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## A Candidate Trans-acting Modulator of Fetal Hemoglobin Gene Expression in the Arab-Indian Haplotype of Sickle Cell Anemia: —*ANTXR1* Variants and HbF—

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### Abstract

Fetal hemoglobin (HbF) levels are higher in the Arab-Indian (AI)  $\beta$ -globin gene haplotype of sickle cell anemia compared with African-origin haplotypes. To study genetic elements that effect

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HbF expression in the AI haplotype we completed whole genome sequencing in 14 Saudi AI haplotype sickle hemoglobin homozygotes—seven selected for low HbF ( $8.2 \pm 1.3\%$ ) and seven selected for high HbF ( $23.5 \pm 2.6\%$ ). An intronic single nucleotide polymorphism (SNP) in *ANTXR1*, an anthrax toxin receptor (chromosome 2p13), was associated with HbF. These results were replicated in two independent Saudi AI haplotype cohorts of 120 and 139 patients, but not in 76 Saudi Benin haplotype, 894 African origin haplotype and 44 Arab Indian haplotype patients of Indian descent, suggesting that this association is effective only in the Saudi AI haplotype background. *ANTXR1* variants explained 10% of the HbF variability compared with 8% for *BCL11A*. These two genes had independent, additive effects on HbF and together explained about 15% of HbF variability in Saudi AI sickle cell anemia patients. *ANTXR1* was expressed at mRNA and protein levels in erythroid progenitors derived from induced pluripotent stem cells (iPSCs) and CD34<sup>+</sup> cells. As CD34<sup>+</sup> cells matured and their HbF decreased *ANTXR1* expression increased; as iPSCs differentiated and their HbF increased, *ANTXR1* expression decreased. Along with elements in cis to the HbF genes, *ANTXR1* contributes to the variation in HbF in Saudi AI haplotype sickle cell anemia and is the first gene in trans to *HBB* that is associated with HbF only in carriers of the Saudi AI haplotype.

### Keywords

ANTXR1; whole genome sequencing; RNA sequencing; erythroid progenitors; HbF

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### Introduction

Fetal hemoglobin (HbF) concentrations in sickle cell anemia are heritable, variable and genetically controlled (reviewed in.[1–4]). The HbF genes, *HBG2* and *HBG1*, are located within the *HBB* gene cluster (11p15.5). HbF levels vary according to the *HBB* gene haplotype suggesting an important element of cis-acting control over HbF gene expression. [5] Nevertheless, patients with the same *HBB* haplotype can have different HbF concentrations implying that trans-acting quantitative trait loci (QTL) also modulate HbF gene expression. Common variants in the trans-acting loci *BCL11A* (2p16) and *MYB*, and cis-acting elements within the *HBB* gene cluster explained 8 to 50% of HbF variation in patients with sickle cell anemia depending on the population examined.[6–11] Although a unique cis-acting sub-haplotype containing putative functional elements was exclusive to the AI haplotype and postulated to have a major effect on expression of their HbF genes, especially *HBG2*, as with all other haplotypes these patients can have widely divergent HbF levels.[9, 12, 13]

To search for novel variants that might modulate HbF expression in AI haplotype sickle cell anemia we examined by whole genome sequencing a highly selected group of these patients who had different HbF levels. Two single nucleotide polymorphisms (SNPs) in an anthrax toxin receptor gene, *ANTXR1*, appeared strongly associated with HbF. This observation was replicated in two independent cohorts of Saudi sickle cell anemia patients with the AI haplotype totaling 259 individuals but not in 76 Saudi Benin haplotype, 894 African origin *HBB* haplotype and 44 Arab Indian haplotype patients of Indian descent. The association of this locus explained about 10% of the HbF variance and was independent of the effect of

*BCL11A*. *ANTXR1* was expressed in erythroid progenitor cells and, like *BCL11A* and *MYB*, appeared to be a suppressor of HbF gene expression.[14–16]

## Methods

### Patients, haplotypes and HbF

Fourteen unrelated patients with sickle cell anemia (homozygosity for rs334, GAG-GTG, E6V) from the indigenous population of the Al-Qatif and Al-Hasa regions along the Western shore of the Persian Gulf in the Eastern Province of the Kingdom of Saudi Arabia were selected for whole genome sequencing. These patients were homozygous for the AI haplotype.

