Enhancement of PRMT6 binding to a novel germline *GATA1* mutation associated with congenital anemia

Yingsi Lu,[1] Qingqing Zhu,[1] Yun Wang,[1] Meiling Luo,[2] Junbin Huang,[2] Qian Liang,[3] Lifen Huang,[2] Jing Ouyang,[1] Chenxin Li,[1] Nannan Tang,[2] Yan Li,[1] Tingting Kang,[1] Yujia Song,[1] Xiaoyu Xu,[1,4] Liping Ye,[1] Guoxing Zheng,[1] Chun Chen[2] and Chengming Zhu[1]

[1]Scientific Research Center, The Seventh Affiliated Hospital of Sun Yat-Sen University; [2]Pediatric Hematology Laboratory, Division of Hematology/Oncology, Department of Pediatrics, The Seventh Affiliated Hospital of Sun Yat-Sen University; [3]Department of Spine Surgery, The First Affiliated Hospital, Shenzhen University, Shenzhen Second People's Hospital and [4]Department of Obstetrics, The Seventh Affiliated Hospital of Sun Yat-Sen University, Shenzhen, Guangdong, China

Correspondence: C. Zhu
zhuchm3@mail.sysu.edu.cn

C. Chen
chenchun69@126.com

G. Zheng
opqsky@126.com

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SUPPLEMENTAL DATA

Detailed materials and methods

Whole exome sequencing (WES) and Sanger sequencing

Genomic DNA extracted from whole blood was ultrasonically fragmented, then selected for 400-600 bp with AMPure XP beads (Agencourt), followed by end repair, sequencing adaptor ligation, and PCR amplification according to standard library construction protocols to obtain a preliminary sequencing library. IDT probes (xGen Exome Research Panel v1) were used to capture exon-targeting regions. The exome libraries of the patient and her parents were then sequenced with the NovaSeq 6000 (Illumina) platform. The sequencing raw reads were filtered by the fastp software for quality control, the Burrows-Wheeler-Alignment (BWA) tool to align with the reference genome GRCh37/hg19, and the Genome Analysis Toolkit (GATK) tool to annotate SNPs and Indels, finally screened for confident variants. The variant effect predictor (VEP) tool was applied in further variant annotation to find the variant associated with the anemia phenotype.

Constructs

Human GATA1 gRNAs were cloned into the lentiCRISPR-v2 puro vector (Addgene plasmid, #52961) as previously described[1]. Full-length human wild-type GATA1 and mutant GATA1 were amplified with the cDNA derived from K562 cells and peripheral blood of the proband, respectively, together with an HA tag, then cloned into either the XbaI and NotI sites of pCDH-MSCV lentiviral vector (applied for invitro model construction) or the NotI and XbaI sites of pCDNA3.1 transient transfection vector (used for protein interaction study). 3xHA-G1WT/G1MU-Turbo-NLS was generated by subcloning the cDNA of the G1WT/G1MU gene from the pCDH vector into the NheI site of pCDNA3-3xHA-TurboID-NLS (Addgene plasmid, # 107171). PRMT6-Flag was generated by the cDNA for PRMT6 subcloned in the pCDNA 3.1-Flag vector. shRNAs targeting PRMT6 were cloned into pLKO.1-hygro vector. All

plasmids had been verified by sequencing before use.

Lentivirus production and infection

Low-passage HEK-293T cells were seeded in 10 ml of DMEM complete media and allowed to reach a 70% confluency in a 10cm culture dish. Lentiviral plasmids were co-transfected with pMD2.G and psPAX2 following polyethyleneimine (PEI) transfection protocol. After 48-hour transfection, the viral supernatant was collected and filtered through a 0.45-μm filter (Merck Millipore). The viral supernatants were then added directly to cells in 6-well plates with polybrene (Sigma-Aldrich) supplemented to a concentration of 2 μg/ml, and the suspension cells were spin-infected at 1500 rpm for 90 min at room temperature. Cells were replaced with a complete medium after 24 hours and selected by corresponding antibiotics the next day.

CRISPR/Cas9-mediated deletion of GATA1 in K562 and HEL cells

GATA1 knock-out in K562 and HEL cells (K562-sgG1 and HEL-sgG1) was performed by lentiviral transduction as described above with lentiCRISPR-v2-sgGATA1 plasmids. 72 hours after infection, cells underwent selection using 2 μg/ml puromycin (Beyotime) for 7 days and were subsequently amplified, then evaluated protein levels of GATA1 by western blot analysis. For details of the sgRNAs sequence see Supplemental Table S4.

Generation of re-expression stable cells

For reconstituted with wild-type GATA1 and GATA1-Leu387fs in GATA1-deficient K562 and HEL cells generated as above mentioned, packaging plasmids were co-transfected with either pCDH-MSCV-HA-G1WT or pCDH-MSCV-HA-G1MU into HEK-293T cells. 48 h post-transfection, GATA1-depleted cells were infected with harvested viral particles for 24 h in the presence of polybrene. Positive stable cell lines were subsequently selected by G418 (70 μg/ml) for 7 days. The GATA1 protein levels of the stable re-expression cells were then evaluated by WB analysis.

