SUPPLEMENTARY APPENDIX

Hydroxyurea differentially modulates activator and repressors of γ-globin gene in erythroblasts of responsive and non-responsive patients with sickle cell disease in correlation with Index of Hydroxyurea Responsiveness

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Supplementary Methods, Figures, Tables and Files

Blood samples of SCD patients and normal donors: Peripheral blood samples of HU low/non responsive SCD patients on blood exchange every other month were obtained at the time of blood exchange when normal donor blood, after removal of nucleated cells including CD34+ cells, was transfused into the patient to replace patient erythrocytes. The nucleated blood cells in patient blood, after apheresis, were returned to the patient. Blood samples of normal donors were aphoresed, mononuclear blood cells no longer to be used for transplantation of cancer patients.

Isolation of CD34+ cells from peripheral blood samples: Peripheral blood samples (30 ml) of SCD patients were centrifuged at 300xg for 30 minutes. The buffy coat between the plasma and sedimented erythrocytes was removed and re-suspended in 15 ml PBS+2% fetal bovine serum (FBS) and layered over 15 ml Ficoll-Paque (density=1.077) in a 50 ml conical tube and centrifuged at 400xg for 30 min at 20^{o}C. The nucleated cells in the interphase were removed and washed twice in PBS+2% FBS and centrifuged at 200xg at 20^{o}C. The cells were then re-suspended in 300 μl IMDM medium and processed for isolation of CD34+ cells using CD34 MicroBead Kit (Miltenyi Biotec) according to vendor protocol. The aphoresed mononuclear cells of normal donors (2 ml), stored frozen at -80^{o}C in 2ml aliquots, were quick-thawed at 37^{o}C, dounced and strained through a cell mesh to remove clumped cells, and re-suspended in 30 ml PBS+2% FBS. The cell suspension was layered over 15 ml Ficoll-Paque as described above to isolate nucleated cells, which were similarly processed using CD34 MicroBead Kit (Miltenyi Biotec) to isolate CD34+ cells.

Ex vivo culture of CD34+ cells to Day 10-12 erythroblasts in the absence or presence of HU: The CD34+ cells, the Day 0 cells (~ 20-50x10^{3} cells) purified from 30 ml of peripheral blood, were re-suspended in 2ml IMDM containing 4mM L-glutamine and 1% penicillin-streptomycin, 15%FBS, 15% human AB serum, SCF (50 ng/ml), IL3 (10 ng/ml) and Epo (2 units/ml). On day 3, the medium was replaced with fresh medium of the same composition, except that IL-3 was eliminated and SCF concentration was lowered to 25 ng/ml. On day 4, the cells were split into two aliquots containing respectively 1/5 and 4/5 of total Day 4 cells. To 4/5 of the cells, freshly prepared stock solution of HU (Sigma) was added to a final concentration of 50 μM, while the remaining 1/5 of Day 4 cells were cultured in the absence of HU. For RT-PCR and Western blots and HPLC analysis, the cells were cultured until Days 10 with the medium replaced every two days and the cell concentration maintained at lower than 10^{6} cells/ml. On day 6, 8 and 10, SCF concentration in the medium was lowered sequentially to 10, 2 and 0 ng/ml and HU was maintained at 50 μM. The yields of Day 10 cells varied with respect to individual SCD patients

and were generally 4-10 million cells in each of the HU-treated and non-treated samples. For cell sickling assays, cells were cultured in Day 10 medium until Day12 to increase the number of enucleated erythrocytes.

RNA and protein analyses by RT-PCR and Western blots: Total cellular RNAs and proteins, isolated respectively from 0.5-1x10^6 Day 10 cells, were analyzed by quantitative real-time qRT-PCR and Western blots as previously described.[21] **q**RT-PCR data for each SCD patient were means of technical triplicates and normalized with respect to the RNA level of β-actin. For RNA analysis by genome-wide RNA-seq, total cellular RNAs (2 μg) isolated from Day 10 patient erythroblasts cultured in the presence or absence of HU were quality-checked in the Bioanalyzer (Agilent Technologies). RNA samples with RIN values of >8.8 were used for construction of cDNA libraries, and sequenced with Illumina HiSseq 2500 (50 base paired-end reads and 50 million reads per sample) by MCG Integrated Genomics Core. Sequence reads were analyzed with respect to Human Reference Genome (hg19) using STAR 2.3.0; gene expression and differential expression were measured using Cufflinks and Cuffdiff 2.1.1. For Western blots, proteins isolated from 0.5-1 million Day 10 cells were loaded in each lane in gel electrophoresis. Protein bands were quantified with the Alpha-Innotech Imager or with infrared fluorescence using the Li-Cor Odyssey Imager and normalized with respect to the protein levels of β-actin. Antibodies were from Santa Cruiz (Dallas, TX): Anti-NF-YA (sc-10779), Anti-GATA1 (sc-265), Anti-GATA2 (sc-9008), Anti-TR4 (sc-9086), Anti-MYB (sc-517), Anti-γ-globin (sc-21756), Anti-β-globin (sc-21757), and Anti-β-actin (sc-130301). Anti-BCL11A was from Novus Biologicals (NB 600-261). Anti-NF-E4 antibody was a gift from Dr. S. Jane.

FACS and HPLC: For FACS analysis, Day 10 erythroblasts (~0.5 million cells) were stained with antibody to transferrin receptor, FITC-conjugated anti-CD71 (BD Pharmingen #555536), and antibody to glycophorin A, APC-conjugated anti-CD235a (BD Pharmingen #551336) and analyzed in a BD LSR II Flow Cytometer. The HbF levels in Day 10 erythroblasts of SCD patients (HbF/HbF+HbS) and normal donors (HbF/HbF+HbA_1+HbA_2) were determined with HPLC by MCG T.H.J. Huisman Hemoglobinpathy Laboratory using an established protocol.[36]

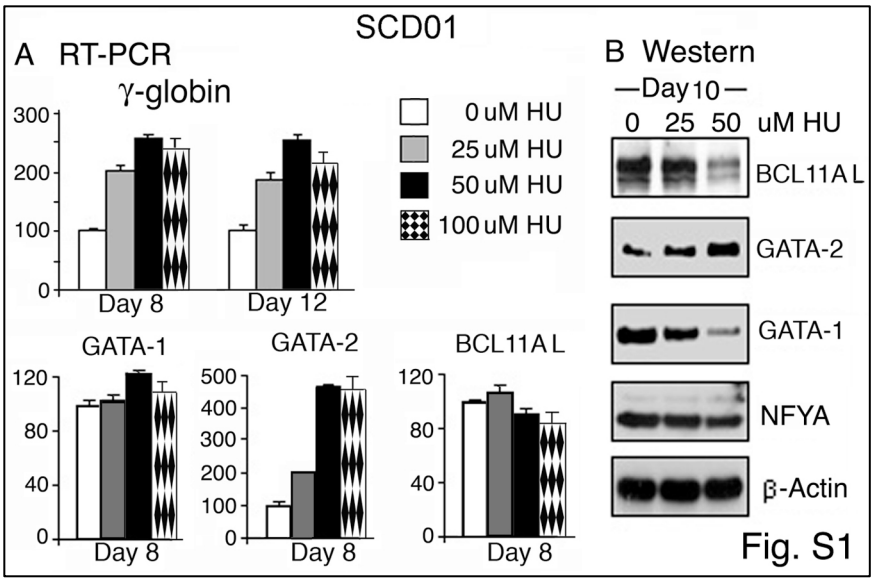


Fig. S1: Optimization of conditions for HU treatment of ex vivo cultured SCD erythroblasts.

