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RESEARCH ARTICLE

A novel mutation in the erythroid transcription factor KLF1 is likely responsible for ameliorating β-thalassemia major

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Abstract

We describe the identification of a novel missense mutation in the second zinc finger of KLF1 in two siblings who, based on their genotype, are predicted to suffer from beta thalassemia major but are, in fact, transfusion-free and in good health. These individuals, as well as two additional members of the same family also carrying this KLF1 mutation, exhibit high levels of fetal hemoglobin (HbF). KLF1 is an erythroid transcription factor, which plays a critical role in the regulation of the developmental switch between fetal and adult hemoglobin by regulating the expression of a multitude of genes including that of *BCL11A*. The mutation appears to be the main candidate responsible for the beta thalassemia-ameliorating effect as this segregates with the observed phenotype and also exogenous expression of the KLF1 mutant protein in human erythroid progenitor cells resulted in the induction of γ-globin, without, however, affecting BCL11A levels. This report adds to the weight of evidence that heterozygous KLF1 mutations can ameliorate the severity of the β-thalassemia major phenotype.

KEYWORDS
fetal hemoglobin, in silico prediction, KLF1, zinc finger, β-thalassemia

1 | INTRODUCTION

Beta (β) thalassemia, probably the best studied hemoglobinopathy, is phenotypically a very heterogeneous disease ranging from an asymptomatic condition to severe anemia due to the effects of genetic and environmental factors. The main predictor of disease severity is the nature of the mutation present in the β-globin gene itself. The major ameliorating factors associated with milder phenotypes of β-thalassemia are the coinheritance of an α-thalassemia mutation and the persistence of the normally repressed fetal hemoglobin (HbF, $\alpha_2\gamma_2$; Stamatoyannopoulos, 2005). HbF expression is dominant during fetal life but the developmental switch in globin gene expression results in the elevation of adult hemoglobin (HbA, $\alpha_2\beta_2$) expression around and after birth. Persistently high levels of HbF may be observed beyond early childhood due to genetic variations within the β-globin locus, such as large deletions and mutations at the promoters of the γ-globin genes (*HBG1* and *HBG2*; Weatherall, 2001). Moreover, various studies have identified genetic factors located outside the β-globin locus, which are responsible for increased levels of HbF. Examples of such genetic factors are single nucleotide polymorphisms (SNPs) in the *BCL11A* gene and the *HBS1L-MYB* intergenic region. *BCL11A* encodes a zinc finger transcription factor which is needed to repress γ-globin expression (Sankaran et al., 2008, 2009; Xu et al., 2010).

The erythroid-specific transcription factor KLF1 is a crucial regulator of the γ- to β-globin switching (Borg et al., 2010; Zhou, Liu, Sun, Pawlik, & Townes, 2010). KLF1 (also known as EKLF) is a zinc finger DNA-binding protein that was first described two decades ago by Miller and Bieker (1993). KLF1 has two distinct domains, an N-terminal trans-activating proline-rich domain and a C-terminal zinc finger domain, which binds to the DNA sequence motif 5′-CCMCRCCCN-3′. Such a motif is located in the regulatory regions of many erythroid-specific genes including the regulatory region of the β-globin gene. The KLF1 zing finger domain consist of three zinc fingers which are essential for the regulation of KLF1 target genes (Siatecka & Bieker, 2011). One of the functions of KLF1 is regulation of the developmental switch between fetal and adult hemoglobin. KLF1 indirectly regulates γ-globin expression by directly regulating the expression of the transcription factor BCL11A (Borg et al., 2010; Zhou et al., 2010). In humans, mutations in KLF1 have been associated with hematological changes and disorders such as the hereditary persistence of fetal hemoglobin (HPFH), the rare In(Lu) blood group phenotype, congenital dyserythropoietic anemia (CDA) type IV, and increased levels of zinc protoporphyrin (Kountouris et al., 2014; Perkins et al., 2016; Stamatoyannopoulos, 2005). Reports on the different KLF1 mutations and the various associated phenotypes have revealed, but also raised questions regarding, the role of KLF1 in human erythropoiesis. Here, we describe and partially characterize a novel KLF1 mutation, p.Ser323Leu, which is responsible for increased levels of HbF in a Cypriot family and appears to have an ameliorating effect on the β-thalassemia major phenotype resulting from the homozygous *IVS1-110*; *HBB*: *c.93–21 G > A* mutation. This is in agreement with an earlier report showing that seven heterozygous KLF1 mutations have the potential to modulate the clinical and hematological severity of β-thalassemia (Liu et al., 2014). However, unlike the family presented here, the patients in the report by Liu et al. (2014) exhibited extended transfusion-free survival rather than transfusion independence.

2 | DESIGN AND METHODS

2.1 | Patients and donors under study

Blood samples were collected from a Cypriot family, whose members exhibited unusually high levels of HbF, for hematological and genetic analyses carried out as part of a series of diagnostic tests. Subsequently, in the course of the current study, additional genetic analyses were performed on the original DNA samples. Blood samples were also collected from healthy donors for the culture of erythroid progenitors. Informed consent was obtained from the healthy donor individuals before sampling. Based on the 23/06/2009 decision of the National Bioethics Committee of Cyprus regarding the creation and use of biobanks and collections of human biological samples for research purposes, no informed consent was necessary for the patients under investigation as their samples and data were collected before 2009 for the purpose of a diagnostic investigation and the patients have been deidentified. Two family members, who were recently required to undergo additional hematological tests as part of this study provided their informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the National Bioethics Committee of Cyprus (Reference number: ΕΕΒΚ/ΕΠ/2013/23).

2.2 | DNA preparation

Genomic DNA was isolated from peripheral blood using the Gentra Puregene Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The DNA concentration and purity were measured using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.3 | Genotyping analysis

All family members were genotypically screened for the presence of mutations at the *HBA1*, *HBA2* (*α-globin*), and *HBB* (*β-globin*) genes as described before (Kountouris et al., 2016). For the investigation of the unusually high levels of HbF, we sequenced (Sanger sequencing) the *KLF1* gene (promoter region, 5′-UTR, coding region, flanking splice junctions, and 238 bp of the 3′-UTR) and the promoter regions of the *HBG1* and *HBG2* (*γ-globin*) genes. The primers used for polymerase chain reaction (PCR) amplification and DNA sequencing are summarized in Table S1. The presence of deletional HPFH was tested by multiplex ligation-dependent probe amplification (MLPA; Harteveld et al., 2005). In addition, family members were genotyped by SNapShot minisequencing assay for 12 SNPs at the *BCL11A* gene and 16 SNPs at the *HBS1L-MYB* intergenic region, which have been associated with variable HbF levels in a number of different populations (Fanis, Kousiappa, Phylactides, & Kleanthous, 2014).

2.4 | Culture of erythroid progenitors and cell lines

Erythroblasts derived from peripheral blood mononuclear cells were immunomagnetically separated using the CD34 MicroBead kit (Miltenyi Biotec Inc., Auburn, CA), expanded and differentiated as described before (Breda et al., 2012). 293T and HEL cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Paisley, UK) and Roswell Park Memorial Institute (RPMI)-1640 cell culture medium, respectively, supplemented with 10% fetal bovine serum (Gibco, Life Technologies).

2.5 | Transient transfection

Transient transfections in 293T cells and western blot analyses were performed as described previously (Fanis et al., 2012) o test the vectors for the expression of the wild-type and mutant HA-tagged KLF1. Briefly, 293T cells were cultured in 6-well plate dishes (Costar, Corning, NY) and after 24 hr were transfected with 4 μg of plasmid DNA using polyethylemine (PEI, Polysciences, Warrington, PA). Four hours after transfection, the culture medium was refreshed and the cells were lyzed for western blot analysis 48 hr later.

