SUPPLEMENTARY APPENDIX

In vitro and *in vivo* induction of fetal hemoglobin with a reversible and selective DNMT1 inhibitor

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Supplemental Information

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Supplemental Methods:

In vitro Maturation of CD34+ Cells to Reticulocytes

$CD34^+$ cells were seeded in T25 flasks in erythroid expansion media: Stemspan H3000 (StemCell Technologies) supplemented with 2 mM L-glutamine, 40 µg/mL human low-density lipoproteins (StemCell Technologies), 10 ng/mL recombinant human (rh) interleukin IL-3, 100 ng/mL rh stem cell factor (R&D Systems), and 0.5 U/mL rh erythropoietin (Invitrogen). Expansion media also included either vehicle (0.3% DMSO) or compound dissolved in DMSO. After 4 days, cells were centrifuged, and media and compounds were replaced. On day 7, cells were collected and plated in a 24 well dish in phase 2 differentiation media:IMDM containing 1X BIT supplement (StemCell Technologies), 3% male AB serum (Invitrogen), 3 U/mL heparin (Sagent Pharmaceuticals), 3 U/mL rhEPO (Invitrogen), and 10 ng/mL rhSCF (R&D Systems), with vehicle or compound. On day 12, media was again exchanged for phase 3 differentiation media: phase 2 media without rhSCF and with 800 µg/mL holo-transferrin, with vehicle or compound. Cells were incubated for an additional 5 days, and were then harvested for flow cytometry analysis of HbF^+ reticulocytes as detailed below.

HbF ELISA

Cryopreserved day 7 EPCs were thawed and cultured with compounds for 5 days after which cells were lysed by freezing and thawing in cell lysis buffer (Invitrogen) supplemented with 1× protease inhibitors. Lysates were then assayed with an anti-HbF ELISA, conducted according to previously described methods. (23)

Cell Growth and Apoptosis Assays

EPCs were cultured as above with compounds or vehicle for 3 days for caspase assay or 5 days for cell growth assay, after which either Caspase-Glo reagents or Cell Titer-Glo (Promega), respectively, were added for 10 min incubation prior to reading luminescence on a ViewLux 1430 (Perkin Elmer).

Global CpG Methylation Assay

Genomic DNA was extracted from cultured EPCs using a genomic DNA isolation kit (Zymo Research). DNA was then fully degraded into nucleosides using the Zymo degradase plus kit and reaction buffer (Zymo Research). DNA hydrolysis samples

were injected onto a HILIC ultra-performance liquid chromatography (UPLC) column (ACQUITY UPLC BEH Amide Column, 130 Å, 1.7 µm, 2.1 mm X 50 mm, Waters) equilibrated in acetonitrile:methanol (90/10, v/v), and eluted with a linear gradient of 10 mM ammonium bicarbonate, pH 9). Cytosine and methylcytosine content was quantified by selected reaction monitoring mass spectrometry, conducted as previously described. (27)

mRNA Analysis

EPCs or bone marrow cells were pelleted into 1.5 mL microcentrifuge tubes, supernatant was removed, and cells were washed with DPBS. Cells were lysed in 500 µL of Trizol reagent and total RNA was isolated using a Direct-zol mRNA isolation kit (Zymo Research). Samples were assessed for total RNA content on a Nanodrop (Thermofisher), and 1 µg of total RNA was then converted into cDNA in a 50 µL reaction using the High Capacity cDNA RT kit + RNase inhibitor (Applied Biosystems). Resulting cDNA was applied to TaqMan (Life Technologies) quantitative PCR assays for human *HBG1*, *HBA1*, *HBB*, or *GAPDH* following vendor's protocols in 50 µL reactions. Reactions were run on a ViiA 7 using TaqMan Fast reagents standard protocol.

