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Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin

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Abstract

The major disorders of β -globin, sickle cell disease and β -thalassemia, may be ameliorated by expression of the fetal gene paralog γ -globin. Uncertainty regarding the mechanisms repressing fetal hemoglobin in the adult stage has served as a puzzle of developmental gene regulation as well as a barrier to rational therapeutic design. Recent genome-wide association studies implicated the zinc-finger transcriptional repressor BCL11A in fetal hemoglobin regulation. Extensive genetic analyses have validated BCL11A as a potent repressor of fetal hemoglobin level. Studies of BCL11A exemplify how contextual gene regulation may often be the substrate for trait-associated common genetic variation. These discoveries have suggested novel rational approaches for the β -hemoglobin disorders including therapeutic genome editing.

The hemoglobin disorders, the most common monogenic diseases in the world, have long served as exemplars of molecular medicine. Upon recognition of a chemically anomalous hemoglobin, Pauling dubbed sickle cell anemia the first molecular disease more than 65 years ago [1]. Less than a decade later, the defining single amino acid substitution in the β -chain was discovered as its etiology [2]. In the 1970s and 1980s, the genetic underpinnings of the thalassemia syndromes, disorders of hemoglobin production rather than structure, were unraveled by molecular cloning, revealing diverse mutations affecting the globin genes [3]. These discoveries emphasized the importance of not only coding sequences but also splice sites, 5' and 3' untranslated regions, proximal promoters, and distal regulatory elements in determining appropriate gene expression.

The Mendelian nature of inheritance of the hemoglobin disorders belies their phenotypic heterogeneity. The chief predictor of clinical severity of the β -hemoglobin disorders is the

level of fetal hemoglobin (HbF, $\alpha_2\gamma_2$). In β -thalassemia, increased levels of γ -globin substitute for absent β -globin and mitigate relative α -chain excess. In sickle cell disease (SCD), γ -globin serves as a potent anti-sickling agent. Clinical observations—including those of rare patients co-inheriting highly elevated HbF (hereditary persistence of fetal hemoglobin), large cohorts with varying HbF levels, and the natural history of infants with waning HbF—unambiguously demonstrate that HbF ameliorates the β -hemoglobin disorders.

The β -globin gene cluster includes 5 paralogous globin genes whose expression is developmentally regulated. These include ϵ -globin, expressed during the early first trimester from the yolk sac, two γ -globin genes ($G\gamma$ and $A\gamma$), expressed during the remainder of fetal life, and the minor δ -globin and major β -globin adult gene products, expressed throughout postnatal life. Hemoglobin switching refers to the process of developmental changes in hemoglobin production. In a transition largely driven at the transcriptional level, HbF, the dominant hemoglobin *in utero*, becomes replaced by HbA ($\alpha_2\beta_2$) as the γ -globin genes are progressively silenced and the β -globin gene is reciprocally activated. In the adult stage, HbA constitutes ~97% of hemoglobin, the minor HbA2 ($\alpha_2\delta_2$) ~2%, and HbF <1%. Residual HbF is unevenly distributed within erythrocytes, limited to a subset of cells termed F-cells.

The mechanisms controlling hemoglobin switching, particularly the fetal-to-adult transition, long remained obscure despite extensive inquiry [4]. The underlying problem is related to cellular identity, and how stage specificity for globin transcription is imposed in an erythroid cell context. More precisely, the crux is how transcript production shifts from the γ -genes to the β -gene in the β -like globin complex. As reactivation of HbF could be curative for the β -hemoglobin disorders, an understanding of the mechanisms of developmental control has immediate and direct clinical implications. An optimal therapeutic maneuver would result in measurable amounts of HbF in all erythrocytes (i.e. pan-cellular F-cell distribution) rather than limited to a subset (i.e. heterocellular F-cell distribution). An understanding of the molecular circuitry controlling HbF is a necessary precondition for rationally designed therapies.

