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

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## Abstract

Mutations in the master hematopoietic transcription factor GATA1 are often associated with functional defects in erythropoiesis and megakaryopoiesis. In this study, we identified a novel *GATA1* germline mutation (c.1162delGG, p.Leu387Leufs*62) in a patient with congenital anemia and occasional thrombocytopenia. The C-terminal GATA1, a rarely studied mutational region, undergoes frameshifting translation as a consequence of this double-base deletion mutation. To investigate the specific function and pathogenic mechanism of this mutant, *in vitro* mutant models of stable re-expression cells were generated. The mutation was subsequently validated to cause diminished transcriptional activity of GATA1 and defective differentiation of erythroid and megakaryocytes. Using proximity labeling and mass spectrometry, we identified selective alterations in the proximal protein networks of the mutant, revealing decreased binding to a set of normal GATA1-interaction proteins, including the essential co-factor FOG1. Notably, our findings further demonstrated enhanced recruitment of the protein arginine methyltransferase PRMT6, which mediates histone modification at H3R2me2a and represses transcription activity. We also found an enhanced binding of this mutant GATA1/PRMT6 complex to the transcriptional regulatory elements of GATA1's target genes. Moreover, treatment of the PRMT6 inhibitor MS023 could partially rescue the inhibited transcriptional and impaired erythroid differentiation caused by the *GATA1* mutation. Taken together, our results provide molecular insights into erythropoiesis in which mutation leads to partial loss of GATA1 function, and the role of PRMT6 and its inhibitor MS023 in congenital anemia, highlighting PRMT6 binding as a negative factor of GATA1 transcriptional activity in aberrant hematopoiesis.

## Introduction

The differentiation process of erythrocytes and megakaryocytes shares the regulation of the hematopoietic transcription factor GATA1.[1] It activates globin genes and hemoglobin (Hb) synthase to support the maturation of Hb in erythroid differentiation during both embryonic development and adult stages.[2,3] Moreover, GATA1 plays a crucial role in regulating cell proliferation and apoptosis in erythroid cells by activating anti-proliferative and anti-apoptotic genes, ensuring stable erythroid development.[4,5] In the case of megakaryocytes, GATA1 is involved in their differentiation and plays a significant role in their proliferation, terminal maturation, and platelet production.[6-8]

Since Nichols[9] first reported a family affected with germline *GATA1* V205M mutation, a series of congenital hematologic disorders caused by *GATA1* mutations have been observed in multiple families.[10-12] It is the discovery of these genetic cases and the exploration of the associated mechanisms that have propelled GATA1 to become one of the most extensively researched hematopoietic transcription factors to date. In earlier studies, the reported mutations were mainly focused on the N-terminal transactivation domain (TAD) and the 2 central zinc fingers (ZF). Mutations in the TAD are mainly related to Diamond-Blackfan anemia (DBA) with a short isoform of GATA1 (GATA1s),[13,14] while those in the zinc fingers are commonly reported in anemia or thrombocytopenia.[9,15] However, very few mutation cases and studies

have been reported in the C-terminus of the GATA1 protein, and the specific function remains not fully defined.

Normal expression of erythrocyte- and megakaryocyte-associated genes depends on GATA1's interaction with co-factors and binding to GATA motifs in downstream genes' transcriptional regulatory regions, both of which are indispensable. A variety of molecules or complexes have been found to interact with GATA1 in transcriptional actions, including FOG1, known as the "Friend of GATA1",[16] the core subunit of the mediator complex MED1,[17] the hematopoietic transcription factor TAL1,[18] CBP/P300 acetyltransferase,[19-22] the SWI/SNF complex and the NuRD complex.[23]

PRMT6, a type I arginine methyltransferase, mediates the production of asymmetric dimethylarginines on histones, including H3R2me2a, which is recognized to be implicated in transcriptional repression.[24] Previous studies have found that PRMT6 binds to the transcription factor RUNX1 before megakaryocyte differentiation but disassociates during megakaryocyte differentiation and is recruited to the promoter of GYPA (CD235a).[25-27] During CD34$^+$ hemopoiesis, PRMT6 inhibits erythroid gene expression and mediates H3R2me2a on GYPA and KLF1.[28] These results suggest that PRMT6 may act as a co-repressor by binding to hematopoietic transcription factors and participating in the regulation of lineage differentiation.

In this study, we identified a novel germline mutation of *GATA1* in a patient with long-term transfusion-dependent anemia and occasional thrombocytopenia. Subsequently, we demonstrated *in vitro* that the mutation impeded the process of normal erythropoiesis and megakaryopoiesis. By employing the Turbo-ID proximity labeling approach to study protein interaction networks in the mutated state, we identified selective alterations in a set of established GATA1-interacting proteins and complexes with increased PRMT6 binding. These alterations are believed to be responsible for the compromised hematopoietic differentiation that results from the mutation.

# Methods

### Ethical considerations

The parents of the proband gave their informed consent for the study and the study was approved by the Ethics Committee of the Seventh Affiliated Hospital, Sun Yat-sen University. The study was carried out according to the principles of the Declaration of Helsinki and the necessary permission from the family was obtained to use clinical information. Bone marrow (BM) aspirates were obtained during diagnosis.

### Cell culture

Cell lines of HEK-293T, HEL, and K562 cells were cultured as previously described.[29] All cell lines had been authenticated by short tandem repeat (STR) analysis and were cultured with routine tests conducted for mycoplasma contamination by polymerase chain reaction (PCR)-based assays (Yeason, Shanghai).

### Constructs, lentivirus production, and infection

Construction methods of plasmids for GATA1 stable knockout, wild-type and mutant GATA1 stable overexpression, GATA1-Turbo-ID assay, PRMT6 overexpression, and knockdown are detailed in the *Online Supplementary Appendix*. For details of the sgRNAs and shRNAs sequence, see *Online Supplementary Table S4*. Lentivirus preparation and modified spin-infection for suspension cells were performed as previously described.[30,31]

### Deletion of GATA1 and the generation of re-expression stable cell models

CRISPR/Cas9-mediated GATA1 knockout in K562 and HEL cells (represented by K562-sgG1 and HEL-sgG1, respectively) was performed by lentiviral transduction with lentiCRISPR-v2-sgGATA1 plasmids. Then, re-expression was performed by lentivirus of wild-type and mutant GATA1, separately, in GATA1-depleted K562 and HEL cells.

### Induction of erythroid and megakaryocytic differentiation

The erythroid induction of K562 and HEL cells was facilitated through hemin (MCE, Shanghai) treatment. Megakaryocytic induction of K562 and HEL cells was achieved by PMA (MCE, Shanghai, China). Benzidine cytochemical staining was performed to assess Hb level in erythroid differentiation as previously described.[32]

### Turbo-ID proximity labeling

Turbo-ID proximity labeling-based western blotting and mass spectrometry were performed with the plasmid carrying a Turbo-ID fused wild-type or mutant GATA1 protein according to the previous description.[33] 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. (Tianjin, China). Complete details of the materials and methods of whole exome sequencing (WES) and Sanger sequencing, FACS analysis, RT-qPCR, western blotting, chromatin immunoprecipitation, and co-immunoprecipitation analysis are described in the *Online Supplementary Appendix*.

### Statistical analysis

Quantification of western blots was performed by ImageJ Software. All statistical tests were performed using SPSS 20.0 (IBM, USA) and GraphPad Prism 9 (GraphPad Software, CA, USA). $P$=0.05 was considered significant. After confirming the Shapiro-Wilk normality test, a $t$ test was performed. Mann-Whitney U test was used to compare sample groups for which normality was rejected.

