

# An intricate regulatory circuit between FLI1 and GATA1/GATA2/LDB1/ERG dictates erythroid vs. megakaryocytic differentiation

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**Abstract.** During hematopoiesis, megakaryocytic erythroid progenitors (MEPs) differentiate into megakaryocytic or erythroid lineages in response to specific transcriptional factors, yet the regulatory mechanism remains to be elucidated. Using the MEP-like cell line HEL western blotting, RT-qPCR, lentivirus-mediated downregulation, flow cytometry as well as chromatin immunoprecipitation (ChIP) assay demonstrated that the E26 transformation-specific (ETS) transcription factor friend leukemia integration factor 1 (Fli-1) inhibits erythroid differentiation. The present study using these

methods showed that while FLI1-mediated downregulation of GATA binding protein 1 (GATA1) suppresses erythropoiesis, its direct transcriptional induction of GATA2 promotes megakaryocytic differentiation. GATA1 is also involved in megakaryocytic differentiation through regulation of GATA2. By contrast to FLI1, the ETS member erythroblast transformation-specific-related gene (*ERG*) negatively controls GATA2 and its overexpression through exogenous transfection blocks megakaryocytic differentiation. In addition, FLI1 regulates expression of LIM Domain Binding 1 (LDB1) during erythroid and megakaryocytic commitment, whereas shRNA-mediated depletion of LDB1 downregulates FLI1 and GATA2 but increases GATA1 expression. In agreement, LDB1 ablation using shRNA lentivirus expression blocks megakaryocytic differentiation and modestly suppresses erythroid maturation. These results suggested that a certain threshold level of LDB1 expression enables FLI1 to block erythroid differentiation. Overall, FLI1 controlled the commitment of MEP to either erythroid or megakaryocytic lineage through an intricate regulation of GATA1/GATA2, LDB1 and ERG, exposing multiple targets for cell fate commitment and therapeutic intervention.

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**Abbreviations:** ATCC, American Type culture collection; ChIP, Chromatin immunoprecipitation; ERG, ETS transcription factor ERG; ETS, E26 transformation-specific; FLI1, friend leukemia integration 1; GATA1, GATA binding protein 1; GATA2, GATA binding protein 2; HSC, hematopoietic stem cell; ITGA2B integrin subunit alpha 2b; ITGB3, integrin subunit beta 3; KLF1, KLF transcription factor 1; LDB1, LIM domain binding 1; LMO2, LIM domain only 2; MEP, megakaryocytic erythroid progenitors; TAL1, T-Cell acute lymphocytic leukemia 1.

**Key words:** friend leukemia integration factor 1, erythroid differentiation/megakaryocytic differentiation, transcriptional regulation, GATA binding protein 1, GATA binding protein 2, LIM Domain Binding 1, erythroblast transformation-specific-related gene

## Introduction

During hematopoiesis, hematopoietic stem cells (HSCs) self-renew and give rise to progenitors and their mature lymphoid and myeloid lineages. This maturation process is controlled by internal and external cues and mediated by specific transcription factors in blood progenitor cells (1,2). Extensive studies have been carried out to uncover the function of hematopoietic transcription factors and their impact on normal development and disease (1,2).

Red blood cells and megakaryocytes arise from a common precursor, designated megakaryocyte-erythroid progenitor (MEP) (3). Commitment of MEPs to the erythroid/megakaryocytic lineage takes place within the bone marrow microenvironment under the influence of multiple regulatory factors. Protein-protein interaction of

heptad transcription factors (TFs) friend leukemia integration factor 1 (Fli-1), GATA binding protein (GATA)1, GATA2, runt-related transcription factor 1 (RUNX1), T-Cell acute lymphocytic leukemia 1 (TAL1) in MEP cells controls lineage specific erythroid and megakaryocytic differentiation (4). In the combinatorial binding of heptad factors in bulk human hematopoietic stem progenitor cells (HSPCs), individual progenitors and cell lines reveal cell-specific changes in the regulatory architecture of these transcription factors during lineage specific differentiation (5-7).

Fli-1 was first identified as the integration sites of provirus, involved in transformation of erythroid cells by friend murine leukemia virus (F-MuLV) (8,9). Fli-1 downregulation in erythroleukemic cells promotes erythroid differentiation (10-12), whereas overexpression or drug-mediated activation of Fli-1 induces differentiation of MEP to megakaryocytic cells (13,14). This is consistent with the fact that in zebra fish FLII acts at the top of the transcriptional network driving both blood and endothelial development (15). In the present study, FLII was found upstream of Gata2, Stem cell leukemia/Tal1), LIM-only protein 2 (Lmo2) and Zebrafish ets-related protein (Etsrp). The expression of the Fli-1 related erythroblast transformation-specific-related gene (*ERG*) has also been implicated in erythroid and megakaryocytic differentiation (16). However, the association between these two genes during the entire maturation process of MEP has not yet been investigated. Studies also implicate the LIM domain binding 1 (LDB1) in the regulation of erythroid differentiation (17-23). Chromatin immunoprecipitation analysis reveals that most DNA bound murine Gata1 and Tal1 proteins are contained within higher order complexes (Ldb1-complexes) that include the nuclear adapters Ldb1 and Lmo2 (17). Notably, FLII in complex with LDB1 was recently shown to regulate megakaryocytic gene expression through interaction with GATA1 in murine erythroleukemic cells (24). LDB1 is deemed to act as a scaffold protein that brings Fli-1 to proximity of Gata1 through DNA looping, leading to activation of megakaryocytic genes (24).

Previous studies identified GATA1 as a direct target of FLII and its expression is suppressed by this TF (12,25). However, the role of GATA2 in erythroid or megakaryocytic differentiation is not fully understood. FLII was shown in the present study to bind the *GATA2* promoter and activates its transcription. By contrast, *ERG* expression was shown to block *GATA2* transcription leading to suppression of megakaryocytic differentiation. Knockdown studies revealed that while GATA1 is critical for erythroid differentiation, GATA2 is mainly required for megakaryocytic differentiation. Moreover, the regulation of LDB1 by FLII, which is mediated through direct regulation of GATA1, played a critical role in its ability to control erythroid and megakaryocytic lineages. The present study provided new insights into the intricate regulatory circuits between FLII and other factors such as GATA1, GATA2, *ERG* and LDB1 that govern erythroid and megakaryocytic differentiation.

