Clinical Cancer Research

ICER Evokes Dusp1-p38 Pathway Enhancing Chemotherapy Sensitivity in Myeloid Leukemia

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Abstract

Purpose: The inducible cyclic adenosine monophosphate (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 quantitative real-time PCR (qRT-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 significantly lowered expression of CREB target genes involved in cell cycle control (CyA1, B1, D1), and in the mitogen-activated protein kinase signaling pathway (ERK, AKT, DUSP1/4), was found after Etoposide treatment. The dual-specificity phosphatases DUSP1 and DUSP4, directly repressed by ICER, activated the p38 pathway, which triggered 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 the 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. *Clin Cancer Res; 17(4); 742–52. ©2011 AACR.*

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; refs.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, inducible cAMP early repressor (ICER) 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 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 mitogen-activated protein kinase (MAPK) family members, which included numerous cellular signaling, such as extracellular regulated kinase1/2 (Erk1/2), c-Jun N-terminal kinase (JNK), and p38, known to transmit different types of signals (14,15). Erk1/2 acts through

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Translational Relevance

The inducible cyclic AMP (cAMP) early repressor (ICER) and cAMP response element binding protein (CREB) are transcriptional regulators of the cAMP-mediated signaling pathway and have recently been found to play a crucial role in leukemogenesis. CREB has been demonstrated to be upregulated in the majority of childhood leukemias, contributing to disease progression, whereas ICER was found to be downregulated. The restored ICER expression in leukemia cell lines has been demonstrated to decrease CREB protein levels and induce a lowered clonogenic potential of myeloid leukemia. We are now presenting novel data about ICER's ability to influence tumor response to chemotherapeutic treatment by controlling the DUSP1 expression that specifically activates the p38 apoptotic pathway. ICER restoration and DUSP1 silencing appears to be a good strategy to sensitize primary cancer cells to conventional chemotherapy and to inhibit tumor growth. The characterization of novel CREB targets is open for further consideration in AML treatment.

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 regulatorycontrol ability, called dual specificity phosphatases (DUSP; refs. 18, 19). The DUSP protein family contains several members with substrate and subcellular localization specificity (20). In particular, DUSP1/4 targets principally p38, and many different stimuli are able to activate their activity (21,22). The balance between the activation and 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 hand, 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) were cultured in DMEM (Invitrogen-Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen-Gibco). Cell lines were grown in suspension diluted to 500×10^6/mL, and after 24 hours were transiently transfected by the Nucleofector system from Amaxa GmbH 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 and colleagues (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 were collected after obtaining written informed consent. AML cells were isolated from BM by hemolysis and cultured in RPMI (Invitrogen-Gibco) supplemented with 100 U/mL penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco), IL-3, IL-6, Flt-3 ligand, and TPO (Sigma). After 24 hours of incubation at 37°C, cells were added to 10% heat-inactivated 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), as well as the scramble negative Sc-siRNA (Sc-siR) 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%.

MTT assay

We performed dose-dependent studies of VP-16, doxorubicin and staurosporine 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) salt was measured. The subtoxic drug dose at which the proliferation of cell lines was not significantly affected was 1 μmol for VP-16, 0.03 μmol for doxorubicin, and 0.24 μmol for staurosporine.

Fluorescence microscopy

HL60 + ICER and HL60 + EV were serum-starved overnight before adding 1 μmol VP-16 for up to 72 hours. To assess time-dependent nuclear morphology perturbations of VP-16-treated and untreated cells, 5×10^5 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 300 F digital camera (Leica Microsystems Ltd.).

Cell cycle analysis and apoptosis assay

HL60 + ICER and HL60 + EV cell lines were serum-starved overnight and then continuously treated with 1 μmol VP-16, 0.03 μmol doxorubicin, and 0.24 μmol

staurosporine. After 6, 24, and 48 hours, 5×10^5 cells were washed twice with PBS, lysed and treated with 50 μg/mL Propidium Iodide (PI) or with annexinV conjugated to fluorescein-isothiocyanate (FITC) and PI. Drugs were solubilized in DMSO, which was found to induce less than 0.5% apoptosis. Cells were analyzed using Cytomics FC500 (Beckman Coulter). Cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems). To determine caspase activation, we used 10 μmol 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 μmol] and a selective inhibitor of MAPK/ERK kinase 1 inhibitor PD98059 (2′-amino-3′-methoxyflavone: Sigma-Aldrich) 120 minutes before VP-16 to selectively block different signals and measured apoptosis by Annexin V/PI staining. In apoptosis assays, cell death of EV was subtracted from 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 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. One microgram of RNA was transcribed using the Superscript II system (Invitrogen-Gibco) in 25 μL final volume following manufacturer's instructions. Quantitative real-time PCR (qRT-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

