Role of Receptor Protein Tyrosine Phosphatase γ in sensing extracellular CO_2 and HCO_3^-

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SUPPLEMENTAL MATERIAL

FULL METHODS

RPTPγ$^{-/-}$ mice

C57bl/6 mice with a targeted disruption of the RPTPγ gene[1] were a generous gift from J. Schlessinger. RPTPγ$^{-/-}$ mice were further backcrossed on a Lab-standard C57bl/6 background for 7-9 generations to attain isogeny amongst the wild-type and knockout mice. Genotypes were confirmed by PCR and mice of both sexes were used in experiments. Mice were allowed access to food and water ad libitum. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University.

Antibody production

We used an epitope consisting of a unique sequence of murine RPTPγ in the CALD domain (DILDHHARVGDEYQELQ, synthesized by Pepceuticals, Enderby, UK), and tagged with a C-terminal cysteine to allow for affinity purification by the free sulfhydryl group and coupled to a keyhole limpet hemocyanin linker (Imject, Pierce, Rockford, IL). Adult female, New Zealand white rabbits (0.5-1 kg, Charles River Laboratories, Wilmington, MA) were immunized every 3 weeks to generate the polyclonal antibody, which we affinity purified from collected serum using SulfoLink resin according to manufacturer's protocol (Pierce). Animals were handled in accordance with approved institutional policies.

Western blotting

We harvested whole kidneys from anesthetized mice (5% isoflurane) and 20 μg of protein was separated by SDS-PAGE on a 4-12% Bis-Tris gel (Invitrogen, NY). Proteins were transferred to PVDF and probed with our RPTPγ antibody (1:1000). Blots were stripped and re-probed with β-actin (loading control). Secondary antibody was conjugated to horseradish peroxidase (HRP) detected with chemiluminescence by imaging using a Fluorochem E (ProteinSimple, Santa Clara, CA).

Fluorescent confocal imaging

We fixed kidneys from deeply-anesthetized mice via abdominal aorta perfusion with PLP (phosphate-lysine-periodate containing 4% paraformaldehyde), after which tissue was embedded in O.C.T. (Sakura Finetek, Torrance, CA) and cryo-sectioned to generate 5-μm slices. Following sectioning, we heated samples in 10 mM citrate buffer (pH 6.0) followed by permeabilization with Triton (0.01% w/v). We then incubated slices with the antibodies directed against RPTPγ (1:100 dilution) and the Na^+-K^+ ATPase α subunit, (1:500 dilution, Abcam, Cambridge, UK). Secondary antibodies were alexa-conjugated goat anti-rabbit (594 nm) and goat anti-mouse (488 nm, Invitrogen). Images were obtained on an Olympus FluoView FV10-ASW confocal microscope.

Tubule perfusion

We dissected kidneys from anesthetized mice, isolated PTs, and perfused PTs at 37°C as described previously [2] with 5% CO_2/22 mM HCO_3^- plus ^{14}C-methoxyinulin [3] as a volume marker. We collected the PT luminal effluent for ^{14}C determination by scintillation counting, and for quantitation of total CO_2 via enzymatically-linked fluorometry, and computed J_V and $JHCO_3$ [2,4].

Indwelling catheters for serial sampling of arterial blood

Seven days prior to treatment (day −7), we anesthetized wild-type (WT) or RPTPγ knockout (RPTPγ$^{-/-}$) mice of either sex with isoflurane (5% induction, 2% maintenance) and implanted catheters in the carotid artery through a small ventral incision in the neck under a dissecting microscope. Mice were kept on heating pads to maintain body temperature. We constructed catheters of stretched PE-10 coupled to Tygon tubing (used to ensure low gas permeability) which were pre-filled with a lock solution (500U/ml heparin, 50% dextrose, SAI Infusion Technologies (Lake Villa, IL). We advanced catheters to the aortic arch and secured them to the vessel with silk suture (6/0) and verified catheter patency. Then, we subcutaneously tunneled the Tygon leader to the back of the head, externalized, and secured the catheter with suture to a polypropylene hub during wound closure. All surgical procedures were performed with aseptic technique; mice with modest weight loss received saline injections, but we terminated and excluded any mice experiencing signs of distress or rapid, excess weight loss at any point in the study. Lines were flushed with heparinized saline (200U/ml) every other day to maintain patency. On days 0, 2, and 7 of the experimental period, we obtained 45 μl of arterial blood for analysis from conscious mice for blood gas analysis. On day 7, an additional sample of blood was taken for hematological analysis along with 300 μl for chemistry prior to terminating the mice. Kidneys were immediately harvested and archived for gene expression analysis.