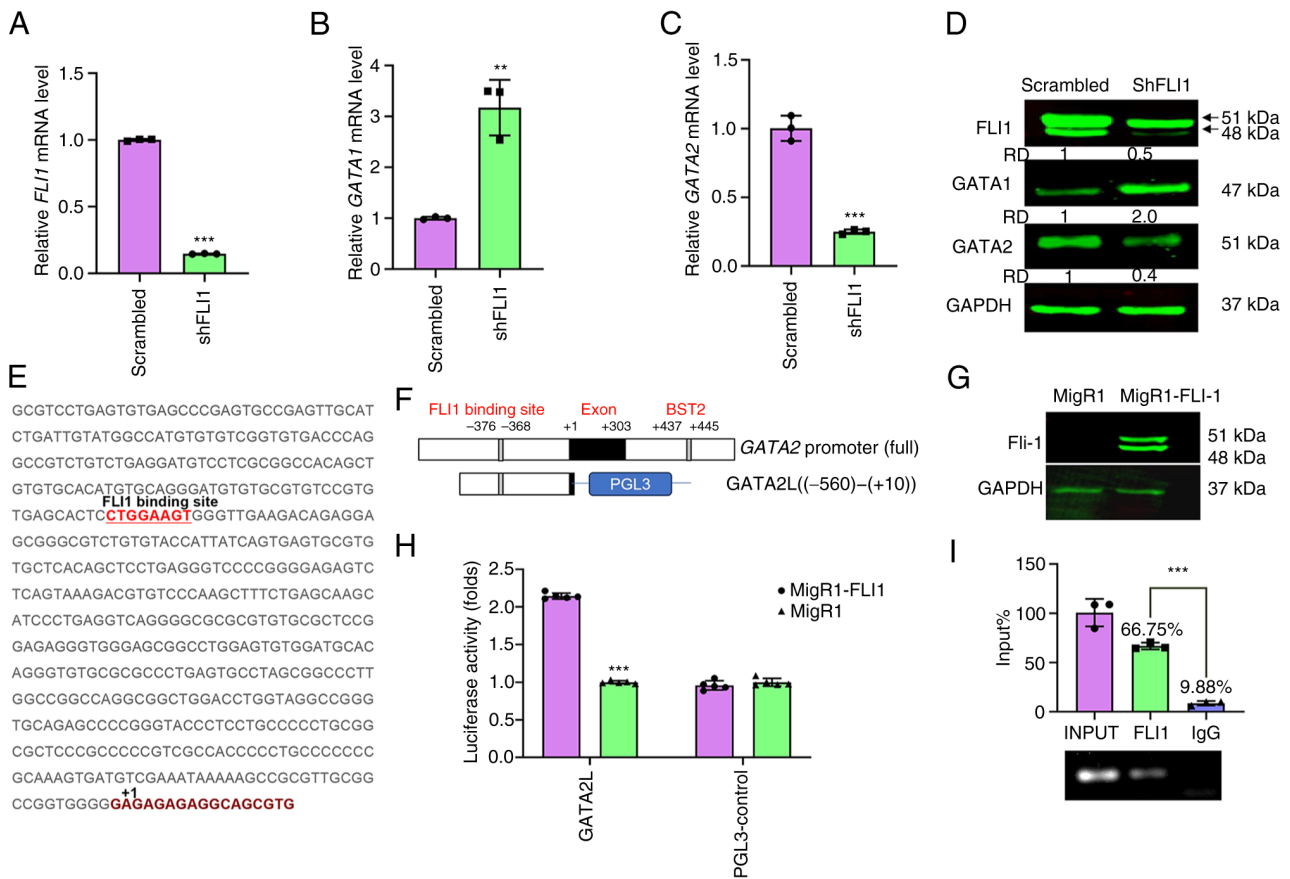
## Materials and methods

**Cell lines and vector.** The human erythroleukemia cell lines HEL (cat. no. ATCC-TIB-180) and 293T (cat. no. ATCC-CRL3216)

[a derivative of the 293T (cat. no. 293tsA1609neo) cell line (cat. no. ATCC CRL-11268)], were previously obtained from ATCC. These cells were cultured and maintained in Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum (HyClone; Cytiva). The luciferase reporter vector PGL3 (PGL3-basic) was purchased from Promega Corporation (cat. no. U47295). The vector PCDNA3 Flag Erg (Addgene, Inc.) was transfected into HEL cells by Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

**Gene cloning, transfections and luciferase activity.** *GATA2* promoter sequences were downloaded from the ensemble genome browser (<https://www.ensembl.org/index.html>) to download. To clone the *GATA2* promoter, the upstream region of the promoter (position -560 to +10; see Fig. 1F and Table SI), containing a potent FLII binding site, was cloned into the luciferase reporter vector PGL3 (Promega Corporation), as previously described (26,27). The promoter cloning was performed by (GenScript Biotech, Cn). The *GATA2* promoter and negative control PGL3 vector DNAs (1.25 µg) with either MigR1 (1.25 µg) or MigR1-FLII (1.25 µg) were mixed well, incubated 12 min at room temperature and transfected into 293T cells at 37°C using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer protocol. Renilla luciferase was used in transfection as internal control to test the transfection efficiency, according to manufacturer recommendations (Promega Corporation). The transiently transfected cells were then plated into 96 well plates and luciferase activity were determined 48 h later, as previously mentioned (27).

**Chromatin immunoprecipitation (ChIP) assay.** In brief, growing HEL cells (4x10<sup>6</sup> cells per reaction) were washed, crosslinked with formaldehyde and then resuspended in 500 µl of lysis buffer (Enzymatic Chromatin IP Kit; Cell Signaling Technology, Inc.). Micrococcal Nuclease (0.5 µl) was added to the fixed cells and incubated at 37°C for 20 min with frequent mixing in order to digest DNA to length of approximately 150-900 bp. The cell lysates were sonicated in three sets of 20-sec pulses using the Sonics Vibra VCX150 (Ningbo Scientz Biotechnology Co., Ltd.) to break the nuclear membrane. As a control, a portion of chromatin aliquot (20 µl) was removed as input DNA. Immunoprecipitations were performed overnight at 4°C with 100 µl process solution and 5 µl of ChIP specific FLII antibody (Abcam) or 1 µl of nonspecific normal rabbit immunoglobulin G (IgG) antibodies (CST). To each IP reaction then added 30 µl of Protein G Magnetic Beads and incubated for 2 h at 4°C with rotation. Precipitates were washed with buffer provided with the kit and reverse crosslinked, using the instructions provided for company's Enzymatic Chromatin IP Kit (CST Biological Reagents Co., Ltd.). Precipitated chromatin was incubated with proteinase K at 65°C for 2 h and used for DNA purification using spin columns from company's Enzymatic Chromatin IP Kit (CST). Quantitative (RT-q) PCR was performed to amplify the indicated promoter regions containing FLII binding sites. The sequences of the ChIP primers were *GATA2L* sense: CGAGTTGCATCTGATTGT ATGG and antisense: GCTCCTCTGTCTTCAACCA. The



**Figure 1.** Direct regulation of the *GATA2* promoter by FLI1. (A) In FLI1 knockout cells designated shFLI1, the expression of (B) GATA1 increased while (C) GATA2 levels decreased, as determined using RT-qPCR. (D) The expression of FLI1, GATA1 and GATA2 in shFLI1 cells by western blot analysis. Arrows show the locations of the FLI1 51 kDa and 48 kDa protein bands. (E) The indicated FLI1 binding site within upstream sequence of the *GATA2* promoter. The promoter sequence was downloaded from ensemble genome browser. (F) The promoter of *GATA2* and its region designated *GATA2L* cloned upstream of the luciferase reporter plasmid PGL3. (G) Expression of FLI1 in 293T cells by western blotting. (H) Luciferase activity of the *GATA2L* promoter co-transfected into 293T cells with either expression vector MigR1-FLI1 or control MigR1. The PGL3 plasmid was used as control. (I) ChIP assay of the *GATA2* promoter for binding to FLI1, by RT-qPCR (top panel). Lower panel is the gel image of the immunoprecipitated PCR-amplified band relative to input. \*\**P*<0.01 and \*\*\**P*<0.001. GATA2, GATA binding protein 2; FLI1, friend leukemia integration 1; sh, short hairpin; RT-qPCR, reverse transcription-quantitative PCR; ChIP, chromatin immunoprecipitation.

percentage of input was calculated by qPCR based upon the intensity of the amplified FLI1 DNA divided by the amplified input DNA. Amplified DNA was also resolved in 2% agarose gel as shown in Fig. 1I.

**RT-qPCR.** Total RNA was extracted from culture of HEL cells ( $4 \times 10^5$ ) using TRIzol® (Thermo Fisher Scientific, Inc.) by using the manufacturer's recommended protocol. RNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific Fisher, Inc.). To generate cDNA, reverse transcription reaction was performed using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.). RT-qPCR was performed using FastStart Universal SYBR Green Master (Roche Diagnostics GmbH) and the Step One Plus Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression was normalized to GAPDH. The  $2^{-\Delta\Delta C_q}$  method was used for relative quantification (28). RNA extraction, cDNA synthesis and RT-qPCR were all performed according to the manufacturer protocols. The primer sequences are in Table SII. A total of three biological triplicates were used for all RT-qPCRs, each in triplicates (n=3).

**Heatmap analysis.** The RNA sequencing for short hairpin (sh)FLI1 in HEL cells has been published previously (29). TBtools software (TBtoolsV1.098) was used for Heatmap analysis (30). The original contributions presented in the study are publicly available and found at <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA682304>.

**STRING, ENCODE and ENSEMBLE database analysis.** STRING database ([www.string-db.org](http://www.string-db.org)) was used for protein protein interaction data analysis. The ENCODE database ([encodeproject.org](http://encodeproject.org)) was used to determine DNA-protein interaction for binding of FLI1 to *GATA2*. The ensemble genome browser (<https://www.ensembl.org/index.html>) was used to extract promoter sequences shown in Fig. 1E.

