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Phosphate transporter regulator that prevents abnormal hyperphosphatemia.

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Short Title: TRAP is a novel regulator of Pi metabolism

Abstract

Renal type II sodium-dependent inorganic phosphate (Pi) transporters NaPi2a and NaPi2c cooperate with other organs to strictly regulate the plasma Pi concentration. A high Pi load induces the phosphaturic hormones parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), enhances urinary Pi excretion and prevents the onset of hyperphosphatemia. How FGF23 is induced from the bones by a high Pi load and the setpoint of the plasma Pi concentration, however, are unclear. Here, we investigated the role of transporter-associated protein (TRAP), found in gene co-expression networks in NaPi2a and NaPi2c function. TRAP is localized in the renal proximal tubules and interacts with NaPi2a. In TRAP-knockout (KO) mice, the serum FGF23 concentration was markedly increased but increased Pi excretion and hypophosphatemia were not observed. In addition, TRAP-KO mice exhibit reduced NaPi2a responsiveness to FGF23 and PTH administration. Furthermore, a dietary Pi load causes marked hyperphosphatemia and abnormal NaPi2a regulation in TRAP-KO mice. Thus, TRAP is thought to be associated with FGF23 induction in bones and the regulation of NaPi2a to prevent an increase in the plasma Pi concentration due to a high Pi load and kidney injury.

Introduction

NaPi2a and NaPi2c (SLC34A1/NPT2A/NaPi2a and SCL34A3/NPT2C/NaPi2c), sodium-dependent phosphate transporters responsible for inorganic phosphate (Pi) reabsorption in the kidney, are essential molecules for regulating the plasma Pi concentration. Both transporters are predominantly expressed at the apical side in the proximal tubules of the kidney^{1, 2}. Parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) are the main contributing hormones regulating the renal NaPi2a and NaPi2c transporters¹⁻⁵. In rodents, NaPi2a plays a central role in Pi reabsorption^{6, 7}. NaPi2a has a PDZ (PSD-95, Disc-large, ZO-1)-binding motif at its C-terminus and binds to Na⁺/H⁺ exchanger regulatory factor (NHERF)1 to form a complex at the apical membrane of proximal tubular cells⁸⁻¹⁰. PTH and FGF23 phosphorylate NHERF1, thereby dissociating the complex, and NaPi2a is endocytosed and degraded in lysosomes^{1, 11-13}. In this way, phosphaturic hormones reduce NaPi2a and enhance urinary Pi excretion.

Dietary Pi intake regulates urinary Pi excretion by altering plasma PTH and FGF23 levels⁵. Both phosphaturic hormones affect NaPi2a and NaPi2c expression in the proximal tubular cells^{1, 2, 5}. With a low Pi diet intake, plasma PTH and FGF23 levels decrease, and the NaPi2a/NaPi2c levels in the proximal tubular cells increase. Therefore, urinary Pi excretion is reduced. In contrast, with a high dietary Pi load, plasma PTH and FGF23 levels are increased to promote the internalization of NaPi2a and NaPi2c in the proximal tubular cells. As a result, urinary Pi excretion is enhanced, and the onset of hyperphosphatemia is prevented. The mechanisms underlying both the induction of phosphaturic hormones by dietary Pi and regulation of the serum Pi concentration, however, remain unclear.

Gene co-expression networks (GCNs) represent gene-gene interactions and while they do not contain information about regulation direction, they allow for the simultaneous analysis of many genes and their potential relationships with each other¹⁴⁻¹⁷. In the present study, we

focused on transmembrane protein (TMEM)174, which is strongly correlated with slc34a1 in the GCNs. TMEM174 (a transporter associated protein; TRAP) is localized in the renal proximal tubule apical membrane. The present study showed that TRAP-knockout (KO) mice exhibit abnormal fluctuations in the plasma Pi levels in response to dietary Pi. TRAP binds to NaPi2a on the cell membrane and is considered to be involved in the regulation of NaPi2a by PTH and FGF23. The roles of TRAP in the control of plasma Pi are discussed.

Results

COXPRESdb search indicates co-expression of transmembrane protein 174 (TMEM174) with renal NaPi transporters. To identify genes co-regulated with slc34a1 or slc34a3 mouse NaPi transporters, we searched for genes using the COXPRESdb v7¹⁶. The top 20 genes are listed in Supplemental Table S1 and S2, and the transmembrane protein 174 (TMEM174) gene was identified as a significant gene co-expressed with slc34a1 and slc34A3. The correlation coefficient (r) for gene expression levels between slc34a1 or slc34a3 and TMEM174 was 0.899717818157933 and 0.36902441214734, respectively (Fig. 1a, 1b).

TMEM174 was originally identified among a large pool of genes by high-throughput cell screening technology to isolate functional genes and provide insight into the mechanisms of gene function¹⁸⁻²⁰. The full-length amino acid sequences of TMEM174 in mouse (NP_080961.1), rat (NP_001019469.1), and human (NP_694949.1) are reported in the NCBI database. The putative TMEM174 protein comprises 243 amino acids with 2 transmembrane domains. In the present study, we named TMEM174 as a transporter-associated protein (TRAP).

Tissue localization of TRAP expression and possible involvement in Pi homeostasis.

Expression of TRAP mRNA was analyzed by real-time polymerase chain reaction (PCR) using

mouse tissues. As reported in human tissue¹⁸, mouse TRAP mRNA was markedly higher in the kidney compared with other tissues (Fig. 2a). TRAP protein expression was detected at the apical membrane of renal proximal tubular cells, but not in the distal tubule (Fig. 2b). Next, we examined whether the renal TRAP protein expression was regulated by dietary Pi regulation and deletion of renal Pi transporters NaPi2a, or NaPi2c (Fig. 2c-2e). A low Pi (LP) diet significantly induced renal TRAP protein expression compared with control Pi (CP) and high Pi (HP) diets, similar to the response of renal Pi transporters to dietary Pi content (Fig. 2c and 2d). Furthermore, deletion of renal NaPi transporters (NaPi2a-KO and NaPi2c-KO mice) significantly reduced the renal TRAP protein expression levels compared with NaPi2a^{+/+} NaPi2c^{+/+} mice (Fig. 2e).

