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"Understanding the mechanism of CREB overexpression in pediatric acute myeloid leukemia"

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“The journey of a thousand miles begins with one step.”

Lao Tzu

A mamma e papa,

Grazie a voi sono quello che sono.

INDICE

SUMMARY

Currently, acute myeoid leukemia (AML) is one of the cancer for which only about half of children and young adults are cured of this disease. Most patients with AML achieve remission after therapy, but nearly half of these patients experience relapse. In addition, despite improvements in supportive care, treatment-related morbidity and mortality remain significant problems. Therefore, the overall goal of current AML programs for children and young adults is to explicate the mechanism of leukemogenesis discovering other oncogenes and molecular targets and to develop novel therapies that overcome drug resistance, decrease relapse rates, and reduce the short- and long-term adverse effects of treatment (1). Recently, the cAMP response element (CRE) binding protein (CREB) has been demonstrated to be overexpressed in the 66 % of leukemic blast cells from patients with AML and in the 84 % of patient with acute lymphoid leukemia (ALL) compared to normal bone marrow or remission samples. CREB overexpression was also associated with a worse prognosis in CREB overexpressing AML patients (2,3). The mechanism of CREB overexpression in leukemia was investigated. First, we analyzed ICER, the endogenous repressor of CREB, and its role in regulating CREB-dependent transcription and its involvement in increasing chemotherapy induced apoptosis in leukemic cell after its forced exogenous expression. ICER was found downregulated in AML cell lines, displaying an inversed correlation with CREB expression. Initially we focused on restored ICER expression in cell lines able to decrease CREB protein and to lower clonogenic potential *in vitro. In vivo*, ICER was able to decrease the extramedullary sites invasion and overall angiogenesis in NOD-SCID mice tail vein injected with HL60 overexpressing ICER, demonstrating therefore it's effect as a suppressor of tumor progression. ICER was found to repress the majority of CREB targets binding on the same sites of interaction on DNA, the CRE motif. An explanation for ICER down regulation in leukemia was found showing that ICER is subjected to degradation through a constitutively active form of the extracellular signal-regulated protein kinase (ERK), upregulated in leukemia and maintained by CREB, which drives it to the proteasome. We then focused on ICER's role in the control of genes involved in apoptosis and MAPK signaling. ICER was found to confer cell enhanced sensibility to drugs when treated with chemotherapics, reducing cell growth and enhancing apoptotic behavior after chemotherapy treatment. A significant lowered expression of CREB target genes involved in cell cycle control (CyA1,B1,D1), and in the MAPK signaling pathway (ERK, AKT, DUSP1/4) was documented. The dual-specificity phosphatases DUSP1 and

DUSP4, directly repressed by ICER, through p38 pathway were identified as main effectors of the enhanced apoptosis. This pathway was confirmed by using p38 directed drugs. The silencing of DUSP1/4 in HL60 confirmed the same enhanced drug sensitivity as established in HL60+ICER. Moreover primary AML cultures showed the same effect.

Given the fact that overexpression of CREB protein did not correlate closely with CREB mRNA levels, suggested that posttranscriptional mechanisms may contribute to its elevated expression in leukemia (4). MicroRNAs that target CREB were identified and investigated as negative regulator of CREB expression. RQ-PCR revealed that miR-34b was expressed significantly less in myeloid cell lines and in AML bone marrow compared to bone marrow controls, showing a inversely correlation with CREB expression. In vitro experiment confirmed the direct regulation of miR-34b on CREB 3'untranslated region, resulting in a reduced CREB protein expression. MiR-34b restored expression caused cell cycle abnormalities, reduced anchorage independent growth, and altered CREB target gene expression, therefore suggesting its role as tumor suppressor. The miR-34b/34c promoter was demonstrated to be hypermethylated in leukemia cell lines, explaining a mechanism of miR-34b down regulation. Then, we further explored the molecular basis of miR-34b as tumor suppressor in AML samples. Primary cultures transiently overexpressing miR-34b showed decreased clonogenicity and increased apoptosis *in vitro*, while *in vivo* miR-34b overexpression in leukemic cell lines downregulated CREB levels, unveiled a reduced leukemia progression in NOD-SCID IL2Rγ null (NSG) mice. Hypermethylation of miR-34b promoter was demonstrated in 65.5 % (74/113) of AML patients, and correlated with elevated CREB protein levels. Bone marrow cells from 49 patients with myelodysplasia (MDS) or juvenile myelomonocytic leukemia (JMML) werefound unmethylated at miR-34b promoter, and CREB expression was not detectable. Three patients with (MDS) that evolved to AML had miR-34b promoter hypermethylation exclusively at the onset of AML. The role of miR-34b/CREB in the evolution of MDS to AML was then analyzed by lowering miR-34b expression in primary healthy samples. Increased CREB levels and upregulation of its target genes expression resulted in increased myelopoiesis and clonogenic capability.

Taken together, these results suggest the important role that ICER cover in regulating CREB transcription and in the chemosensitivity to drugs and suggest that the miR-34b hypermethylation, throu controlling CREB expression, is a critical process for AML pathogenesis widening the set of pathway that can be addressed for the development of new cancer therapies.

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SOMMARIO

Solo la metà dei pazienti in età pediatrica riesce a guarire dalla leucemia mieloide acuta (LAM): la maggior parte dei malati raggiunge la remissione in seguito a chemioterapia, ma, circa metà di questi, ricade. Inoltre, nonostante i miglioramenti nelle terapie di supporto, la morbidità e mortalità connesse al trattamento rimangono un problema importante. Lo scopo, dunque, della ricerca in campo LAM è quello di spiegare i meccanismi di leucemogenesi per scoprire nuovi oncogeni e target molecolari. Il fine di questi studi è quello di sviluppare nuove terapie farmaceutiche, diminuire la frequenza di ricaduta e ridurre gli effetti collaterali indotti dalle terapie correnti (1). Di recente, CREB (*cAMP response element (CRE) binding protein*) e' stato dimostrato essere sovra espresso nell'84 % delle leucemie linfoblastiche acute e nel 66 % delle LAM, ma non in controlli sani ne in midolli di pazienti in remissione. CREB ad alti livelli si è visto essere associato ad una prognosi peggiore (2,3). In questa tesi è stato chiarito il meccanismo dell'overespressione e il ruolo di CREB nella leucemia mieloide pediatrica. In primo luogo è stato studiato ICER, il repressore endogeno di CREB. ICER è stato trovato sottoespresso in linee leucemiche mieloidi, e presenta un andamento inversamente proporzionale all'espressione di CREB. Durante questo dottorato, abbiamo indotto un'espressione forzata di ICER in cellule leucemiche in modo da abbassare la quantità di proteina CREB e col risultato maggiore di ridurre il potenziale clonogenico *in vitro. In vivo,* ICER ha ridotto l'invasione extramidollare e l'angiogenesi in topi NOD-SCID iniettati con una linea leucemica mieloide (HL60) stabilmente esprimente ICER, dimostrando dunque come quest'ultimo sia un soppressore della progressione tumorale anche *in vivo*. ICER reprime la maggior parte dei target di CREB andando a legarsi sul DNA agli stessi siti di interazione, la cosidetta sequenza CRE. L'assenza di ICER nella leucemia è stata inoltre dimostrata dipendere dalla degradazione della stessa proteina via proteasoma grazie alla documentata interazione con ERK (extracellular signal regulated protein kinase). ERK nella leucemia è upregolata e mantenuta attiva da CREB. Successivamente, il ruolo di ICER come fattore di trascrizione nel controllare i geni che sono coinvolti nel meccanismo di apoptosi e di proliferazione, ha dimostrato che i target repressi sono geni coinvolti soprattutto nel ciclo cellulare (CyA1, B1, D1) e nel pathway delle MAPK (ERK, AKT, DUSP1/4). Le fosfatasi DUSP1 e DUSP4 sono state identificate come i principali effettori dell'aumentata apoptosi indotta dopo somministrazione di chemioterapici, attraverso la mancata defosforilazione di P-p38, in quanto, un inibitore di p38 è stato in grado di invertire il fenomeno. Il silenziamento delle

DUSP1/4 nella linea cellulare leucemica HL60 ha confermato gli stessi effetti dell'overespressione di ICER. Fenomeni analoghi si sono inoltre osservati nelle primarie di LAM.

Succesivamente alla scoperta che l'overespressione della proteina CREB e il livello di mRNA non era diretta, abbiamo considerato che un meccanismo posttrascrizionale potesse essere una causa dell'overespressione di CREB nelle LAM (4). A questo scopo abbiamo considerato il possibile ruolo svolto da un miRNA su *CREB*. La caratterizzazione del miR-34b è giunta *in primis* dall'osservazone che questo fosse espresso in maniera ridotta nelle linee leucemiche e nei midolli di pazienti affetti da LAM, con andamento dunque inverso rispetto all'espressione della proteina CREB. Esperimenti *in vitro* hanno validato una regolazione diretta da parte del miR-34b su *CREB*. Il ripristino dell'espressione del miR-34b dunque è stato usato come tecnica per identificare il suo ruolo nelle LAM. Anomalie nel ciclo cellulare, una diminuizione del potere clonogenico e una alterazione dell'espressione dei geni target di CREB ha chiarito un ruolo come soppressore tumorale per il miR-34b. Il promotere del miR-34b/34c e' stato poi scoperto essere ipermetilato nelle linee leucemiche, dando una spiegazione epigenetica alla ridotta espressione del miR-34b nelle LAM. In seguito, il meccanismo molecolare del miR-34b come soppressore tumorigenico è stato approfondito anche in campioni primari di midollo osseo provenienti da pazienti affetti da LAM. Colture primarie overesprimenti in maniera transiente il miR-34b hanno mostrato una diminuita clonogenicità e un aumento dell'apoptosi *in vitro*, mentre *in vivo* l'overespressione stabile del miR-34b in linee leucemiche iniettate in topi NOD-SCID ha rivelato una ridotta progressione del tumore. L'ipermetilazione del promotore del miR-34b, e la conseguente overespressione di CREB, è stata inoltre riscontrata nel 65,5 % (74/113) di pazienti all'esordio di LAM, ma in nessuno dei 49 pazienti affetti da sindrome mielodisplastica (MDS) o JMML (juvenile myelomonocitic leukemia) si è constatata metilazione. Anche i livelli di espressione di CREB in questo gruppo di pazienti non erano rilevabili. Interessante è stato trovare che tre pazienti con MDS evoluta a LAM acquisivano la metilazione solo alla diagnosi di leucemia conclamata. Il ruolo del miR-34b nell'evoluzione della LAM è stato quindi analizzato. Campioni di midollo osseo di donatore che presentano alti livelli di miR-34b e bassi livelli di CREB, sono stati usati come modello di evoluzione in LAM. Inibendo il miR-34b e aumentando l'espressione di CREB e dei suoi target ha alterato la mielopoiesi e la capacità clonogenica del campione sano.

Riassumendo, questi risultati suggeriscono come CREB sia un proto-oncogene nelle LAM e ICER sia il suo soppressore. Inoltre, l'ipermetilazione del miR-34b che controlla l'espressione di CREB, si distingue come un processo critico nella patogenesi della leucemia mieloide acuta aprendo a futuri studi per lo sviluppo di nuove terapie terapeutiche mirate.

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CHAPTER 1

INTRODUCTION

HEMATOPOIESIS

Blood cells have a limited lifespan and, therefore, the maintenance of a constant pool requires persistent supply. This supply is provided by pluripotent hematopoietic stem cells (HSCs), an extremely rare population (0.1-2 %) of nucleated bone-marrow cells, which have the ability to either self-renew, maintaining the pool of HSCs, or to differentiate into various blood cells that lose their self-renewal capacity (1,2). A number of lines of evidence indicate that there is a finite limit to the replicative potential of HSCs, although in healthy individuals, this appears to be sufficient to ensure lifelong production of mature blood cells (3).

According to the current model of hematopoiesis, maturing HSCs give rise to multipotent progenitors, which in turn differentiate into either common lymphoid or common myeloid progenitor cells (CLPs and CMPs, respectively), both of which are lineage-restricted (4).

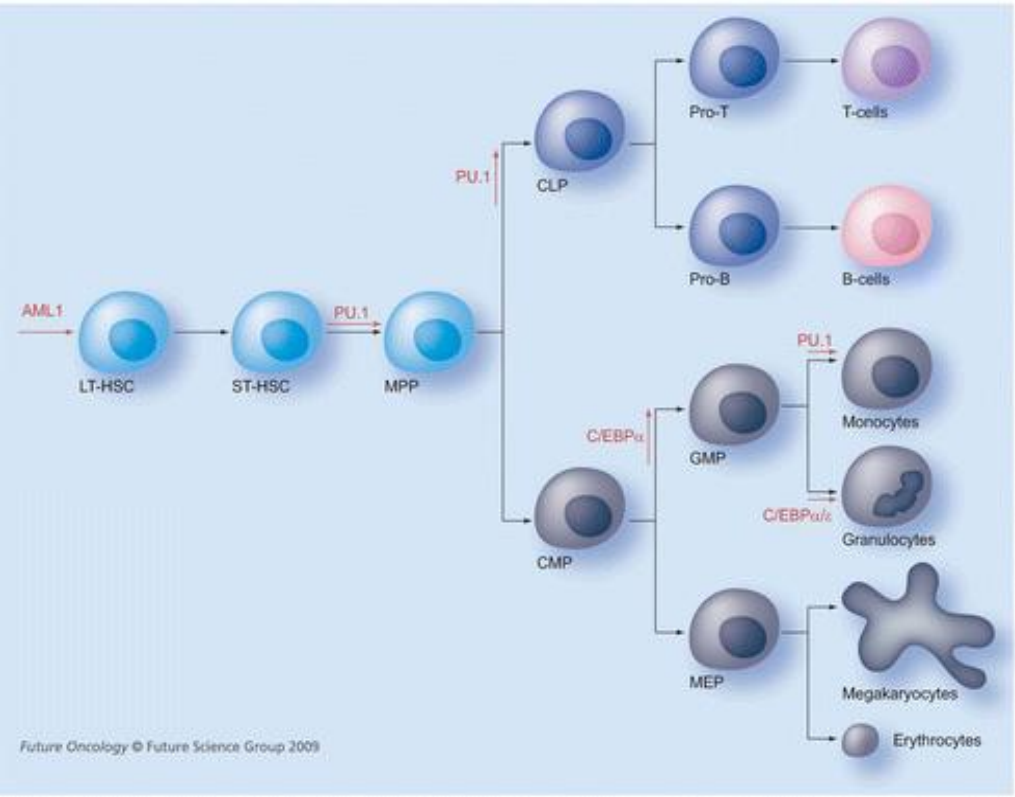

Figure 1.1. Hematopoiesis: From hematopoietic stem cells to mature effectors (5).

CMPs differentiate into either granulocyte–macrophage lineage-restricted progenitors (GMP) or megakaryocyte/erythrocyte lineage-restricted progenitors, which eventually differentiate into

functional end cells, such as monocytes, granulocytes, erythrocytes and platelets. CLPs then further differentiate into pro-B and pro-T cells, which give rise to terminally differentiated B cells and T cells, respectively.

Lineage commitment could be induced either by extracellular factors, including cytokines, direct cell-cell interactions, or other environmental signals. Alternatively, it could be induced by intrinsic mechanisms, such as upregulation of transcription factors, or other regulatory molecules, such as microRNAs. Whether lineage decisions are induced by extracellular cues, by intrinsic events, or by a combination of both, they always involve changes in gene expression programs.

The regulation of gene transcription is critically mediated by the binding of sequence-specific transcription factors to target gene promoters and enhancers. These factors recognize those regions of the genome destined to be transcribed into RNA, and work in part by recruitment of basal transcription factors and RNA polymerase II to target genes. Sequence-specific DNA-binding factors also recruit cofactors to gene regulatory regions, many of which are part of multiprotein enzymatic complexes which facilitate or inhibit gene transcription by modification of chromatin (6). Epigenetic modulation of gene expression represent another level of regulation of this delicate process.

IMPAIRED MYELOPOIESIS

The progression from HSCs to differentiated progeny involves coordinated control of sequential gene expression programs leading to activation or repression of lineage-specific genes. Ineffective hematopoiesis resulting in homeostatic imbalance in the production of blood cells lead to a series of hematological disorders. The interest of this work is addressed to myeloid disorders.

Myeloid malignancies are disorders characterized by acquired somatic mutation in hematopoietic progenitors. They are stem cell-derived clonal disorders and include three broad clinicopathologic categories: myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN) and acute myeloid leukemia (AML). However, such classification is not precise; for example, some patients present features that are characteristic of both MPN and MDS, and are assigned to an ‘‘overlap’’ category of MDS/MPN. The World Health Organization (WHO) has developed a classification to help physician in their work.

Classification of Myeloid Neoplasms According to the 2008 WHO Classification

1. **Myeloproliferative neoplasms (MPN)**
2. **Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB, and FGFR1**
3. **MDS/MPN**
4. **Myelodysplastic syndromes (MDS)**
5. **Acute myeloid leukemia (AML)**

Myelodysplastic syndromes (MDSs) are a group of heterogeneous disorders with an ineffective, inadequate, and dysplastic hematopoiesis. They are characterized by a neoplastic clonal stem cell proliferation which gradually replace and suppress the normal marrow elements. Clinically they are characterized by blood cytopenia due to ineffective hematopoiesis and excessive apoptosis of hematopoietic precursors in the marrow. The impaired production and maturation of hematopoietic cells is manifest by 10 % or more unilineage or multilineage dyslasia. MDS are typical disorder of the elderly, while they represent 2-3 % of the hematological malignancies in childhood. The incidence of MDS appear to be increasing following the use of intensive chemotherapy as secondary or treatment related MDS.

MDSs have an increased risk of evolution to acute leukemia, with approximately 30 % of MDS patients progressing to acute myeloid leukemia (AML) during their course of disease. Myelodysplastic syndromes and the progression into acute leukemia serve as a model for the multistep concept of leukemogenesis. Knudson's model of the 'two hits' provides the basis for the concept of a multistep pathogenesis in the development of MDS and leukemogenesis.

Myeloproliferative neoplasm (MPN) are a spectrum of clonal disorders of the hematopoietic system with an excessive cell proliferation. Common to most MPN is a small but finite risk of disease evolution to an acute leukemia. They are charactherized by mutation of JAK2 and a percentage of blast cells comprises between 10 and 20%.

The myelodisplastic/myeloproliferative neoplasm are rare de novo myeloid neoplasm which display features that are characteristic of both MDS (dyserythropoiesis or dysgranulopoiesis) and MPN (peripheral blood granulocytosis, monocytosis, eosinophilia, or thrombocytosis) at presentation. The bone marrow of patients with MDS/MPN is

characteristically hypercellular. By definition the percentage of blasts in the bone marrow or in the blood must be less than 20 %. This category include the juvenile myelomonocytic leukemia (JMML) which represent less than 2 % of hematopoietic malignancies in the early childhood (7).

Acute myeloid leukemia (AML) is an hematopoietic stem cell disorder relatively rare (10 % of overall leukemia in the childhood) that is characterized by rapid growth of a clonal population of neoplastic cells that accumulate in the bone marrow and other organs as a result of acquired distinct but cooperative genetic mutation that confer a proliferative and survival advantage (Class I mutations) and impair differentiation and apoptosis (Class II mutations). The neoplastic myeloblast are 'frozen' in a varety of differentiation stage, depending on the type, with a loss of normal hematopoietic function due to alteration in the mechanism of self-renewal, proliferation, differentiation (8). To be called acute, the bone marrow usually must include greater than 30 % leukemic blasts. AML comprises a heterogeneous group of aggressive myeloid neoplasms characterized by rapid proliferation of granulocytic, monocytic, megakaryocytic, or rarely, erythroid blast cells (9). While MDS is a disease in which ineffective hematopoiesis is thought to be attributable for the most part to defects in apoptosis, AML is a disease in which impaired differentiation and proliferation play a greater role.

THE PATHOGENESIS OF MYELOID DISORDERS

Genetic alterations, including gene mutations, and chromosomal amplifications, deletions, inversions, translocations are hallmarks of the molecular biology of cancer (10). A growing number of evidence has supported the hypothesis that the differential biologic behavior of tumor cells could be explained in terms of inheritable changes in the patterns of gene expression that occur without a change in the primary nucleotide sequence; this regulation of transcriptional activity is the result of an enzyme-mediated reorganization of chromatin three dimensional structure that modulates its accessibility for transcription factors and other proteins involved in the process of gene expression (11). This kind of modifications go under the name of epigenetics. The elucidation of the function and occurrence of leukemogenic genes in leukemia, and the mechanism of pathogenesis of MDS and MPN has led to a two-hit model of AML pathogenesis: the Knudson's model (12). According to this model, AML develops as a result of two classes of genetic alterations (mutations or gene rearrangements) as previously discussed. A brief excursus

elucidates in the following paragraphs some well studied mechanism of pathogenesis in myeloid neoplasms.

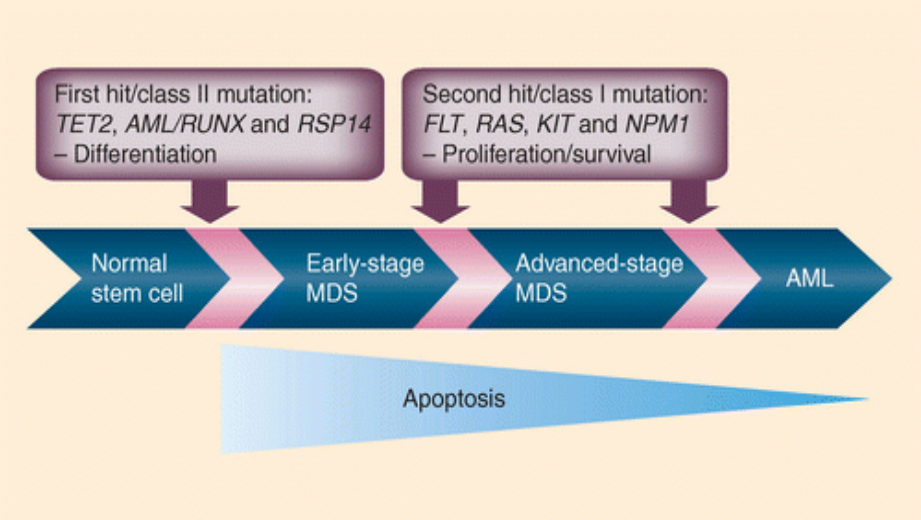

Figure 2.1. Multistep pathogenesis in myelodysplastic syndrome and mechanism of progression to acute myeloid leukemia (13).

Translocations

Chromosomal translocations are a recurring feature in hematological malignancies. Chromosomal aberrations are found in about half of all AML and MDS cases and are grouped into two major subtypes: balanced and unbalanced aberrations (14). In AML, chromosomal translocations often result in loss-of-function mutations in transcription factors that are required for normal hematopoietic development (2). Moreover, translocation often have prognostic significance and detection of these chromosome abnormalities is crucial not only in diagnosis and sub-classification but also in selecting appropriate treatment protocols: for example patient affected with t(8;21), t(15;17), or inv(16) have usually a better prognosis than deletion of chromosome 5, 7 (-5,-7), del(5q) (15). Translocations may lead to the formation of novel fusion proteins or overexpression of transcription factors in inappropriate temporal or developmental patterns. This may lead to global shifts in gene expression, which frequently lead to increased self-renewal in the malignant cells at the expense of normal differentiation. The function of the novel fusion genes may involve tyrosine kinases, such as *ABL*, transcription factors (*AML1*), growth factors (*IL3*) or their receptors (*TAN1*). The four most prevalent oncofusion proteins in leukemia are PML-RARα, AML1-ETO, CBFα-MYH11 and MLL-fusions. Interestingly, the *MLL*

gene at 11q23 and *AML1* at 21q22 have been found translocated with a variety of partner genes, and all of the fusion proteins contributes to leukemogenesis.

Signal transduction

In the classical 'two hit' model (12), the second hit is thought to be caused by alterations in receptor tyrosin kinases (RTKs) signaling and various aberrantly regulated pathways have been described so far. Basically, there is an oncogenic potential in every receptor with tyrosine kinase activity. Structural modifications can lead to constitutive activation of RTKs, subversion of molecular control mechanisms and alterations in signal transduction. Deletions within the extracellular ligand-binding domain alter ligand responsiveness, or eliminate negative control mechanisms that this structure might exert on the kinase domain. Even point mutations are able to induce overall ligand-independent conformational alterations and hence activation of RTKs. Besides genetic alterations, over-expression of the wildtype receptor are known to play an important role in aberrant signal transduction enhancing the survival and proliferation of hematopoietic progenitor cells. Activation of signal transduction in myeloid disorders may occur through a variety of genetic alterations affecting different signaling molecules, such as the *FLT3* and *KIT* and members of the *RAS* family of guanine nucleotide-binding proteins. Activating mutations of *FLT3* and *KIT* are found almost exclusively in acute leukemia. Other class I alterations, which are also seen in different myeloid malignancies, are gain of function mutations of the three main *RAS* isoforms (*N-RAS, K-RAS, HRAS*) (16,17). However, the large number of mutant signaling proteins that have been identified in AML and MDS are likely to reflect activation of a more limited number of downstream effector pathways, such as the RAF/MEK/ERK and PI3K/AKT cascades (18).

MicroRNA

MicroRNAs (miRNAs) have been associated with almost every normal cell function, including proliferation, differentiation and apoptosis. As a consequence, they play a very important role in normal hematopoiesis because they regulate hematopoietic differentiation in almost every stage. Their aberrant expression has been associated with many diseases, including

hematological malignancies. MiRNAs are small (19–24 nucleotide), non-protein-coding single strand RNAs that regulate post-transcriptional gene expression by inhibiting protein translation or destabilizing target transcripts. They recognize target sites predominantly in the 3′-untranslated regions (UTRs) of mRNAs and less often in the 5′-UTRs or coding sequence through perfect (in plants) or imperfect (in mammals) base-pairing. Approximately 30 % of human genes possess conserved miRNA binding sites and are presumed to be regulated by miRNAs. Bioinformatical analysis predicts that the 3′ UTR of a single gene is frequently targeted by several different miRNAs. Many of these predictions have been validated experimentally, suggesting that miRNAs might cooperate to regulate gene expression.

They were found to play a role both as oncogenes through elimination of tumor suppressor proteins, or as tumor suppressor genes by targeting oncogenic mRNAs (19). Various miRNA were found altered in myeloid disorders. Some example are listed in the following table.

miRNA	Location	Expression	Regulated by	Target	*Invitro* effects	*In vivo* effects
miR-155	21q21.3	Upregulated in FLT3-ITD	*NFkB*	*SHIP1*	Blocks megakaryopoiesis	Induces myeloproliferation with MDS changes in mice
				CEBPB	Induced myeloid colonies	
miR-196b	7p15	Up-regulated in t(11q23)/MLL	*MLL*	*HOXB8*	Increases cell survival and proliferation of progenitors	
miR-223	Xq12	Down-regulated in t(8;21)	*RUNX1/RUNX1T1 CEBPA, NFIA*	*MEF2C*	Induces granulocytic differentiation	Granulocytosis in *miR-223* KO mice
miR-29b-1	7q32	Down-regulated in wild-type NPM1 t(11q23)/MLL and -7 and del(7q)	*NFkB,MYC*	*MCL1*	Induces apoptosis	Reduces tumorigenicity
			YY1	*CDK6, SP1*	Inhibits proliferation	
				DNMT3A/ B	Regulates negatively DNA methylation	

Table 1.1. MicroRNA with documented functional role in myeloid leukemogenesis (20).

Epigenetics

Epigenetics, the study of heritable changes in gene function that occur without a change in DNA sequence, is reshaping the way scientists look at traditional genetics. Epigenetic mechanisms comprehend principally DNA methylation and histone acetylation. DNA methylation patterns are perturbed in many human cancers and typically involve regional hypermethylation of CpG islands frequently affecting tumor suppressor genes which are silenced

and occur within an overall setting of genome-wide DNA hypomethylation, which has been linked to genomic instability (21). Cancer cells also show histone deacetylation and a global down-regulation of microRNAs. Hypermethylation of genes involved in cell cycle control and apoptosis is a common feature, particularly in AML and high-risk MDS. Examples of important genes for cell cycle regulation that are hypermethylated in the promoter region of MDS and AML patients are the cyclin-dependent kinase inhibitors p15 (INK4b) and p16 (INK4a). These two genes are rarely mutated or deleted, but transcription of the gene is often silenced due to hypermethylation (22).

Interestingly, DNA methylation microarrays were used by Jiang and coworkers to compare the methylation status of early-stage MDS samples with advanced-stage MDS and AML samples and found an increase of methylated CpG loci in the latter MDS/AML group (23). This led to the suggestion that DNA methylation might be one of the dominant mechanisms for MDS evolution to AML.

Transcription factors

The knowledge that cancer is a results of an accumulation of multiple genetic alterations is currently accepted. Many signaling pathways that are disrupted in cancer converge on transcription factors (TFs), ultimately leading to altered expression of numerous target genes. TFs are activated in response to various stimuli and are able to coordinate and integrate all the information activating or repressing the transcription of target genes. The capacity to regulate different pathway in the cell is due to the characteristic structure of TFs that are able to recognize specific DNA sequences via DNA-binding domain, able to recruit either co-activators or co-repressor to the regulatory regions of genes via protein interaction domains, and able to recruit chromatin remodeling machinery and proteins involved in binding RNA polymerase. Given this critical role, the function of transcription factors is normally closely regulated in the normal cell microenvironmet, often through transient phosphorylation (24). Given the importance of a faithful execution of biological processes such as development, proliferation, apoptosis, survival, and differentiation, a precise and carefully orchestrated set of steps that depend on the proper spatial and temporal expression of genes is necessary.

In hematopoiesis, key transcription factors play central roles in cell fate specification and subsequent differentiation. Importantly, disruption of these factors is often associated with

neoplastic transformation and tumor progression. Moreover, transcription factors are frequently targeted by balanced chromosomal translocations as well as abnormal expression (WT1, ERG) in AML. These include core binding factor (CBF), MLL, RUNX1 and HOX genes. Transcriptional coactivators, such as Creb-binding protein (CBP), p300, and MLL, are also targets of chromosomal translocations in AML (25,26). Furthermore therapies currently adopted in pre-clinical trials arise from research on transcription factors and the results obtained so far, encourage their further study (27).