**shRNA and short interfering (si)RNA expression.** The construction of sh-FLI1 expression construct (shFLI1) has been previously described (29,31). In brief, the shRNA expression plasmid (12 mg) and packaging plasmid psPAX2 (6 mg), pMD2. G (12 mg) (Didier Trono, Addgene plasmid # 12259 and # 12260) were mixed and transfected into HEK293T cells, using Lipofectamine®2000 48 h after transfection, the cell

supernatant was collected for transduction of HEL ( $1 \times 10^6$ ) cells. The positive cells after transduction were selected and cultured for 24 h using RPMI-1640 medium containing puromycin (5 mg/ml; Solarbio, China). Other shRNAs such as shGATA1, shGATA2 and shLDB1 as well as their control scrambled plasmids were generated in a similar fashion. The sequences are shown in Table SIII. Transfection of siRNAs into HEL cells was performed using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, as previously described (27). In brief, Lipofectamine 2000 was used to transfect siRNA (15  $\mu$ L of 20  $\mu$ M) into HEL cells according to manufacturer's instructions (Invitrogen; Thermo Fisher Scientific). After 48 hours, cells were harvested for subsequent assays.

**Overexpression of ERG in HEL cells.** DNA vectors (1.25  $\mu$ g) containing PCDNA3.1(-) (1.25  $\mu$ g; cat. no. V79520; Invitrogen; Thermo Fisher Scientific, Inc.) or PCDNA3 Flag Erg (1.25  $\mu$ g; cat. no. 66977; Addgene, Inc.) were transfected into HEL cells according to the manufacturer's protocol. The transfection was performed using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The medium was changed 48 h post transduction and positive cells were selected for using medium containing G418 (250  $\mu$ g/ml; Beijing Solarbio Science & Technology Co., Ltd.).

**Western blotting.** Western blotting was performed as described elsewhere (14). In brief, cells were collected and protein was extracted using RIPA Lysis Buffer (Beyotime Institute of Biotechnology). After lysis and protein density determination using BCA protein assay kit, 50  $\mu$ g samples were loaded on 10% acrylamide gels and transferred onto the PVDF membrane. Blocked with TBS buffer containing 5% skimmed milk for 1.5 h at room temperature. Polyclonal rabbit antibodies for FLII (cat. no. ab133485) [dilution 1:1,000], GATA1 (ab181544) [dilution 1:2,000], LDB1 (cat. no. ab96799) [dilution 1:1,000] and ERG (cat. no. ab92513) [dilution 1:1,000] were purchased from Abcam; GATA2 (cat. no. 4595S) [dilution 1:500] from CST Biological Reagents Co., Ltd.; GAPDH (cat. no. AB-P-R 001) [dilution 1:1000] from Hangzhou Goodhere Biotechnology Co., Ltd.; secondary antibodies (Anti-rabbit IgG (H+L) (DyLight<sup>™</sup> 800 4X PEG Conjugate)) from CST Biological Reagents Co., Ltd. (cat. no. 5151S) [dilution 1:30,000]. The antibodies were diluted with TBS buffer containing 3% BSA, the primary antibodies were incubated overnight at 4°C, and the secondary antibodies were incubated for 1.5 h at room temperature. The Odyssey Imaging System (LI-COR Biosciences) is used for western blot protein imaging, and the protein density is determined using the software (Odyssey CLX Image Studio 3.1) that comes with the system.

**Flow cytometry.** Immunofluorescence staining was conducted to detect erythroid and megakaryocytic cells, as previously described (14,29). In brief,  $1 \times 10^5$  cells were stained with APC-conjugated antibodies for 40 min at 4°C. Cells were then washed twice and resuspended in 200  $\mu$ L PBS and used for flow analysis. The following primary antibodies were used: Human cluster of differentiation CD41a-APC (cat. no. 559777), human CD61-APC (cat. no. 564174), human CD71-APC (cat. no. 551374) and human CD235a-APC (cat. no. 551336;

all purchased from BD Biosciences). Flow cytometry was performed using a NovoCyte flow cytometer and Novo-express software (ACEC Biosciences Inc.).

The gating strategies were used as followed: FSC-A/SSC-A plots were used to separate live cells from debris. Erythroid cells were differentiated using a scatter/anti-CD71+ and a scatter/anti-CD235a+ gate from unstained control, respectively. Megakaryocytes were differentiated using a scatter/anti-CD41a+ and a scatter/anti-CD61+ gate from unstained control, respectively. Count/anti-CD71+, count/anti-CD235a+, count/anti-CD41a+ and count anti-CD61+ in histograms present the expression of these markers.

**Statistical analysis.** Statistical analysis was carried out using the two-tailed Student t-test or using Welch's ANOVA followed by Tamhane's T2 post hoc test, using Prism 8 software (GraphPad; Dotmatics). Results were expressed as mean  $\pm$  standard deviation from at least three independent experiments.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**FLII directly binds the promoter of GATA2 and regulates its transcription.** The authors have previously reported RNAseq data following lentivirus-shRNA-mediated FLII knockout in erythroleukemia cell line HEL (shFLII), which displays biopotential megakaryopoiesis and erythroid progenitor capacity (29,31). This RNAseq analysis identified a cluster of genes associated with megakaryopoiesis, whose expression is altered following FLII depletion (Fig. S1A) (29). A heatmap of the erythroid differentiation expressed genes (EDEGs), shown in Fig. S1B, identified 52 genes whose expressions was altered in lentivirus-mediated FLII knockout (shFLII) relative to control (Scrambled) cells.

Among DEGs associated with erythroid and megakaryocytic differentiation, GATA1 and GATA2 expression has been previously shown to play pivotal roles in these blood maturation processes, although the underlying mechanism remains to be elucidated (16). GATA1 is negatively regulated by FLII (12), as is shown in the present study by both RT-qPCR and western blot analysis (Fig. 1A, B and D). This is consistent with a previous report demonstrating binding of FLII to a putative site within the promoter of *GATA1* (12). By contrast, GATA2 expression was notably reduced in shFLII compared with scrambled control cells (Fig. 1C and D), suggesting that GATA2 expression may be directly regulated by FLII. Indeed, the present study identified a putative FLII binding site in the promoter of *GATA2* at position -376 to -368 (Fig. 1E). To determine whether FLII is recruited to *GATA2* through this site, the promoter of *GATA2* was cloned into the luciferase reporter gene PGL3 (designated GATA2L; Fig. 1F). Transfection of the GATA2L plasmid together with a FLII expression vector (MigR1-FLII) into 293T cells significantly increased luciferase activity (Fig. 1H). The expression of FLII in 293T cells was verified via western blotting (Fig. 1G). ChIP analysis of HEL cells using primers that flank the putative FLII-binding site within the *GATA2* promoter detected a band that was immunoprecipitated with FLII but not control IgG antibodies (Fig. 1I). These results