2.6 | Western blot analysis and protein quantification

After protein transfer, the nitrocellulose membranes were blocked in 1% bovine serum albumin (BSA), incubated with the appropriate primary antibodies and developed using enhanced chemiluminescence. The membranes were probed with the following primary antibodies: HA (monoclonal F7, sc-7392), γ-globin (monoclonal 51-7, sc-21756) and BCL11A (polyclonal H-116, sc-366825) obtained from Santa Cruz Biotechnology (Santa Cruz, CA), KLF1 (ab2483) from Abcam (Cambridge, MA), and β-ACTIN (monoclonal AC-15, A1978) from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated IgG rabbit anti-goat, goat antimouse, and goat antirabbit (Jackson ImmunoResearch, PA) secondary antibodies were used. The anti-KLF1 antibody was used in 1:500 dilution in TBS/Tween, the anti-γ-globin and the anti-HA antibodies were used in a dilution of 1:1,000 in TBS/Tween, and the anti-ACTIN antibody was used in 1:10,000 dilution in a solution of 1% nonfat dried milk in TBS/Tween. Secondary antibodies were used in a 1:20,000 dilution in nonfat dried milk in TBS/Tween. The levels of each protein were normalized with the corresponding β-ACTIN loading control within the same immunoblot. To avoid repeated blotting and stripping of the immunoblots, the immunoblots were cut into strips containing the proteins of interest, which were then probed with the appropriate primary antibody. Details of the procedure are provided in Figure S4.

Visualization of the immunoblots and acquisition of the images were carried out separately for each immunoblot strip using the UVP BioSpectrum Imaging System (UVP, LLC, Upland, CA). Dynamic total time exposure settings were used, with binning set at 12.1MP, 149% and 1 × 1 interpolation. Exposure times varied depending on the primary antibody used. For anti-ACTIN, a 30 s, single-frame exposure was used. For anti-BCL11A a 2- min exposure was used, whereas for anti-HA and for anti-KLF1 a 5-min exposure was used. For exposure times longer than 2 min, multiple frames were taken.

Quantification of the protein levels was performed using the ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij/).

2.7 | Plasmid constructs and lentivirus-mediated overexpression and knockdown of the wt and mutant *KLF1* genes

The coding sequence of the human *KLF1* gene was amplified from K562 cell line complementary DNA (cDNA) by PCR using the Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), cloned into the pMT2_HA plasmid using *Sal*I and *Eco*RI restriction sites and verified by sequencing. The c.968C>T p.Ser323-Leu mutation was generated using the QuikChange site-directed mutagenesis kit (Agilent technologies, Santa Clara, CA) using the oligonucleotide 5′-GAGATTCGCGCGCTTGGA CGAGCTGACCC-3′ and its complement. Expression of the wild-type and mutant HA-tagged KLF1 was tested by transient transfection in 293T cells. The wild-type and the HA-tagged p.Ser323Leu mutant KLF1 were subcloned into the modified pRRLsin.sPPT.CMV.GFP.Wpre lentiviral vector (Follenzi, Sabatino, Lombardo, Boccaccio, & Naldini, 2002) using the *Xba*I and *Sal*I restriction sites. Three clones targeting the human *KLF1*, obtained from the TRC Mission short hairpin RNA (shRNA) library, were used for knockdown experiments, as well as a nontargeting shRNA control clone. The TRC shRNA clones are described in Table S2. Lentivirus was produced by transient transfection of 293T cells and transduced into erythroid progenitor cells as described before (Zufferey, Nagy, Mandel, Naldini, & Trono, 1997).

2.8 | In silico prediction and structural modeling of the c.968C>T p.Ser323Leu mutated KLF1 zing finger domain

Prediction of the effect of the amino acid substitution on protein function was performed using the PredictSNP program using the default settings (Bendl et al., 2014). The protein structure prediction program I-TASSER (Roy, Kucukural, & Zhang, 2010; Yang et al., 2015; Zhang, 2008) was used to predict the structure of the wild-type and mutated zinc finger domain of KLF1. As a template for the prediction we used the solved structure of protein WT1 complexed with DNA (PDB ID: 2PRT), as it was previously described as the best candidate for constructing a prediction model for KLF1 (Arnaud et al., 2010). The default settings of I-TASSER were utilized. The confidence score of the I-TASSER wild-type KLF1 structure prediction was 0.93 with a structural similarity TN score of 0.84 ± 0.08. The confidence score of the I-TASSER p.Ser323Leu mutated KLF1 structure prediction was 0.92 with a structural similarity TN score of 0.84 ± 0.08. Predicted structures of the wild-type and mutant KLF1 were visualized using the PyMOL software (The PyMOL Molecular Graphics System, Version 1.1 Schrödinger, LLC).

2.9 | Statistical analysis

All statistical analyses were performed with GraphPad Prism 6 software. The p values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Clinical features

During an update study on the status of β-thalassemia in Cyprus (Kountouris et al., 2016), we identified two individuals who were homozygous for the β-thalassemia major *IVS1–110; HBB: c.93–21G>A* mutation but were phenotypically healthy, with near-normal levels of total hemoglobin and extremely high hemoglobin F (HbF) levels (63–66.2%). Interestingly, the two individuals (II.1 and II.2) were siblings (Figure 1a), whereas two additional members of the same family (I.1 and III.2) exhibited high levels of HbF. The clinical features and genotypic information on all family members are presented in Table 1. Initial hematological tests on subjects II.1 and II.2 painted a picture of mild microcytic, hypochromic anemia, that is, typical of

beta thalassemia carriers (trait) in terms of the levels of total hemoglobin, red blood cell (RBC) counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Notable exceptions were the extremely high levels of HbF and the normal levels of HbA_2. Family members I.1 and III.2, who were heterozygous for the *IVS1–110* mutation and for the novel *KLF1* mutation (see section "Genetic features" below), presented a picture typical of beta thalassemia trait, including raised HbA_2 levels but had higher than normal levels of HbF (8.5% and 6.5%, respectively).

Subject I.1 had passed away at an advanced age due to natural causes and subject II.2 had passed away at the age of 62 due to breast and liver cancer. Her medical records indicated that she had developed osteoporosis at the age of 47 and had undergone cholecystectomy at the age of 46. She had no signs of splenomegaly, hepatomegaly, or jaundice and was not being transfused.

More than a decade after the initial identification of this family, further blood tests and a medical examination were carried out as part of this study on the surviving members of the family who exhibited high HbF levels (II.1 and III.2). Subject III.2 (currently 40 years old) was shown to have no laboratory evidence of hemolysis as indicated by normal levels of bilirubin, lactate dehydrogenase (LDH), aspartate transaminase (AST), and reticulocyte counts. She also showed no signs of hepatomegaly, jaundice, or fatigue. There was no indication of thrombosis, leg ulcers, obvious organ damage, or any complications usually associated with nontransfusion dependent thalassemia. Hematological tests showed that she was negative for the a and b antigens of the Lutheran blood group, Lu(a−b−).

Subject II.1 (currently 49 years old) presented signs of mild jaundice, paleness, and fatigue. He had splenomegaly and hepatomegaly, with the spleen and liver palpable at 4 cm and 2 cm below the costal margin, respectively. He was also suffering from osteoporosis but had never presented with thrombosis, ulcers, or obvious organ damage. His latest new blood tests (Table 2) were in agreement with his earlier hematological data. His white blood cell values were within normal parameters. He had reduced total hemoglobin levels, hematocrit (HCT), MCV, MCH, and high red cell distribution width (RDW-CV), confirming hypochromic and microcytic anemia with anisocytosis. Platelet levels were marginally low as were the reticulocyte levels. Interestingly, ferritin levels were normal and he was shown to be Lu(a−b−). The biochemical tests provided evidence of hemolysis as indicated by increased bilirubin levels and in particular by the high levels of direct bilirubin. Normal gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT-SGPT), and aspartate aminotransferase (AST-SGOT) levels indicated normal liver function. Hemostatic parameters did not show significant deviation from normal values but were outside the normal ranges. Subject II.1 was receiving folic acid and vitamin D supplements but no other medication.

