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1 **Title:** All-trans retinoic acid reduces the transcriptional regulation of intestinal sodium-dependent
2 phosphate co-transporter gene (*Npt2b*)
3

4 **Authors:** Masashi Masuda[1]*, Hironori Yamamoto[1,2,3]*, Yuichiro Takei[1,4], Otoki Nakahashi[1,5],
5 Yuichiro Adachi[1], Kohta Ohnishi[1], Hirokazu Ohminami[1], Hisami Yamanaka-Okumura[1], Hiroshi
6 Sakaue[6], Makoto Miyazaki[7], Eiji Takeda[1], and Yutaka Taketani[1]
7

8 **Affiliations:** [1]Department of Clinical Nutrition and Food Management, Institute of Biomedical
9 Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503,
10 Japan. [2]Department of Health and Nutrition, Faculty of Human Life, Jin-ai University, 3-1-1
11 Ohde-cho, Fukui 915-8586, Japan, [3]Department of Nephrology, Faculty of Medical Sciences,
12 University of Fukui, Fukui 910-1193, Japan. [4]Department of Nutrition, University of Kochi,
13 Kochi 780-8515, Japan. [5]Division of Functional Food Chemistry, Institute for Health Sciences,
14 Tokushima Bunri University, Tokushima 770-8514, Japan. [6]Department of Nutrition and
15 Metabolism, Institute of Biomedical Sciences, Tokushima University Graduate School,
16 Tokushima 770-8503, Japan. [7]Division of Renal Diseases and Hypertension, Department of
17 Medicine, University of Colorado Denver, Aurora, Colorado 80045, USA.
18

1 *To whom correspondence should be addressed: Masashi Masuda, Ph.D., Department of Clinical
2 Nutrition and Food Management, Institute of Biomedical Sciences, Tokushima University
3 Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan.
4 **E-mail:** masuda.masashi@tokushima-u.ac.jp; **Tel.:** +81-88-633-7235. Hironori Yamamoto,
5 Ph.D., Department of Health and Nutrition, Faculty of Human Life, Jin-ai University, 3-1-1 Ohde-
6 cho, Fukui 915-8586, Japan. **E-mail:** yamamoto@jindai.ac.jp; **Tel.:** +81-778-43-6549.
7
8 **Running title:** Regulation of *Npt2b* gene promoter activity by RAR/RXR-C/EBP
9
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12 type IIb sodium-dependent phosphate co-transporter (Npt2b)
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14
15

1 **Abstract**

2 Inorganic phosphate (Pi) homeostasis is regulated by intestinal absorption via type II sodium-
3 dependent co-transporter (Npt2b) and by renal reabsorption via Npt2a and Npt2c. Although we
4 previously reported that vitamin A-deficient (VAD) rats had increased urine Pi excretion through
5 the decreased renal expression of Npt2a and Npt2c, the effect of vitamin A on the intestinal Npt2b
6 expression remains unclear. In this study, we investigated the effects of treatment with all-trans
7 retinoic acid (ATRA), a metabolite of vitamin A, on the Pi absorption and the Npt2b expression
8 in the intestine of VAD rats, as well as and the underlying molecular mechanisms. In VAD rats,
9 the intestinal Pi uptake activity and the expression of Npt2b were increased, but were reduced by
10 the administration of ATRA. The transcriptional activity of reporter plasmid containing the
11 promoter region of the rat *Npt2b* gene was reduced by ATRA in NIH3T3 cells overexpressing
12 retinoic acid receptor (RAR) and retinoid X receptor (RXR). On the other hand,
13 CCAAT/enhancer-binding proteins (C/EBP) induced transcriptional activity of the *Npt2b* gene.
14 Knockdown of the *C/EBP* gene and a mutation analysis of the C/EBP responsible element in the
15 *Npt2b* gene promoter indicated that C/EBP plays a pivotal role in the regulation of *Npt2b* gene
16 transcriptional activity by ATRA. EMSA revealed that the RAR/RXR complex inhibits binding
17 of C/EBP to *Npt2b* gene promoter. Together, these results suggest that ATRA may reduce the
18 intestinal Pi uptake by preventing C/EBP activation of the intestinal *Npt2b* gene.

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1 **Abbreviations list:** ATRA, all-*trans*-retinoic acid; BBMV, brush-border membrane vesicle; β-
2 gal, β-galactosidase; C/EBP, CCAAT/enhancer-binding protein; Cr, creatinine; 1,25$(OH)_2D_3$,
3 1α,25-dihydroxyvitamin D_3; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine
4 serum; FEI, fractional excretion index; FGF23, fibroblast growth factor 23; ICP-MS, inductively
5 coupled with plasma-mass spectrometry; Npt, sodium-dependent phosphate co-transporter; Pi,
6 inorganic phosphate; PTH, parathyroid hormone; RAR, retinoic acid receptor; RARE, retinoic
7 acid-response element; RXR, retinoid X receptor; TTNPB, 4-[E-2-(5, 6, 7, 8-Tetrahydro-5, 5, 8,
8 8-tetra-methyl-2-naphtalenyl)-1-propenyl] benzoic acid; VAD, vitamin A-deficient;
9

Introduction

Inorganic phosphate (Pi) homeostasis in mammals is strictly controlled through the balance of intestinal absorption and renal excretion/reabsorption [1, 2]. The uptake of Pi is mediated by type II sodium-dependent phosphate co-transporters (Npt2) in the brush-border membrane of the small intestine and renal proximal tubule [1, 2]. Npt2a and Npt2c are responsible for most Pi reabsorption in the kidney and can be regulated by dietary Pi, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], all-trans retinoic acid (ATRA), and hormonal factors such as parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and tumor necrosis factor-α (TNFα) [1−6]. Npt2b plays a critical role in intestinal Pi absorption and its expression is regulated by dietary Pi, $1,25(OH)_2D_3$, FGF23, epidermal growth factor (EGF), TNFα, and nuclear factor 1 (NF1) [1, 7−14].

Disturbance of Pi homeostasis can cause important clinical disorders. Hyperphosphatemia, which is associated with the pathophysiology of chronic kidney disease (CKD), can lead to vascular calcification, which has been linked to increased cardiovascular morbidity and mortality [15]. The regulation of the intestinal Pi absorption mediated by Npt2b is a possible therapeutic target to control hyperphosphatemia in CKD [16]. In fact, Schiavi *et al.* suggested that targeting Npt2b in addition to using dietary Pi binders may be a therapeutic approach to modulate serum Pi in CKD [17]. Aside from this, the expression of Npt2b has also been detected in various tissues, including the small

1 intestine, lung, kidney, testis, and liver [18]. Npt2b is also involved in the reuptake of phosphate
2 for the synthesis of surfactant proteins in the lung [19, 20]. Mutations in the *Npt2b* gene are linked
3 to pulmonary alveolar microlithiasis (PAM), an autosomal recessive disorder characterized by the
4 deposition of calcium phosphate microlith, and testicular microlithiasis (TM) — a disease that is
5 associated with cancer and infertility [21].
6 Transcription factors precisely control the diversity and specificity of the complex patterns
7 of gene regulation. The transcription factor CCAAT/enhancer-binding proteins (C/EBPs), of
8 which there are six members (C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and C/EBPζ), are
9 involved in adipocyte differentiation, energy metabolism, immunity, and inflammation [22, 23].
10 In addition, C/EBP homologue protein (CHOP), which is activated by endoplasmic reticulum
11 (ER) stress, mediates TNFα- and CKD-induced vascular calcification [24–26]. We recently
12 reported that C/EBPβ contributes to vascular calcification via the upregulation of the expression
13 of type III sodium-dependent phosphate co-transporters (PiT1 and PiT2) [27]. The double-
14 knockout *Cebpa*/*Cebpb* mice generate an early embryonic lethal phenotype [28]. On the other
15 hand, Shibasaki *et al.* demonstrated that the developmental deletion of the *Npt2b* gene leads to an
16 embryonic lethal phenotype [29]. Interestingly, Xu *et al.* suggested that C/EBPα controls
17 surfactant lipid homeostasis by regulating the transcription of the *Npt2b* gene [30].
18 The physiological actions of ATRA, a metabolite of vitamin A, are mediated by specific

nuclear receptors, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs). These receptors are members of the steroid/thyroid hormone nuclear receptor superfamily, which act as ligand-dependent transcriptional factors. RARs and RXRs regulate the transcription of target genes by binding to retinoic acid-response elements (RAREs) in their promoters [31, 32]. We previously reported that the renal expression of Npt2a and Npt2c is decreased in vitamin A-deficient (VAD) rats, and that the transcriptional activity of human *Npt2a* and *Npt2c* genes is upregulated by ATRA and its receptors [4]. However, the effect of ATRA on the intestinal expression of Npt2b and the underlying molecular mechanism remain unclear. Furthermore, in the previous study, we did not examine the effects of ATRA treatment on phosphate homeostasis in VAD rats.