A. Erythroblasts grown from peripheral blood CD34+ cells of patient SCD01 were treated with HU at concentrations 0, 25, 50 and 100 uM starting on Day 4 and continued until Day 8 or12, when the cells were harvested. Total cellular RNAs isolated from Day 8-12 cells treated with 0-100 uM HU were analyzed by real-time RT-PCR with PCR primer pairs specific for γ-globin, GATA-1, -2 and BCL11A genes.[21]

B. Western blots of Day 10 cells treated with 0, 25 and 50 uM HU.

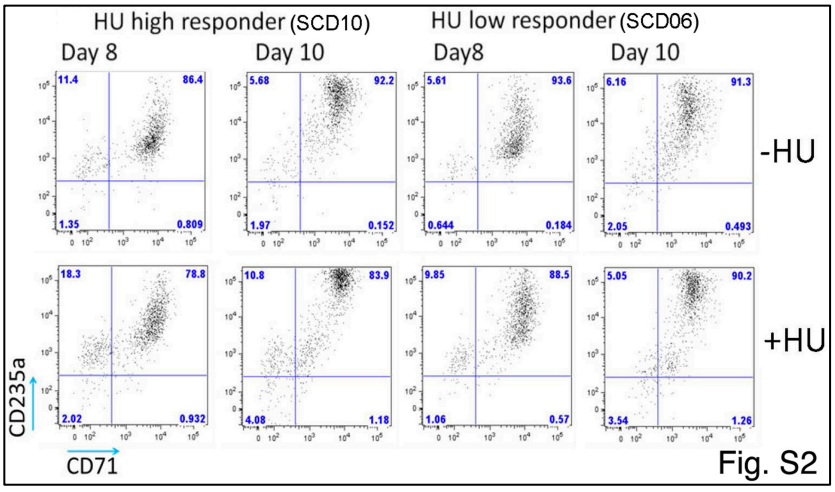


Fig. S2: FACS analysis of Day 8 and Day 10 erythroblasts of HU high and low responsive SCD patients. Day 8 and 10 erythroblasts cultured in the absence and presence of HU, -HU and +HU, respectively, were stained with antibodies CD71 and CD235a and analyzed by FACS.

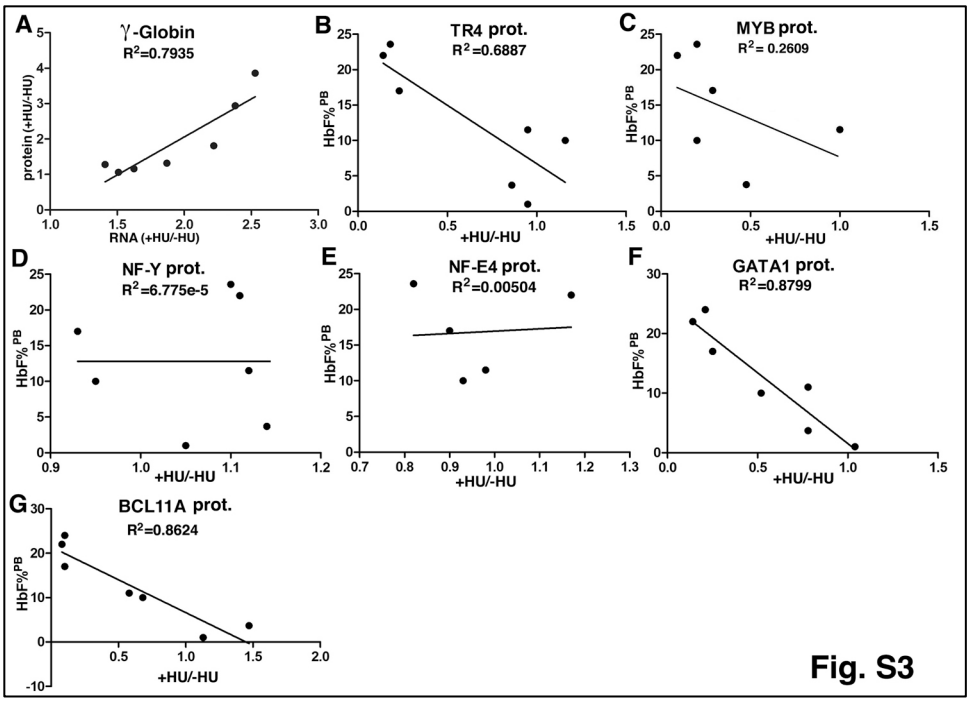


Fig. S3: Correlation analysis by scatter plots of HU-induced fold changes in RNA and protein levels of globin and TFs in Day 10 erythroblasts of SCD patients.

A. Correlation plot of HU-induced fold changes in γ-globin RNA and γ-globin protein of SCD 06-10, 18 and Normal 01 (see Table 1).

B-G. Correlation plots of HU-induced fold changes in protein levels of activator and repressor TFs with respect to HU-induced peripheral blood HbF levels of SCD 06-10, 17 & 18 (Table 1).

Table S1: Blood sample IDs

Blood Samples	Age	Gender	Treatment	HbF% (PB)	HU responsiveness
SCD 01	13	F	HU	12/25	High
SCD 02	15	M	HU	11/30	High
SCD 03	12	M	Bex	4.5/8.8	Low
SCD 04	14	F	HU+Bex	3/6	Low
SCD 05	17	M	Bex	1.5/4.9	Low
SCD 06	20	F	HU+Bex	4/10	Low
SCD 07	32	M	HU+Bex	4.7/11.5	Low
SCD 08	12	F	HU	6.2/17	High
SCD 09	39	M	HU	7/22	High
SCD 10	37	M	HU	12/23.6	High
SCD 11	30	F	Bex	3.2/3.1	Low
SCD 12	43	M	Bex	1.2/2.9	Low
SCD 13	24	F	HU+Bex	2.1/3.5	Low
SCD 14	12	F	Bex	1.1/1.8	Low
SCD 15	44	F	Bex	1.4/2.9	Low
SCD 16	43	M	Bex	2.6/3.8	Low
SCD 17	30	M	Bex	0.7/1	Low
SCD 18	36	M	Bex	2.1/3.7	Low
Normal 01	31	M		0.2	
Normal 02	25	F		0.3	
Normal 03	36	M		0.5	

HU: hydroxyurea; Bex: blood exchange.
HbF% (PB): peripheral blood HbF% pre-HU therapy/post-HU therapy at MTD recorded in the clinics.
High HU responsiveness: HbF% at MTD of ≥20%
Low HU responsiveness: HbF% at MTD of ≤10%
SCD patients on Bex only did not significantly increase HbF levels in response to prior HU trials.

Table S2: Equivalence of HbF induction in cultured Day 10 erythroblasts & peripheral blood of the SCD patients (Paired t-test, two-tailed)

	HbF% (-HU)_Responder	Hb F%^{PB} (-HU)_Responder	HbF% (-HU)_NonResponder	HbF%^{PB} (-HU) _NonResponder
mean	8.33	9.05	3.70	3.38
sd	3.95	2.88	1.12	1.29
p	0.6482		0.2727	
r	0.6887335		0.9011965	

	HbF% (+HU)_Responder	HbF%^{PB} (+HU)_Responder	HbF% (+HU)_NonResponder	HbF%^{PB} (+HU)_NonResponder
mean	20.85	23.50	7.18	6.86
sd	5.63	5.45	2.42	3.32
p	0.09332		0.6417	
r	0.9229771		0.9236116	

Table S3: Comparison of HU-induced changes in RNA and protein levels of TFs in Responders and Non-responders

T-test comparing HU-induced fold changes in RNA levels of TFs in Repsonders and Non-responders

	NFY	GATA2	GATA1	BCL	TR4	MYB	NFE4	γ-globin	IndexHU-3
mean in Nonresponders	0.99	3.00	1.01	0.83	0.91	0.77	1.00	1.30	4.1
mean in Responders	1.07	3.87	1.09	0.80	0.75	0.57	1.00	2.44	4.562
sd in Nonresponders	0.074	0.933	0.240	0.183	0.153	0.287	0.007	0.438	2.2749286
sd inRresponders	0.074	0.781	0.234	0.113	0.214	0.040	0.087	0.291	0.8033492
p	0.206935	0.0786717	0.5633072	0.6433621	0.3319711	0.3503604	0.92965655	4.64E-05	0.5335769