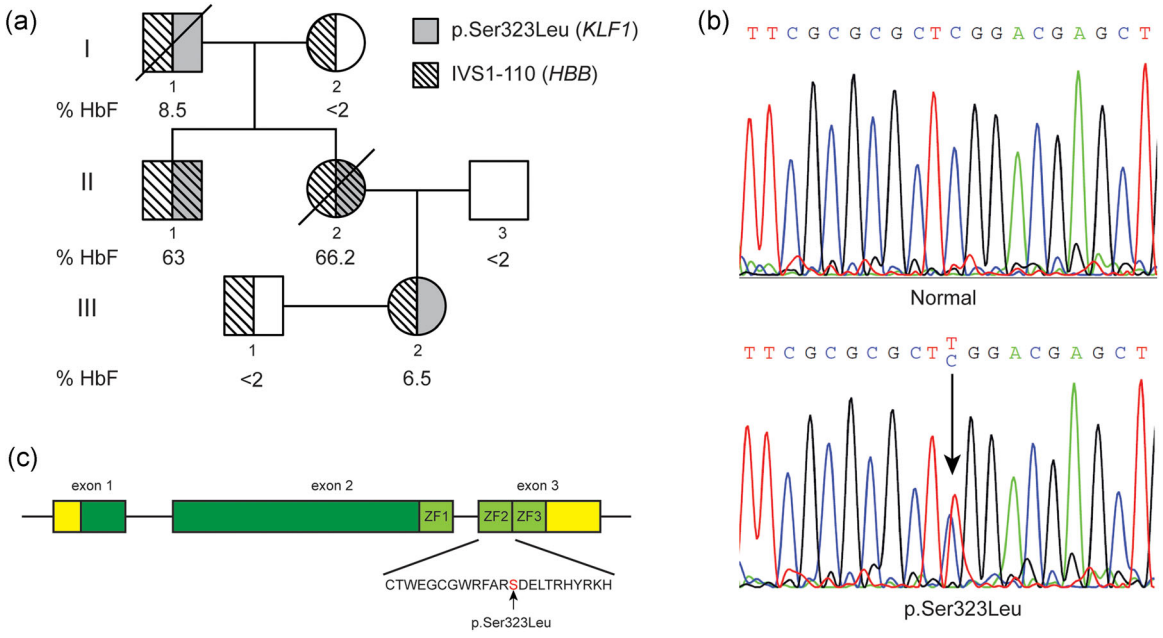

FIGURE 1 Identification of a novel *KLF1* mutation associated with high levels of HbF and ameliorating the β-thalassemia phenotype. (a) Pedigree of the family with KLF1:p.Ser323Leu and *IVS1–110; HBB: c.93–21G>A* mutations. Gray shading indicates the presence of the KLF1:p.Ser323Leu mutation and hatched lines indicate the presence of the *IVS1–110; HBB: c.93–21G>A* mutation. HbF levels are indicated below each individual. (b) Part of the sequencing electropherographs of the *KLF1* gene showing the novel heterozygous mutation (*NM_006563.4:c.968C>T, NP_006554.1:p.Ser323Leu; ithaID: 3138,*) detected in individuals with genotypic β-thalassemia and high HbF. The nonmutated sequence (normal) is depicted above the mutant sequence and corresponds to GenBank accession number U37106.1. (c) Schematic representation of the KLF1 protein. The untranslated regions are indicated with yellow color. The proline-rich N-terminal domain is indicated with dark green color and the zinc fingers are shown with light green color. The amino acid sequence of the second zinc finger is shown, with the location of the p.Ser323Leu mutation indicated by an arrow

TABLE 1 Hematological and genetic parameters of the Cypriot KLF1:p.Ser323Leu family

Case	I.1	I.2	II.1	II.2	II.3	III.1	III.2
Gender	M	F	M	F	M	M	F
Hematological parameters							
Hb (g/dl)	13.2	12.3	13.5	12.7	15.4	13	10.3
RBC (x10^{12}/L)	6.22	6.21	5.86	6.26	5.38	6.61	5.31
MCV (fL)	66	63	69	60.9	85	62	59
MCH (pg)	21.2	19.9	23	20.3	28.6	19.7	19.4
MCHC (g/dl)	32	31.2	33	33.3	33.9	31.7	32.9
HbA2 (%)	5.2	5	2.1	1.9	2.4	5.1	5
HbF (%)	**8.5**	≤2	**63**	**66.2**	≤2	≤2	**6.5**
***A-globin* genotype**							
HBA1	–/–	–/–	–/–	–/–	–/–	–/–	–/–
HBA2	–/–	–/–	–/–	–/–	–/–	–/–	–/–
***B-globin* genotype**							
Large indels (MLPA)	–/–	–/–	–/–	–/–	–/–	–/–	–/–
IVS1–110; HBB: c.93-21G>A	+/–	+/–	+/+	+/+	–/–	+/–	+/–
***KLF1* genotype**							
U37106.1, NM_006563.4:c.968C>T, NP_006554.1:p.Ser323Leu;	**+/–**	–/–	**+/–**	**+/–**	–/–	–/–	**+/–**
***XmnI* polymorphism** (*HBG2:g-158C>T*)							
rs7482144	C/C	C/C	C/C	C/C	C/T	C/C	C/T
***BCL11A* SNPs**							
rs11886868	G/G	G/G	G/G	G/G	A/A	G/A	G/A
rs4671393	T/T	T/C	T/T	T/T	C/C	T/C	T/C
rs7557939	C/C	C/C	C/C	C/C	T/T	C/T	C/T
rs6732518	G/G	G/G	G/G	G/G	A/A	G/A	G/A
rs10189857	T/T	T/C	T/T	T/T	T/T	T/T	T/T
rs6545816	T/T	T/G	T/T	T/T	T/T	T/G	T/T
rs7599488	G/G	G/A	G/G	G/G	G/G	G/G	G/G
rs1427407	A/A	A/C	A/A	A/A	A/C	A/C	A/C
rs766432	G/G	G/T	G/G	G/G	T/T	G/T	G/T
rs10184550	T/C	C/C	T/C	C/C	C/C	T/T	C/C
rs7606173	C/C	C/C	C/C	C/C	G/G	C/G	C/G
rs6706648	G/G	G/A	G/A	G/A	A/A	G/G	G/A
***HBS1L-MYB* SNPs**							
rs28384513	T/G	T/G	T/G	T/G	T/T	T/T	T/G
rs7776054	A/G	A/A	A/A	A/A	A/A	A/A	A/A
rs9399137	C/T	T/T	T/T	T/T	T/T	T/T	T/T
rs9389268	A/G	A/A	A/A	A/A	A/A	A/A	A/A
rs4895441	A/G	A/A	A/A	A/A	A/A	A/A	A/A
rs6929404	C/C	C/C	C/C	C/C	C/C	C/C	C/C
rs9402686	A/G	G/G	G/G	G/G	G/G	G/G	G/G
rs1320963	A/A	A/A	A/A	A/A	A/A	A/A	A/A
rs6904897	G/T	G/T	G/T	G/T	T/T	T/T	G/T
rs35959442	C/G	C/C	C/C	C/C	C/C	C/C	C/C
rs936090	C/T	T/T	T/T	T/T	T/T	T/T	T/T
rs4895440	A/T	A/A	A/A	A/A	A/A	A/A	A/A

(Continues)

TABLE 1 (Continued)

Case	I.1	I.2	II.1	II.2	II.3	III.1	III.2
rs9494142	C/T	T/T	T/T	T/T	T/T	T/T	T/T
rs9402685	C/T	T/T	T/T	T/T	T/T	T/T	T/T
rs11759553	A/T	A/A	A/A	A/A	A/A	A/A	A/A
rs6934903	A/T	T/T	T/T	T/T	T/T	T/T	T/T

Abbreviations: Hb, hemoglobin; HbA2, hemoglobin subunit alpha 2; HbF, fetal hemoglobin; MCH, mean corpuscular; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell.