CREB STORY: A MEMBER OF BZIP FAMILY OF TF

The cAMP response element (CRE) binding protein (CREB) is one of the best studied transcription factor. It's a member of an highly evolutionary conserved family of TF that mediates cyclic AMP (cAMP), growth factor-dependent and calcium-dependent gene expression through the cAMP response element, the CREB/CREM/ATF-1 basic leucine zipper (bZip) family (28-30). In mammals, this family members include *CREB*, the cAMP responsive element modulator (*CREM*), the activating transcription factor (*ATF*) -1, *ATF-2*, *ATF-3*, *ATF4* also known as *CREB2* and and *ATF-5* (31,32).

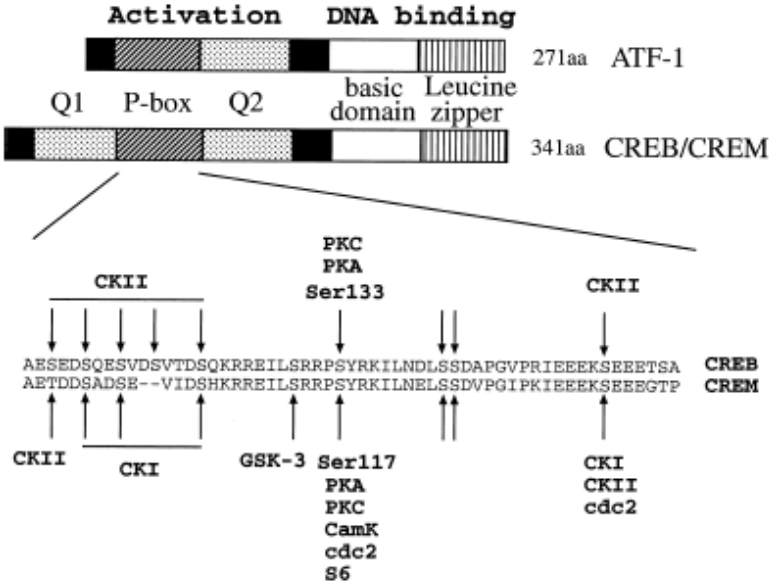

Figure 1.2. Structure of ATF-1, CREB and CREM proteins. The P-box or KID domain, the glutamine-rich domains (Q1 and Q2) and the DNA binding region (leucine zipper and basic domain) are indicated. Phosphorylation sites and kinases responsible for phosphorylation are indicated in the sequence alignment of the KID domain of CREB and CREM (32).

This family of transcription factors present a C-terminal bZip dimerization domain, consisting of an α-helical coiled coil structure, that induce homo or heterodimerization of the transcription factors and binding on the target promoter to a regulatory sequence known as CRE, which has a palindromic consensus sequence 5'-TGACGTCA-3'. This family of transcription factors shares structural features within the transactivating domains. Transcriptional activation is mediated through two regions: one contains several recognitions motifs for protein kinases, it is therefore called kinase inducible domain (KID) or posphorylation box (P-box). The other constitutive activation region, contained in *CREB* and *CREM* genes, consists of two hydrophobic glutamine rich domains, called Q1 and Q2, which flank the KID domain. Mammalian ATF-1 lacks Q1 but contains Q2. Glutamine rich domain can be found in many regulatory, coactivator and basal transcription factors and serve as interaction surfaces for other transcription factors. It has been suggested that CREB and CREM require the KID domain and at least one glutamine rich domain to activate transcription (33,34).

Furthermore, each one of these genes is known to encode for many isoforms generated by several mechanisms, such as alternative splicing, and use of an alternative initiation codon and an alternative, intronic promoter. Among the different isoforms of *CREB* and *CREM*, some proteins are transactivators; others repress transcription. Repressor isoforms are generated by several mechanisms. Alternative splicing, for example, can remove the glutamine-rich regions partially (e.g. Drosophila *CREB2b*) or completely (e.g. mammalian *CREMa*, *CREMb* and *CREMg*) (34,35). Alternatively, it can result from insertion of premature stop codons (e.g. in *CREB* and *Aplysia CREB1*); the truncated proteins lack the DNA-binding domain and the nuclear localization signal (e.g. *CREBg, CREBV and CREBc, and Aplysia CREB1c*) (36).

CREB

CREB has been localized in human to chromosome 2q32.3-q34 (37) and encodes for a 43KDa nuclear transcription factor ubiquitously expressed in all tissues. It is activated through phosphorylation at serine (Ser) 133 in response to a variety of cellular and mitogen stress signals. Phosphorylation of Ser 133 is necessary for signal-induced transcription in vivo. The reason is not fully understood. Probably, phosphorylation of CREB may stabilize the protein, it may promote the translocation of cytoplasmic CREB to the nucleus, (although CREB is primarily

nuclear) or it may promote the ability of CREB to dimerize with other bZIP partners and bind to DNA.

Upon activation, CREB binds as a dimer to the CRE, 5'-TGACGTCA-3', or half CRE sites CGTCA/TGACG, where it promotes the recruitment of the transcriptional coactivator CREB binding protein (CBP) and p300. These coactivators serve as a bridge that allows CREB to recruit and stabilize the RNA polymerase and the basal transcription machinery to iniziate transcription of target genes.

Dephosphorylation appears to represent a key mechanism in the negative regulation of *CREB* transcription (38), however the discovery of *CREM* genes opened a new dimension in the study of the transcriptional response to cAMP.

CREM: ANOTHER CREB/CREM/ATF-1 FAMILY MEMBER

CREM, is localized on chromosome 10p11.21. It generates by a process of extensive alternative splicing, both repressors and activators of transcription in a tissue specific and developmentally regulated manner. Using an alternative intronic promoter (P2) the *CREM* genes generates the powerful transcriptional repressor ICER (inducible cAMP early repressor) which deserves a special mention, since it is responsible for its early response inducibility which is unique amongst CRE-binding factors (39). The *ICER* specific promoter P2 contains tandemly repeated CRE-like elements and is rapidly and strongly induced upon activation of the cAMP pathway. ICER comprises the bZip domain of CREM, but lacks the KID domain. After induction, ICER can compete with CREB for its own promoter thus suppressing its own transcription in a negative autoregulatory loop (39). This negative feedback loop serves as a temporal gene controlling mechanism that allows the cAMP-dependent signaling cascade to prepare for subsequent incoming signals. Moreover, *ICER* expression can be activated in a nonCRE-dependent manner (40). Intracellular levels of ICER protein are controlled by transcription regulation and by protein degradation through the ubiquitin–proteasome system (41).

CREB TARGET GENES

Studies have demonstrated that CREB is phosphorylated in response to up to 300 different stimuli (42). The various functions of CREB are mediated by differential regulation of target

genes involved in metabolic function, transcription, cell cycle, survival, DNA repair, growth factors, signaling and immune regulation. Bioinformatics analysis has revealed that approximately 4000 human genes contains conserved CRE sites adjacent to the transcription starting site; 1500 have downstream TATA boxes necessary for optimal induction in reponse to cAMP. Interestingly also the promoter region of human *CREB* has four half CRE site (http://natural.salk.edu/CREB/) two of them have a TATAbox less than 300bp downstream the CRE. Therefore in a positive feedback loop, phosphorylated CREB can induce its own gene via CREs. In support of this suggestion, treatment with serotonin enhanced binding of creb1 to its promoter region and increased mRNA levels of creb1 in Aplysia, therefore CREB is regulated by a positive feed back loop able to prolong its expression and increase CREB mediated transcription (43).

As previously described, CREB is a central mediator of a variety of pathways and its function varies among the tissues or the cell type in which it is expressed. In the liver, for example, CREB regulates gluconeogenesis, through phosphoenol pyruvate carboxykinase (44). In sympathetic and cerebral neurons, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) stimulate survival by activating the expression of the antiapoptotic protein B-cell lymphoma 2 (BCL-2) (45,46). This was further shown by overexpression of a dominant negative form of CREB in these cells resulting in increased cell death. Moreover, this effect was reverted by overexpression of BCL-2. CREB has also been shown to regulate proliferation through *cyclins A1* and *D1* (*CyA1* and *CyD1*) (47,48).

Furthermore, CRE-binding proteins have been shown to play a role in the physiology of the pituitary gland, in regulating spermatogenesis, in the response to circadian rhythm, growth-factor-dependent cell survival, glucose homeostasis and CREB has also been implicated in learning and memory (49). CREB activation is required for the induction of specific genes by growth factors, for example, c-*FOS* by nerve growth factor (NGF) and *EGR*-1 by the granulocytemacrophage colony-stimulating factor (GM-CSF) (50) and therefore it might have a role in myeloid differentiation.

CREB has been shown to stimulate target gene expression by associating with a number of activators including CBP/p300, TORC and TAFII4. However several results suggest that the interaction of phospho-CREB (P-CREB) with those proteins is too weak for cellular gene

activation *per se* and that additional CREB regulatory partners are required for stable recruitment of such cofactors to the promoter.

THE ROLE OF CREB FAMILY IN CANCERS

Although transcription factors are not often directly modified by mutations in cancer cells, they frequently become activated constitutively through mutations affecting "upstream" pathways. By continually driving the expression of key target genes, these oncogenic transcription factors play a central role in tumor pathogenesis.

A potential role for the CREB family in cellular transformation was first appreciated in clear-cell sarcomas of soft tissues (CSSTs), which contain a t(12,22)(q13,q12) translocation that fuses the DNA-binding and leucine-zipper dimerization (bZIP) domain of the transcription factor ATF1 to the Ewing's sarcoma oncogene product (EWS), an RNA-binding protein . The EWS–ATF1 fusion is found in nearly all CSSTs, where it enhances expression of numerous CREB target genes, by functioning as a strong activator (51). Indeed, disrupting EWS–ATF1 activity appears sufficient to block proliferation and to promote apoptosis in these cells (52,53).

Virally encoded oncoproteins such as human T-cell leukemia virus (HTLV-1) tax and hepatitis B virus X also enlist CREB activity in their efforts to promote cellular transformation. Both proteins enhance CREB target gene expression, at least in part, by binding to the CREB bZIP domain and increasing the affinity of CREB for binding to a subset of low-affinity sites on certain cellular promoters (54,55). Tax has also been shown to bind TORCs to potentiate CREB activation and increase the transcription of viral and cellular targets of *CREB* (56). Another oncogenic retrovirus, Hepatitis B virus (HBV), has also been shown to promote cellular transformation by enhancing *CREB* target gene expression in a similar way to HTLV-1. HBV interacts with CREB/ATF2 and p300/CBP to constitutively turn on *CREB* genes. Based on this evidence, CREB would appear to cooperate with other factors, either in the context of a fusion protein or as part of a complex with an oncoprotein, to induce transformation. But whether CREB alone is capable of promoting tumorigenesis remained unclear.

Increased expression and phosphorylation of CREB has been found in non–small cell lung cancer cell lines, compared to nontransformed bronchial epithelial cell lines, and in pathologic samples from tumors compared to normal adjacent epithelium (57).

CREB has also been implicated in many other cancers, some of which include hepatocellular carcinoma, osteosarcoma and leukemias (58). This evidence suggests that CREB can promote cellular transformation (59) and appears to play a direct role in disease pathogenesis and prognosis (24).

Type of cancer	Findings
Acute myeloid leukemia	CREB is overexpressed in AML cells. In transgenic mice that overexpressed CREB in myeloid cells, CREB promotes MPD (Shankar DB et al, Cheng JC et al)
Clear cell sarcoma	Constitutive activation of some CREB targets mediates transforming activity of EWS-ATF1 and EWS-CREB (Brown AD et al, Antonescu CR et al, Davis IJ et al)
Salivary gland tumor	Constitutive activation of CREB targets mediates transforming activity of TORC1-MAML2 (Coxon A et al)
Adult T-cell leukemia	CREB activation is required for transforming activity of human T-cell leukemia virus type I oncoprotein Tax (Smith MR et al, Grassmann R et al)
Hepatocellular carcinoma	Hepatitis B virus oncoprotein X interacts with CREB and promotes CREB-mediated transcription (Cougot D et al)
Peutz–Jeghers syndrome	Inactivating mutations in the *LKB1* gene predispose patients to cancers of epithelial tissue origin. LKB1-AMPK represses CREB by inhibiting TORCs (Shaw RJ et al)
Adrenocortical hyperplasia	cAMP signaling is aberrantly activated and CREB is constitutively phosphorylated as shown in a genome-wide scan (Horvath A et al)

Table 1.2. Evidence in support of a role for *CREB* in oncogenesis. Adapted from *CREB* − a real culprit in oncogenesis (58).

CREB IN LEUKEMIA

In both mouse and humans, CREB is expressed more highly in less differentiated hematopoietic stem cells (HSCs), in common myeloid progenitor (CMP), in common

granulocyte-macrophage progenitor (GMP), in megakaryocyte-erythroid progenitor (MEP), in multipotent progenitor (MPP) cells, compared to more committed cells. Knockdown of *CREB* in normal myeloid progenitor cells results in decreased myeloid proliferation in colony assays and affects short-term engraftment. However, *CREB* downregulation does not have effects on long-term hematopoietic engraftment (48). Therefore, *CREB* is important for the regulation of normal myelopoiesis, although it does not appear to be necessary for hematopoietic reconstitution or definitive HSC activity.

CREB protein has been demonstrated to be overexpressed in the 66 % of leukemic blast cells from patients with AML and in the 84 % of patient with acute lymphoid leukemia (ALL) compared to normal bone marrow or remission samples (47,60). CREB was found upregulated at both protein and mRNA level in CREB-positive (CREB+) primary AML cells, therefore suggesting that *CREB* could be amplified at genomic level. Analysis of primary adult AML blast cells by using fluorescence *in situ* hybridization revealed that three out of four CREB+ AML patients were detected to have more than the two normal copies of *CREB*, and this may be one potential mechanism for CREB overexpression. However, the nature of this amplification has not yet been determined. However, in pediatric samples cytogenetic analysis did not reveal genomic amplification. In all cases in which AML patient bone marrow samples overexpressed CREB, an increased level of phosphorylated CREB was also observed, indicating that the protein is functionally active. Furthermore, CREB overexpression was associated with a worse prognosis (increased risk of relapse and a decreased in event-free survival) in AML patients compared to patients whose bone marrow did not overexpress CREB (47).

In vitro CREB overexpression experiment in leukemia cell line resulted in increased proliferation and survival of those cells. While downregulation of endogenous CREB by siRNA decreased survival and proliferation of leukemia cell lines. This results was confirmed with cell cycle analysis showing a decreased number of cells in S phase. This appears, at least in part, to be due to a decrease in the expression of genes involved in cell cycle such as *cyclins A1* and *D*. Both *CyA* and *D* regulate the G1-to-S transition and have been demonstrated to be CREB target genes (48). Moreover, silencing of CREB in a murine pro-B cell line (Ba/F3) transduced with a fusion protein (Bcr-Abl), characteristic of the translocation t(9,22), and injected into SCID mice resulted in a decreased disease burden and prolonged survival, confirming the role of CREB as a protooncogene (48).

In addition, activation of CREB in leukemia cells appears to be cAMP independent (60, 61). These results demonstrate that CREB is necessary for maximal proliferation of myeloid leukemia cells in vitro and that CREB is probably one but not the only critical target of signaling pathways regulating growth of these cells. However, overexpression of CREB protein did not correlate closely with *CREB* mRNA levels, suggesting that posttranscriptional mechanisms may contribute to its elevated expression.

Therefore, the overexpression of proto-oncogene CREB still remain an open question to be investigated in leukemia. Answers from this open question can lead to the development of new personalized treatment therapy.

CREB KNOCKOUTS AND TRANSGENIC MICE

Various model of CREB/CREM transgenic models have been developed showing the importance of this family. They are summaried in this paragraph.

Creb-/- mice harbouring a deletion in the bZIP domain are fully formed but die at birth from respiratory distress secondary to pulmonary problems (62). Impaired fetal T-cell development is evident in this model; and the number of developing T cells in the thymus is reduced in Creb-/- mice compared with control littermates.

Crem-/- mice, by contrast, survive to adulthood, but males are sterile owing to enhanced apoptosis of post-meiotic germ cells (63). Disruption of circadian rhythms is also evident: Crem-/- mice show similar locomotor activity throughout the circadian cycle; in wild-type littermates, by contrast, locomotor activity follows a circadian periodicity. Crem-/-- mice also show lower anxiety levels in behavioural testing, apparently due to elevated levels of β-endorphin production secondary to loss of ICER-mediated repression in pituitary cells (64).

The absence of a robust phenotype in Creb- or Crem- knockout animals, secondary to functional compensation by other family members (65), has led to the development of transgenic models expressing dominant-negative forms of *CREB.* Such studies have revealed a role for the *CREB* family of activators in control of cell survival and proliferation. A role for *CREB* in growth-factor-dependent survival has been shown in sympathetic and cerebellar neurons (45,46). The cAMP/CREB signalling pathway has been strongly implicated in the synaptic plasticity that is associated with long-term memory. Disruption of CREB in mice, for example, leads to defects in long-term potentiation and long-term memory (36).

Overexpression of a dominant-negative CREB isoform in the fruitfly Drosophila melanogaster blocks memory consolidation in an olfactory learning model, whereas expression of a dominant-active CREB polypeptide accelerates the learning process (66).

Transgenic mice in which CREB is overexpressed in committed granulocyte and macrophage cells under the control of the hMRP8 promoter develop myeloproliferative disease (splenomegaly and aberrant myelopoiesis), but not AML, after a latency of 1 year (47). Those transgenic mice displayed a phenotype similar to AML patients, which included an elevated peripheral monocyte count relative to control littermates. Bone marrow progenitor cells from these mice have increased replating activity and are hypersensitive to growth factors (47). Therefore, CREB plays a major role in the regulation of normal myeloid cell proliferation and differentiation and acts as a proto-oncogene that potentially contributes to leukemogenesis. CREB promotes tumor formation only when other activators are also induced.

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CHAPTER 2

ICER EXPRESSION INHIBITS LEUKEMIA PHENOTYPE AND CONTROLS TUMOR PROGRESSION

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ABSTRACT

ICER and CREB are transcriptional regulators of the cAMP-mediated signalling pathway. CREB has been demonstrated to be upregulated in the majority of childhood leukemias contributing to disease progression, whereas ICER, its endogenous repressor, was found to be downregulated. Our research focus has been the role of restored ICER expression. ICER exogenously expressed in cell lines decreases CREB protein level and induces a lowered clonogenic potentiality *in vitro*. It decreases HL60's ability to invade the extramedullary sites and to promote bone marrow angiogenesis in NOD-SCID mice, demonstrating its potential effects on tumor progression. ICER represses the majority of 96 target genes upregulated by CREB. It binds CRE promoters and controls gene expression restoring the normal regulation of major cellular pathways. ICER is subjected to degradation through a constitutively active form of the extracellular signal-regulated protein kinase (ERK) which drives it to the proteasome. We propose that ICER is downregulated in HL60 to preserve CREB overexpression which disrupts normal myelopoiesis and promotes blast proliferation. These findings define the role of ICER as a tumor suppressor in leukemia. Unbalanced CREB/ICER expression needs to be considered a pathogenetic feature in leukemogenesis. The molecular characterization of this pathway could be useful for novel therapeutic strategies.

INTRODUCTION

The inducible cyclic AMP (cAMP) early repressor (ICER) is a transcriptional factor that derives from an alternative promoter activation of the cAMP response element (CRE) modulator (*CREM*) gene. ICER is the unique CREM isoform that lacks the transactivation domain and conserves the DNA binding domain (1); it recognizes the CRE consensus region which is present in a great number of promoter genes. It homodimerizes or heterodimerizes with the cAMP response element binding protein (CREB) or with CREM modulators mediating a final cAMP responsive genes repression, principally counteracting CREB activity. ICER expression is a transient phenomenon: it competes with CREB by binding and repressing CRE sequences which are present in its own promoter. This negative feedback control of ICER transcription is physiologically important for correct cAMP dependent gene expression in different tissues (2). ICER activity is determined by its intracellular levels rather than by posttranslational modification since it lacks the phosphorylation domain (P-box) typical of its CREB/CREM family member proteins. ICER expression depends on CREB/ICER transcriptional activity and its protein degradation rate (3).

CREB is a nuclear protein that regulates gene expression principally through activation of cAMP dependent cell signal transduction pathways. CREB activity modulation was found to be a result of PKA dependent phosphorylation of CREB at Serine 133. This modification enhances the transactivation potential of CREB promoting the recruitment of several coactivators (4,5). It has been recently demonstrated that aberrant CREB expression in acute leukemia promotes abnormal proliferation, cell cycle progression and cell survival (6).

ICER has been demonstrated to influence cell growth in neuroendocrine tissue, cardiac myocytes and the liver (7-9). Recently it has been proposed as a tumor suppressor gene in

prostate and pituitary cancers (10,11). We previously documented that ICER was downregulated at diagnosis of acute leukemia in a cohort of pediatric patients, whereas CREB protein was found to be overexpressed. On the contrary, the higher ICER expression was found in healthy bone marrow and during remission phases of leukemia therapy, whereas CREB expression was found strongly decreased (12,13). It is still unclear how the interplay of CREB and ICER affects gene expression and, consequently, cell proliferation and survival in leukemia. Considering that CREB sustained expression in leukemia can cause an upregulation of a number of genes involved in different aspects of cell life, and that ICER lowered expression does not allow the physiological CREB dependent gene repression, we chose to consider CREB/ICER balanced expression and regulation as a crucial event in leukemogenesis. We aimed to study the leukemic phenotype after restoring ICER expression in HeLa and HL60 cells that normally do not express it. The second purpose of this study has been to establish how ICER acts on tumor cells and explicate the mechanism that leads to ICER downregulation in leukemia.

Results provide evidence that the period of enhanced expression of ICER corresponds to the lowest level of CREB expression and to a significant modification of cell proliferation and survival *in vivo*. Expression analysis *in vitro* reveals that ICER significantly represses specific CREB target genes. We described ICER degradation by proteasome pathway as one of the possible causes of its lowered expression found in leukemia.

MATERIAL AND METHODS

Cell culture and transfection. HeLa and leukemia cell lines (HL60, FLG, NB4, 697, Jurkat, RS4;11) (American Type Culture Collection, MD, USA) were cultured in DMEM or RPMI (Invitrogen-Gibco, CA, USA) supplemented with 10% fetal bovine serum (FCS; Invitrogen-Gibco). HeLa cells were grown on glass cover slips using DMEM (Invitrogen-Gibco)

supplemented with 10% fetal bovine serum (Invitrogen-Gibco) until 70% confluent, and were transiently transfected with 0.4µg of pEGFPΔ-N1_FLAG-ICERγ combined with 10µl of Efectene (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. After 24 and 48h, cells were stained with anti-FLAG antibody and visualized by confocal microscopy. The empty vector (pEGFPΔ-N1) was used as negative control. HL60 was grown in suspension diluted at $500X10^6$/ml , and after 24h were transiently transfected with 1.5µg of pEGFPΔ-N1_FLAG-ICERγ combined with 100µl of Solution V (Amaxa Biosystems, Koln, Germany) using Nucleofector (Amaxa), according to the manufactur's guidelines. After 24 and 48h, cells were stained with anti-FLAG antibody to visualize ICER nuclear expression by confocal microscopy. Cells were also used for molecular analyses. 1.5µg of pEGFPΔ-N1 was transfected and used as negative control. To obtain a stable clone for ICER, 48h after transfection, cells were cultured in DMEM supplemented with 1.2mg/ml G418 for three weeks. Single cells were sorted and seeded in 96 well plate. Clones were expanded and screened for ICER expression by Western blotting (WB). All analysis discussed in the above were performed in two independent stable clones and within 7 scrambled stable clones all of which provided the same results. We indicated HL60+ICER cell line in those results obtained with ICER stable expression in text and figures. We considered stably empty vector (EV) transfected cell line, defined as HL60+EV, as control.

Plasmid constructs and cloning. The human *ICERγ* mRNA was amplified from HL60 cDNA using primers (PF1-5'ATGGCTGTAACTGGAGATGAAACAG3') and (PR1-CAAGGTCCAAGTCAAAGACAGTTACTCT), cloned in the pCR2.1TOPO vector (Invitrogen-Gibco), sequenced, and compared to the deposited sequence (GenBank AF069065). ICERγ was amplified from the cloned pCR2.1TOPO-ICERγ plasmid, using the T7 primer and a reverse primer which contained the recognition sequence for Not I and also the sequence for the FLAG epitope (Sigma-Aldrich, MO, USA). The PCR product was digested with Not I and EcoR I and

cloned into the pEGFP-N1vector (Clontech, Otsu, Japan) which was excised of GFP (pEGFPΔ-N1) and digested with Not I and EcoR I (pEGFPΔ-N1_FLAG-ICERγ). The empty vector was created from pEGFPN1 digested with EcoR I and Not I. Next, the EV was treated with Klenow enzyme to fill in overhangs and was self ligated (pEGFPΔ-N1_EV). Clones were sequenced with suitable vector primers to confirm the correctness of the reading frame of the fusion gene. The reporter vector (4XCREpGL3/LUC) was constructed using Luciferase gene (LUC) (Promega, WI, USA) as described before (14). All transfections were performed with a second reporter, *Renilla* (REN) gene, as internal control vector for normalization (phRL-TK, Promega). The plasmids were prepared for transfection experiments using the maxiprep endonuclease free kit (Qiagen).

Soft agar colony assay. A total of $2.5x10^3$ cells of HL60+ICER and HL60+EV cell line were used to test the colony-forming cells (CFCs). Cells were plated onto 2-well in a methylcellulose semi-solid medium not supplemented with nutrients or cytokines (StemCell Technologies, Canada) and incubated at 37°C. The colonies were observed every day and counted at day 12. Colony evaluation and enumeration was done *in situ* by light microscopy after 3-(4,55-dimethylthiazol-2-yl)-2,5diphenyltetrazolium,MTT) incorporation.

***In vivo* assay.** We used sex and age matched NOD-SCID mice. Mice received 250 cGy of total body irradiation up to 24h before inoculation of $5X10^6$ of stable HL60+EV or HL60+ICER cells/mouse. Animals were sacrificed after 3 weeks and the bone marrow (BM) from flushing femurs, peripheral blood (PB) and internal organs were collected for histological and immunophenotypic analyses to determine the presence of invading leukemia cells. Leukemia engraftment was monitored in BM and PB by flow cytometric measuring of the amount of positive human-CD45 and murine-CD45 cells. Sections of BM and spleen were processed for histological examination using antihuman MHC class I antibody to evaluate tumor dissemination.

To determine BM angiogenesis, BM sections were stained with anti-CD34 antibody, and vascular morphometric parameters were calculated as previously described (15). The persistence of ICER expression during *in vivo* experiments was checked in stable transfectants *in vitro*.

Transfection and luciferase assay. A mixture of 0.3µg of 4XCREpGL3/LUC, 0.3µg pEGFPΔ-N1_FLAG-ICERγ or 0.3µg pEGFPΔ-N1_EV together with 0.1µg of phRL-TK as internal control were used to transfect HeLa. A mixture of 0.75µg of 4XCREpGL3/LUC, 0.75µg pEGFPΔ-N1_FLAG-ICERγ or 0.75µg pEGFPΔ-N1_EV together with 0.24µg of phRL-TK as internal control were used to transfect HL60. After 24, 48 hours, proteins were extracted and LUC activity was determined following manufacturer's instructions (Promega). Luminescence was measured using a Luminescence counter (Perkin Elmer, CT, USA). Results were normalized to Renilla activity, compensating for variation in transfection efficiency. Three parallels were used in all transfections and all experiments were performed in triplicate.

***In vitro* transcription**. Single assay (Applied Biosystems, CA, USA) was used to test HIF1α and VEGF expression for their role in angiogenesis. A Low-Density Array based on an Applied Biosystems 7900HT Micro Fluidic Card where probe and primer sets were factory-loaded into 384-well plate was then evaluated for wide gene expression analyses (16,17). 96 genes which were cited in the *CREB database* (http://natural.salk.edu/CREB/) for the high predictive value to contain CRE consensus sequence in their promoter were chosen. We mixed 2µL of single-stranded cDNA (equivalent to 100ng of total RNA) with 48µL of nuclease-free water and 50µL of TaqMan Universal PCR Master Mix (Applied Biosystems). After we loaded 100µL of the sample-specific PCR mixture into each sample port, the cards were centrifuged. Cards were analyzed on ABI PRISM 7900HT Sequence detection system and evaluated with the RQ Manager Software for automated data analysis (Applied Biosystems). Experiments were carried out in duplicate and were analyzed together as one relative quantity (RQ) study.

Expression values for target genes were normalized to the concentration of GAPDH which showed the least variation among reference genes in our cell model. Gene expression values were calculated based on the comparative threshold cycle (Ct) method, where each RNA sample was calibrated to values obtained by the transient or stable transfection with empty vector. The Micro Fluidic Cards detect a fold difference in gene expression at a 99% confidence level.

Chromatin Immunoprecipitation (ChIP) Assay. HL60+ICER and HL60+EV cell lines were processed for ChIP assay (Upstate Cell Signaling Solutions, VA, USA) following the manufacturer's instructions. Other reagents required were purchased from the same source. 1×10^6 cells were lysed and sonicated (Fischer Scientific Model 300 Ultrasonic Dismembrator) 3 times for 10s pulses at 40 power over ice. The immunoprecipitation was performed overnight at 4°C with rotation by using an antibody of interest (1:50) and without antibody selection, as negative control. DNA was recovered and used to perform PCR with primers selected from gene promoter sequence (see Supplementary Table 1s for primer sequences). Amplified PCR products were resolved by 2.0% of agarose gel electrophoresis and visualized by ethidium. The difference in DNA promoters immunoprecipitated (IP) by phosphoCREB (P-CREB) in stable HL60+EV cell line was compared to that of HL60+ICER cell line, and was calculated by RQ-PCR. It was performed using the 7900HT technology (AppliedBiosystems) and the SYBR Green method for amplification and detection (Invitrogen-Gibco) (see supplementary Table 1s primer sequences). Expression values were calculated based on the comparative threshold cycle (Ct) method (18).

