

## Development and characterization of K562 cell clones expressing BCL11A-XL: Decreased hemoglobin production with fetal hemoglobin inducers and its rescue with mithramycin

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**Induction of fetal hemoglobin (HbF) is considered a promising strategy in the treatment of  $\beta$ -thalassemia, in which production of adult hemoglobin (HbA) is impaired by mutations affecting the  $\beta$ -globin gene. Recent results indicate that B-cell lymphoma/leukemia 11A (BCL11A) is a major repressor of  $\gamma$ -globin gene expression. Therefore, disrupting the binding of the BCL11A transcriptional repressor complex to the  $\gamma$ -globin gene promoter provides a novel approach for inducing expression of the  $\gamma$ -globin genes. To develop a cellular screening system for the identification of BCL11A inhibitors, we produced K562 cell clones with integrated copies of a BCL11A-XL expressing vector. We characterized 12 K562 clones expressing different levels of BCL11A-XL and found that a clear inverse relationship does exist between the levels of BCL11A-XL and the extent of hemoglobinization induced by a panel of HbF inducers. Using mithramycin as an inducer, we found that this molecule was the only HbF inducer efficient in rescuing the ability to differentiate along the erythroid program, even in K562 cell clones expressing high levels of BCL11A-XL, suggesting that BCL11A-XL activity is counteracted by mithramycin. Copyright © 2015 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).**

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The  $\beta$ -thalassemias are relevant hereditary hematologic diseases caused by nearly 300 mutations of the  $\beta$ -globin gene [1,2], leading to low or no production of adult  $\beta$ -globin and an excess of  $\alpha$ -globin in erythroid cells. This causes ineffective erythropoiesis and low or no production of adult hemoglobin (HbA) [3]. Background information on  $\beta$ -thalassemia is available in excellent reviews outlining the genetics [4], physiopathology [5], and therapeutics [6] of this disease. Together with sickle cell anemia (SCA), thalassemia syndromes are the most important problems in developing countries, in which the lack of genetic counseling and prenatal diagnosis has contributed

to the maintenance of a very high frequency of these genetic diseases in the population [7]. The total annual incidence of symptomatic individuals with  $\beta$ -thalassemia is estimated at 1 in 100,000 throughout the world [3]. Presently, no definitive treatment for  $\beta$ -thalassemia and SCA is available. Clinical management of  $\beta$ -thalassemia patients includes lifelong blood transfusions associated with chelation therapy to remove the excess transfused iron [8] and, in some cases, bone marrow transplantation [9]. However, considering the limitations and side effects of the currently available therapeutic approaches and management of the  $\beta$ -thalassemia patients, novel alternative options for therapy are needed [7]. We have recently reviewed the available literature concerning the development of DNA-based therapeutic strategies for  $\beta$ -thalassemia [7,10–12].

Induction of fetal hemoglobin (HbF) is considered a promising strategy for treatment of  $\beta$ -thalassemia [7,12–14]. This is supported by the fact that hydroxyurea, a powerful inducer of differentiation and HbF, is able to ameliorate the clinical

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parameters of  $\beta$ -thalassemia and SCA patients and, for this reason, has been used in several experimental trials and patient clinical management [12]. However, because of the expected side effects of HU, novel HbF inducers are of great interest in studies aimed at developing novel therapeutic options for  $\beta$ -thalassemia [15–18].

Several recent publications have concurrently reported that the  $\gamma$ -globin gene expression is under strong negative transcriptional control [19–25]. For instance, BCL11A [21–23], KLF-1 [22,23], MYB [24], and Oct-1 [25] have been suggested as important direct or indirect repressors of  $\gamma$ -globin gene transcription. Apart from the theoretical importance, this conclusion indicates the potential therapeutic use of targeting these transcription factors [26,27]. In fact, the obvious hypothesis is that pharmacologic inhibitors of these transcription repressors might contribute in activating  $\gamma$ -globin genes and promoting HbF production.

The zinc finger transcription factor B-cell lymphoma/leukemia 11A (BCL11A) was recently found to be the major repressor of HbF expression in genomewide association studies (GWAS), leading to identification of a new HbF-associated locus on chromosome 2, located within the gene *BCL11A* [27]. The BCL11A isoform predominant in adult erythroid progenitor cells is BCL11A-XL [21,22]. It has been in fact reported that transgenic deactivation of BCL11A induces HbF and corrects a humanized sickle-hemoglobin mouse model [28]; BCL11A knockdown leads to significant HbF induction in human cells [29,30], similar to knockdown of its positive regulator KLF1 [26]. To move from general concept to therapeutic application, controlled and stable shRNA-mediated HbF induction has achieved an efficiency of potential clinical relevance [31,32].

This field of research is of top interest, as several approaches can lead to pharmacologically mediated inhibition of the expression of  $\gamma$ -globin gene repressors, resulting in  $\gamma$ -globin gene activation. Among these strategies, we underline, in addition to the already mentioned delivery of shRNAs, direct targeting of the transcription factors by aptamers or decoy molecules, as well as inhibition of the mRNA-coding  $\gamma$ -globin gene repressors with antisense molecules, peptide nucleic acids (PNAs), and microRNAs [12].

The objective of the work described here was to develop an *in vitro* experimental model system expressing transcription repressors at different levels and verify whether this approach might be useful in identifying HbF inducers acting on these  $\gamma$ -globin gene regulators. We took advantage of the fact that the human erythroleukemia K562 cells express very low levels of BCL11A-XL and, therefore, were expected to be an appealing *in vitro* system for producing clones expressing this gene at different levels. For this reason, we focused, among the different  $\gamma$ -globin gene repressors, on BCL11A-XL using, among the different available erythroid cell lines, K562 cells. This cell line, on the other hand, is well known and characterized by its suitability in studies aimed at the identification of compounds inducing erythroid

differentiation. In addition, this cell line has been extensively used for the screening and characterization of inducers of embryonic and fetal globin genes [33–37], because it does not normally express the adult  $\beta$ -globin genes [26] and does not produce relevant levels of HbA even if treated with erythroid differentiation inducers. As inducers to be screened, we employed hydroxyurea (as it is the reference HbF inducer, in consideration of its extensive use in therapy of  $\beta$ -thalassemia and sickle cell anemia) and mithramycin (one of the best HbF inducers studied for many years in our laboratory and having a different mechanism of action).

## Methods

The full version of the Methods section is provided as a supplement (see [Supplementary Methods](#), online only, available at [www.exphem.org](http://www.exphem.org)).

## Materials

Mithramycin (MTH, Catalog No. M6891), resveratrol (RSV, Catalog No. R5010), rapamycin (RAPA, Catalog No. R0395), hydroxyurea (HU, Catalog No. H8627), butyric acid (BA, Catalog No. B5887), and dimethyl sulfoxide (DMSO, Catalog No. D2650) were from Sigma-Aldrich (St. Louis, MO). 1-Octylthymine (1-OctylT) was a gift from Roberto Corradini (Department of Chemistry, University of Parma, Parma, Italy).

## Cell lines, primary cells, and culture conditions

Human erythroleukemia K562 cells [33–37] and murine Friend leukemia cells (FLCs) [38,39] were cultured as described [33,39]. Cell growth was studied using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). Human primary erythroid precursor cells (ErPCs) were isolated and cultures performed as described [17,18,34–36]. After 7 days of cell culture phase II, cells were treated for additional 5 days with MTH. All cellular treatments with Hb inducers were carried out by adding the compounds at the beginning of culture setup without further additions and changes of the medium. Hemoglobin-containing cells were detected by specific reaction with a benzidine/hydrogen peroxide solution (0.2% in 5 mol/L glacial acetic acid and 10% H<sub>2</sub>O<sub>2</sub>) [35].