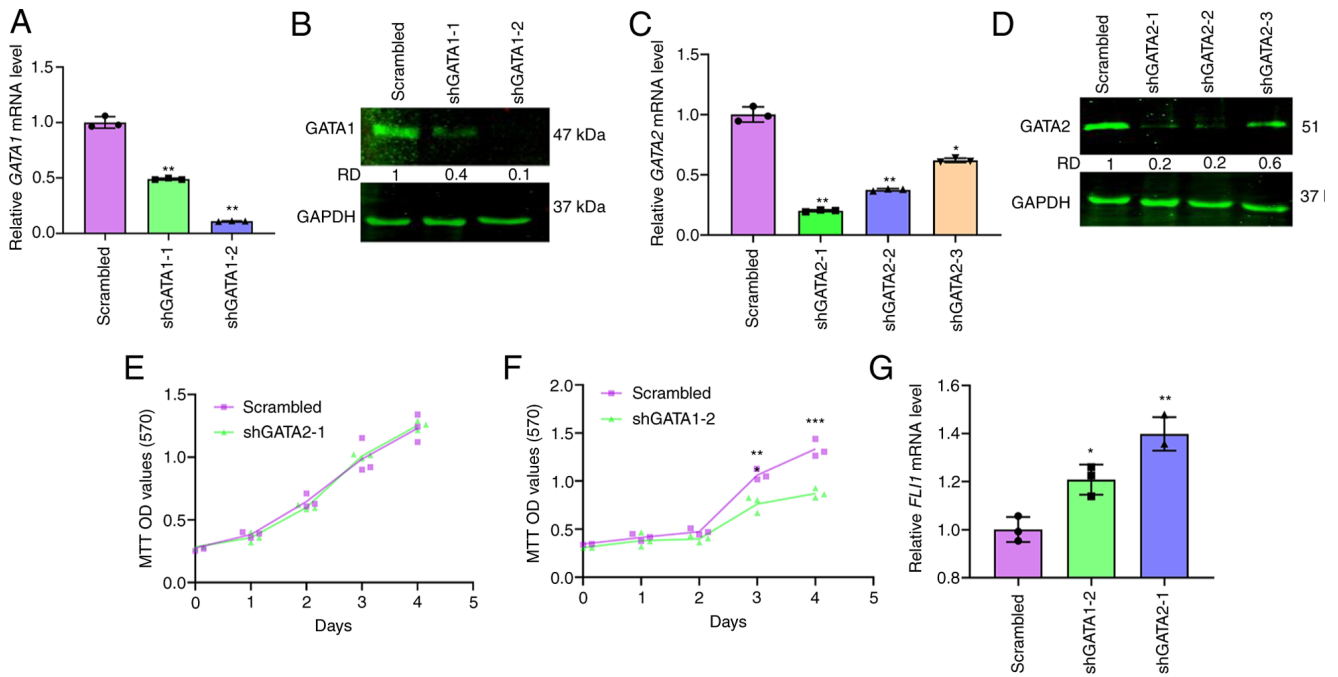


Figure 2. Knockdown of GATA1 and GATA2 in HEL cells and effect on cell proliferation. Lentivirus-mediated knock down of GATA1 (shGATA1-1 and shGATA1-2) in HEL cells as detected by (A) RT-qPCR and (B) western blotting. Lentivirus-mediated ablation of GATA2 (shGATA2-1, shGATA2-2 and shGATA2-3) in HEL cells as measured by (C) RT-qPCR or (D) western blotting. The proliferation analysis of (E) shGATA2-1 and (F) shGATA1-2 vs. scrambled control cells using MTT assay. (G) The expression level of FLI1 in shGATA2-1 and shGATA1-2 cells was detected by RT-qPCR. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . GATA, GATA binding protein; sh, short hairpin; RT-qPCR, reverse transcription-quantitative PCR; FLI1, friend leukemia integration 1.

for the first time point to the human GATA2 promoter as a direct target of FLI1.

**Consequences of FLI1 regulation of GATA1 and GATA2 in erythroleukemic cell proliferation.** To understand the consequences of FLI1 regulation of GATA1 and GATA2 in cell proliferation and differentiation, lentivirus shRNAs were used to knockdown these genes in HEL cells. Significant knockdown was obtained in shGATA1-1 and shGATA1-2 cells at both mRNA (Fig. 2A) and protein (Fig. 2B) levels. Similarly, silencing of GATA2 in HEL cells via shGATA2-1, shGATA2-2 and shGATA2-3 vectors blocked both transcription abundance (Fig. 2C) and protein (Fig. 2D) expression. While knockdown of GATA2 did not alter cell proliferation (Fig. 2E), loss of GATA1 significantly reduced proliferation rate in culture compared to scrambled control (Fig. 2F). Knockdown of GATA1 and GATA2 slightly, but significantly increased FLI1 expression (Fig. 2G). Accordingly, multiple GATA recognition sites were found within the Fli-1 promoter and GATA1 is shown to bind these sequences (32). The expression analysis is also predicted binding of GATA2 to FLI1 (Fig. 3E) that may need further analysis in future studies.

**Effect of GATA1 and GATA2 silencing in erythroid and megakaryocytic differentiation.** To determine the effect of GATA1/GATA2 on erythroid/megakaryocytic lineage development, the present study examined the effect of silencing these genes in HEL cells, using the erythroid (CD71 and CD235a) and megakaryocytic (CD41a and CD61) markers (33,34). It has previously been shown that overexpression of Fli-1 in erythroblasts blocks erythroid differentiation (10,11). Indeed, FLI1 silencing in HEL cells reduced percentage of megakaryocytic

CD41a and CD61 positive cells and increased percentage of late erythroid CD235a cells (Fig. S2A and B). FLI1 knockdown had no effect on expression of early erythroid markers CD71 (Fig. S2A and B).

Knockdown of GATA1 (shGATA1) resulted in a dramatic reduction in expression of the erythroid differentiation marker CD235a, but negligible changes in CD71 levels (Figs. 3A and S3). GATA1 knockdown also suppressed megakaryocytic differentiation as both CD41a and CD61 levels were significantly reduced in shGATA1-2 cells (Figs. 3A and S3). Ablation of GATA1 in HEL cells resulted in downregulation of GATA2 and LDB1, but slightly higher expression of FLI1, as determined by western blotting (Fig. 3B).

GATA2 silencing in shGATA2-1 cells revealed a significant lower percentage of megakaryocytic CD41a and CD61 expressing cells. This analysis also found significant reduction in the percentage of CD71 and CD235a expression in shGATA2-1 cells (Figs. 3C and S4). Indeed, lower expression of megakaryocytic markers *CD61* and *Meis1*, as well as reduced levels of the erythroid *CD235a* marker and the globin genes *HBA1* and *HBA2*, was observed in shGATA2-1 cells (Fig. 3D). Knockdown of GATA2 in HEL cells resulted in downregulation of LDB1, but slightly higher expression of FLI1, as determined by western blotting (Fig. 3E). The expression of GATA1 was slightly reduced in shGATA2-1 cells, probably through increased expression of FLI1 (Fig. 3E). These results suggested a commitment role for GATA2 in megakaryocytic differentiation and some involvement in erythroid maturation via FLI1. GATA1 is probably involved in megakaryocytic differentiation through regulation of GATA2 (Fig. 3B).

Using a protein-protein interaction database (STRING), it was found that FLI1 binds GATA2 as well as other factors

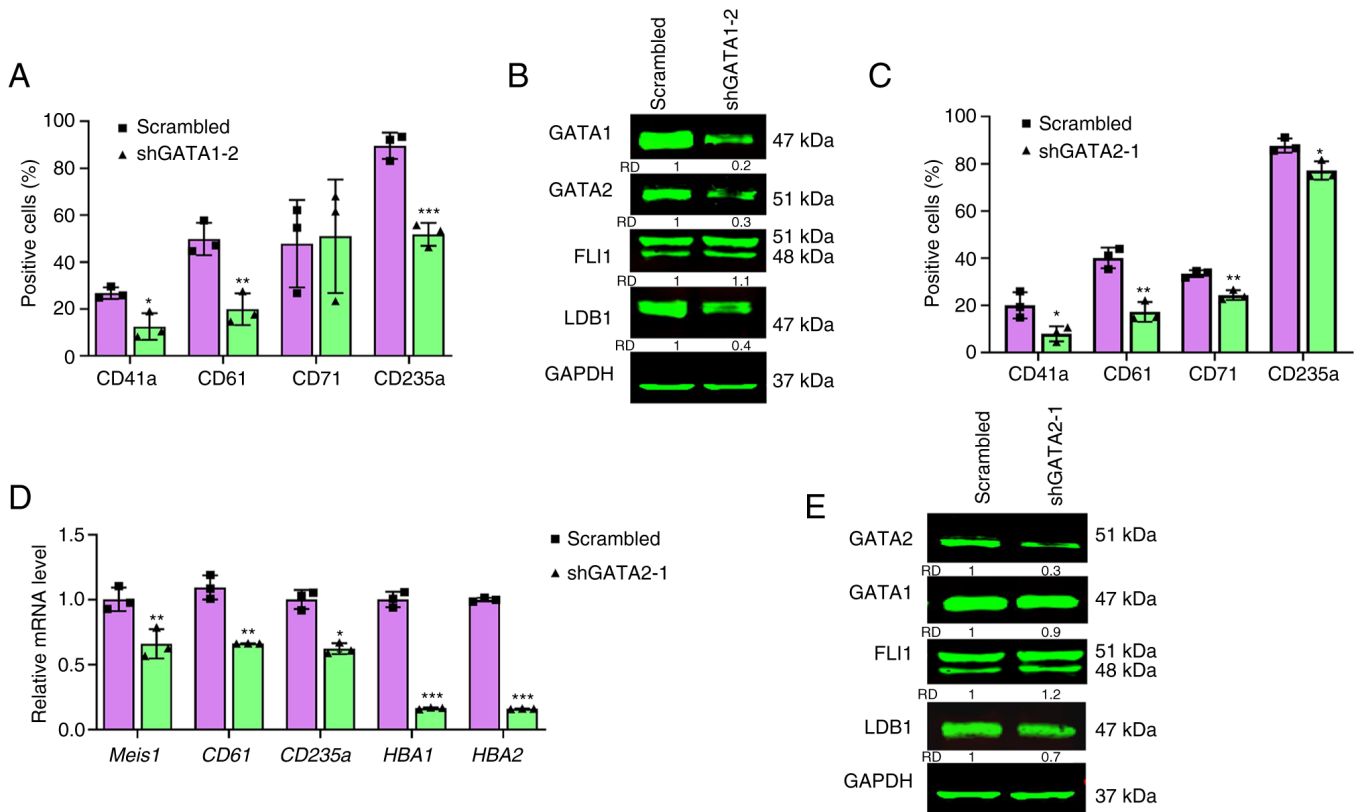


Figure 3. The expression of GATA1 and GATA2 controls erythroid versus megakaryocytic differentiation. (A) Flow cytometry was used to determine the expression of megakaryocytic (CD41a/CD61) and erythroid (CD71/CD235a) markers in shGATA1-2 cells. (B) The expression of the indicated proteins in shGATA1-2 cells as detected by western blotting. RD was detected by densitometer. (C) The expression of megakaryocytic (CD41a/CD61) and erythroid (CD71/CD235a) markers in shGATA2-1 cells was detected by flow cytometry. (D) The expression of the indicated genes via RT-qPCR in shGATA2-1 cells. (E) The expression of the indicated proteins in the shGATA1-2 cells was detected by western blotting. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . GATA, GATA binding protein; CD, cluster of differentiation; sh, short hairpin; RD, relative density.