T-test comparing HU-induced fold changes in protein levels of TFs in Repsonders and Non-responders

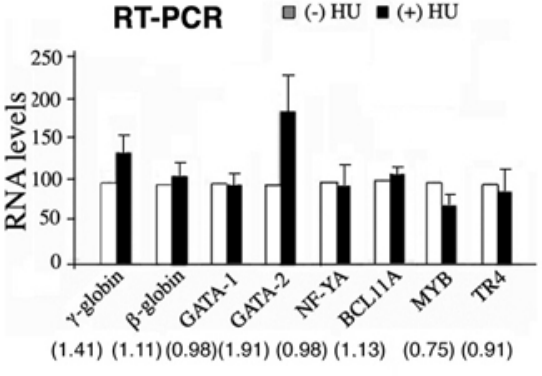
	NFY	GATA2	GATA1	BCL	TR4	MYB	NFE4	γ-globin	IndexHU-3
mean in Nonresponders	1.07	1.13	0.79	0.78	0.98	0.56	0.96	1.15	2.85
mean in Responders	1.05	2.03	0.20	0.12	0.18	0.19	0.96	3.03	81.80
sd in Nonresponders	0.086	1.080	0.459	0.337	0.127	0.405	0.035	0.010	2.175
sd in Responders	0.101	0.802	0.031	0.043	0.045	0.100	0.183	2.420	23.789
p	0.8128562	0.01333	0.00058859	1.19E-05	0.0003529	0.2510425	0.94530835	0.016729	0.00172694

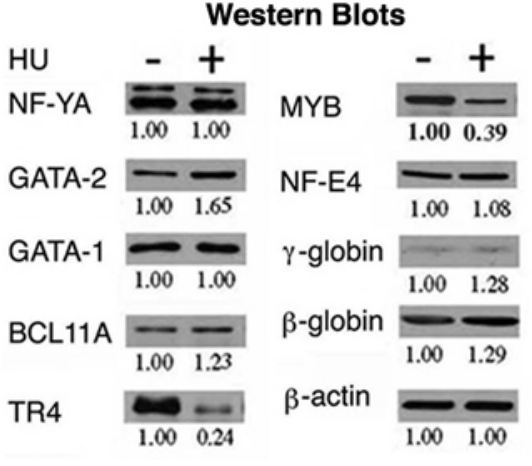
Supplemental File 1: RNA and protein analyses and calculation of IndexHU of SCD patients and normal donors.

Legend: Total cellular RNAs and proteins were purified from Day 10 erythroblasts (SCD 01-18) and normal donors, (normal 01-03) cultured from peripheral blood CD34+ cells of SCD patients and normal donors, in the presence or absence of 50 uM hydroxyurea during days 5-10 culture. RNAs and proteins were analyzed by real-time RT-PCR and Western blots. RT-PCR data were means of technical triplicates from one RNA sample or averages of the triplicate means from two independent RNA samples (SCD 02, 04, 08 and 14). Protein levels in the Western blots were quantified with the Alpha-Innotech Imager and normalized with respect to the protein levels of β-actin. The numerical values of the fold changes induced by HU were ratios of the normalized protein or RNA levels in cells cultured with HU over the normalized levels of the respective protein/RNA in cells cultured without HU. HU-induced fold changes FcGATA-2, FcGATA-1 and FcBCL11A in RNAs and proteins are presented under the blots or the bar graphs. IndexHU's were calculated as (FcGATA-2)/(FcGATA-1)(FcBCL11A). HbF: Percentage of HbF in peripheral blood of SCD patients on HU at MTD obtained from the clinic or of normal donors determined by HPLC (seeTable S1).