## Production of K562(BCL11A-XL) clones

For production of K562 cell clones with integrated copies of a BCL11A-XL-expressing vector, we transfected K562 cells with the pCDNA3.1-BCL11A-XL vector and then cloned and selected positive cells by limiting dilutions in the presence of geneticin.

## FACS analysis

The erythroid differentiation status of K562 cells and K562(BCL11A-XL) clone 12 was investigated by studying transferrin Receptor (trfR) and glycophorin A (GYPA) expression by fluorescence-activated cell sorting (FACS) analysis using anti-human CD71 fluorescein isothiocyanate (FITC)-conjugated antibody (Miltenyi Biotec GmbH, Germany) and anti-human CD235a (glycophorin A) phycoerythrin (PE)-conjugated antibody (Miltenyi Biotec) [36].

### Reverse transcription and quantitative real-time PCR

For gene expression analysis, 300 ng of total RNA (for isolation, see [Supplementary Methods](#), online only, available at [www.exphem.org](http://www.exphem.org)) were reverse transcribed using random hexamers. Real-time quantitative polymerase chain reaction (RT-qPCR) experiments were carried out using the 5'-nuclease assay. Gene-specific primers and fluorescence-labeled probes, reported in [Supplementary Tables E1 and E2](#) (online only, available at [www.exphem.org](http://www.exphem.org)), were purchased from Applied Biosystems (Applied Biosystems, Monza, Italy) and Integrated DNA Technologies (IDT, Coralville, IA).

### Western blotting

Twelve micrograms of protein cytoplasmic extracts were prepared as described under [Supplementary Methods](#) (online only, available at [www.exphem.org](http://www.exphem.org)), denatured for 5 min at 98°C in 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 50 mM dithiothreitol [DTT], 0.01% bromophenol blue, 10% glycerol), and loaded on SDS–polyacrylamide gel in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Western blotting was conducted as described [37] using the BCL11A primary rabbit monoclonal antibody (1:1,000, Catalog No. A300-382A, Bethyl, Montgomery, TX). The primary antibody against p70S6K (Catalog No. 2708, Cell Signaling, Leiden, Netherlands) was used as normalization control.

### Ethics statement

The use of human material was approved by the ethics committee of the Ferrara District, Document No. 06/2013, approved on 20 June 2013. All peripheral blood samples were obtained after receiving written informed consent from patients or their legal representatives.

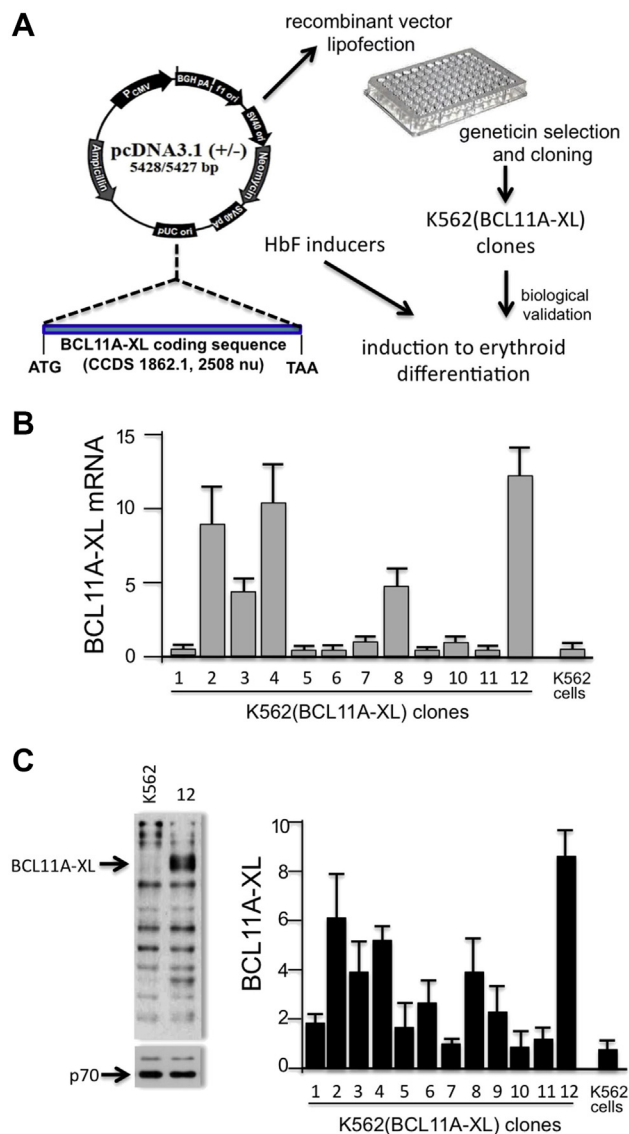
### Statistical analysis

All data were normally distributed and expressed as means  $\pm$  SD. Coefficients of determination ( $R^2$ ) and  $p$ -values were obtained using the GraphPad Prism Software (San Diego, CA). Statistical significance was assumed at  $*p < 0.05$  and  $**p < 0.01$ .

## Results

### Development and characterization of K562(BCL11A-XL) clones

To design an experimental system suitable for screening BCL11A inhibitors (see [Fig. 1A](#) for a flowchart indicating the steps in production and characterization of K562 clones), we produced K562 cell clones with integrated copies of a BCL11A-XL-expressing vector. We characterized 12 K562 clones expressing different levels of BCL11A-XL, as illustrated in [Figure 1B](#), which indicates that most of the different K562(BCL11A-XL) clones obtained express BCL11A-XL mRNA sequences, albeit at very different levels. As known, K562 cells express very low levels of BCL11A-XL [26]. Interestingly, the K562 cell clones producing BCL11A-XL can be grouped into different classes: those expressing BCL11A mRNA at high levels [K562(BCL11A-XL) clones 2, 4, 12],



**Figure 1.** Production and characterization of K562(BCL11A-XL) cells. (A) Scheme describing the strategy. K562 cells were transfected with the pCDNA3.1-BCL11A-XL vector containing BCL11A-XL cDNA under the control of the cytomegalovirus promoter. (B, C) Quantitative analysis of BCL11A-XL expression in 12 K562(BCL11A-XL) clones, determined by real-time quantitative polymerase chain reaction (B) and Western blotting (C). The data represent the average  $\pm$  SD of three independent analyses. Fold expression is relative to K562(BCL11A-XL) clone 10. A representative result of Western blotting is shown on the left in (C) and was performed using protein extracts isolated from K562 and K562(BCL11A-XL) clone 12, as indicated. The BCL11A-XL protein is arrowed. As an internal control, the Western blotting filters were stripped, and binding reaction was performed using an antibody recognizing p70.

