Protein phosphatase 2A orchestrates hematopoietic fate determination via modulation of lactate metabolism

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Abstract

Abnormal hematopoiesis is inherently linked to metabolic reprogramming. Protein phosphatase 2A (PP2A), a master regulator of hematopoietic homeostasis, has been implicated in multiple hematological disorders. However, the precise mechanisms by which PP2A coordinates metabolic networks to govern hematopoietic fate decisions remain poorly defined. Herein, we identify lactate as a critical mediator of myeloid-biased differentiation triggered by PP2A inactivation. Genetic ablation of *PPP2CA*, the catalytic subunit of PP2A, results in aberrant myeloid proliferation and lymphoid depletion. Transcriptomic profiling reveals that *Ppp2ca* deficiency alters the expression of transcriptional regulators governing hematopoietic lineage commitment and energy metabolism. Metabolomic analyses further demonstrate enhanced lactate metabolism in *Ppp2ca*-deficient hematopoietic progenitors. Importantly, either haploinsufficiency or pharmacological inhibition of lactate dehydrogenase A (LDHA) *in vivo* effectively reverses the abnormal hematopoiesis induced by *Ppp2ca* deficiency. Mechanistically, *Ppp2ca* deletion directly promotes the transcriptional initiation of glycolytic genes (e.g., *Ldha*) via RNA polymerase II (Pol II). This leads to heightened lactylation of histone deacetylases (HDAC) at specific residues - lysine 412 in HDAC1 and lysine 451 in HDAC2 - impairing the assembly of the HDAC1/2/SIN3A co-repressor complex on chromatin, enhancing histone acetylation, and ultimately dysregulating hematopoietic gene expression. Collectively, our work establishes the "PP2A-lactate-HDAC lactylation" axis as a pivotal regulator of hematopoiesis and identifies LDHA as a promising therapeutic target for PP2A-associated hematological disorders.

Introduction

Protein phosphatase 2A (PP2A) is a critical serine-threonine phosphatase that is ubiquitously expressed in various cellular subsets.[1,2] As a versatile phosphatase, PP2A has been demonstrated to regulate more than 30 distinct kinases, such as serine/threonine kinase (AKT), protein kinase C (PKC), p70 S6 kinase, adenosine-3',5'-monophosphate (cAMP) dependent kinases, calcium/calmodulin kinases (CAMK), extracellular signal-regulated kinases (ERK), mitogen-activated protein kinases (MAPK), *etc.*[1,3,4] The emerging role of PP2A in the initiation and progression of various cancer and autoimmune disorders has been proposed.[1,3,5] Although separate studies have attempted to elucidate the role of

PP2A in leukemia, the regulation of hematopoietic homeostasis by PP2A remains to be clarified.[6] Moreover, recent research has revealed that PP2A subunits form a complex with the integrator and exert a broader impact on transcription by directly communicating with RNA polymerase (Pol) II, highlighting the diverse actions of PP2A.[7,8]

Cellular metabolism is crucial for hematopoietic homeostasis. Dormant hematopoietic stem cells (HSC) reside within hypoxic niches that exhibit high glycolytic activity and a low yet indispensable rate of fatty acid oxidation (FAO) to maintain quiescence and self-renewal capacity.[9,10] During the expansion process, HSC undergo a metabolic shift from quiescence to an activated state. Enhanced mitochondrial oxidative phosphorylation (OXPHOS) is vital for the proliferation of HSC, supplying precursor molecules for the biosynthesis of amino acids, lipids, and nucleotides.[10-12] Differentiation from multipotent progenitors (MPP) into myeloid or lymphoid progenitors is characterized by a modest increase in OXPHOS. This is followed by a significant rise in mitochondrial membrane potential and ATP levels when these cells further differentiate into lineage-restricted precursors and mature blood cells.[10-12] As a byproduct of glycolysis, lactate plays a crucial role in both physiological and pathological processes.[13,14] Post-translational lactylation has been demonstrated to be involved in the tumorigenesis and inflammation.[15-17] While several studies have reported the involvement of lactate in leukemia, its role in the regulation of hematopoiesis and the associated underlying mechanism remains to be elucidated.[18-20]

In this work, we investigated the impact of PP2A deficiency on hematopoiesis through multiple omics approaches, elucidated the intricate crosstalk between lactate metabolism and epigenetic regulation in driving hematopoietic abnormalities, and proposed a novel metabolic target for therapeutic intervention of PP2A-related diseases.

Methods

Mice

The *Ppp2ca-flox* mice (strain number T018350) and *Ldha-flox* mice (strain number T007813) were purchased from GemPharmatech (Nanjing, China). C57BL/6JSmoc-Gt (ROSA)$^{26Sorem1(SA-FRT-2xpolyA-CAG-FRT-CreERT2-Rox-WPRE-Rox-polyA)Smoc}$ mice (strain number NM-KI-200041) were purchased from Shanghai Model Organisms (Shanghai, China). *Ppp2ca-flox* mice were crossed with C57BL/6JSmoc-Gt (ROSA)26Sor$^{em1(SA-FRT-2xpolyA-CAG-FRT-CreERT2-Rox-WPRE-Rox-polyA)Smoc}$ mice to generate *Ert2*^{cre}*Ppp2ca*$^{f/f}$ mice. *Ert2*^{cre}*Ppp2ca*$^{f/f}$ mice and *Ldha*$^{f/f}$ were crossed to obtain the *Ert2*^{cre}*Ppp2ca*$^{f/f}$*Ldha*$^{f/+}$ mice. All mice were bred and housed under specific pathogen-free conditions.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assays were conducted with the Simple ChIP Plus Enzymatic Chromatin IP (Magnetic Beads) Kit (Cell Signaling Technology) according to the manufacturer's protocol. Briefly, cells were cross-linked in 1% formaldehyde for 10 minutes on ice. Chromatin was fragmented through enzyme digestion and sonication, followed by immunoprecipitation of the lysate using an antibody specific to Phospho-Rpb1 CTD (Ser5) (13523, Cell Signaling Technology) or H3K27ac. Subsequently, protein G magnetic beads were added. After washing and elution steps, the cross-links were reversed by heating. The purified DNA fragments were subjected to quantitative polymerase chain reaction (qPCR) analysis, with input DNA (total chromatin) serving as an internal control. The primer sequences are listed in *Online Supplementary Table S2.*

Extracellular acidification rate and lactate measurement

Primary cells were seeded into XF96-well plates (Agilent) at a density of $2x10^5$ cells per well in six duplicates. Seahorse base media was supplemented with 2 mM glutamine. Then, plates were incubated in a CO_2-free incubator at 37°C for 1 hour, followed by measurement using an XFe96 Analyzer (Agilent). Results were processed with Wave 2.6.0 software. The levels of L-lactate in the cell lysate were quantified using the L-Lactate Assay kit (ab65331, Abcam).

Quantification the enzymatic activities of histone deacetylases

Overexpressed HDAC1-Flag or HDAC2-Flag were immunoprecipitated with an anti-Flag antibody followed by capturing the complex on protein G beads (Cell Signaling Technology). Then, the enzymatic activity of purified HDAC protein was analyzed using the HDAC Activity Assay Kit (colorimetric) (ab1432, Abcam).

Tamoxifen-induced deletion and pharmaceutical intervention *in vivo*

Tamoxifen (75 mg/kg) was intraperitoneally injected into mice for 5 consecutive days to remove the *Ppp2ca* gene and/or haploid deletion of *Ldha* gene. The phenotype was analyzed on day 25. For pharmaceutical intervention, sodium oxamate (500 mg/kg) or stiripentol (200 mg/kg) were applied intraperitoneally every other day from day 6 to day 25. For *ex vivo* cellular studies, mice were administered intraperitoneal injections of tamoxifen daily for 5 consecutive days. Five days after the final injection, BM Lin$^-$ or LSK cells were isolated and sorted for subsequent analysis.

Statistics

For comparisons between two independent groups, statistical differences were evaluated using an unpaired two-tailed Student's *t* test, with *P* values reported in the corresponding figures; a *P* value <0.05 was considered statistically significant. For one-way multi-group analyses (i.e., comparisons involving three or more groups defined by a single independent variable), *P* values were calculated via one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) *post hoc* test to correct for multiple comparisons. For two-way multi-group analyses (i.e., comparisons involving groups defined by 2 independent variables), *P* values were determined using two-way analysis of variance (ANOVA) with subsequent Bonferroni *post hoc* test to account for multiple pairwise comparisons. Statistical analysis was performed using the Graph Prism 7.0 software (GraphPad Software Inc.).

Ethics approval statement

All animal experiments were ethically approved by the Animal Care Committee of Renji Hospital.