including GATA1, RUNX1/2, MYB, SPI1, TAL1, known to play critical roles in erythroid and megakaryocytic differentiation (Fig. S5A). GATA2 is also predicted to interact with LDB1 partner LMO2 that participates in both erythroid and megakaryocytic maturation (17,18). As FLI1 ablation suppresses the expression of ITGAB3 (CD61) (Fig. S2A and B), knockdown of GATA2 causes similar downregulation (Fig. 3D). Accordingly, the present study found that the promoter of *CD61* has a putative GATA2 binding site at position -102 to -96 (Fig. S5B). In the ENCODE database (35), GATA2 strongly binds (Affinity 7.93) to this region of promoter (Fig. S5C). These results confirmed a critical role for GATA2 as a limiting factor in megakaryocytic differentiation.

**Overexpression of *ERG* in HEL cells suppresses megakaryocytic differentiation.** While GATA1 has been shown to control megakaryocytic differentiation, the underlying mechanism remains to be elucidated. In contrast to overexpression of FLI1 in HEL cells, the level of another FLI1-related ETS gene, *ERG*, is negligible (29). These two genes are known to have distinct functions in hematopoiesis (16). The present study found that in both GATA1 and GATA2 knockdown cells, the *ERG* expression was significantly induced, suggesting a negative regulation of *ERG* via these GATA genes in HEL cells (Fig. 4A and B). In common with FLI1 (32), the *ERG* promoter also contains multiple GATA-binding sites

(Fig. S6A). ENCODE analysis indeed found binding of both GATA1 and GATA2 to the *ERG* promoter (Fig. S6B). Thus, GATA1 and GATA2 negatively regulate expression of *ERG* and FLI1 in HEL cells. To further investigate the role of *ERG* in erythroid and megakaryocytic differentiation, *ERG* was overexpressed in HEL cells (Fig. 4C). Higher expression of *ERG* in HEL cells had no significant effect on cell proliferation (Fig. 4D) but resulted in significant downregulation of FLI1 and GATA2 (Fig. 4E) and upregulation of GATA1 (Fig. 4E). Higher *ERG* expression also resulted in strong downregulation of CD41a/CD61 level, indicating that, in contrast to FLI1, *ERG* is a robust suppressor of megakaryocytic differentiation (Figs. 4F and S7). Higher *ERG* expression slightly but significantly induced erythroid CD235a expression, consistent with the higher GATA1 level in these cells. These results for the first time suggest that GATA1/2 control megakaryocytic differentiation through suppression of *ERG*.

**FLI1 negatively regulates LDB1 through GATA1 to control cell differentiation.** The LDB1 gene has also implicated in both erythroid and megakaryocytic differentiation (17,18,24). The present study found that LDB1 is negatively regulated by FLI1 (Fig. S1B), using both RT-qPCR and western blot analysis (Fig. 5A and B). Notably, the LDB1 partner Lmo2 was also negatively regulated by FLI1, and its upregulation in shFLI1 cells was associated with erythroid differentiation

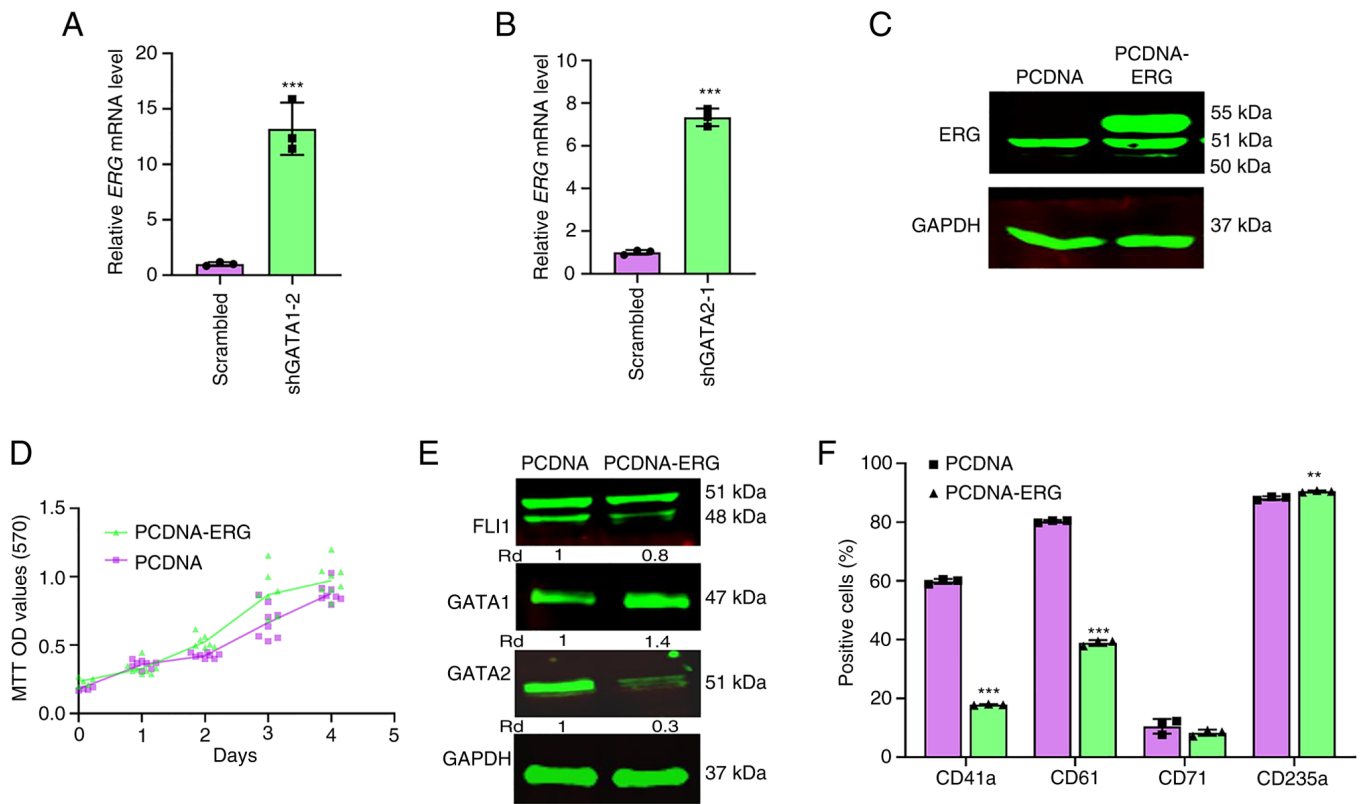


Figure 4. Regulation of erythroid and megakaryocytic differentiation by ERG. The expression of ERG in shGATA1-2 (A) and shGATA2-1 (B) cells was detected by reverse transcription-quantitative PCR. (C) The expression of ERG in HEL cells transfected with PCDNA-ERG or vector PCDNA was detected using western blotting. (D) The proliferation rate of PCDNA-ERG versus control PCDNA cells was determined by MTT. (E) The expression of indicated proteins in PCDNA-ERG and PCDNA cells, as detected via western blot analysis. (F) The flow cytometry was used to detect the expression of megakaryocytic (CD41a/CD61) and erythroid (CD71/CD235a) markers in PCDNA-ERG and PCDNA control cells. An average of three experiments is shown. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . ERG, ETS transcription factor ERG; ETS, E26 transformation-specific; sh, short hairpin; GATA, GATA binding protein; CD, cluster of differentiation; Rd, relative density.

