Inhibition of Polycomb Repressive Complex 2 Through EED Induces Fetal Hemoglobin in Healthy and Sickle Cell Disease Models

Fulcrum Therapeutics

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Abstract

Sickle cell disease (SCD) and β–thalassemias are caused by mutations in the hemoglobin β (HBB) gene that result in a dysfunctional adult hemoglobin protein (HbA) that is the root cause of the increased morbidity and mortality associated with these diseases. Individuals with SCD and β–thalassemias who harbor additional genetic alterations that maintain elevated fetal hemoglobin (HbF) expression during adulthood demonstrate greatly improved disease prognosis and at HbF amounts sufficient to completely restore normal hemoglobin functions can display an asymptomatic disease presentation. Elucidating the regulatory networks that control HbF expression is critical to the discovery of new therapies for SCD and β–thalassemia. BCL11A has been shown to be a master regulator that suppresses fetal hemoglobin gene (HBG) transcription in adults. We show that polycomb repressive complex 2 (PRC2) regulates *BCL11A* expression in erythroid progenitor cells, and thus is a putative upstream regulator of *HBG1/2* gene expression. Moreover, we demonstrate negative modulation of PRC2 activity via multiple different pharmacologic or genetic knockdown approaches targeting PRC2 complex protein embryonic ectoderm development (EED) results in decreased expression of *BCL11A* and concomitant and robust increases in both *HBG* mRNA and HbF protein across *in vitro* and *in vivo* models of SCD. These novel findings further elucidate the regulatory network that underlies fetal hemoglobin gene expression and have important implications for the use of inhibitors of EED as potential therapies for SCD and β-thalassemia. The translation of these findings is underway as FTX-6058, a novel and potent EED inhibitor, is currently being evaluated in clinical studies as a potential treatment for SCD and select hemoglobinopathies (NCT04586985, NCT05169580).

Methods

CD34⁺ in vitro culture

Mobilized peripheral CD34⁺ cells were thawed in expansion media, comprised of IMDM basal medium, 100ng/mL hSCF, 5 ng/mL IL-3, 3 IU/mL EPO, 250 ug/mL Transferrin, 2.5% Normal Human Serum AB, 1% Penicillin-Streptomycin, 10 ng/mL Heparin and 10 ug/mL Insulin. After 7 days of expansion, cells were seeded into fresh expansion media and compound treatment applied (100nM FTX-6058 or DMSO). At day 10, the media was exchanged to differentiation media which contains all expansion media components except for IL-3, and compound treatment reapplied. The cells were differentiated until day 14 when they were harvested.

Townes SCD mouse study[1]

Townes sickle cell disease mice were bought from Jackson Laboratory (Bar Harbor, ME) (B6; 129-Hbatm1(HBA)Tow Hbbtm2(HBG1, HBB*)Tow/J). Townes HbSS mice were 8 weeks of age at the start of the study and received FTX-6274 (5 mg/kg) or vehicle, PO, QD for 21 days. Interim bleeds collected before daily dosing.

Wildtype CD1 mouse study

Male WT CD1 mice were treated at 6-8 weeks and received FTX-6274 (5 mg/kg) or vehicle, PO, QD for 60 days. One-hour post dosing on the last treatment day, whole blood samples were collected by cardiac puncture in isoflurane-anesthetized mice into vacutainer EDTA tubes (BD Biosciences)

Mouse whole blood RNA extraction

Whole blood samples were mixed with 2x DNA/RNA shield. RNA extractions were conducted using the Quick-RNA Whole Blood kit from Zymo Research (# R1201). Concentrations were measured using the Qubit RNA Quantification High Sensitivity Assay (ThermoFisher Scientific # Q32855) and normalized for qPCR input.