Recent work has identified BCL11A as a critical modifier of the hemoglobin disorders. In addition to direct therapeutic relevance for the hemoglobinopathies, these findings emphasize the importance of contextual gene regulation in determining heritable disease severity.

Genetic evidence connecting BCL11A and HbF regulation

The initial breakthrough came from genome-wide association studies (GWAS). These studies identified common variants at three loci associated with HbF level, or the closely associated trait F-cell number [5,6]. The loci included the β -globin gene cluster, intergenic sequences between *HBS1L* and *MYB*, and *BCL11A*. These studies are notable for a number of reasons. First, the genetic architecture is very simple with relatively few genes of large effect. Just three loci account for about half the heritable variation in HbF level [7]. Second, the results have been robust and reproducible across populations of European, African, and

Asian ancestry, as well as in healthy individuals and those with β -hemoglobin disorders [8-11]. Third, the same variants associated with HbF level are associated with the clinical severity of both SCD and β -thalassemia, emphasizing the potential therapeutic importance of the discoveries [8,9,12]. Finally, the results were concordant with prior knowledge, indicating the validity of the approach. Not only were variants recovered at the β -globin gene cluster itself, but also variants in the *HBS1L-MYB* intergenic interval had previously been associated with HbF level by familial linkage studies [13]. Of the three loci, only *BCL11A* was novel.

At the time of its implication in HbF regulation by GWAS, *BCL11A* was known to be a zinc finger transcriptional repressor protein and an oncogene in B-cell malignancies [14,15]. Subsequently it was found to coordinate gene expression during B-lymphopoiesis and neurogenesis [16,17]. Prior to the GWAS, it had not been suspected to play a role in erythropoiesis. Consistent with a function in repressing γ -globin expression, *BCL11A* is expressed in erythroid precursors [5].

Subsequent genetic experiments have demonstrated the critical role *BCL11A* plays as a repressor of HbF. Knockdown of *BCL11A* by RNA interference (RNAi) in primary human erythroid precursors leads to HbF expression without apparent detrimental effect on erythropoiesis [18]. Genetic studies in mice offer additional support but are complicated by the absence of a true fetal stage of globin expression in rodents. Transgenic mouse experiments long ago revealed that human γ -globin genes are expressed roughly coordinately with the endogenous mouse embryonic globins (β h1 and ϵ y) and extinguished at the fetal liver stage rather than after birth [19]. Knockout of *Bcl11a* during mouse development prevents appropriate silencing of endogenous embryonic globins and transgenic human γ -globins [20]. A partial loss of silencing is observed in heterozygotes, suggesting *BCL11A* represses embryonic and fetal globins in a dose-dependent manner. Conditional erythroid knockout of *Bcl11a* greatly relieves repression of mouse embryonic and human fetal globin genes without negative impact on erythropoiesis [21,22]. Inducible deletion of *Bcl11a* in adulthood results in derepression of embryonic and fetal globin genes, indicating that *BCL11A* actively enforces adult-stage globin expression throughout adult life and that repression is reversible.

Additional support for the relevance of *BCL11A* to HbF control is provided by a “preclinical” experiment in which the phenotype of genetically engineered SCD mice is reversed in the context of erythroid-specific *Bcl11a* knockout, a particularly striking result indicating the therapeutic potential of targeting *BCL11A* [21]. In these mice, standard red blood cell parameters normalize, HbF levels are induced in an essentially pancellular fashion, circulating sickle forms are absent from the peripheral blood, and end-organ damage, as indicated by loss of urinary concentrating function, is prevented. These data offered the first experimental evidence that loss of a single gene product could thwart SCD. Overall, the preponderance of experimental genetic data validate *BCL11A* as a therapeutic target for the β -hemoglobin disorders.

