SUPPLEMENTARY APPENDIX

Loss of Forkhead box M1 promotes erythropoiesis through increased proliferation of erythroid progenitors

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Supplemental Data

Supplemental Methods

Lentiviral transduction

The shRNA constructs targeting human FOXM1 were cloned into a pLVTH-GFP vector. Target sequences are listed in Supplemental Table S4. Full-length cDNA encoding human FOXM1 (isoform 2) was obtained by RT-PCR from HEK 293 cells and cloned into pCHD-GFP or pCHD-mCherry vectors. The FOXM1 point mutants were generated from the wild-type mCherry-FOXM1 vector by site-directed mutagenesis.

HEK 293 cells were transfected with lentiviral vectors by using CalPhos Mammalian Transfection Kit (Clontech) according to the manufacturer's instruction. After 3 days of transfection, culture media containing lentivirus were collected and concentrated with ultracentrifugation through a 10% sucrose solution. Viruses were resuspended in 1x Hanks' Balanced Salt Solution (Corning) overnight at 4℃ and kept at -80℃ before use.

Primary CD34+ cells were transduced by spinoculation with lentivirus at a multiplicity of infection score of 10 after 24 hours in culture. Cells were sorted for GFP or mCherry after 5 days of culture using a BD FACSAida (BD Biosciences) and harvested for downstream assays as indicated in the results.

Flow cytometry

For cell surface flow cytometry, cells were washed with PBS and then incubated with indicated antibodies for 20 minutes at room temperature. After washing, cells were analyzed by FACS. Antibodies against CD71-PE (clone MA712;555537), CD71-APC (clone MA712;551373), CD71-BV421 (clone MA712;562995), CD71-APC-H7 (clone MA712;563671), GlyA-APC (clone GA-R2;551336), GlyA-PE-Cy7 (clone GA-R2;563666), CD33-PE-Cy5 (clone WM53;551377), CD11b-PE (clone ICRF44;555388), CD41a-PE (clone

HIP8;555467), CD34-PE (clone581;555822), and CD36-APC (clone CB38;550956) were purchased from BD Biosciences. Antibodies against CD11b-APC (clone ICRF44;17-0118042) and CD123-PE-Cy7 (clone 6H6;25-1239-42) were purchased from eBioscience. Antibody against CD11b-BV605 (clone ICRF44;301332) was purchased from BioLegend. Antibody against CD49d (Integrin alpha4)-PE (clone MZ18-24A9;130-093-282) was purchased from Miltenyl Biotec. Antibody against Band3-APC was kindly provided by Dr. Xiuli An at NYBC.

For intracellular flow cytometry, cells were fixed in 3.2% paraformaldehyde for 10 minutes at 37℃, and permeabilized with 100% methanol for 30 minutes at -80℃. After washing, cells were incubated with indicated antibodies and then analyzed by FACS. Antibody against FOXM1 (ab55006) was purchased from Abcam. Antibodies against p53 (1C12:Alexa Fluor 647 Conjugate:2533) and H3P-ser10 (11D8: Alexa Fluor 647 Conjugated:650805) were purchased from Cell Signaling and BioLegend, respectively.

Data were collected on a FACS Calibur (BD Biosciences) or a DxP10 (Cytek) flow cytometer and analyzed using FlowJo Software (v.10).

RT-qPCR

Total RNA was extracted by using TRIzol reagent (Invitrogen). 500 ng of total RNA was transcribed into first strand cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Real time qPCR reaction was run with iQ^{TM} SYBR Green MasterMix (Bio-Rad) using the CFX384 Touch^{TM} Real-Time PCR Detection System (Bio-Rad). *7SL scRNA* was used as an internal control. Fold change of mRNA was calculated using the $\Delta\Delta C_t$ method. All primer sets used are indicated in Supplemental Table S5.

Immunoblot analysis

Cells were resuspended with RIPA buffer (50 mM Tris-Cl(pH8.0), 150 mM NaCl, 0.1% SDS, 0.5% Deoxycholate, 1% NP-40, 1 mM EDTA, protease inhibitors). Total cell lysates were

resolved in SDS-polyacrylamide gels and transferred to polyvinylidene flouride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST, and primary antibody was bound overnight at 4℃. After washing with TBST, horseradish peroxide (HRP)-conjugated secondary antibody (Bio-Rad) was bound for 1 hour at room temperature. Immunoreactive signals were detected with WesternBright ECL HRP substrate (Advansta).

Antibodies against FOXM1 (clone D12D5;5436), GATA1(clone D52H6;3535), CHK2 (clone D9C6;6334), CDK2 (clone 78B2;2546), MEK1/2 (clone L38C12;4694) and PLK1 (clone 208G4;4513) were purchased from Cell Signaling. Antibody against β-Actin (A5316) was purchased from Sigma-Aldrich and antibody against p21(clone C-19;sc-397) was purchased from Santa Cruz Biotechnology.

Cytospin and Wright-Giemsa staining

$1x10^5$ sorted cells in 500 μl were used to prepare cytospin preparations on coated slides, using the Shandon Southern Cytospin Centrifuge. The slide were stained with Wright-Giemsa solution for 5 minutes, then stained with 25:1 of Phosphate buffer (pH6.8) colorant Selon Giemsa (Harleco) for 10 minutes, then stained with 6:1 of Phosphate buffer (pH6.8):Wright-Giemsa solution for 1 minute, and finally rinsed in water. The cells were imaged using Leica DMLB microscope and INSIGHT SPOT2 software.

Statistical analysis

Data are presented as mean ± s.d. *P* values for statistical significance were obtained using an unpaired Student *t*-test. $P < 0.05$ was considered significant. The data are representative of at least two independent experiments.

Table S1. 3 Phase Liquid Culture System

Basic media	0 ~5 days	6 ~ 10 days	11 days ~
x-Vivo15	50ng/ml SCF	50ng/ml SCF	50ng/ml SCF
10% FBS	50ng/ml TPO	50ng/ml TPO	50ng/ml TPO
1x PSG	50ng/ml FLT-3	50ng/ml FLT-3	50ng/ml FLT-3
	20ng/ml IL-6	20ng/ml IL-6	20ng/ml IL-6
	20ng/ml IL-3	20ng/ml IL-3	20ng/ml IL-3
		3U/ml EPO	3U/ml EPO
		250μg/ml Transferrin	1mg/ml Transferrin

Table S2. Erythroid Medium

Basic media	0 ~5 days	6 ~ 10 days	11 days ~
x-Vivo15	50ng/ml SCF	50ng/ml SCF	50ng/ml SCF
10% FBS	20ng/ml IL-3	20ng/ml IL-3	20ng/ml IL-3
1x PSG	0.5U/ml EPO	3U/ml EPO	3U/ml EPO
		250μg/ml Transferrin	1mg/ml Transferrin