Characterization of TRAP^{-/-} mice fed standard mouse chow. To generate TRAP-null mice, we replaced TRAP exon 1 to part of exon 2 with a neomycin-resistant gene (Supplemental Fig. S1a). We confirmed the mutant genomic DNA isolated from transfected ES clones by Southern blotting, and the mice genotype by PCR analysis (Supplemental Fig. S1b, S1c). Reverse transcriptase-PCR with TRAP-specific primers and Western blotting analysis confirmed the absence of detectable renal TRAP mRNA and protein expression in TRAP^{-/-} mice (Fig. 3a, 3b). Male and female TRAP^{-/-} mice showed similar weight gain compared with TRAP^{+/+} and TRAP^{+/-} mice (Fig. 3c). Between 4 to 5 weeks of age, however, male TRAP^{-/-} mice showed significantly lower body weight than male TRAP^{+/+} and TRAP^{+/-} mice (Fig. 3c left). To measure food intake, and urine and fecal biochemical data, mice were individually placed in metabolic cages. TRAP^{-/-} mice did not show a significant difference in food intake, plasma creatinine, plasma blood urea nitrogen (BUN), blood ionized Ca and plasma Pi concentrations, fecal and urinary Ca, Pi excretion levels, or other blood biochemistry parameters compared with TRAP^{+/+} and TRAP^{+/-} mice (Fig. 3d-l, and Supplemental Table S3).

Trends in Pi-regulating hormones in TRAP^{-/-} mice. Plasma 1,25(OH)₂D₃ levels were not significantly different among the 3 groups (Fig. 4a). Plasma PTH, and especially serum intact FGF23 levels were markedly higher in TRAP^{-/-} mice than in TRAP^{+/+} and TRAP^{+/-} mice (Fig. 4b, 4c). Renal 25-hydroxyvitamin D-1 alpha hydroxylase (Cyp27b1) mRNA levels were not significantly different among the 3 groups, but renal 25-hydroxyvitamin D-24-hydroxylase (Cyp24a1) mRNA levels were significantly higher in TRAP^{-/-} mice than in TRAP^{+/+} and TRAP^{+/-} mice (Fig. 4d, 4e). Parathyroid PTH mRNA levels were not significantly different among the 3 groups (Fig. 4f). FGF23 mRNA was mainly detected in osseous-tissues but has been found in other tissues as well²¹. Expression of FGF23 mRNA in the bone was highly increased in TRAP^{-/-} mice compared with TRAP^{+/+} mice, and to a lower extent in the spleen and thymus, but not in the heart (Fig. 4g). In both TRAP^{+/+} and TRAP^{-/-} mice, FGF23 immunostaining was observed in osteocytes and osteoblasts/preosteoblasts, with no difference in the localization patterns. The number of FGF23-positive cells, however, tended to be increased in TRAP^{-/-} mice (Fig. 4h).

Bone histochemical analysis in TRAP^{-/-} mice. Bone analysis was performed in young (8-week-old) and aged (70-week-old) mice (Supplemental Fig. S2 and Fig. 5). Compared with TRAP^{+/+} mice, neither 8-week-old nor 70-week-old TRAP^{-/-} mice showed any abnormalities in the micro-computed tomography (CT) analysis and morphological measurement using the long bones (Supplemental Fig. S2).

Hematoxylin and eosin staining of the femurs from 8-week-old mice revealed that TRAP^{-/-} mice had slightly more cancellous bone at the metaphysis, and the trabeculae appeared to be slightly thicker and rounded compared with those from TRAP^{+/+} mice (Fig. 5a). In addition,

alkaline phosphatase (ALP)/tartrate-resistant acid phosphatase staining revealed the tendency toward a thicker ALP-positive osteoblast/preosteoblast layer on the trabecular surface in TRAP^{-/-} mice, and a similar number of tartrate-resistant acid phosphatase-positive osteoclasts as in TRAP^{+/+} and TRAP^{-/-} mice, or a slightly higher number than in TRAP^{-/-} mice (Fig. 5b). Furthermore, the bone mineralization and osteoid layer thickness did not differ significantly between TRAP^{+/+} and TRAP^{-/-} mice, whereas mature osteoblasts covered on the bone surface in TRAP^{-/-} mice (Fig. 5c).

Renal NaPi transporter expression in TRAP^{-/-} mice. Immunoblotting analysis using brush border membrane vesicles (BBMVs) and immunofluorescence staining showed the disappearance of only renal NaPi2c protein expression in TRAP^{-/-} mice compared with TRAP^{+/+} mice (Fig. 6a, 6b). In contrast, however, NaPi2a protein expression was not different between TRAP^{+/+} or TRAP^{-/-} mice (Fig. 6a, 6b). Real time-PCR showed significantly decreased slc34a1 and slc34a3 mRNA levels in TRAP^{-/-} mice compared with TRAP^{+/+} mice (Supplemental Fig. S3a, S3b).

Next, we examined the levels of each type of phosphaturic hormone receptor in the kidney. The levels of renal FGF receptor 1 (FGFR1) and PTH receptor (PTHr) mRNA were the same between TRAP^{+/+} and TRAP^{-/-} mice, and renal FGFR4 and α -Klotho mRNA levels were significantly decreased in TRAP^{-/-} mice compared with TRAP^{+/+} mice (Supplemental Fig. S3c-S3f). FGFR1 protein expression was not different between TRAP^{+/+} and TRAP^{-/-} mice, and α -Klotho protein expression was significantly decreased in TRAP^{-/-} mice compared with TRAP^{+/+} mice (Fig. 6c). Phosphorylation of the extracellular signal-regulated kinase (ERK)1/2 also was not different between TRAP^{+/+} and TRAP^{-/-} mice (Fig. 6d).

Scaffold protein levels in the renal proximal tubules of TRAP KO mice. NaPi2a trafficked to

the apical membrane is dependent on its association with PDZ-containing proteins^{1, 8, 9, 22-24}. PTH and FGF23 regulate Pi excretion by controlling the NaPi2a/NHERF1 association^{1, 10}. Immunoblot analysis using renal BBMVs, but not whole homogenate, showed that NHERF1 protein expression was significantly higher in TRAP^{-/-} mice than in TRAP^{+/+} mice (Fig. 7a, 7b).

