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Transcriptional environment and chromatin architecture interplay dictates globin expression patterns of heterospecific hybrids derived from undifferentiated human embryonic stem cells or from their erythroid progeny

Kai-Hsin Chang[1], **Andy Huang**[1], **Hemei Han**[2], **Yi Jiang**[1], **Xiangdong Fang**[2,3], **Chao-Zhong Song**[2], **Steve Padilla**[1], **Hao Wang**[4], **Hongzhu Qu**[3,4], **John Stamatoyannopoulos**[4], **Qiliang Li**[2], and **Thalia Papayannopoulou**[1,*]

[1]Department of Medicine, Division of Hematology, University of Washington, Seattle, WA, 98195, USA

[2]Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA 98195, USA

[3]Laboratory of Disease Genomics and Individualized Medicine, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100029, China

[4]Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA

Abstract

To explore the response of β globin locus with established chromatin domains upon their exposure to new transcriptional environments, we transferred the chromatin-packaged β globin locus of undifferentiated hESCs or hESC-derived erythroblasts into an adult transcriptional environment. Distinct globin expression patterns were observed. In hESC-derived erythroblasts where both ε and γ globins were active and marked by similar chromatin modifications, ε globin was immediately silenced upon transfer, while γ globin continued to be expressed for months, implying that different transcriptional environments were required for their continuing expression. While β globin was silent both in hESCs and in hESC-derived erythroblasts, β globin was only activated upon transfer from hESCs, but not in the presence of dominant γ globin transferred from hESC-derived erythroblasts, confirming the competing nature of γ vs. β globin expression. With time, however, silencing of γ globin occurred in the adult transcriptional environment with concurrent activation of β-globin, accompanied by a drastic change in epigenetic landscape of γ and β globin gene regions without apparent changes in the transcriptional environment. This switching process could be manipulated by overexpression or down-regulation of certain transcription factors. Our studies provide important insights into the interplay between the transcription environment and existing chromatin domains, and offer an experimental system to study the time-dependent human globin switching.

*corresponding author: Dr. Thalia Papayannopoulou, M.D., Dr. Sci. thalp@u.washington.edu. Phone: 206-543-5756. 1705 NE Pacific St. Box 357710. University of Washington. Seattle, WA 98195.

Author Contribution

KHC, AH, HH, YJ, XF, CZS, SP, and HW performed experiments. KHC and TP designed experiments. JS supervised experiments. KHC, HQ and QL analyzed data. KHC, and TP wrote manuscript. The authors declare no competing financial interest.

Keywords

hemoglobin; chromatin; beta-globins; erythroblasts; gene expression

Introduction

Changes in globin gene expression during development are characteristic of all species that use hemoglobin as oxygen carrier. In most species there is only one switch from embryonic to adult, reflecting the two distinct globin expression programs of the primitive and definitive hematopoietic lineages. In humans and several primates there are two globin switches – from embryonic to fetal and from fetal to adult. The human embryonic globin program, characterized by predominant ε gene expression, is confined in the yolk sac erythropoiesis. Fetal as well as low levels of embryonic globin are expressed in the erythroid cells of the fetal livers of early human fetuses, but soon thereafter production of predominantly fetal and low levels of adult globin mark the program of erythroid cells of the fetal liver or the fetal bone marrow erythropoiesis [1]. Switching from fetal to adult starts in the third trimester of gestation and is completed several weeks after birth.

Corresponding to the quantitative change in developmentally specific globin protein and mRNA levels, *cis*-linked changes including histone modifications and DNA methylation surrounding the affected globin regions take place [2–5]. These epigenetic changes may be attributed to the actions of epigenetic modification enzymes recruited to the affected genes. For instance, the recruitment of multiple epigenetic transcriptional corepressors by TR2/TR4 to the silenced globin promoters in adult erythroid cells has been reported [6]. The active role of epigenetic modifications in modulating globin expressions is further illustrated by the reactivation of human fetal or mouse embryonic globin genes upon treating adult erythroid cells with pharmacological agents that target epigenetic modification enzymes including DNA methyl-transferase or histone deacetylase or with RNAi that downregulates histone modification enzyme G9a methyltransferase [4, 7].

Transfer of chromatin-packaged chromosome into a known transcriptional environment can serve as a model system for studying the interaction between transcriptional environment and established chromatin domains. Suzuki and colleagues have built an artificial mini human chromosome containing β globin locus and found that the chromatin-packaged β globin gene cluster can be activated or repressed according to the cellular environment [8]. Alternatively, cell to cell fusion has been used to introduce chromatin-packaged β globin locus into adult type murine erythroleukemia (MEL) cells and a complex globin expression pattern dependent on both donor cell types and time in culture has been observed [9–12]. The transferred β globin locus retains important features including the long range interaction between LCR and active globin promoters [13]. The broad utility of this system has been impeded by the continuous and rapid loss of human chromosome 11 from these hybrids. To overcome this issue, we produced human embryonic stem cells (hESCs) carrying a neo resistance gene on chromosome 11. Heterospecific hybrids generated from fusing MEL cells to these hESCs or their erythroid progenies allowed us to study the response of β globin locus of two distinct chromatin states following its transfer into the adult environment of MEL cells. We confirmed that the existing chromatin domains and transcriptional environment collectively determine the initial outcome of specific globin expression, be it an adult or fetal pattern. The cells that exhibited an initial fetal pattern underwent a temporal fetal to adult switch without significant changes in the overall transcriptional environment. We showed that this system allows molecular analyses that provide insights on the sequential epigenetic changes that occur during development. We further showed that switching in this system can be modulated by drastic changes in the transcriptional

environment. This system can be used in the investigation of transcriptional or pharmacologic elements for therapeutic reactivation of fetal hemoglobin in patients with beta chain hemoglobinopathies.

Materials and Methods

Propagating and differentiation of hESCs into erythroid lineage [14], generation and characterization of heterospecific hybrids [9], and conduction of epigenetic studies [5] have been previously described. Additional details can be found in Supplemental Text. Primers for quantitative reverse transcription polymerase chain reaction (RT-PCR) are summarized in Supplemental Table S1.