Induction of metabolic acidosis (MAc) in mice

Following 1 week of recovery from arterial catheterization (day 0), we randomly assigned WT and RPTPγ$^{-/-}$ mice to control (Ctrl) or 1% (w/v) NH_4Cl (MAc) drinking water groups. Animals in the 4 groups (WT+Ctrl, WT+MAc, RPTPγ$^{-/-}$+Ctrl, and RPTPγ$^{-/-}$+MAc) were monitored over 7 days and no differences were noted with respect to weight or water consumption (i.e., total NH_4Cl load) across the groups (data not shown).

Analysis of arterial blood

As noted above, we sampled 45 μl of blood on days 0, 2, and 7 to perform blood-gas analysis using a clinical analyzer according to manufacturer's instruction (Stat Profile pHOX, Nova Biomedicals, MA). On day 7, we also sampled blood to assess hematology (HemaTrue, Heska, Loveland, CO) and chemistry (Dri-Chem 4000, Heska; please see Supplemental Data). All measured parameters from sampled blood were determined based on standard clinical chemistry definitions and as defined by the manufacturers.

Gene expression profiling by quantitative PCR (qPCR)

Total RNA (tRNA) was isolated from 40 mg kidney tissue preserved in RNAlater (Qiagen, Valencia, CA) using a column-based kit (Aurum, Biorad, Hercules, CA). Samples of tRNA were delivered to the Gene Expression and Genotyping Facility at Case for expression analysis. Reverse transcription to generate cDNA was performed using 1 μg tRNA using the High Capacity cDNA archive kit (ABI, Foster city, CA). Assays for gene targets (Gapdh, used as the housekeeping control; Mm99999915_m1, Ptprg; Mm00477249_m1, Pck1; Mm01247058_m1, Pck2; Mm00551411_m1, Glud1; Mm00492353_m1, Slc38a3; Mm01230670_m1, Slc4a4; Mm01347935_m1, Gls1; Mm01257297_m1, Lrp2; Mm01328171_m1, Slc9a3; Mm01352473_m1, Aqp8; Mm00431846_m1, Car2; Mm00501576_m1, Car4; Mm00483021_m1, Aqp1; Mm01326466_m1, NHE1; Mm00444270_m1) were used according to manufacturer's instructions using the ABI 7900 HT thermocycler (ABI). Samples were run in triplicate and relative expression values and fold change were

calculated using the ΔΔ Ct method. Data is presented as fold change with respect to the indicated comparative group.

Statistical Analysis

Values reported are means ± the standard error of the mean (SEM). For PT data, numbers of tubules is indicated in parentheses and statistical differences were determined by an unpaired, 2 tailed t-test. In vivo data was analyzed by paired t-tests to define time-dependent differences within respective groups and by robust linear regression analysis using day 0 as a covariate and employing an interaction term to estimate the effect of genotype and treatment within groups at each time point. For day 7 analyses (qPCR and clinical chemistry), a 2-way ANOVA using the Bonferroni post-hoc test was employed. For all tests, $p < 0.05$ was set as the criterion for significance.

