

## RESEARCH ARTICLE

# Salubrinal induces fetal hemoglobin expression via the stress-signaling pathway in human sickle erythroid progenitors and sickle cell disease mice

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## Abstract

Sickle cell disease (SCD) is an inherited blood disorder caused by a mutation in the *HBB* gene leading to hemoglobin S production and polymerization under hypoxia conditions leading to vaso-occlusion, chronic hemolysis, and progressive organ damage. This disease affects ~100,000 people in the United States and millions worldwide. An effective therapy for SCD is fetal hemoglobin (HbF) induction by pharmacologic agents such as hydroxyurea, the only Food and Drug Administration-approved drug for this purpose. Therefore, the goal of our study was to determine whether salubrinal (SAL), a selective protein phosphatase 1 inhibitor, induces HbF expression through the stress-signaling pathway by activation of p-eIF2 $\alpha$  and ATF4 trans-activation in the  $\gamma$ -globin gene promoter. Sickle erythroid progenitors treated with 24 $\mu$ M SAL increased F-cells levels 1.4-fold ( $p = 0.021$ ) and produced an 80% decrease in reactive oxygen species. Western blot analysis showed SAL enhanced HbF protein by 1.6-fold ( $p = 0.0441$ ), along with dose-dependent increases of p-eIF2 $\alpha$  and ATF4 levels. Subsequent treatment of SCD mice by a single intraperitoneal injection of SAL (5mg/kg) produced peak plasma concentrations at 6 hours. Chronic treatments of SCD mice with SAL mediated a 2.3-fold increase in F-cells ( $p = 0.0013$ ) and decreased sickle erythrocytes supporting *in vivo* HbF induction.

## Introduction

Sickle Cell Disease (SCD) is a group of inherited blood disorders caused by point mutations in the *HBB* gene on chromosome 11. About 100,000 African Americans are affected with SCD in the United States, while millions suffer worldwide. The clinical symptoms of SCD include chronic hemolytic anemia, recurrent acute pain, and progressive organ damage. However,

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higher fetal hemoglobin (HbF;  $\alpha_2\gamma_2$ ) levels ameliorate clinical severity in people with SCD due to inhibition of hemoglobin S (HbS) polymerization in erythrocytes. Data generated in the Cooperative Study of Sickle Cell Disease, demonstrated that HbF >8.6% correlated with improved long-term survival in adults with SCD [1]. Therefore, reactivation of *HBG* gene expression by pharmacologic agents is an effective therapeutic strategy for treating SCD [2].

Several drugs induce HbF *in vivo* including hydroxyurea (HU), arginine butyrate, and decitabine, among others; however, undesirable side effects have hampered clinical development of some. Hydroxyurea is a short-acting cytotoxic ribonucleotide reductase inhibitor, which increases nitric oxide levels and activates soluble guanylyl cyclase leading to *HBG* activation [3]. Arginine Butyrate is a histone deacetylase inhibitor that rapidly stimulates HbF expression via mRNA stabilization [4]. By contrast, decitabine is a DNA methyltransferase inhibitor mediating hypomethylation of the *HBG* proximal promoters and gene activation [5]. Currently, HU is the only Food and Drug Administration (FDA)-approved drug for inducing HbF synthesis in SCD patients. However, only two-third of adults respond to HU treatment and some patients suffer dose-limiting side effects, such as bone marrow suppression, making this agent less than ideal [6]. After two decades of clinical investigation, three new drugs were recently approved by the FDA for the treatment of pain and anemia in SCD including L-glutamine [7], voxelotor [8] and crizanlizumab [9], however, these agents are not HbF inducers. More recently, novel HbF inducers such as the two anti-inflammatory drugs rapamycin [10] and pomalidomide [11] have been proposed, however, further investigations are needed to demonstrate efficacy *in vivo*. Therefore, we sought to develop a novel, HbF inducing agents with the potential to reduce oxidative stress in SCD erythrocytes.

Salubrinal (SAL) is a semi-permeable synthetic compound and selective inhibitor of protein phosphatase 1 (PP1). The net result of this action is to interfere with the recruitment of phosphorylated eukaryotic initiation factor 2 $\alpha$  (p-eIF2 $\alpha$ ) to PP1 leading to increased p-eIF2 $\alpha$  levels to activate downstream targets such as ATF4 (activating transcription factor 4) [12]. Furthermore, Chen, et al demonstrated that SAL rescues anemia in the DDRGK1<sup>F/F</sup> knockout mouse model [13]. These animals have hematopoietic dysfunction including death of hematopoietic stem cells and acute anemia. More directly related to *HBG* gene regulation, Hahn et al [2] demonstrated in normal erythroid progenitors, that SAL prevents dephosphorylation of p-eIF2 $\alpha$ , thus mediating ATF4 activation to regulate the integrated stress response pathway. In addition, SAL increases HbF production by a posttranscriptional mechanism, without affecting cell proliferation, differentiation, or *HBG* mRNA levels.

To expand these *in vitro* studies, we initially treated K562 cells with SAL mediating *HBG* activation with a simultaneous increase in HbF, p-eIF2 $\alpha$  and ATF-4 protein levels. To investigate the role of ATF4 in the mechanism of HbF induction by SAL, we identified predicted ATF4 binding sites at -835bp upstream of *HBG2*, and the second intron of *HBB* by ChIPseq analysis reported in the ENCODE (encyclopedia of DNA elements) database. Our confirmatory ChIP assay demonstrated *in vivo* ATF4 binding at the -835*HBG2* motif and to a lesser degree for *HBB*. Studies with siATF4 and SAL treatment support HbF induction by ATF4. Therefore, to expand the field we investigated sickle erythroid progenitors generated from peripheral blood mononuclear cells. SAL mediated a significant increase in HbF positive cells (F-cells), the mean fluorescent intensity (MFI) a measure to quantify HbF levels per cell and HbF protein levels by Western blot, along with a significant decrease in reactive oxygen species (ROS). Moreover, SAL increased p-eIF2 $\alpha$  and ATF4 expression in SCD progenitors. Subsequent treatment of the Townes SCD transgenic mice for four weeks with SAL produced a significant increase in F-cells with minimal hematopoietic toxicity. Finally, the percentage of sickled erythrocytes decreased by 66% at four weeks supporting a change in peripheral blood

phenotype. The novel observation of HbF induction in SCD transgenic mice support pre-clinical development of SAL as a treatment option for SCD.

## Materials and methods

### Drug treatments

Salubrinal (SML0951) purchased from Sigma-Aldrich (St. Louis, MO) was reconstituted in 100% dimethyl sulfoxide (DMSO; BP231) for a 50mM stock solution. A second SAL (SC202332) reagent was purchased from Santa Cruz Biotechnology (Dallas, TX) and reconstituted in water at a 1mM concentration stock. SAL dissolved in water was added to the cells containing media at the corresponding concentrations and did not precipitate out of solution at any point in the 48-hour incubation. Hemin (51280), hydroxyurea (H8627) and L-cysteine (168149) were purchased from Sigma-Aldrich. Hemin and hydroxyurea are known HbF inducers and were used as controls. Cysteine is a negative control since it does not induce HbF synthesis.

### Tissue culture

K562 cells cultured in Iscove's Modified Dulbecco medium, fetal bovine serum (10%), penicillin and streptomycin underwent drug inductions for 48 hours, and cell viability assessed by 0.4% Trypan blue exclusion. For primary cultures, human erythroid progenitors were generated from peripheral blood mononuclear cells (PBMCs) isolated from two SCD patients under IRB exempt protocol as previously published [14]. Treatments with SAL, DMSO, cysteine, hemin, HU, and combined SAL/HU occurred on day 8 erythroid progenitors and then harvested on day 10.

### Flow cytometry

K562 cells and erythroid progenitors were fixed with 4% paraformaldehyde, permeabilized and stained with fluorescein isothiocyanate (FITC) anti-HbF antibody (AB-19365, Abcam, Cambridge MA) and FITC-isotype IgG antibody (AB-37406). Flow cytometry was conducted on the FACSCanto (BD Biosciences, San Jose CA) as previously published [15]. To quantify ROS levels, cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) (10 $\mu$ M; Invitrogen, Carlsbad CA) 4 hours before harvest. To determine the effect of SAL on maturation, sickle erythroid progenitors (5x10<sup>5</sup>) were harvested after drug inductions, fixed with 4% paraformaldehyde and then permeabilized in ice-cold acetone/methanol (4:1). The cells were stained with human FITC conjugated transferrin receptor (CD71; ThermoFisher: 11-0719-42) and human FITC conjugated glycophorin A (CD235a; ThermoFisher: 11-9886-42) antibodies and analyzed by flow cytometry.

