Lysine succinylation precisely controls normal erythropoiesis

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Abstract

Lysine succinylation (Ksu) has recently emerged as a protein modification that regulates diverse functions in various biological processes. However, the systemic, precise role of lysine succinylation in erythropoiesis remains to be fully elucidated. In this study, we noted a prominent increase of succinyl-CoA and lysine succinylation during human erythroid differentiation. To explore the functional significance of succinylation, we inhibited succinylation by either knocking down key succinyltransferases or overexpressing desuccinylases. Succinylation inhibition led to suppressed cell proliferation, increased apoptosis, and disrupted erythroid differentiation. *In vivo* overexpression of the desuccinylase SIRT5 delayed erythroid differentiation. Furthermore, integrative proteome and succinylome analysis identified 939 succinylated proteins with 3,562 Ksu sites, distributed across various cellular compartments and involved in multiple cellular processes. Significantly, inconsistencies were observed between protein expression levels and succinylation levels, indicating that the succinylation of certain proteins may function independently of expression. Mechanistically, we implicated KAT2A-mediated succinylation of histone H3 K79, leading to chromatin remodeling and, subsequently, regulation of erythropoiesis. Specifically, we identified CYCS as a key regulator of erythropoiesis, a function that depends on its succinylation sites K28/K40. Taken together, our comprehensive investigation of the succinylation landscape during erythropoiesis provides valuable insights into its regulatory role and offers potential implications for erythroid-related diseases.

Introduction

Red blood cells, the most abundant of circulating blood cells, play crucial roles in gas exchange and in immunity.[1] In a healthy adult, more than $2x10^6$ red blood cells are generated per second through a highly regulated and complex process known as erythropoiesis.[2] This process involves various cellular events, including lineage selection, morphological and structural modifications, cell metabolic changes, and cell cycle regulation.[3-5] Following commitment to the erythroid lineage, hematopoietic stem cells generate the early erythroid progenitor, burst-forming unit-erythroid cells. These latter cells further develop into colony-forming unit-erythroid cells that undergo terminal erythroid differentiation. During terminal erythroid differentiation, proerythroblasts undergo four or five mitotic divisions to generate sequentially ba-

sophilic, polychromatic, and orthochromatic erythroblasts. Finally, the orthochromatic erythroblasts enucleate to generate reticulocytes.[6,7] Disordered erythropoiesis is a critical feature of human diseases such as thalassemias, congenital dyserythropoietic anemias, and myelodysplastic syndromes.[8] Previous studies have established essential roles of transcription factors and cytokine-mediated signal transduction in regulating erythropoiesis, including GATA1,[9] KLF1,[10] and TAL1[11] as well as EPO[12] and TGF-β.[13] With the emergence of the post-genome era, post-translational modifications of proteins, including methylation, acetylation, phosphorylation, and ubiquitination, have increasingly become an area of active investigation within the field of erythroid development.[7,14-19] For example, HDAC5 was reported to regulate chromatin remodeling in late-stage erythroblasts by affecting H4 acetylation.[7] GATA1 can be stabilized by the deubiquitylase USP7 to regulate human terminal erythroid differentiation.[19] Phosphorylated proteins have been mapped to the regulatory network during human erythroid differentiation.[15,16] These studies increase our understanding of post-transcriptional and translational mechanisms during erythroid development. However, the precise, systematic roles of additional post-translational modifications in erythropoiesis largely remain to be defined.

With recent technological advances, novel methods for analyzing protein post-translational modifications have been developed. Lysine succinylation, a recently recognized reversible modification, has garnered a lot of attention. This modification involves the covalent addition of succinyl groups to lysine residues, resulting in a change of protein charge from positive to negative, along with associated structural alterations.[20] The level of succinylation is precisely regulated by succinylation donors, lysine desuccinylases, and succinyltransferases.[21] Disruptions in succinylation have been linked to diverse diseases, including abnormal stem cell development,[22] tumors,[23] cardiac metabolic diseases,[24] and nervous system disorders.[25] However, the role of lysine succinylation in erythropoiesis has not been elucidated.

In the present study, we evaluated changes in succinylation levels during erythroid differentiation *in vitro* and *in vivo* and showed that decreases in succinylation levels disrupt cell proliferation and differentiation, as well as induce apoptosis. Using mass spectrometry, we identified a comprehensive list of succinylated proteins at both early and late stages of erythroid differentiation and uncovered the molecular mechanisms of regulation of erythropoiesis by succinylation. These findings contribute to our increased understanding of novel post-translational modifications involved in the regulation of erythropoiesis.

Methods

Antibodies and reagents

All antibodies and reagents used for flow cytometry, western blotting, and immunofluorescence analyses are listed in *Online Supplementary Tables S1* and *S2*.

Ethical approval

Human blood samples were acquired from Xiangya Hospital with the approval of the Ethics Committee. Informed consent was obtained from all participating subjects as well as their guardians, when applicable. Animal experiments were performed according to protocols approved by the Animal Ethics Committee of Xiangya Hospital.

In vitro differentiation of CD34$^+$ cells and HUDEP2 cells towards the erythroid lineage

Through our previous research, we identified the composition of the culture medium protocol for differentiation of CD34$^+$ cells from peripheral blood mononuclear cells induced to undergo erythroid differentiation.[5] A human umbilical cord-derived erythroid progenitor cell (HUDEP2) cell line (from the Tianjin Blood Research Institute) was induced to undergo erythroid differentiation using the previously described protocol.[26]

Cytospin preparation

A total of 1×10^5 cells in 100 μL DPBS were spun for 5 minutes at 400 rpm onto glass slides using a cytospin apparatus. After airdrying for 1 minute, slides were stained with Giemsa staining solution (Sigma, Darmstadt, Germany) according to the manufacturer's instructions. Stained cells were viewed, and images were acquired with an Olympus BX51 microscope and QCapture Pro 6.0 (Tokyo, Japan).

Plasmid construction, virus preparation and cell infection

Plasmid construction, viral packaging of target genes, and cell infection procedures were conducted using established protocols described in our previous studies.[27] The sequences of shRNA used are listed in *Online Supplementary Table S3*.

Bone marrow transplantation

Bone marrow transplantation was performed using 6- to 8-week-old C57BL6 mice. The mice were intraperitoneally injected with 150 mg/kg of 5-fluorouracil to ablate endogenous hematopoietic stem cells. After 5 days, the mice were sacrificed and bone marrow cells were harvested and then infected with either the MigR1 retrovirus (containing IRES-GFP) or the MIGR-SIRT5 retrovirus. The infected bone marrow cells were then transplanted into sublethally irradiated 6- to 8-week-old recipient C57BL6 mice via retro-orbital injection. For lineage tracing experiments, doxycycline (2 mg/mL) and sucrose (10 mg/mL) were added to the drinking water of recipient mice prior to transplantation and maintained throughout the study.

Liquid chromatography tandem mass spectometry-based analysis of coenzyme A

Cells at different stages of differentiation were obtained as

previously described.[28] Mass spectrometry was used to detect levels of coenzyme A (CoA) in the cells.

Bioinformatics analysis

The succinylated peptides or proteins with valid intensities in at least two replicates were selected for subsequent analysis. We set the criteria for differential proteins/sites as $|\log_2$ fold change$| > 0.5$ and $P<0.05$.[25,29] Functional annotation of the identified proteins was carried out using the online platform Proteomaps (*https://www.proteomaps.net/*).[30] The Motif-X algorithm was applied to analyze motif characteristics within the ten amino acid residues preceding and following lysine residues.[31] Enrichment analysis was performed using the clusterProfiler package[32] and the DAVID tool.[33] Subcellular localization data for proteins were retrieved from The Human Protein Atlas (*https://www.proteinatlas.org/*). To visualize pathway enrichment outcomes, Cytoscape (v3.9.0) and the EnrichmentMap[34] application were used.

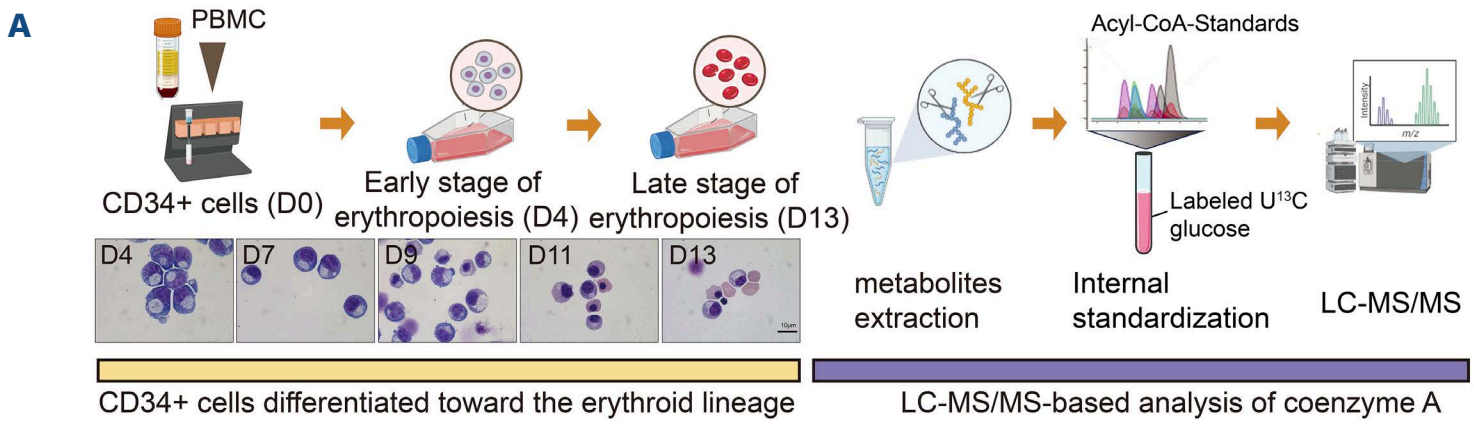
Statistical analysis

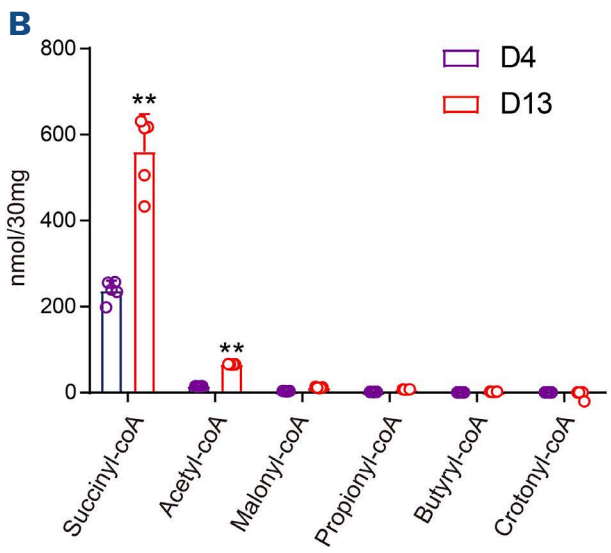
Statistical analyses were conducted on data from three independent experiments, and the bar plots represent the mean ± standard deviation of triplicate samples. Unless otherwise specified, differences between subgroups were assessed using the Student *t* test. Statistical tests, calculations, and visualizations were primarily conducted using R software (v4.0.3) or GraphPad Prism 8 software.