# Results

### Case description: a novel germline *GATA1* mutation (c.1162delGG, p.Leu387Leufs*62) identified in a family associated with congenital anemia

The patient investigated in the study was a 7-month old male child with anemia for more than six months. The first BM evaluation at 45 days found erythroid hyperplasia and thrombocytopenia. The second BM stain at six months of age showed hypoplasia with a lower ratio of granulocytes to erythrocytes (17% *vs*. 1.5%) (*Online Supplementary Figure S1A*). The morphology of all cell types was generally normal in the BM analysis (*Online Supplementary Figure S1A*). Relevant hematologic findings comprised normal Hb electrophoresis results (HbA 78.0%, HbF 19.9%, HbA2 2.1%, at 45 days of age), normal G6PD level (4723 U/L), and negative direct Coombs test. Before transfusion, the patient exhibited consistently low red blood cell count (1.84-3.87x$10^{12}$/L), hematocrit (18-35.3%), Hb (56-110 g/L), and reticulocyte Hb equivalent. The reticulocyte count and mean circulating volume (MCV) were normal (80.8-97.9 fL) (*Online Supplementary Table S1*). Additionally, the patient occasionally had a low platelet count and mean platelet volume (MPV), persistently high lymphocyte count, and low eosinophil count (*Online Supplementary Table S2*). After haploidentical stem cell transplantation (haplo-HSCT) with post-transplant cyclophosphamide (PTCy) at one year old, the patient became transfusion independent. The patient's mother was found to have anemia in the sixth month of gestation. The patient's non-consanguineous parents had three children, and their first male child, who also had anemia and short-term thrombocytopenia, had died of severe anemia two months after birth (*Online Supplementary Table S3*). WES was performed with peripheral blood (PB) from the patient and his parents to confirm the genetic cause of the anemia. A novel *GATA1* mutation (c.1162delGG, p.Leu387Leufs*62, hereon referred to as Leu387fs), which both the patient and his mother carried, was identified and confirmed by Sanger sequencing (Figure 1A). The variant has not been recorded in the ClinVar, HGMD mutation database, or the available literature. Its frequency data are absent in the 1,000 Genomes, ExAC, and gnomAD population database, indicating that the variant might be rare. For this mutation, the anemic proband was hemizygous, mother heterozygous, and father wild-type (Figure 1A, D). This mutation manifested as a double base deletion in the GATA1 coding region, caused a frameshift and extended translation (Figure 1B), and resulted in altered multiple species-conservative amino acids in the C-terminus (Figure 1C).

Given the crucial roles of GATA1 in the erythroid and megakaryocytic differentiation, its germline mutations are often associated with hematologic disorders like anemia and thrombocytopenia,[10,34] consistent with the patient's anemic phenotype and history of platelet reduction. Since its location on the X-chromosome, the germline mutation of the *GATA1* gene has an X-linked inheritance pattern, which is compatible with the family history (Figure 1D). Furthermore, a reduction in GATA1 expression level was found in the patient's BM and peripheral blood (PB) compared with healthy control (Figure 1E). Overall, these results suggest that the proband's anemia may be attributable to a decreased GATA1 dose and not just the mutation itself.

### *GATA1* Leu387fs mutation leads to impaired erythroid differentiation and increased apoptosis during erythropoiesis

To investigate and clarify the effect of the identified *GATA1* mutation, we used the K562 and HEL cells derived from myeloid leukemia patients,[35,36] which express full-length wild-type GATA1,[37] and are capable of targeted differentiation into erythroid or megakaryocytes *in vitro*.[38,39] We first stably depleted GATA1 in K562 and HEL cells via the lentiCRISPR/cas9–v2 system (*Online Supplementary Figure S2A*). Subsequently, we rescued these cells with wild-type or Leu387fs mutant GATA1 to create stable cellular models to assess the mutational effects on erythropoiesis and megakaryopoiesis (Figure 2A, *Online Supplementary Figure S2B, C*).

Given that a low level of spontaneous erythroid differentiation could occur in general K562 cells,[40] during cell culture, we observed that Leu387fs mutant cell pellets displayed a paler red color than the wild-type GATA1 cells and a darker red compared to GATA1-depleted cells (Figure 2B), implying defects of erythropoiesis with cells in the mutant state. To further explore the effect of *GATA1* Leu387fs on erythropoiesis, we introduced hemin to stimulate erythroid maturation in both cell groups. As compared to the wild-type GATA1 controls, the mutant cells showed reduced Hb production before and after induction (Figure 2C, *Online Supplementary Figure S3A, B*). The $CD71^{+}CD235a^{+}$ erythroid populations decreased remarkably during hemin-induced differentiation in mutant cells (Figure 2D, *Online Supplementary Figure S3C*), consistent with the performance of K562 mutant cells without induction (*Online Supplementary Figure S3D*), indicating the Leu387fs mutation contributes to blocked erythroid differentiation.

Moreover, CD235a and the apoptotic marker Annexin V were used to label apoptotic erythrocytes in the flow assay to assess erythroid apoptosis. Both the mutant cells and the GATA1-depleted cells showed a distinct increase in apoptotic cells (AC) and the ratio of erythroid apoptotic cells (%EAC) during differentiation (Figure 2E, *Online Supplementary Figure S3E, F*).

Further evaluation of the transcriptional impact by *GATA1* Leu387fs revealed that a subset group of GATA1 target genes, including a series of globin, heme synthase ALAS2, and ferrochelatase FECH, were detected with reduced expression in the mutant state in comparison to wild-type GATA1 (Figure 2F, *Online Supplementary Figure S3G*), indicating attenuated erythroid transcription activity of the *GATA1* Leu387fs mutation. Taken together, these results demonstrated that the Leu387fs mutation caused a diminished transcriptional