In the present study, we used VAD rats to investigate the effects of ATRA on the expression of Npt2b in the small intestine. We also characterized the *Npt2b* gene promoter with regard to transcriptional regulation through RAR/RXR and C/EBP.

1 **Experimental procedures**

2 **Chemicals and reagents**

3 ATRA, DMSO, and mouse anti-β-actin monoclonal antibody were purchased from Sigma-
4 Aldrich (St. Louis, MO, USA). Buprenorphine hydrochloride was purchased from Otsuka
5 Pharmaceutical Co., Ltd. (Tokyo, Japan). Pentobarbital sodium salt was purchased from Tokyo
6 Kasei Co., Ltd. (Tokyo, Japan). Anti-Npt2b antibody was purchased from Alpha Diagnostics (San
7 Antonio, TX, USA). Goat anti-rabbit IgG(H+L)-HRP conjugate was purchased from Bio-Rad
8 (Hercules, CA, USA). ECL Plus system and poly(dI-dC) were purchased from GE Healthcare
9 (Buckinghamshire, UK). QuikChange® site-directed mutagenesis kit was purchased from
10 Stratagene (La Jolla, CA, USA). 4-[E-2-(5, 6, 7, 8-Tetrahydro-5, 5, 8, 8-tetra-methyl-2-
11 naphtalenyl)-1-propenyl] benzoic acid (TTNPB) was purchased from Biomol Research
12 Laboratories (Boston, MA, USA). Double-strand Stealth RNAi oligos for C/EBPβ and negative
13 control were purchased from Thermo Fisher Scientific (Waltham, MA, USA). [γ-^{32}P] ATP was
14 purchased from ICN (Costa Mesa, CA, USA). $T_{N}T$® Quick Coupled Transcription/Translation
15 System was purchased from Promega Corporation (Madison, WI, USA). T_4 polynucleotide kinase
16 was purchased from Takara (Shiga, Japan). C/EBP consensus oligonucleotide (cebp; catalog
17 number sc-2525) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

18

1 **Animal experiments**

2 The animal work took place in Division for Animal Researches and Genetic Engineering Support
3 Center for Advanced Medical Sciences, Institute of Biomedical Sciences, Tokushima University
4 Graduate School. The animals were housed in pathogen-free conditions and maintained under a
5 standard 12 hr light-dark cycle with free access to water. Briefly, pregnant Wistar rats (Japan SLC,
6 Shizuoka, Japan) were fed an altered vitamin A-deficient AIN93-G diet (Oriental Yeast, Osaka,
7 Japan) containing 0.6% Pi and 0.6% Ca (VAD) or an altered AIN93-G diet (Oriental Yeast)
8 containing 0.6% Pi and 0.6% Ca (control) from the 14th day of gestation until the pups were
9 weaned. Pups (male) were continued on each diet as discussed above until they were killed at 7
10 weeks of age. The VAD group was randomly divided into two groups and intraperitoneally
11 administrated a total of 1 mg/kg body weight of ATRA or DMSO prepared in 500 μl sterile saline.
12 The control group was intraperitoneally administrated the same dose of DMSO. Each group (n =
13 4 per group) of rats was fasted for 18 hr in metabolic cages with water *ad libitum* before sacrifice
14 with a total of 0.1 mg/kg body weight of buprenorphine hydrochloride and a total of 50 mg/kg
15 body weight of pentobarbital sodium salt, and the removal of tissues. Composition of the diets
16 was described previously [4]. The present study was approved by the Animal Experimentation
17 Committee of Tokushima University School of Medicine (animal ethical clearance No. T28-24)
18 and was carried out in accordance with guidelines for the Animal Care and Use Committee of

Tokushima University School of Medicine.

Plasma and urine parameters

Concentrations of Pi, Ca, creatinine (Cr), and vitamin A (retinol) were determined as described previously [4]. Concentrations of plasma 1,25$(OH)_2$D, PTH, and FGF-23 were determined as described previously [6]. Metabolic cages were used for 18 hr urine collection. The fractional excretion indexes for Pi (FEI Pi) and for Ca (FEI Ca) were calculated as urine Pi or Ca/(urine Cr × plasma Pi or Ca).

Feces Pi extraction

Feces samples were collected during the period of 47–49 days to determine intestinal Pi and Ca excretion. The feces were first dried at 110˚C for 12 hr then micropulverized, from which 100 mg samples were ashed at 250˚C for 3 hr, at 350˚C for 3 hr, and 550˚C for 24 hr as previously described [33]. These samples were heated at 100˚C for 15 min with 25 ml of 1% HCl. Extracted Pi and Ca were measured using a standard molybdate assay [34] and inductively coupled with plasma-mass spectrometry (ICP-MS).

Preparation of brush border membrane vesicles (BBMVs) and Pi uptake

1 BBMVs were prepared from rat small intestine and kidney by the Ca^{2+} precipitation method as
2 described previously [3]. The uptake of ^{32}P into BBMVs was measured by a rapid filtration
3 technique. Ten μl of vesicle suspension was added to 90 μl of incubation solution that was
4 composed of 100 mM NaCl or choline chloride, 100 mM mannitol, 20 mM HEPES/Tris, and 0.1
5 mM $KH_2{}^{32}PO_4$, and the preparation was incubated at 20˚C. Na^+-dependent and Na^+-independent
6 Pi uptake were measured as described previously [35].

7

8 **Western blot analysis**

9 Protein samples were heated at 95˚C for 5 min in sample buffer in the presence of 5% 2-
10 mercaptoethanol and subjected to SDS-PAGE. The separated proteins were transferred by
11 electrophoresis to polyvinylidene difluoride transfer membranes (Immobilon-P, Millipore, MA,
12 USA). The membranes were treated with diluted affinity-purified anti-Npt2a (1:5,000), and anti-
13 Npt2c (1:500), and anti-Npt2b (1:2,000) antibody [8]. Mouse anti-β-actin monoclonal antibody
14 was used as an internal control. Goat anti-rabbit IgG(H+L)-HRP conjugate (1:2,000) was utilized
15 as the secondary antibody, and signals were detected using the ECL Plus system.

16

17 **Quantitative PCR analysis**

18 Extraction of total RNA, cDNA synthesis, and real-time PCR were performed as described

previously [4]. The primer sequences (*Npt2b, C/EBPα, and C/EBPβ*) for PCR amplification are shown in Supplementary Table S1. Other primer sequences (*Npt2a*, *Npt2c*, *PiT1*, *PiT2*, and *β-actin*) were described previously [4]. Amplification products were then analyzed by a melting curve, which confirmed the presence of a single PCR product in all reactions (apart from negative controls). The PCR products were quantified by fit-point analysis, and results were normalized to that of *β-actin*.

Reporter plasmid construction

Luciferase reporter plasmids prNp2b-1.8k, prNp2b-800, prNp2b-67, prNp2b-55, pmNp2b-1.7k, pmNp2b-700, pmNp2b-39, and phNp2b-1.5k were constructed by PCR amplification of rat, mouse, or human genomic DNA as a template using gene-specific primers (Supplementary Table S2). These PCR products were subcloned into a pGL-3 or pGL-4.19 vector (Promega, Madison, WI, USA). Reporter plasmid prNp2b-180 was cloned by the digestion of prNp2b-800 using *Xho*I restriction enzyme. Reporter plasmids phNp2b-1.1k, phNp2b-200, and phNp2b+17 were cloned by the digestion of phNp2b-1.5k using *Sac*I/*Hind*III, *Sac*I/*Apa*I, and *Sma*I restriction enzymes, respectively. Mutated reporter plasmids prNp2b-180-Mut-GC-box (rMut-G), prNp2b-180-Mut-C/EBP (rMut-C), prNp2b-180-Mut-E-box (rMut-E), pmNp2b-700-Mut-GC-box (mMut-G), and pmNp2b-700-Mut-C/EBP (mMut-C) were constructed with the QuikChange® site-directed

1 mutagenesis kit using the oligonucleotides shown in Supplementary Table S2. The β-

2 galactosidase expression vector pCMV-β (CLONTECH, Palo Alto, CA, USA) was used as an

3 internal control. Each plasmid was purified with PureYield™ Plasmid Midiprep System

4 (Promega).