Results

Derivation of Neo-resistant human ESCs and ESC-derived erythroblasts

To generate a hESC line that carries a selectable marker on chromosome 11 that is expressed at all stages of differentiation, we targeted the first coding domain of general transcription factor IIH polypeptide 1 (GTF2H1), located ~600 kb upstream of human β globin locus, using adeno associated virus (AAV)-mediated homologous recombination (Figure 1A). Six neomycin resistant H1 colonies were obtained out of 400×10^3 cells and expanded further. Two of the six clones were correctly targeted based on PCR screening (Figure 1B) and were designated as Neo-1 and Neo-2. To test whether the targeting procedure had altered the stem cell properties of these neo-resistant hESC lines, we first determined the expression of stem cell markers and found that both lines expressed high levels of TRA-1-60, TRA-1-81, SSEA-3, and SSEA-4 (Figure 1C), similar to parental H1 cells and other hESC lines [14]. Upon differentiation, the two neo-resistant hESC lines formed cystic embryoid bodies (data not shown) and expressed comparable levels of hematopoietic markers such as CD34, CD117, CD31, CD41, CD45, glycophorin A (GlyA), and CD71 to parental H1 cells (Figure 1D). Erythroid differentiation was studied in Neo-2 and these neomycin-resistant erythroblasts displayed a globin phenotype of high embryonic ε and fetal γ globins with almost no adult β globin (Figure 1E), consistent with the globin expression pattern of hESC- and iPSC-derived erythroblasts previously reported [14–16]. Therefore, AAV-mediated gene targeting of GTF2H1 in hESCs did not affect their stem cell properties or their hematopoietic differentiation characteristics.

Exclusive β globin expression following transfer of the hESC beta globin locus into the adult environment of MEL cells

We have previously reported that, based on the distribution of AcH3 and H3K4me3, the chromatin of entire β globin locus of hESCs is closed [5]. This observation was further confirmed by DNase I hypersensitivity mapping showing that only a minor peak existed at hypersensitive site 2 (HS2) at the LCR (Figure 2A, Supplemental Table S2), which was also observed in various non-erythroid cells we have tested (data not shown). Following transfer of the inactive and DNase I resistant β globin locus into the adult erythroid environment, in all 4 heterospecific-hybrid lines generated, only the β globin gene was activated, which was reflected at the protein level by globin chain-specific monoclonal antibody staining (Figure 2B, left pannels), as well as at the mRNA level by RT-PCR (Figure 2C, upper panel). Thus upon encountering the adult erythroid environment of the MEL cells, the β globin locus of hESCs behaved like the inactive loci of other somatic cells such as fibroblasts or lymphoblasts [10].

Predominant γ globin gene expression following transfer of the β globin locus of hESC-derived erythroblasts into the adult environment of MEL cells

The hESC-derived erythroblasts express an embryonic/fetal globin program [15]. DNAse I mapping experiments showed that the chromatin of 5′ region of the β globin locus was open: the LCR as well as promoters and genes of embryonic ε and fetal γ globins were sensitive to DNaseI cleavage (Figure 2D, Supplemental Table S3) while there was minimal, if any, DNase I sensitivity of the β globin gene. Several changes in the profiles of DNaseI hypersensitive sites (HSs) in the LCR were observed upon transfer of this locus into the adult erythroid environment which included narrowing of the 5 HSs and the increasing of the peak height for HS3, suggesting different trans-factors binding patterns in the LCR between hESC-erythroblasts and the fused hybrids. The broader HSs peak widths in the LCR of hESC-erythroblasts may also be related to the inter-HSs transcription reported previously in these cells [5]. In the globin regions, transfer of the locus resulted in essential abolishment of DNase I sensitivity of the ε globin gene and its promoter (Figure 2D, open arrow). In contrast, DNase I sensitivity of the γ globin gene region remained as high as in the parental hESC-derived erythroblasts (Figure 2D, closed arrow) and the DNAse I sensitivity of the β gene region remained as low as in the parental hESC-derived erythroblasts (Figure 2D and Supplemental Table S3). Consistent with the DNaseI profiles, γ globin mRNA was the predominant globin species found in all seven hybrid lines and consisted between 85 to 99% of total human β locus globin mRNAs, with the remaining being β globin mRNA (Figure 2C, lower panel). ε globin mRNA was all but absent. At the protein level there was a high expression of γ globin chains with minimum β globin chain and no ε globin chain expression (Figure 2B, right panels). Thus, the main event following the transfer of chromatin-packaged β globin locus of hESC-derived erythroblasts into an adult environment is the transition of the locus from an embryonic/fetal program to a predominantly fetal program (Figure 2E).

The β globin locus of hESC-derived erythroblasts undergoes a time-dependent γ to β globin switch

We continued culturing the hESC-erythroblast hybrids for up to 68 weeks. Five out of 7 hybrid lines displayed a progressive, time-dependent γ to β globin switch in both mRNA (Figure 3A, Supplemental Figure S1) and protein levels (Figure 3B and data not shown). Time-wise, this switch closely resembled the in vivo γ to β globin switch in humans and the in vitro γ to β globin switch in hybrids produced by fusing fetal erythroblasts with MEL cells [9]. Two other hybrid cell lines showed transient reactivation of γ globin during culture before their eventual switch to a stable β globin phenotype (Figure 3C).

To determine whether the observed time-dependent γ to β globin switch was simply due to β globin-expressing cells proliferating at a higher rate relative to γ globin-expressing cells, we assessed BrdU incorporation by these two types of cells in hybrid line C3 at week 22 of culture. Similar proportion of γ and β globin-expressing cells incorporated BrdU following a 2- or 4-hour pulse (Figure 3D), suggesting that they proliferated at a similar rate.

The γ to β globin switch reflects epigenetic changes in the β globin locus chromatin

The chromatin configurations before and after switching were examined by digital DNaseI mapping (Figure 4A) and ChIP-seq for H3K27ac, H3K4me3, and H3K4me1 (Figure 4B). The chromatin domain of LCR remained open throughout the culture period as revealed by DNaseI studies and was associated with both histone marks H3K27ac and H3K4me1 but without histone mark H3K4me3, consistent with its role as an active enhancer [17]. Striking changes in chromatin profiles took place in the γ and β globin regions during the culture period. At week 7, when these hybrids expressed a predominantly fetal globin program, high peak of DNase I sensitivity was characteristic of the Aγ/Gγ genes region. At week 53, when

on the basis of globin mRNA levels these hybrids have switched to adult program, the chromatin profile also changed drastically: there were no longer DNase I hypersensitivity peaks in the γ genes while there were prominent peaks in the region of β and δ genes. Similar changes were also observed with histone mark H3K4me3 (Figure 4B).

