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Science **322**, 1839 (2008);
DOI: 10.1126/science.1165409

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26. This work was partly supported by an Action Concertée Incitative "Jeunes Chercheuses et Jeunes Chercheurs" and the trilateral Génoplante GENOSOME program TRIL-046. T.B. was supported by a Ph.D. fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche, and A.P. was supported by the Fundacíon Española para la Ciencia y la Tecnología (Ministerio de Ciencia e Innovacíon) and the Région Ile-de-France. M.T. is supported by the UK Biotechnology and Biological Sciences Research Council and the Gatsby Charitable Foundation and is the recipient of a European Molecular Biology Organization young investigator award and a Royal Society Wolfson Merit award. A.H. is a recipient of a Royal Society Research Fellowship. The authors thank S. Biemelt, D. Zamir, C. Rameau, J. Hofer, B. Letarnec, C. Navarro, E. Kramer, and the Tomato Genetics Resource Centre for providing material; the Cell Biology Laboratory gardener team for their plant care; J. D. Faure, H. Höfte, J. Hofer, A. Mallory, G. Mouille, J. C. Palauqui, F. Parcy, and C. Rameau for their comments on the manuscript; and N. Ori for discussions and sharing results before publication. Sequences have been deposited in GenBank, accessions FJ435156 to FJ435166.

Supporting Online Material
www.sciencemag.org/cgi/content/full/322/5909/1835/DC1
Materials and Methods
Figs. S1 to S10
Tables S1 to S7
References

19 September 2008; accepted 12 November 2008
10.1126/science.1166168

Human Fetal Hemoglobin Expression Is Regulated by the Developmental Stage-Specific Repressor *BCL11A*

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Differences in the amount of fetal hemoglobin (HbF) that persists into adulthood affect the severity of sickle cell disease and the β-thalassemia syndromes. Genetic association studies have identified sequence variants in the gene *BCL11A* that influence HbF levels. Here, we examine *BCL11A* as a potential regulator of HbF expression. The high-HbF *BCL11A* genotype is associated with reduced *BCL11A* expression. Moreover, abundant expression of full-length forms of *BCL11A* is developmentally restricted to adult erythroid cells. Down-regulation of *BCL11A* expression in primary adult erythroid cells leads to robust HbF expression. Consistent with a direct role of *BCL11A* in globin gene regulation, we find that *BCL11A* occupies several discrete sites in the β-globin gene cluster. *BCL11A* emerges as a therapeutic target for reactivation of HbF in β-hemoglobin disorders.

Genome-wide association studies have yielded insights into the genetics of complex diseases and traits (*1*, *2*). In the majority of instances, the functional link between a genetic association and the underlying pathophysiology remains obscure. The level of fetal hemoglobin (HbF) is inherited as a quantitative trait and is of enormous clinical relevance, given its role in ameliorating the severity of the principal hemoglobin disorders, sickle cell disease and β-thalassemia (*3*, *4*). Two recent genome-wide association studies have identified three major loci containing a set of five common single-nucleotide polymorphisms (SNPs) that account for ~20% of the variation in HbF levels (*5*–*7*). Moreover, several of these variants predict the clinical severity of sickle cell disease (*5*), and at least one of these SNPs may also affect clinical outcome in β-thalassemia (*6*). The SNP with the largest effect size is located in the second intron of a gene on chromosome 2, *BCL11A*. Although *BCL11A* has been investigated in the context of lymphocyte development (*8*, *9*), its role in the red blood cell lineage has not been previously assessed.

