

Review Article

Control of globin gene expression by Kruppel-like Factors

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Abstract. Kruppel-like factors (KLFs) are a family of seventeen proteins designated KLF1 to KLF17. KLFs are transcriptional factors that bind GC-rich sequences such as *CACCC* elements. The DNA binds to KLFs via three carboxyl-terminal Cys-2/His-2 zinc fingers. KLFs control cell differentiation and embryonic development. They are also implicated in a number of cellular functions such as erythropoiesis, proliferation and tissue development. This review will focus primarily on KLFs that are involved in haemoglobin control. These include KLF1, KLF2, KLF3, KLF8 and KLF10. The connection between human KLF1 and elevated foetal haemoglobin was first identified in a study done by (Borg et al., 2011) on a large Maltese family with Hereditary Persistence of Foetal Haemoglobin (HPFH) where a nonsense mutation in the Erythroid Kruppel-Like Factor 1 gene (*KLF1*) was identified as the main cause of HPFH. KLF2 is a positive regulator of mouse and human embryonic β -globin genes and it overlaps with KLF1 in embryonic erythropoiesis. KLF3 and KLF8 expression is driven by KLF1 while together KLF3 and KLF8 participate in the silencing of embryonic globin expression during development. KLF10 expression was also shown to be associated with high foetal haemoglobin levels in β thalassaemia patients undergoing hydroxyurea treatment.

Keywords Haemoglobin - Kruppel-like factor 1 - β -thalassaemia - Erythropoiesis.

1 Introduction

The study of haemoglobin (Hb) switching in humans has provided a focus in haematology due in large part to fundamental importance of gene switching in human biology and the clinical significance of the foetal to adult globin switch for developing targeted methodologies to for the treatment of the β -type haemoglobin maladies such as thalassaemia and sickle cell disease (Orkin and Higgs, 2010). Hb is constituted of two α -like and two β -like globin chains, encoded by genes in the *HBA* and *HBB* loci, respectively. Developmental regulation of globin genes results in expression of stage-specific Hb molecules.

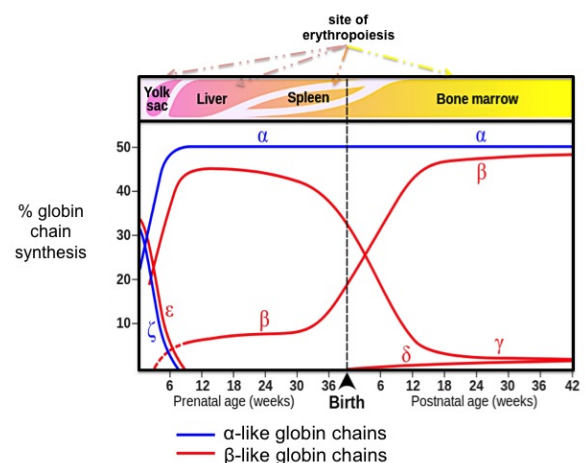


Figure 1: **Developmental Globin Chain Synthesis.** The timeline of the expression of the human globin genes is shown on the X-axis.

2 Developmental Control of Globin Gene Expression

The exact nature of globin gene switching from γ to β is still largely unknown and it is through very specific ques-

tions that one is able to answer parts of this occurrence. Hereditary persistence of foetal haemoglobin (HPFH) has been long sought as a useful contributory 'condition' that can yield additional information on how foetal haemoglobin (Hb F) is regulated and controlled *in vivo* at the adult stage. Hb F ameliorates the symptoms of β -thalassaemia and sickle cell disease (SCD), and reactivation of the *HBG1/HBG2* genes in adults is therefore of substantial interest for the clinical management of β -type haemoglobin disorders. During the development of the human body, seven normal haemoglobin types are expressed in developmental pattern. The production of normal haemoglobin is characterized by two switches (Fig. 1). These are, the embryonic to foetal haemoglobin switch and the foetal to adult haemoglobin switch, the latter being the subject of many critical questions and reviews (Bank, 2006). The embryonic haemoglobin is composed of Hb Gower 1 ($\alpha 2\varepsilon 2$), Hb Gower 2 ($\zeta 2\varepsilon 2$) and Hb Portland ($\zeta 2\gamma 2$). As the expression of ζ - and ε - globin begins to cease after the first two months of gestation, the first haemoglobin switch occurs giving rise to the synthesis of HbF ($\alpha 2^G\gamma 2$) and ($\alpha 2^A\gamma 2$). The site of erythropoiesis also changes from the yolk sac and para-aortic region to the foetal liver (Dover and Boyer, 1980). HbF has a higher oxygen affinity since it binds 2,3-bisphosphoglyceric acid (2,3-BPG) less strongly predominates in the last two trimesters of gestation before the second globin switch takes place (Forget, 1998). The second globin switch which occurs at the time of birth, involves the decline of HbF synthesis coupled with increased synthesis of adult haemoglobin composed of HbA ($\alpha 2\beta 2$) with a minor HbA2 ($\alpha 2\delta 2$) (Brinkman and Jonxis, 1935; Weinberg et al., 1983). This switch is also accompanied by a change in the site of erythropoiesis from the foetal liver and spleen to the bone marrow. However, the anatomical transitions are not thought to be the cause of the gene switching events.