SUPPLEMENTAL BIBLIOGRAPHY

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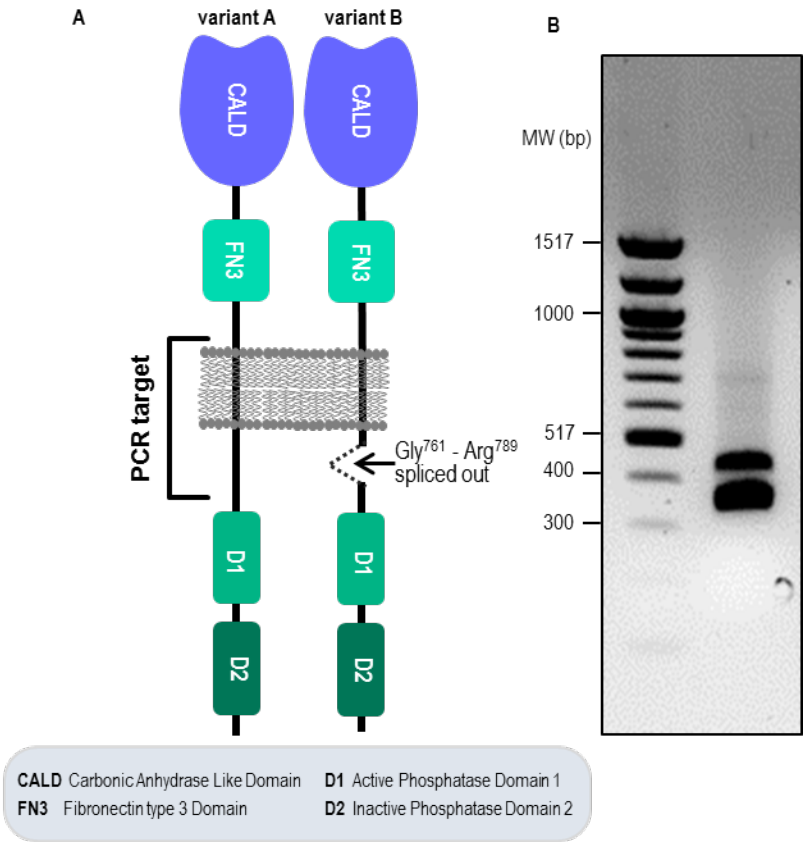
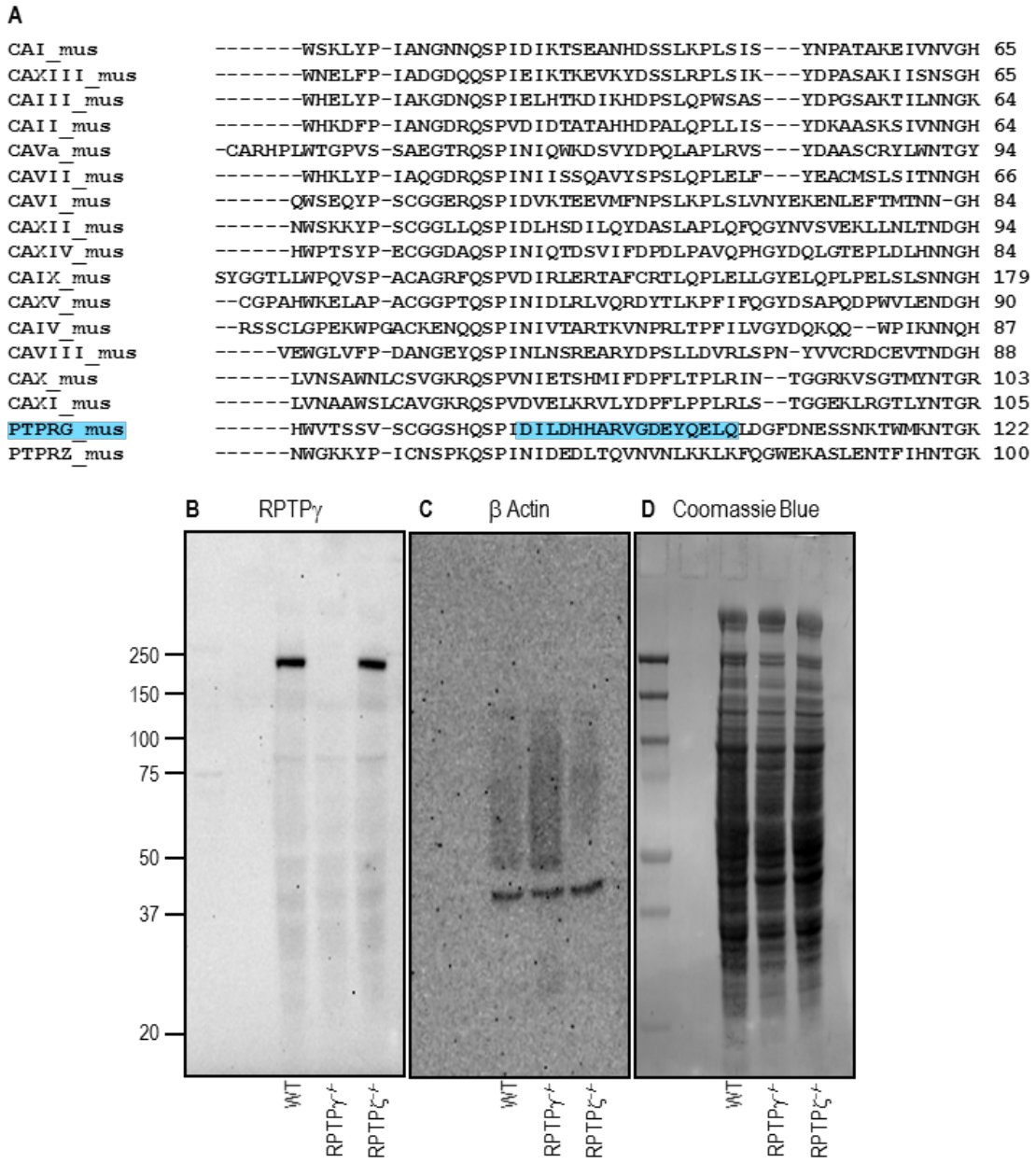


