UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

DOTTORATO DI RICERCA IN BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE XXII ciclo

Structural and functional characterization of CSDA protein complexes involved in the modulation of fetal globin gene expression

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Riassunto

La persistenza ereditaria di emoglobina fetale (HPFH) è una sindrome benigna caratterizzata da alti livelli di emoglobina fetale in età adulta. Questo tipo di condizione è di notevole interesse poichè può migliorare notevolmente il quadro clinico nelle β-talassemie o in altre forme di emoglobinopatie. I livelli di emoglobina fetale sono regolati da meccanismi molecolari molto complessi che possono coinvolgere elementi in cis rispetto al locus β-globinico o fattori esterni a tale locus genico. Allo scopo di individuare fattori trascrizionali potenzialmente coinvolti nella regolazione dell'espressione dei geni β-globinici, abbiamo analizzato il trascrittoma reticolocitario di tre fratelli che, pur presentando lo stesso genotipo β- e β-globinico, mostravano diversi livelli di emoglobina fetale e differente espressione clinica di una forma di β-talassemia intermedia. Mediante esperimenti di differential mRNA display abbiamo identificato il cDNA della cold shock domain protein A (CSDA), un fattore trascrizionale per il quale era già precedentemente ipotizzato un possibile ruolo di modulatore dell'espressione dei geni β-globinici. Studi di espressione nella linea eritroleucemica umana K562 e in cellule eritroidi primarie hanno mostrato una correlazione inversa esistente tra i livelli di espressione della β-globina e quelli di CSDA. Esperimenti di Chromatin Immunoprecipitation (ChIP) e saggi di attività trascrizionale nella linea eritroleucemica umana K562 hanno mostrato che CSDA può legare il promotore β-globinico e reprimerne l'espressione trascrizionale. Dall'analisi dei profili di espressione delle diverse isoforme di CSDA è risultato che le due principali isoforme presenti in cellule di origine eritroidi sono l'isoforma *a* che corrisponde all'intera

sequenza codificante (esoni 2-9) e l'isoforma *b* che, in seguito a un meccanismo di splicing alternativo, differisce dall'isoforma *a* per l'assenza della regione corrispondente all'esone 6. Inoltre, l'isoforma *a* è risultata maggiormente espressa in soggetti adulti normali rispetto a pazienti con HPFH o a cellule eritroidi di tipo embrio-fetali. Poiché le due isoforme differiscono nella regione C-terminale che è coinvolta in meccanismi di interazione proteina-proteina, al fine di identificare gli interattori molecolari delle due isoforme di CSDA, sono stati effettuati esperimenti di immunoprecipitazione su cellule K562 trasfettate con costrutti esprimenti l'isoforma *a* o *b* di CSDA. L'analisi mediante Western blot su tali immmunoprecipitati ha mostrato la presenza di NF-kB e della deacetilasi istonica 2 (HDAC2) solo nelle proteine co-immunoprecipitate con l'isoforma *a* di CSDA ma non in quelle co-immunoprecipitate con l'isoforma *b*.

Inoltre, al fine di valutare il possibile ruolo di NF-kB e di deacetilasi istoniche nel meccanismo di repressione trascrizionale dei geni β-globinici mediato da CSDA, sono stati effettuati trattamenti con Bortezomib, un farmaco che impedisce la traslocazione nel nucleo del complesso p65-p50 di NF-kB, e con la tricostatina A, un inibitore di deacetilasi istoniche. In entrambi i casi, i risultati ottenuti mostrano un aumento dell'espressione dei geni β-globinici, in seguito a questi trattamenti farmacologici, confermando il ruolo di NF-kB e delle deacetilasi istoniche nel meccanismo di repressione trascrizionale dei geni globinici fetali.

Esperimenti di Chromatin Immunoprecipitation hanno inoltre messo in evidenza che tali trattamenti sono in grado di modificare il grado di acetilazione istonica nella regione del promotore β-globinico che lega CSDA.

Nel complesso questi dati dimostrano che NF-kB e HDAC2 partecipano con l'isoforma *a* di CSDA nella formazione di un complesso multiproteico coinvolto nella repressione trascrizionale dei geni globinici fetali attraverso un meccanismo di regolazione del grado di acetilazione istonica.

Introduction

The switch from fetal to adult globin gene expression occurs around birth when fetal globin genes are progressively silenced thereby leading to a gradual decline of fetal hemoglobin (HbF) (Fig. 1 and 2). Impaired hemoglobin switching leads to the persistence of the expression of fetal globin genes throughout adulthood. This condition is without clinical relevance except when co-inherited with hemoglobinopathies. In fact, HPFH (Hereditary Persistence of Fetal Hemoglobin) has great therapeutic potential because high levels of HbF reduce the α/non α globin chain imbalance and thus ameliorate the thalassemic and sickle cell disease phenotypes.[1-4] Consequently, there is a race to clarify the molecular basis of hemoglobin switching and persistence of high HbF levels in the hope of identifying new therapeutic tools for thalassemia and sickle cell disease.

Together with epigenetic factors such as age and gender, several genetic determinants *in cis* to the β-globin gene cluster as well as HPFH quantitative trait loci (QTL) (6q23, 8p, Xp22.2-23) unlinked to the β-globin gene cluster influence HbF levels.[5-12] However, little is known about the *trans*-acting factors mapping on such external loci, although experimental evidence suggests that HBS1L and MYB, whose genes map on chromosome 6q23, and BCL11A, a zinc-finger protein encoded by a more recently described QTL on chromosome 2p15, may modulate HbF production in adult life.[13-20] However, direct interactions between these factors and globin genes have not yet been reported and therefore the molecular mechanisms by which they affect HbF levels remain unclear.

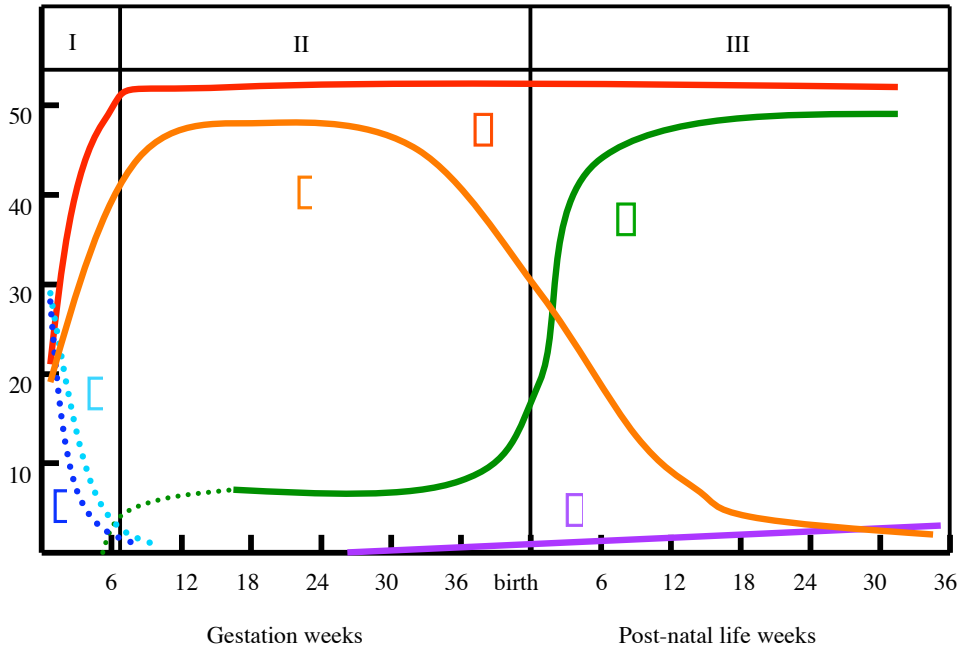


Figure 1: **Schematic representation of the hemoglobin switching during development.**

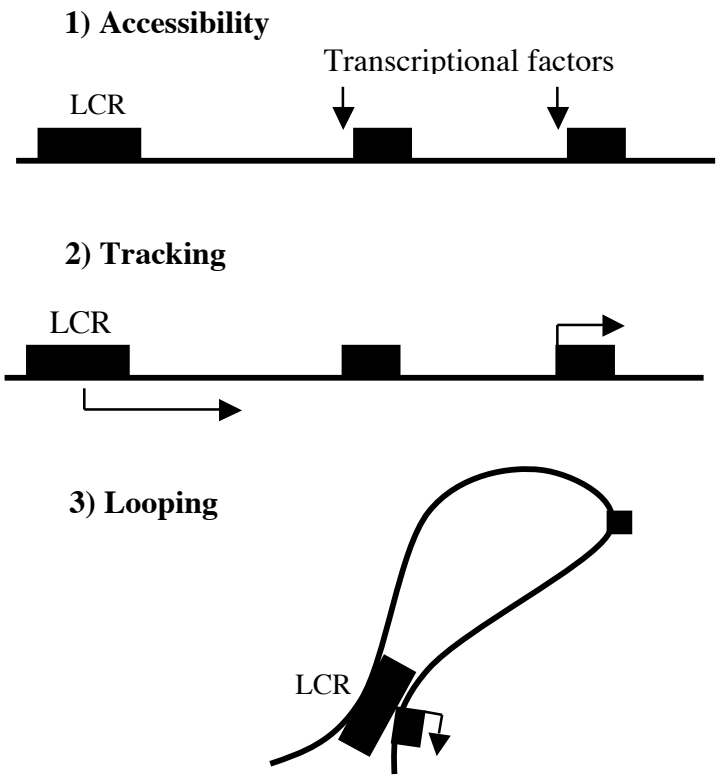


Figure 2: **Hemoglobin switching: models of functional interaction between the LCR and globin gene promoter regions .**

CSDA is a member of the protein family, also called Y-box proteins, characterized by a cold shock domain (CSD) which is highly conserved throughout evolution from bacteria to mammals[40-43]. CSD proteins have three functional domains: an N-terminus, the central CSD and a C-terminal domain. The N-terminus region of the protein contribute to single-stranded DNA binding; the central cold shock domain contains an RNP1 motif that is essential for sequence-specific DNA and RNA binding, whereas the C-terminus of the protein has alternating basic and acidic domains and has been implicated in both no-sequence specific RNA binding and protein-protein interactions with transcriptional regulators like RelA, ZO-1, TATA binding protein, NF-Y, YY-1 and AP-2[42] (Fig. 3). On the basis of all these binding activities, CSD proteins have been shown to be involved in transcriptional activation and repression, as well as post-transcriptional mechanisms of gene expression regulation, including mRNA packaging, transport, localization and stability.