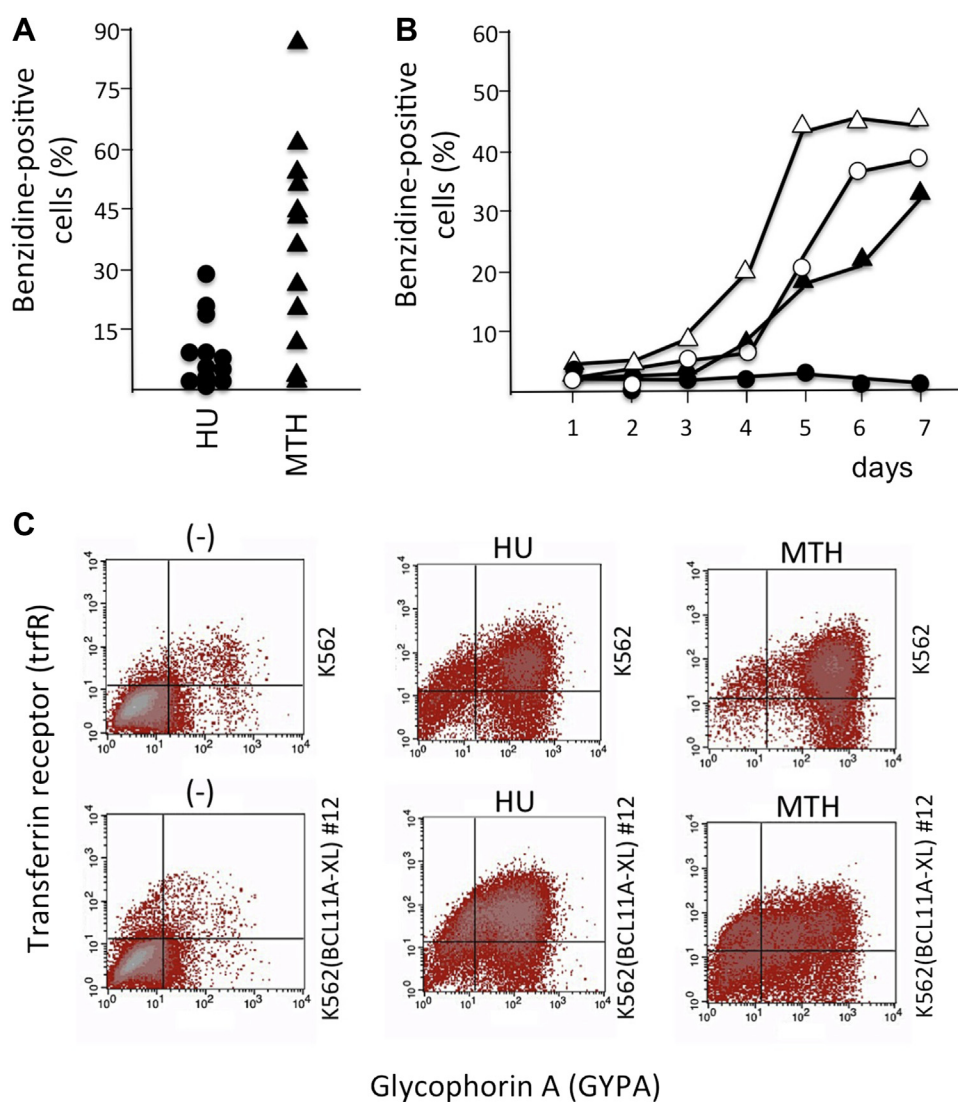
intermediate levels [K562(BCL11A-XL) clones 3, 8] and low levels [K562(BCL11A-XL) clones 1, 5, 6, 7, 9, 10, 11]. The different BCL11A-XL mRNA levels correspond to different levels of BCL11A protein, as illustrated by the Western blot analysis in [Figure 1C](#), which also includes a representative Western blot of proteins produced by

K562(BCL11A-XL) clone 12. The conclusion drawn from this first set of data is that we have generated a panel of K562(BCL11A-XL) clones expressing, unlike the original K562 cells, variable levels of K562(BCL11A-XL) mRNA and protein. The finding that the expression of BCL11A-XL differs among the clones allows studies on the possible relationship between BCL11A-XL and hemoglobinization induced by HbF inducers acting by different mechanisms of action.

#### Ability of K562(BCL11A-XL) clones to undergo induced erythroid differentiation

To determine possible relationships between BCL11A-XL levels and erythroid differentiation stimulated by HbF inducers, we employed two inducers of erythroid differen-

tiation, hydroxyurea (HU) [40] and mithramycin (MTH) [35–37], chosen because they have very different mechanisms of action. In fact, HU inhibits cellular ribonucleotide reductase (critical for DNA synthesis in dividing late erythroid progenitor cells), whereas MTH is a strong DNA-binding compound (selective for GC-rich regions, including important regulatory sequences present in the  $\gamma$ -globin gene promoter). Hemoglobin accumulation was studied with benzidine staining [35], which indicated that when the clones were treated for 4 days with 175  $\mu\text{mol/L}$  HU, only very few of them were able to undergo erythroid induction (Fig. 2A, left). On the contrary, 20 nmol/L MTH appears to induce hemoglobinization (i) in a larger number of K562(BCL11A-XL) clones and (ii) to a greater extent, in terms of percentage of benzidine-positive cells (Fig. 2A,



**Figure 2.** Ability of K562(BCL11A-XL) clones to undergo differentiation. (A) K562(BCL11A-XL) cell clones were treated for 4 days in the absence or presence of 175  $\mu\text{mol/L}$  HU and 20 nmol/L MTH, and the percentage of benzidine-positive (Hb-containing) cells was determined. (B) Kinetics of hemoglobinization of K562 ( $\circ$ ,  $\Delta$ ) and K562(BCL11A-XL) clone 12 ( $\bullet$ ,  $\blacktriangle$ ) treated with 175  $\mu\text{mol/L}$  HU ( $\circ$ ,  $\bullet$ ) and 20 nmol/L MTH ( $\Delta$ ,  $\blacktriangle$ ). (C) Fluorescence-activated cell sorting analysis revealed the expression of trfR and GYPA by K562 cells and by K562(BCL11A-XL) clone 12 treated with 175  $\mu\text{mol/L}$  HU and 20 nmol/L MTH for 4 days, as indicated.



right). This was further confirmed after analysis of the increase in the proportion of benzidine-positive cells in K562(BCL11A-XL) clone 12, exhibiting the highest levels of BCL11A-XL expression (Fig. 1B) and production (see Western blotting analysis illustrated in Fig. 1C). As illustrated in Figure 2B, the increase in the proportion of Hb-producing K562(BCL11A-XL) clone 12 cells is shifted when MTH is used, whereas induction does not occur in the presence of HU. To determine whether the lack of an increase in the proportion of benzidine-positive cells is associated with an inhibitory effect on other erythroid differentiation parameters, the expression of transferrin receptor (trfR) and glycophorin A (GYPA) was determined by FACS analysis in K562 cells and K562(BCL11A-XL) clone 12 cells treated with HU and MTH for 4 days. As illustrated in Figure 2C, the lack of an increase in benzidine-positive cells is not accompanied by a block of the inducer-mediated increase in trfR and GYPA expression. Although this was expected in MTH-treated K562(BCL11A-XL) clone 12 cells (in agreement with the analyses illustrated in Fig. 2B), this was not obvious in the same cells treated with HU. Moreover, it should be noted that the extent of trfR and GYPA expression in HU- and MTH-induced K562(BCL11A-XL) clone 12 cells approaches the values obtained in erythroid-induced K562 cells. When the fluorescence intensity reflecting trfR and GYPA expression in HU- and MTH-induced K562 and K562(BCL11A-XL) clone 12 cells was compared with that measured in untreated cells in three independent experiments, the differences were found to be reproducible and highly significant ( $p < 0.01$ ).