Twenty micrograms from the total protein fraction (Buffer-Biosource International), obtained from HL60 + ICER and HL60 + EV cell lines, were used to perform protein analyses as previously described in Pigazzi and colleagues (11). 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); 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); anti-Caspase8 and anti-Caspase3 (Alexis Biochemicals); anti-CyclinE (Oncogene Research Products); anti-CyclinB, anti-CyclinD1, and anti-Kip1-p27 (BD Bioscience).

Chromatin immunoprecipitation assay

HL60 + ICER and HL60 + EV cell lines were processed for chromatin immunoprecipitation assay (Upstate Cell Signaling Solutions) 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. qRT-PCR was also performed by the SYBR Green method for amplification and detection (Invitrogen-Gibco) by 7900HT technology (Applied Biosystems). Analysis was carried out by the comparative threshold cycle (C_t) method.

Anisomycin treatment

The HL60 parental cell line was treated with anisomycin (Sigma-Aldrich), a p38 activator, 2 μmol 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 20 μmol 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), were introduced in the HL60 cell line (100 nmol in 2 mL of medium) by nucleofection. Scramble siRNA (ScsiR) was used as negative control. mRNA and protein expression were performed to monitor silencing. Apoptosis was measured after VP-16 treatment. VP-16 1 μmol 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 (Fig. 1A) and 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 (Fig. 1B). HL60 + ICER was found to be more sensible to VP-16; its lowered cell proliferation was confirmed up to 72 hours of treatment (Fig. 1C). The cell morphology showed an increased number of apoptotic nuclei after VP-16