Table S3. Myeloid Medium

Basic media	0 day~
x-Vivo15	50ng/ml SCF
10% FBS	50ng/ml TPO
1x PSG	15ng/ml G-CSF
	20ng/ml GM-CSF

Table S4. shRNA Sequences

shRNA	Target Sequence
shLuc	5'- CGCTGAGTACTTCGAAATGTC-3'
shFOXM1(1)	5'- GGACCACTTTCCCTACTTTAA-3'
shFOXM1(2)	5'- GCAAGATCCTGCTGGACAT-3'
shRPS19(1)	5'- CTACGATGAGAACTGGTT CTA-3'

Table S5. Primer Sequences for RT-qPCR

Gene	Forward	Reverse
7SL scRNA	5'-ATCGGGTGTCCGCACTAAGTT-3'	5'-CAGCACGGGAGTTTTGACCT-3'
FOXM1	5'-ATACGTGGATTGAGGACCACT-3'	5'-TCCAATGTCAAGTAGCGGTTG-3'
CD71	5'-GCTGCTTTCCCTTTCCTTGC-3'	5'-CTGCTCGTGCCACTTTGTTC-3'
GlyA	5'-TACGCACAAACGGGACACATA-3'	5'-TCGTTCCAATAACACCAGCCA-3'
CD33	5'-GGTGTGACTACGGAGAGAACC-3'	5'-GGTAGGGTGGGTGTCATTCC-3'
CD11b	5'-ACTTGCAGTGAGAACACGTATG-3'	5'-TCATCCGCCGAAAGTCATGTG-3'
CD41a	5'-GATGAGACCCGAAATGTAGGC-3'	5'-GTCTTTTCTAGGACGTTCCAGTG-3
p21	5'-ATCCCGTGTTCTCCTTT-3'	5'-GCTGGCATGAAGCC-3'
WIG-1	5'-AGAAGCCTTTTGGGCAGGAG-3'	5'-TGCTGCATAGTAATTTCGGAGTT-3'
BAX	5'-TGACATGTTTTCTGACGGCAAC-3'	5'-GGAGGCTTGAGGAGTCTCACC-3'

GADD45A	5'-GAGAGCAGAAGACCGAAAGGA-3'	5'-CAGTGATCGTGCGCTGACT-3'
GATA-1	5'-CCTCATCCGGCCCAAGAAG-3'	5'-CCATCCTTCCGCATGGTCAG-3'
TNF-α	5'-CCCAGGGACCTCTCTCTAATC-3'	5'-AGCTGCCCCTCAGCTTGAG-3'
CDC25B	5'-ACGCACCTATCCCTGTCTC-3'	5'-CTGGAAGCGTCTGATGGCAA-3'
CyclinD1	5'-TATTGCGCTGCTACCGTTGA-3'	5'-CCAATAGCAGCAAACAATGTGAAA-3'
CyclinB2	5'-CCGACGGTGTCCAGTGATTT-3'	5'-TGTTGTTTTGGTGGGTTGAACT-3'
CENPF	5'-ACCTTCACAACGTGTTAGACAG-3'	5'-CTGAGGCTCTCATATTCGGCA-3'
P27	5'-AACGTGCGAGTGTCTAACGG-3'	5'-CCCTCTAGGGGTTTGTGATTCT-3'

Figure S1.

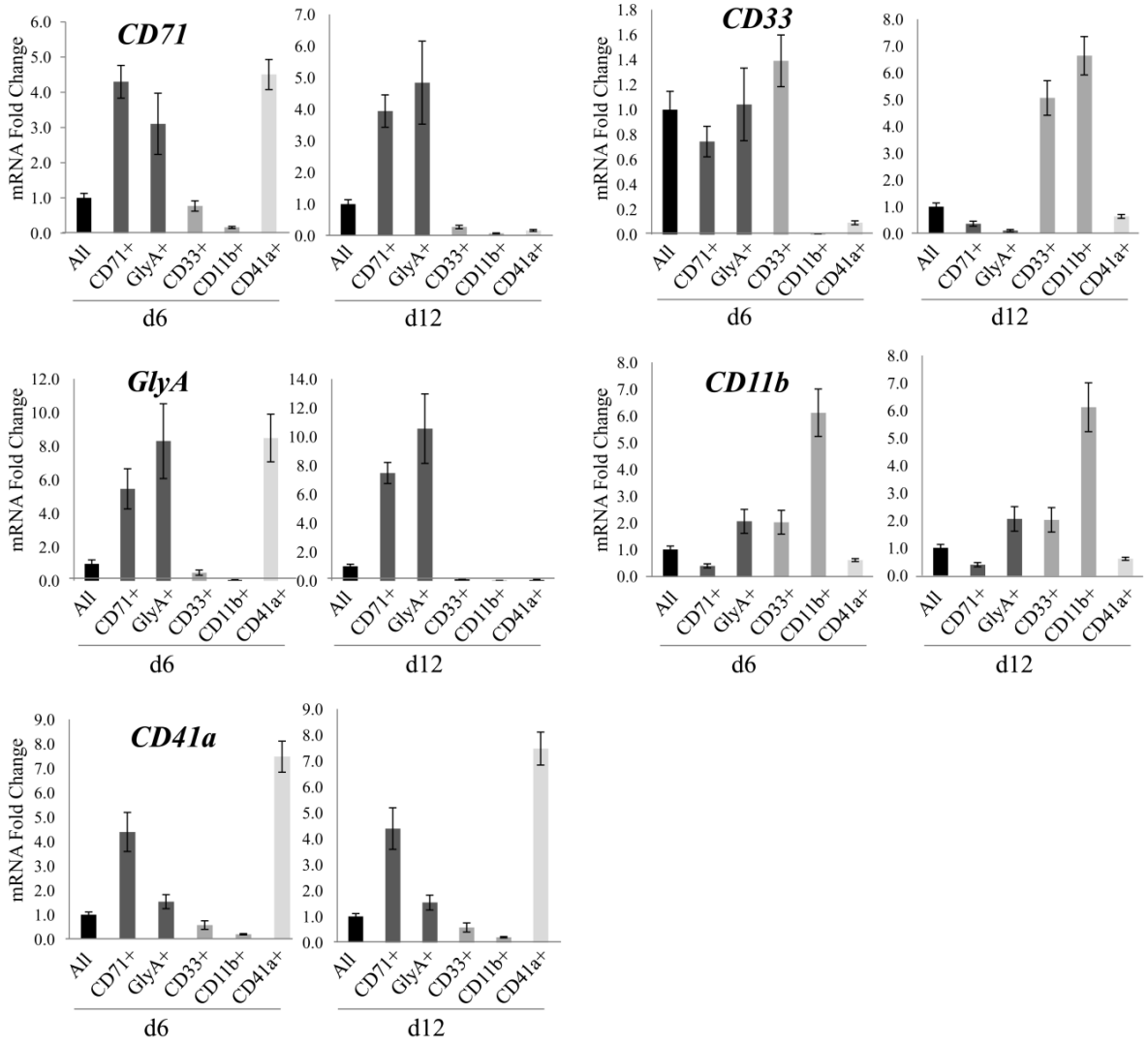


Figure S1. Each cell population expresses its specific cell surface marker. Human cord blood CD34+ hematopoietic progenitor cells were cultured for 6 days or 12 days. At the indicated days, cells were stained with each cell surface marker and sorted. RNA was collected from the indicated populations and analyzed by RT-qPCR.

