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Glucocorticoid-mediated co-regulation of *RCAN1-1*, *E4BP4* and *BIM* in human leukemia cells susceptible to apoptosis

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Abstract

Glucocorticoids (GCs) are known to induce apoptosis of leukemia cells via gene regulatory changes affecting key pro- and anti-apoptotic genes. Three genes previously implicated in GC-evoked apoptosis in the CEM human T-cell leukemia model, *RCAN1*, *E4BP4* and *BIM*, were studied in a panel of human lymphoid and myeloid leukemia cell lines. Of the two *RCAN1* transcripts, the synthetic GC Dexamethasone (Dex) selectively upregulates *RCAN1-1*, but not *RCAN1-4*, in GC-susceptible Sup-B15, RS4;11, Kasumi-1 cells but not in GC-resistant Sup T1 and Loucy cells. *E4BP4* and *BIM* regulation correlated with that of *RCAN1-1*. A putative GRE and four EBPREs were identified within 1500bp upstream from the transcription start site of *RCAN1-1*. GC-refractory CEM C1-15 cells sensitized to GC-evoked apoptosis by ectopic *E4BP4* expression, CEM C1-15mE#3, showed restored *RCAN1-1* upregulation, suggesting that *RCAN1-1* is a downstream target of *E4BP4*. A model for coordinated regulation of *RCAN1-1*, *E4BP4* and *BIM*, and their role in GC-evoked apoptosis is proposed.

Keywords

glucocorticoids; leukemia; apoptosis; *RCAN1*; *E4BP4*; *BIM*

INTRODUCTION

Glucocorticoids (GCs) are used widely in chemotherapy either as a primary cytotoxic drug targeting cancer cells (as in acute leukemia, lymphoma and multiple myeloma) or to reduce inflammation, prevent allergic reactions or reduce the side effects of chemotherapy [1, 2]. The pharmacological actions of GC therapy include, among other effects, the suppression of the immune response and loss of the lymphocyte population via apoptosis. Relapse of leukemia after GC therapy is often associated with development of resistant cells that no longer respond to GC-evoked apoptosis [3]. The primary action of GCs is mediated through their interactions with the GR, a transcription factor from the nuclear receptor family, which modulates up or down regulation of genes containing GC Responsive Elements (GRE) or through interactions with other transcription factors, coactivators and corepressors [2]. We [4, 5] and others [6, 7] have analyzed changes in gene expression profiles induced by GCs in

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an effort to identify candidate genes modulating GC-evoked apoptosis of leukemic lymphoid cells. We have identified a panel of genes, including *RCAN1*, *E4BP4* and *Bcl2L11* (*BIM*) as significantly upregulated in CEM-C7-14 cells which are susceptible to GC-evoked apoptosis, but not in CEM-C1-15 cells, which are refractory to GC-evoked apoptosis [4, 5, 8, 9].

Bim is a BH3-only proapoptotic member of the Bcl-2 family, and its upregulation plays a vital role in apoptosis in response to various stimuli, including GCs, in normal cells as well as in lymphoid and solid tumors [6, 10]. Bim is a direct activator of apoptosis because it can interact with and inhibit the anti-apoptotic members of the Bcl-2 family, as well as promote oligomerization of pro-apoptotic Bak and Bax to open up the mitochondrial apoptosis-induced channel (MAC) [10, 11]. E4BP4 (or NFIL3) is a bZIP transcription factor with a role in anti-inflammatory response, circadian oscillation, apoptosis regulation, and immune cell development [12, 13]. *E4BP4* is homologous to the *C. elegans* pro-apoptotic cell death specification gene *ces-2* [14], which facilitates the downstream upregulation of the pro-apoptotic gene *egl-1*, an ortholog of human *BIM*. In the CEM cell culture model of human leukemia, we have demonstrated that GC-dependent upregulation of *E4BP4* facilitates *BIM* upregulation and subsequent apoptosis [9].

