OSR1-sensitive renal tubular phosphate reabsorption

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Abstract: Background: The oxidative stress-responsive kinase 1 (OSR1) participates in the WNK-(with no K) kinase dependent regulation of renal salt excretion and blood pressure. Little is known, however, about the role of OSR1 in the regulation of further renal transport systems. The present study analyzed the effect of OSR1 on NaPiIIa, the major renal tubular phosphate transporter. Methods: Immunohistochemistry and confocal microscopy were employed to determine renal localization of OSR1 and NaPiIIa. To elucidate the effect of OSR on NaPiIIa activity, cRNA encoding NaPiIIa was injected into Xenopus oocytes with or without additional injection of cRNA encoding OSR1, and phosphate transport was estimated from phosphateinduced currents determined with dual electrode voltage clamp. To elucidate the in vivo significance of OSR1 serum phosphate and hormone concentrations as well as urinary phosphate output of mice carrying one allele of WNK-resistant OSR1 (osr1(tg/+)) were compared to the respective values of wild type mice (osr1(+/+)). Results: NaPiIIa and OSR1 were both expressed in proximal renal tubule cells. Coexpression of OSR1 significantly up-regulated phosphate-induced currents in NaPiIIa-expressing Xenopus oocytes. Despite decreased serum phosphate concentration urinary phosphate excretion was significantly increased and NaPiIIa protein abundance in the brush border membrane significantly reduced in osr1(tg/+) mice as compared to osr1(+/+) mice. Serum PTH and calcitriol levels were similar in osr1(tg/+) mice and in osr1(+/+) mice, serum FGF23 concentration was, however, significantly higher in osr1(tg/+) mice than in osr1(+/+) mice. Conclusions: OSR1 is expressed in proximal renal tubules and participates in the regulation of FGF23 release and renal tubular phosphate transport.

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Abstract

The oxidative stress-responsive kinase 1 (OSR1) participates in the WNK-(with no K) dependent regulation of renal salt excretion and blood pressure. Little is known, however, about the role of OSR1 in the regulation of further renal transport systems. The present study analyzed the effect of OSR1 on NaPiIIa, the major renal tubular phosphate transporter. Immunohistochemistry and confocal microscopy were employed to determine renal localization of OSR1 and NaPiIIa. To elucidate the effect of OSR on NaPiIIa activity, cRNA encoding NaPiIIa was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding OSR1, and phosphate transport was estimated from phosphate-induced current determined with dual electrode voltage clamp. To elucidate the *in vivo* significance of OSR1 serum phosphate and hormone concentrations as well as urinary phosphate output of mice carrying one allele of WNK-resistant OSR1 ($osr1^{tg/+}$) were compared to the respective values of wild type mice ($osr1^{+/+}$). As a result, NaPiIIa and OSR1 were both expressed in proximal renal tubule cells. Coexpression of OSR1 significantly up-regulated phosphate-induced currents in NaPiIIa-expressing *Xenopus* oocytes. Despite a decreased serum phosphate concentration urinary phosphate excretion was significantly increased and NaPiIIa protein abundance in the brush border membrane significantly reduced in $osr1^{tg/+}$ mice as compared to $osr1^{+/+}$ mice. Serum PTH and calcitriol levels were similar in $osr1^{tg/+}$ mice and in $osr1^{+/+}$ mice, serum FGF23 concentration was, however, significantly higher in $osr1^{tg/+}$ mice than in $osr1^{+/+}$ mice. In conclusion, OSR1 is expressed in proximal renal tubules and participates in the regulation of renal tubular phosphate transport.

Key words: Na^+, phosphate cotransporter, FGF23, bone density

Introduction

The oxidative stress-responsive kinase 1 (OSR1) participates in the signaling regulating transport during oxidative and osmotic stress[1-6]. OSR1 is activated by the osmosensitive WNK (with no K) kinase isoforms WNK1 and WNK4 [3;7;8] and up-regulates the thiazide-sensitive Na^+-Cl^- cotransporter (NCC) and the furosemide-sensitive Na^+,K^+,$2Cl^-$ cotransporters (NKCC1 and NKCC2), thus contributing to the regulation of cell volume, transepithelial transport, renal salt excretion, and GABA neurotransmission[1-3;9-14]. Presumably due to their impact on renal tubular salt transport WNK1 and WNK4 [2;15-18] and OSR1 [7;19-22] participate in the regulation of blood pressure.

Nothing is known about a role of OSR1 in other nephron segments and in other renal tubular transport systems. The present study thus explored whether OSR1 influences the activity of the Na^+-coupled phosphate transporter NaPiIIa (SLC34A1), which is the most important carrier accomplishing renal tubular phosphate transport across the apical brush border membrane of proximal renal tubules[23-25]. Renal tubular phosphate reabsorption is tightly regulated by dietary phosphate intake, acid-base status, parathyroid hormone, 1,25-$(OH)_2$ vitamin D_3, FGF23, insulin and insulin-like growth factor IGF1[26-32]. Signaling known to regulate NaPiIIa includes the protein kinases A and C, ERK1/2, Klotho and the PI3K/PKB/GSK3 kinase cascade[33-40].

The present study addressed the putative role of OSR1 in the regulation of NaPiIIa. Coexpression of OSR1 enhanced phosphate-induced currents reflecting electrogenic phosphate transport in NaPiIIa-expressing *Xenopus* oocytes. Immunohistochemistry and confocal microscopy revealed coexpresssion of OSR1 and NaPiIIa in proximal renal tubules. Urinary phosphate excretion was higher and serum phosphate concentration lower in heterozygous OSR1 knockin mice resistant to WNK-mediated activation (*osr1*$^{tg/+}$) than in respective wild type mice (*osr1*$^{+/+}$). Thus, the observations point to a role of OSR1 in the regulation of renal tubular phosphate transport.

Materials and Methods

In vitro expression

For generation of cRNA, constructs were used encoding wild type human NaPiIIa [49] and human OSR1. The cRNA was generated as described previously[50]. The constructs encoding wild type and catalytically-inactive ^{D164A}OSR1 were kindly provided by Dario Alessi (University of Dundee, UK). For electrophysiology, *Xenopus* oocytes were prepared as previously described [51;52]. Fifteen ng of NaPiIIa cRNA were injected on the first day and 10 ng OSR1 cRNA on the second day into *Xenopus* oocytes. All experiments were performed at room temperature 3 days after the second injection. Two electrode voltage-clamp recordings were performed at a holding potential of -50 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments). The control solution (superfusate / ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4. One mM phosphate was added to induce NaPiIIa-dependent currents. The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s.