Results

PP2A modulates hematopoietic lineage commitment

PP2A phosphatase consists of a scaffolding subunit A, a regulatory subunit B and a catalytic subunit C.[7] To investigate the consequences of PP2A deficiency, we employed genetic ablation of the *Ppp2ca* gene, which encodes the catalytic subunit of PP2A. Since mice with hematopoietic deletion of *Ppp2ca* are lethal, we utilized the tamoxifen-induced *Ppp2ca* knockout (KO) mice to investigate the contribution of PPP2CA in hematopoietic lineage commitment. The efficiency of *Ppp2ca* deletion was confirmed (*Online Supplementary Figure S1A*). *Ppp2ca* KO mice displayed splenomegaly, thymic atrophy, and a significant increase in splenocyte counts (Figure 1A, B; *Online Supplementary Figure S1B*). A substantial reduction was found in the percentage of splenic lymphoid cells, including B cells, $CD4^+$ T cells and $CD8^+$ T cells, in *Ppp2ca* KO mice (Figure 1C; *Online Supplementary Figure S1C*). On the contrary, the percentage of splenic myeloid cells, including granulocytes, monocytes and macrophages was found to be increased (Figure 1D; *Online Supplementary Figure S1C*). No significant difference was found in the proportion of splenic dendritic cell (DC) subsets (Figure 1E; *Online Supplementary Figure S1C*). In line with this, a significant reduction in the number of splenic B cells and $CD4^+$ T cells and an elevation in the number of splenic granulocytes, monocytes, macrophages, plasmacytoid DC (pDC) and conventional type 2 DC (cDC2) were observed in KO mice (Figure 1F-H). There was minimal difference in the number of total bone marrow (BM) cells (Figure 1I). Similar to the splenic phenotype, the proportion of B cells was significantly reduced while those of granulocytes and monocytes were found to be markedly increased in the BM of *Ppp2ca* KO mice (Figure 1J-L; *Online Supplementary Figure S1C*). Although the number of BM monocytes was statistically similar between *Ppp2ca* wild-type (WT) and KO mice, the number of BM B cells was reduced and the number of BM granulocytes was increased in *Ppp2ca* KO mice (Figure 1M-O). When we further examined the composition of hematopoietic progenitors in the BM, we found no significant difference between *Ppp2ca* WT and KO mice in the number of lineage negative (Lin^-) cells and the proportion of myeloid progenitors, including granulocyte-monocyte progenitor (GMP), common myeloid progenitor (CMP) and megakaryocyte-erythroid progenitor (MEP) (Figure 1P, Q; *Online Supplementary Figure S1C*). In contrast, both the percentage and the absolute number of common lymphoid progenitor (CLP) were decreased following *Ppp2ca* deletion (Figure 1R, S). Further tracing back to the HSC stage, there were no statistical differences observed in $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells and their subsets, including long-term HSC (LT-HSC), short-term HSC (ST-HSC), MMP2, MMP3, and MMP4 (*Online Supplementary Figure S1D* and *Online Supplementary Figure S2A, B*). Therefore, we focused on the regulatory role of PPP2CA in the myeloid and lymphoid lineage commitment downstream of LSK.

Ppp2ca deficiency leads to dysregulated expression of genes involved in hematopoietic and metabolic pathways

To elucidate the underlying mechanism of PP2A in regulating hematopoiesis, we investigated the transcriptional changes in LSK cells following *Ppp2ca* deletion. Compared with WT mice, the top enriched pathway of downregulated genes in LSK cells from *Ppp2ca* KO mice was the hematopoietic cell lineage pathway (Figure 2A). More precisely, the expression of lineage-related genes associated with HSC, erythroid-megakaryoid and lymphoid lineage was reduced in LSK cells from *Ppp2ca* KO mice (Figure 2B). To be specific, *Flt3, Flt3l, Kit, Il6* and *Il6ra* are critical for the stemness maintenance of HSC. *Il7* and *Il7r*, as well as *Epor*, are respectively critical for the development of lymphoid and erythroid cells (Figure 2B). By contrast, the expression of myeloid-determining genes, including *Cebpα, Cebpβ, Cebpε, Csf2ra, Csf2rb* and *Csf2rb2*, was increased in LSK cells from *Ppp2ca* KO mice (Figure 2B).

The majority of upregulated genes in *Ppp2ca*-deficient LSK cells were enriched in various metabolic pathways, such as amino acid biosynthesis, carbon metabolism, the pentose phosphate pathway (PPP), glycolysis, *etc.* (Figure 2C). Of note, there was a significant increase in the expression of genes associated with glycolysis and the mitochondrial electron transport chain (Figure 2D). Gene set enrichment analysis (GSEA) further demonstrated that the potential of T-cell differentiation, and B-cell differentiation was impaired, while pathways related to the myeloid cell development, glycolysis, OXPHOS, MTORC1 signaling, Myc targets and Myeloid CEBPA network were enhanced in LSK cells following *Ppp2ca* deletion (Figure 2E-G).

To further characterize the global transcriptomic divergence between WT and KO LSK cells, we conducted principal component analysis (PCA). As an unbiased analytical approach, PCA effectively validated the reliability of transcriptomic differences between the two groups, ensuring that the observed variations were driven by the genetic background rather than technical artifacts (*Online Sup-*