(Fig. S1B). The *LDB1* promoter does not have a canonical FLI1 binding site (data not shown) and therefore its induction in shFLI1 cells is likely through GATA1 and/or GATA2 (Fig. 3B and E).

To uncover the role of *LDB1* in erythroid and megakaryocytic differentiation, its expression was silenced using lentivirus-shRNA (Fig. 5C). shLDB1-3 cells with efficient *LDB1* knockdown exhibited strong reduction in FLI1 and GATA2 and robust induction of GATA1 expression (Fig. 5D and E). Nonetheless, *LDB1* silencing in HEL cells had no effect on cell proliferation in culture (Fig. 5F).

Flow cytometry showed that knockdown of *LDB1* significantly reduced the percentage of cells expressing megakaryocytic markers CD41a and CD61. *LDB1* ablation resulted in a lower percentage of erythroid CD71 and CD235a expressing cells (Figs. 5G and S8). These results were further supported by lower expression of erythroid hemoglobin genes *HBA1*, *HBA2* and lower megakaryocytic markers *CD41* and *Meis1*, by RT-qPCR (Fig. 5H). Notably, while FLI1 knockdown in HEL cells accelerated erythroid and moderately decelerated megakaryocytic differentiation (Fig. S2), *LDB1* knockdown suppressed FLI1 (Fig. 5D), induced GATA1 expression (Fig. 5D) and unexpectedly inhibited erythroid maturation (Fig. 5G).

The above results suggested that a certain threshold level of *LDB1* expression may be necessary to enable FLI1 to block

erythroid differentiation. To test this possibility, *LDB1* expression was knocked down using siRNAs (siLDB1-1-siLDB1-4) in shFLI1 cells (Fig. 6A). Downregulation of *LDB1* in shFLI1 by siLDB1-1 resulted in a lower percentage of CD41a and CD61, but slight and insignificant reduction of CD235a expressing cells (Fig. 6B and C). However, in RT-qPCR, siLDB1-1 significantly inhibited expression of erythroid markers *HBA1*, *HBA2*, *HBG1* and *HBG2* (Fig. 6D). These results showed the essential role of *LDB1* in controlling erythroid and megakaryocytic commitments via FLI1.

*Interplay between FLI1 and KLF1 during erythroid differentiation.* Finally, the KLF1 (EKLF) transcription factor controls erythroid differentiation through binding to CACCC motifs within various globin gene promoters (36). KLF1 and FLI1 were both previously reported to negatively regulate each other (37,38). KLF1 is also known to positively regulate GATA1, leading to erythroid differentiation (37-39). In GATA1 knockdown cells (Fig. 7A), *KLF1* expression was considerably suppressed (Fig. 7B), supporting a direct regulation of KLF1 by GATA1. Notably, a negligible change in *KLF1* expression was detected in the *LDB1* knockdown cells (Fig. 7C). Consistent with inhibition of megakaryopoiesis, FLI1 expression was reduced (Fig. 5D), whereas *ERG* expression was strongly elevated in shLDB1-3 cells (Fig. 7D). These results further confirmed the important role of *LDB1* in controlling

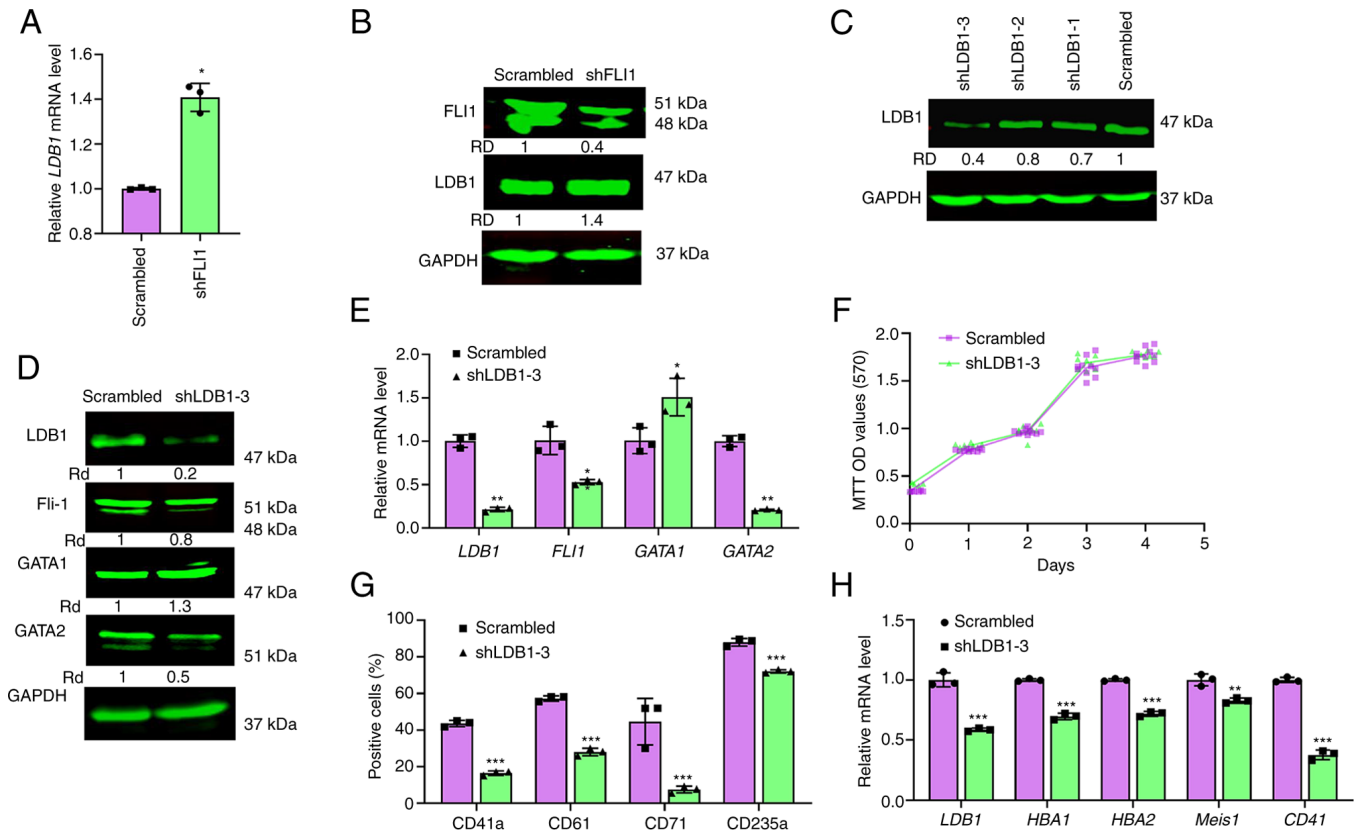


Figure 5. Regulation of erythroid and megakaryocytic differentiation by LDB1. The expression of LDB1 in shFLI1 cells as detected by (A) RT-qPCR or (B) western blotting. (C) Expression of LDB1 protein in HEL cells transfected with shRNA lentiviruses (shLDB1-1, shLDB1-2 and shLDB1-3), as determined by western blotting. (D) The expression of FLI1, LDB1, GATA1 and GATA2 in shLDB1-3 cells by western blotting. GAPDH was used as the loading control. (E) The expression of genes in shLDB1-3 relative to scrambled control cells, as determined by RT-qPCR. (F) Proliferation rate of shLDB1-3 and scrambled control cells, as determined by MTT. (G) The flow cytometry analysis for expression of megakaryocytic (CD41a/CD61) and erythroid (CD71/CD235a) markers in shLDB1-3 cells versus scrambled control cells. Average of three experiments was indicated. (H) The relative expression of indicated genes was determined by RT-qPCR. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . LDB1, LIM domain binding 1; sh, short hairpin; RT-qPCR, reverse transcription-quantitative PCR; GATA, GATA binding protein; FLI1, friend leukemia integration 1; CD, cluster of differentiation; RD, relative density.

erythroid and megakaryocytic differentiation in cooperation with FLI1 and ERG. As depicted in Fig. 7E, transcriptional regulation of GATA1, GATA2, LDB1 and ERG by FLI1 is critical for commitment to either erythroid or megakaryocytic differentiation. In HEL cells, LDB1 silencing decreased FLI1 and increased GATA1 levels (Fig. 5D). This increase in GATA1 expression unexpectedly failed to induce erythroid differentiation. Conversely, reduced LDB1 expression led to GATA2 downregulation, resulting in lower megakaryocytic differentiation. Thus, cooperation between FLI1 and LDB1 is necessary for proper regulation of erythroid differentiation.