Figure S2.

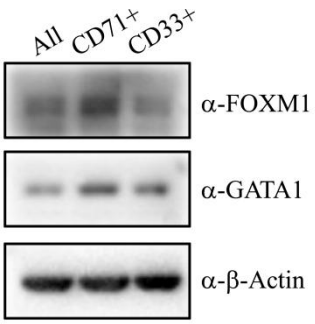


Figure S2. FOXM1 is highly expressed in erythroid progenitors. Human cord blood CD34+ cells were cultured *in vitro* in differentiation media. At day 6, cells were stained with CD71 or CD33 cell surface marker and sorted. Protein was collected from each population and analyzed by immunoblot analysis. GATA1 was used as an erythroid-specific marker protein. β-Actin was used as a loading control.

Figure S3.

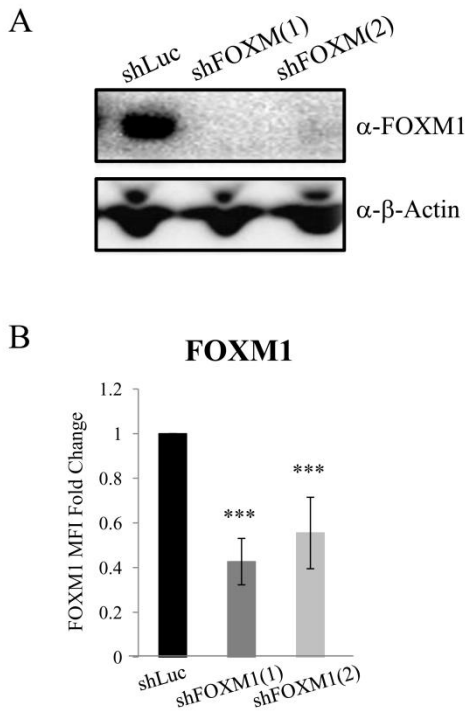


Figure S3. FOXM1 knockdown decreased FOXM1 protein level. (A) K562 cell line was transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Protein was collected and analyzed by immunoblot analysis at 5 days after transduction. β-Actin was used as a loading control. (B) Human cord blood CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. At 5 days after transduction, cells were stained with FOXM1 antibody and were analyzed by flow cytometry. ***P<0.001

Figure S4.

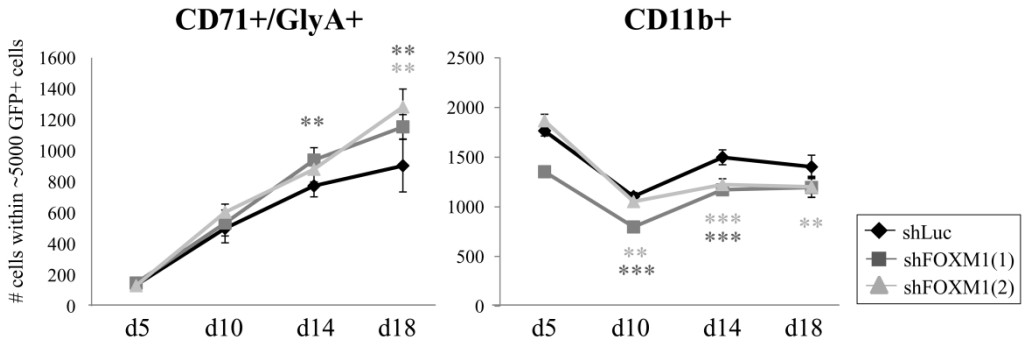


Figure S4. FOXM1 downregulation increases the erythroid population. Human cord blood CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Cells were analyzed for CD71, GlyA, and CD11b expression by flow cytometry at the indicated days after transduction. The numbers of cells expressing CD71, GlyA, and CD11b within about 5000 GFP+ cells are shown. **P<0.01, ***P<0.001

Figure S5.

