Lysine succinylation precisely controls normal erythropoiesis

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Supplemental Methods

$CD34^+$ Cell culture and manipulation

$CD34^+$ cells were purified from peripheral blood mononuclear cells (PMBC) by positive selection with the magnetic-activated cell sorting system (Miltenyi Biotec), according to the manufacturer's instructions. The cell culture procedure was divided into three phases. The cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Life Technologies) containing 200 g/ml human holo-transferrin (sigma-Aldrich), 2% human AB plasma, 10 g/ml insulin (sigma-Aldrich), 3% fetal bovine serum (Gibco FBS, thermofisher), 3 IU/ml heparin (qilu-pharma), and 1% penicillin/streptomycin (Thermofisher) on day 0– day 6, $CD34^+$ cells at a concentration of 10^5/ml were supplemented with 1 ng/ml interleukin 3 (IL-3), 10 ng/ml stem cell factor (SCF, STEMCELL Technologies), and 3 IU/ml EPO. On day 7– day 11, the cells were supplemented with 1 IU/ml EPO and 10 ng/ml SCF alone. day 11–14, the cell concentration was adjusted to 10^6/ml on day 11 and to 5×10^6/ml on day 14. The cells were maintained at 37℃ at the presence of 5% CO_2, and were split into fresh culture medium every 2 days.

HUDEP2 cells towards erythroid lineage

Begin culture of HUDEP2 cells from frozen stock by plating in HUDEP2 expansion media at 100,000 cells/mL. The HUDEP2 cells typically double every 24–36 h. Perform media changes every 3–4 days, while ensuring that the cell density remains below 800,000 cells/mL. The cells were cultured with expansion medium: StemSpa Serum-Free Expansion Medium (SFEM,Stemcell Technologies), 2% PenicillinStreptomycin solution (10000 U/mL stock), 50 ng/mL recombinant human stem cell factor (SCF), 3 IU/mL EPO, 0.4μg/mL dexamethasone, 1 μg/mL doxycycline. Culturing HUDEP2 Cells for Erythroid Differentiation Phase, Transfer the cells from expansion medium, then the cells were cultured in Iscove's modified Dulbecco's medium (IMDM), 1% L-glutamine (this is in addition to the L-glutamine present in IMDM), 2% PenicillinStreptomycin solution (10,000U/mL stock concentration), 330μg/mL human holo-transferrin, 10μg/mL recombinant human insulin solution, 2 IU/mL heparin, 5% inactivated human plasma, 3 IU/mL EPO.

Cytospin preparation

A total of 1×105 cells in 100 μL DPBS were spun for 5 minutes at 400 rpm onto glass slides using the cytospin apparatus. After airdrying for 1 minute, slides were stained with Giemsa staining solution (Sigma, Darmstadt, Germany) according to 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

Primers of two human shRNA were annealed, followed by subcloning into pLKO.1 vectors at AgeI and EcoRI restriction enzyme sites. KAT2A, CPT1A and HAT1 were amplified and cloned into MSCV-puro at AgeI and EcoRI restriction enzyme sites. Cycs overexpression plasmid was cloned into pcl20-N-2*flag-Blasticidin at EcoRI restriction enzyme sites.

Virus preparation

For lentivirus production, pLKO.1 shRNA or pcl20-N-2*flag-Blasticidin together with package plasmids pMDG.2 and PSPAX2 were transfected into 293T cells by PEI (polysciences). For retrovirus production, MSCV vector combined with PECO package vector, were transfected into 293T cells by PEI. The supernatant medium containing virus was collected after 48 and 72 h. The virus medium was filtered by 0.45 μM filter (Millipore) before use.

Cell infection

Cells were mixed with virus medium, 4 μg/ml polybrene was added and cells were spinoculated for 3 h at 32 °C. In the following day, spinoculation was repeated. Then 1 μg/ml puromycin (Solarbio) and 20 μg/ml Blasticidin (Beyotime) were added to select the positive cells.

Western blot analysis

Protein extracts were subject to polyacrylamide gels (Bio-Rad), transferred to a NC membrane, and incubated in blocking buffer (5% non-fat milk in PBST). Antibody staining was visualized using the Bio-rad Imaging System. For western blotting, the primary antibody was diluted to 1:500 by blocking buffer with a final concentration of 2 μg/mL. The secondary antibody (goat anti-rabbit or anti-mouse IgG [H+L] with HRP (abbkine) was diluted to 1:5000 by blocking buffer with a final concentration of 0.4 μg/mL. The SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was used for antibody signal detection.

Co-immunoprecipitation

Total cell lysates were prepared from HUDEP2 cells using RIPA IP buffer (Beyotime). the cell lysate was precleared with Pierce Protein A/G magnetic beads (Bimake) for 30min then incubated with the antibody overnight. This was followed by incubation for 2 h with fresh Protein A/G magnetic beads. The beads were then washed three times with IP buffer, and 2× SDS-PAGE sample buffer was added. Samples were boiled for 10 min then centrifuged, and the supernatants were subjected to Western blot analysis. Immunoprecipitation was conducted using an anti-KAT2A antibody.

Immunofluorescence

Sections were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, treated with 3% BSA and 3% goat serum, 0.1% Triton, and 0.05% Tween-20 in PBS for 1 h, and then incubated with anti-succinyllysine antibody overnight at 4°C. Sections were then washed extensively, incubated with the secondary antibody for 2 h at room temperature, mounted on glass slides, and visualized with a confocal laser scanning microscope (OLYMPUS).

Chromatin Immunoprecipitation (ChIP)-PCR

ChIP was performed with anti-succ-H3K79 antibody (PTM BIO) in HUDEP2 cells using Chromatin Immunoprecipitation (ChIP) Kit (9003S, Cell Signaling Technology, CST) according to the manufacturer's protocol. Quantitative PCR analysis was performed on the immunoprecipitated DNA using SYBR qRT-PCR analysis Master Mix (Vazyme). The PCR primers are listed in supplemental Table 4.

Cleavage Under Targets & Tagmentation (CUT&Tag) assay and sequencing data analysis

$CD34^+$ cells were collected to perform the CUT&Tag assay by using NovoNGS CUT&Tag 4.0 High-Sensitivity Kit (for Illumina®) (Novoprotein, N259-YH01). The library sequencing was performed by Illumina novaseq 6000 platform (Haplox Genomics center). FastQC software was used for quality control. The clean reads were aligned to hg38 genome using bowtie2 (v2.3.5.1) with the options (--very-sensitive--end-to-end). Then, the low-quality mapping reads were removed using SAMtools with the option (-q 35). Peak calling was performed by Macs2 (v.2.2.7.1). BW files were visualized using IGV software. Heatmaps were then generated using DeepTools (v.3.5.1) tool. The R package ChIPseeker was used to perform annotation and functional enrichment analysis for differential binding peak between groups.

BrdU assay

The medium was removed from $1x10^6$ cells and replaced with BrdU-labeled solution. After 3 hours incubation at 37°C, the cells were washed twice with PBS and resuspended in 100 μL BD Cytofix/Cytoperm buffer (BD Biosciences), and then incubated on ice for 15-30 minutes until cooling. The cooled cells were washed with 1 mL 1x BD Perm/Wash buffer (BD Biosciences). The cells were centrifuged at 5000 rpm for 5 min and the supernatant was removed. Subsequently, the cells were resuspended in 100 μL BD Cytoperm Permeability Stability Buffer Plus (BD Biosciences) and incubated on ice for 10 min and then washed with 1 mL 1x BD Perm/Wash buffer, centrifuged at 5000 rpm for 5 min and the supernatant removed. Following the up step, the cells were incubated in 100 μL BD Cytofix/Cytoperm buffer on ice for 5 min followed by washing with 1 mL 1x BD Perm/Wash buffer, centrifuged at 5000 rpm for 5 min and the supernatant removed. Immediately, the cells were treated with DNase (300 μg/mL working concentration diluted in DPBS from 6 mg/mL stock solution (Sigma-Aldrich), to expose incorporated BrdU. 100 μL of the diluted DNase (300 μg/mL in DPBS) was used to resuspend the cells and incubated at 37°C in the dark for 1 hour. After this, the cells were washed with 1 mL 1x BD Perm/Wash buffer, centrifuged at 5000 rpm for 5 min and the supernatant removed. the cell pellet was resuspended in 50 μL 1x BD Perm/Wash buffer with the addition of BrdU antibody (ThermoFisher) at 5 μL per 10^6 cells. Then the cells were incubated for 20 min at room temperature and washed with 1 mL 1x BD Perm/Wash buffer, centrifuged at 5000 rpm for 5 min and the supernatant removed. Finally, the cells were stained with 100 μL FACS buffer containing 1 μL of 1 mg/mL DAPI (BioSharp) and incubated in the

dark for 30 min. The stained cells were then used for flow cytometric analysis.

