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Protein-L-isoaspartate O-methyltransferase is required for *in vivo* control of oxidative damage in red blood cells

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ABSTRACT

Red blood cells (RBC) have the special challenge of a large amount of reactive oxygen species (from their substantial iron load and Fenton reactions) combined with the inability to synthesize new gene products. Considerable progress has been made in elucidating the multiple pathways by which RBC neutralize reactive oxygen species via NADPH driven redox reactions. However, far less is known about how RBC repair the inevitable damage that does occur when reactive oxygen species break through anti-oxidant defenses. When structural and functional proteins become oxidized, the only remedy available to RBC is direct repair of the damaged molecules, as RBC cannot synthesize new proteins. Amongst the most common amino acid targets of oxidative damage is the conversion of asparagine and aspartate side chains into a succinimidyl group through deamidation or dehydration, respectively. RBC express an L-isoaspartyl methyltransferase (PIMT, gene name *PCMT1*) that can convert succinimidyl groups back to an aspartate. Herein, we report that deletion of *PCMT1* significantly alters RBC metabolism in a healthy state, but does not impair the circulatory lifespan of RBC. Through a combination of genetic ablation, bone marrow transplantation and oxidant stimulation with phenylhydrazine *in vivo* or blood storage *ex vivo*, we use omics approaches to show that, when animals are exposed to oxidative stress, RBC from *PCMT1* knockout undergo significant metabolic reprogramming and increased hemolysis. This is the first report of an essential role of *PCMT1* for normal RBC circulation during oxidative stress.

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Introduction

Dysfunction of red blood cells (RBC) is a component of numerous diseases and oxidant stress is a key component to both normal RBC aging and also pathological dysfunction.[1] In addition, approximately one of every 70 Americans is transfused with packed RBC each year.[2] RBC are stored for up to 42 days prior to transfusion out of logistical necessity, resulting in oxidative damage that affects their ability to function upon transfusion,[3] thus impacting a wide variety of diseases for which transfusion is essential, including trauma, issues of chronic hemostasis, cancer, and chronic bone marrow disorders.[2] Thus, damaged or dysfunctional RBC are a widespread factor in human disease and therapy.[4]

RBC face particular metabolic challenges regarding generation and mitigation of oxidative damage. First, RBC have a unique source of oxidative stress because of the high load of iron associated with hemoglobin (RBC account for appriximately 66% of bodily iron),[4] which drives radical-generating Fenton reactions. Second, unlike most other tissues, repair of oxidant stress in RBC cannot involve new protein synthesis owing to the lack of nuclei or ribosomes. As such, elucidating and understanding the nonsynthetic pathways by which RBC manage oxidative stress can provide unique clues on cellular responses to oxidant injury. Although reactive oxygen species (ROS) are highly reactive, they preferentially attack particular chemical groups. When damaging proteins, the side chains of asparagine and aspartate are particularly susceptible to oxidative conversion into a succinimidyl group through deamidation or dehydration, respectively.[5] The succinimidyl group is unstable, and

decays approximately 70-85% of the time into an L-isoaspartyl residue, impacting the protein structure (backbone orientation) and function. Clarke, Ingrosso and colleagues identified L-isoaspartyl groups on RBC membrane proteins in response to aging or pathological oxidative stress, such as in glucose 6-phosphate dehydrogenase deficiency or Down syndrome.[6–12] Thus, accumulation of L-isoaspartyl groups in essential RBC proteins correlates with RBC dysfunction and pathology.

A specific repair pathway exists for asparagines and aspartates that have been oxidized into L-isoaspartyl groups. The enzyme L-isoaspartyl methyltransferase (PIMT, gene name *PCMT1*)[13] methylates L-isoaspartyl groups to form an isoaspartyl methyl ester, which can then spontaneously decompose into a normal aspartyl group (returning aspartate to its original stucture and converting asparagine into aspartate). However, the isoaspartyl methyl ester can also decompose back into the "damaged" L-isoaspartyl group, which can again be methylated by PIMT. Due to the stochastic nature of isoaspartyl methyl ester decomposition (as well as ongoing oxidation of aspartates and asparagines by ROS), multiple and ongoing cycles of PIMT-dependent methylation are required – a process that is constrained both by sufficient PIMT activity and a sufficient pool of methyl donors as co-factors (e.g., of S-Adenosyl-methionine [SAM]).

Recently, we have observed that oxidant stressors from either environmental insults or genetic disease results in the extensive methylation of at least 116 RBC proteins in proximity to their active sites.[14] Moreover, tracing experiments with $^{13}C^{15}N$-methionine (substrate for the synthesis of SAM – the methyl group donor in PIMT-dependent reactions) revealed that the formation of isoaspartyl ^{13}C-methyl-esters was increased in response to oxidative insults. Thus, a model has emerged in which oxidative damage induces formation of L-isoaspartyl groups, which are then methylated by PIMT, resulting in repair of oxidized protiens without the need for resynthesis. However, each of the observations that support his model are correlative. The goal of this paper is to test the causal role of the PIMT pathway in RBC oxidant-damage repair through genetic ablation of *PCMT1*.

Methods

Animal studies with mice

All the animal studies described in this manuscript were reviewed and approved by either the BloodworksNW Research Institute IACUC or the University of Virginia Institutional Animal Care and Use Committee. *PCMT1*$^{+/-}$ founders[15] were acquired from the National Institutes of Health mouse embryo repository and were bred with C57BL/6 females. Interbreeding of *PCMT1*$^{+/-}$ (henceforth PCMT1 heterozygous) mice was designed to generate *PCMT1*$^{-/-}$ (*PCMT1* KO), as confirmed by genotyping. The use of Ubi-GFP and HOD mice have been previously described in prior work from our group.[16] Whole blood was drawn by cardiac puncture as a terminal procedure for *PCMT1* KO or WT mice, followed by harvesting of organs (10 mg of tissues from brain, heart, kidney, liver and spleen). Tissues and blood were snap frozen in liquid nitrogen and stored at -80°C until subsequent analysis. For transfusion studies, fresh RBC (never frozen) were used.

Diamide treatments

RBC from WT and PCMT1 KO mice were incubated with diamide (0.5 μM, Sigma Aldrich) at 37°C for 0, 3 and 6 hours (h), as previously described,[17] prior to metabolomics analyses.

Bone marrow transplantation and phenylhydrazine treatment

BMT was performed as previously described, but with WT or *PCMT1* KO donors and green fluorescence protein (GFP) recipients.[18] Engraftment was monitored by the appearance of GFP-negative RBC and the dissappearance of GFP-positive RBC. On average, GFP-positive RBC were undetectable after approximatley 56 days, consistent with the known RBC lifespan of mice.

Prior to treatment with phenylhydrazine (PHZ) [Sigma Aldrich, USA], mice were injected daily for 3 consecutive days with biotin-XSE (ThermoFisher, Waltham, MA, USA Cat# B1582) until 100% biotinylation of RBC was achieved. Each injection consisted of 1 mg biotin-XSE in a 8% solution of dimethyl sulfoxide (DMSO) in phosphate buffered saline. Mice were then given two intraperitoneal injections (6 h apart) of PHZ to achieve a final dose of 0.01 mg/g body weight. Perpheral blood was then harvested longitudinally and RBC stained with avidin-APC to allow enumeration of RBC that had been present at time of PHZ treatment and to distinguish them from RBC generated by hematopoeisis after PHZ injection.

Storage under blood bank conditions

RBC were collected, processed, stored, transfused and post-transfusion recovery was determined as previously described.[19]

Tracing experiments with labeled glucose and methionine

RBC from WT and *PCMT1* KO mice (100 μL) were incubated at 37°C for 1 h in the presence of 1,2,3-$^{13}C_3$-glucose or ^{13}C-methionine, prior to determination of lactate isotopologues +2/+3 (as markers of pentose phosphate pathway to glycolysis fluxes) and ^{13}C-SAM (as marker of methyltransferase activity[14]), as previously described.[20]

Sample processing and metabolite extraction

A volume of 50 μL of frozen RBC aliquots was extracted in 450 μL of methanol:acetonitrile:water (5:3:2, v/v/v). After vortexing at 4°C for 30 minutes (min), extracts were separated from the protein pellet by centrifugation for 10 min at 10,000 RPM at 4°C and stored at –80°C until analysis.

