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RESEARCH ARTICLE

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Rate of hydrolysis of the phosphate esters of B vitamins is reduced by zinc deficiency: In vitro and in vivo

Hana Yuasa[1] | **Kiyoshi Miyazaki**[1] | **Takamasa Kido**[2] | **Katsutoshi Nishino**[1] | **Miku Shiotsu**[1] | **Tsutomu Fukuwatari**[3] | **Machi Suka**[2] | **Masaya Nagao**[1] | **Taiho Kambe**[1]

[1]Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, Japan

[2]Department of Public Health and Environmental Medicine, The Jikei University School of Medicine, Tokyo, Japan

[3]Department of Nutrition, School of Human Cultures, The University of Shiga Prefecture, Hikone, Japan

Correspondence
Taiho Kambe, Division of Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan.
Email: kambe.taiho.7z@kyoto-u.ac.jp

Present address
Katsutoshi Nishino, School of Bioscience and Biotechnology, Tokyo University of Technology, Tokyo, Japan

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Abstract

Extracellular hydrolysis of the phosphate esters of B vitamins (B1, B2, and B6) is crucial for their cellular uptake and metabolism. Although a few zinc-dependent enzymes have been implicated in these processes, their exact mechanisms of action remain largely unknown. This study investigated the potential involvement of phosphate group hydrolyzing enzymes in the hydrolysis of B vitamin phosphate esters. We evaluated enzyme activity in membrane lysates prepared from cells transiently transfected with these enzymes or those endogenously expressing them. Specifically, we investigated how zinc deficiency affects the rate of hydrolysis of B vitamin phosphate esters in cellular lysates. Assessment of the activities of zinc-dependent ectoenzymes in the lysates prepared from cells cultured in zinc-deficient conditions and in the serum of rats fed zinc-deficient diets revealed that zinc deficiency reduced the extracellular hydrolysis activity of B vitamin phosphate esters. Furthermore, our findings explain the similarities between several symptoms of B vitamin and zinc deficiencies. Collectively, this study provides novel insights into the diverse symptoms of zinc deficiency and could guide the development of appropriate clinical strategies.

KEYWORDS

B vitamins (vitamins B1, B2, and B6), ectoenzyme, hydrolysis, phosphate ester, zinc deficiency

Abbreviations: ALP, alkaline phosphatase; ENPP, ectonucleotide pyrophosphatase/phosphodiesterase; FAD, flavin-adenine dinucleotide; FCS, fetal calf serum; FMN, flavine-mononucleotide; HPLC, high-performance liquid chromatography; IAP, intestinal alkaline phosphatase; IBD, inflammatory bowel disease; NT5E, ecto-5′-nucleotidase; PBS, phosphate-buffered saline; PL, pyridoxal; PLAP, placental alkaline phosphatase; PLP, pyridoxal 5-phosphate; RF, riboflavin; SMPDL, sphingomyelin phosphodiesterase acid-like; TDP, thiamine pyrophosphate; TMP, thiamine monophosphate; TNAP, tissue nonspecific alkaline phosphatase; VB1, Vitamin B1; VB2, vitamin B2; VB6, vitamin B6.

1 | INTRODUCTION

Vitamins B1 (VB1), B2 (VB2), and B6 (VB6) (B vitamins hereafter) are water-soluble coenzymes that are essential for normal physiological function and various biological activities.[1] VB1 plays a crucial role in carbohydrate metabolism along with other enzymes such as pyruvate and α-ketoglutarate dehydrogenases.[2] VB2 acts as a cofactor for several redox enzymes involved in the tricarboxylic acid cycle, fatty acid oxidation, and homocysteine metabolism,[3] and also affects the metabolism of other nutrients including VB6, VB12, and iron.[4] VB6 is vital for amino acid, lipid, and carbohydrate metabolism, and also for neurotransmitter synthesis and the one-carbon metabolic pathway.[5] Deficiencies (or suboptimal levels) in these B vitamins negatively affect human health, whereas optimal levels promote body function.[6,7] Like other vitamins, humans cannot synthesize B vitamins and must obtain them from dietary sources. VB1, VB2, and VB6 can exist in both their active phosphate esters or nucleotide forms, such as thiamine pyrophosphate (TDP), thiamine monophosphate (TMP), riboflavin-5′-adenosine diphosphate (FAD), riboflavin-5′-phosphate (FMN), and pyridoxal 5′-phosphate (PLP), and dephosphorylated forms, including thiamine, riboflavin (RF), pyridoxal (PL), and pyridoxamine. In their intracellular active state, these B vitamins are predominantly present in phosphate ester forms.[1,7,8]

Transporters play pivotal roles in the cellular uptake of B vitamins. For instance, thiamine transporters THTR1 and THTR2 mediate the cell-surface transport of VB1[9,10] and also facilitate the uptake of pyridoxine, an analog of PL.[11] Additionally, some organic cation transporters can transport thiamine.[12] For VB2, the plasma membrane RF transporters RFVT1, RFVT2, and RFVT3 have been identified and characterized.[13] These transporters specifically transport the dephosphorylated forms of vitamins. Therefore, the extracellular hydrolysis of the phosphate esters of B vitamins before their cellular uptake is crucial for their systemic and cellular metabolism.[5,14,15] However, some cells are equipped with transporters that can directly transport their phosphate ester forms. For example, the choline transporter SLC44A4, a thiamine pyrophosphate transporter, can absorb TDP into colon cells through the apical side.[16]

The GPI-anchored enzymes tissue-nonspecific alkaline phosphatase (TNAP) and CD73 (or ecto-5′-nucleotidase) can hydrolyze the phosphate esters of B vitamins.[15,17] TNAP, a broad-spectrum enzyme hydrolyzes phosphate compounds, such as inorganic pyrophosphate (PPi), glucose-6-phosphate,[18] ATP, ADP, and AMP.[19] Specifically, TNAP hydrolyzes TDP to thiamine via TMP,[20] FMN to RF,[15] and PLP to PL.[21] CD73, the only functional 5′-nucleotidase responsible for hydrolyzing extracellular AMP to adenosine, also hydrolyzes FAD to FMN.[15] Additionally, other pyrophosphatases and phosphatases in the extracellular space may contribute to the hydrolysis of the phosphate esters of B vitamins, given their widespread presence and activity.[22,23]

The ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) and sphingomyelin phosphodiesterase acid-like (SMPDL) families include several candidate ectoenzymes.[24–27] ENPP1 and ENPP3 hydrolyze ATP to AMP and PPi, respectively.[24,28,29] ENPP2, also known as autotaxin, is an extracellular lysophospholipase D.[30,31] ENPP4 hydrolyzes diadenosine triphosphate into AMP and ADP and diadenosine tetraphosphate into AMP and ATP.[32,33] ENPP5 cleaves nicotinamide adenine dinucleotide[34]; however, little is known about this process. ENPP6 is a choline-specific glycerophosphodiesterphosphodiesterase.[35] ENPP7 is an alkaline sphingomyelinase that hydrolyzes sphingomyelin into ceramide and phosphocholine.[36,37] Of these, ENPP1, 3, 4, 5, 6, and 7 are membrane-bound forms, with ENPP6 being GPI-anchored, whereas ENPP2 is secreted. Of the SMPDLs, SMPDL3A and SMPDL3B possess nucleotide hydrolase activities[26,27] with SMPDL3A being secreted[38] and SMPDL3B GPI-anchored.[39] In contrast, sphingomyelin phosphodiesterase 1 is located in the lysosomes.[40] These ENPP and SMPDL ectoenzymes are zinc-dependent.[25,26] Additionally, some other enzymes that act as phosphatases include prostatic acid phosphatase (PAP) and CD39, a representative ectonucleoside triphosphate diphosphohydrolase. CD39 contributes to the hydrolysis of extracellular ATP and ADP to AMP,[41] whereas PAP is involved in AMP hydrolysis[42]; however, these proteins are not zinc-dependent ectoenzymes.

Although the functions of ENPPs and SMPDL3s as phosphatases have been explored extensively, their specific contributions to the cellular uptake and metabolism of B vitamins remain unclear. Therefore, this study aimed to investigate the involvement of these enzymes in the hydrolysis of B vitamin phosphate esters using lysates prepared from cells transiently transfected with or endogenously expressing each enzyme. Additionally, we assessed the effect of zinc deficiency on the rate of hydrolysis of B vitamin phosphate esters using lysates prepared from cells cultured under zinc-deficient conditions and serum from rats fed zinc-deficient diets. Our findings elucidate possible mechanisms underlying B vitamin metabolism and its relationship with zinc status.

2 | MATERIALS AND METHODS

2.1 | Plasmid construction

For plasmid construction, various cDNAs were inserted into the expression plasmid harboring different promoters.