LC-MS/MS analysis and database search

The peptides were separated using a NanoElute ultra-high-performance liquid chromatography system. Once separated, the peptides were ionized by injection into a Capillary ion source and analyzed using the timsTOF Pro mass spectrometer. The ion source voltage was set at 1.65 kV, and high-resolution TOF was used to detect and analyze both the peptide precursor ions and their fragment ions. The secondary mass spectrometry scan range was set to 400-1500 m/z. Parallel accumulation serial fragmentation (PASEF) mode was used for data acquisition, where 10 PASEF mode scans were performed to collect the secondary spectra of precursor ions with charges between 0-5 after collecting one primary mass spectrum. The dynamic exclusion time for tandem mass spectrometry scanning was set to 30 seconds to avoid repeat scans of precursor ions. Protein identification was performed using MaxQuant software (v1.6.15.0), and a total of 20,395 Homo sapiens sequences were downloaded from the UniProtKB database (Release 2021-01) for database searching. Carbamidomethyl (C) was set as a fixed modification for cysteine alkylation, and variable modifications included oxidation of methionine and acetylation of protein N-termini. For succinylome, succinylated lysine residue was added as a variable modification. The false discovery rate (FDR) threshold for protein, peptide, and modification site was set to 1%. The peptide mass tolerance and fragment mass tolerance were set to 10 ppm and 0.02 Da, respectively. The probability for site localization was set to >0.75.

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Supplemental Tables and Figures

Supplemental Table 1. All antibodies used in this study

Antibodies	**Catolog**	**Company**	**Use**
Anti-Succinyllysine Mouse mAb	PTM-419	PTMBIO	Western blot
Anti-HAT1 Mouse mAb	PTM-5195	PTMBIO	Western blot
Anti-Succinyllysine Rabbit pAb	PTM-401	PTMBIO	IF
Anti-Succinyl-Histone H3 (Lys79) Rabbit pAb	PTM-412	PTMBIO	Western blot, IP and ChIP
SIRT5 Rabbit pAb	A5784	abclonal	Western blot
SIRT7 Rabbit pAb	A0979	abclonal	Western blot
Lamin B1 Rabbit pAb	A1910	abclonal	Western blot
HBB Polyclonal antibody	16216-1-AP	proteintech	Western blot
Cytochrome c Polyclonal antibody	10993-1-AP	proteintech	Western Blot
GCN5L2 (KAT2A) Rabbit mAb #3305	C26A10	Cell Signaling Technology	Western Blot and IP
CPT1A (D3B3) Rabbit mAb #12252	D3B3	Cell Signaling Technology	Western blot
Histone H3 Antibody #9715	9715S	Cell Signaling Technology	Western blot
GAPDH antibody(0411)	sc-47724	Santa Cruz Biotechnology	Western blot
beta Actin antibody (C4)	sc-47778	Santa Cruz Biotechnology	Western blot
Anti-DDDDK(FLAG)-tag antibody	M185-3L	MBL	Western blot
BD Pharmingen™ 7-AAD	559925	BD Biosciences	Flow cytometry
BV421 Mouse Anti-Human CD235a	562938	BD Biosciences	Flow cytometry
BD Pharmingen™ PE Mouse Anti-Human CD71	555537	BD Biosciences	Flow cytometry
BD Pharmingen™ DAPI Solution	564907	BD Biosciences	Flow cytometry
BrdU Monoclonal Antibody (BU20A), FITC	11-5071-42	Invitrogen	Flow cytometry

Abbreviations: IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; IF: immunofluorescence.

Supplemental Table 2. Specific reagents used.

Product name	**Company**	**Catalog #**	**Applications in the present study**
CD34 Microbead kit	miltenyibiotec	130-046-702	Cell separation
IMDM	thermofisher	31980030	Cell Culture
holo-transferrin	sigma-Aldrich	11096-37-0	Cell culture
insulin	sigma-Aldrich	11061-68-0	Cell culture
fetal bovine serum	thermofisher	30044333	Cell culture
heparin	qilu-pharma		Cell culture
penicillin/streptomycin	thermofisher	15140122	Prevent bacterial contamination of cell cultures
stem cell factor	STEMCELL Technologies	78064	Cell culture
EPO	Sinovac Biotech Ltd		Cell culture
StemSpan serum-free medium STEMCELL Technologies	STEMCELL Technologies	09600	Cell Culture
Doxycycline	selleck	S5159	Cell Culture
dexamethasone	selleck	S1322	Cell Culture
HEK293T cells	ATCC® CRL-11268™		Cell culture
DMEM	thermofisher	12430054	Cell Culture
PEI	polysciences	02371-100	non-viral vector carriers
0.45 μM filter	millipore	HAWP04700	Filtered plasma
puromycin	Solarbio	P8230	screening strains
Blasticidin	beyotime	ST018-5ml	screening strains
Pierce Protein A/G magnetic beads	bimake	B23201	Antibody purification and immunoprecipitation
BD Cytofix/Cytoperm buffer	BD Biosciences	51-2090KZ	Brdu cell proliferation assay

1× BD Perm/Wash buffer	BD Biosciences	554723	Brdu cell proliferation assay
BD Cytoperm Perme Stability Buffer Plus	BD Biosciences	51-2356KC	Brdu cell proliferation assay
BrdU(dilution of dnase	sigma-Aldrich	32160405	Brdu cell proliferation assay
DAPI	biosharp	BS097-10mg	Flow cytometry and immunofluorescence.
Chromatin Immunoprecipitation (ChIP) Kit	Cell Signaling Technology	9003S	Detect protein-DNA interactions
BCA assay kit	Thermo Scientific	23225	determination of protein concentration
SuperSignal™ West Pico PLUS	Thermo Scientific	34580	Protein Electrophoresis & Western Blotting
Annexin V-FITC/PI Apoptosis Detection Kit	Vazyme	A211-01	apoptosis assay
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Thermo Scientific	78835	Nuclear and Cytoplasmic Extraction

Supplemental Table 3. Oligonucleoties used in this study.