BCL11A participates in multiprotein transcriptional complexes in erythroid cells (Figure 1) [18,23,24]. Its protein partners, as ascertained by affinity purification and protein

microsequencing, include DNA-binding erythroid transcription factors (TFs) such as GATA1, FOG1, RUNX1, IKZF1, and SOX6. In addition, it binds to numerous chromatin regulators and transcriptional corepressors. These include members of the NuRD, LSD1/CoREST, NCoR/SMRT, SIN3, and SWI/SNF complexes. Notable interactors include the Mi2 β (CHD4) helicase, HDAC1 and HDAC2 histone deacetylases, LSD1 lysine demethylase, DNMT1 DNA methyltransferase, and NCoR1 and BCoR corepressors. BCL11A occupies erythroid chromatin at the β -globin gene cluster [18,23]. It binds at numerous sites, including within the locus control region (LCR) distal enhancer elements as well as to the ε -globin gene but it does not bind to β -globin or the γ -globin genes themselves. Therefore it appears to exert its repressive function of γ -globin at a distance. BCL11A is required for configuring the β -globin locus in erythroid cells, promoting long-range interactions between the LCR and β -globin gene at the expense of LCR- γ -globin interactions, in keeping with a competitive mode of globin gene regulation [23]. Inhibition of many of the protein partners of BCL11A results in HbF induction, particularly when assayed by RNAi knockdown in CD34⁺ hematopoietic stem and progenitor cells subjected to *ex vivo* erythroid maturation [24,25]. However none of the partners tested to date has the specificity of BCL11A on HbF regulation. These protein partners tend to have more widespread effects on erythroid gene expression as compared to BCL11A itself whose effects are quite restricted to the globin genes. Inhibition of BCL11A's protein partners (unlike BCL11A itself) typically results in impaired erythroid maturation. These results suggest that BCL11A participates in only a subset of the functions and/or complexes of its partners. Moreover, pharmacologic HbF inducers, such as inhibitors of DNA methyltransferase, histone deacetylase, and LSD1 demethylase, are much more effective in the absence of BCL11A [21,24]. These results suggest that residual BCL11A serves as a barrier to alternative strategies for HbF reinduction.

Effects of common genetic variation on *BCL11A*

Although GWAS have been very successful in identifying thousands of loci associated with human traits and disease susceptibility, there remains a dearth of knowledge for most of these associations regarding the identity of the causal variants and affected genes and biologic pathways. Underscoring this challenge, due to linkage disequilibrium, it can be very difficult to distinguish causal variants from mere associated markers. Like many SNPs highlighted by GWAS, the common genetic variants associated with HbF level fall in non-coding sequences. The trait-associated variants at *BCL11A* reside within its large second intron. These variants, co-inherited as haplotypes, cluster at a region marked by an erythroid enhancer chromatin signature (Figure 1) [26]. This region possesses typical enhancer-associated histone modifications, such as H3K4me1 and H3K27ac, erythroid TF occupancy, DNase I sensitivity, and long-range promoter interactions. Fine-mapping of trait-associated variants demonstrates that the most highly trait-associated variants fall directly within DNase I hypersensitive sites (DHSs). The DHS residing variants fully account for the trait association of the previously identified lead variants, suggesting these were merely markers in linkage disequilibrium with enhancer variants. The most highly trait-associated variant rs1427407 disrupts a composite half E-box/GATA motif, a TF recognition site highly bound by GATA1/TAL1 complexes in erythroid cells. This variant is associated with modest

reduction in TF binding and modest reduction of BCL11A expression in primary human erythroid precursors. The DHSs harboring these variants confer adult developmental stage-specific and erythroid lineage-restricted gene expression in classical ectopic enhancer assays. Genome editing experiments demonstrate that the enhancer is strictly required for BCL11A expression in the erythroid lineage but dispensable in non-erythroid contexts [26].