Specifically, ENPP2, ENPP4, ENPP6, ENPP7, SMPDL3A, SMPDL3B, CD39, PAP, and TNAP cDNAs were inserted into pA-puro plasmid, which uses a β-actin promoter.[43] ENPP1, ENPP3, ENPP5, placental alkaline phosphatase (PLAP), Intestinal alkaline phosphatase (IAP), and TNAP cDNAs were inserted into pcDNA3, which is driven by a CMV promoter. CD73 cDNA was inserted into a pTARGET vector, another CMV-driven plasmid. TNAP was inserted into the two expression plasmids to eliminate potential expression bias. Among these, the plasmids for CD73, TNAP, PLAP, ENPP1, ENPP2, ENPP3, CD39, and PAP were developed in previous studies,[19,44–47] while those for ENPP4, ENPP5, ENPP6, ENPP7, IAP, SMPDL3A, and SMPDL3B were constructed in this study. ENPP4, ENPP6, ENPP7, IAP, and SMPDL3A cDNAs were purchased from GE Healthcare (Buckinghamshire, UK). ENPP5 cDNA (IRAK034P02) was purchased from RIKEN BRC (Ibaraki, Japan).[48–51] SMPDL3B cDNA was amplified by RT-PCR using human brain cDNA (DV Biologics, Costa Mesa, CA, USA) as a template. *Cypridina* Luc cDNA was subcloned from the pMCS-*Cypridina* Luc plasmid (Thermo Fisher Scientific, Waltham, MA, USA) into the pcDNA3 plasmid and used as an internal control for transient transfection.

2.2 | Cell culture

HeLa,[52] MIA PaCa-2,[53] PLC/*PRF*/*5* (JCRB Cell Bank), and HepG2[54] cells were maintained at 37°C in a humidified 5% CO_2 incubator using DMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biosera, Kansas City, MO, USA), 100 U/mL penicillin, and 100 μL/mL streptomycin (Nacalai Tesque, Kyoto, Japan). Caco2 cells[55] were maintained in DMEM with 10% heat-inactivated FCS (Thermo Fisher Scientific). RPMI 1640 (FUJIFILM Wako Pure Chemical Corporation) was used to maintain SK-MEL-2 (JCRB Cell Bank)[56] and KU812 (JCRB Cell Bank)[19] cells, while Iscove's modified Dulbecco medium (Nacalai Tesque) was used for HAP1 cells (Horizon Discovery, Tokyo, Japan).

HeLa cells, with high TNAP expression and limited activity in hydrolyzing FAD to FMN, were used to study this reaction as FMN was quickly hydrolyzed to RF. SK-MEL-2 cells, lacking alkaline phosphatase (ALP) activity, were used to assess the hydrolysis of TDP to thiamine via TMP, FMN to RF, or PLP to PL, minimizing reaction interference. MIA PaCa-2, HepG2, KU812, and PLC/*PRF*/*5* cells, expressing CD73 or ENPPs (ENPP1 and ENPP3) were used to evaluate the ability of the enzymes to hydrolyze FAD. HeLa, HAP1, PLC/*PRF*/*5*, and Caco2 cells, expressing TNAP or IAP, were used to assess the ability of the enzymes to hydrolyze FMN, TDP, TMP, or PLP (Figure S1).

To generate a zinc-deficient culture medium, FCS was treated with Chelex-100 (CX) resin (Bio-Rad, Hercules, CA, USA) as described previously[57] and filter-sterilized. A cell culture medium containing 4 or 20 μM $ZnSO_4$ was used for zinc supplementation experiments. Transient transfections were performed by co-transfecting HeLa or SK-MEL-2 cells with each expression plasmid and a *Cypridina* Luc expression plasmid (internal control) at a 10:1 ratio using Lipofectamine 2000 (Thermo Fisher Scientific) to normalize transfection efficiency.[58]

2.3 | Transient transfection

Transient transfection was performed as described previously.[47] HeLa cells or SK-MEL-2 cells were seeded in 6-well plates (1.0×10^5 cells/well) and cultured for 24 h (for HeLa cells) or 48 h (for SK-MEL-2 cells). The cells were then transfected with 1 μg of empty pcDNA3 or plasmids harboring each cDNA with 0.1 μg of *Cypridina* Luc expression plasmid in Opti-MEM (Thermo Fisher Scientific) for normalization of transfection efficiency, using Lipofectamine 2000 (Thermo Fisher Scientific). The transfection medium was replaced after 4 h with the corresponding culture medium, and the cells were cultured for an additional 24 h prior to the experiments.

2.4 | Preparation of membrane lysates

The cultured cells were washed twice with ice-cold phosphate-buffered saline (PBS), collected using a cell scraper, and pelleted by centrifugation (2300 *g* and 4°C for 5 min). Membrane lysates were obtained from the pelleted cells, resuspended in 1.4 mL of cold homogenizing buffer (0.25 M sucrose, 20 mM HEPES, and 1 mM EDTA), and homogenized with 30 strokes of a 7 mL Dounce homogenizer (Wheaton, Millville, NJ, USA). The homogenate was centrifuged at 2300 *g* and 4°C for 5 min to remove the nuclei. The post-nuclear supernatant was centrifuged at 20400 *g* and 4°C for 30 min. The pellet was washed once with Tris-buffered saline and lysed in Pi buffer (10 mM Tris–HCl, 0.5 mM $MgCl_2$, and 0.1% NP40, pH 7.5). The protein concentration was determined using a DC protein assay (Bio-Rad) with bovine serum albumin as the standard.

2.5 | High-performance liquid chromatography analysis of TDP, TMP, and thiamine

Five micrograms of membrane lysates in the lysis buffer or 5 μL serum from rats was incubated with TDP or TMP

(final concentration of 12.5 μM prepared using 50 mM Tris–HCl and 0.5 mM $MgCl_2$; pH 7.5) at 37°C for 1–3 h. The samples were then stored on ice. High-performance liquid chromatography (HPLC) analysis was performed on a Chromaster HPLC system (Hitachi High-Technologies, Tokyo, Japan). The separation of the single peaks of TDP, TMP, and thiamine was performed using an Inertsil ODS-3 column (5 μm, 4.6 mm × 150 mm; GL Sciences, Tokyo, Japan). The chromatographic conditions included a gradient of 10 mM NaH_2PO_4 (pH 7.0) and methanol (9:1 to 6:4, v/v) for up to 8 min, followed by 10 mM NaH_2PO_4 (pH 7.0) and methanol (9:1, v/v) for up to 11.5 min, and then held at 10 mM NaH_2PO_4 (pH 7.0)–methanol (9:1, v/v) for up to 15 min at 40°C. The flow rate was maintained at 0.7 mL/min, and sample elution was detected at excitation and emission wavelengths of 375 and 435 nm, respectively. The levels of TDP, TMP, and thiamine were calculated using the standard curve developed by assessing the peak areas of thiamine pyrophosphate (Sigma-Aldrich, St. Louis, MO, USA), thiamine monophosphate chloride dihydrate (Sigma-Aldrich), and thiamine hydrochloride (Tokyo Chemical Industry, Tokyo, Japan) at 435 nm.

2.6 | HPLC analysis of FAD, FMN, and RF

Five micrograms of membrane lysates (prepared from HeLa or SK-MEL-2 cells) or 5 μL of rat serum was incubated with FAD or FMN (final concentration 12.5 μM prepared using 50 mM Tris–HCl and 0.5 mM $MgCl_2$, pH 7.5) at 37°C for 0.5–1 h. The samples were inactivated at 80°C for 10 min and stored on ice. Analyses were performed on a Chromaster HPLC system fitted with an Inertsil ODS-3 column (5 μm, 4.6 mm × 150 mm). The separation of FAD, FMN, and RF as single peaks was performed under an isocratic condition [65% buffer containing 10 mM NaH_2PO_4 (pH 5.5) plus 35% methanol] at 40°C for 12 min. The flow rate was maintained at 0.7 mL/min and sample elution was detected at excitation and emission wavelengths of 445 and 530 nm, respectively. Flavin adenine dinucleotide disodium salt (Tokyo Chemical Industry), riboflavin 5′-monophosphate sodium salt (Tokyo Chemical Industry), and VB2 (Nacalai Tesque) were used to prepare the standard curve, which was used to estimate the concentrations of FAD, FMN, and RF in the samples.

2.7 | HPLC analysis of PL and PLP

Five micrograms of membrane lysates or 5 μL rat serum was incubated with PLP (at a final concentration of 100 μM prepared using 0.5 mM $MgCl_2$) at 37°C for 0.5–1 h and stored on ice. HPLC analyses were performed on a Chromaster HPLC system. The peaks of PLP and PL were separated using an Inertsil ODS-3 column (5 μm, 4.6 mm × 150 mm) and the following chromatographic conditions: 10 mM NaH_2PO_4 (pH 5.5)–methanol (9:1, v/v) for 2 min, followed by 10 mM NaH_2PO_4 (pH 5.5)–methanol gradient (82:18, v/v) up to 10 min at 40°C. The flow rate was maintained at 0.7 mL/min, and sample elution was detected at excitation and emission wavelengths of 325 and 425 nm, respectively. Pyridoxal 5-phosphate (Tokyo Chemical Industry) and pyridoxal hydrochloride (Tokyo Chemical Industry) were used to prepare a standard curve, which was used to evaluate the levels of PLP and PL in the samples.