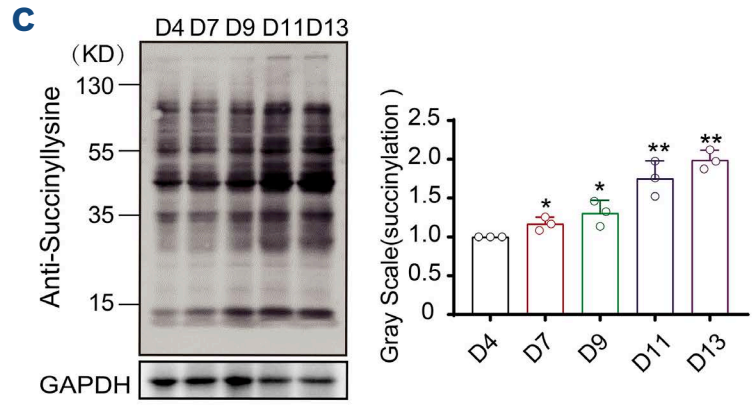
Results

Succinylation is increased during erythropoiesis

Acyl-CoA, essential metabolic intermediates derived primarily from glucose, fatty acids, and amino acids, exert a significant influence on post-translational modifications by adding acyl groups to lysine residues of proteins and modulating their functions.[35] To investigate the potential role of acyl-CoA in erythropoiesis, we initially conducted targeted metabolomics to assess the concentrations of different acyl-CoA types in day 4 (early stage) and day 13 (late stage) erythroid cells (Figure 1A). Our investigation revealed the presence of various common acyl-CoA, including acetyl-, succinyl-, malonyl-, crotonyl-, butyryl-, and propionyl-CoA, in human erythroid cells. Interestingly, we noted that succinyl-CoA levels were most abundant and significantly upregulated during erythroid differentiation (Figure 1B). Subsequently, our investigations were extended to examining whether protein succinylation levels mirrored the observed succinyl-CoA dynamics. Western blot showed a progressive rise in succinylation levels during erythroid differentiation of human CD34$^+$ cells (Figure 1C), whereas other post-translational modifications, such as methylation and acetylation, remained largely unchanged (*Online Supplementary Figure S1A-F*). Furthermore, we corroborated this observation using HUDEP2 cells, an erythroid progenitor cell line derived from CD34$^+$ cells (Figure 1D). To comprehensively assess the subcellular distribution







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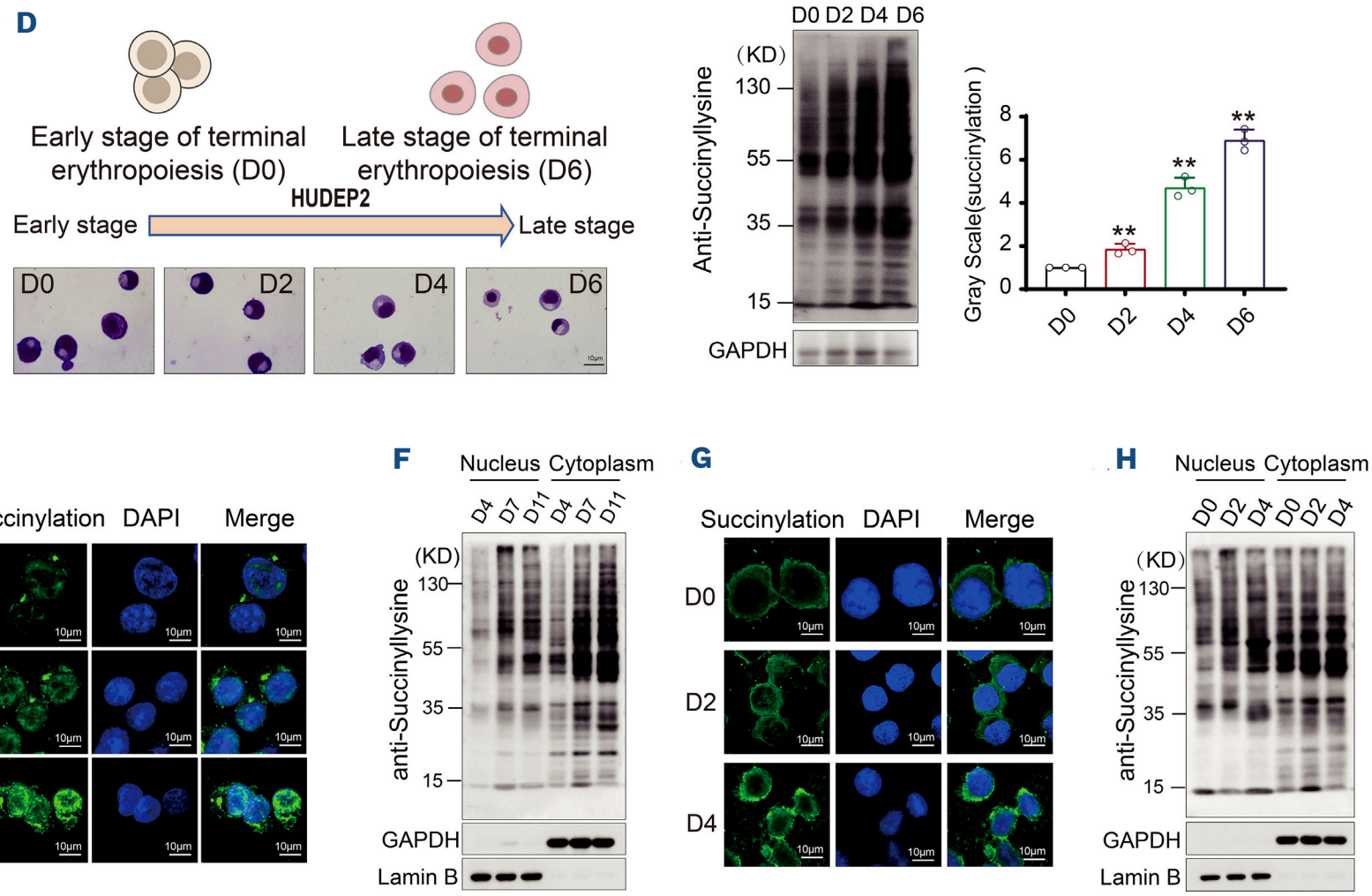

Figure 1. Succinylation accumulates during erythropoiesis. (A) *Left panel*. Illustration of the CD34⁺ cell culture protocol and Giemsa staining defining each developmental stage of human erythroid differentiation. *Right panel*. Schematic representation of targeted metabolomics assays for coenzyme A (CoA). (B) Profiling of short-chain CoA across various stages of human erythroid differentiation using liquid chromatography tandem mass spectrometry (mean ± standard error of mean, N=5 samples). (C) Isolation of CD34⁺ cells from each developmental stage of human erythroid differentiation for cell lysis and subsequent detection of global lysine succinylation through western blot, with GAPDH as the loading control. (D) *Left panel*. Schematic outline of the HUDEP2 cell culture protocol and Giemsa staining defining each developmental stage of human erythroid differentiation. *Right panel*. Collection of HUDEP2 cells from different stages of human erythroid differentiation for cell lysis and assessment of global lysine succinylation by western blot, with GAPDH as the loading control. Quantitative analysis of protein expression data from three independent experiments. (E, G) Immunofluorescence analysis depicting overall lysine succinylation levels during human erythroid differentiation at each developmental stage in CD34⁺ cells (E) and HUDEP2 cells (G) (scale bars, 10 μm). (F, H) Separation of nuclear and cytosolic fractions from each human erythroid differentiation stage in CD34⁺ cells (F) and HUDEP2 cells (H) to determine succinylation levels between these fractions, with confirmation of fraction purity using specific markers. Statistical analysis was conducted on data from three independent experiments, and the bar plot represents the mean ± standard deviation of triplicate samples. $^{*}P<0.05$, $^{**}P<0.01$ *versus* control based on a Student *t* test. PBMC: peripheral blood mononuclear cells; LC-MS/MS: liquid chromatography tandem mass spectrometry; HUDEP2: human umbilical cord-derived erythroid progenitor cells; DAPI: 4′,6-diamidino-2-phenylindole.

of succinylation throughout erythroid differentiation, we performed immunofluorescence imaging on nuclear and cytoplasmic fractions. A robust increase of succinylation was seen in both compartments (Figure 1E-H). Collectively, these dynamic changes in the levels of succinylation suggest that this post-translational modification has an important role in erythropoiesis.