Animals

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Blood was drawn, urine collected or tissue isolated from sex- and age-matched 3-8-month-old heterozygous OSR1 knockin mice ($osr1^{tg/+}$) and respective wild type mice ($osr1^{+/+}$), kindly provided by Dario Alessi. As described earlier[53], in the knockin mice the T-loop Thr residue in OSR1 (Thr185) was mutated to Ala to prevent activation by WNK isoforms. Homozygous OSR1 knockin mice ($osr1^{tg/tg}$) are not viable[53]. Mice had free access to control diet (sniff, Soest, Germany) containing 7000 mg/kg phosphorus or to phosphate-depleted diet (Altromin, Lage, Germany) containing 131 mg/kg phosphate and to tap drinking water ad libitum. To obtain serum, mice were anaesthetized with diethylether (Roth, Karlsruhe, Germany) and blood was withdrawn into capillaries by puncturing the retrobulbar plexus.

To determine creatinine clearance, urinary flow rate and urinary excretion of phosphate, the mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) as described previously[54]. They were allowed a 2 days habituation period during which food and water intake, urinary flow rate and phosphate excretion were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently, 24 h collection of urine was performed for three consecutive days in order to obtain the urinary parameters. This procedure was repeated under low-phosphate diet. To assure quantitative urine collection, metabolic cages were siliconized, and urine was collected under water-saturated oil.

The phosphate concentration was determined colorimetrically utilizing a commercial diagnostic kit (Roche Diagnostics, Mannheim, Germany). The urinary concentration of Ca^{2+} was measured by flame photometry (Eppendorf, Hamburg, Germany). The creatinine concentration in urine was determined using the Jaffe reaction (Labor und Technik, Berlin, Germany). The serum intact parathormone concentration was measured using an ELISA kit (Immunotopics, San Clemante, USA) as was the concentration of $1,25(OH)_2D_3$ (IDS Diagnostics, Frankfurt/Main, Germany) and of FGF23 (Immunotopics).

Brush border membrane vesicles (BBMV) preparation and immunoblotting

BBMVs were prepared from mouse kidney cortex and outer medulla using the Mg^{2+} precipitation technique as described previously[55;56]. The total protein concentration was measured using the Bio–Rad Protein Assay kit (Bio–Rad, Hercules, CA, USA). BBMVs were stored at −80°C until further use. Five micrograms of renal brush border membrane proteins were solubilized in loading buffer containing dithiothreitol (DTT) and separated on 8% polyacrylamide gels. For immunoblotting, the proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 for 60 min, the

blots were incubated with the primary antibodies: rabbit polyclonal anti-NaPiIIa (1:6000) [57] and mouse monoclonal anti-β-actin antibody (42 kD; Sigma, St. Louis, MO; 1:5000) either for 2 h at room temperature or overnight at 4°C. Membranes were then incubated for 1 h at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies 1:5000 linked to alkaline phosphatase (Promega, Mannheim, Germany) or to horseradish peroxidase (HRP) (Amersham, Freiburg, Germany). The protein signal was detected with the appropriate substrates (Millipore, Schwalbach, Germany) using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analysed using the software Advanced Image Data Analyser AIDA, Raytest to calculate the protein of interest/ β-actin ratio.

Western blotting of whole kidney

To determine protein abundance in renal tissue, mice were anesthetized with diethylether. The kidneys were removed and immediately shock-frozen in liquid nitrogen. Renal tissue was homogenised with an electric homogenizer at 4°C in lysis buffer (54.6 mM HEPES; 2.69 mM $Na_4P_2O_7$; 360 mM NaCl; 10% [vol/vol] Glycerol; 1% [vol/vol] NP40) containing phosphatase and protease inhibitors (Complete mini, Roche, Mannheim, Germany). Homogenates were clarified by centrifugation at 20000 g for 20 min. Total protein (100 µg) was separated by SDS-PAGE (10% Tris-Glycine), transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), blocked for 3 h in blocking buffer (5% fat-free milk in Tris-buffered saline (TBS) containing 0.1% Tween), and incubated overnight with an anti Klotho antibody (kindly provided by Akiko Saito from the Kyowa Hakko Kirin Co., Ltd., Japan) (1:1.000 in 5% fat free milk in TBS). After incubation with a horseradish peroxidase-conjugated anti-rat secondary antibody (Cell Signaling, Germany), the bands were visualized with enhanced chemiluminescence according to the manufacturer's instructions. Homogenates were also probed with a primary GAPDH (Cell Signaling) antibody as loading control. Densitometric analysis of Klotho and GAPDH was performed using Quantity One software (Bio-Rad Laboratories).

Immunhistochemistry

For immunofluorescence microscopic detection of NaPiIIa and OSR in proximal tubule immunolabeling was performed on cryostat or paraffin sections of 5 µm thickness, blocked with 5% milk powder in PBS, incubated for 2 h with primary antibodies (sheep anti-OSR antibody [1:500 dilution; University of Dundee] and rabbit anti–NaPiIIa [1:400 dilution; kind gift of J. Biber, Zurich]), followed by secondary antibodies (Cy3-coupled donkey-anti sheep IgG and Cy2-coupled swine-anti rabbit IgG [Dianova]). Specificity of the double-staining procedures was controlled by parallel incubation of consecutive sections, each incubated only with one single probe. Fluorescence signals were detected by confocal laser scanning microscopy (LSM 510 META; Carl Zeiss, Jena, Germany) with a Plan Neofluar 100×/1.3 oil objective.[58;59]

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using unpaired Student’s t-test or ANOVA, as appropriate. Only results with $p < 0.05$ were considered statistically significant.

Results

Immunohistochemistry was employed to explore whether OSR1 is expressed in proximal renal tubules. As illustrated in Fig. 1, OSR1 protein could indeed be detected in the proximal nephron. Concomitant labelling of the proximal tubular phosphate transporter NaPiIIa revealed co-localization of the kinase with the carrier in a subset of proximal tubules (Fig. 1).

To possibly disclose an effect of OSR1 on NaPiIIa, cRNA encoding NaPiIIa was injected into *Xenopus* oocytes with or without cRNA encoding OSR1 and electrogenic phosphate transport determined by the dual-electrode voltage-clamp technique. Addition of phosphate (1 mM) to the bath induced only a small inward current in non-injected or water-injected *Xenopus* oocytes, indicating that *Xenopus* oocytes do not express endogeneous electrogenic phosphate transport (Fig. 2). In *Xenopus* oocytes expressing NaPiIIa, phosphate induced an inward current (I_P) consistent with electrogenic entry of Na^+ together with phosphate (Fig. 2). As illustrated in Fig. 2, I_P was significantly enhanced by additional coexpression of OSR1. In contrast, coexpression of the inactive mutant ^{D164A}OSR1 did not significantly modify I_P in NaPiIIa-expressing *Xenopus* oocytes (Fig. 2).

