mice** In order to prove that the injected hormone had triggered the expected systemic effects, we first analyzed its impact on the renal mRNA expression of *Cyp27b1* and *Cyp24a1*, the hydroxylases chiefly involved in 1,25(OH)$_2$ vitamin D$_3$ metabolism, as well as on its own receptor. As expected, administration of 1,25(OH)$_2$ vitamin D$_3$ reduced the renal mRNA expression of *Cyp27b1* as compared with vehicle treated groups, though the reduction reached statistical significance only in *Slc34a2*-deficient mice (Fig. 1A). In contrast, the renal expression of the catabolic *Cyp24a1* (Fig. 1B) as well as the *Vdr* (Fig. 1C) were upregulated in 1,25(OH)$_2$ vitamin D$_3$ treated mice. The expression of the three genes was similar in wild type and *Slc34a2*-deficient mice treated with vehicle, and each gene was regulated in a comparable way in both genotypes upon 1,25(OH)$_2$ vitamin D$_3$ injection. Upregulation of Vdr, though not of Cyp24a1, was also confirm at the protein level (Fig. 1D), with similar basal values and hormonally-induced changes found in wild type and *Slc34a2*-deficient mice. Thus, 1,25(OH)$_2$ vitamin D$_3$ injection had a similar effect on the enzymes and receptor regulating 1,25(OH)$_2$ vitamin D$_3$ activity in both genotypes.

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ inversely regulates plasma levels of FGF23 and PTH in wild type and intestinal-specific S*lc34a2* deficient mice** 1,25(OH)$_2$ vitamin D$_3$ regulates in opposite directions the plasma levels of FGF23 and PTH, two hormones that induce a phosphaturic response in states of Pi excess. Therefore, we next compared the response of both phosphaturic hormones to 1,25(OH)$_2$ vitamin D$_3$ administration in the absence or presence of intestinal NaPi-IIb. Injection of 1,25(OH)$_2$ vitamin D$_3$ resulted in the expected increase of plasma intact FGF23 not only in wild type but also in S*lc34a2*-deficient mice (Fig. 2A). This increase was paralleled by higher circulating levels of the C-terminal FGF23 fragment (Fig. 2B) and higher ratios of intact to C-terminal fragment (data not shown). Treatment with 1,25(OH)$_2$ vitamin D$_3$ also produced the expected reduction in the plasma levels of intact PTH, an effect that was detected in both genotypes (Fig. 2C). The expression of *Fgf23* mRNA in bones was upregulated in the groups treated with 1,25(OH)$_2$ vitamin D$_3$, though it reached significance only in mutant mice (Fig. 2D). In contrast, the mRNA abundance of *Galnt3* (Fig. 2E) and *Phex* (Fig. 2F), two regulators or FGF23 stability and/or expression, was comparable in vehicle- and hormone-treated mice in both genotypes.

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ specifically increases active intestinal transport of Pi in wild type mice, and the effect is abolished in intestinal-specific *Slc34a2* deficient mice** To compare the effect of 1,25(OH)$_2$ vitamin D$_3$ administration on the intestinal capacity to absorb Pi in

wild type and *Slc34a2* deficient mice, we measured Pi absorption across jejunum and/or ileum combining *in vivo* and *ex vivo* approaches. Absorption of Pi across the ileum, the segment with the highest expression of NaPi-IIb in mice, was measured *in vivo* by the *in situ* ligated loop technique, using luminal concentrations of Pi that allow discrimination between the active (100 μM) and passive/paracellular processes (70 mM). As shown in Fig. 3A, in the presence of 100 μM luminal substrate, the active absorption of Pi in *Slc34a2* deficient mice was reduced to about 20% of the values detected in wild types (both in vehicle- and 1,25$(OH)_2$ vitamin $D_3$ treated animals). In contrast, similar paracellular absorption was measured in both genotypes in the presence of 70 mM luminal substrate (Fig. 3B). Although there was a trend for higher active and passive transport in the ileum of wild type mice treated with 1,25$(OH)_2$ vitamin $D_3$ as compared with vehicle-treated animals, the differences were not significant.

The effect of 1,25$(OH)_2$ vitamin $D_3$ in active and passive fluxes of Pi across jejunum (the segment reported to exhibit higher 1,25$(OH)_2$ vitamin $D_3$ sensitivity (Marks *et al.*, 2006)) and ileum of wild type and *Slc34a2* deficient mice was next compared by means of Ussing chambers. This *ex vivo* technique allowed us to process both segments and use two luminal substrate conditions (100 μM and 70 mM) in parallel. As shown in Fig 3C, active transport measured as fluxes at 100 μM was almost 10 times higher in jejunum of wild type mice treated with 1,25$(OH)_2$ vitamin $D_3$ compared with vehicle-treated animals. This transport component was similarly low in wild type and *Slc34a2* deficient mice treated with vehicle, reflecting the fact that under standard conditions the expression of NaPi-IIb in this intestinal segment is very low. However, unlike in wild types, active jejunal transport in *Slc34a2* deficient mice was not stimulated by the hormonal treatment. In contrast, basal jejunal passive paracellular transport was similar in both genotypes and in both cases it was insensitive to 1,25$(OH)_2$ vitamin $D_3$ administration (Fig. 3D). Active transport in ileum of wild type mice treated with vehicle was far bigger than in jejunum, but the ileal activity was not significantly regulated by 1,25$(OH)_2$ vitamin $D_3$ administration (though there was a trend for higher transport) (Fig. 3E). As expected, the active ileal component was strongly reduced in *Slc34a2* deficient mice and the remaining activity was insensitive to hormonal treatment. Again, the passive paracellular transport in the ileum was not affected by 1,25$(OH)_2$ vitamin $D_3$ treatment in both genotypes (Fig. 3F). However, this passive component was reduced in *Slc34a2* deficient mice compared with wild types.

To further support the lack of contribution of the paracellular pathway to the stimulation of intestinal Pi absorption induced by 1,25$(OH)_2$ vitamin $D_3$, we next analyzed individual apical to basolateral (AP-BL) and basolateral to apical (BL-AP) paracellular permeabilities for Pi, $Na^+$ and $Cl^-$. For that, we performed dilution potential measurements in fragments from jejunum and ileum mounted in Ussing chambers and changed separately the ion composition on the apical and

basolateral sides. In both segments, similar AP-BL and BL-AP permeabilities for Pi were measured in samples from vehicle- and 1,25(OH)$_2$ vitamin D$_3$ injected mice (Fig. 3 G,H), and no differences between genotypes were found either. Similarly, in both segments, AP-BL and BL-AP paracellular permeabilities for $Na^+$ and $Cl^-$ were comparable in vehicle- and hormone treated groups, without difference between genotypes (Table 2). Administration of 1,25(OH)$_2$ vitamin D$_3$ had no effect in transepithelial potential differences, neither in wild types not in mutant mice (Table 2), though AP-BL values in ileum were higher in mutant mice than in wild type animalas injected with vehicle. No differences between treatments and genotypes were found in transepithelial resistance (Table 2).

In agreement with the Ussing chamber data (Fig. 3C), the expression of NaPi-IIb protein in total jejunal membranes from wild type mice was upregulated in animals treated with 1,25(OH)$_2$ vitamin D$_3$ as compared with vehicle injected mice, and no signal was detected in *Slc34a2* deficient mice (Fig. 3I). Higher expression of NaPi-IIb was also detected in homogenates from ileum of wild type mice treated with 1,25(OH)$_2$ vitamin D$_3$ (Fig. 3J), though the change in active transport (Fig. 3A and 3E) was not significant. As in the jejunal samples, no NaPi-IIb signal was detected in ileum from *Slc34a2* deficient mice (Fig. 3H).

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ triggers hyperphosphaturia, hypercalciuria and hypercalcemia in wild type and intestinal-specific S*lc34a2* deficient mice** Next, we investigated if the changes of intestinal absorption described above resulted in an alteration of Pi levels either in feces, urine or plasma. Because 1,25(OH)$_2$ vitamin D$_3$ (as well as PTH and FGF23) in addition to Pi also regulates $Ca^{2+}$ metabolism, the levels of the cation were measured in parallel. As shown in Fig. 4A, the fecal excretion of Pi was comparable in all four analysed groups. Instead, 1,25(OH)$_2$ vitamin D$_3$ administration triggered an increase in the urinary excretion of Pi in both genotypes (Fig. 4B) suggesting that, despite the absence of detectable changes in fecal excretion, the hormonal treatment resulted in a systemic Pi load. As expected, phosphaturia was significantly smaller in vehicle-treated *Slc34a2* deficient mice than in wild type littermates, though the difference disappeared upon hormonal administration. Plasma Pi levels were similar in vehicle- and 1,25(OH)$_2$ vitamin D$_3$-treated groups, and no differences between genotypes were detected either (Fig. 4C).

As for Pi, fecal excretion of $Ca^{2+}$ was similar in all groups (Fig. 4D), whereas the hormonal treatment resulted in 8-10 higher urinary excretion of the cation (Fig. 4E) and hypercalcemia (Fig. 3F), reflecting the well-known activation of intestinal absorption of $Ca^{2+}$ induced by 1,25(OH)$_2$ vitamin D$_3$. No differences with regard to $Ca^{2+}$ levels in stool, urine and plasma were detected between wild types and *Slc34a2* deficient mice, with both genotypes responding similarly to the hormonal treatment.

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ has a minor or no effect on the mRNA expression of intestinal *Slc34a2* and *Slc20* Na$^+$/phosphate cotransporters** To correlate changes in transport activity with cotransporters abundance, the intestinal mRNA abundance of *Slc34a2* and *Slc20* cotransporters was next quantified. The mRNA expression of each transporter along the whole intestinal tract of wild type mice injected with vehicle was measured simultaneously (Fig. 5A, C and E). In addition, for each individual segment, each transcript was simultaneously quantified in the 4 experimental groups, and the expression normalized to the vehicle-treated group (Fig. 5B, D and F); raw data is presented in Fig. 5G. As expected, NaPi-IIb/*Slc34a2* mRNA abundance increased along the small intestine from duodenum to ileum and faded down in the large intestine (Fig. 5A). Also as expected, its expression was almost absent from the whole intestinal tract of *Slc34a2* deficient mice (below 0.5% of the wild type values in all segments except in duodenum where it amounted to 7%). Administration of 1,25(OH)$_2$ vitamin D$_3$ did not alter NaPi-IIb/*Slc34a2* mRNA expression in the small intestine of wild type mice, but induced its upregulation in both proximal and distal colon (Fig. 5B).