TABLE 2 Current, in-depth hematological parameters of subject II.1

White blood cell parameters	Relative values (%)	Reference values (%)	Absolute values (cells/L)	Ref. values (10^9 cells/L)
WBC			7.62×10^9	3.91–8.77
NEU	60.0	40.3–74.8	4.57×10^9	1.82–7.42
LYM	29.4	12.2–47.1	2.24×10^9	0.85–3.00
MON	8.4	4.4–12.3	0.64×10^9	0.19–0.77
EOS	2.1	0.0–4.4	0.16×10^9	0.03–0.44
BAS	0.1	0.0–0.7	0.01×10^9	0.01–0.05
Red blood cell parameters	**Values**	**Reference values**		
RBC ($x10^{12}$/L)	5.77	4.50–6.50		
Hb (g/dl)	↓12.3	13.5–17.5		
HCT (%)	↓ 34.9	40.0–54.0		
MCV (fL)	↓ 60.5	77.0–93.0		
MCH (pg)	↓ 21.3	27.0–32.0		
MCHC (g/dl)	↑35.2	31.0–35.0		
RDW-CV (%)	↑ 24.1	11.0–16.0		
Platelet parameters	**Values**	**Reference values**		
PLT ($x10^9$/L)	↓ 138	150–450		
Reticulocyte parameters	**Values**			
RET (%)	1.33			
Biochemical tests	**Values**	**Reference values**		
Ferritin (ng/ml)	131	20–300		
Uric Acid (UA; mg/dl)	6.3	3.5–7.2		
Urea (mg/dl)	40	17–43		
Creatinine (mg/dl)	0.68	0.67–1.17		
Calcium (mg/dl)	9.4	8.8–10.6		
Proteins-Biuret (g/dl)	6.8	6.6–8.3		
Albumins (ALB; g/dl)	4.6	3.5–5.2		
Globulins (GLOB; g/dl)	2.2	1.4–4.8		
Total bilirubin (TbilT; mg/dl)	↑ 2.29	0.30–1.20		
Direct bilurubin (D-BIL; mg/dl)	↑ 0.6	0.1–0.2		
Indirect bilirubin (IBIL; mg/dl)	↑ 1.7	0.2–1.0		
Alkaline phosphatase (ALP; U/L)	36	30–120		
Gamma-glutamyl transferase (GGT; U/L)	11	9–55		
Alanine aminotransferase (ALT-SGPT; U/L)	14	3–41		
Aspartate aminotransferase (AST-SGOT; U/L)	19	3–38		
Lactate dehydrogenase (LDH; U/L)	436	208–480		
C-reactive protein (CRP; mg/l)	0.45	0.00–5.00		

(Continues)

TABLE 2 (Continued)

White blood cell parameters	Relative values (%)	Reference values (%)	Absolute values (cells/L)	Ref. values (10^9 cells/L)
Immunoglobulin G (IgG; mg/dl)	↓ 761	800–1,800		
Immunoglobulin A (IgA; mg/dl)	155	100–450		
Immunoglobulin M (IgM; mg/dl)	116	60.00–250.00		
Hemostasis	**Values**	**Reference values**		
Prothrombin time (PT; s)	↑ 15.1	10.0–14.0		
International normalized ratio (INR)	↑ 1.3	0.9–1.25		
Activated partial thromboplastin Time (APTT; s)	↑ 40.8	25.0–36.0		
Fibrinogen (FBG; mg/dl)	↓ 180	220–420		

Abbreviations: BAS, basophil; EOS, eosinophil; Hb, hemoglobin; LYM, lymphocyte; MCH, mean corpuscular; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MON, monocyte; NEU, neutrophil; RBC, red blood cell; RDW-CV, red cell distribution width; WBC, white blood cell.

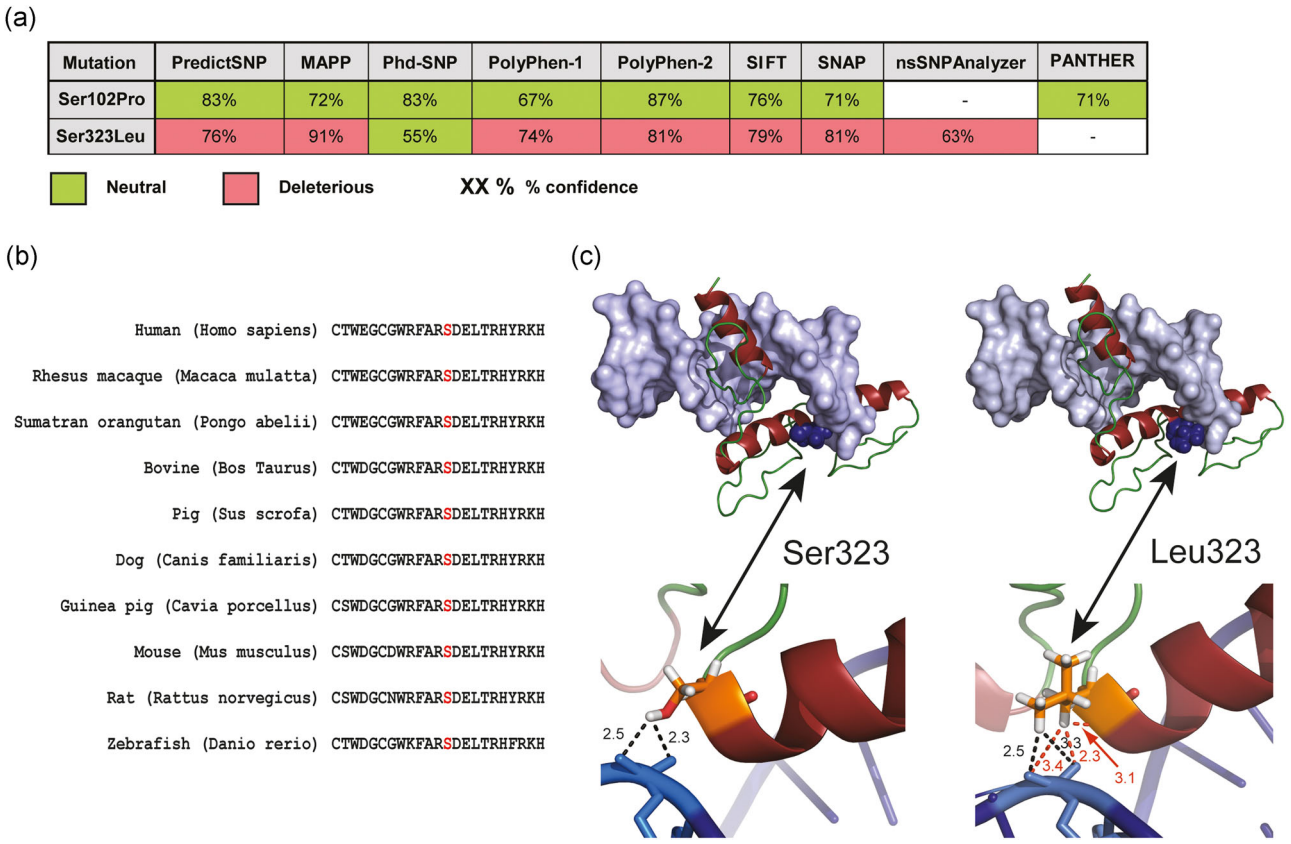

FIGURE 2 Model structure of the p.Ser323Leu mutation. (a) Prediction analysis of the p.Ser323Leu KLF1 mutation. Percentage of confidence is shown by various prediction methods under the PredictSNP tool. Neutral and deleterious prediction effects are shown in green and red, respectively. (b) Multiple sequence alignment of the zinc finger 2 domain of KLF1 from various species. The conserved serine amino acid at position 323 is indicated by red color. (c) An in silico structural model of the wild-type (left) and p.Ser323Leu mutant (right) KLF1 DNA-binding domain based on PDB ID: 2PRT. The KLF1 protein structures are shown in association with the DNA backbone. The change of serine to leucine at position 323 is highlighted with dark blue spheres and indicated by an arrow. The structures are shown in the same scale and orientation. At the bottom panels, in close-up view, the changed amino acids are indicated by sticks. The distances between the amino acid side chains and the DNA are shown with dashed lines. The corresponding numbers are in Angstrom (Å) units