The CSDA gene, located at position 12p13.1, contains 10 exons spanning 24 kb of genomic DNA, with the cold shock domain being encoded by exons 2-5. The C-terminal domain is instead encoded by exons 5-8, with exon 6 alternatively spliced. As consequence of alternative splicing, different C-terminal domains may be expressed, potentially able to take part to distinct protein complexes. CSDA is able to recognize H-DNA structures generated by homopyrimidine tracts[46]. HPFH point mutations (Gγ-202 C→G or C→T) occur in a region with homopyrimidine tracts resulted to be essential for γ-globin gene repression[38] and abolish the high-affinity binding sites for CSDA[46] by disrupting the formation of the intramolecular triplex. In this

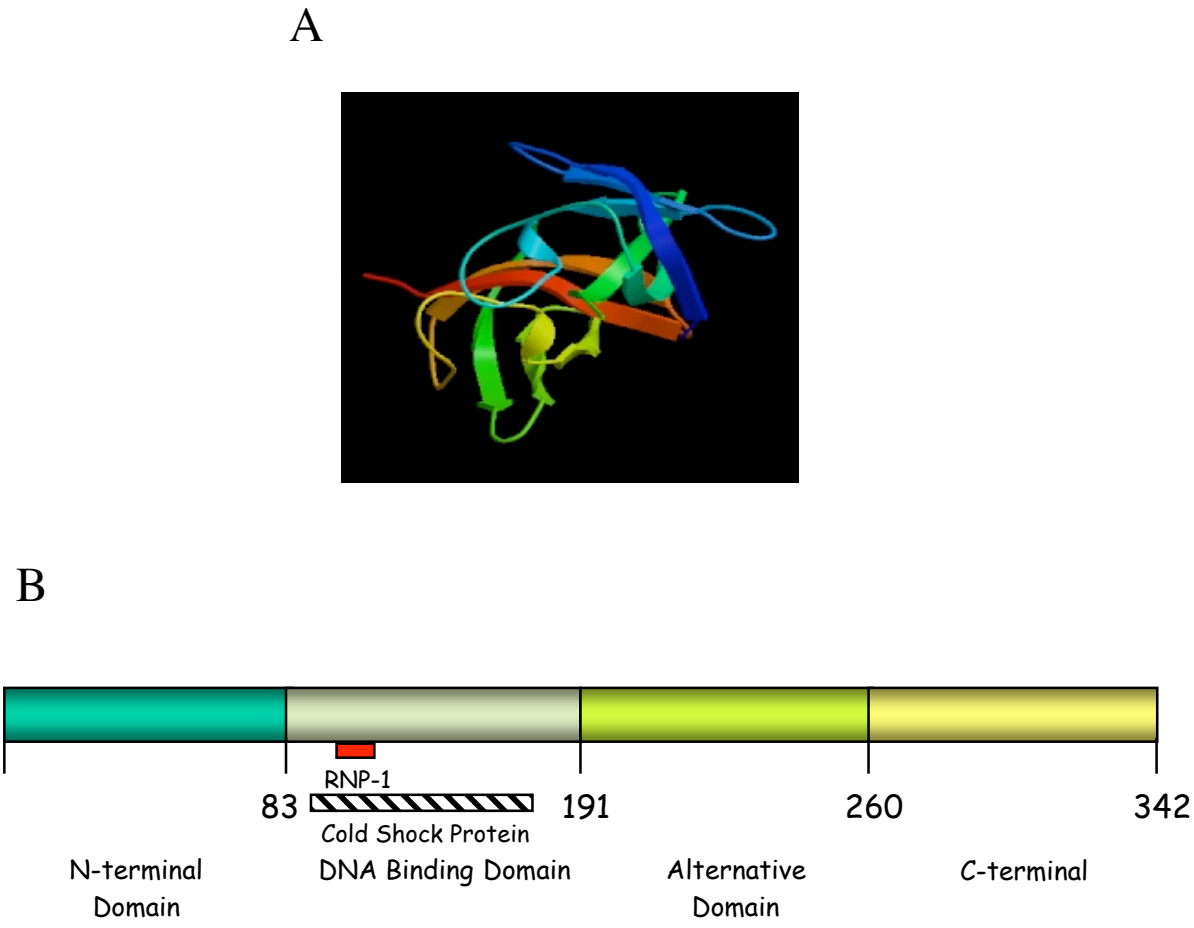


Figure 3: **Protein structure CSDA.** (A) 3D model of CSDA protein; (B) schematic representation of CSDA protein domains.

way, reduced binding of CSDA in the -200 Gγ-promoter region leads to persistent expression of γ-globin genes in adult life (Fig. 4).

This study was aimed at identifying factors and characterizing protein complexes putatively involved in modulation of γ-globin gene expression. Our findings provide experimental evidence that CSDA expression levels modulate γ-globin gene expression in adult life. Furthermore, characterization of CSDA interactors shed light on the molecular mechanisms involved in this regulation and could lead to innovative gene therapy approaches in hemoglobinopathies.

Horwitz et al. JBC, 1994 (269): 14130-9

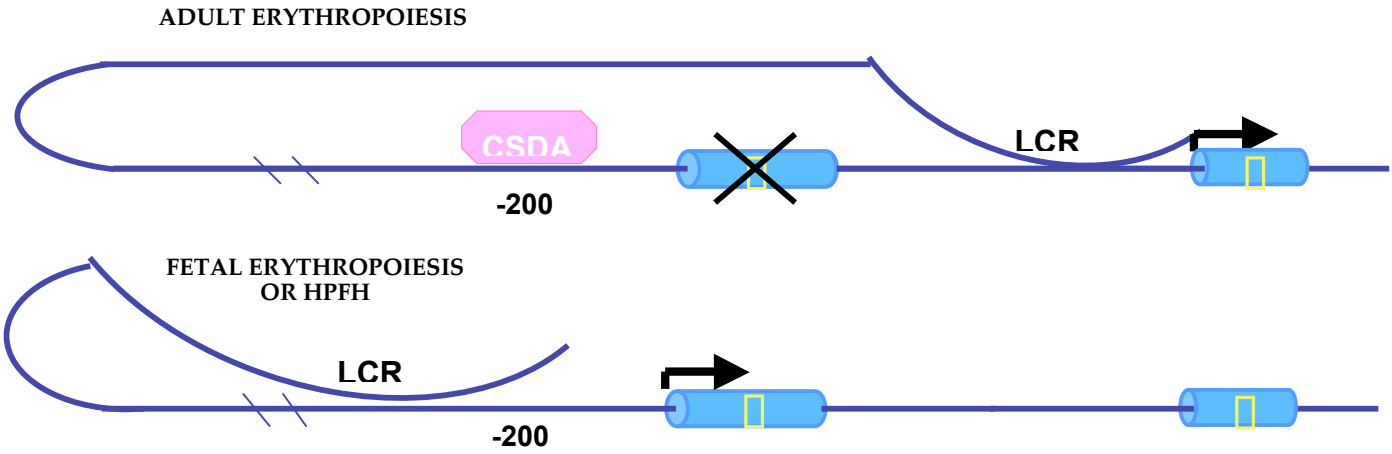


Figure 4: **Schematic representation of a functional model between CSDA and the β-globin promoter.**

Materials and methods

Patients and hematological data

Thalassemia intermedia was diagnosed in three adult siblings. The eldest brother underwent splenectomy at the age of 33 years and was on chronic transfusion therapy since his thirties. His two sisters received blood transfusions only occasionally during their pregnancies. Other five thalassemic patients were enrolled as control in this study. Table 1 lists the hematological and clinical data of all examined patients.

Genetic analysis of α- and β-globin gene clusters

Screening for α- and β-globin gene mutations and polymorphisms was carried out on DNA obtained from peripheral leucocytes by reverse dot-blot, sequencing or Southern blot analysis as reported elsewhere after local Ethics Committee approval and written informed consent were obtained. The β-globin gene cluster haplotypes were determined as reported previously.[21]

RNA isolation, differential mRNA display, quantitative real-time RT-PCR and Western blot

Total RNA was isolated from peripheral reticulocytes according to a recently described procedure.[22] Differential mRNA display analysis was performed using the Delta Differential Display kit (BD Biosciences-Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. cDNA for quantitative real-time analysis of CSDA and β-globin gene mRNAs was synthesized using an MMLV reverse transcriptase (Invitrogen, Carlsbad, CA,

Table 1: Clinical, hematological data at diagnosis and β-globin gene genotypes of the patients examined in this study.

Patient	Sex	Age (yr)	β-thalassemia genotype	Transfusion therapy	Chelation therapy	Hb (g/dL)	HbF (%)	Hb A2 (%)	Splenectomy/ Cholecystectomy	β-thalassemia phenotype
I-1	M	58	IVSI-6/ IVSI-6	Occasionally transfused until his thirties, transfusion dependent for the last 20 years	Yes	6.5	6.0	5.9	yes/yes	severe intermedia
I-2	F	40	IVSI-6/ IVSI-6	Occasionally	No	8.9	27.6	6.0	no/no	intermedia
I-3	F	38	IVSI-6/ IVSI-6	Occasionally	No	8.6	14.5	6.8	no/yes	intermedia
C1	M	40	IVSI-6/ IVSI-6	Transfusion-dependent since his childhood	Yes	6.2	2.9	7.2	yes/yes	severe intermedia
C2	F	45	β°39/β°39	Occasionally transfused until her thirties, transfusion dependent for the last 7 years	Yes	7.8	above 90%	5.2	yes/yes	severe intermedia
C3	M	42	IVSI-6/ IVSI-6	Occasionally	No	8.1	13.2	6.2	no/no	intermedia
C4	M	37	IVSI-6/ IVSI-6	Occasionally	No	8.5	12.8	6.3	no/no	intermedia
C5	M	38	β°39/β°39	Transfusion-dependent since his infancy	Yes	5.4	above 90%	3.8	yes/yes	thalassemia major

USA) from 1 μg of total RNA. Selected bands were cloned in a pGEM T-vector (Promega, Madison, WI, USA) and sequenced on a ABI-Prism 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Comparative sequence searches were performed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Quantitative real-time RT-PCR was carried out with an iCycler instrument (Bio-Rad Laboratories, Hercules, CA, USA) using the SyBR Green master mix (Bio-Rad Laboratories) and the manufacturer's protocol. Primers were designed using the Primer Express 2.0 program (sequences available on request). All data were normalized using endogenous β_2-microglobulin mRNA as control.[23, 24]

CSDA protein levels were evaluated by Western blot with the 4D9 monoclonal antibody against CSDA (Sigma-Aldrich, Saint Louis, MO, USA) using a procedure described previously. [25]

Cell cultures

K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum plus 4 mM glutamine, 10 U/mL penicillin and 10 mg/mL streptomycin at 37°C in a humidified 5% CO_2-containing atmosphere. Cell cultures were kept sub-confluent and transfected for CSDA RNA interference (RNAi) or over-expression analysis.

