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HDAC1 is required for GATA-1 transcription activity, global chromatin occupancy and hematopoiesis

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ABSTRACT

The activity of hematopoietic factor GATA-1 is modulated through p300/CBP-mediated acetylation and FOG-1 mediated indirect interaction with HDAC1/2 containing NuRD complex. Although GATA-1 acetylation is implicated in GATA-1 activation, the role of deacetylation is not studied. Here, we found that the FOG-1/NuRD does not deacetylate GATA-1. However, HDAC1/2 can directly bind and deacetylate GATA-1. Two arginine residues within the GATA-1 linker region mediates direct interaction with HDAC1. The arginine to alanine mutation (2RA) blocks GATA-1 deacetylation and fails to induce erythroid differentiation. Gene expression profiling and ChIP-seq analysis further demonstrate the importance of GATA-1 deacetylation for gene activation and chromatin recruitment. GATA-1^{2RA} knock-in (KI) mice suffer mild anemia and thrombocytopenia with accumulation of immature erythrocytes and megakaryocytes in bone marrow and spleen. Single cell RNA-seq analysis of Lin$^-$ cKit$^+$ (LK) cells further reveal a profound change in cell subpopulations and signature gene expression patterns in HSC, myeloid progenitors, and erythroid/megakaryocyte clusters in KI mice. Thus, GATA-1 deacetylation and its interaction with HDAC1 modulates GATA-1 chromatin binding and transcriptional activity that control erythroid/megakaryocyte commitment and differentiation.

INTRODUCTION

GATA-1 is a tissue specific transcription factor that is essential for terminal differentiation of erythrocytes and megakaryocytes (1). GATA binding sites are present in the regulatory regions of virtually all erythroid-specific genes, suggesting that GATA-1 represents an erythroid-specific master regulator (1–4). Genetic mutations in GATA-1 are associated with various forms of anemia and myeloid malignancy (5–10). GATA-1 has been reported to interact with a variety of coactivators and corepressors, including the HDAC1/2-containing NuRD or MeCP1 corepressor complexes, through its interaction with FOG-1 (2,11–13). The NuRD complexes appear to be important for GATA-1 mediated activation and repression (11,12,14). We recently reported that HDAC1 is acetylated during erythropoiesis and acetylated HDAC1 loss deacetylase activity (15,16). More importantly, acetylated HDAC1 converts the NuRD corepressor complex to coactivator complexes (15,17), supporting the notion that NuRD is required for GATA-1 activation (14). Therefore, HDAC1 is a critical component in regulating GATA-1 function.

GATA1 undergoes acetylation by p300/CBP on several clusters of lysine residues adjacent to the two DNA-binding zinc fingers (18,19). Mutating these sites dramatically impairs GATA-1 function, suggesting that acetylation of GATA-1 plays a role in its transcriptional activity. The mechanistic basis for GATA-1 regulation by acetylation remains controversial; while one study showed direct enhancement of DNA binding by acetylation (18), another study showed enrichment in chromatin binding without affecting DNA binding (20). GATA-1 acetylation may also mediate recruitment of the BET family bromodomain factor Brd3, which plays a critical role in promoting

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GATA-1 occupancy of erythroid target genes (21). However, it remains unknown whether and how GATA-1 acetylation is regulated in contributing to its activities in hematopoiesis.

In this study, we found that GATA-1 can be deacetylated by HDAC1 through direct interaction independently of FOG-1. This interaction is required for GATA-1 deacetylation and GATA-1 chromatin occupancy. Furthermore, the disruption of the GATA-1-HDAC1 interaction represses GATA-1 mediated differentiation programs, including HSC, myeloid progenitors, erythroid and megakaryocytic differentiation, suggesting essential roles for the GATA-1 and HDAC1 interaction in modulating GATA-1 function.

MATERIALS AND METHODS

Acetylation and deacetylation *in vitro*

Flag-tagged p300 and HDAC1 were expression and purified from baculoviral vector. The *in vitro* acetylation and deacetylation assays were described previously (16).

Affinity pull-down assay

Bacterial-expressed GST-tagged wild type GATA-1 and GATA-1 mutants were purified immobilized. The protein level were determined by SDS-PAGE. The GST pull-down assay was performed as previously described (17).

Generation of GATA-1 2RA knock-in mice

A targeting vector harboring GATA-1 R243, 247A (2RA) mutation was generated for gene targeting by homologous recombination. The detail method is provided in Supplementary Methods.

RNA sequencing (RNA-seq) and single cell RNA-seq

Total RNA was isolated and DNAse I treated using RNA purification kit (Genesee Scientific, San Diego). RNA libraries were prepared using the TruSeq RNA sample prep kit (Illumina, San Diego) and sequenced on the Illumina HiSeq 2000 according to the manufacturer's instructions. Reads were analyzed with Tophat (22), Cuffdiff (23) pipeline. Raw data and normalized gene expression data were deposited to Gene Expression Omnibus (GEO) under accession number GSE161607. RT-qPCR were performed to validate RNA-seq results on representative GATA-1 target genes. Primers used are listed in Supplemental Table S1.

For scRNA-seq, Lin^- $cKit^+$ mice bone marrow cells were sorted and loaded to 10× Genomics platform with a target output of 7000 cells. scRNA-seq libraries were constructed using chromium single cell 3′ reagent kits v2 (10× Genomics) and sequenced on the NovaSeq 6000 Sequencing System (Illumina). The analysis was performed using Cell Ranger pipeline (10× Genomics) and 'Seurat' package (24) in R language. The scRNA-seq dataset has been deposited to GEO (accession number GSE161729).

Details of the methods and analysis are described in Supplementary Methods.

Chromatin immunoprecipitation (ChIP) assays and ChIP-seq

ChIP assays were performed as previously described (15). ChIP primers are listed in Supplemental Table S1. ChIP-seq data were mapped to the reference mouse genome (mm10) using Bowtie2 (25). Peaks were identified using the MACS program (26). Binding sites annotation were performed using the ChIPseeker (27). The ChIP-seq dataset has been deposited to GEO (accession number GSE161608). The detailed method is described in Supplementary Methods.

Colony forming assays

Primary mouse bone marrow and spleen cells were harvested from 2RA KI or C57 mice. Methylcellulose mix was prepared follow the manufacture's instruction (Stem cell Technology, Cat # M3134). 1×10^5 bone marrow cells or 5×10^5 spleen cells were added to methylcellulose medium and plated in triplicate. The cells were incubate at 37°C for 6 days and colonies were scored by light microscopy.

RESULTS

HDAC1, but not the FOG-1/NuRD corepressor complex, deacetylates acetylated GATA-1

It has been well documented that FOG-mediated interaction with the HDAC1/2-containing NuRD corepressor complex is required for GATA-1 mediated repression and activation (2,11–15). Since GATA-1 is an acetylated protein and acetylation is positively linked to its activity (21), we examined whether the FOG-1-associated NuRD complex mediates GATA-1 deacetylation. The NuRD complex was immunoprecipitated with FOG-1 antibody from undifferentiated MEL cells and subsequently incubated with in vitro acetylated GATA-1. To our surprise, the NuRD-containing FOG-1 complex did not deacetylate GATA-1 (Figure 1A). As a positive control, the NuRD complex deacetylated histone H4 effectively (Figure 1B), indicating that the purified NuRD complex exhibits active deacetylase activity. Of note, recombinant HDAC1 or HDAC2 can deacetylate acetylated GATA-1 and histone efficiently (Figure 1C, Supplemental Figure S1A and B). Thus, these results indicate that HDAC1/2 can deacetylate GATA-1 *in vitro* in a FOG1/NuRD complex independent manner.

Since HDAC1 deacetylates a number of transcription factors through direct interaction (28), we hypothesized that HDAC1 could deacetylate GATA-1 through a direct interaction. GST pull down was carried out by incubating GST-GATA-1 with ^{35}S-labeled HDAC1 to show that GATA-1 physically and directly interacts with HDAC1 *in vitro* (Figure 1D). The interaction surface in GATA-1 mediating interaction with HDAC1, was further determined by using a series of GST-GATA-1 deletion mutants. In order to eliminate potential contamination of FOG-1 complex in reticulocyte lysates when used to synthesize ^{35}S-labeled HDAC1, Flag-tagged HDAC1 purified from baculovirus expressing insect cells was used for subsequent pull down assays. The interaction region was found to be located within the linker region between the N- and C- zinc-fingers of GATA-1