Next, we examined the interaction between TRAP and NaPi2a/NHERF1 using renal BBMVs of wild-type (WT), TRAP-KO, and NaPi2a-KO mice (Fig. 7c, 7d). NHERF1 immunoprecipitation analysis revealed a NaPi2a/NHERF1 interaction in both WT and TRAP-KO mice. In contrast, NHERF1 immunoprecipitation analysis detected TRAP protein in WT mice, but not in NaPi2a-KO mice (Fig. 7c). These findings suggest that TRAP binds to NHERF1 in the presence of NaPi2a but cannot interact in the absence of NaPi2a. TRAP immunoprecipitation analyses showed a direct interaction between TRAP and NaPi2a (Fig. 7d).

Response to the dietary Pi content in TRAP KO mice. We examined fluctuations in the plasma Pi levels due to differences in the dietary Pi content (Fig. 8a). TRAP^{-/-} mice fed the HP diet had markedly higher plasma Pi levels than TRAP^{+/+} mice. Furthermore, TRAP^{-/-} mice fed the CP or HP diets had extremely high serum FGF23 levels compared with TRAP^{+/+} mice fed an equivalent diet (Fig. 8b). In addition, renal NaPi2a, but not NaPi2c, protein expression levels were significantly higher in TRAP^{-/-} mice fed the LP, CP, or HP diet compared with TRAP^{+/+} mice fed the same diet (Fig. 8c). In contrast, the CP diet and HP diet significantly suppressed renal NaPi2c protein expression levels in TRAP^{-/-} mice compared with TRAP^{+/+} mice (Fig. 8c). NaPi2a immunofluorescence staining was strongly detected at the apical membrane of proximal tubular cells both in TRAP^{+/+} and TRAP^{-/-} mice fed the LP diet (Fig. 8d). The HP diet suppressed NaPi2a staining at the apical membrane in TRAP^{+/+} mice. In contrast, TRAP^{-/-} mice fed the HP diet maintained NaPi2a strong immunostaining at the apical membrane of the proximal tubular cells

(Fig. 8d).

Response to FGF23 in TRAP KO mice. Next, to confirm the effect of the phosphaturic action of FGF23 in TRAP^{-/-} mice, mice were fed the LP diet to reduce endogenous FGF23. Exogenous FGF23 was expressed using the Naked-DNA method, as described previously²⁵⁻²⁷. As shown in Supplemental Figure S4a, the LP diet significantly suppressed serum FGF23 levels in both TRAP^{+/+} and TRAP^{-/-} mice. We confirmed the exogenous FGF23 mRNA (hFGF23) expression in the liver of the FGF23 groups in both TRAP^{+/+} and TRAP^{-/-} mice at 4 days after Naked-DNA injection (Supplemental Fig. S4b)²⁵⁻²⁷. In TRAP^{+/+} mice, but not in TRAP^{-/-} mice, FGF23 increased the level of ERK1/2 phosphorylation compared with the control group (Fig. 9a). Interestingly, the ERK phosphorylation level was higher in the control TRAP^{-/-} mice compared with the control TRAP^{+/+} mice. FGF23 groups of both TRAP^{+/+} and TRAP^{-/-} mice showed significantly lower levels of renal α -Klotho protein expression compared with their control groups (Supplemental Fig. S4c). Renal Cyp27b1 mRNA levels were significantly suppressed, and Cyp24a1 mRNA levels were significantly increased in the FGF23 groups of both TRAP^{+/+} and TRAP^{-/-} mice compared with their control groups (Supplemental Fig. S4d, S4e). Slc34a1, but not slc34a3, mRNA levels were significantly suppressed in FGF23 groups of both TRAP^{+/+} and TRAP^{-/-} mice compared with the control group (Supplemental Fig. S4f, S4g). Urinary Pi excretion levels were slightly but significantly increased in FGF23 groups of both TRAP^{+/+} and TRAP^{-/-} mice compared with the control group (Fig. 9b). In TRAP^{+/+} mice, both renal NaPi2a and NaPi2c protein levels were significantly suppressed after FGF23 Naked DNA injection compared with the control group (Fig. 9c). In TRAP^{-/-} mice, FGF23 significantly suppressed only NaPi2c protein expression and not NaPi2a protein expression levels (Fig. 9c).

Response to PTH in TRAP KO mice. Next, we investigated the effect of PTH on NaPi2a protein expression in TRAP^{+/+} and TRAP^{-/-} mice. The LP diet significantly suppressed plasma PTH levels in TRAP^{+/+} and TRAP^{-/-} mice, and the plasma PTH levels did not differ significantly between TRAP^{+/+} and TRAP^{-/-} mice fed the LP diet (Supplemental Fig. S4h). To confirm that there was no difference in PTH signaling, we measured the renal cAMP level 15 min after administration of PTH. PTH induced cAMP in the kidney of TRAP^{+/+} mice, but not in TRAP^{-/-} mice (Fig. 9d). Like FGF23, renal cAMP levels were slightly, but significantly higher in the control TRAP^{-/-} mice than in the control TRAP^{+/+} mice. Urinary Ca excretion was significantly increased 1 h after PTH administration in both TRAP^{+/+} and TRAP^{-/-} mice (Fig. 9e). Urinary Pi excretion, however, was significantly increased 1 h after PTH administration in TRAP^{+/+} mice, but not in TRAP^{-/-} mice (Fig. 9f). Furthermore, PTH significantly suppressed renal NaPi2a protein expression in TRAP^{+/+} mice, but not in TRAP^{-/-} mice (Fig 9g). We confirmed NaPi2a internalization in the kidney of TRAP^{+/+} mice administered PTH at 15 min and 1 h, as described previously (Fig. 9h, upper)²⁸. In TRAP^{-/-} mice, NaPi2a remained localized at the apical membrane after administration of PTH (Fig. 9h, bottom).