DNMT Biochemical Methyltransferase Assays

Break light format *in vitro* methyltransferase assays [33] for DNMTs were conducted in 384 well format, using black non-binding surface microplates (Corning). A final concentration of 40 nM human full length DNMT1 (produced internally) was added to substrate mixture containing final concentrations of 125 nM hemi methylated oligonucleotide (synthesized at ATD Bio; 5'-FAM-ATCTAGCG**5**ATCAGTTTTCTGATG**5**G**5**TAGAT-Dabcyl-3' where 5 = methyl deoxycitidine) and 2 µM ultrapure SAM (Cisbio #62SAHZLD). Negative control wells included all other reagents but 0 µM SAM. All reagents were made up in 1× assay buffer (20 mM Tris pH 6.8, 25 mM NaCl, 0.5mM $MgCl_2$, 0.01% Triton X100 and 1 mM DTT). The reaction was incubated at 26 °C for 45 min and then stopped with the addition of detection reagent. Detection reagent, made up in 1× Gla assay buffer (20mM Tris pH 8.0, 80 mM NaCl, 0.75mM $MgCl_2$, 0.01% Triton X100 and 1mM DTT) has a final concentration of 100 µM SAH (Sigma) and 0.0008 units/µl Gla1 restriction endonuclease (Sibenzyme #E494). Following addition of the detection reagent the

plate was incubated in the dark at room temperature for 5 hr. Fluorescence intensity was then measured on the PHERAstar FS (BMG Labtech) at Ex 485 nm and Em 520 nm, with a gain of 400 and an integration time of 100 ms.

DNMT3A and DNMT3B break light assays were as above with the following differences. DNMT3A and DNMT3B (produced internally), were used at final concentrations of 600 nM and 300 nM respectively). Negative control wells for two assays included all other reagents but included 200 µM SAH or 40 µM SAH respectively. For the DNMT3A, all reagents were made up in 1× assay buffer (20 mM Tris pH 7.4, 100 mM NaCl, 1.5 mM EDTA, 0.1mM $MgCl_2$, 1mM CHAPS and 1 mM DTT) and the reaction was incubated at 37 °C for 90 min. For the DNMT3B assay, all reagents were made up in 1× assay buffer (20 mM Tris pH 6.8, 75 mM NaCl, 1.5 mM EDTA, 0.5mM $MgCl_2$, 1mM CHAPS and 1 mM DTT) and the reaction was incubated at 37 °C for 90 min. Detection reagents were made up in 1× Gla assay buffer as above, but with inclusion of 200 µM SAH or 40 µM SAH respectively.

Bisulfite sequence analysis

Genomic DNA was extracted in duplicate from EPCs cultured for 3 days in the presence of compound using the Maxwell 16 Cell DNA Purification Kit on the Promega Maxwell 16 (Promega). Isolated DNA was then bisulfite converted using a Zymo EZ DNA Methylation Kit (Zymo Research). Primer sets for bisulfite treated regions were designed from HBG1 and HBG2 sequence using ABI's Methyl Primer Express Software (ThermoFisher). Analyzed regions included nine previously described sites of DNMT1-dependent DNA methylation [37]. The PCR amplicons were between 253bp and 295 bp. Multiplex PCR amplification of all samples was performed using 48×48 Integrated Fluidic Circuit chips (Fluidigm) as recommended by the manufacturer in combination with the Kapa HiFi+ Uracil Polymerase. The resulting amplicons were harvested and barcoded according to the Fluidigm instrument's guidelines. After barcoding, samples were purified using Ampure XP (Beckman Coulter) and sizing was evaluated using the Agilent High Sensitivity DNA kit on the 2100 Bioanalyzer (Agilent). The samples were then normalized using the Kapa Illumina Quantification kit (Illumina) and prepared for massively parallel sequencing using a MiSeq V3 600 bp Reagent Kit (Illumina) with paired-end

sequencing (2x 251cycle) according to the manufacturer's guidelines. An average of ~2.3 million reads were generated per sample. Low-quality nucleotides and adapter sequences were trimmed during QC. The output data was quality-checked using a pre-alignment raw data QC workflow in ArrayStudio v8 (Omicsoft) and assembled to reference with variant calling performed using the CLC-Genomics Workbench v8.1 program (Qiagen).