Residual amounts of Hb F continue to be synthesized throughout adult life and expressed by F-erythrocytes (Hosoi, ; Stamatoyannopoulos and Grosfeld, 2001). In the majority of adults, Hb F consists of less than 2% to total Hb, but there is considerable variation (Thein et al., 2009). Genetic studies have revealed at least three loci that could control Hb F levels in adults: *HBB* (11p15.4) (Gilman and Huisman, 1985; Craig et al., 1996), *HBS1L-MYB* (6q23.3) (Close et al., 2004; Craig et al., 1996; Garner et al., 1998) and *BCL11A* (2p16.1) (Menzel et al., 2007; Lettre et al., 2008). Together, these loci account for less than 50% of the variation in Hb F, indicating that additional loci may be involved. The *HBB* locus in particular contains an important promoter sequence variation at position -158 five primer (5') to the γ globin gene, called *Xmn1* site and other globin gene rearrangements (such as $G\gamma$ - $G\gamma$, $A\gamma$ - $A\gamma$ or multi-

ple γ globin genes) that have shown to effect Hb F levels and $G\gamma$: $A\gamma$ ratios in normal individuals and/or individuals with anaemic stress (Thein et al., 2009).

Clinical syndromes such as the β -thalassaemias, hereditary persistence of foetal haemoglobin (HPFH) and $\delta\beta$ -thalassaemias have all proved to be unique clinical models underlying the pathophysiology of globin gene expression and control in haemoglobinopathies. Genetic analysis of HPFH families is a particularly powerful approach to identify novel modifiers of Hb F levels (Close et al., 2004).

Indeed early clues (Weatherall and Clegg, 1975; Huisman et al., 1975) had indicated that important DNA regulatory sequences are present in between the foetal γ globin genes and adult β globin genes. The various types of γ globin gene re-arrangements and deletional HPFH encompassing DNA regions of the $A\gamma$ - δ intergenic region characterized this. A poly-pyrimidine rich region (PYR) is located in this intergenic region and attracts numerous protein complexes that together form a repressor-like complex that silences the foetal γ globin genes (Bank, 2006). A subset of important and critical genes that act in concert with other molecules and bring about control and regulation of haemoglobin are the Kruppel-like factor genes, most notably Kruppel-like factor 1 (KLF1), Kruppel-like factor 3 (KLF3) and Kruppel-like factor 10 (KLF10).

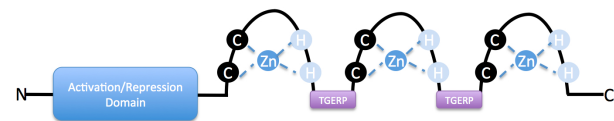


Figure 2: **Schematic diagram of the KLF1 molecule.** The three C-terminal C2H2 zinc fingers are shown, each chelating a single zinc ion. The fingers are linked together by "TGERP"-like motif, which assists in binding to target DNA. The activation/repression domain is found at the N-terminus of the molecule (Adapted from (Pearson et al., 2011))

3 Kruppel-like factor 1

Kruppel-like factors are a subset of genes that code for transcription factors designated KLF1 to KLF17 important in transcriptional regulation and control of a number of other genes. KLFs are implicated in many cellular functions such as erythropoiesis, cell differentiation, proliferation and tissue development (McConnell and Yang, 2010). A closely-knit network of KLFs interacts together to regulate the switch from foetal to adult haemoglobin. A duplicated *CACCC* sequence, known to attract and bind Kruppel-like factors, in the β -globin gene is located between -90 to -105 and is also present in the ε and γ genes (Nienhuis and Maniatis, 1987). The α -globin gene promoter (Liebhaber et al., 1980) also comprises a *CACCC* similar sequence box of the β -globin promoter at position -84 to -89. The *CACCC*-box sequences (*CCACACCCT*) (Donze et al.,

1995) are frequently found in erythroid-specific gene promoters. Two such sites are present in the human and mouse β -globin promoters. KLF1 (Figure 2) is active in primitive and definitive haematopoiesis and not required for yolk sac erythropoiesis and erythroid commitment (Nuez et al., 1995; Perkins et al., 1995), suggested that it is important for the transition from foetal to adult globin expression in humans. Additionally, single base substitutions in the KLF1 binding sites in the β -globin gene promoter cause β -thalassaemia (Orkin et al., 1982). (Schoenfelder et al., 2010) found that mouse *Hbb* and *Hba* associate with hundreds of active genes from nearly all chromosomes in nuclear foci known as ‘transcription factories’. The 2-globin genes preferentially associated with a specific and partially overlapping subset of active genes. (Schoenfelder et al., 2010) also noted that expression of the *Hbb* locus is strongly dependent upon KLF1, while expression of the *Hba* locus is only partially dependent on KLF1. Immunofluorescence examination of mouse erythroid cells displayed that most KLF1 concentrated to the cytoplasm and that nuclear KLF1 was present in isolated sites as clusters. Erythroid cells from KLF1 *null* mice specifically showed a disruption of the association of KLF1-regulated genes within the *Hbb*-associated network. KLF1 knockout more insipidly disrupted interactions within the specific *Hba* network. (Schoenfelder et al., 2010) revealed that KLF1-regulated genes share KLF1-containing transcription factories and that KLF1 is required for the clustering of these co-regulated genes. It was suggested that transcriptional regulation involves a complex 3-dimensional network rather than factors acting on single genes in isolation.