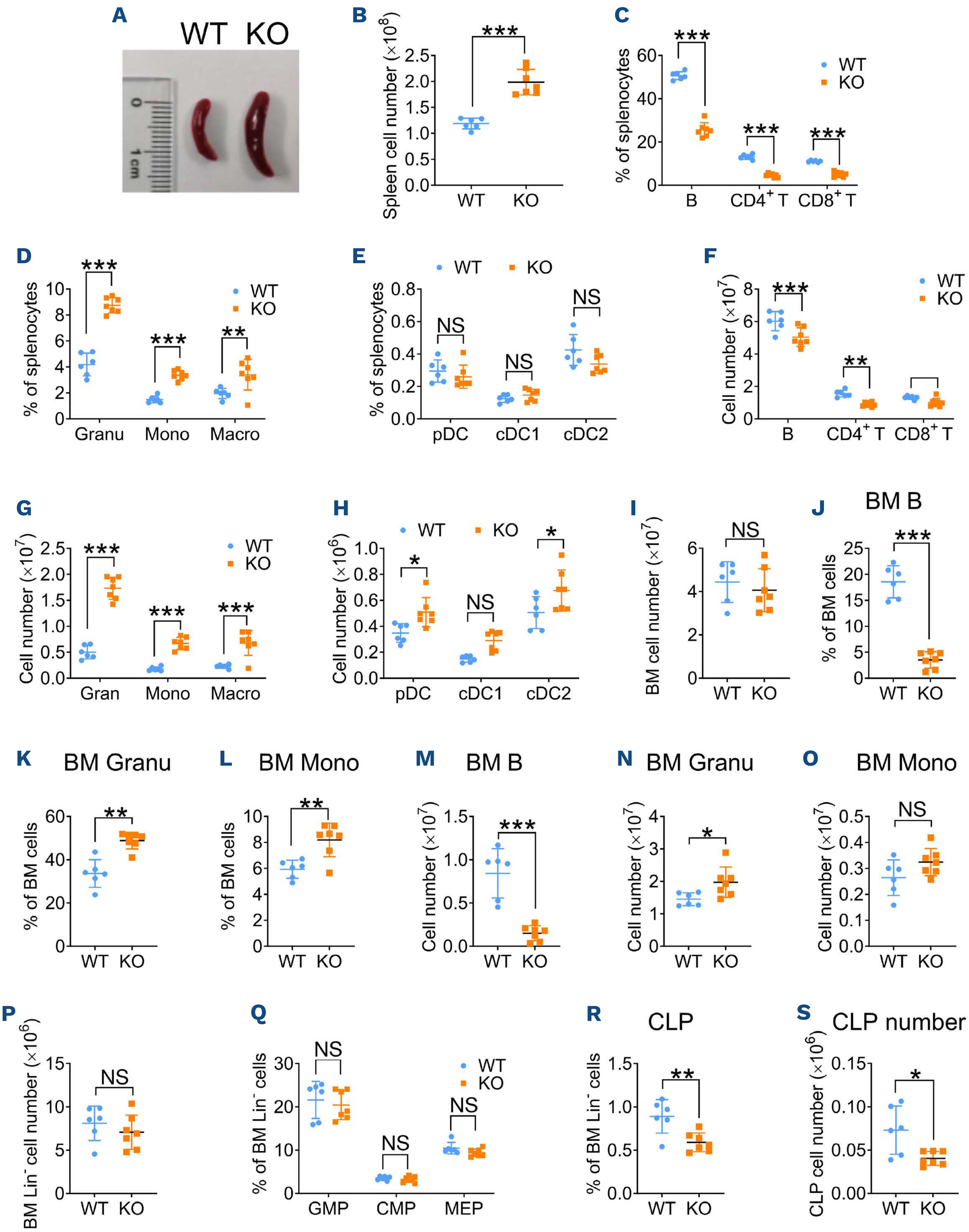

Figure 1. *Ppp2ca* modulates hematopoietic lineage commitment. *Ppp2ca*$^{f/f}$ (wild-type [WT]) or *Ert2*^{cre}*Ppp2ca*$^{f/f}$ (knockout [KO]) mice were treated with tamoxifen for 5 consecutive days, and the phenotype was analyzed on day 10 following last treatment. (A) The splenomegaly observed in *Ppp2ca*-deficient mice. (B) The number of splenocytes. (C) The proportion of splenic B cells, CD4$^+$ T cells and CD8$^+$ T cells. (D) The proportion of splenic granulocytes (Granu), monocytes (Mono) and macrophages (Macro). (E) The proportion of splenic plasmacytoid dendritic cells (pDC), conventional type 1 DC (cDC1) and cDC2. (F) The number of splenic B cells, CD4$^+$ T cells and CD8$^+$ T cells. (G) The number of splenic granulocytes, monocytes and macrophages. (H) The number of splenic pDC, cDC1 and cDC2. (I) The number of total bone marrow (BM) cells. (J-L) The proportion of B cells (J), granulocytes (K) and monocytes (L) in the BM. (M-O) The number of B cells (M), granulocytes (N) and monocytes (O) in the BM. (P) The number of BM lineage-negative (Lin$^-$) cells. (Q) The proportion of granulocyte/monocyte progenitors (GMP), common myeloid progenitors (CMP) and megakaryocyte/erythrocyte progenitors (MEP) in BM Lin$^-$ cells. (R) The proportion of common lymphoid progenitors (CLP) in BM Lin$^-$ cells. (S) The number of CLP. N=6-7. For panels (B, I-P, R and S) *P* values were determined by a two-tailed unpaired *t* test. For panels (C-H, Q), *P* values were determined by two-way ANOVA followed by Bonferroni *post hoc* test. Data are presented as mean ± standard error of the mean. *P<0.05; **P<0.01; ***P<0.001; NS: not significant.

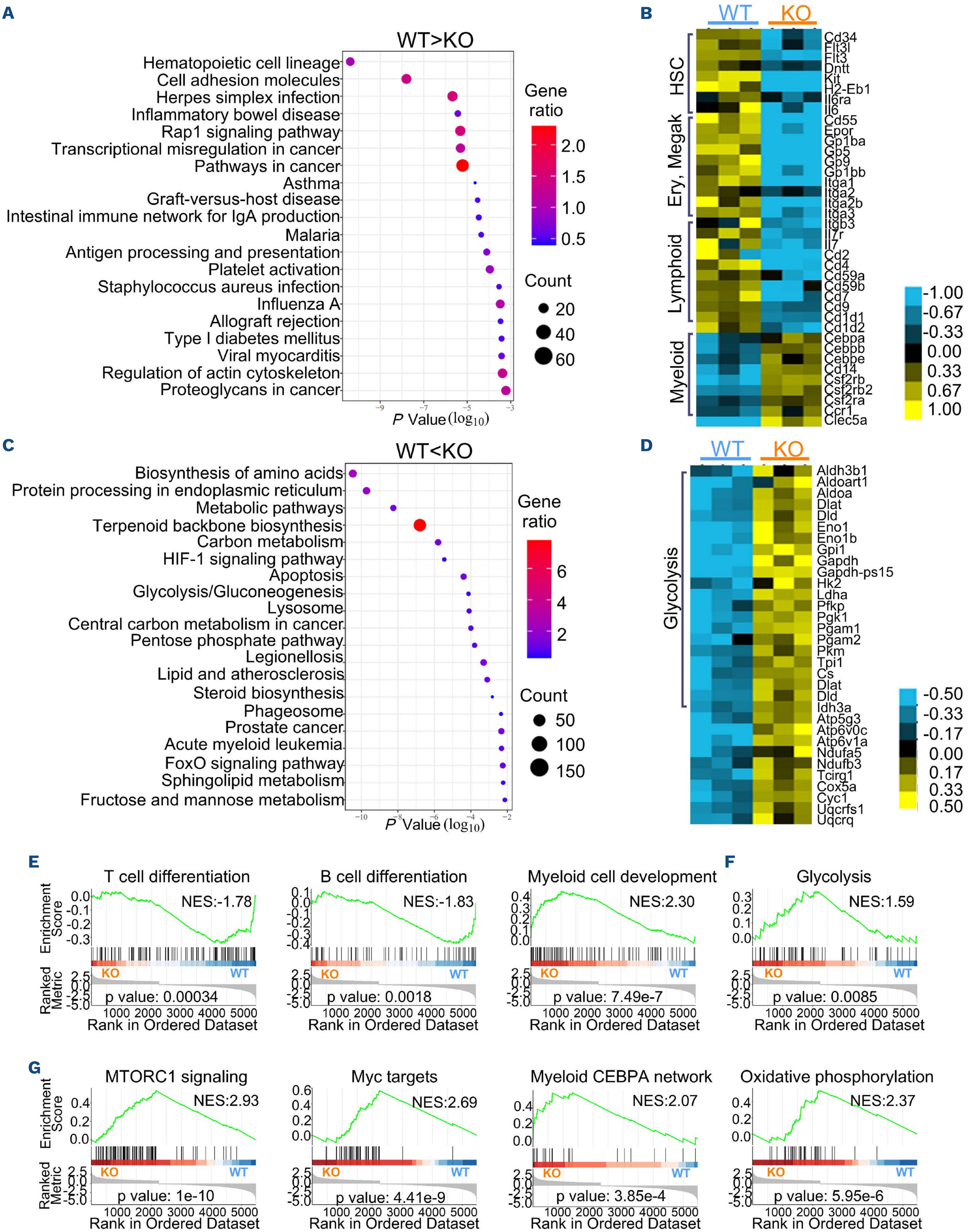

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Figure 2. *Ppp2ca* deficiency leads to dysregulated expression of genes involved in hematopoietic and metabolic pathways. LSK cells, isolated from the bone marrow (BM) of tamoxifen pre-treated *Ppp2ca*$^{f/f}$ (wild-type [WT]) and *Ert2*^{cre}*Ppp2ca*$^{f/f}$ (knockout [KO]) mice, were subjected to RNA-sequencing. (A) KEGG analysis of downregulated genes in *Ppp2ca* KO LSK. (B) A heatmap showing the changes in the expression of genes related to the hematopoietic lineage. (C) KEGG analysis of upregulated genes in *Ppp2ca* KO LSK. (D) Heatmap depicting the alterations in the expression levels of genes associated with energy metabolism. (E-G) Gene set enrichment analysis (GSEA) was performed to identify gene sets enriched in T-cell differentiation, B-cell differentiation and myeloid cell differentiation (E); glycolysis and oxidative phosphorylation pathways (F); and the MTORC1 signaling pathway, Myc target genes, and myeloid CEBPA network (G). N=3.

plementary Figure S2C). Moreover, unbiased identification of differentially expressed genes (DEG) revealed additional candidate regulators, such as *Gimap6, Six1*, and *Gas6*, which are potentially implicated in mediating the hematopoietic regulatory network disrupted by *Ppp2ca* deficiency (*Online Supplementary Figure S2D*). Additionally, we confirmed that the protein levels of key glycolytic enzymes - including HK2, PFKP, PKM2, and LDHA - were elevated in KO LSK cells (*Online Supplementary Figure S2E*).

Ppp2ca deficiency results in metabolic abnormalities

Considering the extensive regulation of metabolic pathways by *Ppp2ca*, we subsequently investigated the impact of *Ppp2ca* deficiency on LSK cell metabolism and found an increase in metabolites associated with energy production and material synthesis, including glycolysis, PPP, fatty acid oxidation (FAO), the tricarboxylic acid cycle (TCA), *etc*. (Figure 3A, B). Seahorse analysis revealed a significant increase in glycolytic activity in *Ppp2ca*-deficient LSK cells, as evidenced by elevated extracellular acidification rate (ECAR) (Figure 3C). The accumulation of lactate and an increased ratio of NAD^+/NADH were observed following *Ppp2ca* deletion (Figure 3D, E). It was consistent with the RNA profiling data that genes encoding key glycolytic enzymes were upregulated in *Ppp2ca*-deficient LSK cells, such as *Hk2, Pfkp, Pkm, Ldha*, *et al* (Figures 2D and 3A). Interestingly, the expression of *Tigar* and *G6pdx*, two enzymes previously implicated in the PPP-dependent development of B-cell malignancies, was also found to be increased after *Ppp2ca* deletion (Figure 3A).[21] Furthermore, increased levels of intracellular reactive oxygen species (ROS) - encompassing both cytosolic and mitochondrial fractions - were detected in LSK cells with *Ppp2ca* deficiency (Figure 3F).