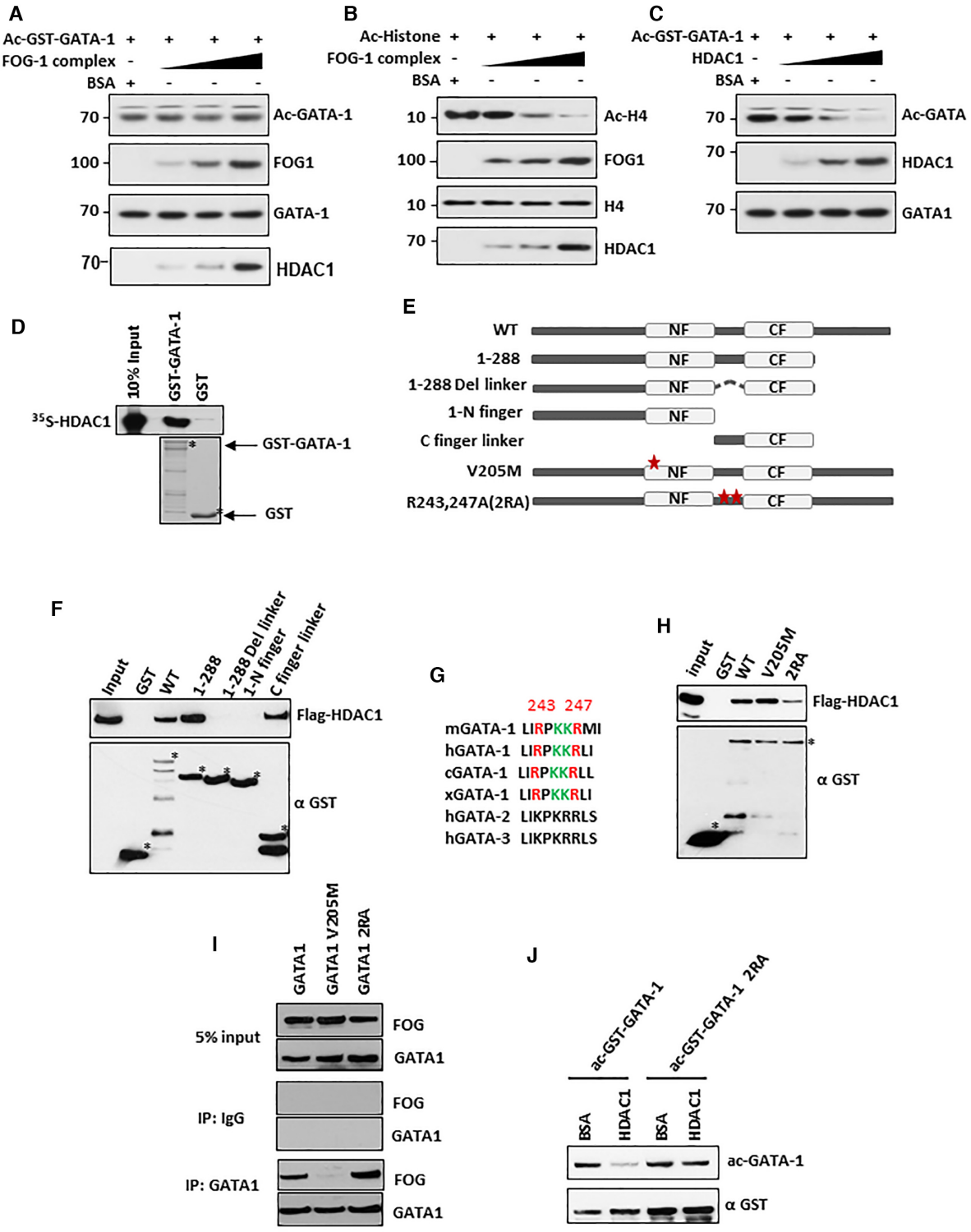


Figure 1. GATA-1 can directly interact with HDAC1 and be deacetylated by HDAC1/2 but not the FOG-1 complex. (**A**) GST-GATA-1 was *in vitro* acetylated by recombinant p300 and then incubated with increasing level of FOG-1 complex purified from MEL cells by immunoprecipitation with FOG-1 antibody. The acetylation level of GATA-1 is evaluated by western blot with anti-acetyl-lysine antibody. BSA is as a control. (**B**) ac-histone was incubated with increasing level of FOG1 complex. (**C**) ac-GATA-1 was incubated with increasing level of HDAC1. (**D**) GST or GST GATA-1 was incubated with ^{35}S-labeled HDAC1. Associated HDAC1 were subjected to SDS-PAGE and autoradiography. (**E**) Schematic representation of GATA-1 and mutant constructs. (**F**) GST tagged GATA-1 and mutant constructs were incubated with Flag tagged recombinant HDAC1. (**G**) Conservation of HDAC1 binding sites. Two arginines which mediate HDAC1 binding are in red. Lysines in green are major acetylation sites at the linker region. (**H**) GST-GATA-1 or mutant were incubated with recombinant HDAC1. The associated Flag-HDAC1 was subjected to western blot with Flag antibody. (**I**) GATA-1 and FOG-1 expression plasmids were transfected into 293 cell and nuclear extracts were immunoprecipitated with anti GATA-1 antibody. (**J**) Acetylated GST-GATA-1 or mutant were incubated with recombinant HDAC1 or BSA.

(Figure 1E, F). Through secondary structure analysis, potential charged surface residues were mutated and two evolutionally conserved arginine residues at aa 243 and 247 of GATA-1 were identified as being important for binding to HDAC1 (Figure 1G). Mutating arginine to alanine at 243 and 247 (2RA mutant), significantly reduced the interaction with HDAC1 (Figure 1H) without affecting GATA-1 DNA binding *in vitro*, or its association with TAL1 (Supplemental Figure S1C and D). The 2RA mutation is also defective in HDAC2 binding (Supplemental Figure S1E), suggesting that both HDAC1 and HDAC2 bind to this linker region. To investigate whether 2RA mutation affects interaction with FOG-1, wild type GATA-1 and 2RA mutant, as well as the FOG-1 binding defective GATA-1 V205M mutant, were overexpressed with FOG-1 in HEK293 cells. GATA-1 or 2RA mutant, but not V205M, can co-precipitate FOG-1(Figure 1I), indicating the 2RA mutation, which lost direct interaction with HDAC1, is still able to interact with FOG-1. These results indicate that the 2RA mutant only affects the direct interaction with HDAC1, but not the interaction with FOG-1/NuRD. Furthermore, the GATA-1 V205M mutant, which is defective in FOG-1/NuRD binding, maintains *in vitro* binding to HDAC1 using nuclear extract from murine erythroleukemic (MEL) cells (Supplemental Figure S1F). Finally, we tested whether the 2RA mutant affects GATA-1 deacetylation. The GATA-1 2RA protein can be acetylated as the wild type protein, however, the 2RA mutation blocks deacetylation (Figure 1J). Therefore, our results indicate that there are two distinct binding modes of GATA-1 to HDAC1: while the V205M mutation affects indirect interaction with HDAC1/NuRD through FOG-1, the 2RA mutation blocks direct binding to HDAC1 and subsequent GATA-1 deacetylation.

HDAC1/GATA-1 direct interaction is required for GATA-1 mediated erythroid differentiation

In order to test the role of FOG-1 independent HDAC1 recruitment in GATA-1 mediated erythroid differentiation, we carried out functional complementation assays in the GATA-1 null G1E proerythroblastic cell line, a well-established *in vitro* model system to study terminal erythroid differentiation following restoration of GATA-1 expression, for example, by retroviral transduction (29,30). We stably expressed in G1E cells estrogen inducible GATA-1 constructs by fusing an estrogen receptor ligand binding domain to wild type GATA-1, V205M or 2RA mutants and to the 2RA/V205M double mutant (Figure 2A, Supplemental Figure S2A). Cells expressing GATA-1 2RA showed increased GATA-1 acetylation, as this mutant is defective in HDAC1 binding (Figure 2B). Immunoprecipitation assays showed that the 2RA/V205M double mutant has undetectable interaction with HDAC1, compared to reduced HDAC1 interaction in the 2RA mutant (Figure 2C), confirming the previous result that HDAC1 interacts with two distinct motifs of GATA-1.

Given that GATA-1 acetylation is important for the binding of GATA-1 to chromatin and subsequent GATA-1 transcriptional activation (20,31), we anticipated that the GATA-1 2RA mutant would promote erythroid differentiation. Surprisingly, we found that the 2RA mutation blocks erythroid differentiation when compared to G1E cells expressing wild type GATA-1 (Figure 2D, Supplemental Figure S2B). Similar effects were shown for the V205M mutation, as previously observed (11,32) (Figure 2D, Supplemental Figure S2B). The 2RA/V205M double mutant also prevents erythroid differentiation (Figure 2D, Supplemental Figure S2B). Therefore, blocking GATA-1 and HDAC1 direct interaction results in inhibition of GATA-1 mediated erythroid differentiation in the G1E cellular model. We next examined protein stability of 2RA mutant. The experiment shows that 2RA mutation results in the increase of protein degradation after cycloheximide (CHX) treatment (Supplemental Figure S2C, D). This result is consistent with previous finding that GATA-1 acetylation reduces protein stability (33).

Blocking the HDAC1/GATA-1 direct interaction alters expression patterns of both GATA-1 activated and repressed genes

We next performed RNA-seq to examine the effects of disrupting HDAC1/GATA-1 direct interactions on global gene expression in G1E GATA-1 2RA cells, compared to expression patterns in control G1E cells and G1E cells expressing WT GATA-1 or the GATA-1 V205M mutant. A total of 1685 genes exhibited at least two-fold differential expression in G1E cells expressing WT GATA-1 compared to control G1E cells (Figure 3A) and, among these GATA-1 regulated genes, approximately one thousand genes were affected by either the 2RA or the V205M mutation (Figure 3B). The altered gene expression profile in GATA-1 2RA cells is largely different from that of cells expressing GATA-1 V205M, suggesting that GATA-1 regulates a distinct set of target genes by differentially interacting directly with HDAC1/2 or via FOG-1 associated NuRD complex (Figure 3A–C). We further narrowed down the gene list to GATA-1 directly regulated genes that possess GATA-1 binding sites in their enhancers/promoters (34). Over 30% of the GATA-1 bound genes in the G1E GATA-1 cells was affected by the 2RA mutation, whilst over 40% of the genes were affected by the V205M mutation. Within these affected genes, about half of them are differentially affected by the 2RA and V205M mutations (Figure 3D, Supplemental Figure S3A). We further analyzed pathways affected by these mutations. Thus, the 2RA mutation affects genes involved in pathways regulating glucose metabolism and translation initiation (Figure 3E and F), whereas V205M affects nucleotide metabolism (Figure 3G). These results suggest the non-redundant functions of these two interaction domains of GATA-1 in erythropoiesis.