The *RCAN1* gene is located on chromosome 21q22.12, near the minimal critical region implicated in the Down Syndrome phenotype. The gene consists of six exons, and codes for two major protein isoforms, each with a unique first exon, exon 1 (*RCAN1-1*) or exon 4 (*RCAN1-4*) and shared exons 5-7 [15]. Additionally, exon 1 has two translation start codons, corresponding to a long and short variant: *RCAN1-1L* and *RCAN1-1S*. All *RCAN1* isoforms share a highly conserved central region, the FLISPP motif, a potential target for serine phosphorylation, and a C-terminal calcineurin binding motif (PKIIQT), through which it inhibits the calcineurin phosphatase (PP3C) activity [16]. *RCAN1-1* expression is upregulated in response to oxidant- stress while Ca^{2+} -induced stress causes *RCAN1-4* upregulation [16]. Increased abundance of both isoforms has been reported in Down syndrome, and may contribute to the phenotype of cardiac and immune dysfunction [16, 17]. Down syndrome patients are also susceptible to early onset Alzheimer's disease, and chronic *RCAN1* expression has been shown to promote formation of neurofibrillary tangles and amyloid beta plaques [17, 18]. Transient expression of *RCAN1* has been shown to mediate the adaptation to and protection from oxidative and calcium-induced stress [18]. *RCAN1* has been shown to be protective in Huntington disease, primarily because of its ability to inhibit calcineurin activity and prevent dephosphorylation of the mutated huntingtin protein, reducing its toxicity [19]. We have previously reported GC-induced upregulation of *RCAN1-1*, but not *RCAN1-4* in CEM cells [8], and have demonstrated that *RCAN1-1* binds to and inhibits calcineurin PP3C activity.

In studies reported here, we extended our investigations to a panel of lymphoid and myeloid leukemia cell lines, and demonstrate a correlation between sensitivity to GC-evoked apoptosis and upregulation of *RCAN1-1*, *E4BP4* and *BIM*. We have identified a novel GC response element (GRE), and potential E4BP4 response elements (EBPRE) within the *RCAN1-1* promoter, in addition to previously reported ones [20]. Using a previously characterized [9] mouse E4BP4 expressing CEM C1-15 cell line (CEM C1-15mE#3), we

demonstrate that E4BP4 regulates *RCANI-1* expression. We present a model by which *E4BP4*, *BIM* and *RCANI-1* coordinately regulate GC-evoked apoptosis in lymphoid cells.

MATERIALS AND METHODS

Leukemic Cell Lines

Sup-B15, RS4;11, Kasumi-1, Sup T1, and Loucy cell lines were obtained from the American Type Culture Collection. **Sup-B15** is a human B cell ALL line with a t(9;22) translocation (Philadelphia chromosome). **RS4;11** is a human acute leukemia cell line with a t(4;11) chromosomal rearrangement exhibiting B-cell and myeloid lineage. The **Kasumi-1** cell line has a t(8;21) translocation representing human AML. The **Sup T1** line is a human T lymphoblastic leukemia cell line expressing multiple T- cell markers including CD1a, CD3, CD4, CD5, CD7 and CD8. **Loucy** cells are human T-cell ALL cells bearing a t(16;20) translocation. **CEM C7-14** and **CEM C1-15** cells, are derived from CCRF-CEM cells, and are sensitive and resistant, respectively, to GC-evoked apoptosis, and are generously donated by Dr. E. Brad Thompson (UTMB, Galveston). **CEM C1-15mE#3** cells are obtained by stable transfection of mouse E4BP4 in CEM C1-15 cells, rendering them sensitive to GC-evoked apoptosis [9], in correlation with *BIM* upregulation.

Cell Culture

RS4;11, Sup T1, Kasumi-1, Loucy and CEM cells were cultured in RPMI 1640 medium supplemented with 5% (CEM) 10% (RS4;11, Sup T1, Loucy) or 20% (Kasumi-1) FBS. Sup-B15 cells were cultured in Isocove's modified DMEM with 4mM L-glutamine, 1.5g/L sodium bicarbonate, 0.05mM 2-mercaptoethanol and 20% FBS. All cell lines were maintained at 37°C in a humidified 5% CO₂ incubator in log phase between 3×10⁵ cells/ml and 3×10⁶ cells/ml.