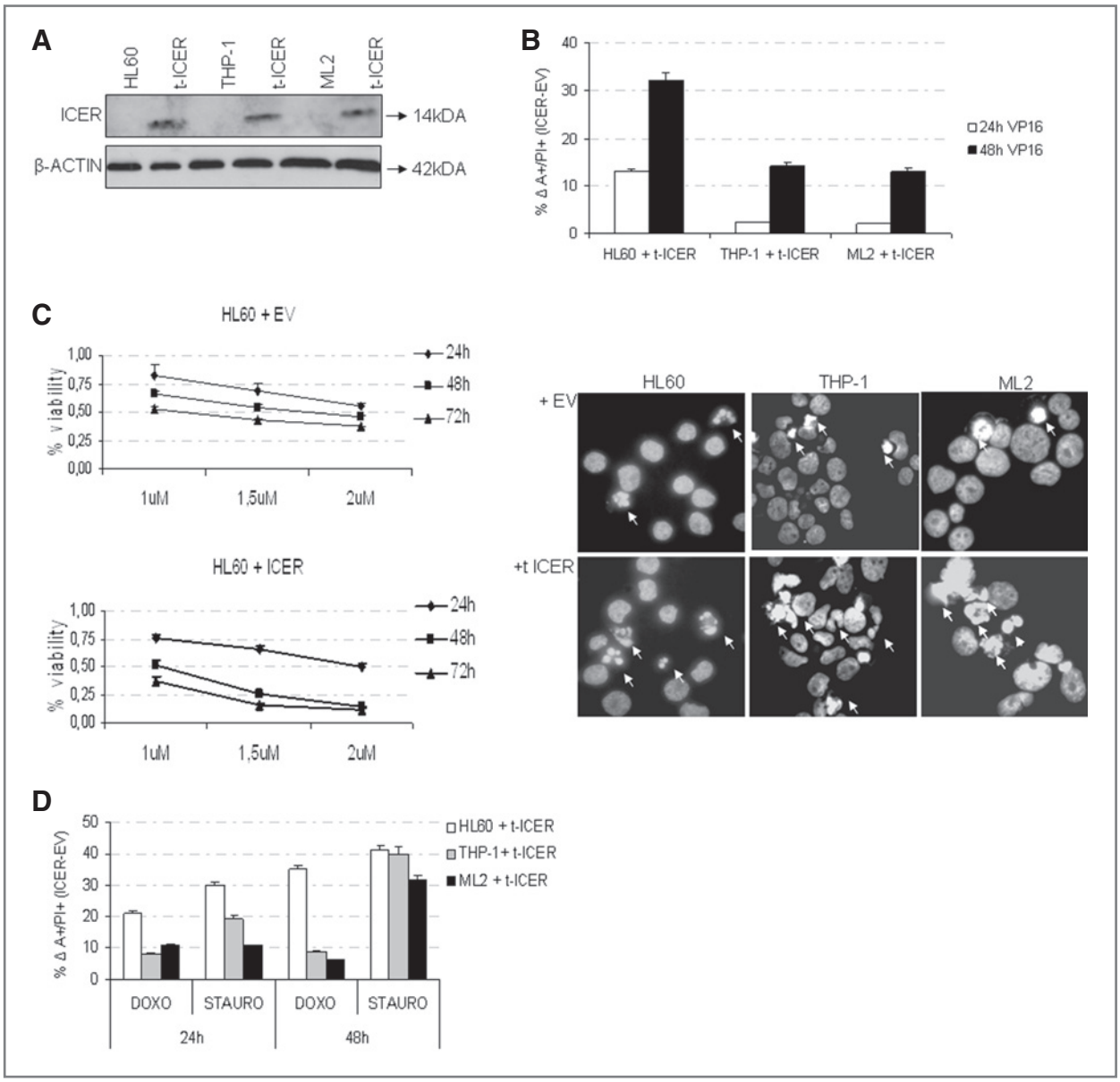

Figure 1. (A) Western blot analysis of ICER transient expression (t-ICER) induced in HL60, THP-1, and ML2 at 48 hours posttransfection. (B) The increase in apoptotic of ICER expressing cell lines after VP-16 treatment is shown with respect to cells transfected with EV in the same treatment conditions (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 hours ($n = 3$). 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 hours of drug treatments. Apoptosis is significantly increased at 24 and 48 hours of treatment in cell lines overexpressing ICER ($n = 3$, $^*p < 0.05$).

treatment in ICER expressing cell lines, confirming the HL60 to be more sensitive to the drug (Fig. 1C). To evaluate if the increase in apoptosis was due exclusively to VP-16, and not to ICER overexpression, two different compounds, staurosporine and doxorubicin, were also tested. 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%, and 10.8% when exposed to doxorubicin; 30%, 19.3%, and 10.7% when exposed to staurosporine. At 48 hours of treatment 35%, 8.9%, and 6.4% were for doxorubicin and 41%, 40%, and 32% were for staurosporine. 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 (Fig. 1D, $n = 3$, $^*p < 0.05$).

