Fetal hemoglobin induction in sickle erythroid progenitors using a synthetic zinc finger DNA-binding domain

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Running Title: *Activation of γ-globin expression by a synthetic DNA-binding domain*

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Key Words: sickle cell disease, γ-globin, fetal hemoglobin, F-cells, transcription, synthetic zinc finger DNA-binding domain.

Supplemental Methods, Figures, and Tables

Subject Recruitment and Blood Processing. After obtaining Institutional Review Board approval for our protocol and informed consent, blood samples drawn from individuals with homozygous sickle cell anemia (HbSS), followed in the Sickle Cell Clinic at Augusta University, were processed. Patients recruited were never treated with hydroxyurea or had not received a blood transfusion 3 months prior to blood draw. A medical record review was completed to obtain complete blood counts, differential, and reticulocyte count, which was performed in the clinical laboratory. The percentage of HbF was determined by high performance liquid chromatography. Peripheral blood mononuclear cells were isolated from blood drawn in EDTA using histopaque separation and stored in DMSO at -80ºC until use for *in vitro* erythroid differentiation cultures.

Primary Erythroid Culture and Direct Protein Delivery. Peripheral blood mononuclear cells were cultured in a modified two-phase liquid culture system as recently published (1). During phase 1, cells were grown in Iscove's Modified Dulbecco medium with 15% fetal bovine serum, 15% human AB serum, 10 ng/mL interleukin-3, 50 ng/mL stem cell factor and 2 IU/mL erythropoietin (Sigma, St. Louis, MO). Phase 2 commenced on day 7 with a similar medium except stem cell factor and interleukin-3 were removed. Trypan blue exclusion staining was used to monitor cell viability throughout the culture period. Erythroid progenitors treated on day 8 with the different ZF-DBD proteins were harvested on day 10 for analysis. Direct protein delivery of ZF-proteins was performed as described by Gaj *et al*. (2) with modifications described by Hossain *et al*. (3). Briefly, ZF-DBDs were incubated with erythroid progenitors at day 8 of culture at a cell density of $1x10^6$ cells/ml in the presence of complete growth media.

Reverse transcriptase-Quantitative PCR (RT-qPCR). Total RNA was isolated from erythroid progenitors using TRIzol (Thermo Fisher, Waltham MA) per the manufacture's protocol. RT-qPCR was performed as previously published for γ-globin and β^S-globin (1,6). We also performed RT-qPCR for human CD71 (PPH00990H) and CD235a (PPH09014B) using gene-specific primers in the RT² qPCR Primer Assay (Qiagen, Germantown, MD). We used published methods for CD71, CD235a, and γ-globin RT-qPCR (1). The α-globin gene (forward: GGTCAACTTCAAGCTCCTAAGC; reverse: GCTCACAGAAGCCAGGAACTTG) and the internal control GAPD gene (forward: GAAGGTGAAGGTCGGAGT; reverse: GAAGATGGTGATGGGATTTC) primers, were purchased from Thermo Fisher.

Western Blot. Cytoplasmic and nuclear protein extracts were prepared from erythroid progenitors 48 h after delivery of the ZF-DBDs as described by Hossain *et al*. (3) using NE-PER Nuclear and Cytoplasmic Extraction Kit per the manufacture's protocol (Pierce, Waltham, MA). Western blot analysis was performed as previously published (1). The primary antibodies used were HbF (51-7) and TATA binding protein (TBP, N-12) (Santa Cruz Biotechnology, Dallas, Texas), FLAG (F3165, Sigma, St. Louis, MO), Myc (MA121316, Thermo Fisher Scientific, Waltham, MA) and β-actin (A5316, Sigma, St. Louis, MO).

Chromatin Immunoprecipitation (ChIP). ChIP assays were performed as previously published (1,4,5). Briefly, erythroid progenitors cultured for 8 days were treated for 48 h with different concentrations of the -567GγZF-DBD, crosslinked with 1% formaldehyde, and nuclei were isolated using cell lysis buffer (5 mmol/l PIPES, 1,4-piperazinediethanesulfonic acid, pH 8.0, 85 mmol/l KCl, 0.5% NP-40, octylphenoxypolyethoxylethanol and 1× CPI). Subsequently, nuclei