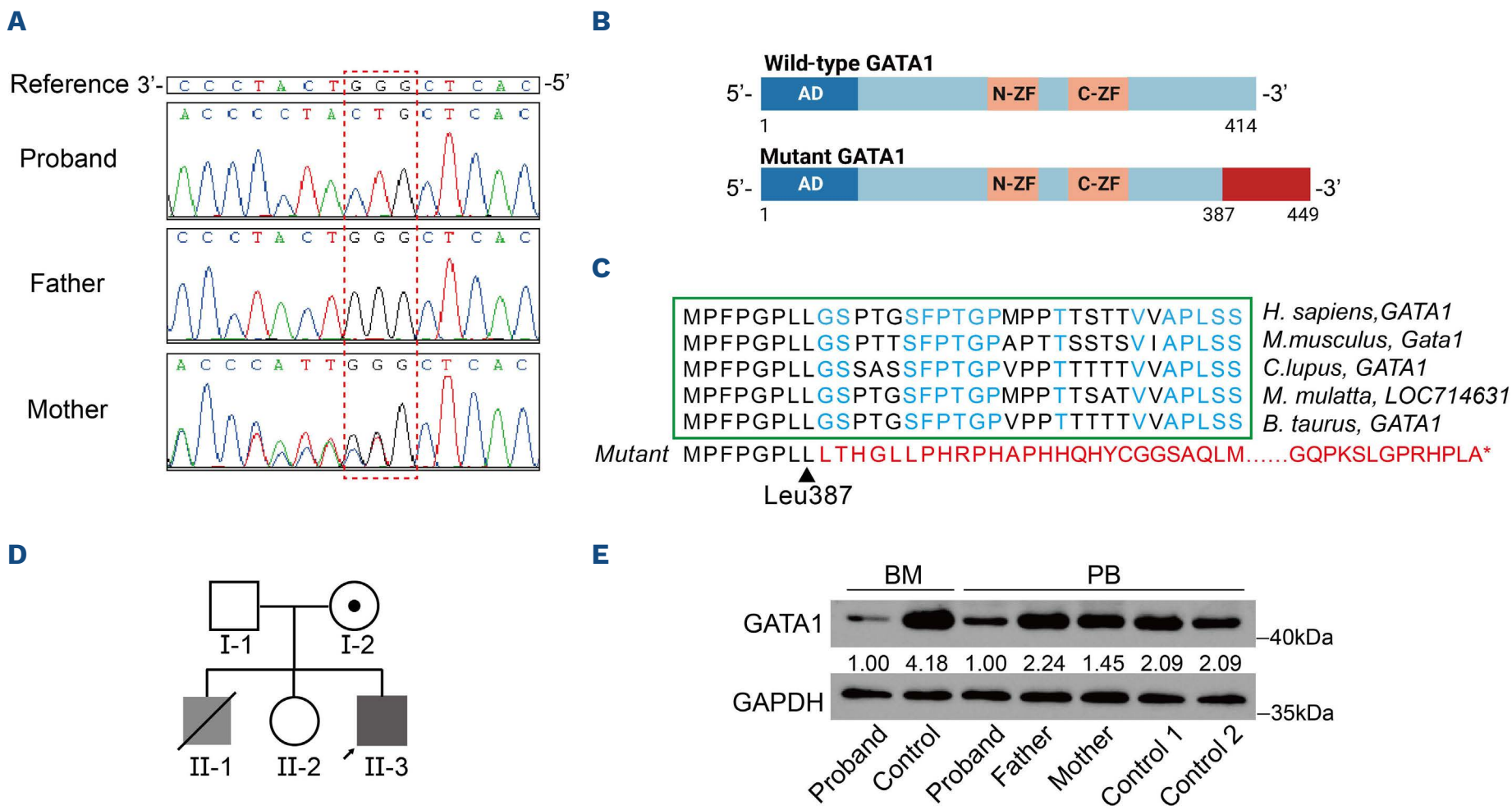


**Figure 1. A novel germline mutation of *GATA1* was identified in a patient with congenital anemia.** (A) Sanger sequencing confirmed the existence of the novel *GATA1* mutation (c. 1162delGG). The proband and his mother were found to have hemizygous and heterozygous mutations in GATA1, respectively, with the double guanine base deletion identified at the codon position highlighted in the red box. (B) Schematic presentation of the wild-type and mutated (p. Leu387Leufs*62) forms of GATA1 protein was provided; red region indicates area of translation frameshift due to the mutation. AD: activation domain; N-ZF: N-terminal zinc finger; C-ZF: C-terminal zinc finger. (C) As indicated by the black triangle, the mutation occurred at the carboxyl terminus of the GATA1 protein, resulting in a frameshift in translation (amino acids in red) with changes of multiple conserved amino acids (blue). (D) A two-generation pedigree of the family shows the anemic proband carrying the hemizygous mutation (black arrow), the proband's mother carrying the X-linked heterozygous mutation (dotted circle), and the proband's older brother who died of severe anemia with an unknown genotype (light gray box with slash). (E) Immunoblot analysis of GATA1 expression levels in bone marrow (BM) and peripheral blood (PB) of the proband and healthy controls. Bands were quantified by Image J software, and intensities were normalized to the proband.

activity of GATA1 and a reduction in Hb synthesis, which led to an impeded erythroid differentiation process and was associated with increased cell apoptosis.

### *GATA1* Leu387fs mutation affects megakaryocytic differentiation

To investigate the effect of the Leu387fs mutation on megakaryocyte differentiation, we used PMA as an inducer of megakaryocyte differentiation and treated both groups of K562 and HEL cells for 96 hours (hr). Both groups of cells showed a significant reduction in $CD41^{high}$ megakaryocytic population in mutant cells (Figure 3A). To analyze ploidy changes during megakaryocytic differentiation in both groups under Leu387fs mutation, measurement of cellular DNA content was performed by flow cytometry; analysis of PI dye showed a significant decrease in high ploidy populations (≥8N) by the *GATA1* Leu387fs mutation, suggesting a defect in polyploidization during megakaryocyte differentiation (Figure 3B). Moreover, the transcriptional expression of megakaryocyte- and platelet-related genes, including platelet agglutinin GPIIb/GPIIIa (ITGA2B and ITGB3), thrombopoietin (TPOR, c-MPL), and megakaryocyte-related transcription factor FLI1, were further analyzed in both groups of model cells. The mutation suppressed the expression of ITGA2B and TPOR compared to wild-type GATA1, while the expression of megakaryocyte transcription factors FLI1 was diminished only in K562 cells by the mutation (Figure 3C). Altogether, these results indicated that the Leu387fs mutation could block megakaryocytic differentiation and impact some platelet-functional gene expression.

### Alterations of the proximity protein networks by *GATA1* Leu387fs mutation were found via Turbo-ID proximity labeling

During specific transcriptional activities, GATA1 is required to assemble into transcriptional complexes by recruiting multiple co-factors/repressors. To investigate the exact mechanism of the hematopoietic defects and how the original protein networks would be changed in the *GATA1* Leu387fs mutant state, the biotin ligase Turbo-ID proximity

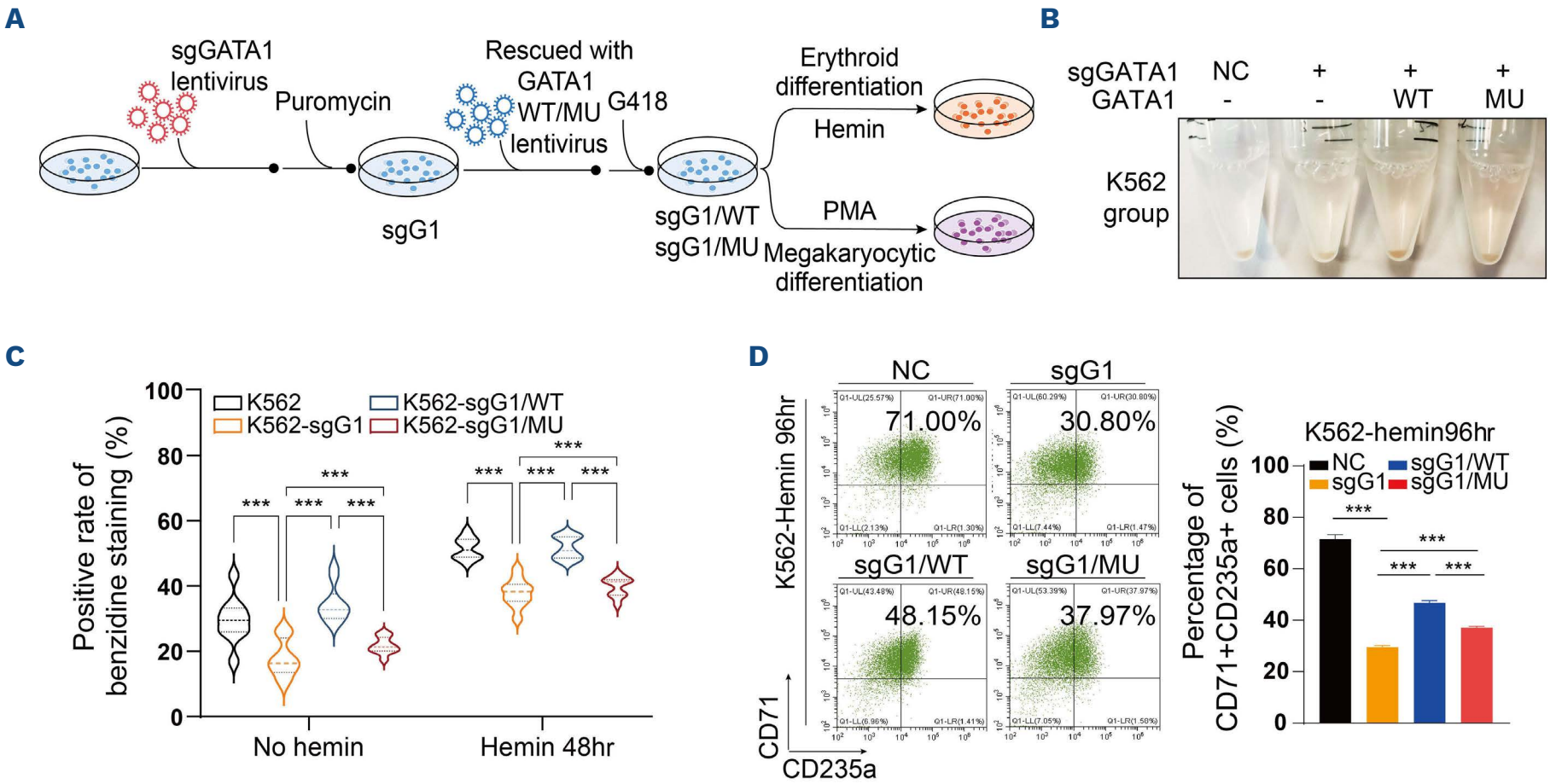


**Figure 2. The Leu387fs mutation in *GATA1* resulted in decreased differentiation, increased cell death, and transcriptional abnormalities during erythropoiesis when compared to wild-type cells.** (A) Schematic workflow of constructing re-expression mutant GATA1 model in K562 and HEL cells. The obtained GATA1 depleted cells, wild-type, and mutant GATA1 complement cells were denoted as sgG1, sgG1/WT, and sgG1/MU, respectively. The process of erythropoiesis and megakaryocytic differentiation were simulated using hemin (40 μM) and PMA (5 nM), respectively. (B) Cell appearance of the constructed K562 re-expression model for the *GATA1* mutation was observed during spontaneous erythroid differentiation. (C) The positive rate of benzidine staining for hemoglobin (Hb) of K562 cells was measured through a violin diagram, which indicated significant differences between the hemin-treated and untreated groups. (D) Flow cytometric analysis of erythroid differentiation was carried out to quantify the percentage of surface $CD71^{+}CD235a^{+}$ population in K562 cells treated with hemin. (E) Flow cytometric analysis of erythroid apoptosis in mutant K562 cells. Representative FACS plots displaying the percentages of AnnexinV and CD235a expressed in mutant and wild-type cells after hemin treatment for 48 hours (hr): $AnnexinV^{+}$ as apoptotic cells (%AC), and measured $AnnexinV^{+}CD235a^{+}/CD235a^{+}$ as erythroid apoptotic cells (%EAC). Bar graphs show the statistical analysis of apoptotic cells (%AC) and erythroid apoptotic cells (%EAC). (F) RT-qPCR was performed on HEL mutant group cells to assess the expression of target genes known to play a role in erythroid differentiation after 96-hr hemin induction. Results were normalized with each value of the housekeeping gene GAPDH. All results presented as mean ± Standard Deviation of 3 independent replicates. **P*<0.05; ***P*<0.01; ****P*<0.001; ns: not significant.