Western Blot (WB). 20μg from total protein fraction (Buffer-Biosource International, CA, USA) obtained from HeLa and HL60 transiently transfected, as well as for stable HL60+ICER and HL60+EV cell lines were used to perform protein analyses. Protein concentration was determined using the BCA method (Pierce, IL, USA). Samples were subjected to 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.2μm

PVDF membranes (GE-healthcare, IL, USA) for immunodetection with a series of antibodies followed by horseradish peroxidase (HRP)-coniugated goat anti rabbit or mouse IgG (Upstate Biotechnology, NY, USA). Antibodies used included anti-β-Actin, anti-FLAG and anti-CyclinA1 (Sigma-Aldrich); anti-BCL-2(C2), anti-NFkBp50, anti-NFkBp65, anti-STAT3 (Santa Cruz Biotechnology, CA, USA); anti-CREB, anti-PhosphoCREB, anti-FOS, anti-CIP1-p21, anti-PhosphoSGK (Upstate Biotechnology). HL60 treated with MG132 as proteasome inhibitor (10μM) or with staurosporin as PKC inhibitor and consequently of MAPKs (1nM) were probed with anti-Phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling technology, MA, USA) and anti-CREM1 (Santa Cruz Biotechnology). The specific bands of target proteins were visualized by enhanced chemoluminescence (ECL advance) according to the manufacturer's instructions (GE-Healthcare), and the density of bands were quantified using the ScionImage software.

Immunoprecipitation. Cells were treated with preoteasome inhibitor MG132 (10μM) for 4h to have the higher ICER concentration. Then, cells were lysed in 200μl of ice-cold 1X CHAPSO lysis buffer (137mM KCl; 5mM $MgCl_2$; 1mM EDTA; 1mM EGTA; 20mM TrisHCl) containing protease and phosphatase inhibitor cocktail (SIGMA). 500μg of protein extracts were precleared with 30μl of Protein A-Sepharose (SIGMA) immunoprecipitated with 2μg of antibody anti-CREM1 (Santa Cruz Biotechnology) and 30μl of protein A-sepharose. Immunoprecipitates were washed three times with 200μl of ice-cold 1X CHAPSO lysis buffer and eluted with Laemlii buffer 10' at 100°C and subjected to SDS-PAGE. Proteins were transferred onto PVDF membranes, and analyzed by immunoblotting with anti-Phospho-ERK1/2 (Cell Signaling technology) and anti-CREM1 (Santa Cruz Biotechnology) followed by Protein A horseradish peroxidase (SIGMA) and detected by enhanced chemo luminescence reagent (GE-Healthcare).

Data analysis. Values were presented as mean±s.d. Significance between experimental values was determined by Student's unpaired *t-test*, and one-way ANOVA was used to test differences in repeated measures across experiments. $p<0.05$ was considered significant.

RESULTS

ICER decreases CREB expression in HeLa and HL60. Previous findings from our laboratory have showed that CREB protein was overexpressed in patients at diagnosis of leukemia whereas ICER mRNA was downregulated. In the present study we analyze CREB and ICER protein expression in HeLa as a good model for transfection experiments, and a variety of leukemic cell lines (Figure 1A). We also included two healthy BM after sorting of CD34+ and CD34- subpopulation cells in the study. Results demonstrated that CREB protein levels were high in HeLa and in all leukemia cell lines (Figure 1B), but undetectable in sorted normal hematopoietic stem cells. On the contrary, ICER was not detected in any of analyzed cell lines, but was expressed in normal hematopoietic cells (CD34-), supporting the association of CREB expression with leukemic tissue (19) and ICER with healthy tissue. The stable expression of ICER (HL60+ICER in Figure 1A), as well as the transient transfection of ICER (t ICER in Figure 1C and 1D) in HeLa and HL60 induced the reduction of the active phosphorylated form of CREB (P-CREB) compared to transfected controls (t EV).

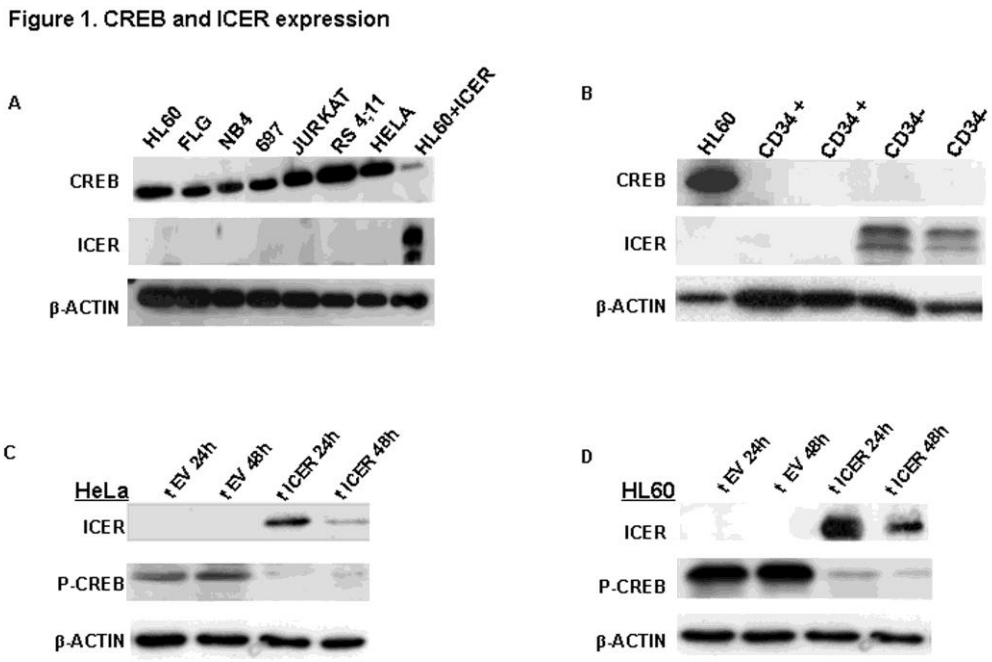

Figure 1. A) Western blot analysis of CREB and ICER expression in a series of leukemic cell lines including HeLa: high level of CREB was observed while ICER was undetectable except when it is exogenously expressed in HL60 (HL60+ICER). B) Western blot shows hematopoietic stem cells sorted from two healthy pediatric bone marrows. CREB was completely absent in both precursor and mature normal cells (CD34+, CD34-), whereas ICER was expressed during the normal hematopoietic development. C) Western Blot analysis of whole cell protein extract from HeLa after 24h or 48h post transient transfection with pEGFPΔ-N1_FLAG-ICERγ (+t ICER) or empty vector (+t EV). ICER, phosphoCREB (P-CREB) and B-ACTIN proteins were detected. Exogenous ICER expression downregulated P-CREB expression in both, HeLa and HL60 cell lines (D).

ICER controls HL60 colony formation *in vitro* and blasts dissemination *in vivo*. In order to understand if ICER induced CREB downregulation could be implicated in leukemia, the *in vitro* clonogenicity assay was performed. On day 12 after plating HL60+ICER formed colonies were found to be significant lower in number (Table 1; n=3, $p<0.05$), as well as more diffuse and smaller compared to HL60+EV colonies formed (control cell line). To establish if ICER plays a role as tumor suppressor in leukemia, as it has been recently found in other solid tumors, *in vivo* experiments were performed. NOD-SCID mice were inoculated with HL60+ICER and with HL60+EV cells. At sacrifice the weight of HL60+ICER inoculated mice was significantly higher

(22.25±0.85g) than the EV inoculated mice (19.75±0.25g, p=0.031). BM engraftment and invasion of extramedullary sites were evaluated.

Table1. ICER suppresses HL60 tumorigenicity *in vitro* and *in vivo*

		HL60+EV	**HL60+ICER**	***p value***
in vitro				
Colonies number		270	140	*0.018**
in vivo				
BM engraftment	$hCD45^+$	18 ± 2.7	23.8 ± 6.8	*0.26*
PB invasion	$hCD45^+$	60.5 ± 31.4	41 ± 4.9	*0.04**
HL60 dissemination *in spleen*				
Area of infiltrating leukemic cells (μm^2)		1857 ± 315.6	612.9 ± 105.3	*0.03**
BM Angiogenesis				
Microvessels count		57.5 ± 7.5	33.5 ± 14.5	*0.036**
Perimeter (μm)		152 ± 4	110.8 ± 15.7	*0.048**
Major axis length (μm)		45.7 ± 5	32.2 ± 1.4	*0.085*
Minor axis length (μm)		15.6 ± 0.7	13.1± 0.6	*0.025**
Total vascular area (μm)		1419 ± 290	559 ± 48	*0.008**

Table 1. HL60 cells were stably transfected with both ICER (HL60+ICER cell line) or EV (HL60+EV cell line).*In vitro* experiments showed a significant lower colonies formation number. *In vivo* cell lines were both injected in NOD-SCID mice. Five mice per group were sacrificed 3 weeks after injection and tumorigenity was analyzed. The engraftment was quantified by flow cytometry analyzing the positivity to the CD45 human ratio antigen ($hCD45^+$) versus the murine $CD45^+$ cells in BM and PB. Results are expressed as means±SEM. The presence of leukemia cells or normal hematopoiesis was evaluated in slides by immunohistochemistry using anti hMHCI (H-300, Santa Cruz) antibodies in BM and spleen tissues, fixed, paraffined and sectioned at 6μm onto charged slides. Blast invasion was measured by considering positive area in μm^2 in comparison to the normal hematopoietic area which was negative for the staining. BM angiogenesis was determine by the use of anti-CD34 antibodies. Vascular morphometric parameters were quantified following the procedure by Pillozzi et al. (13) with the Leica DC Viewer Software. Results were analyzed independently by two investigators in slides at the magnitude of 40 x. Student's t test was used for p values reported in table. *defines statistical significance $p<0.05$.

Results revealed that HL60+ICER and HL60+EV cells similarly engrafted the BM (Table 1), but HL60+ICER cells significantly reduced PB invasion ($p=0.04$), as well as spleen invasion ($p=0.03$). BM slides were also evaluated for vascular morphometric parameters. HL60+ICER inoculated mice showed significant differences in microvessel numbers ($p=0.036$) and total vascular area ($p=0.008$) compared to HL60+EV inoculated mice, giving to ICER the capability to reduce BM angiogenesis in HL60.

ICER recognizes CRE elements when expressed in HL60. ICER is the endogenous repressor of CREB silencing promoter genes. Because ICER is not expressed in cell lines the balance between activation and repression of CRE target genes in leukemia has never been described yet. We considered that the exogenous expression of ICER in our model, both in transient and stable transfected cells, could modify gene expression. To validate this hypothesis, a gene reporter assay was performed. A vector with a reporter gene (LUC) driven by the 4XCREs sequences in the promoter was used. ICER transiently transfected HeLa and HL60 cells, were used and the LUC activity was measured. Results showed that reporter activity significantly decreased when ICER (transient ICER expression, t ICER in the figure) was transiently expressed compared to control (transient Empty Vector expression, t EV) (Figure 2). In particular, after 24h of ICER transfection (white bars in the figure), LUC activity was decreased from 12.5 (t EV) to 1.2 (t ICER) in HeLa cell line and from 32.7 (t EV) to 13.6 (t ICER) in HL60. At 48h (black bars) LUC activity was decreased from 6.5 (t EV) to 1 (t ICER) in HeLa cell line, and from 30.3 (t EV) to 2.3 (t ICER) in HL60 ($p<0.05$). LUC activity was lower also in stable HL60+ICER cell line compared to HL60+EV cell line ($p<0.05$) (data not shown) confirming that its exogenous expression was able to influence CRE sequence binding.

Figure 2. Exogenous ICER controls CRE promoter

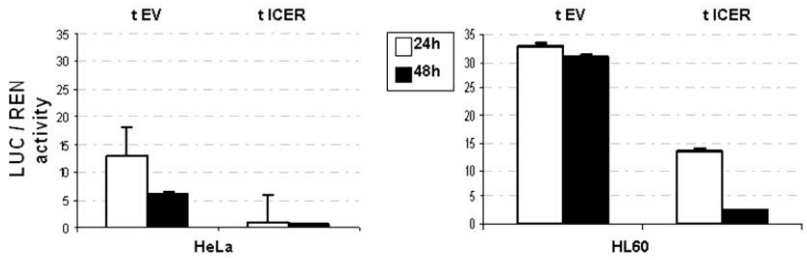

Figure 2. Luciferase (LUC) activity was measured in HeLa and in HL60 at 24h (white bars) and 48h (black bars) of transient co-transfection of 4XCREpGL3/Luc with pEGFPΔ-N1 (+ EV) or the pEGFPΔ-N1_FLAG-ICERγ (+ ICER) plasmids. Results depict a significant decrease ($p<0.001$) of LUC activity after ICER transient transfection, documented also in stable ICER transfection (data not shown). Results (mean±s.d.) are representative of three independent experiments after normalization with Renilla (REN) activity.

ICER represses CREB dependent gene expression. To test if ICER exogenous expression modifies CREB dependent gene expression, a low density array was designed. 96 CREB target genes were preferentially chosen (20). We compared gene expression alterations occurring over time and in different cell systems in untreated HeLa and HL60 cells with those in cells transfected with pEGFPΔ-N1_FLAG-ICERγ or pEGFPΔ_N1. In order to monitor the effect of ICER on gene expression over time, transfected cells were kept in culture for up to 72h. ICER expression was documented to determine the downregulation of the expression of a wide number of genes. Focusing on HL60, 80/96 genes displayed detectable mRNA expression. 71/79 and 65/79 genes were downregulated at 48h and 72h post transient transfection, along with 40/79 genes in the stable HL60+ICER cell line (Supplementary Table 2S; HL60+EV cell line was used as calibrator and Relative Quantity (RQ) of gene expression was considered =1 for each gene

detected. In HL60+ICER cell line, gene expression was downregulated if RQ<1 in respect to the calibrator, whereas gene expression was upregulated if RQ>1 in HL60+ICER cell line compared to calibrator). Among those genes, 40 genes (50%) were significantly downregulated or upregulated by more than 2-fold (RQ≤0.5 or RQ≥2.0, $p<0.05$; Table 2); 18 of these 40 were also significantly downregulated in stable HL60+ICER cell line (highlighted in Table 2). Gene ontology analysis (GO), by KEGG database (http://www.genome.jp/kegg/tool/) (21) allowed us to identify significant genes inherent to the cell cycle-apoptosis pathway (*ATM,CDKN1A (p21),CDKN1B (p27), CDKN2B (p15),*and *RB1*); to MAPK signaling pathway (F*OS, NR4A1, JUN, NFKB2, DUSP1* and *DUSP4)* and to acute myeloid leukemia pathway (*PPARD, STAT3, PBK, RELB, TP53BP1)*. Considering that angiogenesis was documented to have been decreased in mice inoculated with HL60+ICER cell line, we analyzed *HIF1α* and *VEGF* genes, that play a crucial role in angiogenesis, which have CRE sequences in their promoters. Gene expression on *HIF1α* and *VEGF* mRNAs revealed that *HIF1α* expression was reduced by 30% in HL60+ICER cell line respect to the HL60+EV cell line, as well as *VEGF* expression that was reduced by 56% (data not shown).

Table2. Genes significantly modulated by ICER in HL60.

Genes	ncbi-id	GO	RQ 48h	RQ 72h
ATM	NM_138292	Protein kinase	n.s.	0.368
CCR4	NM_005508.4	Receptor	10.106	0.226
CDC14B	NM_003671.2	Phosphatase	n.s.	0.266
CDKN1A	NM_078467	Select regulatory molecule	0.031	0.162
CDKN1B	NM_004064	Select regulatory molecule	0.359	0.449
CDKN2B	NM_004936	Select regulatory molecule	0.259	0.488
CLK1	NM_001024646	Protein kinase	n.s.	0.458
CREM	NM_181571	Transcription factor	0.368	n.s.
CSNK1D	NM_139062	Protein kinase	0.434	n.s.
DUSP1	NM_004417.2	Phosphatase	0.083	0.114
DUSP4	NM_001394.5	Phosphatase	0.095	0.191
EGR4	NM_001965.1	Transcription factor	0.031	0.073
FER	NM_005246	Protein kinase	n.s.	0.418
FOS	NM_005252	Transcription factor	0.096	0.137
FRAT1	NM_181355	Select regulatory molecule	n.s.	0.464
HTATIP2	NM_006410	Protein kinase	0.472	n.s.
IFNGR2	NM_005534.2	Receptor	0.249	0.355
IL6	NM_000600.1	Signaling molecule	0.086	0.176
JAK3	NM_000215.2	Protein kinase	0.186	0.014
JUN	NM_002228	Transcription factor	0.332	0.406
JUNB	NM_002229	Transcription factor	0.349	0.414
MAP3K7	NM_145333	Protein kinase	n.s.	0.417
NBL1	NM_182744	Nucleic acid binding	n.s.	0.149
NFKB2	NM_002502	Transcription factor	0.221	0.217
NR3C1	NM_001018074.1	Transcription factor	0.452	0.392
NR4A1	NM_173158.1	Transcription factor	0.257	0.486
NR4A2	NM_173171.1	Transcription factor	0.255	0.23
NR4A3	NM_173198.1	Transcription factor	0.038	0.01
PBK	NM_018492.2	Protein kinase	0.322	0.490
PCSK1	NM_000439.3	Protease	n.s.	0.066
PPARD	NM_177435.1	Transcription factor	0.165	0.114
PTPN14	NM_005401.3	Phosphatase	0.19	0.195
RB1	NM_000321	Transcription factor	0.327	0.381
RELB	NM_006509.2	Transcription factor	0.151	0.269
RPS6KA5	NM_182398	Protein kinase	n.s.	0.4
SGK	NM_005627	Protein kinase	0.017	0.074
SLA2	NM_032214.2	protein kinase	0.295	0.206
STAT3	NM_213662	Transcription factor	0.376	n.s.
TNFAIP3	NM_006290.2	Nucleic acid binding	0.207	0.318
TP53BP1	NM_005657	Transcription factor	n.s.	0.497

Table defines the Relative Quantity (RQ) of gene expression post 48 or 72 hours of ICER transient transfection. 40 genes significantly repressed ($RQ \geq 2.0$ or $RQ \leq 0.5$; $p<0.05$) in Hl60+ICER compared to HL60+EV are shown. 18/40 genes, grey highlighted in the table, were significantly downregulated also in stable HL60+ICER cell line (see supplementary Table 2S). Abbreviations: n.s., not significant ($0.5 \leq RQ \leq 2.0$).

ICER defines a series of relevant CREB target genes in leukemia. To verify the binding of ICER to CRE sequences at gene promoters, experiments were performed in HL60+ICER and HL60+EV cell lines. 8 genes were chosen for immunoprecipitating chromatin at their promoters. P-CREB was used in stable HL60+EV cell line, and both, P-CREB and anti-FLAG_ICER antibodies in HL60+ICER cell line. DNA pulled down was amplified by PCR and results demonstrated that all promoters were recognized and activated by CREB in HL60+EV (Figure 3A). In HL60+ICER cell line the immunoprecipitation with P-CREB and ICER revealed that ICER, when stably expressed, affects P-CREB binding to CREs. To better quantify ICER's ability to destabilize CREB binding on promoters, we performed real time quantification of DNA immunoprecipitated by P-CREB in HL60+EV and in HL60+ICER cell lines. In particular, the amount of DNA immunoprecipitated by CREB was significantly decreased for *BCL-2* ($p=0.001$), *p21* ($p=0.030$) and *FOS* ($p=0.001$) (Figure 3A at the right), as well as for *STAT3*, *SGK*, *NFkBp50/p65* in HL60+ICER compared to HL60+EV cell line, confirming that ICER counteracted CREB on same promoter genes, and that the disruption of gene expression discussed above depended on ICER expression. The fact that promoters bound both, P-CREB and ICER factors, could be explained by the fact that ICER decreased CREB expression, not nullified it. In this way CREB and ICER can heterodimerize, exerting a repressive activity, similar to that of ICER/ICER homodimer.

To evaluate if the downregulation of the mRNA could also correlate to a reduction of protein level, WB analyses after 48h of transient and stable ICER expression were performed. Results revealed that exogenous expression of ICER affected mRNA expression and consequently protein levels. In particular, less protein abundance was observed for SGK, NFkBp65, FOS and STAT3 (Figure 3B, $p<0.05$ after densitometric analyses) in transiently transfected HL60+ICER

compared to HL60+EV transfected cells. A general downregulation of these proteins in ICER stable clones was also observed (data not shown).

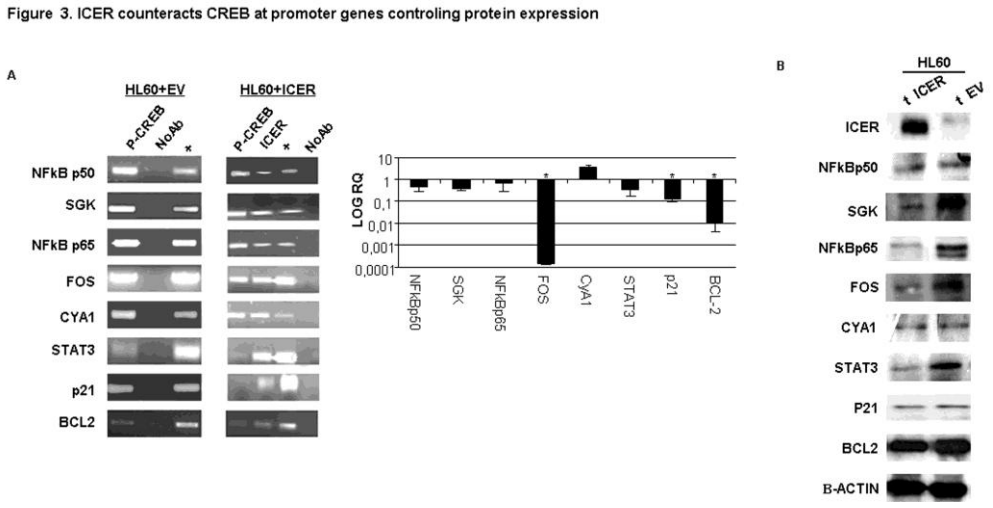

Figure 3. ICER's role in gene expression downregulation investigated by ChIP using PhosphoCREB (P-CREB) in stable HL60+EV, and both, P-CREB and FLAG_ICER, antibodies from HL60+ICER cell line. A) PCR agarose gels of promoters of target genes immunoprecipitated. A portion of negative control without antibody selection (NoAb) and of the total imput (+) was also examinated. Results indicated that stable ICER expression decreased the P-CREB binding to promoters: for some genes ICER totally disrupted CREB binding (STAT3, p21 and BCL-2), whereas in others it heterodymerized with CREB leading to a final repression of transcription. Quantification of DNA promoters (right graphic), bound to P-CREB in HL60+EV cell line and in HL60+ICER cell line, was performed by Sybr green in RQ-PCR, and analyzed by the comparative ΔΔct method. Each promoter quantification has been normalized to the NoAb amplification value. Relative quantity (RQ) of promoters imunoprecipitated in HL60+ICER has been calibrated to the values found in the HL60+EV (LOG RQ= 1). Induced ICER expression significantly decreased P-CREB binding for almost all studied genes. Bars illustrate mean values from triplicate measurements of PCR runs. *defines differences statistically significant ($p<0.05$). B) Western Blot of whole cell lysates collected 48h post transient transfection with pEGFPN1Δ_FLAG-ICERγ (HL60+ t 48h ICER) and pEGFPN1Δ (HL60+ t 48h EV). Results showed a general proteins downregulation 48h after ICER has been exogenously expressed. The same results were obtained in the ICER stable cell line.

ICER interacts with ERK and leads it to proteasome degradation. We supposed that ICER lowered expression could be a result of its degradation. We used the proteasome inhibitor to evaluate if ICER was degraded in a proteasome dependent manner. Results revealed that ICER protein increased during MG132 treatment (Figure 4A). The fact that its degradation has been linked to the inhibition of MAPK kinases, forced us to use staurosporin that inhibits PKC and

consequently the MAPK dependent pathway. We found that while ERK1-2 expression was delayed, ICER expression was increased during treatment (Figure 4B). This link between ICER and ERK was confirmed by co-immunoprecipitation experiment indicating that ICER might be driven to the proteasome by ERK1/2 in HL60 (Figure 4C), suggesting that it could be a reason for ICER lowered expression in leukemia blasts.

Figure 4. ICER is degradated by the proteasome in HL60

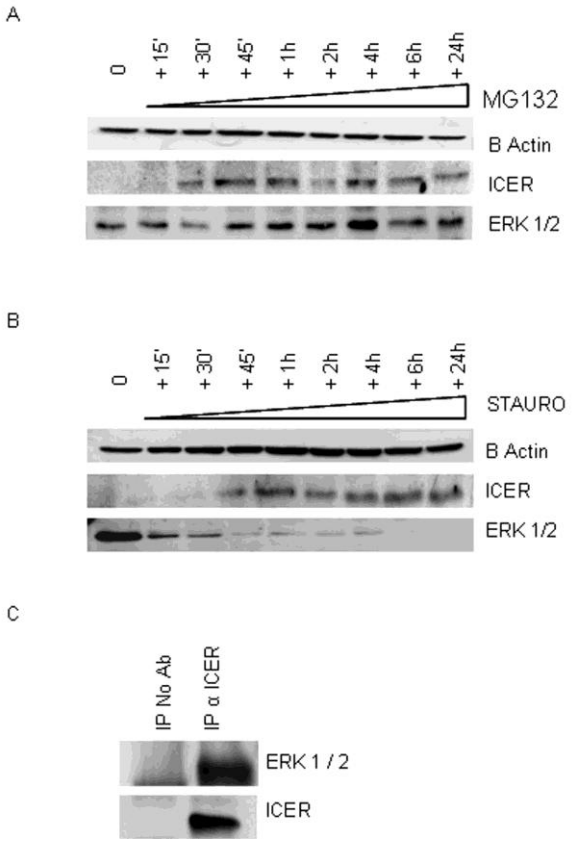

Figure 4. A) Western blot analysis of ICER and ERK1/2 expression in HL60 cell line after proteasome inhibitor MG132 treatment. ICER expression was observed 30' after MG132 introduction, confirming proteasome dependent degradation involvement. After 4 hours of treatment both proteins are at their maximum level of expression. B) HL60 treatment with staurosporin which blocks ERK1/2, showed that while ERK1/2 are disappearing, ICER increased its expression. C) 4h cells treatment with proteasome inhibitor after which ICER was immunoprecipitated. Membrane was probed with anti-ERK1/2 which pointed out that ICER migrates to the proteasome forming a complex with ERK1/2.

DISCUSSION

Transcription factors have been described as being the major protein class whose alterations influence cell proliferation, differentiation and survival (22,23) and their inappropriate activation has been demonstrated to play a significant role in acute leukemia (24,25). Therapies currently adopted in pre-clinical trials take advantage from those molecules and results encourage their knowledge to be improved in the future (26,27).

In this research we present data about two transcription factors that represent prospective targets of therapy in human disorders. We describe that exogenous ICER expression in HL60 cells, normally unexpressed in this cell line, potentiality repressed leukemia *in vitro* and *in vivo*. The role of ICER as a tumor suppressor in acute leukemia is described for the first time. ICER is demonstrated to influence transcriptional activity and turns out to be a crucial controller of the cAMP dependent pathway activated by CREB in this system. Its lowered expression in leukemia might be considered the result of selective proteasome degradation through ERK1/2. We support the idea that ICER downregulation found in leukemia tissue must be considered to be a pathogenetic feature.

The evidence that ICER acts as a tumor suppressor gene in leukemia cell line HL60, as previously reported for other solid tumors (28-30), firstly comes from the decreased clonogenicity ability of HL60+ICER cells *in vitro*. We used immunodeficent mice in order to confirm these data, and reveal that HL60+ICER injected mice have a mild leukemia phenotype for the reduced dissemination of tumor in extramedullary sites. Results confirm that ICER is an important factor to be elucidated in leukemia process. We considered blasts dissemination to be a fundamental step in disease progression. The observed reduced bone marrow angiogenesis induced by HL60+ICER cells, suggested the possibility that ICER played a role in this phenomenon. Vascularization of neoplastic tissue is, in fact, a complicated balance of angiostatic

and angiogenic factors that come from different signaling pathways, including cAMP/CREB (31). Our results show that ICER represses the expression of two genes' major involvement in angiogenesis, *HIF1α* and *VEGF*. Moreover, ICER caused a reduction of *Jak3* and *Stat3* expression as well as of the final effector *IL6* (32,33). The disruption of the VEGF signaling pathway, along with the deregulation of many other genes (*ATM, NFkB, RelB*) (34) explain how ICER might be able to control cell activities contrasting leukemia through gene regulation.

The restoration of ICER expression in HL60 establishes its ability to modulate CREB transactivation potential, confirming results found in other models (35-37). We document that ICER represses CRE at specific gene promoters, giving CREB and ICER expression a direct role in determining leukemic or healthy gene expression. The characterization of genes heavily influenced by ICER exogenous expression, points us to novel targets that could improve the understanding of the molecular pathways involved in the leukemogenic process. Genes related to cell cycle and apoptosis pathways such as *ATM,CDKN1A, CDKN1B, CDKN2B, RB1* were severely compromised, as well as genes involved in acute leukemia pathway such as *PPARD, STAT3, PBK, RELB, TP53BP1*already known to influence tumor susceptibility (38-40). In particular, *STAT3* is persistently activated in many human cancers and enhances transformation or blocks apoptosis in cell cultures. *TP53BP1* is a key transducer of the DNA damage checkpoint signal; *PBK* has recently been demonstrated to be involved in leukemic growth arrest (41). CREB and ICER expression might be considered useful to monitor leukemia activity (42). The possibility to manipulate CREB and ICER reciprocal expression, influencing the regulation of the transcription of their target genes, could potentially establish innovative therapeutic opportunities (43). Furthermore, the downregulation of genes of the MAPK signaling pathway (*FOS, NR4A1, JUN, NFKB2, DUSP1, DUSP4*) might be used to elucidate ICER involvement in chemotherapy response and cell death (44-46).

Finally, we investigate the mechanism of ICER downregulation. Because ICER activity is mainly determined by its intracellular concentration rather than posttranslational modification, we focused on the protein degradation mechanisms (47). Results indicate that ICER immunoprecipitates with ERK1/2 in HL60 and that this complex migrates to the proteasome. This might be considered to be a possible mechanism that could explain CREB overexpression in leukemia. Therefore, we assume that the rapid degradation of ICER blocks its ability to control gene expression triggering to leukemia phenotype. Moreover, ICER lowered expression prevents CREB/ICER heterodimerization which would lead CREB protein to degrade (48,49). Future studies will implement this mechanism to influence CREB/ICER expression in tumor (50).