5

6 **Transfection and luciferase assay**

7 NIH3T3 cells were obtained from Riken Cell Bank, Tokyo, Japan. NIH3T3 cells were cultured in

8 DMEM at 37°C in an atmosphere containing 5% CO_2. The growth medium was supplemented

9 with 10% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate.

10 Mouse RARα, RARβ, and RARγ expression vectors (pSG5-RARα, pSG5-RARβ, and pSG5-

11 RARγ), and mouse RXRα expression vector (pSG5-RXRα) were kindly provided by Dr. P.

12 Chambon. Mouse C/EBPα and C/EBPβ expression vectors (pcDNA3.1-C/EBPα and pcDNA3.1-

13 C/EBPβ) were constructed as previously described [36]. Transfection was performed as described

14 previously [4]. Cells were then treated with several concentrations of ATRA, TTNPB or ethanol

15 as vehicle control for an additional 16 hr. Luciferase assay was performed as described previously

16 [4] .

17

18 **Stealth RNAi**

1 For the application of RNA interference technology, double-strand Stealth RNAi oligos designed

2 using RNAi designer software (https://rnaidesigner.thermofisher.com/rnaiexpress/) were

3 synthesized by Thermo Fisher Scientific. The target sequence for C/EBPβ (NM_009883) is as

4 follows: 5'-AGACCCATGGAAGTGGCCAACTTCT-3'. For the control, Stealth RNAi Negative

5 Control Duplexes were used.

6

7 **Coupled transcription/translation assays**

8 Each C/EBPβ, RARβ, and RXRα proteins were synthesized with the T_NT® Quick Coupled

9 Transcription/Translation System at 30°C for 90 min in the presence of 20 μM methionine.

10 Generated proteins were used for EMSAs.

11

12 **EMSA**

13 EMSA was performed as described previously [3]. Double-stranded nucleotides for Npt2b-C/EBP

14 and Npt2b-C/EBP-Mutant (Mut) were synthesized (Supplementary Table S3). Purified DNA

15 fragments were radiolabeled with [γ-^{32}P] ATP (110 TBq/mmol) using T_4 polynucleotide kinase.

16 Nuclear extracts (RARα, pSG5-RXRα, and C/EBPβ) were prepared as described previously [4].

17 Briefly, the NIH-3T3 cells were cultured in 35-mm dishes to 90% confluence and transfected with

18 pSG5-RARα, pSG5-RXRα, and pcDNA3.1-C/EBPβ. Prepared nuclear extracts (15 μg) were

1 incubated with the radiolabeled probe in binding buffer [10 mM (Tris-HCl), pH7.5, 1 mM DTT,

2 1 mM EDTA, 10% Glycerol, 1 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, 2.5 μg/ml salmon

3 sperm DNA and 2 μg poly(dI-dC)] in a final volume of 20 μl for 30 min at room temperature.

4 Specificity of the binding reaction was determined with a 100-fold molar excess of the indicated

5 cold competitor oligonucleotide. The reaction mixture was then subjected to electrophoresis on a

6 5% polyacrylamide gel with 0.25 × TBE running buffer for two hr at 150 V. The gel was dried

7 and analyzed with a Fujix Bio-imaging analyzer (BAS-1500, Fuji-film, Tokyo, Japan).

8

9 **Statistical analysis**

10 Data were collected from more than 2 independent experiments and were reported as the means

11 ± S.E.M. Statistical analysis for 2-group comparison was performed using a 2-tailed Student's *t*–

12 test, or one-way ANOVA with a Student-Newman post-hoc test for multi-group comparison.

13 All data analysis was performed using GraphPad Prism 5 software (Graphpad

14 Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

15

Results

ATRA alleviates VAD-induced hyperphosphaturia through the upregulation of the *Npt2* gene expression in the rat kidney

Previously, we reported that VAD rats had decreased Pi uptake, however, the effect of ATRA on the intestinal Pi uptake remains unclear [4]. Furthermore, we did not examine the effects of ATRA treatment on phosphate homeostasis in VAD rats. We made VAD rats and these rats were treated with a total of 1 mg/kg body weight of ATRA. Because ATRA cannot be converted to retinol, plasma levels of retinol were undetectable in both VAD and VAD+ATRA rats, which was in line with our expectations (control: 511 ± 12.2 μg/dl). Although the plasma Pi and Ca levels were not changed among three groups of rats, ATRA treatment significantly reduced the urine Pi/Cr and Ca/Cr ratios, which had been increased by VAD (Table 1). Levels of plasma Pi-regulating hormones (1,25[OH]$_2$D$_3$, PTH, and FGF23) did not differ between VAD and control rats. In VAD rats, the plasma PTH level was not affected by ATRA, whereas the plasma 1,25(OH)$_2$D$_3$ and FGF23 levels were significantly reduced by ATRA (Table 1). The Na$^+$-dependent Pi uptake activity—but not the Na$^+$-independent Pi uptake activity—in renal BBMVs was markedly decreased in VAD rats, which was significantly increased by ATRA treatment (Figure 1A). As we previously reported, Western blotting revealed that the expression levels of renal Npt2a and Npt2c proteins in VAD rats were significantly decreased in comparison to controls. Furthermore, the

1 decreased expression of Npt2c protein—but not Npt2a—in the kidney of VAD mice was partially
2 restored by ATRA treatment (Figure 1B). Next, we performed real-time PCR to measure the renal
3 *Npt2a* and *Npt2c* mRNA expression. The decreased mRNA levels of renal *Npt2a* and *Npt2c* in
4 VAD rats were significantly increased by ATRA treatment (Figure 1C).
5
6 **ATRA reduces VAD-induced Pi absorption through the expression of the *Npt2b* gene in the**
7 **rat jejunum**
8 Because Pi homeostasis is strictly controlled by intestinal absorption and renal excretion, we next
9 investigated whether ATRA regulates the Pi absorption and Npt2b expression in the intestine. We
10 used the jejunum for this analysis because the levels of Npt2b protein and mRNA in the jejunum
11 are higher than those in the duodenum and ileum of rats [10]. As expected, the fecal Pi excretion
12 in VAD rats was significantly decreased in comparison to controls (Table 1). The Na^+-dependent
13 Pi uptake activity in intestinal BBMVs was markedly increased in VAD rats, and this was
14 significantly reduced by ATRA treatment (Figure 2A and Supplementary Figure S1). Western
15 blotting revealed that ATRA treatment blocked the VAD-induced Npt2b protein expression in the
16 jejunum (Figure 2B). As shown in Figure 2(C), real-time PCR demonstrated that the VAD-
17 induced jejunal *Npt2b* mRNA levels were significantly suppressed by ATRA. However, the
18 jejunal *PiT1* and *PiT2* mRNA expression levels were not changed among three groups (data not

shown).

Because the expression of the *Npt2b* gene has been detected in various tissues, including the small intestine, lung, kidney, and liver [18], we also investigated the effects of ATRA on the *Npt2b* mRNA expression in several extraintestinal organs by real-time PCR. Unlike the jejunum, the *Npt2b* mRNA expression in the duodenum and ileum was low, and was not changed among three groups of rats (Figure 2C). In the lung, the *Npt2b* mRNA expression was considerably high, but was not changed among three groups. The hepatic *Npt2b* mRNA expression was regulated by ATRA, similarly to the jejunum. ATRA did not affect the reduced expression of the renal *Npt2b* mRNA in VAD rats. The expression of *Npt2b* mRNA was almost undetectable in the spleen.