HbF is a tetramer of two adult α-globin polypeptides and two fetal β-like γ-globin polypeptides. During gestation, the duplicated γ-globin genes constitute the predominant genes transcribed in the β-globin cluster. After birth, γ-globin is replaced by adult β-globin (*4*), a process referred to as the "fetal switch." The molecular mechanisms responsible for this switch have remained largely undefined. Moreover, the extent to which γ-globin gene expression is silenced in adulthood varies among individuals (*5*, *6*). In nonanemic individuals, HbF makes up <1% of total hemoglobin. However, in those with sickle cell disease and β-thalassemia, higher levels of γ-globin expression partially compensate for defective or impaired β-globin gene production, which ameliorates the clinical severity in these diseases. The results of recent genetic association studies provide candidate genes to test for involvement in control of the γ-globin genes. In light of the strong association of SNPs within the *BCL11A* locus with HbF levels in disparate populations (*5*–*7*, *10*), we explore here the hypothesis that the product of the *BCL11A* locus, a multi–zinc finger transcription factor, encodes a stage-specific regulator of HbF expression.

As a first step in seeking how variation at the *BCL11A* locus might relate to γ-globin expression, we examined expression of *BCL11A* in erythroid cells (*11*). In primary adult human erythroid cells, BCL11A is expressed as two major isoforms at the protein and RNA levels (Fig. 1A). These isoforms (designated XL and L) differ only in usage of the 3′ terminal exon and function similarly in other settings (*9*). We have recently fine-mapped the *BCL11A*-HbF association signal to a variant in close linkage disequilibrium (LD) with the SNP rs4671393 (*5*). Because this association has been confirmed in multiple independent European and African diasporic populations, we examined expression of the XL and L isoforms of *BCL11A* as a function of the genotype at rs4671393 in lymphoblastoid cell lines from the HapMap European (CEU) and African (YRI) groups. The utility of this strategy has been shown in prior studies examining the consequences of common genetic variation on gene expression (*12*–*14*). We observed a striking difference in expression for both isoforms between individuals of different SNP genotypes (Fig. 1B). Cells homozygous for the "high-HbF" allele expressed a lower level of *BCL11A* transcripts than those homozygous for the "low-HbF" allele or heterozygous for both alleles. The difference in expression between the "high" and "low" HbF–associated *BCL11A* alleles is 3.5-fold. Hence, relatively modest differences in *BCL11A* expression appear to be associated with changes in HbF expression.

To our surprise, we observed that the embryonic erythroleukemia cell line K562 expressed very little, if any, of the XL and L isoforms but, instead, expressed shorter variant proteins (Fig. 1C). To assess whether the difference between adult erythroblasts and K562 cells reflected developmental stage–specific control of *BCL11A* or the malignant nature of these cells, we examined stage-matched, CD71$^+$/CD235$^+$ erythroblasts isolated from adult bone marrow, second-trimester fetal liver (FL), and circulating first-trimester primitive cells. FL and primitive erythroblasts, which both robustly express γ-globin (*15*), expressed predominantly shorter BCL11A variants (Fig. 1C). Although we continue to investigate the structure of these variant proteins, our findings indicate that the *BCL11A* locus is developmentally regulated, such that full-length XL and L isoforms are expressed almost exclusively in adult-stage erythroblasts. Independently, the genetic data strongly argue that the level of XL and L isoforms is influenced by sequence variants in the *BCL11A* gene.

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To better understand potential mechanisms through which BCL11A might act in erythroid cells, we characterized interacting proteins. We affinity-tagged versions of BCL11A in mouse erythroleukemia (MEL) cells, adult-type erythroid cells that express exclusively adult globins (*16*) (Fig. 2A). We observed no major global transcriptional changes by microarray analysis when tagged versions of BCL11A were expressed in these cells (fig. S1). After affinity purification of protein complexes containing tagged BCL11A and mass spectrometric peptide sequencing, we identified numerous peptides of BCL11A (*9*), as well as all components of the nucleosome remodeling and histone deacetylase (NuRD)–repressive complex (Fig. 2B). The strong association between BCL11A and the NuRD complex in erythroid cells is consistent with prior observations in B cells and of the homolog BCL11B in T cells (*17*), as well as the presence of an N-terminal motif that recruits the NuRD complex (fig. S2) (*18*, *19*). We also found that the nuclear matrix protein, matrin-3 (*20*), consistently copurified with BCL11A, which may account in part for the localization of BCL11A to the nuclear matrix (*9*) (Fig. 2B). Prior work has shown that the β-globin locus is closely associated with the nuclear matrix until later stages of erythropoeisis, when high-level globin gene transcription occurs (*21*).