Low and comparable expression levels of Pit-1/*Slc20a1* mRNA were found in the small intestine and proximal colon of wild type mice injected with vehicle, whereas the distal colon displayed the highest levels (Fig. 5C). The mRNA abundance of this transporter was similar in wild type and *Slc34a2* deficient mice (Fig. 5G), and in both genotypes it was also comparable in vehicle- and 1,25(OH)$_2$ vitamin D$_3$ treated mice (Fig. 5D). Also the expression of Pit-2/*Slc20a2* transcripts in vehicle-treated wild type mice tended to be higher in the colon than in the small intestine, though the difference was not as pronounced as for Pit-1 (Fig. 5E). Pit-2/*Slc20a2* mRNA expression was comparable in wild type and *Slc34a2* deficient mice treated with vehicle, except for a small up regulation in duodenum (Fig. 5G), and in both genotypes was unaltered by 1,25(OH)$_2$ vitamin D$_3$ treatment (Fig. 5F).

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ regulates the expression of genes involved in intestinal transport of Ca$^{2+}$ in wild type and intestinal-specific S*lc34a2* deficient mice** Quantifications for these transcripts were performed as indicated above for the Pi transporters. The expression of *Trpv6* transcripts in vehicle-treated wild type mice was higher is duodenum, lower/undetectable in jejunum and ileum, then increasing from proximal to distal colon (Fig. 6A). 1,25(OH)$_2$ vitamin D$_3$ treatment massively increased the abundance of the channel in the colon, but not in the rest of the intestinal segments (Fig. 6B and G). Both the channels basal levels as well as its mRNA expression upon hormonal treatment were similar in wild type and *Slc34a2*-deficient mice. (Fig. 6B and G).

The highest mRNA expression of Calbindin-D$_{9k}$/*Calb3* in vehicle-injected wild type mice was detected by far in duodenum, with all other segments having about 3 orders of magnitude lower levels (Fig. 6C). Its basal expression was similar in wild type and *Slc34a2* deficient mice (Fig 6G), and in both

genotypes it was upregulated in all intestinal segments upon administration of 1,25(OH)$_2$ vitamin D$_3$, with the effect particularly massive in ileum and large intestine (Fig. 6D).

Pmca-1b/*Atp2b1* mRNA expression in vehicle-treated wild type mice was also bigger in duodenum than in all other intestinal segments (Fig. 6E). Its basal mRNA abundance was similar in wild type and *Slc34a2*-deficient mice (Fig 6G), and in both genotypes it remained unchanged upon administration of 1,25(OH)$_2$ vitamin D$_3$ (Fig. 6F and G). Similarly, the mRNA expression of Ncx1/*Slc8a1* was insensitive to the hormonal treatment, though unlike the ATPase, the higher expression of the exchanger was found in the large intestine (data not shown). The mRNA abundance of the *Vdr* was homogeneous along the whole intestinal tract, with no differences between wild types and *Slc34a2*-deficient mice (data not shown). Unlike in the kidney (Fig. 1A), the expression of *Vdr* in intestine was not regulated by 1,25(OH)$_2$ vitamin D$_3$ (data not shown).

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ reduces the protein abundance of renal Slc34a Na$^+$/Pi cotransporters NaPi-IIa and NaPi-IIc in wild type and intestinal-specific S*lc34a2* deficient mice** To correlate changes in phosphaturia with the abundance of renal cotransporters, we measured the protein expression of NaPi-IIa and NaPi-IIc in renal BBM. In agreement with the higher urinary excretion of Pi, the abundance of both cotransporters was reduced in samples from wild type and intestinal specific S*lc34a2* deficient mice treated with 1,25(OH)$_2$ vitamin D$_3$ compared with vehicle-injected animals (Fig. 7A). The basal expression levels of both transporter, as well as the extent of downregulation induced by the hormonal treatment, was comparable in wild type and mutant mice. In both genotypes, the effect on NaPi-IIa protein levels was accompanied by reduced mRNA expression, though transcriptional downregulation was not apparent for NaPi-IIc (Fig. 7B). Similar mRNA levels of Pit-1 and Pit-2 were measured in vehicle- and 1,25(OH)$_2$ vitamin D$_3$ treated mice, and no differences between genotypes were detected (Fig. 7B).

**Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ upregulates the expression of several genes involved in renal Ca$^{2+}$ reabsorption in wild type and intestinal-specific S*lc34a2* deficient mice** In both genotypes, the renal mRNA expression of the apical channel *Trpv5* (Fig. 8A), the cytoplasmic Ca$^{2+}$-binding protein calbindin-D$_{28K}$/*Calb1* (Fig. 8B), as well as the basolateral exchanger Ncx1/*Slc8a1* (Fig. 8C) was upregulated in samples from 1,25(OH)$_2$ vitamin D$_3$-injected mice. Instead, the expression of the basolateral Pmca-1b/*Atp2b1* pump was not affected by the hormonal treatment (Fig. 8D). For all four genes, their basal mRNA levels as well as their hormonal responses were similar in wild type and mutant mice.

## Discussion

Intestinal absorption of Pi takes place via two distinct mechanisms, an active/saturable process that depends to a major extent on the luminal presence of $Na^+$, and a passive/paracellular route. NaPi-IIb, a high affinity Pi transporter with an estimated apparent affinity for Pi of about 50 µM (Hilfiker *et al.*, 1998), plays a major role in active transport (Sabbagh *et al.*, 2009; Hernando *et al.*, 2015), though additional transporters contributing to the process have been proposed (Candeal *et al.*, 2017). Little is known about the molecular identity of the passive pathway, though the whole intestinal tract is able to conduct Pi paracellularly (Knoepfel *et al.*, 2019). Intestinal absorption of Pi and expression of NaPi-IIb in the gut are under control of 1,25$(OH)_2$ vitamin $D_3$ (Hattenhauer *et al.*, 1999; Xu *et al.*, 2002; Marks *et al.*, 2006), but whether or not the cotransporter is the only molecular target for hormonal regulation remains unknown. Therefore, here we compared the effect on Pi homeostasis of 1,25$(OH)_2$ vitamin $D_3$ administration (i.p injections once per day for two consecutive days) in wild type and intestinal-specific NaPi-IIb/*Slc34a2* deficient mice.

The effectiveness of the treatment was first confirmed by quantifying its impact on the mRNA expression of *Cyp27b1* and *Cyp24a1*, the anabolic and catabolic hydroxylases responsible for the renal control of plasma 1,25$(OH)_2$ vitamin $D_3$ levels, as well as on the mRNA abundance of the *Vdr*, since the hormonal effects on the transcription of *Cyp27b1* and *Cyp24a1* require the presence of the receptor (for review see (Christakos *et al.*, 2016)). Administration of 1,25$(OH)_2$ vitamin $D_3$ triggered the expected adaptive renal response, i.e repression of *Cyp27b1* and stimulation of *Cyp24a1* and *Vdr*, and these changes were similar in the presence or absence of intestinal NaPi-IIb.

1,25$(OH)_2$ vitamin $D_3$ stimulates the production and release of FGF23 by osteocytes (Kolek *et al.*, 2005; Saito *et al.*, 2005), while inhibiting PTH synthesis by the chief cells of the parathyroid glands (Silver *et al.*, 1985; Demay *et al.*, 1992a). 1,25$(OH)_2$ vitamin $D_3$ treatment triggered a similar increase of FGF23 and reduction of PTH levels in wild type and NaPi-IIb/*Slc34a2* deficient mice. In both genotypes, the rise in FGF23 levels was probably due to stimulated production, rather than reduced degradation, since *Fgf23* mRNA expression in bones was upregulated in the groups treated with 1,25$(OH)_2$ vitamin $D_3$, whereas the mRNA expression of *Galnt3*, the polypeptide GalNAc-transferase that confers proteolytic resistance to FGF23 (Topaz *et al.*, 2004; Benet-Pages *et al.*, 2005; de las Rivas *et al.*, 2020), and of *Phex*, an endopeptidase that indirectly downregulates FGF23 protein abundance (Liu *et al.*, 2003) were not modified. On the other hand, reduction of PTH levels may have been caused both by high 1,25$(OH)_2$ vitamin $D_3$ itself as well as by the high $Ca^{2+}$ levels associated with hormonal treatment, since both the VDR and the $Ca^{2+}$-sensing receptor negatively regulate PTH secretion and/or gene transcription/mRNA stability (Demay *et al.*, 1992b; Chen & Goodman, 2004).

Changes in circulating levels of 1,25$(OH)_2$ vitamin $D_3$, PTH and FGF23 represent the final response of the so-called kidney-parathyroid-bone axis, by which these three organs coordinate their hormonal production to prevent alterations in plasma levels of Pi (for review see (Blau & Collins, 2015; Levi *et al.*, 2019)). Under physiological conditions, renal synthesis of 1,25$(OH)_2$ vitamin $D_3$ increases in response to hypophosphatemia since its role is to promote intestinal Pi absorption; in contrast, production of the two phosphaturic hormones FGF23 and PTH is repressed in response to low plasma Pi, thus reducing urinary Pi loss. As previously reported (Marks *et al.*, 2006), administration of 1,25$(OH)_2$ vitamin $D_3$ to wild type mice stimulated intestinal active transport of Pi not in the ileum, the segment which higher basal NaPi-IIb expression, but in jejunum. Active transport was severely reduced in mice lacking NaPi-IIb, and the hormonal treatment failed to change the remaining activity. Moreover, in both types of mice, the passive transport was insensitive to the hormonal treatment, thus suggesting that NaPi-IIb is the main if not the only 1,25$(OH)_2$ vitamin $D_3$ target for intestinal absorption of Pi. This conclusion is further supported by the similar AP-BL and BL-AP permeabilities for Pi measured in intestinal segments (jejunum and ileum) of animals injected with vehicle and 1,25$(OH)_2$ vitamin $D_3$. Furthermore, the hormonal treatment did not influence the transepithelial potential difference neither in jejunum nor in ileum. Potential differences were negative in both segments under all conditions, except in the AP-BL determination in ileum of wild type mice injected with vehicle. Because similar potential differences along the intestinal axis have been reported (Hajjar *et al.*, 1969; Inagaki *et al.*, 2005), and since negative values were also measured in the BL-AP determination, this finding should be further consolidated. It has been recently reported that lithocholic acid increases intestinal Pi absorption in a way that is Vdr-dependent but independent on $Na^+$ ions and NaPi-IIb, and it was suggested that the effect was mediated by an increase in paracellular permeability (Hashimoto *et al.*, 2020). The well-known effect of 1,25$(OH)_2$ vitamin $D_3$ on NaPi-IIb abundance and intestinal $Na^+$/Pi transport (Hattenhauer *et al.*, 1999; Katai *et al.*, 1999; Xu *et al.*, 2002; Segawa *et al.*, 2004; Marks *et al.*, 2006) together with the data presented here, suggest that lithocholic acid and 1,25$(OH)_2$ vitamin $D_3$ act via different downstream mechanisms. Surprisingly and in contrast with previous reports (Knoepfel *et al.*, 2019), passive fluxes across the ileum measured in Ussing chambers was reduced in *Slc34a2*-deficient mice as compared with wild types. Although we cannot explain this discrepancy at the moment, the observation that passive ileal fluxes measured by *in situ* loops and passive jejunal fluxes and permeabilities measured by Ussing chambers were similar in both genotypes, suggests that depletion of NaPi-IIb does not influences passive permeability. The absence of an effect of 1,25 $(OH)_2$ vitamin $D_3$ treatment on paracellular Pi absorption is also in agreement with a human study in patients with chronic kidney disease and 1,25 $(OH)_2$ vitamin $D_3$ deficiency. Restoration of 1,25 $(OH)_2$ vitamin $D_3$ levels in these patients stimulated

active Pi absorption in jejunum but failed to affect paracellular absorption (Davis *et al.*, 1983). Our results in mice explain these findings on the molecular level.