There are a total of 315 GATA-1 direct target genes that are affected by both GATA-1 2RA and V205M, such as the beta-globin, GATA-2 or cKit genes (Figure 3D, Supplemental Figure S3B). The majority of genes in this category are GATA-1 activated genes and are down regulated by both 2RA and V205M mutation (Figure 3H). Similarly, within GATA-1 repressed genes which is up regulated by 2RA, are also upregulated by the V205M mutation with only a few exceptions (Figure 3H). Pathway analysis shows that both interacting domains are important for the regulation of cell cycle, apoptosis, heme biogenesis and histone deacetylation (Figure 3I, J). Therefore, the two mutations, which abolish direct or indirect inter-

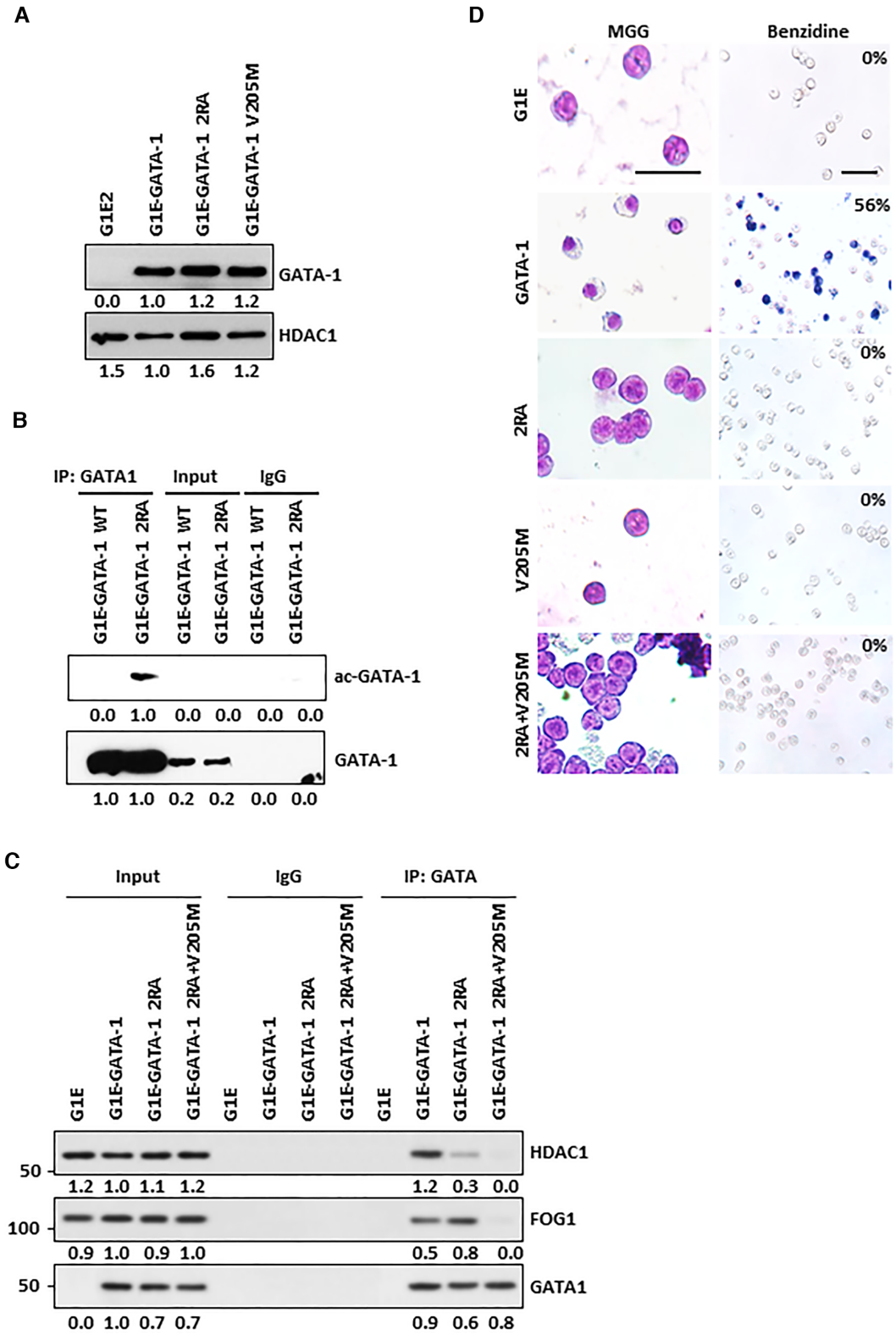


Figure 2. HDAC1/GATA-1 direct interaction is required for erythrocyte differentiation. ER-GATA-1 wt, 2RA, V205M and 2RA + V205M mutants were cloned into a retroviral vector and stably expressed in G1E2 cells. Single clones were selected. Cells were treated with estradiol for 72 h. (**A**) The expression of nuclear GATA-1 from G1E cells was examined by western blot. The band intensity was quantified by ImageJ. (**B**) Cell extracts were immunoprecipitated with GATA-1 and western blotted with anti-acetyl-lysine and GATA-1. (**C**) G1E2 cells expressing ER-GATA-1 wt, 2RA and 2RA/V205M mutants were collected after 72 h estradiol induction. Cell extracts were immunoprecipitated with anti GATA-1 antibody with cross link IP method. The resulting products were subjected to western blot with indicated antibodies. (**D**) G1E cells described above were stained with MGG or Benzidine. The percentage of hemoglobin-positive cells is indicated in the upper right corner of each Benzidine stain panel. Bar = 35 μM