No detectable changes in the transcriptional profile of the hybrids during switching

The changes in the globin programs of the hybrids could conceivably reflect changes in transcriptional environments during culture. Murine transcriptome studies were performed on hybrids before (weeks 4–8), during (week 27), or after (week 53) switch (Figure 5A). Only 4 murine genes (Styx, Tmem176b, Slc22a3, and Bnip3) were found to be expressed at significantly different levels during the switch as compared to early (weeks 4–8) hybrids, and 7 murine genes (Bex4, Brdt, Prkcq, F2rl2, P2rx7, Angpt1, and F2r) were found to be expressed differentially when comparing the switched hybrids (week 53) to those undergoing switch (week 27). None of these genes have previously reported roles in regulating hemoglobin expression. Cytogenetic studies showed no significant linkage between switching and the loss of any particular human chromosome (Supplemental Table S4). Human transcriptome studies confirmed the findings of cytogenetic studies (data not shown). Western blotting, which detected both human and mouse origin of transcription factors known to be critical to γ and β globin expression did not detect consistent trends of either up or down regulation during culture (Figure 5B). These results suggest that the observed γ to β globin switch in the hybrid system may not be due to time-related changes in the transcriptional environment.

We also tested potential involvement of transcriptional factors in the two distinctly different globin expression patterns obtained following the transfer of the β globin locus from hESCs or hESC-derived erythroblasts into MEL cells (Figure 2). mRNA and protein levels by RT-PCR and western blottings detected no significant differences in the expression levels of several known γ globin repressors/β globin activators including EKLF [18, 19], BCL11A [20, 21], MYB [22–24], and SOX6 [25, 26], as well as γ globin activators such as HDAC9 [27] and KLF11 [28] between these two types of hybrids (Figure 5C, D)

The globin phenotype of the hybrids can be modulated by overexpression of EKLF

We then studied whether globin expression in hybrids may be influenced by transcription factors, particularly EKLF [18, 19, 29, 30] with known functions in modulating fetal/adult globin expression. We transfected hESC-erythroblast hybrid line C3 before (at week 8) or during (at week 23) switching with either wild type EKLF (WT), or EKLF with its DNA binding domain or transactivating domain removed (ΔDB and ΔTA respectively) (Figure 6A). To allow for the identification of transfected cells and to gauge for the level of EKLF overexpression at the single cell level, polycistronic vectors were constructed utilizing self-cleaving 2A peptides (Figure 6A) that linked EKLF and GFP together. We found that while overexpressing mutant EKLF (both ΔDB and ΔTA) had relatively little effect on the globin expression phenotype of the hybrids, overexpressing wild type EKLF drastically decreased the proportion of γ globin expressing cells and increased the proportion of β globin expressing cells, in both week-8 and week-23 hybrids (Figure 6B). At week 8, the frequency of the γ-positive hybrids (γ+β− and γ+β−) was ~90% in the control hybrids with no EKLF/GFP overexpression, ~70% in hybrids having low EKLF overexpression and ~60% in hybrids with high EKLF expression. At week 23 the frequency of γ-positive hybrids was ~60% in the control, ~35% in hybrids with either low EKLF overexpression or high EKLF overexpression. γ only population (γ+β−) decreased in a EKLF dose-dependent manner which reached ~90% in both week 8 and week 23 hybrids with high EKLF overexpression. Interestingly, with low EKLF overexpression, the γ-only population decreased by ~50% in week 8 hybrids (from 84.97 ± 2.89 to 40.26 ± 3.61) but decreased by ~67% in week 23

hybrids (from 52.30 ± 2.62 to 17.09 ± 1.40), suggesting that week-23 hybrids responded more readily than week-8 hybrids to the inhibitory effect of EKLF. These data indicate that EKLF accelerated γ to β globin switch in these hybrids in a dose dependent fashion and the switching hybrids more readily submitted to its effect than the pre-switch hybrids.

The γ globin gene of the switched hybrids can be reactivated by Bcl11A suppression and treatment with Decitabine

To study whether once switched, γ globin can be reactivated by down regulation of transcription factors such as Bcl11A, we transduce switched, β globin expressing hybrids with lenti-viruses carrying a previously published shRNA sequence for murine Bcl11A [25]. Bcl11A shRNA decreased the Bcl11A protein level in the hybrids as compared to scrambled shRNA, although it did not completely obliterate Bcl11A expression (Figure 7A). Inducing these Bcl11A shRNA transduced hybrids did not result in higher γ globin expression level than those transduced with control scrambled shRNA (Figure 7B). When these hybrid cells were induced in the presence of HDAC inhibitor decitabine, a more prominent effect of γ globin reactivation was observed in cells transduced with Bcl11A shRNA compared to cells transduced with the scrambled shRNA construct, suggesting a synergistic effect between HDAC inhibitor decitabine and γ globin repressor Bcl11A to bring about induction of γ globin expression.

Discussion

Previous studies of ESCs have identified distinct epigenetic markings with implications in cell fate decisions and transcriptional competency upon differentiation at the promoters of developmentally critical genes and at the intergenic region and enhancers of tissue-specific genes [31–33] including murine β globin locus [34]. However, such epigenetic markings are not found at the β globin locus of hESCs [5]. By transferring chromatin-packaged β globin locus from hESCs to adult erythroid environment of MEL cells, our current studies provided further evidence that despite being pluripotent in nature, the β globin locus of hESCs is essentially closed like that of lymphocytes and of fibroblasts[10] without displaying epigenetic program for the sequential expression of β locus globins. The acquisition of permissible chromatin modifications at the specific globin genes is likely initiated later at the $CD34^{+}CD38^{-}$ hematopoietic progenitor stage [35] when hESCs undergo step-wise differentiation to become erythroid cells [36].

The silencing of embryonic ε globin gene but perseverance of fetal γ globin upon transferring β globin locus from hESC-derived erythroblasts to MEL cells is consistent with previous studies fusing embryonic blood of transgenic mice carrying human β globin locus with MEL cells [11, 12]. It has originally been proposed that epigenetic modifications surrounding embryonic ε globin gene may be less stable than those surrounding fetal γ globin gene [11, 12], but this is unlikely as we and others have identified similar histone codes along with the same looping mechanism utilized by actively transcribed embryonic ε globin and fetal γ globin genes in hESC-derived erythroblasts [5, 37, 38]. Rather, adult transcriptional environment either lacks factors that actively maintain an open chromatin domain surrounding ε globin, or possess factors that actively repress ε globin expression. Several repressive factors have been previously identified, including TR2, TR4 [39, 40] GATA1 together with YY1 [41], and MBD2 [42]. G9a methyltransferase-dependent H3K9me2 and H3K27me2 domain formation and DNMT3A-mediated DNA methylation have also been proposed to be involved in closing ε globin region for transcription [7, 43].