Isolation and culture of primary erythroid cells

$CD34^+$ cells were isolated in high purity using MACS magnetic separation system (Miltenyi Biotec GmbH, Bergisch, Germany), according to manufacturer's instructions, from buffy coats of healthy blood donors at our

local Transfusion Centre, after informed consent had been obtained. To induce erythroid differentiation, cells were kept for one week in IMDM medium (Mascia Brunelli, Milan, Italy) supplemented with 20% fetal calf serum (HyClone, Thermo Scientific, Logan, UT, USA) plus 10 ng/ml Stem Cell Factor (Peprotech, Hamburg, Germany), 1u/ml erythropoietin (Sigma-Aldrich, St. Louis, MO, USA), 1ng/ml interleukin 3 (Peprotech), 10 U/mL penicillin (BioWhittaker, Basel, Switzerland) and 10 mg/mL streptomycin (BioWhittaker) at 37°C in a humidified 5% CO_2-containing atmosphere, according to procedures previously described. [26, 27]

Cells were stained with anti CD34 phycoeritrin (PE)-conjugated (Pharmigen/Becton Dickinson, San Diego, CA) and CD71 fluorescein isothiocyanate (FITC)-conjugated (Pharmigen/Becton Dickinson) for 20 min at room temperature. Then cells were washed and analysed with a flow cytometer (FACScan Becton Dickinson).

CSDA RNA interference and over-expression in K562 cell line and in primary erythroid cells

Transfection of K562 cells was performed with Lipofectamine 2000 (Invitrogen) as transfectant agent. The day before transfection, cells were plated into 12- or 6-well plates at a density of $2.5x10^5$/ml in Optimem medium (Invitrogen) according to the manufacturer's instructions. Primary erythroid cells were transfected at day 7 of culture with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. The day before transfection, cells were diluted at a density of $3x10^5$/ml in the culture medium containing serum and antibiotics and incubated under normal

growth conditions. On the day of transfection cells were plated into 24well plates at a density of $2x10^5$/ml in the culture medium. CSDA RNAi was achieved by transiently transfecting cells with double-stranded small interfering RNAs (stealth™ siRNAs) from Invitrogen. Two different siRNA oligos were used for CSDA isoform *a* knock-down as follows (sense strand shown): siRNA 1 GCCUUACCACGUGGGACAGACCUUU; siRNA 2 ACCUUUGACCGUCGCUCACGGGUCU; an RNAi negative control (High GC, Invitrogen), certified by the manufacturer to have no matches with mRNAs sequences in GenBank, was used as mock control. Cells were collected 48 h after transfection for total protein or RNA isolation procedures, as previously described, in order to perform Western blot or real-time RT-PCR analysis.[22, 25] CSDA silencing was verified at protein level by Western blot with the 4D9 anti-CSDA antibody, whereas quantitative real-time RT-PCR analyses were carried out to evaluate variations of CSDA as well as of □- and □-globin gene mRNA levels, using the procedure described above.

For over-expression experiments, the cDNA of CSDA isoform *a* (Genbank accession number: NM_003651.4) and isoform *b* (Genbank accession number: NM_001145426.1) were amplified by PCR using the HotStart Taq DNA Polymerase (Qiagen) and cloned in a p3xFLAG-CMV 7.1 plasmid vector (Sigma-Aldrich). K562 cells were transiently transfected with 2 □g of each p3xFLAG-CSDA expression vector. Cells were collected 48 h after transfection for total protein or RNA purification in order to perform Western blot or real-time RT-PCR analysis. Western blot analysis was carried out with an antibody against the FLAG epitope (Sigma-Aldrich)

to check exogenous CSDA over-expression, whereas quantitative real-time RT-PCR analysis for ε- and γ-globin gene mRNA were performed as above described in order to evaluate effects on fetal and embryonic globin gene expression.

Plasmid constructs and reporter gene assays

The recombinant plasmid –268γ-luc containing the firefly luciferase reporter gene was obtained by cloning the -268 bp fragment of the proximal Gγ-globin gene promoter in a pGL4 vector (Promega). To normalize the luciferase assay, 0.1 μg of the pRL-CMV vector (Promega) coding for the *Renilla* luciferase was transiently co-transfected with 0.9 μg of construct –268γ-luc in K562 cells at the density of $1.5x10^5$ cells per well in 24-well dishes. The pGL4-null and p3XFlag-null empty vectors (0.9 μg) were used as negative controls whereas the pCMVluc (0.9 μg) was the positive control for the assay. To evaluate the effect of CSDA, cells were co-transfected with 0.5, 1 or 2 μg of the expression vector p3xFLAG-CSDA described above. Luciferase activity was measured 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega) on a $20/20^n$ luminometer (Turner Biosystems, Sunnyvale, CA, USA), according to the protocols of the manufacturers.

Chromatin ImmunoPrecipitation (ChIP) analysis

Chromatin from K562 cells ($12.5x10^6$) was purified and immunoprecipitated with antibodies against CSDA (Sigma-Aldrich), p65, p50, HDAC2 (Upstate Biotechnology) and with immunoglobulin G (IgG) (Sigma-Aldrich) as

described previously.[24] Analysis of histone modification was carried out as recommended by the manufacturer using an antibody against acetyl-histone H3 (acH3, 06-599; Upstate Biotechnology). Antigen-DNA complexes cross-links were reversed over-night at 65°C after addition of RNase (10 ng/μl) in a 100 μl final volume. SDS (0,5% final concentration) and proteinase K (0,6 μg/μl final concentration) were then added and the mix was incubated at 50°C for 3h followed by phenol-chloroform extraction and EtOH precipitation. DNA pellets were resuspended in 30 μl of distilled water. Real-time PCR detection of the proximal Gγ- and β-globin gene promoter regions were performed in a 20 μl reaction mix containing 1X SYBR Green I PCR Master mix (Bio-Rad Laboratories), 20 μM of each primer mix (gamma For: ACTACAGGCCTCACTGGAG and gamma Rev: TGGAACTGCTGAAGGGTGC; beta For: TGTACTGATGGTATGGGGCC and beta Rev: TGATACCAACCTGCCCAGGG; GAPDH For: GGTCGTATTGGGCGCCTGGTCACCA and GAPDH Rev: CACACCCATGACGAACATGGGGGC), 1/10 volume of purified DNA and nuclease-free water on an iCycler instrument (Bio-Rad Laboratories). The thermal profile consisted of 1 cycle at 95°C for 3 minutes followed by 40 cycles at 95°C for 15 seconds, 58°C for 30 seconds, 72°C for 20 seconds. Real time PCR data analysis followed the methodology described in a recent report. [27]

ImmunoPrecipitation (IP) and Western blot analysis in K562 cell line

K562 cells transfected with plasmid vectors p3xFLAG-CSDA isoform *a* and p3xFLAG-CSDA isoform *b* and collected after 48 hours from trasfection, washed two times with PBS 1X and lysed in buffer containing 150 mM NaCl, 50 mM Tris (pH 8), 0.1% NP-40, 10% glicerol, 1mM EDTA, 0.5 mM PMSF, and 1:100 protease inhibitors cocktail (Sigma-Aldrich). Protein extracts, containing the FLAG-tagged CSDA isoforms *a* and *b*, were immunoprecipitated with anti-FLAG-M2 affinity gel (Sigma-Aldrich), a highly specific monoclonal antibody covalently attached to agarose resin, according to the manufacturer's protocol. Immunoprecipitated proteins were separated by SDS-PAGE and analysed by Western blot using the anti-p65 (AB1604a, Millipore), anti-p50 (AB1602b, Millipore) and anti-HDAC2 (sc-7899, Santa Cruz Biotechnology) antibodies.

Treatment with Bortezomib and Trichostatin A (TSA) in K562 cell line

K562 cells, 5 hours after transfection with the p3xFLAG-CSDA isoform *a* and isoform *b* expression vectors, were treated with increasing concentrations of Bortezomib (0.1 nM, 1 nM and 10 nM) or Trichostatin A (80 nM, 160 nM and 240 nM) for 48 h and were analyzed by quantitative Real Time PCR. K562 cells treated with 1 nM Bortezomib or with 160 nM TSA for 48 h were used for ChIP assays as described previously. Immunoprecipitation was carried out using 5 μg of anti-p65, anti-p50, anti HDAC2, anti-CSDA and anti-acetyl-H3 antibodies.

Results

Genetic analysis of β- and γ-globin gene clusters

We identified three siblings affected by different severity degrees of β-*thalassemia intermedia*. Characterization of β-thalassemic defects revealed β^{+}IVSI-6 (T→C) in homozygosis in all of them. For both alleles, the mutation was associated to Orkin's haplotype VI.[28] To search for genetic determinants of different levels of γ-globin gene expression we first carried out an extensive sequence analysis of putative regulatory regions within the β-globin gene cluster potentially involved in these mechanisms. We examined sequence variations in binding sites for transcriptional factors or in polymorphic sequences within the hypersensitive site-2 (HS-2) of the locus control region (LCR) and the promoter region of the Gγ- and γ-globin genes, all of which have been reported to be involved in modulation of globin gene expression (Table 2). [5, 7, 29-35] We found the same β-globin gene cluster genotype in the three patients, and were able to exclude known HPFH mutations and hypothetical parental germ line rearrangements within this locus. Screening for deletional and non-deletional β-globin gene mutations revealed a normal set of β-globin genes in all three siblings (data not shown).