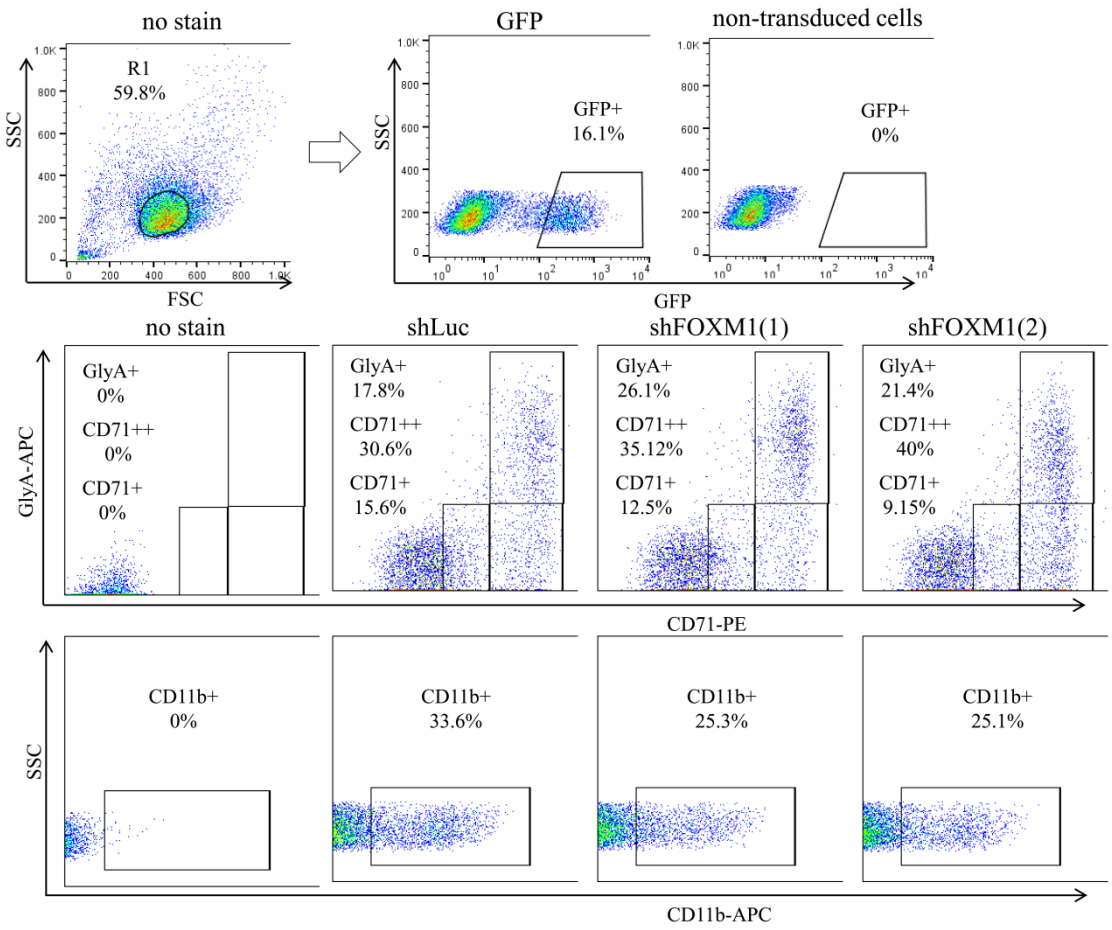


Figure S5. FOXM1 knockdown increases the CD71+/GlyA+ population and decreases the CD11b+ cell population. Human cord blood CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Cells were analyzed for CD71, GlyA, and CD11b expression by flow cytometry at 14 days after transduction. Sample flow plots are shown.

Figure S6.

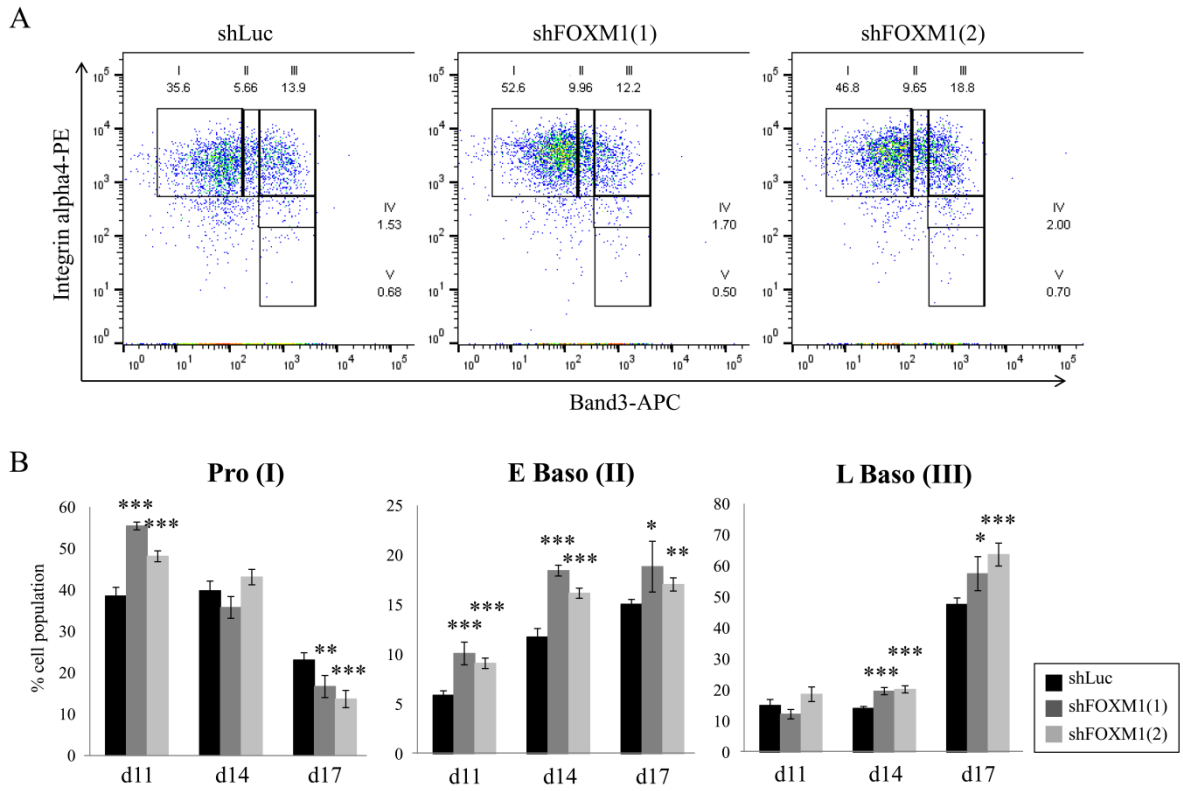


Figure S6. FOXM1 downregulation increases specific stages of later erythroid population. Human cord blood CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Cells were analyzed for GlyA, CD49d, and Band3 expression by flow cytometry at the indicated days after transduction. (A) Sample flow plots at 11 days are shown. (B) The percentages of each population are shown. *p<0.05, **p<0.01, ***p<0.001

Figure S7.

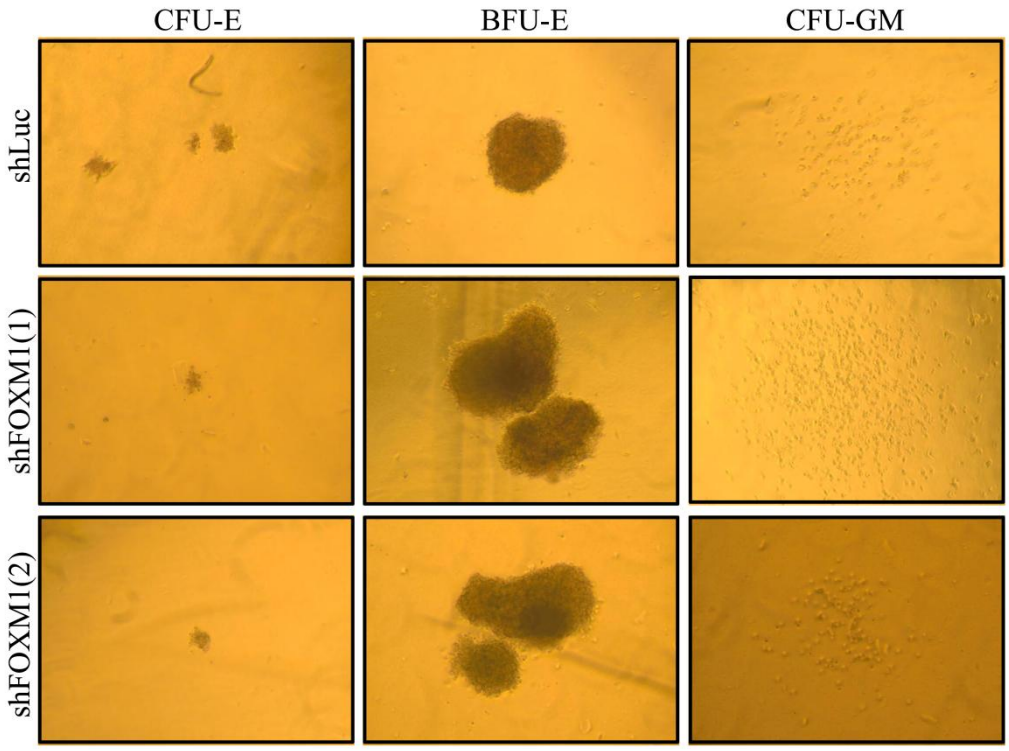


Figure S7. FOXM1 downregulation does not affect morphological changes of colonies. Human cord blood CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Cells were sorted for GFP+ at 5 days after transduction. 1000 cells of GFP+ cells were plated in methylcellulose media and cultured. Colonies were imaged using a phase contrast microscope at 5 x magnification.