Ultra-high-pressure liquid chromatography-mass spectrometry metabolomics

Analyses were performed using a Vanquish ultra-high-pressure liquid chromatography-mass spectrometry (UHPLC) coupled online to a Q Exactive mass spectrometer (MS) (Thermo Fisher, Bremen, Germany). Samples were analyzed using a 3 min isocratic condition or a 5, 9 and 17 min gradient as described.[21] Solvents were supplemented with 0.1% formic acid for positive mode runs and 1 mM ammonium acetate for negative mode runs. MS acquisition, data analysis and elaboration was performed as previously described.[21–23]

Proteomics

Proteomics analyses were performed via filter aided sample preparation (FASP) digestion and nano UHPLC-MS/MS identification (nanoEasy LC 1000 coupled to a QExactive HF, Thermo Fisher), as previously described.[24]

Statistical analyses

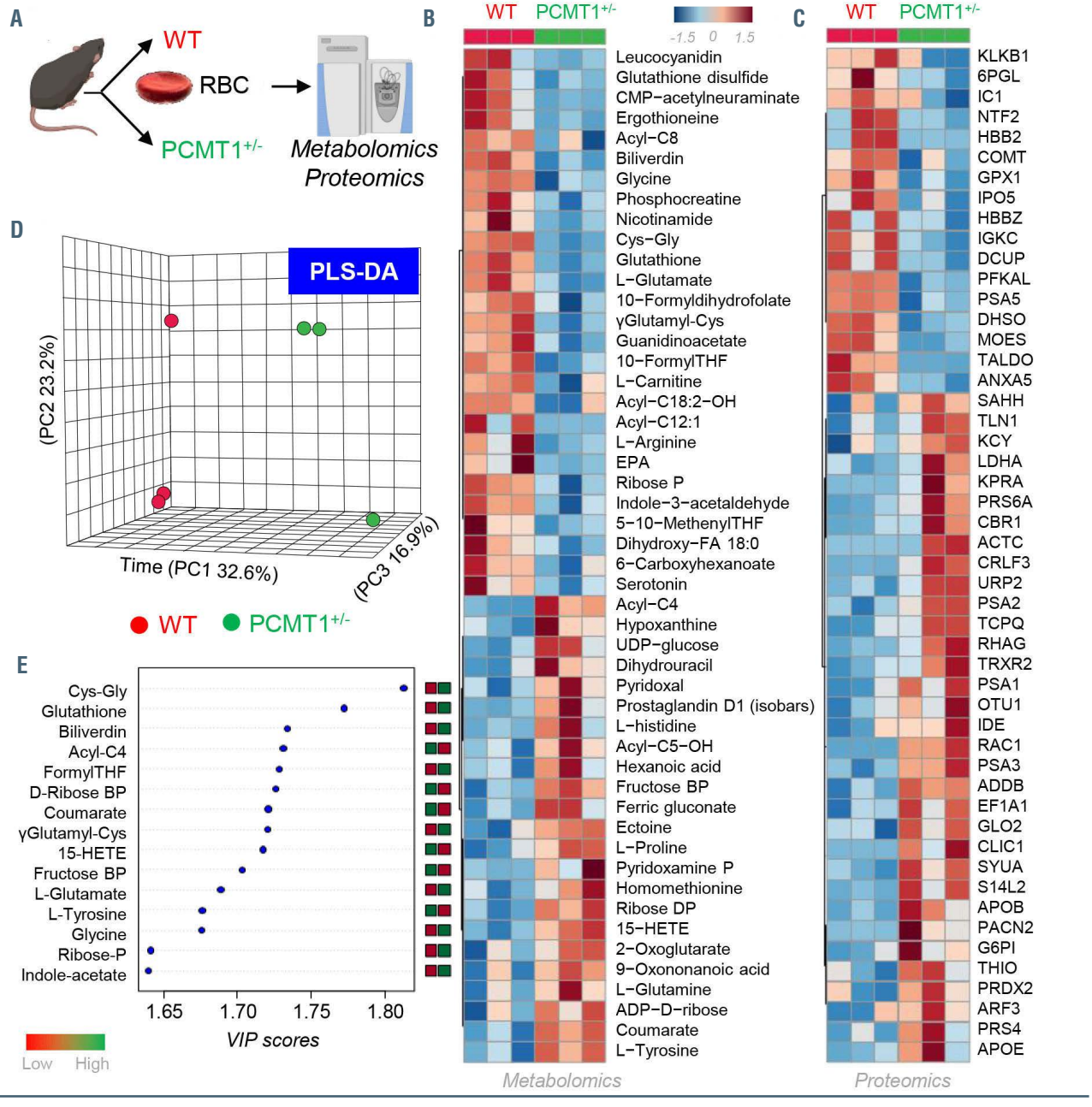
Graphs and statistical analyses (either *t*-test or repeated measures ANOVA) were prepared with GraphPad Prism 5.0 (GraphPad

Software, Inc, La Jolla, CA) and MetaboAnalyst 4.0, which allows to calculate false discovery rate (FDR)-corrected *P*-values from *t*-test and ANOVA analyses.[25] Heat maps include color are graphed as Z-score normalized values for each metabolite/protein (ranges included as legends within each panel containing a heat map); each single box in the heat maps indicates a distinct biological replicate per each group. All the raw data from the omics analyses reported in this study are included in the *Online Supplementary Table S1*.

Results

Lack of a single copy of *PCMT1* results in significant alterations in the metabolome and proteome of red blood cells compared to wild type red blood cells

PCMT1$^{+/-}$ RBC were compared to WT controls (Figure 1A). As predicted, phenotypes between the two mouse strains differed significantly both at the protein and metabolite level (Figure 1B and C report the top 50 significant metabolites and proteins by *t*-test, respectively). Unsupervised analyses revealed a significant impact of PCMT1 heterozygosity on the omics phenotypes of RBC, with approximately 33% of the total variance between the two groups explained by a single principal component (PC1 in Figure 1D). Metabolites with the highest loading weights are highlighted in Figure 1E, showing a significant impact on pathways relevant to redox/NADPH homeostasis (glutathione, ribose phosphate, biliverdin, formyl-tetrahydrofolate, cysteinyl-glycine, gamma-glutamyl-cysteine) and amino acids (glutamate, tyrosine, glycine) in *PCMT1*$^{+/-}$ RBC. Of note, lower levels of enzymes involved in these pathways were detected in *PCMT1*$^{+/-}$ RBC, includ-



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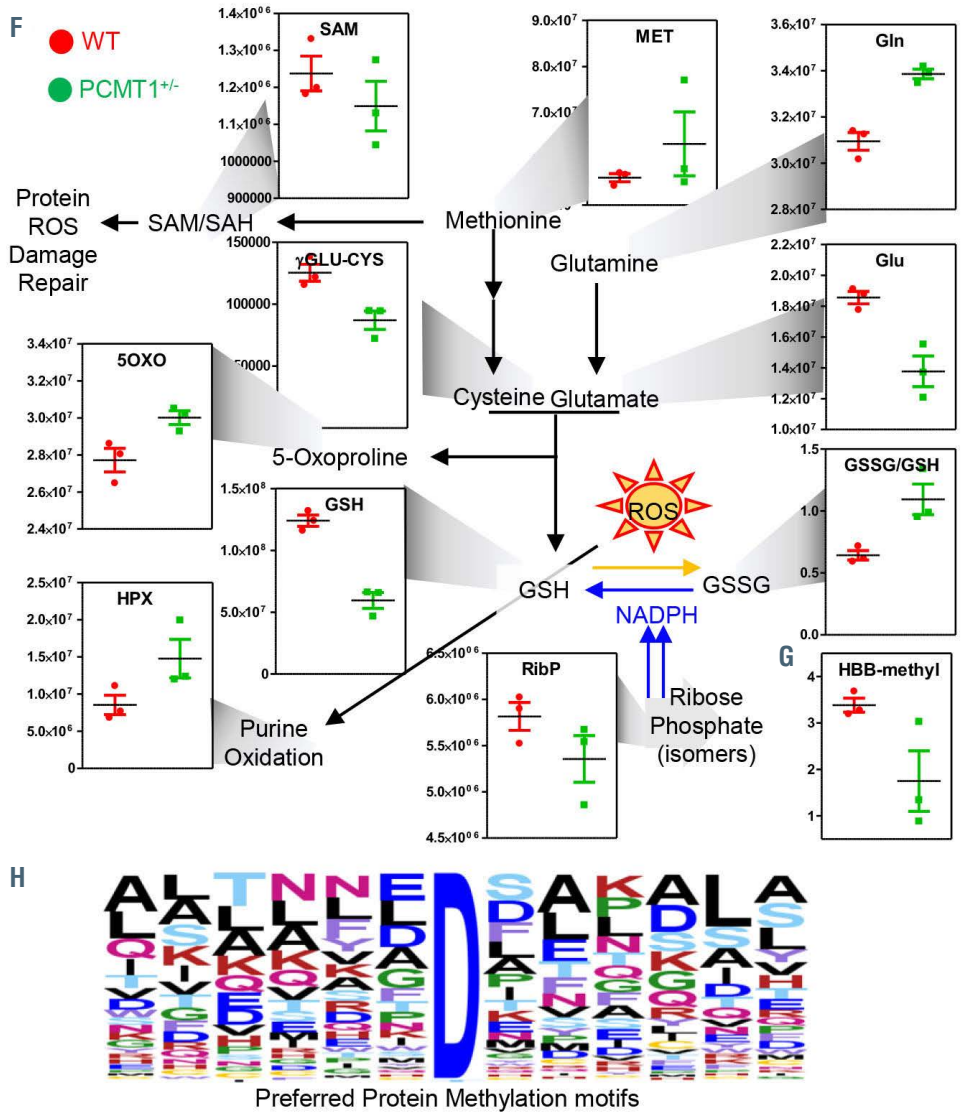


Figure 1. Metabolomics and proteomics of heterozygous *PCMT1*$^{+/-}$ mouse red blood cells. (A) Metabolomics and proteomics of heterozygous *PCMT1*$^{+/-}$ (heterozygous *PCMT1*) mouse red blood cells (RBC); (B) and (C) top 50 metabolites and proteins by *t*-test between wild-type (WT) and heterozygous *PCMT1* mice, respectively; (D to F) Partial Least Square-Discriminant Analysis, Variable Importance in Projection and methionine metabolism in RBC from WT and heterozygous *PCMT1* mice; (G) quantitative analysis of methylated deamidated asparagine residues in hemoglobin – as representative for the RBC proteome; (H) preferred protein methylation motifs as identified in the RBC proteome of WT *vs.* heterozygous *PCMT1* mice.