MEL adult erythroid transcriptional environment failed to activate the silent fetal γ globin gene from hESCs yet sustained the established open chromatin configurations of the fetal γ globin gene from hESC-erythroblasts. While the sustained expression of γ globin could be

due to activation of fetal/embryonic program of MEL cells upon exposure to transcription factors from hESC-erythroblasts, such exposure did not sustain the expression of ε globin. As there were no differences in the levels of several known fetal γ globin repressors such as EKLF and Bcl11A, or activators such as KLF11 and HDAC9 in the γ globin expressing- versus β globin expressing-hybrids, the differential γ globin expression was unlikely to be dependent on these factors. The recently reported LIN28B is also unlikely to be involved as it appears to exert its HbF inducing function through Bcl11A downregulation [44]. Instead, together with previous studies [9, 10], our results suggest that MEL adult transcriptional environment lacks transcription factors that activate γ globin expression, but contains factors required for maintaining an active γ globin state at least for several months even in the absence of factors required for its initial establishment.

The prolonged γ globin expression in an otherwise β-globin favoring environment demonstrates the robustness of the chromatin positive feedback loops [45, 46]. Nevertheless, these positive feedback loops can be negated by the overexpression of strong γ globin repressors such as EKLF. The dose- and chromatin state- (as in pre-switch vs. switching hybrids) dependent inhibitory effect of EKLF overexpression suggests that the outcome of γ and β globin expression and switching is determined, at least partially, by the balance between the positive feedback loops of chromatin modifications and the suppressive effects of transcription factors. As transcriptome studies and western blot analyses suggest a relatively stable murine transcriptional environment before and after switch, the time-dependent transition of chromatin surrounding γ globin gene from an active state to a silent state resembles the switching of the mating-type region of fission yeast which occurs approximately once every 200 days per cell without apparent changes in the transcriptional environment [47]. The stochastic model derived from yeast studies may provide a framework for the observed time-dependent γ to β globin switch in hESC-erythroblast-hybrids, and possibly for the gradual silencing of γ-globin in Bcl11A-null adult mice [48]. As the expression of γ and β globin is competitive in nature [49], the silencing of γ globin results in the expression of β globin, or switching, within an environment already favoring its activation.

The reactivation of γ globin in adult erythroid cells in vitro and/or in vivo via knocking down γ globin repressors, such as Bcl11A and EKLF, is well documented [21, 30, 50]. A recent study using MEL cells carrying two reporter genes inserted into a 183-kb intact human β-globin locus under the control of γ globin promoter and β-globin promoter also shows that silencing Bcl11A leads to the activation of the reporter gene driven by γ globin promoter [51]. We found little γ globin reactivated with Bcl11A knockdown alone without decitabine treatment. The differences between our findings and those by Chan et al. [51] may be partially due to the degree of Bcl11A knockdown achieved. Furthermore, as none of the studies have attained a globin expression pattern resembling that of fetal liver derived erythrocytes, the mere down regulation of γ globin repressors without γ globin activators may not be sufficient to fully re-activate γ globin expression, and the activation may not be sustained over a prolonged period of time [48]. Identification of factors responsible for the initial establishment of an active γ globin domain may aid in the treatment of hemoglobinopathies [20, 52].

The extraordinary stability of fetal γ globin chromatin conformation in the adult transcriptional environment has important implications in attempts to generate adult-globin expressing erythroid cells from patient specific iPSCs. Thus far, the majority of studies find hESC- or iPSC-derived erythroid cells expressing mainly embryonic ε and fetal γ globins although ε globin expression may decrease with prolonged stromal coculture [36]. While some increase in β globin expression has been noted, the level is generally small in comparison to γ globin expression. This is consistent with the observation that fetal cells

transplanted into adult recipients continue to express a fetal globin program [53, 54]. However, it is in stark contrast with a recent study by Kobari et al. [55] which demonstrates that the switch from fetal to adult hemoglobin is completed within 3 days after infusion into NOD/SCID mice nucleated erythroblasts derived from iPSCs. Whether the β globin detected in this study is a result of contaminating human red cells previously injected to block macrophage function is unclear.

Finally, our studies here present a model system, improved from the previous heterospecific hybrid by inserting a selectable marker into chromosome 11, for the study of fetal to adult globin switching. Two important characteristics of the fetal to adult switch are its occurrence in the cells of a single hematopoietic lineage – the definitive hematopoiesis, and its strict dependence on developmental time. The fetal to adult globin switch has been studied with a variety of experimental systems: in vivo, in primates, sheep and goats that display switches from γ to β globin expression; in transgenic mice carrying globin gene plasmids, cosmids, YACS or BACS; in various types of primary human or primate erythroid cell cultures; and in cell lines which express human globins, typically γ and ε. All these systems have certain disadvantages. Having an experimental system closely resembling the in vivo human γ to β globin switch has the advantages of providing the opportunity to investigate the contribution of trans-acting factors to the control of switching or to globin gene silencing in the adult erythropoiesis and for confirmation of the effects of potential pharmacological inducers of fetal globin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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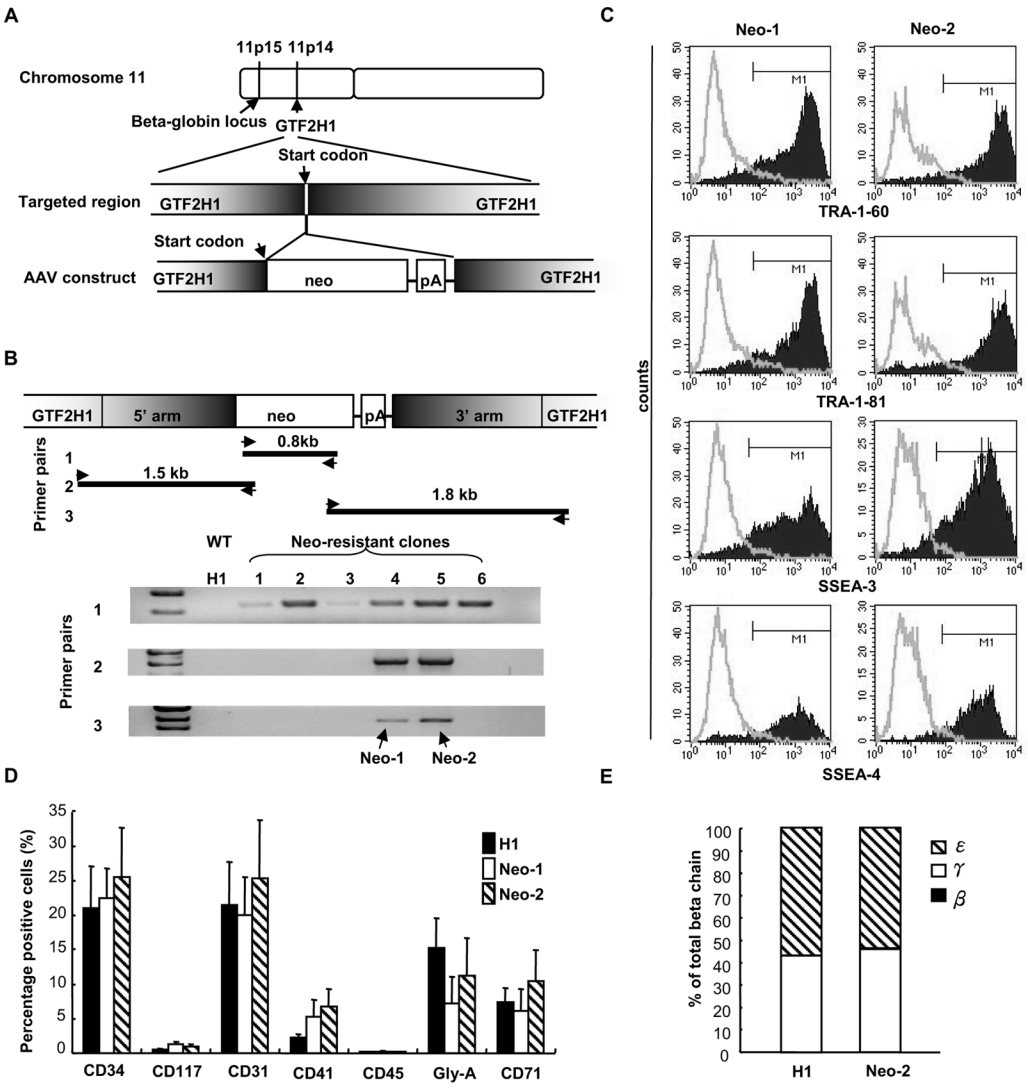