Name	Sequence (5' to 3')	Type
shKAT2A-1-F	CCGGGCTGAACTTTGTGCAGTACAACTCGAGTTGTAC TGCACAAAGTTCAGCTTTTTG	shRNA
shKAT2A-1-R	AATTCAAAAAGCTGAACTTTGTGCAGTACAACTCGAG TTGTACTGCACAAAGTTCAGC	shRNA
shKAT2A-2-F	CCGGCCACCTGAAGGAGTATCACATCTCGAGATGTGA TACTCCTTCAGGTGGTTTTTG	shRNA
shKAT2A-2-R	AATTCAAAAACCACCTGAAGGAGTATCACATCTCGAG ATGTGATACTCCTTCAGGTGG	shRNA
shHAT1-1-F	CCGGGCTACATGACAGTCTATAATTCTCGAGAATTATA GACTGTCATGTAGCTTTTTG	shRNA
shHAT1-1-R	AATTCAAAAAGCTACATGACAGTCTATAATTCTCGAG AATTATAGACTGTCATGTAGC	shRNA
shHAT1-2-F	CCGGCCGTGTTGAATATGCATCTAACTCGAGTTAGATG CATATTCAACACGGTTTTTG	shRNA
shHAT1-2-R	AATTCAAAAACCGTGTTGAATATGCATCTAACTCGAGT TAGATGCATATTCAACACGG	shRNA
shCPT1A-1-F	CCGGCGTAGCCTTTGGTAAAGGAATCTCGAGATTCCT TTACCAAAGGCTACGTTTTTG	shRNA
shCPT1A-1-R	AATTCAAAAACGTAGCCTTTGGTAAAGGAATCTCGAG ATTCCTTTACCAAAGGCTACG	shRNA
shCPT1A-2-F	CCGGCGATGTTACGACAGGTGGTTTCTCGAGAAACCA CCTGTCGTAACATCGTTTTTG	shRNA
shCPT1A-2-R	AATTCAAAAACGATGTTACGACAGGTGGTTTCTCGAG AAACCACCTGTCGTAACATCG	shRNA
shCYCS-F	CCGGAGGGCAGACTTATGATTAGACTTCGTTAGTAATC TATTAAGTCTGCCCTTTTTTG	shRNA
shCYCS-R	AATTCAAAAAAGGGCAGACTTAATAGCTTATCTCGAG ATAAGCTATTAAGTCTGCCCT	shRNA

Abbreviations: F, forward; R, reverse.

Supplemental Table 4. CHIP-qPCR Primers used in this study.

Name	**Sequence (5' to 3')**
CHIP-PCR primer for hFOXO3	tttaattcagaaagatgtactcaatatttaattaaagatatgagatctaacgatgtaggcaggctgcgggag cagggagtatgccctgtgtaaggactccattc
CHIP-PCR primer for hHDAC6	tgaatgagagaatgaacgagtggttgaatggggaaatgagtggtagaggaaactggcaactgttgggtt gggggtgtctatggggattgacttctccaactctcttacctctttttcttc
CHIP-PCR primer for hXPO7	tcttctatagcatcaaataaagagacctataaatggttattaggtgaatgttacccaggttctcctcagttcag aagcaattctttctttactgattatt
CHIP-PCR primer for hHNRNPU	actattgttccccaaaggtccttagtaataaaggacttagtaataggaaaactttcttggaagagatacatgc aatatgtcacatatcacagatacacatatacaaa
CHIP-PCR primer for hCTLA	cctagg ctcgagaagc ctgttcggtt ctcagcatgt ttgagtgcttctgggcgcgg gcggagcgag aaagcaagtgtagggtggcaggctccggagccggaagaagcccgttcaattcagcaacttttcattaag catttgctgtgcctttagtccggtctctgaagcaaccgcattggcgcagtttttccagacttataagcttataa gtctgagccgagcacagaactcgttattagaaaaggagggcggaaaaaaataagaatggaaatatcgtt tttgagagatacaaacaaaagtagcaatgcagttcagcatttaagcacttaaggtgtacagagtgttggatt acgaggaggaaggagggagtagggaactcgaaagatgatctaggtctggagaaagaactgccaagc gcggtaggagtttgctatcgttgagcgattgatttacagctaggacttctggagctcttcctctgggaacag ctttggtaggcaatattgcctaagca

Supplemental Table 5. Succinylated proteins only in Day 0

UNIPROT	SYMBOL	GENENAME	sites
O00411	POLRMT	RNA polymerase mitochondrial	K402
O14874	BCKDK	branched chain keto acid dehydrogenase kinase	K184, K233, K89, K192
O43766	LIAS	lipoic acid synthetase	K318
O75879	GATB	glutamyl-tRNA amidotransferase subunit B	K529
P21583	KITLG	KIT ligand	K42
P82921	MRPS21	mitochondrial ribosomal protein S21	K40
Q14197	MRPL58	mitochondrial ribosomal protein L58	K153, K118, K94, K98
Q14644	RASA3	RAS p21 protein activator 3	K15
Q14CZ7	FASTKD3	FAST kinase domains 3	K471, K481
Q1512	PDK3	pyruvate dehydrogenase kinase 3	K278

0			
Q53R41	FASTKD1	FAST kinase domains 1	K785, K482, K360, K478, K236
Q5T5X7	BEND3	BEN domain containing 3	K816, K821, K822, K824
Q6P4F2	FDX2	ferredoxin 2	K184
Q6PML9	SLC30A9	solute carrier family 30 member 9	K487, K223, K234
Q7Z3T8	ZFYVE16	zinc finger FYVE-type containing 16	K435
Q86WA6	BPHL	biphenyl hydrolase like	K126, K257, K191, K271
Q86YH6	PDSS2	decaprenyl diphosphate synthase subunit 2	K285
Q8IVH4	MMAA	metabolism of cobalamin associated A	K88, K323
Q8N8R5	C2orf69	chromosome 2 open reading frame 69	K346
Q8NCN5	PDPR	pyruvate dehydrogenase phosphatase regulatory subunit	K854, K100, K219, K307, K218
Q96C01	FAM136A	family with sequence similarity 136 member A	K18
Q96GC5	MRPL48	mitochondrial ribosomal protein L48	K64
Q96I51	RCC1L	RCC1 like	K209
Q96PE7	MCEE	methylmalonyl-CoA epimerase	K114, K60, K150
Q99595	TIMM17A	translocase of inner mitochondrial membrane 17A	K56
Q9BYN8	MRPS26	mitochondrial ribosomal protein S26	K185
Q9HC36	MRM3	mitochondrial rRNA methyltransferase 3	K251, K167, K153, K136, K122, K237
Q9HD34	LYRM4	LYR motif containing 4	K44, K47
Q9NWS8	RMND1	required for meiotic nuclear division 1 homolog	K240, K249
Q9UGM6	WARS2	tryptophanyl tRNA synthetase 2, mitochondrial	K333, K354, K234, K198
Q9UHN1	POLG2	DNA polymerase gamma 2, accessory subunit	K463, K288
Q9Y2	MRPS1	mitochondrial ribosomal protein S17	K21