Benzidine staining

Benzidine cytochemical staining was performed to assess the level of hemoglobin[2]. Briefly, cells were washed once with PBS and resuspended in a ratio of 1:1 with benzidine solution (1.46 ml acetic acid, 48.54 ml ddH_2O, 100 mg benzidine dihydrochloride (Sigma-Aldrich)) and a final concentration of 0.1% H_2O_2. Stained cells were incubated in the dark at room temperature for 2 min. Positive staining cells (dark blue or brown) were photographed and quantified via an upright microscope (Leica, DM4B) at magnification x100, with positively stained cells manifesting blue or dark brown. Over 100 total cells were counted for each image.

FACS analysis

The cultured cells were washed with PBS and were blocked with 0.5% FBS for 15 minutes on ice before staining. Erythroid differentiation examinations utilized APC-CD235a (eBioscience) and either PE-Cyanine7-CD71 (eBioscience) or FITC-CD71 (Biolegend). Megakaryocytic induction analysis was investigated using APC-CD41a (eBioscience). To assess erythroid apoptosis, Annexin V analysis was performed wherein cells were washed with 1x Annexin V binding buffer and then stained with FITC-Annexin V (BD, 556420) and APC-CD235a (eBioscience) following the manufacturer's protocols. For Megakaryocytic ploidy analysis (DNA content analysis), cells were permeabilized using cold 70% ethanol and stained with propidium iodide (50 μg/mL) along with RNase A. Flow cytometry was conducted utilizing the CytoFLEX (Beckman Coulter) platform, and subsequent data analysis was performed using CytExpert software (Beckman Coulter) or Flowjo software.

Real-time quantitative PCR

Real-time quantitative PCR was performed by isolating total RNA from cells, performing reverse transcription, and the quantitative PCR assay according to the manufacturer's instructions (AG, China). All primers are listed in Supplementary Table

S3. Normalization was done to the housekeeping gene GAPDH for gene expression levels. The quantitative PCR was analyzed on Bio-Rad CXF96, and the supporting software (Bio-Rad CXF Manager) was used for analysis.

Western blot analysis

The cell pellets were lysed with RIPA buffer (Epizyme, Shanghai) supplemented with a protease inhibitor cocktail (Beyotime, Shanghai) for 30 minutes on ice. Proteins were subsequently collected through 12000 rpm centrifugation for 10 minutes at 4 ℃. Quantitation was carried out by making use of the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and followed by 10% or 12.5% SDS-PAGE electrophoresis separation. The antibodies and the dilution multiple utilized in the study were as follows: anti-GATA1 (Abcam, ab181544, 1:10000), anti-FOG1 (Abcam, 1:1000), anti-GAPDH (Proteintech, 60004-1, 1:10000), anti-HA (CST, C29F4,1:1000), anti-histone-H3 (Proteintech, 17168-1-AP, 1:1000), anti-HBG1 (Proteintech,25728-1-AP,1:500), anti-PRMT6 (Proteintech, 15395-1-AP, 1:1000), anti-H3R2me2a (ABclonal, A3155, 1:1000), anti-FLAG (CST, 9A3, 1:1000), HRP Streptavidin (BioLegend, 405210, 1:1000). For secondary antibodies, Dylight 800-goat anti-rabbit IgG (Abbkine, A23920, 1:10000) or Dylight 800-goat anti-mouse IgG (Abbkine, A23910, 1:10000) were employed based on the species.

Turbo-ID proximity labeling

To verify the cellular location of biotinylated proteins by immunofluorescent staining, HEK-293T cells were seeded on glass coverslips in a 24-well plate for transfection the following day. 1 μg Turbo-ID fusion constructs were introduced through transfection via PEI for each well. The medium was replaced with 50 μM biotin (MCE)-supplemented complete DMEM media for 30min further culture post-36h-transfection. After this, the labeling reaction was ceased by removing the media and rinsing with 500μL cold PBS five times. Immunostaining was performed post-fixation and cell permeabilization using paraformaldehyde and cold methanol using rabbit mAb

anti-HA-tag primary antibodies (CST, #3724). The following secondary antibodies were used: Alexa Fluor 488 Streptavidin (Yeason, #35103ES60), goat anti-rabbit IgG (H+L) cross Alexa Fluor Plus 594 (Thermo Fisher, #A32740). Lastly, 1 μg/ml DAPI was utilized to stain the nuclei.