Additionally, BCL11A complexes contain peptides derived from GATA-1, the principal erythroid transcription factor (*22*) (Fig. 2B). By immunoprecipitation (IP), we confirmed that GATA-1 specifically associates with BCL11A in erythroid cells (Fig. 2C). Moreover, we found that the GATA-1 cofactor FOG-1 (*23*) specifically associates with BCL11A and confirmed the interaction with NuRD components in erythroid cells (Fig. 2C). Prior work has shown that FOG-1 binds to the NuRD complex (*19*), and our results suggest that BCL11A may synergize with this interaction in the context of specific loci. From size fractionation of erythroid nuclear extracts, we observed considerable overlap between NuRD components and BCL11A in megadalton protein complexes, with less extensive overlap with GATA-1 and FOG-1 (fig. S3). It is possible that only a minor fraction of these factors are bound within the BCL11A and NuRD complexes. Alternatively, in vivo association might be greater, but dissociation of the components of protein complexes occurs during gel filtration. GATA-1 and FOG-1 immunoprecipitated with BCL11A when expressed exogenously in nonerythroid cells, which suggests that these proteins directly interact (Fig. 2, D and E). We used this approach to map the determinants mediating association of GATA-1 with BCL11A to the zinc fingers of GATA-1 (Fig. 2F). Together, the proteomic data indicate that BCL11A binds the NuRD complex along with GATA-1 and FOG-1 in erythroid cells. These associated factors are likely to be critical for the action of BCL11A as a transcriptional repressor in erythroid cells.

Genetic, developmental, and biochemical approaches support a potential role for *BCL11A* in γ-globin gene silencing. To test this hypothesis, we modulated the level of *BCL11A* in primary human erythroid cells. As a cellular system in which to perform experiments, we expanded and differentiated erythroid precursors from purified $CD34^+$ human hematopoietic progenitors. We began by examining the effect of transient introduction of small interfering RNAs (siRNAs) that target *BCL11A* mRNA. When siRNAs were introduced into erythroid progenitors at day 0 of differentiation, 40 to 45% depletion (knockdown) of *BCL11A* mRNA levels was achieved, as assessed on day 4 of differentiation. With this knockdown, we observed a 2.3-fold increase in the level of γ-globin RNA (compared with total β-like globin RNA) on day 7 of differentiation (Fig. 3A). We found that, as these siRNAs were introduced at later time points during erythroid differentiation, induction of the γ-globin gene was observed to be less (with 1.7- and 1.4-fold average γ-globin induction seen by adding siRNAs on days 1 and 2 of differentiation). The results we observed from siRNA knockdown of *BCL11A* could be due to a broad effect on the cellular differentiation state, which has been shown to alter γ-globin expression (*3*) or could reflect more direct action at a limited number of targets, including the γ-globin gene. To distinguish between these possibilities, we performed microarray expression profiling of the cells after knockdown of *BCL11A* and subsequent differentiation. The transcriptional profiles of genes in the quantitative range of the array (which excluded the globins) were remarkably similar between cells on day 7 after treatment with *BCL11A* siRNAs and non-targeting (NT) siRNAs on day 0, with a correlation coefficient (r^2) of 0.9901 for the $\log_2$ normalized intensities (Fig. 3B). The expression of the well-characterized transcriptional regulators of the globin genes, GATA-1, FOG-1, NF-E2, and EKLF (*4*), in this microarray data set, was also comparable between the groups (with all *P* values > 0.05 and average fold changes of <1.1-fold). This observation suggests that the effect of *BCL11A* on γ-globin gene regulation is unlikely to be mediated via an effect on the expression of one of these previously characterized transcriptional regulators. Additionally, the morphology of these two groups of cells was indistinguishable throughout differentiation. Together, these results suggest that knockdown of *BCL11A* affects γ-globin expression without causing global changes in the differentiation state of the cells.