Figure 1. Marking chromosome 11 with a neomycin-resistant gene

A) General transcription factor 2H polypeptide 1 (GTF2H1), ~600 kb upstream of β globin locus was targeted by adeno-associated virus (AAV)-mediated homologous recombination. An 859 bp neo-pA cassette was inserted at the first coding region of GTF2H1. B) Six neo-resistant clones were obtained and subjected to PCR screening with the three pairs of primers shown to separate homologous recombination events from random integrations. Notice that two clones: Neo-1 and Neo-2 were correctly targeted. C) Successfully targeted neomycin-resistant hESCs expressed pluripotent stem cell markers during propagations as demonstrated by flow cytometry analyses. D) Neomycin-resistant hESCs retained the ability to differentiate into hematopoietic cells. After placed in embryoid body formation conditions for 14 days, differentiated neomycin-resistant hESCs and parental H1 cells expressed comparable levels of hematopoietic markers. Data are expressed as means standard error of means (SEM). N = 4 for H1, 5 for Neo-1, and 4 for Neo-2. E) Erythroid cells derived from neomycin-resistant hESCs expressed high levels of embryonic ε and fetal γ globin as the parental H1 line did.

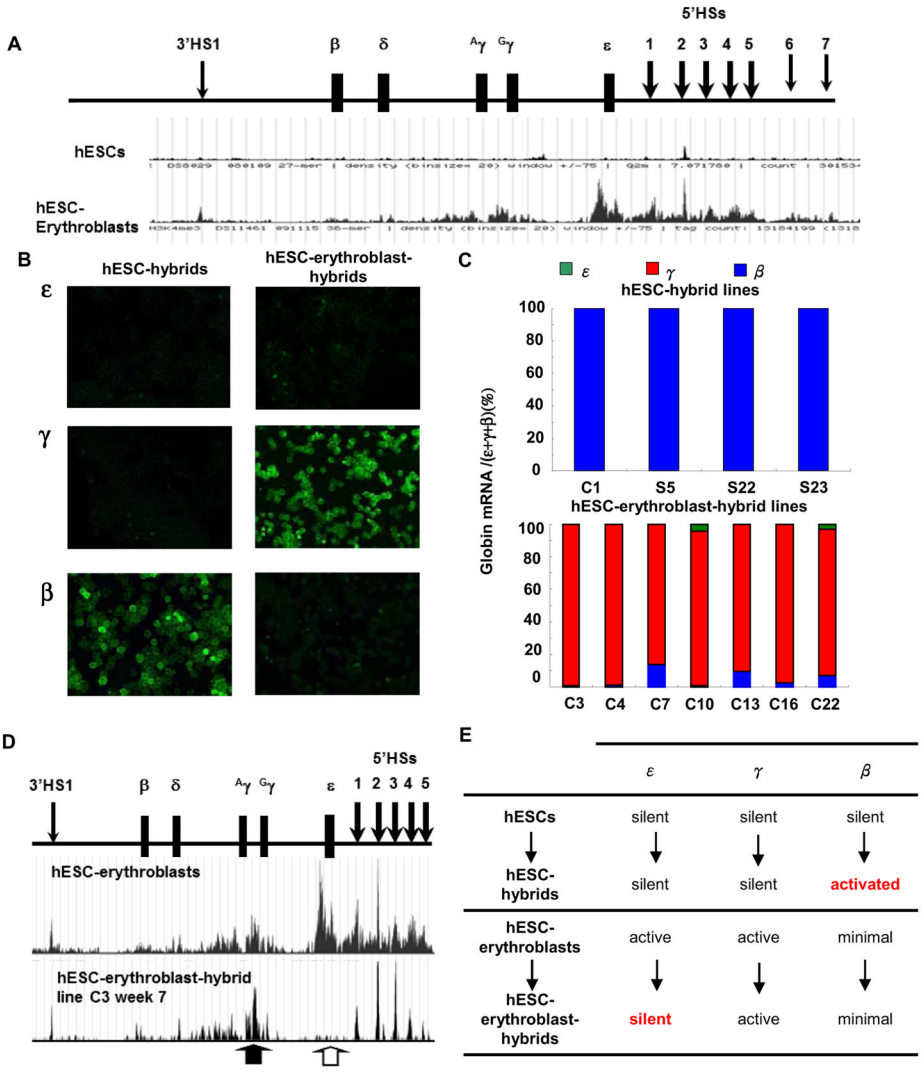

Figure 2. Response of chromatin-packaged β globin locus upon transfer from its original transcription environment into the adult erythroid transcriptional environment of murine erythroleukemia cells (MEL)