## Discussion

The present study showed that FLI1 controls the transcription of GATA1, GATA2 and LDB1, thereby coordinating the erythroid versus megakaryocytic cell differentiation in HEL cells. While FLI1 negatively controls GATA1 to block erythroid differentiation, the present study showed that direct GATA2 transcriptional regulation by FLI1 is essential in promoting the differentiation of the MEP-like erythroleukemia cell line HEL toward megakaryocytic lineage maturation. LDB1 plays a broader role in commitment of progenitors to both erythroid and megakaryocytic differentiation via FLI1. These results

provided novel insights into an intra-regulatory role of FLI1 and its accessories during erythroid and megakaryocytic differentiation. However, further studies on animal models may be necessary to confirm the function of these TFs *in vivo*.

The association between FLI1 and GATA2 was previously observed, but it is not known whether this regulation is direct or indirect (15). The present study showed that regulation of GATA2 by FLI1 is critical for megakaryocytic commitment. RNAseq analysis of FLI1 knockdown cells indeed identified at least 47 genes associated with megakaryocytic differentiation, among them *ITGB3* (*CD61*), *ITGA2B* (*CD41*) and *Meis1* (Fig. S1A). The human *ITGAB3* promoter contains binding site for GATA2, further confirming a role for GATA2 in megakaryocytic differentiation. Indeed, in high-risk acute myeloid leukemia, chromosomal rearrangements between 3q21 and 3q26 is often associated with elevated platelet and megakaryocyte numbers (40). The 3q rearrangements reposition a GATA2 enhancer near the *EVII* (or *MECOM*) locus, which results in both *EVII* and *GATA2* overexpression leading to higher number of megakaryocytic cells. Deleting GATA2 enhancer in mice also results in significant reduction in differentiation of progenitors to megakaryocytes and erythrocytes (41). Moreover, in *inv(16)* leukemia, the *CBF $\beta$ -MYH11* fusion inhibits megakaryopoiesis by blocking the expression of

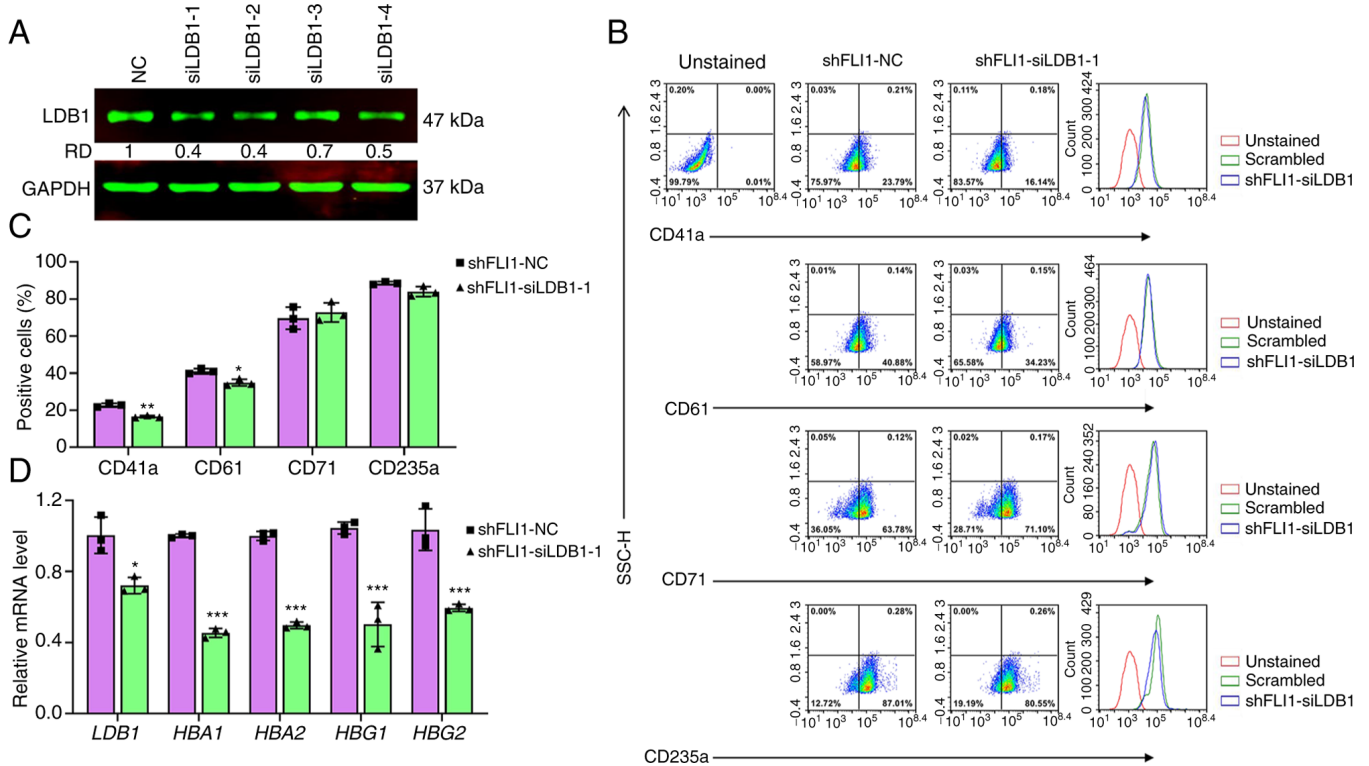


Figure 6. Inhibition of erythroid and megakaryocytic differentiation in shFLI1 cells by LDB1 siRNA. (A) Downregulation of LDB1 in shFLI1 cells using indicated siLDB1s, as detected by western blotting. (B) Flow cytometry for expression of megakaryocytic (CD41a/CD61) and erythroid (CD71/CD235a) markers in shFLI1 cells versus scrambled controls after treatment with siLDB1-4. The same unstained cells were used in each analysis. (C) Average of three experiments. (D) Relative expression of the indicated erythroid differentiation genes in shFLI1-siLDB1-1 cells vs. control by reverse transcription-quantitative PCR. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . sh, short hairpin; FLI1, friend leukemia integration 1; LDB1, LIM domain binding 1; si, short interfering; CD, cluster of differentiation.

GATA2/KLF1 and interfering with a balanced transcriptional program involving these two factors (42). Although K562 erythroleukemic cells do not express FLI1 (14), overexpression of GATA2 in these cells induces megakaryocytic differentiation and blocks erythroid maturation (43). Overall, the results of the present study indicated that FLI1 regulation of GATA2 is essential for megakaryocytic differentiation.

During differentiation, progenitor cells undergo maturation processes to become mature blood cells. This process involves several cell divisions that are probably controlled by TFs. GATA1 expression controls erythroid differentiation. When GATA1 is knocked down in HEL cells, there was a reduction in erythroid differentiation that is also associated with a significant reduction in the rate of cell proliferation (Fig. 2F). As Fli-1 is an oncogene, its activation negatively regulates GATA1 expression resulting in downregulation of this transcription factor and blockage of erythroid differentiation that eventually leads to the development of erythroleukemia. Indeed, several studies have reported a role for GATA1 in cell proliferation and differentiation in various types of cancer involving several growth promoting genes including PI3K (44,45). While the present study provided a correlation between differentiation and proliferation, at least for GATA1, the other transcription factors that showed no change in the rate of proliferation may require additional events to control cell division.

FLI1 homologue gene ERG is also implicated in both hematopoietic stem cell expansion and hematopoiesis (16).

However, the expression of FLI1 and ERG varies in different hematopoietic cells, suggesting distinct or overlapping function (16). Similar to FLI1, overexpression of ERG in hematopoietic cells affected both erythroid and megakaryocytic differentiation (46). Notably, ERG expression is negligible in erythroleukemic cells overexpressing FLI1 (29). This raises the possibility that FLI1 and ERG may exert opposite functions during erythroid differentiation and transformation. The present study showed that, in contrast to FLI1, ERG blocks the expression of GATA2 and its overexpression in erythroleukemic cells suppressed megakaryocytic differentiation. It also showed that FLI1 suppressed ERG expression through downregulation of GATA1. These results pointed to the opposite roles of FLI1 and ERG in erythroid and megakaryocytic differentiation. The present study, for the first time to the best of the authors' knowledge, suggested that GATA1/2 may control megakaryocytic differentiation through suppression of ERG.