Renal injury in TRAP KO mice. Finally, we investigated the plasma Pi and BUN concentrations in a folic acid (FA)-induced acute kidney injury (AKI) model for 7 days. As shown in Fig. 10a, the deletion of TRAP shortened the lifespan of the AKI model mice. There were no significant differences in BUN levels at 24 h and 7 days after administration of FA between TRAP^{+/+} and TRAP^{-/-} mice (Fig. 10b, 10c). AKI-TRAP^{-/-} mice, however, had significantly higher levels of plasma Pi at 24 h after FA administration compared with AKI-TRAP^{+/+} mice, and the hyperphosphatemia was maintained only in TRAP^{-/-} mice until 7 days after FA treatment (Fig. 10d, 10e). Furthermore, serum intact FGF23 levels were markedly higher in TRAP^{-/-} mice at 24

h after FA treatment compared with TRAP^{+/+} mice, and the markedly high levels of serum FGF23 were maintained only in TRAP^{-/-} mice until 7 days after FA treatment (Fig. 10f, 10g). Renal NaPi2a protein levels were significantly reduced in TRAP^{+/+} mice by FA administration, but only slightly reduced in TRAP^{-/-} mice (Fig. 10h).

Discussion

In the present study, we investigated the roles of a strongly correlated molecule (TRAP) in the GCNs and Pi metabolism. The TRAP protein was extremely highly expressed in the kidney and localized at the apical membrane of the renal proximal tubules. Dietary Pi content regulates the renal TRAP protein levels the same as NaPi2a. Immunoprecipitation experiments suggest that TRAP interacts with the NaPi2a/NHERF1 complex (directly with NaPi2a, but not with NHERF1). TRAP^{-/-} mice showed markedly increased serum FGF23 levels and significantly increased plasma PTH levels. Interestingly, renal NaPi2a protein levels were not decreased despite the marked increase in serum FGF23 levels. Based on studies of dietary Pi responses, the marked increase in plasma Pi levels in TRAP^{-/-} mice are due to high Pi loading and may result from a resistance to NaPi2a regulation by PTH and FGF23. Thus, an abnormal dietary Pi response in TRAP^{-/-} mice leads to dysregulation of plasma Pi levels.

TRAP^{-/-} mice are characterized by 1) abnormally high serum FGF23 levels and 2) dietary Pi response abnormalities in the regulation of the plasma Pi concentration. The present findings suggest that TRAP binds to NaPi2a on the cell membrane and is involved in the internalization of NaPi2a. In TRAP^{-/-} mice, no decrease in NaPi2a was observed despite high FGF23 and PTH concentrations. On the other hand, NaPi2c was significantly decreased. In addition, NHERF1 was significantly elevated in TRAP^{-/-} mice. For these reasons, TRAP is considered to be a component of the NaPi2a/NHERF1 complex that receives signals from PTH and FGF23.

Although TRAP deficiency affects the NaPi2a/NHERF1 system, vitamin D-metabolizing enzymes and NaPi2c regulation are considered normal. PTH and FGF23 downregulate the NaPi2a/NHERF1 binary complex by activating 2 distinct signaling pathways that converge at NHERF1¹. The internalization and degradation of NaPi2a increase Pi excretion and depend on activation of the ERK1/2 and serum/glucocorticoid- regulated kinase-1 pathways, resulting in phosphorylation of NHERF1²⁹. PTH signals activate protein kinases A and C^{13, 30, 31}. Triggered by phosphorylation of NHERF1, NaPi2a dissociates from NHERF1 and is then internalized¹³. Not all signals from the receptor have been examined in detail, but some signals were activated. Considering the changes in NaPi2c and vitamin D-metabolizing enzymes (cyp24a1, cyp27b1) in TRAP^{-/-} mice and the effects of PTH/FGF23 administration, it is expected that they are functioning normally except for the signal to NaPi2a. The interaction of NaPi2c with NHERF3 (PDZK1) is more important than that with NHERF1^{32, 33}. In fact, NaPi2c expression is suppressed in NHERF3-KO mice³³. We previously reported differences in signals between the phosphaturic action of FGF23 and the inhibitory effect on vitamin D synthesis³⁴. Therefore, it is considered that the effect of TRAP deficiency is limited to the control function of NaPi2a. More detailed studies on the role of TRAP in NaPi2a regulation, such as the effect of NHERF1 on phosphorylation, are needed.

Another feature of TRAP^{-/-} mice is enhanced FGF23 induction from the bone. High serum FGF23 levels cause the pathology observed in a mouse model of X-linked hypophosphatemia rickets (Hyp mice)^{35, 36}. On the other hand, TRAP^{-/-} mice did not exhibit the abnormal bone morphology seen in Hyp mice and we speculate that this is because TRAP^{-/-} mice do not develop hypophosphatemia. The bone analysis data suggest that a high PTH concentration affects fluctuations in the numbers of osteoblasts and osteoclasts. More detailed studies will help to clarify the role of TRAP in bone.

High FGF23 induction in TRAP^{-/-} mice is improved by a low Pi diet. Therefore, renal TRAP is expected to signal dietary Pi levels to bone FGF23. On the other hand, in an FA-induced renal disorder model, a further increase in serum FGF23 concentration was observed in TRAP^{-/-} mice. FGF23 induction is known to be independent of dietary Pi signals in an FA-acute kidney injury model³⁷. Therefore, the increase in FGF23 in TRAP^{-/-} mice may be independent of the signal of renal damage.

The relationship between α -Klotho and TRAP as a mediator from the kidney remains unclear. α -Klotho plays an important role in phosphate regulation by FGF23 as a co-receptor for FGFR1 in the kidney^{38, 39}. In TRAP^{-/-} mice, renal α -Klotho levels are reduced by approximately 50%. Previous studies reported that a decrease in α -Klotho in the kidney triggers the induction of FGF23 from the bone^{38, 39}. Pi retention, progressive hyperphosphatemia, rising FGF23 levels, and low α -Klotho expression are all observed in patients with progressive chronic kidney disease⁴⁰⁻⁴². The cause of the decrease in α -Klotho in the TRAP^{-/-} mice kidney may be the high concentration of serum FGF23. We reported that renal α -Klotho levels are significantly reduced in NaPi2a/NaPi2c double-KO mice, but no increase in plasma FGF23 is observed²⁷. The reason for the increase in FGF23 in TRAP^{-/-} mice cannot be explained by the decrease in α -Klotho alone. A low-Pi diet suppresses the induction of FGF23 from the bone in α -Klotho-KO mice as well, however, suggesting that it is mediated by the same signal^{43, 44}. The relationship between TRAP and α -Klotho in the kidney was not investigated in the present study.