ATRA downregulates the transcriptional activity of the rat *Npt2b* gene promoter

We previously reported that the transcriptional activities of human *Npt2a* and *Npt2c* genes are controlled by ATRA and its receptors [4]. To investigate the molecular mechanisms underlying the regulation of the *Npt2b* gene expression by ATRA, we examined the responsiveness of rat *Npt2b* gene promoters to ATRA using a luciferase assay. Because we thought that the effects of ATRA on the *Npt2b* gene expression differ among tissues, we used NIH3T3 cells for a luciferase assay to eliminate tissue specific factor. prNp2b-1.8k, phNp2b-1.5k, and pmNp2b-1.7k reporter constructs, which respectively contained the promoter and exon 1 fragments of the

rat, human, and mouse *Npt2b* gene, were utilized for a luciferase assay in NIH3T3 cells. While ATRA had little impact on the transcriptional activity of prNp2b-1.8k without the overexpression of RAR/RXR in NIH3T3 cells, its activity was markedly inhibited by co-overexpressing RARs (RARα, RARβ, or RARγ)/RXR (Figurer 3A). Furthermore, ATRA additively reduced the promoter activity of rat *Npt2b* that was reduced by the overexpression of RARs/RXR in NIH3T3 cells (Figure 3A). Next, ATRA dose-dependently reduced the rat *Npt2b* gene promoter activity in NIH3T3 cells overexpressing RARα/RXR, as well as TTNPB, an RAR-specific agonist (Figure 3B). The phNp2b-1.5k and pmNp2b-1.7k reporter constructs exhibited similar responses to ATRA and TTNPB, as did prNp2b-1.8k (Supplementary Figure S2). A histone modification, a covalent post-translational modification (PTM), such as histone acetylation and methylation, is involved in regulating the transcription [37]. To test whether the inhibition of the *Npt2b* gene promoter activity by ATRA is associated with the effects of histone acetylation and methylation, we determine the luciferase activity of prNp2b-1.8k with Trichostatin A (histone deacetylase inhibitor, TSA) or 5-aza-2deoxycytidine (methylation inhibitor, 5-Aza-2dc). Neither TSA nor 5-Aza-2dc affected the suppression of the luciferase activity of prNp2b-1.8k by ATRA (Figure 3C).

The deletion analysis of the *Npt2b* gene promoter

In the search for conserved putative regulatory elements, the sequence of the rat *Npt2b* gene

1 promoter region (−207 to +33) relative to the transcriptional start site was compared to the
2 corresponding regions of the human (−146 to +83) and mouse (−172 to +49) sequences. As shown
3 in Figure 4(A), highly conserved nucleotide sequences were determined among human, rat, and
4 mouse *Npt2b* gene promoters with a minimal sequence similarity of 73%. Interestingly, a search
5 for transcription factor binding motifs within this region suggested some potential consensus
6 binding site such as GC-box, C/EBP, and E-box (Figure 4A). In order to understand the molecular
7 mechanisms underlying the responsiveness of these *Npt2b* genes to ATRA, several reporter
8 constructs lacking portions of the 5'-promoter region of the human, rat, and mouse *Npt2b* genes
9 were tested in NIH3T3 cells overexpressing RAR/RXR, with or without ATRA. These deletion
10 analyses suggest that the C/EBP binding site in the *Npt2b* gene promoter is involved in the
11 downregulation of the transcriptional activity of the *Npt2b* gene by ATRA and its receptors (Figure
12 4B).
13
14 **RAR/RXR blocks the binding of C/EBP to the *Npt2b* gene promoter**
15 To further test whether the C/EBP binding site is the first target of the downregulation of
16 the *Npt2b* gene promoter by ATRA and its receptors, we determined the luciferase activity of
17 prNp2b-Mut-GC-box (rMut-G), prNp2b-Mut-C/EBP (rMut-C), and prNp2b-Mut-E-box (rMut-
18 E), the sequences and constructs of which are shown in Figure 5(A). These mutation analyses

1 showed that the C/EBP binding site—but not GC-box or E-box—was essential for the repression
2 of the *Npt2b* gene promoter activity by ATRA (Figure 5B and Supplementary Figure S3C). Next,
3 we investigated how the transcriptional factor C/EBP actually contributes to the suppression of
4 the transcriptional activity of the *Npt2b* gene by ATRA and its receptors. The overexpression of
5 C/EBPα or C/EBPβ each increased the promoter activity of the rat *Npt2b* construct to more than
6 double the original level. However, ATRA additively diminished the transcriptional activity of
7 the *Npt2b* gene, which was reduced by the overexpression of RAR/RXR (Figure 5C). Next, we
8 analyzed levels of mRNA expression of C/EBPα and C/EBPβ in NIH3T3 cells using qPCR
9 analysis with the absolute standard curve method. Unlike C/EBPβ, C/EBPα gene was not
10 expressed at all in NIH3T3 cells. Therefore, we selected C/EBPβ for gene knockdown
11 experiments, but not C/EBPα. We generated NIH3T3 cells with the knockdown of *C/EBPβ*,
12 endogenous *C/EBPβ* mRNA levels of which were reduced by more than 50%, using *C/EBPβ*-
13 specific siRNA (data not shown). As shown in Figure 5(D), the luciferase activity of prNp2b-180
14 was reduced by the siRNA-mediated knockdown of *C/EBPβ* in NIH3T3 cells. Furthermore,
15 *C/EBPβ* siRNA significantly ameliorated the reduction of the *Npt2b* gene promoter activity by
16 ATRA (Figure 5E). Next, to elucidate how ATRA and its receptors downregulate the
17 transcriptional activity of the *Npt2b* gene through the action of C/EBP, we examined whether
18 RAR/RXR affects the binding of C/EBP to the *Npt2b* gene promoter by an EMSA analysis.

1 As shown in Figure 5(F), a radiolabeled oligonucleotide containing an Npt2b-C/EBP probe
2 detected a band in nuclear extracts prepared from NIH3T3 cells overexpressing C/EBPβ, but not
3 RAR/RXR. Although these complexes are susceptible to competition with unlabeled Npt2b-
4 C/EBP, consensus C/EBP, and an antibody against C/EBPβ, unlabeled mutated oligonucleotide
5 (Mut: mutated Npt2b-C/EBP) did not compete with these complexes. The formation of these
6 complexes with C/EBPβ was inhibited in the presence of nuclear extracts prepared from NIH3T3
7 cells overexpressing RAR/RXR (Figure 5F). Likewise, although *in vitro* synthesized
8 C/EBPβ recombinant protein bound to this probe, this DNA-protein complex was inhibited by
9 RARβ/RXRα recombinant protein and an antibody against C/EBPβ (Supplementary Figure S4).

Discussion

In the present study, we have determined that the reduction of the intestinal Pi uptake activity and the Npt2b expression in VAD rats were ameliorated by ATRA treatment. Furthermore, we revealed that ATRA reduced the transcriptional activity of the *Npt2b* gene by inhibiting the binding of C/EBP to the *Npt2b* gene promoter. Previously, we reported that VAD induced hyperphosphaturia through the downregulation of the *Npt2a* and *Npt2c* gene expression in the kidney, without changing the plasma Pi levels [4]. From these inconsistent results, we hypothesized that ATRA might affect not only renal Pi reabsorption but also intestinal Pi absorption. In this study, we found that ATRA increased the renal Pi uptake through the induction of the expression of the *Npt2a* and *Npt2c* genes, the levels of which are reduced by VAD, whereas the VAD-induced uptake of Pi via the *Npt2b* gene expression in the jejunum of VAD rats was reduced by the administration of ATRA. Together, these results suggested that ATRA did not change the plasma Pi levels because of the opposite effects of ATRA on the intestinal Pi uptake and the renal Pi uptake. This is the first report to demonstrate the presence of a Pi regulating factor that has opposite effects on the expression patterns of Npt2a and Npt2c in the kidney and Npt2b in the intestine. However, intestinal Pi absorption is mediated by two pathways: sodium-dependent Pi transport by Npt2b and PiT1/2 via a transcellular pathway; and sodium-independent Pi transport via a paracellular pathway [16]. It was recently reported