ing 6-phosphogluconolactonase (6PGL), transaldolase (TALDO), glutathione peroxidase 1 (GPX1) (Figure 1C). Conversely, the levels of other enzymes involved in alternative redox regulation pathways were increased in *PCMT1*$^{+/-}$ RBC, including peroxiredoxin 2 (PRDX2), thioredoxin reductase 2 (TRXR2), hydroxyacyl glutathione hydrolase (GLO2), glucose 6-phosphate isomerase (G6PI), lactate dehydrogenase B (LDHB) (Figure 1C). Accordingly, we focused on these pathways to provide a more detailed overview of the metabolic changes observed in RBC from these mice (Figure 1F). Specifically, we noted an accumulation of methionine (the main methyl group donor precursor - Figure 1F), consistent with a decreased PIMT levels and activity. Likewise, differential D-methylation patterns were observed between WT and *PCMT1*$^{+/-}$ RBC, especially in regions in which aspartyl groups are flanked by other negatively charged residues (D, E, S) in proximity of deamidation susceptible asparaginyl groups (N – representative methylation of deamidated N is shown for the most abundant protein, hemoglobin - Figure 1G, the full list is stated in the *Online Supplementary Table S1*). Notably, significantly higher levels of band 3 methylation of residues D329 and 368 was observed in WT mice compared to heterozygous mice (*Online Supplementary Table S1*).

Lack of both copies of *PCMT1* exacerbates the phenotypes observed in heterozygous mouse red blood cells

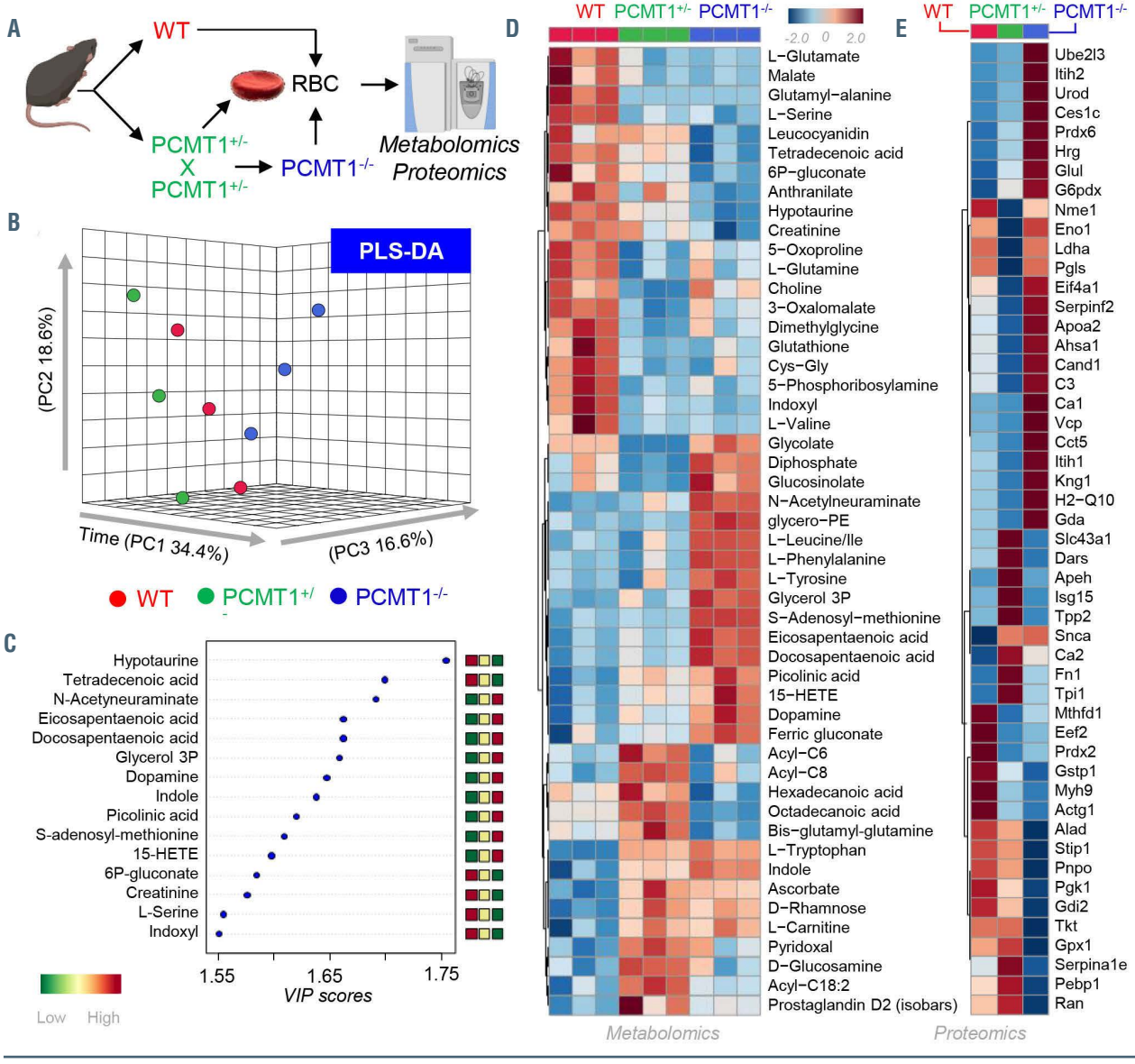
PCMT1 KO mice die at approximately 6-8 weeks from seizures due to accumulated protein damage in brain tissue.[15,26] *PCMT1* KO mice were obtained by crossing of *PCMT1* heterozygous mice and were characterized at 5 weeks of age (prior to the onset of seizures) compared to age-matched WT mice; to ensure that the same background genetics were present in each group, *PCMT1* KO, WT and heterozygous mice were all obtained from the same colony of interbreeding heterozygous mice (Figure 2A). Unsupervised principal component analysis (PCA) revealed a progressive alteration of the metabolic phenotype of RBC from WT to heterozygous and KO mice (Figure 2B), with the main metabolic discriminants across groups being pentose phosphate pathway (PPP) metabolites (6-phosphogluconate, sedoheptulose phosphate, ribose phosphate), polyunsaturated fatty acids (eicosa- and docosahexaenoic acid), oxylipins (15-HETE), tryptophan and tyrosine metabolites (including several indoles, picolinic acid, dopamine – Figure 2C). Changes in the metabolome and proteome (Figure 2D to E) extended the observations from the heterozygous mouse RBC, showing an apparent gene

dose effect for many analytes. In particular, methionine consumption and utilization of S-adenosylmethionine were progressively impaired in the heterozygous and KO mice (Figure 2E), a finding that we confirmed with tracing of stable isotope labeled ^{13}C-methionine (^{13}C-SAM/^{13}C-SAH levels increase almost 3-log fold in KO mice compared to WT - Figure 2G). However, in some cases, loss of a single copy of PCMT1 resulted in a change that was not further exacerbated by deletion of both PCMT1 copies (e.g., decreased GSH, G3P, and 5OXO as well as increased Glucose, DPG, PEP). These findings suggest a threshold effect for some metabolites in response to loss of a single copy of PCMT1. Overall, altered glutathione homeostasis (decreased levels of reducing equivalent pools) was accompanied by an apparent compensatory decrease in glycolysis (steady state levels of glycolytic intermediates in Figure 2E) and activation of the PPP (Figure 2E), which we confirmed with tracing of lactate isotopologues[27] +2/+3 upon incubation with 1,2,3-$^{13}C_3$-glucose (Figure 2F).

Unexpectedly, genetic ablation of *PCMT1* had a dose response effect on tryptophan and tyrosine metabolism (*Online Supplementary Figure S1*). Specifically, accumulation of tryptophan and the products of its breakdown (indole) and oxidation (picolinic acid and quinolinic acid, but not anthranilate) were observed in RBC of heterozygous and KO mice (*Online Supplementary Figure S1*). Similarly, accumulation of tyrosine and dopamine was observed in heterozygous and KO mice, in which the levels of taurine, hypotaurine and acetylcholine were decreased (*Online Supplementary Figure S1*).