R5	7		
Q9Y3D5	MRPS18C	mitochondrial ribosomal protein S18C	K131, K134

Supplemental Table 6. Succinylated proteins only in Day 6

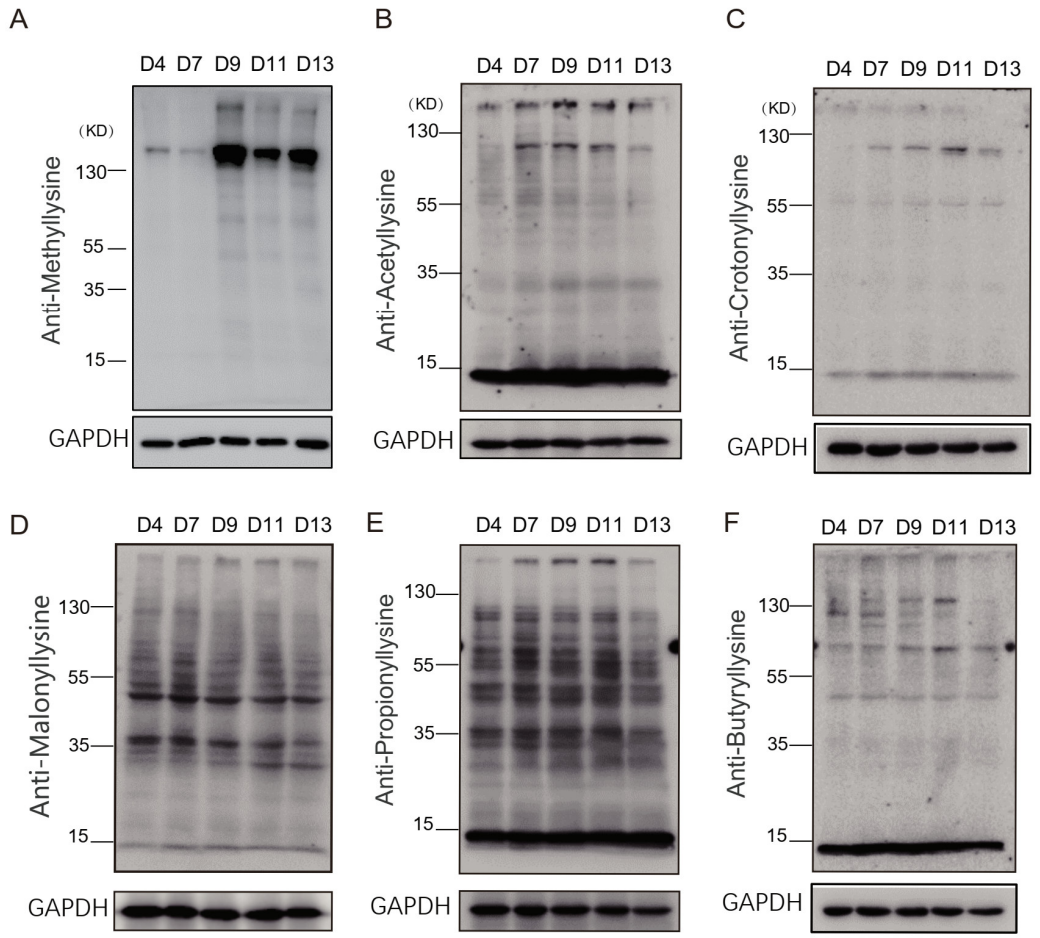
UNIPROT	SYMBOL	GENENAME	sites
P02671	FGA	fibrinogen alpha chain	K202, K89, K476, K148, K157
P02675	FGB	fibrinogen beta chain	K374
P02679	FGG	fibrinogen gamma chain	K231
P78540	ARG2	arginase 2	K229, K241
Q4G176	ACSF3	acyl-CoA synthetase family member 3	K534, K563
Q9HD23	MRS2	magnesium transporter MRS2	K93

Supplemental Table 7. Enrichment analysis of stage-specific Ksu proteins

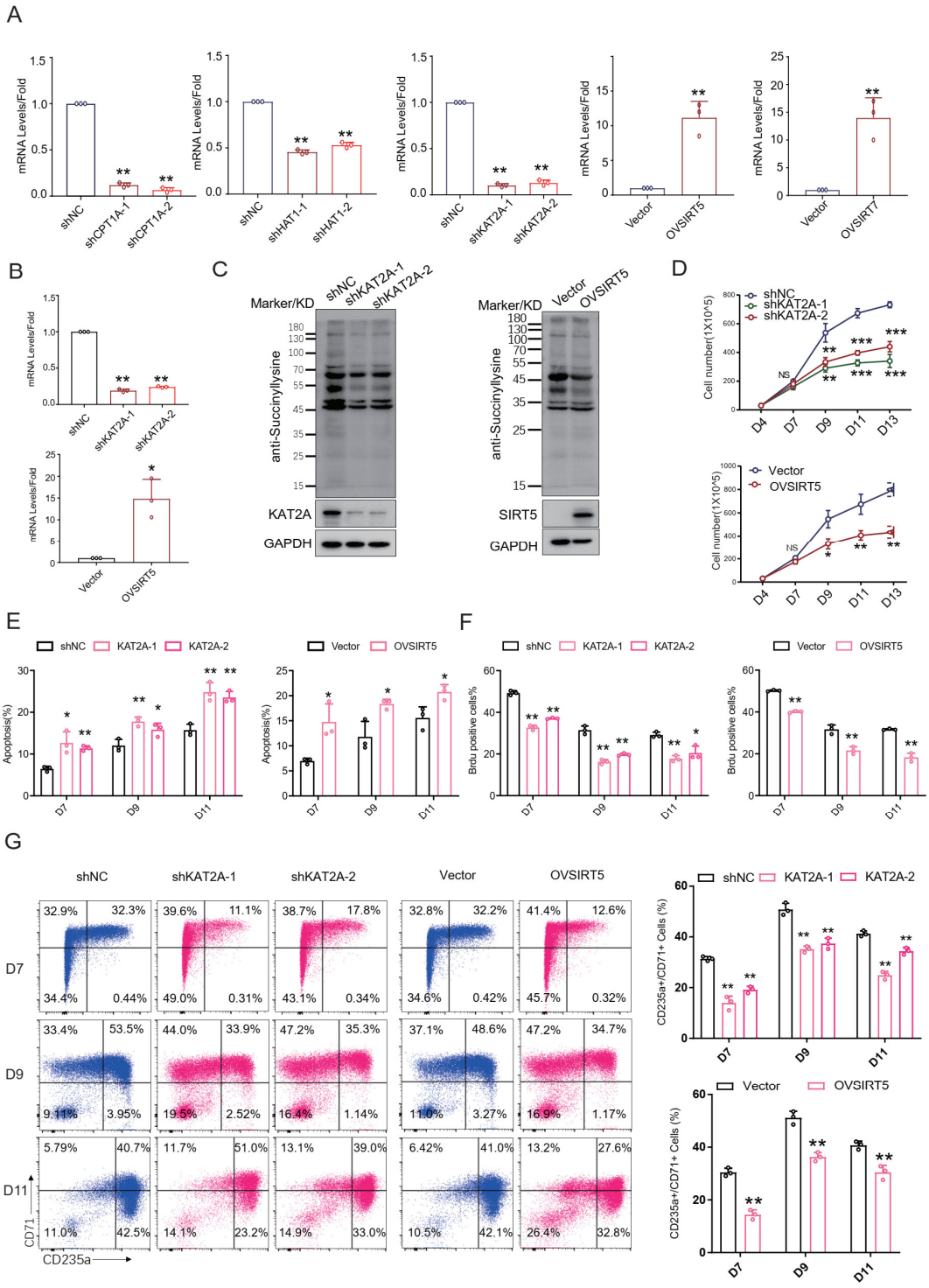
Enrichment analysis of early-stage specific proteins					
ID	Description	pvalue	p.adjust	qvalue	Count
GO:0140053	mitochondrial gene expression	3.16E-16	1.18E-13	9.69E-14	10
GO:0032543	mitochondrial translation	1.25E-13	2.33E-11	1.91E-11	8
GO:0000959	mitochondrial RNA metabolic process	9.22E-07	0.000115	9.41E-05	4
GO:0070131	positive regulation of mitochondrial translation	1.48E-06	0.000139	0.000114	3
GO:0070129	regulation of mitochondrial translation	6.54E-06	0.000489	0.0004	3
Enrichment analysis of late-stage specific proteins					
ID	Description	pvalue	p.adjust	qvalue	Count
GO:0034116	positive regulation of heterotypic cell-cell adhesion	8.21E-09	1.11E-06	2.32E-07	3
GO:0072378	blood coagulation, fibrin clot formation	8.21E-09	1.11E-06	2.32E-07	3
GO:0072376	protein activation cascade	1.23E-08	1.11E-06	2.32E-07	3
GO:0042730	fibrinolysis	3.65E-08	1.87E-06	3.93E-07	3
GO:0031639	plasminogen activation	4.14E-08	1.87E-06	3.93E-07	3

GO:00341 14	regulation of heterotypic cell-cell adhesion	4.14E -08	1.87E-06	3.93E-07	3

