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# *In vivo* genetic evidence for suppressing vascular and soft tissue calcification through the reduction of serum phosphate levels, even in the presence of high serum calcium and 1,25-dihydroxyvitamin-D levels

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## Abstract

**Background—***Klotho* knockout mice *(klotho*$^{-/-}$) have increased renal expression of sodium/phosphate co-transporters (NaPi2a), associated with severe hyperphosphatemia. Such serum biochemical changes in *klotho*$^{-/-}$mice lead to extensive soft tissue anomalies and vascular calcification. To determine the significance of increased renal expression of the NaPi2a protein and concomitant hyperphosphatemia and vascular calcification in *klotho*$^{-/-}$mice, we generated *klotho* and *NaPi2a* double knockout (*klotho*$^{-/-}$/*NaPi2a*$^{-/-}$) mice.

**Methods and Results—**Genetic inactivation of NaPi2a activity from *klotho*$^{-/-}$mice reversed the severe hyperphosphatemia to mild hypophosphatemia or normophosphatemia. Importantly, despite significantly higher serum calcium and 1,25-dihydroxyvitamin D levels in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice, the vascular and soft tissue calcifications were reduced,. Extensive soft tissue anomalies and cardiovascular calcification were consistently noted in *klotho*$^{-/-}$mice by 6 weeks of age; however, these vascular and soft tissue abnormalities were absent even in 12-week-old double knockout mice. *Klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice also regained body weight and did not develop the generalized tissue atrophy often noted in *klotho*$^{-/-}$single knockout mice.

**Conclusion—**Our *in vivo* genetic manipulation studies have provided compelling evidence for a pathologic role of increased NaPi2a activities in regulating abnormal mineral ion metabolism and soft tissue anomalies in *klotho*$^{-/-}$ mice. Notably, our results suggest that serum phosphate levels are the important *in vivo* determinant of calcification, and that lowering serum phosphate levels can reduce or eliminate soft tissue and vascular calcification, even in presence of extremely high serum calcium and 1,25-dihydroxyvitamin D levels. These *in vivo* observations have significant clinical importance and therapeutic implications for chronic kidney disease patients with cardiovascular calcification.

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**Keywords**

Klotho; Vitamin-D; NaPi2a; Calcification

## Introduction

Understanding the molecular regulation of phosphate homeostasis has enormous clinical and biological importance, as it is involved in numerous essential biochemical reactions, including cell signaling process and energy metabolism. Adequate bone mineralization is closely dependent on the status of phosphate metabolism. Abnormal regulation of phosphate homeostasis can cause myopathy, cardiac dysfunctions, hematological abnormalities, and vascular/soft tissue calcifications [1-3]. Recent studies have found that klotho, a transmembrane protein, is actively involved in regulation of mineral ion metabolism by affecting the functionality of ion channels and co-transporter proteins in the kidney [4,5]. The *in vivo* importance of klotho in regulation of mineral ion metabolism is further evident in *klotho* knockout mice, as these mice have severely impaired mineral ion homeostasis [5,6].

The *klotho*$^{-/-}$mice develop severe hyperphosphatemia by three weeks of age, and remain hyperphosphatemic throughout their life [7,8]. The hyperphosphatemia in *klotho*$^{-/-}$ mice is associated with increased renal expression of the NaPi2a co-transporter protein in the proximal tubular epithelial cells [3,7,9]. To assess the significance of increased renal expression of NaPi2a in soft tissue anomalies and vascular calcification in *klotho*$^{-/-}$ mice, we have generated a new mouse model by genetically ablating both the *klotho* and *NaPi2a* genes.

Hyperphosphatemia and reduced serum levels of 1,25-dihydroxyvitamin D are the major biochemical changes detected in patients with chronic kidney disease (CKD). The current treatment approach of reducing serum phosphate levels and providing vitamin D analogs in patients with CKD often poses a dilemma, as studies linked vitamin D treatment to subsequent vascular calcification. Importantly, about 50% of mortalities in CKD patients undergoing dialysis treatment are due to the cardiovascular complication of vascular calcification. Our current study provides the *in vivo* beneficial effects of reducing serum phosphate levels on preventing vascular calcification, even in the presence of extremely high serum 1,25-dihydroxyvitamin D.

## Materials and methods

### Generation of double mutant mice

We have crossbred heterozygous *klotho* mutants [Lexicon Genetics; Mutant Mouse Regional Resource Centers, University of California at Davis, CA] with heterozygous *NaPi2a* mutants to obtain compound heterozygous animals, which were then interbred to generate the desired double homozygous mutants [*klotho*$^{-/-}$/*NaPi2a*$^{-/-}$] [7,9-11]. Routine PCR was used to identify the genotypes of various mice (Supplementary Fig. 1). All studies performed were approved by the institutional animal care and use committee at the Harvard Medical School, Boston, MA.

### Gross phenotype and body weight

The total body weight of each of wild-type, *klotho*$^{-/-}$, *NaPi2a*$^{-/-}$, and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice was taken every week starting at 3 weeks of age until 20 weeks of age. The maximum survival of *klotho*$^{-/-}$ mice was around 15 weeks.

### Biochemical measurements

Blood was obtained by cheek-pouch bleeding of wild-type, *klotho*$^{-/-}$, *NaPi2a*$^{-/-}$, and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice. Serum was isolated by centrifugation at 3000 *g* for 10 minutes and stored at −80°C. Serum and urinary phosphorus and calcium were determined by colorimetric measurements using the Stanbio Phosphorus Liqui-UV Test and Calcium (Arsenazo) LiquiColor Test, respectively, as used in our earlier publications [12]. The level of 1,25-hydroxyvitamin-D [1,25$(OH)_2D_3$] was measured in serum obtained from wild-type, *klotho*$^{-/-}$, *NaPi2a*$^{-/-}$, and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice using a commercial kit (Immunodiagnostic Systems Ltd. Fountain Hills, AZ). The serum level of parathyroid hormone (PTH) was measured using a commercial kit (Immutopics, Inc. San Clemente, CA). The serum level of Fgf23 was measured by ELISA using a commercial kit (Kainos Laboratories, Japan), described in a previous publication [7].