In functional ablation studies in mice, LDB1 has been shown essential for embryonic erythropoiesis and blood island formation (47). LDB1 facilitates nuclear organization of its erythroid partners on the  $\beta$ -globin gene promoter to initiate transcription during erythroid differentiation (48). In contrast to these reports, LDB1 and LMO2 were shown in the present study to function as negative regulators of erythroid differentiation in erythroleukemic cells (49). The present study also showed that FLI1 knockdown in erythroleukemic cells induced higher expression of both human LDB1 and LMO2, supporting a role for these

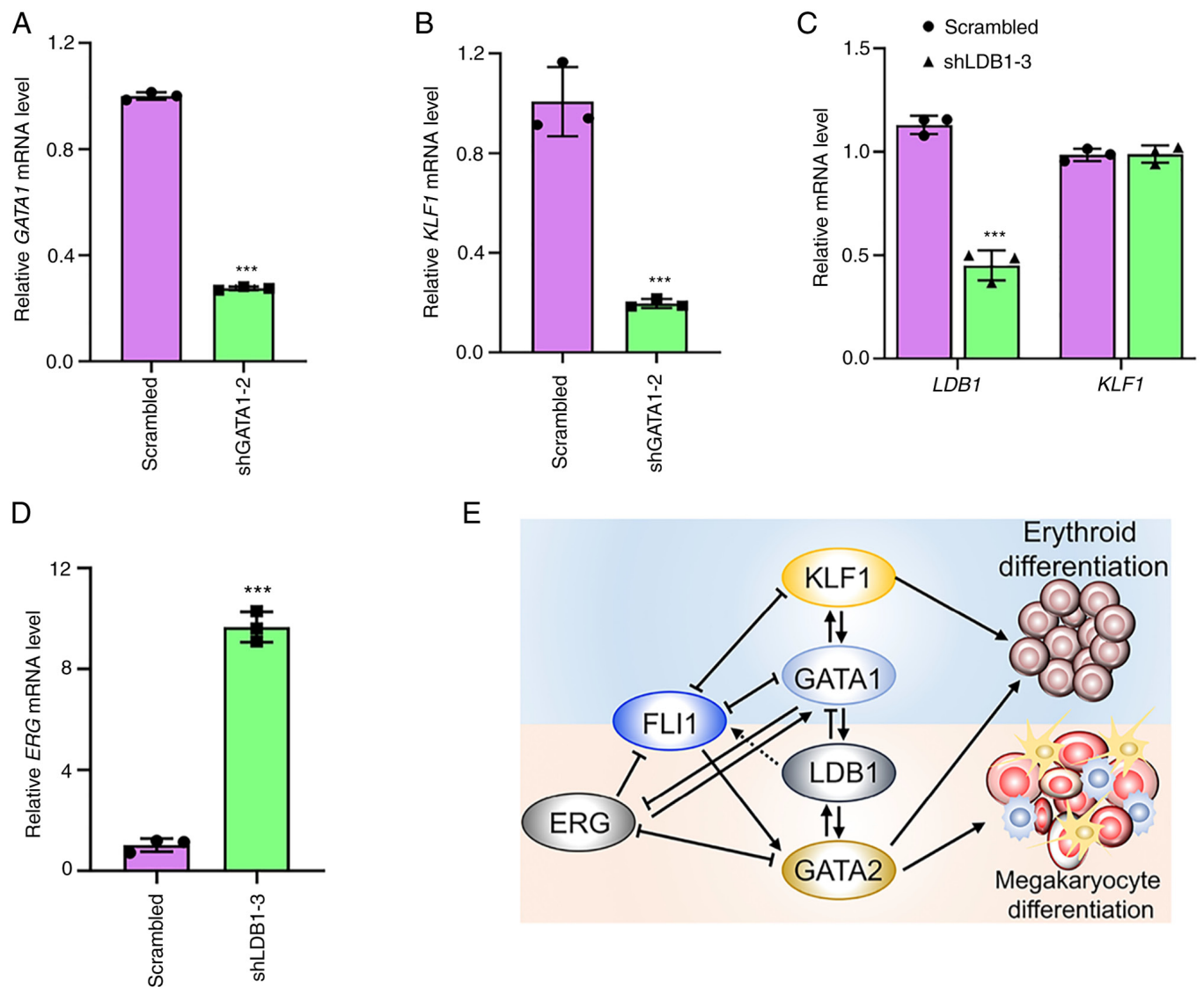


Figure 7. KLF1 transcription regulated by FLI1 controls erythroid differentiation. (A,B) The expression of (A) GATA1 and KLF1 (B) in shGATA1-2 cells as determined by RT-qPCR. (C,D) The expression of (C) KLF1 and (D) ERG in shLDB1-2 cells, as determined by RT-qPCR. (E) The intricate regulatory circuit of FLI1 and other transcription factors leading to erythroid and megakaryocytic differentiation in erythroleukemia HEL cells. Depicted model shows that FLI1 loss through activation of GATA1 induces erythroid differentiation. FLI1 loss suppresses GATA2 transcription, leading to reduce megakaryocytic differentiation. Loss of GATA1 and GATA2 activates ERG which in contrast to FLI1, blocks megakaryocytic differentiation. LDB1, through negative regulation by FLI1, plays a critical during erythroid or megakaryocytic differentiation. Dotted line shows indirect regulation. \*\*\* $P < 0.001$ . KLF1, KLF transcription factor 1; FLI1, friend leukemia integration 1; GATA, GATA binding protein; sh, short hairpin; RT-qPCR, reverse transcription-quantitative PCR; ERG, ETS transcription factor ERG; ETS, E26 transformation-specific; CD, cluster of differentiation.

genes in promoting erythroid differentiation. Accordingly, shRNA-mediated downregulation of LDB1 resulted in reduced expression of erythroid differentiation markers, suggesting a positive role for LDB1, and probably LMO2, in erythroid differentiation. A study by Giraud *et al* (24) reveals that interaction between FLI1 and LDB1 is critical to activate megakaryocytic genes in erythroleukemic cells. Notably, in the erythroleukemic cells of the present study, LDB1 knockdown induced both downregulation of FLI1 and its target GATA2, two essential factors for megakaryocytic differentiation. Accordingly, LDB1 ablation resulted in suppression of megakaryopoiesis associated with downregulation of CD41a/CD61 markers and expression of MEIS1. This result points to LDB1 and GATA2 as positive regulators of megakaryocytic differentiation, controlled by FLI1. The LDB1 ablation experiments also revealed that

a certain threshold level of LDB1 expression enables FLI1 to block erythroid differentiation. While LDB1 itself is not a transcription factor, it may affect FLI1, ERG and other factors through protein-protein interactions. Indeed, LDB1 has been reported to interact with FLI1 in erythroleukemic cells (24). Moreover, FLI1 inhibition is reported to regulate its own transcription (50). Overall, combination of transcription factors and LDB1 can affect the fate of MEP towards either erythroid or megakaryocytic differentiation. These factors together create a complex network due to protein-protein interaction that can affect the fate of MEP that may need further analysis in future studies.

Finally, the transcription factor KLF1, a master regulator of erythroid differentiation (36-39), is confirmed in the present study to be negatively regulated by FLI1 and positively by

GATA1, KLF1 and GATA1 expression both cooperate to actuate erythroid gene expression in erythroid cells (39). Notably, in LDB1 knockdown cells, while the levels of GATA1 were significantly high, these cells lost commitment to erythroid differentiation. As FLI1 complex with LDB1 is critical for megakaryopoiesis (24), this interaction may also control commitment of progenitor cells to the erythroid lineage, a notion that should be addressed in future studies. While different complex binding of these factors is known to be critical for commitment of MEPs to different lineages, the present study provided a new perception into the regulatory circuit that fine tuning the level of these transcription factors required during erythroid and megakaryocytic differentiation.

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### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

CW, MH, WL, YK, AH, KY, LH and XX contributed to the conception, design of the study as well as data acquisition and interpretation. CW performed the experiments and wrote the manuscript. CW, MH were involved in data analysis and statistics. YB, EZ, XX and BG contributed to the conception, design of the study as well as reviewing the manuscript critically. XX and YB confirm the authenticity of all the raw data. YB supervised and designed the study. All authors contributed to interpretation of findings, reviewed and edited the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable

### Patient consent for publication

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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