Normal 01

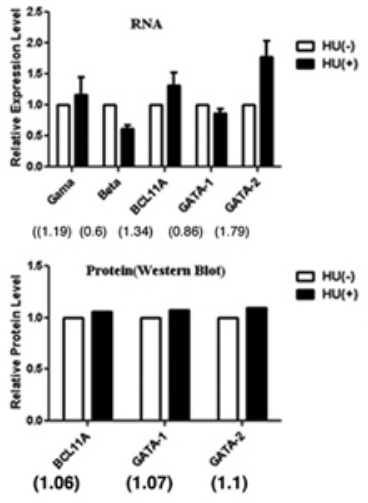
HbF : 0.2% **IndexHU: 1.4**

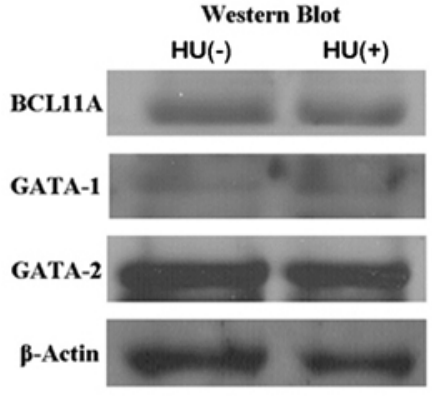




Normal 02

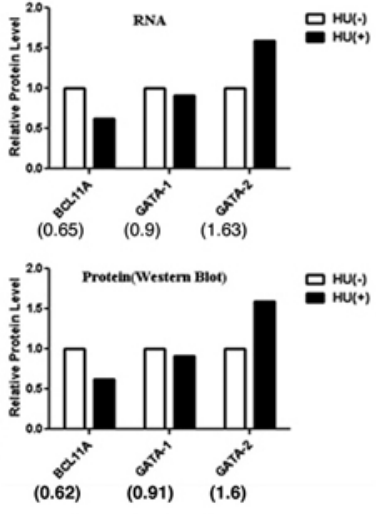
HbF:0.3% **IndexHU: 0.96**

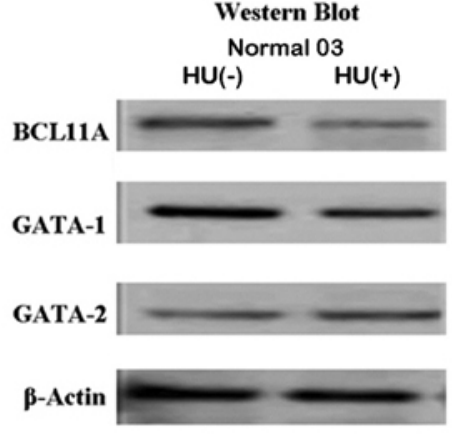




Normal 03

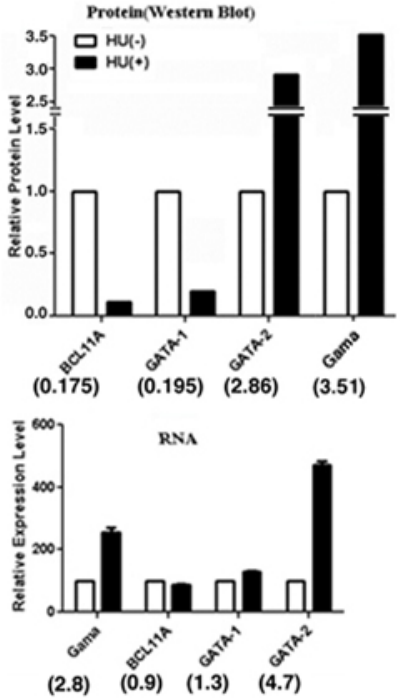
HbF:0.5% **IndexHU: 2.8**

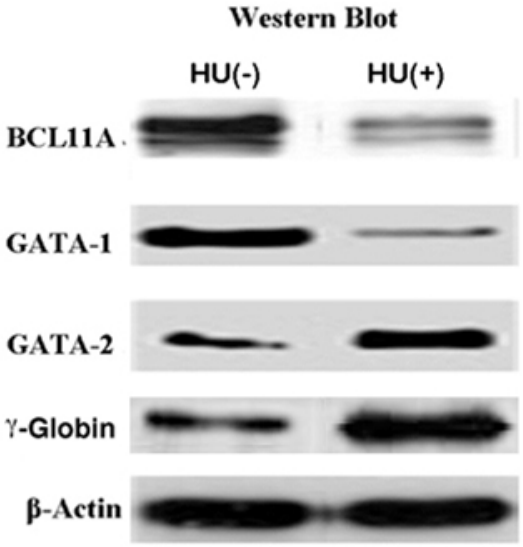




SCD 01

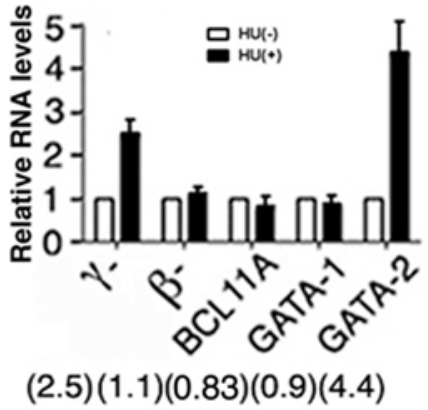
HbF:25% **IndexHU: 83**

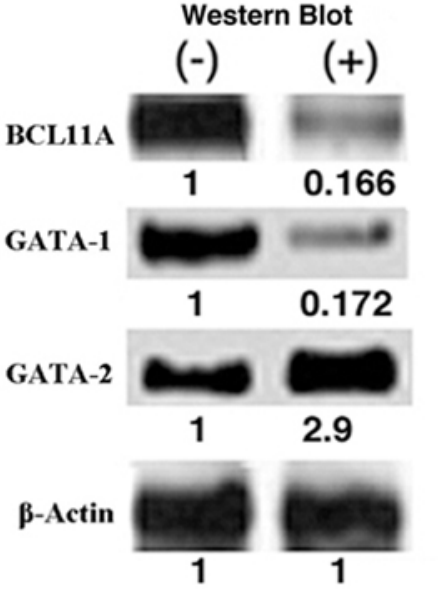




SCD 02

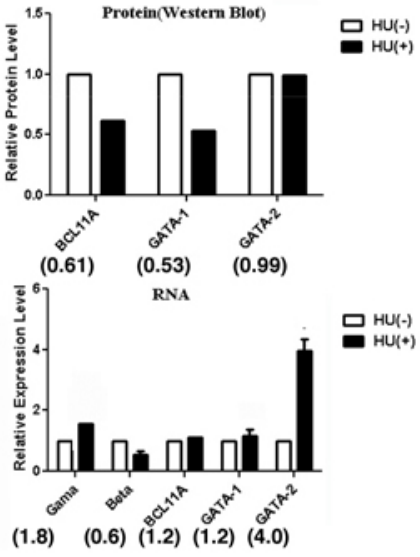
HbF: 30% **IndexHU: 101.6**

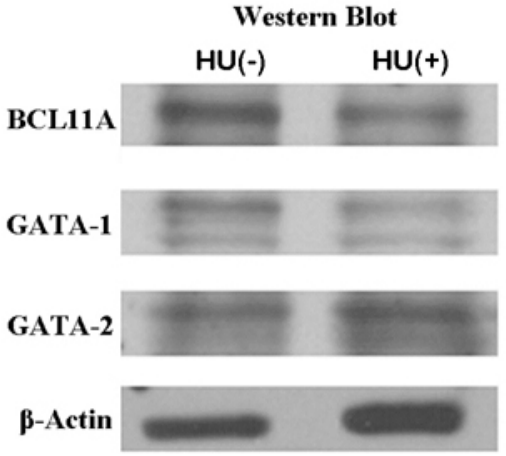




SCD 03