### Western blot

Western blot was performed as previously published [16] with antibodies purchased from Santa Cruz Biotechnology including HbF (SC-21756), HbS (SC-21757), eIF2 $\alpha$  (SC-11386) and Tubulin (SC-53646).  $\beta$ -actin (AM4302) was purchased from Invitrogen (Waltham, MA) and phospho-eIF2 $\alpha$  (Ser51) (CS-3398S) and ATF4 (CS-11815S) from Cell Signaling Technology (Danvers, MA).

### Reverse transcription-quantitative PCR (RT-qPCR) analysis

The RT-qPCR assays were performed as previously published [17]. Gene-specific primers were used to quantify mRNA levels of *HBG*, *HBB*, *ATF4*, *BCL11A* and internal control

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); all mRNA levels were normalized to *GAPDH* for comparative Ct (threshold cycle) statistical analysis. A summary of primer sequences is shown in [S1 Table](#). ATF4 gene silencing was achieved using ATF4 siRNA (SC-35112, Santa Cruz Biotechnology) (200 nM) to transfect K562 cells for 24 h using the Dharmafect 1 system; scrambled siRNA (sc-37007, Santa Cruz Biotechnology) was used as control. After transfections, K562 cells were treated with SAL (24 $\mu$ M) in triplicates for 48 hours and analyzed by RT-qPCR.

### Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed using the Active Motif ChIP-IT High Sensitivity kit (Carlsbad, CA) per the manufacturer's instruction. Briefly, DNA was cross-linked with 1% formaldehyde, and sonicated to shear DNA to ~500 bp. Immunoprecipitations were performed using ATF4 antibody (SC-390063), mouse IgG and anti-RNA polymerase II. Chromatin enrichment was quantified by qPCR using primer pairs designed with Primer-BLAST software. A summary of primer sequences can be found in [S1 Table](#).

### Pharmacokinetics protocol

SCD mice received a single intraperitoneal (IP) injection of SAL 5mg/kg and blood was drawn at 15 min, 30 min, and 1, 2, 3 6 and 24 hours for liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM MS) at Augusta University. The optimal collision energy and retention factor lens were determined using standards and transitions established for SAL was 481/187, 481/129 and 481/189. The integrated peak areas for transitions were calculated (Skyline software, version 20.0).

### Townes transgenic mouse treatment

All animal work was done according to the rules of animal services and under an IACUC protocol at Augusta University; Approval Number: 2012–0437. A standard protocol was used for anesthesia that was approved by animal care at Augusta University. The Townes SCD mouse completes hemoglobin switching from human  $\gamma$ -globin to  $\beta^S$ -globin shortly after birth [18]. These mice display the same clinical symptoms as human SCD patients including erythrocyte sickling, hemolysis and anemia, splenomegaly, and vaso-occlusive pain. Before starting chronic treatments, mice injected with a single dose of SAL 5 mg/kg and were sacrificed to confirm SAL did not precipitate in the peritoneum. Drug treatments in SCD mice (4–6 months old) included: 1) water control (vehicle), 2) HU 100 mg/kg/day (positive control), 3) SAL 3 mg/kg/day, and 4) SAL 5 mg/kg/day, dissolved in water. Intraperitoneal injections occurred 5 days a week for 4 weeks, for two independent replicates (sample size 10 mice per group). Blood collected in EDTA tubes by tail bleed at week 0, 2, and 4 was analyzed for complete blood count and differential (Micros 60 machine; HORIBA Medical/ABX Diagnostics) and F-cells and MFI quantified by flow cytometry as previously published [19]. Blood stained with BD retic-count reagent (acridine orange) quantifies reticulocytes by flow cytometry.

### Giemsa staining

Erythroid progenitors generated from sickle peripheral blood mononuclear cells and SCD mouse blood (10 $\mu$ L) were collected for blood smears and stained with the Sure-Stain Wright-Giemsa (CS434D; Fisher Scientific). The number of sickled erythrocytes per high power field were counted for 500 cells in triplicate per treatment condition for the mouse studies.

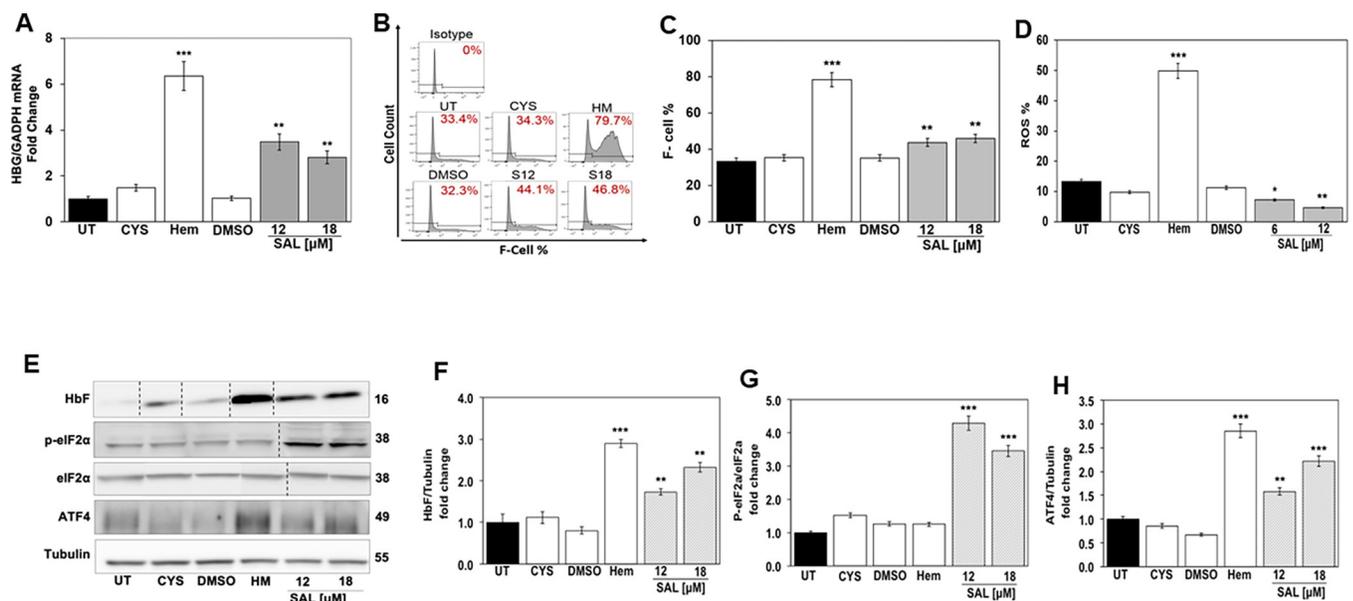
## Statistical analysis

Tissue culture experiments repeated for 3–5 independent studies with three replicates per run. The unpaired student's *t*-test was performed and  $*p < 0.05$  was considered statistically significant. The data are plotted as the mean  $\pm$  standard error of the mean (SEM). For the SCD mouse studies one-sample paired student's *t*-tests were performed to determine differences in blood counts, reticulocytes, F-cells, and MFI at week 0 compared to week 2 and 4 within groups ( $n = 10$ ). To compare the effect across treatment groups, pairwise comparisons analysis of variance (ANOVA) was performed. A level of  $p \leq 0.05$  was used for statistical significance.