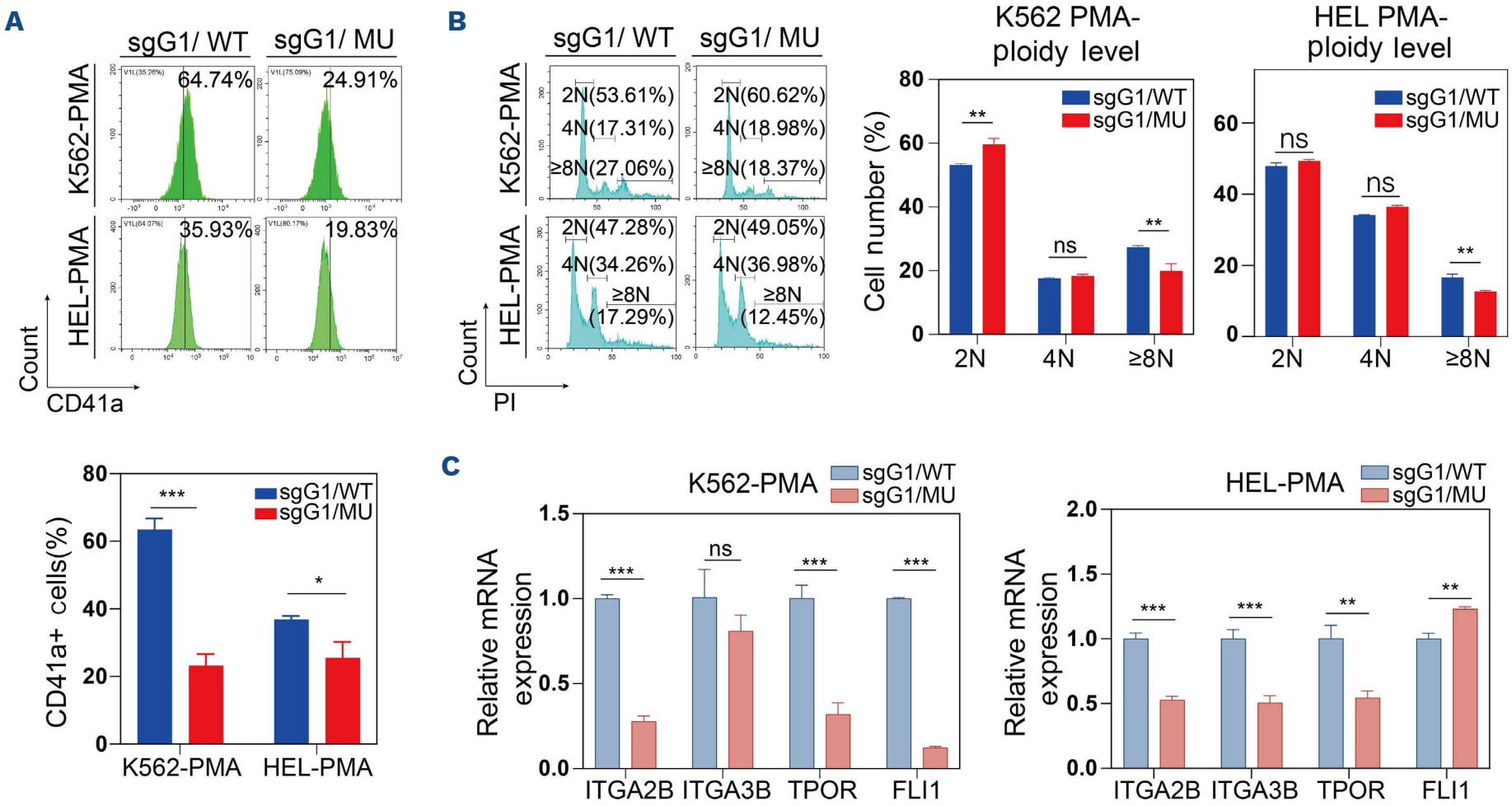


**Figure 3. *GATA1* Leu387fs mutation impedes megakaryocytic differentiation.** (A) (Top) Flow cytometry plots display the megakaryocytic CD41a$^{high}$ proportion in K562 and HEL cells treated with PMA for 96 hours (hr). (Bottom) Statistical results are shown in bar graph. (B) Ploidy analysis of mutant cells during PMA-induced megakaryocytic differentiation represented by flow cytometry analysis of DNA content staining with PI dye. Left peak arose from 2N (G1) cells, middle peak from 4N (G2) cells, and right peak from ≥8N (polyploid cells). Bar graph quantified the percentage of each peak in flow cytometry. (C) RT-qPCR analysis shows expression levels of megakaryocyte and platelet-related genes in mutant cells after PMA induction for 4 days, normalized to the housekeeping gene *GAPDH*. All data are shown as mean ± Standard Deviation of 3 replicates. **P*<0.05; ***P*<0.01; ****P*<0.001; ns: not significant.

labeling method was applied. The wild-type and Leu387fs mutant GATA1-turboID fusion proteins were over-expressed separately in the GATA1-depleted K562 cells. Then the biotin-labeled proximal proteins were collected for mass spectrometry to identify the differential proteins of proximal networks between the wild-type and the mutant GATA1, seeking to discover the critical factors in the process of cell differentiation under hematopoietic abnormality (Figure 4A). Preceding the mass spectrometry assays, the labeling system was tested to evaluate its efficiency, optimize the labeling time, and confirm that the fused Turbo-ID proteins remained in nuclear after transfection (*Online Supplementary Figure S4A, B*). Upon acquisition of mass spectrometry results, we found that the proximal proteins of the wild-type and mutant GATA1 were similar in properties, with only 7 proteins unique to either group out of over 3,000 identified in each sample (*Online Supplementary Figure S4C*). When comparing the quantitative results of proximal proteins in the 2 groups, significantly increased and decreased proteins were found in the *GATA1* Leu387fs group (*Online Supplementary Figure S4D*).

Subsequent analysis revealed that the reduced proximal protein in the Leu387fs mutation comprised a subset of established GATA1 co-factors, including FOG1, an essential co-factor of GATA1, ATF2, a member of the CBP/P300 histone acetyltransferase complex that activates the promoter of GATA1 target genes, MED1, a core subunit of the intermediate complex that mediates the linkage between transcription factors, and POLR1A and POLR2B, 2 subunits encoding RNA polymerases (Figure 4B). Moreover, multiple subunits of the SWI/SNF chromatin remodeling complex and 2 subunits of the NuRD complex, MTA2 and CHD4, were also remarkably reduced in the Leu387fs mutation (Figure 4C). These data on altered binding of the known GATA1-interacting proteins/complexes appear to explain part of the findings in the above study, whereby changes in the binding of the *GATA1* Leu387fs mutation to these known co-factors lead to impaired GATA1-dominated transcriptional activity, ultimately causing dysfunctional differentiation.

