various donor strains to promote Mpf and T6SS activation with P. aeruginosa, which is similar to what we observed in our RP4 mutant analysis (Fig. 1D). P. aeruginosa mutants defective in the attack-sensing pathway genes tagT and pppA (10) also exhibited greater conjugation efficiency as recipient strains (Fig. 3A). Examination of mixtures of T6SS+ P. aeruginosa and E. coli RP4+ donor cells by means of fluorescent microscopy revealed rounding and blebbing of E. coli cells—a response that is typical of T6SS-mediated bacterial killing (Fig. 3B). Thus, inhibition of the conjugative transfer of pPSV35 was likely due to killing of E. coli cells through a donor-directed T6SS attack by P. aeruginosa.

The fact that multiple secretion systems can induce a T6SS counterattack suggested that the signal initiating this response really is a generalized disruption of the P. aeruginosa membrane. Accordingly, we asked whether polymyxin B—an antibiotic known to disrupt Gram-negative bacterial membranes by binding the lipid A component of lipopolysaccharides (24–26)—could induce T6SS activity in P. aeruginosa. We used a P. aeruginosa strain carrying a ClpV1-GFP and fluorescent time-lapse microscopy to monitor T6SS organelle formation and dynamics (9, 10) after exposure to polymyxin B. Cells exhibited a sixfold increase in the average number of visible ClpV1-GFP foci per cell within 90 s of being spotted onto agar pads containing 20 μg/mL of polymyxin B (Fig. 4, A and C, and movie S1). After this increase in T6SS activity, most ClpV1-GFP foci disappeared over the next 3 min, with the remaining foci becoming nondynamic (Fig. 4A and movie S1). The loss of dynamics likely reflects consumption of intracellular adenosine 5’-triphosphate pools after prolonged exposure to polymyxin B intoxication. This increase in T6SS activity was not observed when cells were spotted onto agar pads lacking polymyxin B (Fig. 4, A and D, and movie S1). Additionally, this increase in ClpV1-GFP foci was not observed in tagT mutants even in the presence of polymyxin B (Fig. 4, A, E, and F; and movie S2), suggesting that the same attack-sensing pathway that senses T4SS and T6SS attacks is responding to this antibiotic and mediates activation of the T6SS.

These studies support a model in which the donor-directed T6SS attack response in P. aeruginosa likely involves detection of perturbations in the cell envelope caused by the invasive components of the T4SS conjugation machinery. T6SS may represent a type of bacterial “innate immune system” that can detect and attack invading infectious elements not by recognizing their molecular patterns [such as nucleic acid sequences, as do the clustered regularly interspaced short palindromic repeat (CRISPR) elements (27, 28); or unmethylated DNA, as do restriction enzymes (29)] but rather by recognizing “transfer-associated patterns” (TAPs), including membrane disruptions that occur during interactions with other cells deploying T6SS and T4SS translocation machines. Broad-host-range conjugative elements represent infectious bacterial “diseases” that can cause metabolic stress on their newly acquired hosts and thus represent a fitness burden to bacterial populations unable to combat their acquisition. The donor-directed T6SS attack paradigm may represent a strategy for suppressing the movement of horizontally transferred genetic elements in bacterial populations regardless of their signature molecular patterns (such as nucleic acid chemical structures or primary sequences).

References and Notes

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Supplementary Materials www.sciencemag.org/content/342/6155/250/suppl/DC1 Materials and Methods Table S1 References (30, 31) Movies S1 and S2 25 July 2013; accepted 6 September 2013 10.1126/science.1243745

An Erythroid Enhancer of BCL11A Subject to Genetic Variation Determines Fetal Hemoglobin Level

Daniel E. Bauer,1,2,3 Sophia C. Kamran,3,4 Samuel Lessard,5 Yuan Xu,1,2,3 Yuko Fujiwara,1 Carrie Lin,1 Zhen Shao,1 Matthew C. Canver,1 Elenoec C. Smith,2 Luca Pinello,6 Peter J. Sabo,9,8 Jeff Vierstra,7,8 Richard A. Voit,9 Guo-Cheng Yuan,10 Matthew H. Porteus,9 John A. Stamatyannopoulos,7,8 Guillaume Lettre,7 Stuart H. Orkin1,2,3,4

