SUPPLEMENTAL MATERIAL

Methods

Human Primary Erythroid Cell Differentiation Media. Following GFP sorting, CD34+ cells were incubated in IMDM supplemented with 20% fetal bovine serum, 10ng/ml of SCF, 1u/ml EPO, 1ng/ml IL-3, 1μM Dexamethasone, 1μM Estradiol, and 2% Penicillin/Streptomycin for three days. The cells were then changed to a differentiation media consisting of IMDM supplemented with 20% fetal bovine serum, 1u/ml EPO, 10ng/ml Insulin, and 2% Penicillin/Streptomycin and cultured for a period of 10 days. Cells were spun down and fresh differentiation media was added every other day.

Co-Immunoprecipitation Assays. Immunoprecipitation assays with 10 million CID cells per group were carried out using a commercial kit (SIGMA, FLAG Immunoprecitipation kit cat# FLAGIPT1); rProtein G beads were purchased from Invitrogen (#15920-010). Prior to the experiment, the protein G beads were washed 4 times by spinning down at 500g for 30 seconds 4°C, discarding the supernatant and adding equal volume of 1X wash buffer (40μL of beads were used per reaction). 1mL of lysis buffer containing protease inhibitors was added per 10 million cells. Samples were vortexed and placed on a rotator at 4°C for a half an hour. The samples were then spun down at 1300g for 10 minutes at 4°C and the supernatant was used for pre-clearing as follows: 40μL of protein-G beads were added to each 1mL of supernatant, and samples were placed on a rotator for 2 hours. Samples were then spun at 5000g for 30 seconds at 4°C. 30μL of supernatant were then removed and stored in -80° as

input control. The remaining supernatant was then divided and incubated either with 15μg of MBD2 antibody (Santa Cruz) or 15μg of normal goat IgG. Samples were placed on a rotator overnight at 4°C, then washed 5 times by spinning at 5000g for 30 seconds 4°C, and re-suspended in 1mL of wash buffer. After adding 2x loading buffer samples were then boiled for 5 minutes, spun down at 13000rpm for 30 seconds at room temperature and supernatants were subjected to western blotting as described[25]. Antibodies used for western blot include MBD2 (Santa Cruz sc-1244), p66α (Upstate #07-365), HDAC-2 (Millipore #05-814), RbAp48 (abcam ab79416), MTA-2 (Santa Cruz sc-28731).

High Performance Liquid Chromatography (HPLC). 10 million human erythroid (CD235a and CD71 double positive) cells were collected from either control shSCR or shMi2β treated cultures at day 10 of differentiation. Cells were spun at 300xg for 5 minutes, lysed, and hemolysates were subjected to HPLC for hemoglobin variant testing in the VCUHS Clinical Hematology Laboratory under CLIA certified assay conditions.

ChIP Assay Coordinates. DNA was sonicated sufficiently to obtain an average fragment size of ~700bp. Primers used to detect Mi2β enrichment at the BCL11A and KLF1 promoter region/first exon are included in Table S1. BCL11A primers were designed to generate PCR products that extend from +134bp to +337bp relative to the transcriptional start site. KLF1 primers were designed to generate PCR products that extend from -476bp to -399bp relative to the transcriptional start site.

KLF1 over-expression. The KLF1 expression vector (pSG5/KLF1) was a kind gift from Dr. James Bieker. 5 x 10^6 CID cells were transfected with 5μg of plasmid and 1 μMolar Mi2β siRNA (QIAGEN) via nucleofection (Lonza) as previously described. Cells were collected at 24 hours in order to test KLF1 expression levels, compared to a scramble control and again at 72 hours in order to test the effect on γ-globin RNA level by qPCR.

Antibodies. Antibodies used for Western blots include MBD2 (Santa Cruz, Dallas, TX, USA, sc-1244), CHD4/Mi2β (Abcam, Cambridge, MA, USA ab54603), KLF1/EKLF (Abcam ab88417), FOG-1 (Santa Cruz sc-9361), BCL11A (Abcam ab19487). Antibodies used for fluorescence activated cell sorting (FACS) profiling include anti-human CD71 (eBioscience, San Diego, CA, USA #17-0719-42) and Anti-human CD235a (eBioscience #12-9987-82). Antibodies used for ChIP assays consisted of 7μg of either ChIP-grade Mi2β antibody (Abcam ab70469), or Normal Mouse IgG (Millipore #12-371).