Ppp2ca keeps the transcription of glycolytic genes in check

We next explored the mechanism by which PPP2CA regulates metabolism. Recent studies have unveiled that PPP2CA forms a complex with integrator and exerts a transcriptional inhibitory effect by directly communicating with RNA polymerase (Pol) II.[7] In order to verify the involvement of this novel paradigm in hematopoiesis, we detected the chromatin binding of PPP2CA by using the cleavage under targets and tagmentation (CUT&Tag) technology. Consistent with the previous report,[7] a majority of PPP2CA binding sites (63.98%) were found to be located within the promoter region, primarily in the core promoter regions (≤1 kb) (*Online Supplementary Figure S3A, B*). Global analysis of associated genes revealed that the metabolic pathway was significantly enriched and ranked as the top pathway (*Online Supplementary Figure S3C*). Upon conducting a more in-depth analysis of metabolic genes, we discovered that these genes were enriched in the biosynthesis of amino acids, central carbon metabolism in cancer, glycolysis, Hif-1 signaling, and the PPP pathways (Figure 4A). Specifically, the promoter loci of key glycolytic enzymes (*Hk2, Pfkp, Pkm, Ldha, etc.*) could be occupied by PPP2CA (Figure 4B). In contrast, PPP2CA failed to bind to the promoter regions of the hematopoietic lineage-related genes (*Online Supplementary Figure S3D*). Furthermore, the occupancy of phosphorylated Ser^5 within the Pol II C-terminal domain (CTD), a well-established indicator of transcription initiation, was validated to be increased at the promoters of metabolic genes rather than at those of lineage-associated genes (Figure 4C). Collectively, *Ppp2ca* deficiency directly promotes the transcriptional initiation of glycolytic genes in LSK cells.

Haploid deletion of the *Ldha* gene alleviates the abnormal hematopoiesis in *Ppp2ca*-deficient mice

The metabolomics analysis revealed a remarkable accumulation of lactate in *Ppp2ca*-deficient LSK cells. Lactate plays a pivotal role in coordinating diverse physiological and pathological processes, either through direct action or epigenetic lactylation.[13] To investigate the contribution of lactate to the hematopoietic abnormalities in *Ppp2ca*-deficient mice, we evaluated the impact of the *Ldha* gene, which encodes the A subunit of lactate dehydrogenase enzyme responsible for pyruvate to lactate conversion, on this process. The elevation of lactate levels was observed following *Ppp2ca* deletion (Figure 5A). In contrast, haploid deletion of *Ldha* restored the lactate levels in *Ppp2ca*-deficient hematopoietic progenitor cells to normalcy (Figure 5A). The deletion of *Ppp2ca* resulted in a substantial decline in the populations of BM B cells, as well as splenic B and T-cell subsets (Figure 5B, C). Concomitantly, there was an increase in the proportion of granulocytes and monocytes within the BM and spleen, along with an elevation in the proportion of macrophages in the spleen (Figure 5B, C). Conversely, the composition of DC subsets remained largely unchanged, except for a slight decrease in splenic pDC in mice with haploid deletion of *Ldha* (Figure 5C). Meanwhile, it was discovered that the proportion of CLP decreased (Figure 5D). Significantly, haploid deletion of the *Ldha* gene was found to be sufficient to alleviate the defects caused by *Ppp2ca* deficiency, thereby restoring the normal proportions of B cells, granulocytes, monocytes, and CLP in the BM, as well as the those of B cells, T cells, granulocytes, monocytes and macrophages in the spleen (Figure 5B-D). Additionally, the composition of other progenitors, including CMP, GMP and MEP, was unaffected upon *Ppp2ca* deletion together with or without *Ldha* haploid insufficiency

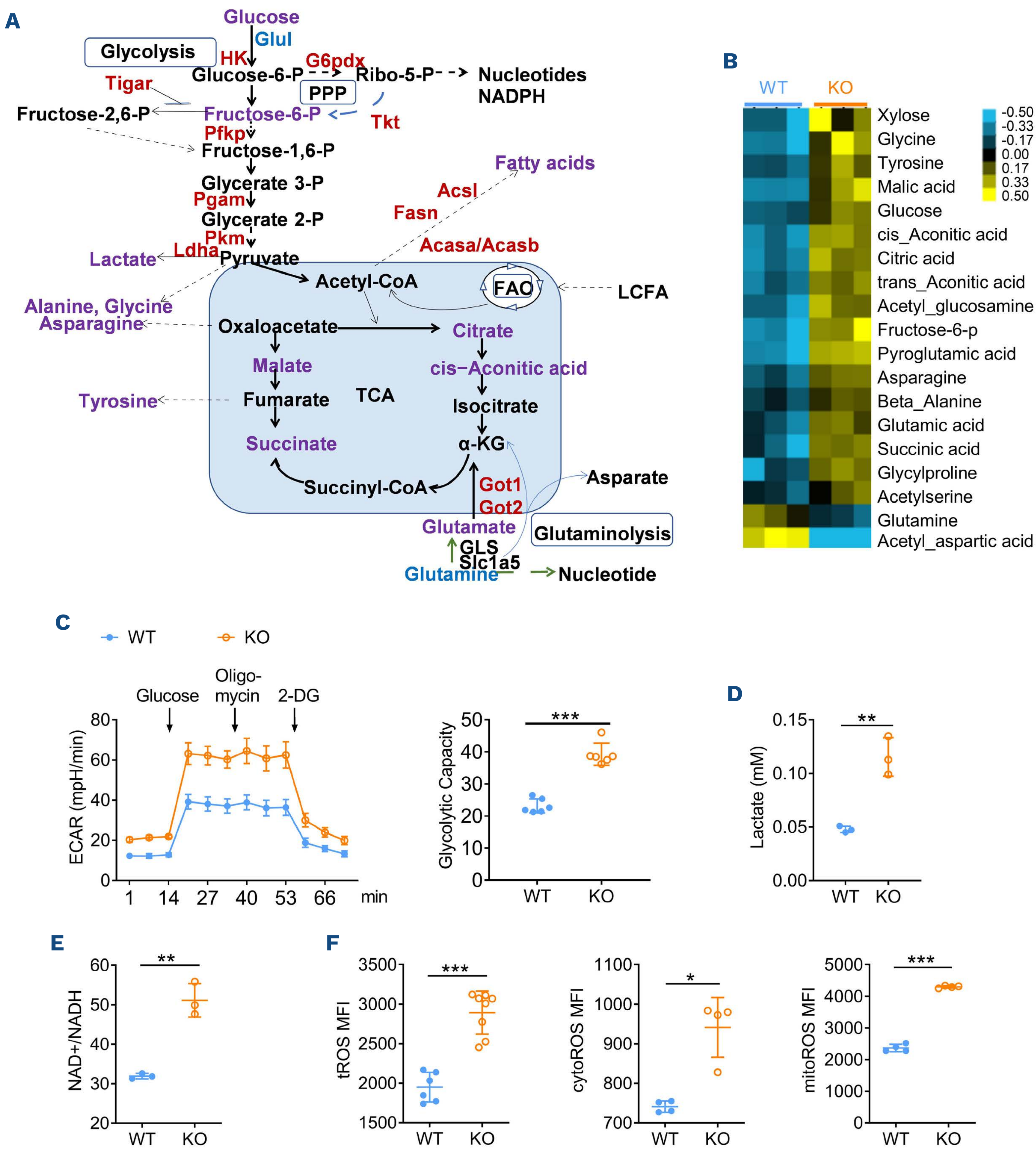

Figure 3. *Ppp2ca* deficiency leads to metabolic abnormalities. LSK cells were isolated from the bone marrow (BM) of tomoxifen-pretreated *Ppp2ca*$^{f/f}$ (wild-type [WT]) and Ert2cre*Ppp2ca*$^{f/f}$ (knockout [KO]) mice, and then subjected to liquid chromatography-tandem mass spectrometry analysis. (A) Overview of metabolomics. Metabolites increased in *Ppp2ca*-deficient LSK cells are represented in purple color. Metabolic genes upregulated in *Ppp2ca*-deficient LSK cells are indicated in red color. Decreased metabolites and metabolic genes are shown in dark blue. (B) Heatmap depicting the alteration in the levels of metabolites. N=3. (C) Analysis of extracellular acidification rate (ECAR) and glycolytic capacity of LSK cells. N=6. (D) The levels of lactate in LSK cells. N=3. (E) The ratios of NAD$^+$ to NADH. N=3. (F) The levels of total reactive oxygen species (tROS), cytosolic ROS (cytoROS) and mitochondrial ROS (mitoROS) in LSK cells. N=4-8. Data are representative of 3 independent experiments. *P* values were determined by a two-tailed unpaired *t* test. Data are presented as mean ± standard error of the mean. *P<0.05; **P<0.01; ***P<0.001. MFI: mean fluorescense intensity.

(Figure 5D). Therefore, *Ldha* is the key enzyme mediating the regulation of *Ppp2ca* on hematopoietic differentiation.