The overall conclusion of the experiments is that K562(BCL11A-XL) clones are induced to increased Hb production with low efficiency with HU, even if treated with high concentrations of the inducer and even if they express low levels of BCL11A-XL. On the contrary, most of the clones expressing BCL11A-XL and resistant to HU hemoglobin induction are able to increase Hb content with 20 nmol/L MTH, suggesting that this inducer might rescue the BCL11A-XL-mediated decrease in expression of globin genes (see, e.g., the data relative to clone 12 illustrated in Fig. 2B). We can conclude that the program of induced erythroid differentiation is not blocked in BCL11A-XL-expressing clones cultured with HU or MTH, as trfR and GYPA are not expressed during HU and MTH treatment, even when using as a model system the K562(BCL11A-XL) clone 12, expressing the highest level of BCL11A-XL (Fig. 2C).

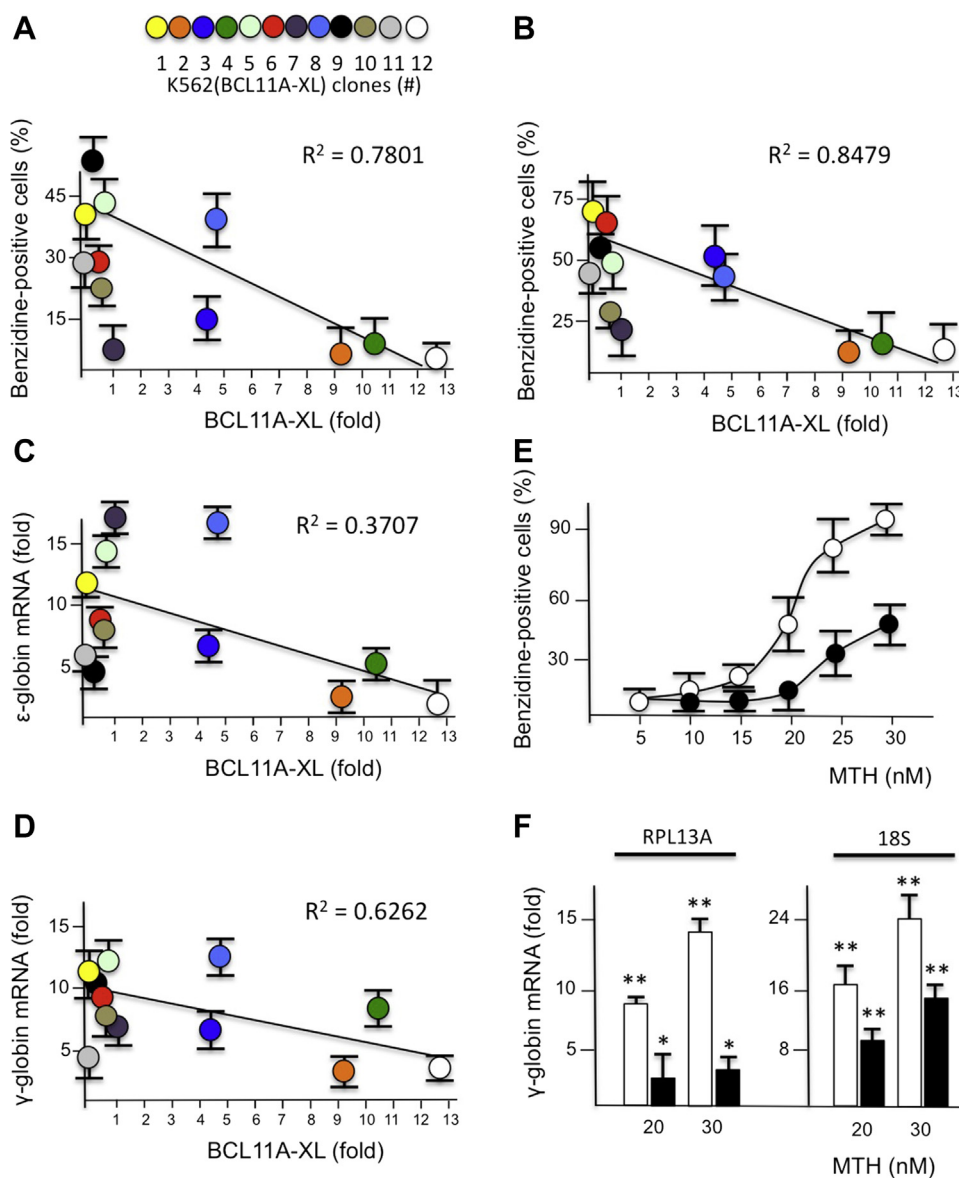
*BCL11A-XL production in K562(BCL11A-XL) clones: Possible correlation between BCL11A-XL, expression of globin genes, and hemoglobin production*

To acquire information supporting possible relationships between BCL11A-XL levels, hemoglobin induction, and globin gene expression, the experiments illustrated in

Figure 3A–D were performed using 20 nmol/L MTH. Figure 3A and B illustrates that an inverse trend does exist between the proportion of benzidine-positive cells and BCL11A-XL mRNA expression, when cells are treated with MTH for 4 (Fig. 3A) and 5 (Fig. 3B) days. In agreement, Figure 3 presents data supporting a possible inverse relationship between  $\epsilon$ -globin (Fig. 3C) and  $\gamma$ -globin (Fig. 3D) mRNA production and BCL11A-XL mRNA expression. The expression of  $\beta$ -globin genes was not considered because K562 cells express very low levels of this gene [26]. These results confirm that the small increase in hemoglobin production in MTH-treated K562(BCL11A-XL) clones expressing high levels of BCL11A-XL (Fig. 3A, B) is associated with low levels of accumulation of  $\epsilon$ -globin (Fig. 3C) and  $\gamma$ -globin (Fig. 3D) mRNAs. In particular, it should be underlined that treatment with 20 nmol/L MTH is able to induce a great increase in  $\gamma$ -globin mRNA, even in K562 clones expressing intermediate/high levels of BCL11A-XL. Altogether, these results suggest that BCL11A-XL activity is counteracted by MTH. This is further sustained by the experiments illustrated in Figure 3E and F, which were performed by treating K562 and K562(BCL11A-XL) clone 12 cells with increasing concentrations of MTH, as indicated. As is clearly appreciable, high MTH concentrations were able to induce increases in the proportions of both benzidine-positive cells (Fig. 3E) and  $\gamma$ -globin mRNA production (Fig. 3F), in a dose-dependent manner ( $p < 0.05$  when cells treated with 25 and 30 nmol/L MTH were compared with untreated cells in three independent experiments). In conclusion, the MTH-mediated increase in  $\gamma$ -globin mRNA might occur even in the presence of high BCL11A-XL expression, particularly when high MTH concentrations are employed. We therefore selected K562(BCL11A-XL) clone 12, which produces the highest level of BCL11A-XL, and determined the effects on these cells of MTH treatment, using high concentrations of the drug (30 nmol/L) to force the erythroid induction pathway.