Table S1

	Forward	Reverse	Probe
Human γ-globin	GTG GAA GAT GCT GGA GGA GAA A	TGC CAT GTG CCT TGA CTT TG	FAM/AGG CTC CTG GTT GTC TAC CCA TGG ACC /BHQ
Human β-globin	GCA AGG TGA ACG TGG ATG AAG T	TAA CAG CAT CAG GAG TGG ACA GA	FAM/CA GGC TGC TGG TGG TCT ACC CTT GGA CCC
Murine α-globin	AAT ATG GAG CTG AAG CCC TGG	ACA TCA AAG TGA GGG AAG TAG GTC T	
Murine glycophorin A	GCC GAA TGA CAA AGA AAA GTT CA	TCA ATA GAA CTC AAA GGC ACA CTG T	FAMTTGACATCCAATCTCCTGA GGGTGGTGA /BHQ
Murine MBD2	TTT GAC TTC AGG ACC GGC AAG ATG	ATT GCT CGG GTG GTT CGT GAA TTT	
Murine Mi2β	GAA CCA CAG GGA GTT AAT GAG	CTT ATA GAG GGA GTA GAG GAA GAC	
Murine Mi2α	GAT GAA GAC TTT GAT GAG CGT	ACA CAT AGG CCT TAA ACT CCT	
Murine α-1- spectrin	TTA GCA CCA CAT ACA AAC AC	AAA CAT ATC CTT TCC TCC CTG	
Murine aminolevulinate dehydratase (Alad)	GAG TTC CCA AGG ATG AAC AG	CTC CTC TGC TAG GAA TGC TC	
Murine Ferrochelatase	GCG AGG TGG TCA TTC TGT TT	ACT GGA CCA ACC TTG GAC TG	
Murine erythropoietin (EPO) Receptor	CCC AAG TTT GAG AGC AAA GC	TGC AGG CTA CAT GAC TTT CG	
Murine transferrin Receptor	ATA AGC TTT GGG TGG GAG GC	CTT GCC GAG CAA GGC TAA AC	
Murine GATA-1	CTG GGA TCG CCT ACA ACC TC	CTG CCA CAA GGT CAA GGC TA	
Murine glucose-6-phosphate dehydrogenase (G6PD)	TCG AAA TTG TAG GGG CAG CG	CCA TTT AAC GCA AGA GGG CG	
Murine uroporphyrinogen III synthase (Uros)	GCT GCC TCT TCT CTT TCC GT	TGC ATG CTT TCC ATG GGG AT	
Murine cyclophilin A	GAG CTG TTT GCA GAC AAA GTT C	CCC TGG CAC ATG AAT CCT GG	
Murine FOG-1	TTC TGG TGG ACT GCT ATA TGT G	AAG ACG TCC TTG TTG ATG AC	
Human KLF1	GCA AGA GCT ACA CCA AGA G	GTG TTT CCG GTA GTG GC	
Human γ-globin promoter	GCC TTG ACC AAT AGC CTT GAC A	GAA ATG ACC CAT GGC GTC TG	
Human Mi2β	ATA AAG ATA AGC CAT TGC CTC C	GAC ATA TGC CTT GAA CTC TTT CTC	
Human BCL11A	AAC CCC AGC ACT TAA GCA AA	GGA GGT CAT GAT CCC CTT CT	
Human BCL11A Promoter	TCC TTC TTT CTA ACC CGG CTC	CTG CGC GCT CTC GTG ATT AT	
Human GAPDH Promoter	TCC CCT TCC TGC AGA CAG CTC C	AGG GAG GGC AGC ATA CCG GG	
Human KLF1 Promoter	GCC TGG GCC CCC ACC TGA TA	GAC TTG GCA CGA GCT CCC CG	

Table S1. Primers used for all experiments described in the methods section.

Table S2

shSCR

Procedure:	Ref Range:	Units:	
Hemoglobin A	[95.0-99.0]	%	95.6
Hemoglobin A2	[1.2-3.0]	%	3.1
Hemoglobin F	**[0.0-2.0]**	**%**	**1.3**
Mi2β mRNA		**%**	**100**

shMi2β

Procedure:	Ref Range:	Units:	
Hemoglobin A	[95.0-99.0]	%	84.8
Hemoglobin A2	[1.2-3.0]	%	2.0
Hemoglobin F	**[0.0-2.0]**	**%**	**13.2**
Mi2β mRNA		**%**	**74.8**

Table S2

Mi2β knockdown increases HbF levels in human primary erythroid cells.

Results obtained by high performance liquid chromatography assay carried out in the VCUHS CLIA approved clinical hematology laboratory showing a >10-fold increase in HbF after Mi2β knockdown (shMi2β) compared to scramble control (shSCR). qPCR assay showed the level of knockdown in the shMi2β treated primary erythroid cells was ~25%.