The newly discovered role of ICER in mediating leukemia gives new insight into future treatment strategies for clinical implementation. Therefore, further investigation into the CREB/ICER connection could improve our knowledge of this novel molecular pathway involved in acute leukemia development.

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CHAPTER 3

ICER EVOKES DUSP1-P38 PATHWAY ENHANCING CHEMOTHERAPY SENSITIVITY IN MYELOID LEUKEMIA

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ABSTRACT

Purpose: The Inducible cAMP early repressor (ICER) is found downregulated in acute myeloid leukemia (AML), failing to control cAMP response element binding protein (CREB) transcriptional activity, recently demonstrated to mediate AML progression. We aimed to characterize ICER's role in drug sensitivity by treating myeloid cell lines and primary AML with chemotherapics.

Experimental design: The effects on CREB target genes induced by ICER restoration and drug treatment were studied by Real Quantitative-PCR (RQ-PCR) and Western blot. Cell cycle and apoptosis analysis were performed. Possible ICER evoked pathways were investigated *in vitro*. The mechanism involved in enhanced drug sensitivity was described in primary AML cultures by silencing ICER main target genes.

Results: AML cell lines reduced cell growth and enhanced apoptotic behavior after chemotherapy treatment if ICER was expressed. A significant lowered expression of CREB target genes involved in cell cycle control (CyA1,B1,D1), and in the mitogen-activated protein kinase (MAPK) signaling pathway (ERK, AKT, DUSP1/4) was found after Etoposide treatment. The dual-specificity phosphatases DUSP1 and DUSP4, directly repressed by ICER, activated p38 pathway which triggered an enhanced caspase-dependent apoptosis. The silencing of DUSP1/4 in HL60 confirmed the same enhanced drug sensitivity induced by ICER. Primary AML cultures, silenced for DUSP1 as well as restored of ICER expression, showed DUSP1 downregulation and p38 activation.

Conclusion: ICER mediates chemotherapy anticancer activity through DUSP1-p38 pathway activation and drives cell program from survival to apoptosis. ICER restoration or DUSP1 inhibition might be possible strategies to sensitize AML cancer cells to conventional chemotherapy and to inhibit tumor growth.

INTRODUCTION

Transcriptional regulation via the cyclic adenosine monophosphate (cAMP) dependent pathway is controlled principally by the cAMP response element binding protein (CREB) and by the cAMP response element modulator (CREM) (1,2). CREB is a transcriptional activator of the downstream target of hematopoietic growth factor signaling, and its role in leukemogenesis was recently described (3). CREB was found to be overexpressed in myeloid leukemia cell lines and in patients at diagnosis, contributing to disease progression, and to improve tumor proliferation and survival *in vitro* (4-6). The CREM gene generates positive and negative transcription regulators. In particular, ICER (inducible cAMP early repressor) is driven by an alternative promoter (P2), which directs the transcription of a truncated product (7). Through its bZIP domain, it can either recognize cAMP response element (CRE) consensus elements on gene promoters, impeding their transcription, or it can dimerize with CREB, impeding CREB phosphorylation of the residue of Serine 133, triggering gene repression and CREB destabilization (8-10). Previous studies demonstrated that exogenous ICER expression decreased CREB protein levels and induced a lowered clonogenic potential *in vitro* and *in vivo*, demonstrating its potential role as tumor suppressor in leukemia as well as in prostate tumors. ICER was shown to repress many target genes upregulated by CREB in acute myeloid leukemia (AML), restoring the normal regulation of the main survival cellular pathways (11-13). The impact of the restored ICER on leukemic cell activity and its ability to suppress tumors is under investigation here. Considerable attention has been focused on the role played by ICER in different kinase cascades, specifically in the control of apoptosis. We focused on the MAPK family members which included numerous cellular signaling, such as extracellular signal regulated kinase (Erk1/2), c-Jun N-terminal kinase (JNK), and p38 known to transmit different types of signals (14,15). Erk1/2 acts through mitogenic stimuli promoting cell proliferation,

whereas p38 and JNK are stress factors related to cell growth inhibition and apoptosis (16,17). The outcome of MAPK activation depends on the level and period of the phosphorylation status of the proteins involved, which are mostly controlled by a specific family of phosphatases with negative regulatory control ability, called dual specificity phosphatases (DUSPs) (18,19). The DUSP proteins family contains several members with substrate and subcellular localization specificity (20). In particular, DUSP1/4 target principally p38, and many different stimuli are able to activate their activity (21,22). The balance between the activation or inactivation of the MAPK mediated by DUSPs modulates the proliferative or apoptotic cell phenotype in several tissues. Increased levels of DUSP1 have been found in ovarian carcinoma, breast and prostate cancer (23-25). On the other end, we previously demonstrated that CREB stabilized extracellular signal regulated kinase (ERK), which is responsible for lowered ICER expression levels (11) by driving it to the proteasome (26), permitting overexpression of DUSP1/4 and thus influencing MAPKs. These facts highlight an intricate interplay between CREB/ICER transcription factors and the signaling of MAPK in the control of myeloid leukemia cell fate (27-29).

The aim of this study was to elucidate whether and how the restoration of ICER expression leads to increased sensitivity of myeloid leukemic cells to chemotherapy treatment, resulting in apoptosis.

MATERIAL AND METHODS

Cell culture and transfection. HL60, ML2, THP-1 cell lines (American Type Culture Collection, MD, USA) was cultured in DMEM (Invitrogen-Gibco, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100U/ml penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco). Cell lines were grown in suspension diluted to 500X106/ml , and after 24 h were transiently transfected by Nucleofector system from Amaxa GmbH (Cologne, Germany) with 1.5 μg of pEGFPΔ-N1_FLAG-ICERγ or pEGFPΔ-N1-EV (the Empty Vector) used as

control. We used the stable HL60+ICER and HL60+EV cell lines previously described in Pigazzi et al. (11).

Primary cell cultures. Three untreated AML patients were included in the present study. The study was approved by the local Ethics Committee, and samples collected after obtaining written informed consent. AML cells were isolated from BM by hemolysis and cultured in DMEM (Invitrogen-Gibco) supplemented with 100U/ml penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco), IL-3, IL-6, Flt-3 ligand and TPO (Sigma). After 24 h of incubation at 37 ° C, cells were added to 10% heat-inactivated fetal calf serum (FCS) to be transiently transfected by nucleofection (Amaxa) with 3.5 μg of pEGFPΔ-N1_FLAG-ICERγ or pEGFPΔ-N1-EV. Silencing of DUSP1 was also performed in the same cells by using 400 nMol (in 2 ml of total volume) of oligonucleotides (Dharmacon Industries, Lafayette, CO), as well as the scramble negative Sirna (Sir-sc) used as control at the same concentration. To establish the time of experiments, we observed cell viability. The three patients were pediatric, age < 18 years; the FAB classifications were one M4, one M1 and one M5. Two of them had normal karyotype; the M4 was inv16-CBFB-MYH11 rearranged. BM infiltration was up to 70%.

3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assay. We performed dose-dependent studies of VP-16, doxorubicin (doxo) and staurosporine (stauro) by growing cells in the presence or absence of increasing concentrations of the drug at 37 °C for up to 72 hours, and reduction of the MTT (Sigma-Aldrich, MO, USA) salt was measured. The subtoxic drug dose at which the proliferation of cell lines was not significantly affected was 1 μM for VP-16, 0.03 μM for doxorubicin and 0.24 μM for staurosporine.

Fluorescence Microscopy. HL60+ICER and HL60+EV were serum-starved overnight before adding 1 μM VP-16 for up to 72 hours. To assess time-dependent nuclear morphology perturbations of VP-16-treated and untreated cells, $5x10^5$ cells were spotted every 24 hours and

incubated in 10% FCS-PBS with DAPI nucleic acid stain (1:1000). The cells were observed at 63/0.75 numerical aperture with a Leica DMBL microscope; images were obtained with a Leica DC 300F digital camera (Leica Microsystems Ltd., Germany).

Cell cycle analysis. HL60+ICER and HL60+EV cell lines were serum-starved overnight and then continuously treated with 1 μM VP-16, 0.03 μM Doxo and 0.24 μM Stauro. After 6, 24 and 48 hours, 5x105 cells were washed twice with PBS, lysed and treated with 50 μg/ml Propidium Iodide (PI) in 1 ml PBS overnight at 4 °C. Cells were analyzed by using Cytomics FC500 (Beckman Coulter, FL, USA). Cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems, CA, USA).

Apoptosis assays. Cell lines were serum-starved overnight and then continuously treated with 1 μM VP-16, 0.03 μM Doxo and 0.24 μM Stauro over a 48 hours period. Drugs were solubilized in DMSO, which was found to induce less than 0.5 % apoptosis. Cells were stained with annexinV conjugated to fluorescein-isothiocyanate (FITC) and PI, according to the manufacturer's instruction (Boehringer, Mannheim, Germany) and analyzed by Cytomics FC500 (Beckman Coulter). To determine caspase activation, we used 10 μM Z-VAD-fmk (Sigma-Aldrich) to study the apoptosis induction pathway 120 minutes before VP-16 treatment. We administered the specific p38 inhibitor SB203580 (4-(4´-fluorophenyl)-2-(4´-methylsulfinylphenyl)-5-(4´-pyridyl) imidazole) (20 μM) and a selective inhibitor of MAPK/ERK kinase 1 inhibitor PD98059 (2´-amino-3´-methoxyflavone) (Sigma-Aldrich) 2 hours before VP-16 to selectively block different signals and measured apoptosis by Annexin V/PI staining. Cell lines with empty vector (EV) expression were treated at same drug concentration and during the same period. In apoptosis assay cell death of EV was subtracted to ICER expressing cells, whereas in western blot the same antibodies were evaluated in the EV protein lysates.

RNA isolation and SYBR green quantitative real-time RT-PCR (QRT-PCR) assays. Total RNA was isolated using TRIzol (Invitrogen) from cell lines after being treated with VP-16 up to 48 hours or without being treated. 1 μg of RNA was transcribed using the Superscript II system (Invitrogen-Gibco) in 25 μl final volume following manufacturer's instructions. RQ-PCR was performed with 1 μl cDNA in 20 μl using the Sybr Green method (Invitrogen-Gibco) and analyzed on an ABI PRISM 7900HT Sequence detection system (Applied Biosystems).

Western Blot (WB). 20 μg from the total protein fraction (Buffer-Biosource International, CA, USA), obtained from HL60+ICER and HL60+EV cell lines, were used to perform protein analyses. Protein concentration was determined using the BCA method (Pierce, IL, USA). Samples were subjected to 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.2 μm PVDF membranes (GE-healthcare, IL, USA) for immunodetection with a series of antibodies followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse Ig (Upstate Biotechnology, NY, USA). Antibodies used included anti-β-Actin, anti-FLAG and anti-CyclinA1 (Sigma-Aldrich); anti-BCL-2(C2), anti-DUSP1, anti-DUSP4, anti-IL6 (Santa Cruz Biotechnology, CA, USA); anti-CREB, anti-PhosphoCREB, anti-CIP1-p21, anti-Bak (Upstate Biotechnology); anti-CdK2 (78B2), anti-BAX, anti-Bcl-xl, anti-Phospho-p44/42 Map kinase (Thr202/Tyr204), anti-Phospho-p38 (Thr180/Tyr182), anti-JNK (T183/Y185), anti-AKT (S473), anti PARP, anti-RB (Cell Signaling technology, MA, USA); anti-Caspase8 and anti-Caspase3 (Alexis Biochemicals, CA, USA); anti-CyclinE (Oncogene Research Products, MA, USA); anti-CyclinB, anti-CyclinD1 AND ANTI-Kip1-p27 (BD Bioscience, NY, USA). The specific bands of target proteins were visualized by enhanced chemiluminescence (ECL advance) according to manufacturer's instructions (GE-Healthcare), and the density of bands were quantified using ScionImage software.

Chromatin immunoprecipitation assay. HL60+ICER and HL60+EV cell lines were processed for chromatin immunoprecipitation assay (Upstate Cell Signaling Solutions, VA, USA) following the manufacturer's instructions. The immunoprecipitation was performed overnight at 4 °C with rotation by using an antibody of interest (CREB, ICER, FLAGM2, RNAPOL) and without antibody selection (NoAb) and Immunoglobulin (Ig), as controls. Imput DNA was used as positive control. DNA was recovered and used to perform PCR. Agarose gel electrophoresis was performed to observe promoter activity. RQ-PCR was also performed by the SYBR Green method for amplification and detection (Invitrogen-Gibco) by 7900HT technology (Applied Biosystems) and analyzed by comparative threshold cycle (Ct) method.

Anisomycin treatment. The HL60 parental cell line was treated with anisomycin (Sigma-Aldrich), a p38 activator, 2 μM final concentration up to 3 hours. Cell proliferation and apoptosis were measured as described above. For rescue experiments, the p38 mitogen-activated protein kinase phosporylation inhibitor SB203580 (Sigma-Aldrich) was used at 20μM concentration 2 hours before anisomycin exposure or VP-16 treatment.

siRNA experiments. Exogenous small interfering RNAs (siRNAs) specific for the DUSP1 and DUSP4 genes (Dharmacon Industries, Lafayette, CO) were introduced in the HL60 cell line (100 nM in 2 ml of medium) by nucleofection. Scramble Sirna (Sc-Sir) were used as negative control. mRNA and protein expression were performed to monitor silencing. Apoptosis was measured after VP-16 treatment. VP-16 1 μM was added after 14 hours of silencing and continuously treated for 24 hours.

Data analysis. Values are presented as mean ± s.d. Significance between experimental values was determined by Student’s unpaired t-test, and one-way ANOVA was used to test differences in repeated measures across experiments. $p < 0.05$ was considered significant.

RESULTS

ICER expression enhances chemotherapy susceptibility of leukemic cell lines. In the present study, we used myeloid leukemic cell lines (HL60, THP-1, ML2,) that were confirmed to express CREB at high levels and ICER at nondetectable levels by Pigazzi and colleagues (11). We induced ICER transient (t-ICER) expression in these cell lines (Figure 1A) and we treated them with a chemotherapeutic agent to investigate the cellular response. We found that exogenous ICER expression in HL60, THP-1 and ML2 mediated an increase in apoptosis with respect to EV (whose % apoptosis was subtracted from the value presented in the figure) after 24 (13 %, 2.2 % and 2.1 %, $n = 3$, $p < 0.05$ for HL60) and 48 hours (32.2 %, 14 % and 13 % $n = 3$, $p < 0.05$ for all cell lines) of VP-16 treatment (Figure 1B). HL60+ICER was found to be more sensible to VP-16, its lowered cell proliferation was confirmed up to 72 hours of treatment (Figure 1C). The cell morphology showed an increased number of apoptotic nuclei after VP-16 treatment in ICER expressing cell lines, confirming the HL60 to be more sensitive to the drug (Figure 1C). To evaluate if the increase in apoptosis was due exclusively to VP-16, and not to ICER overexpression, two different compounds, Staurosporine (stauro) and Doxorubicin (doxo), were also used. The annexin assay showed an increase in apoptosis after treatment in all of the cell lines that overexpress ICER. At 24 hours, the increase in apoptosis for HL60, THP-1 and ML2 overexpressing ICER was 20.8 %, 8 %, 10.8 % when exposed to doxo; 30 %, 19.3 %, 10.7 % when exposed to stauro. At 48 hours of treatment 35 %, 8.9 %, 6.4 % for doxo and 41 %, 40 %, 32 % for stauro. Results are obtained subtracting apoptosis drug mediated in cell line transfected with EV. Results showed that all drugs significantly increased cell death in the ICER expressing cell lines, demonstrating that ICER role in cell death was regardless of the type of drug used (Figure 1D, $n = 3$, * $p < 0.05$).

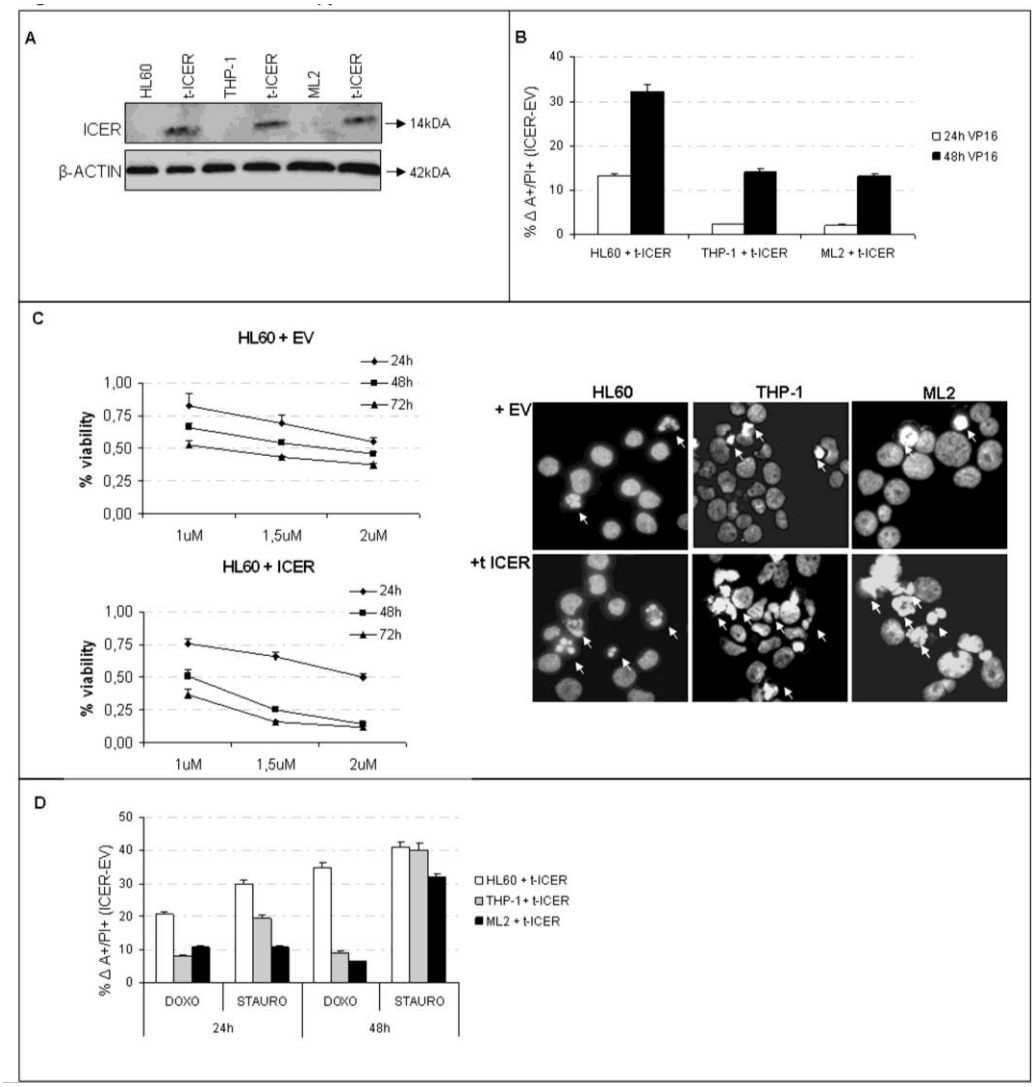

Figure 1. ICER enhances chemotherapy induced cell death. A) Western blot analysis of ICER transient expression (t-ICER) induced in HL60, THP-1 and ML2 48h post transfection. B) The increase in apoptotic of ICER expressing cell lines after VP-16 treatment is shown with respect to cells transfected with EV in the same treatment conditions (its value has been subtracted in the figure, n = 3, * p < 0.05). C) Proliferation of ICER or EV transfected cell lines after VP16 treatment (with respect to transfected but untreated cell lines). Treatment and ICER expression conferred the lowest proliferation values after 48 and 72 h (n = 3). On the right, DAPI nuclear staining after VP-16 treatment in cell lines +ICER reveals chromatin condensation, and the number of rounded and fragmented nuclei increased with respect to cells +EV in the same conditions. D) Apoptosis of cell lines +EV and +ICER after different drug treatments is shown. Histogram represents the percentages of A+/PI+ (to which the value of apoptosis induced by DMSO was subtracted) after 48 h of drug treatments. Apoptosis is significantly increased at 24 and 48 h of treatment in cell lines overexpressing ICER (n = 3, * p < 0.05).

ICER counteracts CREB gene expression. By studying the ICER transcriptional repressor activity by RQ-PCR (11), we revealed that the MAP-phosphatases DUSP1 and DUSP4, the survival molecules ERK1/2 and AKT, as well as controllers of the cell cycle progression, Cyclin A1, B1 and D1, were significantly less expressed in HL60+ICER cells with respect to HL60+EV cells, or HL60 after VP-16 treatment (Figure 2A, $n = 3$, $* p < 0.05$). The mRNA repression correlated with protein reduced levels, particularly after 48 hours of VP-16 treatment, which were not found in the HL60+EV cells treated at the same conditions (Figure 2B). To further investigate the role of cyclin down regulation, cell cycle analysis was performed. Results revealed mainly an accumulation of sub G0 (apoptotic) HL60+ICER cells after drug treatment. A block in the G2-M phase was evidenced mainly after VP-16 and doxo treatment; whereas a rapid ongoing to apoptosis mainly after stauro treatment was shown. We documented cell cycle regulators p21 and Cdk2 activation up to 24 hours of VP-16 treatment, supporting the G2 block of cell cycle progression; their reduction at 48 hours established the maximum effect of treatment linked to higher cell death (Figure 2C, $n = 2$, $p < 0.05$, ctr = untreated cells).

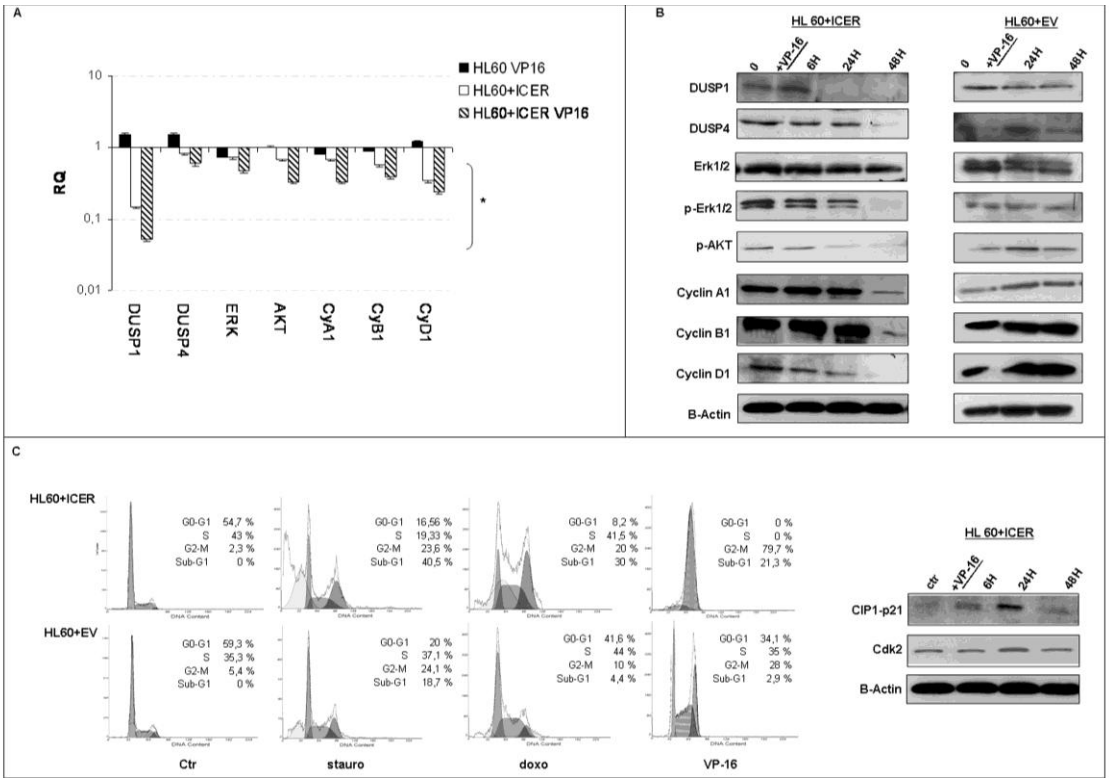

Figure 2. ICER controls gene expression influencing cell cycle and MAP Kinases. A) Histogram shows the relative quantitation (RQ) of CREB/ICER target genes of HL60+EV and of HL60+ICER at 48 h of VP-16 treatment compared to the untreated HL60+EV cell line used as calibrator (RQ = 1 in the figure, n = 2, * p < 0.05). B) Western blot analysis of target proteins which were found decreased in HL60+ICER treated with VP16. HL60+EV cell line, treated at same conditions, did not show reduction in protein levels. C) Cell cycle analysis of HL60+ICER and HL60+EV cell lines, treated with VP-16, doxo, or stauro, were performed after 48h of treatment. Histograms show that HL60+ICER cells treated with drugs present a increased sub-G1 phase or block in G2 phase compared to the untreated cells (ctr). Western blot analysis: p21 and Cdk2 are activated 24 h post treatment in HL60+ICER, consistently with the block in cell cycle progression and with the reduction of cyclins observed in panel 2B.

ICER enhances chemotherapy induced apoptosis by DUSP1/4-p38 pathway. The apoptosis previously discussed was confirmed in the HL60+ICER cell line after 24 hours of treatment (Figure 3A) by the increase of expression of the following: active caspase-3 (active fragments p20, p19 and p17), caspase 8 (active fragments p43/41) and cleaved poly (ADP-ribose) polymerase (PARP) (cleaved fragment p89).

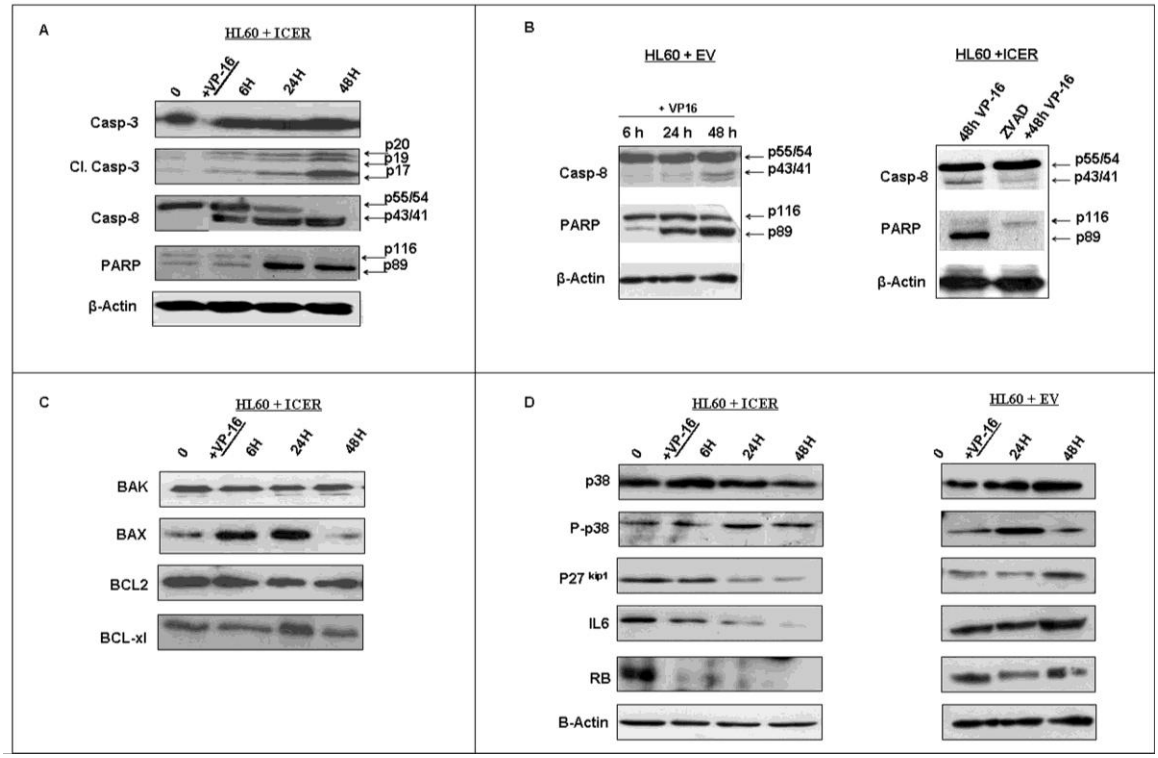

Figure 3. ICER evokes caspases mediated external apoptosis by p38 pathway. A) Western blot shows the activation cleavage mediated of inactive proteins into mature fragments of caspase 3 (p17, p19, p20), caspase 8 (p43, p41) and PARP (p89) starting from 24 h of treatment and up to 48 h. B) Western blot analysis showed that apoptosis enforced by ICER was found to be caspase-mediated (increase of fragments p43, p41 of caspase 8 and of p89 fragment of PARP). By the use of ZVAD as caspase inhibitor, delayed caspase 8 and PARP activation was shown. C) Western blot analysis was conducted on the mitochondrial protein fraction (Mt). The presence of BAK confirm that mitochondria are undamaged. The expression of antiapoptotic BCL-2 and BCL-XL over the treatment period is not influenced by VP-16 in HL60+ICER cell line. D) DUSP 1 and 4 are shown to be repressed over treatment (Figure 2B), whereas p38 was kept phosphorylated during the same period of time. The impaired expression of downstream p38 substrates, such as p27, IL6 and RB is documented. Same proteins reduction was not observed in HL60+EV treated at same conditions.

We observed that caspase 8 and PARP activation was markedly induced in HL60+ICER with respect to the HL60+EV cell line treated in the same conditions. Caspase 8 and PARP activation was controlled by the use of the broad inhibitor z-VAD, confirming that ICER evoked caspases to trigger apoptosis (Figure 3B). In an attempt to determine the sequence of events involved in

ICER mediated apoptosis in HL60, we excluded mitochondrial involvement by demonstrating the maintenance of BAK, BCL-2, BAX, and BCL-XL protein expression during VP-16 treatment. We checked the mitochondrial protein fraction for purity and as loading control, and the presence of BCL-2/BCL-XL demonstrated the mitochondrial membrane integrity up to 48 hours (Figure 3C). We then investigated the role of the phosphatases DUSP1/4 impaired gene and protein expression mediated by ICER repression, and investigated their main target, the proapoptotic p38 protein. Results showed the upregulation and maintenance of the phosphorylated form of p38 expression during VP-16 treatment of HL60 + ICER. The activation of p38 was confirmed by looking at specific substrates, p27, RB and IL6, whose expression was found severely compromised, supporting the observed cell apoptosis (Figure 3D).