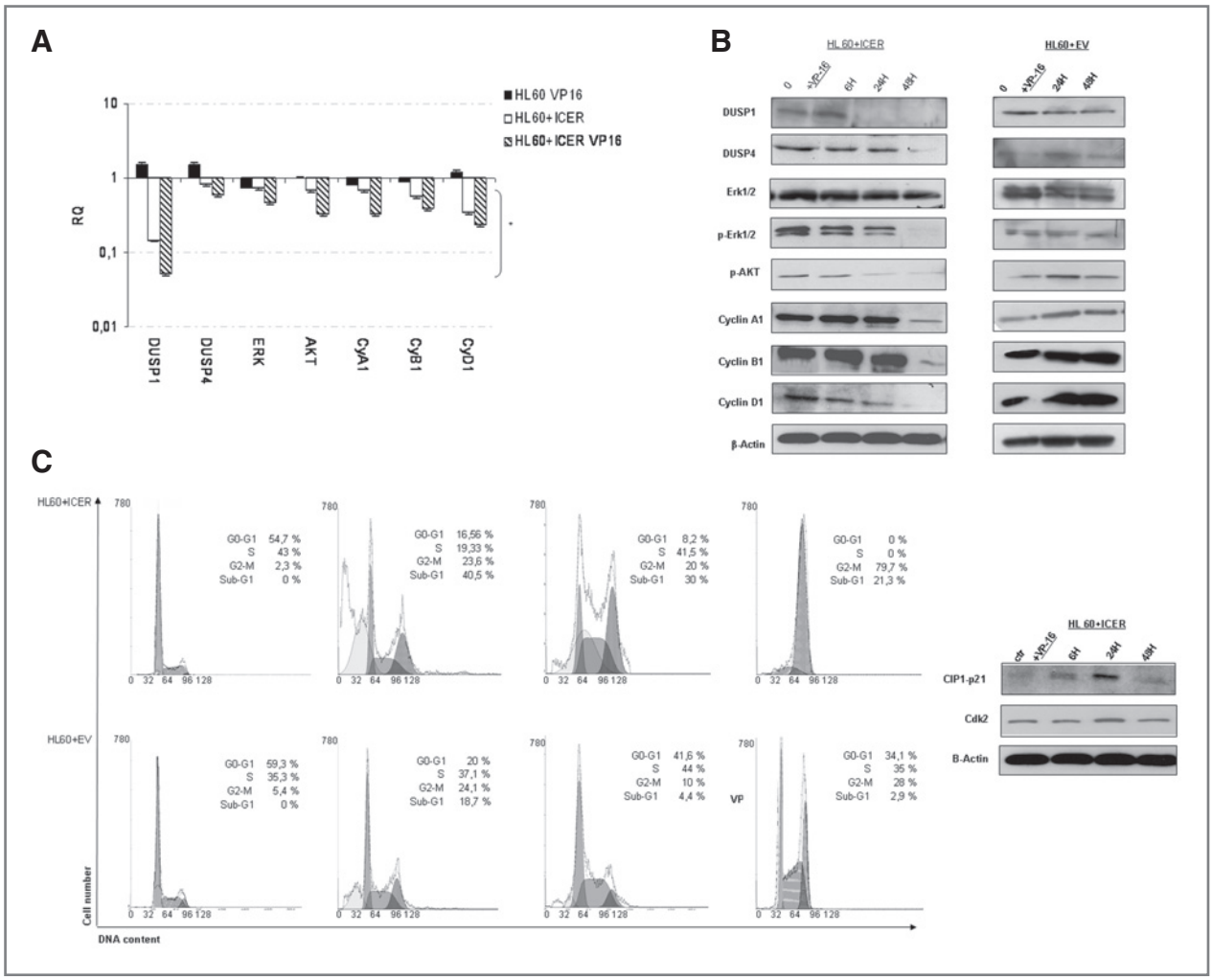

Figure 2. (A) Histogram shows the relative quantitation (RQ) of CREB/ICER target genes of HL60 + EV and of HL60 + ICER at 48 hours of VP-16 treatment compared to the untreated HL60 + EV cell line used as calibrator (RQ = 1 in the figure, $n = 2$, $^*p < 0.05$). (B) Western blot analysis of target proteins which were found decreased in HL60 + ICER treated with VP16. HL60 + EV cell line, treated under the 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, doxorubicin, or staurosporine, were performed after 48 hours 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 hours posttreatment in HL60 + ICER, consistent with the block in cell cycle progression and with the reduction of cyclins observed in panel 2B.

ICER counteracts CREB gene expression

By studying the ICER transcriptional repressor activity by qRT-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 cells after VP-16 treatment (Fig. 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 under the same conditions (Fig. 2B). To further investigate the role of cyclin downregulation, 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 doxorubicin treatment; whereas a rapid ongoing to apoptosis mainly after staurosporine 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 (Fig. 2C, $n = 2$, $p < 0.05$, ctr = untreated cells).