### Histological analyses

Soft tissues, obtained from wild-type, *klotho*$^{-/-}$, *NaPi2a*$^{-/-}$, and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice at 9-12 weeks were fixed with 4% paraformaldehyde, 10% buffered formalin, or Carnoy's solution and were subsequently embedded in paraffin. Four to six-micrometer paraffin sections of various tissues were mounted on SuperFrost Plus slides. Sections were then routinely stained with hematoxylin and eosin, and von Kossa [13,14]. Histological changes were observed by light microscopy.

### Calcification analyses

To determine the effects of hyperphosphatemia on soft tissue and vascular calcification in *klotho*$^{-/-}$ mice, sections were prepared from heart, lung, kidney, liver, spleen, aorta and the gastrointestinal tract, and were stained with von Kossa to visualize mineralized tissues by light microscopy. The von Kossa stained sections of *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice were compared with similarly stained sections from wild-type, *klotho*$^{-/-}$, and *NaPi2a*$^{-/-}$ mice. The von Kossa staining procedure is detailed in an earlier publication [15].

### Immunofluorescence staining

Immunostaining was performed as described previously [16-18]. Briefly, kidneys obtained from wild-type and *klotho*$^{-/-}$ mice were embedded in OCT and stored at −80°C. Frozen sections were incubated in a blocking solution for 30 minutes and then overnight with polyclonal anti-NaPi2a antibody (dilution 1:100; Alpha Diagnostic, San Antonio, TX) at 4°C. The slides were washed with PBS and incubated with fluorescein isothiocyanate-labeled anti-rabbit secondary antibody (dilution, 1:100) for 30 minutes. After a PBS wash, cover-slips were placed on slides using 4,6-diamidino-2-phenylindole (DAPI)-containing mounting media. The expression of NaPi2a was visualized using an immunofluorescence microscopy. Rabbit serum, in place of primary antibody, was used as a negative control. Kidney sections prepared from *NaPi2a*$^{-/-}$ mice were simultaneously stained for NaPi2a, and used as additional negative control.

### Quantitative real-time PCR

Total RNA isolated from the at least three or more kidneys and aortas of wild-type, *klotho*$^{-/-}$, *NaPi2a*$^{-/-}$, and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice was used to detect the relative expression of Ennp-1, ANK, Pit-1, and RUNX2 mRNA as described previously 12,[19]. Real-time PCR was performed in duplicate. The quantity of mRNA was calculated by normalizing the CT (threshold cycle value) of Ennp-1, ANK, Pit-1 or RUNX2 to the CT of the housekeeping gene *GAPDH*. The sequences of the primers used to detect expression patterns of various genes are reported in our earlier publications [7].

### Statistical analysis

Statistically significant differences between groups were evaluated either by the Student's t-test or by Mann-Whitney U-test for a comparison between two groups. All values were expressed as mean ± SE. A p value of less than 0.05 was considered to be statistically significant. All analyses were performed using Microsoft Excel.

## Results and discussion

The identification of specific phosphate transporters has enhanced our understanding of the regulation of renal and intestinal phosphate handling. The type II family of NaPi co-transporters consists of three highly homologous isoforms: type IIa (NaPi2a) and type IIc (NaPi2c), which are mostly expressed in the brush-border membrane of the renal proximal tubules [20], and type IIb (NaPi2b), which is expressed in the epithelial cells of the small intestine and is thought to be involved in intestinal phosphate absorption. Renal phosphate transport through NaPi2a is an important mechanism for maintaining systemic phosphate balance.

To determine the expression pattern of NaPi2a in *klotho*$^{-/-}$ mice, we stained kidney sections from wild-type and *klotho*$^{-/-}$ mice with NaPi2a antibody. Compared with wild-type mice, increased expression of NaPi2a protein, in the luminal side of the proximal tubules, was noted in sections from *klotho*$^{-/-}$ mice (Supplementary Fig. 2). It is presumed that increased expression of NaPi2a is associated with increased renal reuptake of phosphate [21], resulting in hyperphosphatemia and extensive vascular calcification in *klotho*$^{-/-}$ mice (Supplementary Fig. 3). To test this hypothesis, we have generated *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice and compared their physical, morphological, and biochemical phenotype with *klotho*$^{-/-}$mice.

As mentioned, genetic deletion of *klotho* results in hyperphosphatemia [8,9]. To determine whether hyperphosphatemia in *klotho*$^{-/-}$ mice is a NaPi2a-dependent process, we generated a new mouse model that is deficient in both *klotho* and *NaPi2a* genes (*klotho*$^{-/-}$/*NaPi2a*$^{-/-}$), by interbreeding heterozygous *klotho* mice with heterozygous *NaPi2a* mice. The *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice were viable and larger in size to *klotho*$^{-/-}$ mice. At birth, *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice were indistinguishable from their littermates of other genotypes. At 3 weeks of age, *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice were slightly larger in size than *klotho*$^{-/-}$ mice (12.8±0.5 g vs. 10.4 ±0.5 g), but smaller than wild-type mice (16.0±0.5 g). Double knockout mice were similar in size to *NaPi2a*$^{-/-}$animals (13.4±0.27 g). At 9 weeks of age, *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice were still smaller than their wild-type littermates (20.2±0.85 g vs. 28.8±1.1 g), but their body weight was significantly higher than that of *klotho*$^{-/-}$ mice (11.3±0.9 g). At 9 weeks of age, the average body weight of *NaPi2a*$^{-/-}$ mice was 25.7±0.76 g (Fig. 1). Furthermore, *klotho*$^{-/-}$ mice had a maximum survival of around 15 weeks, while all the *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice were capable of survival beyond 15 weeks and were alive until 20 weeks of observation period.