To examine the effects of more persistent reduction in *BCL11A* expression, we utilized lentiviral short hairpin RNA (shRNA)–mediated knockdown of *BCL11A* expression with drug selection of transduced cells (*24*). We chose two independent shRNA constructs. When cells were infected with the two *BCL11A* shRNA lentiviruses and selection was imposed on the initiation of differentiation, we

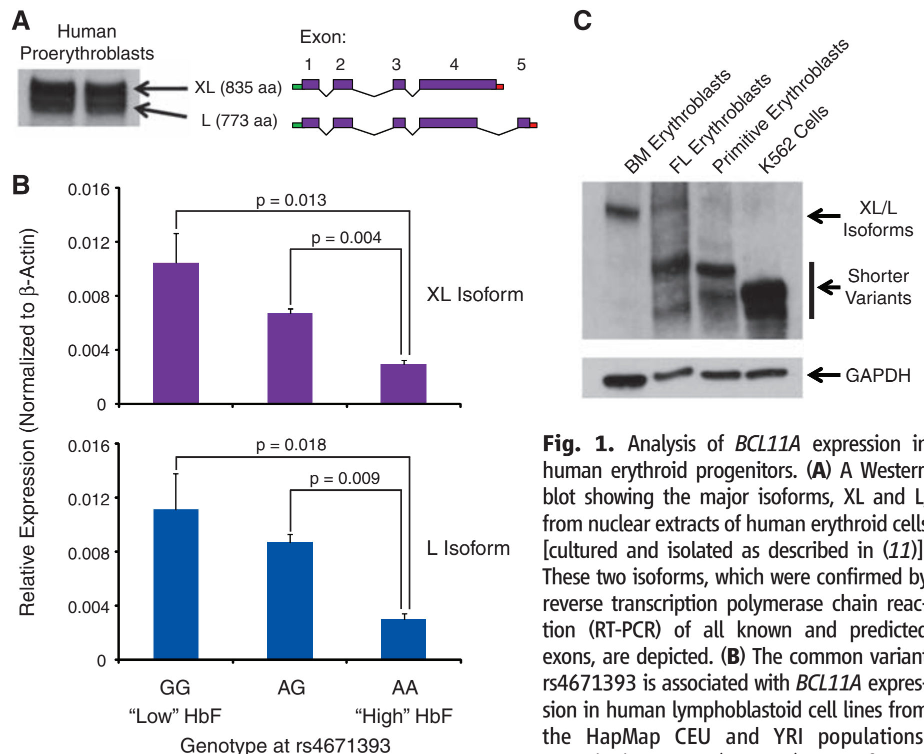

Fig. 1. Analysis of *BCL11A* expression in human erythroid progenitors. (**A**) A Western blot showing the major isoforms, XL and L, from nuclear extracts of human erythroid cells [cultured and isolated as described in (*11*)]. These two isoforms, which were confirmed by reverse transcription polymerase chain reaction (RT-PCR) of all known and predicted exons, are depicted. (**B**) The common variant rs4671393 is associated with *BCL11A* expression in human lymphoblastoid cell lines from the HapMap CEU and YRI populations. Quantitative RT-PCR (qRT-PCR) was performed on RNA from these cell lines and normalized to the level of human β-actin (5, 3, and 3, from left to right). Results are means ± SEM. Significance of differences between genotypes was calculated using the Student's *t* test. (**C**) Western blots of lysates of primary human bone marrow (BM) erythroblasts, second-trimester FL erythroblasts, first-trimester circulating primitive erythroblasts, and K562 cells. Primary human stage–matched erythroblasts were isolated by sorting for the CD235 and CD71 double-positive population. The XL and L bands migrate together here as a result of reduced separation on this blot.