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 (Fig. 3A) by the increase of expression of the following: active

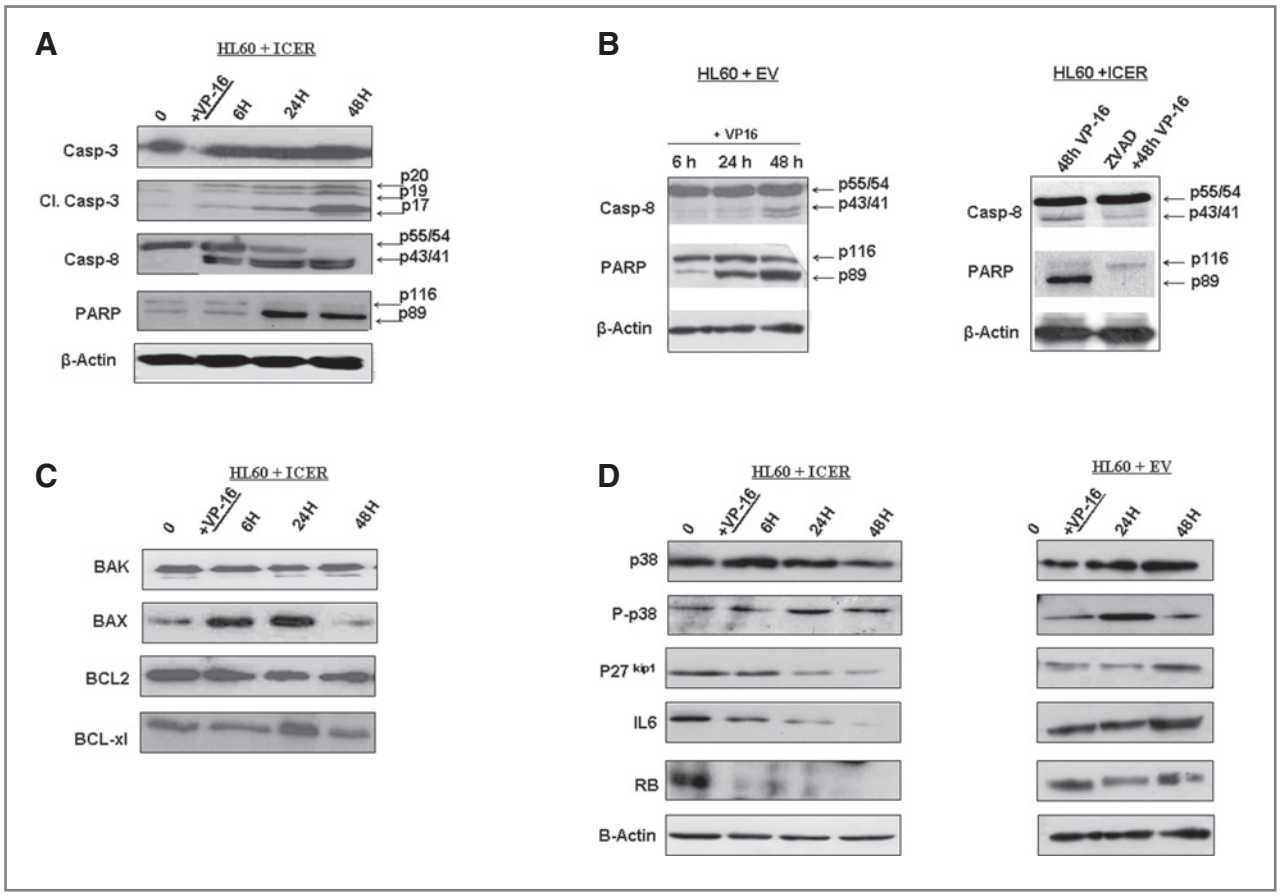

Figure 3. (A) Western blot shows the cleavage-mediated activation of inactive proteins into mature fragments of caspase 3 (p17, p19, p20), caspase 8 (p43, p41), and PARP (p89) starting from 24 hours of treatment and up to 48 hours. (B) Western blot analysis shows that apoptosis enforced by ICER was found 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 were shown. (C) Western blot analysis was conducted on the mitochondrial protein fraction (Mt). The presence of BAK confirms 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 (Fig. 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. The same reduction of proteins was not observed in HL60 + EV treated at same conditions.

caspase 3 (active fragments p20, p19, and p17), caspase 8 (active fragments p43/41), and cleaved PARP (cleaved fragment p89). 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4A). Results of qRT-PCR were interpreted by the ΔΔct method considering HL60 + EV as calibrator [relative quantitation (RQ) = 1]. The amount