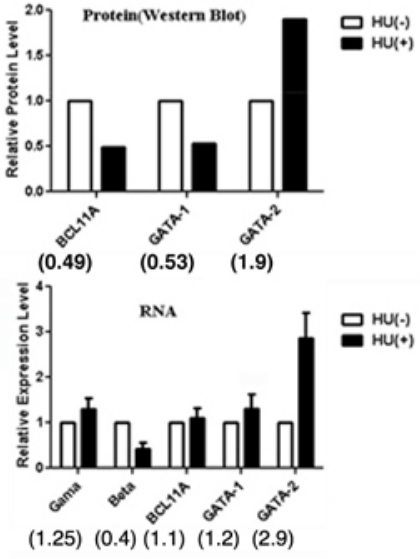
HbF: 4.5% **IndexHU: 3.01**

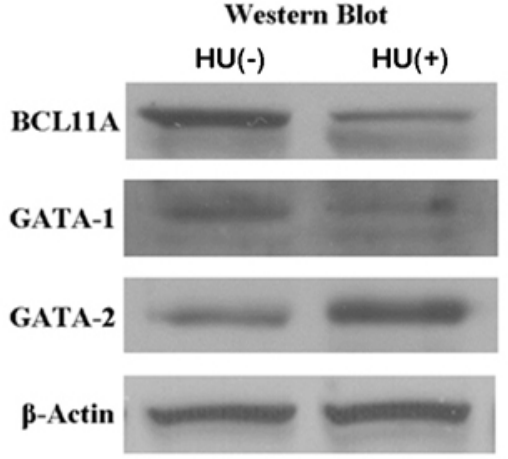




SCD 04

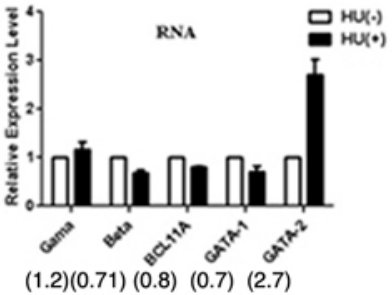
HbF: 6% **IndexHU: 7.33**

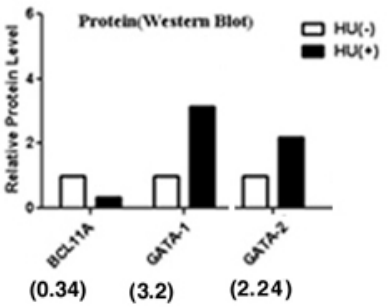


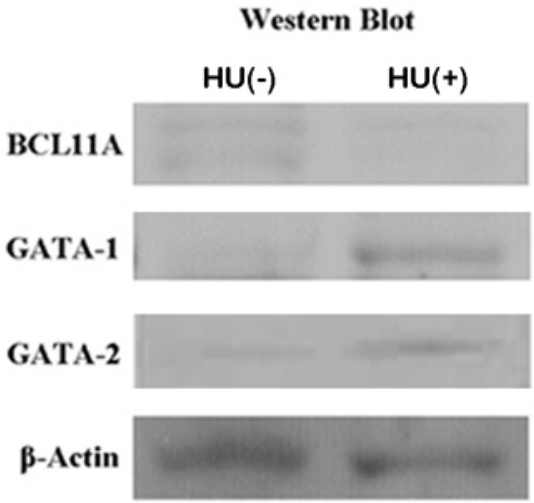


SCD 05

HbF: 4% IndexHU: 2.1

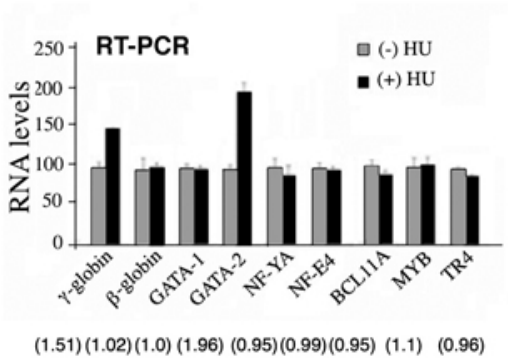


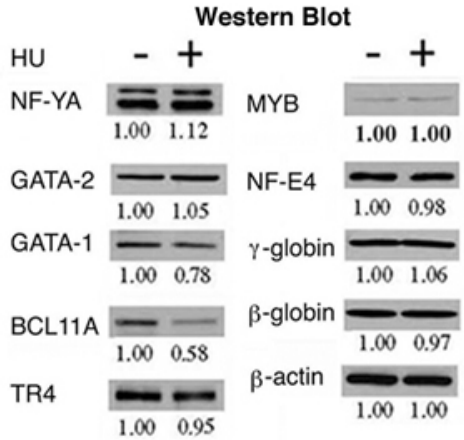




SCD 07

HbF: 11.5% IndexHU: 2.3





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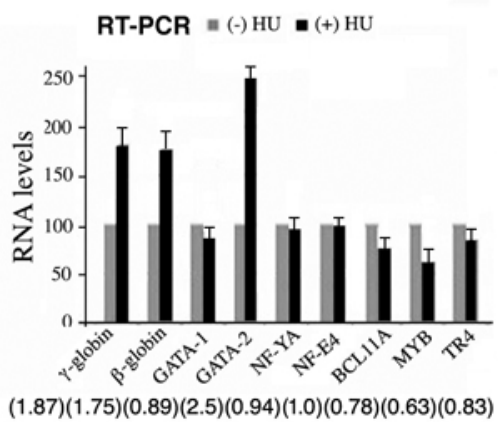
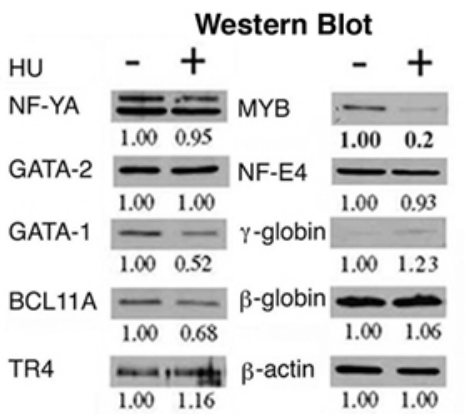
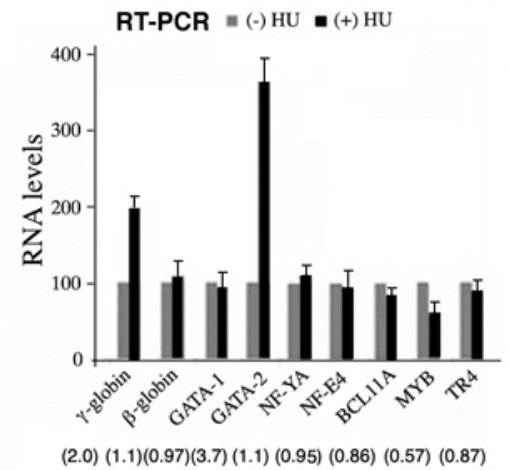
HbF: 10% IndexHU: 2.8