To validate and replicate the results of whole genome sequencing in the Saudi AI haplotype we studied two independent cohorts of similar Saudi sickle cell anemia patients with the AI haplotype. Cohort 1 consisted of 120 Saudi AI haplotype patients; ascertained before 2014 and Saudi Cohort 2 had 139 patients ascertained between 2014 and 2015. Both cohorts were recruited from the Al-Qatif and Al-Hasa regions in the Eastern Province of the Kingdom of Saudi Arabia. We also studied 76 Saudi Benin haplotype patients, 44 Indian AI haplotype patients and 894 African American patients from the Cooperative Study of Sickle Cell Disease (CSSCD) with diverse *HBB* haplotypes of African origin (Table 1). Ascertainment of the AI haplotype and HbS homozygosity was previously described and the *HBB* haplotypes associated with the HbS gene is shown in Fig. S1.[9] These studies were approved by the IRB of all participating institutions.

HbF was measured in Saudi Arabia, India and Boston using high performance liquid chromatography. HbF in the CSSCD and in the patient homozygous for the Senegal haplotype studied by whole genome sequencing was measured by alkali denaturation. Patients were studied after age 5 years when HbF levels had stabilized and they were not prescribed hydroxyurea.

### Whole genome sequencing

DNA was randomly sheared to fragments with a median size range of 300 bp. The extracted DNA was amplified by ligation-mediated PCR and paired-end 100 bp reads were sequenced at 40x coverage on the Illumina HiSeq 2000 platform. High-coverage whole genome reads were aligned to the Human Reference (GRCh37) using the Burrows-Wheeler Aligner version 0.5.9 which achieved an average alignment of 97%. The Genome Analysis Toolkit (GATK) was used to provide duplicate removal, base quality score recalibration, sample level local realignment around indels, genotyping, and variant recalibration. Further details are given in the Supplementary Methods. The scheme used to filter these samples is shown in Figure S2.

### Genotyping and imputation

One-hundred twenty-eight SNPs associated with HbF in the whole genome sequencing analysis were genotyped in the Saudi AI Cohort 1, in the Indian AI haplotype, and in African American sickle cell anemia cases using the KASP reagent (Supplemental

Methods). Only rs4527238 was genotyped in the 139 cases of Saudi AI Cohort 2. For additional analysis, 76 Saudi Benin haplotype patients and 894 African Americans were imputed to the 1000 Genomes reference panel using IMPUTE2. A subset of SNPs was further validated by Sanger sequencing.

### Functional Annotation of candidate variants

Variants in *ANTRX1* were annotated using the GTEX portal (<http://gtexportal.org>)[17] and RegulomeDB (<http://www.regulomedb.org>). To identify SNPs associated with regulatory protein or transcription factors we used SCAN (<http://www.scandb.org/newinterface/about.html>), a large-scale genomic database for functional and physical-based SNP annotation.[18] [19], Hembase (<http://hembase.niddk.nih.gov>), a database for human erythroid gene activity[20] and ErythronDB (<http://www.cbil.upenn.edu/ErythronDB/>) that provides transcript level information on expression patterns of genes involved in the mammalian erythropoiesis.[21] Splice-site analysis for the *ANTRX1* intronic SNPs was conducted using the Human Splice Finder. STRING 9.1 (<http://string-db.org>) was used to model predicted protein interactions of ANTRX1.[22]

### Induced pluripotent stem cell (iPSC) and CD34<sup>+</sup> cell culture

iPSCs were reprogrammed from lymphocytes from the blood of 2 normal donors and CD34<sup>+</sup> cells were isolated from the blood of these same individual for studies using RNA sequencing. iPSCs cells from blood of patients with sickle cell anemia[23] were also reprogrammed from peripheral blood lymphocytes and driven toward erythroid differentiation. Both iPSC and CD34<sup>+</sup> cells were differentiated into erythroid lineage cells as previously described.[24, 25], (Supplementary Methods)

### Gene expression

RNA isolated from iPSCs and CD34<sup>+</sup> cells was sequenced using the Illumina HiSeq-2000 platform. Library preparation, RNA sequencing and data analysis was as described.[26, 27] (Supplementary Methods) For normalizing read depth and length data were expressed as read counts per million (RPKM); raw counts are also displayed to show expression of protein coding splice variants.