2.8 | Immunoblotting

Immunoblotting was performed as described in a previous study.[56] Briefly, the blotted polyvinylidene fluoride membranes (MilliporeSigma, Bedford, MA, USA) were blocked for 1 h with 5% skim milk and 0.1% Tween 20 in PBS or for 30 min with SuperBlock blocking buffer (Thermo Fisher Scientific) and 0.1% Tween 20 in PBS. Then the membranes were incubated with the following primary antibodies diluted in blocking solution overnight at 4°C: anti-CD73 [D7F9A] (1:3000; Cell Signaling Technology, Beverly, MA, USA), anti-ENPP1 [NBP2-27561] (1:3000; Novus Biologicals, Littleton, CO, USA), anti-ENPP3 [HPA043772] (1:3000; Sigma-Aldrich), anti-TNAP [F-4] (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IAP [ab97532] (1:3000; Abcam, Cambridge, UK), and anti-calnexin [10427-2-AP] (1:3000; Proteintech Group Inc., Chicago, IL, USA). Subsequently, immunoreactive bands were detected using 1:3000-diluted horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (NA931 or NA934; Cytiva, Marlborough, MA, USA), Immobilon Western Chemiluminescent HRP substrates (Millipore), or SuperSignal West Femto Maximum Sensitivity substrate (Thermo Fisher Scientific). Chemiluminescent images were obtained using ImageQuant LAS 500 (Cytiva).

2.9 | Measurement of Cypridina Luc activity

Cypridina Luc activity was measured using a Pierce™ Cypridina Luciferase Glow Assay Kit (Thermo Fisher Scientific) and Synergy H1 Hybrid Multimode Microplate Reader (BioTek, Winooski, VT, USA)

according to the manufacturer's instructions (Figure S2). Briefly, membrane lysates (5 μg) prepared from transfected cells were mixed with 40 μL working solution comprising 10 μL 100X Vargulin to 1 mL Cypridina Glow Assay Buffer.

2.10 | Animal care and dietary zinc manipulation

Zinc enzymes such as CD73, TNAP, ENPP1, and ENPP3 are naturally present in blood. CD73 and TNAP (and other ALPs) are released into the circulation through the hydrolysis of the GPI-anchor, and ENPP1 and ENPP3 are converted into soluble forms through proteolytic cleavage.[19,24,59,60] In this study, we used serum obtained from 5-week-old male Sprague–Dawley rats (180–200 g; Japan SLC, Shizuoka, Japan) to examine the hydrolysis of B vitamin phosphate esters. The animals were housed in separate cages at 22°C under a 12/12 h light/dark cycle in accordance with the protocols and guidelines approved by the Animal Experimentation and Ethics Committee of the Jikei University School of Medicine (approval number: 2020-022C1) and the Animal Research-Animal Care Committee of Kyoto University (approval number: Lif-K23011). After dietary manipulation, blood samples were collected via the rat tail vein using heparinized capillary glass tubes. The rats were divided into distinct groups to assess the effects of zinc deficiency as described below:

- *Long-term zinc deficiency*. The rats were divided into two uniform groups ($n = 3$ rats/group): one group was fed a standard diet [17 g/day, containing 0.01% zinc, control] (Oriental Yeast, Tokyo, Japan) and the other was fed a zinc-deficient diet (no zinc) for 2, 4, and 6 weeks. Serum zinc concentrations were determined based on a previous study[61]: 113.5 ± 19.5 μg/dL in the control group and 31.84 ± 12.6 μg/dL in the deficient group.
- *Short-term zinc deficiency*. Rats were divided into two uniform groups ($n = 3$ rats/group): one group was fed a standard diet (17 g/day, containing 0.01% zinc) (Oriental Yeast) and the other was fed a zinc-deficient diet (no zinc) for 2 or 5 days.

2.11 | Serum zinc levels

Serum zinc levels were evaluated as described previously.[61,62] To obtain the serum, blood was collected in a 15-mL centrifuge tube and centrifuged at 1690 *g* for 10 min. Serum Zn levels were quantified using an ACCURAS AUTO Zn kit (Shino-test, Kanagawa, Japan).

2.12 | Statistics and reproducibility

Statistical analyses were performed using GraphPad Prism software (version 9.0.0; GraphPad Software, Boston, MA, USA; https://www.graphpad.com/). All data are expressed as the mean ± standard deviation (SD) from triplicate experiments. Statistical significance was determined using one-way analysis of variance followed by Tukey's multiple comparisons or Student's *t*-test (comparison between two groups). A *p*-value of <0.05 was considered significant.

3 | RESULTS

3.1 | Evaluation of ectoenzymes involved in TDP and TMP hydrolysis

We first identified the ectoenzymes involved in the hydrolysis of the phosphate ester of TDP to thiamine via TMP and that of TMP to thiamine using HPLC (Figure S3). The levels of thiamine and TMP generated from TDP hydrolysis and that of thiamine generated from TMP hydrolysis were evaluated by transient transfection of the expression plasmids for TNAP, CD73, ENPP1-7, SMPDL3A, SMPDL3B, CD39, and PAP into SK-MEL-2 cells (Figure 1)—the results are shown in Figure S4. Thiamine was generated through TDP (Figure 1A) and TMP (Figure 1B) in the membrane lysates prepared from cells expressing TNAP. These results indicated that the use of different promoters (CMV promoter in pcDNA3 and actin promoter in pA-puro) did not affect the rate of hydrolysis. Furthermore, our findings showed that TNAP and its isozymes, namely IAP and PLAP, hydrolyzed TDP to TMP and thiamine, and TMP to thiamine (Figures 1C,D and S4). These results suggest that ALPs hydrolyze VB1 regardless of their isozymes.

3.2 | Evaluation of ectoenzymes involved in FAD and FMN hydrolysis

Next, we investigated the ectoenzymes involved in the hydrolysis of FAD to FMN and FMN to RF using HPLC (Figure S5). Through transient transfection of the enzyme expression plasmids into HeLa and SK-MEL-2 cells, we investigated the resulting levels of RF and FMN generated from FAD hydrolysis (Figures 2A and S6). RF was generated from FAD (via FMN) in the membrane lysates of HeLa

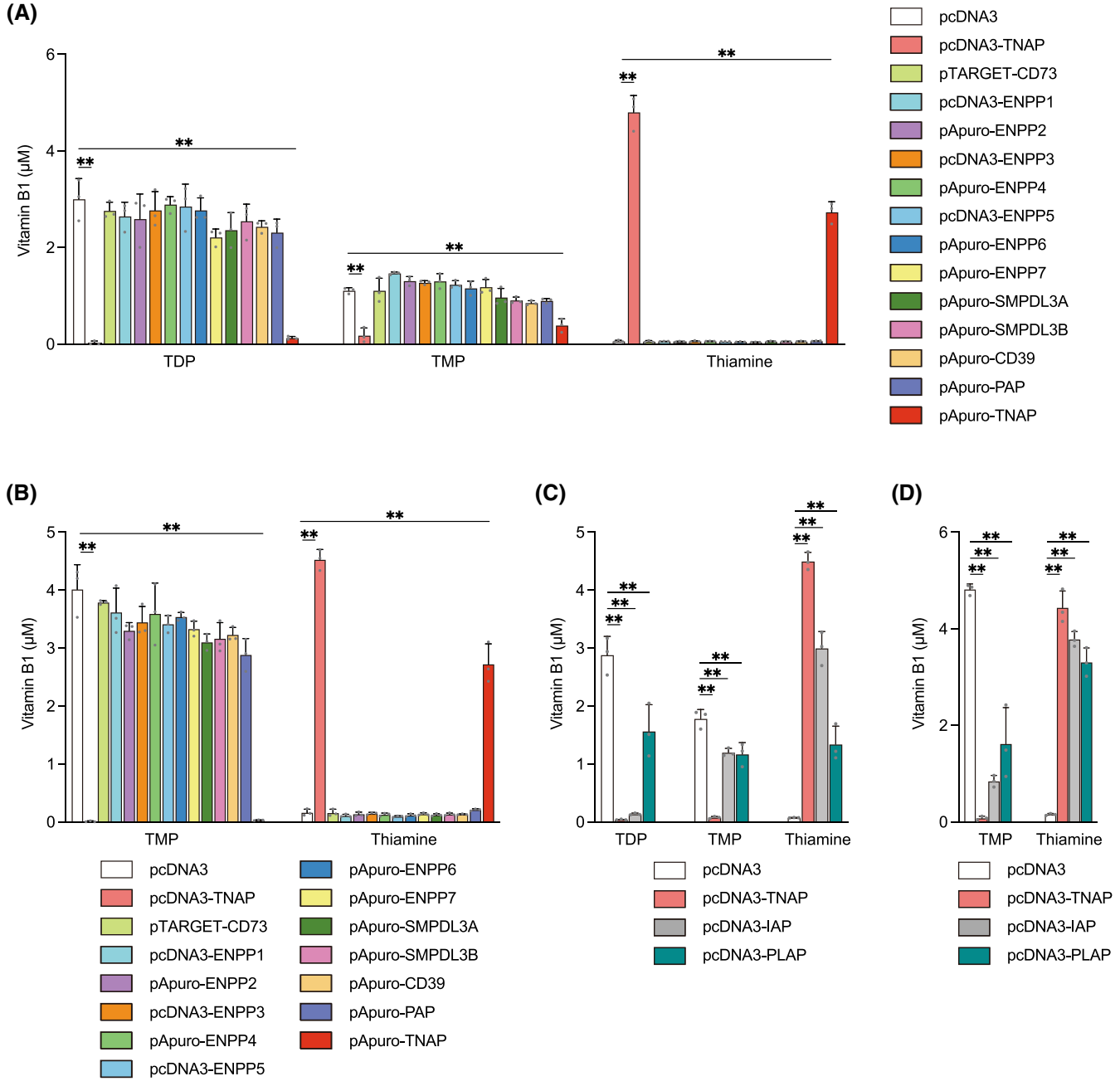

FIGURE 1 ALPs are involved in the hydrolysis of TDP and TMP to thiamine. (A, B). Rate of hydrolysis of TDP and TMP for each enzyme transiently transfected into SK-MEL-2 cells. The levels of TDP, TMP, and thiamine in a reaction solution containing TDP (A) or TMP (B) as a substrate were measured using HPLC. (C, D). The same reaction was performed using membrane lysates prepared from SK-MEL-2 cells transfected with the indicated plasmid. TDP, TMP, and thiamine concentrations were measured as in (A) and (B). Data are presented as the mean ± SD ($n = 3$). $^{**}p < 0.01$ versus control (transfected with pcDNA3) determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Cypridina* Luc activity used as an internal standard for transient transfections is shown in Figure S2.