Finally, phosphaturic hormone is secreted in response to an excessive Pi load and acts on the kidneys to promote Pi excretion. The NaPi2a/NHERF1 complex has an important role. TRAP is expected to regulate the amount of NaPi2a in response to a Pi deficiency or excess and regulates the responsiveness of phosphaturic hormone. For example, vitamin D treatment in Hyp mice restores serum Pi levels by causing FGF23 resistance to NaPi2a/NHERF1^{34, 45}. Thus, TRAP, a

strongly correlated molecule with NaPi2a in the GCNs, is thought to be involved in the regulation of NaPi2a by phosphaturic hormones in the kidney and the prevention of hyperphosphatemia in response to a high dietary Pi load (Figure 11).

Materials and Methods

Experimental animals

All experimental procedures involving animals were conducted in accordance with the Tokushima University School of Medicine and Osaka University Graduate School of Medicine guidelines. This study was also carried out in compliance with the ARRIVE guidelines. All procedures involving the use of animals were subjected to approval from Tokushima University School of Medicine (T2019-126) and Osaka University Graduate School of Medicine (19-064-02) ethics committee.

Male and female C57B6/J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). NaPi2a-KO and NaPi2c-KO mice were maintained as described previously^{7, 46}. Mice were weaned at 4 weeks of age and provided free access to water and standard mouse chow containing 0.8% phosphorus (Oriental MF; Oriental Yeast, Osaka, Japan).

Generation of TRAP-KO mice

TRAP-deficient mice were generated by gene targeting. A targeting vector was constructed by replacing the 129 genomic TRAP loci (exon1 through part of exon2) with neo^r (Supplemental Fig. S1a). The targeting vector was introduced into 129 days 14 embryonic stem (ES) cells by electroporation, and clones that underwent homologous recombination were confirmed by Southern blot analysis (Supplemental Fig. S1b). Genomic DNA was extracted from tail clippings and amplified by PCR using specific primers (Supplemental Fig. S1c, Table 1).

337

338 Dietary adaptation

339 For the dietary adaptation study, mice were fed a test diet based on modified AIN93G (low Pi;
340 0.02 or 0.1% Pi, control Pi; 0.6% Pi, and high Pi; 1.2% Pi), as described previously⁴⁷⁻⁴⁹.

341

342 Effect of FGF23 and PTH on the regulation of NaPi transporter degradation

343 For exogenous FGF23 expression, TransIT-EE Hydrodynamic Delivery Solution was used as the
344 TransIT in vivo gene delivery system (Takara, Osaka, Japan) as described previously^{26, 27}. For
345 PTH injection, mice were administered bovine PTH (amino acid 1–34; Sigma) at a dose of 7.5
346 µg/100 g body weight, as described previously^{50, 51}.

347

348 Metabolic cages to collect urine and fecal samples

349 Mice were individually placed in the metabolic cages at 10:00 for quantitative urine and fecal
350 collection for 24 h with free access to food and water. Fecal samples were ashed according to a
351 modified protocol, as described previously^{34, 47, 52, 53}.

352

353 Biochemical measurements

354 Concentrations of Pi, Ca, and creatinine and BUN were determined using commercial kits (Wako,
355 Osaka, Japan). The fractional excretion index for Ca (FEICa) and Pi (FEIPi) was calculated as
356 follows: urinary Ca or Pi/ (urinary creatinine /serum Ca or Pi). Concentrations of serum FGF23,
357 plasma PTH, and 1,25(OH)₂D₃ were determined using the FGF23 ELISA kit (KAINOS
358 Laboratories, Tokyo, Japan), intact PTH ELISA kit (Immunotopics Inc., San Clemente, CA, USA),
359 and 1,25-(OH)₂ Vitamin D ELISA Kit (Immundiagnostik, Bensheim, Germany), respectively.
360 Other blood clinical parameters were analyzed by automated methods. The cAMP levels were

measured using Cyclic AMP Select EIA Kit (Cayman Chemical, Ann Arbor, MI, USA).

RNA extraction, cDNA synthesis, and quantitative PCR

Mouse tissues were sampled, immediately submerged in RNAlater (Sigma-Aldrich, St. Louis, MO, USA), and stored at -20°C until use. Total cellular RNA from the sampled tissues was extracted and purified using ISOGEN (Wako, Osaka Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized as described previously^{34, 47, 52, 53}. The template DNA was omitted for the negative control (-) for all PCR experiments. The PCR reaction was examined without reverse transcriptase (data not shown). Quantitative PCR was performed using ABI PRISM 7500 (Applied Biosystems, Foster City, CA) as described previously^{34, 47, 49, 52, 53}. The reaction mixture consisted of 10 µl of SYBR Premix Ex Taq, ROX Reference Dye II (Perfect Real Time, Takara, Osaka, Japan) with specific primers (Table 2).

Protein sample purification and immunoblotting

BBMVs prepared using the Ca²⁺ precipitation method, cortical membrane, and whole homogenate were obtained from mouse kidneys and used for immunoblotting and immunoprecipitation analyses as described previously^{34, 49, 52, 53}. Protein samples were heated at 95°C for 5 min in sample buffer in the presence of 2-mercaptoethanol and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred by electrophoresis to Immobilon-P polyvinylidene difluoride (Millipore, Billerica, MA, USA) and treated with diluted antibodies. Signals were detected using Immobilon Western (Millipore)^{34, 49, 52, 53}.

Immunofluorescence staining

Mouse kidneys were fixed with the 4% paraformaldehyde solution (pH 7.2), overnight at 4°C, washed with PBS, cryoprotected with 10% and 20% sucrose at 4°C, embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan), and frozen in hexane at -80°C. Frozen sections (5 µm thick) were collected onto MAS-coated slides (Matsunami Glass IND, Ltd., Osaka, Japan) and air-dried⁴⁸. For immunofluorescence microscopy, serial frozen sections were incubated with primary antibodies overnight at 4°C. Alexa Fluor 488 anti-rabbit (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 568 anti-mouse (Molecular Probes) were used as secondary antibodies for 60 min at room temperature^{48, 49, 51-53}. Thereafter, the sections were mounted with DAPI Fluoromount G™ (Southern Biotech, Birmingham, UK). Images were taken with an A1R confocal laser scanning microscope system (Nikon, Tokyo, Japan).