To pull down the biotinylated proteins for western blot analysis, 5×10^6 post-transfected cells were incubated with 50 μM biotin for 30 minutes to label proximal proteins, subsequently washed with cold PBS, and lysis with 250 μL RIPA lysis buffer. After sonication and centrifugation at 12,000 rpm for 10 minutes, the supernatant was collected, and the protein concentration was then measured using the BCA assay kit (Thermo Scientific, #23225). The input sample was acquired from the lysate. Subsequently, 25 μL of streptavidin magnetic beads (MCE, #HY-K0208) were incubated with 500 μg protein from lysates overnight at 4℃ to enrich biotinylated proteins. Beads were then washed in order with RIPA lysis buffer (1 ml, twice, 2 minutes each), 0.1 M Na2CO3 solution (1ml, once for 10s), 2M urea in PH 8.0 Tris-HCl (1ml, once for 10s), and RIPA lysis buffer (1 ml, twice, 2 minutes each) at last. The beads were then resuspended in 3 x protein loading buffer supplemented with 2 mM biotin and 20 mM DTT, boiled at 100 ℃ for 10 minutes. Finally, the eluate was collected by a magnetic rack and analyzed by western blotting to confirm biotinylated proteins.

To perform the Turbo-ID proximity labeling-based mass spectrometry assays, the plasmid of Turbo-ID fused wild-type or mutant GATA1 protein was transfected in stable GATA1-depleted K562 cells using liposomal transfection reagents (Yeason, #40802ES02) according to the product instructions, respectively. Following individual biotin labeling according to the abovementioned conditions, 1×10^7 cells were collected for protein collection and quantification. 5 mg total protein was incubated overnight at 4°C with 100 μL streptavidin magnetic beads. After the enrichment of biotinylated proteins, the beads were washed with RIPA lysis buffer and ready for mass spectrometry.

Label-free mass spectrometry for protein identification and quantification.

The EASY-nLCTM 1200 (Thermo Fisher, Germany) coupled with Orbitrap Exploris 480 (Thermo Fisher, Germany) platform was utilized for label-free mass spectrometry detection at Novogene Co., Ltd. (Beijing, China). Employing Proteome Discoverer, the mass tolerance for precursor ion was ten ppm, and the mass tolerance for production was 0.02 Da. Fixed modifications for carbamidomethyl, dynamic modification for methionine (M) oxidation, and loss of methionine at the N-terminal were specified. Up to 2 missed cleavage sites were permitted. To ensure high-quality analysis results, PD software was used to filter retrieval results to identify Peptide Spectrum Matches (PSMs) with more than 99% credibility. Identified proteins were required to contain at least one unique peptide, and both the identified PSMs and proteins were retained and subjected to FDR no more than 1.0%. T-tests were employed for statistical analysis of protein quantitation results, with differentially expressed proteins (DEP) defined as those whose quantitation was significantly different between experimental and control groups ($p < 0.05$ and $FC > 1.25$ or $FC < 0.75$ [foldchange, FC]).

Co-immunoprecipitation and immunoprecipitation analysis

HEK-293T cells were transfected with appropriate plasmids and lysed by co-immunoprecipitation lysis buffer NETN (20 mM Tris-HCl (pH 8.0), 0.5% NP-40, 100 mM NaCl, 0.5 mM EDTA) complemented with the protease inhibitor cocktail (Beyotime, Shanghai). The cell lysates were then incubated with a primary antibody and protein A/G agarose (ABmart, A10001M). The agarose beads were washed three times with the NETN buffer and subjected to SDS-PAGE.

Chromatin immunoprecipitation

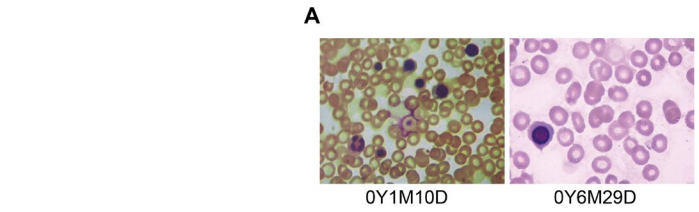
ChIP was performed with the BeyoChIP™ ChIP Assay kit (Beyotime, P2080S) and the following antibodies: anti-H3R2me2a (ABclonal, A3155), anti-PRMT6 (Proteintech, 15395-1-AP), anti-HA (CST, C29F4), anti-H3 (Proteintech, 17168-1-AP) and normal

rabbit IgG (CST, #2729). All primers are listed in Supplemental Table S3.