Further investigation was conducted to analyze the enriched pathway and interaction network in the differential proximity proteins. These results indicated that the *GATA1* Leu387fs mutation interfered with various mRNA-related pathways, which may further explain the transcriptional abnormalities caused by the mutation (*Online Supplementary Figure S4E, F*). The interacting networks significantly reduced by

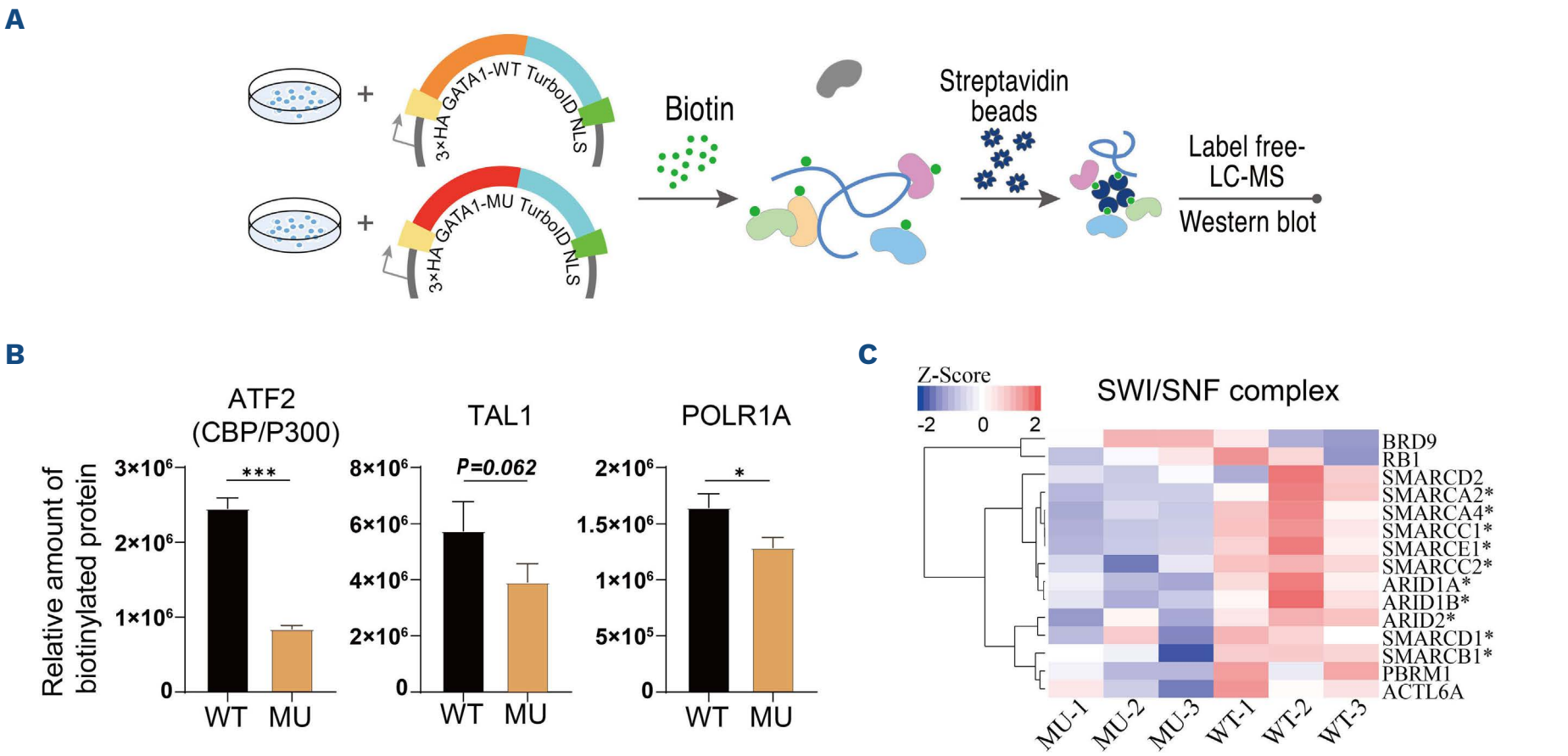


**Figure 4. Turbo-ID proximity labeling was used to identify the altered interaction co-regulators of the *GATA1* Leu387fs mutation.** (A) Schematic of Turbo-ID proximity labeling workflow. Wild-type and Leu387fs GATA1-Turbo fusion protein were over-expressed in GATA1-depleted K562 cells separately. After transfection, the cells were pulsed with biotin for 30 minutes, and the biotinylat-

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ed proteins were subsequently collected and isolated by streptavidin beads. These proteins were then subjected to mass spectrometry and verified for interest regulators by western blot analysis. (B) Mutant GATA1 altered the proximity to a set of known co-factors/interacting proteins and RNA polymerase. Bar graphs show the relative amounts of biotinylated proximal proteins. **P*<0.05; ****P*<0.001. (C) Relative amounts of subunits of the switch/sucrose non-fermentable (SWI/SNF) complex and the nucleosome remodeling complex (NuRD) complex proximal to the wild-type and mutant GATA1 were also compared (upper and lower heat maps, respectively). Z-Score shows fold differences in relative quantitative protein values and color intensity (high: red; low: blue). Asterisks indicate proteins with a significant difference. (D) The significantly decreased/increased proximal proteins were uploaded with GATA1 to STRING for network analysis. A medium level of confidence (0.400) was established for the database searches. Connected lines indicate protein-protein interactions and unconnected nodes in the network were hidden. The significantly reduced proximal proteins were involved in the networks of transcriptional regulatory activity, erythroid/megakaryocyte differentiation, mRNA splicing, and electron transport chain, while the significantly increased proximal proteins were involved in networks such as methylation, mRNA stability regulation, and hemoglobins.

Leu387fs mutation included regulation of transcriptional activity, erythroid/megakaryocyte differentiation, mRNA splicing, and the electron transport chain, whereas, in the increased networks, we found alterations in Hb, regulation of mRNA stability and methylation-related signals (Figure 4D). Within the mutation-increased methylation network of proximal proteins, we identified PRMT6, a member of the protein arginine methyltransferase family (PRMT), which mediates the repressive histone H3R2 asymmetric dimethylation (H3R2me2a), previously reported to be closely related with erythroid and megakaryocytic differentiation, implying its enhanced binding to mutant GATA1 may play a role in the aberrant differentiation caused by the mutation (Figures 4D, 5A).

### Recruitment to PRMT6 was enhanced by the *GATA1* Leu387fs mutation

According to the search results of the Human Protein Atlas and Bloodspot databases, PRMT6 had low tissue specificity (*Online Supplementary Figure S5A*). Still, in BM, PRMT6 showed high expression in erythroid cells (*Online Supplementary Figure S5B, C*), particularly in megakaryocyte-erythroid progenitors (*Online Supplementary Figure S5D, F*), implying that PRMT6 plays a role at this stage of differentiation. We then examined the binding of wild-type GATA1 to PRMT6 in K562 cells that underwent induced erythroid or megakaryocytic differentiation. As a result, we observed attenuated binding of GATA1 to PRMT6 under both differential conditions, which, however, could be due to the reduced PRMT6 expression after differentiation (Figure 5B).

To validate the mass spectrometry results, we performed the pull-down WB analysis with GATA1-depleted K562 cells based on the Turbo-ID method. Consistent with the mass spectrometry findings, the *GATA1* Leu387fs mutation had an increased proximal PRMT6 compared to the wild-type (Figure 5C). We also found the labeled biotinylated PRMT6 in the negative control, suggesting that PRMT6 may be a naturally biotinylated protein (Figure 5C).

Subsequently, co-immunoprecipitation analysis revealed that Leu387fsGATA1 interacts with PRMT6 and is enhanced compared to wild-type GATA1. At the same time, the binding of mutant GATA1 to FOG1 was also weakened in the immunoprecipitation test (Figure 5D). Interaction studies using *E.coli*-produced GST-PRMT6 with *in vitro*-translated HA-tagged GATA1 indicated a weak direct interaction between the mutated GATA1 and GST-PRMT6 but this was significantly enhanced compared to wild-type GATA1 (Figure 5E). Preliminary exploration of the interaction domain between PRMT6 and mutant GATA1 by immunoprecipitation assays revealed that the mutant GATA1 interacts with amino acids 86-375 of PRMT6 (Figure 5F), while PRMT6 could interact with the N-terminal zinc finger of GATA1 (amino acids 84-258), the C-terminus of wild-type GATA1 (amino acids 292-413), and the C-terminus of mutant GATA1 (292-449) (Figure 5G).