Figure S1

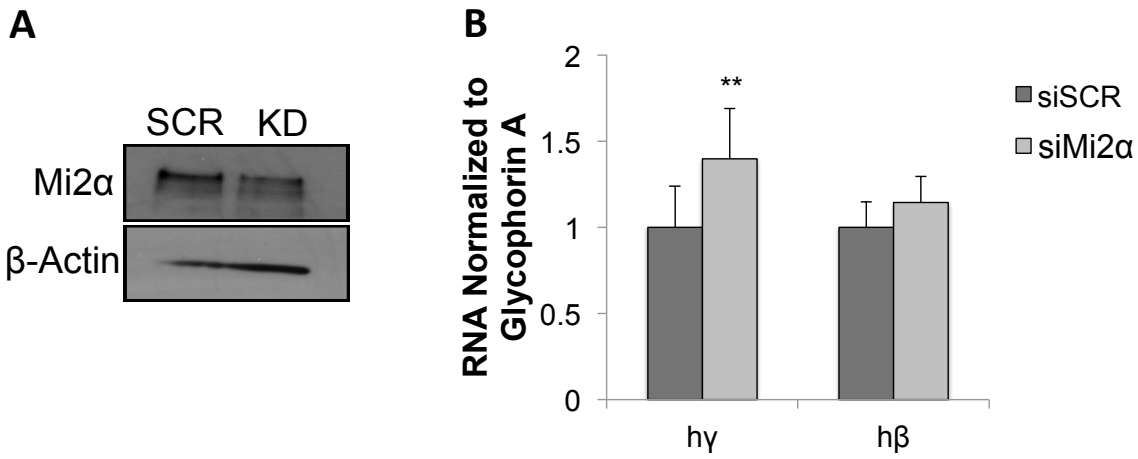

Figure S1

Mi2α knockdown exerts only a small effect on γ-globin gene expression in CID cells.

(A) Western blot showing Mi2α protein level knockdown in CID cells. (B) qPCR data showing Mi2α knockdown leads to a ~1.4-fold induction of γ-globin (hγ) gene expression and no significant increase in β-globin (hβ) gene expression. **signifies $p < 0.02$ according to the student's t-test.