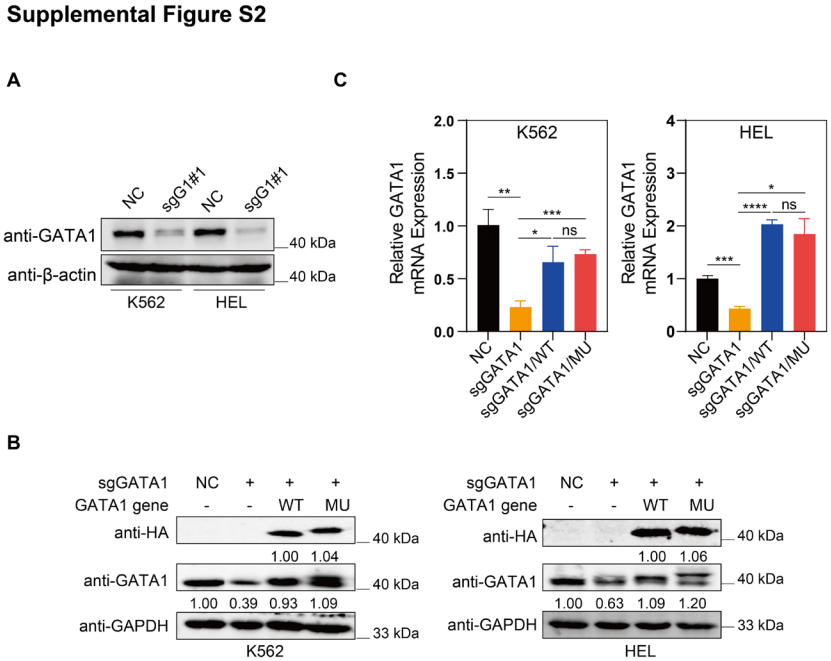
Supplemental References

1. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nature methods*. 2014/08/01 2014;11(8):783-784. doi:10.1038/nmeth.3047
2. Orkin SH, Harosi FI, Leder P. Differentiation in erythroleukemic cells and their somatic hybrids. *Proc Natl Acad Sci U S A*. Jan 1975;72(1):98-102. doi:10.1073/pnas.72.1.98