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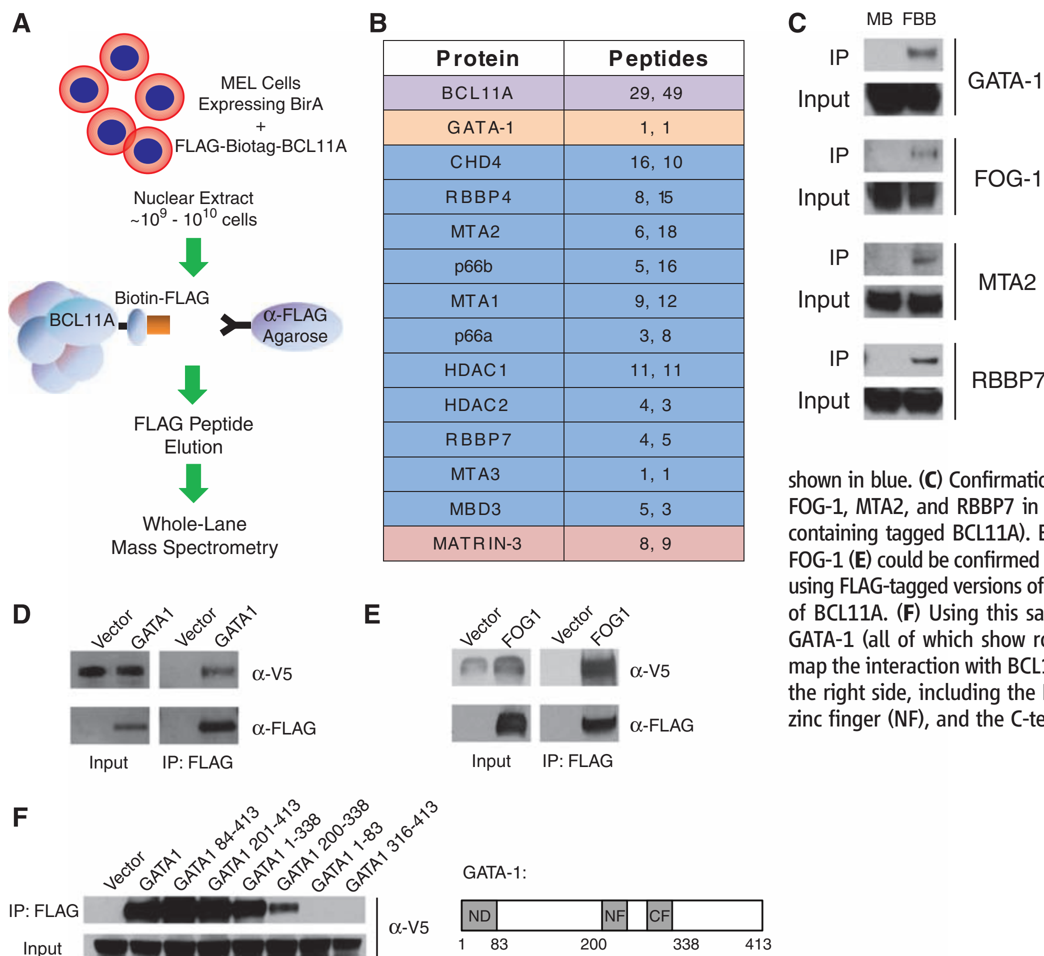
observed an average of 97 and 60% knockdown of BCL11A at the protein level by day 5 of erythroid differentiation (Fig. 3C). No morphological differences between the groups of cells were noted during differentiation, again suggesting that *BCL11A* knockdown did not perturb overall erythroid differentiation (Fig. 3D). The level of γ-globin at day 7 of differentiation was dramatically elevated by 6.5- and 3.5-fold (from an average of 7.4 % in the control to 46.8 and 26%) for the two shRNAs as compared with the control infected cells (Fig. 3E). This robust effect is likely to be the result of elimination of nontransduced cells, as well as the continuous expression of the shRNAs after viral transduction. Induction of γ-globin RNA was accompanied by corresponding levels of mature HbF, as shown by hemoglobin high-performance liquid chromatography (HPLC) and electrophoresis (Fig. 3F and fig. S4). The HPLC revealed that a substantial fraction of the mature hemoglobin in these cells was HbF (with an average level of 35.9 and 23.6%, compared with undetectable levels in the control). On the basis of the differing extents of knockdown of *BCL11A* in the siRNA and shRNA experiments and the concomitant degree of γ-globin induction observed, it appears that BCL11A functions as a molecular rheostat to control silencing of the γ-globin genes.

