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Epigenetic Determinants of Erythropoiesis: Role of the Histone Methyltransferase SetD8 in Promoting Erythroid Cell Maturation and Survival

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Erythropoiesis, in which committed progenitor cells generate millions of erythrocytes daily, involves dramatic changes in the chromatin structure and transcriptome of erythroblasts, prior to their enucleation. While the involvement of the master-regulatory transcription factors GATA binding protein 1 (GATA-1) and GATA-2 in this process is established, the mechanistic contributions of many chromatin-modifying/remodeling enzymes in red cell biology remain enigmatic. We demonstrated that SetD8, a histone methyltransferase that catalyzes monomethylation of histone H4 at lysine 20 (H4K20me1), is a context-dependent GATA-1 corepressor in erythroid cells. To determine whether SetD8 controls erythroid maturation and/or function, we used a small hairpin RNA (shRNA)-based loss-of-function strategy in a primary murine erythroblast culture system. In this system, SetD8 promoted erythroblast maturation and survival, and this did not involve upregulation of the established regulator of erythroblast survival Bcl-x_L. SetD8 catalyzed H4K20me1 at a critical *Gata2 cis* element and restricted occupancy by an enhancer of *Gata2* transcription, Scl/TAL1, thereby repressing *Gata2* transcription. Elevating GATA-2 levels in erythroid precursors yielded a maturation block comparable to that induced by SetD8 downregulation. As lowering GATA-2 expression in the context of SetD8 knockdown did not rescue erythroid maturation, we propose that SetD8 regulation of erythroid maturation involves multiple target genes. These results establish SetD8 as a determinant of erythroid cell maturation and provide a framework for understanding how a broadly expressed histone-modifying enzyme mediates cell-type-specific GATA factor function.

The capacity of stem and progenitor cells to generate multiple cell lineages is orchestrated by cell-type-specific transcription factors that instigate lineage-specific genetic networks. These factors function with a cadre of broadly expressed transcription factors and coregulators, including chromatin-remodeling and -modifying enzymes. Cell-type-specific factors endow broadly expressed factors with activities important for establishing and/or maintaining the specialized transcriptome. Despite this paradigm, the functions of many broadly expressed chromatin-remodeling and -modifying enzymes have not been investigated in cell type-specific contexts. Considering the feasibility of devising small-molecule strategies to target enzymes, it is instructive to identify enzymatic components mediating important biological processes. We have been addressing this problem by asking how GATA factors with specialized expression patterns and functions utilize broadly expressed coregulators to mediate cellular transitions required for development of hematopoietic stem cells (HSCs), progenitors, and differentiated progeny, including the erythrocyte.

The family of dual zinc finger GATA transcription factors (1) recognize DNA with a WGATAR consensus (2, 3). GATA-2 is expressed predominantly in hematopoietic stem/progenitor cells (HSPCs), mast cells, endothelial cells, and neurons (4–8). Through its actions to induce HSC generation (9, 10) and to regulate HSPC function (11–13), GATA-2 mediates multilineage hematopoiesis. Mutations that alter the *GATA2* coding region (14–16) or an essential *cis* element 9.5 kb downstream of the *Gata2* 1S promoter (+9.5 site) (17, 18) cause a primary immunodeficiency syndrome (MonoMAC) commonly associated with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). The +9.5 site enhances *Gata2* transcription and induces HSC generation from hemogenic endothelium in the aorta gonad mesonephros (AGM) region of the developing embryo (9). LIM domain binding protein 1 (LDB1) and the chromatin remodeler Brahma related gene 1 (BRG1) confer activation through the +9.5 site (19). GATA-2 occupancy at this site in the transcriptionally active human and murine *Gata2* loci suggests positive autoregulation (20–22).

GATA-1 is expressed predominantly in erythroid cells, megakaryocytes, mast cells, and eosinophils (6, 23–25) and is essential for controlling the development of these cells (26–29). GATA-1 utilizes its cofactor Friend of GATA-1 (FOG-1) to activate and repress most target genes, including *Gata2* (30, 31). Some GATA-1 target genes have little or no FOG-1 requirement for regulation (31, 32). Since GATA-2 is expressed in multipotent hematopoietic precursors, its chromatin occupancy commonly precedes that of GATA-1. As GATA-1 levels rise during erythropoiesis, GATA-1 displaces GATA-2 from chromatin sites (29). These "GATA switches" occur at numerous sites in the genome, including 5 sites at the *Gata2* locus, and are often associated with

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altered transcriptional output (21, 33–36). GATA-1/FOG-1 recruit the histone acetyltransferase CBP/P300 (37) and the nucleosome-remodeling and deacetylase (NuRD) complex (38–40), and we demonstrated that the chromatin-modifying enzyme SetD8 (PR-Set7) is a context-dependent GATA-1 corepressor at select GATA-1 target genes (41).

SetD8 is the sole enzyme known to monomethylate histone H4 at lysine 20 (H4K20me1) (42). Targeted disruption of murine *Setd8* is embryonic lethal between the 4- and 8-cell stages (43). SetD8 levels are regulated during the cell cycle, and its degradation is required for cell cycle progression (44, 45). While the precise biochemical consequences of H4K20me1 are not established, this histone mark has been reported to correlate with activation and repression. H4K20me1 localizes to inactive heterochromatic regions of *Drosophila* polytene chromosomes (42). H4K20me1 can promote chromatin compaction directly, as well as through subsequent di- and trimethylation (43, 46). Loss of H4K20me1 from H4K20me1-encriched genes increases transcription (47). In support of SetD8 and H4K20me1 involvement in transcriptional activation, the genomic H4K20me1 profile in human T lymphocytes and $CD36^+$ erythroid precursor cells correlates with transcriptional activity (48–50). We analyzed endogenous SetD8 function in a genetic complementation assay in GATA-1-null erythroid precursor cells (G1E-ER-GATA-1) (41). In this system, ER-GATA-1 induces a physiologically relevant window of erythroid maturation over a 2-day time course (51, 52). The G1E-ER-GATA-1 studies provided evidence that SetD8 confers repression of a subset of GATA-1-repressed target genes, and SetD8 almost exclusively mediates repression (41). We also demonstrated that H4K20me1 levels increased across a broad region of repressed, but not activated, loci, which provided evidence for H4K20me1 as a repressive chromatin mark in erythroid cells (41). These results led to the hypothesis that SetD8 controls erythroid cell maturation or function physiologically. Using an *ex vivo* murine fetal liver erythroblast culture system, we establish SetD8 as a positive regulator of primary erythroid cell maturation and survival and developed mechanistic insights of broader relevance to SetD8 function in diverse contexts.

MATERIALS AND METHODS

Cell culture. Primary fetal liver erythroid progenitor cells were maintained at a density of 2.5×10^5 cells/ml in StemPro-34 (Gibco) supplemented with 10% nutrient supplement (Gibco), 2 mM L-glutamine (Cellgro), 1% penicillin-streptomycin (Cellgro), 100 μM monothioglycerol (Sigma), 1 μM dexamethasone (Sigma), 0.5 U/ml of erythropoietin, and 1% conditioned medium from a Kit ligand-producing CHO cell line for expansion. For differentiation, cells were cultured at a density of 1×10^6 cells/ml in embryonic stem cell Iscove's modified Dulbecco medium (ES IMDM; glutamine-free; HyClone) containing 10% fetal bovine serum (FBS; Gemini), 10% plasma-derived serum (PDS; Animal Technologies), 5% protein-free hybridoma medium II (PFHM II; Gibco), 2 mM L-glutamine (Cellgro), 1% penicillin-streptomycin (Cellgro), 100 μM monothioglycerol (Sigma), and 5 U/ml of erythropoietin. Cells were grown in a humidified incubator at 37°C with 5% carbon dioxide. All percentages are vol/vol unless otherwise noted. G1E-ER-GATA-1 cells were cultured in IMDM (Gibco) containing 15% FBS (Gemini), 1% penicillin-streptomycin (Gemini), 2 U/ml erythropoietin, 120 nM monothioglycerol (Sigma), 0.6% conditioned medium from a Kit ligand-producing CHO cell line, and 1 μg/ml puromycin (Gemini). Estrogen receptor (ER)–GATA-1 activity was induced by treating cells with 1 μM β-estradiol (Steraloids, Inc.).

Quantitative real-time RT-PCR. Total RNA was purified from TRIzol per manufacturer instructions (Invitrogen). cDNA was prepared by annealing 300 ng of RNA with 250 ng of a 1:5 mixture of random hexamer and oligo(dT) primers heated at 68°C for 10 min. This was followed by incubation with murine Moloney leukemia virus reverse transcriptase (RT; Invitrogen) combined with 10 mM dithiothreitol (DTT), 20 U RNasin (Promega), and 0.5 mM deoxynucleoside triphosphates (dNTPs) at 42°C for 1 h. The mixture was heat inactivated at 95°C for 5 min and diluted to a final volume of 50 μl. RT-PCR mixtures (20 μl) contained 1 μl of cDNA, appropriate primers, and 10 μl of Power SYBR green master mix (Applied Biosystems). Product accumulation was monitored by SYBR green fluorescence using either a StepOnePlus or a Viia7 instrument (Applied Biosystems). A standard curve of serial dilutions of cDNA samples was used to determine relative expression. mRNA levels were normalized to 18S rRNA.

ChIP assay. Quantitative chromatin immunoprecipitation (ChIP) was conducted as described previously (21, 53) using antibodies specific for monomethylated H4K20 (Millipore), GATA-1 (54), Scl/TAL1 (55), LDB1 (N18, sc11198; Santa Cruz Biotechnology), and acetylated H4 (Upstate). Samples were analyzed by quantitative real-time PCR using either a StepOnePlus or a Viia7 instrument (Applied Biosystems), and product was quantitated by SYBR green fluorescence. The amount of product was determined relative to a standard curve generated from a serial dilution of input chromatin. Dissociation curves revealed that primer pairs generated single products.