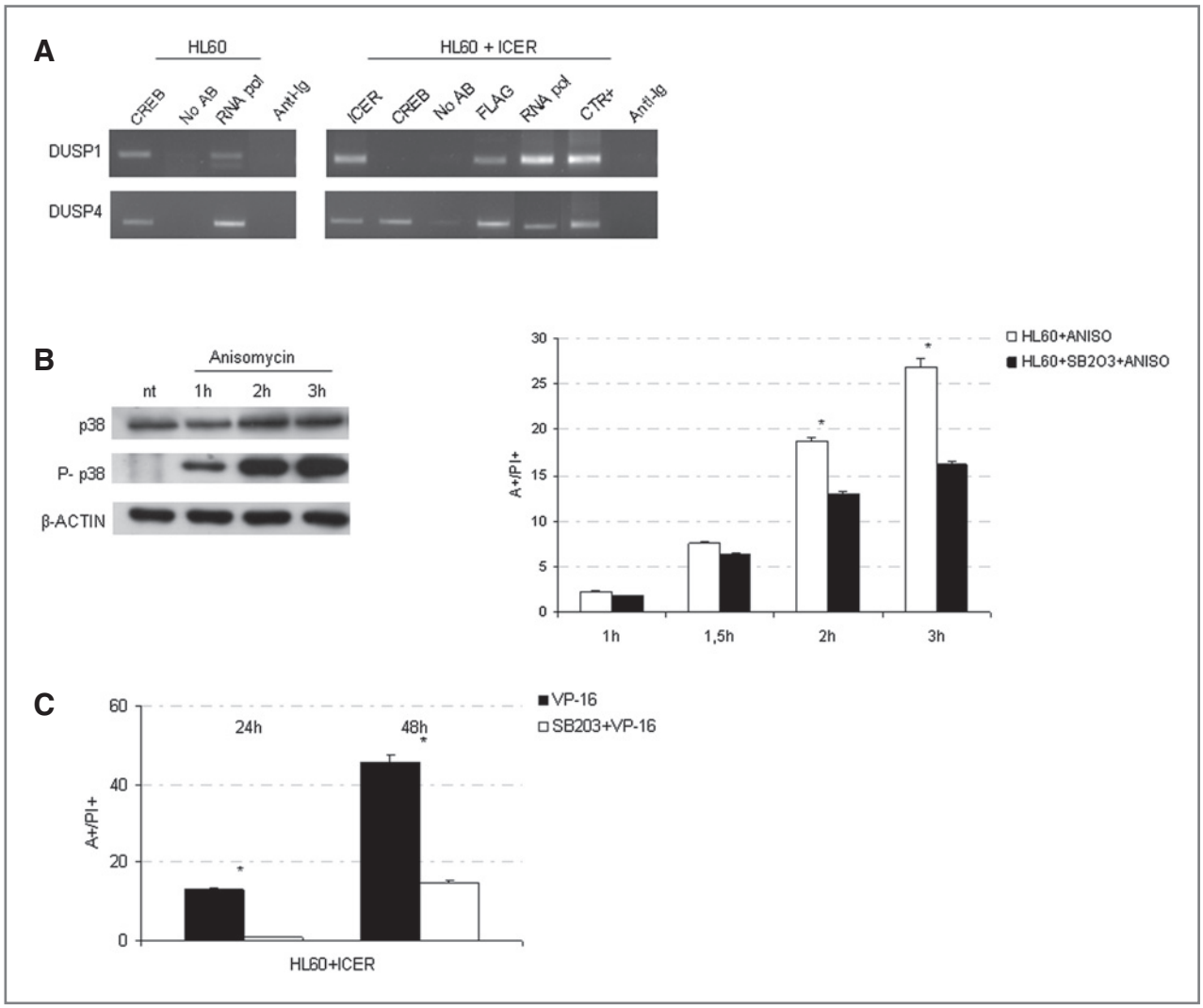

Figure 4. (A) Chromatin immunoprecipitation was performed using CREB, ICER, or FLAGM2 antibody in both cell lines. FLAG antibody was used to pull down ICER exogenous protein, while RNAPOL was used to assess active promoters. Positive control is the imput DNA (ctr+) while negative control is obtained 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 (SB203), 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 SB203 pretreatment. Results show a rescue in apoptosis when cells are pretreated with SB203.