cells expressing ENPP1, ENPP3, or CD73. Then, the generation of RF from FMN hydrolysis was evaluated using the same membrane lysate from SK-MEL-2 cells used in Figure 1. TNAP and its isozymes, IAP and PLAP, showed phosphate hydrolysis activity (Figures 2B,C and S6). These results suggest that, in addition to CD73 and ALPs, several ENPPs participate in the two-step hydrolysis of VB2.

3.3 | Determination of ectoenzymes involved in PLP hydrolysis

The PL level generated from PLP hydrolysis was quantified (Figure S7) to identify the ectoenzymes involved in the hydrolysis of the phosphate ester of PLP (Figure 3). TNAP (Figures 3A and S8) and the isozymes IAP and

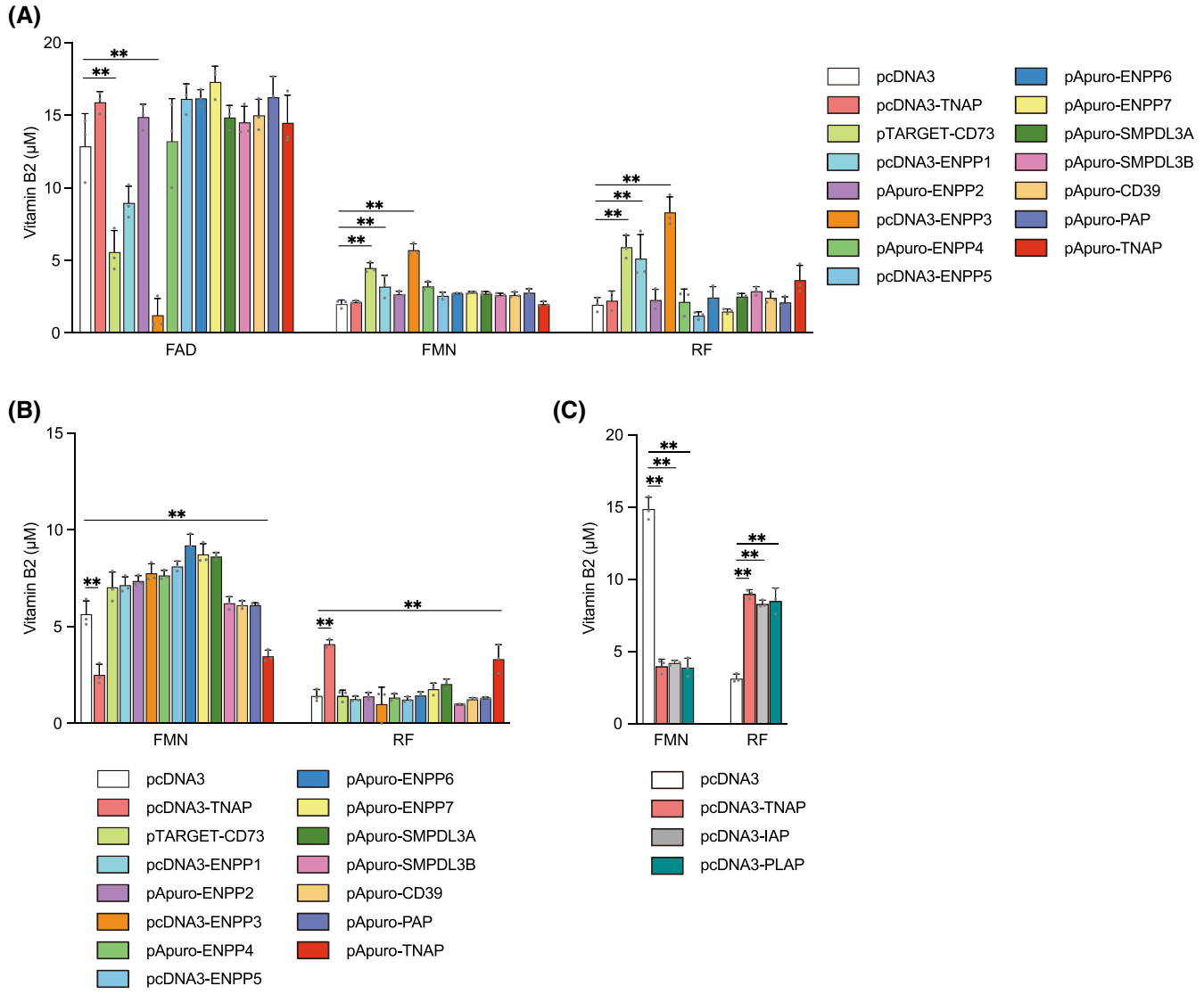

FIGURE 2 CD73, ENPP1, and ENPP3 hydrolyze FAD to FMN, whereas ALPs hydrolyze FMN to RF. (A). Hydrolysis of FAD to FMN and RF by each enzyme transiently transfected in HeLa cells. FAD, FMN, and RF concentrations in the reaction containing FAD as a substrate were measured using HPLC. (B). Hydrolysis of FMN to RF by each enzyme transiently transfected into SK-MEL-2 cells. FMN and RF concentrations in the reaction containing FMN as a substrate were measured as in (A). (C). Hydrolysis of FMN to RF by each enzyme transiently transfected into HeLa cells. FMN and RF concentrations in the reaction containing FMN as a substrate were measured as in (A). Data are presented as the mean ± SD ($n = 3$). $^{**}p < 0.01$ versus control (transfected with pcDNA3) determined using ANOVA followed by Tukey's post hoc test. *Cypridina* Luc activity used as an internal standard for transient transfection is shown in Figure S2.

PLAP hydrolyzed PLP to PL (Figures 3B and S8), suggesting that ALPs hydrolyze VB6 regardless of their isozymes, similar to their hydrolysis of VB1.

3.4 | Rate of hydrolysis of VB1, VB2, and VB6 reduced in membrane lysates prepared from cells cultured in a zinc-deficient medium

To investigate the effects of zinc deficiency on the hydrolysis of VB1, VB2, and VB6, we used membrane lysates from cells expressing TNAP, CD73, ENPP1, and ENPP3 (Figure 4), whose activities are inhibited in zinc-deficiency experiments using other substrates.[19,45,63] HeLa and SK-MEL-2 cells were transiently transfected with expression plasmids for these enzymes to assess the effects on B vitamin hydrolysis. Membrane lysates from SK-MEL-2 cells transfected with TNAP plasmids were used to evaluate the hydrolysis of TDP, TMP, FMN, and PLP (Figures 4A–D and S9). Similarly, membrane lysates prepared from HeLa cells transfected with CD73, ENPP1, or ENPP3 expression plasmids were used to evaluate the hydrolysis of FAD (Figures 4E and S9).