In agreement with the transport data, and confirming previous reports (Marks *et al.*, 2015), the hormonal treatment increased the protein abundance of NaPi-IIb in jejunal epithelia. Moreover, the protein expression of the cotransporter was upregulated in the ileum, though transport activity was not significantly changed in this segment. This could be due to the fact that the basal expression level of NaPi-IIb (and thus transport activity) in ileum is high, and therefore may require comparatively larger increases in the transporter abundance in order to be reflected at the level of activity. On the other hand, the discrepancy regarding the hormonal effect on the expression of the cotransporter in ileum between this work and previous reports (Marks *et al.*, 2015) is most probably the consequence of the different experimental protocols applied in both studies. In the present work, the hormone was administered once a day over two consecutive days and tissues extracted 48 hours after starting the treatment, while in the previous study samples were analysed 24 hours after two daily injections. This would suggest that jejunum and ileum are both responsive to changes on $1,25(OH)_2$ vitamin $D_3$, though the first one would respond earlier. Neither in jejunum nor in ileum did the hormone-induced upregulation of NaPi-IIb associate with changes in its mRNA expression. In fact, the transcript abundance along the whole small intestine was insensitive to $1,25(OH)_2$ vitamin $D_3$ administration, though it was upregulated in the proximal and distal colon. In this regard, early studies suggested that the large intestine had the capacity to absorb Pi (Martin *et al.*, 1987; Hu *et al.*, 1997), which was more recently demonstrated (Marks *et al.*, 2015). Nevertheless, due to the small basal expression of NaPi-IIb in the colon, the potential contribution of this segment to stimulated intestinal absorption of Pi in conditions of high $1,25(OH)_2$ vitamin $D_3$, needs further investigations. Studies addressing whether the effect of $1,25(OH)_2$ vitamin $D_3$ on the abundance of NaPi-IIb involves transcriptional changes have provided conflicting results. On one hand, the human promoter contains a VDR responsive element and its activity is increased *in vitro* upon incubation with the hormone (Xu *et al.*, 2002). Furthermore, the intestinal expression of the cotransporter at the mRNA and/or protein levels is reduced in *Vdr* deficient mice (Segawa *et al.*, 2004; Kaneko *et al.*, 2011). However, transcriptional upregulation of NaPi-IIb by $1,25(OH)_2$ vitamin $D_3$ has been shown only in young animals (Xu *et al.*, 2002), whereas its regulation in adults does not require changes on mRNA (Hattenhauer *et al.*, 1999; Marks *et al.*, 2006). Our data suggest that the mechanism of regulation may differ between the small and large intestine. In addition to NaPi-IIb, the two Slc20 orthologues Pit-1 and Pit-2 are expressed in intestine, and in rats their expression is regulated by dietary Pi(Giral *et al.*, 2009; Candeal *et al.*, 2017), though Pit-2 does not seem to contribute significantly to intestinal absorption of Pi in mice (Pastor-Arroyo *et al.*, 2020). Their mRNA expression along the whole intestine was insensitive to the hormonal treatment.

The increase in the intestinal absorption of Pi in wild type mice promoted by 1,25$(OH)_2$ vitamin $D_3$ treatment did not seemingly alter fecal excretion. This is not surprising, since we estimated that under standard dietary conditions, the daily fecal excretion of Pi in wild type mice amounts to about 40% of the ingested Pi. Due to this considerable basal excretion, large changes on intestinal absorption are required in order to be detectable as intestinal Pi loss, thus explaining the apparent lack of differences between treatments and even between genotypes. Instead, the hormonal administration triggered a hyperphosphaturic response in both genotypes. As reported (Sabbagh *et al.*, 2009; Hernando *et al.*, 2015), basal phosphaturia was reduced in *Slc34a2*-deficient mice, reflecting the appropriate renal response to impaired intestinal absorption, though the difference was lost upon hormonal treatment. The hyperphosphaturic response of wild type mice to 1,25$(OH)_2$ vitamin $D_3$ administration was probably due to both higher intestinal absorption as well as to higher plasma levels of FGF23; however, only the hormonal component can be responsible for the hyperphosphaturia in mutant mice. FGF23, as well as PTH, elicits a phosphaturic response by promoting endocytosis and degradation of NaPi-IIa and NaPi-IIc, the two Slc34 cotransporters expressed in the BBM of proximal tubules (Bacic *et al.*, 2006; Gattineni *et al.*, 2009; Tomoe *et al.*, 2010). Indeed, urinary Pi loss associated in wild type as well as in *Slc34a2*-deficient mice with lower protein expression of NaPi-IIa and NaPi-IIc. NaPi-IIa was also down regulated at the mRNA level, whereas the mRNA expression of NaPi-IIc and the two Slc20 homologues was similar in vehicle- and 1,25$(OH)_2$ vitamin $D_3$- treated mice. Despite the renal loss of Pi, short-term hormonal administration did not alter plasma Pi and even *Slc34a2*-deficient mice remained normophosphatemic in defiance of impaired active intestinal absorption. In this regard, it should be noted that in patients with hypoparathyroidism, increases in FGF23 secondary to 1,25$(OH)_2$ vitamin $D_3$-treatment also resulted in reduced renal reabsorption of Pi and reduced plasma levels of Pi (though plasma Pi remains higher than normal) (Collins *et al.*, 2005). In the same report it was also postulated that the phosphaturic effect of FGF23 is diminished in the absence of PTH.

Because the effects of 1,25$(OH)_2$ vitamin $D_3$ on intestinal and renal (re)absorption of $Ca^{2+}$ are well characterized (for review see (de Barboza *et al.*, 2015; Christakos *et al.*, 2016)), as a proof for the effectiveness of the 1,25$(OH)_2$ vitamin $D_3$ treatment we also analysed the response of several $Ca^{2+}$-related parameters. We estimated that fecal excretion of $Ca^{2+}$ in mice fed on standard chow represents around 90% of the ingested $Ca^{2+}$, and in consequence, the lack of differences between treatments is not surprising. Instead, the stimulation of intestinal absorption manifested in both genotypes as marked hypercalciuria and hypercalcemia in the 1,25$(OH)_2$ vitamin $D_3$-treated groups. Furthermore, administration of the hormone evoked the expected upregulation of the mRNA expression of several intestinal and renal genes involved in active transport of $Ca^{2+}$. This includes

apical channels (Trpv6 and Trpv5, respectively) as well as intracellular $Ca^{2+}$-binding proteins (Calbindin-$D_{9k}$/*Calb3* and calbindin-$D_{28K}$/*Calb1*). Exceptions were the lack of regulation of Trpv6 in duodenum and of Pmcab1/*Atp2b1*, for which transcriptional regulation was previously shown (Balesaria *et al.*, 2009; Lee *et al.*, 2015). Differences in the extent of the treatment (6 hours in previous reports vs. 48 hours in the present study) may be responsible for the discrepancy.

In summary, we have shown that the rise in Pi absorption by the small intestine triggered by 1,25$(OH)_2$ vitamin $D_3$ is due specifically to stimulation of the active transport component, and that this effect is fully dependent on the presence of NaPi-IIb in the intestinal epithelia. Instead, paracellular absorption of Pi is not regulated by 1,25 $(OH)_2$ vitamin $D_3$. Despite this finding, the renal adaptation to 1,25$(OH)_2$ vitamin $D_3$ administration was strikingly similar in wild type and intestinal *Slc34a2*/NaPi-IIb-deficient mice, probably due to the masking effect of high FGF23. Although short-term treatment with 1,25$(OH)_2$ vitamin $D_3$ did not alter plasma Pi levels in mice with compromised active intestinal absorption, chronic administration may lead to Pi imbalance.

## Legends

**Figure 1. Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ regulates the mRNA expression of renal *Cyp27b1* and *Cyp24a1* in wild type and in intestinal-specific *Slc34a2* deficient mice.** Real time RT-PCRs to quantify the renal mRNA expression of (**A**) *Cyp27b1* (n= 6-7-5-6), (**B**) *Cyp24a1* (n= 6-7-5-6) and (**C**) *Vdr* (n= 5-5-4-5) in samples from wild type (WT) and *Slc34a2* deficient mice (KO) injected either with vehicle (C) or with 1,25(OH)$_2$ vitamin D$_3$ (D3). The expression of the analyzed transcripts was normalized to the abundance of *18S*. (**D**) Western blots on renal homogenates with antibodies against Cyp24a1 (top) and Vdr (middle) and beta-tubulin (bottom) (n= 6-7-5-6). Graphs show the quantification of Cyp24a1 (left) and Vdr (right) normalized to tubulin. Statistical significances were calculated with ANOVA test. (A) **P= 0.0016; (B) ****P≤ 0.0001 and ***P= 0.0004; (C) **P= 0.0015 for WT and 0.0016 for KO; (D) **P= 0.0043 for Cyp24a1; *P= 0.0291 and ***P= 0.0006 for Vdr in WT and KO respectively.

**Figure 2. Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ inversely regulates plasma levels of FGF23 and PTH in wild type and intestinal-specific S*lc34a2* deficient mice**. ELISA assays to quantify the plasma levels of (**A**) intact FGF23 (n= 7-8-7-8), (**B**) C-terminal FGF23 fragment (n= 7-6-7-7) and (**C**) PTH (n= 9 in all groups) in samples from wild type (WT) and *Slc34a2* deficient mice (KO) injected either with vehicle (C) or with 1,25(OH)$_2$ vitamin D$_3$ (D3). Real time RT-PCR to quantify the mRNA expression in bone of (**D**) *Fgf23* (n= 7-8-7-8), (**E**) *Galnt3* (n= 8-7-7-8) and (**F**) *Phex* (n= 8-8-7-8), with the expression of the three transcripts normalized to the abundance of *18S*. Statistical significances were calculated with ANOVA test. (A, B, C) ****P≤ 0.0001; (D) *P= 0.0263.