### Immunofluorescence studies

Sickle iPSCs at days 15, 20 and 26 of erythroid differentiation were fixed with methanol and incubated in 1% BSA-0.1% Tween20 phosphate buffer for 1 hour to permeabilize cells and block non-specific binding. Cells were incubated with antibodies to ANTRX1 (1:50 dilution) and HbF-FITC for 2 hours at room temperature. After washing, the 2nd antibody Rhodamine-conjugated AffinityPure F (ab')<sub>2</sub> Fragment Donkey anti-Rabbit IgG (H+L) was used at 1:200 dilution for 0.5 hour. Unstained controls without the 1st antibody added were carried out simultaneously in every immunostaining experiment for each cell type at different times during the cultures and all were negative (data not shown). As ANTRX1 showed specific expression patterns in the cultured CD34<sup>+</sup> and iPSCs, isotype controls were not done. Prior to immunostaining the 2nd antibody was optimized as to concentration, incubation time, whole antibody vs. F(ab')<sub>2</sub> fragment and antibody competitions when co-

staining with anti-HbF Slides were mounted in DAPI medium for and examined under a fluorescent microscope.

### Statistical analysis

Association of SNPs with HbF in the whole genome sequencing experiment was tested using simple linear regression with an additive genetic model for common SNPs ( $MAF > 0.05$ ), and the Sequence Kernel Association Test (SKAT) [28] using sliding windows of 5 SNPs for rare SNPs. PLINK [29] was employed to remove all SNPs in high linkage disequilibrium (LD;  $r^2 > 0.80$ ). The association of the 128 most significant SNPs with HbF was tested using data imputed to the 1000 Genomes reference panel in the African American cohort and genotyped data in the other cohorts. Cubic root transformation of HbF was used as in [30] and a regression model of the allele dosages was used, adjusted for age and sex. The data from 76 Saudi Benin were analyzed using a linear mixed effect model to adjust for relatedness as in [31]. To determine the variation in HbF jointly explained by *ANTXR1* and *BCL11A* in Saudi AI haplotype sickle cell anemia and their additive effects, these 3 regression models were fitted: 1) HbF with rs1427407 in *BCL11A*, 2) HbF with rs4527238 in *ANTXR1* and 3) HbF with both rs4527238 and rs1427407 (all models adjusted by age and sex). All analyses were conducted using the statistical program R v.3.2.

## Results

### Patients

The *HBB* haplotype, number of cases in each patient cohort, age and HbF levels are shown in Table 1. AI haplotype carriers had higher HbF levels than other haplotypes.

### *ANTXR1*, a candidate gene for HbF regulation in Saudi AI haplotype sickle cell anemia

Single SNP analysis or SKAT-based SNP-set analysis of the 14 Saudi AI haplotype HbS homozygotes subjected to whole genome sequencing did not identify any SNPs with genome-wide significance ( $p\text{-value} < 10E-8$ ). We identified 465 SNPs with  $p = 10^{-4}$  for association with HbF that we investigated further (Table S1). One-hundred twenty of these variants were selected for follow up analysis in the replication cohorts based on lack of pairwise correlation, and 99 of these SNPs were successfully genotyped (Table S2). Following analysis of these SNPs, rs4527238 and rs35685045 ( $D' = 1$ ) in intron 9 of *ANTXR1* (anthrax toxin receptor 1, 2p13.1) remained associated with HbF after correction for multiple comparisons ( $p = 0.00011$ ,  $3.34E-05$ , Table 2). As these SNPs were in perfect linkage disequilibrium (LD) the remaining studies focus on rs4527238.