The description of the Active Chromatin Hub (ACH) gave a 3D picture of the human and mouse β -globin loci (Tolhuis et al., 2002; Palstra et al., 2003; Patrinos et al., 2004) and revealed a dynamic structure that communicates enhancers, promoters and specific regulators and co-regulators to execute gene transcription. In this model, the intervening sequences and non-transcribed genes are looping out of the active site of transcription, supporting a looping model for transcriptional activation of the globin genes.

The first transcription regulator that was shown to influence the formation of the ACH to execute correct expression of genes found within the β -globin locus was KLF1. In the absence of KLF1, a fully functional ACH cannot be formed (Drissen et al., 2004). Together with the observation that in KLF1 knockout mice loss of 5'HS3 and β maj-promoter chromatin accessibility (Wijgerde et al., 1996) occurs, this implied that KLF1 is crucial for hypersensitive site formation and involvement of the Locus Control Region (LCR) and the β -globin promoter in the ACH, possibly through

interactions with a SWI/SNF chromatin remodeling complex (Armstrong and Emerson, 1998). GATA1 was also shown to be essential for LCR-gene contacts (Vakoc et al., 2005) in contrast with Ctf that was found to be dispensable for such interactions and globin gene expression (Splinter et al., 2006). A conceivable role of the LCR loop formation in RNAPolIII loading to the promoters of the globin genes has been proposed (Johnson et al., 2001). Still, in KLF1 knockout erythrocytes, RNAPolIII is loaded on the promoter of β -globin but the levels of Ser2 phosphorylated PolII, as a mark of active transcription, are reduced. This explains the decrease in β -globin expression (Bottardi et al., 2006). Thus, it is more likely that the recruitment of RNAPolIII, at least to the β -globin promoter, is LCR independent while the transition from the initiation to the elongation step of active transcription is KLF1-dependent LCR formation (Sawado et al., 2003).

Mutations in human KLF1 were first reported by (Singleton et al., 2008) where it was found that 9 different loss-of-function KLF1 mutations were responsible for a rare In(Lu) blood group. One of the mutations results in a loss of 1 of 3 possible GATA1 binding sites in the human KLF promoter. The other 8 different mutations were found in the KLF1 coding sequence. The effect of these mutations considerably overlapped with those reported in KLF1 null mouse studies (Drissen et al., 2005; Hodge et al., 2006; Pilon et al., 2008). These mutations resulted in individuals with a slightly elevated HbF (1-3%) and their red cells showed gross reduction in expression of Lutheran blood group glycoprotein together with a reduction in Cluster of Differentiation (CD)44 (Singleton et al., 2008).

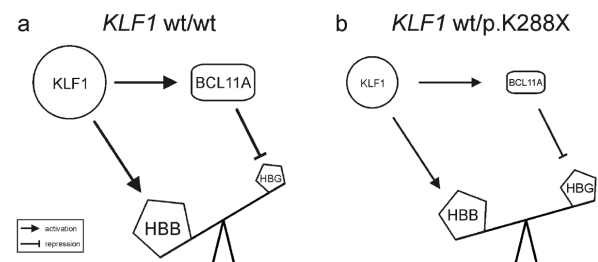


Figure 3: Model for the regulation of β -like globin expression by KLF1 in adults. Figure a shows that KLF1 preferentially activates the HBB gene and the BCL11A gene, while the BCL11A protein silences the HBG1/HBG2 (HBG) genes in normal adults. Figure b shows that in patients with KLF1 p.K288X mutation KLF1 activity is reduced. This decreases expression of BCL11A and the diminished amount of BCL11A protein alleviates repression of the HBG1/HBG2 genes. Adapted from Haploinsufficiency for the erythroid transcription factor KLF1 causes Hereditary Persistence of Foetal Haemoglobin, by (Borg et al., 2010).

The role of *in vivo* KLF1 in elevated HbF and control of human haemoglobin switching was shown for the first time in a large Maltese family (Borg et al., 2010). Ten