**Figure 3. Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ specifically increases active intestinal Pi absorption in wild type mice but not in intestinal-specific *Slc34a2* deficient mice**. *In vivo* absorption of $^{33}$P by the ileum measured in *in situ* ligated loops in the presence of (**A**) 100 µM (n= 4-4-4-5)or (**B**) 70 mM luminal P (n= 8-8-4-5) in samples from wild type (WT) and *Slc34a2* deficient mice (KO) injected either with vehicle (C) or with 1,25(OH)$_2$ vitamin D$_3$ (D3). *Ex vivo* fluxes of $^{32}$P in (**C, D**) jejunum (n= 6-7-7-6 and 5-7-7-6, respectively) and (**E, F**) ileum (n= 6-6-7-5 and 6-7-5-6, respectively) measured in Ussing chambers in the presence of (**C, E**) 100 µM or (**D, F**) 70 mM luminal P. Statistical significances were calculated with ANOVA test. (A) *P= 0.0103, ***P= 0.0007; (C) **P= 0.0040 between WT and 0.0030 between treated WT and KO; (E) *P= 0.0193 and **** P≤ 0.0001; (F) *P= 0.0382 and 0.0141 for vehicle and VD3 injected, respectively. Absolute apical to basolateral (AP-BL) and basolateral to apical (BL-AP) permeabilities for Pi in jejunum (G) and ileum (H) from wild type (WT) and *Slc34a2* deficient mice (KO) injected either with vehicle (C) or with 1,25(OH)$_2$ vitamin D$_3$ (D3). Sample size was 6-6-6-6 for WT and 4-5-5-5 for KO in jejunum and 6-6-6-5 for WT and 5-5-5-5

for KO in ileum. Statistical significances were calculated with ANOVA test. Western blot on 30 μg of (**I**) total membranes from jejunum and (**J**) total homogenates from ileum from wild type (WT) mice injected either with vehicle (C) or with 1,25$(OH)_2$ vitamin $D_3$ (D3) (n= 6-6); samples from single KOs were also included. Blots were incubated with antibodies against NaPi-IIb (top) and actin (bottom). Graphs show the abundance of the cotransporter normalized to actin. Statistical significances were calculated with t-test. **P= 0.0067 and ****P≤ 0.0001.

**Figure 4. Short-term administration of 1,25$(OH)_2$ vitamin $D_3$ triggers hyperphosphaturia, hypercalciuria and hypercalcemia in wild type and intestinal-specific S*lc34a2* deficient mice**. Determination of Pi (**A-C**) (n ≥7) and $Ca^{2+}$ (**D-F**) (n ≥7) concentrations in (**A, D**) stool, (**B, E**) urine and (**C, F**) plasma from wild type (WT) and *Slc34a2* deficient mice (KO) injected either with vehicle (C) or with 1,25$(OH)_2$ vitamin $D_3$ (D3). Fecal excretions were normalized to food intake, and urinary excretion to the creatinine output. The sample size was 9-9-8-8 for fecal determinations, 10-10-9-9 for urinary samples and 8-9-7-8 for plasma. Statistical significances were calculated with ANOVA test. (B) *P= 0.0439 between WT and 0.0361 between genotypes, **P= 0.0026; (E) **P= 0.0012, ***P= 0.0009; (F) ****P≤ 0.0001.

**Figure 5. Short-term administration of 1,25$(OH)_2$ vitamin $D_3$ has a minor or no effect on the mRNA expression of intestinal *Slc34* and *Slc20* $Na^+$/Pi cotransporters.** (**A, C, E**) Real time RT-PCRs to quantify the mRNA expression of (**A**) NaPi-IIb/*Slc34a2*, (**C)** Pit-1/*Slc20a1* and (**E**) Pit-2/*Slc20a2* in duodenum (D), jejunum (J), ileum (I), proximal colon (PC) and distal colon (DC) of vehicle-treated wild type mice (n=5 for all segments and genes, excepted for *Slc34a2* in duodenum with n=4). The expression of each gene was measured simultaneously in all intestinal segments and normalized to the abundance of 18S. Statistical differences between segments were calculated with ANOVA: (A) ****P≤ 0.0001, (B) ***P= 0.0006 between D and DC, ***P= 0.0002 between J and DC and I and DC, **P= 0.0011 between DC and PC, (C) *P= 0.0204 between J and DC and *P= 0.0352 between I and DC. (**B, D, F**) mRNA expression of (**B**) NaPi-IIb/*Slc34a2*, (**D**) Pit-1/S*lc20a1* and (**F**) Pit-2/S*lc20a2* in the 5 intestinal segments of wild type (WT) and *Slc34a2* deficient mice (KO) treated either with vehicle or with 1,25$(OH)_2$ vitamin $D_3$. The sample size was 5 for all groups, except: (B) duodenum of $VD_3$-treated mice with n=8, (D) all segments from vehicle-treated KO with n= 4, (F) all segments vehicle-treated KO with n=4 in D, J, I and DC and n=3 in PC). Upon normalization to 18S mRNA, for each genotype and segment the expression in vehicle-treated animals was considered as 100%. For each gene, all samples were measured simultaneously. Statistical significances versus vehicle treated mice were calculated with t-test. *P= 0.0430 and **P= 0.0026**.** (**G**) Raw data of real time RT-PCRs from which values in B,D,F were calculated. Statistical differences between groups were calculated with ANOVA. Significances on the expression of NaPi-IIb were: *P=0.0123 (between WTs) and *P= 0.0120

(between KOs) in duodenum, **P= 0.0024 in jejunum, ****P ≤ 0.0001 in ileum, **P= 0.0026 and ****P ≤ 0.0001 in proximal colon, and **P=0.0078 in distal colon. Significance in the expression of Pit-2 were: *P= 0.0322 in duodenum, 0.0264 in ileum and 0.0144 in proximal colon.

**Figure 6. Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ regulates the expression of genes involved in intestinal transport of $Ca^{2+}$ in wild type and intestinal-specific S*lc34a2* deficient mice.** (**A, C, E**) Real time RT-PCRs to quantify the mRNA expression of (**A**) *Trpv6*, (**C**) calbindin 9/*Calb3* and (**E**) Pmca1b/*Atp2b1* in duodenum (D), jejunum (J), ileum (I), proximal colon (PC) and distal colon (DC) of vehicle-treated wild type mice (n= 4-5-5-5-5 in A and 5-5-5-5-5 in C and D). Statistical differences between segments were calculated with ANOVA: (A) *P= 0.0335 and **P= 0.0024, (C) ****P≤ 0.0001, (E) ****P≤ 0.0001, ***P= 0.0004 and **P= 0.0016. The expression of the analysed transcripts was normalized to the abundance of 18S. (**B, D, F**) mRNA expression of (**B**) *Trpv6*, (**D**) calbindin 9/*Calb3* and (**F**) Pmca1b/*Atp2b1* in the 5 intestinal segments of wild type and *Slc34a2* deficient mice treated either with vehicle or with 1,25(OH)$_2$ vitamin D$_3$. Sample size (WT-vehicle, WT-VD$_3$, KO-vehicle, KO-VD$_3$) was: (B) 5-5-4-5 in D and DC and 6-6-4-6 in PD; (D) 5-5-4-5 in all segments except in DC with n= 6-6-5-6; (F) 5-5-4-5 in D, J and I, 6-6-4-6 in PC and 6-7-5-6 in DC. Upon normalization to *18S* mRNA, for each genotype and segment the expression in vehicle-treated animal was considered as 100%. For each gene, all samples were measured simultaneously. Statistical significances versus vehicle treated mice were calculated with t-test. (B): **P= 0.0019, ***P= 0.0002; (D) **P= 0.0062 and ***P= 0.0004 in duodenum, **P= 0.0077 and ***P= 0.0005 in jejunum, *P= 0.0159 and **P= 0.0038 in ileum, **P= 0.0044 and **P= 0.0035 in the proximal colon of WT and KO respectively, *P= 0.0204 and ***P= 0.0006 in distal colon**. .** (**G**) Raw data of real time RT-PCRs from which values in B,D,F were calculated. Statistical differences between groups were calculated with ANOVA. Significances on the expression of *Trpv6* were: **P= 0.0019 and ***P=0.0002 in distal colon. Significances on the expression of *Calb3* were: **P= 0.0062 and ***P= 0.0004 in duodenum; **P= 0.0077 and ***P=0.0005 in jejunum; *P= 0.0159 and **P=0.0038 in ileum; **P= 0.0044 and 0.0035 in proximal colon of WT aand KO, respectively; *P= 0.0204 and ***P=0.0006 in distal colon.

**Figure 7. Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ reduces the protein abundance of the Slc34a $Na^+$/Pi cotransporters in renal BBM in wild type and intestinal-specific S*lc34a2* deficient mice. (A)** Western blots on renal BBM from wild type (WT) and *Slc34a2* deficient mice (KO) injected either with vehicle (C) or with 1,25(OH)$_2$ vitamin D$_3$ (D3) (n= 6-7-5-6). The blot was incubated with antibodies against NaPi-IIa (top), NaPi-IIc (middle) and beta-tubulin (bottom). Graphs show the quantification of NaPi-IIa (top) and NaPi-IIc (bottom) normalized to tubulin. Statistical significances were calculated with ANOVA test: (**B**) Real time RT-PCRs to quantify the renal expression of NaPi-IIa/*Slc34a1*, NaPi-IIc/*Slc34a3*, Pit-1/*Slc20a1* and Pit-2/*Slc20a2* (n= 5-5-4-5 for all genes). The

expression of the transporters was normalized to the abundance of 18S. Statistical significances were calculated with ANOVA test. (A) *P= 0.0101 for NaPi-IIa and 0.0238 for NaPi-IIc, **P= 0.0047; (B) *P= 0.0263.