A) DNase I mapping of hESCs and hESC-derived erythroblasts. Undifferentiated hESCs had an inactive β globin locus while the erythroblasts derived from hESCs showed prominent DNase I cleavages at LCR and ε and γ globin regions. B) Globin expression patterns of hybrids derived from hESCs or from hESC-erythroblasts. Representative images of immuno-fluorescent staining of week 4–5 hybrids with monoclonal antibodies against ε, γ, or β globin chain followed by a FITC-conjugated anti-mouse-IgG antibody are shown. Pictures were taken under a Leica DMLB microscope with a 40 X objective lens using a Leica camera with Leica LAS software (version 2.4.1R1) (Leica Microsystems, Buffalo Grove, IL). C) The β locus globin mRNA expression levels of each hybrid line were determined using RT PCR by plotting against standard curves generated using transgenic mouse tissue with known human β locus globin expression levels. Upper panel: hybrids produced by fusing MEL cells with human ES cells; notice the exclusive β globin mRNA expression. Lower panel: hybrids produced by fusing MEL cells with hESC-derived erythroblasts; notice the predominant γ globin mRNA expression. D) DNase I mapping of the β globin locus. Comparison of the β globin locus in the pre-fusion hESC-derived

erythroblasts to the β globin locus of hybrids derived by fusing these erythroblasts with MEL cells. Notice the silencing of ε globin gene (open arrow) and the continued expression of γ globin genes (closed arrow) following the transfer of the β globin locus of the hESC-erythroblasts into the MEL cells. E) Summary of the changes in β locus globin expression before and after transfer of the locus from its native environment into the MEL environment.

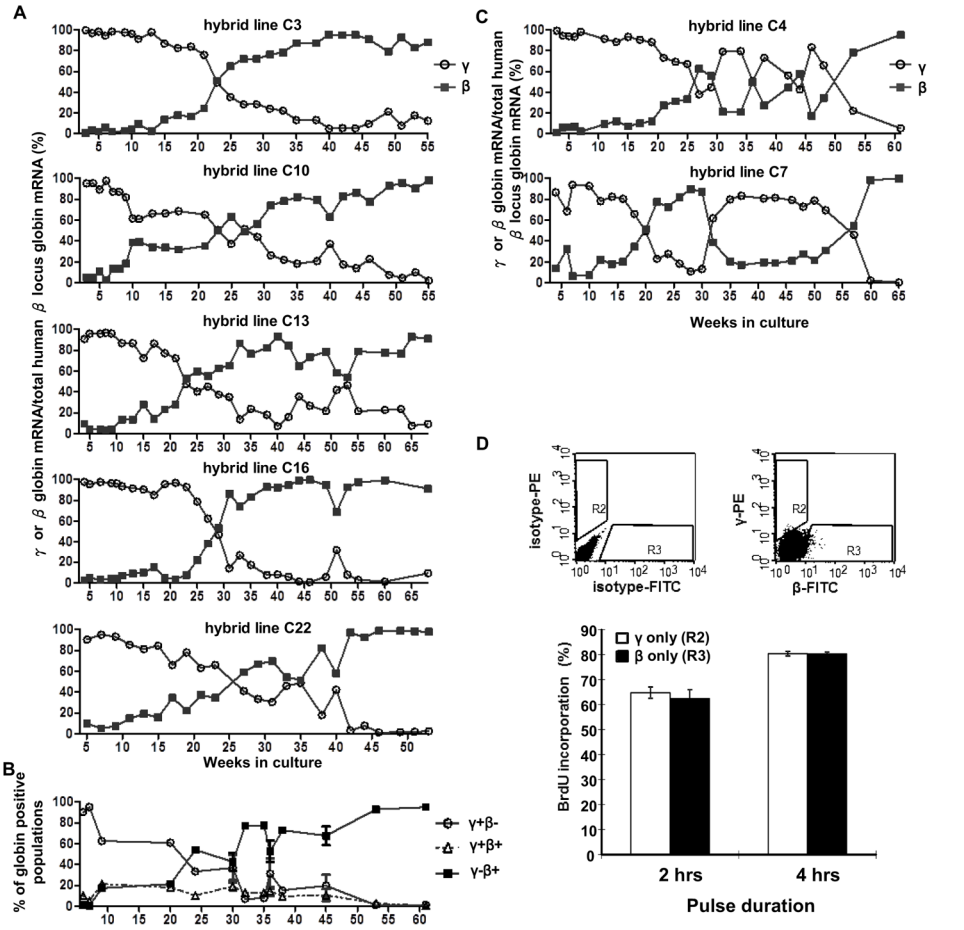

Figure 3. Gradual and time-dependent globin gene switch of hybrids derived from hESC-erythroblasts

A) Five hESC-erythroblast-hybrid lines that showed a characteristic switch consisting of a gradual increase in β globin and a decrease in γ globin expression at mRNA levels with time in culture. Notice the similarity with the in vivo globin switching in humans. Data are expressed as percentage of total human β locus globin mRNA. Human globin mRNA levels normalized to mouse a globin mRNA levels are shown in Supplemental Figure S1. B) The γ to β globin switch is also reflected in parallel changes at the protein level as determined by flow cytometry. Also notice the transient γ and β globin doubly positive population. C) Two hybrid lines, C4 and C7, at culture weeks 20–25 showed a transient reactivation of γ globin gene expression; however, both lines eventually switched. Notice in hybrid line C7, the characteristic reciprocal changes in γ and β globin mRNA expression. D) Similar proliferation rate was found in γ globin producing and β globin producing populations of hybrid line C3 at week 22 of culture as demonstrated by BrdU incorporation for either 2 or 4 hours. Data are presented as mean ± SEM. N=3.