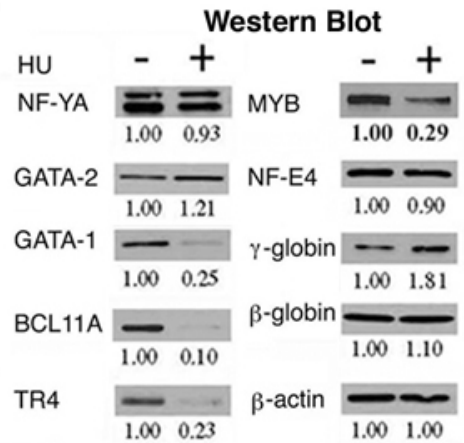




SCD 08

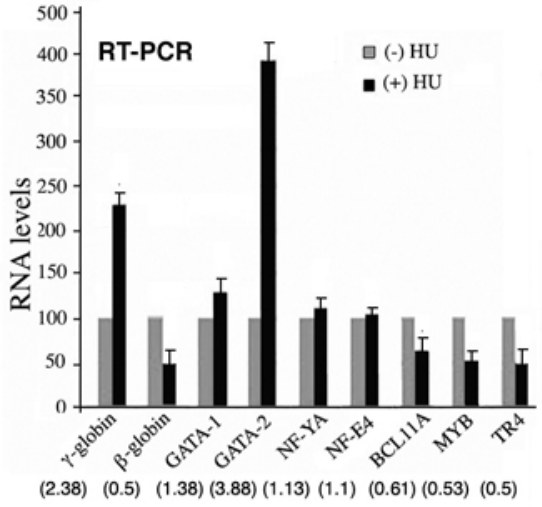
HbF: 17% IndexHU: 42

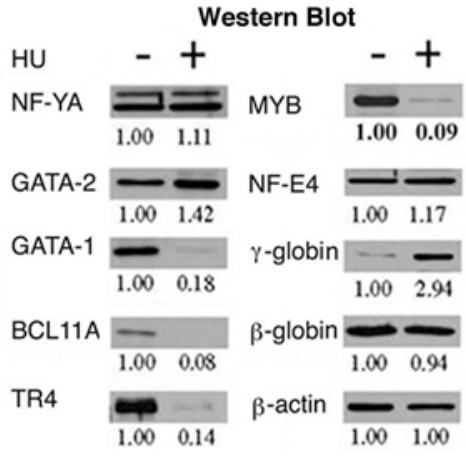




SCD 09

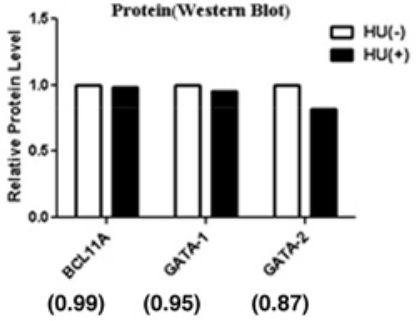
HbF: 22% IndexHU: 98.6

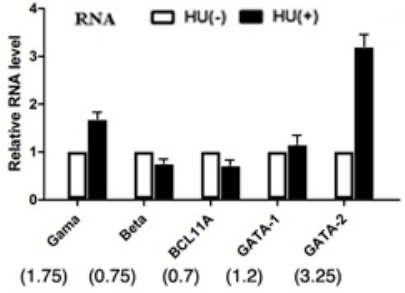


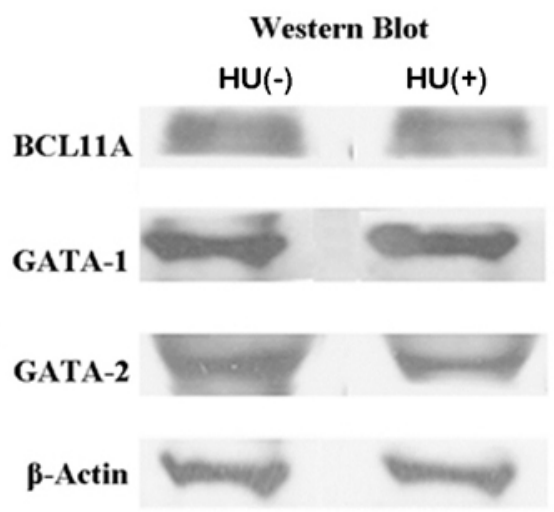


SCD 11

HbF: 3.2% IndexHU: 0.9

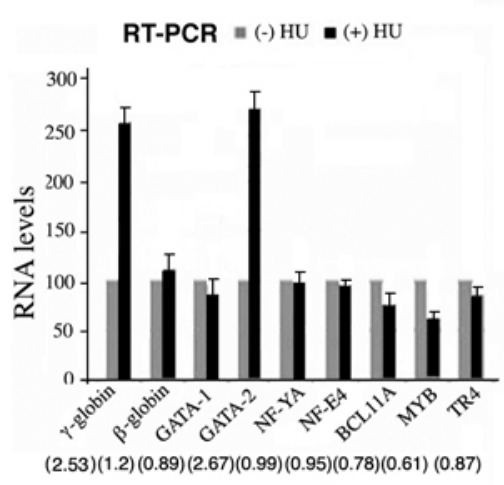


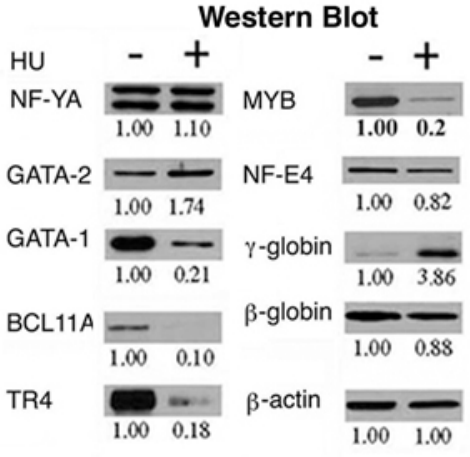




SCD 10

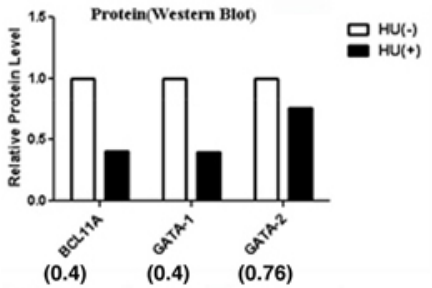
HbF: 24% IndexHU: 83

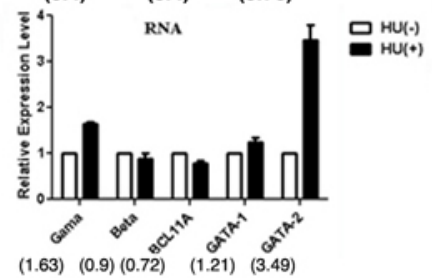


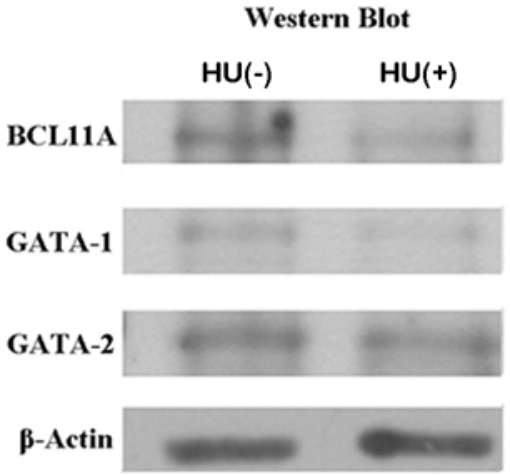


SCD 12

HbF: 2.9% **IndexHU: 4.7**

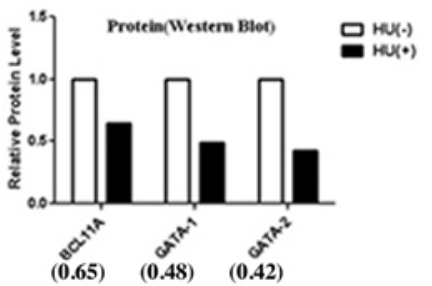


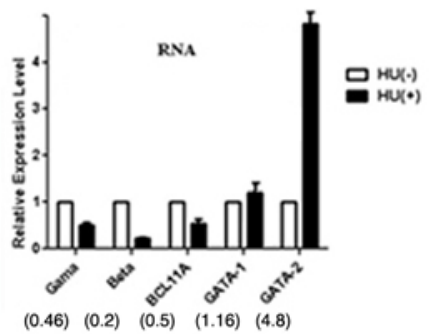


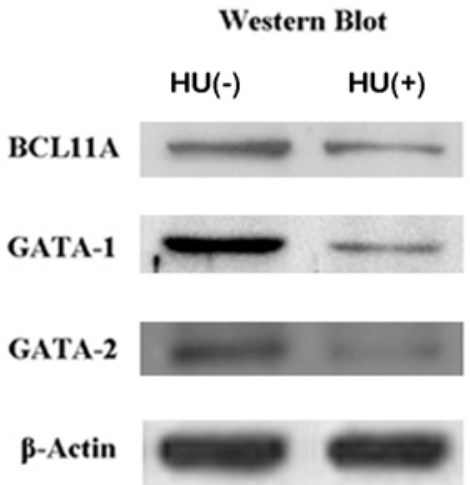


SCD 13

HbF: 3.5% **Index Hu: 1.35**

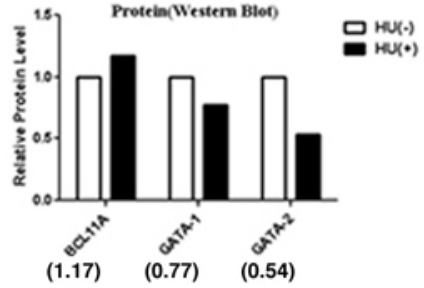


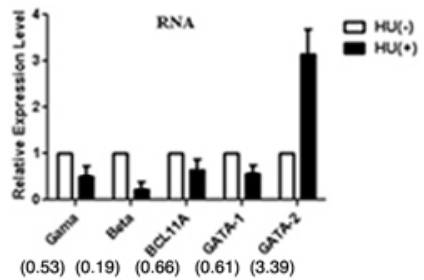


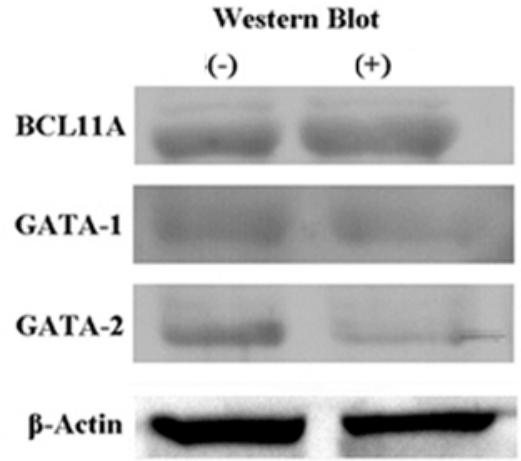


SCD 14

HbF: 1.8% **IndexHU: 0.6**

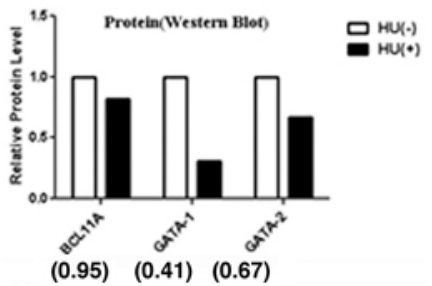


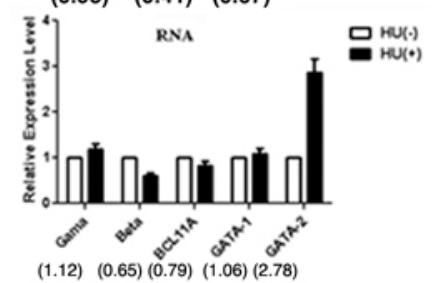


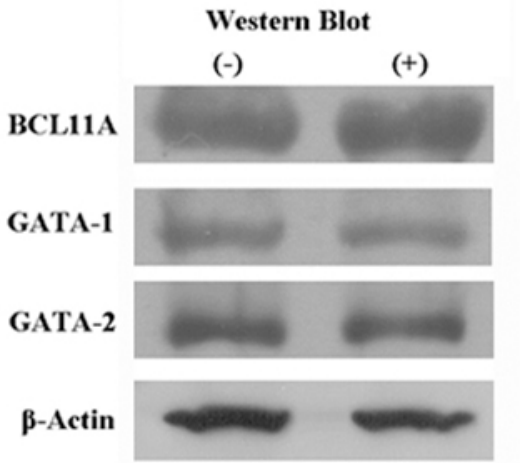


SCD 15

HbF: 2.9% **IndexHU: 1.7**

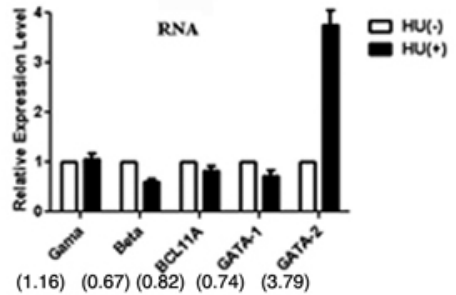