ICER activated p38 by repressing DUSP1/4 transcription triggering cell apoptosis in HL60 and primary cultures. The chromatin at the DUSP1/4 promoter was immunoprecipitated. Pulled-down DNA showed the binding of CREB in the HL60+EV cell line partially or totally substituted by ICER when exogenously expressed in the HL60+ICER cell line (Figure 4A). Results of RQ-PCR were interpreted by the ΔΔct method considering HL60+EV as calibrator (RQ = 1). The amount of DNA immunoprecipitated by CREB was significantly decreased for DUSP1 (RQ = 0.46) and for DUSP4 (RQ = 0.80) in HL60 + ICER with respect to HL60 + EV, confirming that ICER might work preferentially on DUSP1 promoter ($p < 0.05$) (data not shown). To further emphasize the importance of p38 in regulating stress-induced AML cell death, HL60 was treated with anisomycin a specific activator of p38. Western blot showed that increased phospho-p38 levels contributed to higher apoptosis in the HL60 cell line. By using the specific p38 inhibitor SB203580 we prevented apoptosis in the same context (Figure 4B, n = 3, * $p < 0.05$).

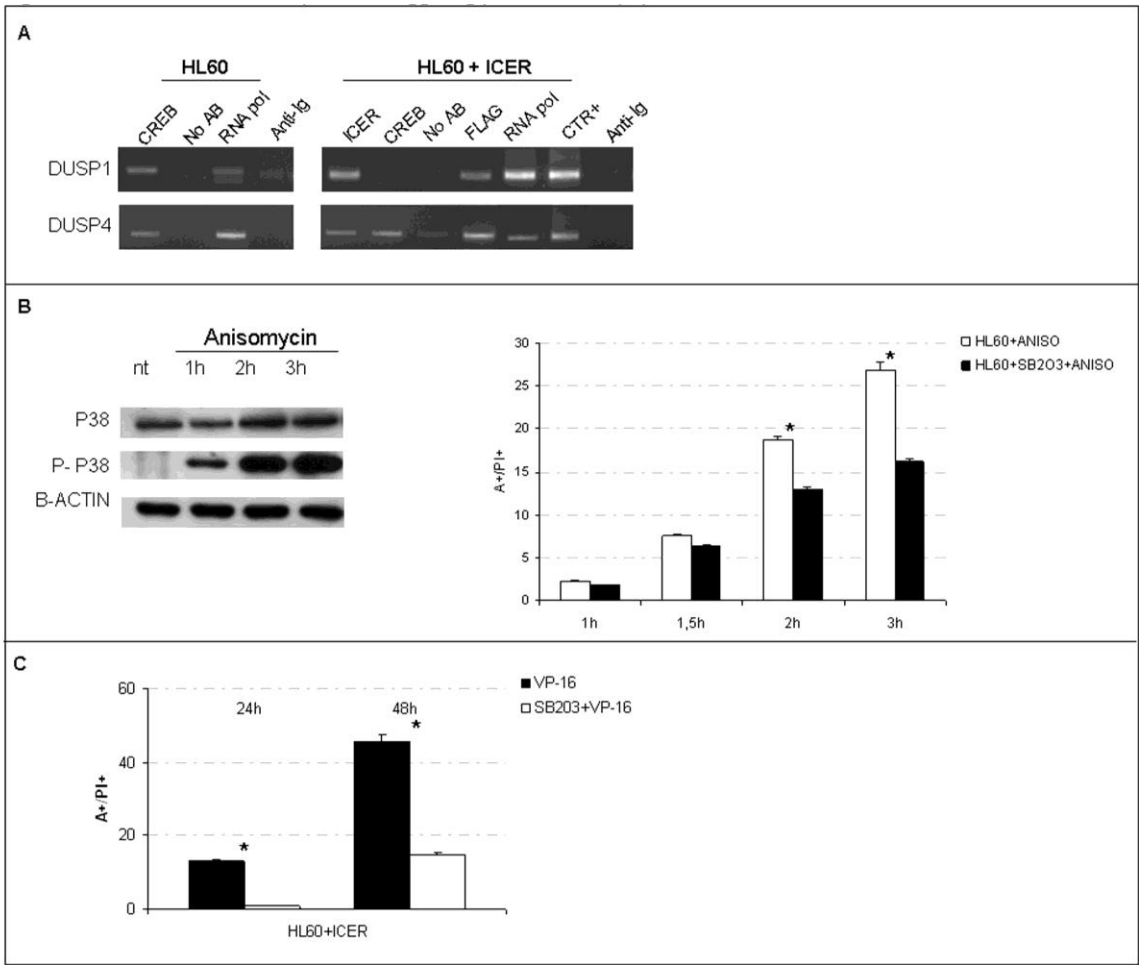

Figure 4. ICER controls DUSP1/4 promoter triggering p38 mediated apoptosis. A) Chromatin immunoprecipitation was performed using CREB, ICER or FLAGM2 antibody in both cell lines. FLAG antibody was used to pull down ICER exogenous protein, while RNAPOL was used to assess active promoters. Positive control is the imput DNA (ctr+) while negative control is obtained without any antibody (NoAb) or using an anti-IgG antibody. B) Western blot shows the increase of phosphorylated p38 (p P38) expression after the use of the specific drug anisomycin (aniso). The treatment increases HL60 apoptosis (A+/PI+) as the consequence of specific p38 induction. By using p38 inhibitor (SB203580), apoptosis was rescued (n = 3, *p < 0.05). C) Apoptosis assay (% of annexin V and PI positive cells) was performed in HL60+ICER or +EV cell after VP-16 treatment with or without SB203580 pretreatment. Results show a rescue in apoptosis when cells are pretreated with SB203.

To prove that the VP-16 enhanced sensitivity induced by ICER expression was mediated by the p38 pathway, a specific inhibitor of all p38 homologues (p38α, p38β and p38β2), SB203580, was used. Apoptosis was found reduced from 13.1 % to 1 % in HL60+ICER 24 hours

post treatment and from 45.7 % to 14.8 % at 48 hours (Figure 4C, n = 3, $p < 0.05$), suggesting that p38 activation was directly involved in the apoptosis mediated by ICER restoration. Apoptosis was rescued by SB20358 also in HL60 + EV treated with VP-16, but with lower efficacy (data not shown).

DUSP1 and DUSP4 silencing phenocopies ICER's role in parental HL60 cell line. The silencing of both DUSP1 and DUSP4 was induced in the HL60 cell line by using small interfering RNAs. To evaluate their ability their ability to induce the same effect mediated by ICER restoration in the HL60+ICER cell line. The decreasing expression of DUSP1 and DUSP4 mRNA was measured with the ΔΔCt method considering scramble siRNA as calibrator (RQ = 1). After 16 hours of DUSP1 silencing, the RQ was strongly decreased (RQ = 0.58), whereas DUSP 4 silencing was inefficient (RQ = 0.87). After 30 hours, DUSP1 mRNA was maintained reduced (RQ = 0.65), whereas DUSP4 was strongly diminished (RQ = 0.37). Protein levels followed the same trend of mRNA. Protein levels followed the same trend of mRNA. DUSP1/4 silencing was documented to increase phospho-p38 levels (p-p38) after 20 hours; apoptosis was slightly improved as well (data not shown). We then treated the DUSP1/4-silenced HL60 cell line with VP-16. Results showed that silenced cells had a decreased proliferation and an increased apoptosis with respect to the scRNA used as control. Therefore, the parental DUSP1/4-silenced HL60 cell line became more sensitive to the drug treatment, as established in the HL60+ICER cell line (Figure 5B, n = 3, * $p < 0.05$).

DUSP1 silencing or ICER exogenous expression promote apoptosis in AML primary cultures.

We silenced DUSP1 in primary BM cultures of AML at diagnosis. In the same cultures, we also restored ICER exogenous expression. DUSP1 silencing was more intense after 24 hours (RQ = 0.27) (calibrated to Sc-Sir, RQ = 1), with respect to 48 hours (RQ = 0.72).

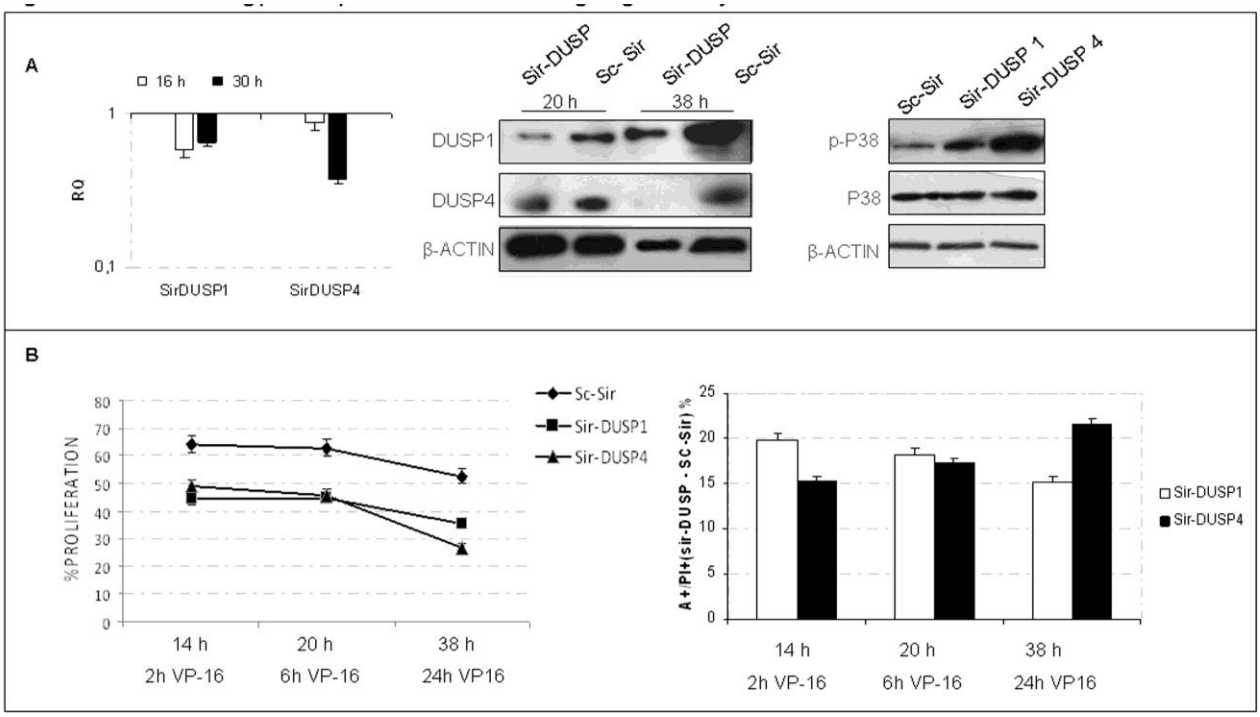

Figure 5. DUSP1/4 silencing phenocopies ICER role in enhancing drug sensitivity. A) RQ of DUSP1/4 after 16 and 30 h of silencing is shown, results are calibrated to Sc-Sir mRNA (RQ = 1) used as control. Western blot shows that silencing of DUSP1 and DUSP4 in HL60 cell line decreased their protein expression compared to the scramble siRNA oligonucleotide used as control (Sc-Sir) at 20 and 48 h post transfection. An increase in phospho-p38 (p P38) was also found concomitantly with silencing of DUSP proteins. B) HL60 cells after 14 h of silencing were treated with VP-16. Their proliferation rate decreases over time. The % of apoptosis significantly increases after DUSPs silencing with respect to cells transfected with Sc-Sir (sc-Sir value has been subctrated) and treated at the same VP-16 concentration (n = 3, * p < 0.05 for all time points presented).

Protein expression was impaired as well. We monitored the effect on p38 levels and found its phosphorylation increased, confirming the pathway activation after DUSP1 silencing (Figure 6A). We restored ICER expression in the same primary AML cultures by transiently transfecting the pEGFPΔ-N1_FLAG-ICERγ plasmid as well as the Empty Vector as control (EV). DUSP1 mRNA and protein expression were found decreased after transfection, confirming that DUSP1 is a downstream target of ICER in myeloid leukemia cells. p38 phosphorylation increased (Figure 6B) promoting cell death and supporting the same activation pathway in patients of the myeloid

cell lines. The scheme in figure 6C summarizes a new view of how CREB might influence the survival signaling in myeloid leukemia.

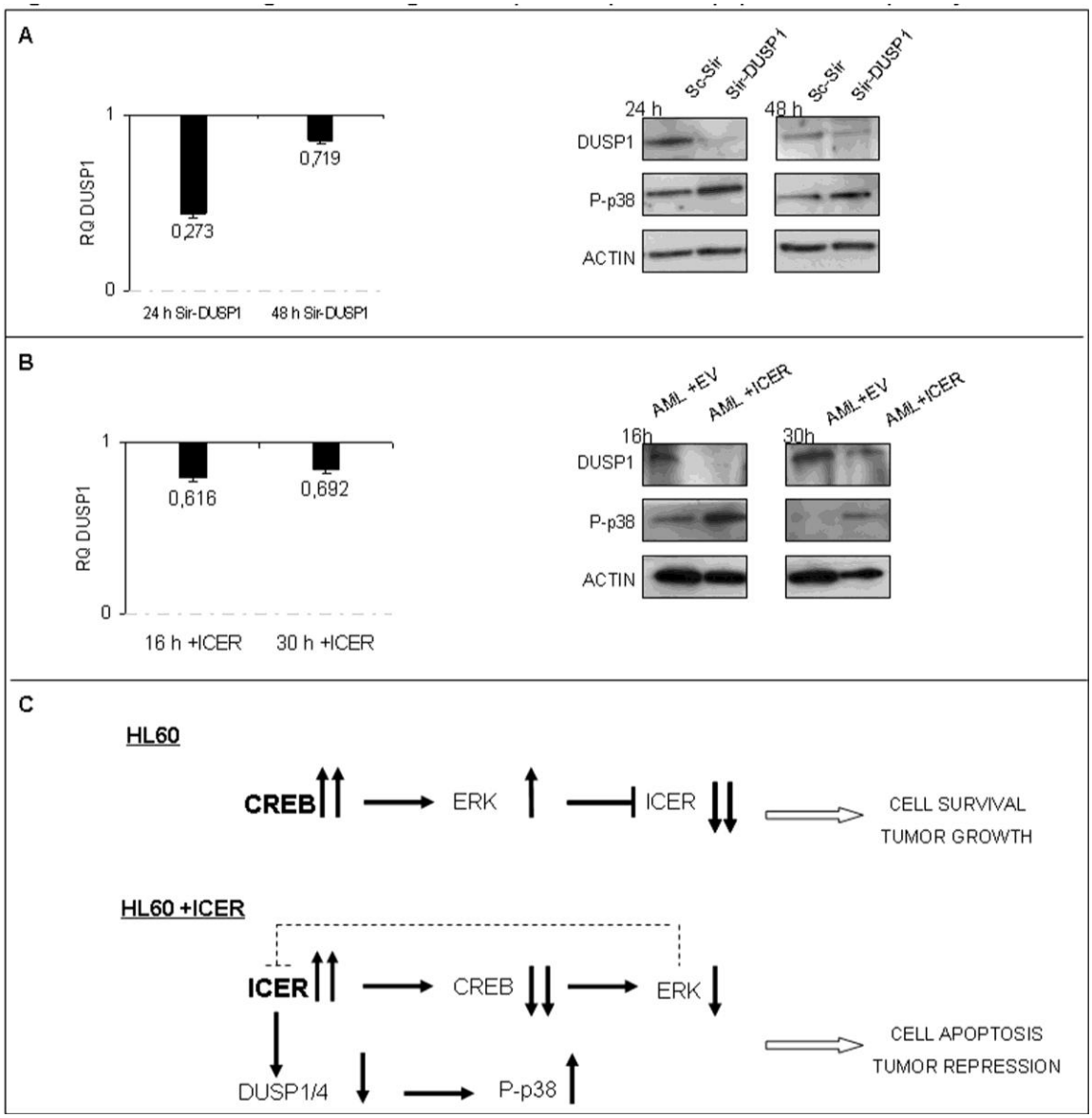

Figure 6. DUSP1 silencing or ICER expression promote apoptosis in AML primary cultures. A) Primary cultures were used to silence DUSP1. A decrease of DUSP1 mRNA after 24 and 48 h (ScSir used as calibrator, RQ = 1) is shown. DUSP1 protein levels were lowered and phospho-p38 (P-p38) activated after silencing. B) ICER expression was introduced in primary AML bone marrow: DUSP1 mRNA was found reduced (RQ = 0.6 after being calibrated to EV) and protein levels as well. p38 activation was confirmed (P-p38). C) Proposed scheme of the cross-talk between CREB/ICER and the DUSPs/p38 pathways in leukemic myeloid cells.

DISCUSSION

AML is a heterogeneous tumor, specifically for its clinical outcome and molecular features. Although many chromosome abnormalities have been recently characterized, such as gene mutations, expression profiles and microRNAs, alternative treatment is needed because resistance to therapy and relapse still occur (30). Several approaches are under experimentation to increase cell apoptosis and a large number of kinases and phosphatases are under examination in the hematological field. Sensitizing cell to drugs could help to improve treatment response, and the characterization of novel molecules is urgent (31–33). We previously reported that ICER expression in leukemic cell lines induces significant antiproliferative effects (6,11). In this regard, results presented here show that restored ICER expression confers an enhanced drug susceptibility to leukemic cells. We described DUSP1 expression to be considered as a crucial target in AML treatment response. Focusing on DUSP1 and DUSP4 repression as ICER's main targets, we established their role in p38 activation, and elucidated the apoptotic signaling evoked by ICER in myeloid leukemia.

Multiple pathways might be addressed to be responsive to ICER restoration in leukemia. In this article, ICER-dependent regulation of pro- and antiapoptotic genes are demonstrated to enhance apoptosis with respect to that observed in leukemia cells without ICER. Cyclins and genes of the MAPK signaling pathway were specifically found severely downregulated by both ICER and chemotherapy treatment leading myeloid leukemia to a different predisposition to cell death. In particular, we found a novel link between ICER and DUSP1/4 phosphatases, whose expression has already been reported to be high in different types of tumors, though never previously discussed in the leukemia field (34). ICER-mediated transcriptional DUSP1/4 repression was demonstrated to contribute to increase p38 phosphorylation, triggering proapoptotic signals. The involvement of p38 in mediating apoptosis was confirmed by the use of

p38 inhibitor SB203580, which interrupts the ongoing process of apoptosis. The induction of DUSP downregulation and p38 activation have been demonstrated to activate a cascade of different stimuli (35-37). The downregulation of the cell survival signaling of ERK and AKT might concur to enforce the p38-mediated apoptosis (23,38). Furthermore, the severe decrease of other downstream factors, such as p27, IL6 and RB, confers the final anti-survival cellular response. The fact that IL6 and RB transcription depends on CREB activity, whereas their phosphorylation on MAPK, confers to these two intricate pathways the ability to converge and collaborate for cell growth and AML progression (39,40). The finding of CREB and ICER transcriptional regulation of DUSP1/4 reveals a novel role in maintaining the balance between the activity of stress and survival kinases, which modulate leukemia cell fate.

DUSP1 and 4 repression appeared to be the crucial event for drug response of leukemia cell lines. The silencing strategy was also used in primary bone marrow cultures of AML at diagnosis to assume the same condition of the HL60+ICER cell line. DUSP1/4 down regulation lowered cell proliferation, phospho-p38 activation and an increased apoptosis of AML patients phenotyping the HL60+ICER cell line behavior. The proposed pathway was confirmed to modulate chemotherapeutic susceptibility of myeloid leukemia. The *in vivo* strategy was also used to exogenously reintroduce ICER expression in primary AML giving the same results as DUSP1 silencing. ICER as controller of DUSP1 expression and of p38 pathway activation in AML patients confirmed our hypothesis, opening for further investigation in future therapy strategy.

Taken together, our results describe a novel apoptotic pathway in myeloid leukemia, summarized as a working model in Figure 6C: in HL60, CREB overexpression maintains high ERK levels, which takes ICER to degradation (11); DUSP1 and 4 dephosphorylate p38, supporting survival and leukemia growth. In the HL60+ICER cell line, ICER is highly expressed.

It decreases CREB expression and promotes gene repression, in particular of DUSP1/4, which in turn allows p38 to remain phosphorylated and to trigger apoptosis. Lowered CREB levels and high p38 levels maintain ERK downregulated, preventing ICER degradation and contributing to tumor suppression (41,42). The identification of this pathway, confirmed in AML patients at diagnosis, offers novel targets to be considered in leukemia treatment. In particular, we support the idea that DUSP1 inhibition by ICER is a good strategy to sensitize cancer cells to conventional chemotherapy and to inhibit tumor growth. With regards to leukemogenesis, blocking CREB or inducing ICER might be further considered as phenomena involved in malignant transformation.

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CHAPTER 4

MIR-34B TARGETS CAMP RESPONSE ELEMENT BINDING PROTEIN (CREB) IN ACUTE MYELOID LEUKEMIA

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ABSTRACT

The cAMP response element binding protein (CREB) is documented to be overexpressed in leukemia, but the underlying mechanism remains unknown. Here, microRNAs (miRNAs), that act as negative regulators of gene expression principally through translational repression, are investigated for the mediation of high CREB protein levels. A series of miRNAs which target CREB were identified. RQ-PCR revealed that miR-34b was expressed significantly less in myeloid cell lines, previously known for high CREB protein levels. Exogenous miR-34b expression was induced, and results revealed a direct interaction with the CREB-3'untranslated region, with the consequent reduction of the CREB protein levels *in vitro*. MiR-34b restored expression caused cell cycle abnormalities, reduced anchorage independent growth, and altered CREB target gene expression, suggesting its suppressor potential. Using reverse phase protein array, CREB target proteins (BCL-2, Cyclins A1, B1, D, NfKB, JAK1, STAT3), as well as many downstream protein kinases and cell survival signaling pathways (AKT/mTOR, ERK) usually elicited by CREB, were observed to have decreased. The miR-34b/34c promoter was demonstrated to be methylated in the leukemia cell lines used. This epigenetic regulation should control the observed miR-34b expression levels in order to maintain the CREB protein overexpressed. In addition, the inverse correlation between miR-34b and CREB expression was found in a cohort of 78 pediatric patients at diagnosis of acute myeloid leukemia, supporting this relationship also *in vivo*. Our results identify a direct miR-34b target gene, provide a possible mechanism for CREB overexpression, and provide new information about myeloid transformation and therapeutic strategies.

INTRODUCTION

MicroRNAs (miRNAs) are a class of small non-coding RNAs able to influence gene expression by targeting mRNA. 30% of human genes possess conserved miRNA binding sites and are presumed to be controlled by this regulation mechanism (1). Generally, miRNAs bind mRNA sequences located at the 3'-untraslated region (UTR) with imperfect complementarity. They usually avoid the interaction of a target mRNA with polysomes, blocking translation, or promoting mRNA degradation. Consensus miRNA sequences are described also in 5'UTRs and in coding sequences, but their role in gene regulation has been demonstrated to be less efficient (2). Hundreds of miRNAs have been identified to date, but their specific functions and target mRNAs have been assigned for only a few (3). MiRNA expression has been demonstrated to be tissue specific, and to control cellular differentiation, proliferation and survival, and changes in their expression have been associated with many pathologies, including human cancer (4). A role of miRNAs in several tumors has recently been recognized, with intrinsic suppressor or oncogenic functions (5). Little is known about their role in acute myeloid leukemia (AML). There is considerable evidence that supports a crucial role for miRNAs in chronic lymphocytic leukemia (6,7), and an involvement of miR-223 and miR-155 has already been proposed in the pathogenesis of AML (8,9). The knowledge and the characterization of novel miRNAs should be expanded in order to elucidate the pathophysiological events that cause myeloid transformation, which is considered to accumulate genomic alterations that act as consecutive transforming events in the leukemic clone during leukemic development (10). Multiple mutations have recently been investigated in signaling molecules, such as growth-factor receptors and transcription factors, and have been associated to specific leukemia phenotypes and sometimes to treatment response (11). The principle aim is to improve AML knowledge by exploring the mechanism of genetic disruptions to improve the traditional cytogenetic markers in clinical use.

CREB is a nuclear protein that regulates gene expression principally through the activation of cAMP dependent cell signal transduction pathways, after being phosphorylated at Serine 133 usually via protein kinase A (12,13). This modification enhances the transactivation potential of CREB and promotes the recruitment of two major cofactors, CREB binding protein (CBP) and p300 (14,15). CREB recognizes the conserved cAMP-responsive elements (CREs), which occur either as a full palindrome (TGACGTCA) or half site (CGTCA/TGACG) at gene promoters in a cell-type specific manner, controlling the expression of genes involved in cell proliferation, differentiation, and survival signaling pathways (16). Now, more than 4000 human genes are known to contain CRE consensus regions, and the ability of CREB to activate or not target gene transcription depends on recruited co-factors and on the cellular gene regulation program (17). CREB overexpression in leukemia has been demonstrated to cause the upregulation of its target genes, influencing leukemia phenotypes; in particular, it promotes abnormal proliferation, cell cycle progression, and higher clonogenic potential *in vitro* and *in vivo* (18,19). CREB overexpressing transgenic mice have been demonstrated to develop myeloproliferative disorders, suggesting that CREB plays a role during the leukemogenic process (20).

Here, we report studies of CREB carried out in order to understand the molecular mechanism that controls its protein overexpression in leukemia. We consider that the inappropriate expression of candidate miRNAs could be a possible mechanism of protein regulation. The CREB 3'-UTR sequence has many miRNA consensus seeds, but this research demonstrates that miR-34b is responsible for CREB expression through the control of its translation.

MiR-34b belongs to the evolutionary conserved miRNA family miR-34s (21), known for its role in the p53 tumor suppressor network (22). MiR-34s have been found controlled in a tissue

specific manner by p53, to have an anti-proliferative potential in cell lines, and to be downregulated in human tumors (23,24). Efforts have been made to identify miR-34 target genes, but apart from the few genes known to be miR-34a targets (25,26), there are no confirmed target genes for miR-34b/34c in leukemia.

In this research, CREB is shown to be an miR-34b target. A series of CREB target proteins and major related pathways are demonstrated to be influenced after miR-34b restoration. The promoter of miR-34b/34c is found methylated, which should explain the lowered miR-34b expression observed in myeloid tissue. Thus, miR-34b suppresses the CREB network, inhibiting tumor growth and should be further considered during AML development.

MATERIALS AND METHODS

Cell culture and transfection. Human acute myeloid leukemia cell line HL60 (American Type Culture Collection) was cultured in DMEM (Invitrogen-Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FCS; Invitrogen-Gibco). Human myeloid cell lines NOMO1, NB4, ML2 and Human CML-derived B-cell like cell line K562 (American Type Culture Collection), were cultured in RPMI (Invitrogen-Gibco) supplemented with 10% FCS. Cells were treated with 2 μM 5-aza-2'-deoxycytidine (DAC-Sigma, St Louis, MO). Cell transfection was performed using the Nucleofector systems (Amaxa Biosystems, Koln, Germany), according to the manufacturer's guidelines. Transfection conditions were optimized to result in more than 70% transfection efficiency with a cell viability of more than 80%. We analyzed bone marrow samples from 78 patients with newly diagnosed pediatric acute myeloid leukemia (AML), and 17 from healthy pediatric bone marrow. The diagnoses of leukemia were made according to standard morphologic criteria on the basis of immunohistochemical,

immunophenotyping, and cytogenetic studies following the AIEOP-2002 AML pediatric protocol. Informed consent in compliance with the Helsinki protocol was obtained.

MiRNA target prediction. The analysis of miRNA predicted targets was carried out using the algorithm miRanda (http://www.microrna.org/microrna/home.do.). The algorithm produced a list of hundreds of miRNAs on the *CREB 3'UTR* sequence gene, and miR-25, miR-32, miR-34b, miR-124, miR-367 were analyzed. We focused on miR-34b for its significant lower expression in the leukemia cell lines used.

RNA extraction and Real-time PCR for miRNA analysis. RNA from cell lines and from bone marrow patients were isolated using a mirVana miRNA isolation kit (Ambion, Austin, TX) according to manufacturer's instructions. RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent technologies, Tokyo, Japan) and then used for PCR. TaqMan miRNA assays (Applied Biosystems, Forest City, CA) were carried out using the stem-loop method to detect the expression level of mature miRNAs. For the retro-transcription reaction, 10 ng of total RNA was used in each reaction and mixed with the specific stem-loop primers (Applied Biosystems). All PCR reactions were run in triplicate and gene expression, relative to U6 snRNA (RNU6B), was calculated by comparative the ΔCt method (27).

Methylation analysis. Genomic DNA was extracted according to manufacturer's instructions (Gentra Autopure LS, Qiagen, Hilden, Germany) from myeloid cell lines. Briefly, 1 μg of DNA was diluted in 50 μl of distilled water and denatured by adding 5.5 μl 3 M NaOH. On ice, 520 μl of bisulphite solution at pH 5, and 30 μl of 10 mM hydroquinone were mixed. The DNA was recovered and desulphonated by adding 5.5 μl 3 M NaOH. The solution was neutralized by adding 55 μl of 6 M ammonium acetate at pH 7. The DNA was ethanol-precipitated and used with methylation specific primers and PCR protocol (28).

Cloning of 3'-UTR of CREB1 mRNA. 100 ng of cDNA from myeloid cell line served as template to amplify CREB 3'-UTR (NM_004379, gi 222194459 from nt 1078 to nt 1359). The amplified PCR product was gel-purified and subcloned into pCR2.1 (Invitrogen-Gibco). The insert was excised with HindIII and SacI restriction enzymes, sequenced and ligated into the pMIR-REPORT miRNA Luciferase (LUC) reporter vector (Ambion) at the 3' of the LUC gene. We refer to pMIR-LUC-3'UTR-CREB in experiments performed using this construct. A mutant 3'UTR of CREB was also synthesized by PCR.