**Figure 8: Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ upregulates the mRNA expression of several transcripts involved in $Ca^{2+}$ reabsorption.** Real time RT-PCRs to quantify the mRNA expression of (**A**) *Trpv5*, (**B**) calbindin 28/*Calb1*, (**C**) Ncx-1/*Slc8a1* and (**D**) Pmca1b/*Atp2b1* in kidneys of wild type (WT) and intestinal-specific S*lc34a2* deficient mice (KO) injected either with vehicle (C) or with 1,25(OH)$_2$ vitamin D$_3$ (VD3) (n= 5-5-4-5 for all genes). The expression of the analysed transcripts was normalized to the abundance of *18S*. For each transcript, all samples were measured simultaneously. Statistical significances were calculated with ANOVA test. (A) *P= 0.0277; (B) **P= 0.0052 for WT and 0.0013 for KO; (C): *P= 0.0280, **P= 0.0066**.**

**Table 1: Source or sequences of primers and probes used for real time RT-PCR analysis**

**Table 2**: **Short-term administration of 1,25(OH)$_2$ vitamin D$_3$ does not regulate intestinal apical to basolateral (AP-BL) and basolateral to apical (BL-AP) permeabilities for $Na^+$ and $Cl^-$, transepithelial potential differences and resistance.** Absolute AP-BL and BL-AP permeabilities for $Na^+$ and $Cl^-$ (cm/sec), transepithelial potential differences (PD, in mV)) and transepithelial resistances (R, in $\Omega*cm^2$) were measured in jejunum and ileum from wild type (WT) and *Slc34a2* deficient mice (KO) injected either with vehicle (C) or 1,25(OH)$_2$ vitamin D$_3$ (D3). Sample size for permeabilities as in Fig. 3G-H; for PD: n= 5-6-5-5-5-5-5-5 in jejunum and 5-6-4-4-4-5-5-5 in ileum; for resistance: 6-6-6-6-5-5-5-5 in jejunum and 6-6-6-5-5-5-5-5 in ileum. Statistical significances were calculated with ANOVA test. a: **P= 0.0051 between the AP-BL PD of WT and KO mice injected with vehicle.

**Conflict of interest**
None

**Data availability**
Data available on request from the authors

**Author contributions**
- Conception: NH, JM, CAW
- Acquisition, analysis, interpretation: NH, EMPA, JM, US, TK, CB
- Drafting and critical revision: NH, JM, TK, CAW

All authors
- Approved the final version of the manuscript
- Agree to be accountable for all aspects of the work
- Qualify as authors

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**Table 1**

| Gene | Forward | Reverse | Probe |
|---|---|---|---|
| Cyp27b1 set | Thermofisher | | |
| Cyp24a1 | CCAGCGGCTAGAGATCAAAC | CACGGGCTTCATGAGTTTCT | TACGGGCTGATGATCCTGGAAGGAC |
| Vdr | AGGCCCACACTCAGCTTCT | ACAGGTCCAGGGTCACAGAG | TACACCCCCTCACTGGACATGATGG |
| Fgf23 | GTATGGATCTCCACGGCAAC | AGTGATGCTTCTGCGACAAGT | TTTTTGGATCGCTTCACTTCAGCCC |
| Galnt3 | GAGAAAGAGCGAGGGGAAAC | GTGGACCATGCTTCATTGTG | ACACCCGACCACCTGAATGTATTGAAC |
| Phex | GCTGCCAGAGAACAAGTGC | TCCTCAGCTATGCCAGGAAG | CAGTCCTCCACAATTTAGGGTCAATGGTG |
| Slc34a1/NaPi-IIa | TGATCACCAGCATTGCCG | GTGTTTGCAAGGCTGCCG | CCAGACACAACAGAGGCTTCCACTTCTATGTC |
| Slc34a2/NaPi-IIb | GAAGACCCTGAATACTGATTTCC | GATATGCCCTCTCAATGCTG | AGGCATGACCTTCATCGTGCAAAGC |
| Slc34a3/NaPi-IIc | CAGCGGTATTACCAGCAACA | CTGTCCTCCTCTGGAGATGC | GTGGCCTCTTCAGCTCTTGCACAGA |
| Slc20a1/Pit-1 | CGCTGCTTTCTGGTATTATGTCTG | AGAGGTTGATTCCGATTGTGCA | TTGTTCGTGCGTTCATCCTCCGTAAGG |
| Slc20a2/Pit-2 | AGGAGTGCAGTGGATGGAGC | ATTAGTATGAACAGCACGCCGG | ATTGTCGCCTCCTGGTTTATATCGCCAC |
| TRPV6 | TTCTGCAGATGGTTCCACAG | TCTCTGGTGCACCTCACATC | CAGCAGAAGAGGATCTGGGAATC |
| TRPV5 | CGTTGGTTCTTACGGGTTGAA | GTTTGGAGAACCACAGAGCCTCTA | TGTTTCTCAGATAGCTGCTCTTGTACTTCCTCTTTGT |
| Calb3/Calb-9k | GCTCTCCAAGGAGGAGCTAAA | CTCCATCGCCATTCTTATCC | CTCCTGAAGGCTTCAAGTACTCTG |
| Calb1/Calb28k | GACGGAAAGCTGGAACTGAC | TGAACTCTTTCCCACACATTTTGAT | ACCAGTGCAGGAAAATTTCCTTCTTAAATTCCA |
| Atp2b1/Pmca1b | AAATTCCTTCAGTTCCAGCTT | GTAGCCAGAGCCAGGGAAG | CAAGACTCGCCACTTAAGGC |
| Slc8a1/NCX | CCATCCTAGGCGAGCACAC | GCTCTCTCCAGCTGTTGGTC | CGAATTCAAGAGCACTGTGG |
| 18S | Thermofisher | | |

**Table 2**

| Genotype | Protocol | Jejunum $P_{Na}$ | Jejunum $P_{Cl}$ | Jejunum PD | Jejunum R | Ileum $P_{Na}$ | Ileum $P_{Cl}$ | Ileum PD | Ileum R |
|---|---|---|---|---|---|---|---|---|---|
| WT | AP-BL-C | 35.20 ± 10.66 | 17.80 ± 6.36 | -2.56 ± 1.07 | 49.91 ± 15.16 | 35.95 ± 4.91 | 19.73 ± 8.45 | 0.86 ± 1.97 | 44.79 ± 5.78 |
| WT | BL-AP-C | 31.49 ± 3.92 | 21.90 ± 3.92 | -1.32 ± 0.83 | 46.07 ± 0.0 | 30.80 ± 6.06 | 26.28 ± 7.66 | -0.91 ± 0.65 | 44.79 ± 8.98 |
| WT | AP-BL-D3 | 32.53 ± 4.86 | 18.64 ± 3.72 | 2.32 ± 0.98 | 48.63 ± 6.27 | 42.12 ± 5.05 | 20.46 ± 8.35 | -0.20 ± 1.06 | 40.31 ± 6.76 |
| WT | BL-AP-D3 | 31.97 ± 7.43 | 30.32 ± 9.14 | -1.13 ± 0.77 | 40.95 ± 7.93 | 37.55 ± 12.70 | 32.47 ± 11.86 | -1.58 ± 0.20 | 38.39 ± 12.14 |
| KO | AP-BL-C | 45.26 ± 14.67 | 18.91 ± 5.42 | -1.96 ± 0.53 | 36.33 ± 15.46 | 49.20 ± 7.49 | 24.33 ± 8.44 | -2.01 ± 0.56[a] | 33.89 ± 4.28 |
| KO | BL-AP-C | 40.74 ± 8.16 | 30.72 ± 9.59 | -1.25 ± 0.51 | 35.76 ± 8.05 | 41.21 ± 6.95 | 33.06 ± 7.72 | -0.51 ± 1.16 | 34.29 ± 7.57 |
| KO | AP-BL-D3 | 41.60 ± 5.17 | 24.52 ± 9.09 | -1.68 ± 0.20 | 38.26 ± 7.27 | 41.45 ± 12.28 | 19.40 ± 6.00 | -1.87 ± 0.94 | 41.70 ± 7.91 |
| KO | BL-AP-D3 | 39.11 ± 7.13 | 33.16 ± 7.73 | -0.73 ± 0.54 | 35.19 ± 7.51 | 38.47 ± 8.18 | 27.66 ± 6.71 | -1.31 ± 1.00 | 38.11 ± 6.30 |