Figure S8.

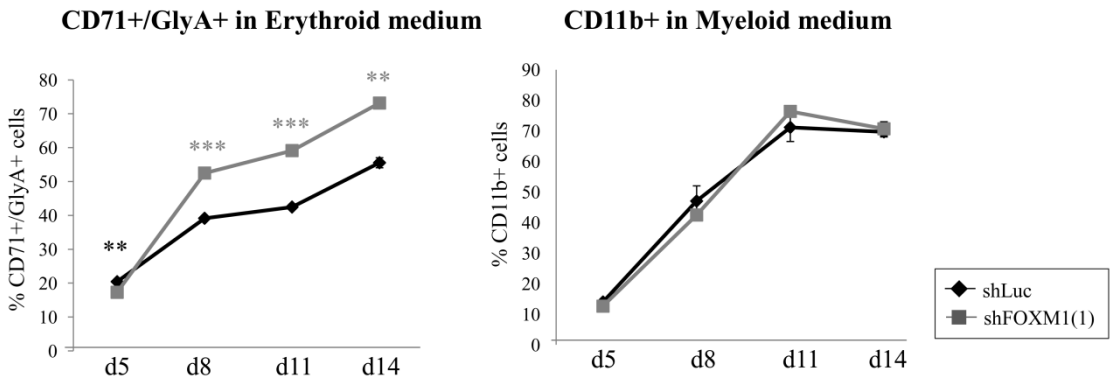
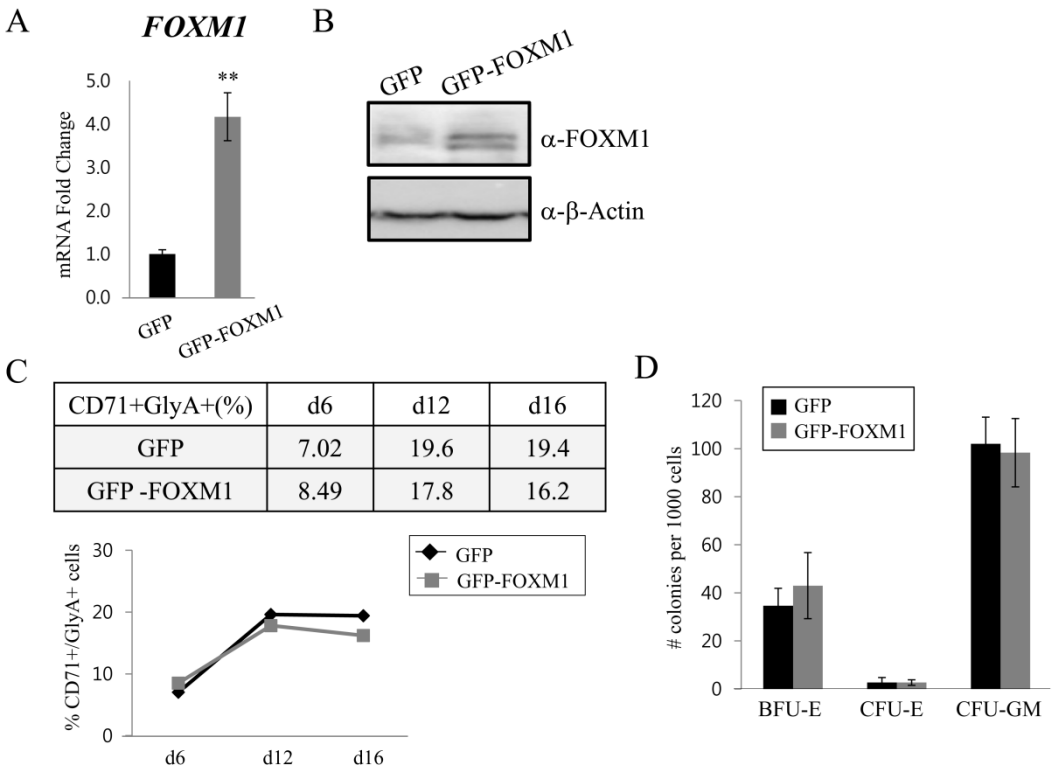


Figure S8. FOXM1 has the direct effect on erythroid cells. Human cord blood CD34+ cells were cultured in erythroid medium or myeloid medium. At 1 day after culture, cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control and were cultured for additional days in each media. Transduced cells were analyzed for CD71, GlyA, and CD11b expression by flow cytometry at the indicated days after transduction. **p<0.01, ***p<0.001

Figure S9.



CD71+GlyA+(%)	d6	d12	d16
GFP	7.02	19.6	19.4
GFP -FOXM1	8.49	17.8	16.2

Figure S9. FOXM1 overexpression does not affect erythroid differentiation. Human cord blood CD34+ cells were transduced with lentivirus carrying full-length human FOXM1 cDNA or GFP control. Cells were sorted for GFP+ at 5 days after transduction. (A) RNA was collected and analyzed by RT-qPCR. (B) HEK 293 cell line was transfected with FOXM1 cDNA or GFP control. Protein was collected and analyzed by immunoblot analysis at 5 days after transduction. β-Actin was used as a loading control. (C) Transduced cells were analyzed for CD71 and GlyA expression by flow cytometry at the indicated days after transduction. (D) 1000 cells of GFP+ cells were plated in methylcellulose media and cultured for 2 weeks. Colonies were counted by an investigator blinded to the conditions. **p<0.01

Figure S10.

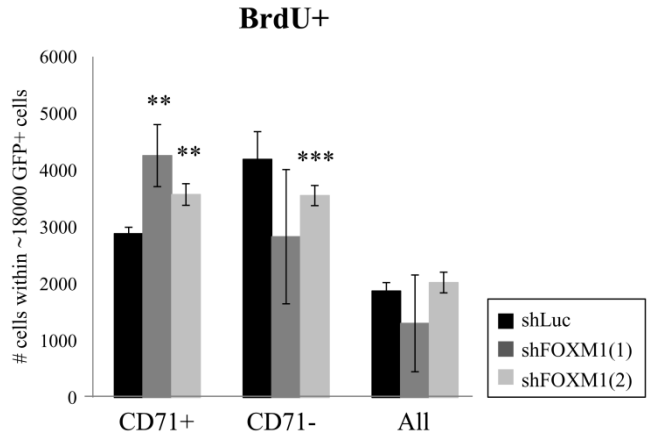


Figure S10. FOXM1 downregulation increases the proliferation of erythroid progenitors. Human cord blood CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Cells were sorted for CD71+/GFP+ (for CD71+ group), CD71-/GFP+ (for CD71- group), or GFP+ (for all group) cells at 5 days after transduction. Sorted cells were cultured for an additional 8 days. Cells were incorporated with BrdU, and then stained with BrdU and CD71 antibodies. Stained cells were analyzed by flow cytometry. The numbers of cells expressing CD71, GlyA, and CD11b within about 18000 GFP+ cells are shown. **P<0.01, ***P<0.001

Figure S11.