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 (Fig. 4B, $n = 3$, $^*p < 0.05$). 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 posttreatment and from 45.7% to 14.8% at 48 hours (Fig. 4C, $n = 3$, $p < 0.05$), suggesting that p38 activation was directly involved in the apoptosis mediated by ICER restoration. Apoptosis was rescued by SB203 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 to induce the same effect mediated by

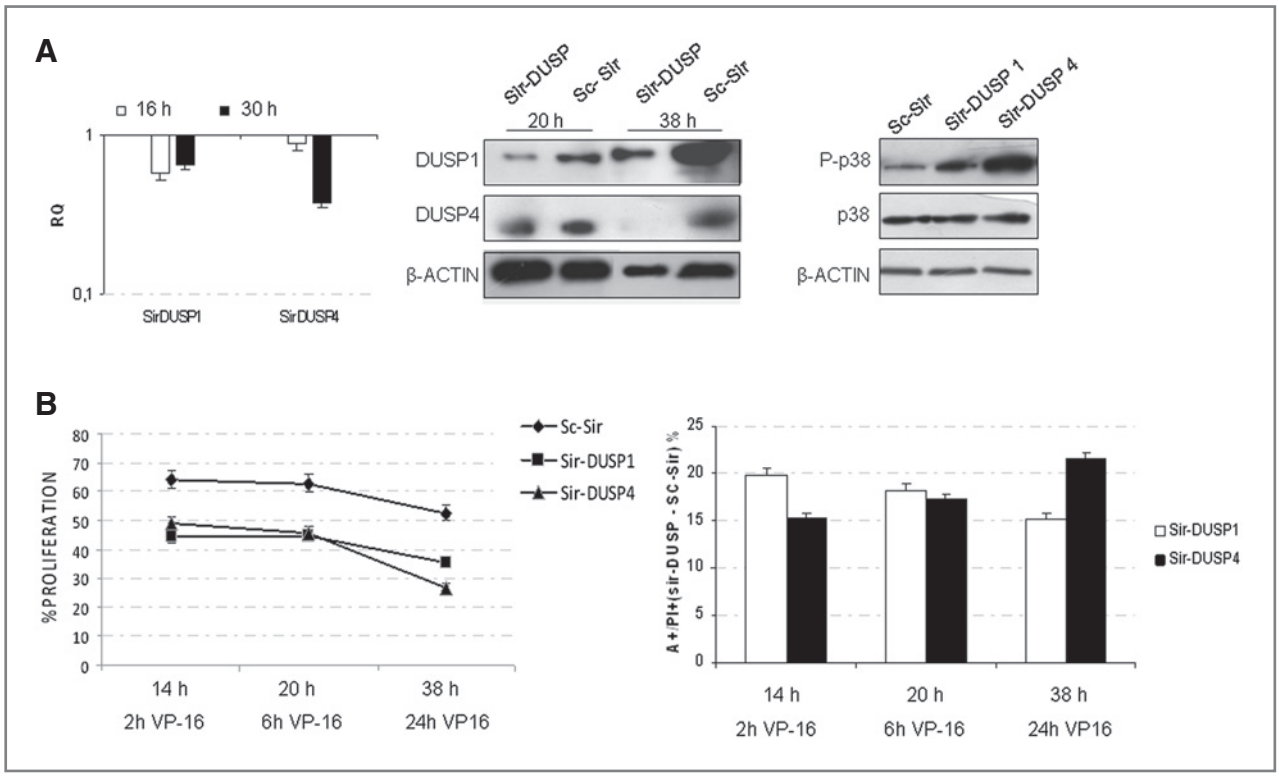

Figure 5. (A) RQ of DUSP1/4 after 16 and 30 hours of silencing is shown, results are calibrated to Sc-SiR mRNA (RQ = 1) used as control. Western blot shows that silencing of DUSP1 and DUSP4 in HL60 cell line decreased their protein expression compared to the scramble siRNA oligonucleotide used as control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

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 DUSP4 silencing was inefficient (RQ = 0.87). After 30 hours, DUSP1 mRNA was maintained reduced (RQ = 0.65), whereas DUSP4 mRNA was strongly diminished (RQ = 0.37). Protein levels followed the same trend of mRNA. DUSP1/4 silencing was documented to increase phospho-p38 levels (p-P38) after 20 hours (Fig. 5A); 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 decreased proliferation and increased apoptosis with respect to the scRNA used as control. Therefore, the parental DUSP1/4-silenced HL60 cell line became more sensitive to drug treatment, as established in the HL60 + ICER cell line (Fig. 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). 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 (Fig. 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 (Fig. 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.