Identification of CSDA as a putative modulator for γ-globin gene expression

Based on the foregoing data, it was conceivable that HbF level variations were mostly associated to genetic determinants not linked to the β-globin gene cluster. To explore this hypothesis, we analyzed the reticulocyte transcriptome from the three patients using a differential mRNA display approach. We found several bands that were differentially expressed in the

Table 2: α- and β-globin gene cluster genotypes in the three siblings affected by *thalassemia intermedia*. For each examined putative regulatory transcription site within the β-globin gene cluster, the nucleotide position, the consensus sequences or the polymorphic variants and the corresponding patient genotypes are reported (the described polymorphic nucleotides are underlined). The N or N' sequences at position –10623/-10570 of HS2 are as reported elsewhere.[33]

			HS2			5'-Gγ				5'-β		
Patient	**α-globin gene genotype**	**β-globin gene genotype**	**-10924 CCTGT E**	**-10905 CCACC Sp1**	**-10623/-10570 $(AT)_xN(AT)_y$ Hox2H**	**-1280 TGATAG GATA1**	**-1225 TGACGTCA CRE**	**-202 C/T/G**	**-158 C/T (Xmn I)**	**-551 C/T**	**-530 $(AT)_x(T)_y$ BP1**	**-521 C/T**
I-1	αα/αα	IVS1-6/IVS1-6	T/T	A/G	$(AT)_8N(AT)_{13}$/ $(AT)_8N'(AT)_{13}$	G/G	G/G	C/C	C/C (-/-)	T/T	$(AT)_6(T)_9$/ $(AT)_6(T)_8$	C/C
I-2	αα/αα	IVS1-6/IVS1-6	T/T	A/G	$(AT)_8N(AT)_{13}$/ $(AT)_8N'(AT)_{13}$	G/G	G/G	C/C	C/C (-/-)	TT	$(AT)_6(T)_9$/ $(AT)_6(T)_8$	C/C
I-3	αα/αα	IVS1-6/IVS1-6	T/T	A/G	$(AT)_8N(AT)_{13}$/ $(AT)_8N'(AT)_{13}$	G/G	G/G	C/C	C/C (-/-)	T/T	$(AT)_6(T)_9$/ $(AT)_6(T)_8$	C/C

sample from patient I-1, the more severely affected sibling, compared with his two sisters (Fig. 5). Selected bands were cloned in a pGEM T-vector and sequenced. Among clones, a complete homology was found between the cDNA sequence of the Cold Shock Domain Protein A (CSDA) known to act as a repressor factor for several hematopoietic genes and clones originated from two different up-regulated bands of patient I-1. CSDA expression levels were evaluated by real time PCR analysis in the three siblings and confirmed the differential display results (Fig. 6A). Also, protein levels of CSDA were found consistent with these findings (Fig. 6B). Therefore, in this family, there was an inverse relationship between CSDA and □-globin gene mRNA levels comparable to that found for peripheral blood values of HbF (Fig. 6C). To further confirm this correlation we examined by real time RT-PCR and Western blot (Fig. 7) three other unrelated homozygotes for the IVSI-6 mutation along with two homozygotes for the □°39 mutation affected by varying degrees of clinical conditions (Table 1). In all the cases examined we found that severity of clinical conditions and HbF values inversely correlated with CSDA levels, thus reinforcing our previous findings in the three siblings.

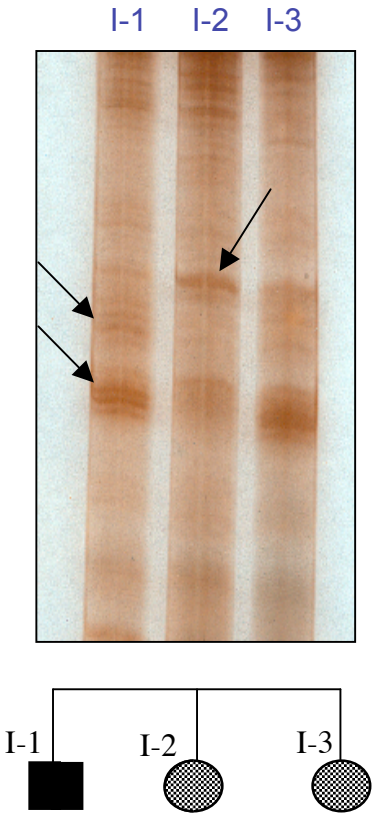
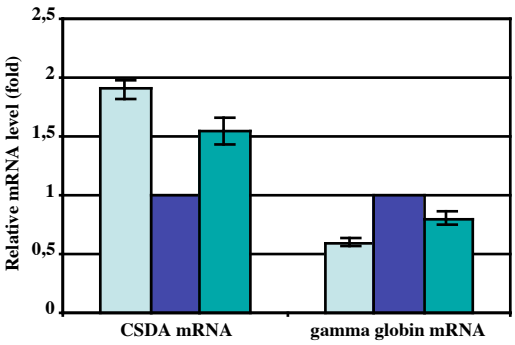


Figure 5: **Identification of transcripts differently expressed in peripheral reticulocytes isolated from the examined patients.** Silver stained differential mRNA display electropherogram and family tree. The arrows indicate bands differently expressed among the three patients.

A



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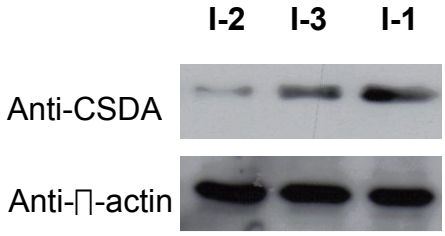

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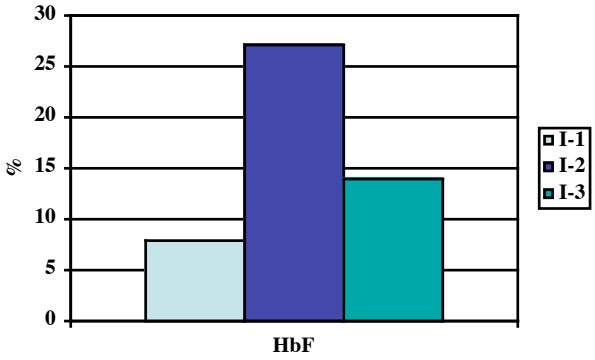
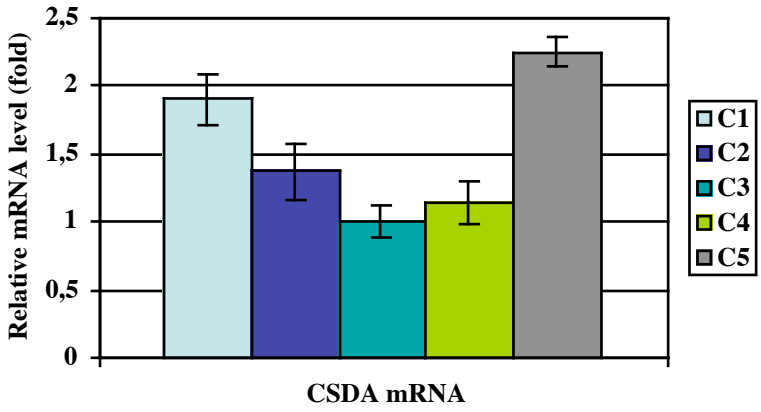


Figure 6: **Correlation between CSDA mRNA levels and fetal globin gene expression.** (A) Inverted correlations between reticulocyte CSDA and β-globin mRNA levels evaluated by quantitative real-time PCR in the three siblings; (B) Western blot analysis with anti-CSDA or anti-β-actin antibodies on protein extracts isolated from peripheral reticulocytes; (C) HbF values detected in the peripheral blood.

A



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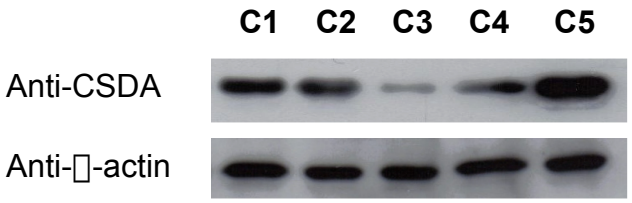

Figure 7: **Evaluation of CSDA expression levels in thalassemic patients.** (A) Quantitative real-time PCR of CSDA mRNA levels isolated from peripheral reticulocytes. (B) Western blot analysis with anti-CSDA or anti-actin antibodies on protein extracts isolated from peripheral reticulocytes.

Effects of CSDA knock-down and over-expression on β-globin gene expression in K562 and primary erythroid cells

We first examined the role played by CSDA in the regulation of β-globin gene expression using transient RNAi in the K562 cell line and two siRNAs specific for CSDA isoform *a* mRNA sequences. Cells were collected 48 h after transfection and subjected to total protein and RNA extraction procedures. Knock-down of CSDA was verified at protein and mRNA levels by Western blot and real-time PCR procedures, respectively (Fig. 8). The effects of CSDA silencing on ε- and γ-globin gene expression were evaluated by quantitative real-time PCR in cells treated with specific or mock siRNAs. The expression level of the γ-globin gene was on average four fold higher when CSDA isoform *a* mRNA levels were reduced to about 40-50% (Fig. 8A and 8B). CSDA isoform *a* knock-down did not affect expression levels of the ε-globin gene, suggesting that, within the β-globin gene cluster, CSDA could specifically act as a repressor of the γ-globin gene.

To verify the quantitative effects of CSDA levels on the regulation of γ-globin gene expression in K562 cells, we over-expressed two recombinant FLAG-tagged CSDA proteins, corresponding to CSDA isoform *a* and *b*, respectively. CSDA over-expression was evaluated in total protein extracts by Western blot with an anti-FLAG antibody, whereas the effects on ε- and γ-globin gene expression were evaluated by real-time PCR analysis. As shown in Figure 9A and 9B, increased CSDA levels resulted in a substantial reduction (around 50%) of γ-globin gene mRNA levels versus untreated cells, whereas no variations were found on the expression levels of the ε-