1 that tenapanor, an inhibitor of the sodium/hydrogen exchanger (NHE3), significantly reduced
2 intestinal Pi absorption in healthy volunteers and improved hyperphosphatemia in both
3 rodents and humans with CKD [16]. The conformational change of the tight junctions in the
4 enterocytes by NHE3 inhibition leads to increased transepithelial electrical resistance, which
5 contributes to the reduction in permeability to phosphate. Interestingly, it has been reported
6 that retinoic acid can enhance the intestinal epithelial barrier by increasing tight junction
7 protein levels [38, 39]. It was suggested that ATRA reduces intestinal Pi absorption through
8 not only a reduction of the Npt2b expression but also the enhancement of the tight junction
9 function in intestinal enterocytes. These data and reports suggest that ATRA regulates Pi
10 homeostasis in the body through the positive and negative regulation of Pi (re)absorption in the
11 kidney and jejunum. Interestingly, it has been suggested that a gut-derived factor called intestinal
12 phosphatonin is released in response to ingestion of dietary Pi and rapidly modulates renal Pi
13 reabsorption to prevent large post-prandial fluctuations in serum phosphate levels [16]. Although
14 a large part of intestinal phosphatonin remains unclear, ATRA may affect this unknown factor.
15 Intestinal Pi absorption is modulated by $1,25(OH)_2D_3$ as a positive regulator and by FGF-23
16 as a negative regulator [9, 11]. In the present study, the levels of plasma $1,25(OH)_2D_3$ and FGF-
17 23 did not differ between VAD and control rats, as previously reported [4], whereas the treatment
18 of VAD rats with ATRA reduced the plasma $1,25(OH)_2D_3$ and FGF-23 levels. We considered that

1 ATRA might reduce the plasma FGF23 levels in VAD rats through the reduction of the plasma

2 $1,25(OH)_2D_3$ levels, because $1,25(OH)_2D_3$ is positive regulator of the FGF23 gene expression

3 [40]. However, it also remains unclear why ATRA reduced the plasma $1,25(OH)_2D_3$ levels in VAD

4 rats. Interestingly, an experiment to investigate the effects of retinol and retinoic acid on

5 metabolism of 25(OH)D (which is converted to $1,25[OH]_2D_3$ by CYP27B1 and which is

6 converted to $24,25(OH)_2D$ by CYP24A1) in kidney cell culture may answer to this question.

7 Treatment with retinoic acid—but not retinol—for 6 h reduced the production of $1,25(OH)_2D_3$

8 and increased the production of $24,25(OH)_2D$ in kidney cells [41]. These findings suggest that the

9 administration of ATRA to VAD rats might reduce plasma $1,25(OH)_2D_3$ levels through the

10 reduction of the conversion of 25(OH)D to $1,25(OH)_2D_3$. Furthermore, these observations suggest

11 that the VAD-mediated upregulation of the *Npt2b* gene expression was independent of the plasma

12 $1,25(OH)_2D_3$ and FGF-23 levels, whereas the ATRA-mediated downregulation of the *Npt2b* gene

13 expression in VAD rats could be partially induced by decreased plasma $1,25(OH)_2D_3$. Although

14 it has been reported that $1,25(OH)_2D_3$ induces *Npt2b* gene promoter activity, a classical vitamin

15 D responsive element (VDRE) in the *Npt2b* gene promoter has not been identified [42].

16 We reported that the transcriptional activity of human *Npt2a* and *Npt2c* genes was upregulated

17 by ATRA and its receptors [4]. Thus, we hypothesized that ATRA might control the expression of

18 the *Npt2b* gene through the regulation of transcription. While ATRA had little impact on the

1 transcriptional activity of prNp2b-1.8k without the overexpression of RAR/RXR in NIH3T3 cells,

2 its activity was markedly inhibited by co-overexpressing RARs/RXR without the addition of

3 ATRA. Furthermore, ATRA additively reduced the promoter activity of rat *Npt2b* that was

4 reduced by the overexpression of RARs/RXR in NIH3T3 cells. These results may suggest that

5 RAR/RXR is more important for the downregulation of the transcriptional activity of rat

6 *Npt2b* gene than ATRA. On the other hand, the promoter activity of the *Npt2b* gene was increased

7 by either C/EBPα or C/EBPβ. Xu et al. reported that regulation of mouse *Npt2b* gene transcription

8 was activated by C/EBPα in a lung epithelial cell line using transient transfection promoter assays

9 [30]. Schwarz et al. reported that liganded RAR inhibits adipogenesis by blocking the C/EBPβ-

10 mediated induction of downstream genes [43]. Although we have investigated whether ATRA

11 regulates the C/EBP expression in the small intestine of rats, the jejunal *C/EBPα* and *C/EBPβ*

12 mRNA expression levels remained unchanged in VAD rats and VAD rats treated with ATRA

13 (data not shown). Furthermore, ATRA treatment did not affect the *C/EBPβ* mRNA expression

14 in NIH3T3 cells (data not shown). Next, although we have investigated whether RAR could

15 displace C/EBP from binding to a canonical C/EBP binding site, an EMSA analysis showed

16 that RAR/RXR could not bind to oligonucleotide containing an Npt2b-C/EBP probe.

17 Surprisingly, it has been reported that C/EBPβ could bind to the glucocorticoid receptor and

18 RAR [44, 45]. We also demonstrated that RAR/RXR inhibited the binding of C/EBP to the

1 promoter of the *Npt2b* gene using an EMSA analysis. These data suggest that liganded
2 RAR/RXR inhibits the binding C/EBPα or C/EBPβ to the C/EBP binding site in the *Npt2b*
3 gene promoter, resulting in the suppression of the transcription activity of the *Npt2b* gene.
4 Interestingly, we found that ATRA has a distinct effect on the expression patterns of *Npt2b*
5 mRNA in different organs. C/EBPs (C/EBPα, β, δ, ε, γ, and ζ), which are expressed at
6 different levels in each tissue, respectively regulate the diversity and specificity of the
7 complex patterns of gene regulation [22, 23]. That is to say, the difference in the C/EBPs gene
8 expression levels in each tissue may contribute to a distinct effect on the *Npt2b* mRNA
9 expression patterns by VAD and ATRA treatment in these tissues. We also reported that a
10 complex of C/EBPβ and activating transcription factor-4 (ATF4) activates the expression of type
11 III sodium-dependent Pi co-transporters (PiT1 and PiT2) through transcriptional regulation [27].
12 Because C/EBP might be involved in the regulation of Pi homeostasis in various ways, further
13 studies are necessary to fully elucidate the underlying mechanisms.

14 Hyperphosphatemia, which is associated with the pathophysiology of CKD, can lead to
15 vascular calcification, which has been linked to increased cardiovascular morbidity and mortality
16 [15]. From studies on the regulation of intestinal Pi transport and metabolism, researchers have
17 targeted Npt2b for alleviating the hyperphosphatemia of CKD [17]. Niacin and its derivative
18 nicotinamide have been reported to reduce the Npt2b expression and lower the plasma Pi levels

1 in CKD animals [46, 47]. However, the exact mechanism through which these compounds reduce
2 intestinal Pi absorption has not been completely elucidated. Interestingly, Fujimori et al. showed
3 that niacin reduced the expression of *C/EBPβ* mRNA in the early phase of adipogenesis [48]. In
4 other words, the molecular mechanism through which niacin diminishes the expression of Npt2b
5 may be associated with the reduction of the C/EBPβ expression by niacin. In the present study,
6 we indicated that ATRA reduces the transcriptional activity of the *Npt2b* gene by inhibiting the
7 binding of C/EBP to the *Npt2b* gene promoter. That is to say, ATRA and C/EBP may become
8 therapeutic targets for the prevention of hyperphosphatemia in CKD. Interestingly, elevated
9 plasma retinol, ATRA, and retinol binding protein 4 (RBP4) levels have been observed in CKD
10 patients and animal models [49, 50]. However, the effects of ATRA on intestinal Pi absorption
11 focusing on the expression of Npt2b through the action of C/EBP in CKD are not understood. The
12 clarification of these effects would be valuable for advancing the treatment of CKD.
13 In conclusion, our findings reveal that the downregulation of the intestinal *Npt2b* gene and
14 the upregulation of the renal *Npt2a* and *Npt2c* gene expression by ATRA contributes to Pi
15 homeostasis in rats. Furthermore, it is clear that ATRA and its receptors can suppress the
16 transcriptional activity of *Npt2b* gene promoters by blocking the C/EBP-mediated induction of its
17 gene promoter activity.
18

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Author Contribution

M. Masuda and H. Yamamoto conceptualized the study. M. Masuda, Y. Takei, O. Nakahashi, Y. Adachi, K. Ohnishi, and H. Ohminami analyzed the data. M. Masuda, H. Yamamoto, H.