Supplemental Figure S1

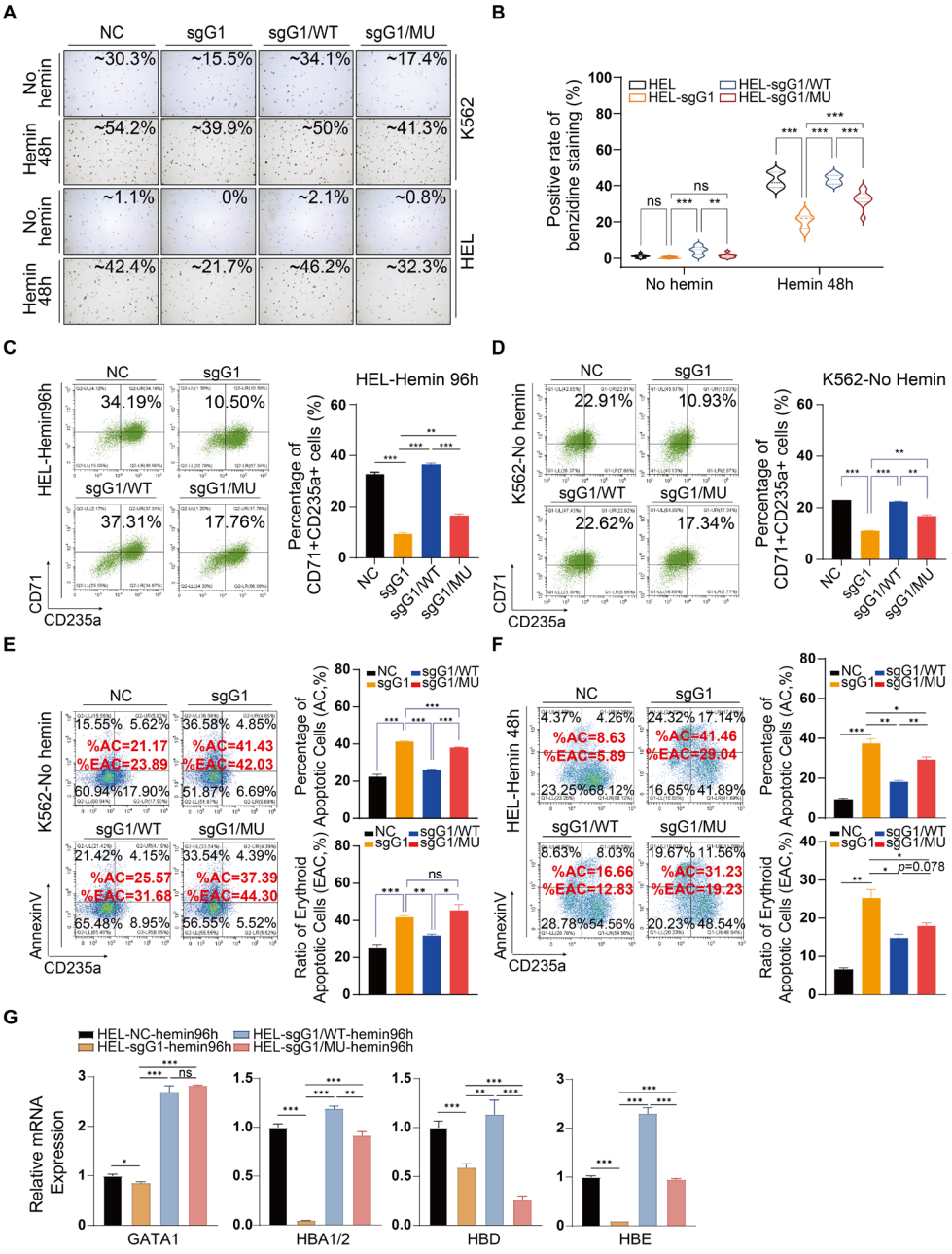


Supplemental Figure S2



Supplemental Figure S3

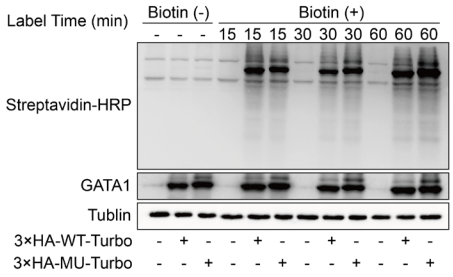
Supplemental Figure S3



Supplemental Figure S4

Supplemental Figure S4

A



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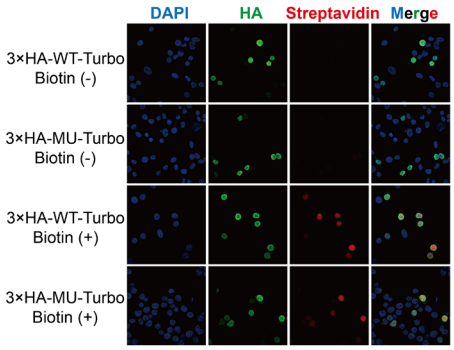

C

Streptavidin pulldown proximity labeling proteins	Turbo-3xHA-GATA1-WT			Turbo-3xHA-GATA1-MU		
Replicate number	#1	#2	#3	#1	#2	#3
Total number of identified proteins	3473	3414	3400	3417	3443	3410
HBG1	0	0	0	2691464.794	1889042.761	2016545.286
MPC1	0	0	0	2295566.46	1950745.601	2592849.807
NDUFS6	2421278.25	1622373.123	1704854.652	0	0	0
FBXL12	738894.3125	704674.6836	803811.6507	0	0	0
PIGK	516467.1875	1043007.591	1004770.237	0	0	0
SKA1	2203040	1959978.64	1608287.985	0	0	0
ITGA2B	672053.5625	741038.8296	784033.81	0	0	0
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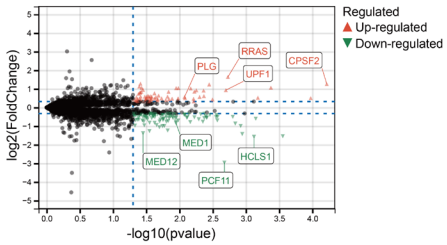
D



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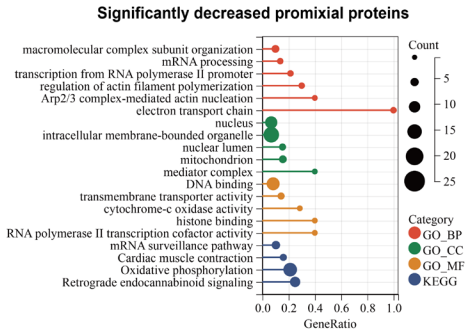