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Figure 1

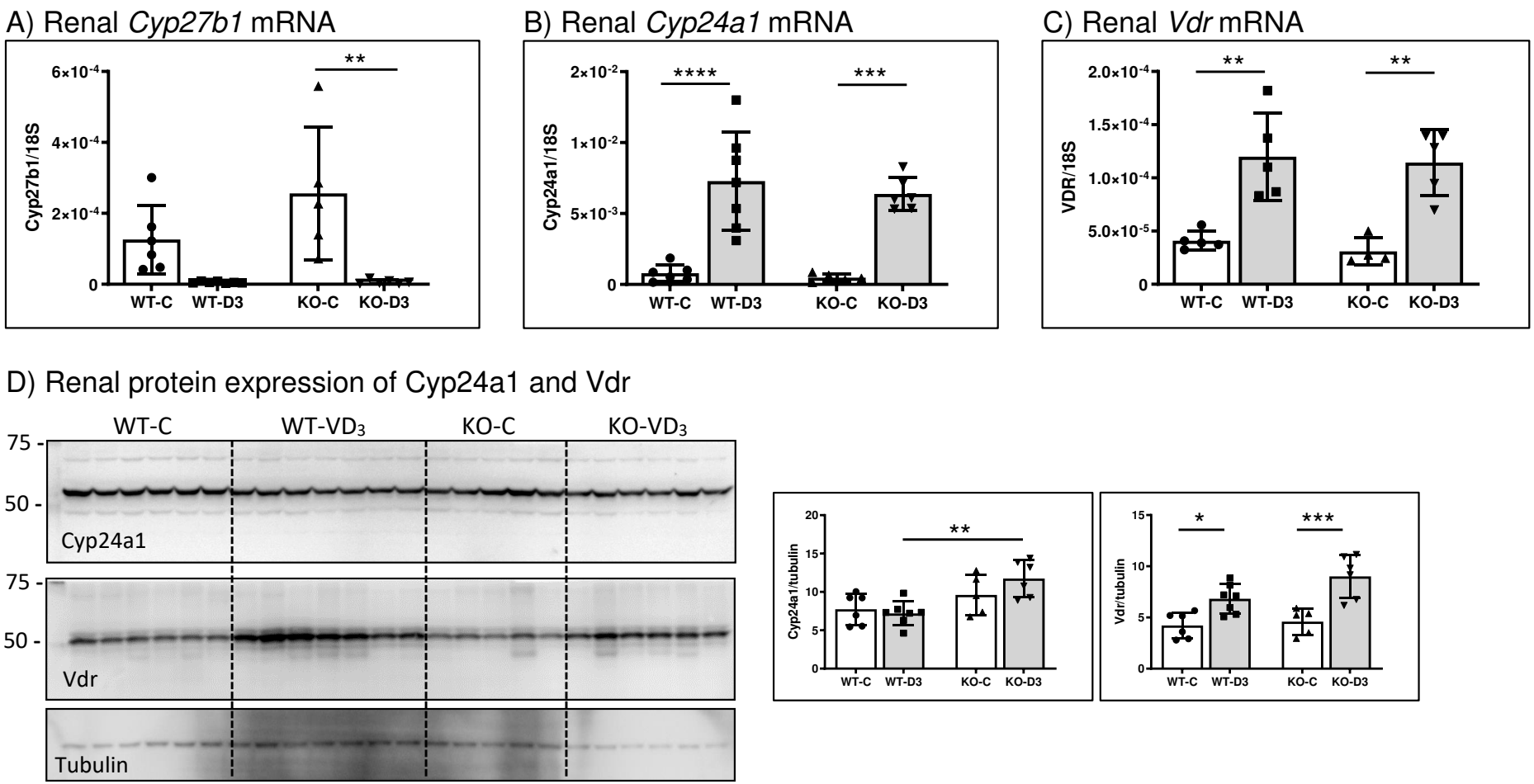

A) Renal *Cyp27b1* mRNA
Cyp27b1/18S
6×10-4
4×10-4
2×10-4
0
**
WT-C
WT-D3
KO-C
KO-D3
B) Renal *Cyp24a1* mRNA
Cyp24a1/18S
2×10-2
1×10-2
5×10-3
0
****
***
WT-C
WT-D3
KO-C
KO-D3
C) Renal *Vdr* mRNA
VDR/18S
2.0×10-4
1.5×10-4
1.0×10-4
5.0×10-5
0
**
**
WT-C
WT-D3
KO-C
KO-D3
D) Renal protein expression of Cyp24a1 and Vdr
WT-C
WT-VD3
KO-C
KO-VD3
75 -
50 -
Cyp24a1
75 -
50 -
Vdr
Tubulin
Cyp24a1/tubulin
20
15
10
5
0
**
WT-C
WT-D3
KO-C
KO-D3
Vdr/tubulin
15
10
5
0
*
***
WT-C
WT-D3
KO-C
KO-D3

Figure 2

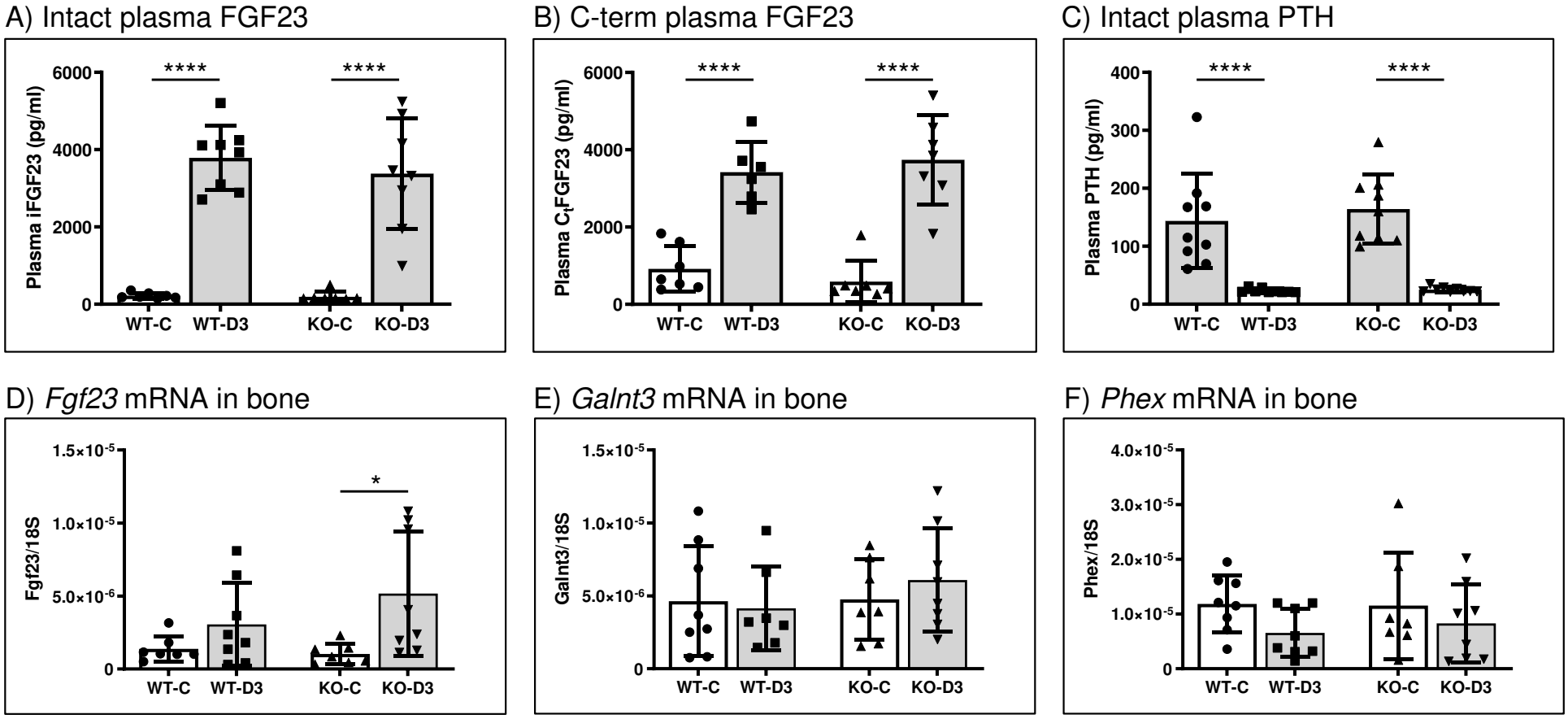

A) Intact plasma FGF23
Plasma iFGF23 (pg/ml)
6000
4000
2000
0
****
****
WT-C
WT-D3
KO-C
KO-D3
B) C-term plasma FGF23
Plasma C-FGF23 (pg/ml)
6000
4000
2000
0
****
****
WT-C
WT-D3
KO-C
KO-D3
C) Intact plasma PTH
Plasma PTH (pg/ml)
400
300
200
100
0
****
****
WT-C
WT-D3
KO-C
KO-D3
D) Fgf23 mRNA in bone
Fgf23/18S
1.5×10-5
1.0×10-5
5.0×10-6
0
*
WT-C
WT-D3
KO-C
KO-D3
E) Galnt3 mRNA in bone
Galnt3/18S
1.5×10-5
1.0×10-5
5.0×10-6
0
WT-C
WT-D3
KO-C
KO-D3
F) Phex mRNA in bone
Phex/18S
4.0×10-5
3.0×10-5
2.0×10-5
1.0×10-5
0
WT-C
WT-D3
KO-C
KO-D3

Figure 3

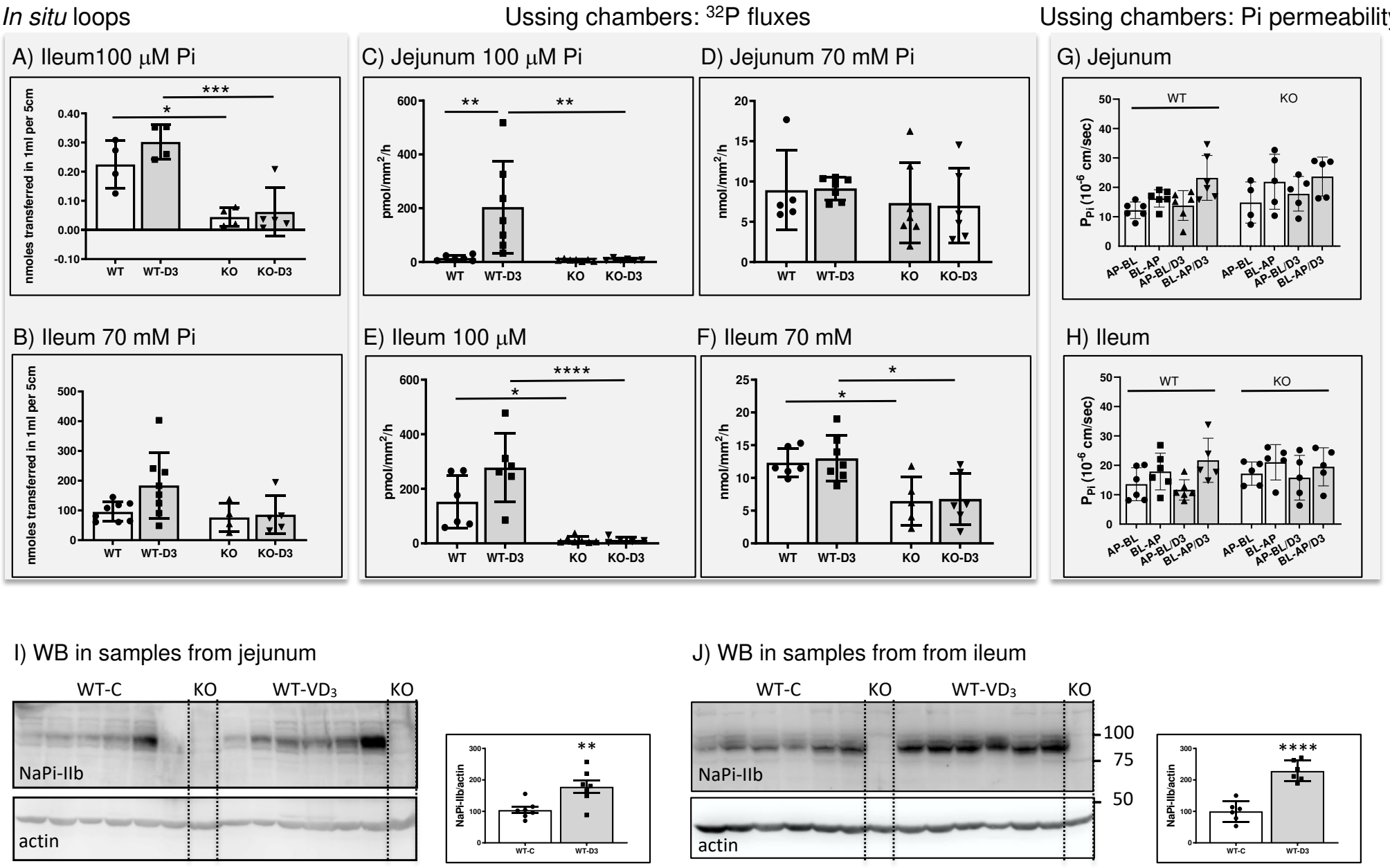

*In situ* loops
Ussing chambers: $^{32}$P fluxes
Ussing chambers: Pi permeability
A) Ileum100 μM Pi
B) Ileum 70 mM Pi
C) Jejunum 100 μM Pi
D) Jejunum 70 mM Pi
E) Ileum 100 μM
F) Ileum 70 mM
G) Jejunum
H) Ileum
nmoles transferred in 1ml per 5cm
pmol/mm²/h
nmol/mm²/h
P_Pi (10⁻⁶ cm/sec)
WT
WT-D3
KO
KO-D3
AP-BL
BL-AP
AP-BL/D3
BL-AP/D3
I) WB in samples from jejunum
J) WB in samples from from ileum
WT-C
KO
WT-VD3
NaPi-IIb
actin
NaPi-IIb/actin
100
75
50