Metabolic characterization of organs from wild-type and *PCMT1* knockout mice

Since picolinic and quinolinic acid are neurotoxicants[28] and *PCMT1* KO mice are characterized by early mortality due to seizures, the metabolomes of different organs from WT and KO mice were characterized, namely brain, heart, kidney, liver and spleen (Figure 3A). PCA



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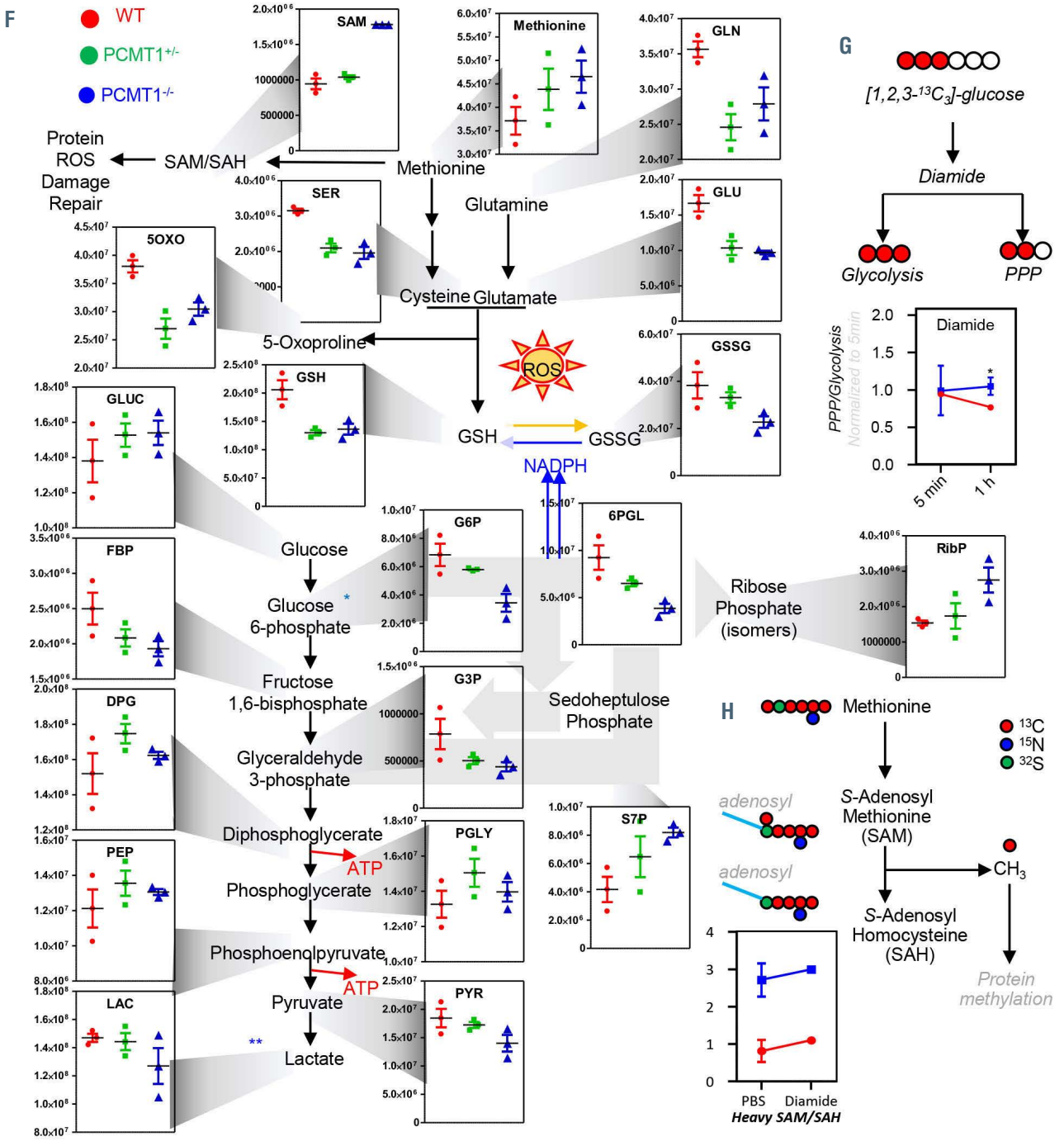


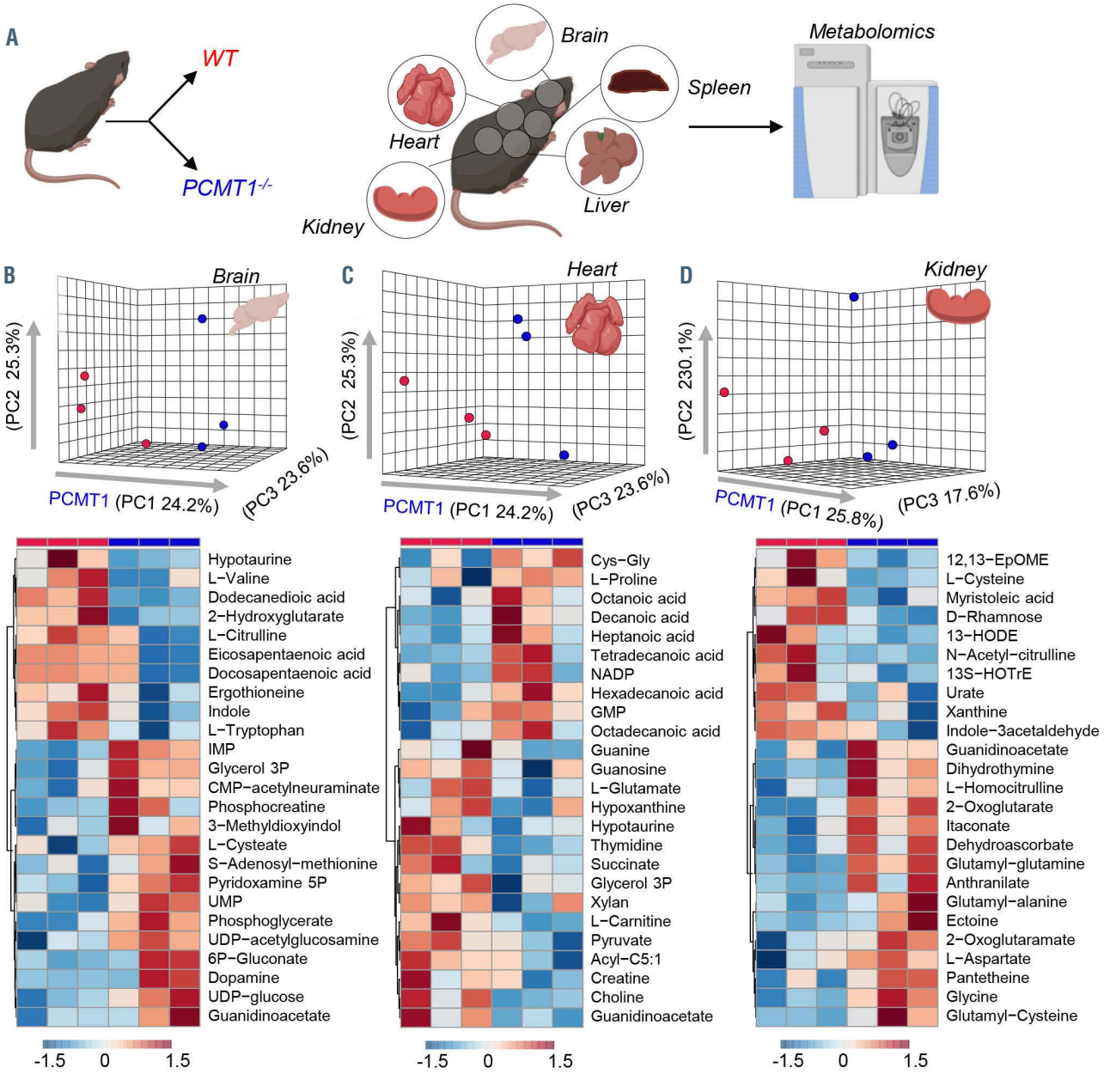
Figure 2. Metabolomics and proteomics of *PCMT1*$^{-/-}$ knockout mouse red blood cells. (A) Metabolomics and proteomics of *PCMT1*$^{-/-}$ knockout (*PCMT1* KO) mouse red blood cells (RBC); (B and C) Partial Least Square-Discriminant Analysis, Variable Importance in Projection; (D and E) top 50 metabolites and proteins by *t*-test between wild-type (WT), heterozygous *PCMT1*$^{+/-}$ (heterozygous *PCMT1*) and *PCMT1* KO mice, respectively (only group averages are shown for proteomics data); (F) an overview of glycolysis, methionine metabolism, the pentose phosphate pathway and glutathione homeostasis in WT, heterozygous and *PCMT1* KO mice; (G and H) tracing experiments with 1,2,3-13C3-glucose and ^{13}C-methionine in WT and *PCMT1* KO RBC prior to and after oxidant challenge with diamide. ROS: reactive oxygen species.

and heat maps (top 25 significant metabolites by t-test) for each organ are provided in alphabetical order in Figure 3B to F. Of note, differences in brain phenotypes were comparable to those observed in RBC, including decreases in polyunsaturated fatty acids, hypotaurine and increases in PPP metabolites and SAM in the KO mice (Figure 3B). However, *PCMT1* KO mouse brains were characterized by lower tryptophan and indole. In contrast, all the other organs showed metabolic phenotypes clearly distinct from RBC and brains. In particular, hearts from KO mice were characterized by significant increases in the levels of several saturated fatty acids (7:0, 8:0, 10:0, 14:0, 16:0, 18:0) and decreased levels of purines (guanine, guanosine, hypoxanthine) and Krebs cycle metabolites (succinate – Figure 3C). Kidneys had similar trends with respect to purines (decreases in hypoxanthine, xanthine and urate), but an opposite phenotype with respect to several fatty acids and related oxidation

products, which decreased in KO mice, whereas carboxylic acids increased (2-oxoglutarate, itaconate - Figure 3D). Comparably to kidneys, similar increases in several fatty acids were noted in livers from KO mice (myristoleic, palmitoleic, dodecanedioic and tetradecenoic acids), though *PCMT1* KO livers were also characterized by significant decreases in the levels of several amino acids (leucine, lysine, cysteine, phenylalanine, threonine – Figure 3E). Spleens from KO mice had lower levels of several 18 carbon-chained fatty acids (similarly to brains and kidneys, opposite to RBC, heart and liver – Figure 3F). In summary, while different organs each had distinct metabolic phenotypes, all organs from KO mice were characterized by (i) lower levels of methionine utilization, (ii) decreased pools of glutathione and glutathione precursors, and (iii) compensatory activation of the PPP (as inferred from steady state levels of intermediates, especially in brain and spleen), suggestive of an altered capacity to cope with oxidant stress in KO mice.