## Results

### SAL induces HbF expression through the integrated stress response pathway in K562 cell line

To gain insight into mechanisms of HbF induction by SAL, studies were conducted in K562 erythroleukemia cells that carries an embryonic/fetal phenotype and expresses endogenous  $\epsilon$ -globin and  $\gamma$ -globin chains [20]. After treatment with SAL and other control agents for 48 hours, RT-qPCR determined *HBG* mRNA levels normalized to *GADPH*. A significant 3.8-fold ( $p = 0.002$ ) and 2.8-fold ( $p = 0.008$ ) increase in *HBG* mRNA was observed at SAL 12  $\mu$ M and 18  $\mu$ M respectively (Fig 1A) compared to DMSO vehicle control. As expected, hemin (Hem) produced a significant 6.2-fold increase ( $p = 0.00013$ ) in *HBG* synthesis. Subsequent flow cytometry with anti-FITC HbF antibody was conducted. Shown in Fig 1B and 1C are



**Fig 1. SAL increased *HBG* transcription and HbF synthesis.** K562 cells were grown without treatment (UT) or treated with Cysteine 10  $\mu$ M (CYS; negative control), hemin 50  $\mu$ M (Hem; positive control) or salubrinal (SAL) 12  $\mu$ M and 18  $\mu$ M and vehicle control dimethyl sulfoxide (DMSO). Total RNA was isolated and whole cell lysates generated for RT-qPCR and Western blot analysis (See [Material and Methods](#)). All data are shown as the mean  $\pm$  SEM ( $n = 6$ ) and  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$  was considered statistically significant. **A)** Shown in the bar graph is the *HBG*/*GADPH* normalized mRNA levels generated by RT-qPCR analysis for each treatment conditions. **B)** Shown are flow cytometry histograms of K562 cells under the different conditions after staining with FITC-conjugated anti-HbF antibody. **C)** Shown in bar graph is the percentage of F-cells (F-cell %)-using FACS Diva software. **D)** Shown in the bar graph is the ROS percentage in the different conditions after staining with DCFDA (See [Materials and Methods](#)). **E)** Western blot analysis determined HbF, p-eIF2 $\alpha$  and ATF4 protein levels compared with tubulin (internal loading control); shown is a representative blot. The vertical black lines represent the change in band order to represent the data. Quantitative data generated by densitometry analysis measured expression levels of **F)** HbF normalized to tubulin, **G)** p-eIF2 $\alpha$  normalized to total eIF2 $\alpha$ , and **H)** ATF4 normalized to tubulin.

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representative histograms and quantitative F-cell levels respectively compared to untreated (UT) K562 cells; note, SAL 12 $\mu$ M and 18 $\mu$ M increased F-Cells by 1.3-fold ( $p = 0.003$ ) and 1.4-fold ( $p = 0.004$ ) respectively.

To determine how quickly SAL modulated  $\gamma$ -globin transcription we performed time course studies. K562 cells treated with cysteine 10 $\mu$ M (CYS), Hemin 50 $\mu$ M (Hem), DMSO, SAL 12  $\mu$ M, and SAL/Hem (SAL12 $\mu$ M/HM50  $\mu$ M) were harvested at 6 and 24 hours. Subsequent RT-qPCR at 6 hours showed no change in  $\gamma$ -globin mRNA levels (S1A Fig), however at 24 hours a significant 1.4-fold ( $p = 0.034$ ) and 1.4-fold ( $p = 0.032$ ) increase in  $\gamma$ -globin by SAL and Hem ( $p < 0.05$ ) respectively was observed (S1B Fig). Furthermore, by Western blot analysis, SAL induced HbF at 6 hours 1.7-fold ( $p = 0.041$ ) and 2.2-fold ( $p = 0.0078$ ) by 24 hours (S1C and S1D Fig). These results demonstrate SAL induces HbF early time points but to see maximal effects we chose 48 hours based on previous *in vitro* experiments across all cells. These data contrast findings by Hahn and Lowrey [2] where lower SAL concentrations than used in our study, did not activate globin gene transcription in normal primary erythroid cells. The level of  $\gamma$ -globin mRNA was not measured in K652 cells by their study.

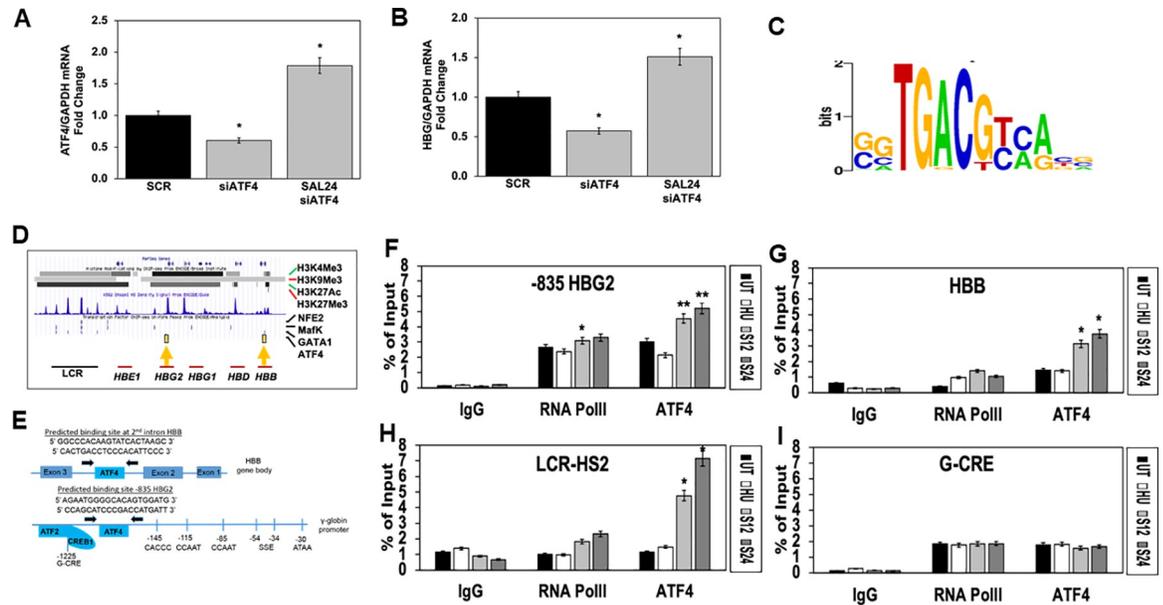
To measure oxidative stress, K562 cells were treated with DCF-DA (10  $\mu$ M) and analyzed by flow cytometry which, showed a decrease in ROS by 50% ( $p = 0.025$ ) and 60% ( $p = 0.008$ ) at SAL 6 $\mu$ M and 12 $\mu$ M compared to 5-fold ( $p = 0.0001$ ) increase by hemin, known to induce oxidative stress (Fig 1D). To gain insight into the role of stress signaling in HbF induction by SAL, Western blot (Fig 1E) confirmed the ability of SAL to induce HbF by 2.5-fold ( $p = 0.005$ ) and increase p-eIF2 $\alpha$  by 3.8-fold ( $p = 0.00015$ ) and ATF4 by 2.0-fold ( $p = 0.00062$ ) (Fig 1F–1H). Interestingly, hemin induced ATF4 by 2.9-fold ( $p = 0.00045$ ) related to its ability to generate high levels of ROS in cells (Fig 1H).

To further support a functional role of ATF4 in *HBG* regulation, we performed siATF4 gene silencing studies. K562 cells were transfected with scrambled siRNA (Scr) or 200nM siATF4 24 hours, followed by the addition of SAL (24 $\mu$ M) RNA for 48 hours. As shown in Fig 2A, using Scr as control normalized to one, siATF4 200nM silenced decreased  $\gamma$ -globin significantly by 50% ( $p = 0.045$ ). Next, we performed rescue experiments and showed SAL treatment overcame siATF4 inhibition and increase endogenous ATF4 levels by 3-fold ( $p = 0.025$ ). We also analyzed the effects on *HBG* levels under the same treatment conditions and showed siATF4 decreased *HBG* mRNA levels 47% ( $p = 0.019$ ), which was overcome by SAL treatment, activating *HBG* 2.1-fold ( $p = 0.04$ ) compared to siATF4 (Fig 2B). Moreover, SAL 24 $\mu$ M increased *HBG* by 2.5-fold above siATF4 treatment levels. We concluded SAL can overcome the silencing effect of siATF4 to activate *HBG* transcription supporting a role for ATF4 in *HBG* activation.

### SAL increase ATF4 binding in the *HBB* locus *in vivo*

To achieve *HBG* activation, we predicted ATF4 binding *in vivo* after SAL treatment, therefore potential ATF4 consensus motifs were identified using ISMARA (integrated system for motif activity response analysis) software. A consensus ATF4 (5'-TGACGTCA-3') motif was identified in the G-CRE at -1225 upstream of *HBG2* (Fig 2C). We previously demonstrated ATF2/CREB1 binding to the G-CRE that was required for HbF induction by sodium butyrate [21]. Since ATF2 is a known binding partner of ATF4 [22], we examine ChIPseq data generated in K562 cells by the ENCODE project. We observed strong ATF4 binding at -835bp upstream of *HBG2* and a weaker binding site in the second intron *HBB* (Fig 2D); this data was used to design qPCR primers (Fig 2E).