Genome-wide association studies (GWAS) have ascertainment numerous trait-associated common genetic variants, frequently localized to regulatory DNA. We found that common genetic variation at BCL11A associated with fetal hemoglobin (HbF) level lies in noncoding sequences decorated by an erythroid enhancer chromatin signature. Fine-mapping uncovers a motif-disrupting common variant associated with reduced transcription factor (TF) binding, modestly diminished BCL11A expression, and elevated HbF. The surrounding sequences function in vivo as a developmental stage–specific, lineage-restricted enhancer. Genome engineering reveals the enhancer is required in erythroid but not B-lymphoid cells for BCL11A expression. These findings illustrate how GWAS may expose functional variants of modest impact within causal elements essential for appropriate gene expression. We propose the GWAS-marked BCL11A enhancer represents an attractive target for therapeutic genome engineering for the β-hemoglobinopathies.

Genome-wide association studies (GWAS) have identified numerous common single-nucleotide polymorphisms (SNPs) associated with human traits and diseases. However, advancing from genetic association to causal biological process has been challenging (1). Recent genome-scale chromatin mapping studies have highlighted the enrichment of GWAS variants in regulatory DNA elements, suggesting many causal variants may affect gene regulation (2–6). GWAS

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To further understand how common genetic variation affects BCL11A, HbF level, and β-globin disorder severity, we compared the distribution of the HbF-associated SNPs at BCL11A with deoxyribonuclease I (DNase I) sensitivity, which is an indicator of chromatin state suggestive of regulatory potential. In primary human erythroblasts, three peaks of DNase I hypersensitivity were observed in intron-2, adjacent to and overlapping the HbF-associated variants (Fig. 1A). We term these DNase I hypersensitive sites (DHSs) +62, +58, and +55 based on distance in kilobases from the transcription start site (TSS) of BCL11A. Brain and B-lymphocytes, two tissues that express high levels, and T-lymphocytes, which do not express BCL11A, showed distinct patterns of DNase I sensitivity at the BCL11A locus, with a paucity of hypersensitivity overlapping the trait-associated SNPs (Fig. 1A and fig. S1).

Chromatin immunoprecipitation sequencing (ChIP-seq) demonstrated histone modifications with an enhancer signature overlapping the trait-associated SNPs at BCL11A intron-2, including the presence of H3K4me1 and H3K27ac and absence of H3K4me3 and H3K27me3 marks (Fig. 1A and fig. S1). The major erythroid TFs GATA1 and TAL1 also occupy this enhancer region. ChIP–quantitative polymerase chain reaction (PCR) confirmed three discrete peaks of GATA1 and TAL1 binding within BCL11A intron-2, each falling within an erythroid DHS (Fig. 1B). A common feature of distal regulatory elements is long-range interaction with cognate promoters. We evaluated the interactions between the BCL11A promoter and fragments across 250 kb of the BCL11A locus using a chromosome conformation capture assay. The greatest promoter interaction was observed within the region of intron-2 containing the trait-associated SNPs (Fig. 1C).

We hypothesized that the causal trait-associated SNPs could function by modulating critical cis-regulatory elements. Therefore, we performed extensive genotyping of SNPs within the three erythroid DHSs +62, +58, and +55 in 1263 DNA samples from the Cooperative Study of SCD (CSSCD) (21). We used 1178 individuals and 38 SNPs for association testing (fig. S2A). Analysis of common variants [minor allele frequency (MAF) > 1%] revealed that rs1427407 in DHS +62 had the strongest association to HbF.