Primary fetal liver erythroid progenitor cell isolation. Primary erythroid precursor cells were isolated from embryonic day 14.5 (E14.5) fetal livers using the EasySep negative-selection mouse hematopoietic progenitor cell enrichment kit (StemCell Technologies). Briefly, fetal liver cells were resuspended at a concentration of 5×10^7 cells/ml in phosphate-buffered saline (PBS) containing 2% FBS, 2.5 mM EDTA, and 10 mM glucose. The EasySep mouse hematopoietic progenitor cell enrichment cocktail was added at 50 μl/ml, supplemented with 2.5 μg/ml biotin-conjugated CD71 antibody (eBioscience) for removal of proerythroblasts. After 15 min of incubation on ice, cells were washed once by centrifugation for 5 min at 1,200 rpm at 4°C. Cells were resuspended at a concentration of 5×10^7 cells/ml in PBS containing 2% FBS, 2.5 mM EDTA, and 10 mM glucose, and EasySep biotin selection cocktail was added at 100 μl/ml. After 15 min at 4°C, EasySep mouse progenitor magnetic microparticles were added at 60 μl/ml. After 10 min at 4°C, cells were resuspended to a total volume of 2.5 ml and incubated with a magnet for 3 min. Unbound progenitor cells were carefully transferred into a 15-ml tube and used for subsequent experiments.

Retroviral infection. The microRNA 30 (miR-30) context *Setd8* and *Gata2* shRNAs were cloned into MSCV-PIG vector, kindly provided by Mitchell Weiss, using BglII and XhoI restriction sites. Retroviruses expressing shRNA targeting luciferase (control), *SetD8*, and *Gata2*, containing a *Gata2* cDNA (56) or an empty vector, were produced by transfecting 3×10^6 293T cells with 15 μg of both MSCV-PIG vector and pCL-ECO viral packaging vector. Retrovirus-containing supernatant was harvested 24 or 48 h posttransfection. Primary erythroid precursor cells were spinfected with 100 μl of retrovirus supernatant and 8 μg/ml Polybrene in 400 μl of fetal liver expansion medium at 1,200 × *g* for 90 min at 30°C. After centrifugation, 500 μl prewarmed fetal liver expansion medium was added, and cells were incubated at 37°C overnight.

The miR-30 context *SetD8* shRNA sequence is TGCTGTTGACAGTGAGCGCAAGCACTGTTCTCCTGCTCAATAGTGAAGCCACAGATGTATTGAGCAGGAGAACAGTGCTTTTGCCTACTGCCTCGGA; the miR 30 context *Gata2* shRNA sequence is TGCTGTTGACAGTGAGCGCAAGGAGTAGGCAAGAAGAAAATAGTGAAGCCACAGATGTATTTTCTTCTTGCCTACTCCTTTTGCCTACTGCCTCGGA.

Erythroid maturation assay and fluorescence-activated cell sorting (FACS). Cells were washed with PBS once, and 1×10^7 cells were stained with 0.8 μg of anti-mouse Ter119–APC and anti-mouse CD71–PE (eBioscience) at 4°C for 30 min in the dark. After staining, cells were washed

three times with 2% bovine serum albumin (BSA) in PBS. Samples were analyzed using a FACSAria II cell sorter (BD Bioscience). Cells were gated on green fluorescent protein (GFP) to ensure retroviral expression. DAPI (4′,6-diamidino-2-phenylindole) exclusion was utilized for live/dead discrimination. Cells in the R1, R2, R3, and R4/5 populations were sorted into 5-ml round-bottom tubes and immediately processed for RNA isolation and cytospin or were cross-linked for subsequent ChIP experiments.

Apoptosis analysis. One million cells were washed with PBS, followed by a wash with 1× annexin binding buffer (Life Technologies). Cells were resuspended in 100 μl annexin binding buffer and labeled with 5 μl annexin V-Alexa Fluor 350 conjugate (Life Technologies) and propidium iodide (PI). Samples were analyzed using an LSRFortessa or LSRII cytometer (BD Biosciences). As an additional method of analyzing apoptosis, fetal liver cells were stained for active caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP). Two million cells were washed with PBS, fixed in 1% paraformaldehyde, and stored overnight in 70% ethanol at −20°C. Following permeabilization in 0.25% Triton X-100 (Sigma), cells were stained with anti-active caspase-3 phycoerythrin (PE)-conjugated antibody (BD Pharmingen number 51-68655X) and anti-cleaved PARP allophycocyanin (APC)-conjugated antibody (BD Pharmingen clone F21-852). Fluorescence was monitored using a FACSAria or LSRII cytometer (BD Biosciences).

Proliferation analysis. Cell proliferation was measured using the CellTrace Violet cell proliferation kit per manufacturer instructions (Life Technologies). One million cells were labeled with 10 μM CellTrace Violet dye for 30 min, washed with prewarmed media, and cultured for 24 h. After 24 h, CellTrace Violet fluorescence intensity was measured using an LSRII cytometer (BD Bioscience). To determine the fluorescence intensity of the first cellular generation, a control sample was labeled with 10 μM CellTrace Violet 30 min prior to sample analysis. The percentage of cells in each daughter generation was determined using ModFit LT software (Verity Software House).

Protein analysis. Protein samples were isolated by centrifugation of 1×10^6 cells from each condition, washed with cold PBS, and lysed in 1× SDS sample buffer (25 mM Tris, pH 6.8, 2% β-mercaptoethanol, 3% SDS, 0.005% bromophenol blue, 5% glycerol). Samples were boiled for 15 min and stored at −80°C. Samples were resolved by SDS-PAGE, and proteins were detected by semiquantitative Western blotting with ECL 2 Western blotting substrate (Thermo Scientific). The antibodies used were anti-SetD8 (07-316; Millipore), anti-α-tubulin (clone DM1A, 05-829; Millipore), and anti-β-actin (3700S; Cell Signaling Technology). Secondary antibodies included goat anti-mouse IgG–horseradish peroxidase (HRP) and goat anti-rabbit IgG–HRP (sc-2005 and sc-2004; Santa Cruz Biotechnology).

Statistical analysis. Student's *t* tests were conducted using GraphPad software or Microsoft Excel.

Primers. The following primers were used for quantitative RT-PCR (5′ to 3′): 18S rRNA, CGCCGCTAGAGGTGAAATTCT and CGAACCTCCGACTTTCGTTCT; SetD8 mRNA, TAGCTGGAATCTACAGGAAGCGA and GTGTCTTTGCTCTTCTGTTCATCTG; Bcl2l1 mRNA, GACAAGGAG ATGCAGGTATTGG and TCCCGTAGAGATCCACAAAAGT; Bax mRNA, TGAAGACAGGGGCCTTTTTG and AATTCGCCGGAGACACTCG; Tal1 mRNA, CGAGCGCTGCTCTATAGCCTT and TCACCCGGTTGTTGTT GGT; Vim mRNA, CGAAAGCACCCTGCAGTCAT and AAGGTCAAGAC GTGCCAGAGA; Gata2 mRNA (1), GCAGAGAAGCAAGGCTCGC and CAGTTGACACACTCCCGGC; Gata2 mRNA (2), GGCTCTACCACAAGA TGAATGGA and AGGTGGTGGTTGTCGTCTGAC; Gata1 mRNA, GGCC CAAGAAGCGAATGATT and GGTTCACCTGATGGAGCTTGA; Fog1 mRNA, CCTTGCTACCGCAGTCATCA and ACCAGATCCCGCAGTCT TTG; Klf1 mRNA, CACGCACACGGGAGAGAAG and CGTCAGTTCGTC TGAGCGAG; Pu.1 mRNA, GGCAGCGATGGAGAAAGC and GGACATG GTGTGCGGAGAA; Kit mRNA, AGCAATGGCCTCACGAGTTCTA and CCAGGAAAAGTTTGGCAGGAT; Lyl1 mRNA, AAGCGCAGACCAAGC CATAG and AGCGCTCACGGCTGTTG; MyoD promoter, GGGTAGAGG ACAGCCGGTGT and GTACAATGACAAAGGTTCTGTGGGT; Eif3k promoter, GTGATTTCCTTCCAGCAGTTGTAA and CTCACGCTATTGGTC TCTTTTAAGTG; Gata2 +9.5 Site −933 bp, CTTGCTGCTGGCTCTGA GAAC and AGTCCAGGGTCTTTTAAGGATAAATTC; Gata2 +9.5 Site −480 bp, AACCTTCAAATGCAGACACTTCAC and GAATCCGCCAGAA CGAAGAC; Gata2 +9.5 Site, GACATCTGCAGCCGGTAGATAAG and CAT TATTTGCAGAGTGGAGGGTATTAG; Gata2 +9.5 Site +446 bp, GCCGAG GGAGTTCAGTGCTA and AGCGCTACTCCTGTGTGTTCTTC; Gata2 +9.5 Site + 880 bp, TCCTGGCGACTCCTAGATCCTA and GAAAGCCC TGAGGAAGTTGGA; Gata2 −77 Site, CTTTACCACATCAGGATACAG AGCA and CACCGCACAGCAGTGATAGATAGT; Gata2 −22 Site, GCTT TATCAGGCCACAGCTTG and GCACAGTCCTGGCAAAGTTCTC; Gata2 −3.9 Site, GAGATGAGCTAATCCCGCTGTA and AAGGCTGTATTTTTCC AGGCC; Gata2 −2.8 Site, GCCCTGTACAACCCCATTCTC and TTGTTCCC GGCGAAGATAAT; Gata2 −1.8 Site, GCATGGCCCTGGTAATAGCA and CA GCCGCACCTTCCCTAA; Gata2 1S Promoter, CCCCTCGAAGTGATGT CGAA and TCTGGCTGTCTCTCGGTTCC; Gata2 1G Promoter, AGATACC CAGAAGGTGCACGTC and GCAGACCCTGCACCCCT; β-Globin HS2, AG GGTGTGTGGCCAGATGTT and ACCCAGATAGCACTGATCACTCAC; Lyl1 Exon 1, TCAGCATTGCTTCTTATCAGCC and CGCAGAGGCCAGAG GATG; and Kit −114 kb, GCACACAGGACCTGACTCCA and GTTCTGAGA TGCGGTTGCTG.