In order to elucidate the *in vivo* significance of OSR1-sensitive renal tubular phosphate transport, experiments were performed in OSR1 knockin mice (*osr1*$^{tg/+}$), which were heterozygously carrying a WNK-insensitive ^{T185A}OSR1 mutant. The animals were compared to respective wild type mice (*osr1*$^{+/+}$). On a standard diet, urinary phosphate excretion was significantly higher in *osr1*$^{tg/+}$ mice than in *osr1*$^{+/+}$ mice (Fig. 3). A low-phosphate diet decreased the urinary phosphate excretion in both, *osr1*$^{tg/+}$ and *osr1*$^{+/+}$ mice (Fig. 3). During the low-phophate-diet, however, urinary phosphate excretion was still significantly higher in *osr1*$^{/tg/+}$ mice than in *osr1*$^{+/+}$ mice (Fig. 3).

In theory, the phosphaturia of *osr1*$^{tg/+}$ mice could have been due to an increased serum phosphate concentration. Conversely, phosphaturia due to decreased renal tubular phosphate transport should result in a decrease of serum phosphate concentration. Thus, serum phosphate concentration was determined. As illustrated in Fig. 4, the serum phosphate concentration was significantly lower in *osr1*$^{tg/+}$ mice than in *osr1*$^{+/+}$ mice. Thus, the phosphaturia of *osr1* $^{tg/+}$ mice was not due to hyperphosphatemia but obviously resulted in hypophosphatemia. Dietary phosphate depletion decreased the serum phosphate concentration in both, *osr1*$^{tg/+}$ mice and in *osr1*$^{+/+}$ mice and further enhanced the difference between the genotypes.

To study whether the partial OSR1 deficiency results in lower apical expression of NaPiIIa, BBMVs from the proximal tubule were retrieved and NaPiIIa protein expression determined. As shown in Fig. 5, BBMVs from *osr1*$^{tg/+}$ mice indeed expressed NaPiIIa to a significantly lesser extent than BBMVs from *osr1*$^{+/+}$ mice.

At least in theory, the decreased renal tubular phosphate transport could have resulted from altered hormone levels. Thus, the serum concentration of parathyroid hormone (PTH), calcitriol ($1,25(OH)_2D_3$), and fibroblast growth factor 23 (FGF23) were determined. As shown in Fig. 6, the serum concentrations of PTH (Fig. 6A) and of $1,25(OH)_2D_3$ (Fig. 6B) were similar in *osr1*$^{tg/+}$ mice and in *osr1*$^{+/+}$ mice. The serum FGF23 concentration was, however, significantly higher in *osr1*$^{tg/+}$ mice than in *osr1*$^{+/+}$ mice (Fig. 6C). Dietary phosphate depletion resulted in a significant increase in the $1,25(OH)_2D_3$ concentration of both, *osr1*$^{tg/+}$ mice and *osr1*$^{+/+}$ mice and tended to decrease the serum concentrations of PTH and FGF23, effects, however not reaching statistical significance (Fig. 6).

As FGF23 signaling depends on Klotho, additional experiments were performed to elucidate whether partial OSR deficiency modifies renal Klotho expression. To this end, renal Klotho protein was determined in *osr1*$^{tg/+}$ mice and *osr1*$^{+/+}$ mice (Fig. 7). As a result, no significant difference of Klotho protein abundance could be observed between *osr1*$^{tg/+}$ mice and *osr1*$^{+/+}$ mice.

Discussion

The present observations reveal that oxidative stress-responsive kinase 1 (OSR1) is expressed in proximal renal tubules and stimulates renal tubular phosphate transport. Moreover, WNK resistance of OSR1 led to phosphaturia. Accordingly, renal tubular phosphate reabsorption was decreased and thus renal phosphate excretion increased in OSR1 knockin mice carrying one allele of WNK-insensitive ^{T185A}OSR1 mutant (*osr1*$^{tg/+}$) as compared to the respective wild type mice (*osr1*$^{+/+}$).

The electrophysiological recordings with the two-electrode voltage clamp in *Xenopus* oocytes heterologously expressing NaPiIIa with and without OSR1 pointed to a stimulatory effect of OSR1 on NaPiIIa activity. Along those lines, partial deficiency of OSR1 activity in *osr1*$^{tg/+}$ mice led to lower NaPiIIa surface abundance in brush border membrane vesicles iolated from the proximal tubule.

The phosphaturia of *osr1*$^{tg/+}$ mice was not secondary to an increased serum phosphate concentration, which was actually decreased. In theory, the phosphaturia *osr1* $^{tg/+}$ could result from an increased serum level of PTH, a hormone increasing urinary phosphate output by stimulation of internalization and subsequent degradation of NaPiIIa[30;34]. The PTH serum concentration was, however, not significantly different between *osr1*$^{tg/+}$ and *osr1*$^{+/+}$ mice. This observation, does not rule out minor alterations of the serum PTH level. Renal tubular phosphate transport and serum phosphate concentration could further be modified by calcitriol ($1,25(OH)_2D_3$). The serum $1,25(OH)_2D_3$ concentration was, however, again not significantly different between between *osr1*$^{tg/+}$ mice and *osr1*$^{+/+}$ mice. The renal 1α-hydroxylase and thus the formation of $1,25(OH)_2$ D_3 are stimulated by PTH [41] and by cellular phosphate depletion[42]. The present observations confirm the increase in serum $1,25(OH)_2D_3$ following dietary phosphate depletion and confirm the decrease of urinary phosphate output in phosphate-depleted animals.

Our study further shows that the FGF23 serum concentration was higher in *osr1*$^{tg/+}$ mice than in *osr1*$^{+/+}$ mice. FGF23 is known to be up-regulated by $1,25(OH)_2D_3$ and in turn to decrease the formation of $1,25(OH)2D_3$[43;44]. In view of the serum $1,25(OH)_2D_3$ levels, the slightly but significantly enhanced FGF23 serum level in *osr1*$^{tg/+}$ mice cannot be explained by $1,25(OH)_2D_3$-dependent stimulation of FGF23 expression.

FGF23 is secreted by osteoblasts and decreases renal tubular phosphate transport,[45]. The increased serum FGF23 concentrations could thus contribute to the phosphaturia of the *osr1*$^{tg/+}$ mice. Since FGF23 formation is stimulated by PTH, increased phosphate intake and hyperphosphatemia[46-48], the enhanced FGF23 serum level of *osr1*$^{tg/+}$ mice cannot be explained by renal phosphate loss and hypophosphatemia. In theory, the enhanced FGF23 release in *osr1*$^{tg/+}$ mice could be secondary to the effect of OSR1 on NaCl-co-transporter NCC. The carrier is expressed in osteoblasts, fosters bone differentiation and increases bone mineral density[23-25]. OSR1 activates and phosphorylates NCC[1-3;9-14]. Alternatively, OSR1 could be effective through inhibition of renal NCC or by more directly modulating FGF23 release in osteoblasts. Further studies are warranted addressing the mechanisms causing the elevated FGF23 plasma concentration in *osr1*$^{tg/+}$ mice .

The effect of FGF23 on renal cells requires expression of Klotho, which thus similarly influences phosphate homeostasis[38]. The determination of total renal Klotho protein expression did, however not reveal any significant differences between *osr1*$^{tg/+}$ mice and *osr1*$^{+/+}$ mice.