These results have furthered our understanding of globin gene regulation and demonstrate that the hemoglobin disorders continue to be paradigmatic of advances in molecular medicine. The role of BCL11A in repressing HbF emphasizes the importance of heritable modifiers, even for apparently monogenic conditions. The fact that common genetic variation impacts *BCL11A* through an erythroid-specific enhancer appears to be just one example of how context-specific gene regulation is a major determinant of human traits and disease susceptibility. Numerous chromatin mapping efforts have identified substantial enrichment of common variants related to human traits and disease susceptibility by GWAS within distal regulatory elements, particularly enhancers active in cell types relevant to the trait [27-29]. For example, one recent report estimated that 60% of the causal variants associated with autoimmunity reside within immune cell enhancers [30]. This mechanism of regulatory variation is conceptually appealing in that many genes are pleiotropic and under substantial evolutionary constraint. Therefore although trans-acting gene-encoded regulatory factors are highly conserved, the cis-regulatory landscape upon which these factors act may be subject to much greater evolutionary turnover [31,32]. This could result in both inter- and intra-species phenotypic variation that could escape excess negative selection. For example, mice with *Bcl11a* deficiency demonstrate perinatal lethality, presumably due to the critical role of BCL11A in development of the central nervous system. Humans haploinsufficient for *BCL11A* due to intrachromosomal deletions demonstrate severe neurodevelopmental phenotypes, such as autism, mental retardation, and speech and language disorder [33-35]. Analysis of exome-sequencing data from healthy individuals show that *BCL11A* is among the 5% of genes under the highest evolutionary constraint, with relative absence of missense as compared to synonymous mutations. Variants at *BCL11A* have been associated with type 2 diabetes mellitus and BCL11A coordinates pancreatic gene expression [36]. BCL11A, subject to recurrent gene amplification in triple-negative breast cancer, is required for mammary stem and progenitor cell function [37]. These results indicate that BCL11A plays varied roles in different cellular lineages and that coding variation at *BCL11A* is highly deleterious [38]. In contrast, HbF-associated erythroid enhancer variants at *BCL11A* are very common; e.g. the most highly associated variant rs1427407 has a 20% allele frequency [39].

In addition, the example of *BCL11A* highlights the importance of studying protective genetic variation. Many mutations and many pharmacotherapies act in a loss-of-function manner. Therefore, phenocopying protective inactivating genetic variants with rational therapeutics may be a favorable approach as compared to attempts to reverse disease-associated inactivating mutations. Other examples of protective inactivating mutations that have prompted investigation of novel therapeutic strategies include *PCSK9* in heart disease and *CCR5* in AIDS [40,41].

Outstanding questions

There are several questions that remain regarding mechanisms of HbF repression and how recognition of the critical role of BCL11A may be harnessed for therapeutic advantage.

First, how does BCL11A actually repress γ -globin transcription. BCL11A has several potentially functional domains, including six C2H2 zinc fingers, one C2HC zinc finger, a NuRD-interacting domain, an acidic domain, and a proline-rich domain [23]. There are several splice variants of BCL11A, with the XL-transcript producing the dominant isoform in erythroid cells. As described above, BCL11A has numerous protein partners. It remains to be defined which domains and partners are required for its function. Careful structure-function analysis of BCL11A may highlight critical protein-protein interactions. Although BCL11A has been reported to bind DNA *in vitro*, it is uncertain if it directly binds DNA *in vivo*, or is recruited by other TFs. Motif analyses have yielded conflicting results as to sequence binding preference emphasizing that an obvious DNA binding function of BCL11A has yet to be demonstrated [42-44]. The DNA binding preference for the paralog BCL11B has been similarly challenging to define [45,46]. Perhaps function of BCL11A at the LCR and putative regulatory sequences intergenic to γ - and δ -globin genes are sufficient to account for its effects [47]. It is also possible that BCL11A directly interacts with factors that in turn may bind to the γ -globin genes.