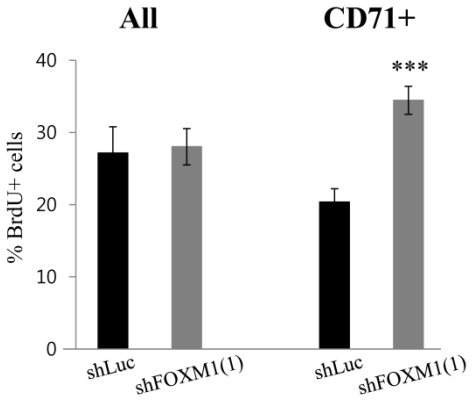


Figure S11. FOXM1 knockdown in erythroid progenitors increases BrdU+ population. Human cord blood CD34+ cells were cultured for 5 days and then sorted for CD71+. Sorted cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. After culturing an additional 8 days, cells were incorporated with BrdU, and then stained with BrdU. Stained cells were analyzed by flow cytometry. ***$p<0.001$

Figure S12.

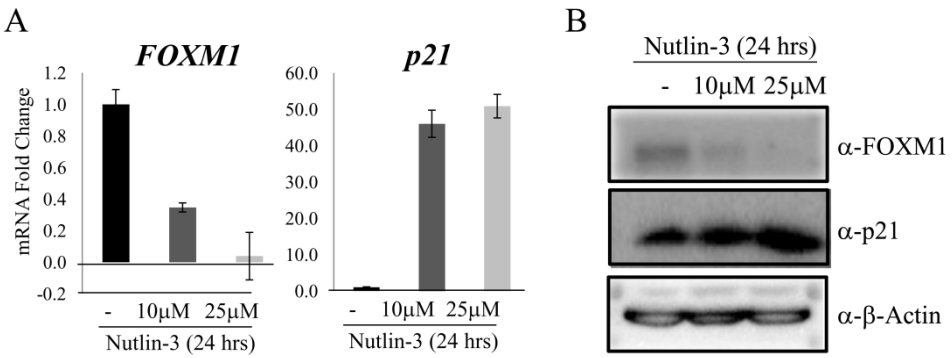


Figure S12. p53 negatively regulates FOXM1 expression. Human cord blood CD34+ cells were cultured for 4 days and then treated with Nutlin-3 (N6287, Sigma-Aldrich) for an additional 24 hours. (A) RNA was collected and analyzed by RT-qPCR. *p21* was used as a positive control. (B) Protein was collected and analyzed by immunoblot analysis. p21 was used as a positive control and β-Actin was used as a loading control.

Figure S13.

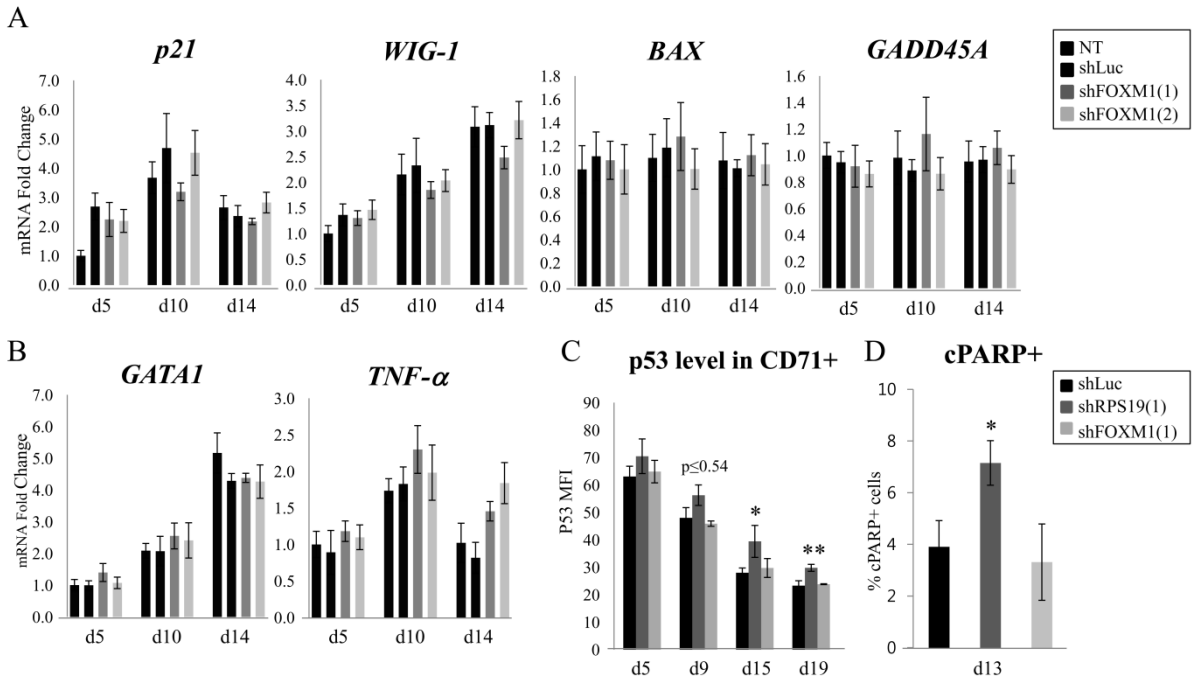


Figure 13. FOXM1 function on erythropoiesis is independent of the p53 pathway. Human cord blood CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. (A and B) Cells were sorted for GFP+ at the indicated days after transduction. RNA was collected and analyzed by RT-qPCR. (C) Transduced cells were stained with p53 and CD71 antibodies at the indicated days after transduction. Stained cells were analyzed by flow cytometry. RPS19 downregulation was used as a positive control. (D) Sorted cells were cultured for an additional 8 days. At 13 days post-transduction, cells were stained with cPARP antibody and analyzed by flow cytometry. RPS19 downregulation was used as a positive control. *p<0.05, **p<0.01

Figure S14.