Immunoprecipitation

Renal BBMVs of mice were lysed for 30 min at 4°C in TNE lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% TritonX-100, pH7.5), centrifuged for at 12,000 x g, 10 min at 4°C, and then supernatants were collected for immunoprecipitation. Immunoprecipitation samples were adjusted to 200 µg proteins/ ml in tubes, and anti-NHERF1 (LS-C46891, LIFESPAN BIOSCIENCES, Seattle, WA, USA) or anti-TRAP antibodies were added to tubes and rotated at 4°C overnight. Next, protein A agarose beads (Santa Cruz Biotechnology, Dallas, TX, USA) were added to the tubes and rotated at 4°C for 1 h. Protein A agarose beads were centrifuged at 3000 x g for 1 min at 4°C and washed with TNE lysis buffer 4 times before removing the supernatant and eluting in SDS sample buffer. Loading samples were heated at 95°C for 5 min and then analyzed by SDS-PAGE using antibodies against NHERF1, TRAP, and NaPi2a.

Antibodies

Rabbit anti-NaPi2a and NaPi2c polyclonal antibodies were generated as described previously and used for immunoblotting and immunohistochemistry^{7, 48, 51, 52}. Rabbit anti-TRAP polyclonal antibodies were generated against mouse TRAP C-terminal fragments in rabbits as described previously⁵⁴. Briefly, purified GST-TRAP C-terminal (residues 90-243) fusion proteins were used as antigens in rabbits. The purified IgG fractions were obtained from serums of the immunized animals. Mouse anti-actin monoclonal antibodies (Millipore) were used as an internal control. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was utilized as the secondary antibody (Jackson Immuno Research Laboratories, Inc, West Grove, PA, USA). The diluted antibodies for immunoblotting were as follows: anti-NaPi2a (1:15000), anti-NaPi2c (1:1500), anti-TRAP (1:1000), and anti-actin (1:10000). The diluted antibodies for immunofluorescence staining were as follows: anti-NaPi2a (1:500), anti-NaPi2c (1:1000), anti-TRAP (1:200), anti-villin (Millipore; 1:300), and anti-NHERF1 (LIFESPAN BIOSCIENCES; 1:1000).

Bone analysis

Histochemical analysis was performed as described previously^{34, 55}. The femora of all the groups were fixed with 4% paraformaldehyde overnight at 4°C, decalcified for 4 weeks with 10% EDTA, and then embedded into paraffin for immunohistochemical examinations. For von Kossa staining, tibiae were immersed in a mixture containing 2% paraformaldehyde and 2.5% glutaraldehyde diluted in a 0.067 M cacodylate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in a 0.067 M cacodylate buffer for 4 hr at 4 °C. After post-fixation, the tibiae were embedded in epoxy resin (Epon 812, Taab, Berkshire, UK) and sliced to semithin sections with 500 nm thickness using an ultramicrotome (Sorvall MT-5000; Ivan Sorvall, Inc., Norwalk, CT). Epoxy resin slides from undecalcified specimens were embedded in an aqueous solution of silver nitrate until a dark

brown/black staining of the bone tissue was discernible under light microscopy.

Immunohistochemical analyses of mouse bone sections were performed as described previously. Briefly, the sections were immersed into 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase. To reduce nonspecific binding, 1% bovine serum albumin (BSA; Serologicals Proteins Inc., Kankakee, IL, USA) in PBS (1% BSA-PBS) was applied to the sections for 20 min. The sections were then incubated with rabbit polyclonal antisera against tissue-nonspecific ALP diluted at 1:200⁵⁶. A general method for rapid purification of soluble versions of glycosylphosphatidylinositol-anchored proteins expressed in insect cells: an application for human tissue-nonspecific alkaline phosphatase or rabbit polyclonal anti-dentin matrix protein-1 (Takara Bio, Shiga, Japan) diluted at 1:200 with 1% BSA-PBS at room temperature for 2 h. The treated sections were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA) for 1 h, and the immunoreactivities were visualized by using diaminobenzidine tetrahydrochloride as a substrate. For double detection of ALP and tartrate-resistant acid phosphatase, the sections immunodetected for ALP were incubated in a mixture of 8 mg of naphthol AS-BI phosphate (Sigma-Aldrich), 70 mg of red violet LB salt (Sigma-Aldrich), and 50 mM L (+) tartaric acid (0.76 g; Nacalai Tesque, Kyoto, Japan) diluted in 60 ml of a 0.1 M sodium acetate buffer (pH 5.0) for 20 min at room temperature. Methyl green was used for counterstaining in all sections.

Statistical Analysis

Data are expressed as means $\pm$ SE. Differences among multiple groups were analyzed by analysis of variance followed by the Scheffe test. Differences between 2 experimental groups were established by analysis of variance followed by Student's *t* test. A *P* value of less than 0.05 was considered significant.

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Author contributions

Conceived of and designed the research; S.N., Y.K., K.M., and H.S. Performed the experiments; S.S., Y.S., A.H., K.K., M.K., K.T., M.U., I.K., T.H, Y.K., P.W., S.N. and H.S. Analyzed the data; S.S., Y.S., S.N., T.H., N.A., and H.S. Prepared Figures: S.S., H.S., Y.S., and T.H. Drafted the manuscript; Y.S., H.S., and K.M. Reviewed and approved manuscript; K.M., T.H., N.A., S.N., Y.K., and H.S.