out of twenty seven family members exhibited Hereditary Persistence of Foetal Haemoglobin (HPFH) with HbF levels ranging from 3.3% to 19.5%. A genome wide linkage analysis on the 27 family members revealed a consistent haplotype at 19p13.12-13 co-segregating with the high HbF. DNA sequencing revealed two linked mutations in KLF1 that were found in all individuals with HPFH. The first mutation was inferred to be a neutral substitution (p.M39L), as mouse KLF1 contains a leucine at this position (Miller and Bieker, 1993). The second mutation is the p.K288X mutation, heterozygous nonsense mutation that involves an alanine (A) to thymine (T) transversion resulting in a lys288-to-ter (K288X) premature stop codon. This mutation is predicated to ablate the complete zinc finger domain of the protein (Feng et al., 1994), perturbing its function. A random sample of 400 individuals drawn from the general Maltese population did not find KLF1 p.K288X variant. A genome wide expression analysis was carried out on RNA isolated from erythroid progenitors cultured from peripheral blood from four family members with HPFH and four without. These data showed that mild hypochromic microcytic indices shown by individuals with HPFH was due to deregulation of these KLF1 target genes. It was also noted that embryonic *Hbb-y* and *HBE1* genes were highly regulated whereas the expression of *BCL11A* was downregulated in these HPFH individuals. *BCL11A* is a foetal globin repressor (Sankaran et al., 2008). Downregulation of *BCL11A* and expression of *HBG1/HBG2* genes was confirmed by quantitative RT-PCR (qPCR). KLF1 knockdown in human erythroid progenitor cells (HEPs) derived from healthy donors was investigated and the quantitative S1 nuclease protection assays showed that knockdown of KLF1 give rise to an increase in *HBG1/HBG2* expression and also a decrease in *BCL11A* expression both at a protein level and at mRNA level. HEPs were transduced with lentiviral vectors that expressed either the KLF1 p.K288X truncation mutant or full length KLF1. After transduction with full length KLF1, levels of *BCL11A* protein were increased. This result was not observed after transduction with either Green Fluorescent Protein (GFP) or truncated KLF1 lentiviral vectors. This shows that KLF has a dual role (Figure 3) in the regulation of foetal-to-adult globin gene switching. Primarily it acts directly on the *HBB* locus as a preferential activator of the *HBB* gene as reported by (Wijgerde et al., 1996) and secondly it acts indirectly by activating the expression of *BCL11A* which in turns represses the *HBG1/HBG2* genes (Borg et al., 2010; Zhou et al., 2010). In this study it was concluded that haploinsufficiency of KLF1 give rise to HPFH and a fruitful approach in raising HbF levels in individuals with β -type haemoglobinopathies can be achieved by the attenuation of KLF1.

When compared to primitive erythroid progenitors, in definitive erythroid progenitors it was shown that the level of mouse KLF1 increases threefold (Zhou et al., 2006). When carrying out chromatin immunoprecipitation (CHIP) experiments, it was found that this temporal change in KLF1 abundance is due to the differential binding of KLF1 to embryonic/foetal and adult globin gene promoters during development (Zhou et al., 2010). To study the hypothesis that a decrease in KLF1 levels in adult erythroid progenitors will give rise in reactivation of foetal globin gene expression (Zhou et al., 2010) deleted the 50-base pair HS1 enhancer of the mouse KLF1 gene and the mutant allele strain was bred with mice containing a bacterial artificial chromosome carrying a 100-kb insert spanning the human globin locus. It was found that there was an increase in endogenous mouse $\epsilon\gamma$ 2-globin/ β -globin and BAC derived human γ -globin/ β -globin gene expression ratios in livers of embryonic (E) 14.5 animals that were homozygous for the enhancer deletion. It showed that an increase in definitive erythroid progenitors is essential for correct globin gene switching.

The *BCL11A* RNA and *BCL11A* protein levels were examined in adult erythroid progenitors from *KLF1^{EHS1Δ/EHS1Δ}* mice and it was found as already concluded by (Borg et al., 2010) that *Bcl11a* expression is decreased, which in turn augments γ -globin gene expression. When carrying out Chip-quantitative PCR (qPCR), (Zhou et al., 2010) found that in adult bone marrow erythroid cells, KLF1 binds to the CACCC box in the mouse *BCL11A* promoter, whilst (Borg et al., 2010) showed the same in humans. Therefore *BCL11A* expression is directly regulated by KLF1 *in vivo*.

4 KLF1 mutations and Haemoglobin A₂

One of the most reliable haematological findings for identification of β -thalassaemia carriers is increased haemoglobin A₂ (HbA₂) between 3.8% to 6.0%. This is not always the case because some atypical carriers have borderline HbA₂ levels those between 3.3% - 3.8% (Galanello et al., 1994; Mosca et al., 2008; Giambona et al., 2008). Decreased mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) are usually associated with borderline A₂ levels and these are usually the result of mild β^+ -thalassaemia mutations, co-inherited δ and β -thalassaemia, β -promoter mutations or coexisting iron deficiency anaemia (Harthoorn-Lasthuizen et al., 1999; Galanello et al., 1981). Genetic determinants such as triplication of the α -globin genes, β -promoter mutations and some *HBD* and *HBB* gene variants can give rise to borderline HbA₂ with normal MCV and MCH %

(Galanello et al., 1994; Mosca et al., 2008; Giambona et al., 2008). (Perseu et al., 2011) for the first time found that KLF1 gene mutations are responsible for individuals with border line HbA2 and normal MCV and MCH.