In conclusion, OSR1 participates in the regulation of FGF23 release and renal tubular phosphate transport. OSR1 stimulates NaPiIIa, and partial loss of OSR1 activity leads to phosphaturia and increased formation of FGF23. The present observations thus disclose a completely novel, powerful element in the regulation of mineral metabolism and a novel functional role of oxidative stress-responsive kinase 1.

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Statement of competing financial interests

The authors declare that they have no competing financial interests to disclose.

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Figure legends

Fig. 1. Distribution of the oxidative stress responsive kinase 1 (OSR1) in wild type (*osr1*$^{+/+}$) kidneys.
A. Confocal images showing double-labeling of OSR1 (green signal) and NaPiIIa (red signals) in proximal tubules from wild type kidneys. Merged images demonstrate co-localization of OSR1 and NaPiIIa in a subset of proximal tubules, whereas some proximal tubules express either OSR1 or NaPiIIa.
B. Higher resolution images demonstrating co-localization of OSR1 (green signal in the apical cellular compartment) and NaPiIIa (red signal in the brushborder membrane) in proximal tubules from wild type kidney; nuclei are counterstained in blue on the merged image.

Fig. 2: Coexpression of OSR1 up-regulates electrogenic phosphate transport in NaPiIIa-expressing *Xenopus* oocytes
A. Representative original tracings showing phosphate (1 mM)-induced current (I_P) in *Xenopus* oocytes injected with water (a) as well as expressing NaPiIIa without (b) or with additional coexpression of wild type OSR1(c) or inactive mutant ^{D164A}OSR1 (d).
B. Arithmetic means ± SEM (n = 17-62) of phosphate (1 mM)-induced current (I_P) in *Xenopus* oocytes injected with water (H_2O), or expressing NaPiIIa without (2nd bar) or with additional coexpression of wild type OSR1 (3rd bar) or of the inactive mutant ^{D164A}OSR1 (4th bar). *** ($p<0.001$) indicates statistically significant difference from the absence of OSR1. ### ($p<0.001$) indicates statistically significant difference between wild type OSR1 and mutant ^{D164A}OSR1.

Fig. 3: Urinary phosphate excretion in *osr1*$^{+/+}$ and *osr1*$^{tg/+}$ mice
Arithmetic means ± SEM (n = 14-16) of urinary phosphate excretion in heterozygous OSR1 knockin mice (*osr1*$^{tg/+}$, black bars) and wild type mice (*osr1*$^{+/+}$, white bars) under normal diet (left bars) and following dietary phosphate depletion (right bars). * ($p<0.05$), ** ($p<0.01$) indicate significant difference from *osr1*$^{+/+}$ mice, ### ($p<0.001$) indicates significant difference from normal diet.

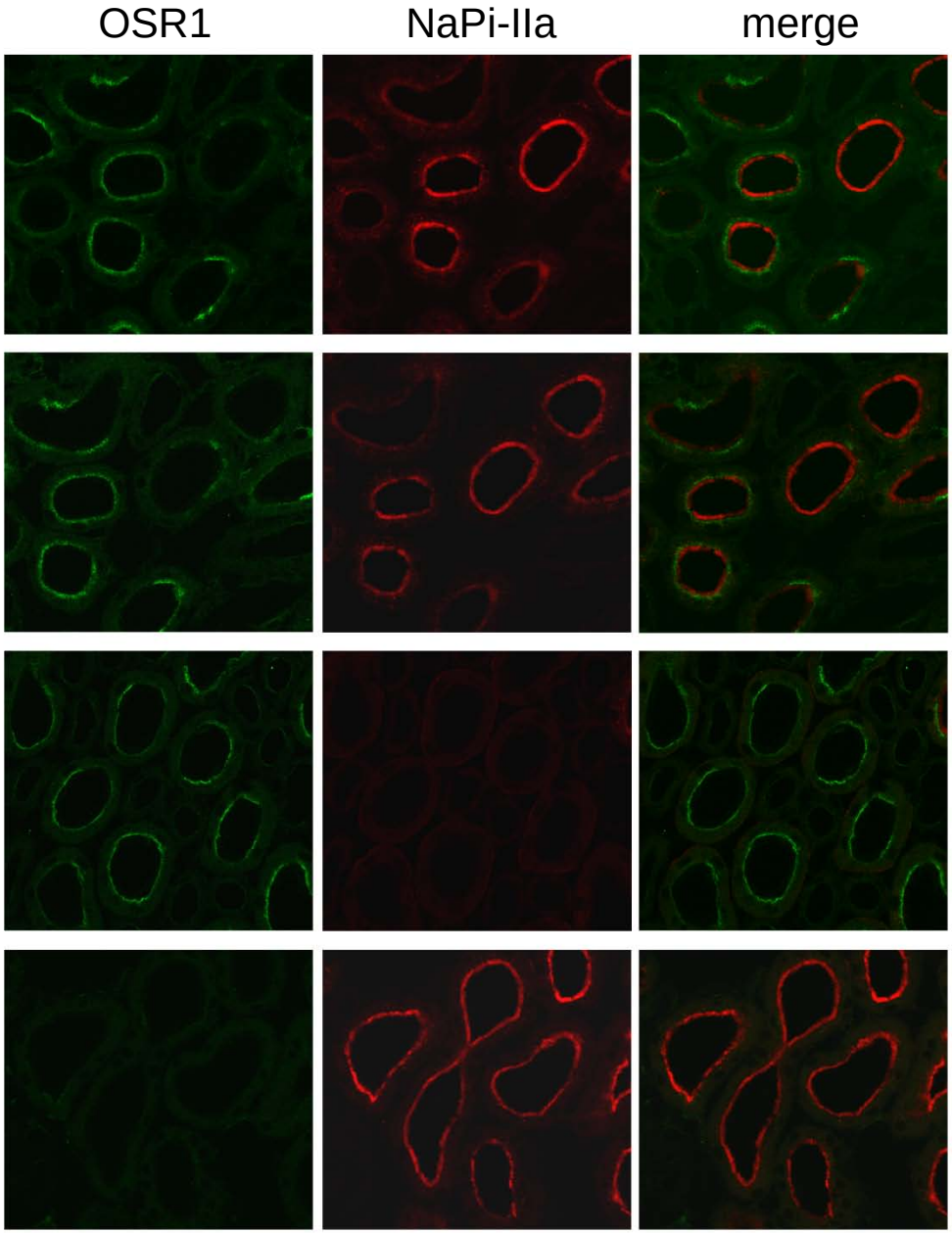
Fig. 4: Serum phosphate concentration in *osr1*$^{+/+}$ and *osr1*$^{tg/+}$mice
Arithmetic means ± SEM (n = 14-16) of the serum phosphate concentration in heterozygous OSR1 knockin mice (*osr1*$^{tg/+}$, black bars) and wild type mice (*osr1*$^{+/+}$, white bars) under normal diet (left bars) and following dietary phosphate depletion (right bars). * ($p<0.05$), ** ($p<0.01$) indicate significant difference from *osr1*$^{+/+}$ mice, # ($p<0.05$), ## ($p<0.01$) indicate significant difference from normal diet.