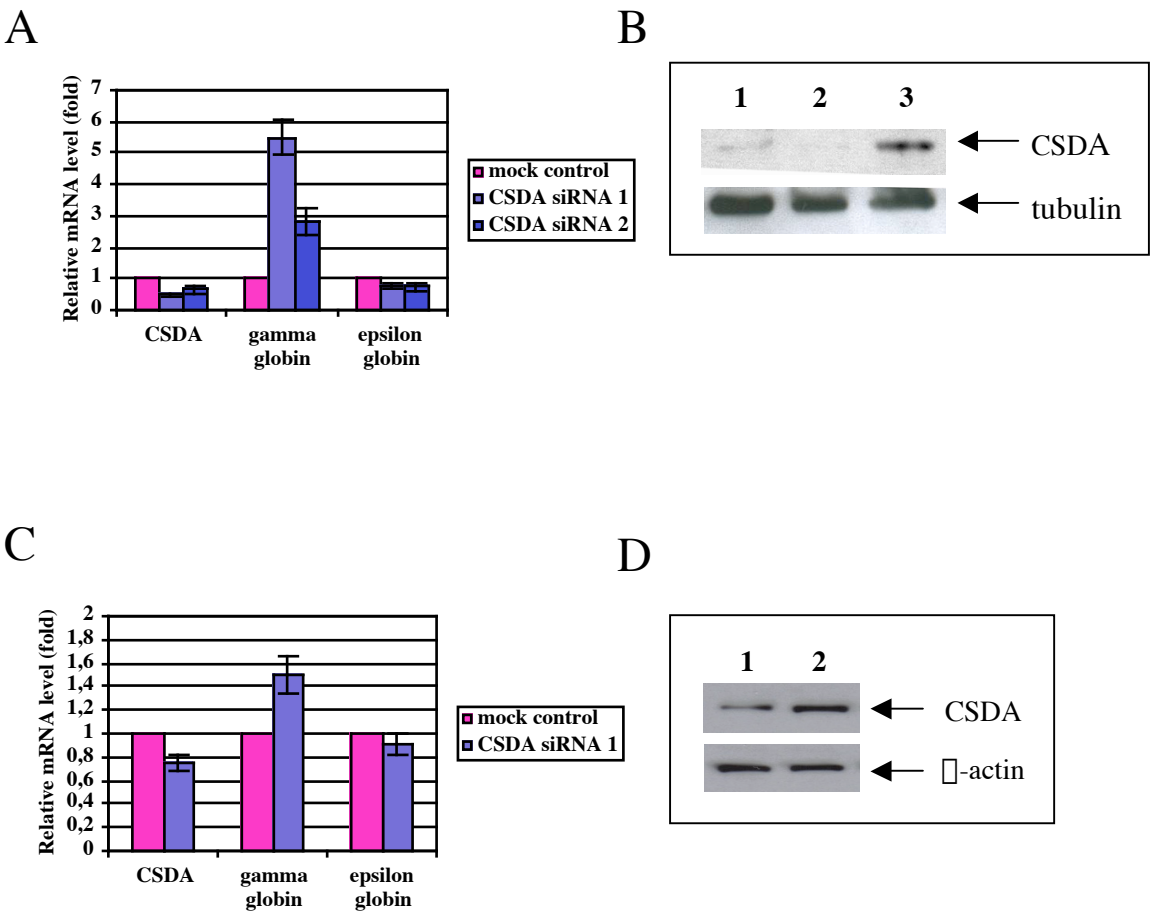

Figure 8: **Knock-down of CSDA in K562 and in primary erythroid cells and evaluation of the corresponding variations of - and -globin mRNA levels.** (A) Quantitative real-time PCR analysis of CSDA, - and -globin gene mRNA levels in K562 cells transfected with CSDA siRNA 1, CSDA siRNA 2 or a siRNA negative control (mock); (B) Western blot analysis with anti-CSDA or anti-tubulin antibodies on protein extracts from K562 cells transfected with CSDA siRNA 1 (lane 1), CSDA siRNA 2 (lane 2) or a siRNA negative control (lane 3); (C) Quantitative real-time PCR analysis of CSDA, - and -globin gene mRNA levels in primary erythroid cells transfected with CSDA siRNA 1 or a siRNA negative control (mock); (D) Western blot analysis with anti-CSDA or anti--actin antibodies on protein extracts from primary erythroid cells transfected with CSDA siRNA 1 (lane 1) or a siRNA negative control (lane 2). Results depicted are representative of 3 independent experiments.

globin gene. Moreover, this effect is specific for CSDA isoform *a* because over-expression of CSDA isoform *b* did not affect β-globin gene expression levels (Fig. 9).

Effects of CSDA knock-down and over-expression on β-globin gene expression were also evaluated in primary erythroid cells in which similar results were found (Fig. 8C, 8D, 9C and 9D), although CSDA silencing apparently enhanced β-globin gene expression more efficiently in K562 cells than in primary erythroid cells (Fig. 8A and 8C).

CSDA interacts with the region at –200 bp of the ^{G}γ-globin gene promoter

To verify whether CSDA plays a transcriptional role on the β-globin gene promoter, we performed reporter gene assays by transiently transfecting into K562 cells a plasmid construct containing a luciferase gene driven by the –268 bp fragment of the ^{G}γ-globin gene promoter. When K562 cells were co-transfected with a CSDA expression vector (p3XFLAG-CSDA), the luciferase activities showed a slight but statistically significant decrease in a manner dependent on the amount of the CSDA vector transfected (Fig. 10B), thus providing further evidence that CSDA acts as a repressor factor of β-globin gene expression. To verify that CSDA is capable of binding to the proximal promoter of the ^{G}γ-globin gene we performed ChIP analysis on chromatin from K562 cells immunoprecipitated with anti-CSDA antibody. Primers specific for the ^{G}γ-promoter revealed a strong enrichment compared with IgG controls. As control we examined the proximal β-globin gene promoter region for which no chromatin enrichment was found (Fig. 11). Therefore, CSDA specifically interacts *in vivo* with the proximal promoter

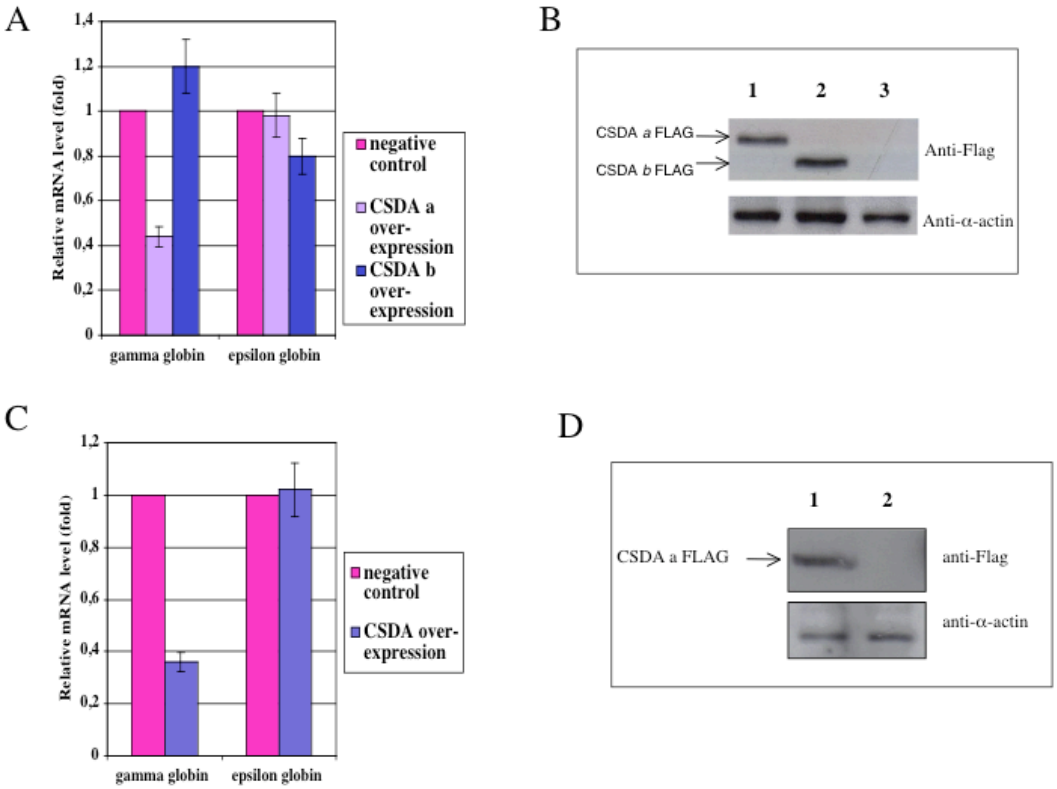

Figure 9: **CSDA over-expression in K562 and in primary erythroid cells and evaluation of the corresponding variations of [illegible]- and [illegible]-globin mRNA levels.** (A) Quantitative real-time PCR analysis of [illegible]- and [illegible]-globin gene mRNA levels in K562 cells transfected with the 3xFLAG-CSDA expression plasmid vectors for CSDA isoform *a* or isoform *b* (p3xFLAG-CSDA*a* or p3xFLAG-CSDA*b*) or with a 3xFLAG empty plasmid vector (p3xFLAG) used as negative control; (B) Western blot analysis with anti-FLAG or anti-[illegible]-actin antibodies on protein extracts from transfected K562 cells with p3xFLAG-CSDA*a* (1), p3xFLAG-CSDA*b* (2) or p3xFLAG (3); (C) Quantitative real-time PCR analysis of [illegible]- and [illegible]-globin gene mRNA levels in primary erythroid cells transfected with a 3xFLAG-CSDA expression plasmid vector (p3xFLAG-CSDA*a*) or with a 3xFLAG empty plasmid vector (p3xFLAG) used as negative control; (D) Western blot analysis with anti-FLAG or anti-[illegible]-actin antibodies on protein extracts from transfected primary erythroid cells with p3xFLAG-CSDA*a* (1) or p3xFLAG (2). Representative data from 3 independent experiments are shown.

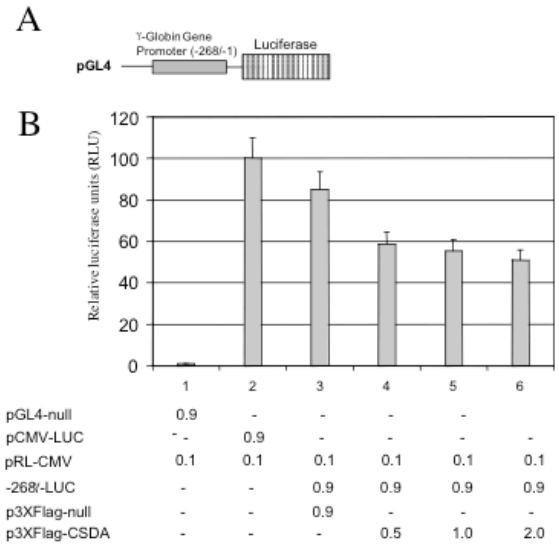

Figure 10: **CSDA suppresses transcriptional activity of the β-globin gene in K562 cells.**
(A) Schematic representation of the –268β-luciferase construct (–268β-LUC) used. (B) Relative luciferase activities measured 48 h after transfection in K562 cells. The –268β-LUC costruct was used as reporter plasmid (lanes 3-6). Co-transfection of plasmid p3XFlag-CSDA represses the reporter gene in a dose dependent manner (lanes 4-6). pGL4-null empty vector indicates the background reporter activity (lane 1), pCMV-LUC and p3XFlag-null were used as positive (lane 2) and negative (lane 3) controls, respectively, pRL-CMV plasmid was used to normalize the results. The mean activities from 3 independent experiments are shown.