In principle, *BCL11A* might influence globin gene expression either directly by interacting with cis-regulatory elements within the β-globin cluster or indirectly by affecting cell cycle or other pathways that ultimately impinge on HbF expression. To discriminate between these possibilities, we used chromatin immunoprecipitation (ChIP) in primary human erythroid progenitors. Occupancy of neither the γ- nor β-globin proximal promoters was detected. Rather, we observed robust binding in several other regions of the β-globin cluster (Fig. 4). These include the third hypersensitivity site (HS3) of the locus control region (LCR) (*25*), the region of the high HbF–associated Corfu deletion upstream of the δ-globin gene (*4*), and another region downstream of the Aγ-globin gene that is commonly deleted in certain forms of hereditary persistence of fetal hemoglobin (*4*). Of particular note, all of these cis elements have been suggested to play a role in γ-globin silencing. Our results strongly argue that *BCL11A* acts within the β-globin cluster. A fuller accounting of mechanism will necessitate comprehensive analysis of chromatin occupancy of BCL11A and partner proteins in the β-globin cluster. We speculate that the shorter BCL11A variants present in cells that actively express γ-globin may participate in other aspects of transcriptional regulation within the β-globin cluster. As such, we propose that *BCL11A*, at different levels and in its variant forms, reconfigures the β-globin locus at different development stages.

The molecular studies of globin switching during ontogeny serve as a paradigm for the developmental control of mammalian genes. Our results indicate that *BCL11A* is itself a developmentally regulated and critical modulator of this process. We have shown that silencing of γ-globin gene expression in primary adult human erythroid cells depends on the presence of full-length (XL and L isoforms) *BCL11A*. Our protein data suggest that BCL11A functions in concert with the NuRD-repressor complex, GATA-1, and FOG-1. Of note, inhibitors of histone deacetylases (HDACs) induce some HbF in patients with hemoglobin disorders (*26*). HDAC1 and HDAC2 are both core components of the NuRD complex, and its association with BCL11A suggests that this complex may be the molecular target of these therapies. On the basis of findings of human genetics studies, we postulate that directed downregulation of *BCL11A* in patients would elevate HbF levels and ameliorate the severity of the major β-hemoglobin disorders (*5*–*7*). As a stage-specific component involved in the silencing of γ-globin expression, *BCL11A*, therefore, emerges as a new therapeutic target for reactivation of HbF in sickle cell disease and the β-thalassemias.



Protein	Peptides
BCL11A	29, 49
GATA-1	1, 1
CHD4	16, 10
RBBP4	8, 15
MTA2	6, 18
p66b	5, 16
MTA1	9, 12
p66a	3, 8
HDAC1	11, 11
HDAC2	4, 3
RBBP7	4, 5
MTA3	1, 1
MBD3	5, 3
MATRIN-3	8, 9