Pharmacological interventions targeting lactate metabolism reverse the hematopoietic defects caused by *Ppp2ca* deficiency

Since the genetic intervention targeting elevated lactate reversed the hematopoietic disorders caused by *Ppp2ca* deletion, we further investigated the *in vivo* effects of two distinct *Ldha* inhibitors, oxamate and stiripentol, on the treatment of *Ppp2ca*-deficient mice (*Online Supplementary Figure S4A*).[20,22,23] The application of stiripentol alleviated the phenotypic defects by restoring the proportion of B cells, granulocytes, and monocytes to normal levels (*Online Supplementary Figure S4B-D, G* and *H*). In contrast, administration of sodium oxamate exhibited relatively modest

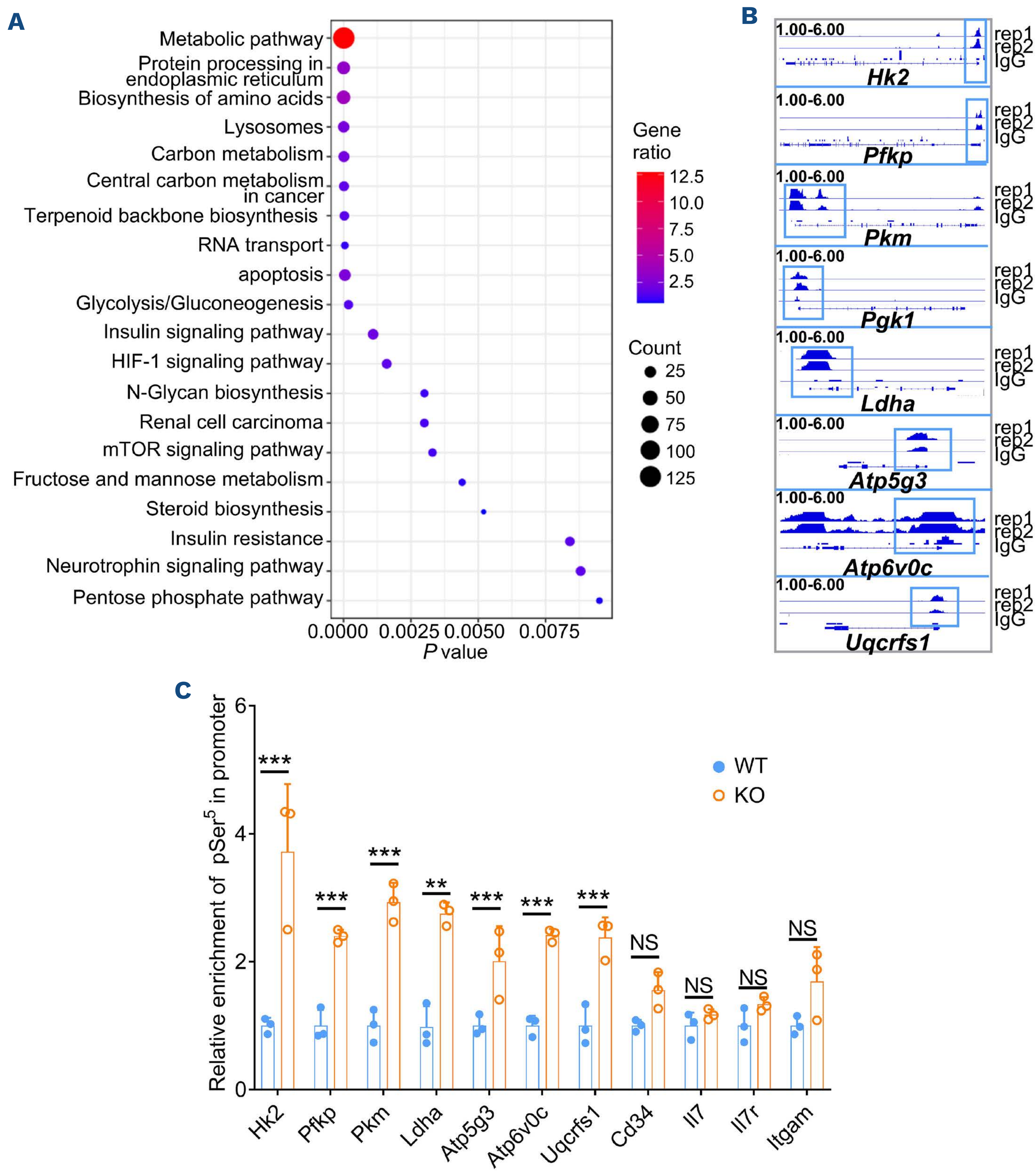

Figure 4. *Ppp2ca* keeps the transcription of glycolytic genes in check. LSK cells were isolated from the bone marrow (BM) of wild-type (WT) mice, and subjected to CUT&Tag analysis. (A) Enriched metabolic pathways of genes associated with the binding loci of PPP2CA. (B) The genomic browser track presenting the PPP2CA signal at the loci of representative target genes. The blue rectangles indicate the peak regions of PPP2CA occupancy on the promoters of target genes. Repeated experiments are presented. rep: repeat. (C) LSK cells isolated from the BM of tamoxifen-pretreated $Ppp2ca^{f/f}$ (WT) and $Ert2^{cre}Ppp2ca^{f/f}$ (knockout [KO]) mice were subjected to chromatin immunoprecipitation-quantitative polymerase chain reaction analysis. The occupancy of $pSer^5$ at the promoter of target genes is shown. pSer,[5] a well-recognized marker of transcription initiation. N=3. *P* values were determined by a two-tailed unpaired *t* test. Data are presented as mean ± standard error of the mean. $^{**}P$<0.01; $^{***}P$<0.001; NS: not significant.

therapeutic efficacy, failing to fully restore the normal differentiation of granulocytes and monocytes within the spleen (*Online Supplementary Figure S4G, H*). Although neither oxamate nor stiripentol could completely mitigate the decline in T-cell subsets, their application resulted in a partial recovery of the T-cell proportion (*Online Supple-*

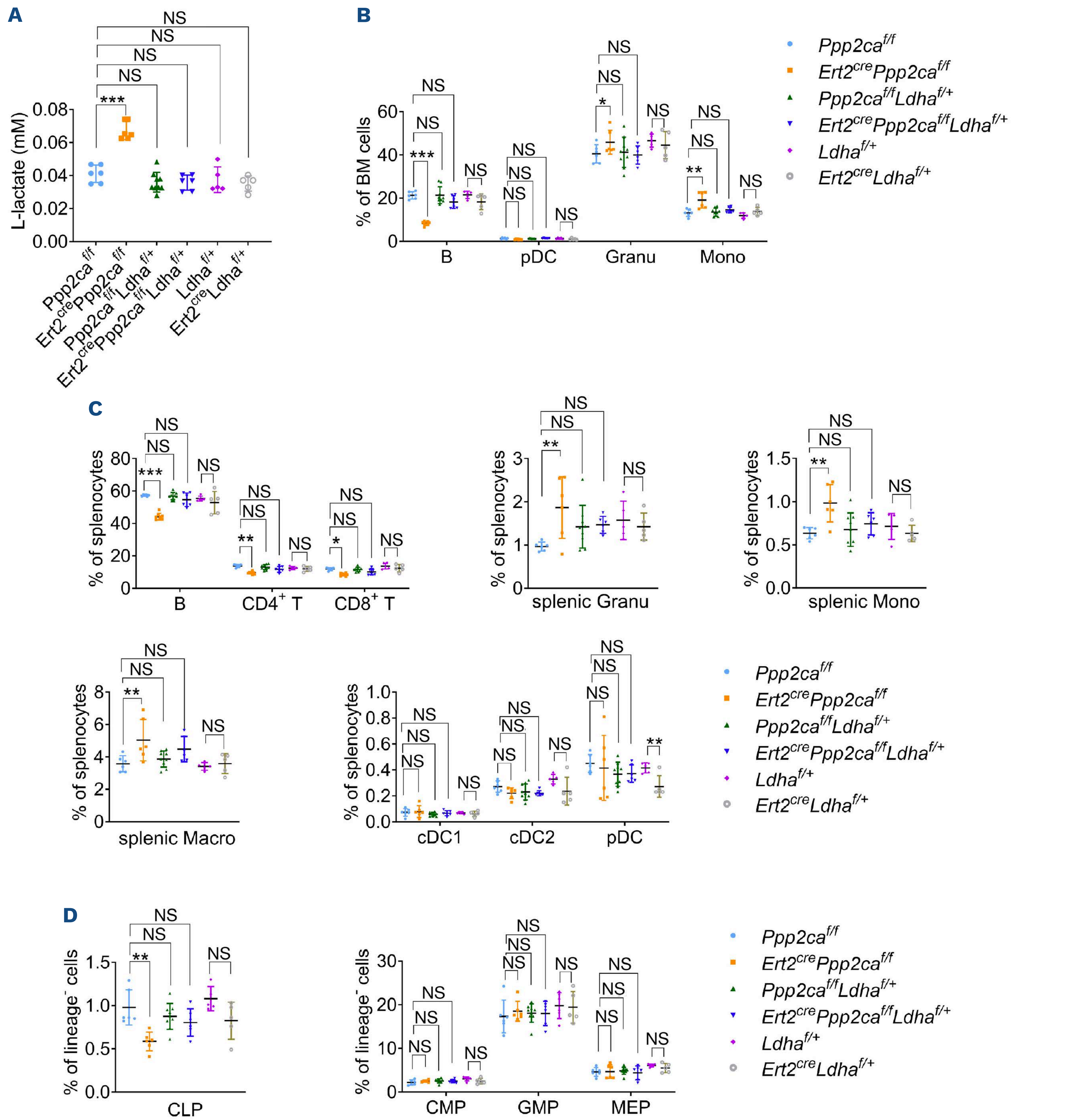

Figure 5. Haploid deletion of *Ldha* gene alleviates the abnormal hematopoiesis in *Ppp2ca*-deficient mice. (A) The levels of L-lactate in the Lin⁻ cells of bone marrow (BM). *P* values were determined by one-way ANOVA followed by Tukey's honestly significant difference (HSD) *post hoc* test. Data are presented as mean ± standard error of the mean. N=5-9. ****P*<0.001; NS: not significant. (B) The proportion of B cells, plasmacytoid dendritic cells (pDC), granulocytes (Granu) and monocytes (Mono) in the BM. (C) The proportion of B cells, T cells, granulocytes, monocytes, macrophages (Macro), conceventional type 1 DC (cDC1), cDC2 and pDC in the spleen. (D) The proportion of common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte/monocyte progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MEP) (MEP) in the BM. N=5-9. *P* values were determined by two-way ANOVA followed by Bonferroni *post hoc* test. Data are presented as mean ± standard error of the mean. **P*<0.05; ***P*<0.01; ****P*<0.001; NS: not significant.