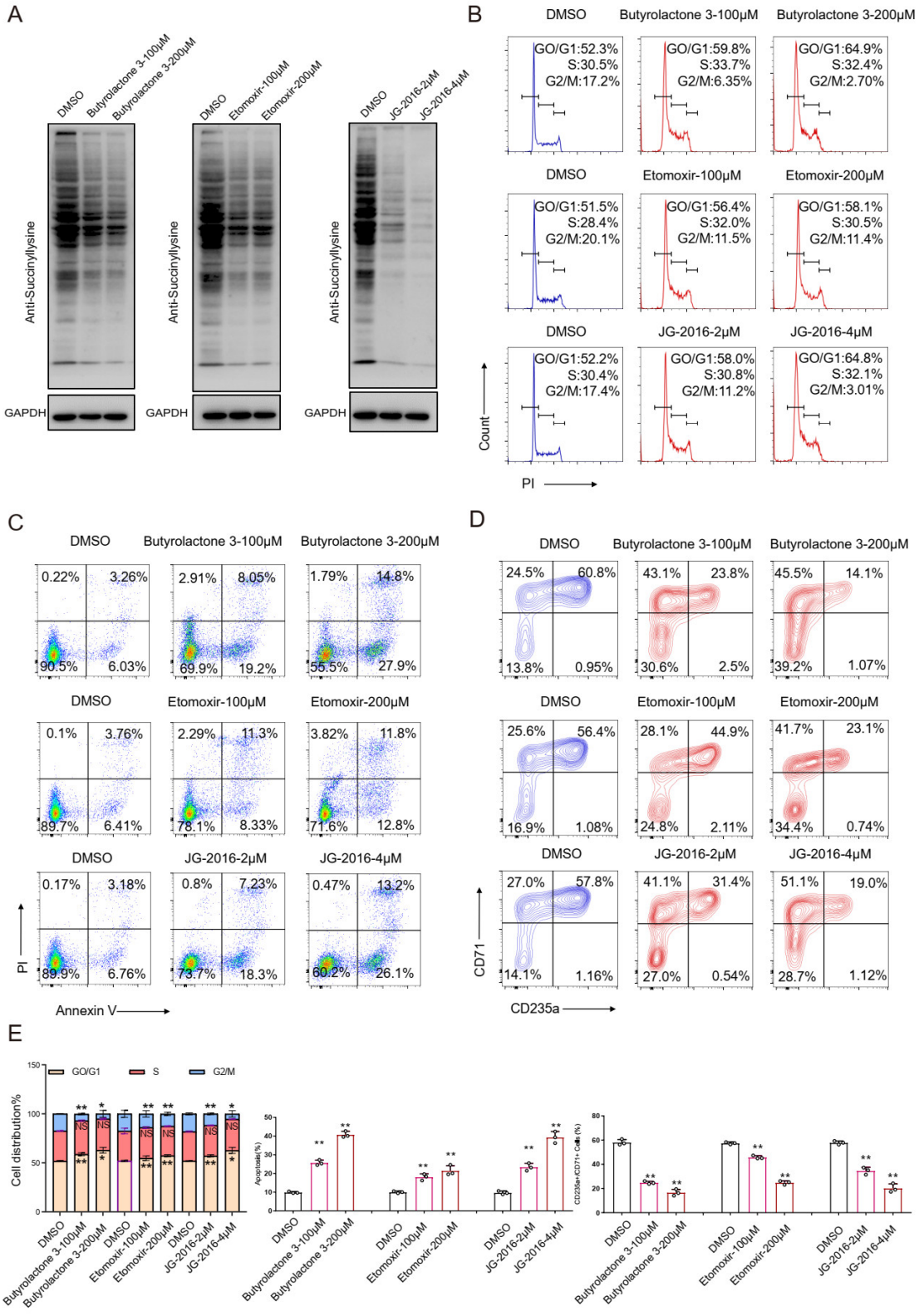
Supplementary Figures



Supplementary Figure S1. Protein post-translational modifications during CD34+ cells erythroid differentiation. (A-E) CD34+ cells were collected at each developmental stage of human erythroid differentiation for cell lysis, and posttranslational modifications were detected, including methylation (A), acetylation (B), crotonylation (C), malonylation (D), propionylation (E) and butyrylation (F). GAPDH was used as a loading control.

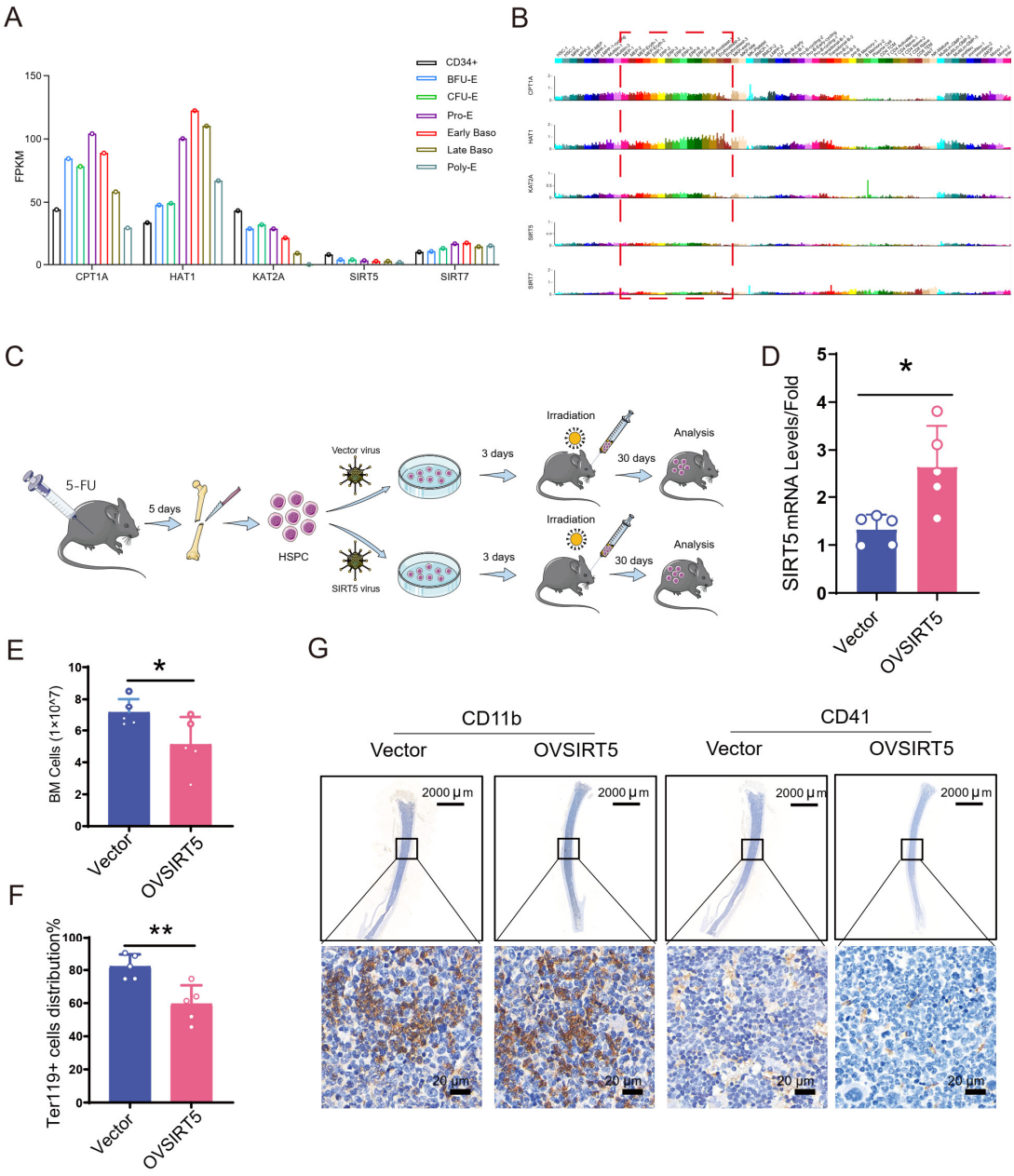

A
mRNA Levels/Fold
shNC
shCPT1A-1
shCPT1A-2
shHAT1-1
shHAT1-2
shKAT2A-1
shKAT2A-2
Vector
OVSIRT5
OVSIRT7
B
C
Marker/KD
anti-Succinyllysine
KAT2A
SIRT5
GAPDH
D
Cell number(1X10^5)
D4
D7
D9
D11
D13
NS
E
F
shNC
KAT2A-1
KAT2A-2
Apoptosis(%)
Brdu positive cells%
G
D7
D9
D11
CD71
CD235a
CD235a+/CD71+ Cells (%)
H
HAT1
CPT1A
HBA
HBB
HBG