Transfection and luciferase reporter assay. MiR-34b oligonucleotide and a miRNA from the Arabidopsis thaliana genome used as negative control (referred to as miR-neg) were purchased from Dharmacon (Lafayette, CO). A mixture of pMIR-LUC-3'UTR-CREB, Renilla plasmid (REN), and of mature miR-34b oligonucleotide were used to co-transfect cell lines. A mixture of pMIR-LUC-3'UTR-CREB, REN, and miR-neg was used as control. After 24 hours, RNA and proteins were extracted. RQ-PCR was used to test miR-34b expression, and protein lysates were analyzed for LUC and REN activity levels using the Dual Luciferase Assay System (Promega, Madison, WI). LUC activity was normalized to REN activity, compensating for variation in transfection efficiency. Experiments were performed in triplicate.

Western Blot. Western blot analyses were carried out as previously described (19). Briefly, 20 μg of the total protein fraction (Buffer-Biosource International, Camarillo, CA) isolated from transfected cells was used. Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Antibodies used were antiPhospho-CREB (Upstate Biotechnology, Lake Placid, NY) and anti-β-actin (Sigma-Aldrich). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti rabbit or mouse IgG (Upstate Biotechnology). Proteins were detected using enhanced chemoluminescence (ECL) and films (GE-healthcare, IL).

RNA isolation and SYBR green quantitative real-time RT-PCR (QRT-PCR) assays. Total RNA was isolated from cell lines (2 to 5 x10^6 cells per sample) using TRIzol (Invitrogen). 1 μg of RNA was transcribed using the Superscript II system (Invitrogen) in 25 μl final volume following manufacturer's instructions. RQ-PCR was performed with 1 μl cDNA in 20 μl using the Sybr Green method (Invitrogen) and analyzed on an ABI PRISM 7900HT Sequence detection system (Applied Biosystems). Experiments were carried out in triplicate and were analyzed with respect to standard curves in a relative quantity (RQ) study. Expression values for target genes (Table 1S) were normalized to the concentration of GUS, which showed the least variation among reference genes in our cell model.

Reverse Phase Protein Microarrays. The experiments were performed as described by Espina et al. (29,30). Briefly, cell lysates were diluted in a mixture of 2x Tris-Glycine SDS Sample Buffer (Invitrogen-Gibco), plus 5% of β-Mercaptoethanol and loaded onto a 384-well plate and serially diluted with lysis buffer into four-point dilution curves ranging from undiluted to 1:8 dilution. Samples were printed in duplicate onto nitrocellulose-coated slides (FAST slides, Whatman Schleicher & Schuell, Florham Park, NJ) with a 2470 Arrayer (Aushon BioSystems, Burlington, MA). Slides were stained with Fast Green FCF (Sigma) according to the manufacturer's instruction and visualized (ScanArray 4000, Packard). Arrays were stained with antibodies (Cell Signaling) on an automated slide stainer (Dako Autostainer Plus, Dako Cytomation, Carpinteria, CA) using a Catalyzed Signal Amplification System kit (CSA kit, Dako Cytomation) according to the manufacturer's recommendations. Antibody staining was revealed using DAB. The TIF images of antibody- and Fast Green FCF-stained slides were analyzed using Microvigene Software (VigeneTech Inc, Boston, MA). For each sample, the signal of the negative control array (stained with the secondary antibody only) was subtracted from the

antibody slide signal, and then the resulting value was normalized to the total protein value. The data processing generated a single value for each leukemia sample relative to each protein.

Cell cycle analysis. Cell lines were transfected with oligonucleotide miR-34b or miR-neg. After 24h of incubation, 5 x 10^5 cells were washed twice with PBS, lysed, and treated with 50 μg/ml Propidium Iodide (PI) in 1 ml PBS overnight at 4 °C. Cells were analyzed using Cytomics FC500 (Beckman Coulter, FL, USA). Cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems, CA).

siRNA experiments. Exogenous small interfering RNAs (siRNAs) specific for the CREB gene (Dharmacon Industries, Lafayette, CO) were introduced in myeloid cell lines (100 nM in 2 ml of medium) (18). A scramble of all 4 siRNAs (siRNAsc) was also used.

Anchorage-independent assay. To determine anchorage-independent growth of transfected cells, a total of 2.5 x 10^3 cells were seeded in a methylcellulose semi-solid medium not supplemented with nutrients and cytokines after miRNA and siRNA transfection (StemCell Technologies, Vancouver, Canada). Colony evaluation and enumeration was done *in situ* by light microscopy after 3-(4,55-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) incorporation.

Data Analysis. Statistical analyses were performed with Mann Whitney or unpaired two-tailed t test. A p value less than 0.05 was considered significant.

RESULTS

MiRNAs expression in cell lines. We studied the expression of 5 mature miRNAs predicted to target the *3'-UTR* region of the *CREB* gene by informatic tools (Figure 1A). Expression analyses revealed that miR-124a and miR-367 were not detectable in our samples, whereas miR-25, miR-32, and miR-34b were downregulated compared to healthy sorted myeloid

sub-populations (CD3-/CD19-) in all cell lines (Figure 1B). Among them, miR-34b was significantly less expressed (10^{-12} –fold) and was selected to be studied *in vitro*.

MiR-34b promoter region is methylated in leukemia. The miR-34b promoter region was studied in leukemic cell lines for hyper-methylation of the CpG island, as recently described in colon cancer (28). After treatment with DAC, miR-34b expression by RQ-PCR was found to have increased in the cell lines used, from 3.34 to 19.67 fold induction, confirming that miR-34b expression might be controlled by methylation in myeloid cancer cells (Figure 1C). MSP revealed that the miR-34b/c was methylated in all cell lines tested. By contrast, no methylation was detected in the sample made up of three normal bone marrow samples collected from healthy donors, indicating that methylation of the miR-34b/c region is a tumor-specific phenomenon (Figure 1D).

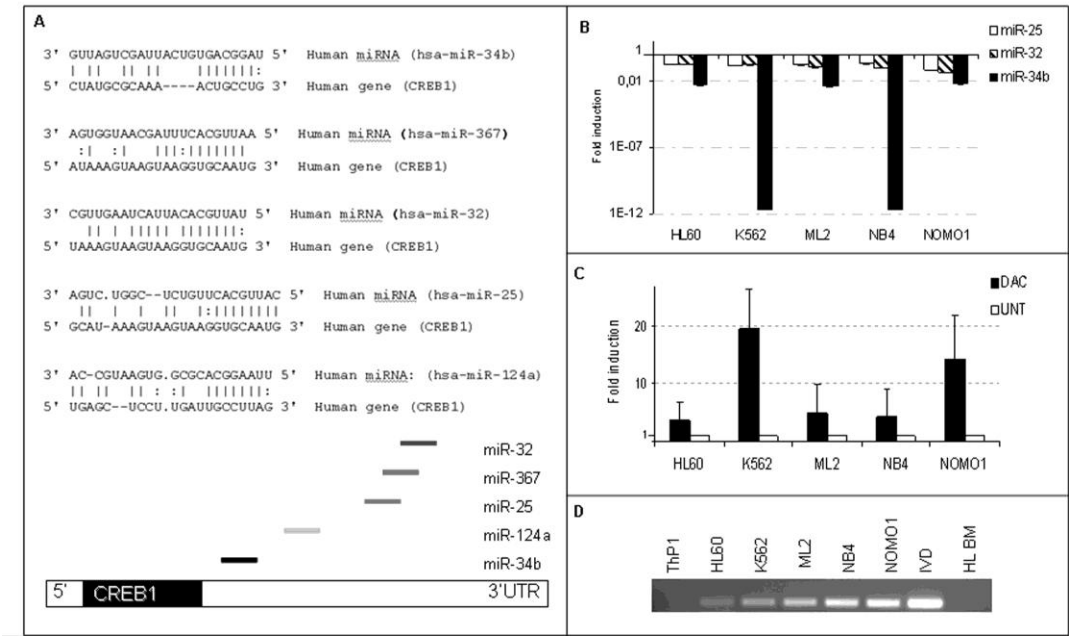

Figure 1. A) Schematic structure of the CREB gene with the predicted mature miRNAs allineated on the 3'UTR region. B) Analysis of miR-25, miR-32, and miR-34b expression in myeloid cell lines are presented. The fold induction expression with respect to the myeloid population sorted from a healthy bone marrow (value = 1 in figure) is calculated using the comparative ΔCt method (n = 3, p < 0.01). C) MiR-34b expression in myeloid cell lines before and after treatment with DAC. Expression after treatment (DAC, black bars) is shown relative to the untreated cells (UNT, white bars), expression value 1. Increase of miR-34b expression by DAC is statistically significant in all cell lines used (p < 0.01). D) MSP analysis of the miR-34b/c CpG island in a set of myeloid cell lines and a sample made up of three mixed healthy bone marrow samples (HL BM). *In vitro* methylated DNA (IVD) and ThP1 cell line were used as positive and negative controls, respectively.

Mir-34b suppresses CREB expression and inhibits cell growth. To assess the role of miR-34b in CREB expression and cell growth, HL60 cell line was transfected with the mimic oligo miR-34b with either control mirna (miR-neg) at 60, 120 and 180 nM up to 72 h. The MTT based cell proliferation assay revealed that miR-34b inhibits cell growth in a dose dependent manner. Using 180 nM miR-34b, cell proliferation was reduced to 58% at 48 h with respect to miR-neg, whereas at 120 nM, cell growth was always up to 70% after 72 h treatment (n = 3, p <

0.05). 120 nM miR-34b concentration was the treatment that gave the maximum effect on CREB translation inhibition (Figure 1S).

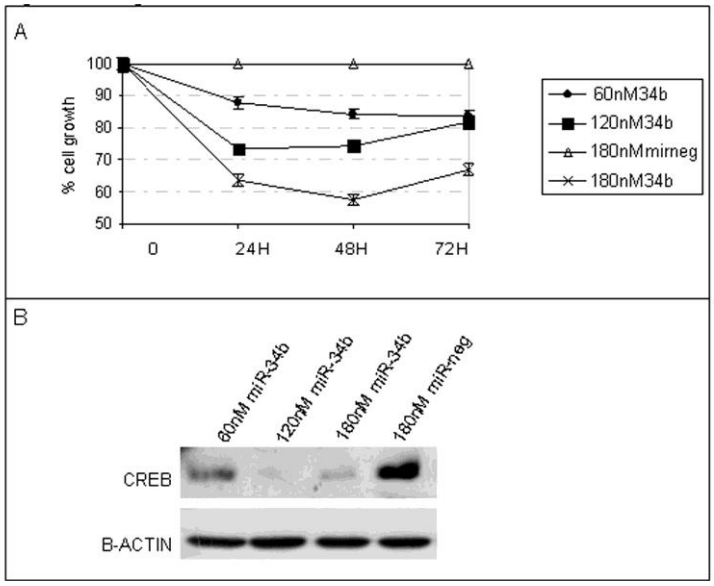

Figure 1S. A) Transfection of miR-34b into HL60 cell line inhibits cell growth measured by MTT cell proliferation assay. Cell viability respect to miR-neg transfection at 60, 120 and 180 nM are presented up to 72 h. B) Western blot analysis revealed that at 120 nM concentration, CREB protein expression was strongly reduced, while cells were still viable up to 70%.

MiR-34b targets CREB 3'-UTR. To validate *CREB* as a miR-34b target gene in different myeloid cell lines, the potential base pairing between miR-34b and *CREB 3'-UTR* was investigated. A portion of CREB *3'-UTR* was cloned and introduced into myeloid cell lines along with the miR-34b oligonucleotide. The ectopic expression of miR-34b was confirmed by RQ-PCR, which revealed a higher miR-34b expression, up to 10^4 fold induction, with respect to the basal condition (data not shown).

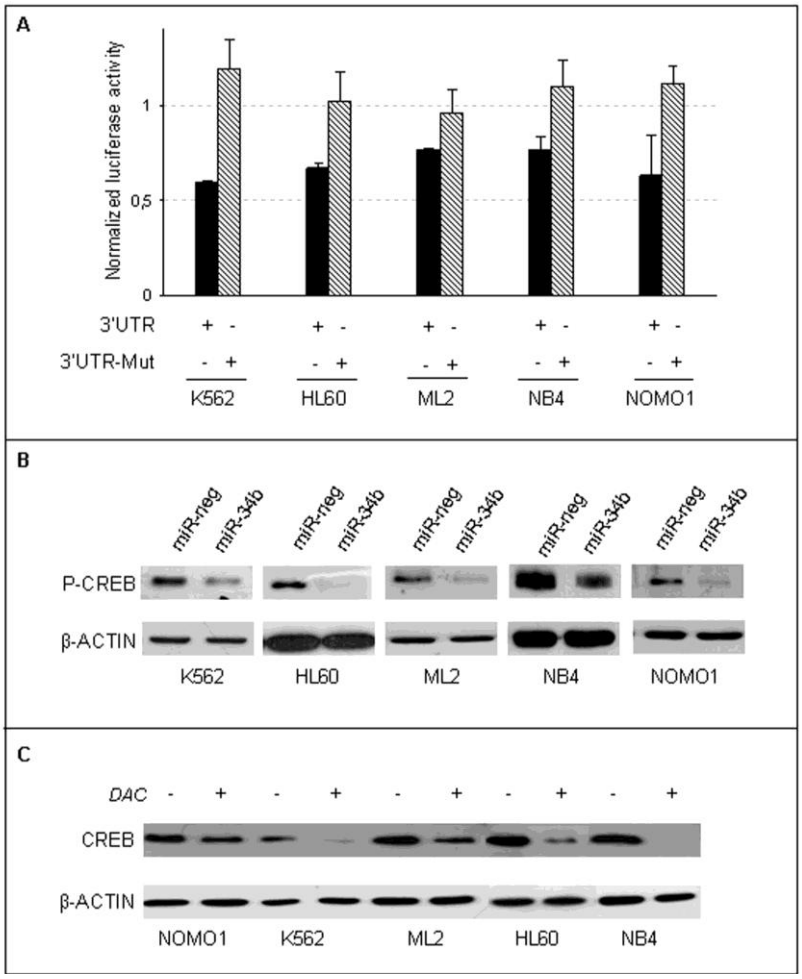

Figure 2. A) pMIR-LUC-3'UTR-CREB was transfected together with a miR-34b oligo (black columns). 3'UTR-Mut indicates the mutated CREB 3'UTR plasmid (black and white columns). Luciferase activity reduction by exogenous miR-34b was statistical significant in all cell lines (n = 3, p < 0.05). B) Western blot after miR-34b restoration is shown. A decrease of the active phosphoCREB (P-CREB) protein expression is iduced by miR-34b whereas miR-neg transfection does not modify CREB expression (n = 3, p < 0.05). C) CREB protein expression after DAC treatment was found reduced (n = 3, p < 0.05).

A decrease of 40% to 25% of luciferase activity was observed compared to that of miR-neg introduction, suggesting that *CREB 3'-UTR* carries a miR-34b regulatory element. In contrast, exogenous miR-34b expression did not reduce LUC activity with the mutated *CREB 3'-UTR*, further suggesting that CREB expression is typically regulated by miR-34b (Figure 2A). To discover the miR-34b role in translational CREB, western blots were performed. Results

confirmed that miR-34b, not miR-neg, inhibited CREB protein expression, which was found drastically reduced in all myeloid cell lines studied. The active phosphoCREB protein was demonstrated to be reduced after miR-34b expression, suggesting a cascade effect on its transcriptional activity (Figure 2B). Moreover, the expression of CREB was studied after DAC treatment. The demethylating treatment provoked a decrease of CREB protein (Figure 2C) as a result of miR-34b increased expression (Figure 1C), confirming CREB as a miR-34b target gene and its promoter to be epigenetically controlled.

MiR-34b influences CREB transcriptional activity which controls leukemia phenotype. To evaluate if restored miR-34b expression influences CREB transcriptional activity, we monitored the mRNA expression of a series of CREB target genes. Results revealed that the transcription of *cyclin A1, B1, D1, BCL-2, STAT3, JAK1 and NFkB* were decreased in our model (Figure 3A, $n = 3$, $p < 0.05$). Further, we used protein array in order to evaluate if CREB target gene repression was also able to influence protein expression levels. The HL60 cell line, after being transfected with the miR-34b oligonucleotide, showed a decreased expression of a series of CREB target proteins (BCL2, CyclinA1, CyclinB, CyclinD1, STAT3, NFkB, JAK1) and a series of kinases and molecules that control cell proliferation (ERK, AKT, mTOR, PKA, Smac-Diablo, SMAD1) ($n = 2$, $p < 0,05$, Figure 3B). The results of the gene and protein expression alteration described above were sought in cell growth and in cell clonogenic potential in an anchorage-independent manner, which are hallmarks of tumorigenicity. Results showed that cell growth (Figure 1S) and proliferation was compromised, as a lowered number of cells in S and G2/M phases was observed (Figure 3C). Furthermore, clonogenicity assay revealed a significant lowered number of colonies for NB4 and HL60, whereas K562 colonies were significantly larger in dimension and morphology ($n = 3$, $p < 0.05$, Figure 3D).

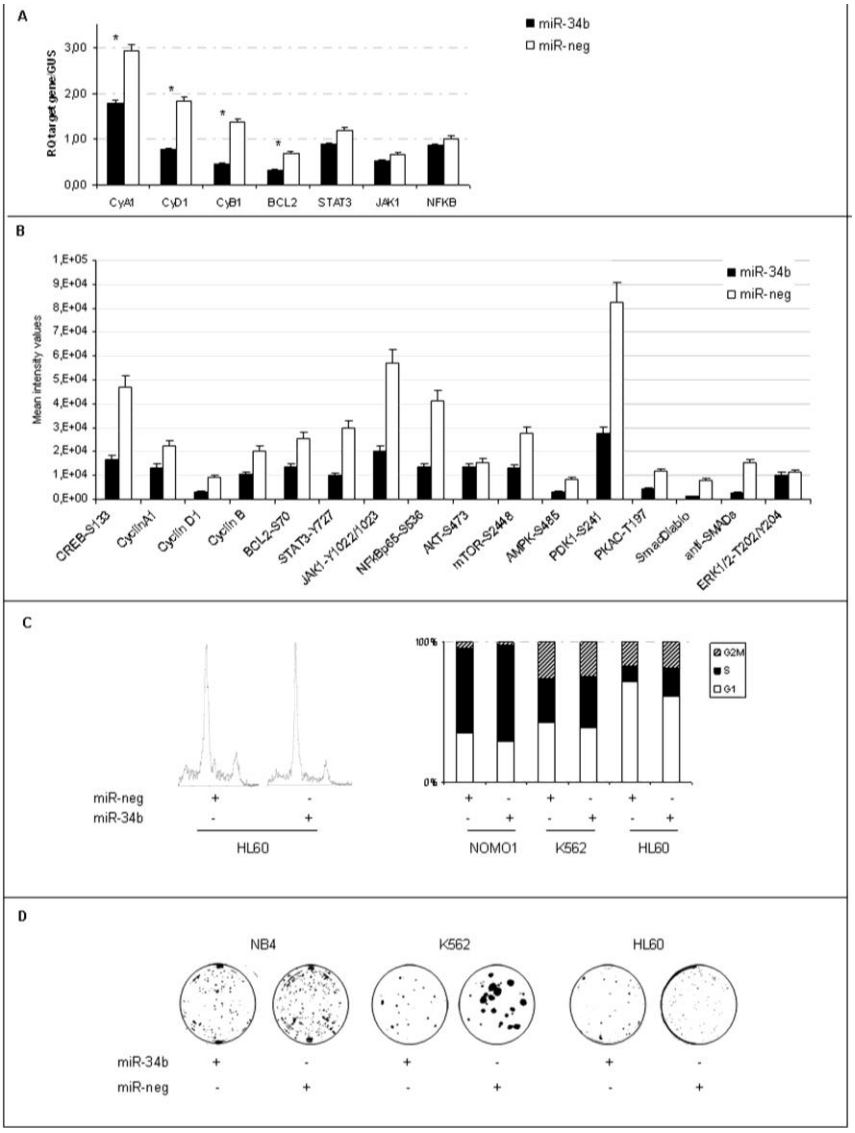

Figure 3. A) RQ-PCR for a series of CREB target genes was performed. Lowered gene expression was detected in HL60 after being transfected with oligo miR-34b (black bars) compared to miR-neg (white bars), as the consequence of the reduced p-CREB protein levels (n=3, * p < 0.05). B) Phosphoprotein expression assay using reverse phase protein array. Comparison of staining intensities that were statistically different between HL60 after miR-34b exogenous expression and miR-neg are shown. Normalized intensity values represent averages of two independent experiments (n = 2, p < 0.05). C) HL60, NOMO1 and K562 cell cycle analyses are represented. The number of cells in S and G2-M phases were observed lowered by miR-34b exogenous expression. D) Representative results from a colony formation assay using the indicated cell lines showed a lower colony formation efficiency if miR-34b is expressed relative to miR-neg induction (n = 3, p < 0.05).

CREB silencing suppresses myeloid cell proliferation. We used small interfering RNAs to downregulate expression of endogeneous CREB in HL60 and K562. The addition of different CREB siRNAs, but not the siRNA-neg used as control, significantly inhibited CREB expression and myeloid cell line proliferation. CREB mRNA expression significantly decreased from 35% to 75% in HL60 ($n = 2$, $p < 0.05$). In the K562 cell line, mRNA decreased CREB was found lower respect to HL60; in fact, the decrease was from 10% to 60% (Figure 4A). The effect of CREB silencing strongly influenced protein expression, which was found reduced for all siRNAs used. The siRNA 3 for HL60 and siRNA 1 for K562 gave the strongest CREB translation inhibition (figure 4B, $n = 2$, $p < 0.05$). Finally, cell line proliferation and tumorigenicity assays revealed that CREB silencing induced a significantly reduced cell proliferation and clonogenicity (Figure 4C, $n = 2$, $p < 0.05$), as previously demonstrated by miR-34b restoration. These results suggested that CREB regulates growth and survival of myeloid leukemia cells.

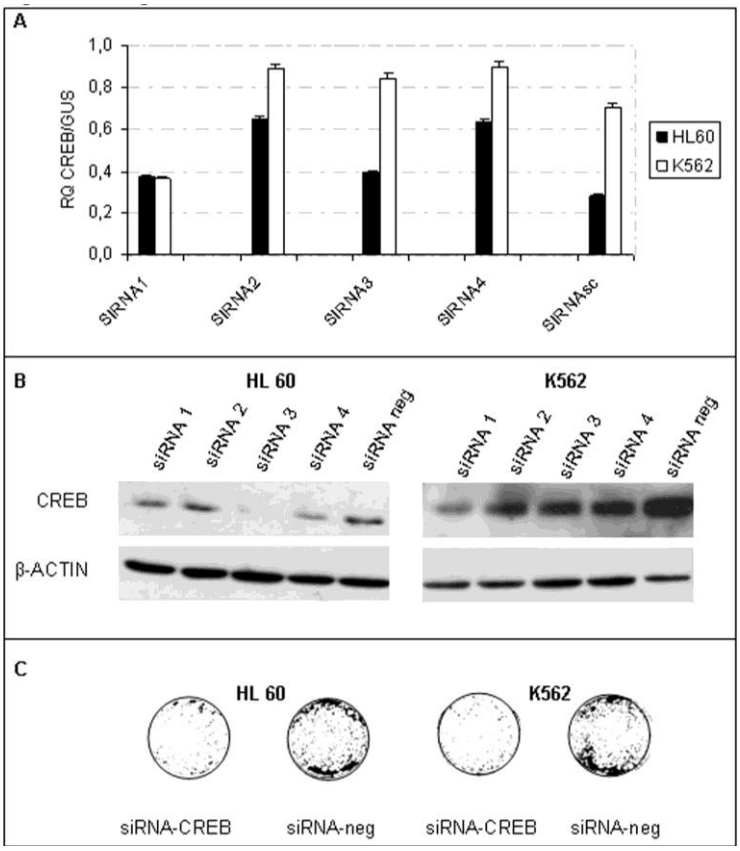

Figure 4. A) RQ-PCR for CREB expression after small interfering RNA (siRNA) transfection was performed. CREB mRNA was reduced after siRNA transfection introduction in HL60 and K562. B) Western blot analysis shows CREB reduced levels after being silenced. C) CREB siRNA inhibits growth of colonies in methylcellulose with respect to colonies formed after siRNA-neg transfection (n = 2, p < 0.05).

MiR-34b expression in AML patients. CREB pathological overexpression was previously reported for a large percentage of AML patients at diagnosis (19). The distribution of miR-34b expression in 78 AML patients at diagnosis (Table 2S) was heterogeneous but always significantly downregulated with respect to 17 HL BM, which revealed a higher miR-34b expression (Figure 5A, $p < 0.001$). The mean of miR-34b expression in patients was 1.07 ± 0.23 compared to 12.48 ± 4.26 of the HL BM. In order to explain miR-34b heterogeneity in patients,

we considered their CREB protein levels as previously discussed (19). Results demonstrated that patients with higher CREB protein levels (CREB+) had the lowest miR-34b expression (mean 0.41 ± 0.17), whereas patients with lower CREB protein levels (CREB-) had an heterogeneous and highly miR-34b expression (0.95 ± 0.43), confirming a strong relationship between CREB and miR-34b (Figure 5B, $p < 0.001$). Next, we considered miR-34b expression among different cytogenetic groups of AML patients at diagnosis; in particular, patients with rearranged MLL and without cytogenetic markers (NEG) were observed for the lowest detected level of miR-34b (Figure 5C, $p < 0,001$) as previously described (31).

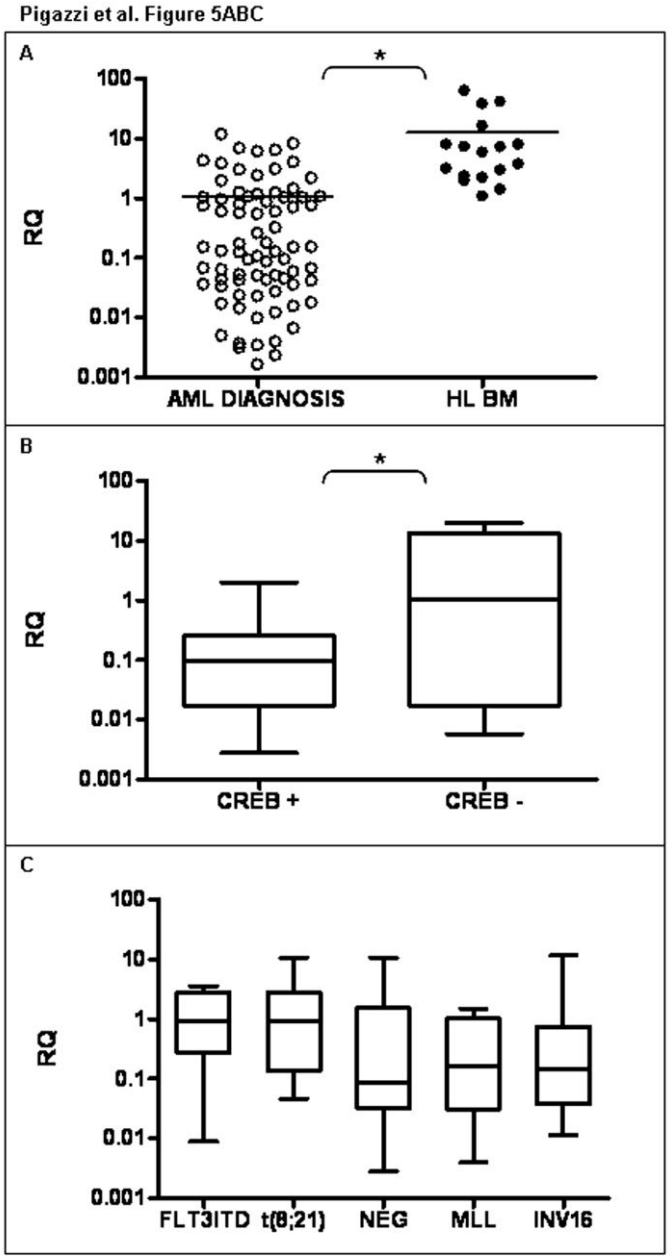

Figure 5. RQ-PCR analyses were performed in patients at diagnosis of leukemia (AML DIAG) and in bone marrow of healthy children (HL BM). A) The Relative Quantity (RQ) of miR-34b expression was significantly higher in HL BM with respect to AML DIAG ($p < 0.05$). B) Patients with high CREB protein level (CREB+) showed the lowest miR-34b expression with respect to patients with lower CREB protein levels (CREB-) ($p < 0,001$). C) MiR-34b expression is considered for different cytogenetic subgroups of patients. Its expression was significantly lowered in NEG and in the MLL subgroups. (n=3, * $p < 0.05$; black lines, define the average).

DISCUSSION

MiRNA's mRNA partners, their function, and their tissue specificity are being continuously investigated in normal and diseased samples to increase our understanding of tumorigenesis and improve cancer therapy. The extent of miRNA regulation is under scrutiny. MiRNAs have been demonstrated to control a variety of cellular pathways by influencing the expression of specific target genes (32,33) and are considered here in an effort to explore the mechanism that causes CREB protein overexpression. CREB has recently been defined as an oncogene (34), and it was found overexpressed in acute leukemia, as well as proving able to induce myeloproliferative syndrome in transgenic mice (19,20).

In silico and gene expression analyses conducted in this research, focus on miR-34b as a possible candidate to target CREB. MiR-34b is documented to be less expressed in myeloid leukemia cell lines compared to healthy bone marrow, in agreement with other experiments that described low levels of the expression of the miR-34 family of miRNA in other human cancers (21). The forced expression of miR-34s has previously been demonstrated to decrease cell growth and induce senescence in mouse embryonic fibroblasts (35), as well as to control cell proliferation and clonogenical potential in OSE (36), indicating that miR-34 expression plays an important role in influencing tumorigenesis in diverse cell types, probably through the control of different mRNA targets. However, their p53 dependence and target genes remain to be determined (37).

In this regard, our study establishes CREB as one of miR-34b's targets in myeloid leukemia. MiR-34b binds directly to the 3'UTR region of CREB mRNA with specificity to the seed region, as mutation in this region eliminates this phenomenon. Furthermore, the restoration of miR-34b expression changes the leukemia phenotype, confirming its possible role as tumor suppressor. Until now, there is no genomic evidence of miR-34b downregulation, apart from the

frequent deficiency of functional p53 that drives their transcription in several cancer cells. A recent investigation in colon rectal cancer found a methylated miR-34b/34c promoter at chromosome 11q23, which might exclude p53 transcriptional activity on this region (28). Our findings about increased miR-34b expression in leukemic cell lines after treatment with a demethylation agent, and the study of the CpG island at the miR-34b/34c promoter, suggest that this mechanism might be considered in myeloid leukemia for explaining miR-34b downregulation (38). Moreover, myeloid cell lines have been demonstrated to increase miR-34b expression and decrease CREB protein levels after demethylation treatment. These data lead us to suppose that myeloid leukemia cells might epigenetically maintain miR-34b downregulated in order to sustain CREB protein overexpression as a possible hypothesis for observed leukemia progression.