Reagents

Dexamethasone (Dex) was purchased from EMD Biosciences (Madison, WI). TRIzol reagent was from Invitrogen Life Technologies (La Jolla, CA). Reagents for reverse transcription, endpoint PCR and Real-time qPCR (RT-qPCR), including M-MLV reverse transcriptase, oligo(dT)₁₅ primer, RNasin® Ribonuclease inhibitor, dNTP mix, and Taq DNA polymerase were purchased from Promega Life Sciences (Madison, WI). SYBR® JumpStart™ TaqReadyMix (Cat #4438) was from Sigma-Aldrich (St. Louis, MO). Other reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich.

Assessment of Cell viability

Cells were plated at a density of 1–5 × 10⁵ cells/ml and treated for 96h with 0.1% ethanol or Dex at a final concentration of 10nM, 100nM, 1µM, or 10µM. Aliquots were removed at 24 h intervals for cell counts. Viable cells were counted by the trypan blue dye exclusion method using a Hemocytometer.

Apoptosis Assay

To confirm that cell death occurred via apoptosis, we employed CFTM594 Annexin V and Hoechst 33342 dyes from Biotium (Hayward, CA). Cells were treated with ethanol or 1 μ M Dex for 33h, washed and incubated for 30 min with CFTM594 Annexin V (detects exposed phosphatidylserine) and Hoechst 33342 (stains nuclei), and observed using Texas Red (ex/em 593/614) and Hoechst (ex/em 350/461) filters on an Accu-Scope 3025 epifluorescence microscope equipped with a ProgRes MF camera (JENOPTIK, Germany), and the 2.7 ProRes software.

RNA Extraction and Reverse Transcription

All cells were treated at a density of 5×10^5 cells/ml for 24h with either 1 μ M Dex or 0.1% ethanol. RNA was extracted from approximately 2.5×10^7 cells using TRIzol reagent. For reverse transcription, 7 μ g of total RNA was incubated for 3h at 42°C in the presence of 0.5 μ g of oligo(dT)₁₅, 1 μ l (~200U) of M-MLV reverse transcriptase, 0.5mM dNTP mix, and 100U of RNase inhibitor.

Endpoint and Real-Time Quantitative PCR (RT-PCR) Analysis

End-point PCR was performed for 25 cycles on a GeneAmp[®] PCR System 9700 (Applied Biosystems, Foster City, CA) using 1 μ l of the reverse transcription product, 500nM each of the forward and reverse primers (Table 1), 250 μ M of dNTP mix, 1.5mM MgCl₂, and *Taq* DNA polymerase at 25 U/ μ l. Amplicons were resolved on a 1% Agarose gel. For RT-qPCR, 1 μ l of the reverse transcription product was mixed with SYBR[®] Green JumpStart Taq Ready mix and the appropriate primers (Table 1) in a final volume of 25 μ l, and run on an Applied Biosystems 730 real-time PCR instrument. Data were analyzed using the freeware program LinRegPCR, to determine efficiency (E) and cycle threshold (CT) values. Relative expression levels and fold induction were quantitated using the Pfaffl method: $(E)^{CT_{\text{target}}(\text{control-sample})} / (E)^{CT_{\text{reference}}(\text{control-sample})}$, where β -actin was the reference gene.

RESULTS AND DISCUSSION

In the T-lymphoblastic leukemia model of CEM cells, we have previously reported a correlation between GC-evoked apoptosis and upregulation of *E4BP4* and *BIM*, as well as upregulation of *RCANI-1* but not *RCANI-4*. We have also demonstrated that E4BP4 at least partially modulated *BIM* expression. To determine whether genetic pathways for GC sensitivity were comparable in different types of leukemias, we evaluated a panel of well characterized human T- B- and myeloid leukemia cell lines for their sensitivity and dose dependence to Dex, and correlated this with the expression of the two transcripts of *RCANI* as well as *E4BP4* and *BIM*.

Susceptibility of leukemic cell lines to Dex-evoked death

Sup-B15 cells were responsive to Dex in a dose-dependent manner, with 10nM Dex reducing viable cell numbers to 32% of ethanol treated controls by Day 2 and 1 μ M Dex causing 100% loss of cell viability by day 4. RS4;11 cells were also susceptible to Dex-induced death, with 62% viable cells by day 2 in the 10nM Dex treated group compared to ethanol treated controls, and greater than 95% loss of cell viability at day 4 with 1 μ M Dex.