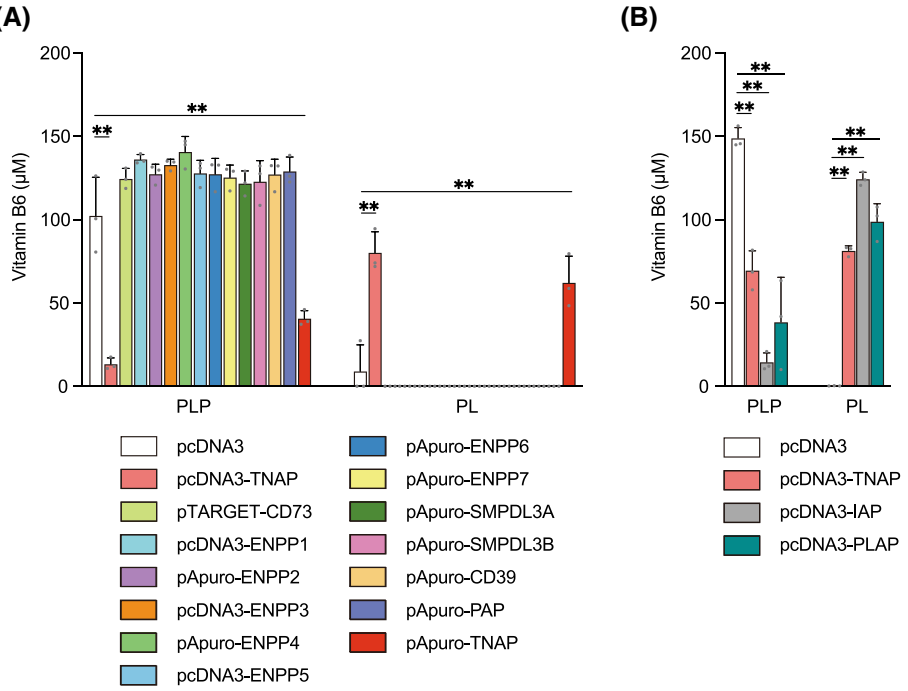

FIGURE 3 ALPs are involved in the hydrolysis of PLP to PL. (A). Hydrolysis of PLP to PL by each enzyme transiently transfected into SK-MEL-2 cells. PLP and PL concentrations in the reaction containing PLP as a substrate were measured using HPLC. (B). The same reaction using membrane lysates prepared from SK-MEL-2 cells transfected with TNAP, IAP, and PLAP. PLP and PL concentrations were measured as in (A). Data are presented as the mean ± SD ($n = 3$). $^{**}p < 0.01$ versus control (transfected with pcDNA3) determined using ANOVA followed by Tukey's post hoc test. *Cypridina* Luc activity used as an internal standard for transient transfection is shown in Figure S2.

Next, to confirm the specific effects of zinc on the activities of each enzyme, we used a zinc-deficient medium, containing CX-treated FCS and a zinc-supplementation medium supplemented with 4 or 20 μM $ZnSO_4$ (which mimics zinc concentrations in the normal culture medium or human serum). The rate of hydrolysis of the phosphate esters was reduced in the membrane lysates of cells cultured in a zinc-deficient medium compared with that in those obtained from cells cultured in a normal medium (Figure 4A–E).

To complement the cell transfection experiments, we confirmed that the endogenous activities of TNAP, CD73, ENPP1, and ENPP3 during the hydrolysis of B vitamins were altered by zinc levels in non-transfected human cells. Based on previous screenings,[19] we selected specific cell lines expressing zinc-dependent enzymes (Figure S1). Membrane lysates were prepared from pancreatic carcinoma MIA PaCa-2, hepatoma PLC/*PRF/5* and HepG2, cervical cancer HeLa, myeloma HAP1, KU812 basophils, and intestinal epithelial Caco2 cells expressing IAP (Figures 5 and S10) to examine their hydrolytic activity using HPLC. The hydrolysis of FAD and FMN (VB2) was impaired in cells cultured under zinc-deficient conditions (Figure 5A,B). This diminished activity caused by zinc deficiency was completely recovered following zinc supplementation (4 or 20 μM $ZnSO_4$; Figure 5A,B). We also examined the effects of zinc deficiency on the hydrolysis of TDP (VB1), TMP (VB1), and PLP (VB6) using membrane lysates from PLC/*PRF/5*, HeLa, HAP1, and Caco2 cells (Figures 6A–C and S11). The activities of all enzymes were substantially reduced in cells cultured under zinc-deficient conditions, which was reversed by zinc supplementation (Figure 6). These findings indicate that the rate of hydrolysis of B vitamins [TDP to thiamin via TMP (VB1), FAD to RF via FMN (VB2), and PLP to PL (VB6)] is significantly impaired in zinc-deficient conditions.

3.5 | Rate of hydrolysis of VB1 and VB2 reduced in serum prepared from rats fed a zinc-deficient diet

Assessment of the hydrolysis of B vitamin phosphate esters in the serum of rats exposed to long-term zinc-deficiency conditions revealed a significantly diminished rate of hydrolysis of VB1 (TDP to Thiamine) and VB2 (FAD to RF) (Figures 7A–D and S12), mirroring the reductions in serum zinc concentrations. However, we could not measure the hydrolysis of VB6 under the assay conditions.

Next, to investigate the effects of short-term zinc deficiency conditions on hydrolysis of B vitamin phosphate esters, we performed the same experiments using

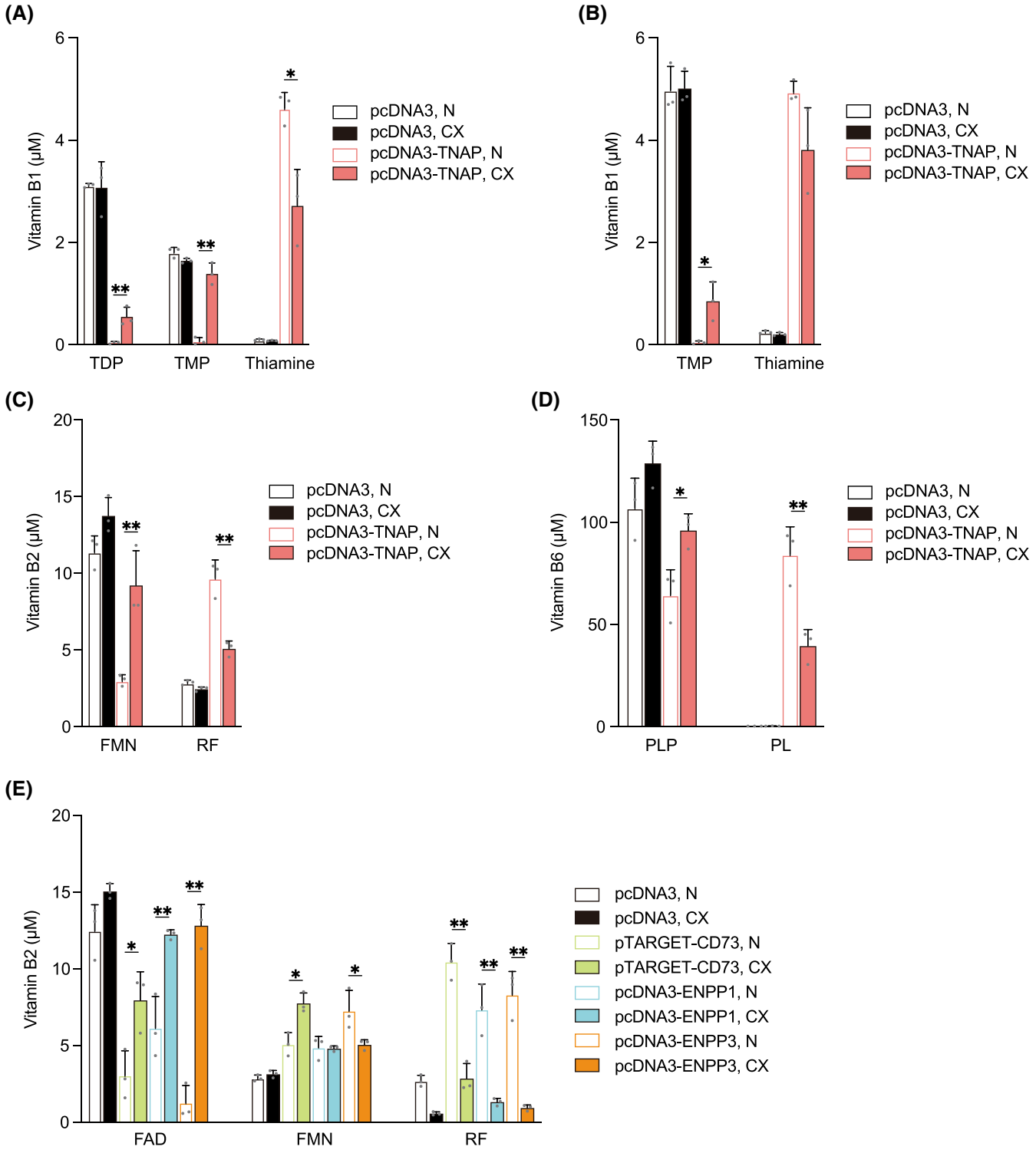

FIGURE 4 Zinc deficiency affects B vitamin hydrolysis by overexpressed zinc enzymes. (A, B). Hydrolysis of TDP and TMP to thiamine by TNAP transiently overexpressed in SK-MEL-2 cells was impaired in zinc-deficient conditions. The concentrations of TDP, TMP, and thiamine were measured as described in Figure 1. (C, D). Hydrolysis of FMN to RF or PLP to PL by TNAP transiently overexpressed in SK-MEL-2 cells was impaired in zinc-deficient conditions. The same membrane lysates as those described in A and B were used. The concentrations of FMN and RF (C) and those of PLP and PL (D) were measured as in Figures 2B and 3A. (E). Hydrolysis of FAD by CD73, ENPP1, and ENPP3 transiently overexpressed in HeLa cells was impaired in zinc-deficient conditions. FAD, FMN, and RF concentrations were measured as in Figure 2A. Membrane lysates were prepared from transfected HeLa and SK-MEL-2 cells cultured in normal (N) or zinc-deficient medium obtained using CX-treated FCS (CX). Data are presented as mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$ (normal [N] vs. zinc-deficient [CX] medium) determined using Student's *t*-test. *Cypridina* Luc activity used as an internal standard for transient transfection is shown in Figure S2.