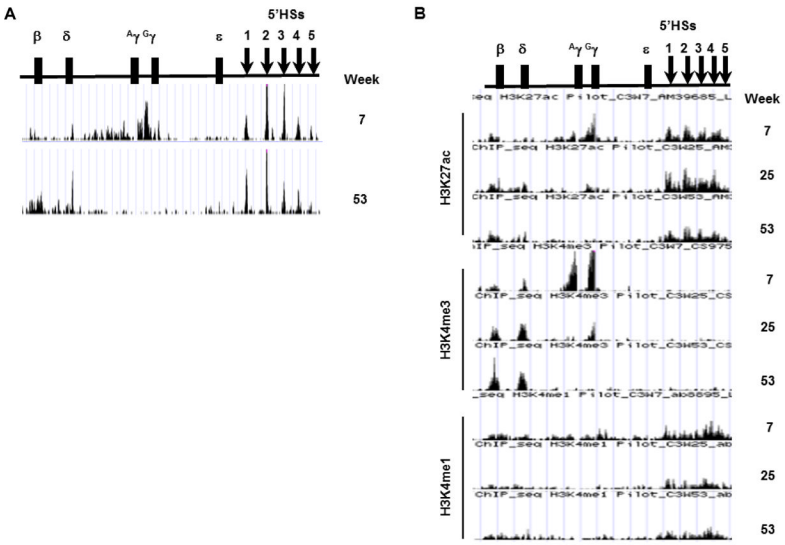

Figure 4. Dynamic epigenetic changes in the β globin locus throughout culture

A) DNase I sensitivity of hESC-erythroblast–hybrids at the time of predominant γ globin expression (week 7) and after the completion of γ to β switch (week 53). Notice that the DNaseI hypersensitive sites (DHSs) of the LCR remained unchanged while there were drastic changes in DNase I sensitivity of the γ and β globin regions. The week 7 hybrids were induced while the week 53 were uninduced. B) ChIP-seq was carried out to determine the histone configurations of human β globin locus in hybrids before, during or after they switched from γ globin producing cells to β globin producing cells. Notice the changes in H3K27ac and H3K4me3 in the γ and β gene regions. All hybrids were induced.

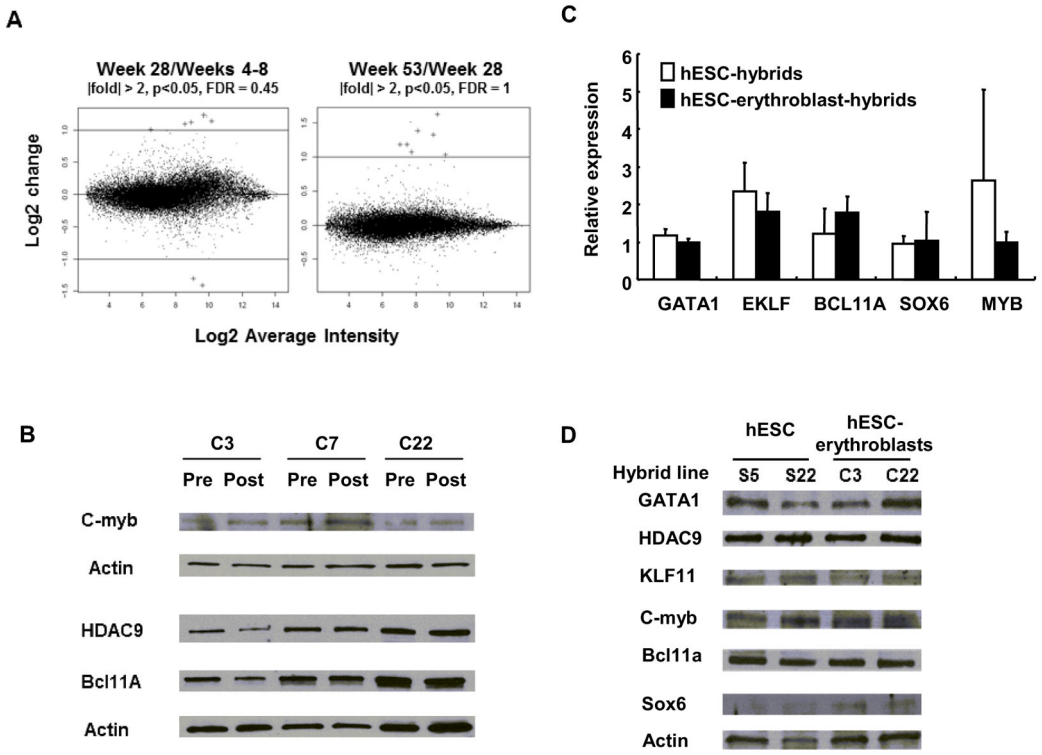

Figure 5. Trascriptome profiles of hybrids with different globin expression patterns

A) Differentially expressed genes were identified using microarray when comparing switching (Week 28) to pre-switch (Week 4–8) hESC-erythroblast-hybrids (left panel) and when comparing post-switch (Week 53) to switching (Week 28) hESC-erythroblast-hybrids (right panel). Three samples (lines C3, C10, and C22) at each time point were studied. B) Protein levels of γ globin gene repressors Bcl11A and C-MYB were determined by western blotting before (Pre) and after (Post) γ to β globin switch in 3 hESC-erythroblast-hybrid lines C3, C7, and C22. C) The expression levels of several known γ globin repressors were determined using real time RT-PCR. There were no differences in the expression of these factors between the β globin expressing hESC-hybrid lines and the γ globin expressing hESC-erythroblast-hybrid lines. Data are presented as mean ± SEM. N=4. D) The protein levels of several known γ globin repressors and activators in hESC- and hESC-erythroblast hybrid lines were determined by western blotting. For Bcl11A, the 125 kD XL isoform, known to mediate γ globin silencing, was most prominent in all samples. There was little other isoforms detected. Representative blots are shown.