1 Yamanaka-Okumura, H. Sakaue, E. Takeda, and Y. Taketani discussed and interpreted the results

2 from the study. M. Masuda, M. Miyazaki, and Y. Taketani wrote the original draft. M. Masuda,

3 H. Yamamoto, and Y. Taketani approved final version of manuscript.

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Figure Legends

Figure 1. Effects of ATRA treatment on the expression of renal Npt2a and Npt2c in VAD rats. Seven-week-old male rats with VAD induced by a VAD diet were randomly divided into two groups and treated with DMSO (VAD) or ATRA (VAD + ATRA). (**A**) The Na^+-dependent and Na^+-independent Pi transport activity were assessed by measuring the uptake of Pi in renal BBMVs. (**B**) Western blotting of Npt2a and Npt2c in renal BBMVs. Each lane was loaded with 25 μg of BBMVs. β-actin was used as an internal control. (**C**) The *Npt2a* and *Npt2c* mRNA levels in renal BBMVs were analyzed by quantitative PCR. β-actin was used as an internal control. Values are the mean ± S.E.M. ($n = 3–4$). *$P < 0.05$, **$P < 0.01$ (one-way ANOVA with a Student-Newman post-hoc test).

Figure 2. Effects of vitamin A on the expression of intestinal and extraintestinal Npt2b in rats. (**A**) Na^+-dependent and Na^+-independent Pi transport activity was assessed by measuring the uptake of Pi in intestinal BBMVs. (**B**) Western blotting of Npt2b in BBMVs. Each lane was loaded with 25 μg of BBMVs. β-actin was used as an internal control. (**C**) The mRNA expression levels of *Npt2b* in various tissues (e.g., duodenum, jejunum, ileum, lung, liver, kidney, and spleen) of rats were determined by RT-PCR (lower) and quantitative PCR (upper). β-actin was used as an internal control. Values are the mean ± S.E.M. ($n = 3–4$). *$P < 0.05$, **$P < 0.01$ (one-way ANOVA with a Student-Newman post-hoc test).

Figure 3. Suppression of rat *Npt2b* gene promoter by ATRA and its receptors in NIH3T3 cells. (A) A Schematic illustration of the rat *Npt2b* gene promoter in the upper panels. prNp2b-1.8k and pCMV-β were transfected with pSG5-RAR (α, β, γ) and pSG5-RXRα, or empty vector and incubated in the presence of 100 nM ATRA or ethanol as a vehicle control for 24 h in NIH3T3 cells. *$P < 0.05$ vs. empty vector. $^{\#}P < 0.05$ vs. vehicle (one-way ANOVA with a Student-Newman post-hoc test). (**B**) NIH3T3 cells were transfected with prNp2b-1.8k, pSG5-RARα, pSG5-RXRα, and pCMV-β and treated with the indicated concentrations of ATRA (white circles) or TTNPB (black circles) for 24 h. (**C**) NIH3T3 cells were transfected with prNp2b-1.8k, pSG5-RARα, pSG5-RXRα, and pCMV-β and incubated in the presence of 100 nM ATRA with 100 nM TSA or 5-Aza-2dc (5-Aza). Each point represents the average of quadruplicate analyses ± S.E.M. normalized for β-gal activity. Similar results were obtained from three independent experiments. *$P < 0.05$, N.S. = not significant (one-way ANOVA with a Student-Newman post-hoc test).

Figure 4. Nucleotide sequence of 5'-flanking region in the *Npt2b* gene and the deletion analysis. (**A**) The sequences of the human, rat, and mouse *Npt2b* gene promoter regions (human,

1 −146 to +83 bp; rat, −207 to +33 bp; mouse: −172 to +49 bp). The boxes indicate the putative
2 binding sites for various transcription factors (STAT, GC-box, C/EBP, TATA-box, E-box).
3 Asterisk indicates homology of sequences in the *Npt2b* gene promoter among these mammalians
4 species. (**B**) Transcriptional activity of deletion constructs of human, rat, and mouse *Npt2b* gene
5 promoters (phNp2b-1.5k, prNp2b-1.8k, and pmNp2b-1.7k). Deletion constructs are illustrated in
6 the panels on the left. NIH3T3 cells were transfected with the indicated human, rat, or mouse
7 *Npt2b* gene reporter constructs and pSG5-RARα, pSG5-RXRα, and pCMV-β and treated with
8 100 nM ATRA or ethanol (NT) for 24 h. Each point represents the average of quadruplicate
9 analyses ± S.E.M. normalized for β-gal activity. Similar results were obtained from three
10 independent experiments. **P* < 0.001 vs. NT (two-tailed unpaired Student's *t*-test).
11
12 **Figure 5. C/EBP binds to the C/EBP binding site in the *Npt2b* gene promoter and the**
13 **mutation analysis of the *Npt2b* gene.** (**A**) Transcription factor binding sites mutated in the rat
14 *Npt2b* gene promoter region are underlined. rMut-G, rMut-C and rMut-E targeted the binding
15 sites for transcription factors GC-box, C/EBP and E-box, respectively. A schematic diagram
16 showing the wild-type (rWT) rat *Npt2b* reporter plasmid as well as reporter plasmid with
17 mutations (rMut-G, rMut-C, and rMut-E) in transcription factors sequences, shown with an X
18 through the mutant sequences. (**B**) Each rat *Npt2b* reporter plasmid (rWT, rMut-G, rMut-C, and
19 rMut-E) was transfected with pSG5-RARα, pSG5-RXRα, and pCMV-β into NIH3T3 cells. Cells
20 were treated with vehicle (NT: ethanol) or 100 nM ATRA and cell lysates were assessed for β-gal
21 and luciferase activities 24 h later. **P* < 0.01 vs. NT (two-tailed unpaired Student's *t*-test). (**C**)
22 NIH3T3 cells were transfected with prNp2b-180, pCMV-β, pSG5-RARα, pSG5-RXRα,
23 pcDNA3.1-C/EBP (α or β), or empty vector and incubated in the presence of 100 nM ATRA or
24 ethanol and cell lysates were assessed for β-gal and luciferase activity 24 h later. **P* < 0.01 (one-
25 way ANOVA with a Student-Newman post-hoc test). (**D**) NIH3T3 cells were transfected with
26 prNp2b-180 and pCMV-β 48 h after transfection with C/EBPβ siRNA (10 or 100 pmol) or
27 control (Cont.) and cell lysates were assessed for β-gal and luciferase activity 24 h later. **P* <
28 0.01 vs. Cont. (two-tailed unpaired Student's *t*-test). (**E**) NIH3T3 cells were transfected with
29 prNp2b-180, pCMV-β, pSG5-RARα, pSG5-RXRα, or empty vector 48 h after transfection with
30 C/EBPβ siRNA (100 pmol) or control and incubated in the presence of 100 nM ATRA or ethanol
31 and cell lysates were assessed for β-gal and luciferase activity 24 h later. **P* < 0.01 (one-way
32 ANOVA with a Student-Newman post-hoc test). (**F**) EMSAs using ^{32}P-labelled Npt2b-C/EBP as
33 probes. EMSAs were performed with nuclear extracts (N.E.) from NIH3T3 cells overexpressing
34 C/EBPβ, RARβ, and RXRα, with the addition of unlabeled competitor oligonucleotides as
35 indicated. A 100-fold molar excess of each competitor was used. The location of the DNA-protein
36 complex band is indicated by an arrowhead. cebp, C/EBP-binding sequence; Mut, mutated Npt2b-

1 C/EBP; αC/EBP, C/EBPβ-specific antibody. Each point represents the average of quadruplicate
2 analyses ± S.E.M. normalized for β-gal activity. Similar results were obtained from three
3 independent experiments.
4