Figure 1 shows the distribution of HbF by genotype of rs4527238 in 120 Saudi AI haplotype patients (Cohort 1), an additional independent set of 139 Saudi AI haplotype patients (Cohort 2), 76 Saudi Benin haplotype patients, 44 Indian AI haplotype HbS homozygotes, and 894 African Americans. The C allele of rs4527238 was associated with lower HbF only in the Saudi AI sickle cell patients, but not in the Indian AI, Saudi Benin, or African American patient cohorts. There was no LD between SNPs in *ANTXR1* and 6 SNPs in *BCL11A* including the sentinel SNP rs1427407 ( $D' = 0.006-0.152$ ) in Saudi Cohort 1. *BCL11A* variants were not genotyped in Saudi Cohort 2.

In joint analysis, both rs4527238 (*ANTXR1*) and rs1427407 (*BCL11A*) were significantly associated with HbF in AI haplotype patients. The regression model using rs1427407 in *BCL11A* explained about 8% of HbF variability. The regression model of HbF with rs4527238 in *ANTXR1* explained about 10% of HbF variability. A model including both *BCL11A* and *ANTXR1* SNPs explained about 15% of HbF variability. *BCL11A* rs1427407 and *ANTXR1* rs4527238 have independent, additive effects on HbF as shown by the lack of difference between the SNP effect when we compared the unadjusted and adjusted model, and the genetic effect of rs4527238 (2.60) is slightly larger than that of rs1427407 (2.23). (Table S3)

### ***ANTXR1* is conserved in vertebrates, expressed in iPSC and CD34<sup>+</sup> derived erythroid progenitors and its protein levels vary inversely with HbF expression**

Functional annotation using the GTEX portal showed that rs4527238 is a significant eQTL for *ANTXR1* in whole blood, and *ANTXR1* expression increased with each copy of the its C allele of rs4527238 ( $p=0.04$ ; Figure S3) that was associated with significantly lower level of HbF. SNP rs4527238 had no effect on the expression of *BCL11A* ( $p=0.3$ ; data not shown).

*ANTXR1* is widely expressed with highest levels in bone marrow (Figure S4). Rs35685045, which is in perfect LD with rs4527238, is conserved in vertebrates [32] and is located on a promoter-flanking region with possible regulatory feature (Ensemble Regulation (ENSR00001543322, Chr2: 69096821–69100420). RegulomeDB analysis predicted binding sites for TBP and MEF-2. Further, this SNP is located within the 5 kb region upstream of miR-3126 (Chr2: 69,103,682–69,103,755).

Expression of *ANTXR1* as RPKM and its splice variants in iPSCs and CD34<sup>+</sup> cells are shown in Supplement Figure S5. In CD34<sup>+</sup> and iPSCs isolated from two different normal subjects, expression increased over time in CD34<sup>+</sup> erythroid cells and decreased over time in iPSC-derived erythroid progenitors; only the long splice variant of *ANTXR1* was expressed.

Figure 2 shows immunofluorescence co-staining for ANTXR1 and HbF during the erythroid differentiation of sickle iPSCs. ANTXR1 showed specific expression patterns in the cultured CD34<sup>+</sup> cells and iPSCs, which cannot be explained as non-specific binding. As hemoglobin expression increased fetal ( $\gamma$ ) and embryonic ( $\epsilon$ ) globin chains (not shown) are the predominant non- $\alpha$  globin. iPSCs displayed increasing expression of  $\gamma$ -globin chains as *ANTXR1* immunofluorescence fell.

### **Predicted protein interactions with *ANTXR1***

The predicted protein interactions of ANTXR1 using String 9.1 are shown in Figure S6. ANTXR1 interacted directly with LRP6[33], HDAC2[34], BCLX and BRAC1. The highest level of confidence of these interactions was with LRP6 (0.95). Nine other proteins had scores of 0.551 and higher, including HDAC2 (0.61).