Next, we measured serum phosphate and calcium levels in 3-, 6-, and 9-week-old wild-type, *klotho*$^{-/-}$, *NaPi2a*$^{-/-}$, and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice. The double knockout mice were slightly hypophosphatemic by 6 weeks of age (7.2±0.41 mg/dl, n=10), compared to wild-type mice (8.5±0.69 mg/dl, n=5). The low serum phosphate levels were also noted in age-matched *NaPi2a*$^{-/-}$ mice (4.6±0.14 mg/dl, n=10). Serum phosphate levels were slightly high in *klotho*$^{-/-}$ mice (12.1±0.64 mg/dl, n=11) (Fig. 2). Significant reduction of serum phosphate levels were also noted in 9-week-old double knockout mice, compared to *klotho*$^{-/-}$ mice. Collectively, these findings suggest that inactivation of NaPi2a function can reverse hyperphosphatemia to hypophosphatemia or normophosphatemia at 3-, 6-, and 9-weeks of age.

Serum calcium levels were higher in the *klotho*$^{-/-}$ mice at around 6 weeks of age, as also noted in both *NaPi2a*$^{-/-}$ and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice (Fig. 2). At around 6 weeks of age, serum calcium in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice (11.4±0.44 mg/dl, n=12) was significantly higher than in

wild-type mice (7.6±0.46 mg/dl, n=6). The higher serum levels of calcium were also noted in *klotho*$^{-/-}$ mice (9.8±0.34 mg/dl, n=10) and *NaPi2a*$^{-/-}$ mice (10.0±0.12 mg/dl, n=10) of similar age (Fig. 2).

Despite reversal of serum phosphate level from severe hyperphosphatemia to mild hypophosphatemia or normophosphatemia in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice, the serum 1,25$(OH)_2D_3$ levels in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice were extremely high. Statistically significant increased serum levels of 1,25$(OH)_2D_3$ were also detected in *klotho*$^{-/-}$ and *NaPi2a*$^{-/-}$ mice (Fig. 3). In contrast to 1,25$(OH)_2D_3$, serum PTH levels were markedly reduced in *klotho*$^{-/-}$ and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice (Fig. 3); serum PTH was also extremely low in *NaPi2a*$^{-/-}$ mice. Of relevance, compared to the control (175 pg/ml), serum FGF23 levels were extremely high in the *klotho*$^{-/-}$ mice (7107 pg/ml) at 6 weeks of age [22]. Markedly increased serum levels of FGF23 were also noted in the in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice (7440 pg/ml); the serum FGF23 levels were low in *NaPi2a*$^{-/-}$ mice (53 pg/ml) (Fig. 4).

The major finding of our *in vivo* genetic manipulation studies is that NaPi2a regulates systemic phosphate homeostasis in *klotho*$^{-/-}$ mouse. Inactivation of the *NaPi2a* gene from *klotho*$^{-/-}$ mice reversed severe hyperphosphatemia to hypophosphatemia/normophosphatemia, despite significantly higher serum levels of calcium and 1,25$(OH)_2D_3$.

We then examined the effects of reducing serum phosphate from *klotho*$^{-/-}$ mice on vascular and soft tissue calcification. Extensive vascular and soft tissue calcifications were present in the heart, lung, kidney, aorta and other organs in *klotho*$^{-/-}$ mice, as determined by von Kossa staining (Supplementary Fig. 3). The extensive calcification observed in *klotho*$^{-/-}$ mice by 6 weeks was significantly reduced or eliminated in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice, even at 12 weeks of age (Figs. 5 and 6). These results indicate that high serum phosphate is an important determinant of calcification, and the lowering of serum phosphate can reduce/eliminate calcification, even in presence of higher serum calcium and 1,25$(OH)_2D_3$ levels (Fig. 3).

Lowering serum phosphate levels in *klotho*$^{-/-}$ mice significantly rescued soft tissue anomalies, helped restore fertility, and markedly reduced extensive soft tissue and vascular calcifications in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice (Table 1). These results suggest that the phenotypes of *klotho*$^{-/-}$ mice are mostly derived from high serum phosphate levels. Despite significantly high serum levels of calcium and 1,25$(OH)_2D_3$, the opposing phenotypes of *klotho*$^{-/-}$ and *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice suggest that such dissimilarities are due to phosphate toxicity. Our results provide compelling genetic evidence of the *in vivo* importance of NaPi2a in the regulation of systemic phosphate homeostasis in *klotho*$^{-/-}$ mice, and that abnormal regulation of NaPi2a co-transporters can lead to phosphate toxicity to induce severe soft tissue anomalies, including general tissue atrophy, and reduction of skeletal muscle mass. Of relevance, hyperphosphatemia and severe muscle wasting are common clinical problems encountered in CKD patients.

Extensive vascular and soft tissue calcifications were widely present in the lung, kidney, aorta, and other organs in *klotho*$^{-/-}$ mice, as detected by von Kossa staining (Fig. 5, Supplementary Fig. 3). The extensive calcification noted in *klotho*$^{-/-}$ mice by 6 weeks of age was completely eliminated in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice and was not detected even in 12-week-old double mutant mice. These results indicated that high serum phosphate is an important determinant of calcification, and lowering serum phosphate can reduce/eliminate calcification, even in presence of higher serum 1,25$(OH)_2D_3$ levels (Fig. 3).

Phosphate retention and subsequent hyperphosphatemia, together with reduced circulating levels of 1,25-dihydroxyvitamin D, are the major biochemical changes detected in patients with CKD [23]. Coronary calcification is the single most important pathologic condition that influences the mortality of CKD patients undergoing dialysis treatment [24]. Hyperphosphatemia

is believed to be an important risk factor for such cardiovascular calcification [25]. The current approach of reducing serum phosphate levels and correcting vitamin D insufficiency/deficiency in CKD patients often poses a dilemma, as high doses of vitamin D/analog treatment are believed to affect the subsequent vascular calcification process. Our *in vivo* genetic manipulation study suggests that minimizing phosphate toxicity can reduce vascular calcification, even in presence of extremely high serum 1,25-dihydroxyvitamin D and calcium levels. One limitation of animal study is that the mechanisms of human vascular diseases in renal insufficiency may be different from vascular lesions in *klotho*$^{-/-}$ mice, and established medial and atherosclerotic lesions may persist even after molecular manipulations, as advanced stages of calcification may not be reversible. We believe that lowering serum phosphate levels can delay the progression of vascular lesions, but may not always reverse the established lesions.