*Mithramycin reverses BCL11A-XL-mediated inhibition of  $\gamma$ -globin mRNA production and hemoglobin induction in K562(BCL11A-XL) clone 12*

To verify the effects of different erythroid inducers on K562 cells expressing high levels of BCL11A-XL, K562 and K562(BCL11A-XL) clone 12 cells were treated with hydroxyurea [40], resveratrol [17], butyric acid [15], 1-octylthymine [41], rapamycin [18], and mithramycin [35–37] (Fig. 4). The data obtained indicate that all the inducers employed, as reported in previous studies by our and other groups [17,18,33,35,38–41], increased Hb production in K562 cells (Fig. 4A). In sharp contrast, among this selected group of HbF inducers, the only inducer able to stimulate an increased proportion of Hb-producing cells was mithramycin, when K562(BCL11A-XL) clone 12 was employed

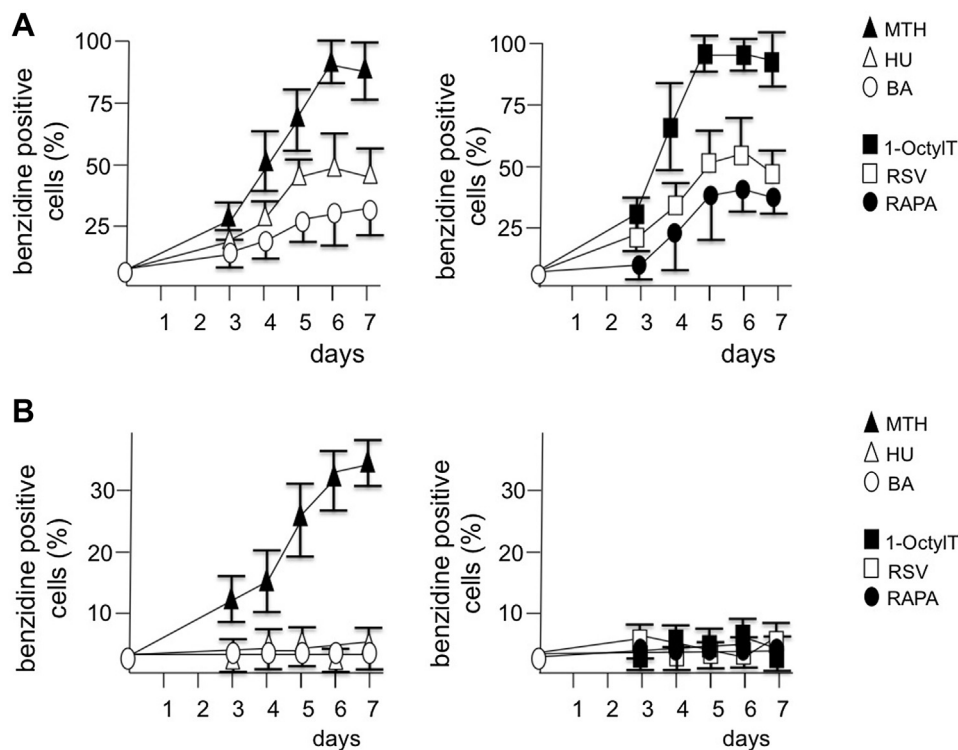


**Figure 3.** Ability of K562(BCL11A-XL) clones to increase HbF production and globin gene expression. (A–D) K562(BCL11A-XL) cell clones were treated for 4 days in the absence or presence of 20 nmol/L MTH, and the relative expression of BCL11A-XL mRNA (fold with respect to wild-type cells) was compared with the percentage of benzidine-positive cells: (A) cells treated for 4 days with MTH; (B) cells treated for 5 days with MTH; (C) fold increase in expression of  $\epsilon$ -globin mRNA; (D) fold increase in expression of  $\gamma$ -globin mRNA. (E) Effects of increasing concentrations of MTH on the percentage of benzidine-positive cells in K562 ( $\circ$ ) and K562(BCL11A-XL) clone 12 ( $\bullet$ ) cell cultures treated for 6 days. (F) Expression of  $\gamma$ -globin mRNA after treatment of K562 (white boxes) and K562(BCL11A-XL) clone 12 (black boxes) cells with 20 and 30 nmol/L MTH for 4 days, as indicated. Fold increase was determined with respect to untreated cells using RPL13A and 18S as reference sequences. The results represent averages  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$ . \*\* $p < 0.01$ .

(Fig. 4B). The conclusion drawn from this set of experiments is that BCL11A-XL-expressing clones are suitable to select K562 inducers possibly interfering with BCL11A, thereby inducing  $\gamma$ -globin mRNA expression and increases in Hb production.

To conclusively verify whether mithramycin induces full activation of the erythroid pattern in the K562(BCL11A-XL) clone 12, cells were treated for 4 days in the absence or presence of 30 nmol/L MTH, and the expression of

$\gamma$ -globin gene was analyzed, together with the expression of other erythroid-associated genes such as those coding *trfR*, *GYP A*, *NFE2L3*, *ALAS-2*, *DNMT* (*EBP49*), and *SCL4A1* (band 3). The results obtained indicate that the MTH-mediated induction in K562(BCL11A-XL) clone 12 is, as in the original K562 cell line, limited not only to the expression of  $\gamma$ -globin genes, but also to all the other erythroid genes analyzed, albeit at different levels (Fig. 5B, C).



**Figure 4.** Increase in proportion of hemoglobin-containing, benzidine-positive cells in K562 and K562(BCL11A-XL) clone 12 cells treated with different fetal hemoglobin inducers. K562 (A) and K562(BCL11A-XL) clone 12 cells (B) were cultured in the presence of 30 nmol/L MTH ( $\blacktriangle$ ), 175  $\mu$ mol/L HU ( $\triangle$ ), 1.5 mmol/L BA ( $\circ$ ), 25  $\mu$ mol/L 1-OctylIT ( $\blacksquare$ ), 150 nmol/L RAPA ( $\square$ ), and 30  $\mu$ mol/L RSV ( $\bullet$ ), and the proportion of benzidine-positive cells was determined. Data represent averages  $\pm$  SD of four independent experiments. The differences between inducer-treated and untreated K562 cells were highly significant ( $p < 0.01$ ) for all inducers, when data relative to 6 and 7 days of induction were considered. On the contrary, only K562(BCL11A-XL) clone 12 cells treated with MTH exhibited significant differences ( $p < 0.05$ ) with respect to untreated cells.

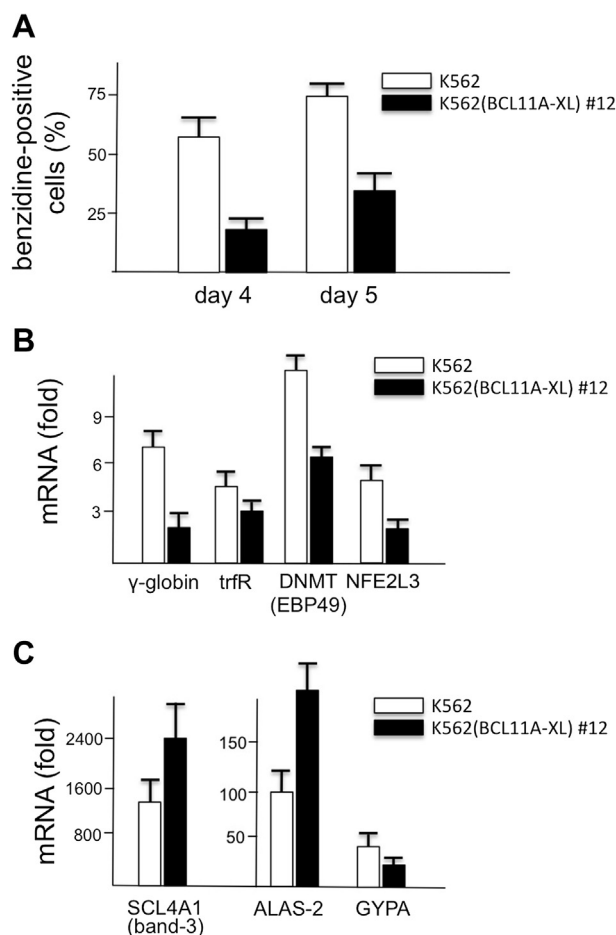
*Effects of MTH treatment on BCL11A-XL and globin gene expression in other erythroid cellular systems: Murine FLCs and human primary ErPCs from  $\beta$ -thalassemia patients*