were lysed (50 mmol/l Tris, pH 8.0, 10 mmol/l EDTA, 0.32% SDS, and 1× CPI). The chromatin was sonicated to an average 400 bp fragment length using a Bioruptor (Diagenode, Denville, NJ). For each immunoprecipitation reaction, chromatin equivalents of 2×10^5 cells were incubated with 0.5 μg FLAG (F3165, Sigma, St. Louis, MO) or normal rabbit IgG antibody (I8140, Sigma, St. Louis, MO) overnight at 4°C. The antibody bound chromatin fragments were captured by protein A/G magnetic beads (Pierce). Purified DNA was analyzed by qPCR with SYBR green (Bio-Rad) using the CFX connect real-time system, (Bio-Rad). Sequences of the primers for qPCR: HS2, forward 5'-CCTTCTGGCTCAAGCACAGC-3', and reverse 5'-ATAGGAGTCATCACTCTAGGC-3'; G-567, forward 5'-GCTGAGATGAAACAGGCGTG-3' and reverse 5'-TGATGGGACACGTCTTAGTCTC-3'. The ChIP procedure for mouse erythroleukemia cells and primers for the murine β-globin locus are described in Stees *et al*. (5).

***In vitro* RBC sickling analysis.** Erythrocyte sickling studies were performed with sickle erythroid progenitors cultured for 8 days as previously published (6) and treated with The -567GγZF-DBD (1μM ZF-DBD or 3μM ZF-DBD) and the negative control ZF-DBD (1μM) as described above. Cells were incubated in 1% oxygen for 24 h at 37ºC in a hypoxic chamber O_2 Tissue Culture Glove Box from Coy Laboratory Products (Grass Lake, MI); control cells were also incubated at normal oxygen (21% O_2). The deoxygenated cells were fixed with 3.7% formaldehyde for 10 min before removal to atmospheric condition for microscopic examination using light microscopy. Bright field images (20x or 10x magnification) of erythroid progenitors were acquired on an EVOS Cell Imaging System (Thermo Fisher Scientific) and the number of sickled erythroid progenitors determined manually.

Generation and analysis of the DNA-binding characteristics of the negative control ZF-DBD. The negative control ZF-DBD was designed to target an 18 bp DNA sequence upstream of the core of murine locus control region (LCR) hypersensitive site 2 (5'HS2). The target sequence is specific for the mouse globin gene locus and not present in the human LCR. PCR assembly of the coding region was performed as described in Hossain *et al*. (3). Figure S1A shows the amino acid sequence of the NC-ZF-DBD as well as the His and Myc tag. Figure S1B shows the DNA-binding characteristic of the NC-ZF-DBD using electrophoretic mobility shift assay (EMSA). The binding curve on the right was used to determine the dissociation constant (Kd), which was determined to be 100 nM. The DNA-binding specificity of the NC-ZF-DBD was determined by ChIP after delivery into murine erythroleukemia (MEL) cells according to a protocol described by Hossain et al. (3; Figure S2). The NC-ZF-DBD interacted with murine HS2 but not with the γ-globin, dematin, or Hmox1-E2 promoters. The primers used for ChIP are described by Stees *et al.* (5)

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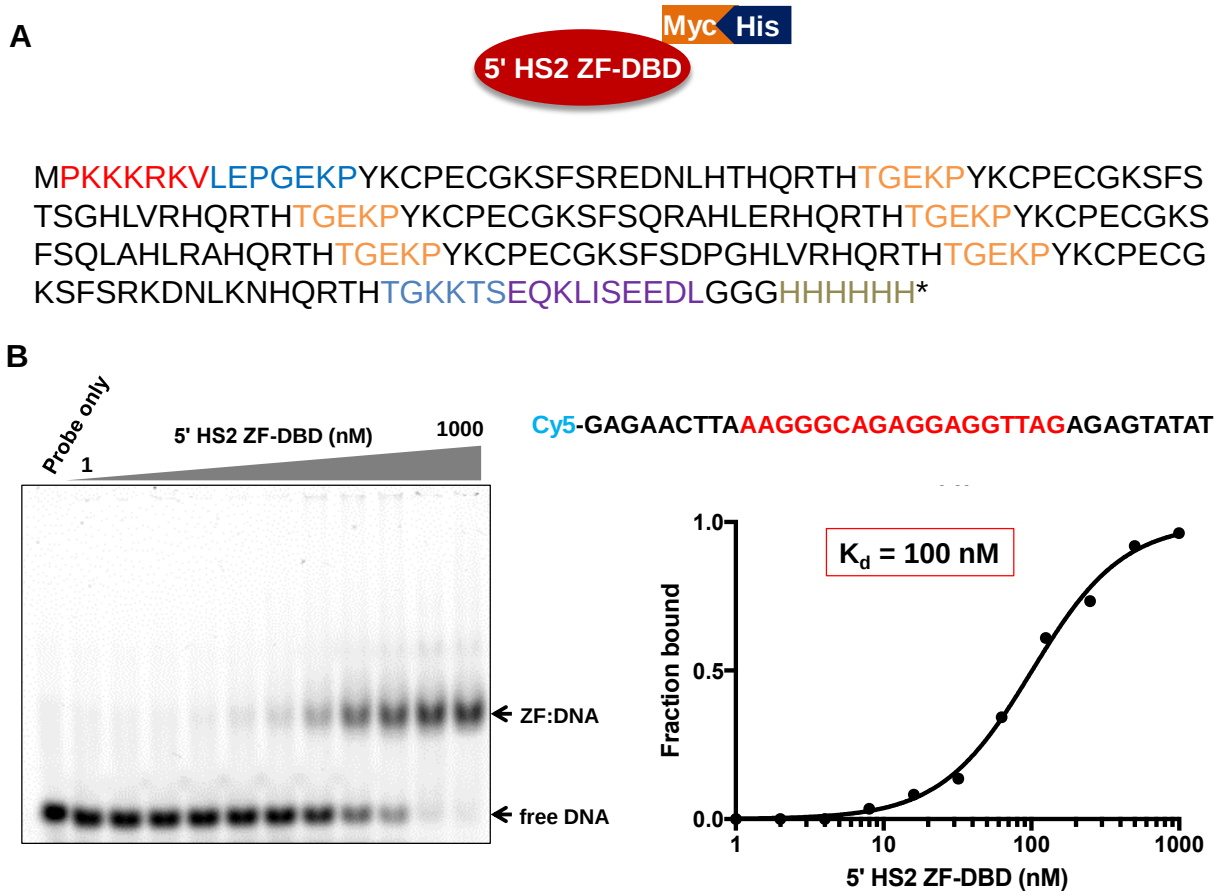