A second major unanswered question is how BCL11A is developmentally regulated. Although the identification of the role of BCL11A in hemoglobin switching was a major advance, on one level this merely redefines the problem as identifying the factors that activate BCL11A during the adult stage. A substantial component of this regulation appears to be transcriptional [23,26]. The erythroid enhancer of *BCL11A* restricts expression to the definitive developmental stage. Fine-mapping the *BCL11A* enhancer would be one approach to elucidate cis- and trans-acting determinants upstream of BCL11A. BCL11A is also expressed in hematopoietic stem and progenitor cells as well as in lymphocyte precursors and various immune cells [48,49]. With the recent appreciation for inflammatory inputs into definitive hematopoietic stem cell (HSC) formation and function [50-53], one possibility to be investigated is the interplay between inflammation, BCL11A, and HSC biology. Several regulators of BCL11A have already been identified, and ongoing work will be needed to determine the role of these during organismal development as well as erythroid maturation. KLF1 is an erythroid TF with an important role in HbF control that binds to the BCL11A promoter and positively regulates BCL11A expression [54,55]. MYB, another key HbF regulator, may itself be upstream of KLF1 [56]. Mi2 β the helicase component of the NuRD complex, may be required for complete BCL11A expression, although other components of NuRD such as MBD2 appear to repress the expression of BCL11A [57]. There also appear to be post-transcriptional controls on BCL11A expression and processing, with short variants detectable prenatally, apparently due to protein processing [18,20]. The microRNA miR486-3p has been reported to destabilize the BCL11A transcript in erythroid cells [58]. An important connection to be determined is how generic developmental programs interface with BCL11A. One such candidate pathway is the heterochronic LIN28/let-7 axis, whereby LIN28 is abundant in many tissues during fetal life and reciprocally let-7 microRNAs are abundant after birth. In fact, LIN28 overexpression has been reported to increase HbF levels

in CD34⁺ cells from healthy donors and SCD patients, although the impact of LIN28 on BCL11A expression has been inconsistent [59,60].

The advances in our knowledge of globin gene regulation and hemoglobin switching have mainly stemmed from observations of human genetics. The existing GWAS account for about half the heritable variation of HbF level, suggesting another half remains to be determined. Whether more highly powered conventional studies, novel study designs, such as restricting analyses to genes and elements most likely to be involved in adult-stage erythropoiesis, and/or investigation of rare informative patients will be required, it seems likely that human genetics has more lessons to teach.

Translating findings to the clinic

One area of clinical need is obtaining a plentiful source of human blood cells suitable for transfusion. Currently altruistic donors are used as sources for blood products. However infectious transmission risk, genetic and immune incompatibilities, and cost are major concerns. Pluripotent and hematopoietic stem cells may be cultured from various donors and induced to differentiate. Current protocols for *in vitro* erythroid maturation, while still not of a scale to support routine clinical transfusion, are advancing [61-63]. One challenge is that pluripotent stem cell derived progeny typically retain an embryonic character, in the case of red blood cells expressing embryonic and fetal forms of hemoglobin. A recent study showed that overexpression of BCL11A is able to convert induced pluripotent stem cell derived lines from the expression of only embryonic and fetal globin genes to predominant expression of adult globins [64]. This finding could be of relevance for efforts to use pluripotent stem cells as a source of adult blood cells.

Current treatments for the hemoglobin disorders are supportive and do not address the fundamental pathophysiologic defects. Hydroxyurea is the only FDA-approved medication for the treatment of SCD. Although this agent can modestly elevate HbF levels and mitigate painful crises, it has inconsistent efficacy, causes dose-limiting myelosuppression, and requires careful laboratory monitoring, which limit its utility. Allogeneic HSC transplantation can be curative, but immune incompatibility restricts its application. Additional therapeutic options are needed. One approach would be to target BCL11A directly at the gene level. This could be accomplished by RNA interference [65] although persistent and specific delivery to erythroid cells would be crucial.

Another possibility would be therapeutic genome editing with targetable endonucleases [66]. Rapid progress in genome editing tools, particularly with the RNA-guided CRISPR/Cas systems, has made precise genome modification increasingly plausible in clinical settings [67,68]. Therapeutic genome editing would offer the potential for autologous transplantation of genetically modified HSCs (Figure 2). Although direct repair of β -globin itself would be the most direct curative strategy for the β -hemoglobinopathies, there could be several advantages to targeting *BCL11A*, a disease modifying gene, rather than β -globin itself. First, in contrast to the single point mutation underlying SCD, β -thalassemia may be caused by hundreds of unique mutations, so developing a universal repair strategy would be difficult. Second, HSCs appear to be particularly refractory to homology-directed repair (HDR),

perhaps because HDR requires an active cell cycle whereas HSCs are quiescent cells [69]. HSCs mainly repair double strand breaks by nonhomologous end-joining, so genetic disruption rather than precise gene correction may be a much more feasible strategy for the hematopoietic system given current technology.