RESULTS

SetD8 promotes a developmental transition required for primary erythroid cell maturation. SetD8 is a context-dependent GATA-1 corepressor at a subset of GATA-1 target genes (41). To evaluate SetD8 functions during erythroid maturation, we conducted an shRNA-based loss-of-function analysis in primary murine fetal liver hematopoietic precursors. Freshly isolated lineage-depleted hematopoietic precursors from E14.5 fetal livers were infected with retrovirus expressing shRNA targeting *Setd8*. After 72 h of culture in medium supporting erythroid precursor cell expansion, we quantitatively analyzed erythroid maturation using flow cytometry with the surface markers CD71 and Ter119 (Fig. 1A). Downregulating *Setd8* mRNA by 70 to 80% (Fig. 1A), which strongly lowered SetD8 protein levels (Fig. 1B), significantly reduced cells in the R3 population (early and late basophilic erythroblasts) (2.5-fold decrease, $P = 0.00001$) and increased the R2 population (proerythroblasts) (1.6-fold increase, $P = 0.0004$) (Fig. 1C). In addition, downregulating SetD8 significantly increased cells in the R1 (CFU-E and BFU-E) and the very low abundance R4 (orthochromatic erythroblasts) and R5 (reticulocytes and erythrocytes) populations (Fig. 1C). In cells cultured under conditions promoting differentiation, SetD8 knockdown induced 4.3-fold ($P = 0.0005$) and 2.4-fold ($P = 0.002$) increases in R1 and R2 populations, respectively, while reducing R3 population cells 1.8-fold ($P = 0.00005$) (Fig. 1C). The accumulation of R2 cells, concomitant with reduced R3 cells, suggests that SetD8 promotes the developmental transition of the immature R2 erythroblast to a more mature R3 erythroblast.

Wright-Giemsa staining of flow-sorted, live R2 cells cultured under expansion conditions indicated that downregulating SetD8 induced profound membrane blebbing (16% of cells, a 160-fold increase over control), which is often an attribute of apoptosis (Fig. 1D). This result suggested that SetD8 might suppress apoptosis in maturing erythroid precursor cells. We tested whether SetD8 downregulation induces apoptosis by staining SetD8 knockdown and control cells with annexin V and propidium iodide (PI) and quantitating the percentage of apoptotic cells using flow cytometry. SetD8 knockdown increased the percentage of early apoptotic cells (PI negative, annexin V positive) 6.8-fold and

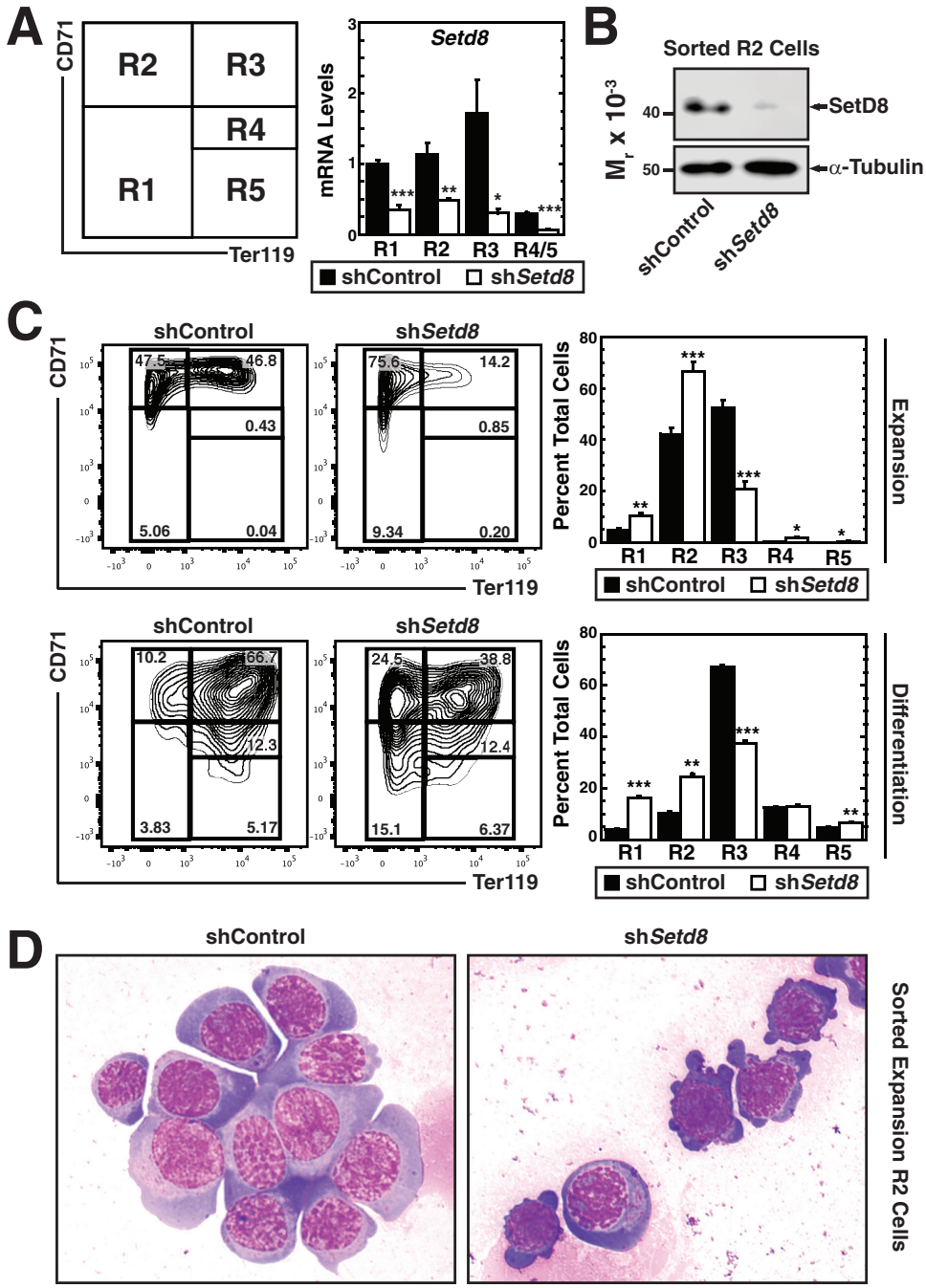


FIG 1 SetD8 promotes maturation of primary erythroid precursor cells. (A) (Left) Diagram depicting R1 through R5 erythroid precursor populations based on Ter119 and CD71 surface expression. (Right) Quantitative real-time RT-PCR analysis of *Setd8* mRNA in FACS-sorted R1, R2, R3, and R4/5 cells ($n = 6$, values are means ± standard errors [SE]). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Analysis of SetD8 protein levels by Western blotting (image representative of 3 independent experiments). (C) Representative plots from flow cytometric analysis of erythroid maturation based on expression of the surface markers CD71 and Ter119 after 3 days of culture in medium promoting progenitor expansion (top) or differentiation (bottom). The average percentage of total cells in the R1, R2, R3, R4, and R5 populations after treatment with control or *Setd8* shRNA is depicted on the right ($n = 6$ for expansion, $n = 3$ for differentiation; values are means ± SE). Asterisks are as defined for panel A. (D) Representative images from Wright-Giemsa-stained flow-sorted R2 cells treated with *Setd8* or control shRNA.

increased late apoptotic cells 4-fold under expansion culture conditions (Fig. 2A). Flow cytometric quantitation of apoptosis using the apoptotic markers active caspase-3 and cleaved PARP indicated that SetD8 knockdown caused a 2.7-fold ($P = 0.0005$) increase in active caspase-3/cleaved PARP double-positive cells (Fig. 2B).

An established erythroblast mechanism to counteract proapoptotic signals involves GATA-1-mediated induction of the antiapoptotic factor Bcl-x_L encoded by *Bcl2l1* (57). In addition, our prior analysis revealed SetD8 repression of the gene encoding the proapoptotic factor Bax in G1E-ER-GATA-1 cells (41). We tested whether SetD8 downregulation deleteriously impacted erythro-