In this study 145 subjects with borderline HbA2 (values between 3.3% and 4.1%) and normal or slightly reduced MCV and MCH were studied while 8 normal people were used as controls. The presence of mutations in the *HBB* promoter, triplicated α -globin genes, and haemoglobin variants were excluded. Out of the 145 subjects, 52 had the non-sense KLF1 p.Ser270X mutation, similar to the one reported by (Borg et al., 2010) but slightly more upstream. The p.Thr280_His283del mutation was found in two subjects while the p.Arg319GlufsX34 frameshift mutation was found in four individuals. Two individuals had the p.Thr327Ser mutation while one individual had the p.Lys332Gln missense mutation; another one had the p.Leu326Arg mutation. The p.Ser270X mutation and the Thr280_His283 del mutation lie in exon 2 and 4, while the p.Arg319GlufsX34, p.Leu326Arg, p.Thr327Ser and p.Lys332Gln lie in exon 3. The p.Ser270X and the p.Arg319GlufsX34 mutations result in haploinsufficiency of KLF1 since they ablate the DNA binding domain. On the other hand the p.Thr280_His283del mutation result in the deletion of cysteine 281 which is crucial for Zn coordination and therefore it eliminates the Zn finger structure and binding to DNA. The other missense mutations that is the p.Leu326Arg, p.Thr327Ser and the p.Lys332Gln mutations affects the amino acids that are adjacent to the residues expected to directly contact DNA. Therefore this might interfere with the binding of KLF1 to DNA. On the other hand these mutations could impair the interaction of KLF1 with Brg1 and Baf156 that were mapped by (Kadam et al., 2000) to the DNA binding domain resulting in the alteration of the chromatin remodeling ability of KLF1.

In the 145 subjects no correlation between HbF levels and known HbF-associated polymorphisms such as the XmnI in the HBG1 gene, rs9399137 in the HBSIL-MYB intergenic region and the rs11886868 in the BCL11A gene was found (Thein et al., 2007; Uda et al., 2008). As previously reported by (Singleton et al., 2008; Borg et al., 2010; Satta et al., 2011) in large series of subjects anaemia was absent in individuals carrying KLF1 mutations. This confirms that one functional KLF1 allele is sufficient to sustain normal human erythropoiesis. Also all the KLF1 mutations were associated with the In(Lu) blood group as reported by (Singleton et al., 2008). This suggests that the amount of KLF1 necessary to regulate Lutheran expression is highly limiting.

5 KLF1 mutations and Foetal Haemoglobin

HPFH is characterized by the presence of elevated foetal haemoglobin in red blood cells of adults. Individuals may be heterozygotes, homozygotes or compound heterozygotes for HPFH (Huisman et al., 1975) and it has been identified in a diverse range of ethnic groups (Giardine et al., 2007). It is well known that HPFH can be caused by deletions within the β -globin gene cluster on chromosome 11 (Henthorn et al., 1990) and point mutations in the promoters of the γ -globin genes (Ottolenghi et al., 1989; Wood, 1993). Single nucleotide polymorphisms (SNPs) or oligonucleotide motifs within the β -globin gene cluster are also associated with HPFH. The C-T polymorphism at position -158 of the γ -promoter which creates an XmnI restriction site is the best known of these mutations (Gilman and Huisman, 1985). As already mentioned two major sites that affect the HbF levels that are unlinked to the β -globin gene cluster that have been identified are the HBSIL-MYB intergenic region on chromosome 6q23 (Thein et al., 2007) and the BCL11A on chromosome 2p16.1 (Uda et al., 2008). An addition potential locus associated with HPFH was identified by (Borg et al., 2010) and this was the KLF1 gene.

To examine whether KLF1 mutations are involved in the increased HbF levels a study by (Gallienne et al., 2011) was carried out. In this study a total of 131 samples with elevated HbF levels (between 1.5-25%) together with 121 normal samples with HbF levels < 1% were tested for KLF1 mutations. Out of 131 patients, 41 were α -thalassaemia carriers, one had Haemoglobin E disease, 6 were carriers for sickle cell trait and 28 were carriers for a β -thalassaemia mutation. In total in these 131 subjects, eleven different KLF1 mutations were identified, nine of which were previously unreported. A polyPhen-2 and SIFT analysis for the mutations in KLF1 identified in 11 out of these 131 subjects predicted to effect gene function. Eight of the eleven mutations were missense mutations. The p.L51R was in exon 1 while p.R301C, R301H, W313C, R328H, R328L, T334K and T334R are in the zinc finger domains. These mutations are expected to disrupt DNA binding. In exon 2, two frame shift mutations were found while an 11 bp deletion, the K54PfxX9, mutation gave rise to new stop codon 8 nucleotides downstream. This mutation is the most severe type of mutation since it gives rise to the loss of all three zinc finger domains and most of exon 2. A G176AfsX179 gave rise to a 7bp insertion producing a stop codon 178 nucleotides downstream. The final mutation identified was a 1 bp nucleotide substitution at the 3' end of exon 2. This mutation is expected to disrupt splicing. In one of the

individuals homozygote for sickle cell mutation a previously unreported KLF1 mutation was identified. This mutation was the c.914-4_-14-1 del CTAG. This individual was completely asymptomatic and maintained a haemoglobin level of 12.7g/dL with HbF level of 20.3%. This shows that the KLF1 mutation is ameliorating the phenotype by increasing the HbF level via reduced γ -globin gene suppression.