Exacerbation of metabolic differences in red blood cells from wild-type and *PCMT1* knockout mice by oxidant stress with diamide *in vitro*

While the above analysis showed a clear effect of *PCMT1* on the metabolome, it was performed in a baseline state of oxidative stress. In order to test the hypothesis that *PCMT1* KO RBC would be impaired in their response to an oxidative insult, RBC were incubated with diamide *in vitro* for up to 6 h (Figure 4A). While the overall metabolic impact of deleting *PCMT1* was greater in the RBC metabolome than the effect of diamide treatment (Figure 4B), we identified a subset of metabolites that had a higher baseline level in WT RBC and which increased further following diamide treatment only in WT but not in KO mice (Figure 4C – blue box).



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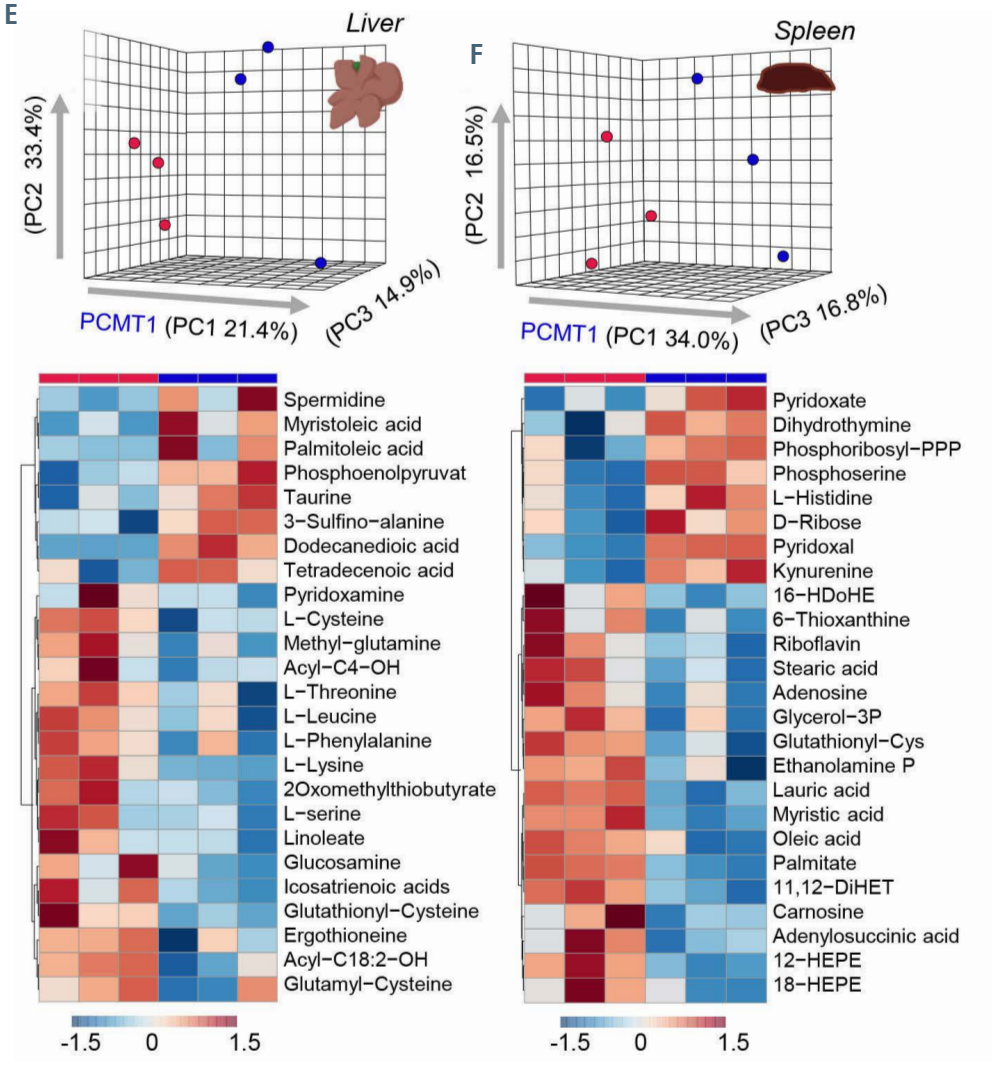


Figure 3. Organ metabolomics in wild-type and *PCMT1*$^{-/-}$ knockout mice. (A) Organ metabolomics in wild-type (WT) and *PCMT1*$^{-/-}$ knockout (*PCMT1* KO) mice; (B) metabolomics of brain; (C) heart; (D) kidney; (E) liver and (F) and spleen in WT and *PCMT1* KO mice. Each panel illustrates the results from Partial Least Square-Discriminant Analyses and the top 25 significant metabolites by *t*-test for each organ.

Highlights of metabolites in this group are presented in Figure 4D including several tryptophan metabolites (kynurenine, nicotinamide, hydroxyindoleacetate), PPP and glutathione homeostasis metabolites (ribose phosphate, glutathionyl-cysteine, ergothioneine). The increase of these metabolites in WT but not *PCMT1* KO mice demonstrate a defect in the ability of *PCMT1* KO RBC to further activate the PPP following additional exogenous insults. Thus, in addition to impairing protein repair, lack of *PCMT1* prevents compensatory metabolic shifts to environmental insults.

Increased *in vivo* hemolysis in response to systemic oxidant stress

In order to allow the study of *PCMT1* KO RBC in adult mice *in vivo*, bone marrow transplant (BMT) was carried out with donors being *PCMT1* KO or WT controls (5 weeks of age prior to seizure onset in *PCMT1* KO) and recipients being Ubi-GFP recipient mice that express GFP in all blood lineages. This allowed monitoring of engraftment by evaluating the progressive decline in GFP positivity in circulating blood cells via flow cytometry (Figure 5A). No significant difference was seen in the rate of RBC engraftment from *PCMT1* KO *versus* WT donors (approximately 56 days) (Figure 5B). Consistent with what was observed for RBC from 5-week-old *PCMT1* KO mice compared to WT controls, the metabolic phenotypes of RBC from *PCMT1* KO→WT mice showed significant accumulation of SAM compared to WT→WT RBC, as well as decreases in glutathione (total pool – including reduced and oxidized) and increases in dopamine and tryptophan metabolites (indole, indole acetate, 3-methyldioxyindol) (Figure 5C). Conversely, BMT *PCMT1* KO→WT RBC showed higher levels of glycolytic intermediates (glucose 6-phosphate, glyceraldehyde 3-phosphate, 2,3-diphosphoglycerate and isobaric isomer, phosphoenolpyruvate) and lower steady state levels of PPP metabolites (6-phosphogluconate and ribose phosphate) or other pathways involved in NADPH homeostasis (folate, pyruvoyl-THF – Figure 5C). Together, these results suggested a lower antioxidant capacity of RBC from *PCMT1* KO donors compared to WT donors; however, this difference did not affect the ability of hematopoietic stem cells to generate RBC or RBC to circulate in an otherwise healthy state.

In order to test the effects of oxidant stress that was increased from normal healthy conditions, mice were treated intravenously with a biotinylating reagent such that 100% of RBC were biotinylated. Mice were then injected with PHZ, which induces oxidant stress and is known to be toxic to RBC (Figure 6A). Clearance of RBC was then monitored by assessing the percentage of Biotin + RBC over time in peripheral blood. Significantly higher clearance of

PCMT1 KO RBC was observed compared to WT RBC following two consecutive (6 h apart) injections of PHZ *in vivo* (Figure 6B), resulting in double the clearance of the *PCMT1* KO RBC within the first 30 h from PHZ injection. Of note, the metabolic phenotypes of *PCMT1* KO→WT RBC upon PHZ-induced stress showed significant decreases in the levels of glutathione pools (both reduced and oxidized) and accumulation of several short chain fatty acids (e.g., 5:0, 6:0, 8:0, 9:0-OH, 12:0) markers of fatty acid breakdown and oxidation in the mitochondria-devoid mature RBC (Figure 6C).