1 **Supplementary Figure Legends**
2 **Figure S1. Effects of vitamin A on intestinal Na^+-dependent Pi transport activity in rats.** Na^+-
3 dependent Pi transport activity was assessed by measuring the uptake of Pi in intestinal BBMVs
4 at 30 s and 60 s. Values are the mean ± S.E.M. (n = 3–4). *$P < 0.01$ vs. Control. $^{\#}P < 0.05$ vs.
5 VAD (one-way ANOVA with a Student-Newman post-hoc test).
6
7 **Figure S2. Suppression of human and mouse *Npt2b* gene promoter by ATRA and its**
8 **receptors in NIH3T3 cells.** (A and C) A schematic illustration of the human and mouse *Npt2b*
9 gene promoter in the upper panels. Each human or mouse *Npt2b* reporter plasmid (phNp2b-1.5k
10 or pmNp2b-1.7k) was transfected with pSG5-RAR (α, β, or γ), pSG5-RXRα, and pCMV-β into
11 NIH3T3 cells. Cells were treated with vehicle (NT: ethanol) or 100 nM ATRA and cell lysates
12 were assessed for β-gal and luciferase activity 24 h later. (**B** and **D**) Each human or mouse *Npt2b*
13 reporter plasmid (phNp2b-1.5k or pmNp2b-1.7k) was transfected with pSG5-RARα, pSG5-
14 RXRα, and pCMV-β into NIH3T3 cells and treated with the indicated concentrations of ATRA
15 (white circles) or TTNPB (black circles) for 24 h. Each point represents the average of
16 quadruplicate analyses ± S.E.M. normalized for β-gal activity. Similar results were obtained from
17 three independent experiments. *$P < 0.01$ and **$P < 0.001$. vs. NT (two-tailed unpaired Student's
18 *t*-test).
19
20 **Figure S3. The mutation analysis of the C/EBP binding site in the mouse *Npt2b* gene**
21 **promoter.** (A) mMut-G and mMut-C targeted the binding sites for transcription factors GC-box
22 and C/EBP, respectively. (**B**) A schematic diagram showing the wild-type (mWT) mouse *Npt2b*
23 reporter plasmid as well as reporter plasmid with mutation (mMut-G and mMut-C) in
24 transcription factor sequences. Mutant sequences are indicated with an X. (**C**) Each mouse *Npt2b*
25 reporter plasmid (mWT, mMut-G, and mMut-C) was transfected with pSG5-RARα, pSG5-
26 RXRα, and pCMV-β into NIH3T3 cells. Cells were treated with vehicle (NT: ethanol) or 100 nM
27 ATRA and cell lysates were assessed for β-gal and luciferase activities 24 h later. Each point
28 represents the average of quadruplicate analysis ± S.E.M. normalized for β-gal activity. Similar results
29 were obtained for three independent experiments. *$P < 0.01$ vs. NT (two-tailed unpaired Student's
30 *t*-test).
31
32 **Figure S4. RAR/RXR inhibits the interaction between C/EBP and the C/EBP binding site in**
33 **the *Npt2b* gene promoter.** EMSAs using ^{32}P-labelled Npt2b-C/EBP as probes. EMSAs were
34 performed with in vitro synthesized C/EBPβ, RARβ, and RXRα, with the addition of unlabeled
35 competitor oligonucleotides as indicated. A 100-fold molar excess of each competitor was used.
36 The location of the DNA-protein complex band is indicated by an arrowhead. cebp, C/EBP-

1 binding sequence; Mut, mutated Npt2b-C/EBP; αC/EBP, C/EBPβ-specific antibody.

Table 1. Effects of vitamin A on plasma, urine Pi levels, and fecal Pi excretion.

	Control	VAD	VAD+ATRA
Plasma			
retinol (μg/dl)	511 ± 12.2	U.D.	U.D.
Pi (mg/dl)	5.58 ± 0.21	5.84 ± 0.37	5.24 ± 0.17
Ca (mg/dl)	11.1 ± 0.13	10.9 ± 0.46	10.7 ± 0.56
PTH (pg/ml)	124 ± 9.73	128 ± 10.7	135 ± 6.74
FGF23 (pg/ml)	296 ± 17.1	258 ± 20.6	164 ± 24.8#
$1,25(OH)_2D$ (pg/ml)	544 ± 19.4	575 ± 48.6	423 ± 5.44#
Urine			
Pi/Cr	1.34 ± 0.30	4.71 ± 0.60*	2.82 ± 0.23#
Ca/Cr	0.26 ± 0.10	0.87 ± 0.03*	0.47 ± 0.04#
FEI Pi	0.25 ± 0.06	0.78 ± 0.10*	0.51 ± 0.05
FEI Ca	0.02 ± 0.01	0.08 ± 0.01*	0.04 ± 0.01#
Fecal			
Pi (mg/day)	46.4 ± 2.09	35.3 ± 2.07*	N.D.
Ca (mg/day)	78.7 ± 6.71	62.3 ± 9.45*	N.D.

Values are means ± S.E.M. (*n* = 3–4). *$p < 0.05$ vs. control; #$p < 0.05$ vs. VAD. (one-way ANOVA with a Student-Newman post-hoc test). U.D.: undetectable. N.D.: not determined.