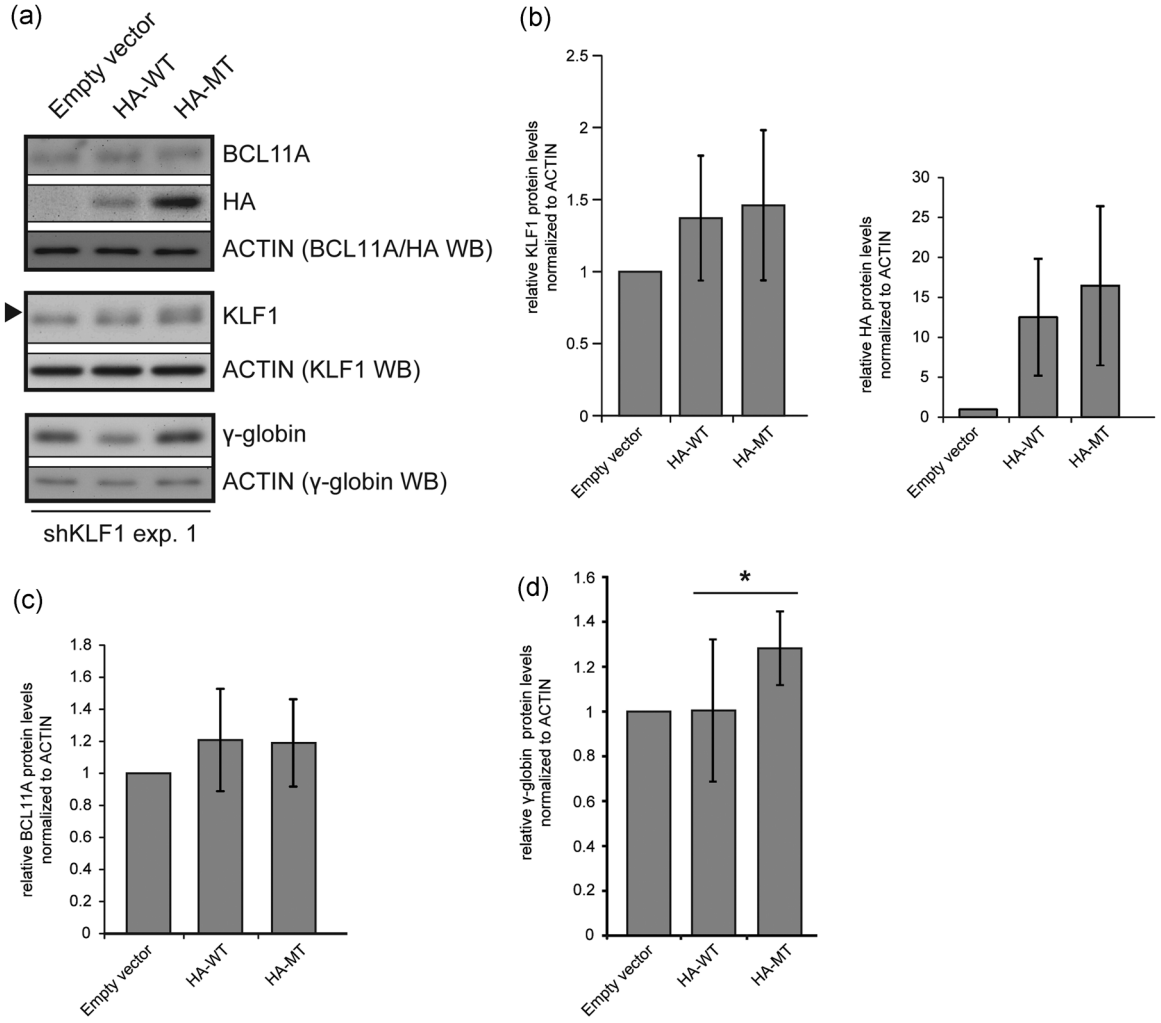

FIGURE 3 KLF1 p.Ser323Leu mutation increase the expression of γ-globin. (a) Cell lysates from human erythroid progenitor cells treated with lentivirus expressing shRNA against KLF1 (shKLF1 exp. 1) and transduced with empty vector, wild-type KLF1 (HA-WT), and p.Ser323Leu mutant KLF1 (HA-MT) analyzed by western blot analysis with the antibodies indicated. Actin staining serves as loading control for each SDS-PAGE gel. Overexpressed KLF1 is indicated by an arrow. Each thick border presents images of strips of membrane derived from the same western immunoblot. The blot membrane was cut into strips containing the proteins of interest and each strip was probed with the primary antibody indicated on the right hand side of the strip. Quantification of (b) KLF1, HA protein levels, (c) BCL11A protein levels, and (d) γ-globin protein levels using the the ImageJ software. Error bars: SD of a triplicate experiment. $^*p < .05$

3.2 | Genetic features

A pedigree analysis showed a dominant pattern of inheritance for the high HbF phenotype in the family (Figure 1a). Initially all individuals of the family were screened for the presence of mutations at the *HBA1*, *HBA2* (*α-globin*), and *HBB* (*β-globin*) genes. The sequencing results of the *HBA1* and *HBA2* genes were normal. *HBB* sequencing revealed the presence of the *IVS1-110; HBB: c.93-21G>A* (NM_000518.5:c.93-21G>A) mutation (Figure 1a; Table 1), as expected. The unusually high levels of HbF in some family members could be linked to *in-cis* variations at the β-globin locus (Weatherall, 2001). However, sequencing analysis of the *HBG1* and *HBG2* (γ-globin) gene promoters revealed no mutations. In addition, multiplex ligation probe analysis (MLPA) identified no deletions in the β-globin locus, thus excluding deletional HPFH delta-beta thalassemia as a possible cause for the high levels of HbF detected (Table 1).

In addition, we performed genotype analysis for all family members by SNaPshot minisequencing for 12 SNPs at the *BCL11A* gene and 16 SNPs at the *HBS1L-MYB* intergenic region, which have been shown to be associated with variable levels of HbF in different populations. Screening for *BCL11A* and *HBS1L-MYB* SNPs revealed variations at a proportion of these SNPs (Table 1) that might have a minor contribution in the increase of HbF levels but were not the main determinant.

3.3 | Identification of a novel KLF1 variant

Sequencing analysis of the *KLF1* gene in the two genotypically thalassemic patients revealed a novel mutation in the second zinc finger (U37106.1, NM_006563.4:*c.968C>T*, NP_006554.1:p.Ser323-Leu; ithaID: 3138, https://www.ithanet.eu/db/ithagenes?ithaID=

3138; Figure 1b,c) a known variation located in exon 2 of the *KLF1* gene (U37106.1, NM_006563.4:*c.304T>C*, NP_006554.1:p.Ser102-Pro). The novel mutation had never been reported in the literature before, was not present in dbSNP, exome variant server or HMGD and was absent in control samples from 100 individuals with normal levels of HbF (< 2%) sequenced for *KLF1*. Investigation by DNA sequencing of all family members revealed the presence of the novel mutation only in the individuals exhibiting high levels of HbF (Figure 1a; Table 1). Moreover, screening of the *KLF1* gene in 165 individuals with high levels of HbF (> 3%) (manuscript in preparation) identified four additional independent individuals with the *c.968C>T* p.Ser323Leu mutation. All these individuals had the novel mutation in the heterozygous state. Together these results suggest that the *c.968C>T* p.Ser323Leu mutation might be the main cause for the high HbF in the family.

3.4 | Evaluation and structural analysis of the novel p.Ser323Leu mutation

To obtain an indication of the relative importance of the novel mutation, we carried out an in silico prediction of the functional outcome of the amino acid change on the protein structure. We also performed an amino acid sequence alignment of the second zinc finger region of KLF1 to multiple species. Prediction analysis using the PredictSNP tool revealed that the p.Ser323Leu mutation is likely to have a deleterious effect on protein function, whereas the known variant p.Ser102Pro (Figure 2a) was shown to have a neutral effect. Alignment analysis showed the serine residue at position 323 to be highly conserved (Figure 2b), suggesting that mutations at this residue might affect the KLF1 protein mechanistically and/or structurally.