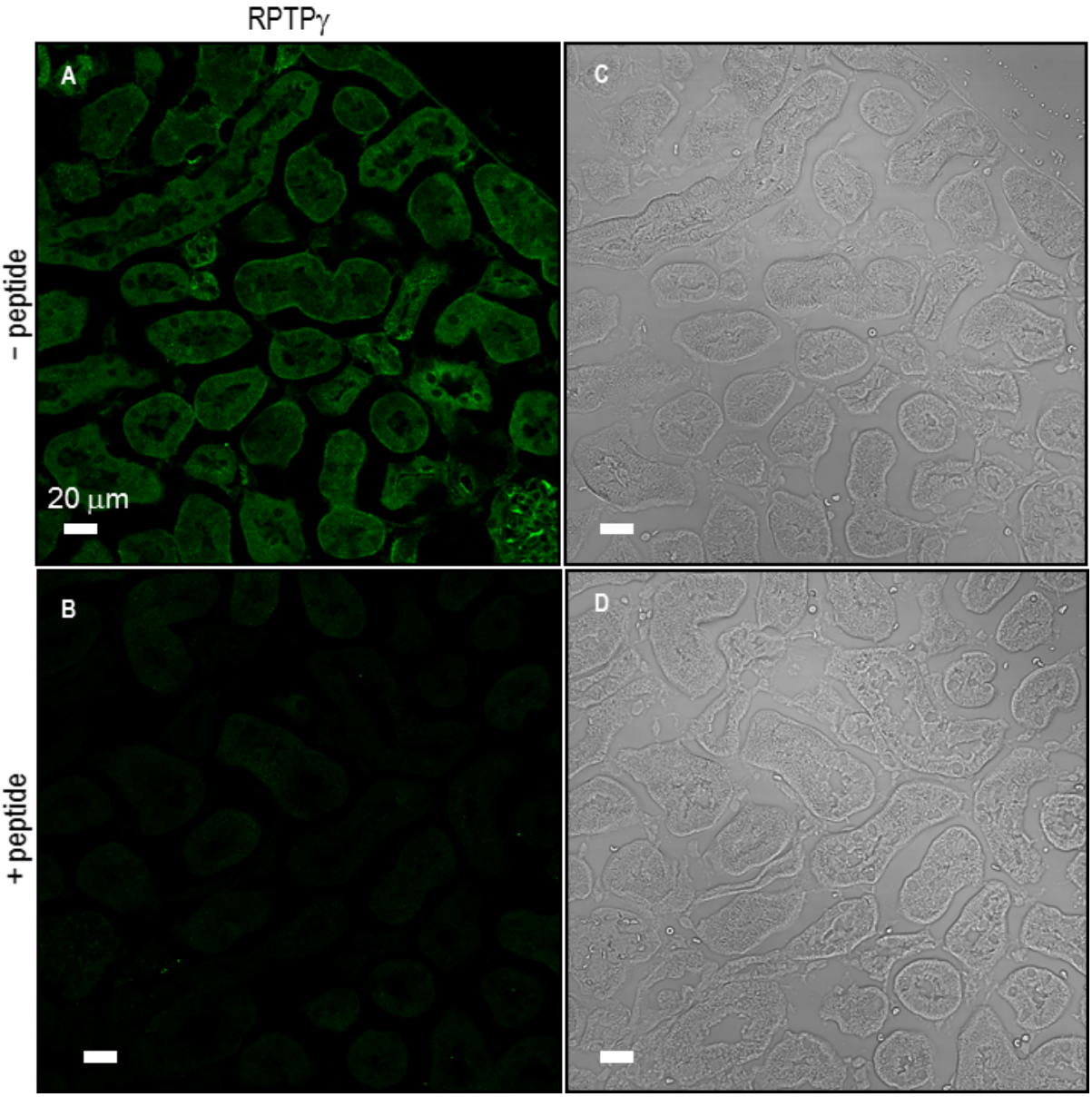
Supplemental Figure 1

RPTPγ structural domains and gene (*Ptprg*) splice variant expression in mouse kidney cortex. (A) Diagram of RPTPγ protein highlighting structural motifs and PCR targets. (B) We isolated RNA from mouse kidney cortex for reverse transcription followed by PCR amplification of the region of interest spanning the splice region indicated. Two rounds of PCR were performed (second-round template was first-round product) and detected on an agarose gel stained with ethidium bromide. As anticipated, we detected a product of 435 bp representing variant A, and a 348 bp band indicative of variant B spanning exons 12-16 of the gene. We cloned and sequenced both products and verified that they match murine *Ptprg* (ref sequence ID: NM_008981.3), with the B product lacking the predicted 29 aa (corresponding to exon 14). Primers used in PCR were 5'agcctatgtcccgaggggaccgattttctgag3' (forward) and 5'tattggaatagagctcaccgatgtg3' (reverse).



Supplemental Figure 2

Epitope design of and immunoblotting with our newly synthesized antibody directed against the CALD region of murine RPTPγ. (A) Homology overlay of murine carbonic anhydrases with the epitope highlighted in blue. **(B)** Western blot of whole kidney lysates isolated from wild-type (WT), RPTPγ$^{-/-}$, or RPTPζ$^{-/-}$ mice using our antibody. Samples were run via SDS-PAGE and membranes were probed with RPTPγ followed by **(C)** actin in addition to **(D)** Coomassie-blue staining of the gel to verify equal loading. As can be seen in panel **(A)** reactivity is apparent in kidneys from WT & RPTPζ$^{-/-}$ mice, but is lacking in material from RPTPγ$^{-/-}$, confirming the specificity of our antibody and demonstrating the expression of the protein in the mouse kidney. The antibody was developed as described in the Methods section.



Supplemental Figure 3

Immunohistochemical verification of our newly developed antibody against RPTPγ in mouse renal cortex. (A) Staining of tissue slices (5 μm) with antibody which was affinity-purified with the peptide used as the epitope demonstrating extensive RPTPγ protein abundance in the mouse kidney. (B) Staining of cortical slices with affinity purified antibody which was then incubated with peptide to block antigen binding providing evidence of the antibody's specificity. (C–D) contrast images of the corresponding renal cortex slices shown in panels (A) & (B). Scale bar represents 20 μm. Immunohistochemistry was performed as described in the Methods section.