Hyperphosphatemia in *klotho*$^{-/-}$ mice is associated with extensive soft tissue calcification (Fig. 5, Supplementary Fig. 3) [7,9,22]. Imbalance between phosphate and pyrophosphate usually determines the ectopic calcification process 26,[27]. We have found that the expression of Ennp-1 (pyrophosphate generator) and ANK (pyrophosphate transporter) is slightly elevated in the kidneys and aortas of *klotho*$^{-/-}$ mice, and such elevation might be a compensatory response in these mutant mice (Supplementary Figs. 4, 5). Since elevation of pyrophosphate regulating molecules can not suppress calcification process, it appears likely that extensive calcification in *klotho*$^{-/-}$ mice is primarily associated with high serum phosphate levels.

In summary, the phenotypes of *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice suggest that: **1**) increased NaPi2a activity is the main cause for the severe hyperphosphatemia and ectopic calcification observed in *klotho*$^{-/-}$ mice, and **2**) NaPi2a-mediated renal phosphate homeostasis is independent of serum $1,25(OH)_2D_3$ levels in mice deficient for *klotho*. Notably, lowering phosphate burden, by reducing serum phosphate levels can modulate vascular and soft tissue calcification, despite the presence of extremely high serum $1,25(OH)_2D_3$ levels. These results provide compelling genetic evidence of the importance of NaPi2a in regulating renal phosphate homeostasis in *klotho*$^{-/-}$ mice, and more importantly, suggest that reducing "phosphate toxicity" should be the single most important therapeutic priority in minimizing the risk of vascular calcification and eventual disease progression [23,28-30].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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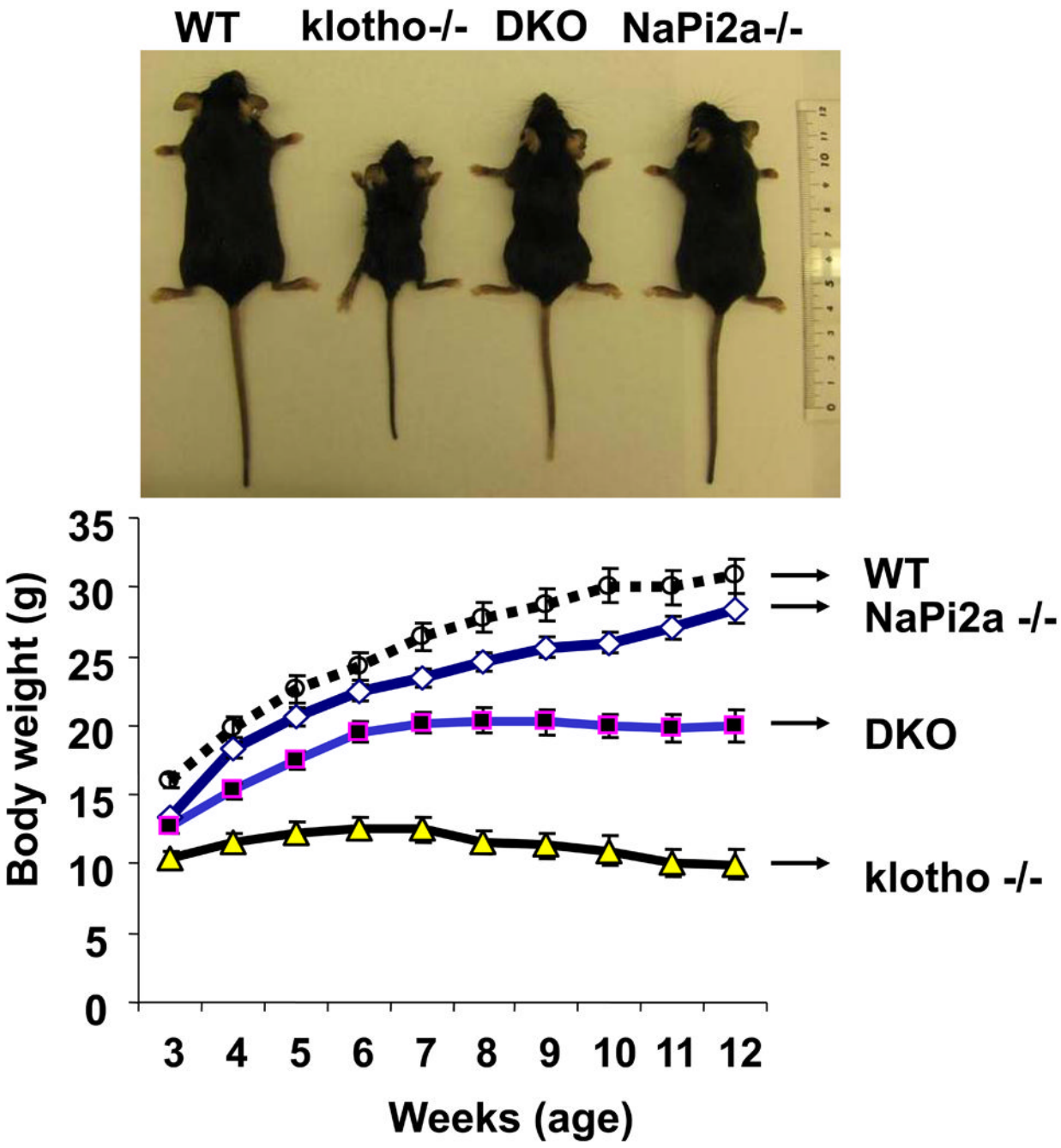


**Figure-1. Macroscopic phenotype of *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice**

Gross phenotype of wild-type (WT), *klotho*$^{-/-}$, *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ (DKO), and *NaPi2a*$^{-/-}$ mice at around 12 weeks of age (**upper panel**). Body weight curves (**lower panel**) for all four genotypes, showing DKO mice (n=34) are smaller than WT mice (n=22), but larger than *klotho*$^{-/-}$ mice (n=23), suggesting that inactivation of NaPi2a function from *klotho*$^{-/-}$ mice helped in regaining the body weight in DKO mice. The average body weight of the *NaPi2a*$^{-/-}$ mice (n=42) is more than DKO mice. The statistical analyses among the groups were compared through Student's unpaired two-tail t-test ($p<0.0001$ at all time points for WT vs. *klotho*$^{-/-}$ mice; $p<0.001$ at all time points for WT vs. DKO mice; $p<0.0001$ at all time points for *klotho*$^{-/-}$ vs. *NaPi2a*$^{-/-}$ mice; $p<0.0001$ at all time points for *klotho*$^{-/-}$ vs. DKO mice).