Disruption of lysine succinylation impairs human erythroid differentiation

Recent findings have documented that succinyltransferases and desuccinylases, including CPT1A,[36] HAT1,[37] KAT2A,[38] SIRT5,[39] and SIRT7,[40] are key regulators of succinylation. To investigate the importance of succinylation within erythroid differentiation, we employed an shRNA-mediated knockdown approach or a lentiviral method for overexpression to interfere with this process. The quantitative polymerase chain reaction (qPCR) results showed that the mRNA levels of the three succinyltransferases were significantly decreased and the two desuccinylases were significantly overexpressed (*Online Supplementary Figure S2A*). Knockdown of succinyltransferases CPT1A, HAT1, and KAT2A resulted in decreased succinylation levels in HUDEP2 cells (Figure 2A). This reduction was accompanied by inhibition of cell proliferation, cell cycle arrest, induction of apoptosis and blockage of erythroid differentiation (Figure 2B-E). Similarly, reduction of succinylation by overexpression of the desuccinylases SIRT5 and SIRT7 led to comparable consequences. Furthermore, we disrupted succinylation in human CD34⁺ cells by knocking down KAT2A or overex-

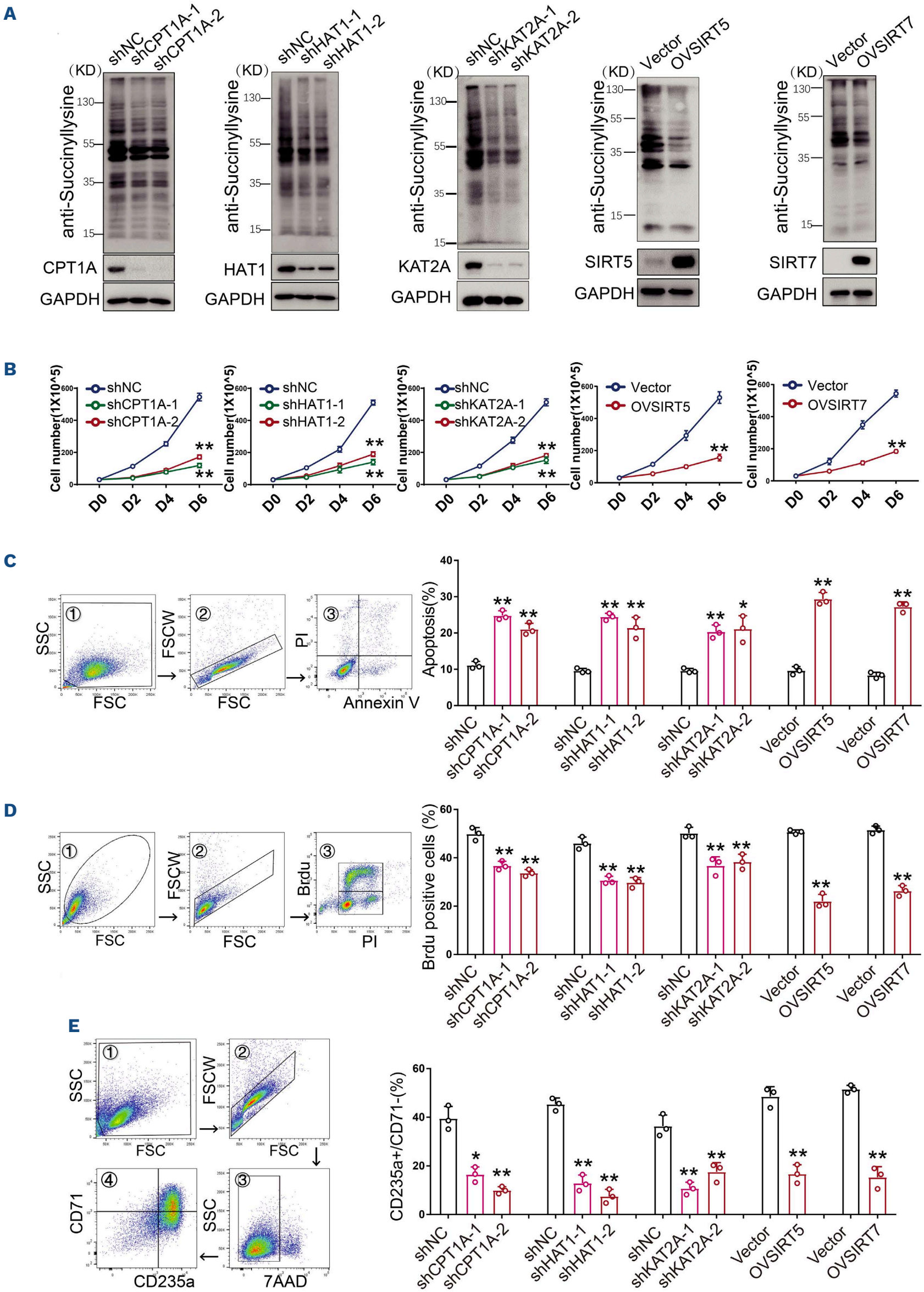

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Figure 2. Impact of global lysine succinylation on human terminal erythroid differentiation. (A) Western blot analysis depicting global lysine succinylation levels in HUDEP2 cells following infection with knockdown of succinyltransferases or overexpression of desuccinylases. (B) Growth curves of erythroid cells at various developmental stages, determined by manual cell counting. (C) *Left panel*. Flow cytometry plots showing gating of apoptotic cells assessed via annexin V/propidium iodide staining. *Right panel*. Cell apoptosis proportions during erythroid differentiation in HUDEP2 cells. (D) *Left panel*. Flow cytometry plots showing gating of S-phase cells assessed via bromodeoxyuridine (BrdU)/propidium iodide staining. *Right panel*. Results of cell-cycle distribution obtained through BrdU assay during erythroid differentiation in HUDEP2 cells. (E) *Left panel*. Flow cytometry plots showing gating of erythroid cells assessed via $CD235a^+$ (GPA) and CD71 staining. *Right panel*. Proportions of GPA and $CD71^-$ cells during erythroid differentiation in HUDEP2 cells. Statistical analysis was conducted on data from three independent experiments, and the bar plot represents the mean ± standard deviation of triplicate samples. Significance levels are denoted as $^*P<0.05$, $^{**}P<0.01$ *versus* control, based on a Student *t* test. shNC: short hairpin negative control; SSC: side scatter; FSC: forward scattered light signal width; PI: propidium iodide; Brdu: bromodeoxyuridine; 7AAD: 7-aminoactinomycin.

pressing SIRT5, resulting in impaired erythroid development (*Online Supplementary Figure S2B-H*). Consistent with these findings, treatment with three succinyltransferase inhibitors (butyrolactone-3, etomoxir, and JG-2016) produced similar results (*Online Supplementary Figure S3A-E*).

Overexpression of the desuccinylases SIRT5 delays erythroid differentiation *in vivo*

Our bulk RNA-sequencing data suggested that among enzymes related to protein succinylation modification, SIRT5 expression is relatively low during erythroid differentiation (*Online Supplementary Figure S4A*).[41] Analysis of public single-cell RNA-sequencing data from bone marrow samples[42] corroborated this finding, showing low SIRT5 expression in the erythroid lineage (*Online Supplementary Figure S4B*). Additionally, SIRT5 is extensively employed as a tool for investigating protein succinylation *in vivo*.[24,43,44] We therefore conducted a bone marrow transplantation study in lethally irradiated B6 recipient mice, wherein retroviral transduction of the expression vector (MigR1) or OV-SIRT5 was utilized. The overall experimental process is illustrated in *Online Supplementary Figure S4C*. Four weeks after transplantation, we assessed SIRT5 expression and succinylation levels in mouse bone marrow cells, revealing a significant reduction in succinylation levels in the bone marrow cells of mice transfected with OV-SIRT5 compared to those transfected with only vector (Figure 3A, *Online Supplementary Figure S4D*). Blood cell count analysis further demonstrated that red blood cell counts and hemoglobin levels were significantly lower in OV-SIRT5 mice compared to vector-transfected animals, while white blood cell counts did not differ significantly (Figure 3B). Skeletal examination of the lower limbs revealed a noticeable pallor in the bones of OV-SIRT5 mice when compared to those of the control mice (Figure 3C). This distinction was also observed in the bone marrow cell pellets. Moreover, the bone marrow cell count indicated a significantly lower number of cells in OV-SIRT5 mice than in the control group (*Online Supplementary Figure S4E*). Flow cytometric analysis confirmed that overexpression of SIRT5 reduced the proportion of $Ter\text{-}119^+$ erythroid cell among bone marrow GFP^+ cells (Figure 3D, *Online Supplementary Figure S4F*). Furthermore, we performed immunohistochemical staining on the bone marrow, and we found that after SIRT5 overexpression, the expression of TER119 in the marrow was reduced, and there was no significant difference in the expression of other lineage-specific markers (Figure 3E, *Online Supplementary Figure S4G*). Subsequently, we distributed erythroblasts into five subpopulations based on the surface marker levels of CD44.[45] Notably, flow cytometry analysis showed that SIRT5 overexpression induced mildly ineffective erythropoiesis, as reflected by decreased reticulocytes in the bone marrow (Figure 3F). Together, our *in vivo* and *in vitro* findings imply that the accurate expression of succinylation plays a crucial role in erythroid development.