HBG1/2, BCL11A and Hbb-bh1 qPCR

Taqman probes (ThermoFisher Scientific #4331182) for HBG1/2, BCL11A and Hbb-bh1 (Hs00361131_g1, Hs00256254_m1, and Mm00433932_g1, respectively) were used and mRNA expression was normalized using TFRC, mouse and human OAZ1 and GAPDH genes (Mm00441941_m1, Mm01611061_g1, Hs01548010_g1, and Mm99999915_g1, respectively). TaqMan 1-Step qRT-PCR Mix (Applied Biosystems # A25602C002) was used for master mix.

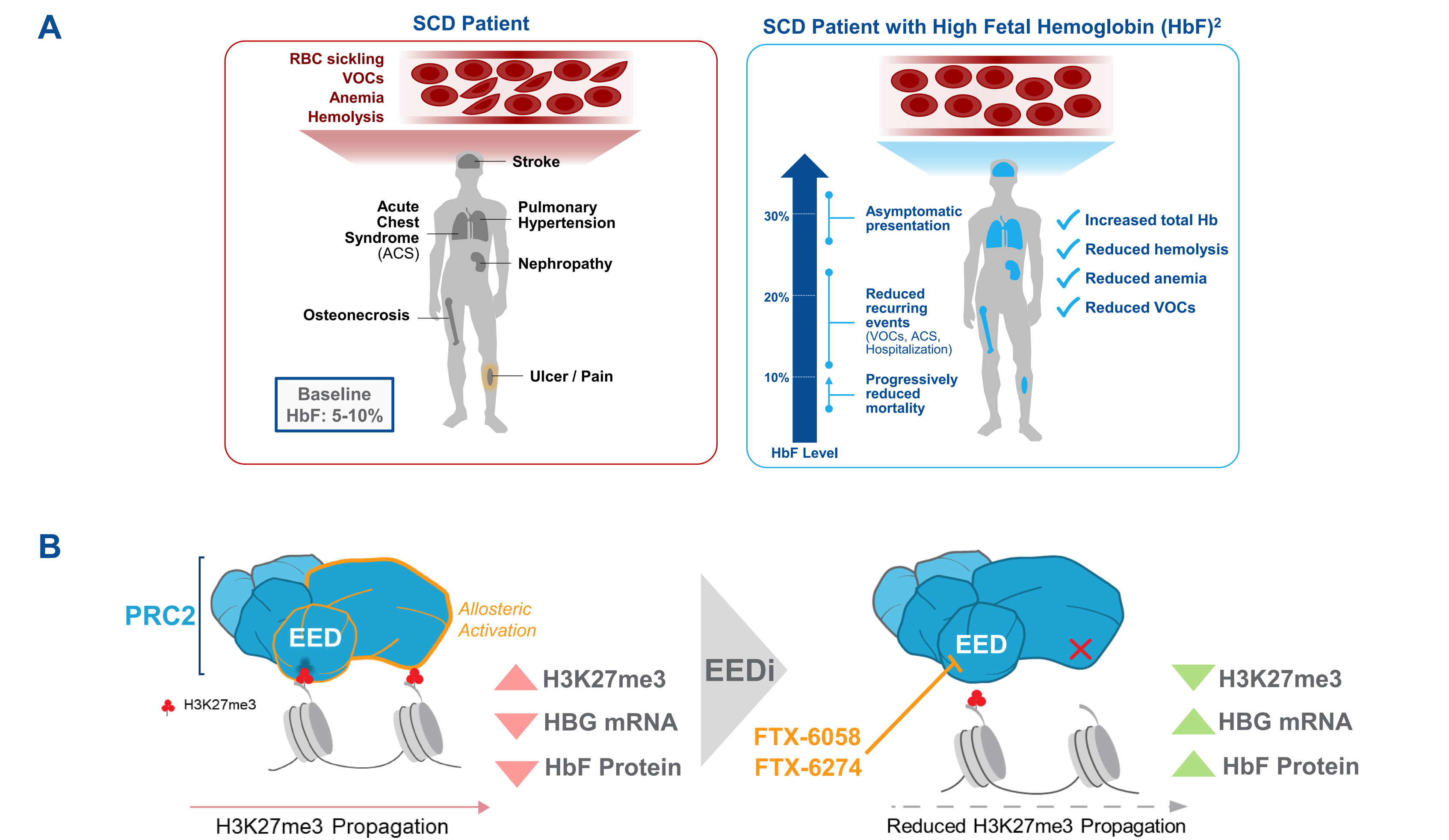
CRISPR experiments

sgRNA target sequences were as follows: Non-Targeting Control (NTC) #A35526 (ThermoFisher), enhancer region of