The molecular mechanism by which miR-34 family miRNAs suppress tumors is currently under consideration in many cancers (39,40). Until now, gene expression analyses have been performed, suggesting that the cause of tumor suppression might be the ability to target genes related to the cell cycle pathway (41). CREB is the first direct miR-34b target gene identified. Reverse-phase protein microarray analysis allows (29,30) the elucidation of the complex cellular signaling that would be influenced by exogenous miR-34b expression and the consequent CREB downregulation. As a transcription factor, CREB controls thousands of genes that are known to contribute to healthy cell life (17). The observed decreased expression of CREB target genes, such as Cyclin A1, Cyclin B, and Cyclin D1, might explain cell cycle abnormalities found in myeloid cell lines. Furthermore, the downregulation of the antiapoptotic BCL2, as well as AKT/mTOR proteins, reads-out for the anti-survival signaling pathway caused by the miR-34b restored expression. Also the documented downregulation of STAT3, NFkBp65 and JAK1, which are directly controlled by CREB, might be considered a critical event in cancer

suppression, principally for the disruption of their downstream pathways. The ERK1/2, PKA, AKT and SMADs and SMAC lowered protein expression might be explained as a cellular response a cascade to miR-34b suppressor activity on CREB. For the first time, protein arrays account for direct signaling pathways and targets to be further studied for leukemogenesis and targeted therapy (42,43).

To better understand whether miR-34b down-regulation of CREB might be considered a primary pathway involved in leukemia cell proliferation, we silenced CREB using small interfering RNAs targeting CREB gene and demonstrated that myeloid cell growth and tumorigenicity were strongly compromised. In this sense we proved that CREB deregulation is a critical phenomenon for leukemia progression and that miR-34b's role on the CREB protein is a direct and fundamental mechanism of tumor regulation.

The relevance of our *in vitro* experiments is substantiated by data obtained in AML patients. The fact that CREB protein was previously documented as overexpressed in a large number of AML patients at diagnosis (18,19) suggests that the inverse correlation between CREB and miR-34b expression. The expression levels of miR-34b is shown here to be lowered in patients when compared to healthy bone marrow samples. Moreover, miR-34b is found to be always at the lowest degree of expression when CREB protein levels are high. We also documented a heterogeneous expression of miR-34b in distinct cell types and during myeloid differentiation (31), suggesting that miR-34b may influence hematopoiesis and play a role in different leukemia phenotypes. Its role as a potential marker might be further evaluated (44,45). In addition, the observation of reduced miR-34b levels in samples that have higher CREB protein levels opens future investigation of miR-34b and CREB as possible diagnostic indicators (46,47), especially when a broad spectrum of miRNAs involved in AML is available.

Based upon the current study, miR-34b targets CREB, mediating biological activity in normal and leukemic tissue. The process of methylation that deregulates miR-34b expression in hematopoietic development leads to pathological outcomes, mainly through CREB protein upregulation, which leads to a combinatorial overexpression of a large number of targets provoking cell proliferation and survival. The silencing of miR-34b/34c genes by methylation in leukemia, supports the possibility that a large series of other target oncogenes that would contribute to disease are upregulated (48-50); these remain to be discovered. Therefore, the ectopic miR-34b expression or the use of demethylating agents in myeloid leukemic cells could reactivate the control on CREB expression, contributing to the reduction of malignancy. MiR-34b restoration turns out to be a fundamental step in treating myeloid cells, prompting consideration in AML pathogenesis and for new therapeutic strategies.

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CHAPTER 5

HYPERMETHYLATION OF MIR-34B IS ASSOCIATED WITH CREB OVEREXPRESSION AND MYELOID CELL TRANSFORMATION

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In preparation.

ABSTRACT

The cAMP-response-element-binding protein (CREB) is a nuclear transcription factor downstream of various stimuli, and its overexpression has been demonstrated to promote leukemia progression. MiR-34b regulates CREB expression, and is downregulated in myeloid leukemia cell lines due to the hypermethylation of its promoter. Here we find that primary AML cells transiently overexpressing miR-34b showed decreased clonogenicity and increased apoptosis. *In vivo*, miR-34b overexpression in HL-60 and K562 cell lines downregulated CREB levels, suppressing leukemia progression in NSG mice. Hypermethylation of miR-34b promoter was demonstrated in 65.5 % (74/113) of AML patients, and correlated with elevated CREB protein levels. Bone marrow cells from 49 patients with aberrant myeloid proliferation were found unmethylated at the miR-34b promoter, and CREB expression was not detectable. Three patients with myelodysplasia (MDS) that evolved to AML had miR-34b promoter hypermethylation exclusively at the onset of AML. CREB target genes differently clustered in these two diseases. We explored the role of miR-34b/CREB in the evolution of MDS to AML by lowering miR-34b expression in primary healthy samples. Increased CREB levels and upregulation of its target genes expression resulted in increased myelopoiesis and colony formation. Taken together, these results suggest that miR-34b hypermethylation is critical for AML pathogenesis.

INTRODUCTION

CREB is a nuclear transcription factor downstream of numerous stimuli that are critical for normal and neoplastic hematopoiesis (1-4). In leukemia, it is overexpressed, promoting tumor proliferation, cell cycle progression, and higher clonogenic potential *in vitro* and *in vivo* through upregulation of specific target genes (5,6). CREB deregulation in acute myeloid leukemia (AML) has been previously shown to depend on genomic amplification (7), and on miRNA control. In particular, the expression of miR-34b, a CREB regulator, was decreased at diagnosis in AML patients, conferring higher CREB protein levels. MiR-34b promoter methylation has been described as one of the reasons accounting for the control of CREB expression in AML (8). The methylation of CpG islands has been defined as the most common epigenetic modification leading to transformation to cancer, mainly through silencing of tumor suppressor genes (9,10). Epigenetic modification of DNA is being increasingly recognized as important in the regulation of normal and tumor cells (11,12). Promoters of various genes involved in DNA repair, cell cycle control, apoptosis and detoxification like the cyclin dependent kinase inhibitors p15 and p16, HIC1 (13), MEG3, SNRPN (14) and others have been shown to be hypermethylated in high risk myelodysplastic syndromes (MDS) and in acute myeloid leukemia (AML). CREB overexpressing transgenic mice developed myeloproliferative syndrome with splenomegaly and aberrant myeloid proliferation in the spleen, highlighting the role of CREB in myeloid cell transformation. Furthermore, CREB knockdown in AML cells resulted in decreased proliferation but no effects on normal myelopoiesis in mouse transduction/transplantation assays (6). Therefore, CREB is required for AML cell proliferation but not proliferation or differentiation of normal myeloid progenitor cells.

The myelodysplastic and myeloproliferative diseases constitute a group of molecularly distinct entities with variable degrees of ineffective hematopoiesis and susceptibility to leukemic

transformation. Recent studies provide some insights into the pathophysiology of MDS and the role of aberrant epigenetic programming in the progression to AML (15-17). In this paper, we report an association of miR-34b methylation and CREB expression that may be involved in transformation from MDS to AML. From our data using *in vivo* models and bone marrow samples from MDS patients developing AML, we describe the role of hypermethylation of miR-34b in promoting leukemogenesis. The correlation of CREB and miR-34b expression was also evaluated in MDS/AML patients revealing an association with non-random genetic aberrations typical of AML.

METHODS

Patients. One hundred thirteen bone marrow (BM) samples from patients with diagnoses of leukemia were analyzed. Diagnosis was made according to standard morphologic criteria on the basis of immunohistochemical, immunophenotyping, and cytogenetic studies following the AIEOP-2002 AML pediatric protocol (18). Informed consent in compliance with the Helsinki protocol was obtained. Bone marrow samples from 49 patients with diagnoses of myelodysplastic syndrome or myeloproliferative disorder (MDS/MPD) were also included in the study. Specifically, 32 had MDS, and 17 Juvenile Myelo-Monocytic leukemia (JMML).The patients analyzed in this study were diagnosed as affected by JMML according to differential diagnostic criteria, as previously published (19). Five patients evolved to AML, and paired material of MDS and of AML was also considered in the analyses (Table 1). Hematopoietic progenitors and myeloid cells including granulocytes, macrophages, and erythroid cells ($CD19^-CD3^-$) of 17 healthy bone marrows were sorted on a BD FACSAria cell sorter (BD Biosciences, Rockville, MD).

Table 1. Clinical features of MDS patients.

	Diagnosis	Days from first diagnosis	% Blasts	karyotypes
Pt-1*	RAEB	0	8%	46,XY
	RAEB	35	19%	
	AML	56	80%	
Pt-2*	RAEB	0	10%	45,XX,-7
	AML	166	75%	
Pt-3	MDS	0	12%	NA
	AML	49	80%	
Pt-4*	RAEB	0	13%	46,XY
	AML	60	78%	
Pt-5*	RC	0	2%	47,XY,der(7)t(1;7)(q21;q22),+8
	AML	19050	90%	

Patients 1,2,3 were used for MS-PCR and miR-34b expression analysis; * patients were used for GEP analysis.

Primary cell culture and transfection. Primary cell cultures were obtained from bone marrow of patients at the onset of AML and from healthy donors. The cells were cultured in RPMI (Invitrogen-Life Technologies) supplemented with 10% fetal bovine serum (FBS; Invitrogen-Life Technologies) and cytokines such as IL-3 (20 ng/mL), IL-6 (20 ng/mL), SCF (50 ng/mL), TPO (50 ng/mL) and FLT-3 Ligand (50 ng/mL). Cell transfection was performed using a Nucleofector (Amaxa Biosystems) according to the manufacturer's guidelines. Exogenous oligonucleotides that mimic the action of miR-34b or a miR-Neg as a control (Dharmacon Industries) were introduced in AML primary cell cultures (300 nM). The healthy primary cell cultures were co-transfected with a miR-34-inhibitor (300 pmol) and 3.5ug pEGFP-N1-ΔGFP-CREB plasmid, or co-transfected with an empty vector (pEGFP-N1-ΔGFP -EV) and a miR-Neg used as controls. Human fetal liver was also used. It was obtained without identification information under federal and state regulations from the University of California, Los Angeles (UCLA) CFAR Gene and Cellular Therapy Core Laboratory and UCLA OB-GYN. CD34+ cells were isolated as previously described (20). Cells were prestimulated in X-Vivo 15 media (Lonza)

supplemented with 10% FBS and cytokines as described above. Cells were seeded into RetroNectin (Takara)-coated plates with 2 % bovine serum albumin 24 h before the lentivirus transduction.

Cell culture. Human AML cell line HL60 (American Type Culture Collection) and human chronic myelogenous leukemia–derived B-cell–like cell line K562 (American Type Culture Collection) were cultured respectively in DMEM and RPMI 1640 (Invitrogen-Life Technologies) both supplemented with 10% fetal bovine serum (FBS; Invitrogen-Life Technologies).

Constructs. pEGFP-N1-ΔGFP-CREB plasmid was obtained cloning within NotI and EcoRI cloning site a full length cDNA for CREB into the pEGFP-N1 vector (Invitrogen). Lentiviral constructs FUGW and FUGW-34b (human sequence) were developed as previously described (21). Fucrw-LUC was obtained digesting the Fucrw vector, downstream the ubiquitin promoter, and the Luciferase amplicon with XbaI and EcoRI. The luciferase sequence was amplified from pMIR-REPORT (5'- ATCTTCTAGACAGGCTCAAGCATGGAAGAC-3', 5'-ATATGAATTCAGCTTACA

CGGCGATCTTTC-3'). Fugw-miR-target (or Fugw-SCR) was constructed as previously described by Brown BD et al. (22). Briefly oligonucleotide sense 1, sense 2, antisense 1 and 2 were annealed and ligated into the BrsGI and EcoRI restriction site in the 3'UTR of the GFP expression cassette in Fugw (sequence are available upon request).

Lentivirus production and transduction. To generate VSV-G-pseudotyped lentiviruses, 2×10^6 293T cells were transfected with pGag-Pol, pVSV-G, and either Fugw-EV, Fugw-miR-34b, Fugw-miR-target, Fugw-miR-Neg or Fucwr-LUC. Transfection was performed with TransIT 293 according to manufacturer's instructions. After 48 h, viral supernatant was harvested and used to spin-infect 7.5×10^5 HL60 or K562 cells or 10^6 fetal liver cells for 1h at 2500rpm.

Polybrene was added to a final concentration of 10 μg/ml prior to spin-infection. Stably transduced cells were then sorted with BD FACSaria II. Flow cytometry sorting was performed in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility.

RNA extraction and real-time PCR for miRNA analysis. RNA from bone marrow patients was isolated using a mirVana miRNA Isolation kit (Ambion) according to the manufacturer's instructions. RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies) and then used for PCR. Taqman miRNA assays (Applied Biosystems) were carried out using the stem-loop method to detect the expression level of mature miRNAs. For the retro-transcription reaction, 10 ng of total RNA were used in each reaction and mixed with the specific stem-loop primers (Applied Biosystems). All PCRs were run in triplicate and gene expression, relative to U6 small nuclear RNA (RNU6B), was calculated by the comparative DCt method (23).

RNA isolation and SYBR Green quantitative real-time reverse transcription-PCR assays. Total RNA was isolated using Trizol (Invitrogen). One μg of RNA was reverse-transcribed into cDNA in a final volume of 25 μl using the SuperScript II (Invitrogen) according to the manufacturer's instructions. RQ-PCR was performed with 1 μL cDNA in 20 μL using the SYBR Green method (Invitrogen) and analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Experiments were carried out in triplicate and gene expression, relative to GUS, was calculated by the comparative DCt method.

Gene expression analysis. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The GeneChip Human Genome U133 Plus 2.0 was used for the microarray experiments (24,25); *in*

vitro transcription, hybridization and biotin labeling processes were performed according to the Affymetrix GeneChip^{R} One Cycle Target Labelling protocol (Affymetrix, Santa Clara, CA, USA). Microarray data (CEL files) were generated using default Affymetrix microarray analysis parameters (GCOS 1.2 software). CEL files can be found at the GEO repository (http://www.ncbi.nlm.nih.gov/geo/). Series Accession Number GSE25300.

CEL files were normalized using robust multiarray averaging expression measure implement in Partek Genomic Suite Software (http://www.partek.com). Gene expression data were analyzed using Partek Genomic Suite Software. Paired t test was applied to identify differently expressed genes between sample pairs. Multiplicity corrections were used to control false discovery rate using FDR q-value; probes with adjusted p-values less than 0.05 were declared significant. Dot plot analysis was generated using GraphPad Software, Prism 3.03 (Graph Pad Software Inc., La Jolla, CA); analysis were generated using paired t-test implement in Prism 3.03 software; p-value less than 0.05 were declared significant.

Methylation analysis. Genomic DNA was extracted from 113 AML patients, 49 MDS/MPD and 3 bone marrows from healthy donors and 2 cord blood samples according to the manufacturer's instructions (Gentra Autopure LS, Qiagen). One µg of DNA was treated with sodium bisulfite using EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH) following the manufacturer's instructions. The DNA eluted was analyzed in a methylation-specific (MS) and unmethylation-specific (UMS) PCR reaction.

Sequencing. To assess the specificity of the methylation assay, the PCR amplicon was sequenced: first it was purified according to Microcon protocol (Millipore) and then amplified with the Big Dye Terminator reaction (Applied Biosystems) according to manufacturer instructions. DNA was then purified (Centrisep, Applied Biosystems). Ten microliters of sample

were diluted with 12 µl of formamide, denatured for 10 minutes at 99°C and loaded in the ABI PRISM^{TM} 310 Genetic Analyser sequencer (Applied Biosystems).

Western blot analysis. Western blot analyses were carried out as previously described (26). Briefly, 20 µg of the total protein fraction (Buffer-Biosource International) isolated from transfected cells were used. Protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce). Antibodies used were anti-phospho-CREB (Upstate Biotechnology), anti-PU.1 (Santa Cruz Biotechnology), anti-MEIS1/2 (Santa Cruz Biotechnology) and anti-Actin (Sigma-Aldrich), and the horseradish peroxidase–conjugated secondary antibody was goat anti-rabbit or mouse IgG (Upstate Biotechnology). Proteins were detected using enhanced chemiluminescence and films (GE Healthcare).

Cell cycle analysis. Cell lines were transfected with oligonucleotide miR-34b or miR-Neg. After 24h of incubation, 5 x 10^5 cells were washed twice with PBS, lysed, and treated with 50 µg/ml Propidium Iodide (PI) in 1 ml PBS overnight at 4 °C. Cells were analyzed using Cytomics FC500 (Beckman Coulter, FL, USA). Cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems, CA). **Colony assays.** To determine anchorage-independent growth of transfected cells, a total of 2 X 10^3 cells were seeded in duplicate in a methylcellulose semisolid medium supplemented with nutrients and cytokines (StemCell Technologies) after miRNA and siRNA transfection. Colony evaluation and enumeration was done *in situ* by light microscopy after 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) incorporation. 2 X 10^3 cells of human fetal liver transduced with lentivirus and sorted, were seeded in duplicate. Cells were then collected from methylcellulose, washed in PBS twice and marked with fluorochrome-linked antibodies to CD33, CD36, CD61, CD15 and Glycophorin-A for fluorescence-activated cell sorting (FACS) analysis of the myeloid compartment as previously described in Shankar DB et al (7).

Xenograft experiments in NOD-SCID IL-2receptor gamma null (NSG) mice. Stable cell lines were washed twice with PBS. Cells were counted and viability was tested using the trypan blue exclusion method. Only cells that were growing with a viability of > 90% were used. NSG mice were 6 to 8 weeks of age at the time of injection. Each mouse was injected with 5×10^6 HL60-miR-34b/LUC (or EV/LUC as a control) or K562-miR-34b/LUC (or EV/LUC as a control) suspended in equal volume of PBS (without FBS or antibiotics) and Matrigel (BD) in 0.1 mL. The mixture was injected using a 28 1/2-guage needle subcutaneously, dorsally off the midline. All mice were euthanized when tumors reached a volume of 1.5 cm^3. The mice were treated according to the NIH Guidelines for Animal Care and as approved by the University of California at Los Angeles Institutional Animal Care and Use Committee.

Bioluminescence Imaging. Each mouse was injected with 5×10^6 HL60-34b/LUC (or EV/LUC as a control) cells suspended in 0.1 mL PBS (without FBS or antibiotics) through the tail vein using a 28 1/2-gauge needle. All experimental manipulations with the mice were done under sterile conditions in a laminar flow hood. After the injection of cells, the mice were imaged at various time points to ensure the presence of disease using an *in vivo* IVIS 100 bioluminescence/optical imaging system (Xenogen). D-Luciferin (30 mg/mL; Xenogen) dissolved in PBS was injected intraperitoneally at a dose of 100 μL/mouse 15 min before measuring light emission (measured in Relative Intensity units (RIU) = photons/sec/cm^2). General anesthesia was induced with 2.5 % isoflurane and continued during the procedure with 2 % isoflurane. Relative intensity units for regions of interest were measured in triplicate and averaged.

Data analysis. Statistical analyses were performed with Mann-Whitney or unpaired two-tailed t test. A p value < 0.05 was considered significant.

RESULTS

MiR-34b suppresses CREB expression and tumor growth in primary AML cultures and *in vivo*. Bone marrow from AML patients at diagnosis were transfected with miR-34b oligonucleotide. miR-34b expression levels were monitored by RQ-PCR and was increased from 10^2 to 10^3 fold compared to miR-Neg used as calibrator. CREB protein levels decreased especially after 48 h of transfection (Figure 1A, n = 3). Cell cycle revealed a higher cell number in sub-G1 phase (25.5 % vs 9.9 %, $p < 0.001$) confirming increased apoptosis after miR-34b exogenous expression compared to miR-Neg. miR-34b restoration as well as CREB silencing decreased the survival and clonogenicity of cells in semisolid medium (Figure 1A, n = 3, $p < 0.05$). To determine whether miR-34b works as a tumor suppressor by inhibiting CREB protein, NSG mice were inoculated subcutaneously with K562 and HL60 stably expressing miR-34b (K562+34b and HL60+34b). Both cell lines showed a decreased CREB protein expression (Figure 1B, n = 3, $p < 0.001$). The tumor induced by K562+34b was smaller (0.68 g) compared to the control cell line (1.18 g) as well as the tumor formed by HL60+34b (0.79 g) compared to HL60+EV (1.39 g) (Mice n = 10 each cohort, $p < 0.05$). We injected 5×10^6 HL60-34b or EV cells into the tail veins of 5 mice per group to analyze the potential effect of miR-34b overexpression on a metastatic model of leukemia. Engraftment and disease progression were monitored by acquiring *in vivo* bioluminescence imaging at least once per week. We quantified bioluminescence intensities between comparable regions of interest. Bioluminescence showed less aggressive disease in mice injected with HL60 overexpressing miR-34b respect to the control cell line. For HL60+EV transduced cells, geometric mean bioluminescent intensity in control mice was 9.62×10^6 RIU, while it was 4.29×10^6 RIU in miR-34b expressing mice at day 21 ($p < 0.01$, Figure 1C).

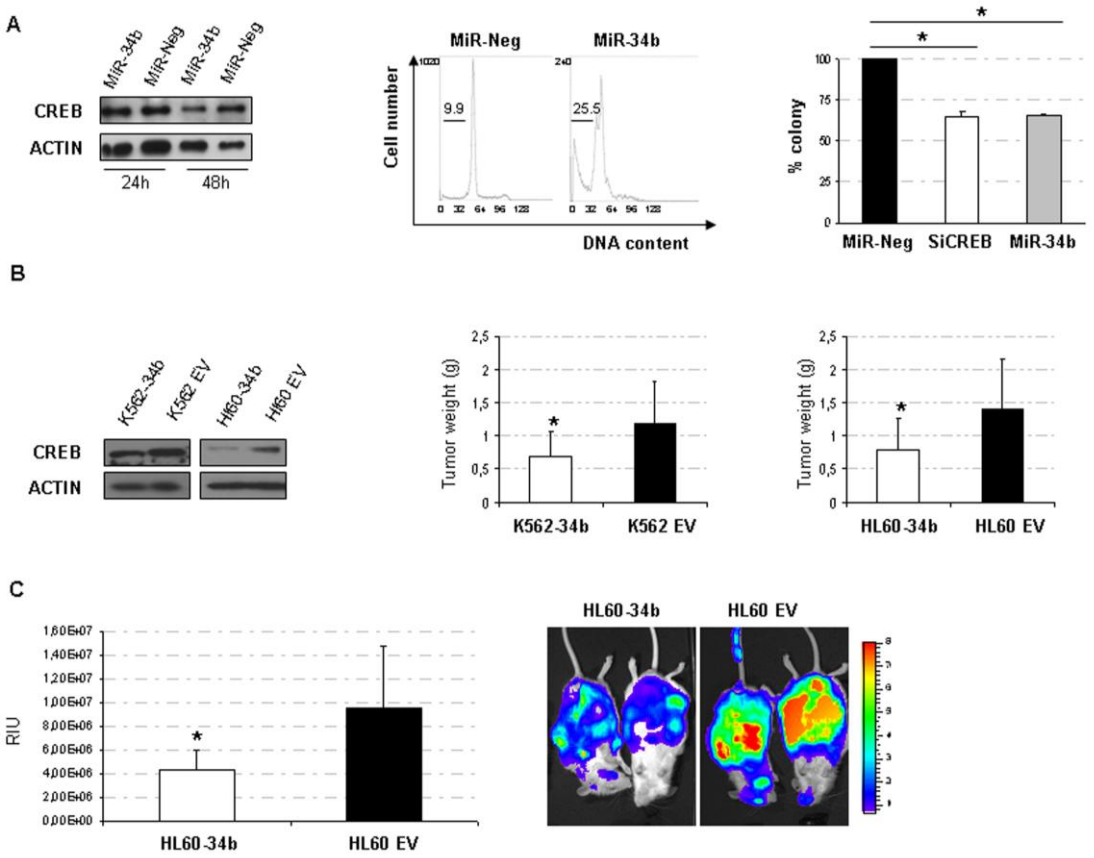

Figure 1. MiR-34b suppresses CREB expression and AML progression in primary cultures and *in vivo*. A. AML primary culture transfected with miR-34b oligonucleotide. (Left) Western blot analysis shows a decrease in CREB protein expression 24 hours and 48 hours after transfection compared to control. (Middle) Representative cell cycle analysis profile. In AML primary cells overexpressing miR-34b, there is a 10% increase of apoptotic cell fraction in SubG1 compared to control. (Right) Representative colony formation assay. A decreased formation of colony is visible in AML primary culture miR-34b and siCREB transfected. *<0.05. B. Flank injection of K562 and HL60 leukemic cell lines overexpressing miR-34b in NSG mice. (Left) Western Blot analysis of leukemic cell lines transduced with Fugw-miR-34b or EV shows a decrease in CREB protein expression compared to control (EV). (Right) Tumor weight of the xenograft model. Ten mice per group were injected subcutaneously with K562-34b and EV or with HL60-34b and EV. MiR-34b overexpressing cell line induce a decreased tumor formation in mice compared to EV. Error bars represent standard deviation. *p<0.05. C. *In vivo* imaging with IVIS 100 bioluminescence/optical imaging system (Xenogen) of mice tail vein injected with 5 x 106 HL60-34b or EV (5 mice each group). On the left, a histogram shows a decrease of the Relative Intensity Units (RIU) of mice injected with leukemic cell line overexpressing miR-34b compared to those injected with the control cell line (p=0.01); on the right a representative image of the two groups of mice showing a less pronounced metastasis in those mice injected with miR-34b overexpressing cell line.

MiR-34b expression is controlled by methylation in AML patients. CREB overexpression was previously reported for a large percentage (66 %) of AML patients at diagnosis (5). The distribution of miR-34b expression in bone marrow from 113 AML patients at diagnosis was heterogeneous, but significantly downregulated (RQ = 0.176) with respect to $CD19^{-}CD3^{-}$ sorted population (RQ = 1, Figure 2A). MiR-34b promoter methylation was studied in bone marrow samples, since it was recently found that miR-34b was hypermethylated in leukemic cell lines (8). MS-PCR revealed 65.5 % (74/113) of bone marrow cells from AML patients methylated at miR-34b promoter. By contrast, no methylation was detected in healthy samples (HL-BM), indicating that methylation of this miR-34b/c region is a tumor-specific phenomenon (Figure 2B). In order to understand if there is a correlation between the methylation status of miR-34b promoter and its expression levels, we subdivided patients by the methylation or unmethylation of miR-34b promoter. Our results demonstrated that methylated patients had lower miR-34b expression (RQ = 0.075), while the unmethylated patients had a significantly higher expression of miR-34b (RQ = 0.373). CREB protein expression correlated to the methylation status of miR-34b promoter ($p < 0.05$, Figure 2C, Meth vs Un-Meth). To prove that the mechanism regulating miR-34b in AML involves promoter methylation, we treated primary cultures with a demethylating agent (AZA). Treatment of cells with AZA resulted in increased miR-34b expression and decreased CREB protein (data not shown), confirming our hypothesis. We aimed to find if miR-34b promoter methylation might confer different outcome to AML patients. There was a trend towards lower overall survival of AML patients with methylated miR-34b promoter (69 % at 4y) compared to the unmethylated patients (61 % at 4y), but these results did not reach statistical significance ($p = 0.34$).

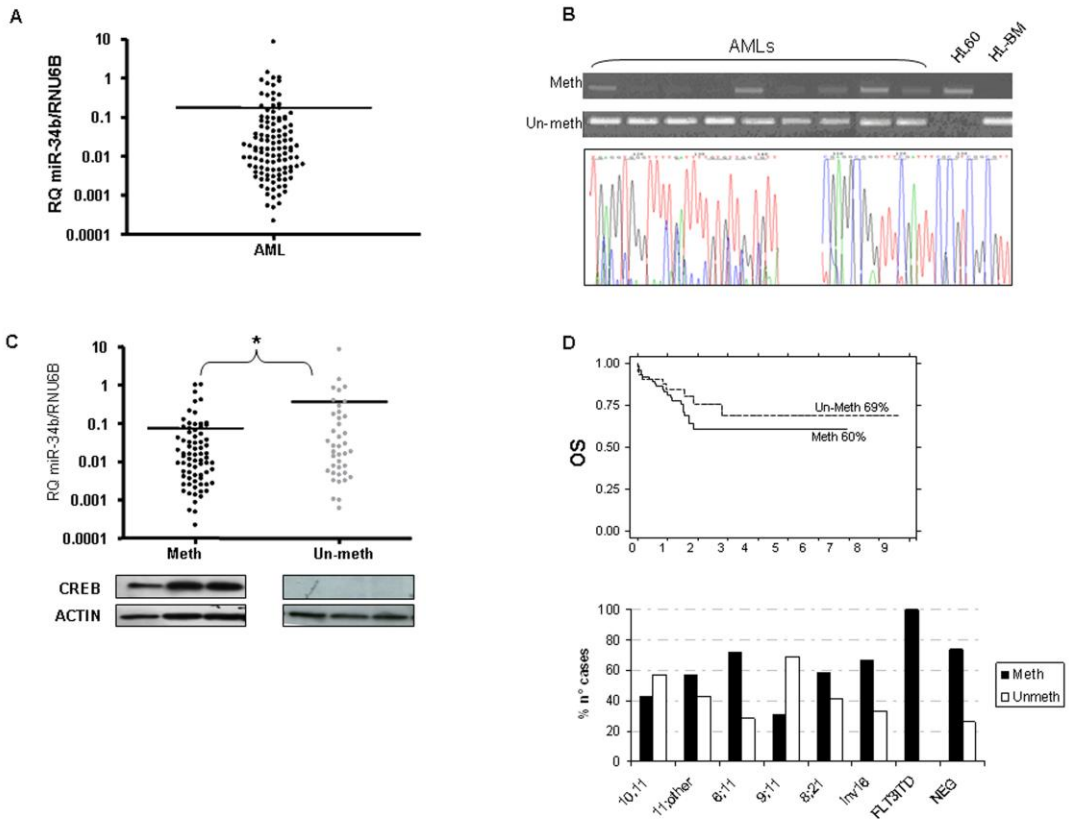

Figure 2. MiR-34b expression was decreased in AML patients due to promoter methylation

A. Levels of miR-34b expression by RQ-PCR of 113 AML patients (RQ = 0.176) normalized to a myeloid CD19-3- populations sorted from healthy bone marrow (RQ = 1). B. Methylation Specific PCR (MS PCR) analysis of the miR-34b/c promoter in a representative set of AML patients samples is shown. HL60 cell line and healthy bone marrow samples (HL-BM) were used as positive and negative controls. Direct sequencing of the miR-34b promoter after bisulfite treatment confirmed methylation of G/C islands. C. (up) RQ-PCR analysis of methylated patients (Meth, RQ = 0.075) and unmethylated patients (Un-Meth, RQ = 0.373). * <0.05. (down) Western blot analysis of AML methylated and unmethylated patients. CREB expression is more pronounced in patients with miR-34b methylated promoter. D. (up) Kaplan-Meier overall survival (OS) curves of AML patients with methylated miR-34b promoter (thick line) and unmethylated miR-34b promoter (broken line) determined by MS-PCR. The unmethylated AML have a better overall survival 69 % compared to methylated (60 %). (bottom) Distribution of methylated and unmethylated patients in AML patients grouped by cytogenetic aberrations (% cases n° = pt meth or un-meth/ tot pt).