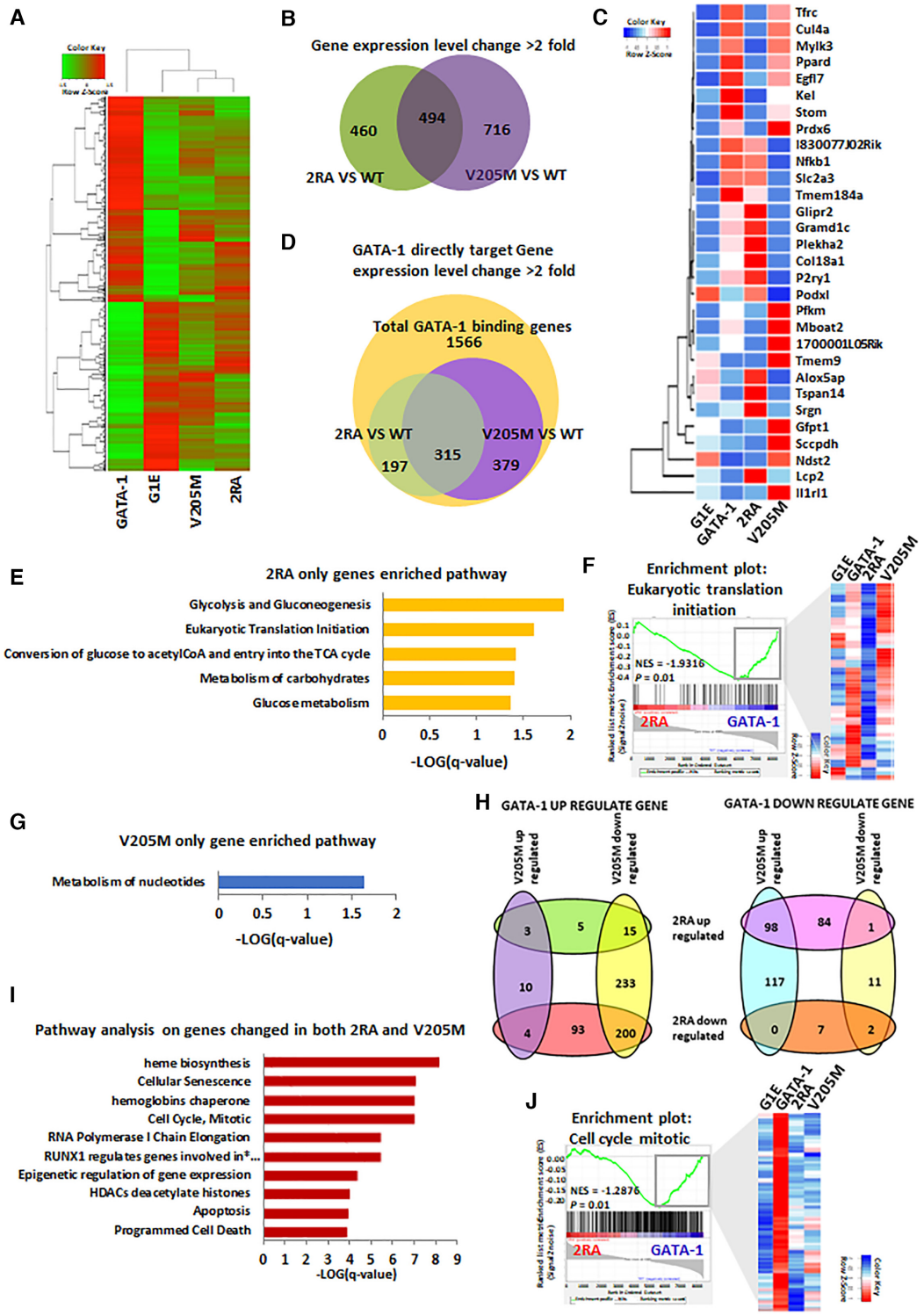


Figure 3. Differential gene expression regulation by V205M and 2RA mutants of GATA-1 in G1E cells. (**A**) A heat map showing genes differentially expressed (over two fold) in G1E cells that stably express GATA-1, V205M or 2RA. (**B**) A Venn diagram showing overlapping and unique gene expression pattern in G1E cells expressing GATA-1 V205M and 2RA. (**C**) Heatmap shows top changed genes differentially regulated by 2RA and V205M mutants. (**D**) A Venn diagram showing GATA-1 direct target genes expression pattern in G1E cells expressing GATA-1 V205M and 2RA. (**E**) Enriched pathway in 2RA only differentially regulated genes. (**F**) GSEA shows 2RA only differentially regulated genes enriched in Eukaryotic translation Initiation pathway. Heatmap shows differential gene expression pattern with 2RA and V205M. (**G**) Enriched pathway in V205M only differentially regulated genes. (**H**) Venn diagrams showing GATA-1 up (left) and down (right) regulated genes in 2RA and V205M mutant cells compared to wild type. (**I**) Enriched pathway in overlapped genes both changed in 2RA and v205M compared with wild type. *RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function. (**J**) GSEA shows overlapped genes enriched in cell cycle mitotic pathway. Heatmap shows similar gene expression pattern with 2RA and V205M.

action with HDAC1, play distinct and overlapping roles in GATA-1 mediated gene regulation, possibly through regulating GATA-1 deacetylation and chromatin deacetylation, respectively.

The HDAC1/GATA-1 direct interaction is important for GATA-1 and HDAC1 recruitment to GATA-1 binding sites

It has been shown that mutations in GATA-1 acetylation sites reduce chromatin binding (20,31). Since the 2RA mutant blocks GATA-1 deacetylation, it is conceivable that the mutation will affect GATA-1 binding to targeted chromatin sites. We therefore performed ChIP-seq analysis to analyze global binding patterns of GATA-1 2RA, as well as its impact on HDAC1 recruitment. The 2RA mutation abolished over 20% of GATA-1 binding (Figure 4A). For the sites where GATA-1 2RA remained bound, the binding activity was largely reduced compared to wild type GATA-1 (Figure 4B, Supplemental Figure S4A). Interestingly, GATA-1 2RA binding is reduced in both GATA-1 activated and repressed genes, consistent with the reduction of GATA-1 mediated gene activation and repression from RNA seq results (Figures 3H and 4B). Although GATA-1 2RA binding is reduced at all GATA binding motifs in different genomic regions (Supplemental Figure S4B), the most profound reduction of binding was observed in proximal promoter regions near TSSs (Figure 4C, Supplemental Figure S4C). HDAC1 ChIP-seq shows that HDAC1 is recruited to GATA-1 binding sites and the 2RA mutation reduces HDAC1 recruitment at these sites (Supplemental Figure S4D). Furthermore, HDAC1 is recruited to both activated and repressed gene promoters at comparable levels in WT G1E GATA-1 cells and, accordingly, the reduction of HDAC1 recruitment by GATA-1 2RA is seen in both GATA-1 activated and repressed genes (Figure 4D and E, Supplemental Figure S4E). However, GATA-1 repressed genes exhibit a greater reduction in HDAC1 binding compared to activated genes (Figure 4D). These results indicate that the GATA-1/HDAC1 interaction is required in both activated and repressed genes.

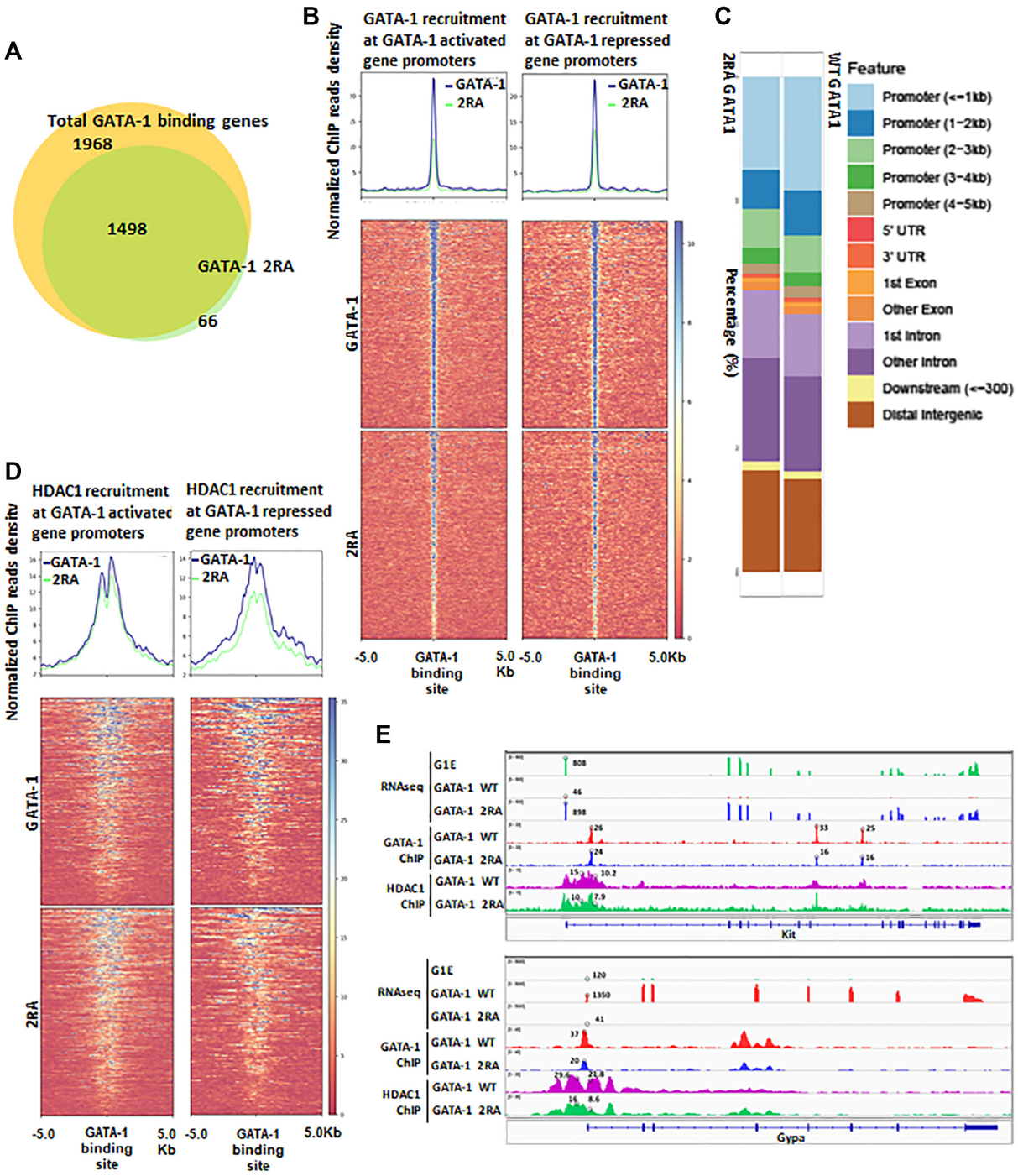
Histone acetylation is associated with activated genes at GATA-1 binding sites and is negatively associated with repressed genes (35) (Supplemental Figure S4F). We then examined how 2RA affects histone acetylation at GATA-1 activated and repressed gene promoters. The β-globin gene expression is not activated in GATA-1 2RA expressing cells (Supplemental Figure S3B). However, loss of HDAC1 interaction in 2RA mutation results in the reduction of histone acetylation on β-globin gene locus (Figure 4F). In contrast, GATA-2 gene expression is not repressed by GATA-1 2RA (Supplemental Figure S3B). Histone acetylation level remain high in GATA-2 gene locus (Figure 4G). Thus, loss of HDAC1 binding does not consistent with histone acetylation level at gene locus. This may suggest that the recruitment of HDAC1 by GATA-1 serves mainly for GATA-1 deacetylation.

GATA-1 2RA knock-in mice suffers from anemia and thrombocytopenia

In order to more comprehensively understand the role of the GATA-1 /HDAC1 interaction in hematopoietic lineage development, GATA-1^{2RA} knock-in mice were generated using a GATA-1 2RA mutated homologous recombination targeting vector. The mice were generated by InGenious Targeting Laboratory (Ronkonkoma, NY). The targeting strategy is illustrated in Figure 5A. Briefly, the arginine residues at aa 243 and 247 of GATA-1 are converted to alanine, with an FRT neo cassette for positive selection inserted between exon IV and exon V. The targeting vector was linearized and then transfected by electroporation in HF4 (129/SvEv × C57Bl/6) (Hybrid) embryonic stem (ES) cells. The correct clones were identified through Southern blot analysis (Figure 5B). Targeted GATA-1^{2RA} ES cell clones were injected into C57Bl/6 wild-type blastocysts to generate GATA-1^{2RA}/wild-type chimeras. Since GATA-1 is located on the X chromosome, resulting male chimeras with a high percentage of agouti coat color were mated to C57BL/6 WT female mice to generate germline GATA-1 2RA knock-in hemizygote mice (Figure 5C). The GATA-1 2RA knock-in allele displayed Mendelian inheritance in all offspring, indicating that the 2RA mutation does not cause any embryonic survival defects (Supplemental Figure S5A).