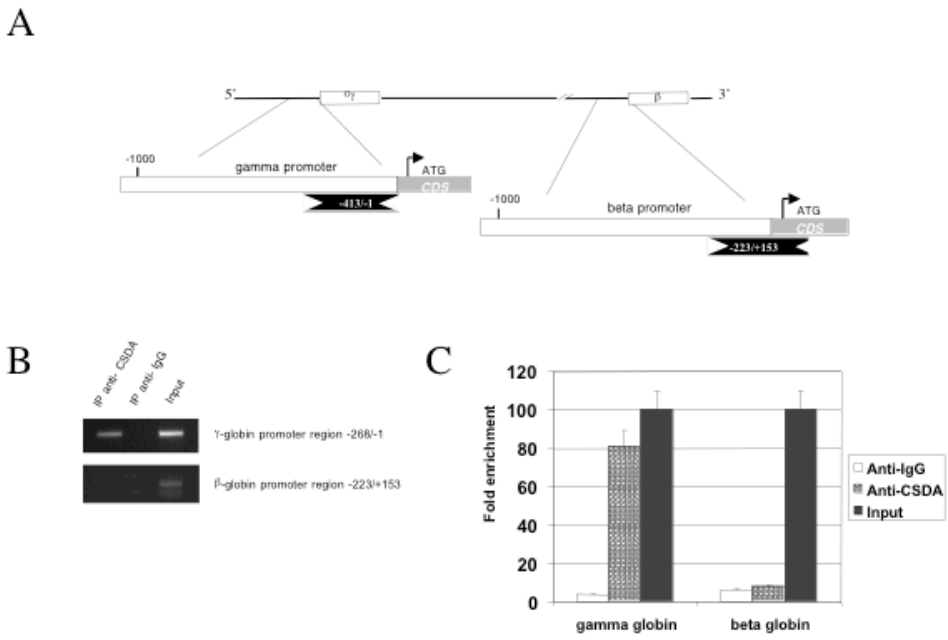

Figure 11: **CSDA interacts with the proximal promoter region of β-globin gene in K562 cells.** (A) Schematic representation of the β-globin gene locus and of the Gγ- and Aγ-globin gene promoter fragments examined in this study. (B) 1.5% ethidium bromide-stained agarose gel showing amplification of the indicated promoter fragments from K562 DNA immuno-precipitated with anti-CSDA or IgG antibodies. (C) Quantitative real-time PCR of the immuno-precipitated samples normalized to input (total genomic DNA) quantities for any given promoter. Representative data from 3 independent experiments are shown.

region of the $^{G}\gamma$-globin gene.

Taken as a whole, these results demonstrate that CSDA suppresses γ-globin gene expression at least in part at the transcriptional level.

Analysis of the expression pattern of CSDA isoforms

To characterize erythroid-specific CSDA isoforms, we amplified full-length cDNAs of CSDA, using primers located at the 5'- and 3'-UTR regions. We found two amplification products in the three patients, which resulted corresponding to CSDA isoform *a* and isoform *b*, respectively. Furthermore, in the patient with the lower HbF level, we found higher expression of CSDA isoform *a* respect to his two siblings, suggesting that this isoform could be more involved in the transcriptional repression of γ-globin gene expression. According to this hypothesis, we also found low expression levels of this isoform in K562 cell line, which shows an embrio-fetal globin gene expression pattern (Fig. 12).

CSDA isoform a interacts with NF-kB and HDAC2

To identify putative molecular interactors of CSDA isoform *a* and isoform *b*, we performed immunoprecipitation experiments with the anti-FLAG antibody in K562 cells over-expressing CSDA isoform *a* or isoform *b*. Whole protein extracts immunoprecipitated with anti-FLAG were analyzed by Western blot. This study showed that the NF-kB heterodimer complex (p65-p50) specifically interacts with CSDA isoform *a*, but not with CSDA isoform *b* (Fig. 13). Since histone deacetylases have been described as molecular partners of NF-kB in repression mechanisms of gene expression[51],

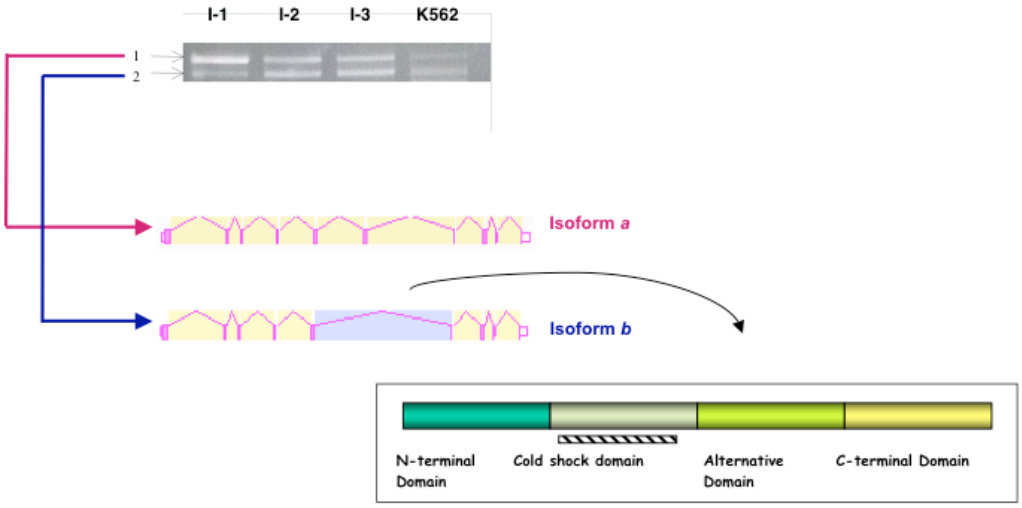

Figure 12: **Characterization of erythroid specific CSDA isoforms.** 0.9% ethidium bromide-stained agarose gel showing PCR products of CSDA full length cDNAs from peripheral reticulocytes of patients I-1, I-2 and I-3 and from K562 cells. 1: amplification fragment of 1560 bp corresponding to isoform *a;* 2: amplification fragment of 1350 bp corresponding to isoform *b*.

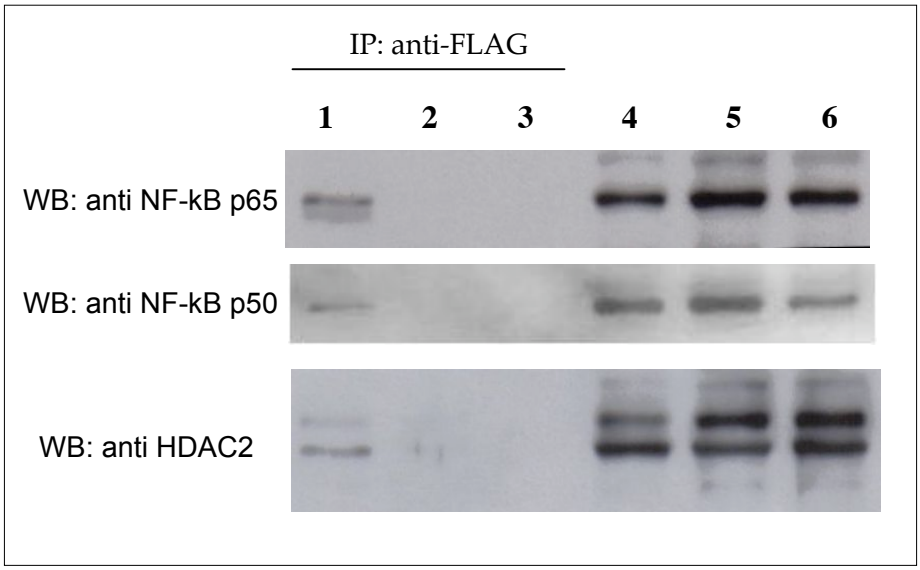

Figure 13: **CSDA isoform *a* associates with p65, p50 and HDAC2**. Western blot analysis (WB) with anti-p65, anti-p50 and anti-HDAC2 antibodies on transfected K562 protein extracts respectively with p3xFLAG-CSDA*a* (lanes 1 and 4), p3xFLAG-CSDA*b* (lanes 2 and 5) or p3xFLAG (lanes 3 and 6) immunoprecipitated with the anti-FLAG antibody (lanes 1, 2, 3) or not immunoprecipitated (lanes 4, 5, 6).

we also analyzed the possible interaction between CSDA and HDAC2. Even in this case we found that HDAC2 specifically interacts with CSDA isoform *a*, but not with isoform *b*. Taken altogether these data support our hypothesis of CSDA isoform *a* as a trascriptional repressor factor for β-globin genes.

NF-kB and HDAC2 form a multiprotein complex with CSDA on the region at –200 bp of the ^{G}γ-globin gene promoter

To investigate if NF-kB and HDAC2 interact with CSDA isoform *a* at the –200 region of the ^{G}γ-globin gene promoter, we performed ChIP assays, using antibodies against p65, p50 and HDAC2. The results demonstrated that NF-kB p65-p50 heterodimer complex and HDAC2 both interact *in vivo* with the region at –200 bp of the ^{G}γ-globin gene promoter containing the CSDA binding site (Fig. 14), indicating that NF-kB and HDAC2 are CSDA partners at this region.

Bortezomib induces β-globin gene transcriptional expression

To examine the role played by NF-kB on transcriptional repression of β-globin gene expression mediated by CSDA, we treated K562 cells, previously transfected with constructs expressing FLAG-CSDA isoform *a* or isoform *b,* with Bortezomib, a proteasome inhibitor which is able to arrest NF-kB complex (p65-p50) nuclear traslocation.

Quantitative analysis by Real Time PCR showed that, following the Bortezomib treatment, β-globin gene expression increased only in K562 cells over-expressing CSDA isoform *a*, whereas no effects on β-globin gene expression were found in K562 cells over-expressing CSDA isoform *b* (Fig.