KLF1 mutations are associated with a spectrum of phenotypes such as the In(Lu)blood group (Singleton et al., 2008), zinc protoporphyria (Satta et al., 2011), Increased HbA2 (Perseu et al., 2011), congenital dyserythropoietic anaemia (CDA) (Arnaud et al., 2010) and hereditary persistence of foetal haemoglobin (Borg et al., 2010).

6 Kruppel-like factor 10 (KLF10)

KLF10 is situated on chromosome 8q22.2 and functions as a transcriptional repressor involved in the regulation of cell growth. Whole-transcriptome analysis identified molecular signatures of HEP cells isolated from two patient groups representing β -thalassaemia and SCD patients that respond well or do not respond well to Hydroxyurea (HU) treatments, and a healthy adult group whose erythroid progenitor cells were grown with or without addition of HU in culture media (Borg et al., 2011). Looking at molecular signatures in response to HU common probe sets revealed 43 common genes that may be involved in developmental regulation of HbF. These genes can act either directly or indirectly leading to an increase in γ -globin synthesis therefore an increase in HbF.

One HU target gene of particular interest that was present in all groups was the KLF10 gene. The KLF10 gene appeared to be significantly associated with high HbF because it appeared in the comparison between low- versus high HbF expressing cells under HU treatment for both the Hellenic β -thalassaemia/SCD compound heterozygotes and the Maltese individuals. It appears that KLF10 is both an HU and a KLF1 target; it appears to influence globin synthesis by acting on genes either directly or indirectly. In the Caucasian populations, within KLF10 four tag SNPs with a minor allele frequency were identified. Two of the SNPs reside in the 3-Untranslated Region (UTR), one is a synonymous-coding SNP in exon 3 and one is intronic. The intronic, SNP namely the rs3191333 polymorphism resides on a regulatory region and therefore it can act as a pharmacogenomic marker by exerting a functional role of KLF10 expression. To see the effect of rs3191333 (c.*141C>T) tag SNP with β -thalassaemia severity (Borg et al., 2011) exploited a large number of

β -thalassaemia major patients with low HbF and well-characterized β -thalassaemia intermedia patients with high HbF levels. An independent β -thalassaemia/SCD compound heterozygous patient sample that received HU as therapeutic routine also was included. It was concluded that the absence of the rare homozygous mutant (TT) SNP (rs3191333) is significantly associated with increased HbF in β -thalassaemia intermedia compared with β -thalassaemia major patients and healthy volunteers. In β -thalassaemia intermedia samples the rare allele (T) is less frequent and only present in heterozygous state. Therefore it can be hypothesized that the presence of the T allele in context of β -thalassaemia could be associated with low HbF levels. An inverse correlation in the homozygous normal C/C in responders and homozygous T/T genotypes in non responders was also noted. From the above one can conclude that in homozygous mutant state the rs3191333 SNP renders the KLF10 transcript unstable and therefore the KLF10 gene expression is decreased.

It has been hypothesized that the KLF10 might be acting through SIN3A, especially since the interactions between the two have been already documented by (Zhang et al., 2001). The *SIN3A* is a very important corepressor gene that works together with HDAC1 and physically binds to KLF1 on acetylated lysine residue at position 302 and in turn this represses KLF1 activity. Repressed activity of KLF1 results in decreased adult stage globin synthesis which in turn facilitates the synthesis of foetal globin (Siatecka and Bieker, 2011). It can be hypothesized that though the interaction with *SIN3A*, KLF10 represses the adult HBB gene leading to a higher HbF level (Borg et al., 2011).

7 Kruppel-like factor 2 (KLF2)

KLF2 is a positive regulator of the mouse and human embryonic β -globin genes (Basu et al., 2005). KLF2 was known as lung KLF or LKLF and plays an important role in T-cell differentiation and blood vessel development (McConnell and Yang, 2010). Within their DNA binding domains, KLF1 and KLF2 have high homology and they reside close to each other on chromosome 19 in human and on chromosome 8 in mouse (Basu et al., 2005). In mouse embryonic β -globin genes, KLF1 and KLF2 can partially compensate for each other. It was found that when both KLF1 and KLF2 are ablated in mice, there is more reduction in ϵ and β H1-globin mRNA than when KLF1 or KLF2 single knockdowns are performed (Basu et al., 2007).

(Alhashem et al., 2011) determined whether KLF1 and KLF2 control the human embryonic and foetal β -globin genes and the mechanistic roles of these two genes in globin gene regulation. For this study a mouse model that has the KLF1 and KLF2 knock

out (KO) alleles on the same DNA homology was generated together with transgenic (Tg) mice that carry the entire human β -globin locus. The Tg-HBB mice were bred with $KLF1^{+/-}$ or $KLF2^{+/-}$ mice and therefore $KLF1^{+/-}$ -Tg-HBB $KLF2^{+/-}$ -Tg-HBB and $KLF1^{+/-}$ - $KLF2^{+/-}$ -Tg-HBB mice were obtained. The mouse embryonic yolk sacs and blood cells were collected followed by RNA and cDNA synthesis and ChIP assays. By comparing the amounts of KLF1 and KLF2 mRNA it was found that the quantities of KLF1 equalled the KLF2 mRNA at E9.5 but KLF1 mRNA increases dramatically by E12.5 whereas KLF2 mRNA levels remain relatively unchanged between E9.5 and E12.5. This shows that at E9.5 both KLF1 and KLF2 regulate the mouse ϵ and γ 1-globin genes but at E12.5 only KLF1 regulates adult β -globin gene expression.