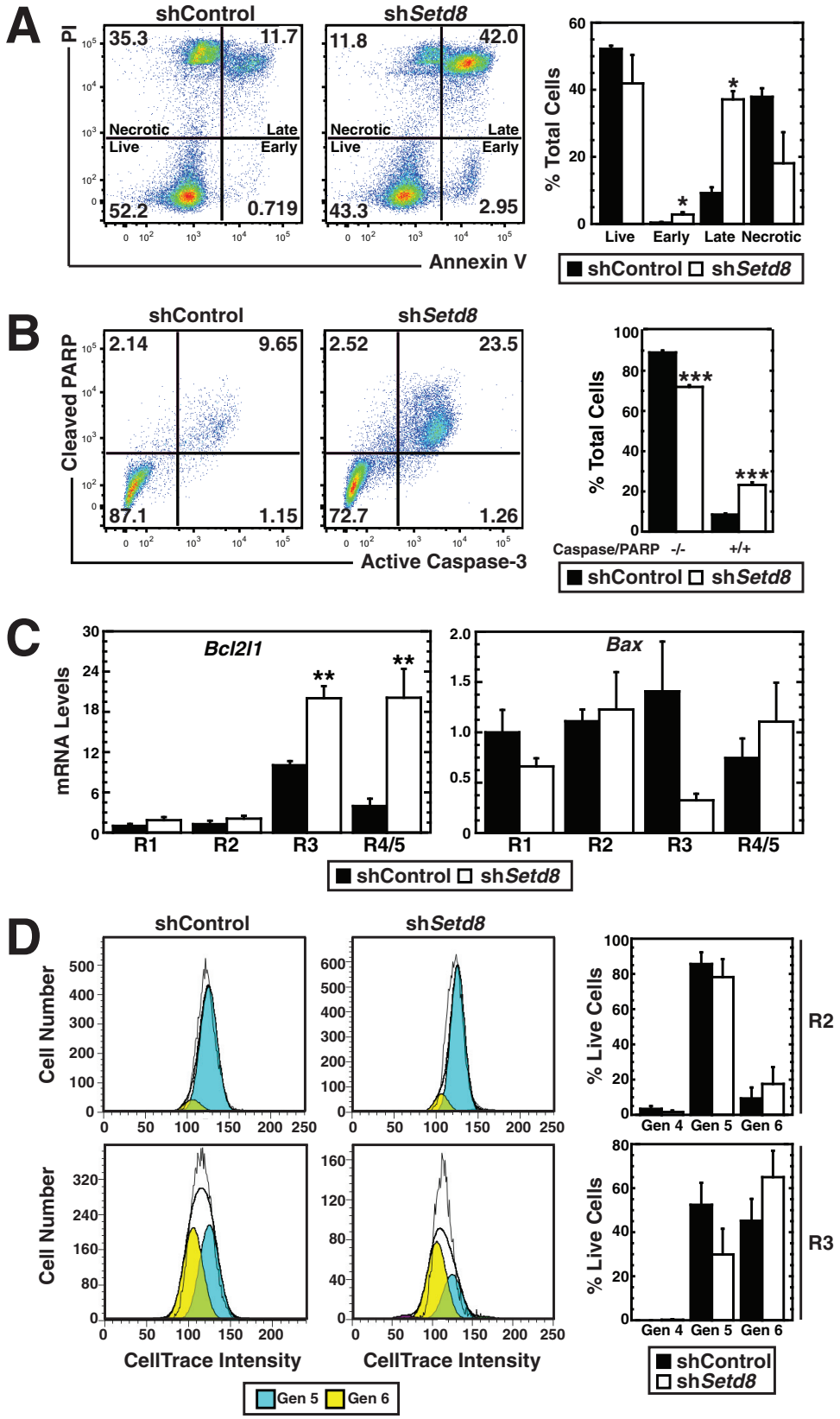


FIG 2 SetD8 confers erythroblast survival. (A) Representative plots from flow cytometric analysis of apoptosis using annexin V and propidium iodide staining in cells treated with *Setd8* or control shRNA under expansion culture conditions. The average percentage of cells in the live, early apoptotic, late apoptotic, and necrotic populations are displayed on the right ($n = 3$; values are means ± SE). (B) Representative plots from flow cytometric quantitation of apoptosis using anti-active caspase-3 and anti-cleaved PARP antibodies. Averages of active caspase-3/cleaved PARP double-negative and double-positive populations are displayed on the right ($n = 3$; values are means ± SE). (C) Quantitative RT-PCR analysis of *Bcl2l1* and *Bax* mRNA in sorted R1, R2, R3, and R4/5 cells treated with control or *Setd8* shRNA ($n = 6$; values are means ± SE). (D) Representative plots from cellular proliferation analysis of proerythroblasts (R2 population, top) and basophilic erythroblasts (R3 population, bottom) using CellTrace Violet dye. The average percentage of total cells in each daughter generation is depicted on the right ($n = 3$; values are means ± SE). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

blast survival by decreasing *Bcl2l1* and/or increasing *Bax* expression in any population. Real-time RT-PCR analysis indicated that SetD8 downregulation increased *Bcl2l1* mRNA 2-fold ($P = 0.005$) and 5-fold ($P = 0.006$) in R3 and R4/R5 cells, respectively, without changing *Bax* expression (Fig. 2C). Thus, reducing SetD8 levels did not induce erythroblast apoptosis via downregulating *Bcl2l1* or upregulating expression of the proapoptotic gene *Bax*.

SetD8 knockdown cells accumulated considerably slower in culture than in cells treated with control shRNA. We tested whether SetD8 inhibits cell proliferation by staining cells with a membrane-intercalating dye and monitoring cell divisions by flow cytometry. After 24 h, the vast majority of cells expressing control shRNA underwent 5 or 6 cell divisions. SetD8 knockdown had little to no effect on the percentage of cells that underwent 6 cell divisions in both the R2 and R3 populations (Fig. 2D). These results suggest that SetD8 confers fetal liver erythroblast survival by counteracting apoptosis, rather than exerting a major influence on proliferation of viable cells.

SetD8-mediated *Gata2* repression in immature erythroblasts. To dissect the molecular mechanism governing the SetD8-dependent R2-to-R3 transition, we tested whether lowering SetD8 levels altered the expression of key erythroid transcription factors and coregulators. To ensure that any potential alterations in gene expression do not reflect changes in cellularity in the hematopoietic precursor cell population, we isolated R1, R2, R3, and R4/5 cell populations by FACS and quantitated gene expression in these populations. While the SetD8 knockdown did not change *Gata1*, *Fog1*, *Klf1*, *Tal1*, and *Pu.1* expression, *Gata2* mRNA increased 2- to 3-fold (primer set 1; $P = 0.0002$) selectively in the R2 population (Fig. 3A). A second primer set targeting a distinct *Gata2* exon-exon junction yielded an identical result; SetD8 downregulation induced *Gata2* mRNA in the R2 population 2.4-fold (primer set 2; $P = 0.0007$). Western blot analysis of FACS-purified R2 cells revealed that reducing endogenous SetD8 protein upregulated GATA-2 protein levels (Fig. 3B). GATA-2 promotes HSC generation and function and regulates hematopoietic progenitor survival and proliferation (9–12). As GATA-2 overexpression in wild-type (WT) hematopoietic precursors inhibits hematopoiesis (58) and GATA switching is considered to be a driver of erythroid maturation (33, 59), SetD8-mediated repression of *Gata2* transcription may be an important determinant of the transition from an immature erythroid precursor cell to an erythroblast destined to undergo enucleation to yield a reticulocyte and subsequently an erythrocyte.

In the R2 population, SetD8 knockdown elevated *Vim* mRNA 2-fold ($P = 0.03$) (Fig. 3A). *Vim* encodes the intermediate filament vimentin, which is strongly repressed upon primary erythroid cell maturation. Previously, we demonstrated that GATA-1 utilizes SetD8 to repress *Vim* expression (41). SetD8-mediated *Vim* repression may have important functional consequences for this developmental transition, as it has been proposed that downregulating vimentin serves a permissive function for erythroid maturation (60, 61). SetD8 knockdown induced a small, but significant, increase in *Kit* mRNA levels in the R2 population, as well as a significant decrease in *Lyl1* mRNA in the R5 population (Fig. 3A). As *Kit* is an established direct GATA-2 target gene (62), the increased *Kit* expression in R2 cells upon SetD8 knockdown may reflect increased GATA-2 levels/activity.

Previously, we reported that *Gata2* mRNA expression was not regulated by SetD8 in G1E-ER-GATA-1 cells under conditions in which ER–GATA-1 was inactive and also under conditions in which ER–GATA-1 was conditionally activated for 24 h. However, our finding that SetD8 represses *Gata2* mRNA in R2 cells but not R1 or R3 cells suggested that SetD8 may be required for initiation but not maintenance of *Gata2* repression. To test this, we infected G1E-ER-GATA-1 cells with control or *Setd8*-specific shRNA and quantitated *Gata2* primary transcripts and mRNA at several time points after β-estradiol-mediated ER–GATA-1 activation. SetD8 knockdown significantly elevated *Gata2* primary transcripts at 0, 1, 2, 4, and 6 h of β-estradiol treatment. However, by 12 and 24 h, *Gata2* primary transcripts were reduced to a level indistinguishable from that of the control (Fig. 3C). Whereas *Gata2* mRNA levels were significantly elevated by SetD8 knockdown at 6 and 12 h after β-estradiol treatment, at 24 h, *Gata2* mRNA was repressed to the same extent as in the control (Fig. 3C). These results support a mechanism whereby SetD8 is required for the initiation but not maintenance of GATA-1-mediated *Gata2* repression and demonstrate that SetD8 regulates *Gata2* in primary cells and in the G1E-ER-GATA-1 genetic complementation assay.

To test whether elevated *Gata2* expression contributed to the maturation blockade resulting from SetD8 downregulation, we infected primary fetal liver erythroid precursor cells with a GATA-2-expressing retrovirus. After 72 h of culture under conditions that promote erythroid precursor expansion, GATA-2 expression induced a 1.7-fold increase in both R1 ($P = 0.007$) and R2 ($P = 0.008$) cells and a 1.5-fold ($P = 0.003$) decrease in R3 cells (Fig. 4A). GATA-2 overexpression was confirmed by Western blotting (Fig. 4B). We tested whether the GATA-2-mediated maturation blockade was associated with changes in the expression of other erythroid transcription factors. GATA-2 expression induced little to no change in *Gata1*, *Klf1*, *Fog1*, and *Pu.1* expression in FACS-purified R1 to R4/5 populations (Fig. 4C). Thus, elevating GATA-2 expression in primary erythroid precursors induced a maturation barricade comparable to that resulting from endogenous SetD8 downregulation.

To test whether reduced GATA-2 is sufficient to rescue the SetD8 knockdown phenotype, we performed a SetD8/GATA-2 double knockdown in fetal liver-derived erythroid precursor cells. Following culture under expansion conditions for 72 h, erythroid maturation was quantitated by flow cytometry. SetD8 knockdown significantly reduced the percentage of cells in R3 and increased the percentage of cells in R2 (Fig. 4D). GATA-2 knockdown induced a profound increase in late-stage erythroblasts; R4 and R5 cells increased 12- and 52-fold, respectively. Thus, endogenous GATA-2 expression strongly blocks maturation. However, other than a slight, but significant, decrease in R1 cells, the SetD8/GATA-2 double knockdown resulted in no significant differences in the percentages of cells in the R2, R3, R4, or R5 populations compared to those in the SetD8 single-knockdown condition (Fig. 4D). Western blot analysis confirmed an efficient knockdown of GATA-2 protein in both single- and double-knockdown conditions (Fig. 4D). Thus, while SetD8 represses *Gata2* mRNA and GATA-2 protein in primary R2 cells, lowering GATA-2 is insufficient to rescue the maturation phenotype in SetD8 knockdown cells.