Supplementary Figure S2. Effects of global lysine succinylation on erythroid differentiation in human CD34$^+$ cells. (A) The qRT-PCR results showing the mRNA expression levels of succinyltransferases and desuccinylases in HUDEP2 cells. (B) The qRT-PCR results showing KAT2A and SIRT5 mRNA expression levels in cultured primary erythroid cells at day 7. (C) Western blotting analysis of global lysine succinylation levels in CD34$^+$ cells infected with KAT2A knockdown and SIRT5 overexpression. (D) Cell growth curves determined by manual cell counting. (E) Cell apoptosis proportions assessed via annexin V/PI staining during erythroid differentiation. (F) Results of cell-cycle distribution obtained through BrdU assay during erythroid differentiation. (G) Flow cytometry analysis of KAT2A knockdown or SIRT5 overexpression during human erythroid differentiation at different stages in human CD34$^+$ cells. (H) Differentiation of HUDEP2 cells after infection with KAT2A or, HAT1 or CPT1A shRNA lentivirus or SIRT5 or SIRT7 overexpression virus. Western blotting images displaying KAT2A, HAT1, CPT1A, SIRT5, and SIRT7 expression in HUDEP2 cells infected with relative lentivirus, along with hemoglobin expression.



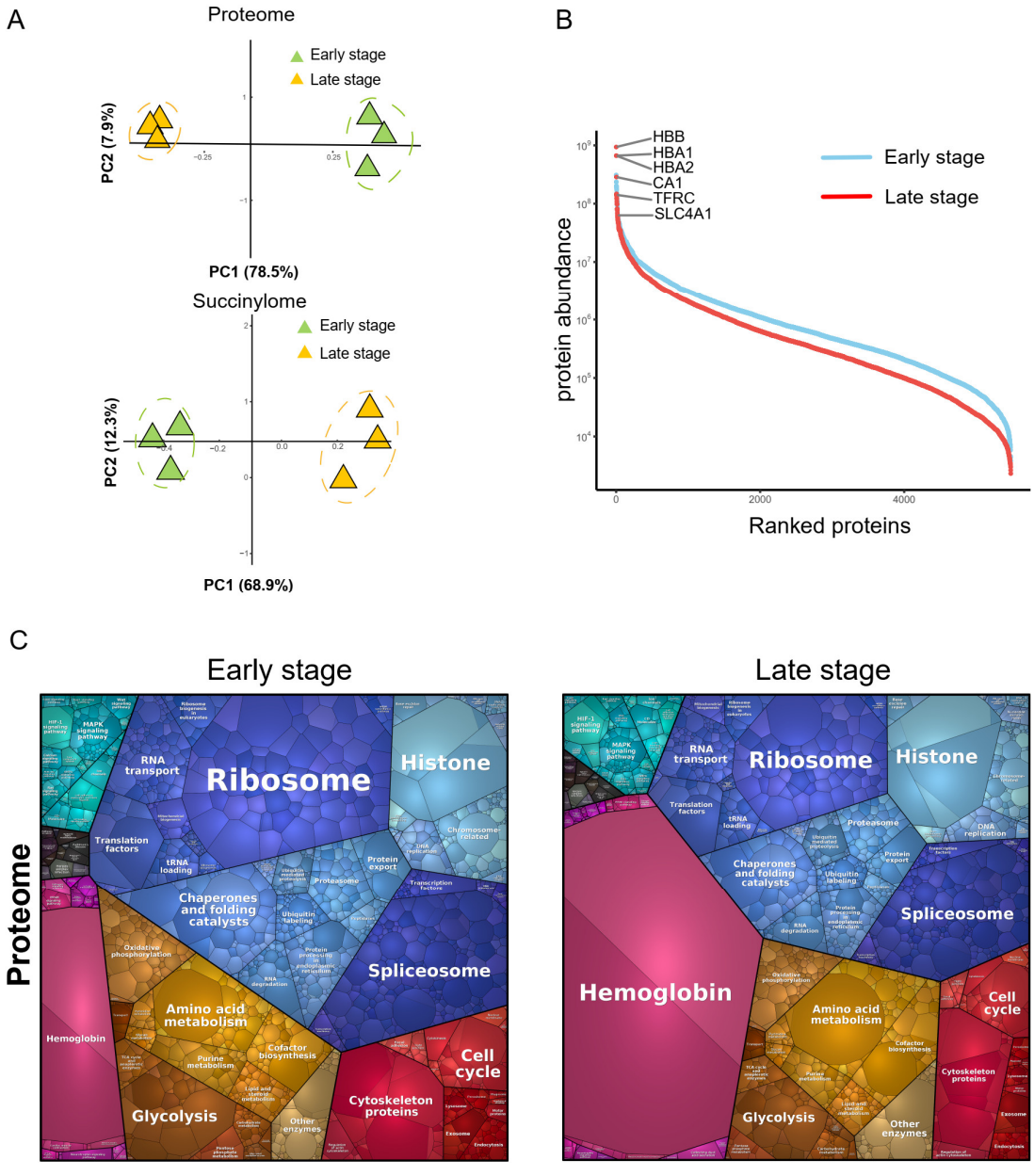
Supplementary Figure S3. Effects of global lysine succinylation on erythroid differentiation following treatment with succinyltransferase inhibitors in human CD34+ cells. (A) Western blot analysis of global lysine succinylation levels in CD34+ cells treated with

succinyltransferase inhibitors - Butyrolactone 3 (a specific small-molecule inhibitor of KAT2A), Etomoxir (an irreversible inhibitor of CPT1A), and JG-2016 (a potent inhibitor of HAT1). (B) Cell-cycle distribution during erythroid differentiation assessed through PI assay. (C) Proportions of apoptotic cells evaluated via annexin V/PI staining during erythroid differentiation. (D) Flow cytometry analysis of erythroid differentiation at various stages in human CD34+ cells treated with succinyltransferase inhibitors. (E) Cell-cycle distribution during erythroid differentiation in CD34+ cells (left), proportions of apoptotic cells during erythroid differentiation in CD34+ cells (middle), and proportions of GPA- and CD71-positive cells during erythroid differentiation in CD34+ cells (right). Statistical analysis was performed on three independent experiments, with bar plots representing the mean ± SD of triplicate samples. Significance levels are indicated as *P < 0.05, **P < 0.01, versus control, based on Student's t-test.



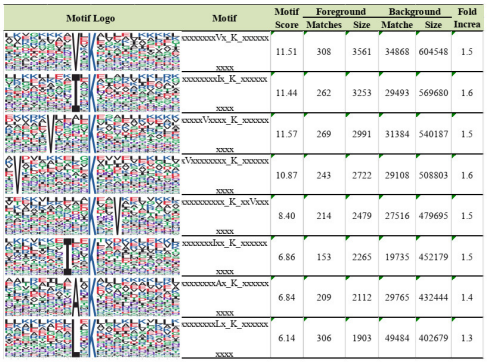
Supplementary Figure S4. Overexpression of SIRT5 impairs erythroid differentiation in vivo. (A) Expression levels of the succinylation-related enzymes during erythroid differentiation in bulk RNAseq. (B) Expression levels of the succinylation-related enzymes in the bone marrow (BM), with the red dotted box highlighting the erythroid lineages. (C) Experimental procedure for mouse bone marrow transplantation. (D) Relative mRNA levels of SIRT5 in BM cells from control and OV-SIRT5 mice, presented as the mean ± SEM (n = 5). β-actin serves as the endogenous control. (E) Upper panel: Bone marrow cell counts of control and OV-SIRT5 mice. Bottom panel: $Ter119^{+}$ cells distribution analysis based on flow cytometry results. (F) The representative vertical sections of mouse BM showing expression of

CD11b and CD41 by immunostaining.