The succinylome and proteome of differentiating erythroid cells

To obtain insights into the succinylation of proteins and their participation in regulating erythroid differentiation, we embarked on proteome and succinylome analyses of differentiating HUDEP2 cells (Figure 4A). Principal component analysis showed the reproducibility of the two -omics datasets (*Online Supplementary Figure S5A*). We identified a total of 58,454 peptides corresponding to 6,132 proteins as well as 3,540 succinylated peptides from 939 unique proteins (Figure 4B). We further noted that nearly all succinylated proteins were also present in the proteomes of the same samples. Evidently, the majority (95.7%) of the commonly succinylated proteins were shared between the early stage and late stage of erythroid differentiation in the succinylome, indicating that erythroid differentiation requires alteration of succinylation instead of *de novo* modification (Figure 4B). The degree of protein succinylation was significantly increased in the late stage of erythroid differentiation (Figure 4C), which was consistent with the observed phenotype in Figure 1C, D. In contrast, the expression of protein levels was decreased in erythroid differentiation (*Online Supplementary Figure S5B*). The most abundant succinylated proteins included hemoglobin and mitochondria-related proteins (Figure 4C). Notably, Spearman analysis revealed inconsistencies between alterations in protein levels and succinylation levels (R = -0.103) (Figure 4D). Proteomap analysis of the total proteins revealed a significantly higher proportion of hemoglobin proteins and lower levels of cell cycle proteins in the late stage of

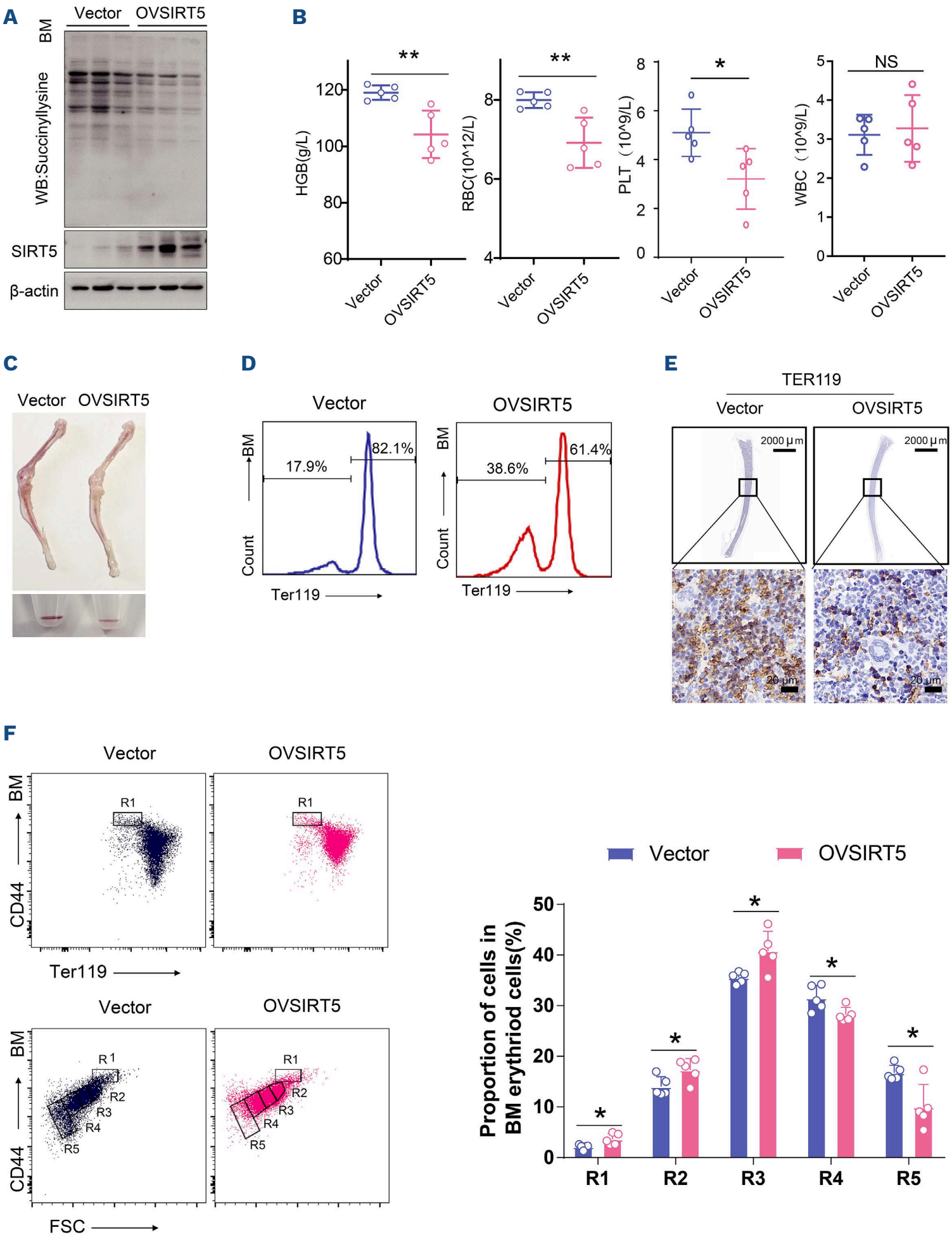


Figure 3. Overexpression of SIRT5 inhibits erythroid development *in vivo*. (A) Immunoblot analysis detecting indicated global lysine succinylation levels in bone marrow (BM) cells of control and OV-SIRT5 mice (N=3). (B) Peripheral blood count analysis in control and OV-SIRT5 mice (N=5). (C) Macroscopic views of the lower limb skeleton and limb BM cell pellets from control and OV-SIRT5 mice. (D) Flow cytometric analysis depicting the percentages of Ter119^{+} cells in BM cells from control and OV-SIRT5 mice. (E) A representative vertical section of mouse BM showing expression of Ter119 by immunostaining. (F) Flow cytometric analysis of erythroblasts in BM of control and OVSIRT5 mice. *Left panel.* Erythroblasts categorized into five subpopulations by surface staining for CD44 and Ter119: Ter119^{med}CD44^{high} proerythroblasts (R1). The bottom panel shows representative plots of CD44 *versus* FSC of Ter119^{+} cells with gating of populations R2, R3, R4, R5 (basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts, reticulocytes). *Right panel.* Cell distribution analysis based on the flow cytometry results. The bar plot represents the mean ± standard deviation of sample numbers. NS: not significant; $^{*}P<0.05$, $^{**}P<0.01$ *versus* control based on a Student *t* test (N=5). HGB: hemoglobin; RBC: red blood cells; PLT: platelets; WBC: white blood cells; FSC: forward scatter.

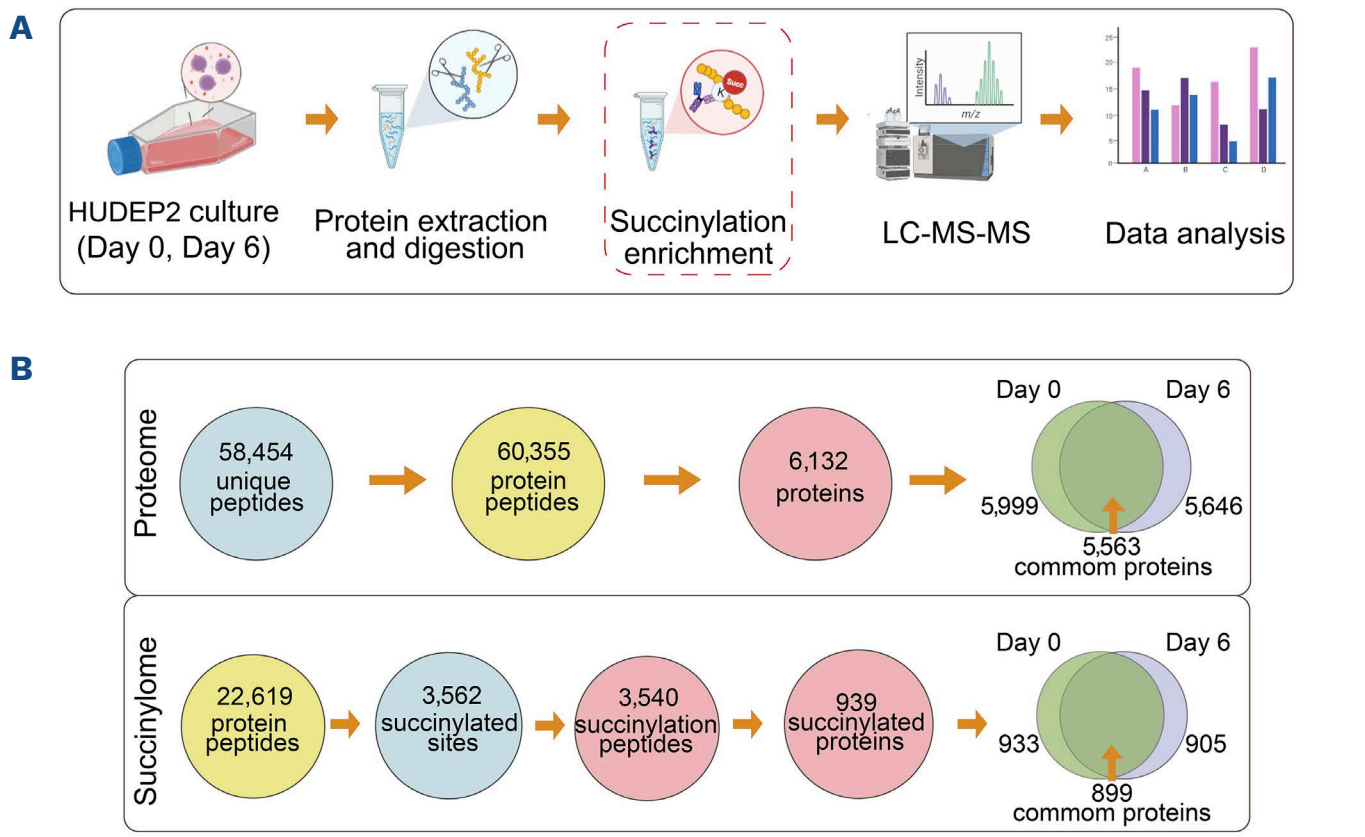


Figure 4. Succinylome and proteome profiling during erythroid differentiation. (A) Experimental workflow showing the 4D-label-free quantification method for both the proteome and succinylome (with an additional succinylation enrichment step). (B) After screening and mining quantitative data, the combined results from six samples of early-stage and late-stage erythroid differentiation revealed 5,563 common proteins and 3,540 quantified succinylated peptides from 939 proteins. (C) The distribution of the succinylome intensity in early or late stages of erythroid differentiation. (D) Association analysis between succinylome and proteome intensities using Spearman rank correlation, after transforming the intensities to z-scores. (E) Distribution of the number of succinylation sites per protein among all succinylated proteins. (F) Analysis of subcellular localization of quantifiable succinylated proteins. HUDEP2: human umbilical cord-derived erythroid progenitor cells; LC-MS-MS: liquid chromatography tandem mass spectrometry; Nuc: nuclear; Cyto: cytoplasm; Mito: mitochondrion.