One of the hypotheses derived from the experiments described in this article is that mithramycin might have a direct effect on BCL11A-XL activity. We therefore wanted to further verify whether increases in globin gene expression might be associated with downregulation of BCL11A-XL in other cellular erythroid model systems, such as murine FLCs [38,39] and human primary ErPCs isolated from  $\beta$ -thalassemia patients [34–36]. FLCs were cultured for 4 days with 400 nmol/L MTH or 2% DMSO, and the proportion of benzidine-positive cells was determined together with the levels of  $\alpha$ -globin, adult  $\beta^{\text{major}}$ -globin, embryofetal  $\beta\text{h1}$ -globin, and BCL11A mRNAs. The results obtained (Fig. 6A) illustrate a decrease in BCL11A mRNA expression in both MTH- and DMSO-treated FLCs, in association with an increase in the proportion of benzidine-positive cells and content of  $\alpha$ -globin, adult  $\beta^{\text{major}}$ -globin, and embryo-fetal  $\beta\text{h1}$ -globin mRNAs (in all cases  $p < 0.01$ ). In addition to the FLC system, erythroid precursor cells from  $\beta$ -thalassemia patients might be a very informative experimental model, because

MTH is a strong inducer of HbF in these cells, as originally reported by our group [35]. Figure 6B illustrates that when ErPCs from three  $\beta^+$ - and four  $\beta^0$ -thalassemia patients were isolated and cultured for 5 days with 30 nmol/L MTH, a sharp decrease in BCL11A-XL and increase in  $\gamma$ -globin mRNA were observed (in both cases  $p < 0.01$ ). Figure 6C depicts the relationship between MTH concentration and BCL11A-XL or  $\gamma$ -globin mRNA content in ErPCs from a  $\beta^039/\beta^039$  thalassemia patient. This experiment clearly underlines that the increase in  $\gamma$ -globin mRNA does not occur until downregulation of BCL11A-XL is induced by mithramycin. Similar results were obtained with ErPCs isolated from  $\beta^+$ -thalassemia patients (Finotti et al., manuscript in preparation).

### Discussion

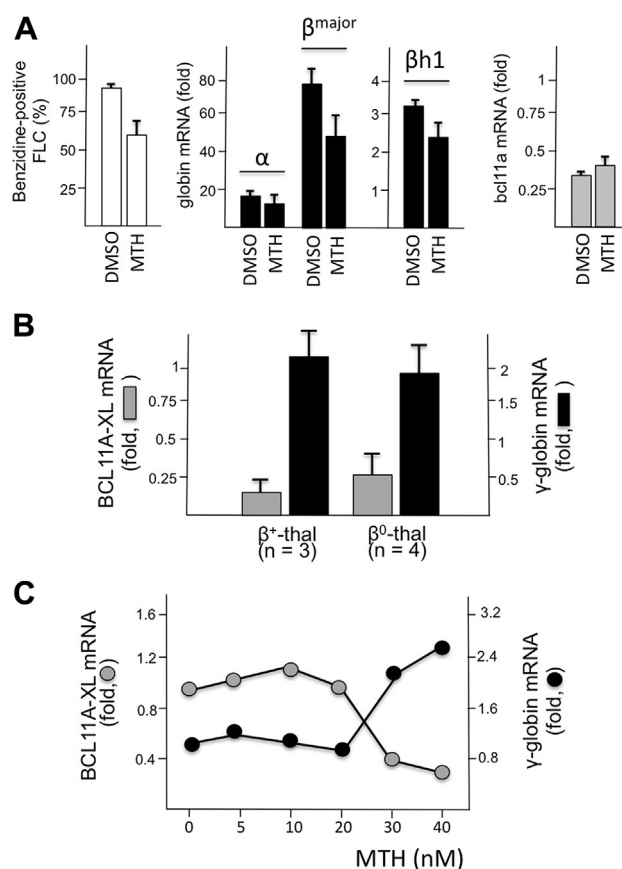
Induction of fetal hemoglobin (HbF) is considered a possible therapeutic strategy in the treatment of  $\beta$ -thalassemia [7,13–18,27], where the production of HbA is impaired by mutations affecting the  $\beta$ -globin gene [1–4]. Therefore, screening systems for the identification of novel lead compounds for induction of HbF in erythroid cells are of great interest in the field of applied hematology [42–44].



**Figure 5.** Effects of 30 nmol/L MTH treatment on erythroid differentiation markers. K562 (white boxes) and clone 12 (black boxes) cells were treated in the absence or presence of 30 nmol/L MTH. (A) Effects on the increase in the proportion of benzidine-positive cells (%) evaluated after 4 and 5 days of cell culture. (B, C) After RNA extraction, expression of the following mRNAs was analyzed in 4-day MTH-treated cells:  $\gamma$ -globin, trfR, DNMT (EBP49), NFE2L3 (B); and SCL4A1 (band-3), ALAS-2, GYPA (C). Data represent averages  $\pm$  SD of four independent experiments.

Recent results suggest that BCL11A-XL is a major repressor of  $\gamma$ -globin gene expression [21–23,29]. Therefore, interference with the binding of the BCL11A-XL transcriptional complex to the  $\gamma$ -globin gene promoter provides a novel approach to induce expression of the  $\gamma$ -globin genes [30–32]. To develop a cellular screening system for BCL11A-XL inhibitors, we produced K562 cell clones with integrated copies of a BCL11A-XL-expressing vector, taking advantage of the fact that very low levels of endogenous BCL11A-XL are present in K562 cells [26].

We characterized 12 K562 clones expressing different levels of BCL11A-XL (Fig. 1) and found that a clear association does exist between the levels of BCL11A-XL and the extent of hemoglobin production obtained by treatment with MTH (Fig. 3). In addition, we found that this molecule was the only one, among a short list of Hb inducers (Fig. 4),



**Figure 6.** Effects of MTH treatment on BCL11A-XL and globin gene expression in murine FLCs and ErPCs from  $\beta$ -thalassemia patients. (A) FLCs were treated for 4 days with 2% DMSO or 400 nmol/L MTH. The proportion of benzidine-positive cells (left), content of globin mRNAs (middle), and content of Bcl11a (right) were evaluated. (B) ErPCs from three  $\beta^+$ - and four  $\beta^0$ -thalassemia patients were isolated and cultured for 5 days with 30 nmol/L MTH. BCL11A-XL and  $\gamma$ -globin mRNAs were analyzed by RT-qPCR. (C) Relationship between MTH concentration and content of BCL11A-XL and  $\gamma$ -globin mRNAs in ErPCs from a  $\beta^{0/39}/\beta^{0/39}$  thalassemia patient. ErPCs were cultured for 5 days with increasing concentrations of MTH, RNA was isolated, and RT-qPCR performed. The data (A) represent averages  $\pm$  SD of three independent cultures.

able to rescue the ability to differentiate along the erythroid program, even when K562 cell clones expressing high levels of BCL11A-XL were used, suggesting that the BCL11A-XL inhibitory activity of the  $\gamma$ -globin gene expression is counteracted by MTH.