Dissecting the mechanism underlying SetD8-mediated erythroid maturation: H4K20me1 accumulation at a GATA switch site restricts Scl/TAL1 occupancy. *cis* elements mediating endogenous *Gata2* expression have been established (63). The +9.5 intronic E-box-spacer-GATA composite element strongly enhances

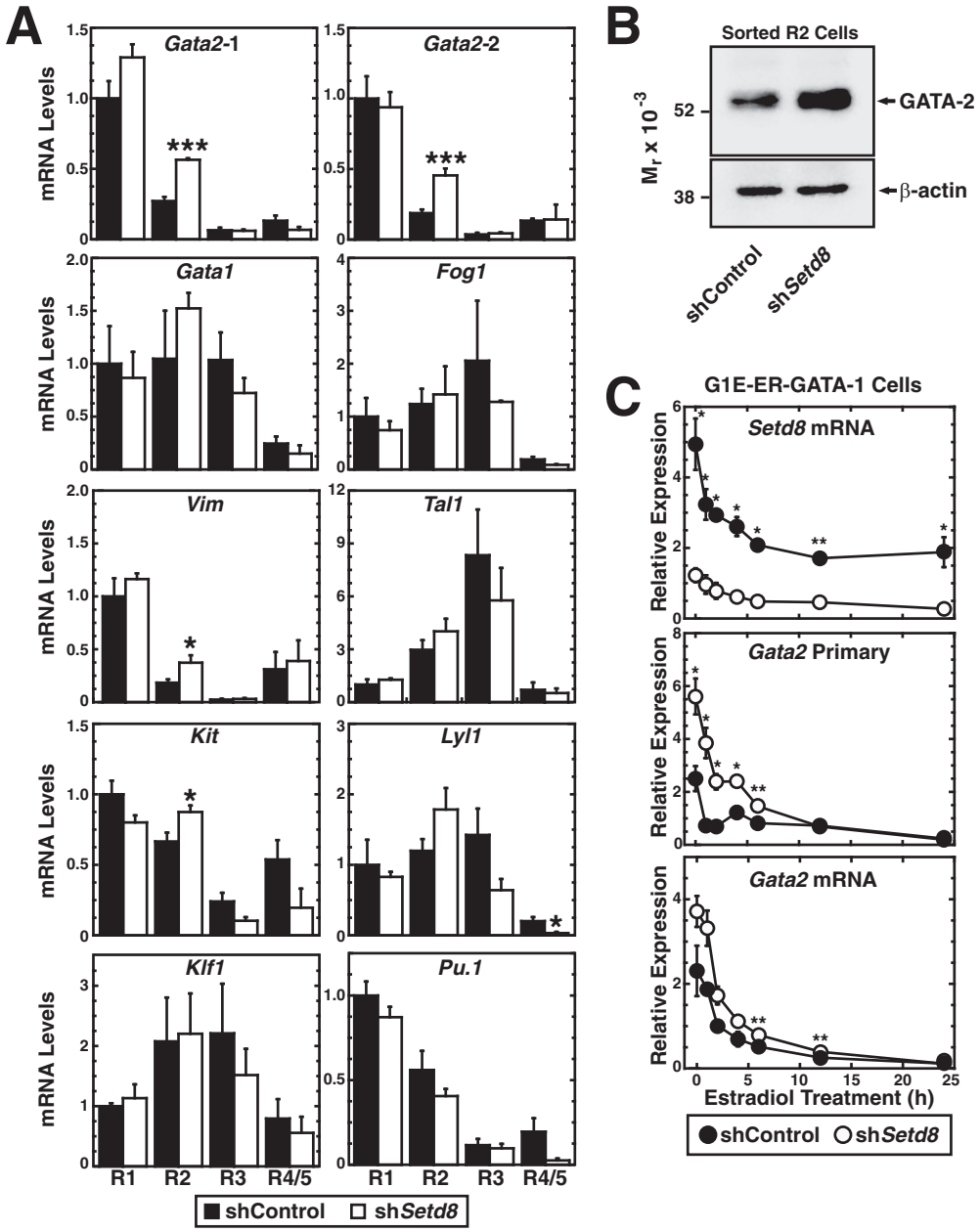


FIG 3 SetD8 represses *Gata2* in the proerythroblast population. (A) Real-time RT-PCR analysis of *Gata2* (2 primer sets targeting distinct exon-exon junctions), *Gata1*, *Fog1*, *Klf1*, *Pu.1*, *Vim*, *Tal1*, *Kit*, and *Lyl1* mRNA in FACS-sorted R1, R2, R3, and R4/5 cell populations ($n = 6$; values are means ± SE). (B) Analysis of GATA-2 protein level in FACS-sorted R2 cells by Western blotting (image representative of 3 independent experiments). (C) Time course of *Setd8* mRNA, *Gata2* primary transcript, and *Gata2* mRNA expression levels following estradiol treatment in G1E-ER-GATA-1 cells ($n = 3$; values are means ± SE). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

endogenous *Gata2* expression in hemogenic endothelial cells of the mouse AGM and in fetal liver HSPCs (9, 17). HSC generation from AGM hemogenic endothelium and the establishment of the fetal liver HSPC compartment are both +9.5 site dependent (9, 17). Furthermore, similar fetal liver results were obtained from a novel conditional *Gata2* knockout strategy using a +9.5 site-containing sequence driving Cre recombinase expression (64). Accordingly, the +9.5 site is essential for embryogenesis, with $+9.5^{-/-}$ embryo lethality occurring at ~E13.5. In contrast, the −1.8 and −2.8 GATA switch sites modestly upregulate *Gata2* expression in HSPCs (65, 66), while the −3.9 site has no apparent role (19); these sites are not critical determinants of hematopoiesis or embryogenesis, at least not in the steady state in the mouse. A leukemogenic chromosomal inversion extracts the −77 GATA switch site (22) from the *GATA2* locus and relocalizes it ~40 megabases away to the promoter of the *EVI1* protooncogene, thereby upregulating *EVI1* expression (67, 68).

We tested whether SetD8 catalyzes H4K20me1 at the essential +9.5 GATA switch site. Whereas the biochemical consequences of H4K20me1 are unresolved, given our demonstration that SetD8

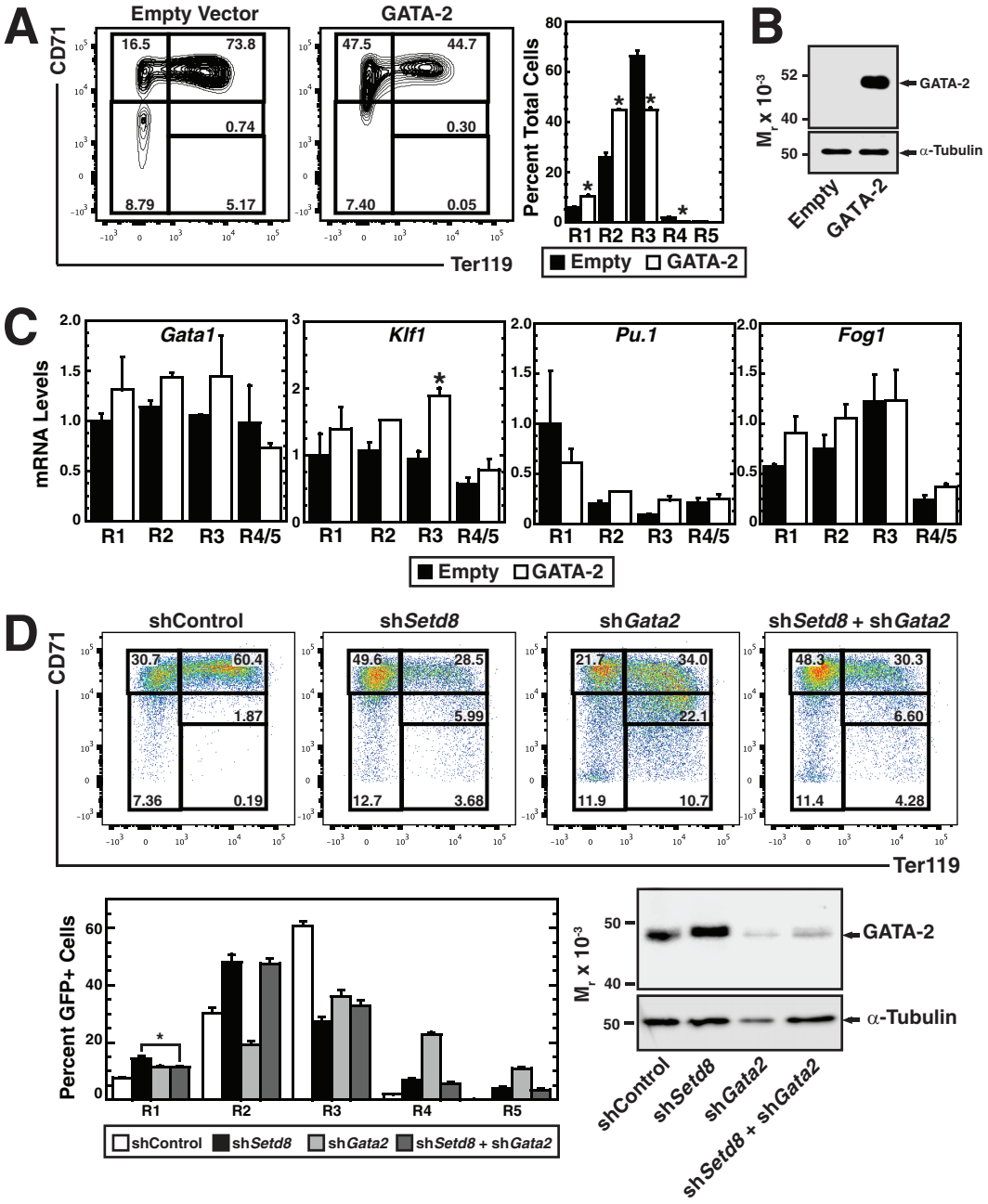
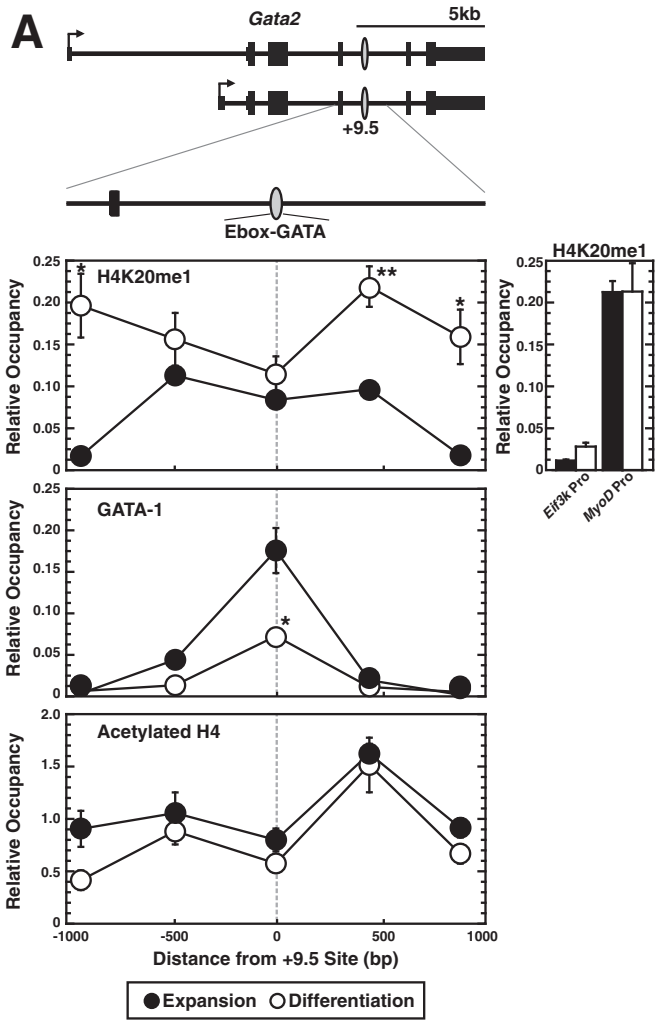