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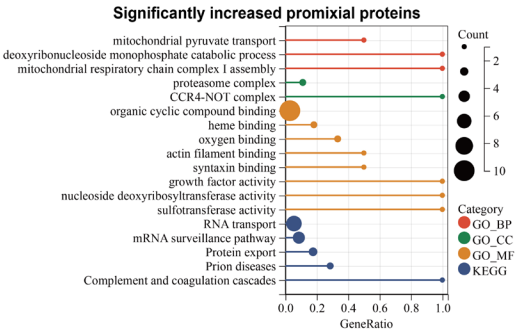
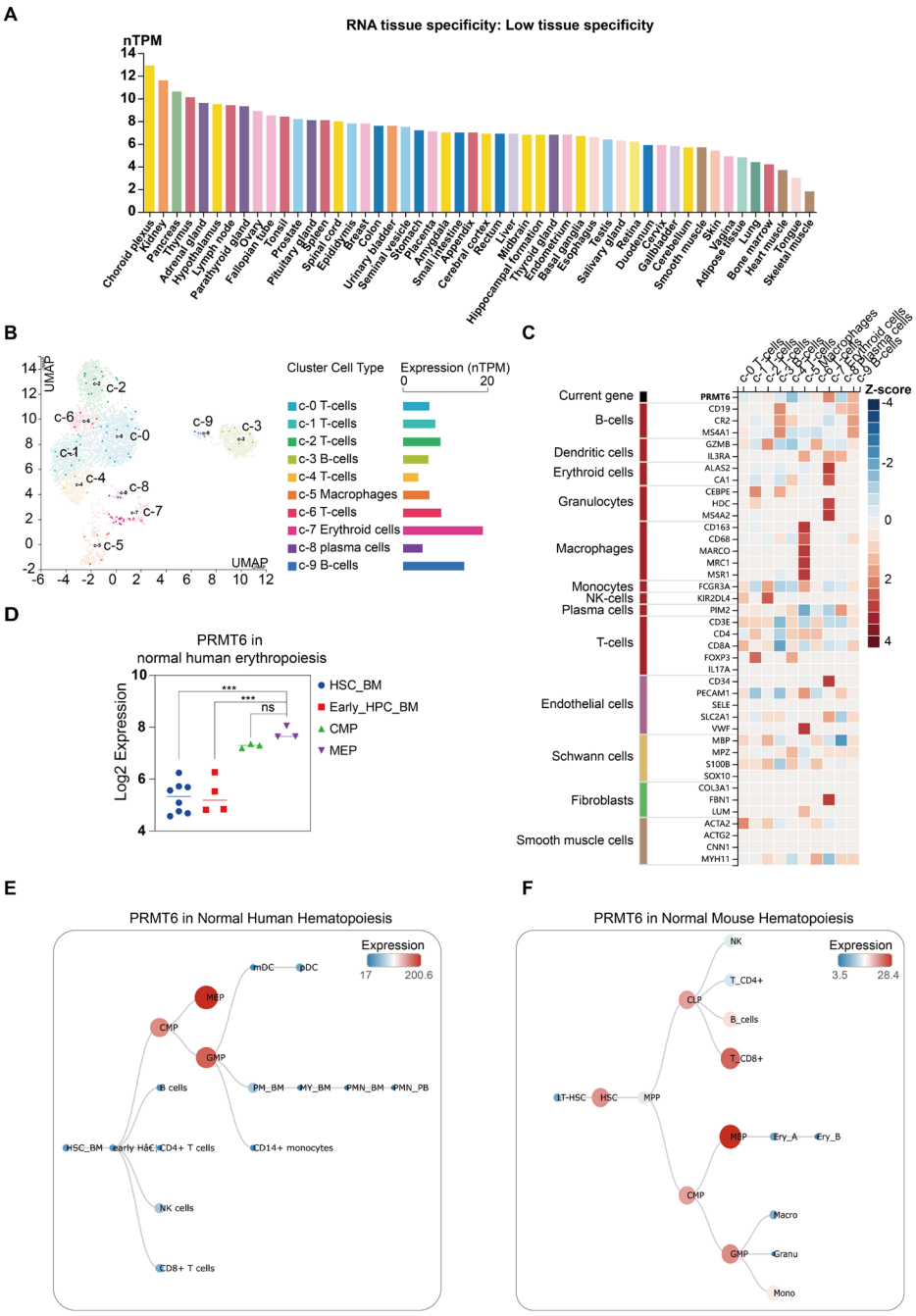
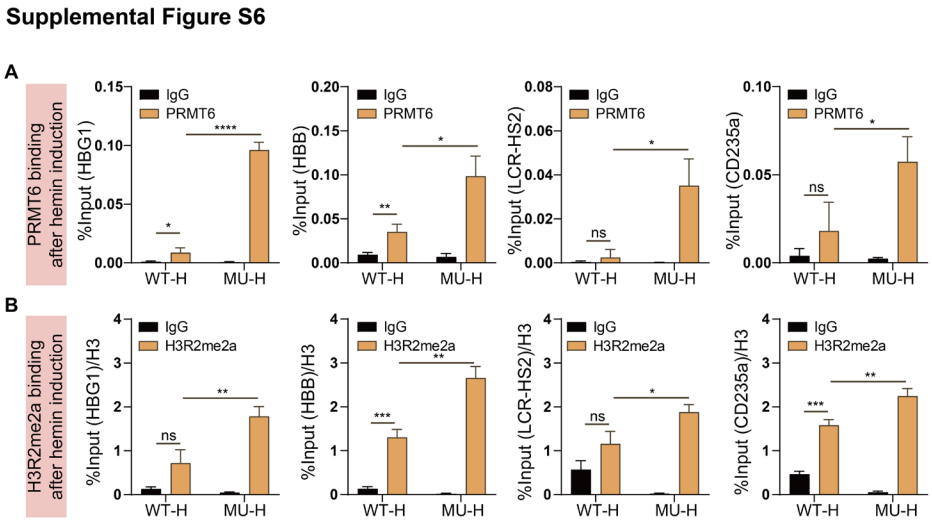


Supplemental Figure S5



Supplemental Figure S6



Supplemental Figure Legends

Supplemental Figure S1. The erythrocyte characteristics were observed in the bone marrow of the proband.

A. To examine erythrocyte characteristics, Wright-Giemsa staining was conducted on bone marrow smears at the first month (0Y1M10D) and the sixth month (0Y6M29D) after birth.

Supplemental Figure S2. Verification of re-expression GATA1 Leu387fs model in K562 and HEL cells by western blot and RT-qPCR.

A. Western blot showed the verification of the CRISPR/cas9-mediated GATA1-depleted effect in K562 and HEL cells, and the wild-type K562 and HEL cells were taken as control, respectively.

B. The re-expression effect of the wild-type and mutant GATA1 was detected through western blotting analysis, with GAPDH used as the loading control. The intensity of bands was quantified and normalized to NC control.