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,

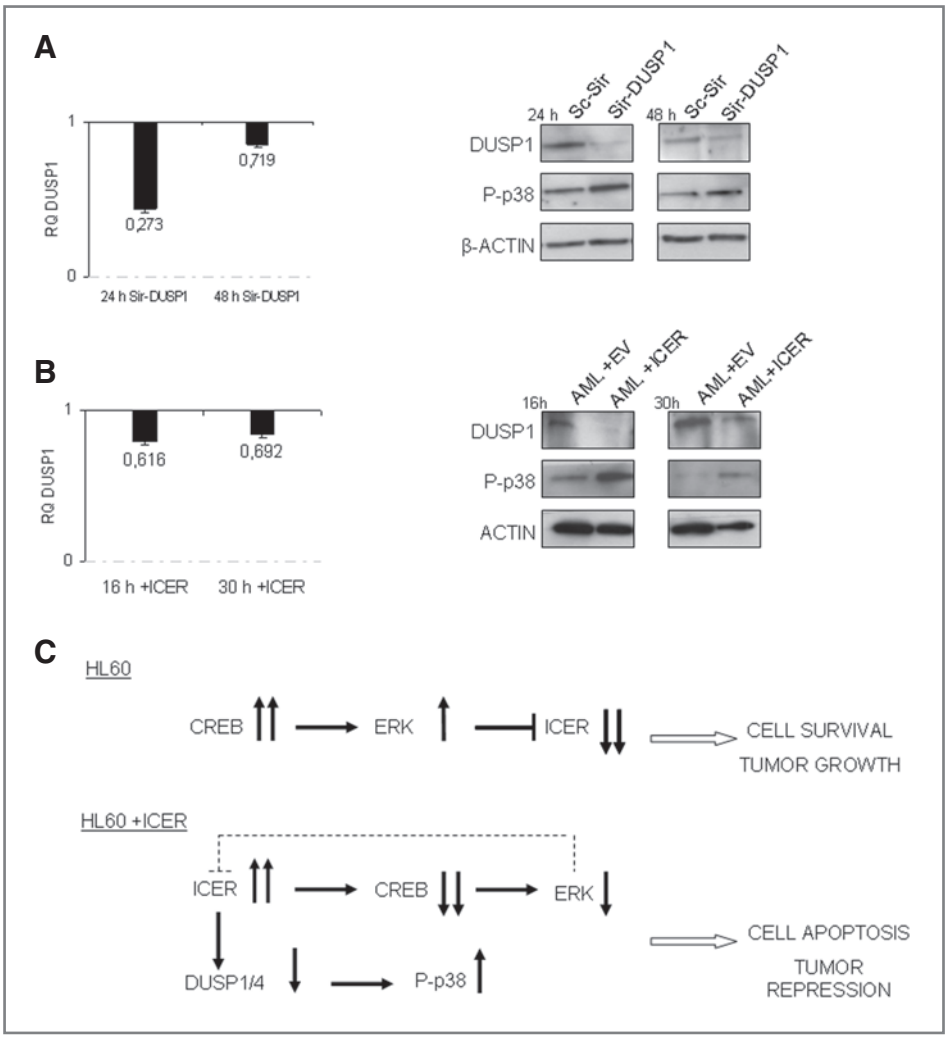

Figure 6. A, Primary cultures were used to silence DUSP1. A decrease of DUSP1 mRNA after 24 and 48 hours (ScSiR used as calibrator, RQ = 1) is shown. DUSP1 protein levels were lowered and phospho-p38 (p-P38) activated after silencing. B, ICER expression was introduced in primary AML bone marrow: DUSP1 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.

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 an increase in 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 has 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 antisurvival 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 that modulate leukemia cell fate.

DUSP1 and 4 repression appeared to be the crucial event for drug response of leukemia cell lines. The silencing of DUSP1/4 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 downregulation 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 of 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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