To investigate *in vivo* DNA-protein interaction, ChIP assay was completed in K562 cells treated with SAL (12 $\mu$ M and 24 $\mu$ M) for ATF4 binding in the -835*HBG2* region, *HBB* gene, LCR-HS2 and G-CRE along with control RNA PolII (gene transcription rate) binding (Fig 2F–2I). Since the *HBG* gene is actively transcribed in K562 cells, we observed 3.2-fold



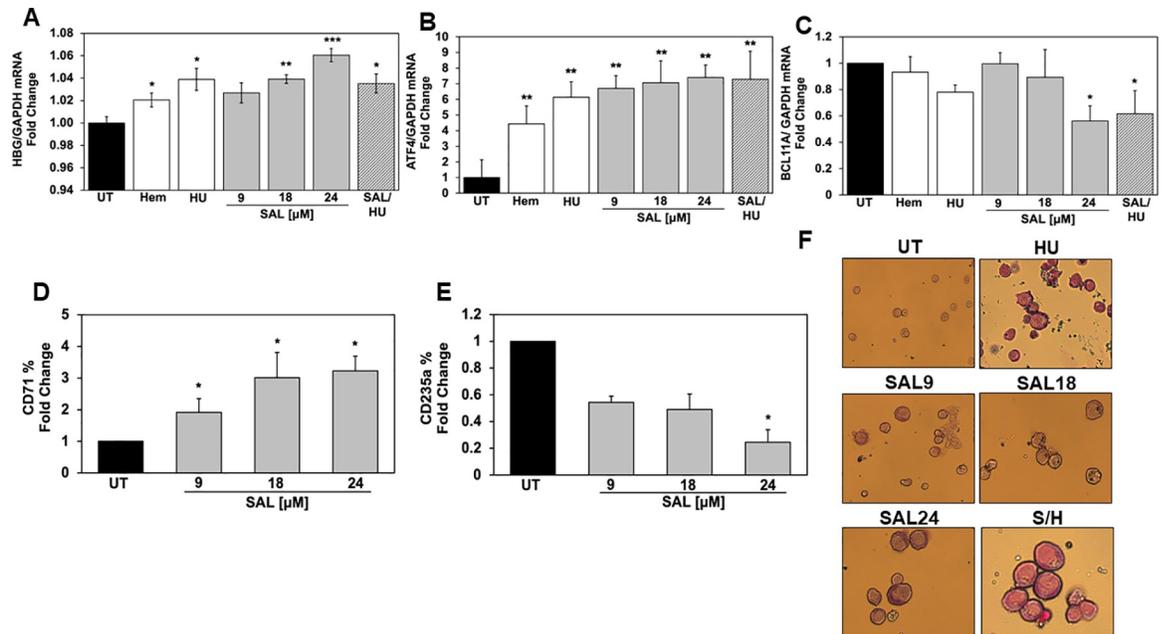
**Fig 2. ATF4 binds to the *HBG* and *HBB* promoters to activate transcription.** K562 cells were transfected with 200nM siATF4 or siRNA scrambled (SCR) control for 24 hours, followed by treatment with SAL (24 $\mu$ M) in triplicates for 48 hours. Total RNA was collected, and RT-qPCR analysis was performed to determine the levels of **A) *ATF4*** and **B) *HBG*** mRNA expression levels after siATF4 transfection. **C)** ISMARA (Integrated System for Motif Activity Response Analysis) software was used to generate the ATF4 consensus binding sequence: 5'GTGA CGT [A/C] [A/G]3'. **D)** ChIPseq for K562 cells was downloaded from the ENCODE database which showed strong ATF4 binding in the region of the predicted motif -835bp*HBG2* promoter and weaker binding in the second intron of the *HBB* gene (yellow arrows). **E)** Schematic of ChIP assay primer sequences used for qPCR analysis. **F-I)** K562 cells were treated with HU (75 $\mu$ M; white bar), SAL 12 $\mu$ M (light grey) and SAL 24 $\mu$ M (dark grey) for 48 hours and used for ChIP assay for IgG (non-specific binding control), RNA polymerase II (PolII) (transcription rate control), and ATF4 binding at the **F) -835*HBG2***, **G) *HBB*** gene, **H) LCR-HS2** and **I) G-CRE** (n = 3 per group). Data are shown as mean  $\pm$  SEM and \*p<0.05; \*\*p<0.01; \*\*\* p<0.001 was considered statistically significant.

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(p = 0.015) chromatin enrichment for RNA PolII and ATF4 up to 5.2-fold (p = 0.011) at -835*HBG2* compared to IgG control (Fig 2F). As expected, there was lower ATF4 binding at the *HBB* site (Fig 2G). Since the LCR-HS2 is a powerful globin gene enhancer element, we investigated ATF4 binding in this region [23]. Interestingly, there was a 7.2-fold (p = 0.022) increase in ATF4 binding in the LCR-HS2 at SAL 24 $\mu$ M (Fig 2H). The predicted ATF4 site in the G-CRE showed 2-fold chromatin enrichment, which was not significantly changed by SAL treatment nor was RNA PolII binding (Fig 2I).

### SAL activates *HBG* and *ATF4* transcription in human sickle erythroid progenitors

Hahn et al previously reported the ability of SAL to induce HbF in normal erythroid progenitors generated from CD34+ stem cells [2]. Therefore, to provide additional support for developing SAL for treatment of SCD, we generated primary erythroid progenitors from PBMCs isolated from sickle cell patients as previously published [14]. These cells are under oxidative stress and undergo hemolysis. After erythroid lineage commitment on day 8, we treated progenitors with SAL (9, 18 and 24 $\mu$ M) dissolved in water alone or combined with HU (75 $\mu$ M) for 48 hours. Cell viability remained >90% for all groups; RT-qPCR was performed and *HBG* mRNA were normalized by *GAPDH*. The calculated *HBG/GAPDH* mRNA ratios showed a significant 1.04-fold (p = 0.004) and 1.06-fold (p = 0.001) increase at SAL 18 and 24 $\mu$ M respectively (Fig 3A), which was not increased further by combination SAL/HU treatment.



**Fig 3. SAL activates *HBG* without changing *HBB* mRNA levels in sickle erythroid progenitors.** Sickle erythroid progenitors were treated on day 8 with Hem 75 $\mu$ M, HU 75 $\mu$ M and SAL 9, 18 and 24 $\mu$ M alone or SAL 18 $\mu$ M combined with HU 75 $\mu$ M (SAL was dissolved in water). After 48 hours of treatment, data were generated and shown as the mean  $\pm$  SEM (n = 6) and \*p<0.05; \*\*p<0.01; \*\*\* p<0.001 was considered statistically significant. **A)** RT-qPCR demonstrated the *HBG* mRNA levels were normalized to *GAPDH* the internal control. RT-qPCR demonstrates **B)** *ATF4* mRNA levels and **C)** *BCL11A* mRNA levels normalized with *GAPDH*. Sickle erythroid progenitors under the different treatment conditions were stained with human FITC-conjugated **D)** CD71 and **E)** CD235a antibody and analyzed by flow cytometry. **F)** Representative images of erythroid progenitors generated at 40-x magnification by light microscopy.

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To define molecular mechanism in sickle progenitors, we quantified *ATF4* mRNA levels, which showed a significant dose-dependent increase of 6.7-fold ( $p = 0.010$ ), 7.1-fold ( $p = 0.004$ ) and 7.3-fold ( $p = 0.0026$ ) by SAL 9, 18 and 24 $\mu$ M, respectively (Fig 3B). Interestingly, treatment with HU produced a 6.1-fold ( $p = 0.025$ ) increase in *ATF4* suggesting an additive effect might not be expected for combination SAL/HU studies (Fig 3B). We next tested the ability of SAL to silence the *HBG* repressor B-cell lymphoma/leukemia 11A (*BCL11A*). Like HU, SAL 24 $\mu$ M produced a 44% decrease in *BCL11A* ( $p = 0.032$ ) expression which was comparable to SAL/HU combination treatment (Fig 3C).