## Discussion

High HbF levels are associated with reduced morbidity and mortality in sickle cell disease. [35, 36] In the Middle East and in India the HbS gene is often on the AI *HBB* haplotype.[37] Amongst these patients mean HbF is two to four times higher than in individuals with African-origin haplotypes, but it can have a wide range of HbF concentrations.[9, 38]

A polymorphism in intron 9 of *ANTXR1*, a type 1 transmembrane protein and receptor for anthrax toxin, was associated with HbF only in Saudi patients with the AI haplotype. The intronic SNPs associated with HbF are found in all four coding variants of the seven splice variants of *ANTXR1*. Only the longest isoform of the coding variants was expressed in iPSC and CD34<sup>+</sup> derived erythroid cells (Figure S5) and rs4527238 is at a potential acceptor splice site (Figure S7). *ANTXR1* has not been previously associated with a hematologic phenotype. Loss of function mutations in *ANTXR1* that has been linked to cell adhesion and migration and angiogenesis causes GAPO syndrome, a rare recessive disorder associated with growth retardation, alopecia, pseudoanodontia and progressive optic atrophy that is characterized by excessive extracellular matrix deposition.[39, 40]

MicroRNAs have been shown to modulate hematopoietic differentiation, proliferation and activity of hematopoietic cells.[41–43] MiR-3126 is embedded within the intron of *ANTXR1* that is the site of the HbF associated variants and might have independent intronic promoters.[41] MiR-221 and miR-222 can regulate HbF expression in erythroid cells by modulating the Kit receptor.[44] Mir-96 regulated HbF expression by direct post-transcriptional inhibition of  $\gamma$ -globin mRNA during adult erythropoiesis, and MiR-15a and miR-16-1 modulated *MYB* expression in human erythroid cells increasing fetal and embryonic hemoglobin gene expression.[45, 46] Whether or not MiR-3126 has any effect on HbF expression is unknown.

As cultured CD34<sup>+</sup> erythroid cells mature HbF synthesis wanes and this is associated with increased *ANTXR1* expression and decreased HbF/HbA and HbF mRNA/HbA mRNA ratios. Conversely, as erythroid progenitors derived from iPSCs matured in culture they begin to produce HbF as these cells have an embryonic/fetal globin gene expression profile. As HbF production increased *ANTXR1* expression declined (Figure S5). These data suggest that *ANTXR1* is a suppressor of HbF gene expression.

How might *ANTXR1* impact HbF gene expression? Bioinformatic analysis suggests two possibilities based on its putative role in hematopoiesis and Wnt signaling and interaction with HDAC2 and globin gene expression. A model for the effect of *ANTXR1* on HbF regulation is shown in Figure S8. Wnt signaling is differentially activated during hematopoiesis.[47] ANTXR1 was predicted to interact with LRP6, a Wnt co-receptor, and with FZD1, another Wnt signaling-related protein (Fig. S6).[33] These interactions might occur at the extracellular von Willebrand Factor A domain of ANTXR1 that is a protein-protein interaction module.[48] [33, 49] [50] *ANTXR1* knockdown decreased the expression of Wnt pathway-associated proteins including LRP6.[51] Low *ANTXR1* expression might reduce Wnt signaling, and through effects on hematopoietic stem cells, indirectly increase HbF as stress erythropoiesis is associated with increased HbF production.[52–54]

*ANTXR1* might also modulate HbF gene expression directly by interactions with HDAC2. [55] *HDAC2* inhibition increased HbF gene expression and was associated with reduced expression of *ANTXR1*. *MYB* has similar dual effects on HbF concentration affecting both erythropoiesis and transcription within the *HBB* gene cluster.[16]

In Saudi sickle cell anemia with the AI haplotype *ANTXR1* is a candidate for an additional trans-acting HbF modulator. Along with cis-acting HbF modulatory elements, *ANTXR1* and other trans-acting modulators like *BCL11A*, might account for the Gaussian distribution of HbF levels in the AI haplotype.[9] Whether targeting the *ANTXR1* pathway is a therapeutic approach to HbF induction will require functional and mechanistic studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Whole genome sequencing results are available on request from the University of Dammam and Boston University.