It was already known by (Basu et al., 2007) that KLF1 and KLF2 regulate the mouse embryonic ϵ and γ 1 during primitive erythropoiesis, (Alhashem et al., 2011) wanted to test whether these two genes also regulate human β -globin gene expression in embryo. In transgenic mouse models, the human embryonic and foetal β -globin genes, the ϵ and γ are both expressed at E10.5 (Jiang et al., 2008). To test this hypothesis dual Tg-HBB and heterozygous KO mice were crossed with heterozygous KO mice to obtain E10.5 $KLF1^{-/-}$ -Tg-HBB, $KLF2^{-/-}$ -Tg-HBB and $KLF1^{-/-}$ - $KLF2^{-/-}$ -Tg-HBB embryos. The amounts of human ϵ - and γ -globin mRNA was measured using qRT-PCR. It was found that ϵ -globin mRNA was significantly reduced to 15 to 49% in $KLF1^{-/-}$ -Tg-HBB and $KLF2^{-/-}$ -Tg-HBB yolk sacs when compared with Tg-HBB. On the hand γ -globin mRNA was reduced to 31% of Tg-HBB in $KLF1^{-/-}$ -Tg-HBB yolk sacs. In $KLF2^{-/-}$ -Tg-HBB yolk sacs the γ -globin gene expression is reduced to 73% showing that KLF2 has a more modest effect on γ -globin mRNA as already discussed by (Basu et al., 2005). When looking at these results one can say that KLF1 appears to have a greater effect on ϵ and γ globin gene expression but with KLF2 also plays an important role. The synergistic regulation of the human ϵ and γ globin genes by KLF1 and KLF2 in transgenic mice models cannot be ruled out.

ChIP assays were carried out to better understand the mechanism by which KLF1 and KLF2 control human and mouse embryonic β -globin gene expression. It was found that at E10.5, KLF1 was significantly enriched at the promoters of the ϵ and β 1-globin genes at mouse 5'HS2 in the LCR. KLF1 was significantly enriched at the promoter of the γ -globin gene, 5'HS2 and 5'HS3 in human β -globin loci. The pattern of KLF1 enrichment at the mouse and human β -globin loci at E11.5 was similar as that at E10.5, the only difference being that the binding of KLF1 to ϵ -globin was also seen at E11.5. KLF2 in contrast to KLF1 was detected at the ϵ but

not at the β 1-globin promoter at E11.5. This is consistent with (Strouboulis et al., 1992) that at E11.5 there is higher expression of the E γ than β 1-globin gene. In the mouse β -globin LCR there was no evidence that KLF2 binds to 5'HS2 and 5'HS3 but in human β -globin locus KLF2 was enhanced by about 2-fold at the γ -globin promoter and at 5'HS2 and 5'HS3. These results by (Alhashem et al., 2011) show that by direct binding to the CACCC elements in the promoters and LCR KLF1 and KLF2 regulates the embryonic and foetal β -globin genes. As in adult erythroid cells, binding of KLF1 and KLF2 to LCR could be necessary for direct contact between the LCR and the β -globin gene promoter (Drissen et al., 2004).

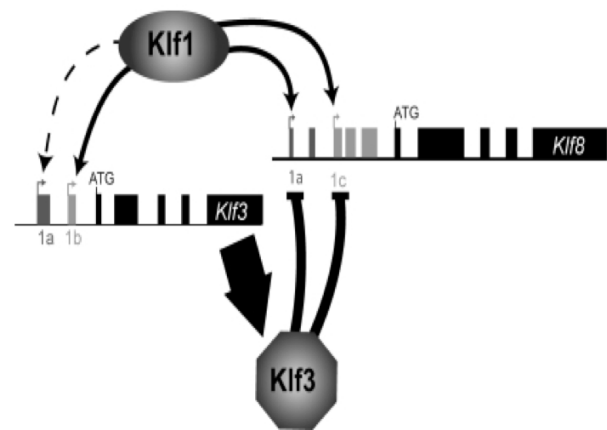


Figure 4: Schematic diagram of cross-regulation between Klf1, Klf3 and Klf8. Klf1 activates expression of the Klf3 and Klf8 gene promoters. Klf3 can also repress the Klf8 promoters. In tissues where both Klf1 and Klf3 are present, such as foetal liver, the proteins compete for binding, and this restricts Klf8 to low levels of expression. In the absence of Klf3, for example in the Klf3 null foetal liver, Klf1 gains access to the Klf8 promoters, resulting in de-repression of the gene and increased Klf8 protein (Adapted from (Eaton et al., 2008)).