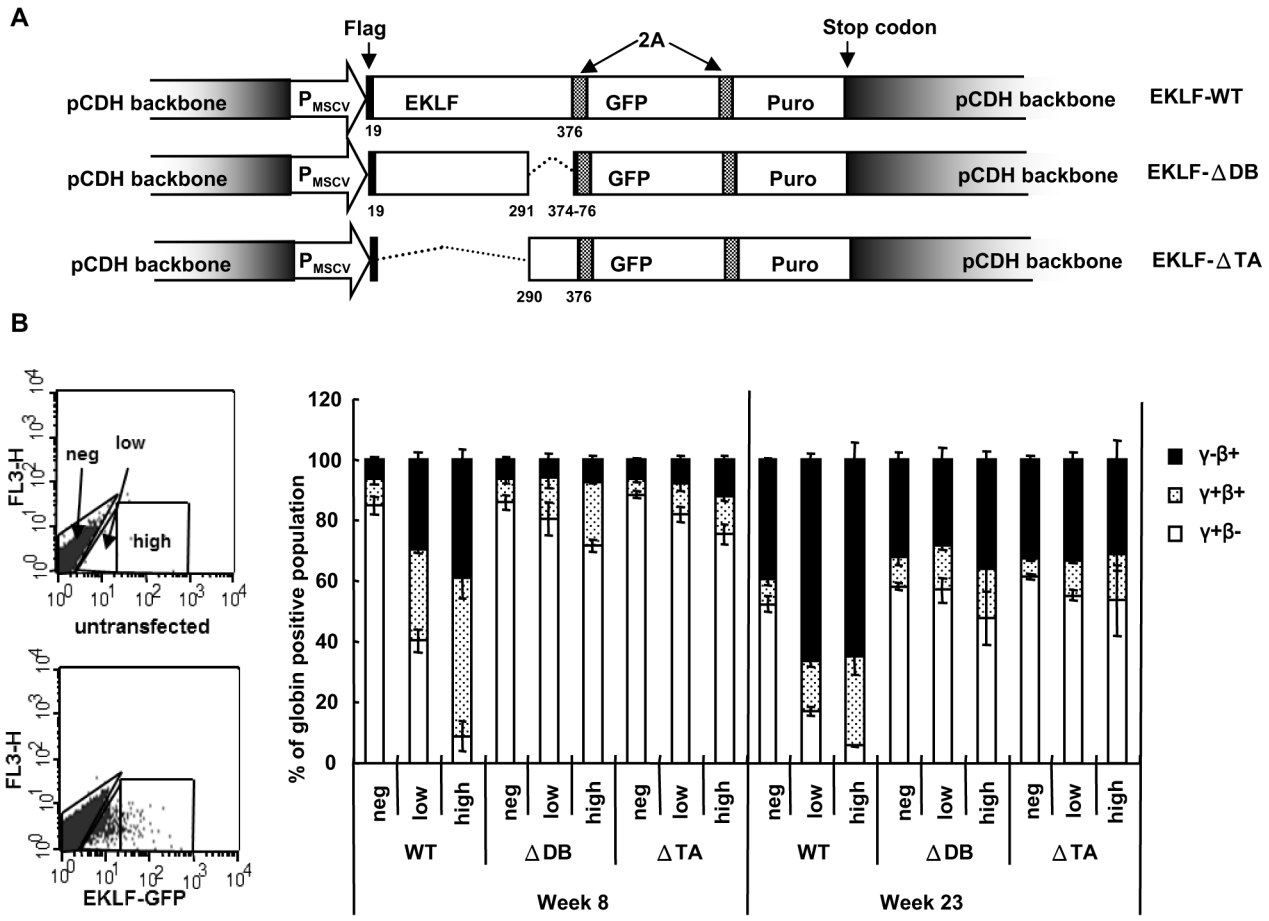

Figure 6. Modulation of globin expression by EKLF

A) Three constructs were made to express wild type (WT) or truncated versions of EKLF with either its DNA binding domain (DB) or transactivating domain (TA) removed. EKLF was linked in-frame to eGFP via DNA sequence encoding 2A peptide. B) Overexpression of WT EKLF but not mutant EKLF resulted in accelerated switching from γ globin producing cells to β globin producing cells as revealed by flow cytometry. WT EKLF overexpression drastically reduced γ globin expression both in the week 8 hybrids showing predominantly γ expression and in the week 23 hybrids which were in the middle of γ to β switch in a dose-dependent fashion. Transfected cells were gated into high EKLF, low EKLF, and no EKLF expressing populations based on GFP intensity as EKLF and GFP were linked by 2A peptide. Data shown are mean ± SEM. All experiments were done in triplicates.

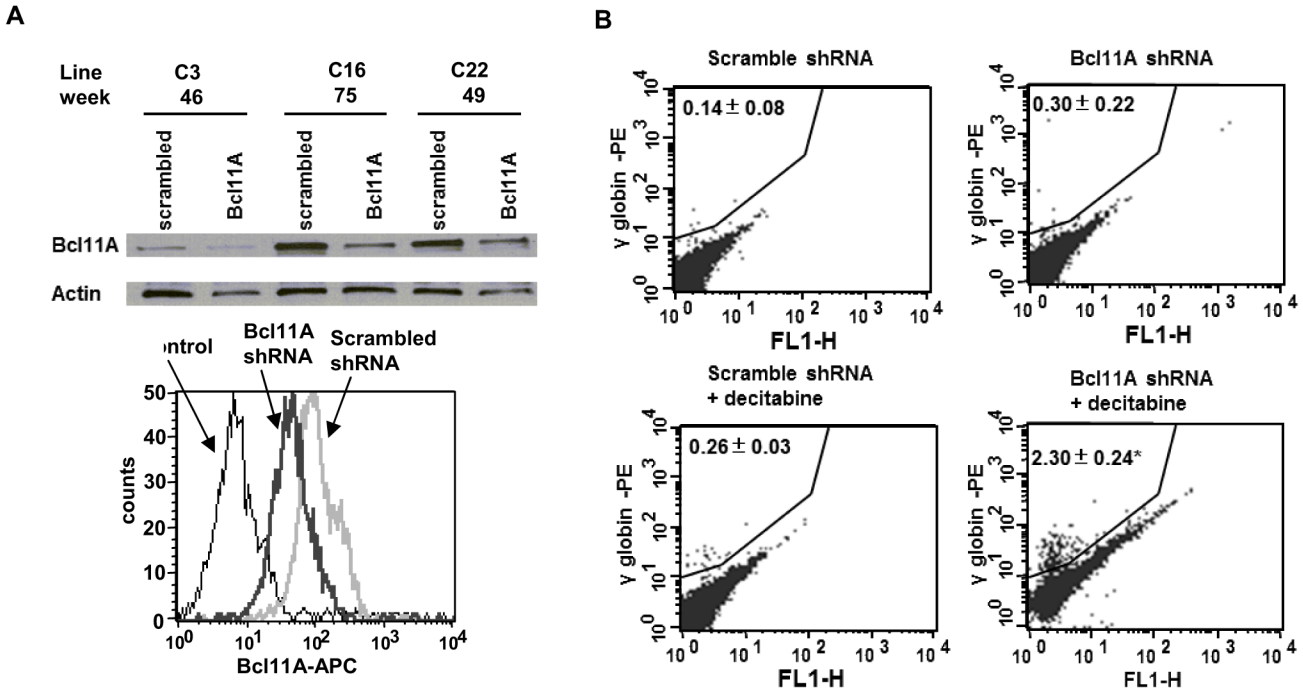

Figure 7. Modulation of γ gene expression by Bcl11A suppression and treatment with Decytabine

A) Bcl11A was knocked down in 3 post-switch hybrid lines via RNA interference. The level of knock down was confirmed by both western blotting (Top panel) and by flow cytometry (bottom panel). Representative blots and plot are shown. B) The reactivation of γ globin in Bcl11A knockdown hybrids vs. control hybrids with or without the addition of decitabine. The γ globin expressing population was only significantly increased in Bcl11A shRNA + decitabine treatment group ($p<0.05$). N=4. Numbers shown are mean ± SEM for percentage of cells expressing γ globin. N=4. * $p<0.05$