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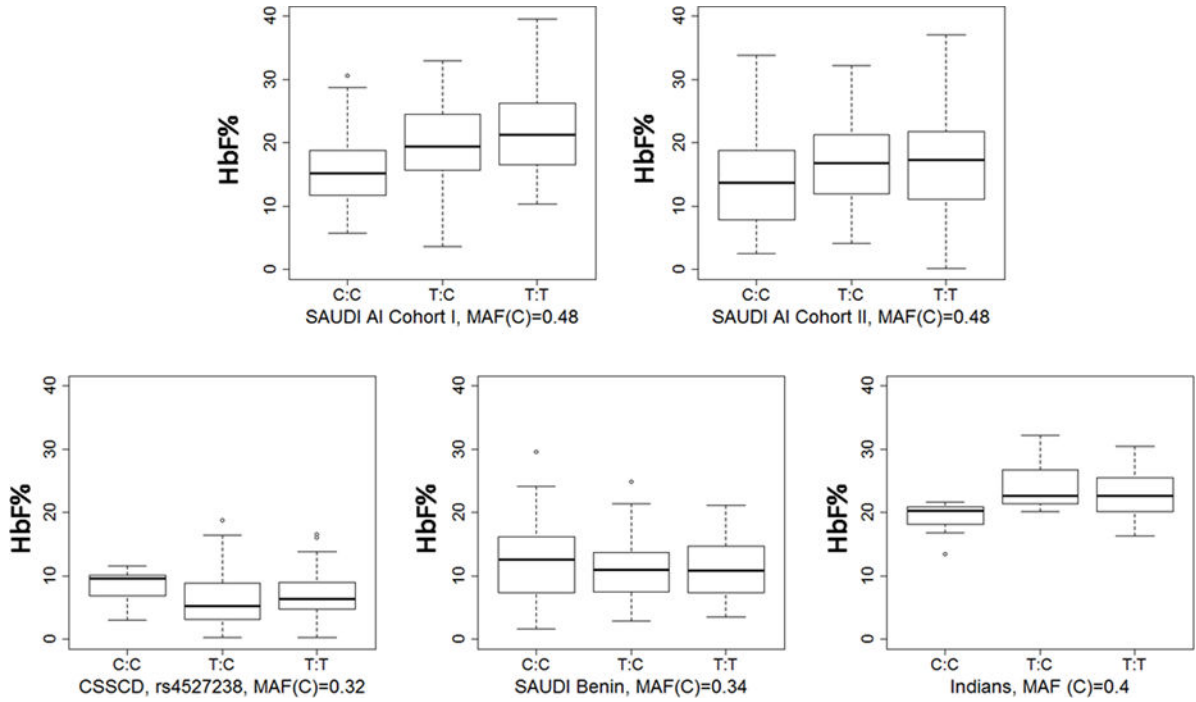
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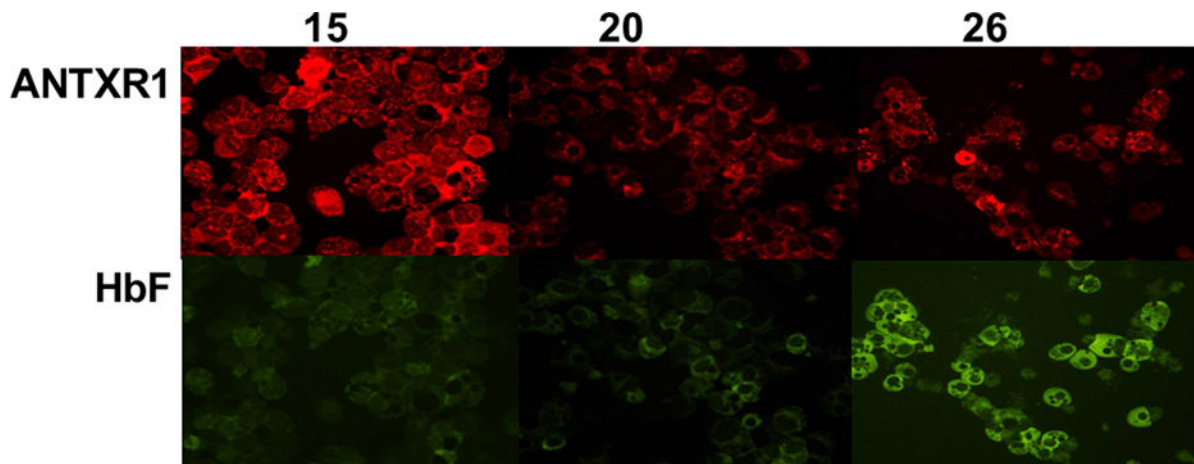
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**Figure 1. Association of rs4527238 in *ANTXR1* with HbF expression in Saudi AI Cohort I (n=120), Saudi AI Cohort II (n=139), Indian AI (n=44), Saudi Benin (n=76) and CSSCD (n=894) datasets**