In silico structural analyses of the KLF1 zinc finger domain containing the p.Ser323Leu mutation and the wild-type KLF1 zinc finger domain offer a possible explanation as to how the mutation may affect the binding activity of the conserved zinc finger 2 domain. The serine at position 323 of KLF1 is directed towards the target DNA. The substitution of the relatively small, polar hydrophilic serine residue by the nonpolar, hydrophobic leucine residue seems not to alter the structure of the rest of the zinc finger domain but mainly affects the distance of the side chain of residue 323 from the DNA backbone. In silico modeling shows that the distance between the mutant 323Leu and the DNA backbone is increased due to the shortness of the leucine side chain compared with the wild-type 323Ser. In addition, the change from a polar serine to a nonpolar leucine residue might affect the formation of hydrogen bonds crucial for the stabilization of the protein itself or transcription factor complexes (Figure 2c). However, it must be stressed that the effect of the mutation on the binding specificity and affinity of KLF1 can only be ascertained using functional studies such as electrophoretic mobility shift assays (EMSA).

3.5 | Characterization of the effect of the p.Ser323Leu mutation on γ-globin production

To investigate the causative effect of the p.Ser323Leu mutation with respect to the high levels of HbF, we performed functional analyses of the p.Ser323Leu mutant KLF1 in human erythroid progenitor (HEP) cells cultured from the peripheral blood of healthy donors. Wild-type and mutant KLF1 were fused to the HA-tag, cloned into a lentiviral expression vector and transduced into the human erythroid progenitor cells (Figure S1). The transduction efficiency of the HA-wild type KLF1 (HA-WT) construct was lower compared with the HA-p.Ser323Leu mutant (HA-MT) construct (Figure S1). To eliminate the effect of the endogenous wild type KLF1 on γ-globin expression, we knocked down the endogenous KLF1 in the erythroid progenitor cells, whereas at the same time expressing the HA-WT or the HA-MT KLF1 using lentiviral vectors. To efficiently knockdown the endogenous KLF1 protein without affecting the exogenously expressed HA-WT KLF1 or the HA-MT KLF1, we used a shRNA that targets the 3′-UTR of *KLF1*. Two independent shRNAs targeting the 3′-UTR and one shRNA targeting the coding sequence of *KLF1* were tested in the Human Erythroleukemia (HEL) cell line. All three shRNAs tested exhibited the same knockdown efficiency (Figure S2). A shRNA containing a scrambled sequence was used as a negative control. γ-globin expression in HEP cells carrying the exogenous HA-MT KLF1 was compared with HEP cells carrying the exogenously expressed HA-WT KLF1. KLF1-knockdown HEP cells transduced with the exogenous HA-WT KLF1 did not exhibit any statistically significant differences in terms of BCL11A and γ-globin expression levels when compared with simple KLF1-knockdown HEP cells (Figure 3a–d; Figure S3). The full-size blots from which Figure 3a was derived are shown in the supplementary materials (Figure S4). In contrast, KLF1-knockdown HEP cells transduced with HA-MT KLF1 exhibited a significant increase in the levels of γ-globin when compared with simple KLF1-knockdown cells and to KLF1-knockdown cells transduced with HA-WT KLF1 (Figure 3a,d; Figure S3). The functional analysis was performed in three separate biological experiments with HEP cells derived from three healthy donors (Figure 3a; Figure S3B and S3C). No significant changes in BCL11A levels were observed following transduction with HA-MT KLF1. These results indicate that the mutant p.Ser323Leu KLF1 activates γ-globin expression through a mechanism independent of BCL11A, possibly resulting from a modification in its DNA-binding specificity and/or affinity. However, to get a more thorough picture of the effects of this mutation, more sensitive protein quantitation methods should be used, where the interactions of the mutant KLF1 with target genes other than BCL11A, such as ZBTB7A/LRF and Sox6 (Norton et al., 2017; Xu et al., 2010), that also play a role in globin gene regulation by *KLF1*, need to be investigated.

4 | DISCUSSION

In this study we report a novel mutation in the KLF1 transcription factor identified in the heterozygous state in two siblings. These individuals are homozygous for a severe β-globin mutation (*IVS1-110; HBB: c.93-21G>A*) which would normally result in a β-thalassemia major phenotype. However, neither sibling requires blood transfusions. Two additional members of this family, bearing the same *KLF1* mutation, show elevated levels of HbF.

Subject II.1, who carries the novel KLF1 mutation (p.Ser323Leu) and is homozygous for *IVS1-110*, showed hematological characteristics akin to a β-thalassemia carrier with reduced Hb, HCT, MCV, and MCH levels and complete transfusion independence. He had no leg ulcers and never suffered from thrombosis. However, his condition was more severe than a carrier's as indicated by the presence of splenomegaly, hepatomegaly, osteoporosis, and hemolysis-related indications such as jaundice and high levels of bilirubin. His overall clinical severity appears to fall between a β-thalassemia carrier and β-thalassemia intermedia (TI) patient. The second member of this family with the same *KLF1* and *HBB* genotype (subject II.2) was also transfusion independent but had passed away at the age of 62 due to cancer.

The study by Liu et al. (2014) looking at the effects of *KLF1* mutations in the Chinese population focuses its attention on 12 β-thalassemia patients (β^0/β^+ or β^0/β^0) with coinheritance in the heterozygous state of the p.Gly176AlafsX179 or the p.His229Asp *KLF1* mutations. In these patients, presence of the *KLF1* mutations led to a dramatic improvement in clinical severity, resulting in a TI phenotype, with reduced transfusion requirements and Hb levels in the range of 6.5–9.2 g/dl. In combination with additional modifiers, such as mutations in the α-globin gene, the two *KLF1* mutations led to a very mild, transfusion-independent TI phenotype in the Chinese β-thalassemia patients. As noted above, the ameliorating effect brought about by the p.Ser323Leu *KLF1* mutation identified in the current study is even more pronounced, as the individual under investigation has a phenotype that is close to thalassemia trait, even in the absence of any additional modifiers, such as α-thal mutations or the *XmnI* polymorphism. Subject II.1 has a hemoglobin level of 12.6 g/dl (even higher than the average Hb level of IVSI-110 heterozygote carriers in the Cypriot population, which stands at 12.0 g/dl–unpublished data) and at 49 years old, much older than any of the subjects in the Liu et al. (2014) study, is still not receiving any transfusions. The precise causes of the differences in the patient phenotypes observed between the two studies are a matter of speculation, as full functional studies have not been performed for any of the mutations mentioned. However, the p.Gly176AlafsX179 mutation, resulting in a truncated KLF1 protein lacking all thee zinc finger motifs, is likely to be either nonfunctional or get degraded by the cells' nonsense-mediated mRNA decay mechanism or the proteasomal degradation systems. Hence the end phenotype is likely to be due to KLF1 haploinsufficiency. The p.His229Asp and the p.Ser323Leu *KLF1* missense mutations, on the other hand, affects directly the KLF1 zinc fingers. Such changes are likely to modify the DNA-binding activity of the resulting mutant KLF1 protein in different ways, causing the observed differences in the patients' phenotype.

The p.Ser323Leu mutation is located on the second zinc finger of the KLF1 protein. KLF1 is a transcription factor that plays a crucial role in erythropoiesis and regulates the expression of several erythroid-specific genes (Perkins et al., 2016; Tallack et al., 2010; Yien & Bieker, 2013). In addition, KLF1 is an important regulator of the levels of HbF as it was demonstrated to be a positive transcriptional regulator of BCL11A (Borg et al., 2010; Zhou et al., 2010). Most, though not all, known KLF1 mutations have been associated with elevated HbF levels in humans. A number of KLF1 mutations have been shown to be responsible for diseases associated with ineffective erythropoiesis or to have no effect on γ-globin expression (Borg, Patrinos, Felice, & Philipsen, 2011; Waye & Eng, 2015).

Several KLF1 mutations have been identified that map close to the novel p.Ser323Leu described here. The p.Glu325Lys mutation, which is also found on the second zinc finger of KLF1, has been shown to cause congenital dyserythropoietic anemia type IV (CDA IV), a rare and severe red blood cell disorder (Arnaud et al., 2010; Jaffray et al., 2013). The neighboring p.Ser323Leu mutation has no such deleterious effects and appears to ameliorate, to a certain degree, the clinical phenotype of β-thalassemia major. Both mutations cause high levels of HbF. A recently described mutation at the same amino acid (p.Ser323Trp) is responsible for the rare In(Lu) blood group phenotype.(Kawai et al., 2017) The (p.Ser323Leu) mutation is also the likely cause of the Lu(a-,b-) phenotype identified in subjects II.1 and III.2.