BCL11A, CTAACAGTTGCTTTTATCACAGG, EED pool of 3 sgRNAs GGTGCATTTGGCGTGTTTGT, ATGGCTCGTATTGCTATCAT, ATGGCTCGTATTGCTATCAT (Horizon Discovery). On culture day seven, CD34cells were electroporated with an Amaxa 384-well HT Nucleofector System (Lonza) with the P3 primary cell kit according to the manufactures protocol using program DG-137.

mRNA sequencing

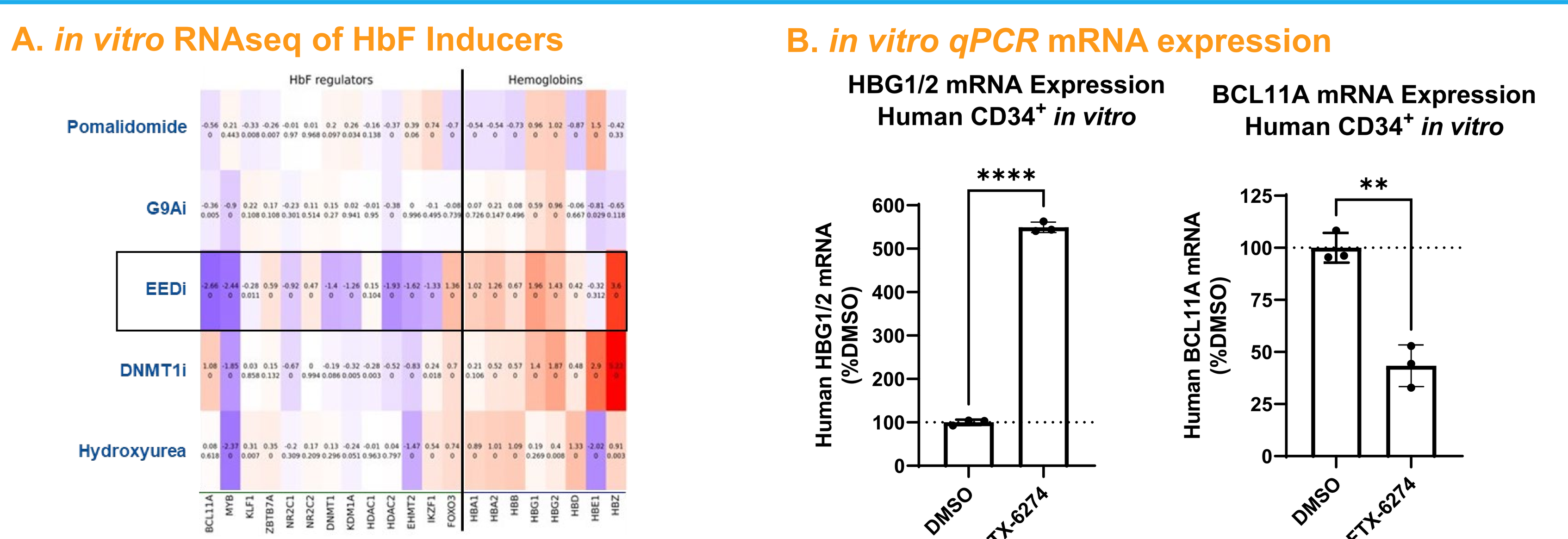
The purified mRNA samples were prepped for bulk sequencing with each other using the Illumina Stranded mRNA Prep Ligation sequencing kit (Illumina #20040534). Sequencing was conducted using the HiSeq model with 150 paired-end reads