The data for Saudi AI Cohort I, Saudi Benin and CSSCD were based on genome-wide SNP analysis imputed to the 1000 Genomes reference panel while rs452728 was genotyped directly in the Indian AI cohort. The CSSCD sample included 47% Benin, 27% Benin/Bantu, 10% Benin/Senegal and 16% other haplotypes. The bar in each rectangle is the median value for the HbF in percentage scale



**Figure 2. Immunofluorescence co-staining studies of ANTXR1 and HbF in sickle iPSCs**  
As ANTXR1 staining falls from culture days 15 to 20 and 26 (top row), HbF staining increases (bottom row). This reflects increased HbF synthesis as iPSCs mature and decreased HbF synthesis as CD34<sup>+</sup> cells differentiate. A similar pattern was seen for the  $\epsilon$ -globin chain (data not shown). Under our culture conditions established for erythroid differentiation of human CD34<sup>+</sup> cells 80–90% of cells are erythrocytes on day 22, based on FACS analysis and Wright-Giemsa-staining (data not show). When the iPSCs were induced toward erythroid differentiation, >80% of cells are erythrocytes on day 15 of culture. Occasional bright ANTXR1 staining cells are non-erythroid based on Wright-Giemsa staining.

**Table 1**  
**Patients selected for whole genome sequencing and in follow-up patient cohorts**

Forty-seven percent of African American patients (CSSCD) were Benin haplotype homozygotes, 27% Benin/Bantu compound heterozygotes, 10% Benin/Senegal compound heterozygote and 16% had other haplotypes including 8 Senegal haplotype homozygotes with HbF levels of 9.2, 2.3, 4.6, 16.0, 16.3, 11, 5.9 and 3.4% and ages of 24, 30, 16, 6, 24, 27, 44 and 23 years, respectively.

	N (cases)	Age (yrs.); SD	HbF (%); SD
<b>Whole genome sequencing</b>			
<i>Saudi AI Haplotype</i>			
<i>(low HbF)</i>	7	34.1; 10.3	8.2; 1.3
<i>(high HbF)</i>	7	25.9; 6.8	23.5; 2.6
<b>TaqMan assays</b>			
<i>CSSCD</i>	894	13.6; 11.3	5.2; 5.6
<i>Saudi AI Haplotype Cohort 1</i>	120	26.7; 10.1	18.0; 7.0
<i>Saudi AI Haplotype Cohort 2</i>	139	28.9; 12.6	16.2; 7.5
<i>Indian AI Haplotype</i>	44	14.6; 4.7	23.0; 4.8
<i>Saudi Benin Haplotype</i>	76	15.9; 9.9	11.4; 5.7



**Table 2**  
**Association of rs4527238 with HbF in AI haplotype Saudi sickle cell anemia and other haplotypes**

There was >95% power to detect a genetic effect if present. CSSCD-Cooperative Study of Sickle Cell Disease, an observational study of African American sickle cell disease.

	<b>Coded Allele</b>	<b>Coded Allele Frequency</b>	<b>Beta (SE)</b>	<b>Pvalue</b>
<b>Saudi AI (Cohort 1)</b>	T	0.52	3.23 (0.84)	0.00011
<b>Saudi AI (Cohort 2)</b>	T	0.52	1.54 (0.87)	0.0795
<b>Saudi Benin</b>	T	0.66	-1.42 (1.03)	0.16819
<b>Indian AI</b>	T	0.6	0.92 (0.95)	0.33435
<b>CSSCD</b>	T	0.66	0.03 (0.07)	0.72168