Peripheral blood analysis reveals that GATA-1^{2RA} knock-in mice suffered from mild anemia and thrombocytopenia with low red cell count, low hemoglobin and low platelet count in both genders (Figure 5D, Supplemental Figure S5B). We therefore analyzed the erythroid and megakaryocyte differentiation profiles. Bone marrow and spleen cells were stained with erythrocyte differentiation markers CD71 and Ter119 and analyzed by FACS (36,37) (Figure 5E). Differentiation and maturation of erythroid progenitors consist of several populations of erythroid cells, including proerythroblasts and early basophilic erythroblasts (I, $CD71^{high}$, $TER119^{low}$), early and late basophilic erythroblasts (II, $CD71^{high}$, $TER119^{high}$), polychromatophilic erythroblasts (III, $CD71^{med}$, $TER119^{high}$) and orthochromatophilic erythroblasts (IV, $CD71^{low}$, $TER119^{High}$) (36,38). In bone marrow, the population of $CD71^{high}$, $TER119^{high}$ erythrocytes (Fraction II), was increased in KI mice compared to the wild type. The later stage Ortho EBs (IV) were decreased in KI mice (Figure 5E, top panel) indicating a block in erythroid differentiation. The analysis of spleen also shows an increase of premature erythroblasts and a decrease of normal mature erythrocytes (Figure 5E, middle panel), resulting in splenomegaly (Supplemental Figure S5C). These results indicate that the 2RA mutation causes a defect in erythropoiesis in the bone marrow and spleen. Notably, there is a significant increase of spleen size due to increased splenic erythropoiesis (extramedullary hematopoiesis) in an effort to compensate for the defect in bone marrow erythropoiesis (Supplemental Figure S5C and D). We also examined erythropoiesis in the fetal liver to investigate GATA-1^{2RA} effects in earlier developmental states. As expected, erythroid progenitor cells from fetal liver fail to produce sufficient mature erythroid cells (Figure 5E, bottom panel).

We then characterized the megakaryocytic differentiation defect in the GATA-1^{2RA} mice. Bone marrow cells were stained with megakaryocytic differentiation markers CD41 and CD150 and analyzed by FACS. The KI mice exhibit more CD41/CD150 positive cells, which represent megakaryocyte progenitor (MKP) cells (39) (Figure 5F). Furthermore, the knock-in mice have reduced polyploidiza-


A
Total GATA-1 binding genes
1968
1498
GATA-1 2RA
66
B
GATA-1 recruitment at GATA-1 activated gene promoters
GATA-1 recruitment at GATA-1 repressed gene promoters
Normalized ChIP reads density
GATA-1
2RA
GATA-1
2RA
-5.0
GATA-1 binding site
5.0 Kb
5.0Kb
C
2RA GATA1
WT GATA1
Percentage (%)
Feature
Promoter (<=1kb)
Promoter (1-2kb)
Promoter (2-3kb)
Promoter (3-4kb)
Promoter (4-5kb)
5' UTR
3' UTR
1st Exon
Other Exon
1st Intron
Other Intron
Downstream (<=300)
Distal Intergenic
D
HDAC1 recruitment at GATA-1 activated gene promoters
HDAC1 recruitment at GATA-1 repressed gene promoters
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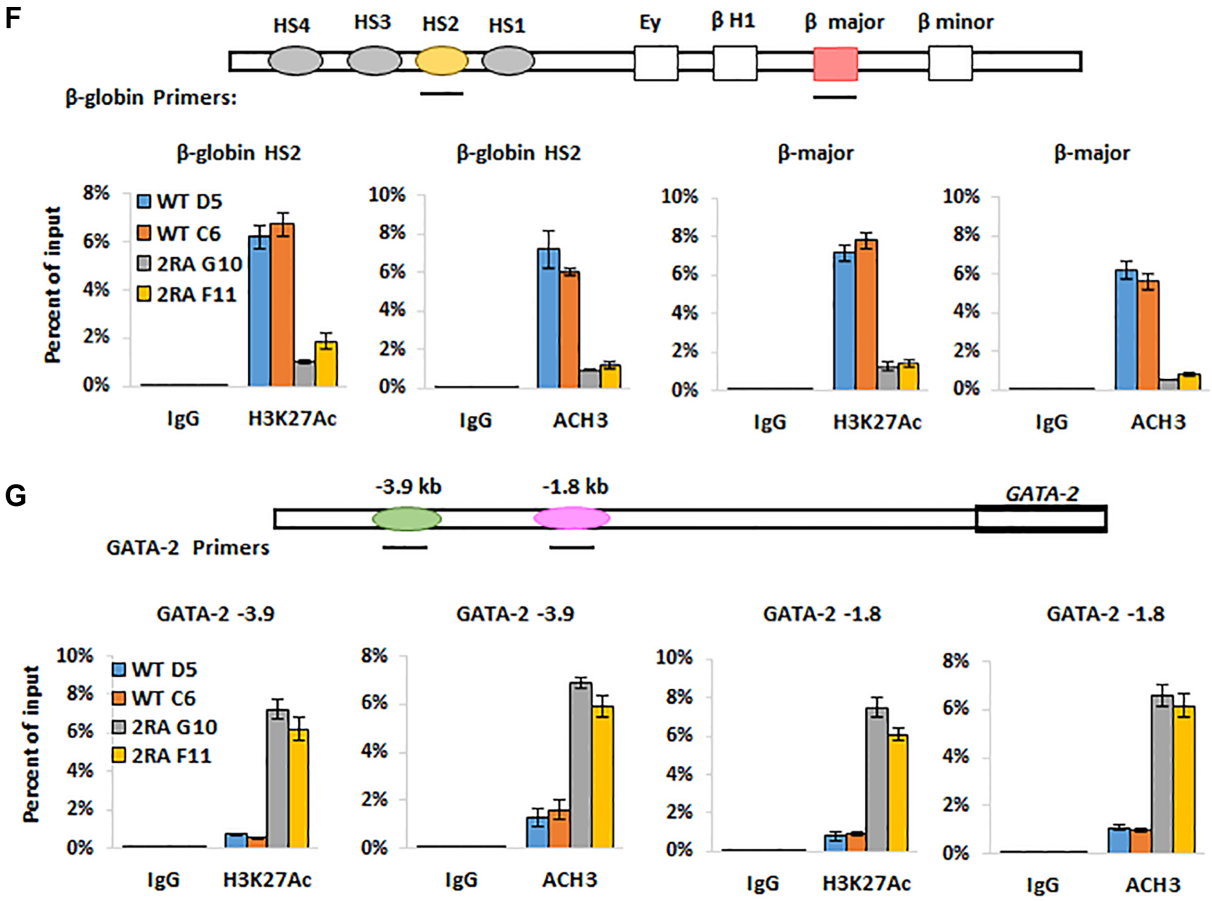


Figure 4. Disruption of GATA-1/HDAC1 interaction resulting in the decrease of chromatin recruitment of GATA-1. (**A**) Venn diagram shows G1E cells expression GATA-1 2RA has reduced binding on GATA-1 binding genes compare to GATA-1 WT expressing cells. (**B**) ChIP-seq data shows disrupting of GATA-1/HDAC1 interaction globally reduced GATA-1 chromatin recruitment at GATA-1 activated and repressed gene promoters. (**C**) Bar graph shows GATA-1 or mutant binding distribution. (**D**) ChIP-seq data shows HDAC1 chromatin recruitment at GATA-1 activated and repressed gene promoters in GATA-1 2RA mutant cells compare to WT. (**E**) Track view shows gene expression and chromatin recruitment at GATA-1 repressed gene *kit* and GATA-1 activated gene *Gypa*. (**F** and **G**) ChIP assay on H3K27AC or H3 acetylation at β-major and β-globin HS2 locus (**F**) and GATA-2 enhancers (**G**). WT D5 and WT C6 are two clones expressing wild type GATA-1, 2RA G10 and 2RA F11 are two single cell clones expressing GATA-1 2RA in G1E cells.

tion (Figure 5G), thus showing that the 2RA mutation blocks megakaryocyte differentiation at the MKP stage and resulting in the reduction of mature megakaryocytes.