### *GATA1* Leu387fs leads to enhancement of PRMT6 and its driven H3R2me2a modification binding at the promoter/enhancer of the erythroid target genes

In our study, the mutation was found to suppress CD235a (GYPA) and multiple globin gene expression. Upon the β-globin gene cluster, DNase I hypersensitive sites (HS) in the locus control region (LCR) serve as distal enhancers that provide binding sites for GATA1, mediating the formation of loop chromatin structures (LCR-promoter loops) and activate downstream globin gene expression.[41,42] To elucidate the involvement of increased recruitment of PRMT6 by *GATA1* Leu387fs in regulating the transcriptional activity of erythroid target genes, chromatin immunoprecipitation assays were performed in re-expression K562 cells. CD235a, HBB, HBG1, and distal enhancer of β-globin genes LCR-HS2 were selected as target genes.

Within the results, we found that the Leu387fs mutation had no discernible impact on the binding of GATA1 itself to the promoters/distal enhancers of the target genes (Figure 6A). Significant binding was detected on these regulatory elements in wild-type and mutant GATA1 with comparable levels (Figure 6A). However, the binding of PRMT6 and its mediated repressive histone modification H3R2me2a to the regulatory elements of these target genes was significantly enhanced in mutant GATA1 cells compared to wild-type cells (Figure 6B, C). Similar results were observed in cells stimulated with hemin (*Online Supplementary Figure S6A*, B). In addition, we found that the binding of PRMT6 and H3R2me2a modifications was almost always reduced upon hemin induction in both wild-type and mutant cells (*data not shown*). Together, these results suggest that the bind-

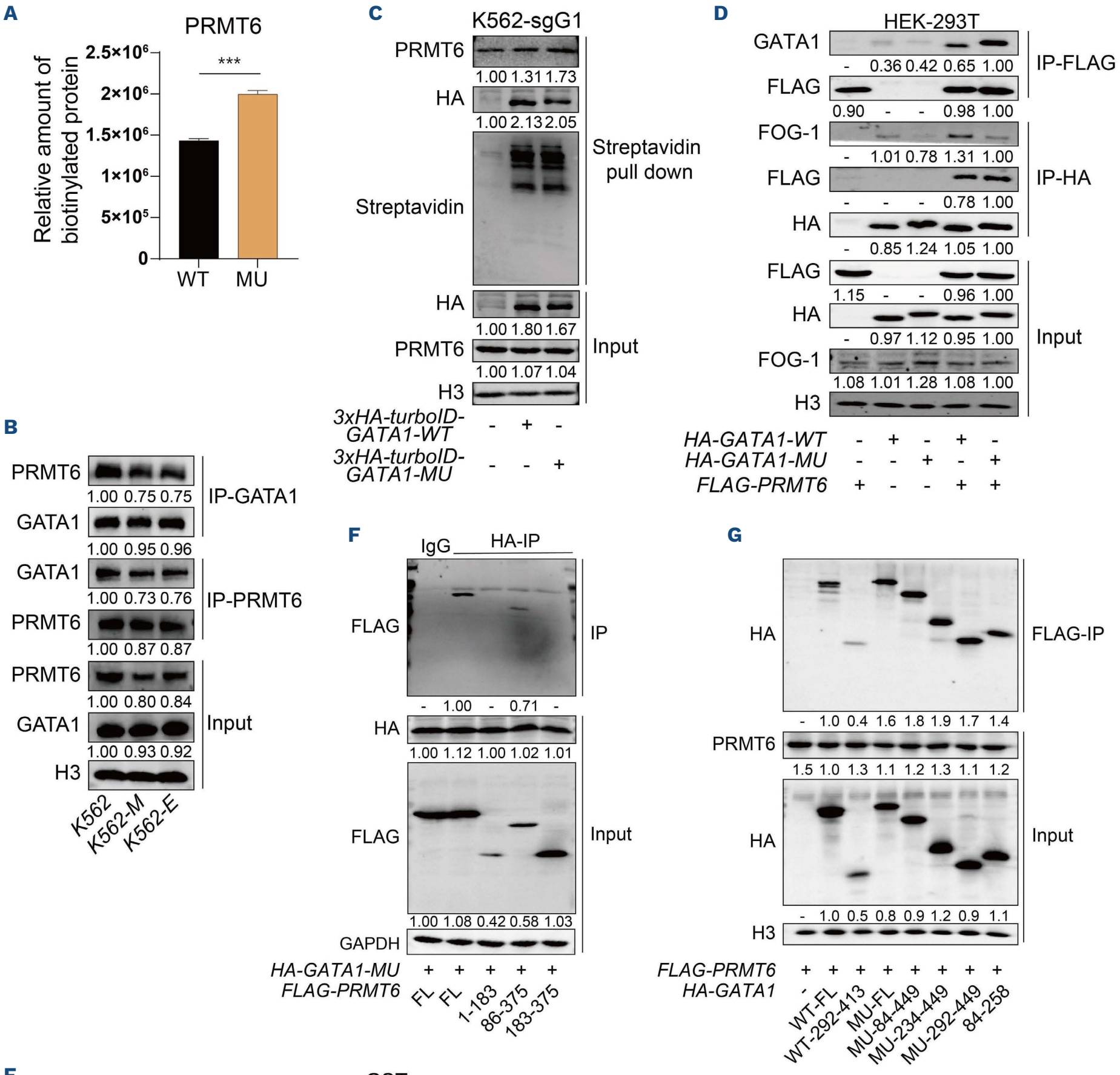


**Figure 5. The Leu387fs mutation in GATA1 resulted in enhanced interaction with arginine methyltransferase PRMT6, compared to wild-type GATA1.** (A) Turbo-ID-mass spectrometry result showed a significant increase in the relative amount of biotinylated PRMT6 protein with the mutant GATA1. ***$P$<0.001. (B) Co-immunoprecipitation analysis of GATA1 binding to PRMT6 during erythropoiesis (E) or megakaryopoiesis (M) in K562 cells. Intensities of bands were corrected by the input H3 and normalized to untreated K562 cells. (C) The proximity between *GATA1* Leu387fs mutation and PRMT6 was confirmed in K562 GATA1-depleted cells, using pull-down immunoblot analysis based on the Turbo-ID method. Intensities of bands were calibrated by the input H3 and normalized to the untransfected K562-sgG1 cells. (D) Co-immunoprecipitation analysis of mutant GATA1 with PRMT6 in HEK-293T cells. Experiments involved

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co-expression of Flag-tagged PRMT6 alongside HA-tagged wild-type or mutant GATA1, with transfected alone as a control. Pull-down assays were performed with Flag and HA tags, respectively, to ascertain PRMT6's enhanced interaction with mutant GATA1. Results of the HA-tagged GATA1 precipitation experiment also revealed a decrease in mutant GATA1's binding ability to FOG1. The intensities of bands were calibrated by the input H3 and normalized to the sample transfected with both mutant GATA1 and PRMT6. (E) GST-pull-down assay was utilized to compare the *in vitro* binding ability of GST-PRMT6 with wild-type or mutant GATA1. Labeled values are quantified intensities of bands normalized to the sample GST-wild-type GATA1. (F) Different Flag-tagged truncated PRMT6 was co-expressed with HA-tagged mutant GATA1 in immunoprecipitation (IP) assay to explore the binding site of mutant GATA1 at PRMT6. Lable values are band intensities calibrated by the internal reference protein and followed normalization. (G) Different HA-tagged truncated GATA1 was co-expressed with Flag-tagged PRMT6 in IP assay to explore the binding domain of PRMT6 at mutant GATA1. Lable values are band intensities calibrated by the internal reference protein and followed normalization. All band intensities were quantified using Image J software.

ing of PRMT6 and H3R2me2a to erythroid gene activation elements impedes the process of erythroid differentiation, and the mutation-induced transcriptional repression may be attributed to the increase of PRMT6 interaction with *GATA1* Leu387fs.