Fig. 2. Proteomic affinity screen identifies BCL11A partner proteins in erythroid cells. (**A**) The scheme used for the affinity-purification in mouse erythroleukemia (MEL) cells is depicted in this diagram. Once FLAG peptide elution was performed, whole-lane mass spectrometry from acrylamide gels was done as described in (*11*). We used a subtractive approach, including a simultaneous pull-down in parental Mel-BirA (MB) cells. (**B**) The results of this subtractive screen are shown with the number of peptides obtained in each trial listed adjacent to the identified protein. The components of the NuRD complex are shown in blue. (**C**) Confirmation of interactions of BCL11A with GATA-1, FOG-1, MTA2, and RBBP7 in erythroid (MEL) cells (FBB is the cell line containing tagged BCL11A). BCL11A interactions with GATA-1 (**D**) and FOG-1 (**E**) could be confirmed by exogenous expression in COS-7 cells by using FLAG-tagged versions of GATA-1 or FOG-1 and V5-tagged versions of BCL11A. (**F**) Using this same strategy in 293T cells, fragments of GATA-1 (all of which show robust expression here) could be used to map the interaction with BCL11A. The structure of GATA-1 is shown on the right side, including the N-terminal domain (ND), the N-terminal zinc finger (NF), and the C-terminal zinc finger (CF).

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Fig. 3. *BCL11A* acts as a silencer of γ-globin gene expression, based on modulation of *BCL11A* levels. (**A**) siRNA-mediated knockdown of *BCL11A* results in elevations of γ-globin mRNA levels (as a percentage of total β-like globin gene expression) in human erythroid progenitor cells at day 7 of differentiation in comparison with NT control siRNAs. (**B**) Microarray profiling of these cells using the Affymetrix U133 Plus 2.0 array reveals that there is close similarity in the expression profile of NT and *BCL11A* siRNA-treated cells (r^2 = 0.9901). Microarray data processing and filtering were performed as described previously (*27*) and in (*11*). (**C**) Lentiviral-mediated shRNA delivery to human erythroid progenitors results in robust knockdown of BCL11A protein. Control samples were infected with lentivirus prepared from the backbone pLKO.1ps vector. (**D**) At day 6 of differentiation, the cells appear to be morphologically indistinguishable; this is also the case at other stages of differentiation. (**E**) The shRNA-mediated knockdown of *BCL11A* results in robust induction of γ-globin mRNA level on day 7 of differentiation (***$P < 10^{-5}$ in comparison with control). (**F**) Hemolysates prepared from cells on day 12 of differentiation show the presence of mature HbF by hemoglobin HPLC. The HbF peaks are labeled with an arrow in each chromatogram, with the first peak corresponding to acetylated HbF (*28*) and the second unmodified HbF. All results are means ± SEM. Statistical significance was calculated using the Student's *t* test.