Figure 4

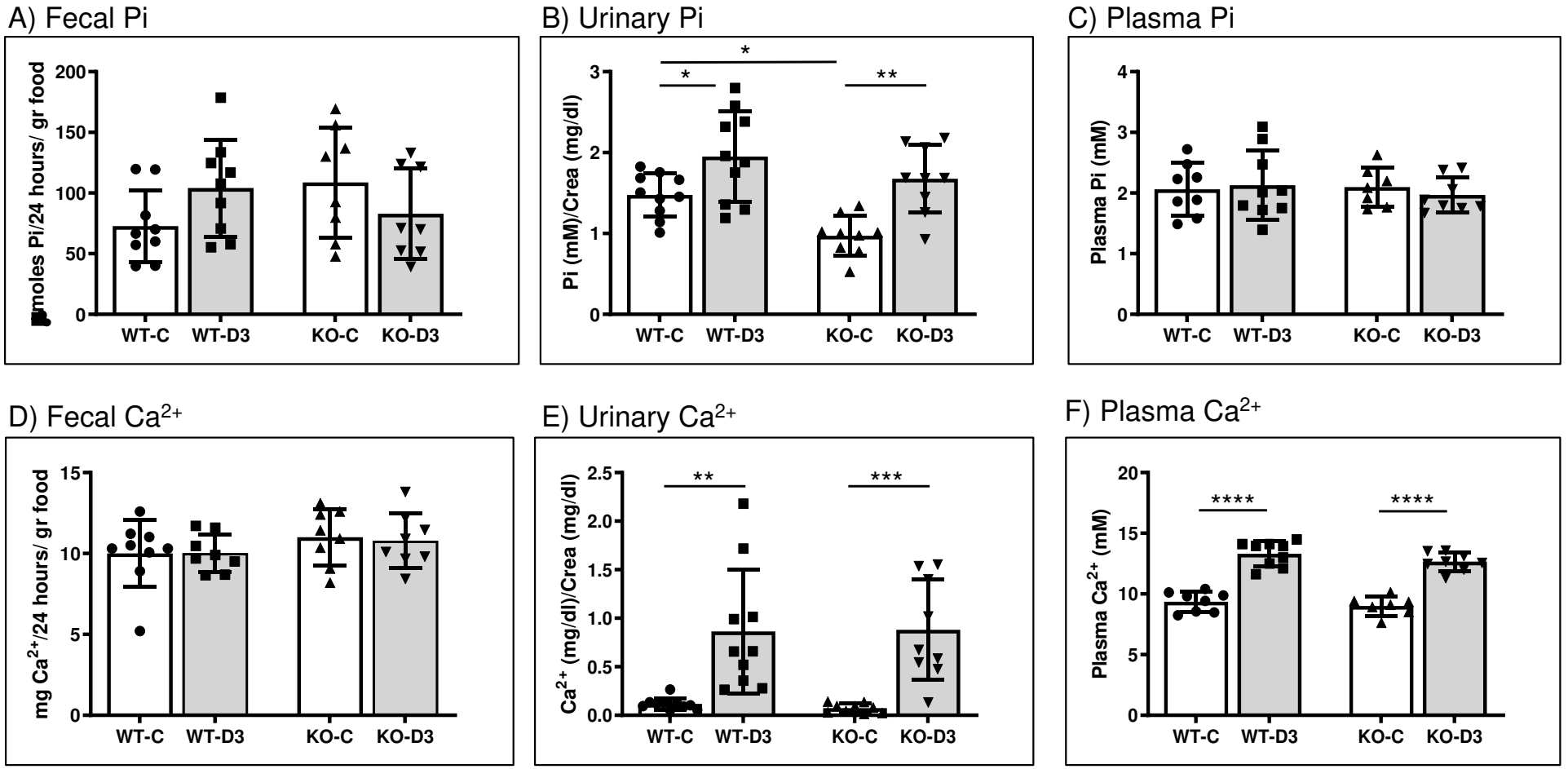

A) Fecal Pi
μmoles Pi/24 hours/ gr food
200
150
100
50
0
WT-C
WT-D3
KO-C
KO-D3
B) Urinary Pi
Pi (mM)/Crea (mg/dl)
3
2
1
0
*
*
**
WT-C
WT-D3
KO-C
KO-D3
C) Plasma Pi
Plasma Pi (mM)
4
3
2
1
0
WT-C
WT-D3
KO-C
KO-D3
D) Fecal Ca2+
mg Ca2+/24 hours/ gr food
15
10
5
0
WT-C
WT-D3
KO-C
KO-D3
E) Urinary Ca2+
Ca2+ (mg/dl)/Crea (mg/dl)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
WT-C
WT-D3
KO-C
KO-D3
F) Plasma Ca2+
Plasma Ca2+ (mM)
20
15
10
5
0
****
****
WT-C
WT-D3
KO-C
KO-D3

Figure 5

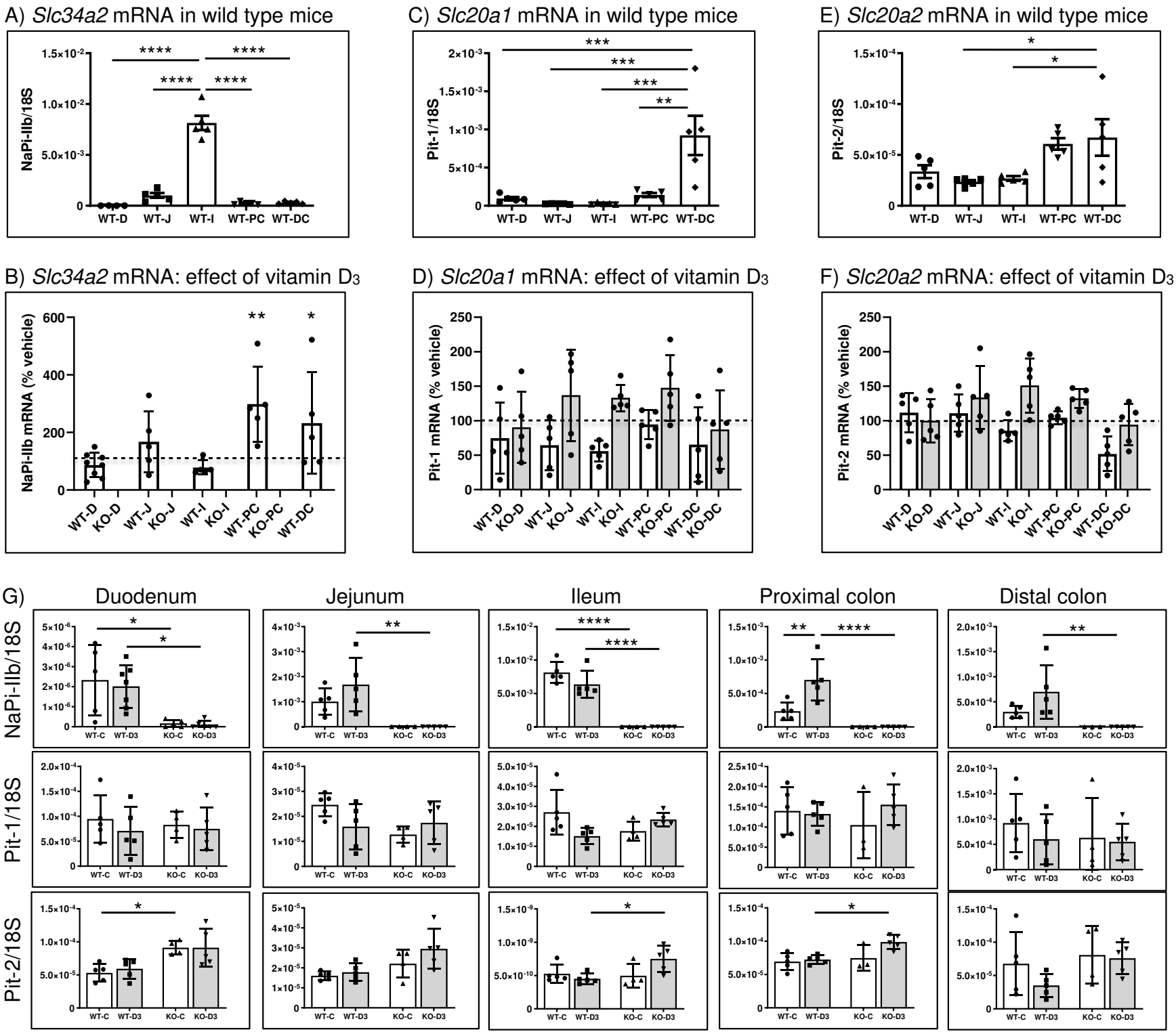

A) Slc34a2 mRNA in wild type mice
NaPi-IIb/18S
WT-D WT-J WT-I WT-PC WT-DC
C) Slc20a1 mRNA in wild type mice
Pit-1/18S
WT-D WT-J WT-I WT-PC WT-DC
E) Slc20a2 mRNA in wild type mice
Pit-2/18S
WT-D WT-J WT-I WT-PC WT-DC
B) Slc34a2 mRNA: effect of vitamin D3
NaPi-IIb mRNA (% vehicle)
WT-D KO-D WT-J KO-J WT-I KO-I WT-PC KO-PC WT-DC
D) Slc20a1 mRNA: effect of vitamin D3
Pit-1 mRNA (% vehicle)
WT-D KO-D WT-J KO-J WT-I KO-I WT-PC KO-PC WT-DC KO-DC
F) Slc20a2 mRNA: effect of vitamin D3
Pit-2 mRNA (% vehicle)
WT-D KO-D WT-J KO-J WT-I KO-I WT-PC KO-PC WT-DC KO-DC
G)
Duodenum
Jejunum
Ileum
Proximal colon
Distal colon
NaPi-IIb/18S
Pit-1/18S
Pit-2/18S
WT-C WT-D3 KO-C KO-D3