mentary Figure S4E and *F*). Besides, when detecting the progenitors, we found that the decrease in CLP was reversed by stiripentol (*Supplementary Figure S4I*). Furthermore, no significant difference was observed in the proportion of myeloid progenitors among the groups (*Online Supplementary Figure S4J*). Therefore, the excessive accumulation of lactate represents the primary pathogenic mechanism underlying hematopoietic abnormalities in *Ppp2ca*-deficient mice.

Increased lactylation of HDAC1 and HDAC2 upon deletion of *Ppp2ca*

To elucidate the underlying mechanism of lactate-driven hematopoietic abnormalities, we conducted comprehensive research utilizing lactyl-modification omics. Mass spectrometry (MS) analysis revealed that upregulated lactylation was observed in 89 of 90 differentially lactyl-modified sites (Figure 6A). These sites were distributed across 67 proteins, among which ten proteins had been proven to be associated with hematopoiesis (Figure 6B). No significant change in histone (H) lactylation was observed upon re-confirmation with immunoblotting screening (*Online Supplementary Figure S5*). Of note, both HDAC1 and HDAC2 were particularly critical. The lactylated lysine residues (K), including the 412th K (K412) in HDAC1 and the 451st K (K451) in HDAC2, along with their respective adjacent peptides, were found to be conserved across human and mouse (Figure 6C, D). Immunoblotting analyses validated the upregulated lactylation of HDAC1 and HDAC2 in *Ppp2ca*-deficient LSK (Figure 6E). When lactate metabolism was blocked by the application of oxamate, a significant reduction in the levels of Klac in LSK cells was observed (Figure 6E). Concurrently, the global acetylation of K (Kac) in *Ppp2ca* deficient LSK was found to be elevated (Figure 6F). These findings strongly suggest that the augmented lactyl-modification may potentially attenuate the deacetylation function of HDAC.

Enhanced lactylation of histone deacetylases contributes to the excessive expression of myeloid-determining genes following *Ppp2ca* deletion

To assess the influence of lactylation on the enzymatic activities of HDAC1 and HDAC2, the K412 in HDAC1 and the K451 in HDAC2 were genetically mutated to arginine (HDAC1 R412 and HDAC2 R451). This approach, which involves substituting K with R at specific residues, is a widely acknowledged and validated experimental strategy for investigating the functional roles of lactylated sites within proteins.[15,24-26] Furthermore, we ascertained that no additional modifications occurred at these two specific sites. Remarkably, the replacement of K by R resulted in a substantial decline in the lactylation levels of HDAC1 and HDAC2 (Figure 6G). When conducting *in vitro* analysis of enzyme activity, we found that these mutations did not compromise the deacetylation capabilities of HDAC1 and HDAC2 (Figure 7A). It has been reported that the K412 residue in HDAC1 is adjacent to the "IACEE" domain, which is required for its interaction with pocket proteins. And, the K451 residue in HDAC2 is situated within the coiled coil domain (CCD), that mediates the binding of HDAC2 to other proteins, including the subunits of co-repressors (Figure 7B).[27,28] Therefore, we postulated that the upregulated lactyl-modification might impede the functional assembly of HDAC repressors on chromatin, and consequently led to excessive histone acetylation.

Class I HDAC are capable of assembling into four distinct protein complexes, namely SIN3,[29] NuRD,[30] CoREST,[31] and MiDac,[32] exerting a role of histone deacetylation. Among them, the SIN3 complex has been identified as indispensable for hematopoiesis.[33] It has been reported that genetic ablation of *Sin3a*, the core subunit of SIN3 complex, results in hematopoietic disorders that phenotypically resemble those in HDAC1 and HDAC2 double-deficient (DKO) mice.[33] More importantly, through an integrative structural analysis of the SIN3A/HDAC complex utilizing Halo affinity capture, chemical crosslinking, and high-resolution mass spectrometry (XL-MS), it was revealed that K412 resides in a region where HDAC1 directly interacts with other subunits, including NGDN, SIN3A, and SAP30L. Additionally, K451 is positioned within a domain responsible for the self-interaction of HDAC2 in the SIN3A/HDAC co-repressor.[34] In light of these findings, we devised co-immunoprecipitation assays to assess the influence of lactylation at these two sites on the interaction between HDAC and SIN3A. Our results indicated that the K412R substitution strengthened the interaction of HDAC1 with endogenous HDAC2 and SIN3A (Figure 7C). Analogously, the K451R mutation promoted the binding of HDAC2 with endogenous HDAC1 and SIN3A (Figure 7C). Collectively, these data suggest that site-specific lactylation of HDAC weakens the assembly of the SIN3A/HDAC complex.

To investigate the consequences of hyper-lactylated HDAC1 and HDAC2, we firstly assessed the alteration of H3K27 acetylation (H3K27ac), a well-characterized chromatin marker of active promoters and enhancers,[35,36] in *Ppp2ca*-deficient LSK. Upon deletion of *Ppp2ca*, a global elevation in H3K27ac levels was detected (*Online Supplementary Figure S6A*). Specifically, the findings demonstrated an increased occupancy of H3K27ac at the promoters of genes crucial for myeloid determination, including *Cebp*α*, Cebp*β*, Cebp*ε*, Csf2ra, Csf2rb* and *Csf2rb2* (*Online Supplementary Figure S6B*). These hyper-acetylated sites were notably enriched in mTOR, G1/S transition and AKT pathways (*Online Supplementary Figure S6C*). Consistently, genes upregulated in *Ppp2ca*-deficient LSK also exhibited heightened H3K27ac (*Online Supplementary Figure S6D*). Moreover, the augmented acetylation of H3K27 at the promoters of Cebpα, Cebpε, Csf2ra, Csf2rb and Csf2rb2 was confirmed by CHIP-quantitative real-time PCR (Figure 7D). Finally, the application of oxamate effectively suppressed the excessive acetylation of H3K27 (Figure 7E). Furthermore, haploid deletion of *Ldha* also alleviated the increased H3K27ac caused by *Ppp2ca* deficiency (Figure 7F). Therefore, these results suggest