Multivariate analysis with cytogenetic abnormalities, WBC, age and FAB-morphology of AML patients did not reveal significant correlation with miR-34b promoter methylation status; whereas methylation occurred mainly in t(6;11)MLL-AF6 and FLT3ITD positive patients and in patients without known cytogenetic aberrations (NEG), three categories known to have a bad prognosis in pediatric AML (Figure 2D). Still, the methylation of miR-34b might have some utility as an independent negative prognostic feature in AML.

MiR-34b promoter is unmethylated in MDS/MPD. Given the adverse effects of loss of miR-34b expression in AML and its function in regulating CREB, we tested miR-34b expression in MDS/MPD samples, which are known to sometimes progress to AML. Forty nine MDS/MPD patients presented higher levels of miR-34b (RQ = 5.5) compared to AML at diagnosis (RQ = 1) (Figure 3A). MiR-34b promoter region was then studied for hypermethylation. MS-PCR revealed miR-34b promoter completely unmethylated in MDS/MPD patient samples (49/49, 100%). Since miR-34b regulates CREB, we also examined CREB expression in MDS/MPD samples. CREB protein expression was not detectable in MDS/MPD patients with miR-34b promoter hypomethylation (Figure 3B). We examined DNA of 3 MDS patients who evolved to AML and whose BM samples were available for miR-34b methylation analysis. MS-PCR revealed that miR-34b promoter was methylated exclusively at the time of transformation to AML, and miR-34b expression levels decreased during evolution (RQ_{mean}-MD = 0.41 vs RQ_{mean}-AML = 0.26; fold reduction for each samples at the AML onset was 15.9, 1.16 and 2.9, Figure 3C). We used RNA to study changes in gene expression profiles (GEP) from MDS to AML stage. Of the 4 paired samples from each patient (one at the MDS stage and the other at the AML stage), supervised analysis of gene expression profiles identified 11 differentially expressed CREB-

target genes: *PRKACB, FDX1, NRXN2, PROSC, ADAM10, RAB7L1, NPR3, ITM2C, LATS2, CDK6 and HOXA7* ($p < 0.001$).

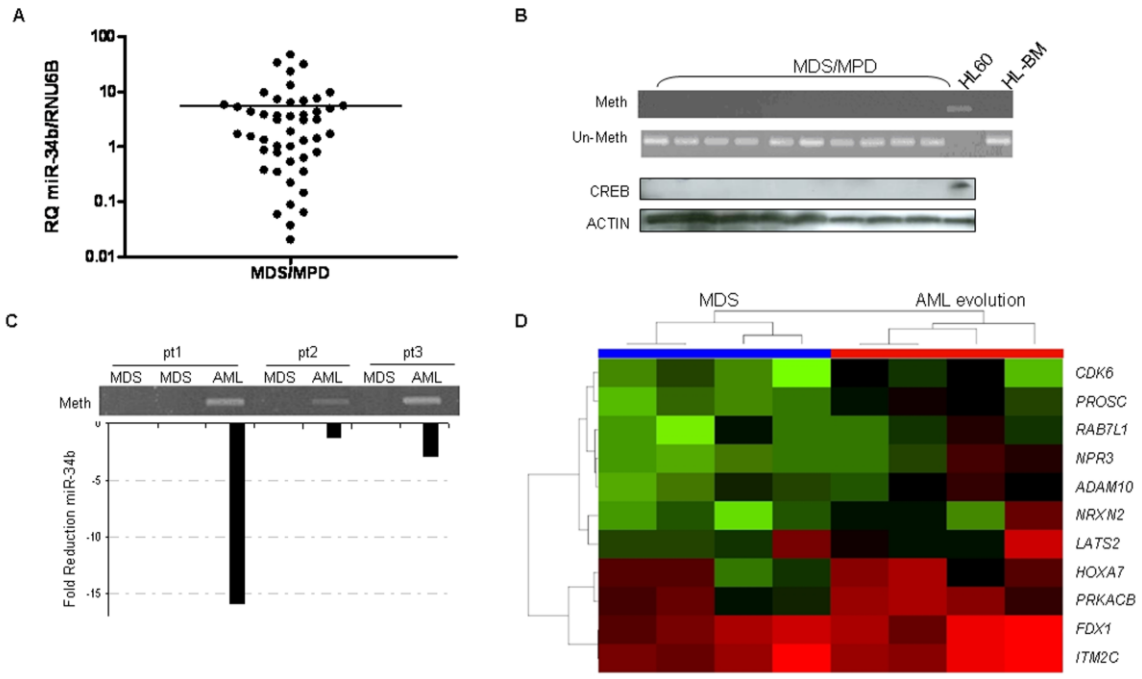

Figure 3. Methylation of miR-34b promoter is associated with MDS transformation to AML

A. Relative levels of miR-34b expression by RQ-PCR in 49 MDS patients (RQ = 5.5) with respect to miR-34b expression in AML (RQ = 1). B. MS PCR analysis of the miR-34b/c CpG island in a representative set of MDS patient samples. HL60 cell line and healthy bone marrow samples (HL-BM) were used as positive and negative controls, respectively. One hundred percent (49/49) of MDS/MPD patient bone marrow samples were unmethylated. Western blot analysis in the same set of MDS patients and in HL60 cells used as positive controls shows a nondetectable level of CREB expression in MDS samples. C. MS PCR analysis of the miR-34b promoter in 3 MDS patients evolved to AML. Methylation of promoter is present just at the onset of AML. Fold reduction of miR-34b expression for each patients samples was 15.9, 1.16, 2.9. D. Hierarchical clustering analysis of 4 patient pairs at diagnosis of MDS (blue) and evolution in AML (red) using the 11 differentially expressed CREB-target genes.

Unsupervised hierarchical clustering analysis using these 11 genes divided the 4 pairs into two separated groups, one clustered with the patients at diagnosis of MDS and the other with

patients who developed AML revealing CREB overexpression may correlate with disease progression from MDS to AML (Figure 3D).

MiR-34b inhibition in healthy cultures conferred a tumor phenotype. Since miR-34b downregulation and CREB overexpression are associated with tumor progression, we inhibited miR-34b and upregulated CREB in healthy primary cultures. By RQ-PCR we confirmed that miR-34b decreased (RQ = 0.54) and that enforced CREB protein expression was induced in healthy bone marrow after transfection. We performed real time PCR for several CREB and miR-34b targets: *BCL2*, *CYCLIN A1*, *B1*, *D1*, *p21*, *NFKB*, *STAT3*, *IL6* previously demonstrated to be CREB targets in AML (26). Most of them were found to be increased in healthy cultures with CREB overexpression and miR-34b downregulation. *MET* has been described as a miR-34b target, but the transient transfection was not sufficient to control its translation. Cell cycle analysis showed a slight increase in number of cells in S phase after CREB expression (57.87 % vs 44.32 %, Figure 4A). To explore if CREB overexpression affects cell growth, we examined clonogenic growth in methylcellulose, and revealed an increased number of colonies (5-fold) as well as increased expression of self-renewal genes (*BMI-1, GATA-2*, *HOXB4 and GFI-1*). Colonies showed a typical erythroid morphology ($p < 0.05$) and FACS analysis showed an increased number of granulocytes and monocytes expressing the CD15, as well as cells with erythroid expression markers (CD36, CD61 and Glycophorin), indicating an expansion of both erythroid and myeloid cells. PU.1 and GATA-1 were found upregulated in cells expressing low levels of miR-34b and overexpressing CREB. We quantified the expression of *GATA-1* dependent gene, *EPO-R*,, confirming its upregulation in miR-34b inhibited cells (RQ = 1.33 after 24 h of transfection, data not shown). *MEIS-1*, the main oncogenic target of CREB, was found also increased by CREB restoration (Figure 4B).

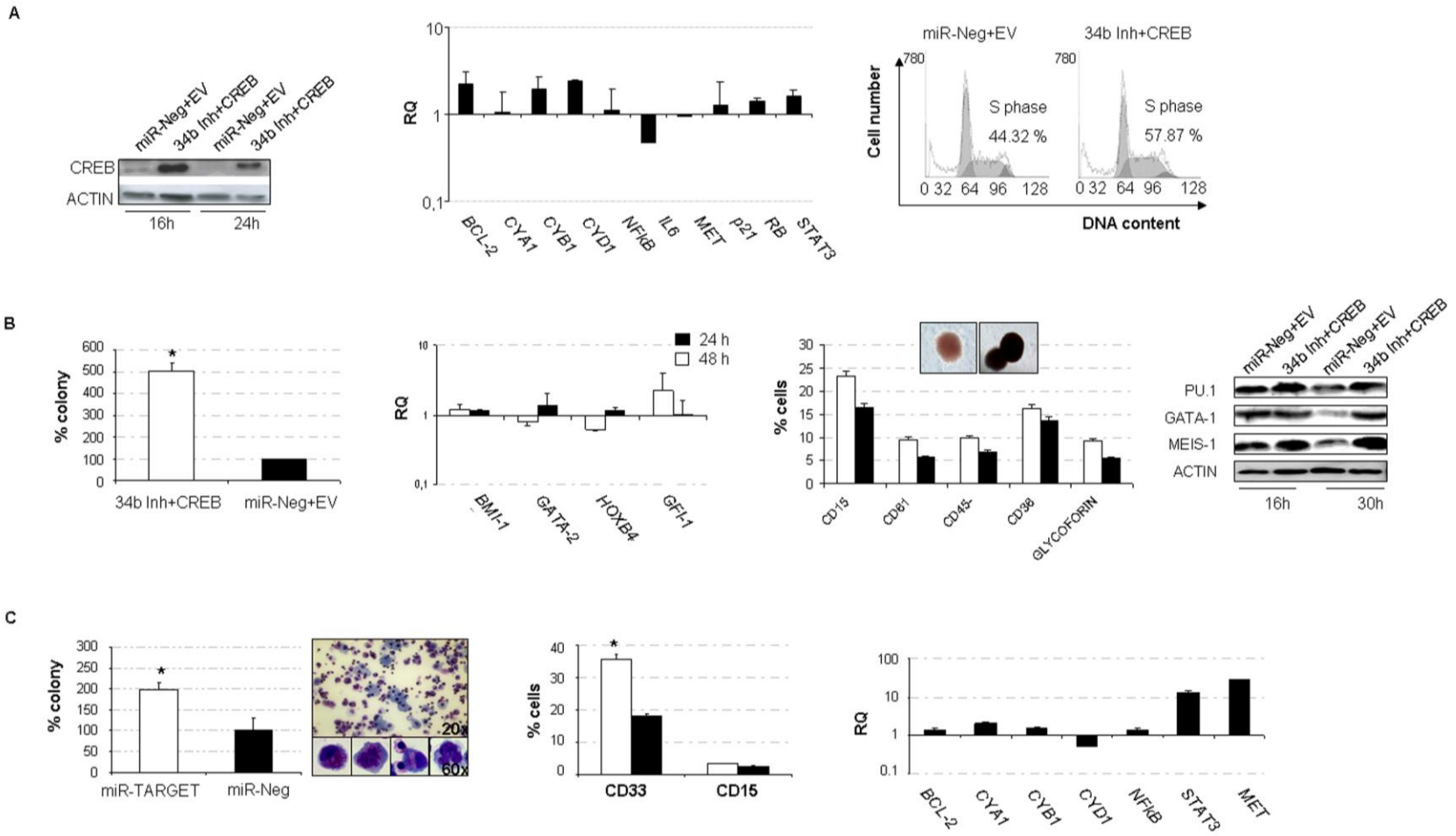
A
miR-Neg+EV
34b Inh+CREB
miR-Neg+EV
34b Inh+CREB
CREB
ACTIN
16h
24h
RQ
10
1
0,1
BCL-2
CYA1
CYB1
CYD1
NFkB
IL6
MET
p21
RB
STAT3
miR-Neg+EV
34b Inh+CREB
Cell number
780
780
S phase
44.32 %
S phase
57.87 %
0 32 64 96 128
0 32 64 96 128
DNA content
B
% colony
600
500
400
300
200
100
0
*
34b Inh+CREB
miR-Neg+EV
RQ
10
1
0,1
24 h
48 h
BMI-1
GATA-2
HOXB4
GFI-1
% cells
30
25
20
15
10
5
0
CD15
CD61
CD45-
CD38
GLYCOFORIN
miR-Neg+EV
34b Inh+CREB
miR-Neg+EV
34b Inh+CREB
PU.1
GATA-1
MEIS-1
ACTIN
16h
30h
C
% colony
300
250
200
150
100
50
0
*
miR-TARGET
miR-Neg
20x
60x
% cells
40
30
20
10
0
*
CD33
CD15
RQ
100
10
1
0.1
BCL-2
CYA1
CYB1
CYD1
NFkB
STAT3
MET

Figure 4. CREB overexpression and miR-34b knockdown in normal bone marrow progenitors.
A. Healthy bone marrow progenitors transiently transfected with miR-34b inhibitor oligonucleotides and a CREB overexpressing vector (34bInh+CREB) or a control (miR-Neg-EV). (Upper Left) Western blot analysis shows a increase in CREB protein expression 16 h or 24 h post transfection. (Lower Left) Representative cell cycle analysis shows an increase in S-phase with healthy bone marrow transfected with 34bInh+CREB. (Right) Gene expression analysis of a series of CREB and miR-34b target genes is calculated using the comparative ΔCt method. Each gene quantification has been normalized to the RQ of healthy BM transfected with miR-Neg-EV (RQ=1). Error bars are SEM of three experiment (n = 3). B. (Upper Left) Histograms represent the number of colony formed as a percentage of the colony number for the 34bInh+CREB (white bar) divided by the Neg+EV (black bar) number (* < 0.05). (Upper Right) Positive regulator of self renewal as BMI-1, GATA-2, HOXB4 and GFI-1 were analyzed in RQ-PCR. Their expression is increased at 48h post transfection in 34bInh+CREB transfected healthy bone marrow. (Lower Left) Examples of erythroid colonies formed with healthy bone marrow transfected with 34bInh+CREB. FACS analysis of myeloid and erythroid markers (CD15, 61, CD45-, CD36, Glycoforin) in healthy primary culture transfected with 34bInh+CREB (white) and miR-Neg+EV (black). Healthy bone marrow with knock down of miR-34b has an increased expression of myeloid end erythroid markers. (Lower Right) Western blot analysis of protein involved in myeloid differentiation is analyzed at 16 h and 30 h post transfection. The Western blot shows an increase in expression of PU.1, GATA-1 and MEIS-1 in 34bInh+CREB transfected healthy bone marrow. C. Fetal liver transduced with Fugw-miR-TARGET or EV. (Left) Histograms represent the number of colonies formed as a percentage of the colony number for the knockdown of miR-34b divided by the EV number. In fetal liver cells with a reduction in miR-34b expression (white) there is a 2-fold increase in colony formation compared to controls (black) (* < 0.05). (Middle) Representative microscope field of a May-Grünwald-Giemsa (MGG) staining (20X) of fetal liver cell with a knock down in miR-34b shows an increase in all the myeloid populations compared to EV (60X). (Right) Graph also shows an increased myeloid compartment in fetal liver cell transduced with miR-TARGET (white bar) compared to control (black bar) as evinced by CD33 and CD15 expression at FACS. (Bottom) Gene expression analysis of a series of CREB and miR-34b target genes is calculated using the comparative ΔCt method. Each gene quantification has been normalized to the RQ of fetal liver transfected with miR-Neg-EV (RQ=1) (n = 3).

To study the specific role of miR-34b knockdown, a stable model was used. We transduced fetal liver cells with a construct that overexpressed microRNA target sequences from polymerase II promoters in order to stably and specifically knock down miR-34b. Infected cells were sorted and showed reduced miR-34b expression (miR-34b RQ = 0.25). The expression of CREB mRNA was found to be increased (RQ = 4.48) compared to fetal liver cells transduced

with the scramble miR-target (miR-Neg) (data not shown). The same sorted cells were seeded in semisolid media and colony formation was increased compared to controls (Figure 4C; $p = 0.001$). Cell morphology was evaluated and almost all cells were of myeloid lineage in sorted cells with miR-34b reduction with respect to the miR-Neg. A large number of myeloid precursors as well as more differentiated cells were present, confirming CREB role in myeloid development (CD33 = 35.4 % vs 18 % ($p < 0.05$) and CD15 = 3.3 % vs 2.5 % ns). We examined expression of CREB targets *BCL2, CYCLINs, STAT3,* and *NFkB* and all were increased in fetal liver cells with miR-34b knock down. *MET*, a known miR-34b target, was also induced in this stable system (Figure 4C). These results correlate increased proliferation and derepression of CREB and its targets with inhibition of miR-34b, implying an important role for miR-34b in the regulation of myeloid cell proliferation.

DISCUSSION

Alterations in miRNA expression in malignant cells have been documented in numerous studies and suggest that miRNAs contribute to the characteristics of tumors by disrupting the expression of specific targets (27). Recently, miR-34 family members are described to play an important role as tumor suppressors in multiple cancers (28), since their loss has been demonstrated to impair cell cycle and apoptosis principally by controlling CDK6, MET, SIRT1 and MYC (29-31) in different cell models. CREB, a direct target of miR-34b, is overexpressed in AML contributing to leukemic progression (8). The role played by this miRNA in CREB regulation and in myeloid transformation was investigated in this paper.

Previously, miR-34b was reported to be hypermethylated and downregulated in leukemic cell lines. MiR-34b overexpression was demonstrated to lower CREB levels, induce apoptosis and decrease the clonogenic potential *in vitro*. Here, forced expression of miR-34b was studied in

its ability to suppress leukemia in primary AML cultures and in mice. Increased apoptosis and decreased clonogenicity in AML primary cultures was documented, as well as a reduced tumor mass and metastatic progression of leukemic cells in NSG mice. These results led us to further explore the molecular basis of miR-34b as tumor-suppressor in human MDS and AML patient samples. In recent years, a number of studies have provided evidence of miRNA downregulation in a variety of human cancers principally linked to epigenetic silencing, such as histone modification and DNA methylation (32). In this study, we showed that the reduction of miR-34b correlates with the methylation status of its promoter in a large series of *de novo* AML patients. Hypermethylation of miR-34b is exclusively characteristic of AML, with a striking lack of the same phenomenon in a large cohort of MDS/MPD patients. This finding convinced us to focus on the possible involvement of miR-34b promoter methylation in the evolution from MDS to AML. MDS patients that evolved to AML were found to gain methylation of miR-34b promoter, which specifically marked the establishment of leukemia. Moreover, treatment of primary cells with a demethylating agent increased miR-34b expression and decreased CREB protein levels, as we expected, since CREB is a direct target of miR-34b in AML cells (8). The regulation of CREB by miR-34b in patient samples suggests a mechanism of how CREB promotes abnormal proliferation, cell cycle progression, and clonogenic potential *in vitro* and *in vivo* (5,6). CpG methylation of miR-34b may be a common event during tumorigenesis, as recently documented in other cancers (29, 33-35); our results suggest it may be a crucial step in the progression to AML. This leads to aberrant expression of downstream CREB target genes as indicated by our gene expression profile. CREB expression tightly clustered to two different diseases confirming that specific pathways evoked by an aberrant expression of miR-34b might support transformation to AML. Functional analysis might delineate which of the CREB targets, *CDK6,*

LATS2, HOXA7 or *ADAM10* (36-38), may be involved in different oncogenic processes such as leukemia burden or numbers of blasts in bone marrow from MDS patients.

The biological and clinical role of DNA methylation in AML has produced controversial results (39-41). We found that miR-34b as single methylation event was able to influence onset of AML principally by controlling CREB expression. The exploration of miR-34b hypermethylation in a large cohort of well-characterized AML patients had the potential to define how miR-34b expression correlated with prognosis. In fact, results demonstrated that hypermethylation of miR-34b defined a group of AML patients with moderate low overall survival. Same patients were positive for genetic aberrations commonly identified in AML with poor prognosis, e.g. normal karyotype, the t(6,11)*MLL-AF6* and *FLT3ITD*, but a larger cohort of patients is warranted. We highlight new putative AML subgroups who might respond to drugs that affect methylation.

In an effort to explore the mechanistic basis of transition of MDS to AML, we knocked down miR-34b in normal bone marrow and fetal liver progenitor cells. Cell morphology after miR-34b downregulation suggested a myelodysplastic process thereby driving cells preferentially to myeloid differentiation in both systems. We observed that the knock down of miR-34b resulted in increased CREB levels leading to alterations in CREB and miR-34b target gene expression, known to be important in cell proliferation and survival (*NFKB, BCL2, STAT3, CYCLINs* and *MET*) (26, 35). An increased cell proliferation and colony forming ability were found, and supported our hypothesis that lowering miR-34b levels increased tumorigenic potential of myeloid cells. Increased CREB levels improved cell growth confirmed by the upregulation of genes that control cell cycle and self-renewal (*BMI-1, GATA-2, HOXB4, GFI-1*) (42,43). Furthermore, deregulation of some master regulator proteins of hematopoiesis, GATA-1, PU.1 and MEIS-1 was observed. Work by others has demonstrated that manipulation of the expression

levels of GATA-1 and PU.1 results in rapid cell fate decisions between the development of leukocytes or red blood cells from a common progenitor. Perturbation of this process can result in a blockade of differentiation that could lead to leukemia. Our results revealed that PU.1 and GATA-1 might play an important role in transformation from MDS to AML. In fact, the erythroid differentiation appeared improved. We observed a large number of erytroid colonies, which might be induced by the increased expression of GATA-1 and its targets (*EPO-R*). Since PU.1 was increased at the same time, the enforced stoichiometry of both proteins might be involved in the impaired myeloid/erythroid differentiation triggered in healthy cultures by CREB exogenous expression (44,45). The increased MEIS-1 expression strengthened the power of CREB transcriptional control during the leukemogenic process (46).

Finally, we describe findings that support the idea that suppression of miR-34b through the hypermethylation of its promoter is critical for the pathogenesis of AML and promotes a malignant phenotype in healthy samples through regulation of CREB-dependent genes. Future studies will focus on studying the role of miR-34b and CREB in myeloid leukemogenesis and as a potentially novel pathway for development of new drugs for therapy.

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CHAPTER 6

CONCLUSION

Transcription factors have been described as the major protein class whose alterations influence cell proliferation, differentiation and survival and their inappropriate activation has been demonstrated to play a significant role in acute leukemia. Therapies currently adopted in pre-clinical trials arise from those molecules and the results obtained so far encourage their further study (1).

The discovery of CREB protein expression at high levels in pediatric bone marrow of patients affected by acute myeloid and lymphoblastic leukemias compared to healthy controls, and the identification of CREB as proto-oncogene (2-4) in the progression of leukemia lead us to start a study in order to unravel the mechanism of CREB overexpression in this system.

We firstly dimostrated the tumor suppressor role of ICER and miR-34b in leukemia, describing their mechanism of action. Interestingly, they both have an expression inversely correlated to CREB expression in leukemia and healthy controls. ICER regulates CREB target genes directly binding CRE elements in their promoter. In addition, ICER can directly influence CREB expression regulating it's own transcription (5). Therefore ICER downregulation in response to ERK1/2 induced proteosomal degradation can partially explain the abundance of CREB protein. Further study showed that ICER confers an enhanced sensibility to drugs when AML cells are treated with chemotherapics, specially through p38 pathway mainly repressing the dual-specificity phosphatases DUSP1 and DUSP4.

Moreover, given the fact that CREB protein overexpression did not closely correlate with CREB mRNA levels, a posttranscriptional mechanisms, like miRNA regulation, was considered. MiR-34b was found to directly target CREB and its absence in leukemia contribute to CREB increased expression. The reason for miR-34b down regulation was explained by the hypermethylation of the CpG island in its promoter. Hypermethylation of miR-34b is found to be exclusively characteristic of AML, with a striking lack of the same phenomenon in a large cohort of MDS or MDS/MPD patients. Furthermore miR-34b promoter methylation has been considered to be associated to the evolution from MDS to AML. This might be a crucial step in cancerogenesis since CpG methylation of miR-34b is a common event recently documented in other cancers (6-9). Finally, we produced findings that support the study of miR-34b and CREB in myeloid leukemogenesis that may lead to the development of new drugs or help in coordination with other molecular markers to better stratify patients. Further study are necessary to better unravel the mechanism of leukemia pathogenesis or the reason for miR-34b/34c

promoter hypermethylation in the evolution to AML. Hence this thesis shed light to new pathways and mechanism that can be targeted and that can improve the response of tumor cells to conventional treatments. Targeting miRNAs to reprogramme miRNA networks in cancer constitutes a reasonable and evidence-based strategy with a strong potential and chances for success.

Concluding, ICER and miR-34b for the first time might be taken into account as broad markers of leukemia. Their use could be important in the treatment and diagnosis of those patients defined as negative, thus lacking a known molecular target, permitting a close follow up during the course of their disease, predicting molecularly their clinical progression. ICER and DUSP1/4 emerged from this thesis as new potential targets that might be used in order to increase the available therapies to treat AML patients, expecially overcoming drug resistance. Furthermore, DUSP1/4 will be analyzed in an *in vivo* model to better unravel their importance in sensibilizing cells to chemotherapic. In addition the ten CREB target genes that permitted to stratify MDS from their evolution to AML can be examined in further study as potential effector of CREB mediated oncogenesis.

A cartoon summarizing the whole new picture is in the following figure (Figure 6.1).

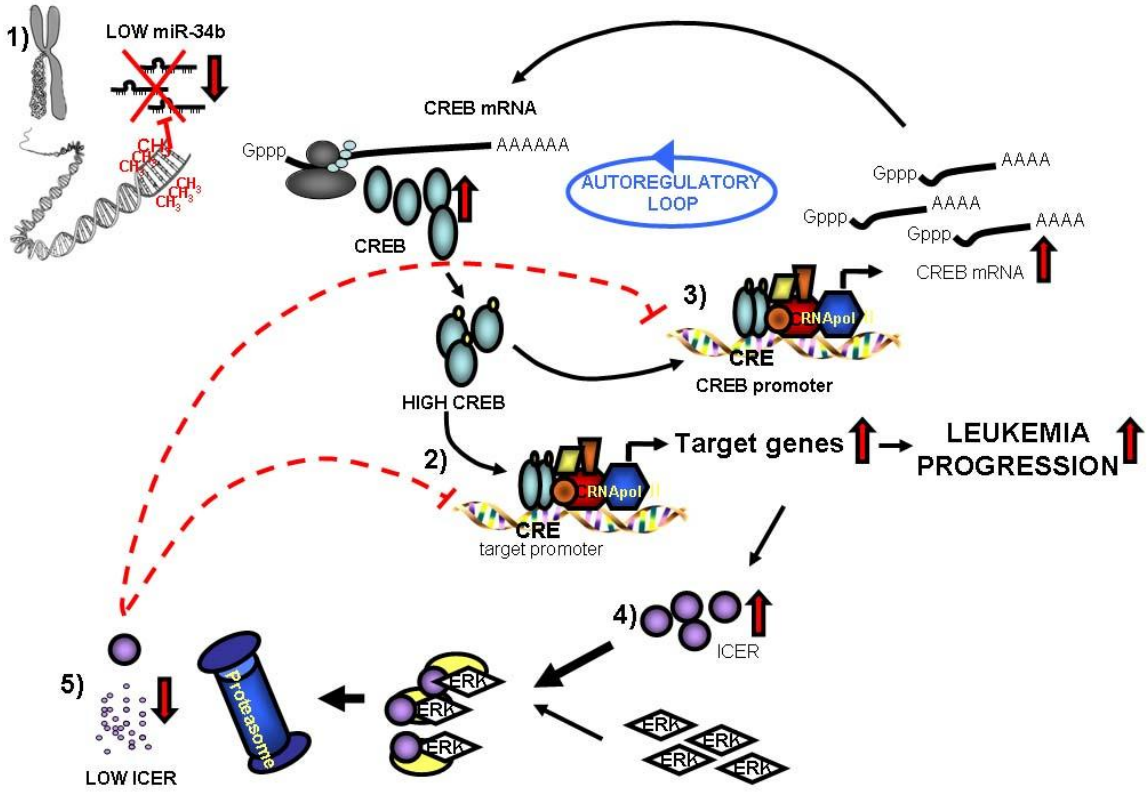

Figure 6.1 Scheme that summarize the mechanism that leads to CREB upregulation in leukemia.

1) Hypermethylation of miR-34b promoter in AML patients leads to a lower expression of the mature form of miR-34b, therefore the inhibition of miR-34b on CREB translation is not effective leading to an increase in CREB protein. 2) High CREB levels induce an overexpression of his target genes and to a progression of leukemia. 3) CRE binding sites in CREB promoter are recognized by CREB in a positive autoregulatory way. 4) ICER is induced by CREB upregulated transcription. ERK interacts with ICER and other protein leading to a proteasomal degradation of ICER. 5) Threfore ICER cannot act as a efficient tumor suppressor on CREB target genes, promoting leukemia phenotype. Abbreviations CRE = cAMP responsive element.

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Publications

Pigazzi M, Manara E, Baron E, Basso G. ICER expression inhibits leukemia phenotype and controls tumor progression. Leukemia. 2008 Dec;22(12):2217-25.

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