Amplicon	**Chr (GRch37/hg19)**	**Target start position**	**Target end position**	**Target Name**	**primer forward**	**Target Name**	**primer reverse**	**amplicon size**
1	chr11	5270406	5270706	1_Methylation5270556_HBG1_1f	ACACTGACGACATGGTTCTACAGATTTTTTGGGAGATGTTATAAA	1_Methylation5270556_HBG1_1r	TACGGTAGCAGAGACTTGGTCTATTACCACTAAATCTCAACCCA	270
2	chr11	5270784	5271084	2_Methylation5270934_HBG1_2f	ACACTGACGACATGGTTCTACATTTGGAAYGTTTGAGGTTATTA	2_Methylation5270934_HBG1_2r	TACGGTAGCAGAGACTTGGTCTCCATAAATAAACAACCAAAAACC	280
3	chr11	5270984	5271284	3_Methylation5271134_HBG1_3f	ACACTGACGACATGGTTCTACAATGTAAATATTTGTTTGAAAYGGT	3_Methylation5271134_HBG1_3r	TACGGTAGCAGAGACTTGGTCTTCCTCCTCTATAAAATAACCCA	258
4	chr11	5271889	5272189	4_Methylation5272039_HBG1_4f	ACACTGACGACATGGTTCTACATTGTGTTAGAAATAAAGTTGTTTAAAG	4_Methylation5272039_HBG1_4r	TACGGTAGCAGAGACTTGGTCTTAACCCAAAATTTTAACRTAACT	262
5	chr11	5274734	5275034	5_Methylation5274884_HBG2_5f	ACACTGACGACATGGTTCTACAGGTAAAGTATGTTTAGGGGTGA	5_Methylation5274884_HBG2_5r	TACGGTAGCAGAGACTTGGTCTAACTTCCCTCAAAACCTAAAAT	274
6	chr11	5275068	5275368	6_Methylation5275218_HBG2_6f	ACACTGACGACATGGTTCTACAATGTTTTAGGGTTTAAGGAGTGTT	6_Methylation5275218_HBG2_6r	TACGGTAGCAGAGACTTGGTCTTATATTTAACCACCAAAATTCCC	253
7	chr11	5275520	5275820	7_Methylation5270934_	ACACTGACGACATG	7_Methylation5270934_HBG	TACGGTAGCAGAGA	274

				HBG2_7f	GTTCTAC AGGATTT GTGGTAT TTTTTGAT T	2_7r	CTTGGTC TACCTTA AAATTCT CAAAATC CA	
8	chr11	5275790	5276090	8_Methylation5270934_HBG2_8f	ACACTGA CGACATG GTTCTAC AGAGTAT TTAGTGA GGTTAGG GG	8_Methylation5270934_HBG2_8r	TACGGTA GCAGAGA CTTGGTC TTTTTACC AAACACA AAATCCT	277
9	chr11	5276060	5276360	9_Methylation5270934_HBG2_9f	ACACTGA CGACATG GTTCTAC AGGTTGA TAAAAGA AGTTTTG GT	9_Methylation5270934_HBG2_9r	TACGGTA GCAGAGA CTTGGTC TCCCCTA ACCTCAC TAAATACT C	295
10	chr11	5276340	5276640	10_Methylation5276490_HBG2_10f	ACACTGA CGACATG GTTCTAC ATTGAAA TTGTTGTT TTATAGG ATT	10_Methylation5276490_HBG2_10r	TACGGTA GCAGAGA CTTGGTC TCCAAAA CTTCTTTT ATCAACC	292
11	chr11	5277060	5277360	11_Methylation5277210_HBG2_11f	ACACTGA CGACATG GTTCTAC AGATTAT GAAGTTT GAAAGGA TTTT	11_Methylation5277210_HBG2_11r	TACGGTA GCAGAGA CTTGGTC TCATATTA ACCACTT AACATAA CAAAAA	253
12	chr11	5279236	5279536	12_Methylation5279386_HBG2_12f	ACACTGA CGACATG GTTCTAC ATGTGGT TTAGATTT TTAGGTT TT	12_Methylation5279386_HBG2_12r	TACGGTA GCAGAGA CTTGGTC TAAAAAT ACATTAC AACTCCC ACT	262

Western Blot

EPCs were cultured with compounds for 24 hours and then lysed in RIPA buffer (Teknova) with phosphatase and protease inhibitors (Roche). Lysates were briefly sonicated on ice, and then clarified by centrifugation at 20,000 RCF for 10 minutes. After determination of protein concentration, equal amounts of total protein were prepared in loading buffer (Invitrogen) and loaded on Tris Acetate gels (Invitrogen). Gels were run at 150V and then transferred to nitrocellulose membranes (iBlot System, Thermofisher). Resulting membranes were blocked for 2 hours with blocking buffer (Odyssey), and then probed with primary antibodies in a 1:1 mix of blocking buffer and PBS + 0.1% TWEEN 20 at 4°C overnight. Antibodies used were

DNMT1, DNMT3A, and Vinculin (Cell Signaling Technologies) and DNMT3B (Sigma). Primary antibody mix was removed, membranes were washed repeatedly with PBS + 0.1% TWEEN 20, and membranes were probed with Odyssey secondary antibodies for 2 hours at room temperature. Blots were again washed repeatedly and were then scanned using Licor IR scanner. Band densitometries were analyzed with Odyssey v2.1.