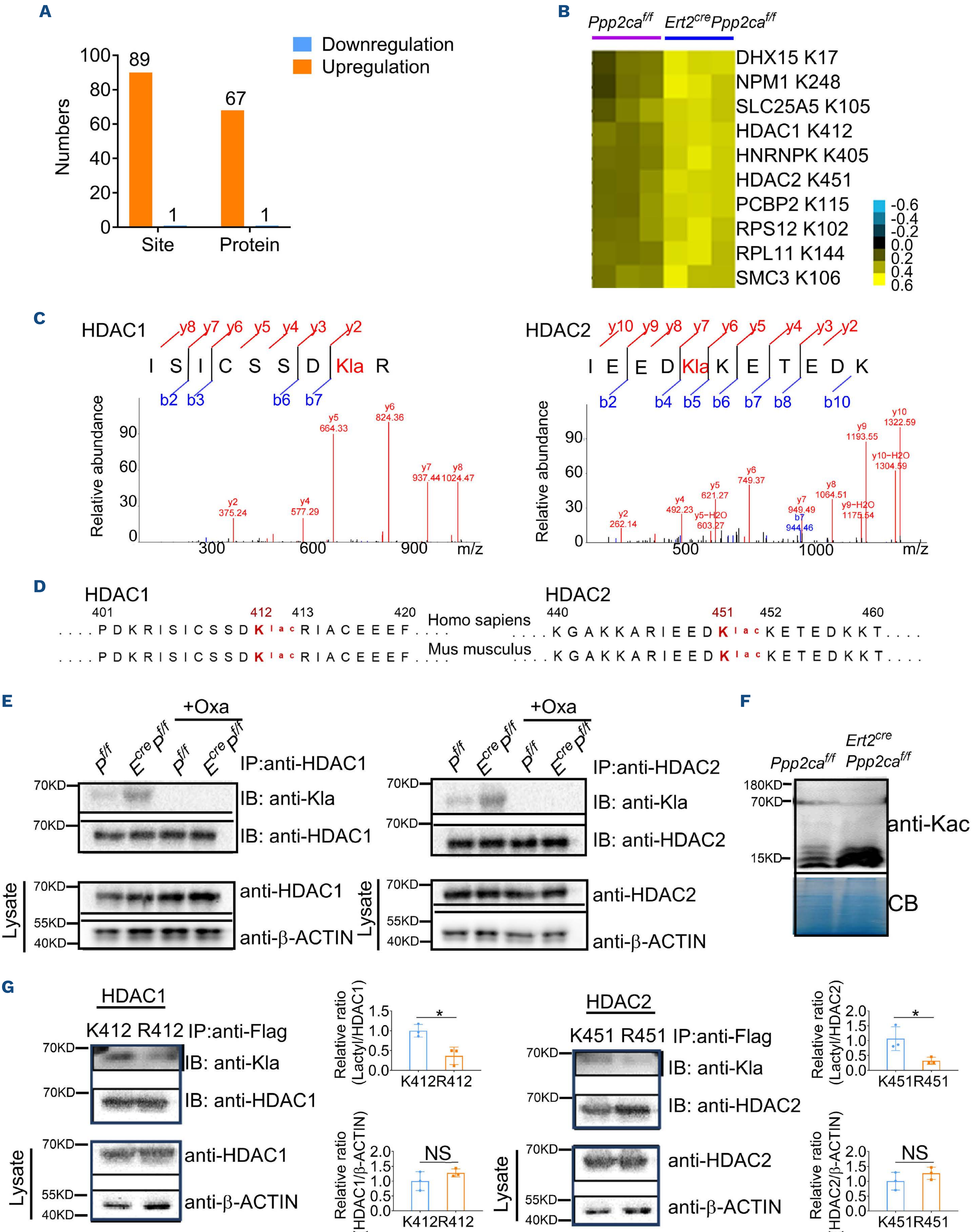

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Figure 6. Increased lactylation of HDAC1 and HDAC2 upon deletion of *Ppp2ca*. (A) LSK cells were prepared from the bone marrow (BM) of tamoxifen-pretreated *Ppp2ca*$^{f/f}$ (wild-type [WT]) and *Ert2*^{cre}*Ppp2ca*$^{f/f}$ (knockout [KO]) mice. Statistical analysis of lactylated sites and proteins. (B) Heatmap depicting the increased lactylation levels of proteins involved in hematopoiesis. N=3. (C) Mass spectrometry analysis of the lactylated histone deacetylases (HDAC) peptides (HDAC1K412la and HDAC2K451la). 'b' refers to the amino-terminal parts of the peptide and 'y' refers to the carboxy-terminal parts of the peptide. Data represent 3 independent experiments. (D) The conservation of lactylated sites in HDAC1 and HDAC2. (E) Lin$^-$ cells isolated from the BM of tamoxifen-treated *Ppp2ca*$^{f/f}$ (P$^{f/f}$) and *Ert2*^{cre}*Ppp2ca*$^{f/f}$ (*E*^{cre}*P*$^{f/f}$) mice were incubated with or without oxamate (Oxa). Subsequently, intracellular HDAC1 and HDAC2 were respectively immunoprecipitated (IP) with anti-HDAC1 and anti-HDAC2 antibodies, followed by detection of lactylation using anti-lactyllysine antibody (anti-Kla). (F) Immunoblot analysis assessing the pan-lysine acetylation (anti-Kac) levels in LSK. Coomassie blue staining (CB) indicates equal loading of protein. (G) Analysis of the site-specific lactylation of HDAC1 and HDAC2. Cell lysates from Lin$^-$ cells with HDAC1-Flag or HDAC2-Flag overexpressing were subjected to IP using an anti-Flag antibody (anti-Flag), followed by detection of lactylation with an anti-lactyllysine antibody (anti-Kla). The relative ratios of lactyl-HDAC to total-HDAC and total-HDAC to β-actin are shown. The experiments were repeated 3 times. *P* values were determined by a two-tailed unpaired *t* test. Data are presented as mean ± standard error of the mean. **P*<0.05; NS: not significant.

that lactate mediates the regulatory effect of PPP2CA on H3K27 acetylation.

Discussion

In this study, we utilized multi-omic approaches to elucidate the metabolic and epigenetic alterations consequent to *Ppp2ca* deletion. The deficiency of *Ppp2ca* promoted RNA polymerase II-mediated initiation of glycolytic genes and lactate accumulation. Subsequently, the elevated lactate metabolism facilitated the lactylation of HDAC. This process impeded the assembly of the HDAC/SIN3A complex, thereby increasing the levels of H3K27 acetylation and upregulating the expression of myeloid-determination genes. As a result, it induced a reduction in lymphoid cells and an expansion of myeloid cells (*Online Supplementary Figure S7*).

Previous studies reported that PP2A redirected glucose utilization towards the PPP metabolism in B cells, promoting the survival of B cells and the progression of B-cell tumors.[21] The balance maintained by the activity of PP2A and B-cell transcriptional factors (TF) PAX5 and IKZF1 plays a gatekeeper role in the B-cell malignancies.[21] However, our findings indicate that PP2A exerts a profound impact on the maintenance of hematopoietic homeostasis and is indispensable for the development of both B and T cells. *Ppp2ca* KO mice exhibit a reduced proportion of CLP and terminally differentiated lymphocytes, while lactate blockade can restore normal hematopoiesis in *Ppp2ca*-deficient mice. Therefore, PP2A and downstream lactate metabolism play important roles in early stage of lineage determination, far beyond their influence on terminally differentiated B cells.

The classical model for PP2A regulation generally focuses on its capacity to dephosphorylate a wide range of proteins, especially the molecules in PI3K-AKT-mTOR pathway.[37-40] However, our findings reveal that *Ppp2ca* deficiency directly facilitated the RNA transcriptional initiation of various metabolic genes mediated by Pol II, including genes significantly enriched in the glycolytic pathway. This result highlights that *Ppp2ca* can serve as a critical regulatory gene that directly orchestrates metabolism. Subsequently, the "Warburg effect" and the accumulation of lactate were observed in *Ppp2ca*-deficient LSK cells, leading to the elevated lactylation of HDAC1 and HDAC2. Thus, our work establishes a novel "PP2A-LDHA-HDAC" axis in the regulation of hematopoiesis.

Both HDAC1 and HDAC2 are required for the maintenance of hematopoietic homeostasis in a functional complementary way.[33,41,42] Single deletion of *Hdac1* or *Hdac2* had little effect on hematopoiesis. Simultaneous loss of *Hdac1* and *Hdac2* (*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{\Delta/\Delta}$) significantly impaired the reconstituting abilities of hematopoietic stem cells.[33,42] Genetic deletion of *Sin3a* exhibited a comparable phenotype to *Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{\Delta/\Delta}$ mice.[33] Conditional KO of *Sin3a* further demonstrated that *Sin3a* was essential for T- and B-lymphocyte development.[33] Likewise, we found that BM inactivation of *Ppp2ca* led to a decline in the expression of genes for hematopoietic stem cell maintenance. And *Ppp2ca* was revealed indispensable for the differentiation of T cell and B cells. The phenotypic resemblance among *Ppp2ca* KO mice, *Sin3a* KO mice and *Hdac1/2* DKO mice strongly corroborates our conclusion that the impaired formation of HDAC/SIN3A complex ultimately mediated the epigenetic remodeling and hematopoietic dysfunction in *Ppp2ca*-deficient HSC. In addition, the direct lactylation of histones has garnered substantial research interest in recent years.[16] Nevertheless, no significant disparities in histone lactylation were detected between the WT and KO Lin$^-$ cells, regardless of whether modification proteomics or immunoblotting assays were used.