### Aberrant phenotypes in erythroid differentiation caused by *GATA1* Leu387fs could be partially rescued with PRMT6 inhibitor MS023

To clarify the impact of increased PRMT6 binding and its mediated H3R2me2a on the *GATA1* Leu387fs, we introduced

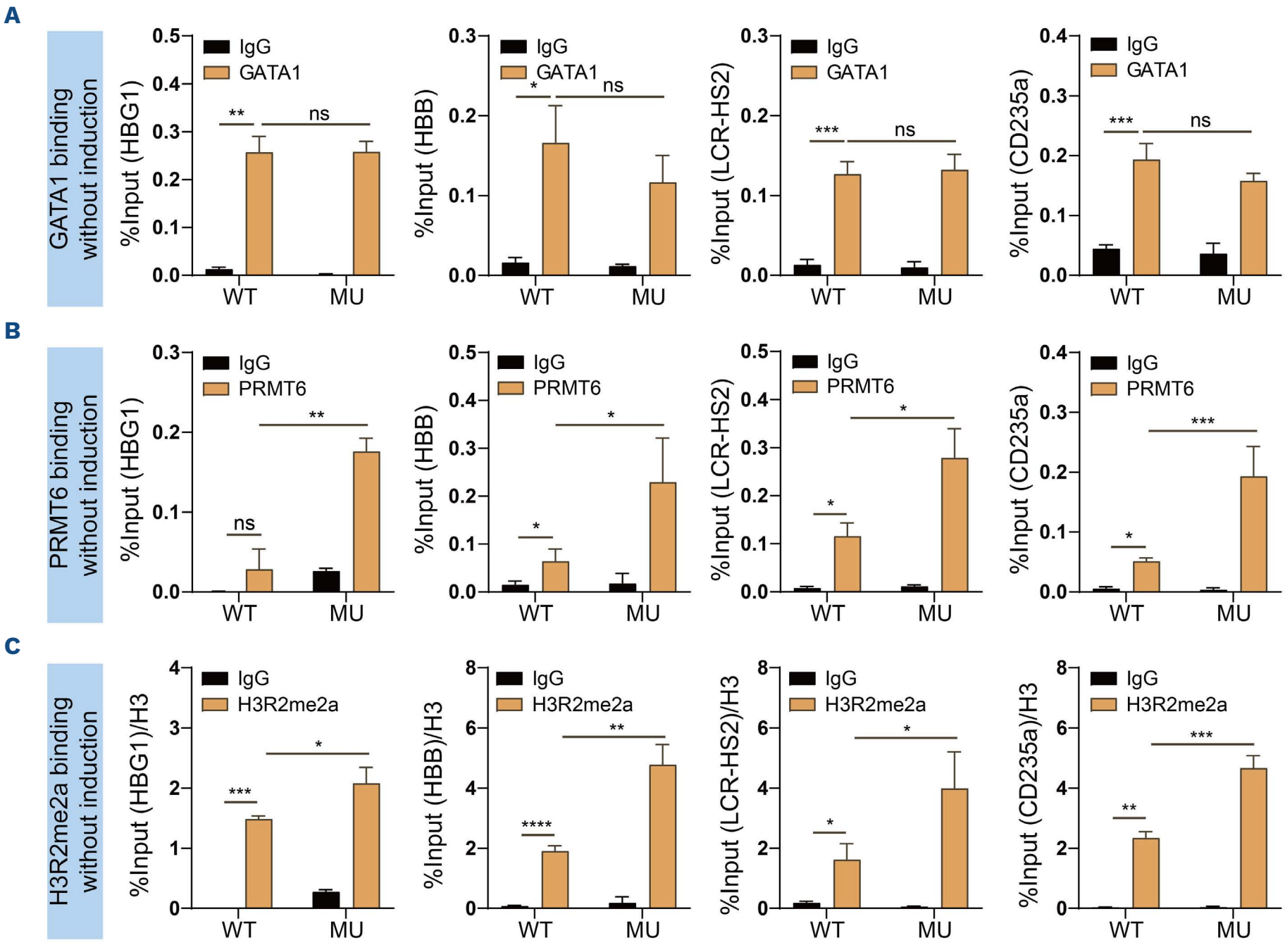


**Figure 6. Occupancy changes in PRMT6 and H3R2me2a modification on the promotor/enhancer of GATA1 erythroid target genes by *GATA1* Leu387fs mutation.** Chromatin immunoprecipitation (ChIP)-qPCR assays were performed on the re-expression wild-type and mutant K562 cells during spontaneous differentiation to determine the binding of HA-tagged GATA1, PRMT6, and H3R2me2a modification on the target genes HBG1, HBB, CD235a (GYPA) promoter, and LCR-HS2 distal enhancer. (A) HA-tagged GATA1 binding results without erythroid induction. (B) PRMT6 binding results without erythroid induction. (C) H3R2me2a binding results with histone H3 serving as the control in spontaneous differentiation. The data are presented as mean ± Standard Deviation of 3 independent replicates by percent enrichment relative to input. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns: not significant.

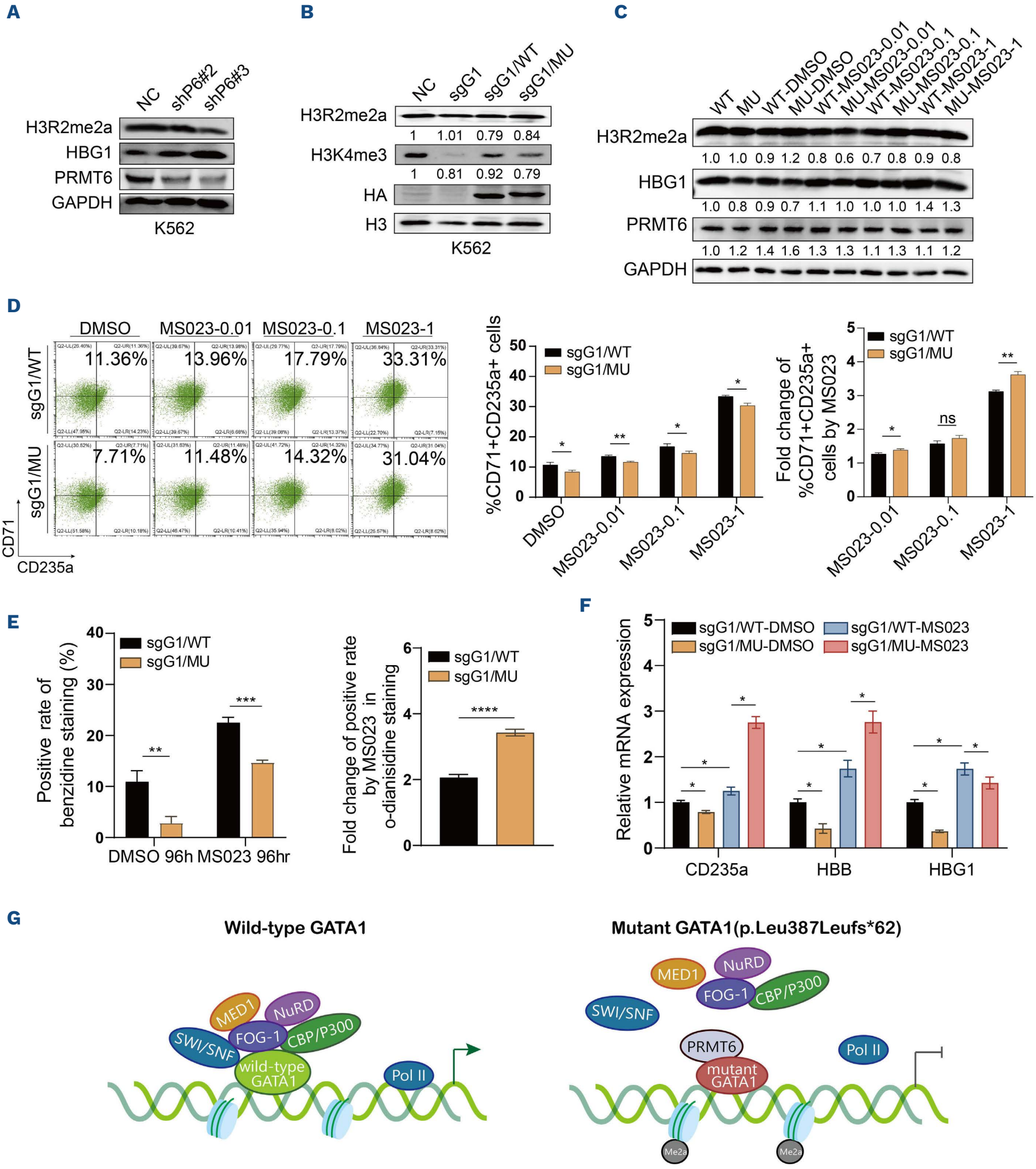


**Figure 7. Abnormal erythroid differentiation caused by *GATA1* Leu387fs mutation can be partially rescued with MS023.** (A) In K562 cells, stable knockdown of PRMT6 led to an increase in γ-globin (HBG1) expression and a decrease in the H3R2me2a modification, as observed in western blot analysis. (B) Western blot analysis was performed to determine the expression levels of histone modifications H3K4me3 and H3R2me2a in mutant GATA1 re-expression cells. The resulting values were measured using Image J