To develop SAL for human trials, a better understanding of its effect on erythroid maturation is desirable. Therefore we measured transferrin receptor (CD71) and glycophorin A (CD235a), which are expressed on the surface of erythroid progenitors during differentiation [24]. As shown in Fig 3D, a dose-dependent 1.92-fold ( $p = 0.030$ ), 3.01-fold ( $p = 0.037$ ) and 3.2-fold ( $p = 0.011$ ) increase in CD71 expression was produced by SAL 9, 18 and 24 $\mu$ M, respectively. In addition, SAL mediated a dose-dependent decrease in CD235a expression by 44%, 51%, and 76% ( $p = 0.027$ ) at SAL 9, 18 and 24 $\mu$ M, respectively (Fig 3E). Shown in Fig 3F are Giemsa stains of progenitors under the different treatment conditions. These data suggest a dose-dependent delay in erythroid maturation by SAL.

### SAL induces HbF expression and reduces ROS in human sickle erythroid progenitors

Individuals with SCD experience oxidative stress produced by hemoglobin polymerization and chronic hemolysis with the release of free heme [25], therefore we investigated the ability

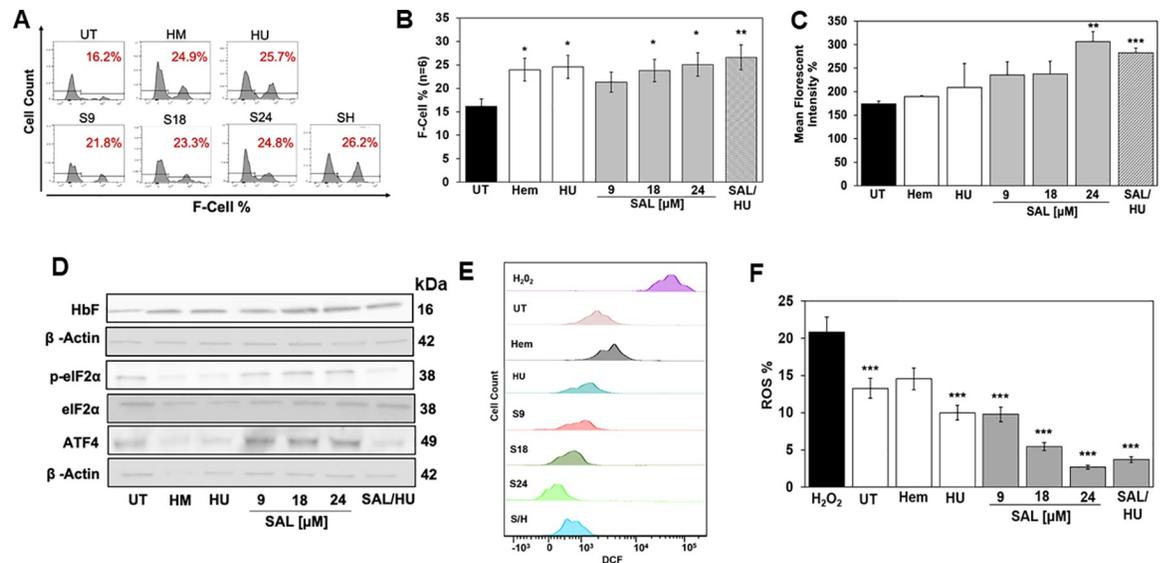
of SAL to induce HbF and decrease ROS in sickle erythroid cells. On day 8, we treated progenitors with SAL (9, 18 and 24 $\mu$ M) or combined with HU (75 $\mu$ M) dissolved in water for 48 hours. Cell viability remained >90% for all groups. As shown in the representative histograms (Fig 4A) and quantitative bar graph (Fig 4B), treatment with SAL 9, 18 and 24 $\mu$ M increases F-cells from 15.3% (untreated) to 21.3%, 23.8% and 25.1% ( $p = 0.021$ ) respectively. Likewise, we observed an increase in HbF levels per cell using MFI (Fig 4C) and Western blot analysis (Fig 4D), reaching significance at the SAL 24 $\mu$ M ( $p = 0.003$ ) concentration; by contrast, HbS expression was not altered by SAL (S2A and S2B Fig). In the presence of SAL 24 $\mu$ M we observed a significant dose-dependent increase of HbF, p-eIF2 $\alpha$  and ATF4 up to 1.6-fold ( $p = 0.0441$ ), 1.8-fold ( $p = 0.043$ ) and 1.5-fold ( $p = 0.013$ ) respectively (S2C–S2E Fig). Lastly, we determined changes in ROS levels, SAL treated cells were stained with DCF-DA (10  $\mu$ M) for 4h and flow cytometry completed. Shown in Fig 4E is representative histograms confirming a dose-dependent decrease in ROS by 30.8% ( $p = 0.0000272$ ), 63.8% ( $p = 0.00000644$ ) and 85% ( $p = 0.000000133$ ) with increasing SAL concentrations (Fig 4F); adding HU did not improve ROS levels further.

### SAL induces HbF expression in SCD transgenic mice

Our long-term goal is to develop a novel agent for treatment of individuals with SCD; therefore, preclinical studies evaluating the pharmacokinetics characteristics, toxicity, and ability of SAL to induce HbF, were completed in Townes SCD transgenic mice [18]. To establish the plasma distribution of SAL, liquid chromatography-multiple reaction monitoring mass spectrometry showed SAL parent ion peak at  $m/z$  481.03 (Fig 5A). Next, we treated mice with a single intraperitoneal (IP) SAL 5mg/kg dose and blood was drawn at 15 min, 30 min, and 1, 3, 6, 12 and 24 hours; SAL peaked at 6 hour (29 fmol) in plasma and decreased to barely detectable levels by 24 hours (Fig 5B and 5C).

To determine *in vivo* efficacy and toxicity, we treated SCD mice 4–6 months old with SAL 3 mg/kg/day (SAL3) and SAL 5 mg/kg/day (SAL5) or HU 100mg/kg/day (HU100) by IP injections, 5 days per week for 4 weeks (S3 Fig). Two independent replicates were completed with five mice each ( $n = 10$  per group). Animals were weighed and peripheral blood drawn at week 0, 2 and 4. Over 4 weeks of treatment, normal daily activity and weight gain was maintained (Fig 5D) and no drug toxicity or significant change in blood counts was observed (S4 and S5 Figs). By contrast, HU produced a sustained significant decrease in granulocyte, monocyte and platelet counts by week 4. To evaluate the effect on erythropoiesis we measured reticulocyte count using acridine orange and flow cytometry analysis. Treatment with SAL3 produced a significant decrease in reticulocyte count from 35.9% to 27.3% ( $p = 0.035$ ) at week 4 which was not observed at the higher dose of SAL (Fig 5E), while treatment with HU100 increased reticulocytes up to 49.15% ( $p = 0.033$ ). To determine whether SAL and HU treatment changed reticulocyte counts significantly compared to vehicle control, ANOVA was performed with  $n = 10$  mice per group. Of note, the minor decrease at SAL3 week 4 was not different from control. By contrast, HU100 significantly increased reticulocytes counts ( $p = 0.00018$ ) compared to water control (Fig 5E, brackets). While significant changes in hemoglobin or hematocrit were not mediated by SAL, more importantly, there was no signs of toxicity on hematopoiesis. The variability of individual response to treatment recapitulates patterns observed in human clinical trials for HU in sickle cell patients.

To establish whether SAL mediated *in vivo* HbF induction, we performed flow cytometry analysis. For SAL3, F-cells increased from 3.7% to 8.5% ( $p = 0.0013$ ) and SAL5 increased F-cells to 8.4% ( $p = 0.0023$ ) (Fig 6A). This level of F-cell induction was comparable to the 8.4% increase for HU100 ( $p = 0.014$ ); shown in Fig 6B, is representative histograms of F-cells data.