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1Division of Hematology/Oncology, Boston Children’s Hospital, Boston, MA 02115, USA. 2Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA. 3Harvard Medical School, Boston, MA 02115, USA. 4Howard Hughes Medical Institute, Boston, MA 02115, USA. 5Montreal Heart Institute, Montreal, Quebec H2X 3W1, Canada. 6Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA 02115, USA. 7Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA. 8Department of Medicine, University of Washington, Seattle, WA 98195, USA. 9Department of Pediatrics, Stanford University, Palo Alto, CA 94304, USA. 10Harvard Medical School, Boston, MA 02115, USA. *Corresponding author. E-mail: stuart_orkin@dfci.harvard.edu

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**Fig. 1. Chromatin state and TF occupancy at BCL11A.** (A) ChIP-seq from human erythroblasts with indicated antibodies. DNase I cleavage densities are from indicated human tissues. Three erythroid DHSs termed +62, +58, and +55 are based on distance in kilobases from BCL11A TSS. BCL11A transcription is from right to left. (B) ChIP–quantitative PCR from human erythroblasts at BCL11A intron-2. DHSs +62, +58, and +55 are boxed. Enrichment at negative (GAPDH and OCT4) and positive control (β-globin LCR HS3 and α-globin HS-40) loci are displayed. (C) Chromosome conformation capture in human erythroblasts using BCL11A promoter as anchor. Error bars indicate SD.
Fig. 2. Regulatory variants at BCL11A. (A) Genotype data obtained in 1178 individuals from CSSCD for 38 variants within BCL11A +62, +58, or +55 DHSs. Shown are most highly significant associations to HbF level among common (MAF > 1%) SNPs (n = 10 variants) before (rs1427407) or after (rs7606173) conditional analysis on rs1427407. SNP coordinates are chromosome 2, build hg19. (B) Chromatin from erythroblasts of individuals heterozygous for rs1427407, immunoprecipitated by GATA1 or TAL1 and pyrosequenced to quantify the relative abundance of the rs1427407-G allele. Composite half E-box–GATA motif previously identified (23) is shown. (C) gDNA and cDNA from erythroblasts of individuals heterozygous for rs1427407, rs7606173, and rs7569946. Haplotyping demonstrated rs7569946-G, rs1427407-G, and rs7606173-C on the same chromosome in each. Pyrosequencing was performed to quantify the relative abundance of the rs7569946-G allele.

Fig. 3. The GWAS-marked BCL11A enhancer is sufficient for adult-stage erythroid expression. (A) A 12.4-kb fragment of BCL11A intron-2 ( +52.0 to +64.4 kb from TSS) was cloned to a lacZ reporter construct. Shown is a transient transgenic mouse embryo from 12.5 dpc X-gal stained. Arrowhead indicates liver. (B) Cell suspensions isolated from peripheral blood (PB) and fetal liver (FL) of stable transgenic embryos at 12.5 dpc X-gal stained. (C) Sorted erythroblasts and B-lymphocytes from young adult stable transgenic mice subject to X-gal staining or RNA isolation followed by quantitative reverse transcription (RT)–PCR. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed relative to T-lymphocytes. Error bars indicate SD.
level ($P = 7.23 \times 10^{-50}$) (Fig. 2A, fig. S2B, and supplementary text). We identified associations to HbF level within the three DHSs that remained after conditioning on rs1427407 (Fig. 2A and fig. S2B), which is consistent with the hypothesis that multiple functional SNPs within the composite enhancer act combinatorially to influence BCL11A regulation. The most significant residual association was for rs7606173 in DHS +55 ($P = 9.66 \times 10^{-11}$).

The SNP rs1427407 falls within a peak of GATA1 and TAL1 binding (Fig. 1, A and B). The minor T-allele disrupts the G-nucleotide of a sequence element resembling a half E-box/GATA composite motif [CTG($n_0$)GATA], a consensus sequence enriched for chromatin bound by GATA1 and TAL1 complexes in erythroid cells (22, 23). We identified five primary erythroblast samples from individuals heterozygous for the major G-allele and minor T-allele at rs1427407 and subjected these samples to ChIP followed by pyrosequencing. As anticipated, we observed an even balance of alleles in the input DNA. However, we detected more frequent binding to the G-allele as compared with the T-allele in both the GATA1 and TAL1 immunoprecipitated chromatin samples (Fig. 2B).