Kasumi-1 cells were responsive to Dex-mediated cell death, with 10nM Dex reducing viable cells to 65% of ethanol treated controls, and 1 μ M Dex causing 100% loss of cell viability by day 4. Sup-T1 and Loucy cells were found to be resistant to Dex-evoked cell death, with Sup-T1 cells having virtually identical growth profiles in ethanol or Dex up to a concentration of 10 μ M. Loucy cells showed a 50% decrease in viable cells by day 1, but subsequently recovered to exhibit a parallel growth profile to ethanol treated control cells.

Cell death occurs via apoptosis

To confirm that the Dex-induced cell death occurred via apoptosis, cells treated with 1 μ M Dex for 33h were evaluated for phosphatidylserine exposure to the outer leaflet of the plasma membrane using CF 594 Annexin V. Hoechst 33342 was used to stain the nuclei. Stained cells were observed under an epifluorescence microscope, using appropriate filters, as described in the methods section. Representative images documenting Dex-induced apoptosis of RS4;11 cells are shown in Figure 1, bottom right.

Dex-mediated upregulation of *RCAN1-1* correlates with apoptosis

RNA extracted from cells treated with ethanol or 1 μ M Dex was subjected to reverse transcription and RT-qPCR as described in the methods section. The data (Figure 2A) show that *RCAN1-1* was induced in cells that are susceptible to apoptosis, but cells that were resistant to Dex treatment showed little to no change in expression of *RCAN1-1*. The data also show that *RCAN1-4* was either not induced or minimally affected by Dex treatment in all cell lines (Figure 2A). Sup-B15 cells showed an 8.8 fold (± 2.7) induction of *RCAN1-1*, and a modest upregulation of *RCAN1-4* (2.5 ± 1.5 fold). *RCAN1-1* was upregulated 5.5 fold (± 0.3) by Dex in RS4;11 cells, while *RCAN1-4* expression was increased 2.7 fold (± 1.3). Expression of *RCAN1-1* in Kasumi-1 cells was increased by 4.5 fold (± 0.3) upon Dex treatment while *RCAN1-4* was induced by 2.2 fold (± 0.4). The induction of *RCAN1-4* by Dex could be a consequence of Dex-evoked increase in intracellular Ca^{2+} levels in correlation with apoptosis. Dex treatment had little to no effect on the Loucy and Sup T1 cell lines. *RCAN1-1* expression decreased by 0.7 fold (± 0.03) in Sup T1 cells, and was 1.2 fold (± 0.5) in Loucy cells in response to Dex. *RCAN1-4* expression after Dex treatment was essentially unaffected in both Sup T1 and Loucy cells.

Dex-mediated upregulation of *BIM* and *E4BP4* in sensitive cells

We [9] and others [6, 21] have previously demonstrated that Dex-evoked apoptosis of leukemic cells is facilitated by upregulation of *BIM*, with *E4BP4* acting as an upstream regulator in the CCRF-CEM human T-lymphoblastic leukemia model [9]. To extend our observations to other T-, B- and myeloid leukemia cell lines, we evaluated the ability of Dex to regulate *BIM* and *E4BP4* in our panel of human leukemia cell lines, using an endpoint PCR assay. We found that *E4BP4* expression was clearly upregulated by Dex in the sensitive RS4;11 and Kasumi-1 cells, but slightly induced in the sensitive B-leukemic cell line Sup-B15. There was a clear Dex-induced upregulation of *BIM* in all three GC-sensitive cell lines (Figure 2B). The GC-refractory Sup T1 and Loucy cells showed no Dex-mediated effect on either *BIM* or *E4BP4* expression. Our data suggest a correlation between GC-

evoked apoptosis and upregulation of *RCAN1-1*, *BIM* and *E4BP4*, except in Sup-B15 cells where *E4BP4* upregulation was minimal.