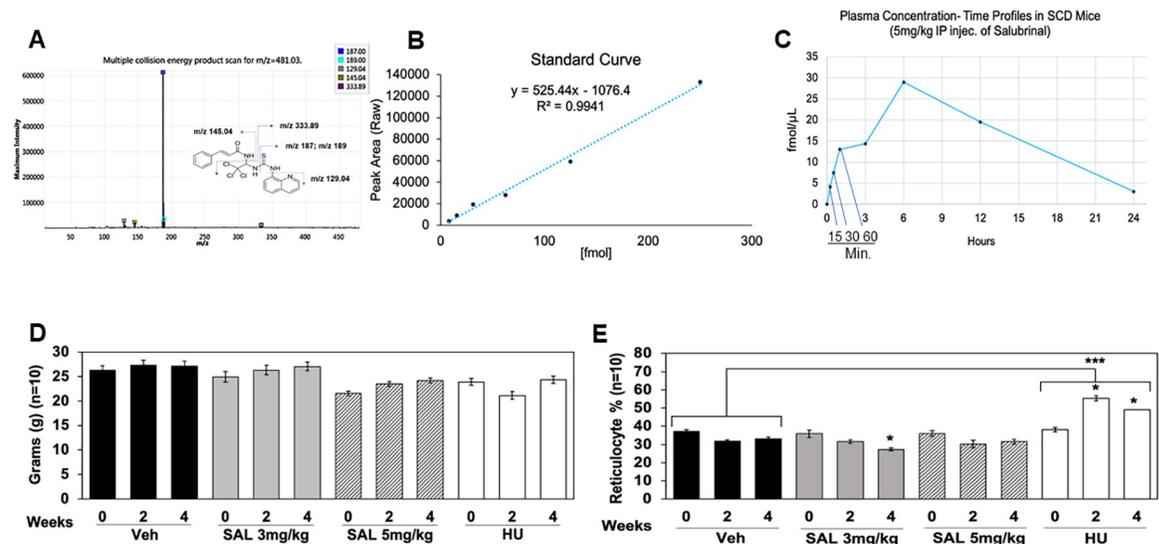
**Fig 4. SAL activates HbF expression and reduces ROS levels in sickle erythroid progenitors.** Sickle erythroid progenitors were grown in our two-phase culture systems and were treated on day 8 with Hem 75 $\mu$ M, HU 75 $\mu$ M and SAL 9, 18 and 24 $\mu$ M alone or SAL 18 $\mu$ M combined with HU 75 $\mu$ M. After 48 hours of treatment, data were generated and shown as the mean  $\pm$  SEM (n = 6) and \*p<0.05; \*\*p<0.01; \*\*\* p<0.001 was considered statistically significant. **A**) Shown is histograms representation of F-cell levels determined by flow cytometry. **B**) Shown is quantitative data generated by FlowJo analysis. **C**) The level of HbF expression was measured by MFI data generated by flow cytometry analysis. **D**) Representative western blots gel of HbF, p-eIF2 $\alpha$  and ATF4. **E**) Shown is a representative histogram overlay of DCF positive cell counts generated by flow cytometry as a measure of ROS levels; hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was used as a positive control. **F**) Shown in the bar graph is the quantitative data of ROS levels compared to untreated cells (UT).

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ANOVA analysis demonstrated significant differences in F-cells for mice treated with SAL3 (p = 0.0357), SAL5 (p = 0.0421) and HU100 (p = 0.0305) compared to water vehicle control. We also quantified HbF levels per cell by MFI analysis, where we observed a significant increase from 297 to 542 units (p = 0.00014) for SAL3 and SAL5 changed from 335 to 502 units (p = 0.0044) (Fig 6C). Similarly, HU increased MFI to comparable levels suggesting SAL might be effective *in vivo* like HU in SCD. To further support SAL clinical development, we tested its ability to achieve an anti-sickling effect. Mouse blood was examined by light microscopy (Fig 6D) and quantitative data confirmed SAL5 reduced the percentage of sickled erythrocytes by 66% (p = 0.00319) (Fig 6E). These findings support the ability of SAL to induce HbF and produce phenotypic changes in the preclinical SCD transgenic mouse model, like HU without significant side effects or toxicity.

## Discussion

Identifying molecular mechanisms involved in *HBG* activation will support development of additional novel therapies for individuals living with SCD. Hydroxyurea is the only FDA-approved HbF inducing drug used in SCD which ameliorates the clinical phenotype of frequent pain episodes, inflammation and hemolysis, and improves long term survival [26]. Other potent HbF inducer tested in clinical trials such as arginine butyrate [27, 28], 5-azacytidine [29], decitabine [30] and short chain fatty acid derivatives [31] function through diverse molecular mechanisms but none have advanced to FDA-approval for SCD. Factors such as a requirement for intravenous administration of arginine butyrate, hindered development since the agent is rapidly inactivated when given orally [32]. Likewise, decitabine mediated HbF induction in a Phase 1 clinical trial when given intravenously, however lack of efficacy by oral administration required combination treatments with tetrahyouridine, which has shown



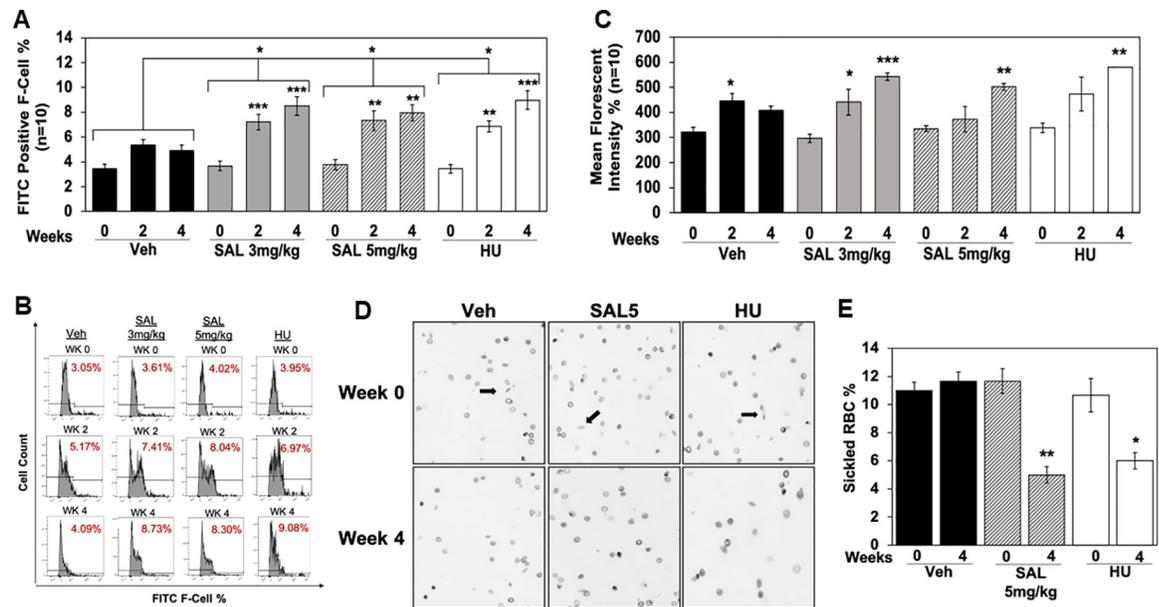
**Fig 5. Plasma level of SAL in SCD transgenic mice and reticulocyte analysis.** A) At the corresponding time points, blood was drawn by tail bleed into an EDTA tube. Plasma was immediately collected and 80 $\mu$ l acetonitrile was added to 20 $\mu$ l of mouse plasma and vortexed for 5 minutes. The samples were then centrifuged at 16,000g for 10 minutes and the supernatant was transferred into a glass vial for liquid chromatography- multiple reaction monitoring mass spectrometry. Multiple collision scan for the SAL molecule shows the different fragmentation by mass spectrometry. B) Shown is the standard curve used to determine plasma concentrations of SAL. C) The *in vivo* SAL levels were calculated from the standard curve (Panel B), using plasma isolated from peripheral blood of SCD mice. After establishing the plasma concentration of SAL, we next treated Townes SCD transgenic mice (4–6 months old) with SAL 3 mg/kg/day and SAL 5 mg/kg/day dissolved in water, for 4 weeks by intraperitoneal (IP) injections. Water vehicle control (Veh) and positive control hydroxyurea 100 mg/kg/day (HU) treatments were completed. All treatment groups consisted of n = 10 mice with an equal sex distribution of 5 males and 5 females. D) Mouse weights in grams were obtained at week 0 (baseline), week 2, and week 4. E) Blood samples collected at weeks 0, 2 and 4 were stained with acridine orange for reticulocyte counts by flow cytometry. Data are shown as the mean  $\pm$  SEM and brackets represent results of the ANOVA analysis across treatment groups. \*p<0.05 and \*\*\* p<0.001 was considered significant.

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promise in clinical trials with SCD patients [33]. Therefore, the discovery of non-chemotherapeutic oral HbF inducers that act by unique molecular and cellular mechanisms for use as a standalone agent or combined with HU support development of additional safe and effective treatment options for SCD.