erythroid differentiation (*Online Supplementary Figure S5C*). To investigate the sequence patterns of succinylation during erythroid differentiation, we identified eight conserved motifs from all succinylated peptides (*Online Supplementary Figure S6A*). We quantified the occurrence of succinylation sites and observed that approximately 60% of the proteins contained two or more succinylation sites (Figure 4E). Furthermore, we performed gene ontology enrichment analysis on proteins with more than ten succinylation sites and identified significant enrichment in pathways such as respiratory electron transport, ATP synthesis, and other related processes (*Online Supplementary Figure S6B*). To assess the specificity of proteins with more than ten succinylation sites, we compared our findings with succinylation data from patients with Alzheimer disease[25] as a non-erythroid reference (*Online Supplementary Figure S6C*). Interestingly, while there was some overlap, we found that highly succinylated proteins in our cohort were enriched in the erythroid lineage. In contrast, proteins highly succinylated in patients with Alzheimer disease were predominantly associated with mitochondrial metabolism, such as ALDH7A1. We ectopically expressed HBB and ALDH7A1 in $CD34^+$ cells, observing that HBB was significantly more succinylated in late erythroid cells than in early cells (*Online Supplementary Figure S6D*). Immunoprecipitation results showed that HBB interacted with CPT1A but not with HAT1 or KAT2A (*Online Supplementary Figure S6D*). Although ALDH7A1 exhibited detectable succinylation when overexpressed, it did not interact significantly with CPT1A, KAT2A, or HAT1 (*Online Supplementary Figure S6E*). This specificity may reflect the distinct metabolic and functional requirements of different cellular processes, such as erythroid development *versus* neurodegenerative diseases.

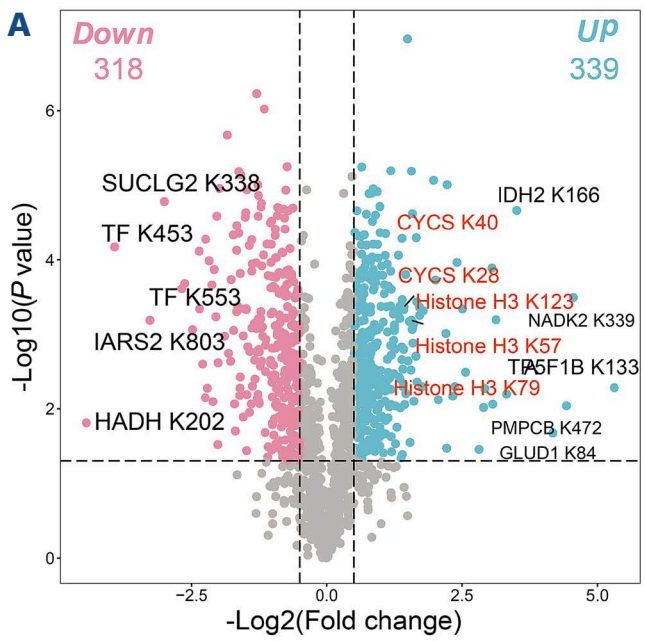
Additionally, we noted that proteins with multiple Ksu sites (N>2) exhibited more succinylation modification at the late stage of erythroid differentiation (*Online Supplementary Figure S6F*), such as SLC4A1, SPTA1, and SPTB. The western blot experiments indicated that these proteins had high succinylation levels (*Online Supplementary Figure S6G*). We further investigated the distribution and role of succinylated proteins by conducting a cellular compartment analysis of the 939 quantifiable succinylated proteins. Our findings revealed that succinylated proteins are present in various subcellular compartments (Figure 4F). Notably, approximately 33.8% (317/939) of the succinylated proteins were localized in the mitochondria, including CYCS, SDHA, and LDHA. In the cytoplasm, the succinylated proteins displayed significant enrichment in processes such as translational initiation, mRNA catabolism, and protein targeting to the endoplasmic reticulum (*Online Supplementary Figure S6H*). The succinylated proteins localized in the nucleus showed significant enrichment in the regulation of mRNA metabolic processes, DNA conformation changes, and hematopoietic stem cell differentiation (*Online Supplementary Figure S6H*). Additionally, the succinylated proteins in the mitochondria were significantly enriched in mitochondrial gene expression, translation, and ATP metabolic processes (*Online Supplementary Figure S6H*). The succinylated proteins in the other compartments (membrane, vesicles, endoplasmic reticulum, etc.) were significantly enriched in endosomal transport, response to endoplasmic reticulum stress, and positive regulation of cell adhesion (*Online Supplementary Figure S6H*). These results collectively suggest a potential regulatory role of succinylation during erythroid differentiation.

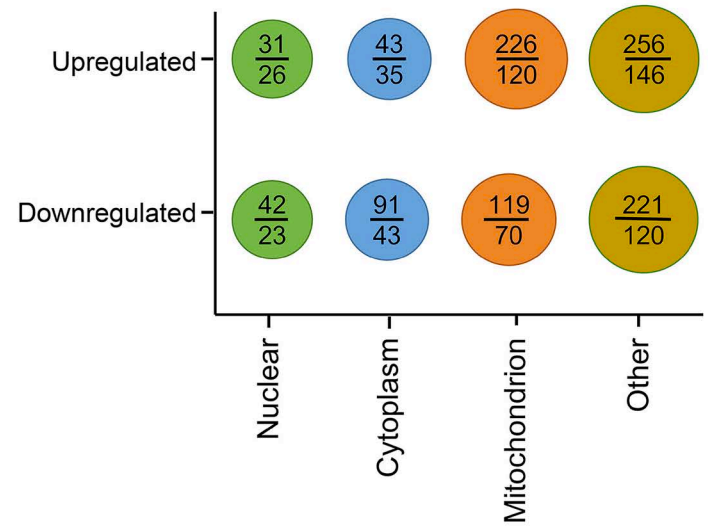
Comparison of the succinylome during early-stage and late-stage erythroid differentiation

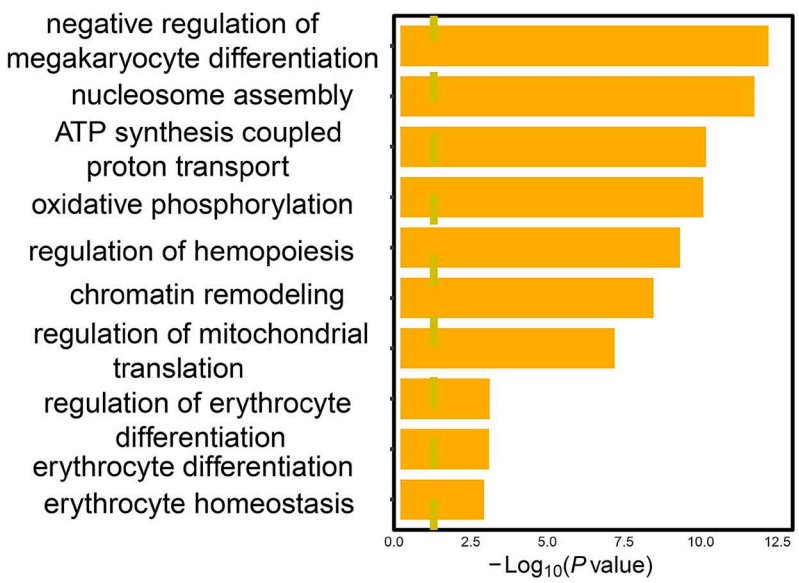
We then investigated the dynamic alterations in succinylation during erythroid differentiation. The volcano plot indicated 339 upregulated Ksu sites along with 318 downregulated Ksu sites (Figure 5A), distributed across the nucleus, cytosol, and mitochondria (Figure 5B). Importantly, gene ontology analysis of the upregulated succinylated proteins demonstrated enrichment in processes critical for erythroid development, including regulation of hematopoiesis, erythrocyte differentiation, and erythrocyte homeostasis (Figure 5C). This enrichment underscores the potential importance of protein succinylation in regulating red blood cell development and function. Conversely, downregulated succinylated proteins exhibited enrichment in metabolic processes such as ribonucleotide biosynthesis, small molecule catabolism, and fatty acid metabolism (Figure 5D), suggesting a shift in cellular metabolism during differentiation. To further reveal the pivotal pathways involving succinylated proteins, we constructed a functional enrichment network (Figure 5E). This analysis reinforced our findings, revealing enrichment in pathways related to hematopoiesis and heme synthesis, including erythrocyte differentiation and homeostasis. Collectively, these results highlight the impact of succinylome changes on erythroid differentiation and its associated pathways, pointing to a potential regulatory mechanism in erythropoiesis mediated by protein succinylation.