Because the common synonymous SNP rs7569946 lies within exon-4 of BCL11A, it can be used to discriminate expression of alleles. We identified three primary erythroblast samples doubly heterozygous for the rs1427407–rs7606173 haplotype and rs7569946. For each sample, we determined by means of molecular haplotypeing that the major rs7569946 G-allele was in phase with the low-HbF–associated rs1427407–rs7606173 G–C haplotype (table S4) (24, 25). Pyrosequencing revealed that whereas the alleles were balanced in genomic DNA (gDNA), significant imbalance was observed in complementary DNA (cDNA) with 1.7-fold increased expression of the low-HbF–linked G-allele of rs7569946 (Fig. 2C and supplementary text).

To understand the context within which these apparent regulatory trait-associated SNPs play their role, we explored the function of the harboring composite element. We cloned a 12.4-kb (+52.0 to 64.4 kb from TSS) human gDNA fragment containing the three erythroid DHSs in order to assay enhancer potential in a mouse transgenic lacZ reporter assay (fig. S4). Endogenous BCL11A shows abundant expression throughout the developing central nervous system, with much lower expression observed in the fetal liver (26). In contrast, we observed in the transgenic embryos LacZ expression largely confined to the fetal liver, the site of definitive erythropoiesis, with weaker expression noted in the central nervous system (Fig. 3A).

A characteristic feature of globin gene and BCL11A expression is developmental regulation (supplementary text). In stable transgenic BCL11A +52.0- to 64.4-kb reporter lines at 12.5 days post coitum (dpc), circulating primitive erythrocytes failed to stain for X-gal, whereas definitive erythroblasts in fetal liver robustly stained positive (Fig. 3B). Endogenous BCL11A was expressed at 10.4-fold–higher levels in B-lymphocytes as compared with erythroblasts. LacZ expression was restricted to erythroblasts and not observed in B-lymphocytes (Fig. 3C). These results indicate that the GWAS-marked BCL11A intron-2 regulatory sequences are sufficient to specify developmentally restricted, erythroid-specific gene expression.

We aimed to disrupt the enhancer to investigate its requirement for BCL11A expression. Because there are no suitable adult-stage human erythroid cell lines, we turned to the mouse erythroleukemia (MEL) cell line. We observed an orthologous enhancer signature at intron-2 of mouse Bcl11a (27). Sequence-specific nucleases can produce small chromosomal deletions via nonhomologous end joining (NHEJ)–mediated repair (28). We engineered transcription activator-like effector nucleases (TALENs) to introduce double-strand breaks to flank the orthologous 10-kb Bcl11a intron-2 sequences carrying the erythroid enhancer chromatin signature (fig. S7A). Three different clones were isolated that had undergone biallelic excision of the intronic segment (figs. S7 and S8 and supplementary text). BCL11A transcript was profoundly reduced in the absence of the orthologous erythroid composite enhancer (Fig. 4A). BCL11A protein expression was not detectable in the enhancer-deleted clones (Fig. 4B). In the absence of the BCL11A enhancer, embryonic globin gene derepression was pronounced, with the ratio of embryonic ε to adult β/γ globin increased by a mean of 364-fold (fig. S9).

To examine potential lineage-restriction of the requirement for the +50.4- to 60.4-kb intronic sequences for BCL11A expression, we evaluated their loss in a nonerythroid context. The same strategy of introduction of two pairs of TALENs to obtain clones with NHEJ-mediated deletion was used in a pre-B lymphocyte cell line. In contrast to the erythroid cells, BCL11A expression was retained in the Δ50.4- to 60.4-kb enhancer deleted pre-B cell clones at both the RNA and protein levels (Fig. 4, A and B). These results indicate that the orthologous erythroid enhancer sequences are essential for erythroid gene expression but are not required in B-lymphoid cells for integrity of transcription from the Bcl11a locus.

The prior identification of BCL11A as a critical repressor of HbF levels has raised new hope for mechanism-based therapeutic approaches to the β-hemoglobinopathies (29). However, the paradox that genetic variation at BCL11A is common, well-tolerated, and disease-protective despite the critical roles of BCL11A in neurogenesis and lymphopoiesis (19, 20, 30) has remained unsolved. We have demonstrated that the HbF-associated variants localize to an erythroid enhancer of BCL11A. Through allele-specific analyses, we show that

![Fig. 4](https://www.science.org)
genetic variation within this enhancer is associated with modest impact on TF binding, BCL11A expression, and HbF level. Relatively small effect sizes associated with individual variants may not be surprising given that most single-nucleotide substitutions, even within critical motifs, result in only modest loss of enhancer activity (31, 32). In contrast, loss of the BCL11A enhancer results in the absence of BCL11A expression in the erythroid lineage. Most trait-associated SNPs identified by GWAS are noncoding and have small effect sizes (I, 33). The impact of GWAS-identified SNPs on biological processes is often uncertain. Our findings underscore how a modest influence engendered by an individual noncoding variant neither predicts nor precludes a profound contribution of an underlying regulatory element.