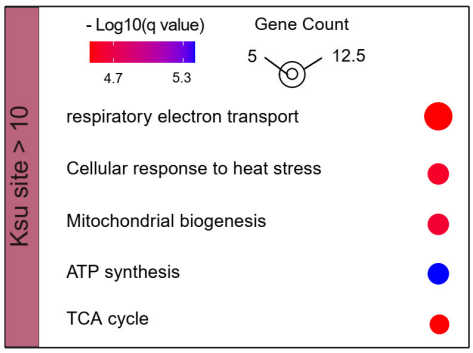
Supplementary Figure S5. Characterization of proteome and motif analysis for succinylated lysine residues. (A) Principal component analysis of all identified proteins in the proteome (upper) and succinylome (lower). (B) Distribution of proteome intensities at different erythroid differentiation stages. (C) Proteomaps demonstrate significant differences in hemoglobin levels during erythroid differentiation in proteomes.

A



Motif Logo	Motif	Motif Score	Foreground Matches	Foreground Size	Background Matche	Background Size	Fold Increa
	xxxxxxxVx_K_xxxxxx xxxx	11.51	308	3561	34868	604548	1.5
	xxxxxxxIx_K_xxxxxx xxxx	11.44	262	3253	29493	569680	1.6
	xxxxxVxxxx_K_xxxxxx xxxx	11.57	269	2991	31384	540187	1.5
	xVxxxxxxxx_K_xxxxxx xxxx	10.87	243	2722	29108	508803	1.6
	xxxxxxxxx_K_xxVxxx xxxx	8.40	214	2479	27516	479695	1.5
	xxxxxxIxx_K_xxxxxx xxxx	6.86	153	2265	19735	452179	1.5
	xxxxxxxAx_K_xxxxxx xxxx	6.84	209	2112	29765	432444	1.4
	xxxxxxxLx_K_xxxxxx xxxx	6.14	306	1903	49484	402679	1.3

B



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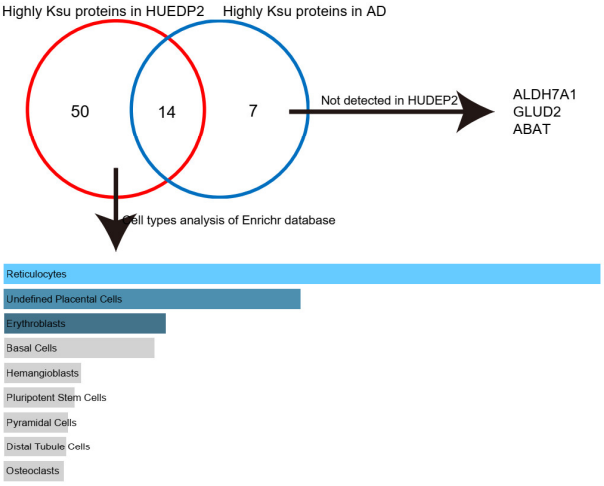

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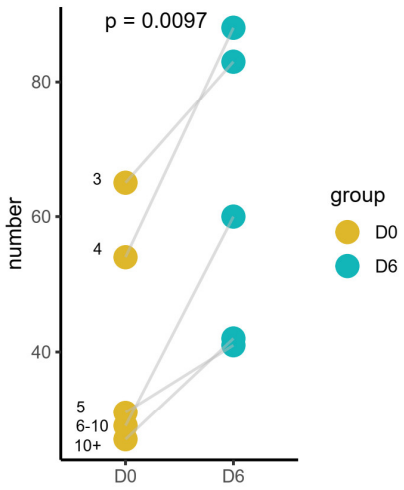

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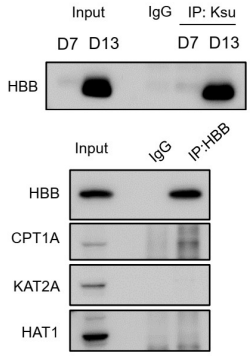

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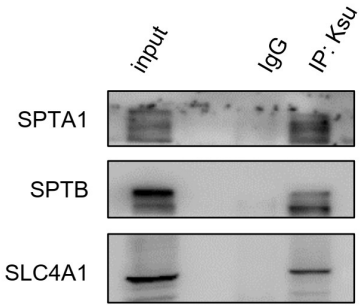

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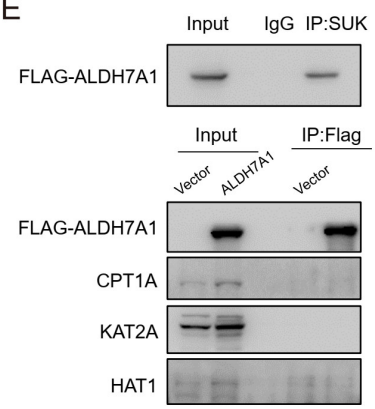