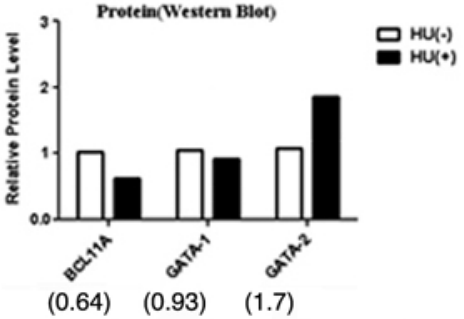


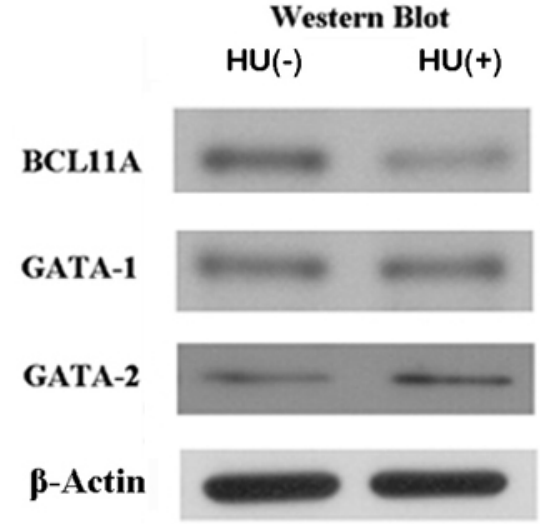


SCD 16

HbF: 3.8% IndexHU: 2.9

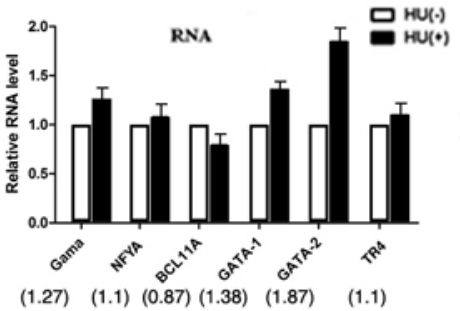


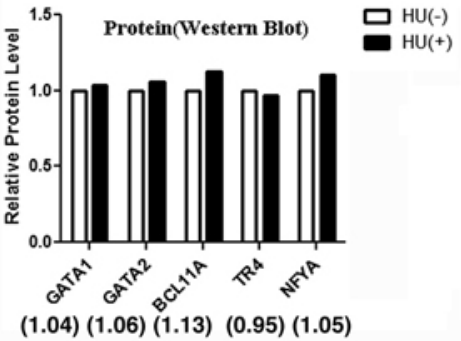


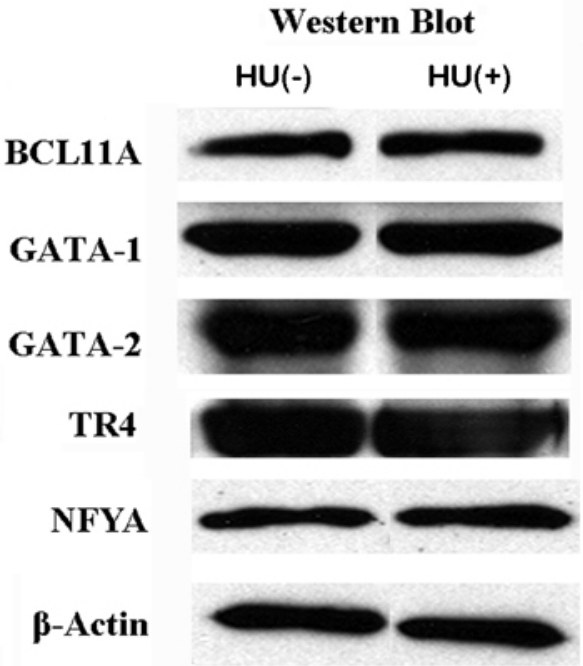


SCD 17

HbF: 1% IndexHU: 0.9



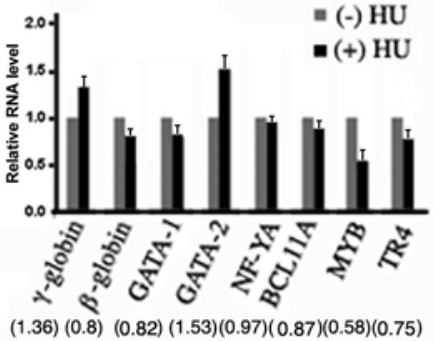


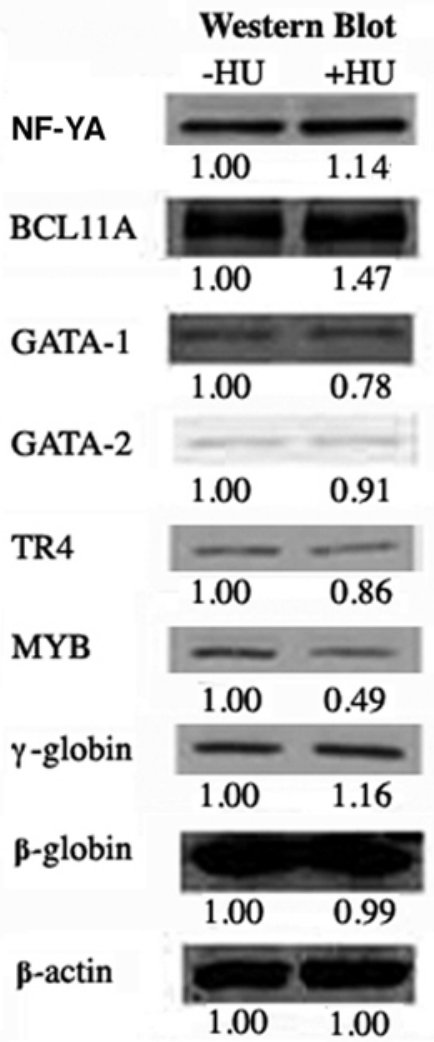


SCD 18

HbF: 3.7%

Index HU- 3 : 0.8
-4 : 0.95
-5 : 1.9





Supplemental File 2

Legend: Genome-wide RNA-seq of hydroxyurea-induced changes in the RNA levels of globin genes and transcription factors that regulate transcription of γ-globin gene. Total cellular RNAs were isolated from Day 10 erythroid cells cultured from CD34+ cells of two high HU-responders (SCD02 and 08) and two low responders (SCD14 and 18). The RNA samples passed quality checks in the Bioanalyzer with RIN values of >8.8 and were sequenced with 50 base paired-end reads and 50 million reads per sample. The RNA-seq data showed that in cultured erythroid cells of both HU high and HU low/non-responding SCD patients, HU did not regulate transcription of GATA-1, NF-Y, TR2 and TR4 genes but regulated transcription of the GATA-2 gene with p values of 0.006-0.02.