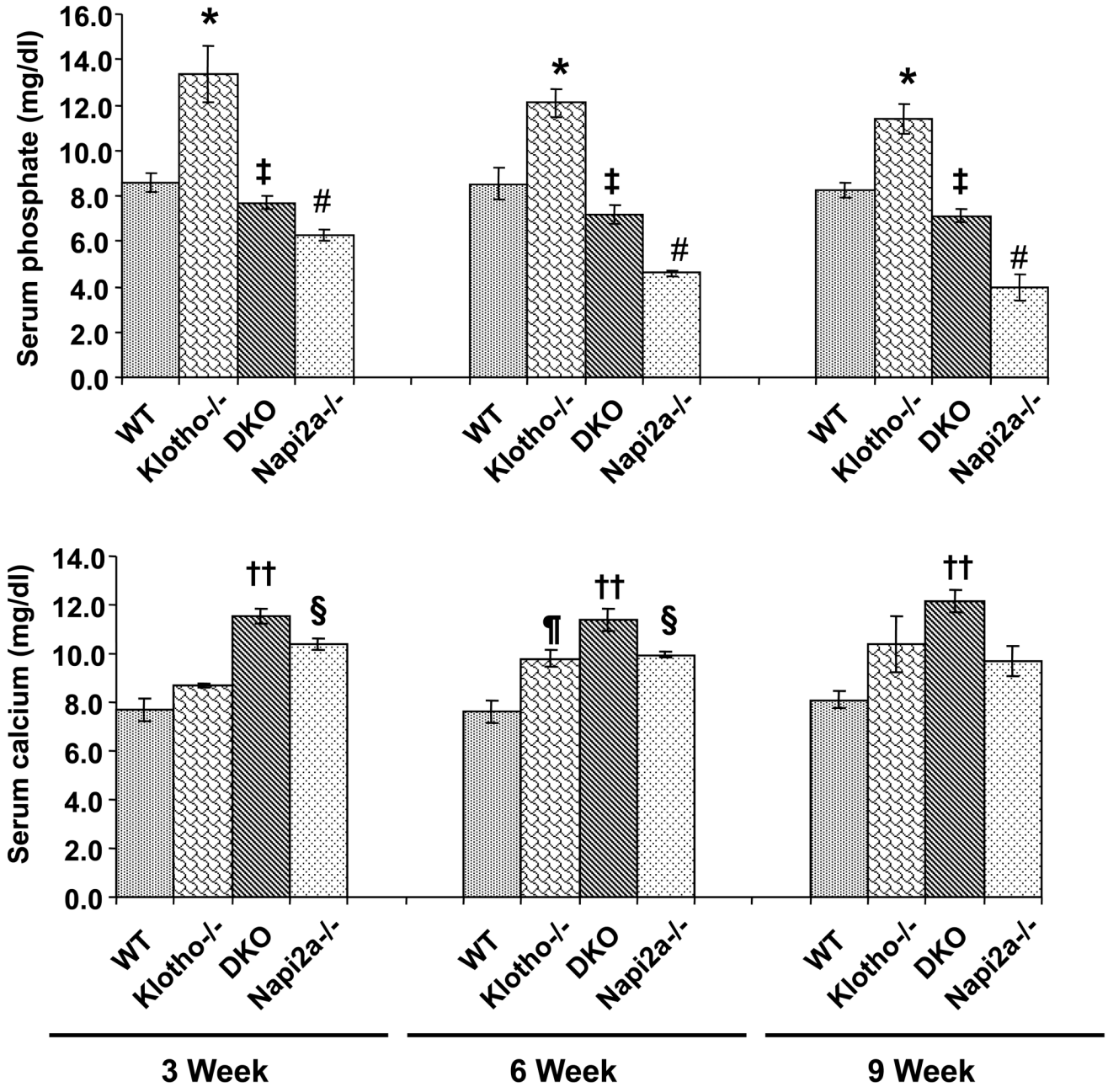


**Figure 2. Biochemical analysis of serum phosphate and calcium**

The serum phosphate (**upper panel**) and calcium (**lower panel**) levels are higher in *klotho*$^{-/-}$ mice, compared to the wild-type (WT) mice at 3-, 6-, and 9- weeks of age. In contrast to the *klotho*$^{-/-}$ mice, serum phosphate levels are markedly reduced in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ (DKO). Serum phosphate levels are also low in *NaPi2a*$^{-/-}$ mice (*: $p < 0.05$, *vs.* WT; #: $p < 0.001$, *vs.* WT; ‡: $p < 0.001$, *vs. klotho*$^{-/-}$). As for serum calcium levels, compared to the WT controls, increased serum levels of calcium are noted in all three mutant mice at different time points. At around 6 weeks of age, serum calcium in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice (11.4±0.44 mg/dl, n=12) is significantly higher than in wild-type mice (7.6±0.46 mg/dl, n=6). The higher serum levels of calcium are also noted in *klotho*$^{-/-}$ mice (9.8±0.34 mg/dl, n=10) and

*NaPi2a*$^{-/-}$ mice (10.0±0.12 mg/dl, n=10) of similar age. The statistical analyses among the groups were compared through Student's unpaired two-tail t-test (¶: $p < 0.01$, *vs.* WT; ††: $p < 0.001$, *vs.* WT; §: $p < 0.001$, *vs.* WT).