Fig. 5: Protein abundance of renal sodium-dependent phosphate cotransporter NaPiIIa in the brush border membrane in kidneys from *osr1*$^{+/+}$ and *osr1*$^{tg/+}$ mice
Western blot for NaPiIIa. The membrane was stripped and reprobed for β-actin to control for loading. Densitometry was performed and the ratio of the protein of interest over β-actin calculated. Bar graphs present data as arithmetic means ± SEM (n = 10). *** indicates significant difference from *osr1*$^{+/+}$ mice (Student's t-test, $p<0.001$).

Fig. 6: Serum concentration of PTH, 1,25(OH)$_2$D$_3$, and FGF23 in *osr1*$^{+/+}$ and *osr1*$^{tg/+}$ mice
Arithmetic means ± SEM (n = 8-11) of serum PTH (A), 1,25(OH)$_2$D$_3$ (B), and FGF23 (C) in osr1$^{+/+}$ mice (white bars) and osr1$^{tg/+}$ mice (black bars) under normal diet (left bars) and following dietary phosphate depletion (right bars). * ($p<0.05$) indicates significant difference from *osr1*$^{+/+}$ mice, ### ($p<0.001$) indicates significant difference from normal diet.

Fig.7 Abundance of Klotho protein in the kidneys from *osr1*$^{+/+}$ and *osr1*$^{tg/+}$ mice
Original Western blots (upper panel) and arithmetic means ± SEM (n = 10) of the Klotho over GAPDH density ratio in osr1$^{+/+}$ mice (white bar) and osr1$^{tg/+}$ mice (black bar).