FIG 4 GATA-2 expression inhibits the proerythroblast to basophilic erythroblast transition. (A) Representative plots from flow cytometric analysis of erythroid maturation, using the surface markers Ter119 and CD71, in cells infected with GATA-2-expressing retrovirus or empty vector control. Population averages are displayed on the right ($n = 3$; values are means ± SE). (B) Western blot analysis of GATA-2 protein levels (image representative of 3 independent experiments). (C) Quantitative RT-PCR analysis of *Gata1*, *Klf1*, *Pu.1*, and *Fog1* mRNA levels in sorted erythroid precursor populations ($n = 3$; values are means ± SE). (D) (Top) Representative plots of flow cytometric quantification of erythroid maturation using cell surface markers CD71 and Ter119. Averages for R1 through R5 populations are depicted (bottom left; $n = 3$; values are means ± SE), and all results for experimental conditions in each population are significantly different from those for the control sample in the same population. The SE comparison displayed on the graph compares the SetD8 single-knockdown condition to the SetD8/GATA-2 double-knockdown condition. (Bottom right) Western blot analysis of GATA-2 protein levels (image representative of 3 independent experiments). *, $P < 0.05$.

functions predominantly as a repressor in erythroid cells, SetD8-catalyzed H4K20me1 might establish repressive chromatin at the GATA switch site, precluding regulatory complex assembly and/or destabilizing the complex. To determine whether SetD8 directly represses *Gata2*, we conducted quantitative chromatin immunoprecipitation (ChIP) analysis for H4K20me1 in primary fetal liver cells under expansion conditions (primarily R2 cells) and differentiation conditions (primarily R3 cells), in which *Gata2* is active and repressed, respectively. Using primers that tile the +9.5 site, we detected a localized region of elevated H4K20me1 within 1 kb (upstream or downstream) from the +9.5 site coinciding with GATA-1 occupancy under expansion condi-



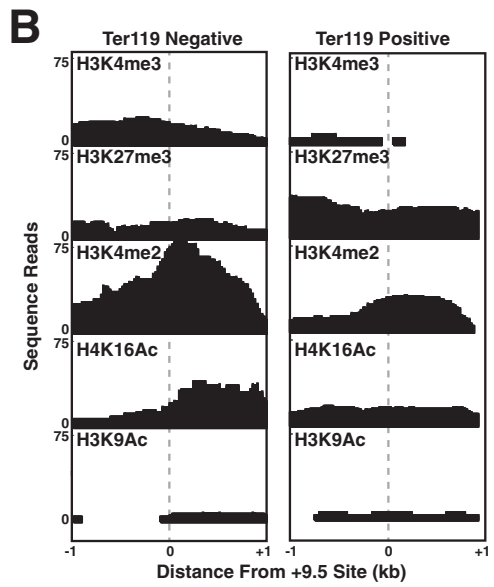


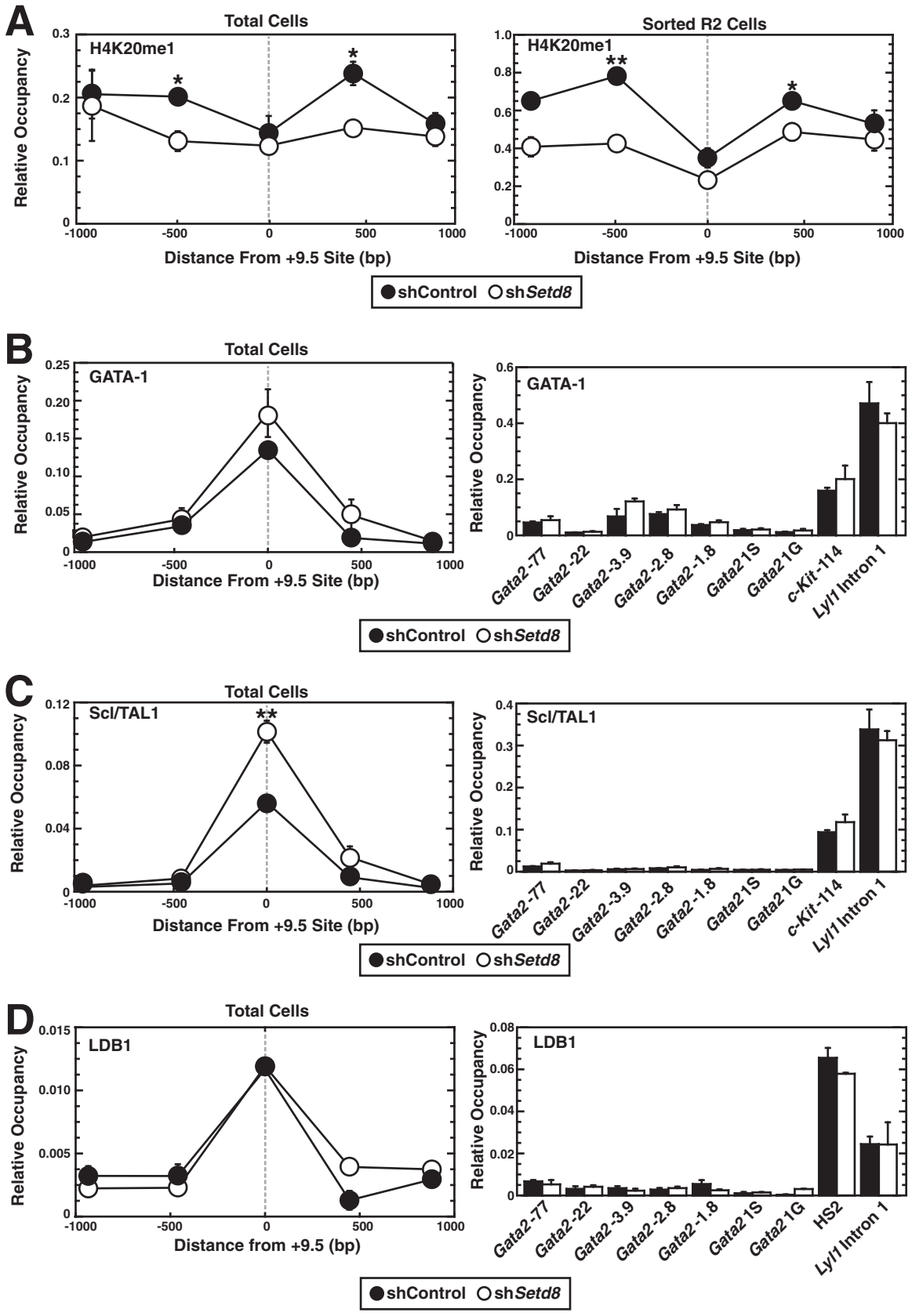
FIG 5 H4K20me1 is induced at the +9.5 site upon repression of *Gata2*. (A) (Top) Schematic diagram depicting the *Gata2* locus and highlighting the critical GATA switch site 9.5 kb downstream of the *Gata2* 1S transcription start site. (Bottom) H4K20me1, GATA-1, and acetylated H4 chromatin occupancy measured by quantitative ChIP at the +9.5 site in expanding and differentiating fetal liver progenitor cells. H4K20me1 levels at the repressed *MyoD* promoter and at the active *Eif3k* promoter serve as controls ($n = 3$; values are means ± SE). *, $P < 0.05$; **, $P < 0.01$. (B) ChIP-seq profiles of H3K4me3, H3K27me3, H3K4me2, H4K16ac, and H3K9ac in Ter119-negative and Ter119-positive cells at the +9.5 site.

tions (Fig. 5A). Attempts to measure SetD8 occupancy with anti-SetD8 antibodies did not yield convincing results, and therefore H4K20me1 was used as a surrogate for SetD8 function; SetD8 is the only enzyme known to catalyze this mark. In differentiating erythroid cells in which *Gata2* is repressed, H4K20me1 was more broadly enriched, consistent with a spreading mechanism. H4K20me1 levels at the +9.5 site in differentiating cells were comparable to H4K20me1 levels at the repressed muscle-specific *MyoD* promoter. Consistent with active transcription, H4K20me1 levels at sites 1 kb upstream or downstream of the +9.5 site in expansion culture were comparable to that at the constitutively active *Eif3k* promoter. Importantly, acetylated histone H4 levels did not change under these conditions, suggesting that increased H4K20me1 did not reflect nucleosome repositioning or deposition of new nucleosomes (Fig. 5A). This result reiterates our prior data in the G1E-ER-GATA-1 cell system, in which repression of direct GATA-1/SetD8 target genes involved broad H4K20me1 spreading (41).

To gain a deeper perspective of the chromatin landscape of the +9.5 site region of the endogenous *Gata2* locus, we mined chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) data in Ter119-negative (R1 and R2) and Ter119-positive (R3, R4, and R5) cells (69) to determine the distribution of histone modifications at the +9.5 site, which infer active transcription or repressive chromatin. Under these conditions, *Gata2* expression was repressed 11.3-fold in R3 cells compared to R2 cells as measured by RNA sequencing (RNA-seq) (69). Compared to the +9.5 site in Ter119-negative cells, the +9.5 site in Ter119-positive cells exhibited increased H3K27me3, consistent with the transcriptional repression. The histone modifications commonly associated with transcriptional activity, i.e., H3K4me3, H3K4me2, and H4K16ac, decreased in Ter119-positive cells, also consistent with a repressive state (Fig. 5B).