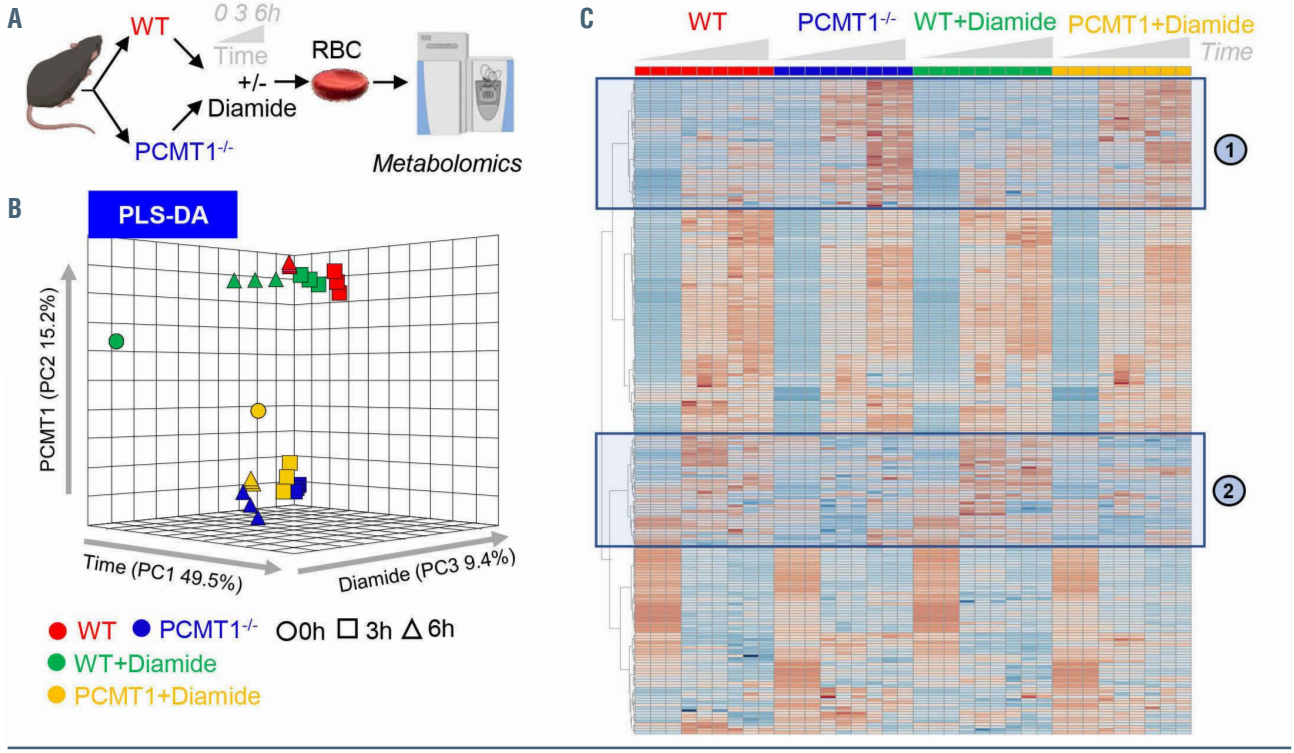
Increased oxidant stress but normal circulation in red blood cells from *PCMT1* mice following blood storage

We hypothesized that RBC from *PCMT1* KO mice would be more susceptible to storage-induced damage (Figure 7A). RBC were collected from WT and *PCMT1* KO mice (at 5 weeks of age prior to seizures) and stored at 2-6 °C for 12 days. Stored RBC were mixed with fresh tracer cells (HOD RBC that express a trackable transgene) in order to control for differences in injection or blood volume. The mixture was transfused into Ubi-GFP+ recipient mice. This approach allows us to determine the percentage of test RBC (GFP- HOD-) as a function of control RBC (GFP-HOD+) that are circulating at 24 h after transfusion, a parameter referred to as post-transfusion recovery (PTR) and one of the Food and Drug Administration-mandated gold standards for RBC storage quality in humans.[29]

Contrary to our prediction, both fresh and stored WT and *PCMT1* KO RBC showed comparable PTR (Figure 7B). Despite the lack of a phenotype with respect to PTR, *PCMT1* KO RBC were characterized by (i) a decrease in methionine consumption and accumulation of SAM, but not SAH (consistent with the lack of *PCMT1* activity); (ii) significantly lower glutathione pools (especially GSSG) and increased oxidation of methionine thiols to sulfoxide; (iii) increased levels of purine and lipid oxidation markers (hypoxanthine; 15-HETE, 12,13-diHOME, 9-oxononanoic acid) (Figure 7C), previously identified as predictors of poor post-transfusion recoveries in mice and humans.[16,30,31] On the other hand, stored *PCMT1* KO mouse RBC showed significantly higher levels of PPP metabolites (gluconolactone phosphate), the CoA precursor pantotheine (indirectly involved in the Lands cycle for the recycling of oxidized lipids) and sphingosine 1-phosphate, at least at baseline (Figure 7C and D).

Discussion

Herein, we report that while *PCMT1* is not required for normal erythropoiesis or RBC circulatory lifespan, upon exposure to oxidative stress, RBC lacking PCMT1 are more rapidly cleared from circulation. Metabolically, the absence of PIMT results in a severe depletion of the glutathione pools and a compensatory activation of the PPP to generate the reducing equivalents required for the recycling of oxidized thiols. This occurs in both RBC and also peripheral organs. Additional metabolic perturbations are also observed, including increased levels of tryptophan oxidation products (especially metabolites in the kynurenine pathway) and dopamine in both RBC and various organs, including the brain. Since metabolites in the kynurenine pathway can be neurotoxicant,[28] it is interesting to note how our metabolomics analysis could provide a potential clue as to the mechanisms that drive early onset of seizures in mice lacking both copies of PCMT1. To our knowledge, this is the first report showing an essential role of PIMT in maintenance of RBC circulatory capability during oxidative stress.



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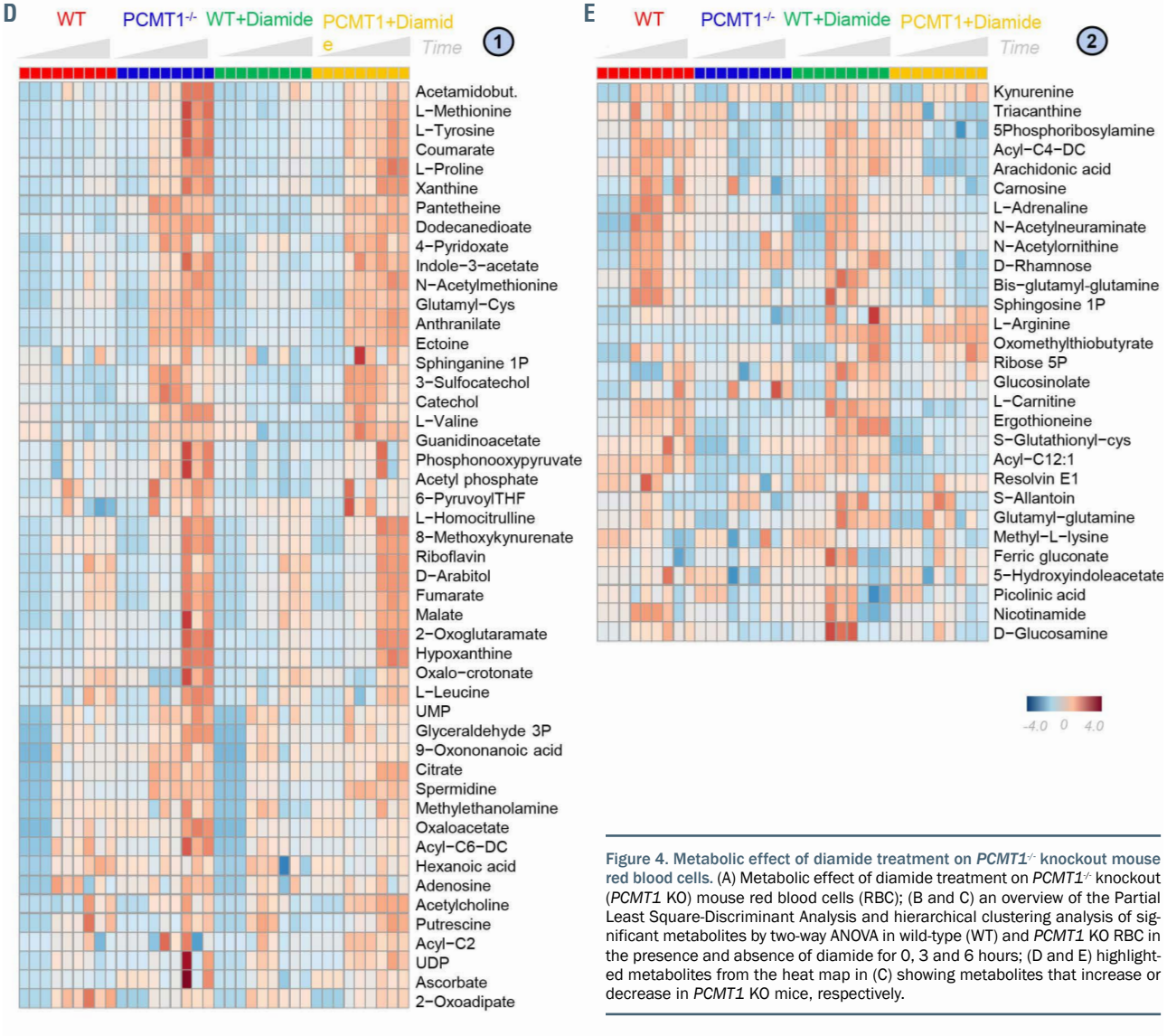

Figure 4. Metabolic effect of diamide treatment on *PCMT1*$^{-/-}$ knockout mouse red blood cells. (A) Metabolic effect of diamide treatment on *PCMT1*$^{-/-}$ knockout (*PCMT1* KO) mouse red blood cells (RBC); (B and C) an overview of the Partial Least Square-Discriminant Analysis and hierarchical clustering analysis of significant metabolites by two-way ANOVA in wild-type (WT) and *PCMT1* KO RBC in the presence and absence of diamide for 0, 3 and 6 hours; (D and E) highlighted metabolites from the heat map in (C) showing metabolites that increase or decrease in *PCMT1* KO mice, respectively.