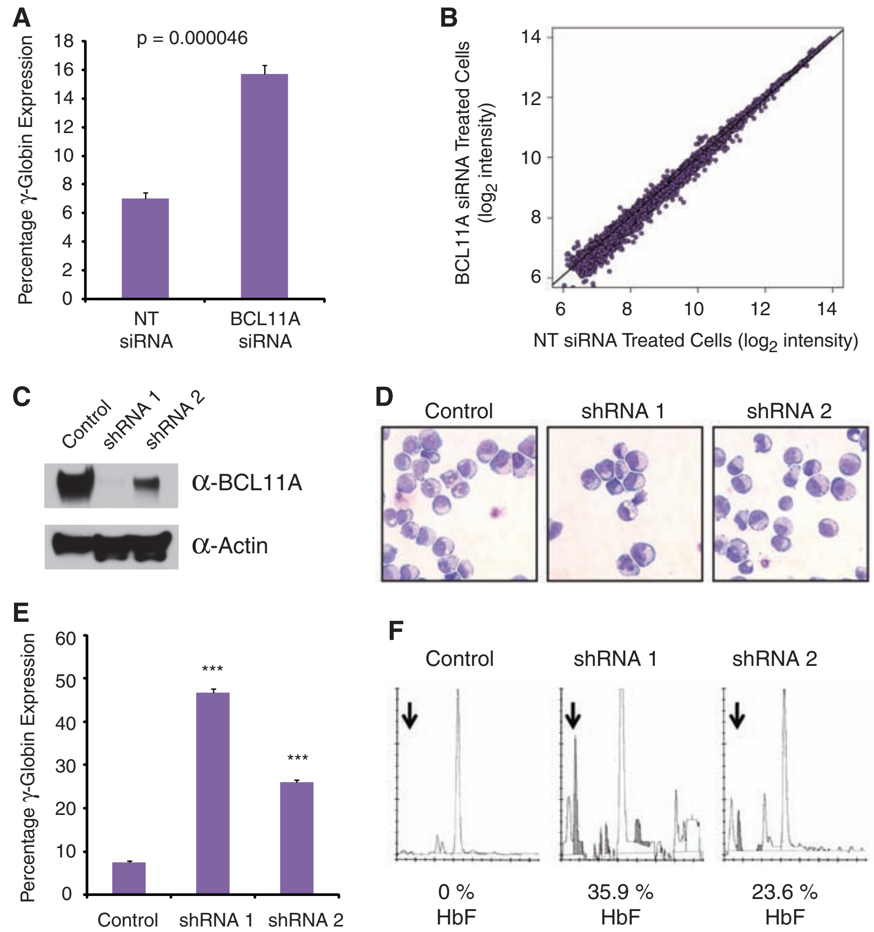
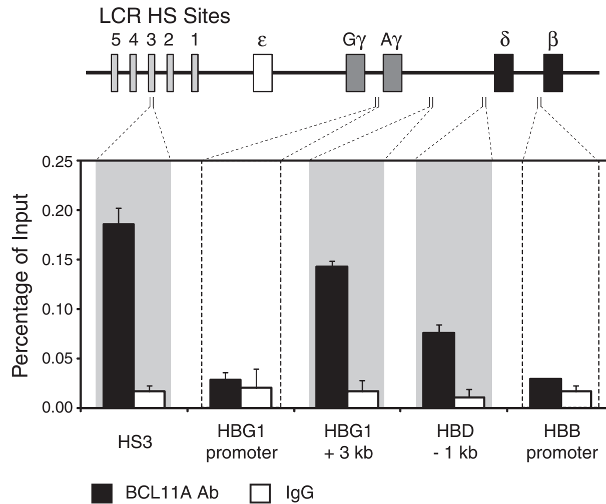


Fig. 4. *BCL11A* occupies discrete regions in the human β-globin locus in adult erythroid progenitors. The human β-globin locus is depicted at the top with regions showing significant binding shaded in gray in the histogram plot below. The results are means ± SD (*n* = 3 per group).



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29. We thank J. Snow, P. Tucker, G. Ippolito, B. Lee, M. Leid, T. Nakamura, D. Altshuler, E. Choy, and S. Bonakdar for providing reagents and suggestions. We are grateful to D. Nathan and A. Michelson for critical comments; D. Shao, N. Yudanin, and A. Soriano for experimental assistance; and C. Chen and V. Sankaran for assistance with data analysis. This work was supported by grants from the National Heart, Lung, and Blood Institute, NIH (HL32262-26 and HL32259-27), a Center of Excellence in Molecular Hematology Award from the National Institute of Digestive and Kidney Diseases, NIH, and the Howard Hughes Medical Institute. T.F.M. was supported by the Leukaemia Research and Kay Kendall Leukaemia Funds. V.G.S. was supported by a Medical Scientist Training Program grant from the NIH. Array datasets are deposited at Gene Expression Omnibus (accession no. GSE 13285). A patent application related to this work was filed, and V.G.S. and S.H.O. are inventors on this patent.

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Figs. S1 to S4
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3 September 2008; accepted 12 November 2008
Published online 4 December 2008;
10.1126/science.1165409
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