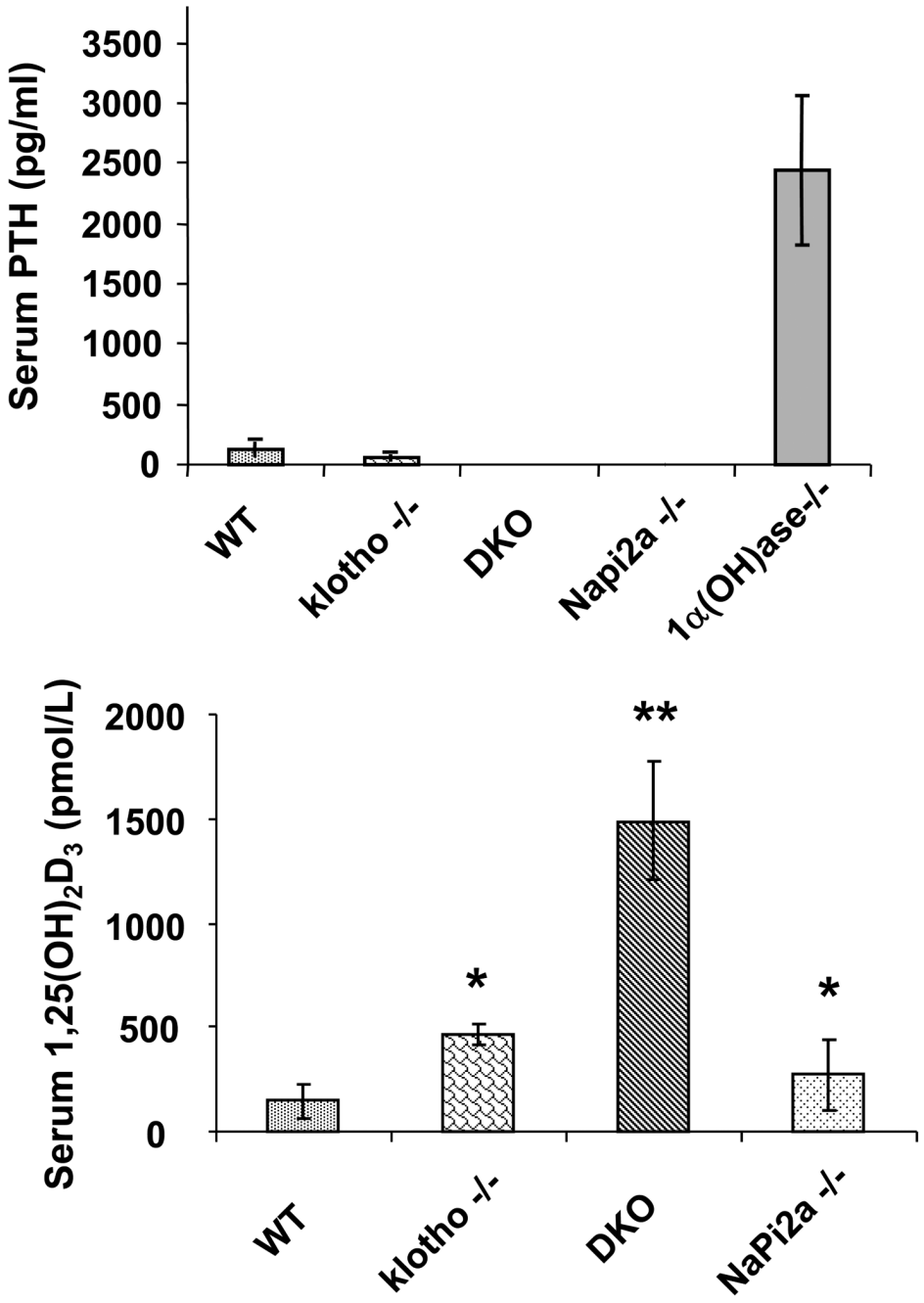


**Figure 3. Biochemical measurements of serum PTH and 1,25(OH)$_2$D$_3$ in various genotypes**

Compared to the wild-type (WT) mice (n=3; 121.8±60 pg/ml), serum PTH levels are markedly reduced in *klotho*$^{-/-}$ mice (n=5; 58.3±28 pg/ml). Serum PTH levels are low and undetectable in both *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ (DKO) and *NaPi2a*$^{-/-}$ mice, respectively. We also measured serum PTH levels in 1-alpha hydroxylase knockout mice as a positive control and found significantly increased levels (n=4; 2443.4±610 pg/ml), compared to the controls. As for serum 1,25 (OH)$_2$D$_3$ levels, compared to the WT mice (n=5; 144.8±77 pmol/L), markedly increased serum levels are noted in all three mutant mice; in *klotho*$^{-/-}$ mice (n=5; 465.4±47 pmol/L), in DKO (n=7; 1487.9±279 pmol/L), and in *NaPi2a*$^{-/-}$ mice (n=5; 272.6±169 pmol/L) (*: $p < 0.01$, *vs.* WT; **: $p < 0.005$, *vs.* WT).