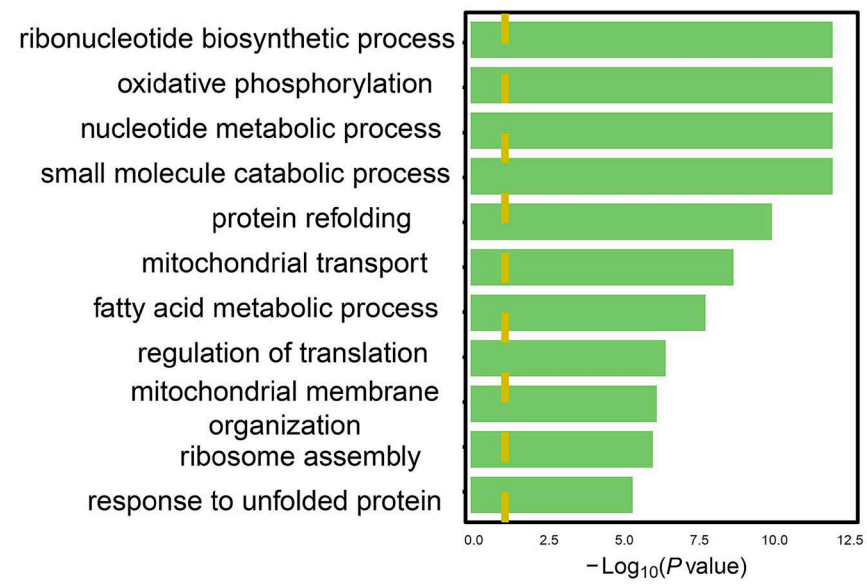
Association analysis of the succinylome and proteome

Given the prevalence of succinylated proteins within mitochondria, we undertook an investigation into the relationship between alterations in metabolism-related protein levels and succinylation levels. As depicted in Figure 6A, a substantial subset of succinylated proteins participated in the pivotal metabolic pathways of erythropoiesis, including glycolysis, oxidative phosphorylation, the tricarboxylic acid cycle, and heme synthesis. Furthermore, we performed a global association analysis of the fold change in succinylation levels and protein levels (Figure 6B). Remarkably, the overall correlation between the fold changes of the two omics was very weak (R = -0.06). We selected the top ten succinylated proteins (including histone H3, CYCS, XPNPEP1, GUF1, FDPS, TARS2, NT5C, ECI2, ATP5F1D, and POLDIP2) to









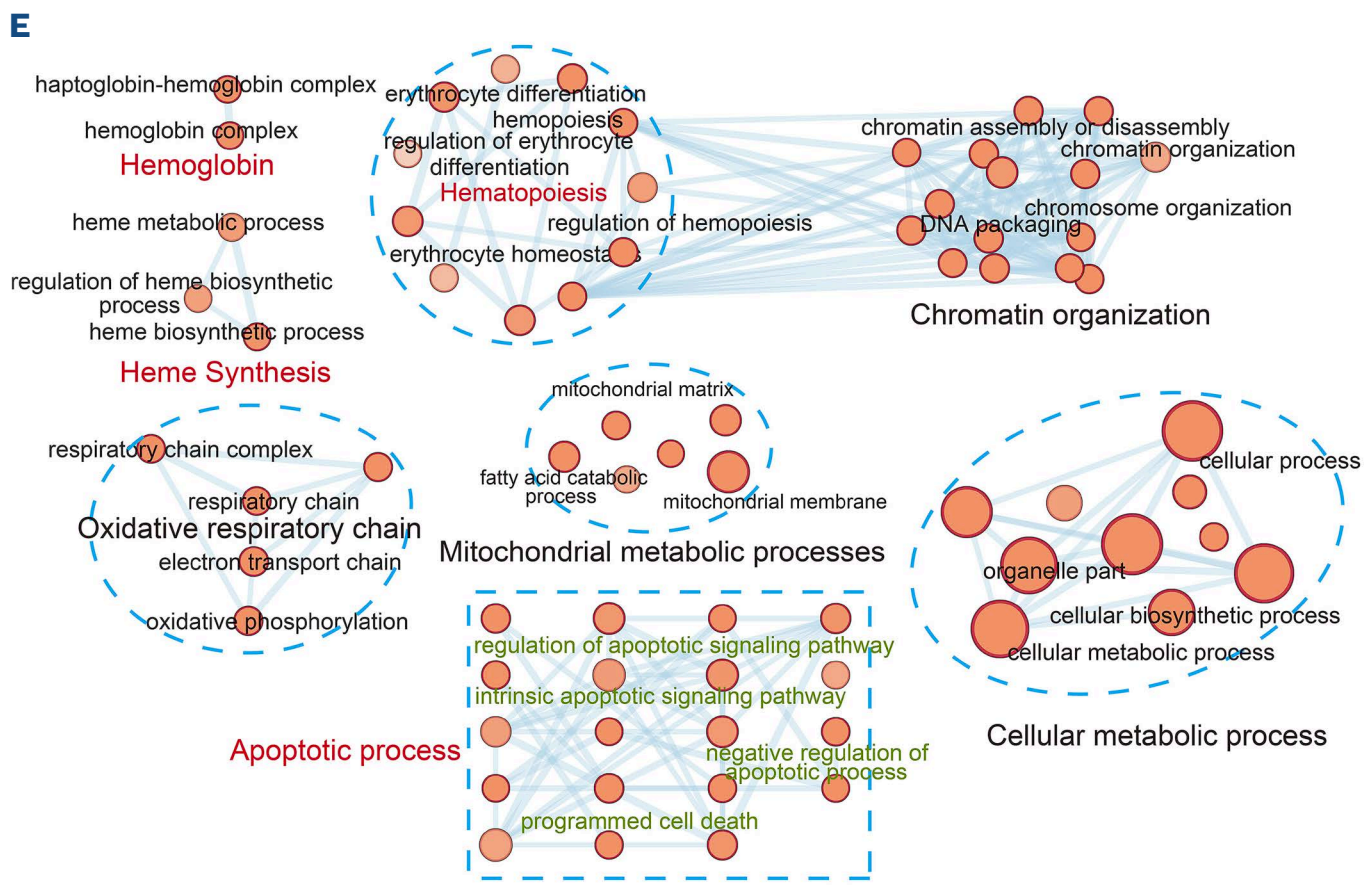


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Figure 5. Differential analysis of the succinylome between early and late erythroid stages. (A) Volcano plot illustrating 939 succinylated proteins in early and late erythroid stages. Each spot represents a specific succinylated peptide. Red symbols to the left of zero indicate significantly downregulated succinylated peptides, while blue symbols to the right of zero indicate significantly upregulated succinylated peptides in the late stage (P<0.05 and $|\log_2(\text{fold change})| \geq 0.5$, Student two-sided t test). (B) Distribution of differential succinylation peptides (numbers above the line) and succinylated proteins (numbers below the line) according to subcellular localization. (C, D) Gene ontology results of upregulated succinylated proteins and downregulated succinylated proteins. (E) Pathway enrichment analysis of the succinylome. The network represents the identified gene ontology and Kyoto Encylopedia of Genes and Genomes (KEGG) terms (nodes) and the relationship between them (edges) based on the similarity of associated genes/proteins. The edge weight reflects the similarity between terms.

visualize using a heatmap, and the results supported the notion that changes in succinylation of these proteins were independent of variations in protein abundance (Figure 6C).

The genome loci of H3K79Ksu mediated by KAT2A in CD34⁺ cells

As the histone H3, which was succinylated by succinyltransferase KAT2A,[38] was the most succinylated protein depicted in the above proteins, we investigated the role of succinylation in H3K79 during erythroid differentiation. Western blot confirmed that the succinylation levels of H3K79 (H3K79Ksu) were significantly increased during erythroid differentiation (Figure 7A, *Online Supplementary Figure S7A, B*). Co-immunoprecipitation proved the interaction between KAT2A and histone H3 in HUDEP2 cells (Figure 7B). Additionally, western blot results demonstrated that KAT2A knockdown reduced the H3K79Ksu (Figure 7C, *Online Supplementary Figure S7C-E*).

To further elucidate the role of H3K79Ksu in the erythrocytes, we performed CUT&Tag of H3K79Ksu after KAT2A knockdown. The normalized density showed that KAT2A knockdown significantly reduced the genomic binding capacity of H3K79Ksu (Figure 6F). Genomic annotation of the affected peaks showed their distribution as follows: 13% in promoters, 31.4% in other introns, and 37.6% in distal intergenic regions (*Online Supplementary Figure S7F*). Representative erythroid gene loci demonstrating H3K79Ksu signal changes after KAT2A knockdown were shown in Figure 6G. Intersection analysis of peaks located in promoter regions identified a total of 2,511 peaks (*Online Supplementary Figure S7G*). Enrichment analysis revealed that these peaks were associated with transcription factor binding, the nuclear envelope, and chromatin remodeling processes (Figure 7F). Previous studies suggested that histones participate in chromatin condensation, enucleation, and reticulocyte maturation processes.[46] To further investigate this, we conducted chromatin immunoprecipitation qPCR experiments using a H3K79 Ksu antibody. Our results suggested that the reduced H3K79Ksu was specifically enriched in genes associated with chromatin remodeling, including XPO7, FOXO3, HDAC6, CLTA, and HNRNPU after KAT2A knockdown (Figure 6H). These findings imply that protein succinylation mediated by succinyltransferases plays an important role in erythroid differentiation.

Succinylation is required for cytochrome C functions in erythropoiesis

Previous literature[47] and our results suggest that proteins with multiple succinylation sites are abundant in mitochondria. Accordingly, we chose cytochrome C (CYCS), which exhibited the most pronounced change in succinylation in mitochondria, for further analysis. CYCS plays a crucial role as an electron carrier in biological oxidation and cellular respiration.[48] Abnormal function of CYCS leads to blood cell-related disease.[49] To investigate the function and succinylation of CYCS during erythroid differentiation, we created shCYCS and succinylation-deficient mutations of CYCS (K28R and K40R) in the modified residues. The western blot confirmed the efficacy of shCYCS and the WT-flag-CYSC or the mutations, and immunoprecipitation assays showed that succinylation levels were downregulated when either K28 or K40 or both were mutated compare to WT-flag-CYCS (Figure 8A). Functionally, knockdown of CYCS led to inhibition of cell proliferation, blocked erythroid differentiation, induced cell apoptosis, and decreased mitochondrial membrane potential (Figure 8B-F, *Online Supplementary Figure S8*). Notably, the overexpression of WT-flag-CYCS rescued these phenotypes of shCYCS but not succinylation-deficient mutants. In summary, these findings reveal that the function of CYCS depends on succinylation in coordinating erythroid differentiation.

Discussion

Our primary findings are illustrated in Figure 8G. In this study, we discovered that succinylation gradually increases during erythroid differentiation. Disrupting succinylation resulted in aberrant erythroid development. Using multi-omics to analyze dynamic changes in the succinylation network, we identified 3,562 succinylation modification sites and 939 succinylated proteins. These proteins are predominantly involved in crucial pathways for erythroid development, including mitochondrial metabolism, heme synthesis, and chromatin remodeling.