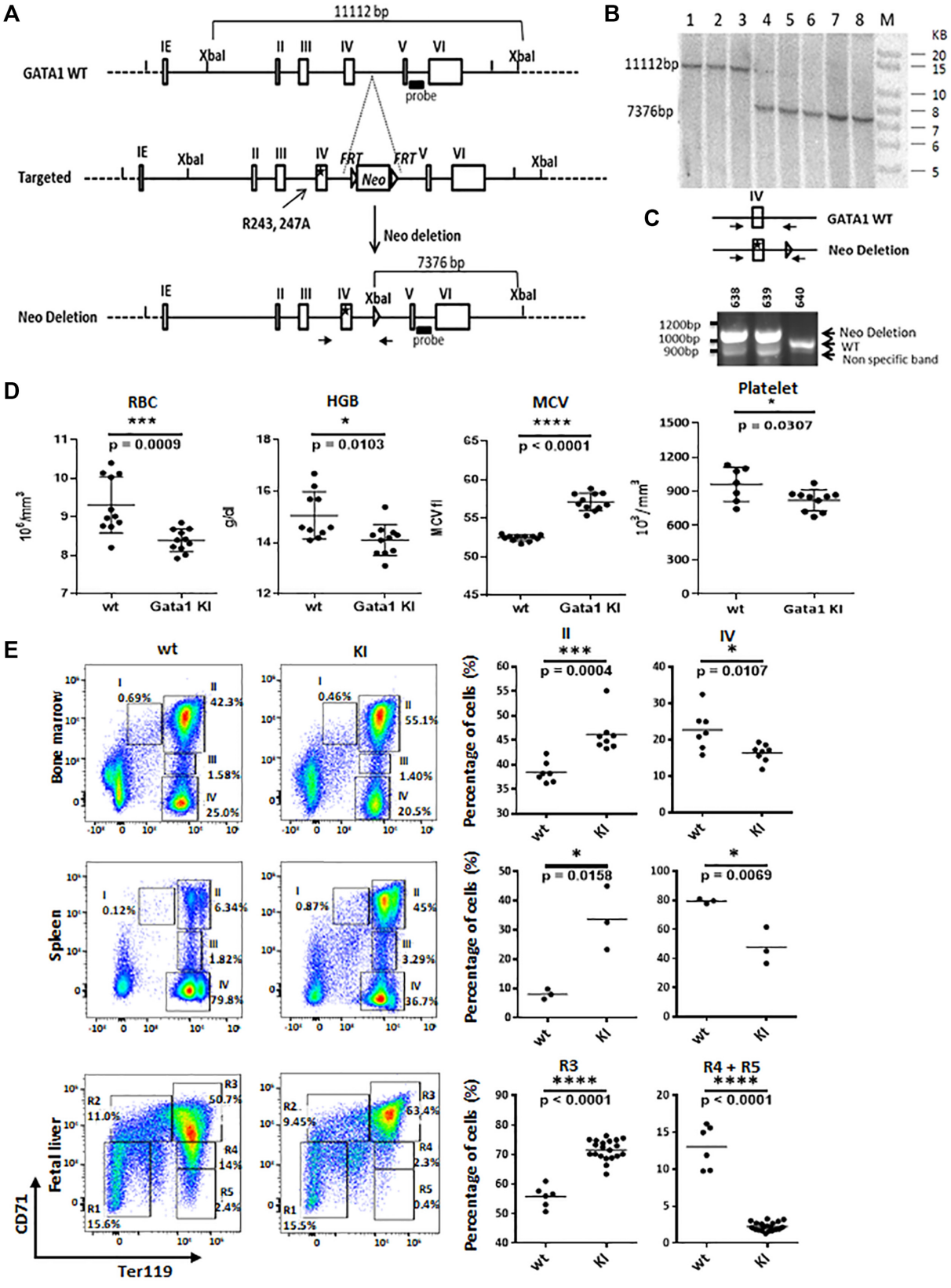
To examine the effect of GATA-1^{2RA} in hematopoietic stem and progenitor cells (HS/PCs), we further assessed the frequencies of colony-forming unit cells (CFU-C) in the bone marrow and spleen cells of WT and GATA-1^{2RA} mice (40,41). Myeloid progenitor CFU-GEMM colonies were increased in the bone marrow and spleen culture in GATA-1^{2RA} mice (Figure 5H), indicating a compensatory expansion of progenitor cells. Although there was no statistically significant difference in the number of erythroid colony-forming units (BFU-E) in the bone marrow, the number was increased several fold in the spleens of GATA-1^{2RA} mice (Figure 5H). These observations support the notion that the 2RA mutation blocks differentiation of hematopoietic progenitors and demonstrate that there is substantial extramedullary erythropoiesis occurring in GATA-1^{2RA} mice.

We next investigated the ability of GATA-1^{2RA} mice to recover under hematopoietic stress conditions. We challenged wild-type and GATA-1^{2RA} mice with phenyl hydrazine, a chemical that induces hemolytic anemia, and assayed the potential of the mice to recover from anemia. All GATA-1^{2RA} mice died within 5 days after the administration of phenyl hydrazine, whereas 75% of wild-type littermates made a full recovery (Figure 5I). These results indicate that the GATA-1 sites required for interaction with HDAC1 are critical for GATA-1-mediated stress hematopoiesis.

GATA-1^{2RA} altered HS/PC hierarchy and inhibited erythroid/megakaryocytic differentiation trajectory

We wished to obtain a comprehensive view of the role of the HDAC1/GATA-1 direct interaction in lineage commitment and differentiation. We thus performed single cell RNA-seq analysis using Lin^- $cKit^+$ (LK) bone marrow cells which are enriched in myeloid progenitors, to study the impact of GATA-1^{2RA} on GATA-1 mediated transcription coherent state of HS/PC and myeloid subpopulations. Single cell transcription profiles of over 6000 cells were analyzed from both wild type and GATA-1^{2RA} mice.

A total of 24 transcriptional clusters were identified on the basis of expressed genes previously reported (42–44) (Figure 6A). The 2RA KI increased the frequencies of spe-


A
11112 bp
GATA1 WT
IE
XbaI
II
III
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VI
XbaI
probe
Targeted
FRT
Neo
FRT
R243, 247A
Neo deletion
7376 bp
Neo Deletion
B
1 2 3 4 5 6 7 8 M
KB
20
15
10
8
7
6
5
11112bp
7376bp
C
GATA1 WT
Neo Deletion
638 639 640
1200bp
1000bp
900bp
Neo Deletion
WT
Non specific band
D
RBC
***
p = 0.0009
HGB
*
p = 0.0103
MCV
****
p < 0.0001
Platelet
*
p = 0.0307
wt
Gata1 KI
E
wt
KI
Bone marrow
Spleen
Fetal liver
CD71
Ter119
II
***
p = 0.0004
IV
*
p = 0.0107
*
p = 0.0158
*
p = 0.0069
R3
****
p < 0.0001
R4 + R5
****
p < 0.0001
Percentage of cells (%)