Interestingly, a site recognized by MTH is present in the  $\gamma$ -globin gene promoter and corresponds to a region specific for the binding of the BCL11A complex (Finotti et al., manuscript in preparation). Additional experiments performed using electrophoretic mobility shift assay (EMSA) as experimental strategy indicated that MTH interferes with the molecular interactions between  $\gamma$ -globin gene promoter and DNA-binding proteins, strongly suggesting that the activity of MTH on  $\gamma$ -globin gene transcription might be, at least in part, ascribed to a direct



inhibitory effect on the binding of molecular protein complexes to the BCL11A binding site of the  $\gamma$ -globin gene promoter (Finotti et al., manuscript in preparation).

To sustain the concept that the increase in expression of  $\gamma$ -globin genes is inversely associated with BCL11A-XL content, murine FLCs [38,39] and ErPCs isolated from  $\beta$ -thalassemia patients were employed, induced by MTH, and analyzed for content of globin and BCL11A-XL mRNAs. Fully in agreement with the conclusions derived from the data on K562(BCL11A-XL) clones, downregulation of BCL11A-XL was observed in FLCs and ErPCs following a MTH-induced increase in globin mRNAs production ( $\beta^{\text{major}}$ - and  $\beta\text{h1}$ -globin mRNAs in FLCs,  $\gamma$ -globin mRNA in ErPCs).

### Conclusions

We have suggested a possible application of K562 cell clones stably transfected with constructs expressing the BCL11A-XL gene in the identification of inducers able to stimulate HbF production via downregulation of the  $\gamma$ -globin gene repressor BCL11A-XL. A possible limitation of the proposed model system is that the use of modifiers acting on the promoter of the endogenous BCL11A gene or on other regulatory pathways of the endogenous BCL11A mRNA might require suitable internal controls and additional experimental strategies. On the other hand, the advantages of this system, unlike primary ErPCs, are: (i) availability of clones expressing different well-characterized levels of BCL11A-XL; (ii) possibility of obtaining large amounts of these cells for molecular studies; (iii) possible use for screening (results available within 1 week); and (iv) absence of genetic variability affecting  $\gamma$ -globin gene expression. In addition, our strategy, at least in theory, might be followed to produce K562 cell clones expressing different levels of other transcription repressors of the  $\gamma$ -globin gene, such as Myb [24,29], Oct-1 [25], KLF-1 [23,26], and LYAR [19].

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## Supplementary methods

### *Cell lines and culture conditions*

Human erythromyeloblastoid leukemia K562 cells [33–37], K562(BCL11A-XL) clones, and murine Friend leukemia cells (FLCs) [38,39] were cultured in a humidified atmosphere of 5% CO<sub>2</sub>/air in RPMI-1640 medium (Gibco Life Technologies, Monza, Italy) supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest, Nuaille, France), 100 U/mL penicillin, and 100 µg/mL streptomycin. Treatment of cells with erythroid differentiation inducers was carried out by adding the appropriate drug concentrations at the beginning of the cultures [(K562 and K562(BCL11A-XL) clone cells were seeded at 30,000/mL; FLCs were seeded at 70,000/mL)]. The medium was not changed during the induction period. Hemoglobin-containing cells were detected by specific reaction with a benzidine/hydrogen peroxide solution containing 0.2% benzidine HCl (Sigma Genosys) in 5 mol/L glacial acetic acid, pre-activated with a 10% (v/v) solution of 30% H<sub>2</sub>O<sub>2</sub> [35].

### *In vitro culture of erythroid progenitors from β-thalassemia patients*

The two-phase liquid culture procedure was employed as previously described [17,18,34–36]. Mononuclear cells were isolated from peripheral blood samples of β-thalassemia patients by Ficoll–Hypaque density gradient centrifugation. After isolation, the mononuclear cell layer was washed three times by adding 1 × phosphate-buffered saline (PBS) solution and seeded in α-minimal essential medium (α-MEM, Sigma Genosys) supplemented with 10% FBS (Celbio, Milan, Italy), 1 µg/mL cyclosporine A (Sandoz, Basel, Switzerland), and 10% conditioned medium from the 5637 bladder carcinoma cell line culture. The cultures were incubated at 37°C, under an atmosphere of 5% CO<sub>2</sub>. After 7 days in this phase I culture (at 37°C, under an atmosphere of 5% CO<sub>2</sub> in air, with extra humidity), nonadherent cells were harvested from the flask, washed in 1 × PBS, and then cultured in phase II medium, composed of α-MEM (Sigma Genosys), 30% FBS (Celbio), 1% de-ionized bovine serum albumin (BSA, Sigma Genosys), 10<sup>-5</sup> mol/L β-mercaptoethanol (Sigma Genosys), 2 mmol/L L-glutamine (Sigma Genosys), 10<sup>-6</sup> mol/L dexamethasone (Sigma Genosys), 1 U/mL human recombinant erythropoietin (EPO) (Tebu-bio, Magenta, Milan, Italy), and stem cell factor (SCF, BioSource International, Camarillo, CA) at the final concentration of 10 ng/mL. Erythroid precursor cell differentiation was assessed by benzidine staining [17,18,34–36].

### *FACS analyses*

The erythroid differentiation status of K562 cells and K562(BCL11A-XL) clone 12 was investigated by studying transferrin receptor (trfR) and glycophorin A (GYPA) expression by fluorescence-activated cell sorting (FACS)

analysis using anti-human CD71 fluorescein isothiocyanate (FITC)-conjugated antibody (Miltenyi Biotec, Germany) and anti-human CD235a (glycophorin A) phycoerythrin (PE)-conjugated antibody (Miltenyi Biotec) [36]. According to the manufacturer's protocol, 10 µL of antibodies were added to freshly isolated cells in 100 µL 1 × PBS and 1% FBS, and cells were incubated on ice for 30 min. Cells were washed twice in 1 × PBS and 1% FBS and analyzed using the BD FACScan system (Becton, Dickinson, Italy).

### *RNA extraction*

Cells were isolated by centrifugation at 500 g for 10 min at 4°C, washed in PBS, and lysed in Tri-Reagent (Sigma-Aldrich), according to the manufacturer's instructions. The isolated RNA was washed once with cold 75% ethanol, dried, and dissolved in DNase/RNase-free water before use.

### *Reverse transcription and RT-qPCR*

For gene expression analysis, 300 ng of total RNA was reverse transcribed to cDNA using the Taq-Man Reverse Transcription PCR Kit and random hexamers (Applied Biosystems) in a 50 µL reaction. Real-time quantitative polymerase chain reaction (RT-qPCR) experiments were carried out using the 5'-nuclease assay with the primers and probes indicated in [Supplementary Tables E1 and E2](#). The cDNA (1 µL) was amplified for 40 PCR cycles using the Taq Man Universal PCR Master Mix 2X (Applied Biosystems) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relative expression was calculated using the comparative cycle threshold method, and the endogenous controls human 18S rRNA, human RPL13A, and murine GAPDH were used as normalizer genes. Duplicate negative controls (no template cDNA) were also run with every experimental plate to assess specificity and to rule out contamination. RT-qPCRs were performed in duplicate for both target and normalizer genes.