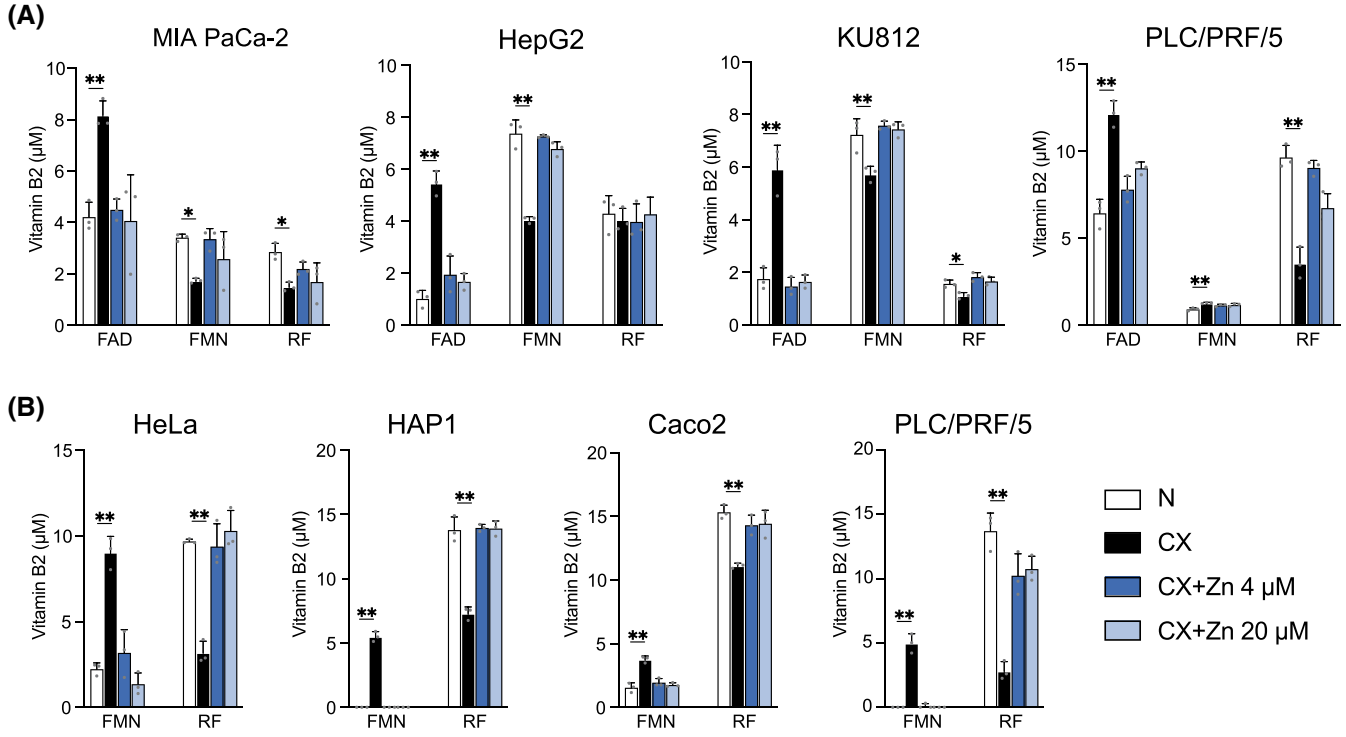

FIGURE 5 Zinc deficiency affects VB2 hydrolysis by zinc enzymes in non-transfected cells. (A). Hydrolysis of FAD to RF via FMN in the membrane lysates prepared from MIA PaCa-2, HepG2, KU812 cells, or PLC/*PRF*/*5* cultured in normal medium (N), zinc-deficient medium generated using CX-treated FCS (CX), and zinc-deficient medium supplemented with 4 or 20 μM $ZnSO_4$ (CX + Zn 4 μM or CX + Zn 20 μM). (B). Hydrolysis of FMN to RF in the membrane lysates prepared from HeLa, HAP1, Caco2 cells, or PLC/*PRF*/*5* cultured in the same conditions as in (A). FAD, FMN, and RF concentrations were measured as in Figure 2. Data are presented as the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus normal (N) determined using one-way ANOVA followed by Tukey's post hoc test.

sera obtained from rats fed the zinc-deficient diet for 2 or 5 days. Serum zinc concentrations tended to decrease (Figure 8A), whereas, the rate of hydrolysis of VB2 reduced significantly in these rats. Specifically, the rate of hydrolysis of FAD reduced significantly (Figures 8B,C and S13), while that of FMN tended to be altered (Figure 8B,C). These results suggest that dietary zinc deficiency reduces the rate of the hydrolysis of TDP, TMN, FAD, and FMN by reducing the activity of zinc enzymes such as CD73, TNAP, ENPP1, and/or ENPP3, thereby affecting B vitamin metabolism in vivo.

4 | DISCUSSION

The membrane transport mechanisms of B vitamins, including their intestinal absorption and cellular uptake, remain largely unclear. In this study, we demonstrated that CD73, ENPP1, and ENPP3 hydrolyzed FAD to FMN, while TNAP, IAP, and PLAP hydrolyzed TDP to TMP, TMP to thiamine, and PLP to PL. Furthermore, under zinc deficiency, the hydrolytic activities of these enzymes, particularly for VB2, were significantly impaired both in vitro and in vivo (Figure 9) because they are all zinc enzymes. These findings provide insight into the metabolism of B vitamins and the interplay between vitamins and minerals.

The cellular uptake of B vitamins requires the hydrolysis of phosphate esters to allow membrane penetration. However, this process is largely uncharacterized, hindering our understanding of the effects of B vitamin phosphate ester hydrolysis on cellular metabolism. Commercially available culture media for mammalian cells often include dephosphorylated forms of VB1, VB2, and VB6 (thiamine, RF, PL, or pyridoxal) as supplements, which may benefit cultures of cells lacking TNAP (IAP), CD73, ENPP1, and ENPP3 because of the rapid uptake of B vitamins. This occurs because the culture media typically contain heat-inactivated FCS, leading to inactivation of TNAP, CD73, ENPP1, and ENPP3. Similar conditions may occur in laboratory animal diets,[64] which commonly contain the dephosphorylated B vitamins. Therefore, further investigation to understand B vitamin phosphate ester hydrolysis is essential.

This study demonstrated that ENPP1, ENPP3, and CD73 are involved in FAD hydrolysis. According to data from the Human Protein Atlas (https://www.proteinatlas.org), CD73 expression is specific to certain cell