Supplemental Table 1. Blood-gas analysis of wild-type (WT) and RPTPγ knockout (RPTPγ $^{-/-}$) mice subjected to 7 days of 1% (w/v) NH_4Cl in drinking water to induce metabolic acidosis (MAc)

Analyte	Day	WT + Ctrl	RPTPγ$^{-/-}$ + Ctrl	WT + MAc	RPTPγ$^{-/-}$ + MAc
pH_a	0	7.41 ± 0.03	7.37 ± 0.02	7.40 ± 0.01	7.38 ± 0.01
	2	7.44 ± 0.01	7.36 ± 0.02 †	7.31 ± 0.02 *♦	7.27 ± 0.01 *†♦
	7	7.42 ± 0.03	7.38 ± 0.02	7.38 ± 0.01 ♦♦	7.29 ± 0.02 *†♦
SBC (mM)	0	20.73 ± 1.00	19.73 ± 0.88	20.12 ± 0.69	19.75 ± 0.73
	2	23.87 ± 0.64	20.37 ± 1.08 †	18.03 ± 0.89 *♦	16.86 ± 0.71 *♦
	7	21.65 ± 1.12	20.27 ± 1.37	19.79 ± 1.04	16.60 ± 0.94
P_{CO2} (mmHg)	0	27.54 ± 1.04	30.32 ± 1.06	27.12 ± 0.43	29.41 ± 1.27
	2	31.35 ± 1.05 ♦	31.70 ± 0.64	31.15 ± 1.66 ♦	31.94 ± 0.72
	7	28.91 ± 1.85	29.87 ± 1.62	28.22 ± 0.92	29.34 ± 1.65
SO_2 (%)	0	96.73 ± 0.36	96.84 ± 0.27	97.29 ± 0.11	96.67 ± 0.16
	2	97.63 ± 0.20	97.28 ± 0.34	96.87 ± 0.38	96.52 ± 0.31
	7	96.63 ± 0.39	96.51 ± 0.56	96.74 ± 0.26	94.69 ± 0.47 *†♦♦
P_{O2} (mmHg)	0	96.70 ± 4.6	101.4 ± 3.7	103.9 ± 2.6	100.0 ± 2.8
	2	93.1 ± 2.6	100.0 ± 3.3	98.2 ± 3.0	97.2 ± 2.6
	7	93.6 ± 2.4	97.6 ± 3.4	97.2 ± 3.1	88.7 ± 2.7 *♦♦
$[HCO_3^-]$ (mM)	0	17.9 ± 0.8	17.7 ± 0.9	17.3 ± 0.6	17.6 ± 0.8
	2	21.8 ± 0.6 ♦	18.6 ± 1.0 †	16.2 ± 0.9 *	15.3 ± 0.8 *♦
	7	19.1 ± 1.0	18.1 ± 1.6	17.3 ± 1.1	14.5 ± 1.1 ♦
TCO_2 (mM)	0	18.7 ± 0.8	18.8 ± 0.9	18.1 ± 0.6	18.4 ± 0.8
	2	22.8 ± 0.6 ♦	19.6 ± 1.0 †	17.1 ± 0.9 *	16.3 ± 0.8 *♦
	7	19.9 ± 1.0 ♦♦	19.1 ± 1.6	18.1 ± 1.1	15.4 ± 1.1 ♦
BEecf (mM)	0	-6.8 ± 1.3	-7.9 ± 1.2	-7.6 ± 0.9	-7.7 ± 1.0
	2	-2.4 ± 0.8 ♦	-7.0 ± 1.4 †	-10.2 ± 1.2 *♦	-11.7 ± 1.1 *♦
	7	-5.5 ± 1.4	-7.2 ± 1.8	-9.0 ± 1.1	-12.2 ± 1.4 ♦
BEb (mM)	0	-4.6 ± 1.3	-5.8 ± 1.2	-5.3 ± 0.9	-5.8 ± 1.0
	2	-0.6 ± 0.7 ♦	-5.1 ± 1.3 †	-7.9 ± 1.2 *♦	-9.7 ± 1.0 *♦
	7	-3.4 ± 1.3	-5.2 ± 1.6	-7.0 ± 1.0	-10.0 ± 1.3 ♦