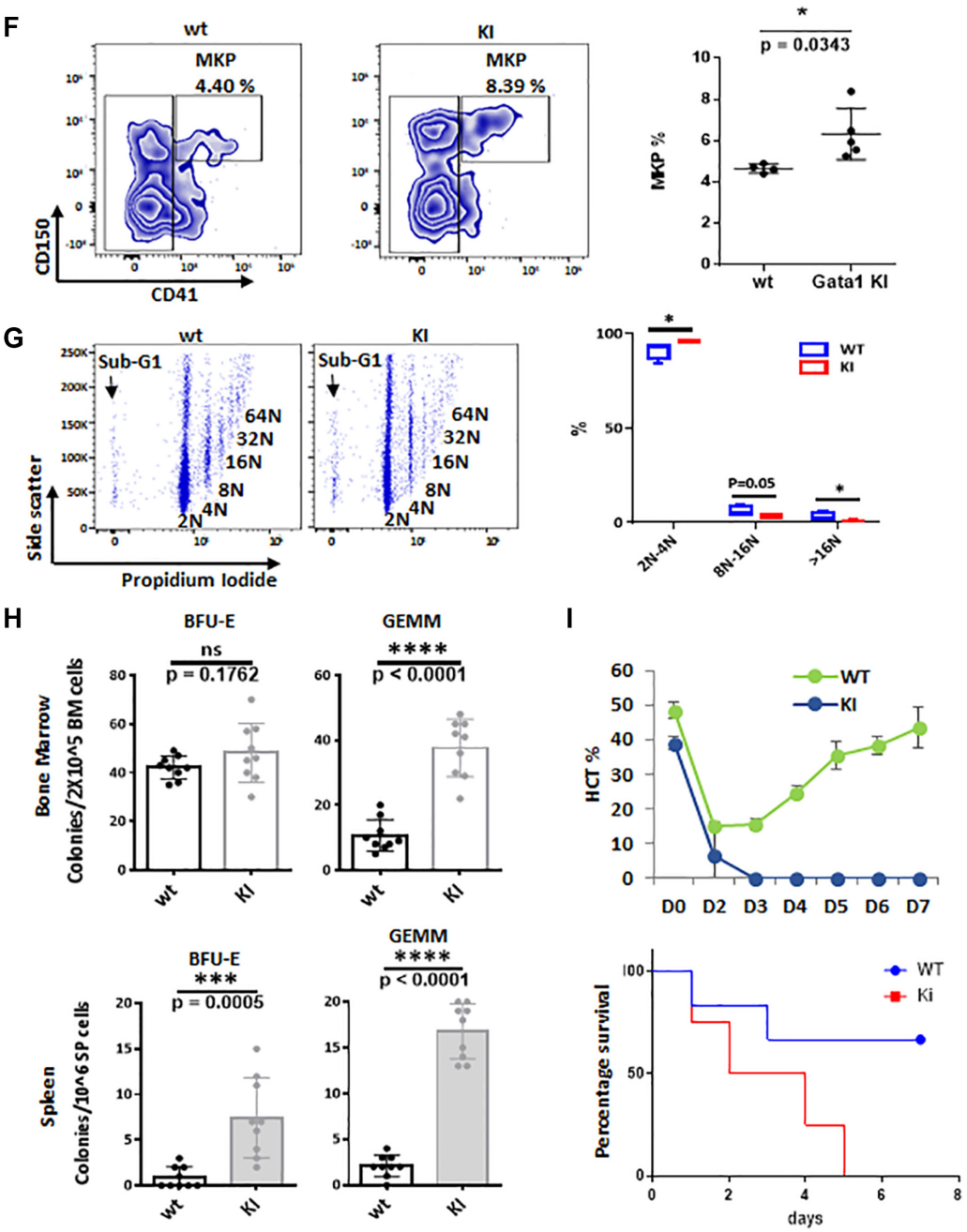

Figure 5. Disruption of GATA-1/HDAC1 interaction leads to hematologic defect in knock in mice, and affects erythropoietic stress response. (**A**) Schematic diagram of the targeting strategy for GATA1^{2RA} knock-in allele. The arginine residue at aa 243 and 247 of GATA-1 is converted to alanine and FRT neo cassette for positive selection was inserted between exon IV and exon V. The targeting vector was linearized and then transfected by electroporation of HF4 (129/SvEv × C57Bl/6) (Hybrid) embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones. The Neo cassette in targeting vector has been removed during ES clone expansion. (**B**) Targeting vector was transfected by electroporation of HF4 (129/SvEv x C57Bl/6) (Hybrid) embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded. Positive clones were further confirmed by Southern Blotting analysis using an internal probe. Clones 4, 5, 6, 7 and 8 were confirmed as correctly targeted. (**C**) Screening for hemizygous male mice. Primer set as indicated was used to screen mice for the deletion of the Neo cassette. The PCR product for the wild-type is 933 bp. After Neo deletion, one FRT site remains (85 bp). A second band with a size of 1018 indicates Neo deletion. (**D**) Hematocrit of peripheral blood from Gata1 WT and 2RA mutant KI mice. (**E**) FACS analysis of bone marrow, spleen and fetal liver cells from the Gata1 WT and 2RA KI mice (left). The relative number in each region as a percentage of gated cells is indicated and summarized in the right panels. (**F**) Mouse bone marrow cells were first gated for Lin$^-$ckit$^+$scal$^-$ population. Then CD41$^+$CD150$^+$ population was gated as MKP. Percentages of MKP cell population were summarized in the right panel. (**G**) Mouse spleen cells were stain with CD41 antibody and PI. CD41 positive cells were first gated then plot for PI and side scatter. Percentages of cell population were summarized in the right panel. $n = 5$ (**H**) Colony forming assays performed with bone marrow (top) and spleen (bottom) cells. (**I**) Top: Hematocrit in the blood of wild-type (WT) and 2RA knock in (KI) mice during recovery from PHZ-induced (35 mg/kg) anemia. One representative experiment is shown. Results are shown as mean ± SD; $n = 2$. Bottom: Survival curve of WT and KI mice following single injection of 35 mg/kg PHZ. $n = 6$ for each group.

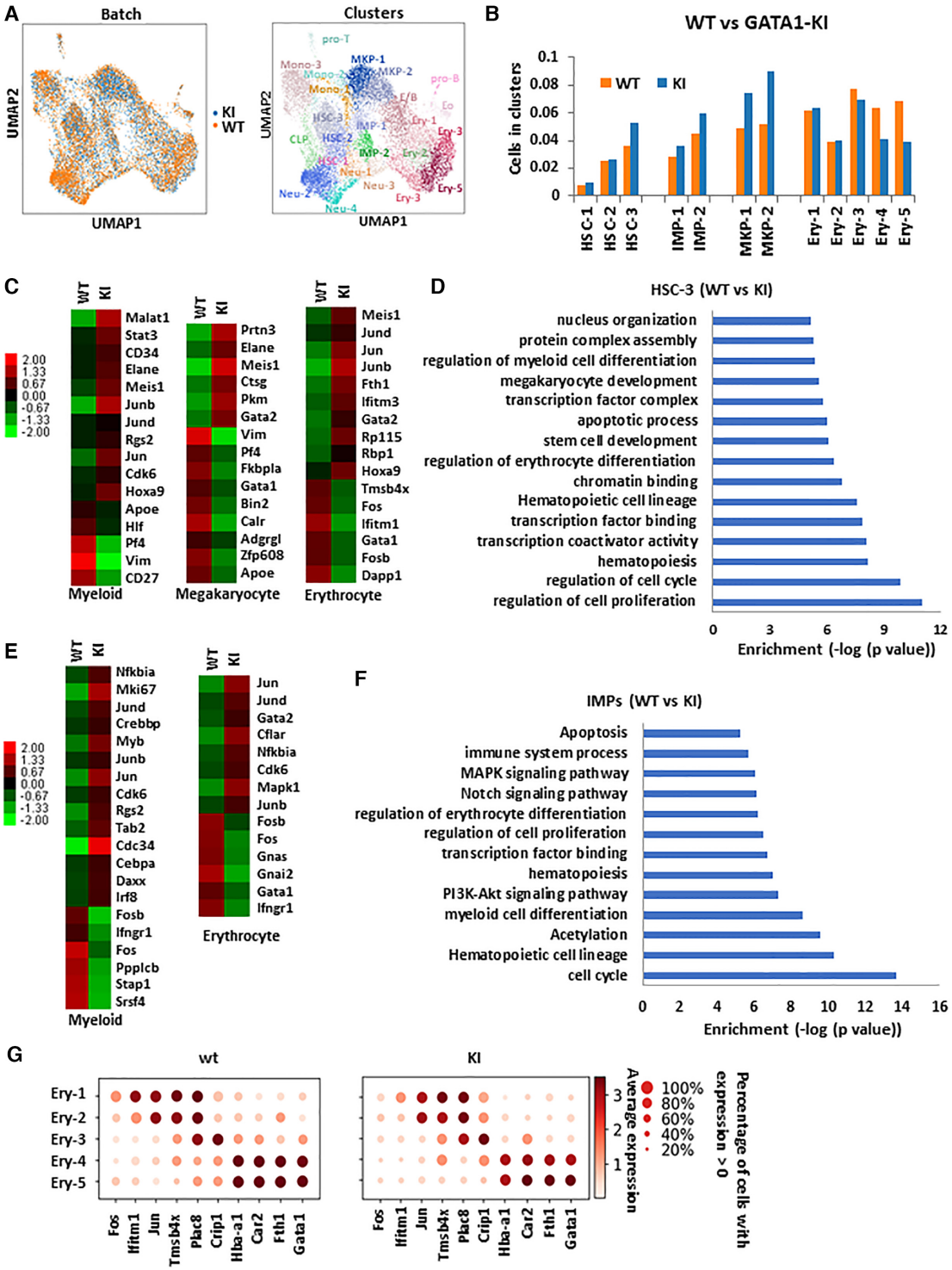

Figure 6. Single-cell RNA-sequencing data integration of batches and clustering to analyze WT and Gata1-KI LK cells. (**A**) Uniform Manifold Approximation and Projection (UMAP) two-dimensional visualization of WT and KI cells isolated from Bone marrow LK cells with the integration of batches analysis. Different colors represent 24 clusters (cell types) defined by the *k*-means clustering algorithm. IMP, immature myeloid progenitor; Mono, monocyte progenitor; Neu, neutrophil/granulocyte progenitor; E/B, erythroid/basophil progenitor; Ery, erythroid progenitor; MkP, megakaryocyte progenitor; CLP, common lymphoid progenitor; Eo, eosinophil progenitor; pro-B, B-cell progenitor; pro-T, T-cell progenitor. (**B**) Relative cell percentage in clusters in WT and KI samples. (**C**) Hierarchical clustering heatmap of differential expression genes in HSC-3 cluster comparing WT and GATA1-KI cells. (**D**) Top GO terms enrichment in HSC-3 cluster comparing WT and GATA1-KI cells. (**E**) Hierarchical clustering heatmap of differential expression genes in IMP cluster comparing WT and GATA1-KI cells. (**F**) Top GO terms enrichment in IMP cluster comparing WT and GATA1-KI cells. (**G**) Top differentially expressed genes (FDR < 0.05, logistic regression with Bonferroni correction) and GATA-1 gene expression when comparing each cell cluster with the remaining clusters corresponding to Ery-pro clusters in WT and KI cells. The dot size encodes the fraction of cells within the cluster that show detectable expression of the gene (UMIs > 0), while the color encodes the average expression level across all cells within a cluster.

cific cell clusters, including the HSC-3 cluster and the immature myeloid progenitor (IMP) clusters IMP1 and IMP2 (Figure 6B). The increase of these populations may reflect a compensatory mechanism for the reduction of mature erythrocytes and megakaryocytes (Figure 6B).

The gene expression heatmap shows that the HSC-3 cluster exhibits myeloid bias as it expresses signature genes for myeloid progenitors, megakaryocytic progenitors and erythroid progenitors, in addition to HSC signature genes (44) (Figure 6C, Supplemental Figure S6A). The HSC-3 associated myeloid biased gene signatures were reduced in GATA-1^{2RA} cells, while the HSC signature and HSC-3 cluster frequency were markedly enhanced and expanded (Figure 6B, C Supplemental Figure S6A). GO term analysis of differentially expressed genes in HSC-3, consistently showed the down regulation of myeloid cell differentiation and erythrocyte/megakaryocyte differentiation (Figure 6D). It has been reported that GATA-1 is expressed in some populations of HSC and directs transcription priming of myeloid biased differentiation (44,45). Our data support the notion that GATA-1 is expressed in the myeloid biased HSC-3 cluster and further showed that GATA-1^{2RA} inhibited the myeloid potential of the HSC-3 cluster by derepressing GATA-2 (Figure 6C). This is consistent with a change in chromatin binding and coactivator recruitment (Figure 4B and D). The GATA-1^{2RA} derived HSC-3 cluster also shows altered transcription profiles of genes associated with the cell cycle, apoptosis and cell survival (Supplemental Figure S6B). Accordingly, GATA-1^{2RA}, enhanced and expanded IMP signatures and frequencies, at the same time inhibiting erythroid signatures and potential (Figure 6E). GO term analysis on differentially expressed genes in the IMP1 and IMP2 clusters, supported the notion that the GATA-1 2RA mutation results in inhibition of myeloid and erythroid differentiation (Figure 6F).