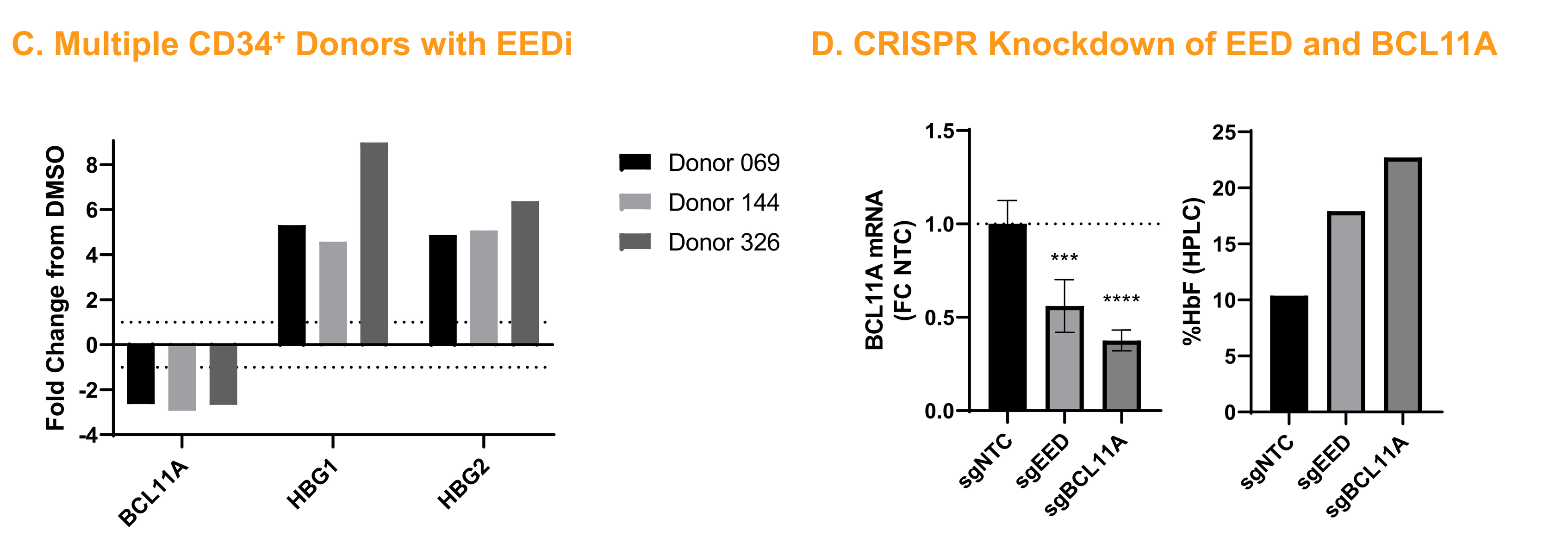
Background



(A) Increased levels of HbF have been shown to reduce pathology of SCD, making it an attractive mechanism for the treatment of SCD[2]. **(B)** PRC2 structure and role in transcriptional silencing, and the inhibition of EED by FTX-6058 and FTX-6274.