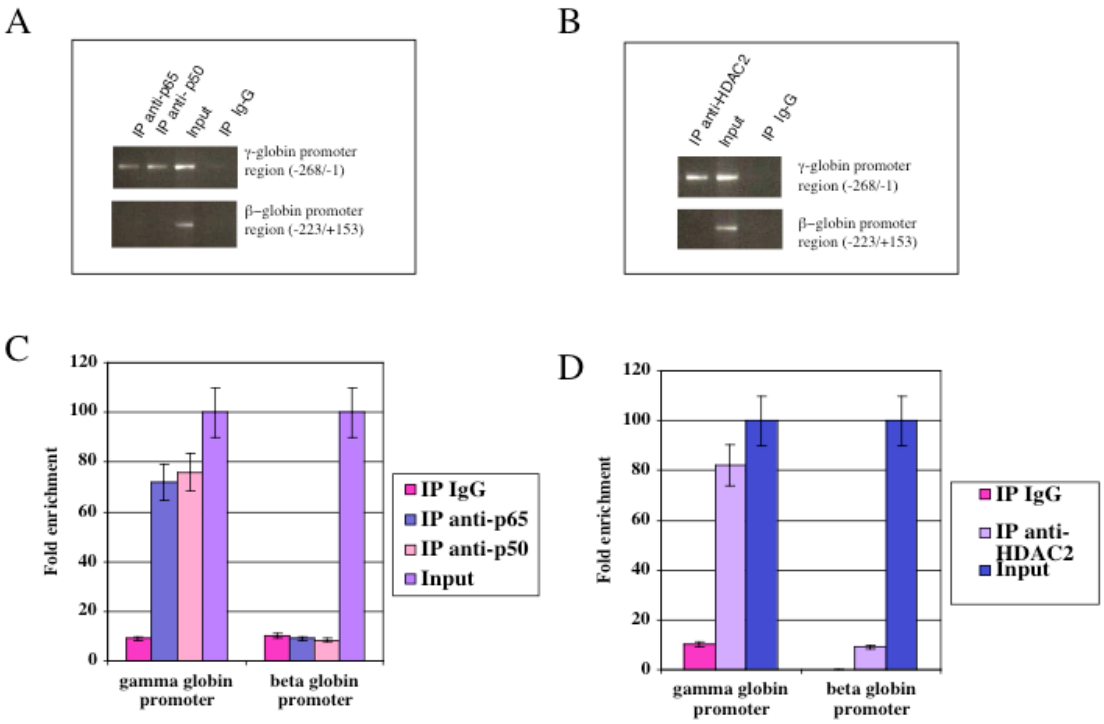

Figure 14: **NF-kB (p65-p50) and HDAC2 interact with the proximal promoter region of -globin gene in K562 cells.** (A, B) 1.5% ethidium bromide-stained agarose gel showing amplification of the indicated promoter fragments from K562 DNA immunoprecipitated with anti-p65, anti-p50, anti-HDAC2 or IgG antibodies. (C, D) Quantitative real-time PCR of the immunoprecipitated samples normalized to input (total genomic DNA) quantities for any given promoter. Representative data from 3 independent experiments are shown.

15A). These findings suggest that NF-kB can interact with CSDA isoform *a* to negatively regulate □-globin expression gene.

By ChIP analysis, we also examined if Bortezomib was able to affect NF-kB binding to the -200 bp region of the ^{G}□-globin gene promoter. Results show a relevant reduction of NF-kB binding to this region following treatment with this drug (Fig. 15 B and C). Therefore, the Bortezomib treatment is able to affect □-globin gene expression by reducing NF-kB binding to this promoter.

Role of histone deacetylase in transcriptional repression of fetal globin gene

To confirm the role of histone deacetylase in transcriptional repression of □-globin gene, we performed TSA treatments in K562 cells. As shown by quantitative real-time PCR, K562 cells showed an increased □-globin gene expression following treatment with the histone deacetylase inhibitor TSA, in a dose-dependent manner (Fig. 16A). These data demonstrated the role played by modulation of histone acetylation on transcriptional repression mechanisms of □-globin gene expression. Therefore, to evaluate variations in histone acetylation levels at the -200 bp of the ^{G}□-globin gene promoter we performed ChIP assays in K562 cells using an anti-acetyl-H3 antibody and analyzed the histone acetylation pattern following the TSA treatment. Enrichment of chromatin immunoprecipitatated with anti-acetyl-H3 at the ^{G}□-globin gene promoter was compared with the GAPDH gene used as endogenous control. Results indicated that the TSA treatment induces a ~15-fold increase in H3 acetylation level at the -200 bp fragment of the ^{G}□-globin gene promoter region (Fig. 16B).

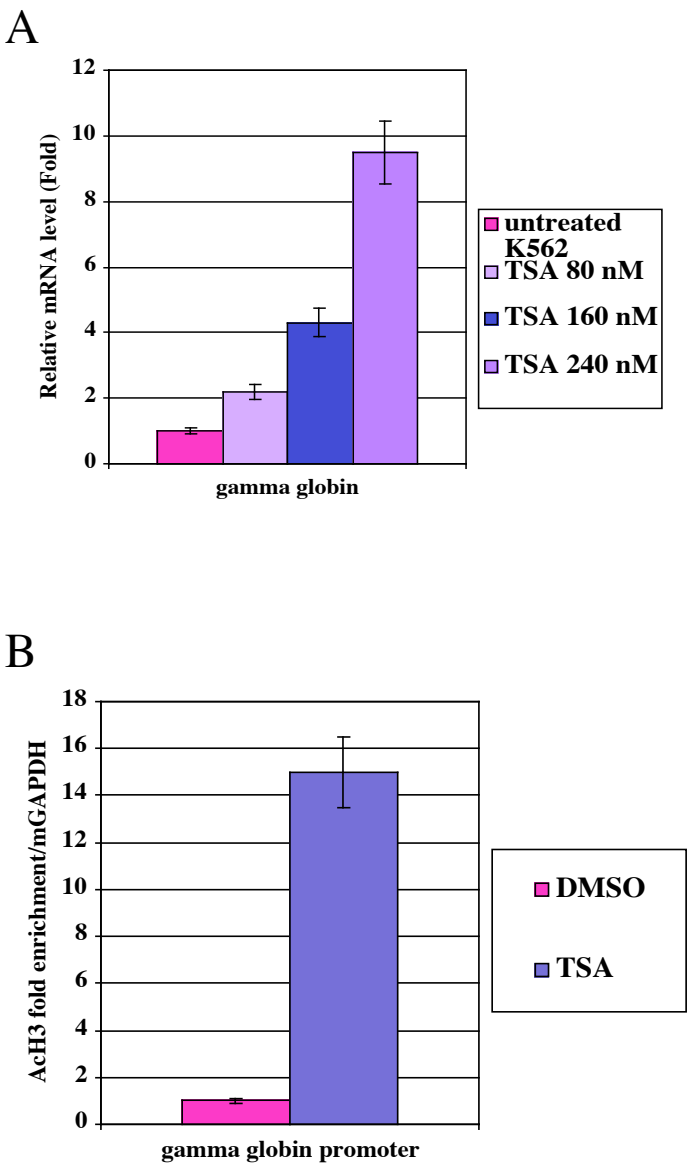

Figure 16: **Treatment with TSA in K562 and evaluation of the corresponding variations of β-globin mRNA and histone H3 acetylation levels.** (A) Quantitative real-time PCR analysis of β-globin gene mRNA endogenous levels after treatment with increased concentration (80, 160 and 240 nM) of TSA in K562 cell line (B) Quantitative real-time PCR of the fragments from K562 DNA immunoprecipitated with anti-acetyl-H3 or IgG antibodies after or without TSA treatment. Immunoprecipitated samples were normalized to input (total genomic DNA) quantities for any given promoter and corrected respect to a reference gene (GAPDH). Representative data from 3 independent experiments are shown.

Bortezomib increases H3 acetylation levels on gamma globin gene promoter

To investigate if the NF-kB inhibition, mediated by Bortezomib, affects the histone acetylation levels at the -200 bp of the $^{G}\gamma$-globin gene promoter, ChIP assays were performed in K562 cell line previously treated with this drug. The results demonstrated a ~10-fold increase in H3 acetylation levels following the Bortezomib treatment in this promoter region (Fig. 17). Taken altogether, these data suggest that NF-kB can affect deacetylase activity in this region which in turn should modulate local chromatin conformation and thus fetal globin gene expression.

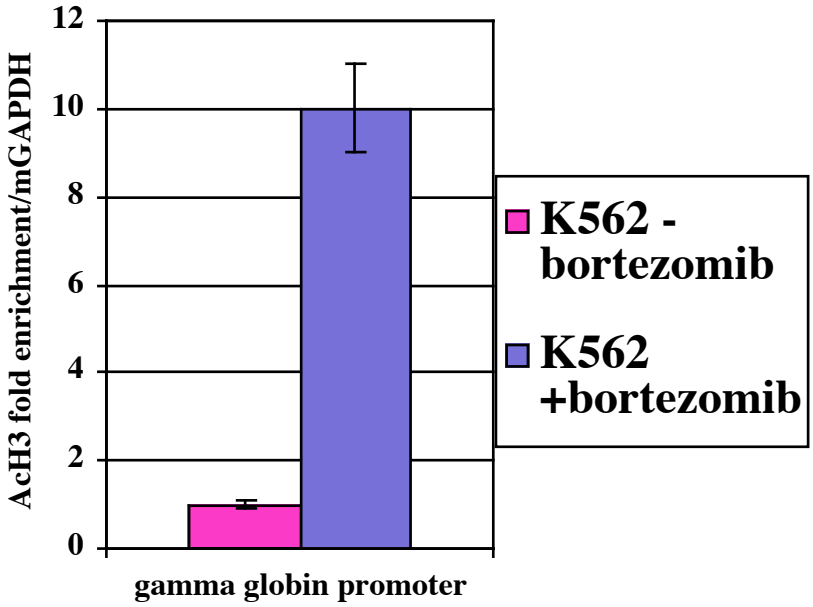

Figure 17: **Evaluation of histone acetylation levels following Bortezomib treatment.** Quantitative real-time PCR of the fragments from K562 DNA immunoprecipitated with anti-acetyl-H3 or IgG antibodies with or without bortezomib treatment. Immunoprecipitated samples were normalized to input (total genomic DNA) quantities for any given promoter and corrected respect to a GAPDH reference gene. Representative data from 3 independent experiments are shown.

Discussion

We examined the reticulocyte transcriptome of three siblings who differed in HbF levels and in β-thalassemia severity although they had the same β- and β-globin gene cluster genotypes. The aim of the study was to identify factors that modulate the level of β-globin gene expression. The two sisters of the family were affected by a milder form of *thalassemia intermedia* and had higher levels of HbF than their elder brother who was affected by a more severe form of the disorder and was transfusion-dependent. Nevertheless, all the three siblings were homozygous for the β^+ IVS I-6 (T→C) mutation and all had a normal set of β-globin genes. Therefore, the different severity of *thalassemia intermedia* was clearly related to the different HbF levels.