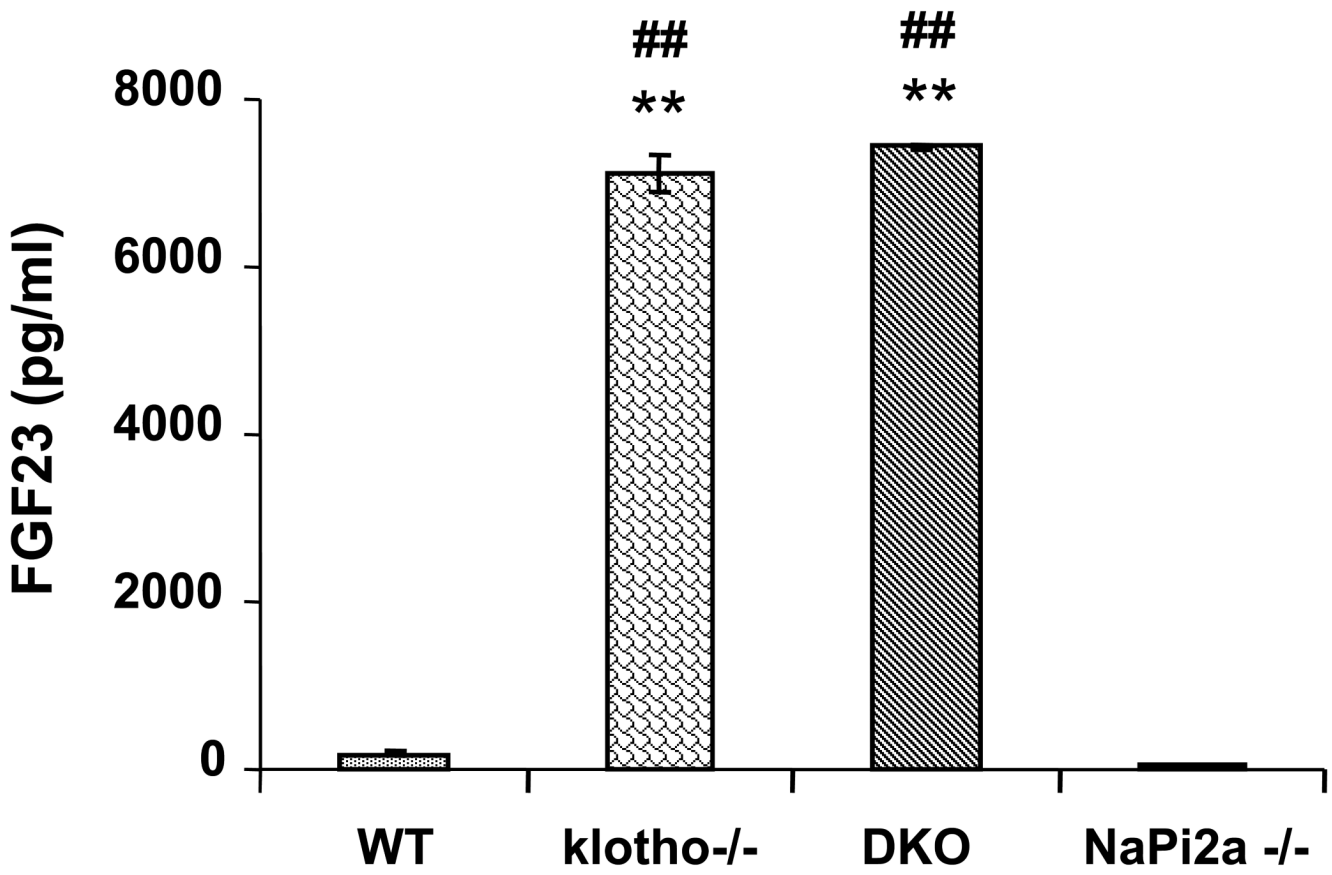


**Figure 4. Biochemical measurements of serum FGF23 in various genotypes**

The average serum levels of FGF23 are higher in *klotho*$^{-/-}$ mice (n=6; 7107 pg/ml) compared to wild-type mice (n=9; 176 pg/ml). Similarly increased FGF23 serum levels are also noted in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ (DKO) mice (n=7; 7440 pg/ml). The serum FGF23 levels is extremely low (n=6; 53 pg/ml) in *NaPi2a*$^{-/-}$ mice. Data presented after adding dilution factors (**: $p < 0.001$, *vs.* wild-type; ##: $p < 0.001$, *vs. NaPi2a*).

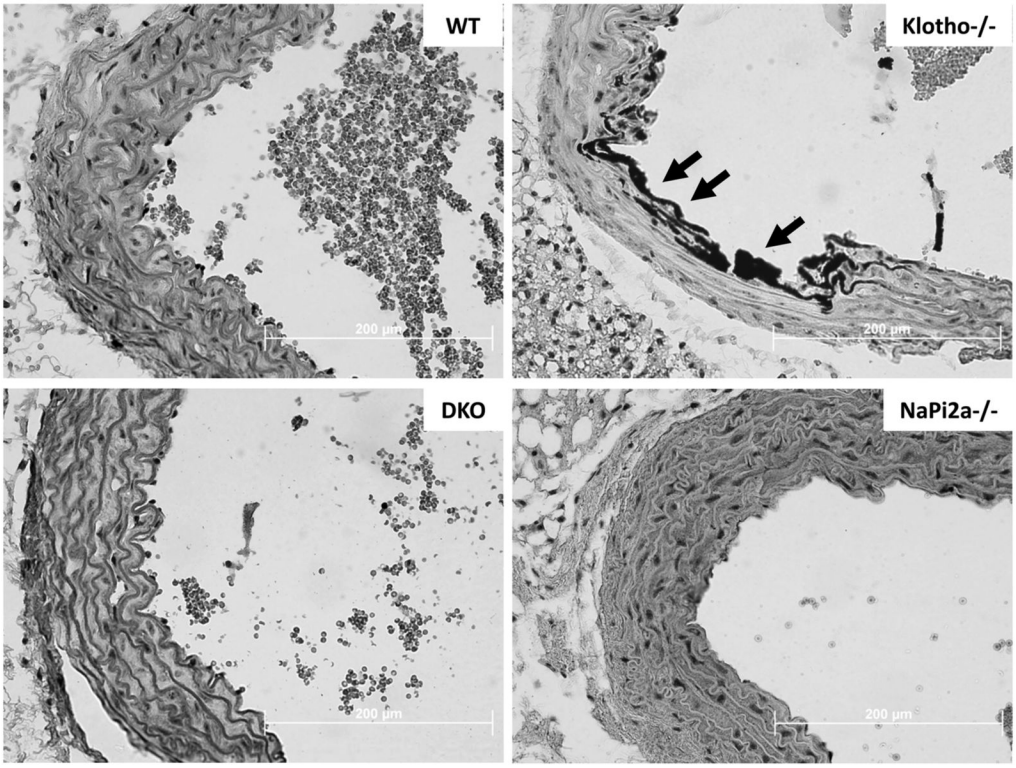


**Figure 5. Aortic calcification**

Sections prepared from aorta of wild-type (WT), *klotho*$^{-/-}$, *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ (DKO) and *NaPi2a*$^{-/-}$ mice showing extensive calcifications in the aortic wall of only in *klotho*$^{-/-}$ mice. No such calcification is detected in aorta obtained from DKO mice or *NaPi2a*$^{-/-}$ mice (von Kossa staining; ×60).