We sampled arterial blood in conscious mice as described in "Methods". Values are the mean ± SEM (n = 8–16). * $P<0.05$ vs. genotype-matched Ctrl (WT+MAc *vs.* WT+Ctrl or RPTPγ$^{-/-}$+MAc *vs.* RPTPγ$^{-/-}$+Ctrl). † $P<0.05$ vs. water-matched WT (RPTPγ$^{-/-}$+Ctrl *vs.* WT+Ctrl or RPTPγ$^{-/-}$+MAc *vs.* WT+MAc). ♦Denotes a statistically significance change from Day 0 and ♦♦denotes significant change from Day 2 (within the respective treatment group, $p<0.05$). Abbreviations: pH_a, arterial pH; SBC, standard bicarbonate; Pc_{O_2} partial pressure of carbon dioxide; SO_2, saturated oxygen; P_{O_2}, partial pressure of oxygen; $[HCO_3^-]$; bicarbonate concentration calculated from pH_a and P_{CO_2}; TCO_2, total carbon dioxide; BE_{ecf}, base excess of extracellular fluid; BE_{eb}, base excess of blood. All parameters were determined based on established calculations in accordance with standardized clinical chemistry definitions.

Supplemental Table 2.Chemistry analysis of plasma from wild-type (WT) and RPTPγ $^{-/-}$ mice subjected to 7 days of 1% (w/v) NH_4Cl in drinking water to induce metabolic acidosis (MAc).

Analyte	WT + Ctrl	RPTPγ $^{-/-}$ + Ctrl	WT + MAc	RPTPγ $^{-/-}$ + MAc
Na (mEq/L)	146.4 ± 2.3	145.9 ± 2.9	147.2 ± 2.3	148.6 ± 2.1
K (mEq/L)	4.2 ± 0.2	4.7 ± 0.2	4.2 ± 0.1	4.8 ± 0.1†
Cl (mEq/L)	112.5 ± 1.9	111.9 ± 2.6	117 ± 2.1	114.9 ± 2.8
Mg (mg/dL)	1.6 ± 0.0	1.9 ± 0.0	2.4 ± 0.7	1.9 ± 0.1
Ca (mg/dL)	9.3 ± 0.1	9.7 ± 0.2	8.6 ± 0.6	9.5 ± 0.7
GLU (mg/dL)	279.8 ± 23.3	241.3 ± 23.1	254 ± 17.2	258.4 ± 14.5
BUN (mg/dL)	18.8 ± 1.8	23.8 ± 2.7	23.9 ± 1.3	22.5 ± 2.9
CRE (mg/dL)	0.20 ± 0.00	0.25 ± 0.03	0.23 ± 0.03	0.25 ± 0.04
ALB (g/dL)	3.1 ± 0.6	3.0 ± 0.5	2.7 ± 0.4	3.0 ± 0.5
IP (mg/dL)	5.4 ± 1.6	6.5 ± 0.8	4.2 ± 0.8	6.5 ± 0.9
TP (g/dL)	6.5 ± 1.7	5.5 ± 0.7 *	8.4 ± 1.0	8.1 ± 1.1

We sampled arterial blood in conscious mice as described in "Methods". Values are the mean ± SEM (n = 8–16). Statistical analysis was performed using a 2-way ANOVA with the Bonferroni post-hoc analysis using $p<0.05$ as the criterion for significance. * $P<0.05$ vs. genotype-matched Ctrl (WT+MAc *vs.* WT+Ctrl or RPTPγ$^{-/-}$+MAc *vs.* RPTPγ$^{-/-}$+Ctrl). † $P<0.05$ vs. water-matched WT (RPTPγ$^{-/-}$+Ctrl *vs.* WT+Ctrl or RPTPγ$^{-/-}$+MAc *vs.* WT+MAc). Abbreviations: GLU, glucose; BUN, blood urea nitrogen; CRE, creatinine; ALB, albumin; IP, inorganic phosphate; TP, total protein. All parameters were determined based on established calculations in accordance with standardized clinical chemistry definitions.