Supplemental Table 1. Effects of alternative decitabine dose regimens *in SCD mouse model*. Results are for 4 studies and reflect mean values for each dose group of %F-cells (flow cytometry) or %HbF (HPLC) in whole blood.

Study	Dose (mg/kg)	Frequency	%F-cell Fold-Change (FC)	%HbF Fold-Change (HPLC)
1	0.1	QD M-F	**1.3**	nd
1	0.3	QD M-F	**2.5***	nd
1	0.9	QD M-F	**nd †**	nd
2	0.3	QD M-F	**5.9†**	**2.9†**
3	0.3	QD M,W,F	nd	**2.0**
4	0.2	QD M,W,F	nd	**1.1**
4	0.4	QD M,W,F	nd	**2.0***
4	0.8	QD M,W,F	nd	**4.8†**

nd: not done

* : P <0.01

most effective tolerated dose

† : not tolerated (≥ 33% mortality)

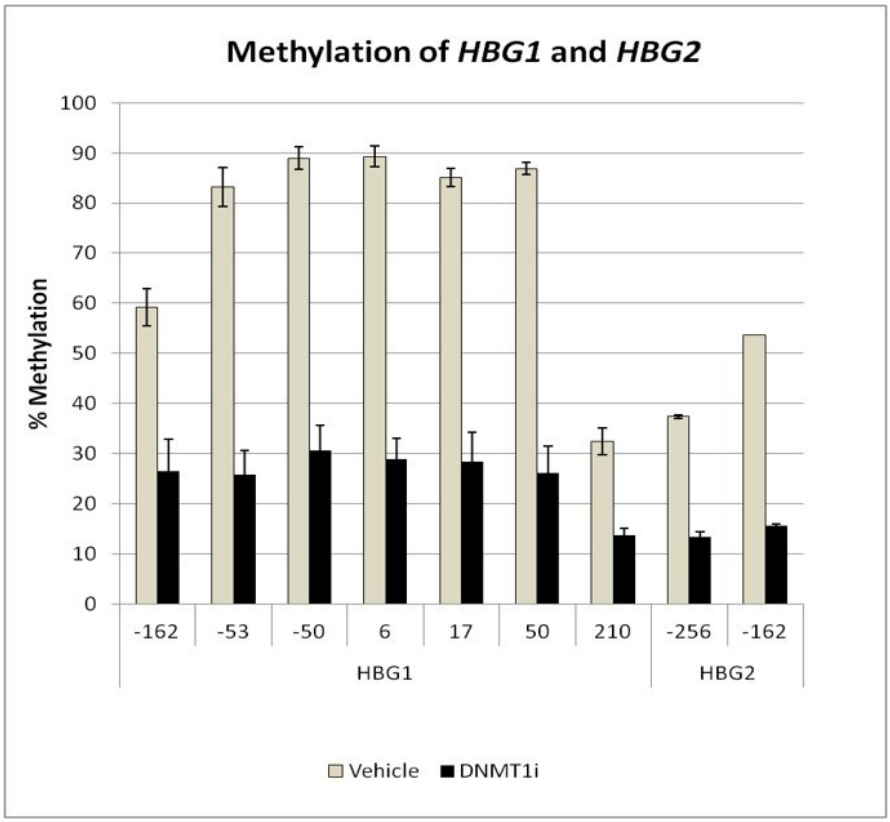
Supplemental Table 2. Effects of decitabine (M,W,F) *in vivo* on peripheral blood. Mean +/- SD are shown. Statistically significant treatment-related differences from vehicle-treated mice are indicated in bold font. Asterisks indicate significance by 1-way ANOVA. (*: P <0.01; **: P <0.001)