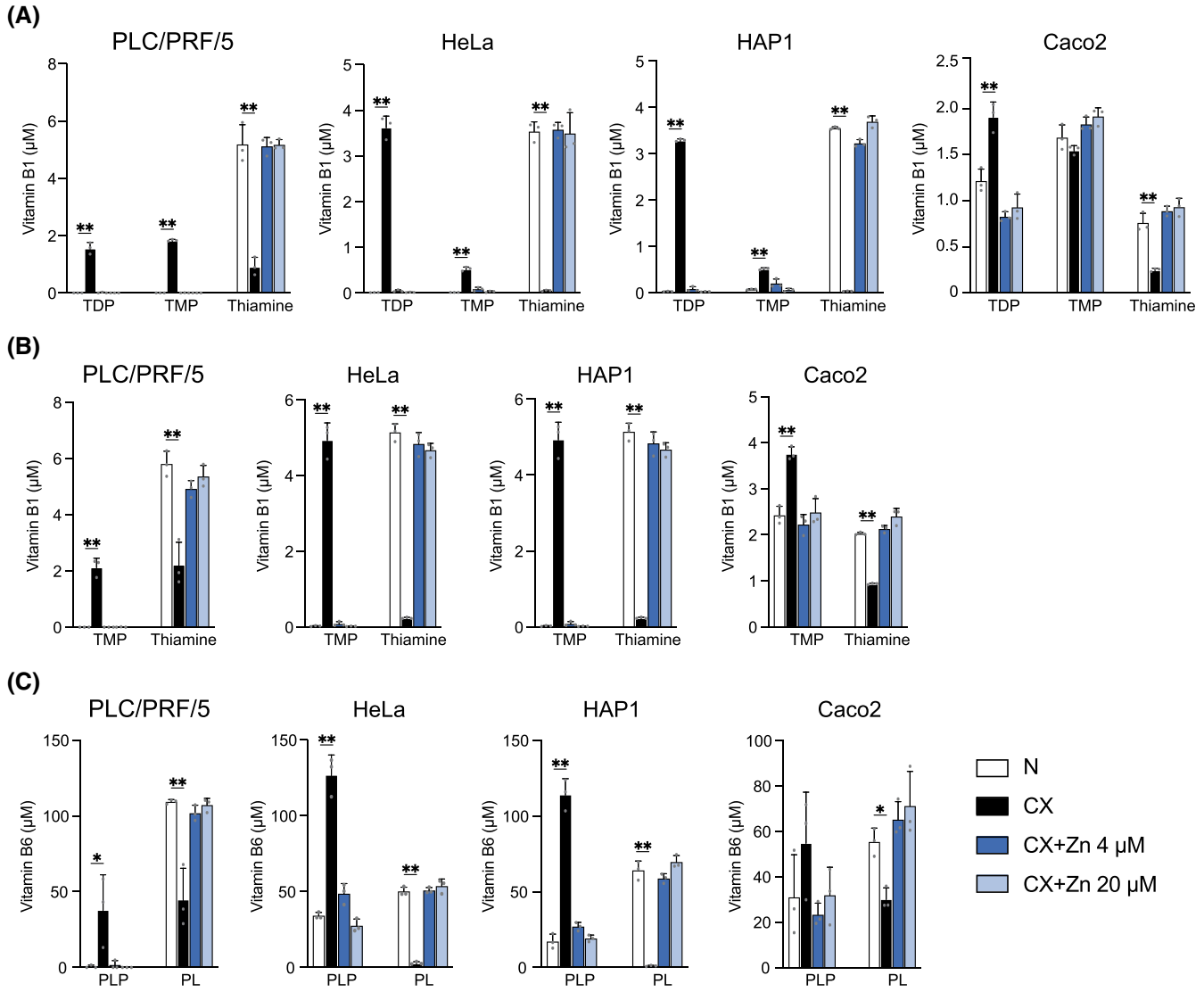

FIGURE 6 Zinc deficiency affects VB1 and VB6 hydrolysis by zinc enzymes in non-transfected cells. (A, B). Hydrolysis of TDP to thiamine via TMP (A) and TMP to thiamine (B) in the same membrane lysates used in Figure 5B, which were prepared from PLC/*PRF*/*5*, HeLa, HAP1, or Caco2 cells. (C). Hydrolysis of PLP to PL in the same membrane lysates. PLP and PL concentrations were measured as in Figure 3. Data are presented as the mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$ versus normal (N) determined using one-way ANOVA followed by Tukey's post hoc test.

types. This suggests that both ENPPs are particularly important for VB2 metabolism in cells that lack CD73, while they also cooperate with CD73 in VB2 metabolism. Furthermore, ENPP3 is highly expressed on the apical membrane of the small intestine,[65,66] similar to CD73,[66,67] which is significant because dietary VB2 is absorbed in the small intestine.[1,4] Consistently, the Human Protein Atlas data also shows that both ENPP3 and CD73 are highly expressed in the small intestine. Additionally, dietary VB1 and VB6 are absorbed in the small intestine,[1,14,68] where IAP is specifically expressed on the apical membrane.[69,70] Collectively, these findings suggest that ENPP3, CD73, and IAP would play important roles in B vitamin absorption.

The activities of TNAP, IAP, CD73, ENPP1, and ENPP3 are sensitive to zinc deficiency, suggesting potential interactions between B vitamin metabolism and zinc status. However, it remains unclear whether the zinc deficiency-induced reduction in B vitamin phosphate ester hydrolysis is associated with symptoms of B vitamin deficiency. Nonetheless, the clinical symptoms suggest a close relationship. Zinc deficiency causes several symptoms, such as dermatitis, diarrhea, alopecia, immune dysfunction, and taste disorders.[71–74] Moreover, it complicates various chronic diseases, including gastrointestinal disorders, renal disease, sickle cell anemia, cirrhosis, cystic fibrosis, atherosclerosis, neurological disorders, age-related degenerative diseases, and febrile seizures.[75–77] Similarly, B

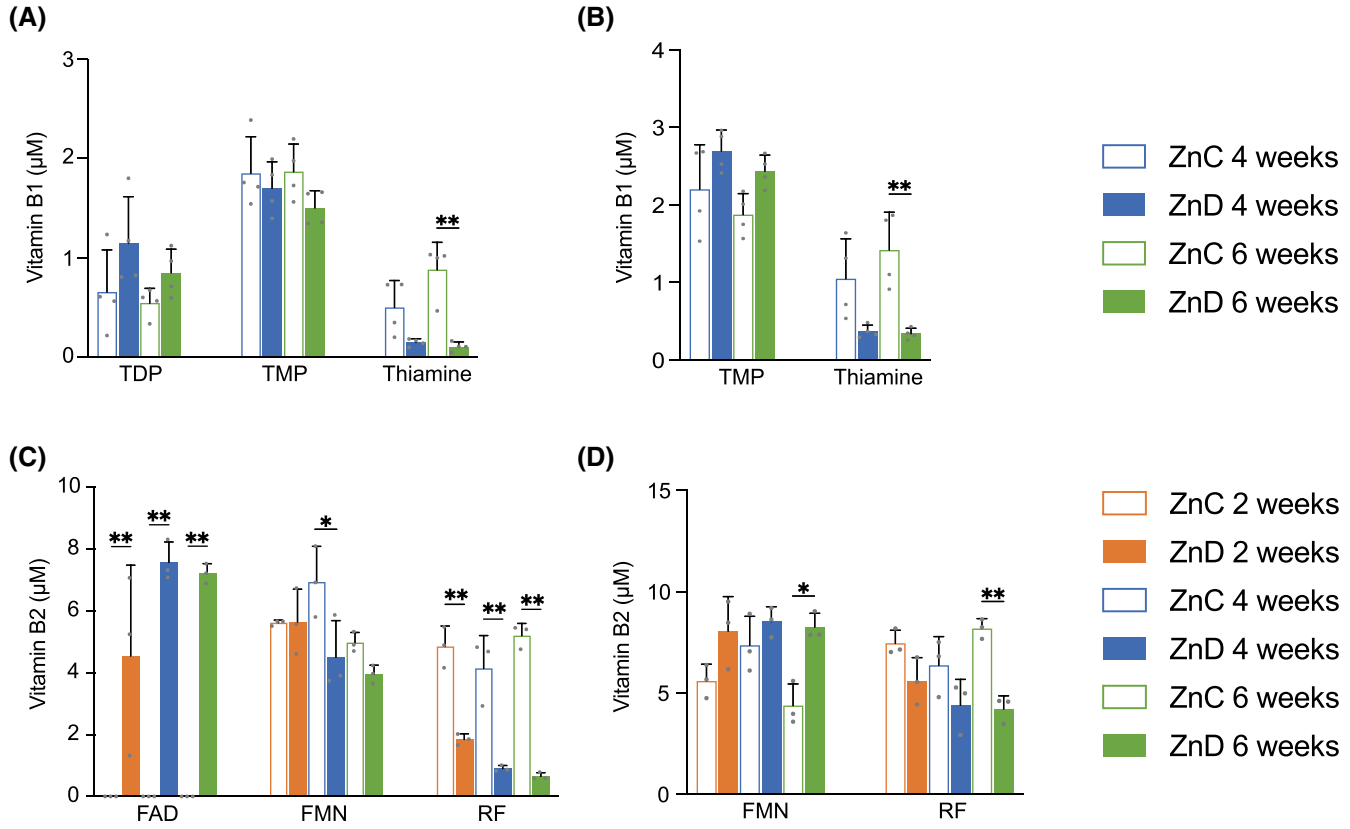

FIGURE 7 Rate of hydrolysis of VB1 and VB2 is reduced in the serum of rats fed a zinc-deficient diet. (A–D). Rate of hydrolysis of TDP (A), TMP (B) (at 4 and 6 weeks), FAD (C), and FMN (D) (at 2, 4, and 6 weeks) in serum prepared from rats fed ZnC (control) or ZnD (zinc-deficient) diets for 2, 4, and 6 weeks. The concentrations of each B vitamin were measured as in Figures 1 and 2. All activities are shown as the mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$ versus ZnC at mentioned time points determined using one-way ANOVA followed by Tukey's post hoc test.

vitamin deficiency presents a range of symptoms.[2,6,7,20,78,79] Deficiencies in VB2 and VB6, in particular, show some similarities to zinc deficiency: VB2 deficiency results in cheilosis, angular stomatitis, glossitis, dermatitis, and anemia,[79] whereas VB6 deficiency results in anemia, dermatitis, glossitis, electroencephalographic abnormalities, depression and confusion, weakened immune function, and seizures.[14,15] The resemblance between zinc and B vitamin deficiencies, particularly for VB2 and VB6, suggests that zinc deficiency affects VB2 and VB6 metabolism in various pathophysiological aspects. Defective TNAP and CD73 expression could reduce intracellular VB2 levels.[15] Consistent with this hypothesis, our findings revealed a significant reduction in the rate of FAD to FMN hydrolysis in sera of rats fed a zinc-deficient diet only for 2 days, even though serum zinc concentrations were not significantly decreased. This finding suggests potential interactions between B vitamin metabolism and zinc status; however, the effects of zinc deficiency on VB2 and VB6 metabolism in vivo were not investigated in this study, which requires extensive investigation in future studies. Specifically, studies should examine the B vitamin absorption rate in the small intestine under zinc deficiency conditions, as this tissue plays a major role in regulating B vitamin status and exhibits high expression of ENPP3, CD73, and IAP.