Competing interests

The authors declare no competing interests

Additional information**Supplementary Information**

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Table 1 Primers for genotyping PCR

Primer name	Sequences (5'-3')
Primer 1	ACATTCATCCTGATCGCTGTGTG
Primer 2	CGTGCAATCCATCTTGTTCAT
Primer 3	GGAATTTAACCAGGGCAGCTTAA

Table 2 Primers for RT-PCR

Primer	Sense (Sequences; 5'-3')	Antisense (Sequences; 5'-3')
Cyp24A1	TGGGAAGATGATGGTGACCC	TCGATGCAGGGCTTGACTG
Cyp27B1	GAGCAAACCTCCAGGAAGCAG	TGAGGAATGATCAGGAGAGG
Mouse FGF23	CCATCTACAGTGCCCTGATG	GCTGAAGTGAAGCGATCC
GAPDH	CTGCACCACCAACTGCTTAGC	CATCCACAGTCTTCTGGGTG
Human FGF23	GCTCTGGGTCTGTGCCTTGT	GTGATACTGAGGAGAGTG
α-klotho	CAATGGCTTTTCCTCCTTTAC	TGCACATCCCACAGATAGAC
PTH	TGAGAGTCATTGTATGTAAAGATGAGGTGTTTGCCCAGGTTG	
slc34a1	AGTCTCATTCGGATTTGGTGTCA	GCCGATGGCCTCTACCCT
slc34a3	TAATCTTCGCAGTTCAGGTTGCT	CAGTGGAATTGGCAGTCTCAA
Tmem174/TRAP	GCCACTTTGCTTTTCTCAGG	GGGACCCTCTCCTCGTTATC

Figure legends

Figure 1 Genes co-expressed with renal slc34a NaPi transporters

The genes co-expressed with slc34a1 or slc34a3 were identified by database search on COEXPRESdb, as listed in Table 1 and 2. Correlation profile of gene expression between (a) slc34a1 (x axis: probe ID 1423279_at), or (b) slc34a3 (x axis: probe ID 1439519_at), and TMEM174 (y axis: probe ID 1435201).

Figure 2 Tissue localization of TRAP expression and possible involvement in Pi homeostasis.

(a) TRAP mRNA level in several tissues of wild-type (WT) mice by real-time PCR. Male mice at 8 weeks of age (n=5–9) were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Values are mean $\pm$ SE. (b) Immunofluorescence staining TRAP (green) in renal section of 8-week-old WT mice. DAPI (blue), Villin (red). Sections were prepared from mouse kidney embedded in OCT compound and frozen. (c, d) Western blotting analyses of the renal brush border membrane vesicles (BBMVs) isolated from the WT mice (n=3–5) fed a low Pi (LP: 0.02%), control Pi (CP: 0.6%), and high Pi (HP: 1.2%) diet. Each lane was loaded with 20 μ g of BBMVs. Actin was used as an internal control. Values are mean $\pm$ SE. ^a p <0.05, ^{a'} p <0.01 vs. LP. Experiments were performed in triplicate. (e) Western blotting analysis of the renal BBMVs isolated from the kidney of 8-week-old NaPi2a^{+/+} NaPi2c^{+/+}, NaPi2a^{-/-}, and NaPi2c^{-/-} mice (n=3–5). Each lane was loaded with 20 μ g of BBMVs. Actin was used as an internal control. Values are mean $\pm$ SE. ^a p <0.05 vs. NaPi2a^{+/+}NaPi2c^{+/+} mice. Experiments were repeated at least 3 times.

Figure 3 Characteristics of TRAP^{-/-} mice.

Expression of TRAP mRNA and protein in TRAP^{+/+}, TRAP^{+/-}, and TRAP^{-/-} mice. PCR and

Western blotting analysis in the kidney of mice. (a) Aliquots of each PCR product were electrophoresed on a 1.5% agarose gel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. (b) Western blotting analysis of BBMVs isolated from the kidneys of TRAP^{+/+}, TRAP^{+/-}, and TRAP^{-/-} mice. Each lane was loaded with 20 µg of BBMVs. Actin was used as the internal control. (c) Growth curves for male and female TRAP^{+/+}, TRAP^{+/-}, and TRAP^{-/-} mice. Values are mean ± SE (n=10-30). **p*<0.05 vs. TRAP^{+/+}, #*p*<0.05 vs. TRAP^{+/-} mice. Metabolic cages were used for measurement of 24-h food intake (g/ day), and collection of urine, and feces from mice. (d) Food intake, (e) plasma creatinine, (f) plasma blood urea nitrogen, (g) blood ionized Ca, (h) fecal Ca excretion, (i) urinary Ca excretion, (j) plasma Pi, (k) fecal Pi excretion, (l) urinary Pi excretion. Male TRAP^{+/+}, TRAP^{+/-}, and TRAP^{-/-} mice at 8–9 weeks of age (n=30–50). Values are mean ± SE.

Figure 4 Effects of Deletion of TRAP on Pi homeostasis

(a-c) Plasma 1,25(OH)₂D₃, plasma intact PTH, serum intact FGF23 levels of Male TRAP^{+/+}, TRAP^{+/-}, and TRAP^{-/-} mice at 8–9 weeks of age. Values are mean ± SE (n=10–20). ^a*p*<0.01 vs. TRAP^{+/+} mice. ^b*p*<0.01 vs. TRAP^{+/-} mice. (d-g) Real-time PCR analysis. GAPDH was used as an internal control. Values are mean ± SE (n=10-15). ^a*p*<0.01 vs. TRAP^{+/+} mice. ^b*p*<0.01 vs. TRAP^{+/-} mice. [#]*p*<0.01. (h) Immunohistochemistry of FGF23 (brown color) in metaphyseal and diaphysis trabeculae bones of TRAP^{+/+} and TRAP^{-/-} male mice (8-week-old). Arrows: osteoblast/preosteoblast, arrow head: osteocyte.

Figure 5 Bone analysis in TRAP^{-/-} mice

Histological analysis of longitudinal femoral sections of 8-week-old TRAP^{+/+} and TRAP^{-/-} mice. (a) Hematoxylin/eosin staining, (b) Double staining of alkaline phosphatase (brown color) and

tartrate-resistant acid phosphatase (red color), (c) von Kossa staining of metaphyseal trabeculae.

Figure 6 Deletion of TRAP and renal NaPi transporters

(a) Western blot analysis of NaPi transporters in TRAP^{+/+} and TRAP^{-/-} mice (8-week-old mice, n=5, respectively). Each lane was loaded with 20 µg of BBMVs. Actin was used as an internal control. Values are mean ± SE. [#]*p*<0.01. Experiments were repeated at least 3 times. (b) Immunofluorescence staining of NaPi2a or NaPi2c (green) in renal sections of 8-week-old TRAP^{+/+} and TRAP^{-/-} mice. DAPI (blue), Villin (red). Sections were prepared from kidneys embedded in the OCT compound and frozen. Scale bar; 100 µm. (c-d) Western blotting analysis of FGFR, α-Klotho (c), and total ERK1/2/phosphorylation ERK1/2 (d) levels. Each lane was loaded with 20 µg of cortical membranes (c) or whole homogenate (d). Actin was used as an internal control. Values are mean ± SE. [#]*p*<0.01. Experiments were repeated at least 3 times.