We investigated the ability of SAL to induce HbF expression through the eIF2 $\alpha$ -ATF4 stress-signaling pathway in normal and sickle erythroid progenitors. SAL is a semi-permeable synthetic compound and is a selective inhibitor of PP1, interfering with the recruitment of p-eIF2 $\alpha$  to PP1 through GADD34 and CREP. Under stress conditions, p-eIF2 $\alpha$  rapidly inhibits general protein synthesis, including globin chains to prevent cell toxicity. Moreover, p-eIF2 $\alpha$  increases translation of selective mRNAs, such as ATF4 to reprogram gene expression for adaptation to stress.

Severe cellular oxidative stress associated with chronic hemolytic states is predicted to promote cell death via ATF4-mediated activation of C/EBP homologous protein [34]. Previously our group established the ATF2/CREB heterodimer binds the consensus G-CRE motif to induce HbF expression in primary erythroid cells [35]. Since ATF4 binds in the G-CRE, we investigated p-eIF2 $\alpha$ -ATF4 stress signaling as a mechanism involved in HbF induction by SAL. To support this speculation, we observed increases in p-eIF2 $\alpha$  and ATF4 levels and *in vivo* binding of ATF4 at the -835*HBG* and *HBB* predicted motifs by ChIP assay, replicating published ENCODE ChIPseq data. In addition, there was an increase of ATF4 binding at the LCR-HS2 enhancer, which is required for DNA loop formation to activate globin gene transcription during hemoglobin switching. The weaker *HBB* motif showed low ATF4 binding



**Fig 6. SAL increases HbF expression and produce anti-sickling effects in SCD mice.** A) Peripheral blood collected at the time points shown was stained with FITC-anti-HbF antibody and flow cytometry performed to quantify F-cells by FlowJo analysis. The paired student's t-tests were used to determine the significance in F-cells at week 0 compared to week 2 and 4 within each group ( $n = 10$ ). ANOVA was used to compare the significance of F-cells between water compared to the two concentrations of SAL and HU group; results are represented by brackets between treatment group. Data are shown as the mean  $\pm$  SEM and \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*  $p < 0.001$  was considered significant. B) Representative histograms demonstrating the increase in the number of FITC-HbF positive cells from week 0 to week 4 for each treatment group. C) The level of HbF expression per cell was measured by MFI data generated by flow cytometry analysis. D) Shown is a representative image of peripheral blood generated at 40-x magnification by light microscopy. E) The levels of sickled red blood cells for the different conditions (black arrows) were quantified for 500 cells in triplicate and shown in the graph.

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however, we observed not increase in HbS *in vitro*. We speculate since the motif is not a promoter or enhancer element along with weaker histone marks, that ATF4 did not support activation of the *HBB* gene.

In all cells tested, SAL mediated a dose-dependent increase of F-cells and HbF expression with a parallel decrease in ROS levels, thus supporting dual beneficial effects of treatment with this agent. In fact, treatment with hemin recapitulates the high oxidative stress produced by free heme; this mechanism for ATF4 activation as a stress response supports a relationship between hemin and ATF4 [36]. To explicate the role of ATF4 in *HBB* activation, siATF4 studies showed the ability of SAL treatment to overcome gene silencing and rescue *HBB* activation. The dose and duration of SAL is critical in achieving its efficacy for enhancing HbF production. We are the first to utilize sickle erythroid progenitors generated from PBMCs from humans to test the ability of SAL to induce HbF expression. To guide our dosing, Suragani et al. suggested concentrations  $>25\mu\text{M}$  SAL further increase p-eIF2 $\alpha$  resulting in inhibition of protein synthesis [36]. To evaluate the effects on erythropoiesis, we observed SAL  $24\mu\text{M}$  treatment increased CD71 and decreased CD235a expression suggesting delay in erythroid progenitor maturation *in vitro*. However, in our SCD mouse studies the slight increase in reticulocytes was not significant, therefore, what was observed in tissue culture did not recapitulate in mice; there were no adverse effects on peripheral blood cell counts.

SCD pathophysiology includes chronic hemolysis, anemia and organ damage caused by HbS polymerization under deoxygenated conditions; as a result, the lifespan of red blood cells is shortened to 14–21 days due to oxidative membrane damage [37]. It is known that chronic

hemolysis leads to excess hemoglobin in plasma and oxidative stress complicated by free heme, haptoglobin deficiency and globin chain precipitation. Since we observed decreased ROS levels with SAL treatment there are added benefits of protecting SCD progenitors against oxidative stress. In addition, SAL produced phenotypic anti-sickling effects in SCD mouse blood, which is clinically relevant.

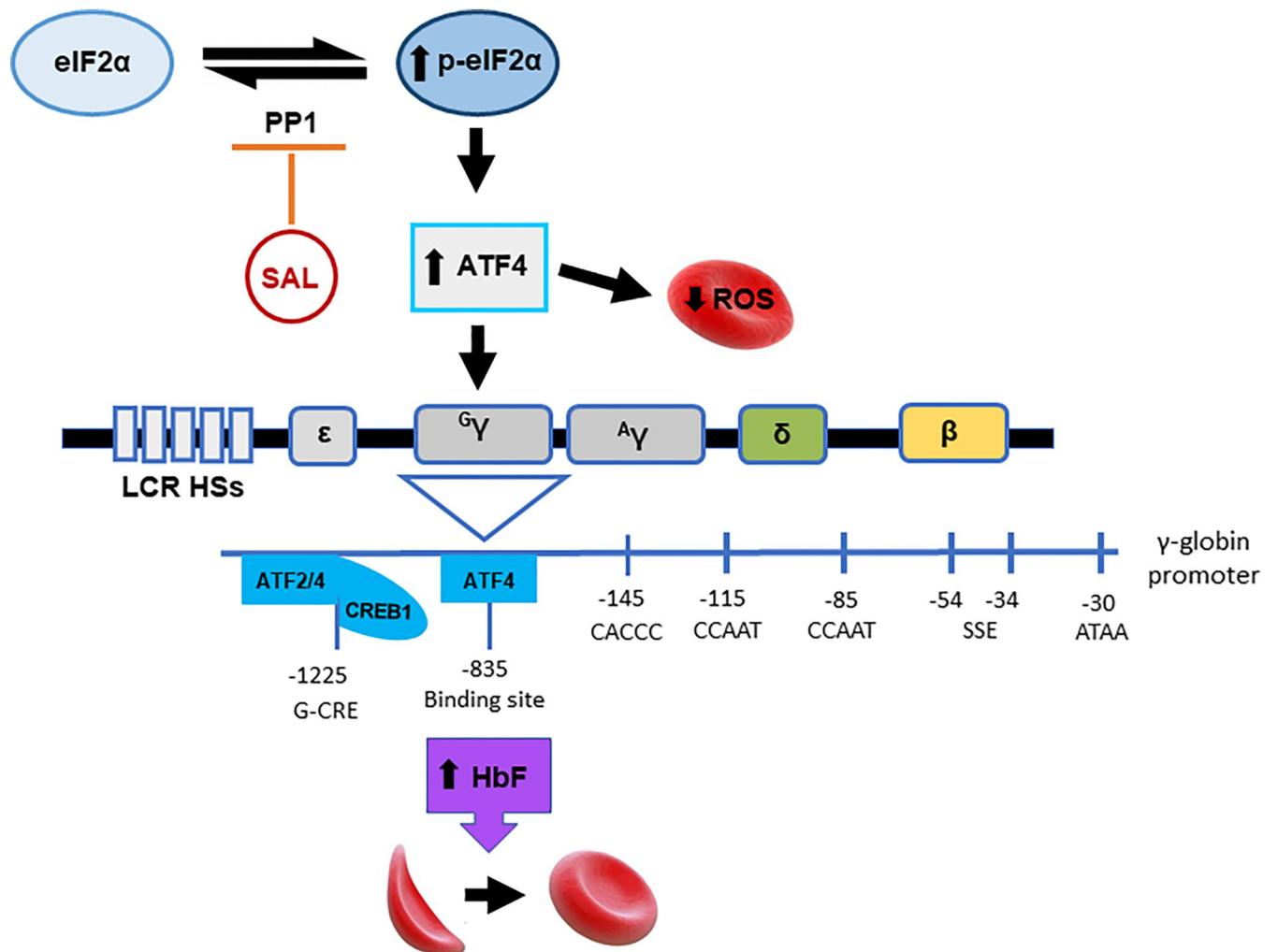
Heme-regulated eIF2 $\alpha$  kinase activates the p-eIF2 $\alpha$ -ATF4 integrated stress response in erythroid precursors to control ROS levels. In iron deficiency states, signaling through this pathway regulates globin chain synthesis and reduce oxidative stress during erythroid differentiation [36]. Increases in p-eIF2 $\alpha$  inhibit globin mRNA translation to prevent chain imbalance due to limited availability of heme. Furthermore, p-eIF2 $\alpha$  enhancement increase protein chaperone reserves in cells, to aide in protein folding during stress [38]. We showed that SAL changes the *HBG/HBB* mRNA ratio at steady state, along with *ATF4* expression.