In a previous study, we demonstrated that extracellular ATP is hydrolyzed to adenosine by TNAP, CD73, ENPP1, and ENPP3 and that the rate of hydrolysis is impaired under zinc deficiency.[19] In this study, we also observed similar results for B vitamins, raising the question of whether these ectoenzymes are also involved in the hydrolysis of other phosphorus-containing compounds. Among the ectoenzymes evaluated in this study, TNAP, IAP, PLAP, and ENPP3 released the phosphate from lipopolysaccharide (LPS; Unpublished results, Taiho Kambe), which may diminish the inflammatory responses associated with LPS, such as those in inflammatory bowel diseases (IBDs)[80–83] and sepsis.[84,85] Furthermore, both IBDs and sepsis worsen with zinc deficiency, and B vitamins play crucial roles in managing these conditions.[68,86,87] Therefore, the involvement of these enzymes in phosphate ester hydrolysis could explain the unique association between zinc deficiency and various diseases.

In conclusion, zinc deficiency reduced the rate of hydrolysis of the phosphate ester forms of B vitamins by TNAP, CD73, ENPP1, and ENPP3, highlighting the essential role of zinc in enzyme activity. The observed interaction

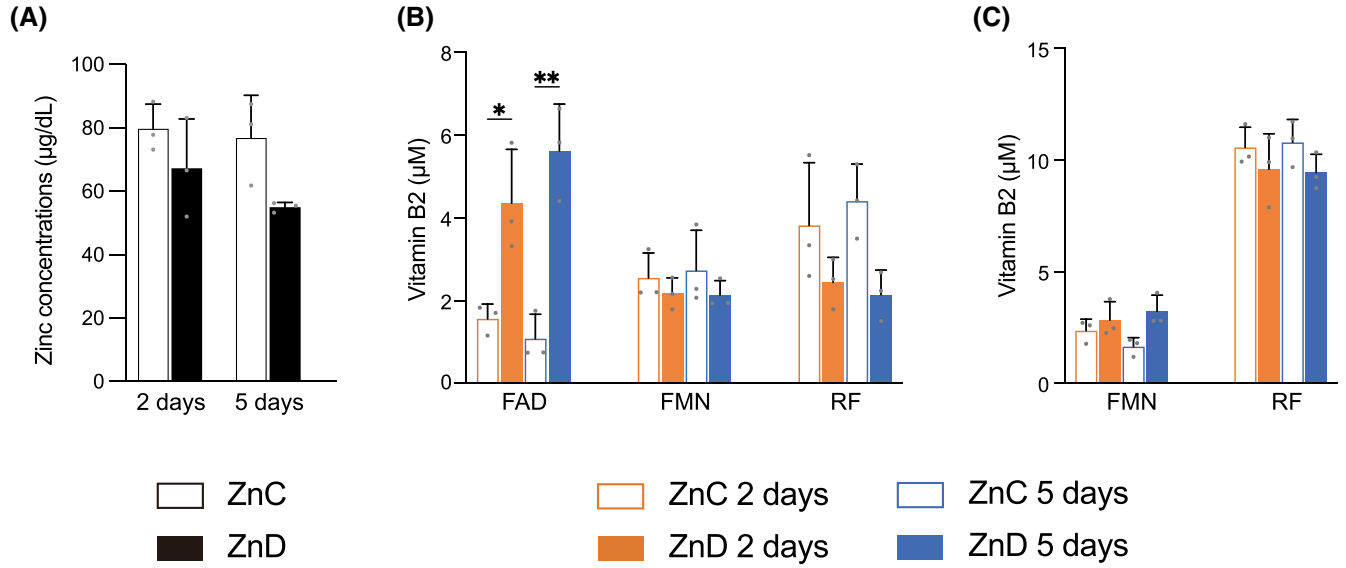

FIGURE 8 Rate of hydrolysis of VB2 is reduced in the serum of rats fed a zinc-deficient diet for a shorter period. (A). Serum zinc concentrations in rats. (B, C). The ability of hydrolysis of FAD (B) and FMN (C) (at 2 or 5 days) in serum from rats fed ZnC (control) or ZnD (zinc-deficient) diets for 2 or 5 days. VB2 concentrations were measured as in Figure 2. All activities are shown as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ versus ZnC at mentioned time points determined using one-way ANOVA, followed by Tukey's post hoc test.

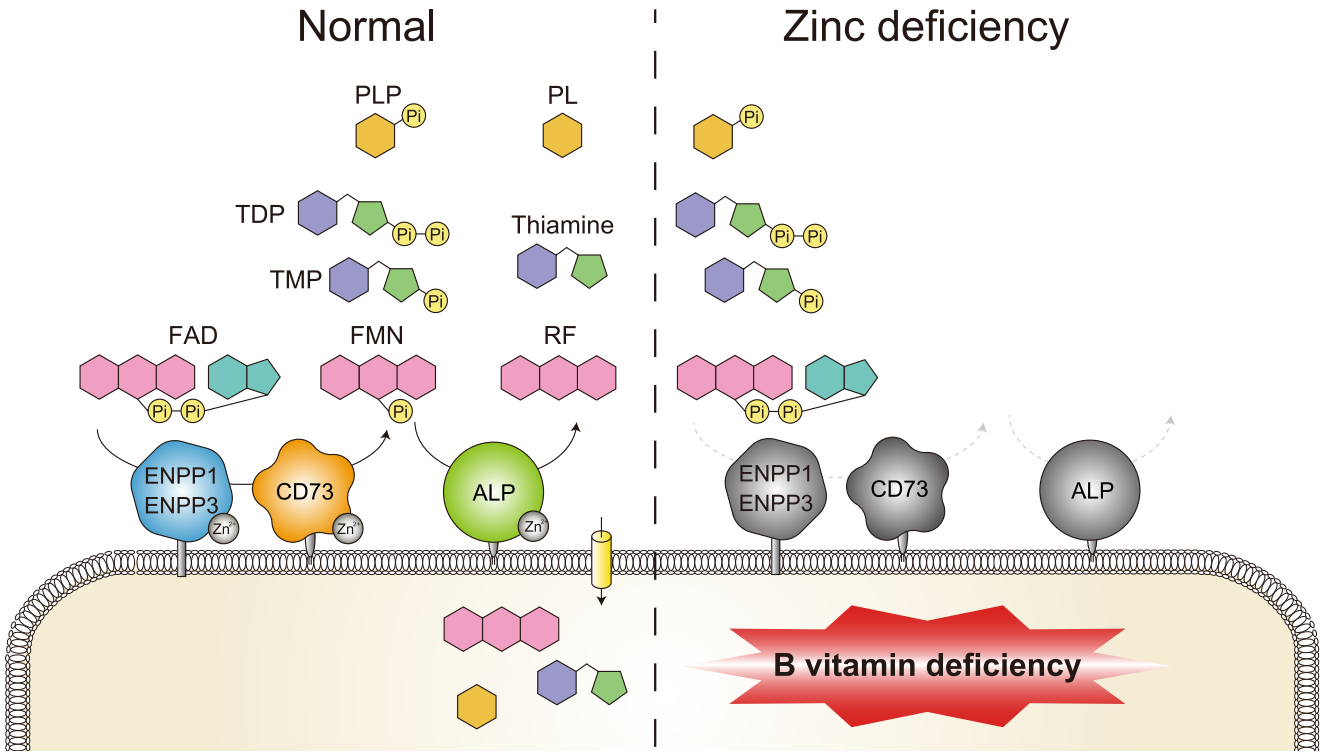

FIGURE 9 Proposed scheme of the mechanism by which the rate of B vitamin hydrolysis is reduced under zinc deficiency. Extracellular FAD is hydrolyzed to FMN by CD73, ENPP1, and ENPP3. FMN is hydrolyzed to RF (VB2) by TNAP (IAP and PLAP) on the plasma membrane and in the extracellular space in the normal status. Extracellular PLP and TDP–TMP are also hydrolyzed to PL and thiamine (VB6 and VB1) by TNAP (IAP and PLAP). The generated RF, PL, and thiamine are taken up into the cytosol across the plasma membrane through specific transporters (*left*). The activities of these zinc enzymes are reduced under zinc deficiency. Consequently, the rate of hydrolysis of FAD and FMN to RF, PLP to PL, and TDP–TMP to thiamine is reduced (*right*), which likely results in the reduced uptake of B vitamins. This suggests that zinc deficiency may affect B vitamin deficiency and that the symptoms of B vitamin deficiency may mimic those of zinc deficiency.

between B vitamins and zinc in this study may provide novel insights into the diverse symptoms of zinc deficiency, especially since both micronutrients are required to maintain good health and normal bodily functions.

AUTHOR CONTRIBUTIONS

H.Y. and T.K. conceived the study and designed the experiments. H.Y., K.M., T.K., K.N., M.S., and T.K. collected, analyzed, and interpreted the data. T.F., M.S., and M.N. provided technical assistance. H.Y., K.M., and T.K. prepared the figures. T. K drafted and edited the manuscript. All the authors reviewed the manuscript.

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DISCLOSURES

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its Supporting Information file or are available from the corresponding author (Taiho Kambe, Kyoto University; E-mail: kambe.taiho.7z@kyoto-u.ac.jp) upon reasonable request. The full-length immunoblots corresponding to the images in Figure S1 are shown in Figure S14.

ORCID

Katsutoshi Nishino https://orcid.org/0009-0003-4790-0006
Taiho Kambe https://orcid.org/0000-0001-9757-063X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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