A



B

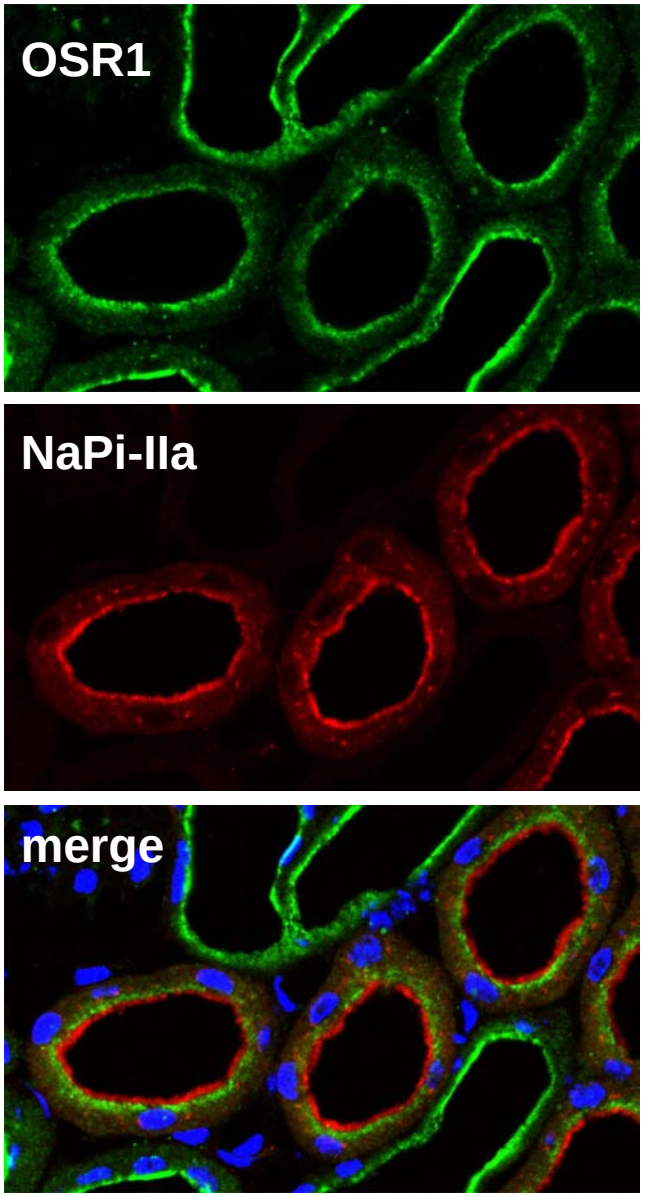


Figure 1

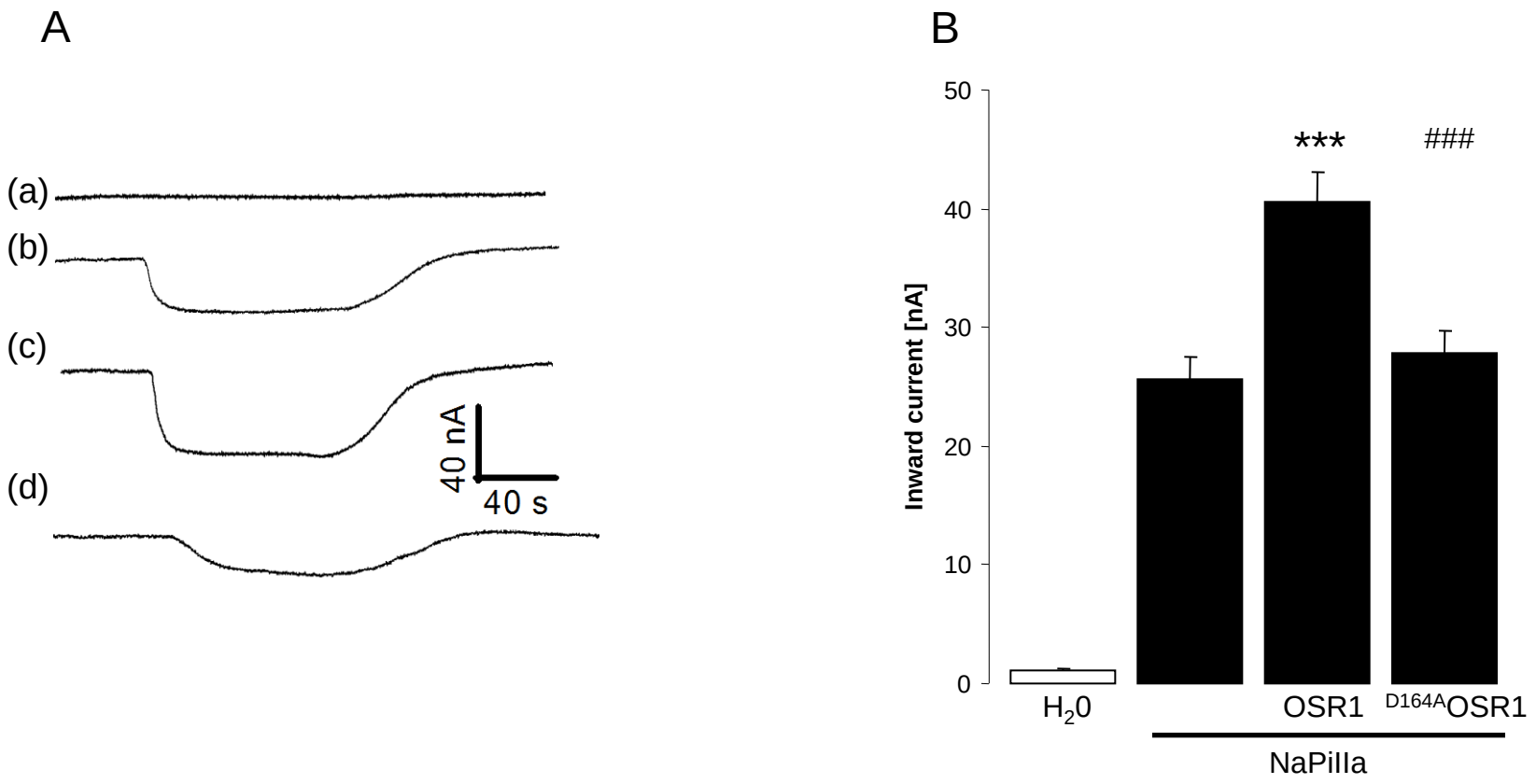

A
(a)
(b)
(c)
(d)
40 nA
40 s
B
Inward current [nA]
50
40
30
20
10
0
***
###
H20
OSR1
D164AOSR1
NaPiIIa

Figure 2

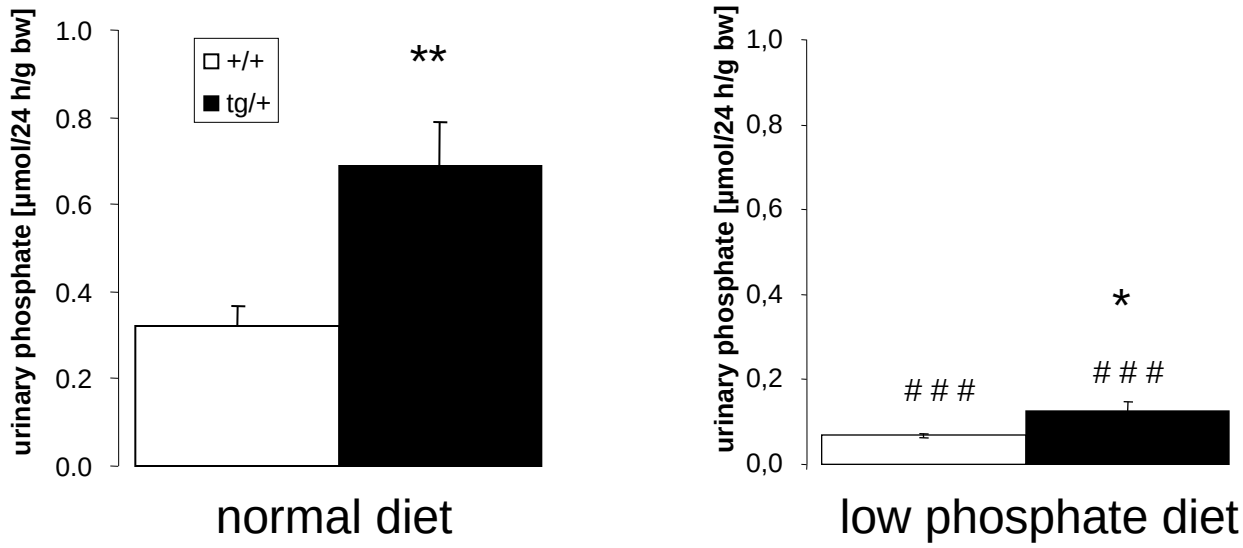
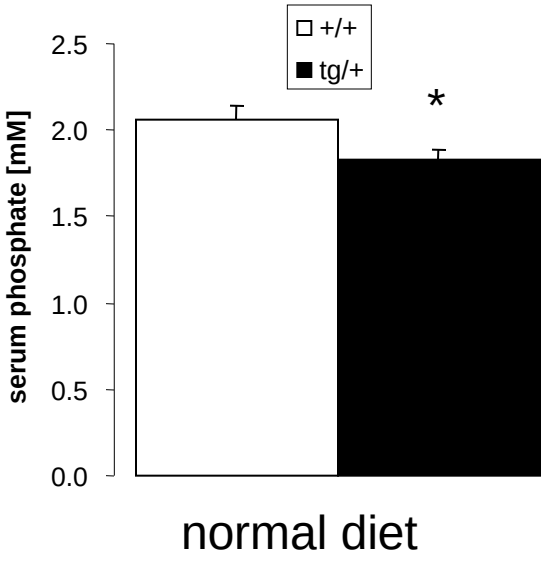
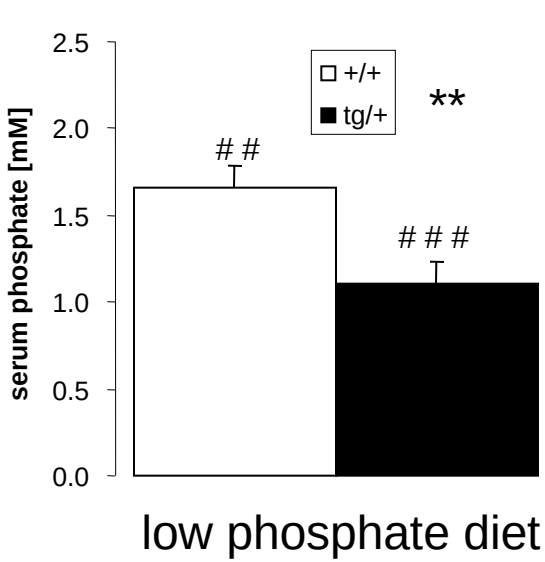

urinary phosphate [µmol/24 h/g bw]
1.0
0.8
0.6
0.4
0.2
0.0
+/+
tg/+
**
normal diet
urinary phosphate [µmol/24 h/g bw]
1,0
0,8
0,6
0,4
0,2
0,0
*
# # #
# # #
low phosphate diet

Figure 3


□ +/+
■ tg/+
serum phosphate [mM]
2.5
2.0
1.5
1.0
0.5
0.0
*
normal diet


□ +/+
■ tg/+
serum phosphate [mM]
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2.0
1.5
1.0
0.5
0.0
**
# #
# # #
low phosphate diet