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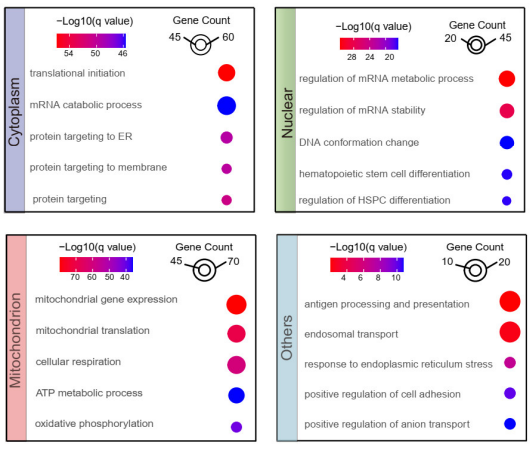
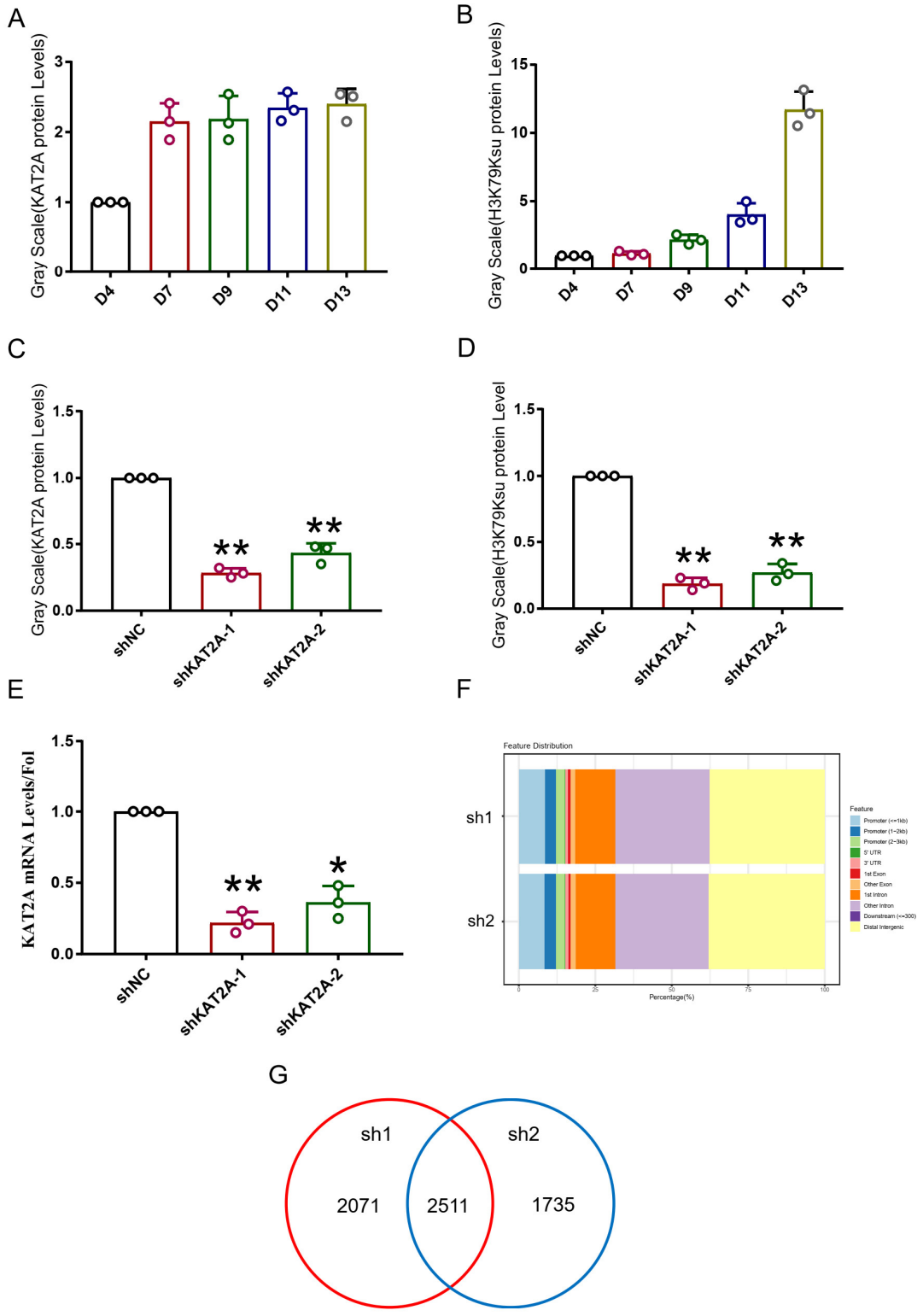
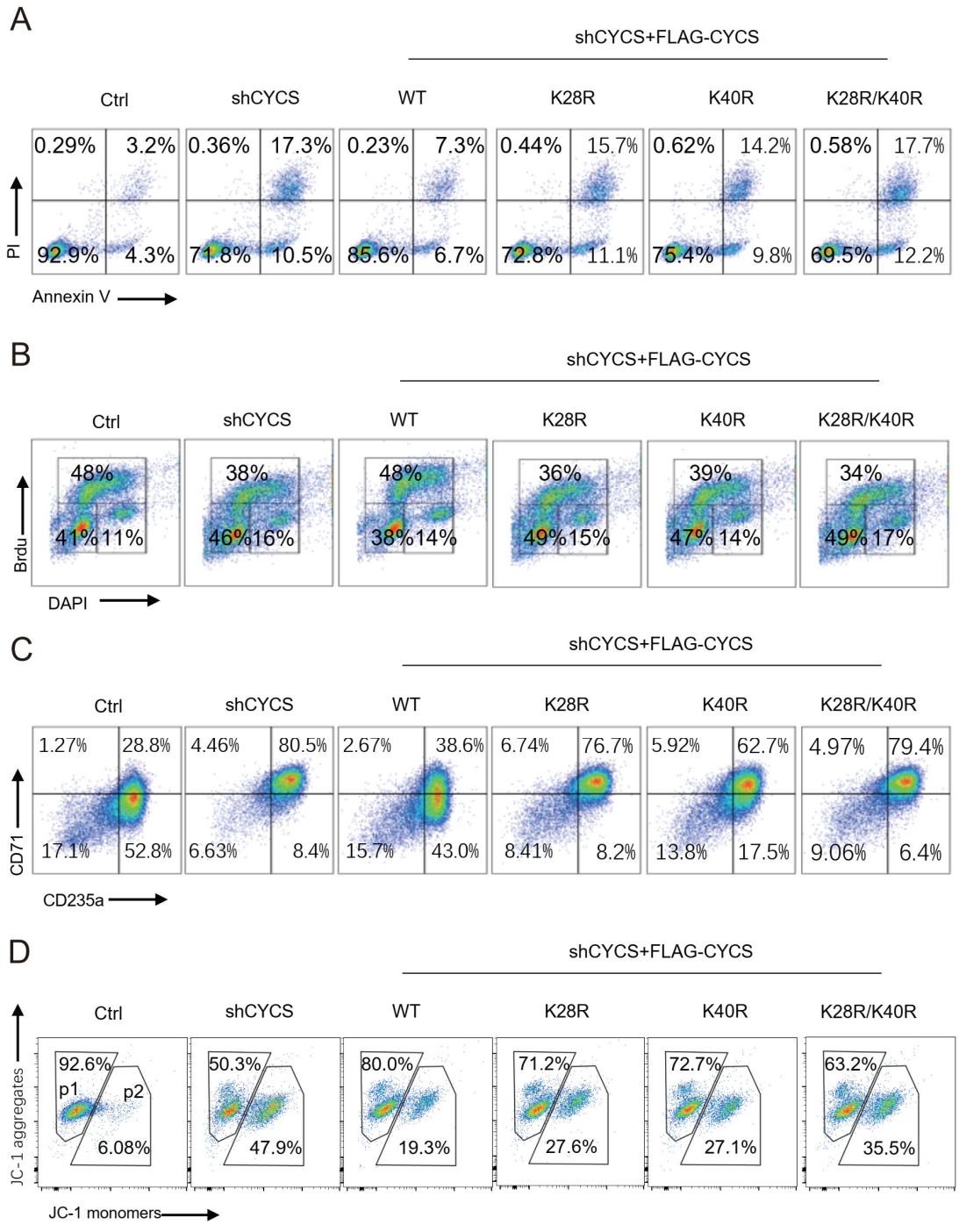


Supplementary Figure S6. Characterization of succinylome. (A) Identification of eight conserved amino acid residue frequencies around succinylated lysine residues using Motif-X during erythroid differentiation. (B) Gene Ontology Biological Process (GO-BP) enrichment analysis of proteins with >10 succinylated sites, showing significantly enriched functions. (C) The Venn plot showing the differences of succinylated proteins with >10 succinylated sites between our data with AD patients. (D) Detection of the succinylation levels of HBB and the interaction between HBB and three succinyltransferases. (E) Detection of the succinylation levels of flag-ALDH7A1 and the interaction between ALDH7A1 and three succinyltransferases. (F) Changes in the number of succinylated lysine residues from early to late-stages of erythroid differentiation. (G) Detection of the succinylation levels of SPTA1, SPTB, and SLC4A1. (H) GO analysis of succinylated proteins localized in the nucleus, cytoplasm, mitochondrion, and others showing significantly overrepresented functions.



Supplementary Figure S7. The CUT&Tag analysis of H3K79Ksu in CD34^{+} cells. (A) Grayscale statistics of KAT2A expression level in erythroid differentiation. (B) Grayscale statistics of H3K79Ksu expression level in erythroid differentiation. (C) Grayscale statistics of KAT2A protein expression level. (D) Grayscale statistics of H3K79Ksu expression level. (E)

Detection of KAT2A mRNA level after knocking down KAT2A. (F) Genomic distribution of affected peaks. (G) The Venn plot showing the overlap of peak located in the promoter. Statistical analysis was performed on three independent experiments, with bar plots representing the mean ± SD of triplicate samples. Significance levels are indicated as *P < 0.05, **P < 0.01, versus control, based on Student's t-test.



Supplementary Figure S8. The succinylation of CYCS affects erythroid differentiation. (A) Representative images of flow cytometry analysis of apoptosis by annexin V/PI staining in HUDEP2 cells. (B) Representative images of flow cytometry analysis of cell cycle by BrdU assay. (C) Representative images of flow cytometry analysis of GPA and CD71 expression. (D) Representative flow cytometry analysis of mitochondrial membrane potential using JC-1 staining.