C. RT-qPCR assays were conducted to evaluate the relative GATA1 expression level in both GATA1-depleted and re-expression cells. The RT-qPCR results are presented as mean ± SD of 3 independent replicates. Statistical significance was determined as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, no statistically significant.

Supplemental Figure S3. GATA1 Leu387fs mutant cells exhibited impaired differentiation during erythropoiesis compared to wild-type cells, as shown in Figure 2.

A. The representative benzidine staining images in both mutant model cell sets under no induction and 48h hemin induction conditions at a 100X magnification.

B. The positive rate of benzidine staining for hemoglobin of HEL cells was measured through a violin diagram, which indicated significant differences between the hemin-

treated and untreated groups.

C. Flow cytometric analysis of erythroid differentiation was carried out to quantify the percentage of surface CD71+CD235a+ population in HEL cells treated with hemin.

D. Flow cytometry analysis of spontaneous erythroid differentiation in K562 mutant cells. The bar graph presents the analyzed percentage of surface CD71+CD235a+ cells.

E. Flow cytometry analysis of erythroid apoptosis in K562 mutant cells without erythroid induction. The representative FACS plots show the percentages of Annexin V and CD235a expressed on the surface of K562 mutant and wild-type cells following 1% serum-induced apoptosis for 48 hours. The bar graphs describe the statistical analysis of apoptotic cells (AC%) and erythroid apoptotic cells (EAC%), as calculated in Figure 2E.

F. Flow cytometric analysis of erythroid apoptosis in erythroid-induced mutant HEL cells. Representative FACS plots displaying the percentages of AnnexinV and CD235a expressed in mutant and wild-type cells after hemin treatment for 48 hours. The bar graphs (right) show the statistical analysis of apoptotic cells (%AC) and erythroid apoptotic cells (%EAC), as calculated in Figure 2E.

G. RT-qPCR was performed on HEL mutant group cells to assess the expression of target genes after 96h hemin induction, related to Figure 2F. Results were normalized with each value of the housekeeping gene GAPDH.

The data are shown as mean ± SD of 3 independent replicates, and the statistical significance is represented by asterisks, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no statistical significance.

Supplemental Figure S4. Proximity labeling experiments by Turbo-ID method, related to Figure 4.

A. Assessment of the Turbo-ID labeling system in HEK293T cells by transfecting either wild-type or Leu387fs mutant GATA1 protein fused with 3xHA-tagged Turbo-ID. Upon transfection, biotin was added for 0, 15, 30, and 60 minutes to enable labeling. We evaluated the transfection level and abundance of biotinylated protein by analyzing

the GATA1 and streptavidin bands, respectively.

B. Confocal fluorescence imaging was performed to visualize the cellular localization of the transfected GATA1-Turbo-ID fusion protein (green) and the biotinylated proteins (red) at a magnification of 200X. Biotin-labeled and unlabeled cells were fixed and stained with anti-HA-Alexa Fluor 488 and streptavidin–Alexa Fluor 594, respectively. The cellular nucleus was marked with DAPI (blue).

C. The label-free quantitative mass spectrometry results showed the most significant differential biotinylated proteins between the wild-type and mutant groups, as illustrated in the summary table. Both groups were the results of three independent replicates.

D. Volcano plot showing the differential proximity proteins in the mass spectrometry result. The y-axis shows the log2-fold change in protein abundance, and the x-axis shows the -log10 adjusted p-value (Student's two-sided t-test with Benjamini–Hochberg adjustment for multiple comparisons). Significantly decreased proximity proteins ($p < 0.05$, Foldchange < 0.75) in the mutant cells are shown by green inverted triangles, increased proximity proteins ($p < 0.05$, Foldchange > 1.25) are shown with red triangles, and proteins with insignificant differences are represented with black dots. Several proteins with marked changes in proximity are indicated.

E, F. GO and KEGG analysis of significantly decreased and increased proximal proteins by GATA1 Leu387fs mutation identified in the Turbo-ID-based mass spectrum assay. The top remarkably enriched processes or terms are shown in the plots.

Supplemental Figure S5. Evaluation of PRMT6 expression in tissue cells and during development.

A. mRNA expression profiles of PRMT6 in various human tissues from the Consensus transcriptomics datasets of Human Protein Atlas (available from https://www.proteinatlas.org/), ranked by normalized expression (nTPM) level.

B. Analysis of PRMT6 expression from the bone marrow scRNA-seq datasets on Human Protein Atlas. 9 single-cell clusters (c1-c9) were observed and visualized by a Uniform Manifold Approximation and Projection (UMAP) plot. The color intensity of

individual cells depends on the percentage of maximum expression (log2(read_count + 1)/log2(max(read_count + 1) * 100) in five different bins (i.e., <1%, <25%, <50%, <75%, and ⩾75%). Bar graph illustrating PRMT6 expression for each bone marrow cell cluster: pTPM, protein-transcripts per million. Image credit: Human Protein Atlas.