Figure 4

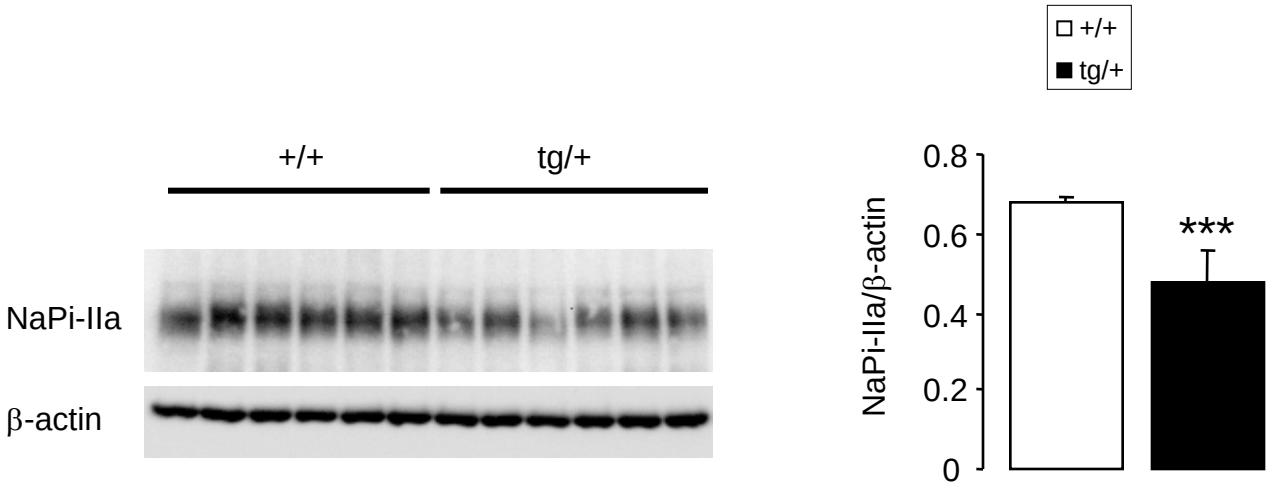
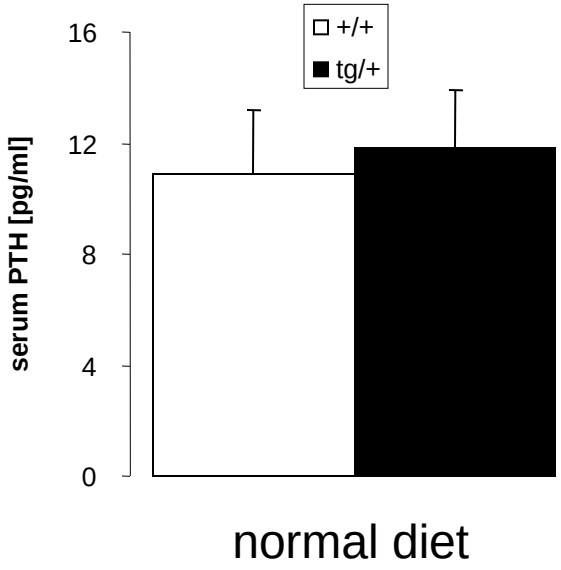
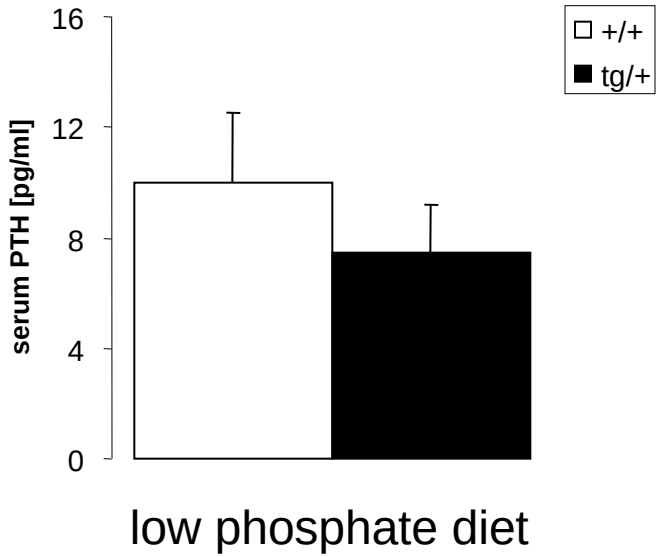


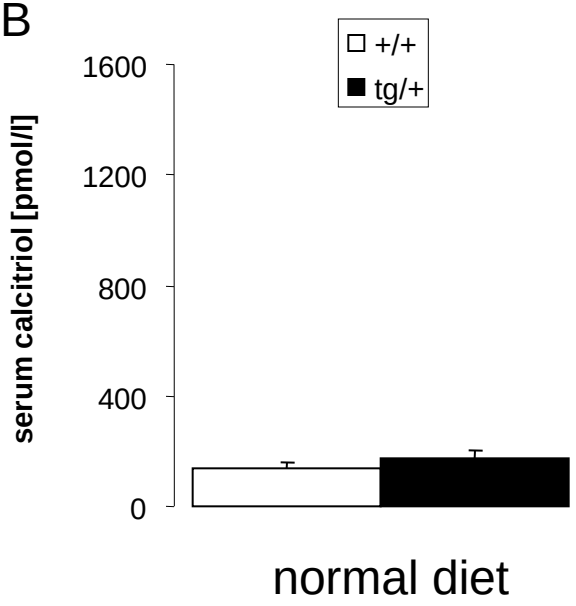
Figure 5

A


+/+
tg/+
serum PTH [pg/ml]
16
12
8
4
0
normal diet


+/+
tg/+
serum PTH [pg/ml]
16
12
8
4
0
low phosphate diet

B


+/+
tg/+
serum calcitriol [pmol/l]
1600
1200
800
400
0
normal diet

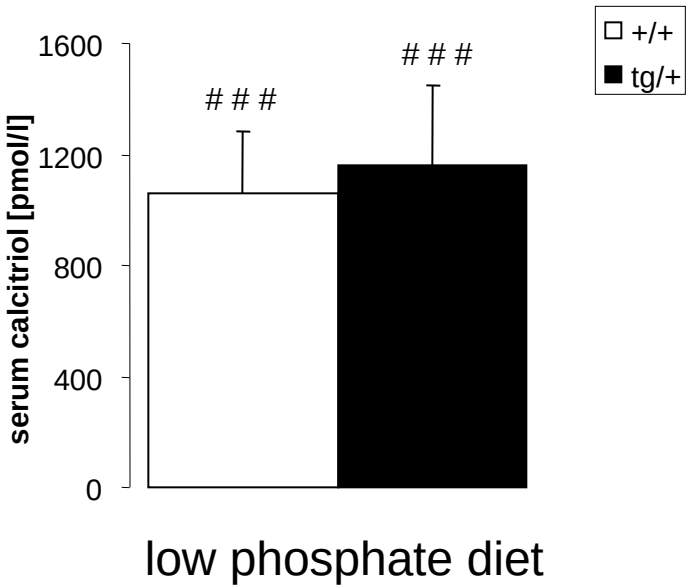

+/+
tg/+
# # #
# # #
serum calcitriol [pmol/l]
1600
1200
800
400
0
low phosphate diet