Detrimental effects of loss of *BCL11A* in the hematopoietic compartment, either on B-lymphocytes or even on stem and progenitor cells would need to be considered [48]. Targeting the erythroid enhancer of *BCL11A*, or alternatively the target sequences bound by *BCL11A* within the β -globin gene cluster (should they be defined adequately), offers the potential for erythroid specificity [26].

Ultimately it would be highly desirable to develop small molecule inhibitors of *BCL11A*, as these might be suitable for use in larger numbers of patients, whereas any strategy employing stem cell transplantation, whether allogeneic or autologous (with RNA interference or genome editing), will be limited to specialized centers. Although more needs to be learned about *BCL11A* structure and function to allow the rational design of such an inhibitor, recent successes in targeting transcriptional regulators offer hope of feasibility [70-73]. One challenge to small molecule inhibitors of *BCL11A* would be to restrict effects to the erythroid lineage. It is possible that there could be a therapeutic window whereby an intermediate level of *BCL11A* inhibition could allow derepression of HbF but not ablate essential extra-erythroid functions of *BCL11A*. Alternatively, it is possible that erythroid-specific aspects of *BCL11A*, such as discrete protein-protein interactions, could be targeted.

Epidemiologic transitions in the developing world, with better control of infectious disease, and accompanying survival of patients with hemoglobin disorders beyond early childhood, are anticipated to result in large increases in the number of patients over the coming decades, with more than 400,000 born each year [74]. We anticipate that these conditions will continue to provide new insights about human genetics and disease and hope they could serve as successful examples of rational therapeutic design.

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Figure 1. The BCL11A–HbF repression axis

BCL11A itself relies on an erythroid enhancer, the subject of common trait-associated genetic variation, for its adult-stage erythroid expression pattern. GATA1 and TAL1 bind to this enhancer, and KLF1 (which may in turn be regulated by MYB) transactivates BCL11A, binding to its promoter. BCL11A is found in a multiprotein transcriptional complex in erythroid precursors, including chromatin regulators such as the NuRD complex (with Mi2β helicase and HDAC1/2 deacetylases), DNMT1 DNA methyltransferase, LSD1 lysine demethylase, SWI/SNF remodeling complex, additional corepressors such as NCoR and BCoR, and erythroid DNA-binding transcription factors such as GATA1, IKZF1, Runx1, and Sox6. BCL11A binds various regions of the β-globin gene cluster, including the locus control region (LCR) distal enhancer elements, to the ε-globin gene itself, and to intergenic sequences between γ- and δ-globin that have been implicated in γ-globin repression. BCL11A does not bind directly to the γ-globin genes. The outcome of BCL11A expression in adult-stage erythroid cells is the predominant expression of β-globin (and thus HbA) at the expense of γ-globin (and HbF).

Figure 2. Therapeutic genome editing of *BCL11A* for the β -hemoglobin disorders

Hematopoietic stem cells may be collected from a β -hemoglobin disorder patient, modified *ex vivo*, and then autologously re-engrafted to the patient. Genome editing technology has been rapidly advancing, and a variety of targetable endonucleases, such as CRISPR/Cas9, could be employed. The target sequences could be the erythroid enhancer of *BCL11A*. This strategies would allow disruption of *BCL11A*'s repression of HbF in erythroid cells while sparing extra-erythroid functions of *BCL11A*. Furthermore, this genetic disruption would rely on the robust nonhomologous end-joining repair pathway, rather than on lower-frequency homology-directed repair.