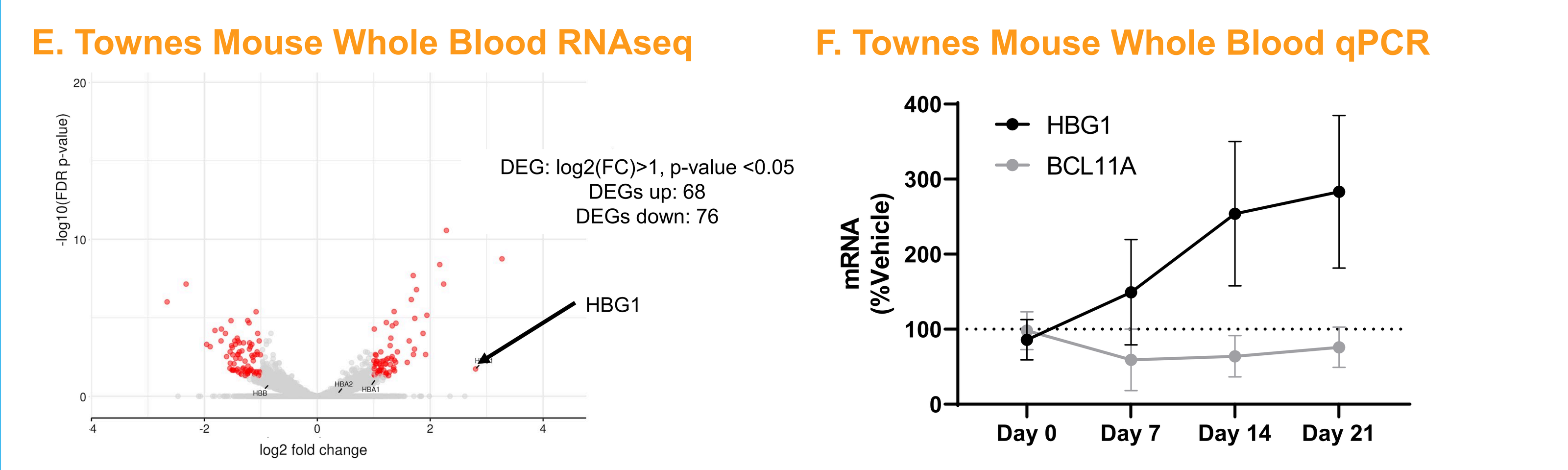
Results

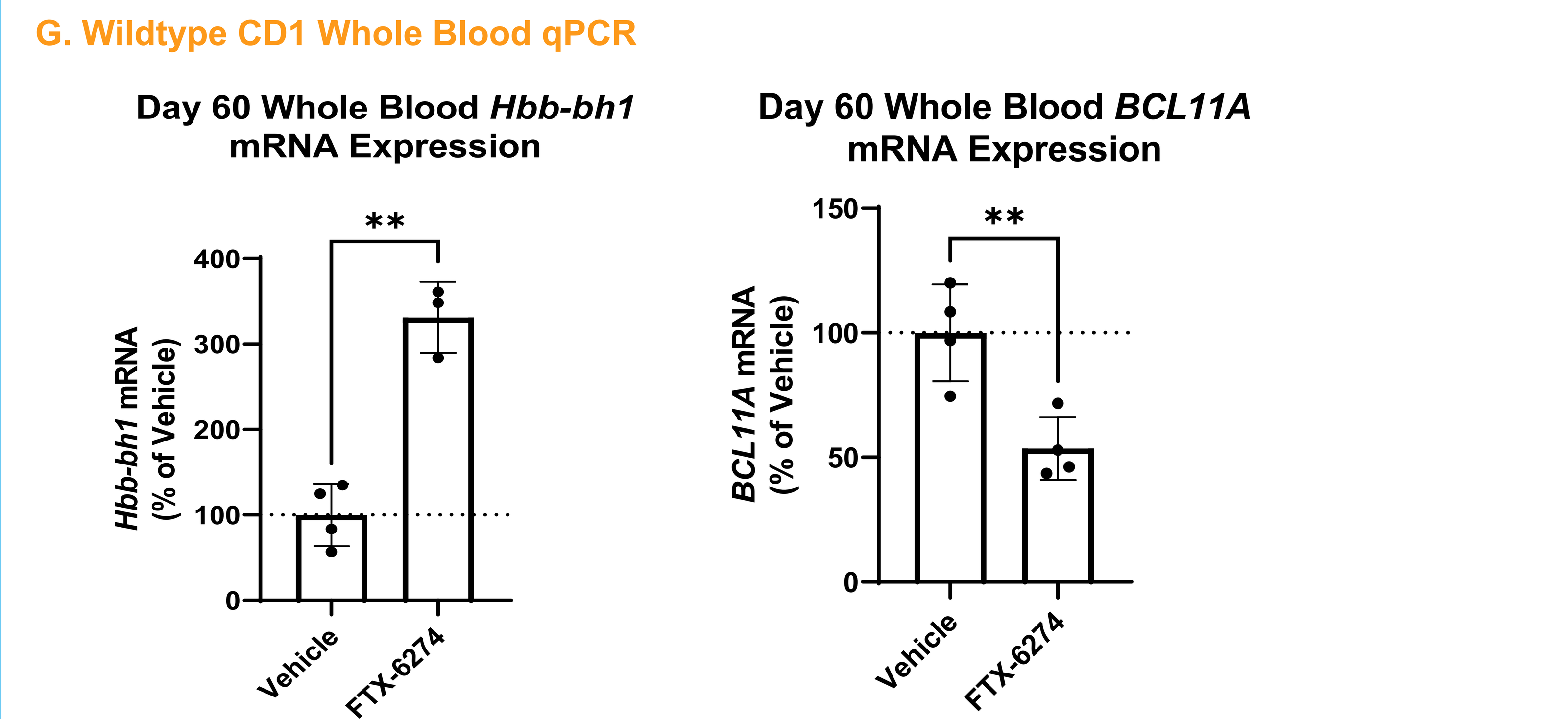




(A) *in vitro* RNA-sequencing of CD34⁺ cells treated with fetal hemoglobin-inducing molecules shows that inhibition of EED potently downregulates expression of master fetal hemoglobin regulator BCL11A and other hemoglobin regulating factors. **(B)** Healthy CD34⁺ cells treated with EED inhibitor FTX-6274 show significant increases in *HBG1/2* mRNA and decreased *BCL11A* expression. **(C)** Profiling of multiple donors confirms transcriptional impact of EEDi on *HBG1/2* and *BCL11A in vitro.* **(D)** CRISPR knockdown of *EED* and *BCL11A* in healthy CD34⁺ cells *in vitro* shows reduction in *BCL11A* transcript with both *EED* and *BCL11A* knockdown and proportional induction of HbF protein by HPLC.

Results (cont.)





(E) RNA-sequencing of whole blood from Townes SCD mice treated with 5mg/kg of EED inhibitor FTX-6274 for 21 days shows significant and selective induction of *HBG1* mRNA **(F)** qPCR analysis of whole blood from Townes SCD mice treated with 5mg/kg FTX-6274 for 21 days shows a time-dependent reduction in *BCL11A* mRNA and a concomitant induction of *HBG1* mRNA. **(G)** qPCR analysis of whole blood from wildtype CD1 mice treated for 60 days with 5mg/kg FTX-6274 shows sustained induction of embryonic globin *Hbb-bh1* and persistent reduction in *BCL11A*.

Conclusion

These studies have shown that EED inhibition elevated fetal hemoglobin expression across all *in vitro* and *in vivo* models profiled, and the reduction in *BCL11A* expression let to a concomitant induction of fetal hemoglobin. BCL11A has been robustly validated as a target for HbF induction, with previously published data showing therapeutic translation from preclinical primary *in vitro* models to people living with sickle cell disease[3]. A small molecule approach to inducing HbF via reduction of *BCL11A* expression could be transformative over genetic therapies using siRNA, CRISPR, or base editing, because there would be no need for myeloablative pre-conditioning and stem cell transplant, making treatment far more accessible for people living with sickle cell disease. These studies have demonstrated the potential of EED inhibition as a novel strategy for fetal hemoglobin induction, and novel EED inhibitor FTX-6058 will continue to be tested in clinical trials as a potentially transformative therapy for people living with sickle cell disease and select hemoglobinopathies (NCT04586985, NCT05169580).

References

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(2) D. Powars, J. Weiss, L. Chan, W. Schroeder, Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia? Blood 63, 921–926 (1984).

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