### *Protein extract preparation*

Treated or untreated K562 cells and K562(BCL11A-XL) clones (2 × 10<sup>5</sup> cells) were washed three times with cold PBS and centrifuged at 300 g for 10 min at 4°C. Then, cellular pellets were suspended in 50 µL cold water, frozen with dry ice for 5 min, and vortexed for 10 sec. This step was repeated four times in succession, samples were finally centrifuged at 14,000 g for 20 sec, and supernatant cytoplasmic fractions were collected and immediately frozen at -80°C.

### *Western blotting*

Twelve micrograms of cytoplasmic extract were denatured for 5 min at 98°C in 1 × SDS sample buffer (62.5 mmol/L Tris-HCl pH 6.8, 2% SDS, 50 mmol/L dithiothreitol [DTT], 0.01% bromophenol blue, 10% glycerol) and loaded on SDS-polyacrylamide gel (10 × 8 cm) in Tris-glycine buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS).

A biotinylated protein ladder (size range: 9–200 kDa) (Cell Signaling, Euroclone, Pero, MI, Italy) was used as standard to determine molecular weight. The electrotransfer to 0.2- $\mu$ m nitrocellulose membrane (Pierce, Euroclone) was performed overnight at 360 mA and 4°C in electrotransfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 5% methanol). The membranes were prestained in Ponceau S solution (Sigma-Aldrich) to verify the transfer, washed with 25 ml TBS (10 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl) for 10 min at room temperature, and incubated in 25 mL of blocking buffer for 2 hours at room temperature. The membranes were washed three times for 5 min each with 25 mL of TBS/T (TBS, 0.1% Tween-20) and incubated with BCL11A primary rabbit monoclonal antibody (1:1,000) (Catalog No. A300-382A, Bethyl, Montgomery, TX) in 15 mL primary antibody dilution buffer, under gentle agitation, overnight at 4°C. The day after, the membranes were washed three times for 5 min each with 20 mL of TBS/T and incubated in 15 mL of blocking buffer, under

gentle agitation for 2 hours at room temperature, with an appropriate horseradish peroxidase-conjugated secondary antibody (1:2000) and a horseradish peroxidase-conjugated anti-biotin antibody (1:1,000) used to detect biotinylated protein marker. Finally, after three washes each with 20 mL of TBS/T for 5 min, the membranes were incubated with 10 mL LumiGLO (0.5 mL 20 $\times$  LumiGLO, 0.5 mL 20 $\times$  peroxide, and 9.0 mL Milli-Q water) (Cell Signaling) under gentle agitation for 5 min at room temperature and exposed to X-ray film (Pierce). As necessary, after the stripping procedure using the Restore Western Blot Stripping Buffer (Pierce), membranes were reprobated with primary and secondary antibodies. X-Ray films for chemiluminescence blots were analyzed with Gel Doc 2000 (Bio-Rad Laboratoires, Milan, Italy) using the Quantity One program to elaborate the intensity data of our specific target protein. The primary antibody against p70S6K (Catalog No. 2708, Cell Signaling) was used as normalization control.

**Supplementary Table E1.** TaqMan assays obtained from Applied Biosystems and PrimeTime qPCR Assays obtained from Integrated DNA Technologies employed in RT-qPCR experiments (primer and probe sets)

Gene name	Species	Company	Assay ID
B-Cell lymphoma/leukemia 11A (BCL11A) <sup>a</sup>	Human	Applied Biosystems <sup>b</sup>	Hs00256254_m1
BCL11A <sup>c</sup>	Human	Applied Biosystems	Hs00250581_s1
$\zeta$ -Globin	Human	Applied Biosystems	Hs00923579_m1
Nuclear factor erythroid 2-like 3 (NFE2L3)	Human	Applied Biosystems	Hs00852569_g1
$\delta$ -Aminolevulinatase synthase 2 (ALAS-2)	Human	Applied Biosystems	Hs00163601_m1
Dematin actin binding protein (DNMT [EBP49])	Human	Applied Biosystems	Hs00157387_m1
Solute carrier family 4 member 1 (SCL4A1 [band3])	Human	Applied Biosystems	Hs00978603_m1
18S ribosomal RNA	Human	Applied Biosystems	4310893E
Ribosomal protein L13a (RPL13A)	Human	Applied Biosystems	Hs03043885_g1
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Mouse	IDT <sup>d</sup>	Mm.PT.39a.1

<sup>a</sup>This assay was used for amplification of BCL11A transcripts in K562(BCL11A-XL) clones.

<sup>b</sup>Applied Biosystems, Monza, Italy.

<sup>c</sup>This assay was used for amplification of BCL11A transcripts in erythroid precursor cells from  $\beta$ -thalassemia patients.

<sup>d</sup>Integrated DNA Technologies, Coralville, IA.



**Supplementary Table E2.** Sequences of primer and probe sets for real-time quantitative polymerase chain reaction

Gene name	Species	Primer forward	Primer reverse	Probe
$\gamma$ -Globin	Human	5'-TGG CAA GAA GGT GCT GAC TTC-3'	5'-TCA CTC AGC TGG GCA AAG G-3'	5'-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3'
$\alpha$ -Globin	Human	5'-CAC GCG CAC AAG CTT CG-3'	5'-AGG GTC ACC AGC AGG CAG T-3'	5'-FAM-TGG ACC CGG TCA ACT TCA AGC TCC T-TAMRA-3'
Transferrin receptor (trfR)	Human	5'-TCA GAG CGT CGG GAT ATC G-3'	5'-TGA ACT GCC ACA CAG AAG AAC A-3'	5'-FAM-TGG CGG CTC GGG ACG GA-TAMRA-3'
GlycophorinA (GYPA)	Human	5'-CGG TAT TCG CCG ACT GAT AAA-3'	5'-AAA GGC AGT CTG TGT CAG GT-3'	5'-FAM-AAA GCC CAT CTG ATG TAA AAC CTC TTC CCC T-TAMRA-3'
$\alpha$ -Globin	Mouse	5'-CTG ACC TCC AAG TAC CGT TAA G-3'	5'-GCT TCT TCC TACTCA GGCTTT AT-3'	5'-/56-FAM/TCT CTC CCT/ZEN/TGC ACC TGT ACC TCT/3IABkFQ/-3'
$\beta$ -Globin (major)	Mouse	5'-GGA AAG GTG AAC TCC GAT GAA-3'	5'-TGA TAG CAG AGG CAG AGG ATAG-3'	5'-/56-FAM/CTT TGG ACC/ZEN/CAG CGG TAC TTT GAT/3IABkFQ/-3'
$\beta$ h1-Globin	Mouse	5'- GGG AAG GCT CCT GAT TGT TTA-3'	5'- TCT TGC CAT GGG CTC TAATC-3'	5'-/56-FAM/TTG GAA ACC/ZEN/TCT CTT CTG CCCTGG/3IABkFQ/-3'
BC111a	Mouse	5'- TTG TAA ACG TCC TTC CCC AC-3'	5'- TGT AGC AAT CTC ACT GTC CAC-3'	5'-/56-FAM/AGT AGC AAG/ZEN/CTC ACC AGG CAC AT/3IABkFQ/-3'