During erythroid differentiation, erythrocytes undergo remodeling such that the main protein component of mature erythrocytes is hemoglobin. To achieve this, approximately 10^9 molecules of hemoglobin must be synthesized, requiring the consumption of 10^{10} molecules of succinyl-CoA.[50] The interaction of succinyl-CoA with the erythroid-specific

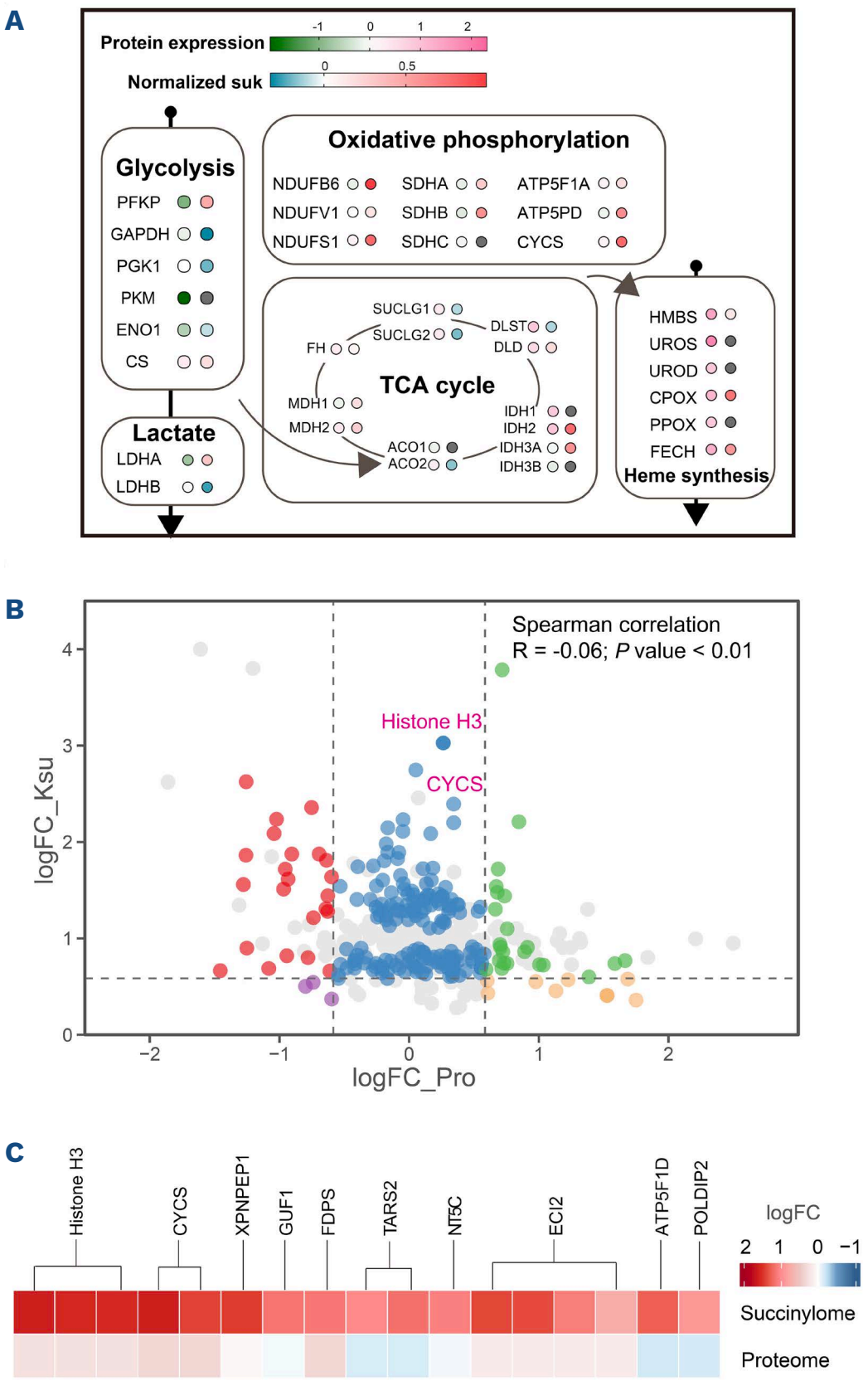

Figure 6. Integrated analysis of the proteome and succinylome. (A) Changes in metabolism-related succinylated proteins in the proteome and succinylome. For each protein, changes in the proteome are shown on the left and changes in succinylation are shown on the right. (B) Comparison of the proteome and succinylome changes. Gray dots indicate statistically not significant results (P>0.05). Blue dots indicate proteins with significantly upregulated succinylation levels but no change in protein levels (P<0.05). Spearman correlation was calculated to determine the correlation between changes in the succinylome and proteome. (C) Heatmap showing the fold change of each succinylated site for the top ten highly succinylated proteins in both the proteome and succinylome. Each square corresponds to a succinylation site. suk: succinylation; TCA: tricarboxylic acid; FC: fold change.

ALAS-E gene is disrupted in sideroblastic anemia.[51] Furthermore, IDH1 mutations can alter downstream succinyl-CoA levels, contributing to the onset and progression of myelodysplastic syndromes.[52] In humans, heme and globin synthesis predominantly occurs in basophilic erythroblasts, with synthesis levels peaking in polychromatic/orthochromatic erythroblasts.[41,50] Interestingly, our mass spectrometry data indicate that succinyl-CoA is the most abundant of the various acyl-CoA and its levels remain elevated throughout terminal erythroid differentiation. Notably, elevated succinyl-CoA levels can result in lysine succinylation of proteins. Succinylation has been suggested to play a role in the regulation of a wide range of biological processes.[20] Although the importance of protein succinylation in pathological conditions, such as leukemia,[53] has been recognized, its role in normal blood development has not been extensively studied. Our study represents the first demonstration that succinyl-CoA serves additional functions during erythropoiesis beyond heme synthesis. We further reveal that succinylation mediated by succinyl-CoA gradually increases during the terminal stages of erythroid differentiation and plays a crucial role.

Protein succinylation levels are regulated by succinyltransferases and desuccinylases. Many of these enzymes, such as KAT2A, HAT1, and SIRT5, have been shown to regulate both succinylation and acetylation.[54] There are also bifunctional enzymes unrelated to acetylation, such as CPT1A.[55,56] Interestingly, when we knocked down succinyltransferas-

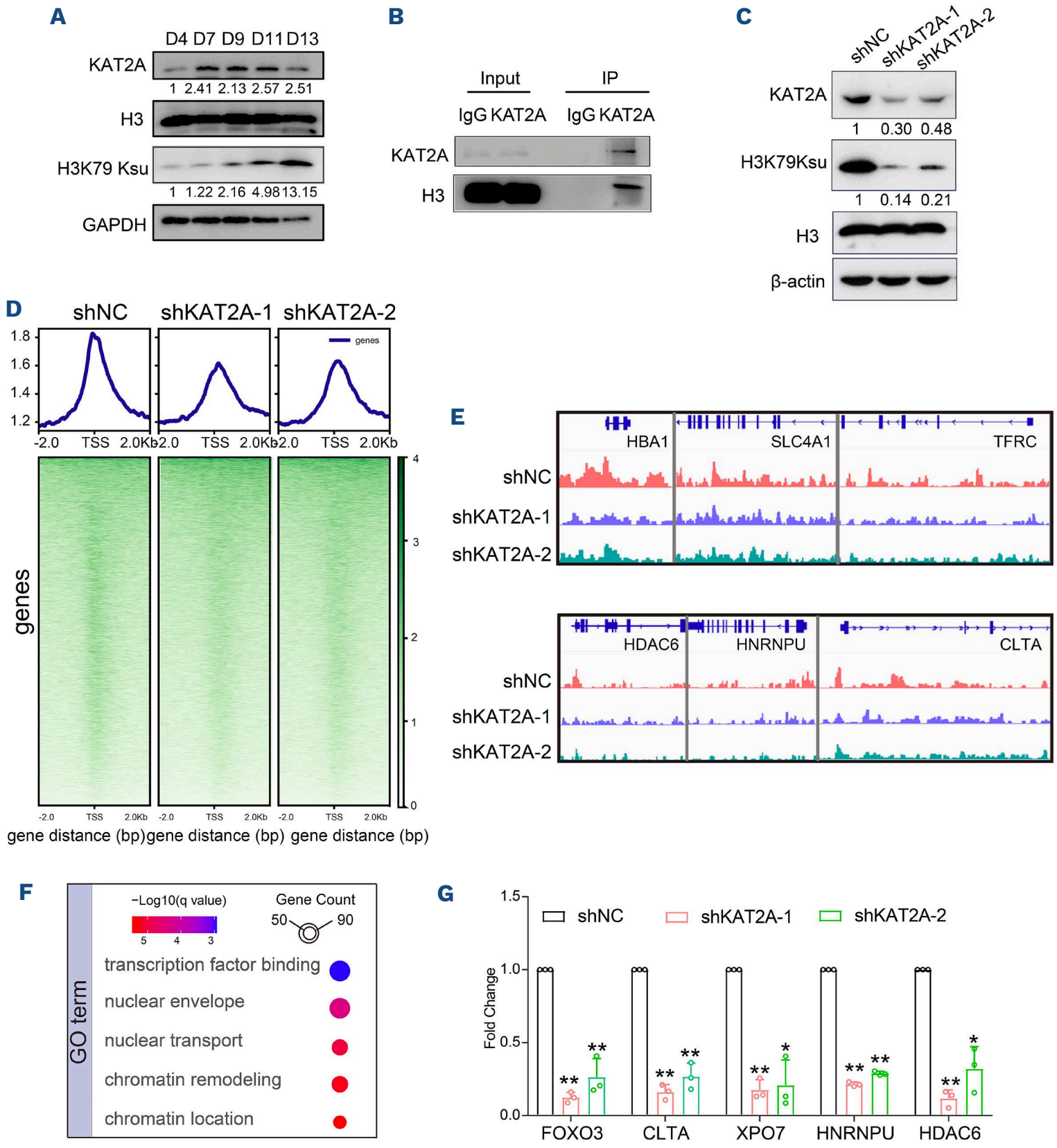

Figure 7. CUT&Tag analysis of H3K79Ksu mediated by KAT2A in CD34⁺ cells. (A) Detection of KAT2A protein level and succinylation level with histone H3 at various stages of erythroid differentiation by western blot. GAPDH was used as a loading control. (B) Co-immunoprecipitation experiment with a KAT2A antibody followed by detection of precipitated proteins using a histone H3 antibody. (C) Western blot showing KAT2A, H3K79 succinylation, and H3 expression in erythroblasts infected with control shRNA or KAT2A shRNA. β-actin was used as a loading control. (D) The peak signal of H3K79Ksu with knocking down KAT2A and control. (E) Representative erythroid genes showing the CUT&Tag signal of H3K79Ksu after KAT2A knockdown. (F) The gene ontology annotation of the affected peaks located in promoter regions. (G) Chromatin immunoprecipitation polymerase chain reaction assay (ChIP-PCR) results for Succ-H3K79 binding to the FOXO3, CLTA-1, HDAC6, XPO7, and HNRNPU promoter regions. ChIP-PCR was performed using Succ-H3K79 and control IgG antibodies in HUDEP2 cells infected with lentivirus containing control shRNA or KAT2A shRNA. IP: immunoprecipitation; shNC: short hairpin negative control; bp: base pairs. CYCS: cytochrome C; GO: gene ontology.