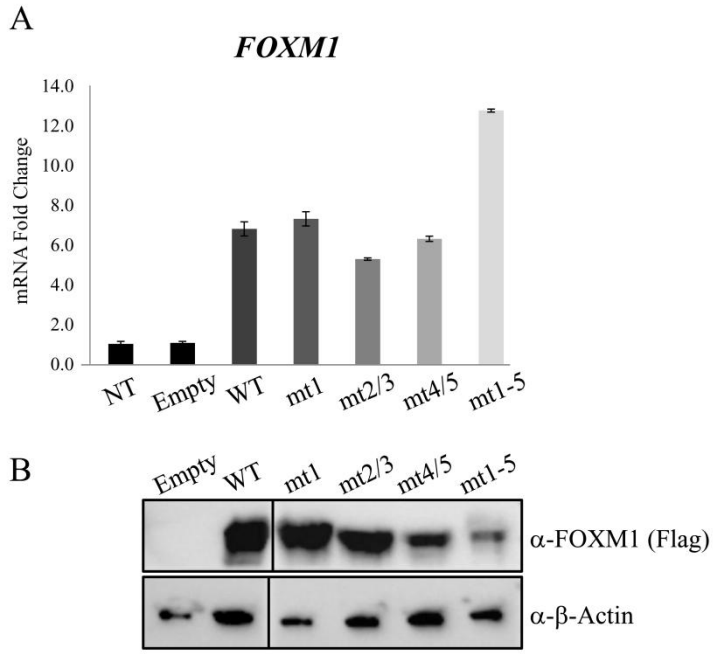


Figure S14. FOXM1 mutant mRNA and protein were expressed in human hematopoietic progenitor cells. (A) Human cord blood CD34+ cells were transduced with lentivirus carrying FOXM1 mutant cDNA. Cells were sorted for mCherry+ at 5 days after transduction. RNA was collected and analyzed by RT-qPCR. (B) HEK 293 cell line was transfected with FOXM1 mutant cDNA. Protein was collected and analyzed by immunoblot analysis at 3 days after transfection. β-Actin was used as a loading control.

Figure S15.

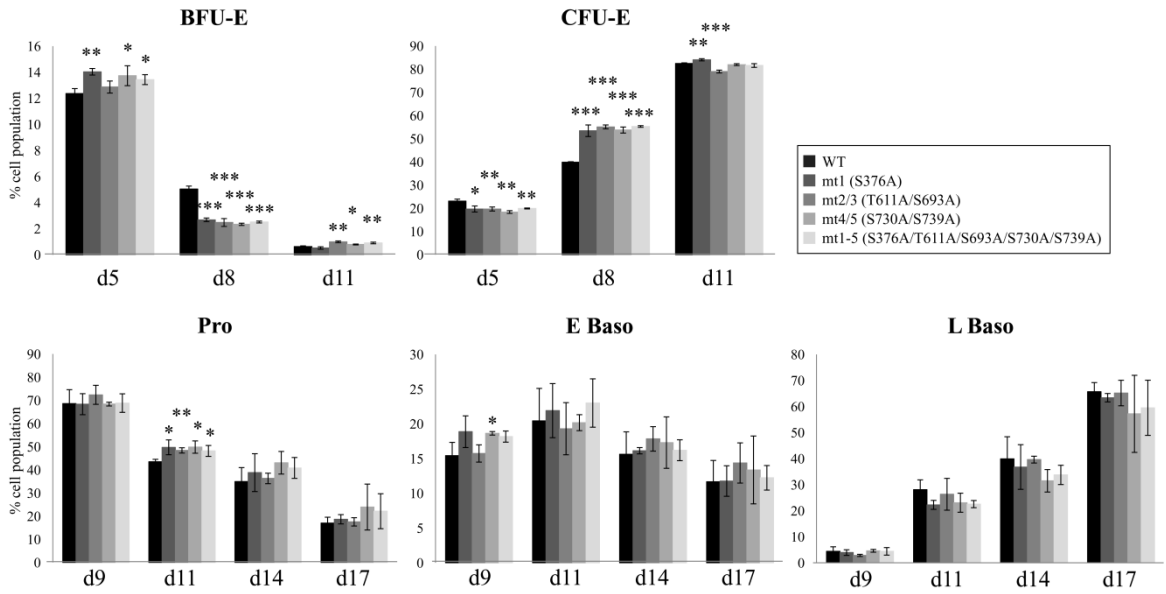


Figure S15. FOXM1 mutants increase specific stages of erythroid population. Human cord blood CD34+ cells were transduced with lentivirus carrying FOXM1 mutant cDNA. Cells were analyzed for CD123, CD34, and CD36 expression or for GlyA, CD49d, and Band3 expression by flow cytometry at the indicated days after transduction. The percentages of each population are shown. *p<0.05, **p<0.01, ***p<0.001

Figure S16.

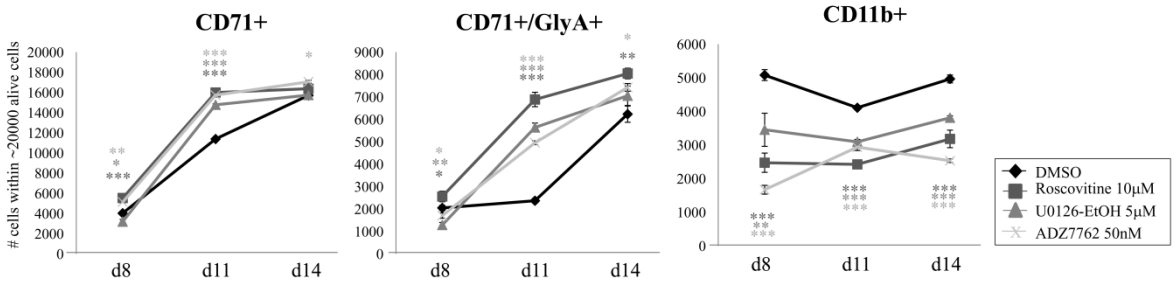


Figure S16. FOXM1 function on erythroid proliferation requires its phosphorylation by CHK2 or CDK1/2 kinases. Human cord blood CD34+ cells were treated with the each kinase inhibitor for 5 days. Cells were analyzed for CD71, GlyA, and CD11b expression by flow cytometry at the indicated days after washing out the drug. The numbers of cells expressing CD71, GlyA, and CD11b within about 20000 alive cells are shown. *p<0.05, **P<0.01, ***P<0.001

Figure S17.

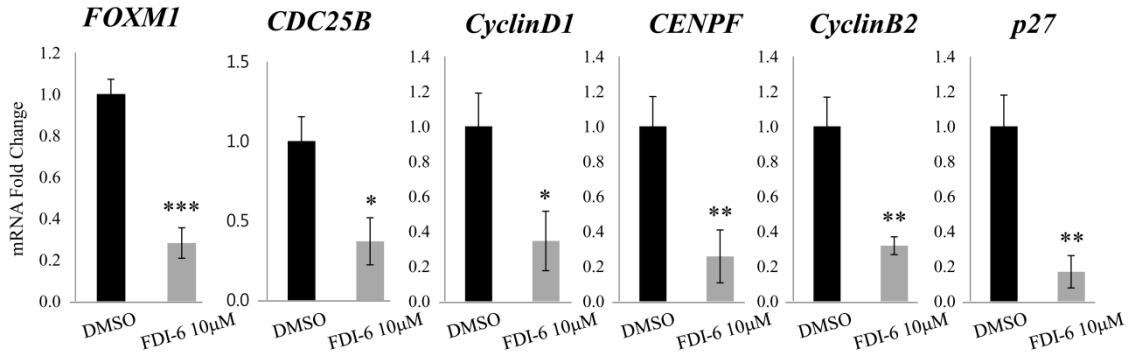


Figure S17. Expression of FOXM1 target genes was decreased by FDI-6 treatment. Human cord blood CD34+ cells were treated with FDI-6. Cells were collected at 5 days after treatment. RNA was collected and analyzed by RT-qPCR. *p<0.05, **p<0.01, ***p<0.001

Figure S18.