SCD02

GENE	LOCUS	STATUS	(-) HU	(+)HU	Fold change	Test Stat	p_value	
HBG1	chr11:5269501-5271087	OK	38.2553	57.7168	1.50872723	-0.530554	0.595728	A gamma-globin
HBG2	chr11:5274420-5276011	OK	99.8612	223.297	2.2360617	-1.16901	0.2424	G gamma-globin
HBB	chr11:5246695-5248301	HIDATA	2.7million	2.5million	1	0	1	beta-globin
HBD	chr11:5254058-5255858	OK	1678.91	1627.13	0.96916284	0.0304036	0.975745	delta-globin
HBA1	chr16:226678-227520	OK	612.785	694.858	1.13393504	-0.175841	0.860419	alpha1-globin
HBA2	chr16:222845-223709	OK	330.766	419.27	1.26757402	-0.347238	0.728412	alpha2-globin
NFYA	chr6:41040706-41108573	NOTEST	6.54674	6.11383	0.93387509	0.11185	0.910943	
NFYB	chr12:104510857-104532040	NOTEST	5.58589	5.33307	0.95473867	0.0622376	0.950374	
NFYC	chr1:41154751-41237275	NOTEST	24.7558	17.8541	0.72121085	0.560817	0.574922	
GATA1	chrX:48644981-48652717	OK	487.556	473.71	0.97160128	0.0355725	0.971623	
GATA2	chr3:128198264-128212030	NOTEST	5.97264	24.6827	4.13262942	-2.36369	0.0180937	
BCL11A	chr2:60678301-60780633	LOWDATA	16.1369	10.5037	0.65091001	0	1	
NR2F2	chr15:96869156-96883492	NOTEST	0	0	1	0	1	CoupTFII
NR2C1	chr12:95414057-95467404	LOWDATA	6.69441	6.29077	0.93970589	0	1	TR2
NR2C2	chr3:14989235-15106816	NOTEST	4.57196	3.63607	0.79529941	0.325235	0.745003	TR4
MYB	chr6:135502452-135540311	LOWDATA	7.20171	9.90327	1.37512713	0	1	

SCD08

GENE	LOCUS	STATUS	(-)HU	(+)HU	Fold change	Test Stat	p_value	
HBG1	chr11:5269312-5271122	OK	201.824	385.562	1.91039107	2.62371	0.00785	A gamma-globin
HBG2	chr11:5274419-5667019	OK	5134.74	12510	2.43634763	2.64967	0.0086	G gamma-globin
HBB	chr11:5246693-5250625	OK	85257.3	132252	1.55120999	0.618382	0.33615	beta-globin
HBD	chr11:5253907-5256600	OK	4352.98	10534.5	2.42005528	2.47746	0.01555	delta-globin
HBA1	chr16:226678-227521	OK	1223.59	2116.08	1.72940528	3.80888	0.0221	alpha1-globin
HBA2	chr16:222845-223709	OK	20414.9	41178.7	2.01709635	2.61152	0.10525	alpha2-globin
NFYA	chr6:40994771-41067715	OK	5.52713	6.12569	1.10829505	0.187167	0.84565	
NFYB	chr12:104510854-104532067	OK	15.4914	13.0895	0.84494773	-0.529102	0.6063	
NFYC	chr1:41157319-41237275	OK	23.6142	21.8426	0.92497546	-0.259138	0.793	
GATA1	chrX:48644961-48652718	OK	404.298	420.464	1.03998535	0.156443	0.87265	
GATA2	chr3:128198269-128221191	OK	7.7403	19.0835	2.46546539	3.28228	0.00635	
BCL11A	chr2:60678301-60780702	OK	30.0656	22.3826	0.7444573	-0.831952	0.4469	
NR2F2	chr15:96670597-96883492	NOTEST	0	0	1	0	1	CoupTFII
NR2C1	chr12:95415668-95467479	OK	25.4421	25.9069	1.01826651	0.0627036	0.95815	TR2
NR2C2	chr3:14860468-15106842	OK	2.69491	2.76371	1.02552823	0.0125446	0.9872	TR4
MYB	chr6:135502452-135540311	OK	30.6551	13.5637	0.44246032	-2.88571	0.0038	

SCD14

GENE	LOCUS	STATUS	(-)HU	(+)HU	Fold change	Test Stat	p_value	
HBG1	chr11:5269501-5271087	NOTEST	15.3246	8.53043	0.55664984	0.616401	0.53763	A gamma-globin
HBG2	chr11:5274420-5276011	OK	41.5831	20.4506	0.49179876	0.880036	0.37884	G gamma-globin
HBB	chr11:5246695-5248301	OK	2349.92	703.478	0.29936138	1.21148	0.225712	beta-globin
HBD	chr11:5254058-5255858	OK	442.318	157.638	0.35639031	1.42379	0.154506	delta-globin
HBA1	chr16:226678-227520	OK	423.972	174.621	0.41186687	1.30193	0.192942	alpha1-globin
HBA2	chr16:222845-223709	OK	191.518	84.7731	0.44263824	1.11524	0.264747	alpha2-globin
NFYA	chr6:41040706-41108573	NOTEST	14.1877	12.2617	0.86424844	0.252446	0.800696	
NFYB	chr12:104510857-104532040	NOTEST	13.9718	13.0112	0.9312536	0.0998711	0.920447	
NFYC	chr1:41154751-41237275	LOWDATA	22.3367	21.7907	0.97555763	0	1	
GATA1	chrX:48644981-48652717	OK	179.227	101.437	0.56596824	0.80612	0.420174	
GATA2	chr3:128198264-128212030	LOWDATA	135.211	356.63	2.63758941	0	1	
BCL11A	chr2:60678301-60780633	LOWDATA	10.475	4.2172	0.40259468	0	1	
NR2F2	chr15:96869156-96883492	NOTEST	0	0	1	0	1	CoupTFII
NR2C1	chr12:95414057-95467404	NOTEST	14.4667	13.999	0.96766883	0.0562396	0.955151	TR2
NR2C2	chr3:14989235-15106816	NOTEST	20.9069	20.4006	0.97578188	0.0353673	0.971787	TR4
MYB	chr6:135502452-135540311	FAIL	233.562	198.154	0.85	0	1	

SCD18

GENE	LOCUS	STATUS	(-)HU	(+)HU	Fold change	Test Stat	p_value	
HBG1	chr11:5269312-5271122	OK	257.061	371.46	1.44502555	1.46845	0.1211	A gamma-globin
HBG2	chr11:5274419-5667019	OK	8139.1	15435.9	1.89651266	1.70865	0.0843	G gamma-globin
HBB	chr11:5246693-5250625	OK	82222.7	71870.7	0.87409803	-0.132385	0.8976	beta-globin
HBD	chr11:5253907-5256600	OK	4796.18	6134.75	1.27908931	0.74779	0.4495	delta-globin
HBA1	chr16:226678-227521	OK	1425.42	1502.91	1.05436545	0.370727	0.8207	alpha1-globin
HBA2	chr16:222845-223709	OK	20724.4	23122.8	1.11572618	0.502948	0.75345	alpha2-globin
NFYA	chr6:40994771-41067715	OK	6.40202	6.71935	1.04956819	0.0897798	0.9282	
NFYB	chr12:104510854-104532067	OK	17.2223	17.3782	1.00905387	0.0286544	0.97645	
NFYC	chr1:41157319-41237275	OK	22.5664	20.9283	0.92740924	-0.245302	0.7952	
GATA1	chrX:48644961-48652718	OK	380.036	366.509	0.96440798	-0.145956	0.8804	
GATA2	chr3:128198269-128221191	OK	6.26721	16.0348	2.55852087	3.3024	0.00655	
BCL11A	chr2:60678301-60780702	OK	23.7062	23.2495	0.98073407	-0.0532149	0.95895	
NR2F2	chr15:96670597-96883492	NOTEST	0	0	1	0	1	CoupTFII
NR2C1	chr12:95415668-95467479	OK	21.889	24.6249	1.12499142	0.386324	0.74365	TR2
NR2C2	chr3:14860468-15106842	OK	3.06949	1.94096	0.63234071	-0.212418	0.78425	TR4
MYB	chr6:135502452-135540311	OK	32.375	22.6016	0.69811761	-1.37815	0.15075	