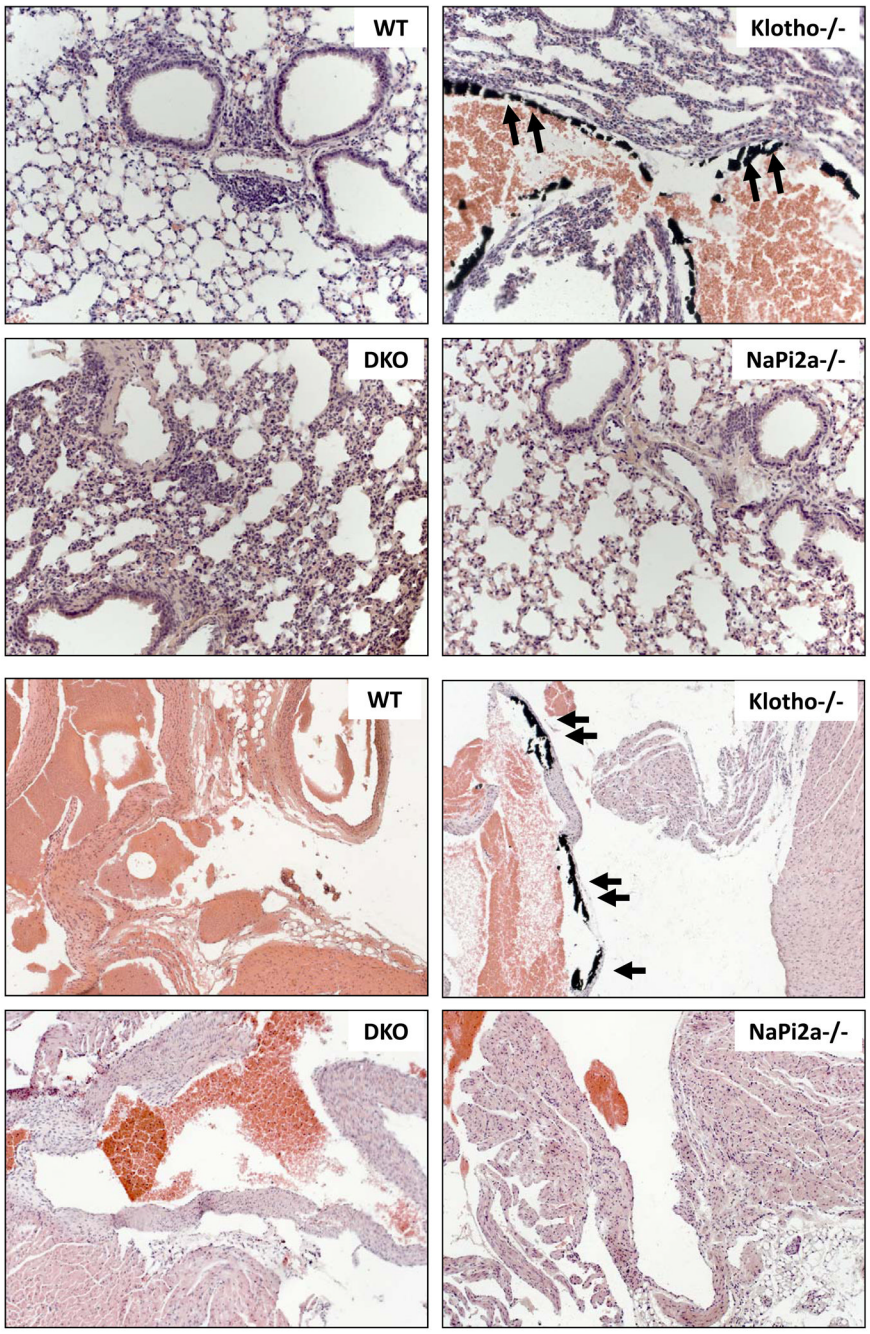


**Figure 6. Vascular calcification in lung and heart**

Lung sections (**upper four panels**) prepared from wild-type (WT), *klotho*$^{-/-}$, *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ (DKO) and *NaPi2a*$^{-/-}$ mice showing extensive calcifications in the lung parenchyma and vessel wall of *klotho*$^{-/-}$ mice. Inactivation of NaPi2a from *klotho*$^{-/-}$ mice reduced such calcification from DKO mice. Similarly, vascular calcification is also noted in heart (**lower four panels**) of *klotho*$^{-/-}$ mice but is absent from DKO mice (von Kossa staining; lung ×20, heart ×10).

## Table-1

Phenotypes of various mutant mice compared to wild-type littermates at 6 to 9 weeks of age.
**E:** extremely; **M:** moderately; **S:** slightly; **D:** diffuse; **F:** focal

| | **Wild-type** | **klotho-/-** | **klotho-/-/NaPi2a-/-** | **NaPi2a-/-** |
|---|---|---|---|---|
| **Gross appearance** | | | | |
| Body weight | Normal | Reduced (E) | Reduced (M) | Reduced (S) |
| Growth retardation | Absent | Present (E) | Present (S) | Present (S) |
| **Generalized atrophy** | | | | |
| Spleen atrophy | Absent | Present (D) | Absent | Absent |
| Muscle wasting | Absent | Present (D) | Absent | Absent |
| Skin atrophy | Absent | Present (D) | Absent | Absent |
| Intestinal atrophy | Absent | Present (D) | Absent | Absent |
| **Morphological changes** | | | | |
| Atherosclerosis/arteriosclerosis | Absent | Present | Absent | Absent |
| Vascular calcifications | Absent | Present | Absent | Absent |
| Emphysema | Absent | Present (D) | Present (F) | Present (F) |
| **Biochemical changes** | | | | |
| Serum $1,25(OH)_2D_3$ | Normal | High | High | High |
| Serum phosphate | Normal | High | Low/normal | Low |
| Serum calcium | Normal | High | High | High |
| Serum PTH | Normal | Low | Low | Low |
| Serum FGF23 | Normal | High | High | Low |
| **Overall affect** | | | | |
| Physical activity | Normal | Sluggish | Normal | Normal |
| Infertility | Absent | Present | Absent | Absent |
| Lifespan (until 20 weeks) | Normal | Short | Normal | Normal |