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software and normalized with the NC group. (C) Western blot analysis was conducted to detect the expression levels of γ-globin (HBG1), PRMT6, and H3R2me2a following treatment with the PRMT6 inhibitor MS023 at indicated concentrations (0.01 μM, 0.1 μM, and 1 μM, respectively) in both the re-expression wild-type (WT) and mutant (MU) K562 cells for 4 days. The empty and DMSO treatment groups were used as controls. Indicated values were quantified using Image J software and normalized with the WT cells without any treatment. (D) Erythroid differentiation was analyzed via flow cytometry following treatment with the indicated concentrations of MS023 in the WT- and MU-complementary cells for 4 days. Percentages of surface marker $CD71^+CD235a^+$ cells were quantified, and the fold changes of $CD71^+CD235a^+$ proportion by MS023 treatment compared to DMSO control were also calculated. The presented data are the mean ± Standard Deviation (SD) of 3 independent replicates. (E) The positive rate of benzidine staining between re-expressing WT and MU K562 cells treated with 1 μM MS023 for 4 days was compared. DMSO was used as the negative control. Fold changes of positive rate by MS023 treatment compared with DMSO control were also calculated. The data presented are the mean ± SD of 3 independent replicates. (F) RT-qPCR analysis was performed to measure the relative expression levels of CD235a (GYPA), β-globin (HBB), and γ-globin (HBG1) at the mRNA level following treatment of cells with 1 μM MS023 for 4 days. DMSO was used as the negative control. Data presented as mean ± SD of 3 independent replicates. (G) Schematic representation of the novel *GATA1* mutation's mechanism. In its WT state, GATA1 engages in transcriptional regulatory complex formations with co-factors, including FOG1, to ensure proper transcriptional activities for the expression of downstream target genes. However, in its mutated state, GATA1 decreases its binding capabilities to certain original co-factors. Still, increased binding to the arginine methyltransferase PRMT6 facilitates a higher level of histone H3R2me2a modification located on the promoter of the GATA1 target genes, which effectively inhibits the regular transcription process. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns: not significant. hr: hours.

the PRMT6 inhibitor MS023 in the mutational complemented cells for investigation. Knockdown of PRMT6 in K562 cells resulted in reduced H3R2me2a and increased levels of γ-globin (Figure 7A). In previous reports, H3R2me2a modification was antagonistic to the activating histone modification H3K4me3,[24] yet we found a significant reduction in H3K4me3 levels, but not of H3R2me2a levels, in both GATA1-depleted and mutant cells (Figure 7B). Decreased expression of H3R2me2a and increased expression of γ-globin were observed in K562 complemented cells treated with different concentration gradients of MS023 (Figure 7C). The expression of γ-globin was reduced in *GATA1* Leu387fs cells compared to wild-type cells, and this difference was noticeably reduced following MS023 treatment (Figure 7C). While MS023 did not entirely reverse the decline in erythroid cells induced by the mutation, a more significant increase in the $CD71^+CD235a^+$ population was observed in 2 dosage groups compared to wild-type cells following MS023 treatment (Figure 7D). A comparable pattern was identified in the analysis of Hb synthesis (Figure 7E). Finally, the transcription levels of GATA1 erythroid target genes before and after MS023 treatment were also analyzed, in which the expression of CD235a and β-globin gene in mutant cells was reversed after MS023 treatment (Figure 7F). Though the expression of the γ-globin gene in the mutant cells remained lower following MS023 treatment than in the wild-type cells, its post-treatment gain in fold change of expression was significantly greater than that of the control cells (Figure 7F). Taking these results together, we concluded that PRMT6 inhibitor MS023 can partially rescue the erythroid defects caused by *GATA1* Leu378fs mutation.

## Discussion

In this study, we identified a novel *GATA1* germline mutation (c.1162delGG, p.Leu387Leufs*62) in a child with persistent anemia and occasional thrombocytopenia. Despite inducing a frameshift and the extending translation, this mutation maintains the complete integrity of the zinc fingers and the N-terminal transactivation domain of GATA1. Very few C-terminal GATA1 mutation reports and pathogenic investigations have been done. Out of the documented cases, T296P was found with thrombocytopenia and insignificant erythroid impact,[43] Stop414Arg caused an erythroid Lu(a-b-) phenotype and mild thrombocytopenia,[44] R307C/H caused mild thrombocytopenia and hemolytic anemia with elevated adenosine deaminase levels.[45] Although these cases varied in appearance, they seemed to have a shared deficiency in platelet production. However, the mutational case we found primarily had anemia, but the thrombocytopenia did not persist, although we did find an impaired megakaryopoiesis by the mutation *in vitro*. The precise mechanism underlying the recovered platelet count of the patient remains unknown at this time and it could be the result of frequent transfusion or drug use.

So far, investigation into the role of mutations in the C-terminal GATA1 has been limited. An early study involving transgenic mice with deletion of the C-terminus showed an impact on embryonic hematopoiesis and regulation of megakaryocyte proliferation while suggesting that this region was trans-activated and had no effect on DNA binding or self-dimerization.[46] An earlier work on the 'bloodless' zebrafish $vlt^{m651}$ with partial deletion of the C-terminal gata1 showed complete inhibition of DNA binding and transcriptional activation of GATA1[47] and, more recently, the R307C/H mutation found in congenital hemolytic anemia showed reduced DNA binding and aberrant nuclear localization.[25,45] In our mutational investigation, we did not observe abnormalities in the cellular localization or the binding of GATA1 to several target genes. Undoubtedly, we still need additional support from global data regarding the DNA-binding of the mutant GATA1, and the specific function of the human C-terminal GATA1 remains to be further clarified.

Changes in the protein-protein interaction network had been the focus of our exploration into the pathogenesis of the novel identified mutation. With Turbo-ID proximity labeling, we found a subset of proteins in the GATA1 transcriptional complex showed reduced interaction with mutant GATA1, including FOG-1, whose binding site is located at the N-terminal zinc finger with GATA1, as previously demonstrated.[48] Meanwhile, for the first time, we reported the protein-protein interaction of PRMT6/GATA1 and identified an increased aberrant binding of PRMT6 to mutant GATA1. We showed that PRMT6 could function as a co-repressor of mutant GATA1, achieving transcriptional repression of target genes through GATA1 binding with increased H3R2me2a modification to the regulatory elements of the GATA1 target genes. A similar mechanism was observed in the transcription factor RUNX1, which controls hematopoietic progenitors and megakaryocytes. When RUNX1 binds to PRMT6, RUNX1-mediated gene expression is blocked in megakaryocytes; however, upon dissociation, the active expression of these genes resumes.[25] These results suggest that PRMT6 acts as a negative transcription regulator during hematopoiesis and is responsible for stabilizing gene expression.

As stated above, the *GATA1* Leu387fs mutation recruits more PRMT6, accompanied by decreased binding of known GATA1 co-factors (Figure 7G). It may explain why we used the PRMT6 inhibitor but could only partially restore erythroid gene expression. We have not yet learned whether PRMT6 competes with these co-factors for binding; this requires further experimental evidence. In addition, the process by which PRMT6 was recruited to specific transcription factors remains to be investigated.

**Disclosures**

*No conflicts of interest to disclose.*

**Contributions**

*YLu, QZ, YW, ML, LH, JO, CL, YLi, TK and YS performed experiments. YL, YW, JH and CZ performed bioinformatics analyses. LY, NT and XX managed and maintained research data. YLu and CZ wrote the manuscripts. YLu, LY and CZ revised the manuscripts. YLu, GZ and CZ designed the study. CC and CZ directed the study. All authors contributed to the research and approved the final version.*

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**Data-sharing statement**

*Processing data of quantitative mass spectrometry are available in Online Supplementary Table S5. All other data are available from the corresponding author upon reasonable request.*

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