The potential role of PIMT in RBC blood storage remains unclear. As with any negative finding, the lack of change in PTR for *PCMT1* KO RBC does not formally rule out that PIMT may be involved in the biology of stored RBC, if one evokes the possibility of redundant pathways. However, given the metabolic changes seen in *PCMT1* KO RBC, we do not believe redundant pathways are likely. More importantly, a number of the metabolites that are increased in *PCMT1* KO RBC have shown a strong inverse correlation to PTR in both human and murine RBC, in particular increased hypoxanthine[30] and oxidized lipid species.[16] This correlation has held under multiple experimental conditions and contexts; as such, oxidized lipids have been posited to play a causal role in post-transfusion clearance of stored RBC. However, the current report instantiates a condition in which there is an increase in hypoxanthine and certain oxidized lipids, but normal PTR. Thus, this counts as importance evidence against the lipid oxidation hypothesis of RBC clearance after storage. On the other hand, it is worth noting that *PCMT1* KO RBC have a compensatory activation of the PPP, which is relevant in the light of the recently reported decreases in PTR in blood donors with G6PD deficiency,[29,32] the most common enzymopathy in humans and thus clinically relevant for ~400 million individuals worldwide. It remains to be assessed whether ablated PIMT activity may result in poor post-transfusion performances of the stored RBC in the context of G6PD deficiency or other genetic factors that have been shown to negatively impact post-transfusion recoveries in mice (e.g., STEAP3[16]). In addition, the murine recipients here were all healthy, which is not consistent with the pro-oxidant environment the stored RBC faces upon transfusion in the critically ill or chronically hypoxic recipient (e.g., trauma or sickle cell patient, respectively).[33,34]

Several unanticipated alterations in metabolism were observed in *PCMT1* KO mice, which serve as grounds for reasonable hypothesis driven speculation in the context of broader pathological consequences. Increased kynurenine pathway metabolites have been suggested to be responsible for neurotoxicity, as reported in Down syndrome.[28] Prior reports had also suggested an increase in isoaspartyl damage and PIMT activation in Down syndrome.[7] Dysregulation of methionine metabolism has also been reported in RBC from individuals with Down syndrome;[35] this has been partially explained by the localization on chromosome 21 of genes coding for enzymes involved in homocysteine metabolism. Of note, alterations of RBC kynurenines and indoles (breakdown product of trypto-

phan metabolism) have been reported in RBC from aging mice and mouse models of parabiosis, raising the possibility of a role for PIMT-associated dysregulation of tryptophan metabolism in the aging process. On the other hand, dopamine – whose metabolism is dependent on NADPH – has been shown to accumulate in RBC from individuals with G6PD deficiency,[32] as well as in response to exercise-induced oxidant stress.[36] These hypotheses will need to be rigorously assessed by subsequent studies, but the pattern that emerges from the current findings are consistent with a possible role of PIMT in these processes.

An important implication of the findings in this report is that there are widespread metabolic effects of deleting PCMT1, far beyond the predicted decrease in methionine consumption as a PIMT substrate. Rather, general shifts were seen in PPP activity, both at baseline as well as in response to oxidative stress. Moreover, as above, alterations in other metabolic pathways were likewise observed. This raises the important question of how PIMT contributes to widespread metabolic effects, since the known activity of PIMT is with regards to repairing oxidative damage once it has occurred and not altering the metabolism of oxidative pathways. The most likely hypothesis is that aspartate and asparagine residues are critically important in a wide range of metabolic enzymes and failure of PIMT to repair them when they are oxidized, results in alterations of these pathways through alteration of enzymatic activity or even inactivation. As isoaspartyl damage would impact protein backbone orientation, while methylation of isoaspartyl groups would affect the charge of side chains, one could speculate that PIMT activity (or lack thereof) could play a role in enzymatic function[37] and RBC structural/function homeostasis through regulation of protein-protein interactions, in like fashion to phosphorylation.[38] A related, but distinct hypothesis is that lack of PCMT1 results in functional alteration of proteins involved

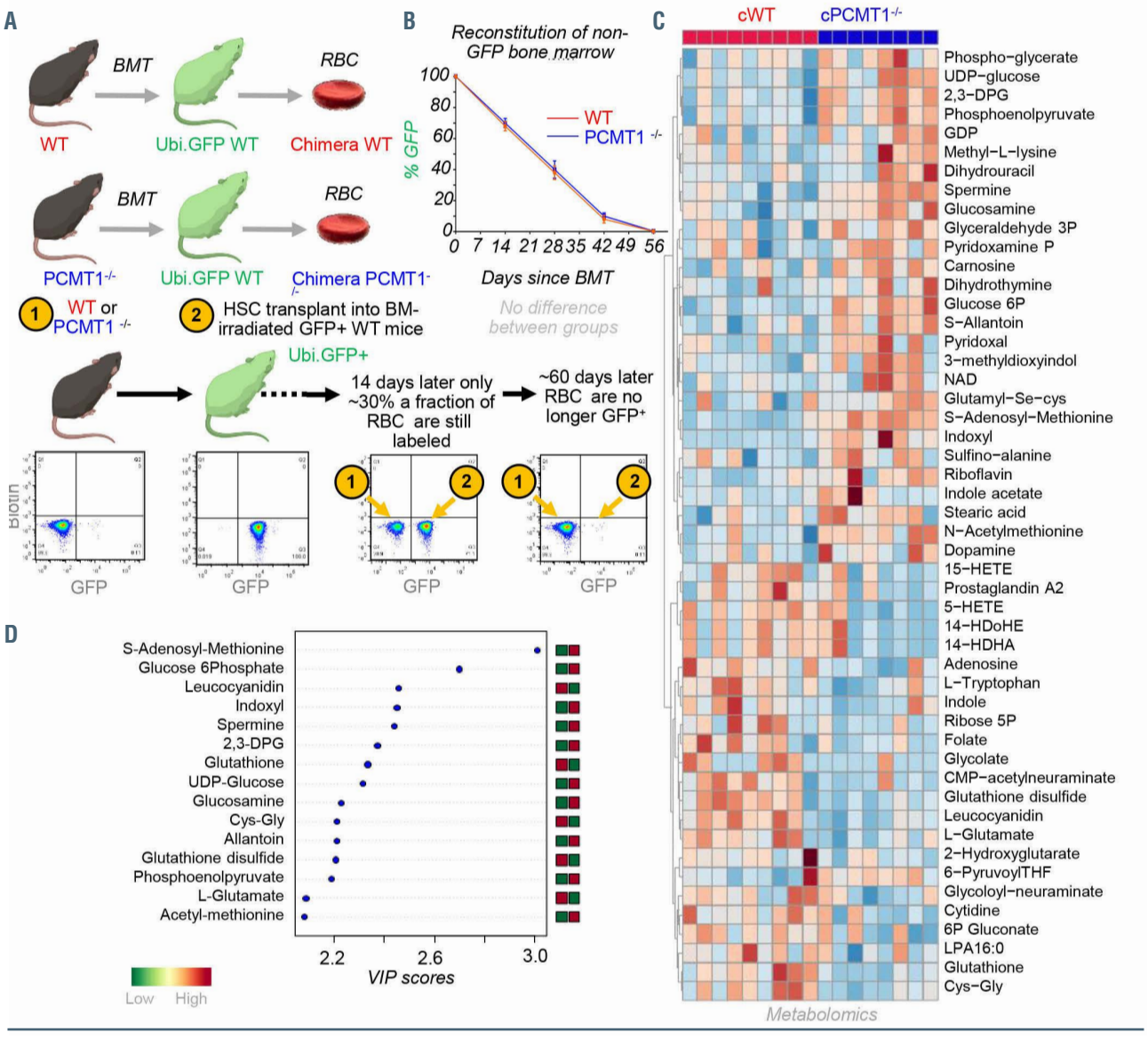

Figure 5. Metabolomics of bone marrow-transplanted wild-type and *PCMT1*$^{-/-}$ knockout x Ubi-GFP.B6 chimerae. (A) Metabolomics of bone marrow-transplanted wild-type and *PCMT1*$^{-/-}$ knockout ((*PCMT1* KO) x Ubi-GFP.B6 chimerae; (B) no significant changes in the time necessary to reconstitute the bone marrow of non-green fluorescence protein (GFP)-labeled red blood cells (RBC) were noted between the two groups, however, significant changes in RBC metabolism was noted between WT→GFP-WT and *PCMT1*→GFP WT mouse RBC; (C and D) show the top 50 significant metabolites (*t*-test) and variable importance in projection from these analyses.

in gene expression (e.g., transcription factors and/or enzymes involved in post-translational modification) as a result of unrepaired oxidation of aspartate and/or asparagine, which then changes the baseline genetic expression of enzymes involved in metabolic pathways. However, this explanation would only apply to nucleated cells, and not mature RBC. Given that the same general changes were seen in both RBC and other organs, this hypothesis is not favored as a general mechanism. A third and even less likely hypothesis, but one we cannot formally rule out based upon existing data, is that PIMT has additional enzymatic activities that have not yet been identified. The exact mechanism(s) by which PIMT activity broadly affects metabolism will require further investigation to elucidate; however, the findings in this manuscript demonstrate that PCMT1 is required for a number of distinct metabolic pathways and is essential for RBC to survive oxidative stress.