Supplemental Table 3. Hematological analysis of wild-type (WT) and RPTPγ $^{-/-}$ mice after 7 days of 1% (w/v) NH_4Cl in drinking water to induce metabolic acidosis (MAc).

Analyte	WT + Ctrl	RPTPγ $^{-/-}$ + Ctrl	WT + MAc	RPTPγ $^{-/-}$-MAc
WBC(10^3/μL)	10.1 ± 1.1	8.8 ± 1.0	11.1 ± 0.5	9.4 ± 0.7
LYM(10^3/μL)	8.1 ± 0.9	6.8 ± 0.8	8.0 ± 0.4	7.3 ± 0.5
MONO(10^3/μL)	0.50 ± 0.07	0.48 ± 0.05	0.67 ± 0.08	0.52 ± 0.05
GRAN(10^3/μL)	1.4 ± 0.2	2.1 ± 0.3	2.4 ± 0.4	1.6 ± 0.3
LYM %	79.8 ± 2.4	68.4 ± 4.4	72.6 ± 3.6	77.6 ± 2.8
MONO %	4.4 ± 0.4	5.3 ± 0.4	5.6 ± 0.4	4.9 ± 0.2
GRAN %	15.7 ± 2.2	26.2 ± 4.0	21.7 ± 3.2	17.4 ± 2.5
HCT %	53.1 ± 1.8	46.6 ± 2.7	50.2 ± 1.6	53.5 ± 2.2
MCV (fL)	51.8 ± 0.6	53.8 ± 1.6	52.2 ± 0.7	54.2 ± 1.8
RDWa (fL)	37.5 ± 0.7	44.5 ± 3.1	40.5 ± 1.5	43.4 ± 3.9
RDW %	18 ± 0.2	20.6 ± 0.6 †	19.5 ± 0.4	19.4 ± 0.8
HGB (g/dL)	15.7 ± 0.5	13.6 ± 0.7	14.7 ± 0.4	15.4 ± 0.4
MCHC (g/dL)	29.5 ± 0.1	29.4 ± 0.4	29.3 ± 0.1	28.9 ± 0.4
MCH (pg)	15.3 ± 0.1	15.8 ± 0.4	15.3 ± 0.2	15.6 ± 0.2
RBC (10^6/μL)	10.2 ± 0.4	8.7 ± 0.6 †	9.5 ± 0.2	9.8 ± 0.3
PLT (10^3/μL)	440.2 ± 57.3	460.1 ± 39.6	513.3 ± 50.6	369.6 ± 38.5 †
MPV (fL)	6.2 ± 0.1	6.2 ± 0.1	6.2 ± 0.2	6.3 ± 0.3

We sampled arterial blood as described in "Methods". Values are the mean ± SEM (n = 8–16). Statistical analysis was performed using a 2-way ANOVA with the Bonferroni post-hoc analysis using $p<0.05$ as the criterion for significance. * $P<0.05$ vs. genotype-matched Ctrl (WT-MAc *vs.* WT+Ctrl or RPTPγ$^{-/-}$+MAc *vs.* RPTPγ$^{-/-}$+Ctrl). † $P<0.05$ vs. water-matched WT (RPTPγ$^{-/-}$+Ctrl *vs.* WT+Ctrl or RPTPγ$^{-/-}$+MAc *vs.* WT+MAc). Abbreviations; WBC, white blood cells; LYM, lymphocytes; MONO, monocytes; GRAN, granulocytes; HCT, hematocrit; MCV, mean RBC volume; RDW, RBC width; HGB, hemoglobin; MCHC, mean cell [HGB]; MCH, mean cell hemoglobin; RBC, red blood cells; PLT, platelets; MPV, mean platelet volume.