Challenges to inhibiting BCL11A for mechanism-based reactivation of HbF include the supposedly “undruggable” nature of transcription factors (34) and its important nonerythroid functions (20, 30). With recent developments in their efficiency and precision, sequence-specific nucleases can be designed to exquisitely target genomic sequences of interest (35–37). We propose the GWAS-identified enhancer of BCL11A as a particularly promising therapeutic target for genome engineering in the β-hemoglobinopathies. Disruption of this enhancer would impair BCL11A expression in erythroid precursors with resultant HbF derepression while sparing BCL11A expression in nonerythroid lineages. Rational intervention might mimic common protective genetic variation.

References and Notes

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Supplementary Materials
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Ancient DNA Reveals Key Stages in the Formation of Central European Mitochondrial Genetic Diversity

Guido Brandt,*† Wolfgang Haak,**† Christina J. Adler,† Anna Szécsényi-Nagy,† Sarah Karminna,† Sabine Möller-Rieke,† Harald Meller,† Robert Gansmeier,† Susanne Friederich,† Veit Dresey,† Nicole Nicklisch,† Joseph K. Pickrell,‡ Frank Sirocco,§ David Reich,§ Alan Cooper,‡† Kurt W. Alt,‡† The Genographic Consortium§

The processes that shaped modern European mitochondrial DNA (mtDNA) variation remain unclear. The initial peopling by Palaeolithic hunter-gatherers ~42,000 years ago and the immigration of Neolithic farmers into Europe ~8000 years ago appear to have played important roles but do not explain present-day mtDNA diversity. We generated mtDNA profiles of 364 individuals from prehistoric cultures in Central Europe to perform a chronological study, spanning the Early Neolithic to the Early Bronze Age (5500 to 1550 calibrated years before the common era). We used this transect through time to identify four marked shifts in genetic composition during the Neolithic period, revealing a key role for Late Neolithic cultures in shaping modern Central European genetic diversity.

T he Central European Neolithic and the subsequent Early Bronze Age (EBA) reflect periods of momentous cultural changes (1–4). However, the extent to which such prehistoric cultural changes were accompanied by differences in the underlying genetics of local populations (1–5) and how such population shifts contributed to the present-day genetic diversity of Central Europe (6–9) are yet to be understood. Ancient DNA studies have revealed genetic discontinuities between indigenous hunter-gatherers and early farmers and between the latter and present-day Europeans (10, 11). Although this confirms the importance of genetic shifts after the arrival of farming, the number and sequence of events and their potential origins and contributions to the genetic composition of modern-day Central Europe remain unclear (5, 6, 12).

We collected samples from 25 sites of the Mittelelle-Saale region in Saxony-Anhalt, Germany, attributed to nine archaeological cultures of the Early, Middle, and Late Neolithic period and the EBA, spanning ~4000 years (Fig. 1A, figs. S1 and S2, and table S1) (13). Mittelelle-Saale played a key role in human prehistory in Central Europe (4, 13), and the continuous settlement activity
An Erythroid Enhancer of *BCL11A* Subject to Genetic Variation Determines Fetal Hemoglobin Level


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*BCL11A* Variants

Recent chromatin mapping data have suggested that trait-associated variants often mark regulatory DNA. However, there has been little rigorous experimental investigation of regulatory variation. Bauer *et al.* (p. 253; see the Perspective by Hardison and Blobel) performed an in-depth study of the *BCL11A* fetal hemoglobin-associated locus. The trait-associated variants revealed a chromatin signature that enhanced erythroid development. The enhancer was required for erythroid expression of *BCL11A* and thus for globin gene expression.

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