To determine if SetD8 catalyzed H4K20me1 at *Gata2*, we knocked down SetD8 in primary fetal liver erythroid precursor cells and quantitated H4K20me1 levels. In the mixed population of erythroid precursor cells, downregulating SetD8 reduced H4K20me1 at sites 480 bp upstream ($P = 0.027$) and 446 bp downstream ($P = 0.041$) from the +9.5 GATA binding site. In addition, in R2 cells isolated by FACS, SetD8 downregulation significantly reduced H4K20me1 at sites 480 bp upstream ($P = 0.001$) and 446 bp downstream ($P = 0.027$) from the +9.5 composite element (Fig. 6A). These data support a direct mechanism of regulation in which SetD8 establishes and/or maintains H4K20me1 at the +9.5 site.

GATA-1 directly represses *Gata2* transcription, and the hematopoietic-specific basic helix-loop-helix transcription factor Scl/TAL1 positively regulates *Gata2* through the +9.5 site. Previously, we demonstrated that Scl/TAL1 chromatin occupancy is reduced at the +9.5 site during GATA-1-mediated repression of *Gata2* (19). At the genomic level, loss of Scl/TAL1 chromatin occupancy correlates with transcriptional repression (70–72). In addition, we


A
Total Cells
H4K20me1
Relative Occupancy
Distance From +9.5 Site (bp)
Sorted R2 Cells
H4K20me1
Distance From +9.5 Site (bp)
shControl shSetd8
B
Total Cells
GATA-1
Relative Occupancy
Distance From +9.5 Site (bp)
GATA-1
Gata2-77 Gata2-22 Gata2-3.9 Gata2-2.8 Gata2-1.8 Gata21S Gata21G c-Kit-114 Ly/1 Intron 1
shControl shSetd8
C
Total Cells
Scl/TAL1
Relative Occupancy
Distance From +9.5 Site (bp)
Scl/TAL1
Gata2-77 Gata2-22 Gata2-3.9 Gata2-2.8 Gata2-1.8 Gata21S Gata21G c-Kit-114 Ly/1 Intron 1
shControl shSetd8
D
Total Cells
LDB1
Relative Occupancy
Distance from +9.5 (bp)
LDB1
Gata2-77 Gata2-22 Gata2-3.9 Gata2-2.8 Gata2-1.8 Gata21S Gata21G HS2 Ly/1 Intron 1
shControl shSetd8

demonstrated that the LIM domain protein LDB1 occupies the +9.5 site and that reactivation of the repressed *Gata2* locus in induced G1E-ER-GATA-1 cells upon removal of β-estradiol from the culture medium requires LDB1 (19). Since SetD8 induces H4K20me1 at the +9.5 site, we reasoned that this modification would reconfigure the chromatin environment to be inhospitable to factors interacting with *cis* elements within this region. To determine if the SetD8-mediated histone modification change alters GATA-1, Scl/TAL1, and LDB1 occupancy at *Gata2*, we quantitated GATA-1, Scl/TAL1, and LDB1 occupancy at the +9.5 site in expansion culture cells (primarily R2) infected with control shRNA- or *Setd8* shRNA-expressing retrovirus. While knocking down SetD8 did not alter GATA-1 or LDB1 occupancy (Fig. 6B and D), the knockdown increased Scl/TAL1 occupancy at the +9.5 site 2-fold ($P = 0.0039$) (Fig. 6C). Scl/TAL1 occupancy was unaltered at the GATA-binding site in intron 1 of *Lyl1*, which is unresponsive to SetD8 knockdown (Fig. 6C). In addition, Scl/TAL1 and GATA-1 occupancy were unchanged at the −77, −3.9, −2.8, and −1.8 sites, the 1S and 1G promoters, a negative-control site 22 kb upstream from the *Gata2* 1S promoter, and the −114 kb GATA-binding element at *Kit* (Fig. 6B and C). LDB1 occupancy was unchanged at the −77, −3.9, −2.8, and −1.8 sites, the 1S and 1G promoters, a negative-control site 22 kb upstream from the *Gata2* 1S promoter, DNase I hypersensitive site 2 (HS2) at the β-globin locus control region, and intron 1 of *Lyl1* (Fig. 6D). Thus, SetD8 limits the extent of Scl/TAL 1 occupancy at the +9.5 site.

To determine if SetD8-mediated *Gata2* repression requires the +9.5 site, we infected freshly isolated HSPCs from an E14.5 mouse heterozygous for the +9.5 element with retrovirus expressing control or *Setd8*-specific shRNA and cultured for 72 h under expansion conditions. After isolating RNA from FACS-purified R2 cells, we utilized allele-specific primers to quantitate transcription from the WT and mutant (Mut) +9.5 alleles (Fig. 7A). Primary transcripts from the wild-type *Gata2* allele were significantly upregulated by SetD8 knockdown. However, the mutant allele lacking the +9.5 element was insensitive to the SetD8 reduction (Fig. 7B). A second set of allele-specific primers yielded an identical result; SetD8 knockdown upregulated primary transcripts from the wild-type but not the +9.5 mutant allele. These results establish a requirement of the +9.5 site for SetD8 to repress *Gata2*. Since Scl/TAL1 occupies the *Gata2* locus (55, 73) and upregulates *Gata2* expression (21, 73), the SetD8 restriction of Scl/TAL1 occupancy at the +9.5 site constitutes an attractive mechanism for how this histone methyltransferase represses *Gata2* transcription. Thus, SetD8 controls erythroid maturation by functioning as a corepressor for GATA-1, repressing *Gata2* transcription, and conferring erythroblast survival (Fig. 8).

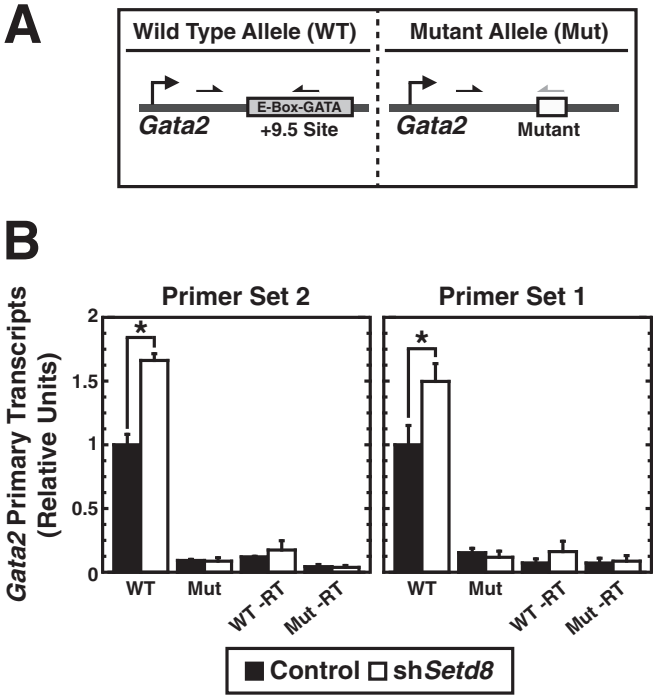


FIG 7 SetD8 requires the +9.5 site to repress *Gata2*. (A) Schematic of the experimental paradigm in which fetal liver erythroid progenitors were isolated from E14.5 mouse embryos heterozygous for the +9.5 site at *Gata2*. Cells were infected with control or *Setd8*-specific shRNA and cultured under expansion conditions for 72 h. GFP^+ R2 cells were isolated by FACS, and *Gata2* primary transcripts were measured using allele-specific PCR. (B) Quantitative RT-PCR analysis of *Gata2* primary transcripts using primers specific for the WT or +9.5 mutant allele. Two different sets of primers were used to confirm results ($n = 3$; values are means ± SE). *, $P < 0.05$.

DISCUSSION

Given the plethora of chromatin-modifying and -remodeling enzymes in the proteome (74, 75), one would assume that these factors are intrinsic regulators of essentially all critical biological processes. However, many of these enzymes have not yet been studied in cell-type-specific and/or physiological contexts. Furthermore, targeted deletions of genes encoding these presumptively critical factors can yield early embryonic lethality that precludes tractable functional analysis. This scenario applies to

FIG 6 SetD8 catalyzes H4K20me1 and restricts Scl/TAL1 occupancy at the +9.5 site. (A) (Left) H4K20me1 chromatin occupancy measured by quantitative ChIP at the +9.5 GATA switch site in total cells treated with control or *Setd8* shRNA ($n = 3$; values are means ± SE). (Right) H4K20me1 chromatin occupancy in FACS-sorted R2 cells treated with control or *Setd8* shRNA ($n = 3$; values are means ± SE). (B) (Left) Quantitative ChIP analysis of GATA-1 at the +9.5 site in cells cultured under expansion conditions and treated with either control or *Setd8* shRNA ($n = 3$; values are means ± SE). (Right) Quantitative ChIP analysis of GATA-1 occupancy at the −77, −3.9, −2.8, and −1.8 GATA switch sites at *Gata2*, the 1S and 1G *Gata2* promoters, a negative-control site 22 kb upstream from the *Gata2* 1S promoter, and the positive-control sites 114 kb upstream from the c-*Kit* promoter and *Lyl1* intron 1. (C) (Left) Quantitative ChIP analysis of Scl/TAL1 at the +9.5 site in cells cultured under expansion conditions and treated with either control or *Setd8* shRNA ($n = 3$; values are means ± SE). (Right) Quantitative ChIP analysis of Scl/TAL1 occupancy at the −77, −3.9, −2.8, and −1.8 GATA switch sites at *Gata2*, the 1S and 1G *Gata2* promoters, a negative-control site 22 kb upstream from the *Gata2* 1S promoter, and the positive-control sites 114 kb upstream from the c-*Kit* promoter and *Lyl1* intron 1 ($n = 3$; values are means ± SE). (D) (Left) Quantitative ChIP analysis of LDB1 at the +9.5 site in cells cultured under expansion conditions and treated with either control or *Setd8* shRNA ($n = 3$; values are means ± SE). (Right) Quantitative ChIP analysis of LDB1 occupancy at the −77, −3.9, −2.8, and −1.8 GATA switch sites at *Gata2*, the 1S and 1G *Gata2* promoters, a negative-control site 22 kb upstream from the *Gata2* 1S promoter, and the positive-control sites DNase I hypersensitive site 2 (HS2) at the β-globin locus control region (LCR) and *Lyl1* intron 1 ($n = 3$; values are means ± SE). *, $P < 0.05$; **, $P < 0.01$.