Figure S1. Characterization of the negative control ZF-DBD. Amino acid sequence of the NC-ZF-DBD (**A**) and DNA-binding characteristics *in vitro* (**B**).

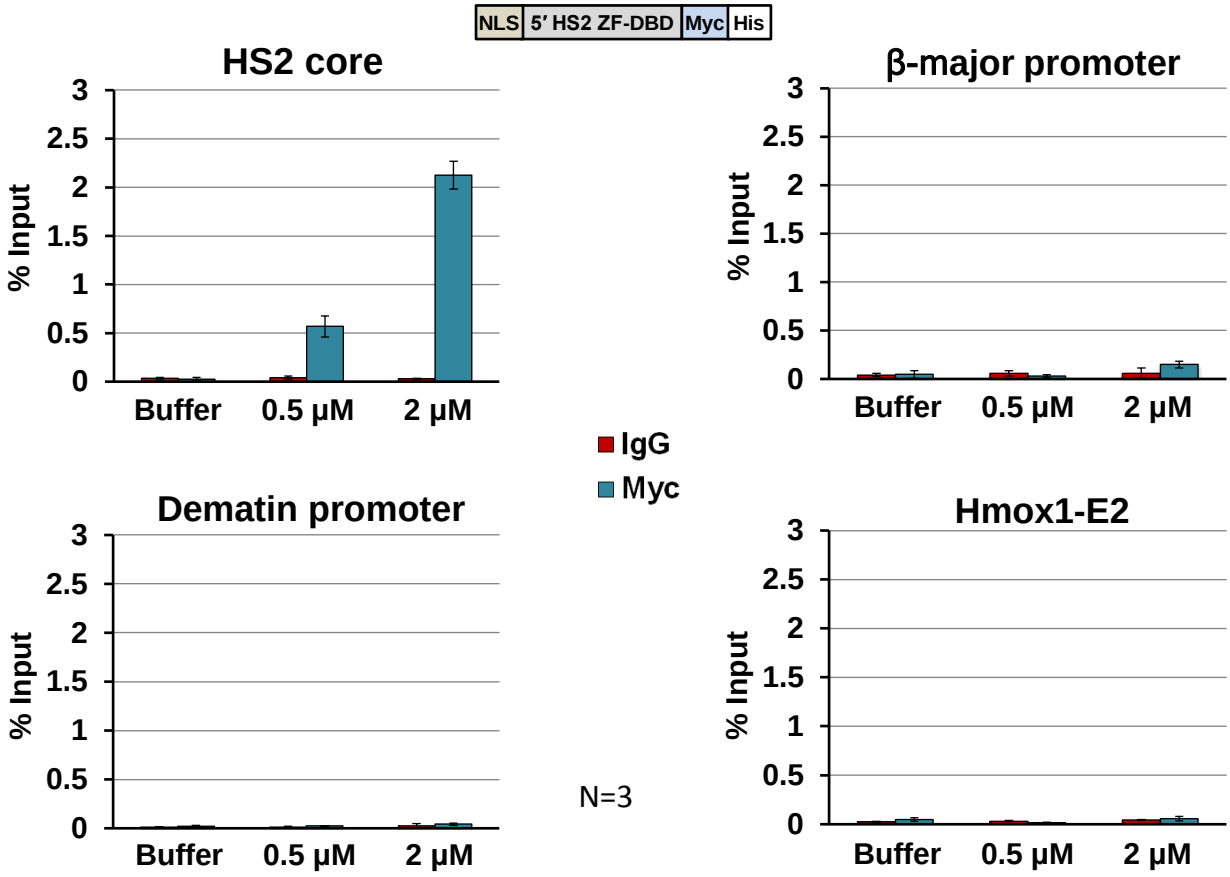

Figure S2. Binding of the NC-ZF-DBD to the murine LCR HS2 after delivery to MEL cells. Cells were subjected to ChIP and DNA was analyzed by qPCR using primers specific for LCR HS2 and the indicated genes.

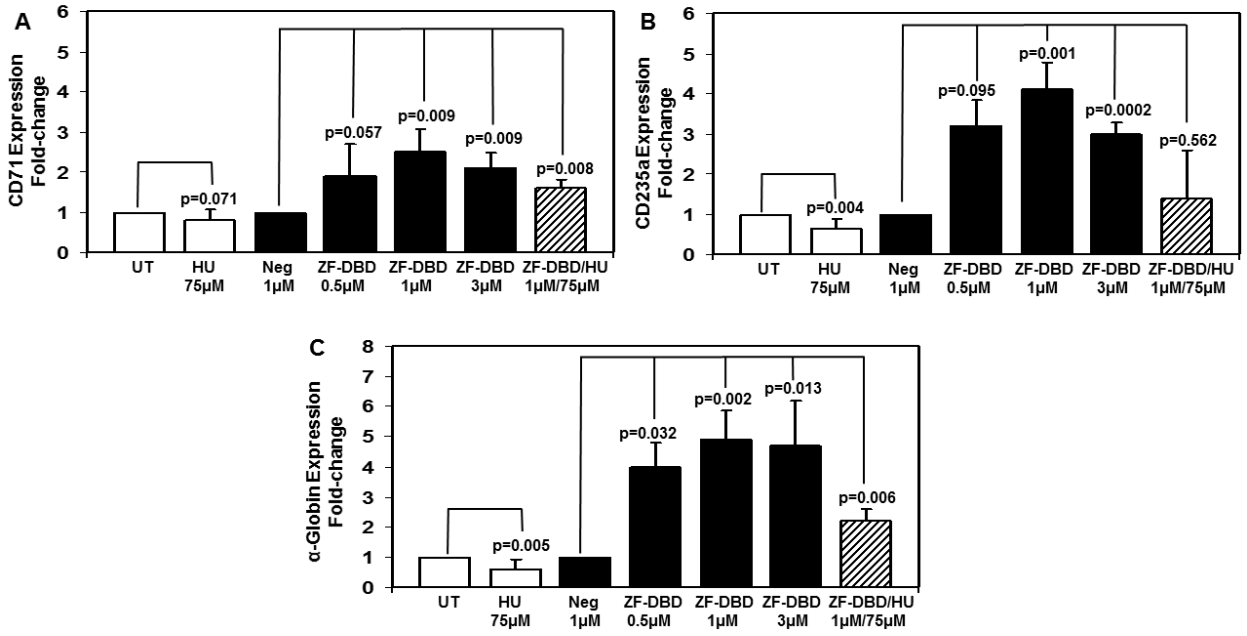

Figure S3. Erythroid marker expression increases during treatment with the -567GγZF-DBD. Sickle progenitors were treated with the various agents on day 8 and harvested on day 10. Cell viability (Table S2) and expression of erythroid markers CD71 (A), CD235a or glycophorin A (B) and α-globin (C) was measured by RT-qPCR (Supplemental Methods). The levels of all genes were normalized to GAPD before fold change was calculated. Data are shown as mean ± standard deviation.