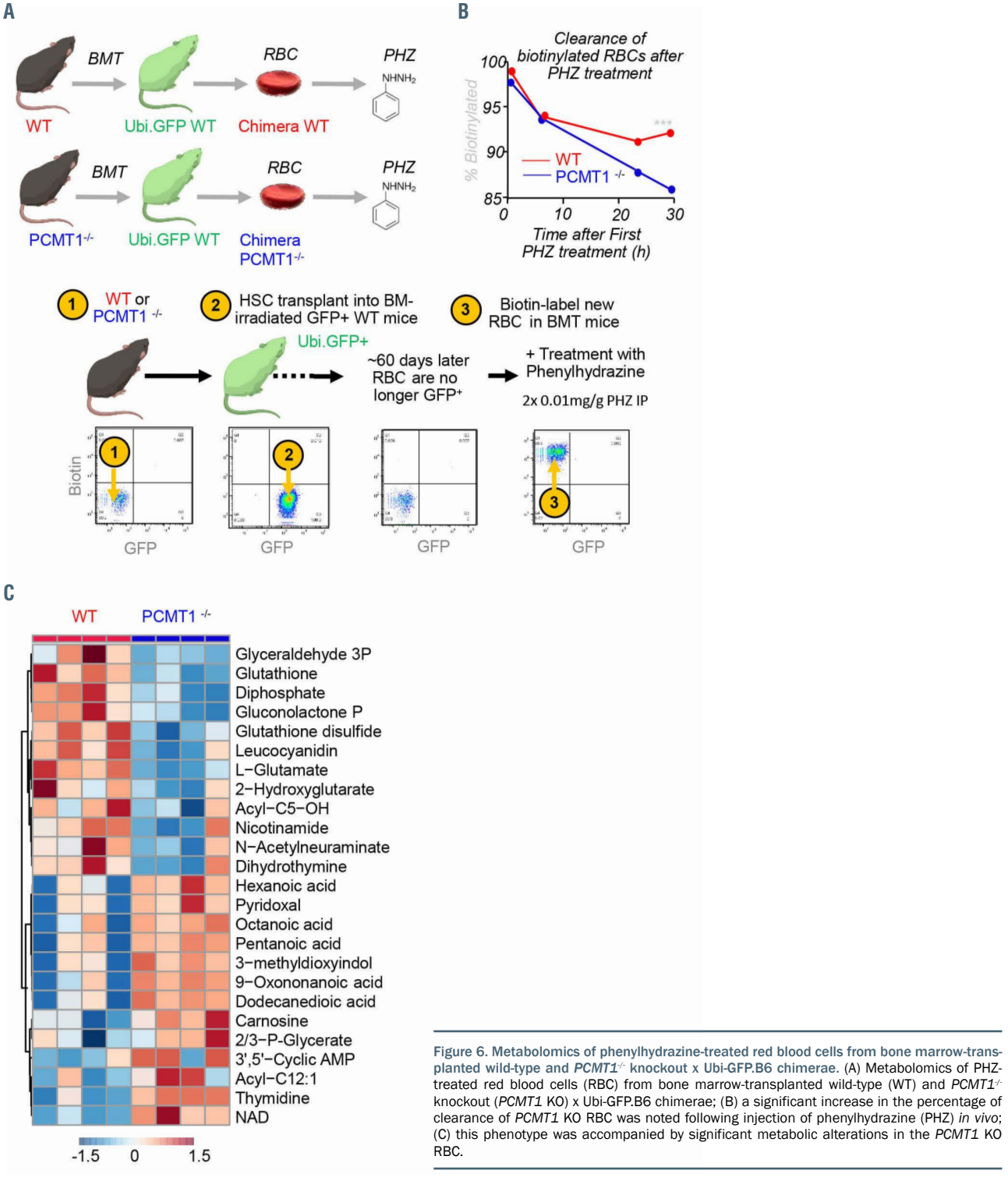

Figure 6. Metabolomics of phenylhydrazine-treated red blood cells from bone marrow-transplanted wild-type and *PCMT1*$^{-/-}$ knockout x Ubi-GFP.B6 chimerae. (A) Metabolomics of PHZ-treated red blood cells (RBC) from bone marrow-transplanted wild-type (WT) and *PCMT1*$^{-/-}$ knockout (*PCMT1* KO) x Ubi-GFP.B6 chimerae; (B) a significant increase in the percentage of clearance of *PCMT1* KO RBC was noted following injection of phenylhydrazine (PHZ) *in vivo*; (C) this phenotype was accompanied by significant metabolic alterations in the *PCMT1* KO RBC.

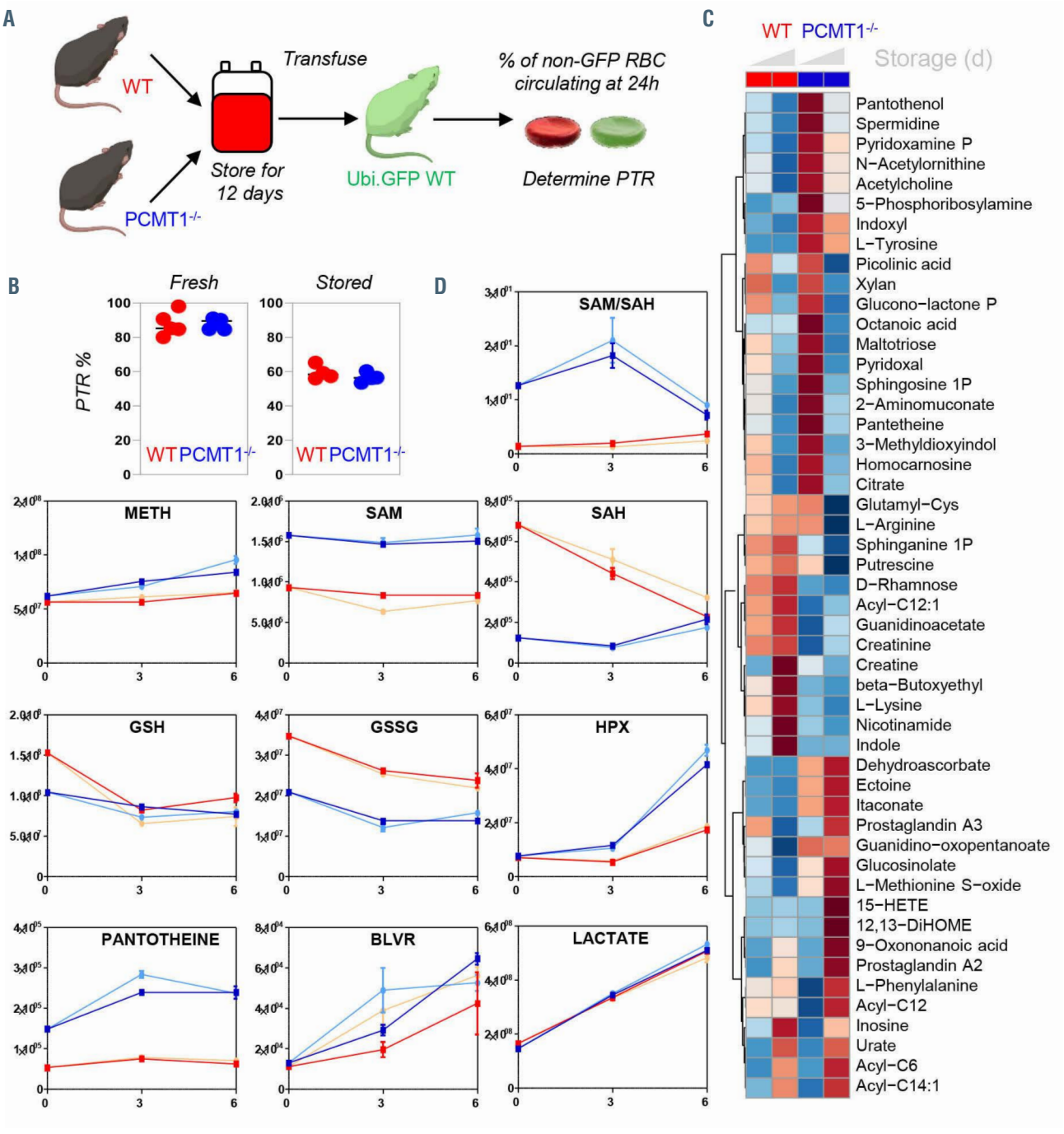

Figure 7. Metabolomics and post-transfusion recovery of stored red blood cells from wild-type and *PCMT1*$^{-/-}$ knockout mice. (A) Metabolomics and post-transfusion recovery of stored red blood cells (RBC) from wild-type (WT) and *PCMT1*$^{-/-}$ knockout (*PCMT1* KO) mice; (B) despite no significant changes in baseline and end of storage post-transfusion recoveries (PTR), *PCMT1* KO RBC showed significant alterations of energy and redox metabolism – as highlighted in the heat map in (C) and line plots in (D) for representative metabolites amongst the significant ones by repeated measures ANOVA as a function of storage duration.

Disclosures

The authors declare that AD and KCH are founders of Omix Technologies Inc and AD of Altis Biosciencens LLC; AD and JCZ are a consultant for Rubius Therapeutics; AD is an advisory board member for Hemanext Inc and FORMA Therapeutics Inc. All the other authors disclose no conflicts of interest relevant to this study.

Contributions

AD and JCZ designed the study; AH, JCZ performed mouse studies. AD, BB, EJM performed metabolomics analyses; MD, KCH performed proteomics analyses; AD and JCZ analyzed data, prepared figures and wrote the first draft of the manuscript; AD and JCZ revised the manuscript. All the authors contributed to the finalization of the manuscript.

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