Figure. 1 Masuda M. et al

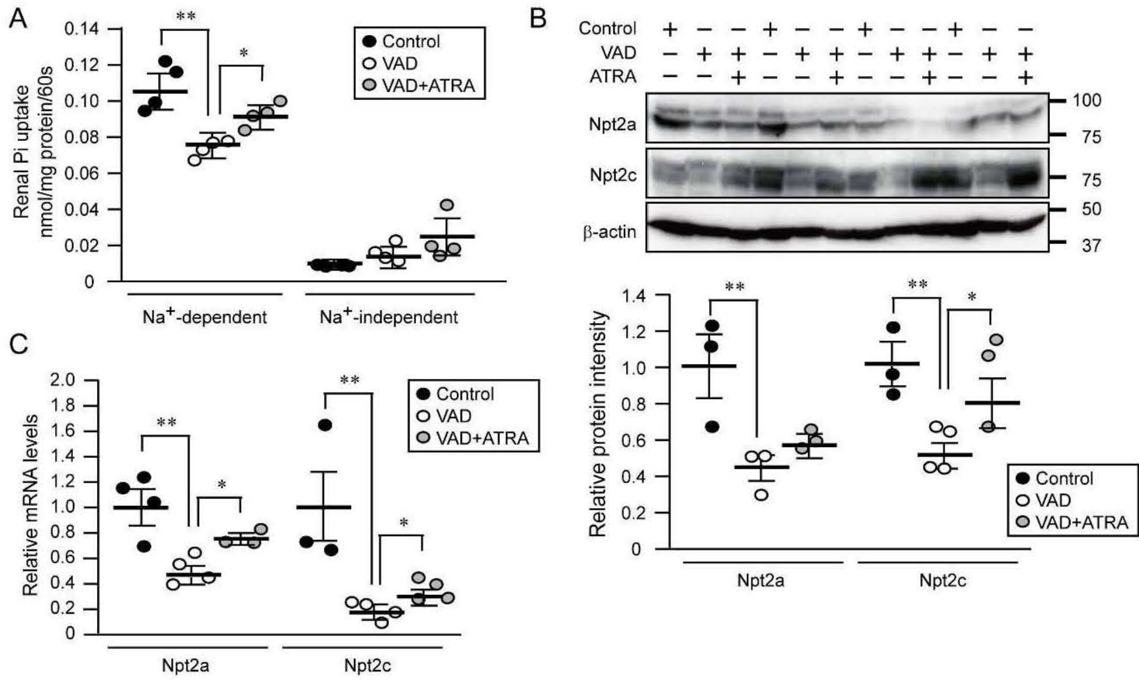


Figure 2. Masuda M. et al

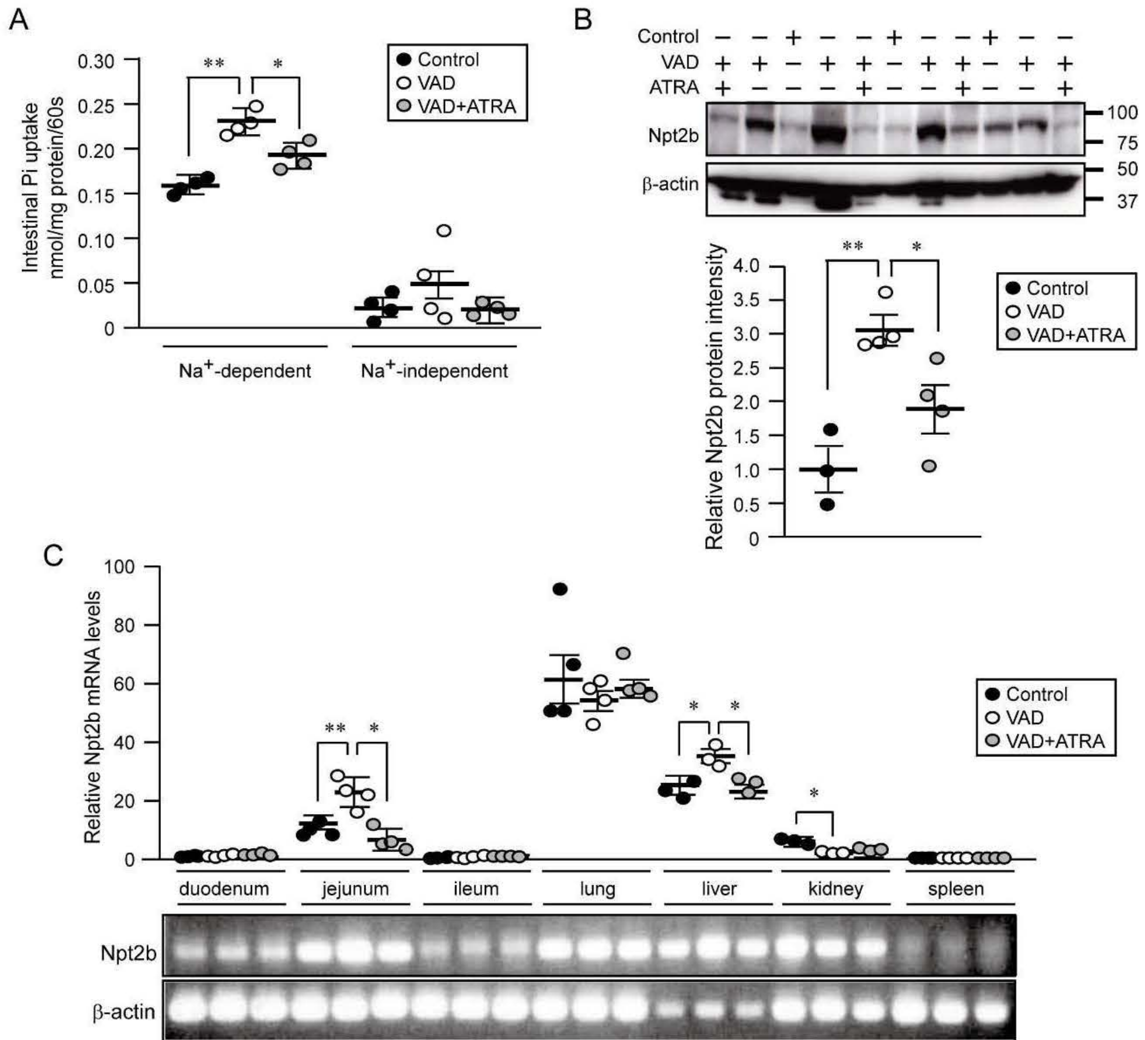


Figure 3. Masuda M. et al

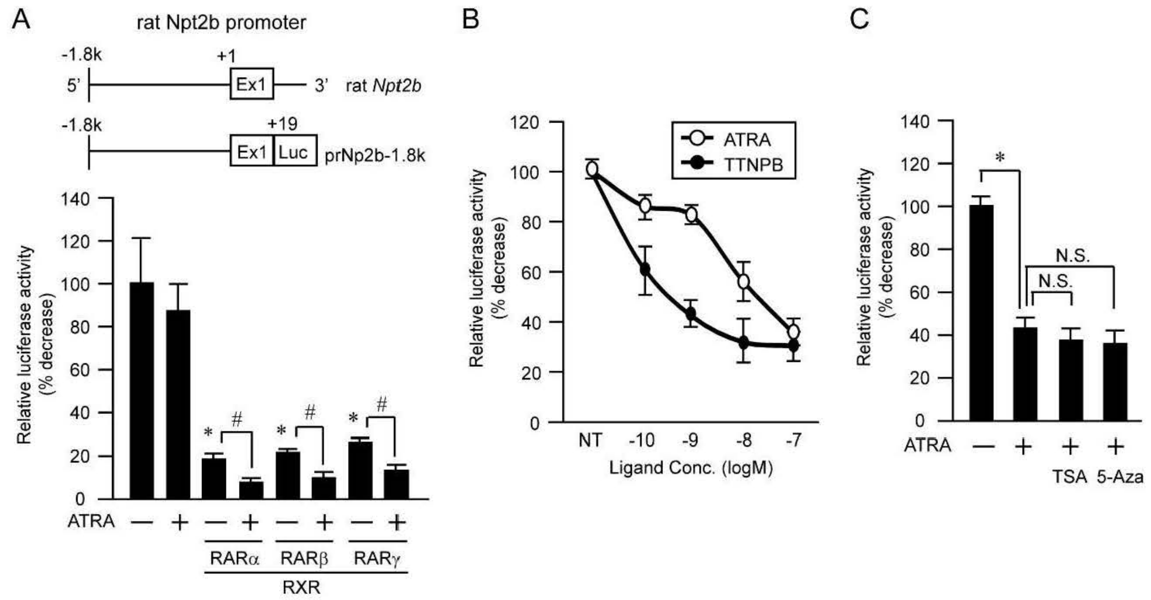
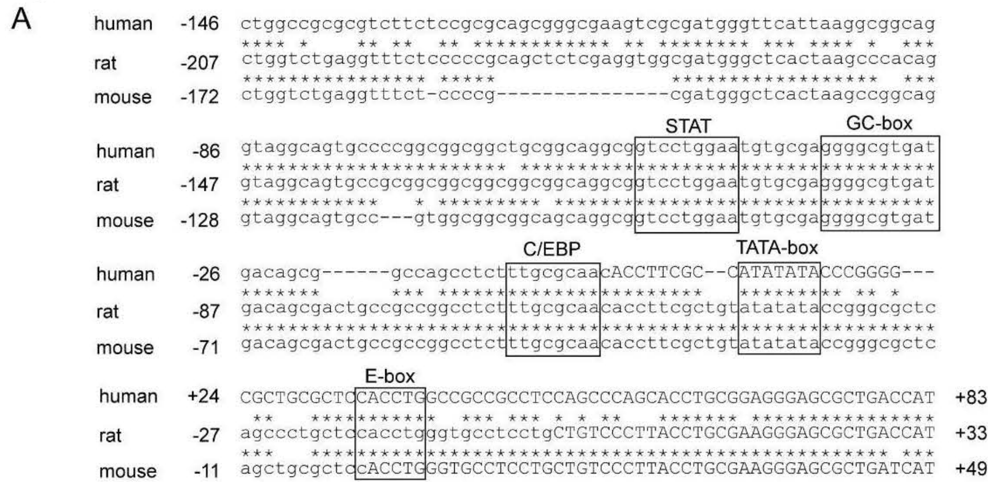


Figure 4. Masuda M. et al

A



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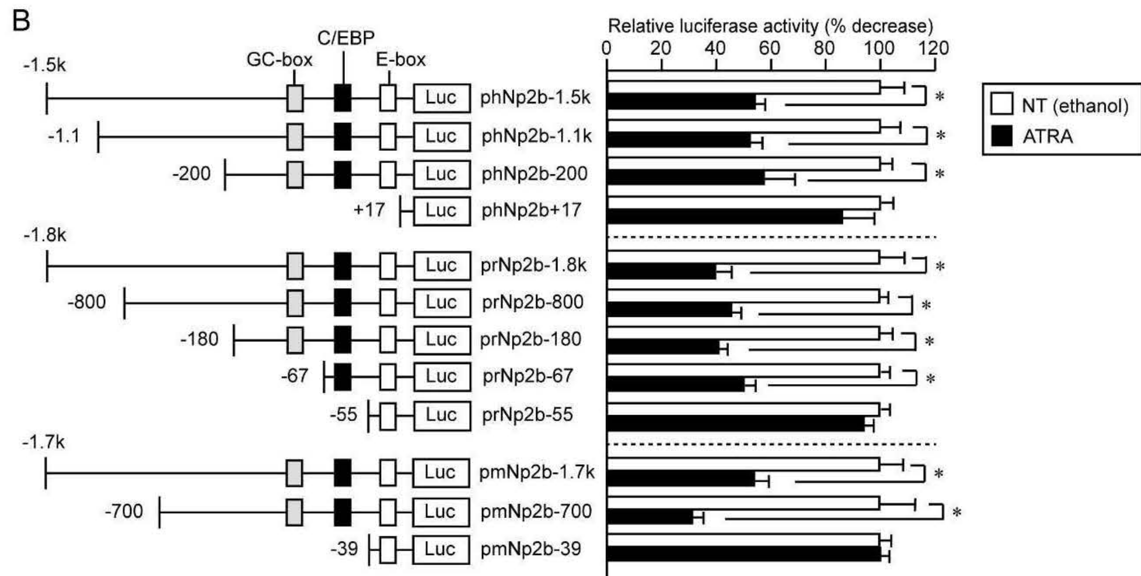

Figure 5. Masuda M. et al