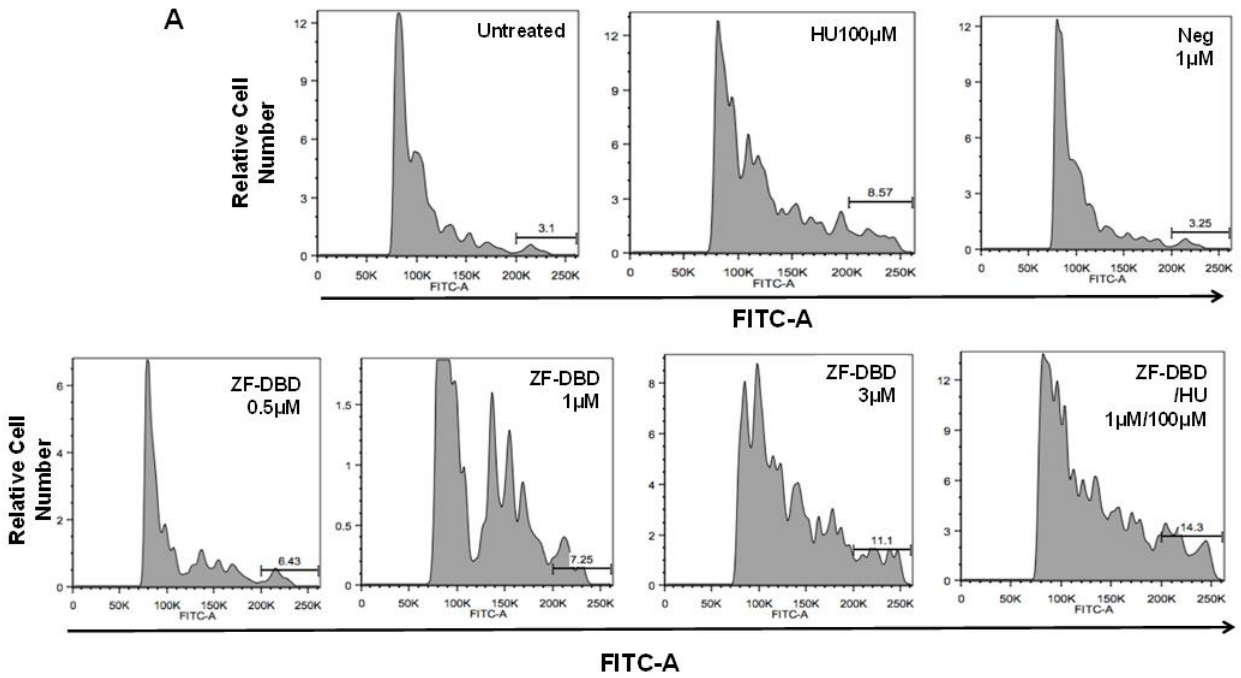

Figure S4. Increased number of F-cells in sickle erythroid progenitors after treatment with the -567GγZF-DBD. Cells were exposed to hydroxyurea, the -567GγZF-DBD (ZF-DBD) at different concentrations (0.5, 1, and 3µM, as indicated), the negative control ZF-DBD (Neg, 1µM), or a combination of 1µM of the -567GγZF-DBD and 75µM of hydroxyurea. Cells were stained with FITC labeled anti-γ-globin antibody and analyzed by flow cytometry. Shown is the number of HbF positive cells (F-cells) after treatment with the various agents.

TABLE S1. Summary of laboratory values of children with sickle cell disease used in the analysis.											
	Age (years)	Diagnosis	HU Therapy	Hgb (gm/dL)	Hct (%)	WBC ($x10^3$)	Neutr	Lymph	Plats	Retic	HbF (%)
ID-033	11	HbSS	No	7.6	21	19.3	9.8	5	321	12.5	4.5
ID-040	12	HbSS	No	8.5	24.9	11.8	3.2	5.5	484	12.2	15.5
ID-050	10	HbSS	No	7.9	22.3	15.8	4.5	9.6	482	11.1	5.3
Abbreviations: HU, hydroxyurea; Hgb, hemoglobin, Hct, hematocrit; WBC, white blood cells, Neutr, neutrophils; Lymp, lymphocytes; Plat, platelets, Retic, reticulocyte count, HbF, fetal hemoglobin											

TABLE S2. Summary of cell viability using trypan blue exclusion.					
	Day 8 (%)	**SD**	**Day 10 (%)**	**SD**	**p value**
Untreated	92.00	3.00	92.33	3.06	0.8993
HU (75µM)	92.00	3.00	85.61	5.77	0.1671
Negative control (1µM)	93.00	3.46	69.67	1.15	0.0004
DBD (0.5µM)	92.00	3.00	83.00	10.58	0.2294
DBD (1µM)	92.00	3.00	81.33	2.31	0.0115
DBD (3µM)	92.00	3.00	74.00	2.65	0.0015
HU (72µM) & DBD (1µM)	92.00	3.00	82.00	5.29	0.0465