Figure 6

C

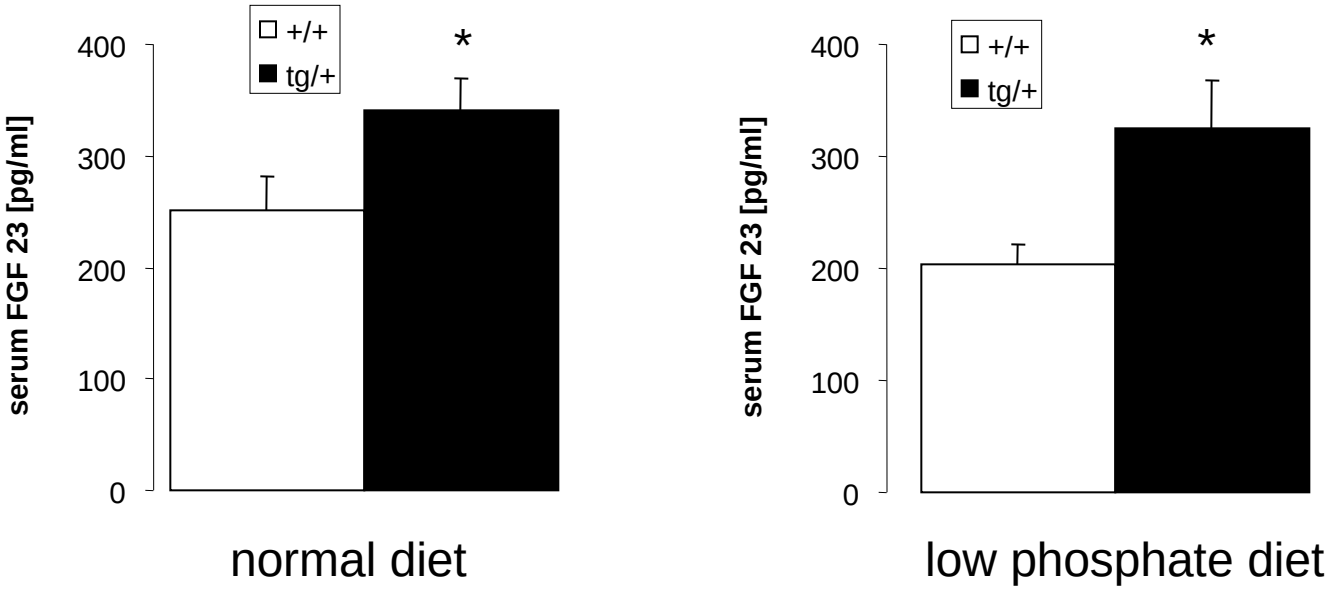

serum FGF 23 [pg/ml]
400
300
200
100
0
+/+
tg/+
*
normal diet
serum FGF 23 [pg/ml]
400
300
200
100
0
+/+
tg/+
*
low phosphate diet

Figure 6

Klotho Protein Expression in OSR1 mice

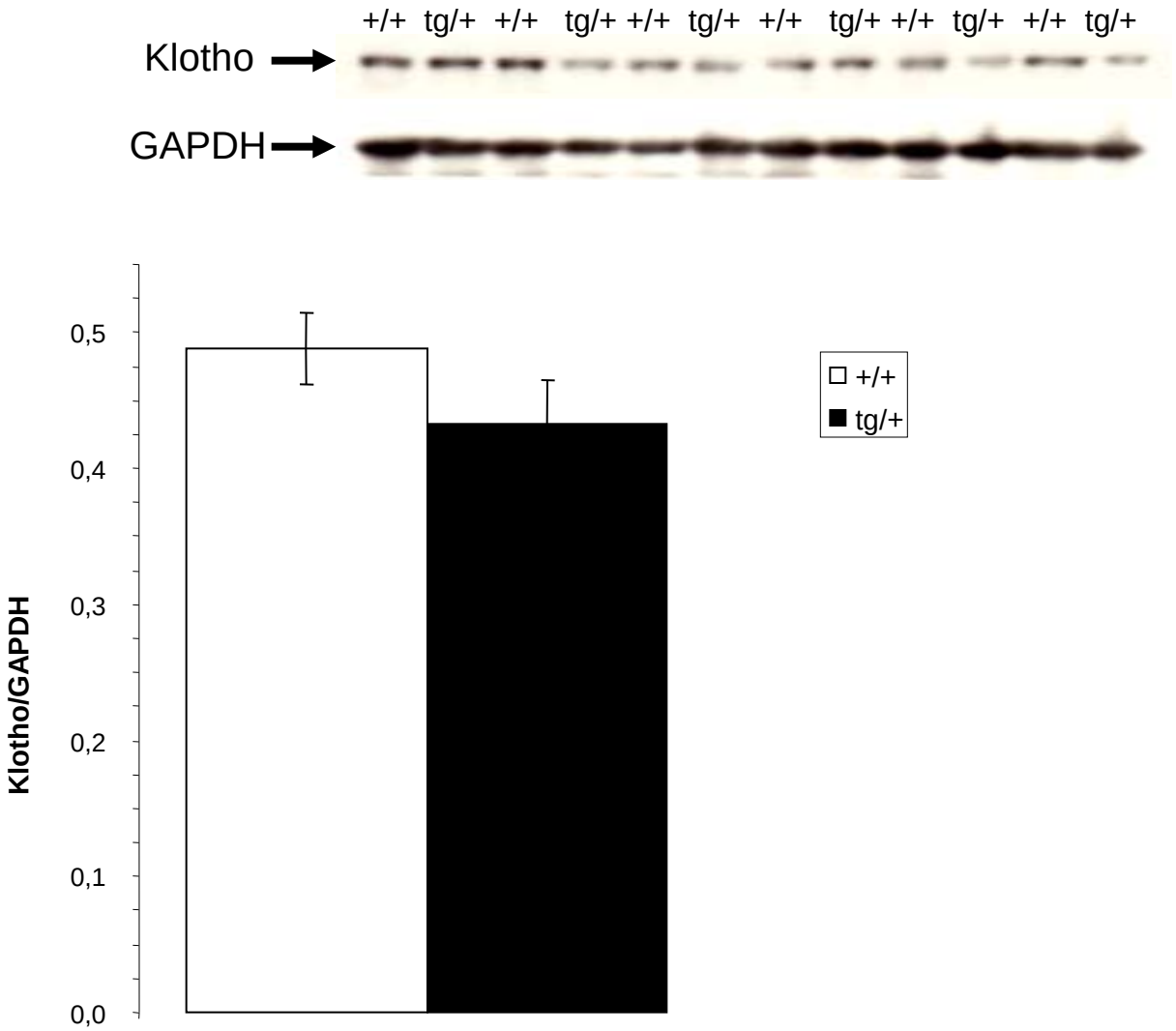


Figure 7