Exogenous expression of the p.Ser323Leu KLF1 mutant in healthy human erythroid progenitor cells depleted of the endogenous KLF1 results in an increase in the levels of γ-globin, confirming our observations of elevated HbF levels in the family under study. Similar findings of increased levels of γ-globin were also observed for the p.Glu325Lys mutation. Arnaud et al. (2010) suggested that the p.Glu325Lys mutation has increased binding affinity to DNA and operates in a dominant negative fashion compared to the wild-type KLF1, resulting to the CDA IV phenotype. However, Singleton et al. (2011) proposed that the disease phenotype is caused by a lower affinity of the p.Glu325Lys mutation, leading to the formation of p.Glu325Lys KLF1 transcription complexes that are easily dissociated from gene promoters in an antagonistic way.

The observed increases in γ-globin gene expression seen in the family under investigation may be brought about by changes in the DNA-binding affinity and specificity of the mutant KLF1 molecule, and thus altered expression of KLF1 target genes. This hypothesis is proposed based on the location of the novel mutation in the second zinc finger domain of the protein, which is part of the DNA-binding domain. The in silico and in vitro work presented here hint at, but do not prove this hypothesis. Definitive proof can only be provided by additional EMSA and binding assays.

The effect of this mutation seems not to be mediated through changes in the level of expression of BCL11A, However, we cannot discount the possibility that more sensitive techniques such as mass

spectrometry might have been able to detect differences in BCL11A levels that are below the detection threshold of western blot analysis used in this study. Similarly, the quantitative real-time polymerase chain reaction (RT-qPCR) can sensitively detect differences in mRNA levels, but such data would still need to be verified at the protein level. It is very important that additional KLF1 target genes such as ZBTBZ7A/LRF, a transcriptional inhibitor of γ-globin expression, and Sox6 are investigated to identify the precise mechanism of action of the KLF1:p.Ser323Leu mutation.

The HEP cells used for our experiments are routinely used by many laboratories for the study of globin gene expression, as their differentiation profile recapitulates many of the characteristics of developmental globin gene regulation. However, it has been noted that HEP cells express relatively high levels of fetal hemoglobin, which may complicate the interpretation of experimental results and can reduce their usefulness as a research tool. This is particularly the case when small changes in γ-globin gene expression need to be evaluated, which may be masked by background levels of expression. Although the use of appropriate controls can overcome some of these issues, the recently developed human umbilical cord blood-derived erythroid progenitor 2 (HUDEP2) (Kurita et al., 2013) cell line is increasingly utilized in favor of HEP cells. Studying the novel p.Ser323Leu KLF1 mutation in this cell line could provide more definitive results.

In the nonthalassemic p.Ser323Leu KLF1 heterozygote family members studied here, as well in four additional individuals identified in the Cypriot HPFH cohort (manuscript in preparation), the levels of HbF vary from 4.2% to 8.5%. Apart from KLF1, other genetic modifiers outside the β-globin locus have been associated with increased levels of HbF. Such modifiers are variations at the promoters of the *HBG1* and *HBG2* genes and SNPs at the *BCL11A* gene and the *HBS1L-MYB* intergenic region (Menzel et al., 2007; Thein et al., 2007). In the family case study presented here, there is no variation at the *HBG1* and *HBG2* gene promoters and the associated SNPs at the *BCL11A* and *HBS1L-MYB* loci do not segregate with the high HbF levels (Table 1). The differences at the HbF levels in these individuals might be due to as yet unknown genetic modifiers involved in the regulation of γ-globin gene expression. In our family case we could not conclude if the p.Ser323Leu KLF1 mutation causes borderline HbA_2 levels (Perseu et al., 2011) because all individuals having the KLF1 mutation were also heterozygous for the *IVS1–110; HBB: c.93–21G>A* mutation (Figure 1a; Table 1), which is known to increase the HbA_2 levels (Steinberg & Adams, 1991).

Although the availability of limited sample quantities of peripheral blood for this experiment allowed the performance of only the most vital hematological and biochemical tests, it is hoped that in the future sufficient material will be collected to allow the generation of HEP cell cultures from subject II.1. This will enable additional functional studies to be performed on the novel p.Ser323Leu *KLF1* mutation. EMSAs will allow us to detect any changes in the DNA-binding affinity of the mutant KLF1 compared with the wild type protein. Potentially, protein binding microarrays or SELEX-seq can be used to characterize the mutant KLF1 DNA-binding sites, which can lead to the identification of genes whose expression is affected by the p.Ser323Leu *KLF1* mutation. In parallel, RNA seq studies can highlight differences in the expression profile of HEP cells from subject II.1 and wild type KLF1 controls (homozygous for the *IVS1–110; HBB: c.93–21G>A* mutation), which, in combination with the DNA-binding data can help resolve the pathways through which the mutation ameliorates the thalassemic phenotype. The causative role of the mutation in elevating HbF levels can be investigated by correction of the mutation using gene editing in HEP cells from subject II.1, where we would expect to see a restoration of HbF to background levels. In addition, gene editing can be applied for the creation of the p.Ser323Leu *KLF1* mutation in HUDEP-2 cells to achieve the high levels of HbF. Finally, as KLF1 plays a critical role in globin switching by being involved in chromatin looping at the β-globin locus, 4C or Hi-C methodologies should be used to study chromosomal interactions within this locus in the patient's HEP cells.

This report shows that a heterozygous *KLF1* mutation contributes to the elevation of HbF and may be part of the reason ameliorating a severe thalassemia genotype such as the homozygous *IVS1–110; HBB: c.93–21G>A*. If future studies show an unequivocal link between the pSer323Leu *KLF1* mutation and a substantially milder thalassemic phenotype, then these findings will need to be incorporated into national β-thalassemia screening and diagnostic programs, as the ameliorating effects of this mutation would be substantial enough to affect the reproductive choices and genetic counseling offered to couples carrying the *IVS1–110; HBB: c.93–21G>A* and the KLF1:p.Ser323Leu mutations. Moreover, the identified p.Ser323Leu KLF1 mutation can be used for designing and optimization of future gene therapy studies for β-thalassemia patients.

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CONFLICT OF INTERESTS

The authors have no financial or nonfinancial conflict of interests to declare.

AUTHOR CONTRIBUTIONS

P. F. designed the experiments; P. F. and I. K. carried out the experiments; M. H., A. K., S. C., and M. S. carried out the patient evaluations, P. F. prepared the manuscript; M. P. and M. K. revised the manuscript critically.

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REFERENCES

Arnaud, L., Saison, C., Helias, V., Lucien, N., Steschenko, D., Giarratana, M. C., …Cartron, J. P. (2010). A dominant mutation in the gene encoding the erythroid transcription factor KLF1 causes a congenital dyserythropoietic anemia. *American Journal of Human Genetics*, *87*(5), 721–727.

Bendl, J., Stourac, J., Salanda, O., Pavelka, A., Wieben, E. D., Zendulka, J., … Damborsky, J. (2014). PredictSNP: Robust and accurate consensus classifier for prediction of disease-related mutations. *PLoS Computational Biology*, *10*(1), e1003440.

Borg, J., Papadopoulos, P., Georgitsi, M., Gutierrez, L., Grech, G., Fanis, P., Phylactides, M., Verkerk, A. J., van derSpek, P. J., & Scerri, C. A.et al (2010). Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. *Nature Genetics*, *42*(9), 801–805.

Borg, J., Patrinos, G. P., Felice, A. E., & Philipsen, S. (2011). Erythroid phenotypes associated with KLF1 mutations. *Haematologica*, *96*(5), 635–638.