8 Other Kruppel-like factors

Although some KLFs such as KLF1 primarily function as activators of transcription (Miller and Bieker, 1993), other KLFs such as KLF3 have been described as transcriptional repressors (Turner and Crossley, 1998). On the other hand KLF8 can act as either activator or repressor depending on the biological context and the gene regulatory region through which it is operating (van Vilet et al., 2000; Wei et al., 2006; Urvalek et al., 2010; Urvalek et al., 2011). It was documented (Funnell et al., 2007; Eaton et al., 2008) by that the transcription of KLF3 and KLF8 is carried out by KLF1 as showed in figure 4.

KLF3 that was previously known as basic Kruppel-like factor (BKLF) silences gene expression by the recruitment of co-repressor C-terminal binding protein (CtBP) (Turner and Crossley, 1998; Pearson et al., 2011). Although KLF3 is expressed widely, since it has an ery-

throid specific promoter that is directly activated by KLF1, it is abundant in erythroid tissue (Tallack et al., 2010; Funnell et al., 2007). *In vitro*, KLF3 exhibits similar DNA-binding preferences to KLF1 and in erythroid cells *in vivo* it is as though many genes that are activated by KLF1 are repressed by KLF3 to fine-tune their expression during erythropoiesis (Funnell et al., 2012). KLF8 is one such gene that is activated by KLF1 and repressed by KLF3 (Eaton et al., 2008). KLF3 and KLF8 proteins share 96% of their sequence in their zinc finger domain (Lomberk and Urrutia, 2005) and both proteins recruit CtBP corepressor to silence gene expression (Turner and Crossley, 1998).

The erythroid roles of KLF1 and KLF3 were already investigated but little was known about the contribution of KLF8 to this regulatory network. Therefore Alister et al., (2013) investigated the erythroid role of KLF8. For this study KLF3^{-/-} were crossed with mice with homozygous disruption of KLF8 (KLF8^{gt/gt}) followed by histological examination of mice, quantitative real time RT-PCR for KLF8, ϵ -globin, β h1-globin, ζ -globin and α -globin. Protein overexpression in COS cells was also performed followed by Western blotting, genotyping, ChIP analysis and microarrays. It was found by (Lahiri and Zhao, 2012) that KLF8 protein is normally expressed at very low levels in non-cancerous tissue, Alister et al., (2013) noted that endogenous KLF8 was also seen in wildtype foetal brain and placental tissue.

It was noted that mice deficient in KLF3 were also viable, but KLF3^{-/-}-KLF8^{gt/gt} double mutant died at around E14.5. This shows that *in vivo* KLF3 and KLF8 have overlapping roles and they partially compensate in each other's absence. When KLF3^{-/-} mice were analyzed, KLF8 expression in several tissues was upregulated but this was also noted also in erythroid tissue. Alister et al., (2013) hypothesized that KLF3 and KLF8 operate in a regulatory network to control gene expression since it is known that both of them are activated by KLF1 (Eaton et al., 2008). This was confirmed by microarray analysis of Ter119⁺ foetal liver cells from single mutant and double mutant embryos. In the absence of KLF3, 64 genes were unregulated and these were not significantly elevated in KLF3^{-/-}-KLF8^{gt/gt} embryos.

By qRT-PCR Alister et al., (2013) confirmed that in KLF3^{-/-} and KLF3^{-/-}-KLF8^{gt/gt} embryonic gene expression is derepressed but adult globin expression is unchanged. This can be due to KLF3, which is the primary repressor of embryonic globin expression and in its absence KLF8 is able to partially compensate. Alister et al., (2013) suggest that together KLF3 and KLF8 participate in the silencing of embryonic globins with other repressors such as SOX6, GATA1, YY1, COUP-TF and DRED (Stamatoyannopoulos, 2005; Tanabe et al., 2002; Filipe et al., 1999; Tanimoto et al., 2000).

In Klf3^{-/-} and KLF3^{-/-}-KLF8^{gt/gt}, BCL11A expression was not found to be unchanged when compared to wildtype. This implies that the repression of embryonic globin genes by KLF3 and KLF8 is not indirectly achieved by altering the transcription of these known regulators in erythroid cells (Alister et al., 2013). In Klf3^{-/-} and KLF3^{-/-}-KLF8^{gt/gt} it was also noted that in addition to upregulation of the β -like embryonic globins there was also unregulation of Hba-x expression. Both KLF3 and KLF8 are a pair of transcription regulators that operate in an erythroid transcriptional network downstream of KLF1. The dominant role in regulating gene expression is carried out by KLF3 while KLF8 is able to partially compensate at some loci.

9 Conclusion

As seen in this review, KLFs are a family of DNA-binding transcriptional regulators that are involved in a wide range of biological processes. KLF1, KLF10, KLF2, KLF3 and KLF8 are involved in haemoglobin control. Trying to understand the complex network between these transcription factors and haemoglobin switching can be a fruitful approach to raise therapeutic HbF levels in individuals with β -type haemoglobinopathies treating the underlying dyserythropoiesis and associated complications.

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