C/EBPα is mainly involved in cell fate decisions towards myeloid differentiation. It acts as a pioneer TF in cooperation with other TF to prime the myeloid gene expression program as early as LT-HSC.[43,44] C/EBPα, in collaboration with PU.1 or C/EBPβ, redirects the lymphoid progenitors towards myeloid progenitors to eventually differentiate into monocytes/granulocytes.[44] C/EBPε, acting downstream of C/EBPα, is required for terminal granulopoiesis.[45,46] The GM-CSF pathway is involved in the generation of granulocytes, macrophages, and dendritic cells from hematopoietic progenitors. Increased expression of GM-CSF receptors on hematopoietic progenitor cells during inflammation

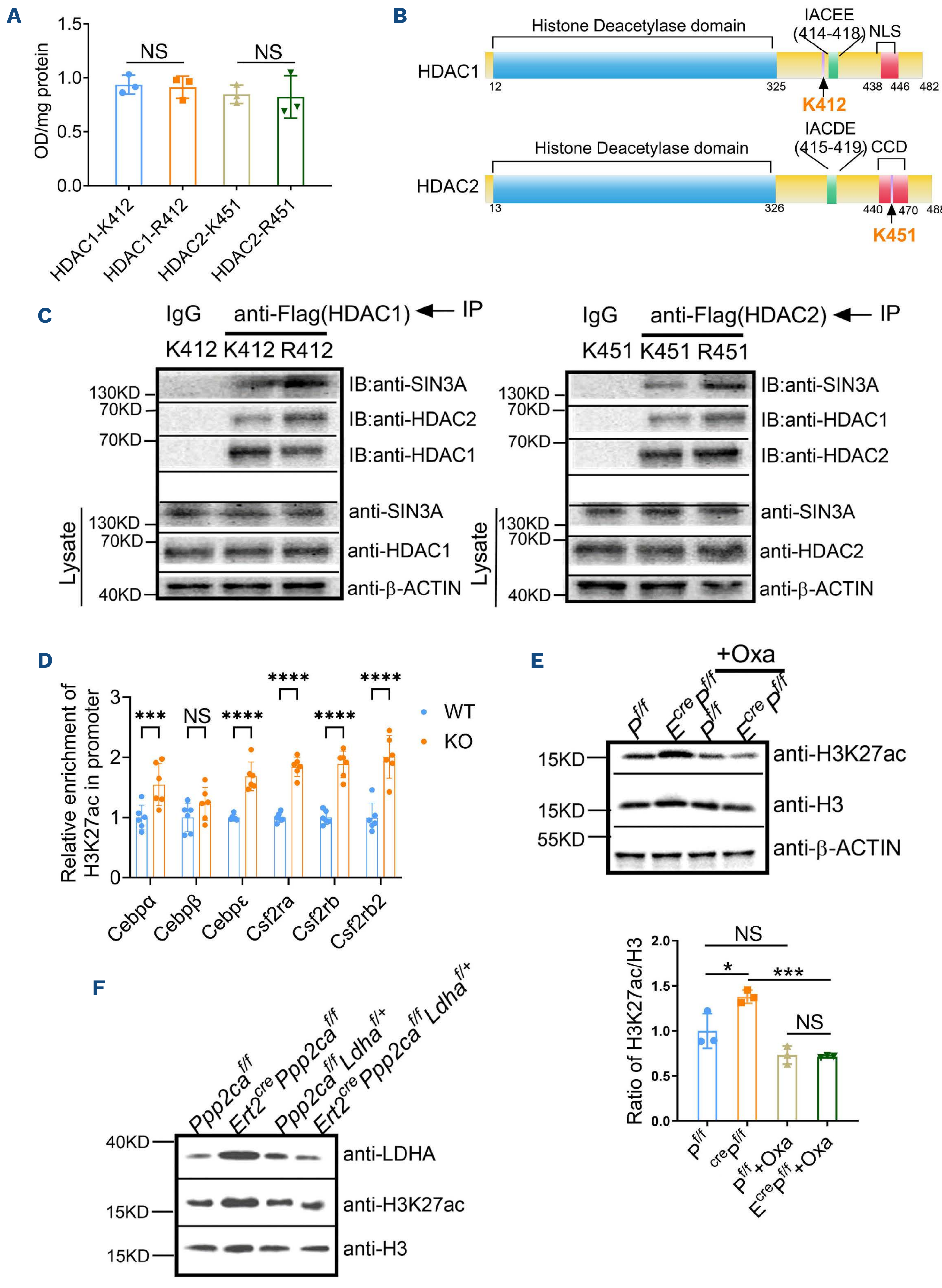

Figure 7. Enhanced lactylation of histone deacetylases contributes to the excessive expression of myeloid determining genes following P*pp2ca* deletion. (A, C) Lin$^-$ cells were transfected with the lentiviral constructs encoding HDAC1-K412-Flag (K412), HDAC1-R412-Flag (R412), HDAC2-K451-Flag (K451) or HDAC2-R451-Flag (R451). (A) Overexpressed histone deacetylases (HDAC) were immunoprecipitated (IP) with anti-Flag antibody. The deacetylase activity of HDAC1 and HDAC2 was detected. N=3. *P* values were determined by one-way ANOVA followed by Tukey's *post hoc* test. Data are presented as mean ± standard error of the mean (SEM). NS: not significant. (B) Schematic diagram of the structural domains of HDAC1 and HDAC2. (C) The impact of site-specific HDAC lactylation on the assembly of SIN3A/HDAC complex. Overexpressed HDAC proteins were IP with anti-Flag antibody, and associated proteins were detected by immunoblotting. Data are representative of 3 independent experiments. (D) LSK cells isolated from the bone marrow (BM) of tamoxifen-pretreated *Ppp2ca*$^{f/f}$ (wild-type [WT]) and *Ert2*^{cre}*Ppp2ca*$^{f/f}$ (knockout [KO]) mice

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were subjected to chromatin immunoprecipitation-quantitative polymerase chain reaction analysis. The relative enrichment of H3K27ac at the promoter of target genes is shown. N=6. *P* values were determined by a two-tailed unpaired *t* test. Data are presented as mean ± SEM. ****P*<0.001; *****P*<0.0001. (E) LSK cells were prepared from the BM of tamoxifen-treated $Ppp2ca^{f/f}$ ($P^{f/f}$) and $Ert2^{cre}Ppp2ca^{f/f}$ ($E^{cre}P^{f/f}$) mice, and then incubated with oxamate (Oxa). The acetylation level of histone H3 at lysine 27 (H3K27ac) was assessed by immunoblotting. β-actin was used as the internal loading control. Data are representative of 3 independent experiments. *P* values were determined by one-way ANOVA followed by Tukey's *post hoc* test. Data are presented as mean ± SEM. **P*<0.05; ****P*<0.001. (F) Immunoblot analysis of the LDHA and H3K27ac levels in the BM LSK cells from $Ppp2ca^{f/f}$, $Ert2^{cre}Ppp2ca^{f/f}$, $Ppp2ca^{f/f}Ldha^{f/+}$ and $Ert2^{cre}Ert2^{cre}Ppp2ca^{f/f}Ldha^{f/+}$ mice.

promotes a striking increase in myelopoiesis at the earliest hematopoietic stages.[47,48] Therefore, the upregulated expression of *Cebpα, Cebpε, Csf2ra, Csf2rb* and *Csf2rb2* in *Ppp2ca*-deficient LSK cells due to enhanced acetylation of H3K27 led to aberrant myelopoiesis. Furthermore, as it has been well-documented that C/EBPα deficiency can result in an augmented quantity and enhanced repopulating capacity of HSC,[43] the upregulated expression of Cebpα may potentially play a role in the downregulation of HSC-related genes within the *Ppp2ca*-deficient LSK.

Notably, the alteration in the proportion of DC is less prominent than that observed in other immune cell subsets in KO mice. There may be two reasons for these results. On the one hand, accumulating studies have confirmed that DC originate from hematopoietic progenitors that are distinct from lymphoid and myeloid progenitors.[49-51] Thus, *Ppp2ca* exerts differential effects on the differentiation potential of distinct progenitor subsets. On the other hand, it has been reported that *Cebpα* - whose expression is elevated in the HSC of KO mice (Figure 2B) - is essential for the differentiation of stem/progenitor cells into common DC progenitors (CDP), but is dispensable for the subsequent transition of CDP to mature DC.[52] Thus, the upregulated Cebpα expression in KO HSC may provide a mechanistic explanation for the mild increase in the numbers of cDC and pDC observed in our study (Figure 1H). Moreover, high levels of Cebpα can drive lymphoid-to-myeloid lineage reprogramming of hematopoietic progenitors.[53] This lineage-shifting effect of Cebpα overexpression may account for the more profound changes in the populations of T cells, B cells, monocytes, and granulocytes - starkly contrasting with the mild alterations in DC subsets.

In addition to *Ldha*, several other genes - including *Gimap6, Six1*, and *Gas6* - are also implicated in regulating the abnormal phenotypes associated with *Ppp2ca* deficiency. *Gimap6* is essential for maintaining T-cell survival and homeostasis.[54] The marked downregulation of *Gimap6* in KO model thus likely contributes to the reduced proportion of T cells, which aligns with the observed immunophenotypic abnormalities. *Six1* has been shown to promote the maintenance of myeloid leukemia stem cells and enhance myeloid proliferation.[55] Consequently, the elevated expression of *Six1* in KO mice may drive the abnormal expansion of myeloid cells. *Gas6* is well characterized as a potent growth factor that supports the robust expansion of HSC.[56] The reduced *Gas6* expression in KO mice is consistent with the diminished expression of HSC lineage-specific genes in *Ppp2ca*-deficient LSK cells.

As the most abundant phosphatase with broad effect, PP2A has emerged as a pivotal therapeutic target for diverse tumors, including leukemia.[57] Moreover, *Ppp2ca* has been identified as a lupus susceptibility gene and the PP2A activator fingolimod/FTY720 has been approved by the Food and Drug Admsintration for the treatment of multiple sclerosis.[58,59] In this work, pharmacological inhibition of lactate significantly ameliorated the abnormalities resulting from PP2A dysfunction. Therefore, our research proposes lactate blockade as a potential novel treatment for a broad range of PP2A-associated disorders.

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