Figure 6

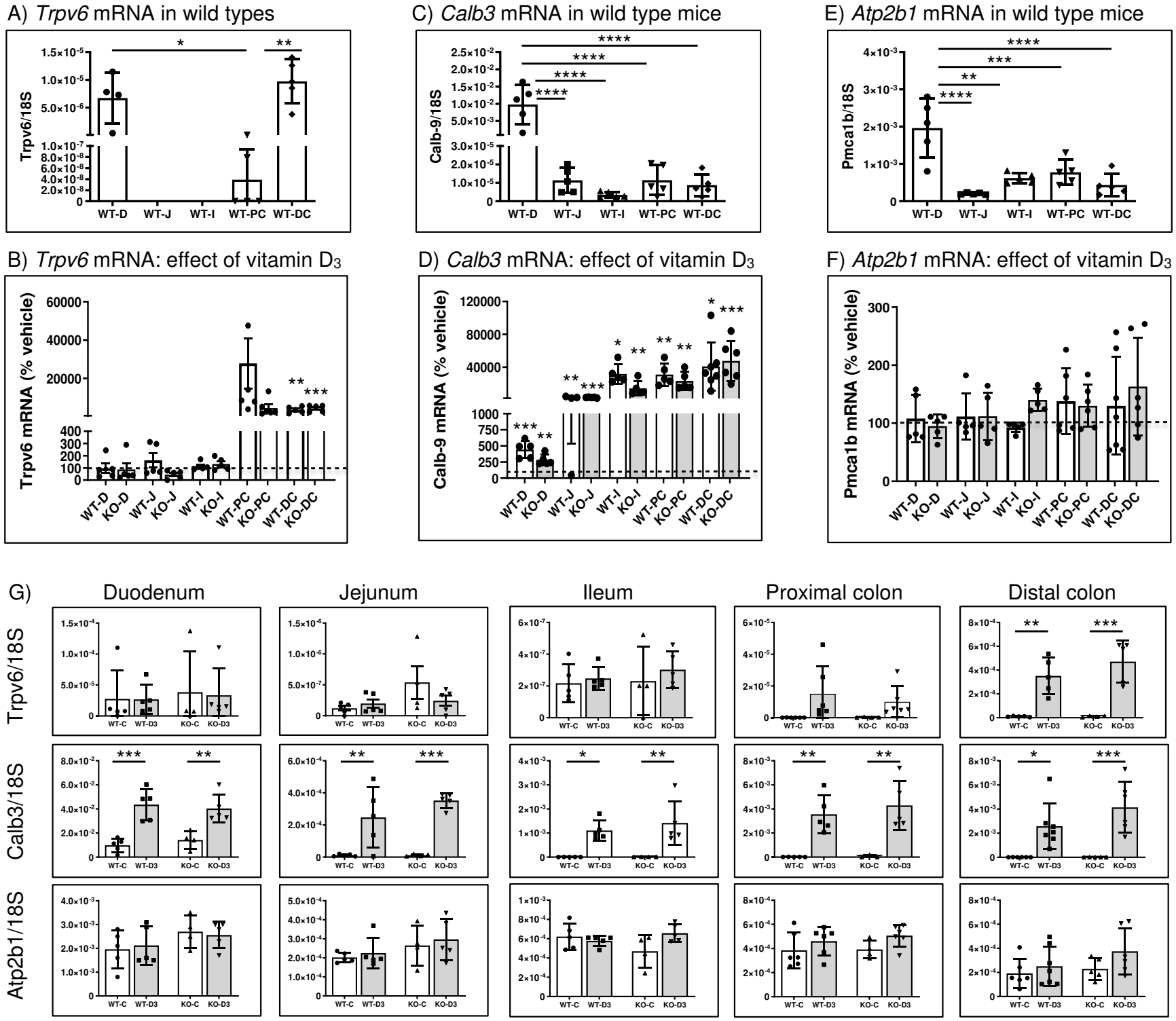
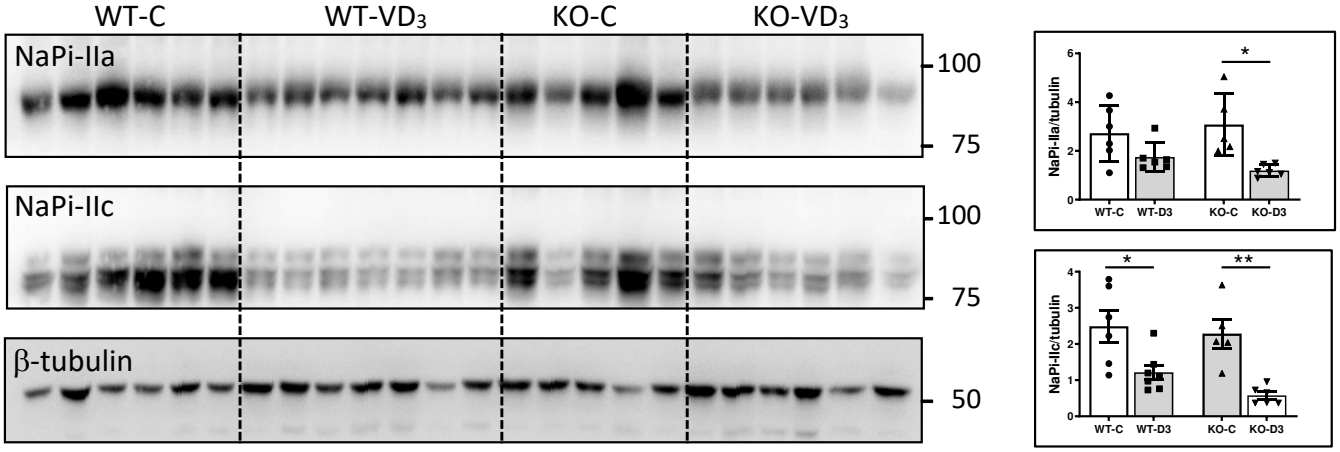

A) Trpv6 mRNA in wild types
Trpv6/18S
WT-D
WT-J
WT-I
WT-PC
WT-DC
C) Calb3 mRNA in wild type mice
Calb-9/18S
E) Atp2b1 mRNA in wild type mice
Pmca1b/18S
B) Trpv6 mRNA: effect of vitamin D3
Trpv6 mRNA (% vehicle)
WT-D
KO-D
WT-J
KO-J
WT-I
KO-I
WT-PC
KO-PC
WT-DC
KO-DC
D) Calb3 mRNA: effect of vitamin D3
Calb-9 mRNA (% vehicle)
F) Atp2b1 mRNA: effect of vitamin D3
Pmca1b mRNA (% vehicle)
G)
Duodenum
Jejunum
Ileum
Proximal colon
Distal colon
Trpv6/18S
Calb3/18S
Atp2b1/18S
WT-C
WT-D3
KO-C
KO-D3

Figure 7

A) Expression of NaPi-IIa and NaPi-IIc proteins in renal BBM




B) Renal expression of *Slc34* and *Slc20* mRNAs

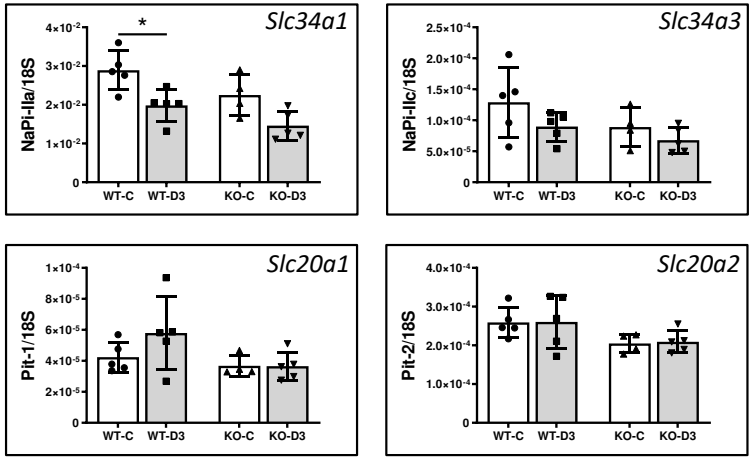
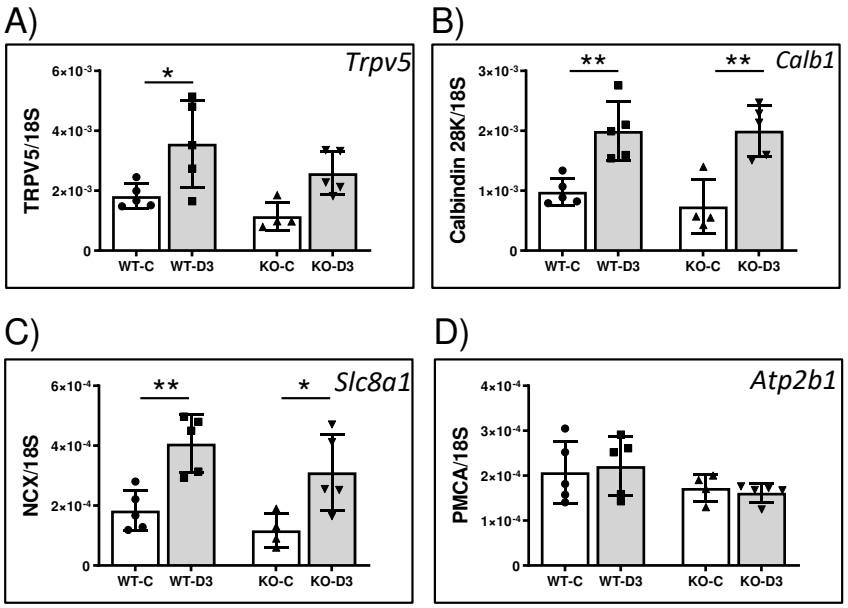


Figure 8


A)
Trpv5
TRPV5/18S
6×10-3
4×10-3
2×10-3
0
*
WT-C
WT-D3
KO-C
KO-D3
B)
Calb1
Calbindin 28K/18S
3×10-3
2×10-3
1×10-3
0
**
**
WT-C
WT-D3
KO-C
KO-D3
C)
Slc8a1
NCX/18S
6×10-4
4×10-4
2×10-4
0
**
*
WT-C
WT-D3
KO-C
KO-D3
D)
Atp2b1
PMCA/18S
4×10-4
3×10-4
2×10-4
1×10-4
0
WT-C
WT-D3
KO-C
KO-D3