Breda, L., Casu, C., Gardenghi, S., Bianchi, N., Cartegni, L., Narla, M., … Rivella, S. (2012). Therapeutic hemoglobin levels after gene transfer in beta-thalassemia mice and in hematopoietic cells of beta-thalassemia and sickle cells disease patients. *PLoS One*, *7*(3), e32345.

Fanis, P., Gillemans, N., Aghajanirefah, A., Pourfarzad, F., Demmers, J., Esteghamat, F., …vanDijk, T. B. (2012). Five friends of methylated chromatin target of protein-arginine-methyltransferase[prmt]-1 (chtop), a complex linking arginine methylation to desumoylation. *Molecular & Cellular Proteomics*, *11*(11), 1263–1273.

Fanis, P., Kousiappa, I., Phylactides, M., & Kleanthous, M. (2014). Genotyping of BCL11A and HBS1L-MYB SNPs associated with fetal haemoglobin levels: A SNaPshot minisequencing approach. *BMC Genomics*, *15*, 108.

Follenzi, A., Sabatino, G., Lombardo, A., Boccaccio, C., & Naldini, L. (2002). Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. *Human Gene Therapy*, *13*(2), 243–260.

Harteveld, C. L., Voskamp, A., Phylipsen, M., Akkermans, N., den Dunnen, J. T., White, S. J., & Giordano, P. C. (2005). Nine unknown rearrangements in 16p13.3 and 11p15.4 causing alpha- and beta-thalassaemia characterised by high resolution multiplex ligation-dependent probe amplification. *Journal of Medical Genetics*, *42*(12), 922–931.

Jaffray, J. A., Mitchell, W. B., Gnanapragasam, M. N., Seshan, S. V., Guo, X., Westhoff, C. M., …Manwani, D. (2013). Erythroid transcription factor EKLF/KLF1 mutation causing congenital dyserythropoietic anemia type IV in a patient of Taiwanese origin: Review of all reported cases and development of a clinical diagnostic paradigm. *Blood Cells, Molecules, and Diseases*, *51*(2), 71–75.

Kawai, M., Obara, K., Onodera, T., Enomoto, T., Ogasawara, K., Tsuneyama, H., …Inaba, S. (2017). Mutations of the KLF1 gene detected in Japanese with the In(Lu) phenotype. *Transfusion*, *57*(4), 1072–1077.

Kountouris, P., Kousiappa, I., Papasavva, T., Christopoulos, G., Pavlou, E., Petrou, M., Feleki, X., Karitzie, E., Phylactides, M., & Fanis, P.et al (2016). The molecular spectrum and distribution of haemoglobinopathies in Cyprus: A 20-year retrospective study. *Scientific Reports*, *6*, 26371.

Kountouris, P., Lederer, C. W., Fanis, P., Feleki, X., Old, J., & Kleanthous, M. (2014). IthaGenes: An interactive database for haemoglobin variations and epidemiology. *PLoS One*, *9*(7), e103020.

Kurita, R., Suda, N., Sudo, K., Miharada, K., Hiroyama, T., Miyoshi, H., … Nakamura, Y. (2013). Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. *PLoS One*, *8*(3), e59890.

Liu, D., Zhang, X., Yu, L., Cai, R., Ma, X., Zheng, C., …Lin, L. (2014). KLF1 mutations are relatively more common in a thalassemia endemic region and ameliorate the severity of beta-thalassemia. *Blood*, *124*(5), 803–811.

Menzel, S., Garner, C., Gut, I., Matsuda, F., Yamaguchi, M., Heath, S., Foglio, M., Zelenika, D., Boland, A., & Rooks, H.et al (2007). A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. *Nature Genetics*, *39*(10), 1197–1199.

Miller, I. J., & Bieker, J. J. (1993). A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. *Molecular and Cellular Biology*, *13*(5), 2776–2786.

Norton, L. J., Funnell, A. P. W., Burdach, J., Wienert, B., Kurita, R., Nakamura, Y., …Crossley, M. (2017). KLF1 directly activates expression of the novel fetal globin repressor ZBTB7A/LRF in erythroid cells. *Blood Advances*, *1*(11), 685–692.

Perkins, A., Xu, X., Higgs, D. R., Patrinos, G. P., Arnaud, L., Bieker, J. J., … Workgroup, K. L. F. C. (2016). Kruppeling erythropoiesis: An unexpected broad spectrum of human red blood cell disorders due to KLF1 variants. *Blood*, *127*(15), 1856–1862.

Perseu, L., Satta, S., Moi, P., Demartis, F. R., Manunza, L., Sollaino, M. C., … Galanello, R. (2011). KLF1 gene mutations cause borderline HbA(2). *Blood*, *118*(16), 4454–4458.

Roy, A., Kucukural, A., & Zhang, Y. (2010). I-TASSER: A unified platform for automated protein structure and function prediction. *Nature Protocols*, *5*(4), 725–738.

Sankaran, V. G., Menne, T. F., Xu, J., Akie, T. E., Lettre, G., VanHandel, B., … Orkin, S. H. (2008). Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science*, *322*(5909), 1839–1842.

Sankaran, V. G., Xu, J., Ragoczy, T., Ippolito, G. C., Walkley, C. R., Maika, S. D., Fujiwara, Y., Ito, M., Groudine, M., & Bender, M. A.et al (2009). Developmental and species-divergent globin switching are driven by BCL11A. *Nature*, *460*(7259), 1093–1097.

Siatecka, M., & Bieker, J. J. (2011). The multifunctional role of EKLF/KLF1 during erythropoiesis. *Blood*, *118*(8), 2044–2054.

Singleton, B. K., Lau, W., Fairweather, V. S., Burton, N. M., Wilson, M. C., Parsons, S. F., Richardson, B. M., Trakarnsanga, K., Brady, R. L., & Anstee, D. J.et al (2011). Mutations in the second zinc finger of human EKLF reduce promoter affinity but give rise to benign and disease phenotypes. *Blood*, *118*(11), 3137–3145.

Stamatoyannopoulos, G. (2005). Control of globin gene expression during development and erythroid differentiation. *Experimental Hematology*, *33*(3), 259–271.

Steinberg, M. H., & Adams, J. G., 3rd (1991). Hemoglobin A2: Origin, evolution, and aftermath. *Blood*, *78*(9), 2165–2177.

Tallack, M. R., Whitington, T., Yuen, W. S., Wainwright, E. N., Keys, J. R., Gardiner, B. B., Nourbakhsh, E., Cloonan, N., Grimmond, S. M., & Bailey, T. L.et al (2010). A global role for KLF1 in erythropoiesis revealed by ChIP-seq in primary erythroid cells. *Genome Research*, *20*(8), 1052–1063.

Thein, S. L., Menzel, S., Peng, X., Best, S., Jiang, J., Close, J., Silver, N., Gerovasilli, A., Ping, C., & Yamaguchi, M.et al (2007). Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(27), 11346–11351.

Waye, J. S., & Eng, B. (2015). Kruppel-like factor 1: Hematologic phenotypes associated with KLF1 gene mutations. *International journal of laboratory hematology*, *37*(Suppl 1), 78–84.

Weatherall, D. J. (2001). *The Thalassaemia Syndromes*. Oxford, UK: Blackwell Scientific.

Xu, J., Sankaran, V. G., Ni, M., Menne, T. F., Puram, R. V., Kim, W., & Orkin, S. H. (2010). Transcriptional silencing of {gamma}-globin by BCL11A involves long-range interactions and cooperation with SOX6. *Genes and Development*, *24*(8), 783–798.

Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., & Zhang, Y. (2015). The I-TASSER Suite: Protein structure and function prediction. *Nature Methods*, *12*(1), 7–8.

Yien, Y. Y., & Bieker, J. J. (2013). EKLF/KLF1, a tissue-restricted integrator of transcriptional control, chromatin remodeling, and lineage determination. *Molecular and Cellular Biology*, *33*(1), 4–13.

Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*, *9*, 40.

Zhou, D., Liu, K., Sun, C. W., Pawlik, K. M., & Townes, T. M. (2010). KLF1 regulates BCL11A expression and gamma- to beta-globin gene switching. *Nature Genetics*, *42*(9), 742–744.

Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., & Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature Biotechnology*, *15*(9), 871–875.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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