es or overexpressed desuccinylases in terminal erythroblasts, both manipulations showed similar phenotypes, inhibiting terminal cell proliferation and differentiation. Among these succinylation-related enzymes, SIRT5 has been reported to have relatively weak deacetylase activity compared to other sirtuins. In fact, SIRT5 exhibits robust desuccinylation activity with its catalytic efficiency being approximately 1,000-fold higher than its deacetylation activity.[39,57] Therefore, SIRT5 is commonly used in succinylation studies due to its preference for desuccinylation over

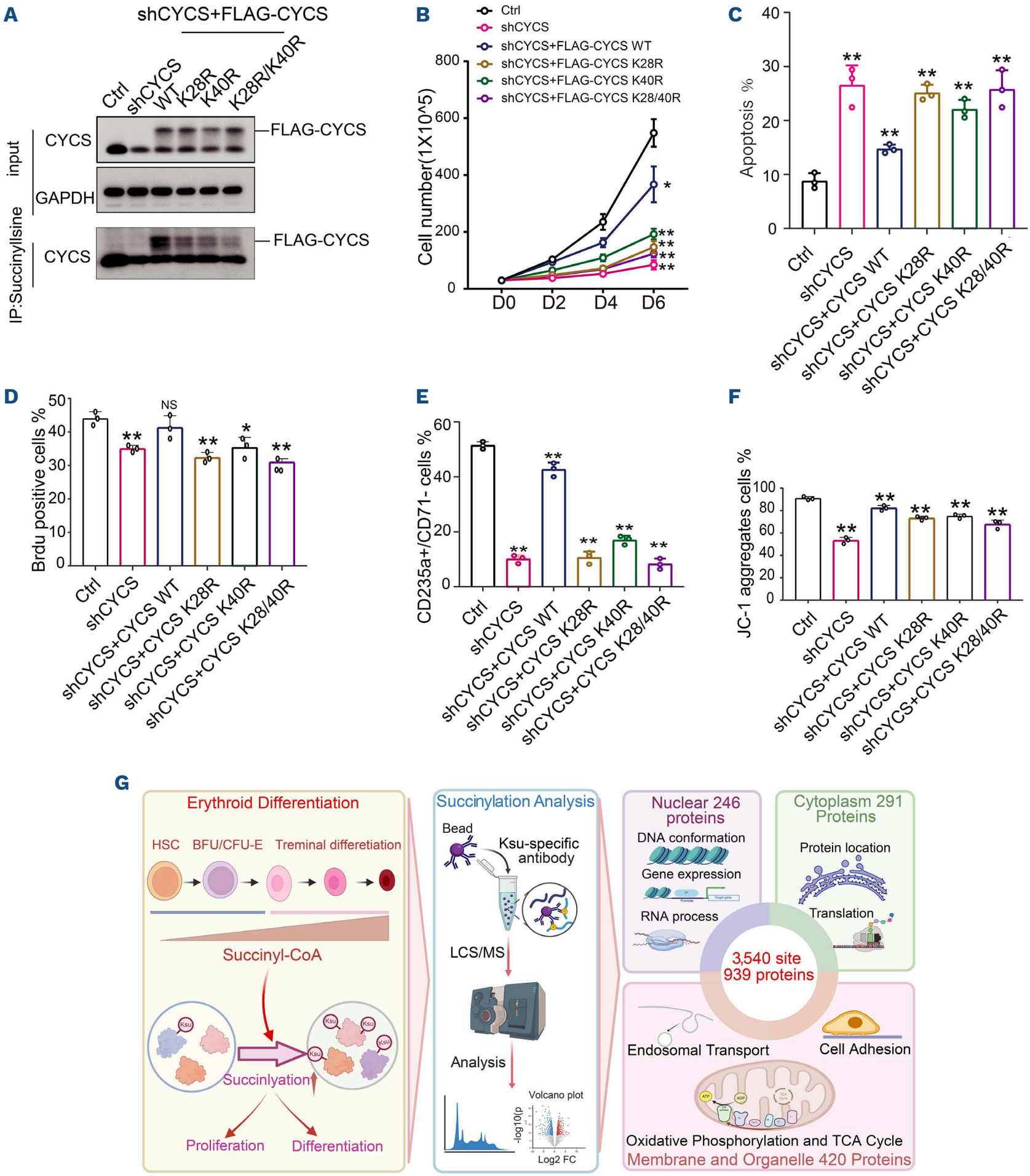

Figure 8. Altered succinylation of cytochrome C impairs erythroid differentiation. (A) Immunoprecipitation of succinylated proteins using pan-Ksu antibody after transfection of flag-tagged vector, flag-WT-CYCS, or its mutants in HUDEP2 cells with shCYCS. (B-E) Transfection of flag-tagged vector, flag-WT-CYCS, or its mutants into HUDEP2 cells with shCYCS, along with control shRNA and flag-tagged vector co-transfected cells as control. Cell growth curves determined by manual cell counting of HUDEP2 cells (B). Representative images of flow cytometry analysis of apoptosis by annexin V/propidium iodide staining in HUDEP2 cells (C). Cell-cycle distribution results of bromodeoxyuridine assay from the above HUDEP2 cells (D). Representative images of flow cytometry analysis of GPA and CD71 expression in HUDEP2 cells (E). (F) Representative flow cytometry analysis of mitochondrial membrane potential using JC-1 staining. (G) Schematic diagram of this study. Statistical analysis was conducted on data from three independent experiments, and the bar plot represents the mean ± standard deviation of triplicate samples. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ *versus* control based on a Student *t* test. CYCS: cytochrome C; IP: immunoprecipitation; Ctrl: control; WT: wild-type; Brdu: bromodeoxyuridine; HSC: hematopoietic stem cells; BFU-E: burst-forming unit erythroid; CFU-E: colony-forming unit erythroid; LCS/MS: liquid chromatography/mass spectrometry; TCA: tricarboxylic.

deacetylation. Furthermore, our investigation revealed that SIRT5 expression remains consistently low across stages of erythroid differentiation (*Online Supplementary Figure S2*), and the phenotype of knockout Sirt5 mice does not exhibit disruption in erythroid development.[58] To deepen our understanding of succinylation, we conducted *in vivo* transplantation experiments in mice by overexpressing SIRT5. Remarkably, this overexpression significantly altered the succinylation patterns of intracellular proteins, resulting in impaired erythroid differentiation and decreased red blood cell production *in vivo*. These findings *in vivo* and *in vitro* underscore the precise regulation of succinylation during erythroid differentiation. Any disturbance in this balance could potentially contribute to erythroid developmental disorders and the onset of erythroid-related diseases.[59]

Prior investigations have predominantly linked succinylation to mitochondrial metabolic enzymes.[60,61] However, subcellular localization analysis of our data reveals that numerous succinylated proteins are distributed across cellular compartments, including the nucleus and plasma. Our findings indicate that succinylation in the nucleus is enriched in pathways such as chromatin remodeling and DNA synthesis, while in the cytoplasm, it predominantly enriches processes such as translation initiation, translocation, and protein synthesis. This suggests the pivotal role of succinylation as a functional modification in erythroid differentiation. Furthermore, our analysis highlights the close association of these protein modifications with red blood cells; many succinylated proteins are associated with erythrocyte functions, particularly within the hemoglobin family. Notably, succinylated forms of hemoglobin comprise 80% of common hemoglobin family members. Remarkably, the succinylation sites we identified align with pathogenic mutation sites reported in HBB and HBA in thalassemia, suggesting a potential role of succinylation in regulating the normal function of hemoglobin.[62,63] We will pursue future studies investigating the relationship between succinylation modification and related diseases. In conclusion, our results establish a bridge between succinylation and erythropoiesis. The quantitative and modification proteomic analyses of the succinylation proteins change during erythropoiesis. Succinylation was found to have an important function in erythroid differentiation and our study provides new mechanistic insights into normal and disordered erythropoiesis.

Disclosures

No conflicts of interest to disclose.

Contributions

JL, YS, LL, and MY conceived and designed the experiments. BH, HG, JZ, YL, DL, YC, LH, HZ, ML, and WX performed experiments and analyzed data. LN collected the blood samples. YN and LS provided the cell line. YS and JL drafted the manuscript. MY, CDH, NM, LL, YS, and JL provided insights into some aspects of the work and edited the manuscript. All authors read and approved the final manuscript.

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Data-sharing statement

The identified succinylated protein data have been deposited in the National Genomics Data Center (https://ngdc.cncb.ac.cn/) with the dataset identifier OMIX004702. Further inquiries may be directed to the corresponding author.

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