C. Heat map of Z-scores for PRMT6 expression in bone marrow cell clusters relative to other established cell type markers. Image credit: Human Protein Atlas.

D. Log2 expression of PRMT6 in different stages of human erythropoiesis (one-way ANOVA, Dunnett test), taken from the Bloodspot database (https://www.bloodspot.eu/). HSC_BM, hematopoietic stem cells from bone marrow; Early_HPC_BM, Hematopoietic progenitor cells from bone marrow; CMP, Common myeloid progenitor cell; MEP, Megakaryocyte-erythroid progenitor cell. The data are shown as mean ± SD, and the statistical significance is represented by asterisks, ***, $p < 0.001$; ns, no statistical significance.

E. The mRNA expression of PRMT6 in normal human and murine hematopoietic cells. The size and color of the dots correlate with the abundance of PMRT6 mRNAs. Data were obtained from the Bloodspot database. HSC_BM, Hematopoietic stem cells from bone marrow; early HPC_BM, Hematopoietic progenitor cells from bone marrow; CMP, Common myeloid progenitor cell; GMP, Granulocyte monocyte progenitors; MEP, Megakaryocyte-erythroid progenitor cell; PM_BM, Promyelocyte from bone marrow; MY_BM, Myelocyte from bone marrow; PMN_BM, Polymorphonuclear cells from bone marrow; PMN_PB, Polymorphonuclear cells from peripheral blood; NK cells, CD56+ natural killer cells; mDC, CD11c+ myeloid dendritic cells; pDC, CD123+ plasmacytoid dendritic cells.

F. The mRNA expression of PRMT6 in murine hematopoietic cells. The size and color of the dots correlate with the abundance of PMRT6 mRNAs. Data were obtained from the Bloodspot database. LT-HSC, Long term hematopoietic stem cell; HSC, Hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte monocyte progenitor; Macro, bone marrow macrophages; Granu, granulocytes; Mono, Monocytes; B_cells,

B cells; T_CD4+, CD4+ T cells; T_CD8+, CD8+ T cells; NK, NK cells; MEP, Megakaryocyte-erythroid progenitor; Ery_A, erythrocytes A (CD71+); Ery_B, erythrocytes B (CD71-).

Supplemental Figure S6. The occupancy changes of PRMT6 and H3R2me2a modification on the promotor/enhancer of GATA1 erythroid target genes by GATA1 Leu387fs mutation, related to Figure 5.

ChIP-qPCR assays were performed for the binding of HA-tagged GATA1, PRMT6, and H3R2me2a modification on the target genes HBG1, HBB, CD235a (GYPA) promoter, and LCR-HS2 distal enhancer in the re-expression wild-type and mutant K562 cells post 40 μM hemin-induced erythroid differentiation for 48h.

A. PRMT6 binding results post hemin induction.

B. H3R2me2a binding results with histone H3 as control after hemin induction.

The data are presented as mean ± SD of 3 independent replicates by percent enrichment relative to input, and statistical significance is denoted by asterisks. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, differences not statistically significant.

Supplemental Table Legends

Supplemental Table S1. RBC parameters at different periods of the proband.

Abnormal parameters are indicated by bold font. Normal reference ranges are shown in the table header. RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW-SD, red cell distribution width-standard deviation; RDW-CV, red cell distribution width-coefficient of variation; RET#, absolute reticulocyte count; RET%, reticulocyte percentage; RET-He, reticulocyte hemoglobin equivalent.

Supplemental Table S2. Mainly hematological indices besides red blood cells of the

proband.

Abnormal parameters are indicated by bold font. Normal reference ranges are shown in the table header. LYMPH#, absolute lymphocyte count; LYMPH%, lymphocytes percentage; NEUT#, absolute neutrophil count; NEUT%, neutrophil percentage; EO#, absolute eosinophils count; BASO#, absolute basophil granulocyte count; PLT, Platelet Count; MPV, mean platelet volume; PDW, Platelet Distribution Width.

Supplemental Table S3. Partial hematology results of the proband's brother.

Abnormal parameters are indicated by bold font. Normal reference ranges are shown in the table header.NEUT#, absolute neutrophil count; PLT, Platelet Count; Hb, hemoglobin concentration.

Supplemental Table S4. Sequences of Oligos applied in this study.

Sequences of Oligos were applied for sgRNA-knockout, shRNA-knockdown, RT-qPCR, and chIP-qPCR experiments in this study.

Supplemental Table S5. Relative protein quantification based on analysis of Turbo-ID mass spectrometry.

Protein: protein IDs identified to the sequence; Gene: gene name; MU_1, MU_2, MU_3: relative protein expression of three individual mutant GATA1 samples; WT_1, WT_2, WT_3: relative protein expression of three individual wild-type GATA1samples; MU.vs.WT FC, fold change of protein expression in the mutant group compared to the wild-type group; MU.vs.WT Pvalue is the p-value for the two-group test; MU.vs.WT log2FC, logarithms of fold change by the mutant group compared to the wild-type group.