Frequencies of megakaryocyte progenitor cells (MKP1, MKP2) are also increased in GATA-1 2RA KI mice with accompanying changes in the expression levels of signature genes (Figure 6B, Supplemental Figure S6C), again consistent with a compensatory effect observed in FACS analysis (Figure 5D). The frequencies of erythroid clusters 4 and 5 (Ery4, Ery5) were decreased in GATA-1^{2RA} mice (Figure 6B). These two clusters represent more mature erythrocytes as they both express high level of alpha-globin (Hba-a1) and GATA-1 (Figure 6G). The GATA-1 2RA mutation reduced Hba-a1 gene and Gata-1 expression, indicating an inhibition of terminal erythroid differentiation (Figure 6G). Taken together, our data demonstrate that the GATA-1 deacetylation by HDAC1 regulates specific HS/PC populations, erythroid and megakaryocytic differentiation programs by controlling GATA-1 transcription activity and GATA-1 mediated change of chromatin landscape (Figure 7).

DISCUSSION

In this study, we found that although the NuRD complex contains active HDAC1/2, it does not deacetylate GATA-1. However, HDAC1/2 on their own can efficiently deacetylate GATA-1. Further study led to the identification of a direct interaction between GATA-1 and HDAC1 mediated through a novel interacting site at the linker region between the two zinc fingers of GATA-1. Our study shows that the interaction between GATA-1 and HDAC1 is required for the deacetylation of GATA-1 and for GATA-1 mediated erythroid/megakaryocytic differentiation. Introduction of a mutation that cripples the GATA-1/HDAC1 direct interaction (2RA mutant), results in GATA-1 constitutive acetylation and impairing erythroid differentiation in G1E cells, by blocking both GATA-1 activated and repressed genes. GATA-1 acetylation has been previously studied and, although it is generally considered that it promotes chromatin binding and activation (2,18–20), GATA-1 acetylation was also shown to positively target GATA-1 for degradation and subsequent suppression of GATA-1 transcriptional activity (33). In this study, 2RA mutation results constitutive acetylation and the protein in deed is less stable compare to the wild type protein. Consistent with previous reported *(19),* GATA-1 2RA mutant protein binds to DNA similarly to the wild type protein *in vitro.* However, chromatin binding is significantly reduced on a genome-wide scale. These defects may either be due to the reduction of GATA-1 protein level, constitutive acetylation of GATA-1, or due to loss of interaction with HDAC1. Although our preliminary studies show that there is no cofactor associated with HDAC1 at this site, uncovering the detailed mechanism underlying contribution of GATA-1 acetylation status to its chromatin binding and transcriptional regulation in hematopoiesis, warrants further investigation. Furthermore, it remains to be determined whether the FOG-1 interacting surface in the N-terminal GATA1 zinc finger and the HDAC1 interacting site cooperate to regulate GATA-1 mediated transcription in hematopoiesis.

The ChIP-seq data shows that HDAC1 is recruited to active promoters by GATA-1 and the level of HDAC1 on promoters are positively correlated to high levels of histone acetylation. Therefore, it is conceivable that HDAC1 may not play a role in histone deacetylation at active promoters but, instead, it may deacetylate GATA-1 or other protein substrates at the promoter region. This data is consistent with an earlier report showing that highly active genes associate with high levels of HATs and HDACs, including HDAC1 (46), as well as high levels of histone acetylation at promoter regions (47). Since HDAC1 and HAT can dynamically compete for the binding to histone H3 tail (48), we speculate that HDAC1 at active gene promoters may be important for balancing HAT activity in order to maintain gene activation at a desired magnitude (46). In addition, HAT can attenuate HDAC1 activity by acetylating HDAC1. Our earlier studies showed that HDAC1 is gradually acetylated during erythropoiesis and that the acetylated NuRD complex positively regulates GATA-1 function (15). Whether HDAC1 itself is acetylated when it directly binds to the GATA-1 linker site to subsequently modulate GATA-1 activity, remains to be determined. In conjunction with this point, it remain to be established whether GATA-1 acetylation/deacetylation is regulated during erythropoiesis, however our present data suggests it does.

The GATA-1^{2RA} knock-in mice suffered from mild anemia and thrombocytopenia, a phenotype also seen in FOG-1 KI mice in which the FOG-11/NuRD interaction was disrupted, suggesting multiple layers of regulation of GATA-1 function (14,49,50). The bone marrow and spleen of GATA-1^{2RA} mice showed an accumulation of immature

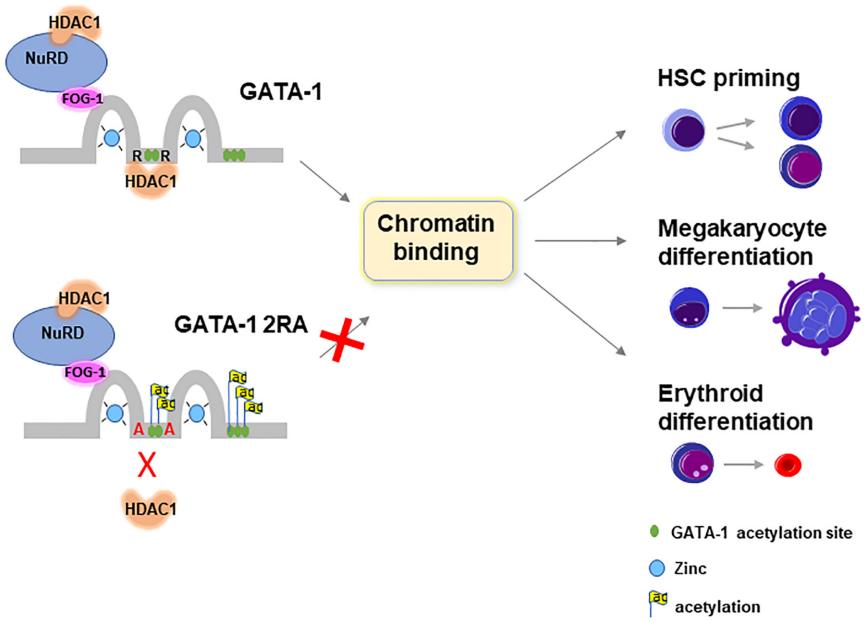

Figure 7. Graphic illustration of function of HDAC1 in regulating GATA-1 function. HDAC1 and GATA-1 direct interaction at the linker region mediates GATA-1 deacetylation. The mutant of GATA-1 that is defective in HDAC1 binding is constitutively acetylated and resulting reduction of chromatin binding at GATA-1 target genes and blockage of GATA-1 mediated gene transcription. In development, loss of HDAC1 binding results in reduction of myeloid biased HS/PC, defective megakaryocyte differentiation and erythroid differentiation.

erythrocytes and megakaryocytes, whereas the single cell RNA-seq analysis from mice bone marrow LK cells revealed profound changes in HSC, myeloid progenitor and erythroid/megakaryocyte lineage cell populations and accompanying signature gene expression. GATA-1 gene expression is reduced in all of these populations. These observations suggest that bone marrow progenitors in GATA-1^{2RA} mice fail to give rise to sufficient numbers of mature erythrocytes and megakaryocytes. Therefore, the GATA-1 /HDAC1 interaction is essential for GATA-1 deacetylation and hematopoiesis. Given that the FOG-1 complex also contains HDAC1, it is possible that FOG-1- dependent and independent HDAC1s coordinate GATA-1 acetylation and chromatin acetylation status in the GATA-1 target genes in hematopoiesis.

It has been reported that GATA-1 is expressed in HS/PCs (45). However, the role of GATA-1 in HS/PC development remain elusive. Our study demonstrates that GATA-1 specifically regulates the transcription state of a subpopulation of HS/PCs that exhibit myeloid bias. This cluster of HS/PCs may represent short term HSCs, expressing signature genes for myeloid progenitors, erythroid progenitors and megakaryocyte progenitors. The expression of GATA-1^{2RA} resulted in increased GATA-2 expression which, in turn, may result in cells remaining in a stemness state, with reduced expression of myeloid/erythroid biased signature genes in HS/PCs. Thus, the role of GATA-1 in HS/PC function warrants further investigation.

DATA AVAILABILITY

RNA-seq data generated in this study are available at NCBI GEO database with the accession number GSE161607. The scRNA-seq dataset has been deposited to GEO (accession number GSE161729). The ChIP-seq dataset has been deposited to GEO (accession number GSE161608).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Author contributions: B.Y. performed most of the experiments; J.Y. and N.C. performed some experiments on knock-in mice, M.K. and T.Y. performed some of the experiments; H.L. contributed to analysis of ChIP-seq and scRNA-seq database; and J.Z., J.S., R.H, S.H. and Y.Q. conceived the project, designed experiments, interpreted results and wrote the manuscript.

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Conflict of interest statement. None declared.

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