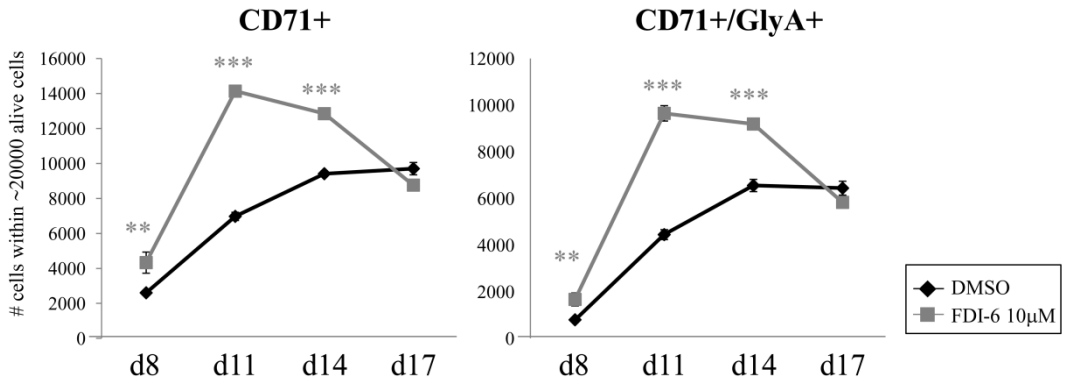


Figure S18. FDI-6 increases the erythroid population. Human cord blood CD34+ cells were treated with FDI-6 for 5 days, and then the drug was washed out. Cells were analyzed for CD71 and GlyA expression by flow cytometry at the indicated days after culture. The numbers of cells expressing CD71 and GlyA within about 20000 alive cells are shown. **P<0.01, ***P<0.001

Figure S19.

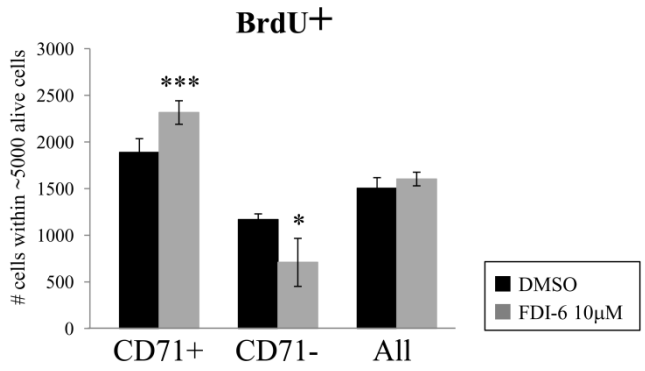


Figure S19. FDI-6 increases proliferation of erythroid progenitors. Human cord blood CD34+ cells were treated with FDI-6 for 5 days. Cells were sorted for CD71+ or CD71- cells at 5 days after drug treatment. Sorted cells were cultured for an additional 4 days in differentiation media. At 9 days post-culture, cells were incorporated with BrdU, and then stained with BrdU and CD71 antibodies. Stained cells were analyzed by flow cytometry. The numbers of BrdU+ cells within about 5000 alive cells are shown. *P<0.05, ***P<0.001

Figure S20.

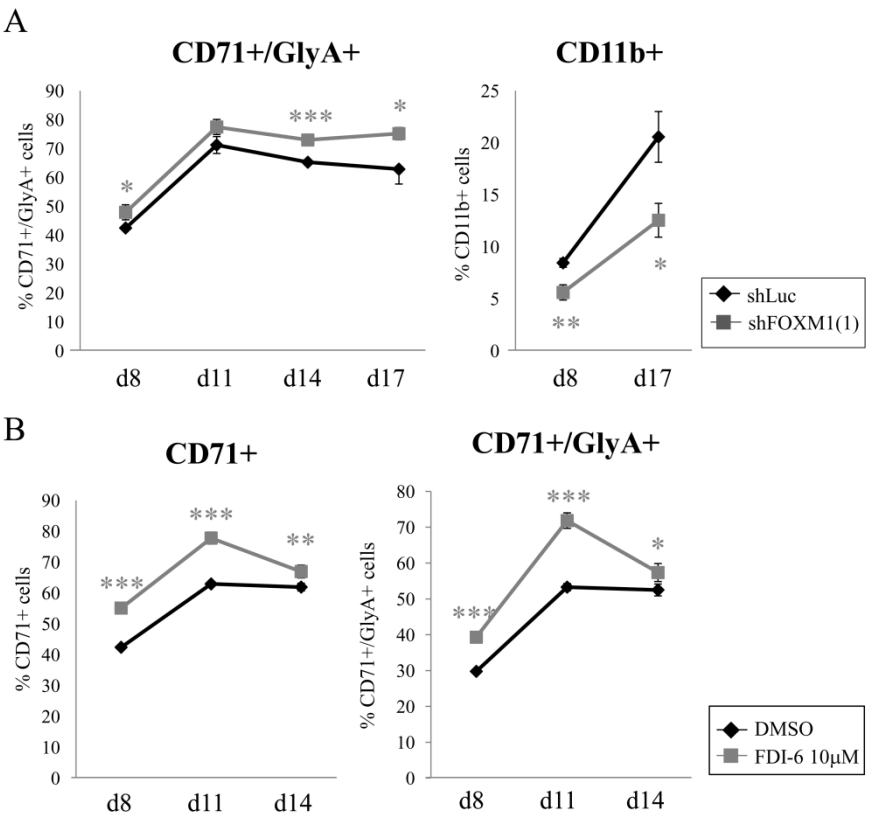


Figure S20. FOXM1 downregulation increases the erythroid population in human bone marrow CD34+ hematopoietic progenitor cells. (A) Human bone marrow CD34+ cells were transduced with lentivirus carrying shRNA against FOXM1 or Luc control. Transduced cells were analyzed for CD71, GlyA, and CD11b expression by flow cytometry at the indicated days after transduction. (B) Human bone marrow CD34+ cells were treated with FDI-6 for 5 days and then the drug was washed out. Cells were analyzed for CD71 and GlyA expression by flow cytometry at the indicated days after culture. *p<0.05, **p<0.01, ***p<0.001