Figure 7 Scaffold protein levels in TRAP^{-/-} mice.

Western blot analysis of scaffold proteins in TRAP^{+/+} and TRAP^{-/-} mice fed normal mouse chow. Each lane was loaded with 20 µg of renal whole homogenate (a) and BBMVs (b). Actin was used as an internal control. Values are mean ± SE. [#]*p*<0.05. Immunoprecipitation of NHERF1 (c) or TRAP (d) from renal BBMVs lysates of WT, TRAP-KO and NaPi2a-KO mice fed the normal mouse chow and immunoblotting with anti-TRAP, -NaPi2a or -NHERF1 antibodies. Arrow indicates the TRAP. Experiments were repeated at least 3 times.

Figure 8 Dietary Pi regulation in TRAP^{-/-} mice

Male TRAP^{+/+} and TRAP^{-/-} mice (n=5) fed a low Pi (LP: 0.02%), control Pi (CP: 0.6%), or high Pi (HP: 1.2%) diet for 12 days. (a) Plasma Pi, (b) Serum intact FGF23, Values are mean ± SE.

[#]*p*<0.05, [#]*p*<0.01 vs same diet, ^a*p*<0.05, ^a*p*<0.01 vs LP, ^b*p*<0.01 vs. CP of same genotype. (c) Western blotting analyses. Each lane was loaded with 20 µg of BBMV. Actin was used as an internal control. Values are mean ± SE. [#]*p*<0.05, [#]*p*<0.01. (d) Immunostaining of renal NaPi2a (Green) localization in the kidney sections of mice fed the LP (0.02% Pi) and HP (1.2% Pi) diet for 7 days. DAPI (Blue), Villin (Red). Experiments were performed in triplicate.

Figure 9 Abnormal regulation of phosphaturic action to renal NaPi2a Pi transporter in TRAP^{-/-} mice.

TRAP^{+/+} and TRAP^{-/-} mice (9-10-week-old mice, n=3–5, respectively) were fed a 0.02% low Pi diet for 7 days to reduce endogenous FGF23. Exogenous FGF23 expression was performed using the Naked-DNA method, as described previously²⁷. (a) Western blotting analysis of total ERK1/2 and phosphorylation ERK1/2 in TRAP^{+/+} and TRAP^{-/-} mice. Each lane was loaded with 20 µg of the whole homogenate. Values are mean ± SE. [#]*p*<0.05 vs control of the same genotype, **p*<0.01 vs same treatment of TRAP^{+/+} mice. ns; not significant. (b) FEIPi. Values are mean ± SE. [#]*p*<0.05 vs control of the same genotype. (c) Western blot analysis of NaPi transporters in TRAP^{+/+} and TRAP^{-/-} mice. Each lane was loaded with 20 µg of BBMV. Actin was used as an internal control. **p*<0.01 vs same treatment of TRAP^{+/+} mice. Values are mean ± SE. [#]*p*<0.01 vs control of the same genotype. ns; not significant. Experiments were performed in triplicate.

TRAP^{+/+} and TRAP^{-/-} mice (9-10-week-old mice, n=3–5, respectively) were fed a 0.1% low Pi diet for 7 days to reduce endogenous PTH. Bovine PTH (1–34) was administered to TRAP^{+/+} and TRAP^{-/-} mice. Samples were collected after 15 min or 1 h administration of PTH. (d) cAMP level of the kidney of mice after 15 min PTH administration. (e) FEICa, (f) FEIPi. Samples were collected after 1 h administration of PTH. Values are mean ± SE. [#]*p*<0.05 vs

control of the same genotype. ** $p < 0.01$ vs same treatment of TRAP^{+/+} mice. ns; not significant. (g) Western blot analysis of NaPi2a in TRAP^{+/+} and TRAP^{-/-} mice. Each lane was loaded with 20 µg of BBMVs. Actin was used as an internal control. Values are mean ± SE. # $p < 0.01$ vs control of the same genotype. * $p < 0.01$ vs same treatment of TRAP^{+/+} mice. ns; not significant. (h) Immunostaining of renal NaPi2a (Green) localization in the kidney sections of mice 15 min and 1 h after administration of PTH. DAPI (Blue). Villin (Red). Experiments were performed in triplicate.

Figure 10 Renal injury in TRAP^{-/-} mice

Male TRAP^{+/+} and TRAP^{-/-} mice (n=10–30) were administered folic acid (FA; 240 µg/kg BW). (a) Survival curve, Plasma BUN at 24 h (b) and 7 days (c), plasma Pi at 24 h (d) and 7 days (e), and serum intact FGF23 at 24 h (f) and 7 days (g) after administration of FA. Values are mean ± SE. # $p < 0.05$, #' $p < 0.01$ vs vehicle of the same genotype, * $p < 0.05$ vs same treatment of TRAP^{+/+} mice. ns; not significant. (h) Western blotting analysis of NaPi2a. Each lane was loaded with 20 µg of renal BBMVs of mice 24 h after administration of FA. Actin was used as an internal control.

Figure 11 The putative role of TRAP in the control of plasma Pi levels

TRAP interacts with NaPi2a and is involved in internalization by PTH and FGF23. Under a high Pi load, urinary Pi excretion is enhanced by internalization of NaPi2a induced by PTH and FGF23, and hyperphosphatemia is prevented. In TRAP-KO mice, internalization of NaPi2a is resistant to PTH and FGF23. As a result, Pi reabsorption is maintained, and plasma Pi concentration increases. TRAP is expected to be a molecule that is associated with both the induction of phosphaturic hormone (FGF23) and the regulation of NaPi2a in the proximal tubules. G; glomerulus, PT; proximal tubule.

12/13/21

820

Figure 1

a

b

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

WT mice

TRAP-KO mice

Supplementary Files

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