Variability in the severity of the disease has been extensively reported in β^+ IVS I-6 (T→C) homozygotes, mainly relied on variations in β-globin chain production. Molecular studies also indicate that such variations cannot be attributed to regulatory elements within the β-globin gene cluster, suggesting a major role for transacting factors able to modulate β-globin gene expression. [30, 36] Therefore, this type of β-thalassemia condition provides an interesting experimental model to evaluate the effects of genetic molecular modifiers of disease severity in β-thalassemia syndromes.

In our study, analysis of genetic loci linked to the β-globin gene cluster and putatively involved in regulation of globin gene expression showed the same genetic background for all the three siblings, and this drew our attention to factors not linked to the β-globin gene cluster. To look for such potential genetic modifiers, we examined the reticulocyte transcriptome of the three patients using a differential mRNA display approach. Among

several bands differentially expressed we focused our attention to those corresponding to human cDNAs coding for factors potentially involved in gene expression or in signal transduction mechanisms. Particularly, in clones originated from two different up-regulated bands of the more severely affected patient (subject I-1) we found homology with the cDNA of CSDA, a cold shock domain (CSD) protein.

CSD proteins have been reported to be involved in transcriptional activation and repression, as well as in post-transcriptional mechanisms of gene expression regulation, including mRNA packaging, transport, localization and stability.[37-43]

Although a general consensus binding site has not been established for CSD proteins, they are generally considered to bind to CT-rich sequences that can generate H-DNA structures.[41] HPFH point mutations (Gγ-202 C→G or C→T) occurring in a region with homopyrimidine tracts reported to be critical for γ-globin gene repression are thought to abolish the high-affinity binding sites for CSDA by disrupting the formation of the intra-molecular triplex and thus the H-DNA structure.[44-46] Therefore, reduced binding of CSDA in the –200 Gγ-promoter region could lead to persistent expression of γ-globin genes in adult life.[43]

In agreement with this proposed model, our differential display results showed that CSDA expression levels were inversely correlated to γ-globin gene expression. Furthermore, by demonstrating that defective levels of CSDA contribute to increase fetal globin gene expression, our study suggested that CSDA could act as a QTL for fetal hemoglobin production. To confirm these findings we firstly examined other patients homozygous for

the β^+ IVS I-6 (T→C) β-thalassemia mutation and affected by varying degrees of clinical conditions. We found that severity of clinical conditions and HbF values inversely correlated with CSDA levels. We also examined two homozygotes for the β°39 mutation who were chosen because affected by different clinical conditions, despite sharing the same β-thalassemic genotype. Also in these cases lower expression levels of CSDA were associated to milder thalassemic conditions and higher values of HbF, thus reinforcing the validity of our previous findings in the three siblings.

The role of CSDA on β-globin gene regulation was also investigated by expression studies in human erythroleukemia K562 cells and in primary $CD71^+$ erythroid cells which demonstrated that CSDA represses β-globin genes and can be considered a QTL for the HPFH phenotype. In fact, in both these cell systems, down- and up-modulation of CSDA levels consistently corresponded to variations of β-globin gene expression levels: CSDA knock-down induced by RNAi resulted in significantly increased expression of β-globin genes, whereas its over-expression was associated with reduced β-globin gene mRNA levels, although silencing of CSDA seemed to enhance β-globin gene expression more efficiently in K562 cells than in primary erythroid cells.

However, at this regard, it is to be considered that primary cells are less efficiently transfectable than stable cell lines and consequently CSDA was silenced at a lower rate in erythroid cells than in K562 cells. Furthermore, the erythroid cells were trasfected at an early stage of differentiation (7-day culture) when β-globin mRNA is highly expressed, as already reported.[28, 47] Therefore it is expectable that such cells could respond to induction of β-

globin expression less efficiently than more differentiated cells in which γ-globin genes expression is declined or constitutively repressed.

The putative mechanism by which CSDA modulates γ-globin gene expression was investigated by gene reporter assays and ChIP analysis in K562 cells. In this way we were able to demonstrate that CSDA is able to modulate γ-globin gene expression at the transcriptional level. Thus, our study provides the first *in vivo* evidence that CSDA is a trans-acting repressor factor of γ-globin gene expression.

We also examined the role played by specific CSDA isoforms on γ-globin gene expression and demonstrated that CSDA isoform *a* specifically acts as a repression factor for these genes. To investigate the molecular mechanism of γ-globin gene silencing mediated by CSDA isoform *a*, we identified CSDA molecular interactors through immunoprecipitation experiments and found that CSDA isoform *a*, NF-kB (p65-p50) and HDAC2 take part to a multi-protein complex on the –200 Gγ-promoter region.

Several studies suggest that NF-kB factors are involved in normal erythropoiesis [52]. The NF-kB factors p105, p50, p100, p52 and p65 are present in early normal erythroid precursors and decline during differentiation, showing a dynamic expression of these components [52]. Moreover, NF-kB contributes to regulate silencing of the human γ-globin gene during the embryo-fetal swiching of globin gene expression [53]. The trans-activation function of NF-kB is also regulated through interaction of the p65 (REL-A) subunit with histone deacetylase (HDAC) proteins, such as HDAC1 and HDAC2, to negatively regulate expression of the NF-kB-dependent genes [54], through a mechanism of protein acetylation and

deacetylation modulation which regulate the entire NF-kB signaling pathway (Fig. 18).[55]

It is well known that histone acetylation changes at the human β-globin locus play a key role in the switching mechanisms of globin gene expression during development.[56]

Our study suggest that CSDA recruits NF-kB on the –200 Gγ-promoter region to modulate local HDAC activity and to repress β-globin gene expression (Fig. 19). This hypothesis is supported by data on different levels of histone acetylation in the -200 Gγ-promoter region, following TSA and Bortezomib treatments, thus confirming the role of HDAC in the transcriptional repression mechanisms of β-globin gene expression and suggesting that NF-kB could modulate HDAC activity.

Taken altogether our results indicate that CSDA multiprotein complexes contribute to modulation of fetal globin gene expression and shed light on the molecular mechanisms involved in globin gene switching. Furthermore, the dose-dependent effects of CSDA repression on fetal globin gene expression suggest that CSDA itself could be under the control of other putative HPFH modulators, which could, therefore, indirectly contribute to the regulation of globin gene expression.

The results of RNAi experiments in K562 and in primary erythroid cells drive attention on CSDA as a potential molecular target for treatment of hemoglobinopathies because silencing of CSDA expression is expected to induce persistence expression of HbF in adult life. Experiments of stable interference of CSDA expression are required to explore the feasibility of such approach and to evaluate the long-term effects of this treatment in an

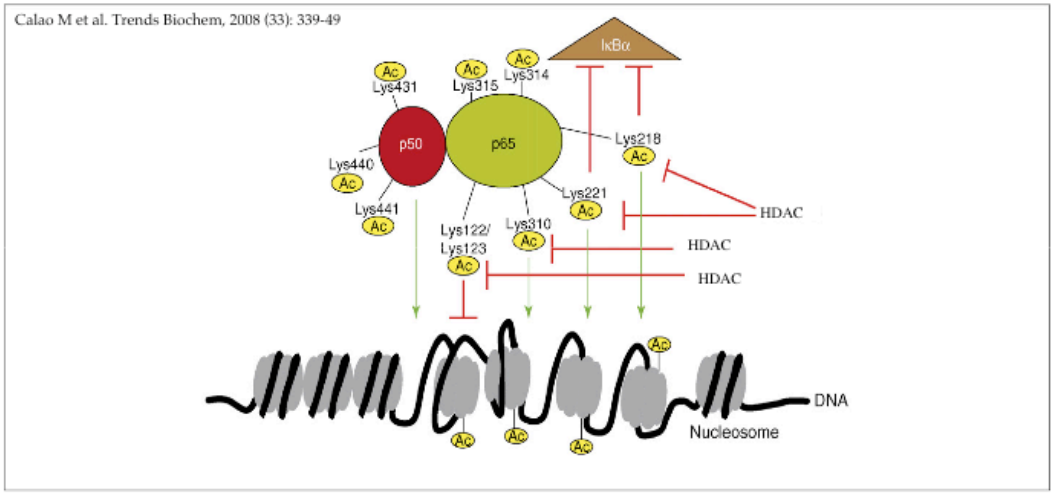

Figure 18: **HDAC deacetylation sites on nucleosome components and NF-kB p50 and p65 subunits.** Acetylation and deacetylation events, in combination with other post-translational protein modifications, generate an "NF-kB-signaling code" and regulate NF-kB-dependent gene transcription in an inducer- and promoter-dependent manner. Indeed, theintricate involvement of histone acetyltransferases and histone deacetylases modulates both the NF-kB-signaling pathway and the transcriptional transactivation of NF-kB-dependent genes.

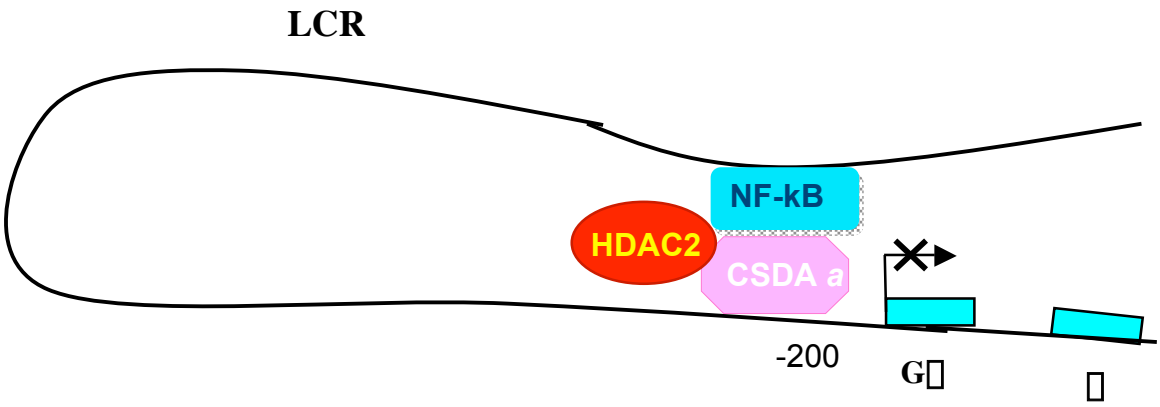

Figure 19: **Schematic representation of the proposed model of the CSDA protein complex involved in modulation of -globin gene expression.**

erythroid environment.

In conclusion, our study helps to elucidate the complex network of factors regulating globin gene expression and could eventually pave the way to novel RNA interference based therapeutics for hemoglobinopathies.

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