Figure S2

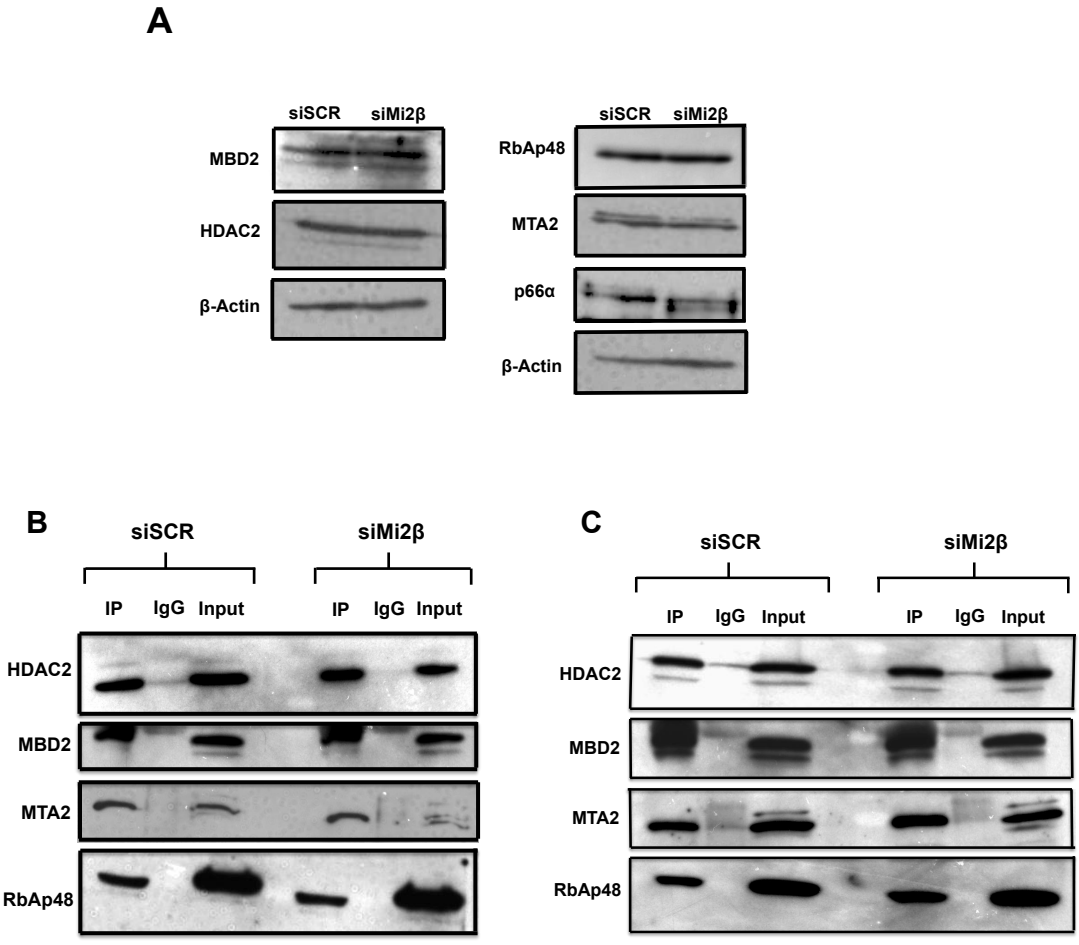

Figure S2

Mi2β knockdown does not lead to destabilization of the MBD2/NuRD complex.

(A) Western blot of CID cell extracts showing MBD2/NuRD complex components 72 hours after Mi2β knockdown. (B-C) Western blots showing MBD2/NuRD complex components following co-immunoprecipitation reactions with either MBD2 antibody or IgG control for samples transfected with either siMi2β or siSCR control. To account for the potential increased stability of preformed MBD2/NuRD complexes, experiments were carried out at both 24 hours (B) and 72 hours (C) post-transfection.

Figure S3

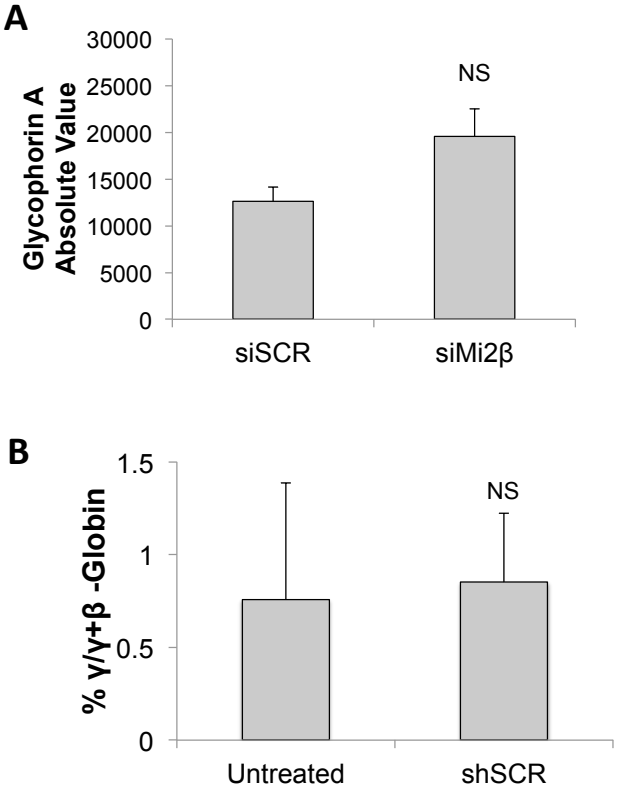

Figure S3

Glycophorin A levels are unchanged in CID cells upon Mi2β knockdown and similar low levels of γ-globin mRNA are present in untreated and Scramble shRNA treated human primary erythroid cells.

(A) Absolute values of Glycophorin A/5ng of RNA determined by qPCR show no significant difference between siScramble control and siMi2β samples in CID cells. (B) Untreated (no shRNA) cells show no significant difference than shSCR control in the expression of γ/γ+β after 10 days of erythroid differentiation in human primary erythroid cells. At this stage, cells show less than 1% γ-globin when normalized to γ+β-globin gene expression. NS = Not statistically significant.