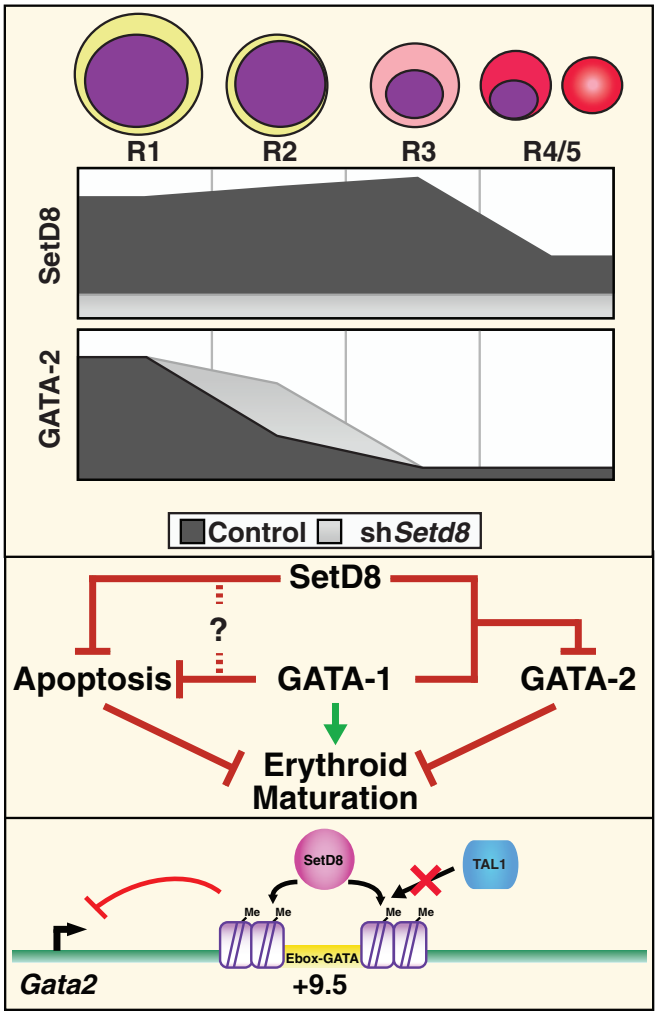


FIG 8 Model depicting SetD8 activity to promote erythroid maturation. SetD8 represses *Gata2* specifically in the immature proerythroblast (R2) population. SetD8 catalyzes H4K20me1 and restricts Scl/TAL1 occupancy at the +9.5 site at *Gata2*. This mechanism limits the capacity of Scl/TAL1 to confer activation through the +9.5 site, which is required for SetD8 to repress *Gata2* transcription. The model assumes that *Gata2* is one of multiple SetD8 targets that collectively contribute to the control of erythroid cell survival and maturation.

SetD8, which has been rigorously studied, especially in model systems such as *Saccharomyces cerevisiae* (76), and its mouse knockout yields embryonic lethality before the 4- and 8-cell stages (43). Unlike histone marks in the epigenome catalyzed by multiple enzymes, intriguingly, H4K20me1 is known to be catalyzed by SetD8 only (76). Based on these attributes, our prior work establishing SetD8 as a context-dependent GATA-1 corepressor (41), and many unanswered questions regarding how committed progenitors progressively mature into erythrocytes (77), it is compelling to ask whether SetD8 has important physiological functions in red cell biology. Given the massive chromatin and nuclear reconfiguration associated with erythroid maturation (77, 78), the erythroid system offers unique potential to elucidate how a histone-modifying enzyme orchestrates and/or negotiates complex nuclear transactions in a specialized, but critical, cell type from a multicellular organism. See the companion article by Malik et al. (79).

Although SetD8 is a context-dependent GATA-1 corepressor, whether SetD8 controls the maturation and/or function of primary erythroid precursor cells was unclear. Here, we demonstrated that SetD8 promotes the maturation and survival of definitive erythroblasts. In our prior G1E-ER-GATA-1 cell analysis, small interfering RNA (siRNA)-mediated SetD8 downregulation did not induce apoptosis (41). While the G1E-ER-GATA-1 system recapitulates a normal window of erythropoiesis, i.e., maturing from proerythroblasts to basophilic erythroblasts (51), these cells do not mature efficiently beyond this stage. As maturation in this system is driven by ER–GATA-1, which is constitutively expressed from an integrated retroviral vector, dynamic control of GATA-1 expression from the endogenous *Gata1* locus in this cell line differs from that in primary erythroid cells. Though SetD8 downregulation did not alter GATA-1 expression, the unique sensitivity of the primary erythroid cells illustrates that the two systems do not share all parameters. Furthermore, G1E-ER-GATA-1 cells were derived from murine ES cells (52), and the cell derivation process involved expression of the antiapoptotic factor Bcl-2, which might impact cell physiology and contribute to differential susceptibility to apoptosis in the two systems.

GATA-1 directly activates *Bcl2l1*, which encodes the antiapoptotic factor Bcl-x_L during erythroid maturation (57). SetD8 repressed *Bcl2l1* (Fig. 2C), suggesting that SetD8-mediated survival does not involve the transcriptional induction of *Bcl2l1*. Loss of SetD8 in murine ES cells causes massive spontaneous DNA damage. SetD8 degradation in early G_1 is required for cell cycle progression through S phase, and SetD8 Ser 29 phosphorylation mediates progression through anaphase (45). SetD8 depletion induces DNA damage as a result of new DNA synthesis (43). As erythroblasts undergo several extremely rapid cell divisions during maturation, apoptosis resulting from SetD8 downregulation in the maturing erythroblast may be linked to DNA damage (80).

A common feature of the G1E-ER-GATA-1 and primary fetal liver erythroblast system is the capacity of SetD8 to repress *Vim* expression (Fig. 3A) (41). Since *Gata2* expression is highly upregulated in uninduced G1E-ER-GATA-1 cells due to the loss of GATA-1 that directly represses *Gata2* transcription (29), not surprisingly, SetD8 knockdown in these cells did not further enhance the already high-level *Gata2* expression. In the primary cells, we established that SetD8 represses *Gata2* and elevating GATA-2 expression induces a maturation blockade similar to lowering SetD8 expression. However, reducing the level of endogenous GATA-2 in the context of SetD8 knockdown did not rescue the maturation blockade. These results suggest that SetD8 activity to promote the proerythroblast to basophilic erythroblast transition involves a multicomponent mechanism, including promoting cell survival, repressing *Gata2*, and repressing other target genes, including *Vim*. SetD8 catalyzed H4K20me1 at the essential +9.5 GATA switch site, and this chromatin modification was associated with suppression of Scl/TAL1 occupancy at this site but at not other genomic sites bound by GATA factors but not functionally impacted by SetD8 downregulation (Fig. 6).

Although our loss-of-function studies utilized a high-efficiency shRNA-based knockdown strategy, residual SetD8 might limit the magnitude of phenotypic alterations. Nevertheless, modest changes in GATA-2 levels translate into important functional consequences. The magnitude of *Gata2* upregulation upon SetD8 knockdown was 2- to 3-fold (Fig. 3A), and studies with *Gata2*$^{+/-}$ mice have demonstrated significant deficits in HSPC function

with only a 2-fold drop in *Gata2* expression (13, 81). *GATA2* overexpression correlates with poor prognosis of pediatric (82) and adult (83) AML.

Composite *cis* elements consisting of an E-box, an 8-bp spacer, and a GATA motif were originally identified as sequences at which GATA-1 assembles a multimeric complex containing Scl/TAL1 and the non-DNA binding components LDB1 and LMO2 (84). GATA-1 and GATA-2 occupy a small percentage of these composite elements in the genome (20, 21). The +9.5 site represents such a composite element, and its targeted deletion in the mouse revealed its requirement for embryogenesis, HSC generation in the AGM, and establishment of the HSPC compartment (9, 17, 22, 55, 85). In certain cases, Scl/TAL1 occupies GATA motif-containing GATA factor-bound chromatin sites lacking the E-box component of the composite element (70).

While it is assumed that the various components of the +9.5 complex all are important mediators of transcriptional activation, whether +9.5 components that limit +9.5 activity exist is unclear. Scl/TAL1 loss from GATA-1-occupied chromatin sites genome-wide correlates with transcriptional repression (70–72). GATA-1, Scl/TAL1, and LDB1 cooccupancy correlates with active transcription. Our analysis demonstrating that SetD8 restricts Scl/TAL1 occupancy at the +9.5 site provides an example of a negative regulatory component that dictates assembly and therefore functionality of an endogenous GATA factor-Scl/TAL1 multimeric complex. As SetD8 catalyzes H4K20me1 at the +9.5 GATA switch site, under conditions in which Scl/TAL1 occupancy is restricted, SetD8-catalyzed H4K20me1 may contribute to the establishment of repressive chromatin, which counteracts mechanisms that ensure +9.5 site accessibility.

SetD8 represses *Gata2* in proerythroblasts, in which *Gata2* is not completely transcriptionally inactive (Fig. 3A). In the more mature basophilic erythroblast, *Gata2* is silenced and insensitive to SetD8 downregulation (Fig. 3A). Since *Gata2* is initially repressed in the proerythroblast population, our findings are consistent with a mechanism in which SetD8 is required for initiation but not maintenance of *Gata2* repression. This is also relevant to *Vim*, as SetD8 represses *Vim* transcription selectively in the proerythroblast population. Previously, we demonstrated that establishment versus maintenance of GATA-1 target genes can be differentially regulated (86). It will be instructive to probe deeply into mechanisms underlying establishment and maintenance phases of GATA factor-mediated transcriptional control, to establish the extent to which SetD8 interfaces with other cellular proteins that establish the repressive state, and to elucidate why certain loci are SetD8 responsive while others are SetD8 insensitive.

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