Study 2-Decitabine	***RBCs*** **($x10^6$/µl)**	***Platelets*** **($x10^3$/µl)**	***Neutrophils*** **($x10^3$/µl)**	***Lymphocytes*** **($x10^3$/µl)**	***Monocytes*** **($x10^3$/µl)**	***% Change DNA Methylation***
Vehicle	7.7 ± 0.7	673 ± 88	3.8 ± 1.2	12.4 ± 6.2	0.7 ± 0.4	0 ± 4.8
0.2 mg/kg	**5.9 ± 0.5 ****	657 ± 122	**2.1 ± 0.4 ***	8.1 ± 1.0	**0.2 ± 0.2 ***	-4.3 ± 6.5
0.4 mg/kg	**4.8 ± 0.9**	553 ± 57	**0.8 ± 0.2**	**5.8 ± 1.7**	**0.2 ± 0.1**	-9.3 ± 11.3

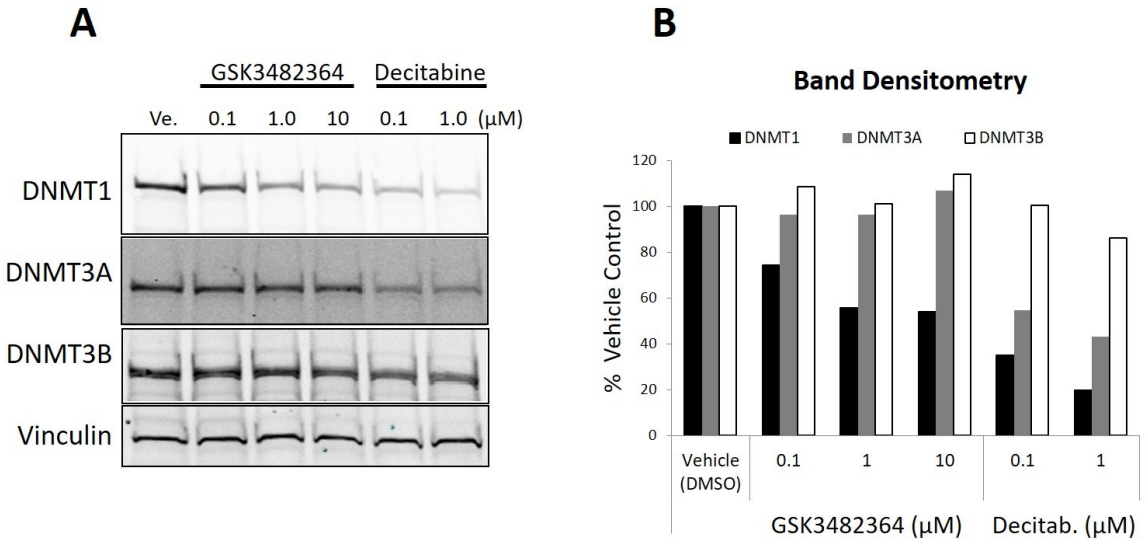
	**		**	*	*	
0.8 mg/kg	**1.7 ± 0.3 ** **	**427 ± 61 ***	**0.09 ± 0.06 ** **	**2.5 ± 1.1 ** **	**0.0 ± 0.0 * ***	**-13.2 ± 6.0**

Supplemental Figure 1. Effect of DNMT1 inhibitor on methylcytosines around *HBG1* and *HBG2* by bisulfite sequencing. Depicts the bisulfite sequencing results for EPCs treated for 3 days with 10 μM GSK3484862 (an active diastereomer of GSK3482364). Sites of methylation are labeled as positions relative to respective start sites for *HBG1* and *HBG2*.

1



Supplemental Figure 2. Effect of GSK3482364 or decitabine on DNMT protein levels. (A) Immunoblots of EPCs treated for 24 hours with GSK3482364 or decitabine. Vinculin is included as a high MW loading control. (B) Densitometry of bands with Odyssey software, corrected for vinculin band signal as loading control.



Supplemental Figure 3. Effects of GSK3482364 *in vivo* on reduced frequency dose schedule and longer duration dosing. (A) %HbF by HPLC in whole blood of transgenic sickle cell mice treated with GSK3682364A for 11days, dosed orally, b.i.d. for either 3 (black) or 5 (gray) weekdays per week. (B) %HbF of transgenic sickle cell mice treated with GSK3682364A for 2 weeks or 4 weeks, dosed orally, b.i.d. for 3 weekdays per week. Bars represent mean %HbF +/- SD from 5-6 mice. (**: P <0.001).