A recent study confirms HRI-eIF2 $\alpha$ -ATF4 signaling suppressed *HBG* transcription by enhancing *BCL11A* expression [39]; however, this mechanism has not been studied in sickled erythroid progenitors under oxidative stress. Huang, et al. [39] demonstrated that ATF4 activates *BCL11A* in HUDEP2 cells, however, the mechanism did not show the same effect in their HRI<sup>-/-</sup> mouse model, concluding that this mechanism can vary between cells. By contrast, in our sickle erythroid progenitor studies, SAL activated ATF4 expression and decreased *BCL11A*, like HU. Furthermore, treatment with siATF4 decreased *HBG* mRNA in K562 cells, which were rescued by SAL supporting a role for ATF4 in SAL mediated HbF induction.

Studies have shown that ATF4 regulates a variety of genes that control cell adaptation to stress conditions, however, long-term stress results in C/EBP homologous protein activation and initiation of apoptosis [40]. As shown in Fig 7, SAL inhibits the negative feedback loop of the integrated stress response pathway to promote increased p-eIF2 $\alpha$  levels and ATF4 activation. Furthermore, limited data exist to demonstrate a role of ATF4 signaling in erythroid differentiation however, our data support the ability of SAL to activate ATF4 and reduce oxidative stress in SCD progenitors. In addition, the pathogenesis of neurodegeneration in brain involves ATF4 expression. Wu et al. [41] showed that treatment of mice with SAL up-regulated ATF4 expression. Subsequent studies with siATF4 showed silencing of the parkin protein required for protection against rotenone cytotoxicity. These data indicate the ATF4-parkin pathway plays an important role in the SAL-mediated neuroprotection of rotenone-induced dopaminergic cell death.

To provide further insight and evidence of efficacy for the development of SAL as a non-chemotherapeutic HbF inducer for individuals with SCD, preclinical studies were completed in the Townes SCD mouse model which has been used to test various agents for their capacity to induce HbF *in vivo* [42, 43]. Theoretically, the 24 $\mu$ M SAL concentration *in vitro* should be equivalent to ~5mg/kg/dose which produced HbF induction *in vivo* in SCD mice. Furthermore, other groups have shown mouse IP injections of SAL 1mg/kg [44] reduced pain in sickle mice. Moreover, chronic treatment demonstrated the ability of SAL to induce HbF and produce an anti-sickling effect without toxicity. We know SCD patients experience endoplasmic reticulum stress due to mast cell activation, mitochondrial dysfunction, and associated oxidative stress to activate the integrated stress pathway [45]. Gupta and colleagues [45] demonstrated the ability of SAL to attenuate pain in the transgenic HbSS-Berk mouse model, with concomitant decrease in ROS and endoplasmic reticulum stress [44].

SAL is under development for hepatic steatosis by altering cellular stress and autophagy through eIF2 $\alpha$  signaling [46], treatment of osteogenesis imperfect [47], and SAL efficiently blocked osteoporosis in mice [48]. To move SAL to clinical trials an oral formulation is required; mice have been treated with SAL in soymilk, which restored primordial follicles production in galactosemia mice [49]. The design of novel and safer alternatives or combination



**Fig 7. Model of HbF induction by SAL.** This schematic illustrates the proposed mechanisms of how SAL induces HbF. To achieve control of the integrated stress response, eIF2 $\alpha$  is phosphorylated when one of four kinases is activated by a stress stimulus. Normally this negative feedback loop recruits p-eIF2 $\alpha$  through the downstream activation of GADD34 or CREP to regenerate active eIF2 to control cellular homeostasis. However, SAL, a known dephosphorylation inhibitor of phoso-eIF2 $\alpha$  inhibits protein phosphatase 1 enzyme function causing a net result of increased phoso-eIF2 $\alpha$  levels, which activates the downstream ATF4 transcription factor. Increased levels of ATF4 protein translocate to the nucleus to bind to the  $\gamma$ -globin promoter at the predicted binding sites, leading to an increase in HbF. In sickle progenitors,  $\gamma$ -globin synthesis is increased upon SAL treatment and simultaneously decreasing ROS and sickling. The dual action of SAL in sickled erythroid progenitors makes it an ideal candidate for SCD and other hemoglobinopathy treatments.

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treatments with HU is highly desirable for people with SCD, along with the expanded understanding of  $\gamma$ -globin gene regulation.

## Supporting information

**S1 Table. Summary of primer sequences.**  
(DOCX)

**S1 Fig. SAL increased  $\gamma$ -globin transcription and HbF synthesis in K562 cells at different time points.** A) Shown in the bar graph is quantified  $\gamma$ -globin/GAPDH mRNA data generated by RT-qPCR for each treatment conditions after 6-hour incubation and B) after 24-hour incubation. C) Western blot analysis determined HbF levels compared with tubulin as the internal loading control after 6 and 24-hour treatments. D) Quantitative data generated by

densitometry analysis shows the expression of protein levels in K562 cells of HbF normalized to tubulin.

(DOCX)

**S2 Fig. SAL increases HbF, p-eIF2 $\alpha$  and ATF4 levels without changing HbS levels in sickle erythroid progenitors.** Panel A and B show Western blot and quantitative data generated by densitometry analysis shows the expression levels of HbS in sickle erythroid progenitors. The vertical black lines represent the elimination of a water control. Quantitative data generated by densitometry analysis shows the expression of protein levels in sickle erythroid progenitors of C) HbF normalized to  $\beta$ -actin, D) p-eIF2 $\alpha$  normalized to total eIF2 $\alpha$ , and E) ATF4 normalized to  $\beta$ -actin.

(DOCX)

**S3 Fig. SAL treatment schematic in the Townes SCD transgenic mice.** A) Schematic showing IP injections administration of SAL, 4–6 months old Townes SCD transgenic mice were treated with 3 or 5 mg/kg of SAL dissolved in water for 4 weeks, water (vehicle) and hydroxyurea (HU; positive control) treatments were completed as controls (N = 10 per group; 5 males and 5 females).

(DOCX)

**S4 Fig. SAL has no effect on white blood cell or platelet counts in Townes SCD transgenic mice.** Peripheral blood was collected in EDTA tubes by tail bleed at week 0, 2, and 4. Blood samples were analysed for automated complete blood counts with differential using a Micros 60 machine (HORIBA Medical/ABX Diagnostics). A-D) WBC, white blood count; Grans, granulocyte; Lymps, lymphocytes; Mono, monocyte; Plts, platelets. In the HU treated group the following had a statistically significant change: WBC (p = 0.0068), Grans (p = 0.041), Lymps (p = 0.023), Mono (p = 0.021), Plts (p = 0.0060 (WK2); 0.0040 (WK4)). Data were generated and shown as the mean  $\pm$  SEM (n = 10) p<0.05; \*\*p<0.01; \*\*\* p<0.001 was considered statistically significant.

(DOCX)

**S5 Fig. SAL has no effect on red blood cell counts in Townes SCD transgenic mice.** Peripheral blood was collected in EDTA tubes by tail bleed at week 0, 2, and 4. Blood samples were analysed for automated complete blood counts with differential using a Micros 60 machine (HORIBA Medical/ABX Diagnostics). A-D) RBC, red blood cells; Hb, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; RDW, red cell distribution width. In the HU treated group the following had a statistically significant change: HCT (p = 0.052), MCV (p = 0.0028 (WK2); 0.0029 (WK4)). Data were generated and shown as the mean  $\pm$  SEM (n = 10) p<0.05; \*\*p<0.01; \*\*\* p<0.001 was considered statistically significant.

(DOCX)

**S1 Raw image. Raw image data set for Fig 1 through Fig 6.**

(DOCX)

**S1 File. Original Western blot gels.**

(DOCX)

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