Figure S4

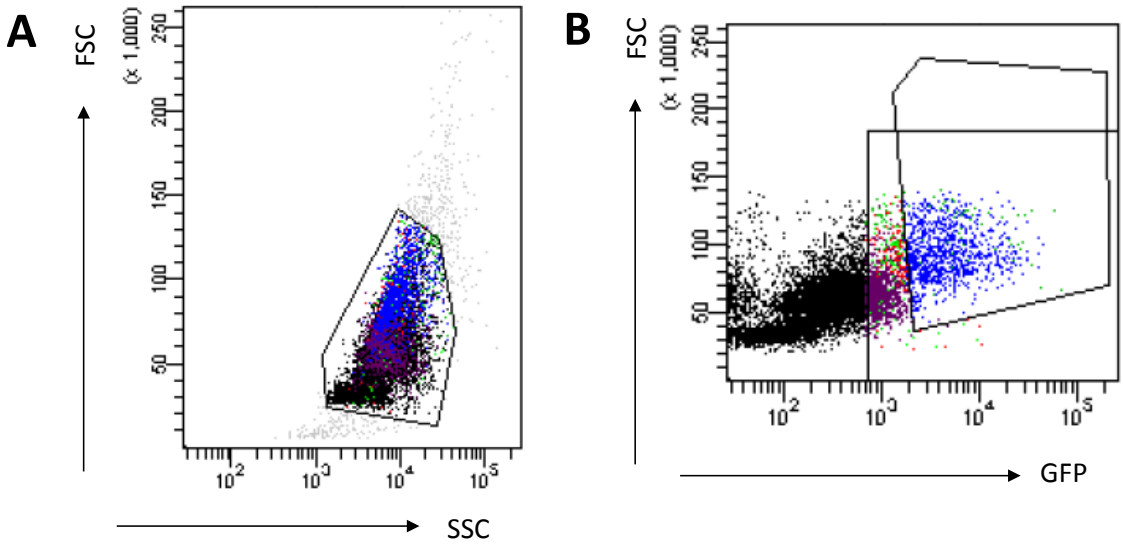

Figure S4

GFP expression in cells undergoing erythroid cell differentiation versus myeloid cell differentiation.

(A) Forward scatter vs. side scatter plots showing that differentiated erythroid cells, depicted in magenta (Figure 3H, Quadrant 2), are smaller in size than cells lacking erythroid markers depicted in blue (Figure 3H, Quadrant 3). (B) GFP expression levels showing that the larger cells (blue) have higher GFP than differentiated erythroid cells (magenta).

Figure S5

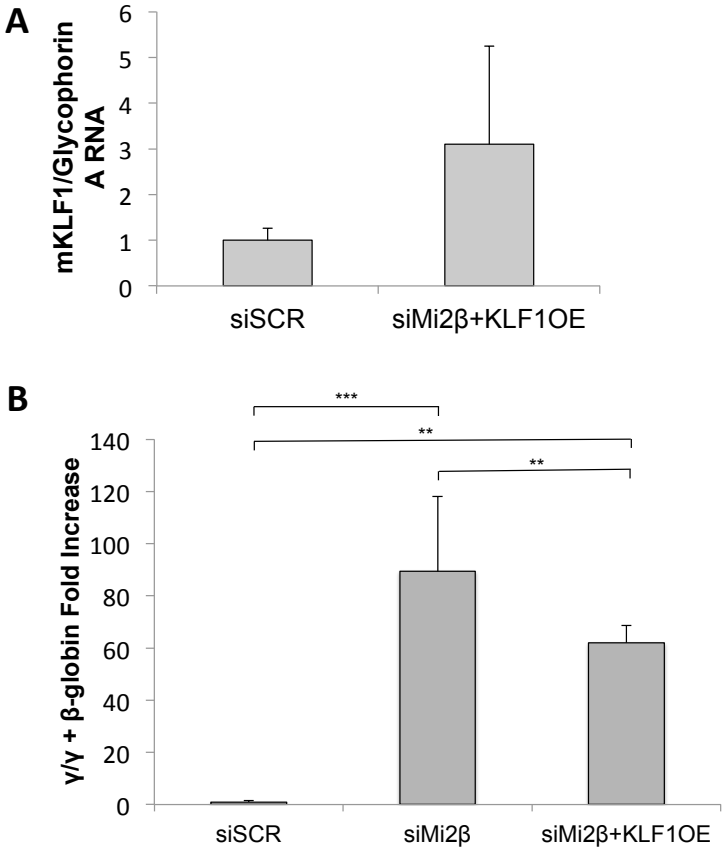

Figure S5

KLF1 overexpression significantly decreases γ/γ+β gene expression after Mi2β knockdown in CID cells.

(A) qPCR results showing that KLF1 RNA is expressed ~3-fold higher in samples transfected with Mi2β siRNA and KLF1 expression vector (siMi2β + KLF1 OE) than scramble control cells (siSCR) at 24 hours post-transfection. (B) qPCR data showing that enforced KLF1 expression in Mi2β knockdown cells leads to a significant decrease in γ/γ+β expression compared to Mi2β knockdown alone at 72 hours (62-fold induction vs. 89-fold induction). **signifies $p < 0.02$, and ***signifies $p < 0.001$ according to the student's t-test.