Crosstalk between E4BP4 and RCAN1-1

Our laboratory is evaluating the hypothesis that E4BP4 is an upstream transcriptional regulator that modulates expression of key pro- and anti-apoptotic genes. Since *RCAN1-1* is regulated coordinately with *E4BP4*, we sought to determine whether there was any crosstalk between RCAN1-1 and E4BP4. Interestingly, the *RCAN1-1* promoter has been reported to have putative E4BP4 response elements (EBPRE) [20]. We searched the *RCAN1-1* promoter for sequences resembling consensus EBPRE (Figure 3B) and found five putative EBPREs, including two that have been previously reported (Figure 3A). It is likely that GC-mediated *RCAN1-1* expression is driven by E4BP4 via these sequences, along with GRE-driven responses. In addition to the half GRE reported by Sun *et al.* [20], we identified a full GRE sequence in the *RCAN1-1* promoter, suggesting that GCs can directly regulate *RCAN1-1* expression. In a preliminary experiment to test whether E4BP4 might regulate RCAN1-1 expression, we utilized the mouse E4BP4 overexpressing CEM C1-15mE#3 cell line [9], derived from the parental GC-resistant CEM C1-15 line, which is sensitized to GC-evoked apoptosis. [9]. Here we demonstrate that CEM C1-15mE#3 have a restored ability for Dex-mediated *RCAN1-1* upregulation (Figure 3C). *RCAN1-1* transcript levels were induced 13.2-fold in CEM C7-14 cells, 1.9-fold in CEM C1-15 cells, and 11.7-fold in CEM C1-15mE#3 cells, suggesting that E4BP4 overexpression restores GC-dependent *RCAN1-1* upregulation. Indeed, *RCAN1* expression is regulated in a circadian fashion, in the same phase as *E4BP4*, according to a study that did not distinguish between *RCAN1-1* and *RCAN1-4* [22]. In cardiomyocytes, the oscillatory expression of *RCAN1-4*, but not *RCAN1-1*, serves as a cardioprotector and a vital mediator of the circadian rhythmicity of cardiovascular susceptibility to ischemia/reperfusion injury [23, 24].

Proposed model for genetic coordination of GC-evoked apoptosis

A schematic outlining the cross-talk among the three GC-induced genes, *BIM*, *E4BP4* and *RCAN1-1*, and their effect on lymphoid cell apoptosis, is presented in Figure 4. GCs are known to modulate their actions via binding to GR in the cytoplasm. The activated GR translocates to the nucleus where it binds as a dimer to conventional GRE sequences on target genes [2] or through other mechanisms. Although the *BIM* promoter lacks a traditional GRE, GCs regulate its expression via a novel intronic GR binding region [25], and through a region in the 3'UTR, by relieving miRNA mediated post-transcriptional repression [26]. GR-mediated upregulation of *E4BP4*, and subsequent induction of *BIM*, has been reported in correlation with apoptosis in lymphoid and osteoblastic cells [9, 27]. GR has been shown to regulate *E4BP4* transcription via a GR binding sequence (GBS) located ~5kb upstream of the transcription start site [28, 29]. We have demonstrated that E4BP4 regulates *BIM* expression [9], however, the mechanism of this regulation is currently being investigated. GREs and EBPREs have been reported in the *RCAN1-1* promoter [20], suggesting that *RCAN1-1* is both a primary and secondary target of GC-dependent regulation. A fundamental step in GC-evoked apoptosis is the release of cytochrome c from the mitochondria. There are primarily two types of channels that facilitate this process [30]. The mitochondrial apoptosis induced channel (MAC) is formed in the outer mitochondrial

membrane by oligomerization of pro-apoptotic members of the Bcl-2 family (Bax and Bak), in the absence of active inhibitory members (Bcl-2, Bcl-xL) [11]. Bim catalyzes the formation of MAC by facilitating Bax oligomerization [11]. Although the precise composition of the mitochondrial permeability transition pore (mPTP) is not clear, there is evidence that adenine nucleotide translocator (ANT), located on the inner mitochondrial membrane, and VDAC (voltage dependent anion channel), located on the outer mitochondrial membrane, form the mPTP [31]. RCAN1-1 is a homolog of the *Drosophila* protein nebula, which binds to ANT [32]. RCAN1-1L also has been proposed to bind to ANT and its sustained overexpression has been shown to open mPTP, stimulate mitochondrial degradation, and reduce cell survival [17]. RCAN1 is known to bind to and inhibit its PP3C phosphatase activity [16]. Since calcineurin is known to protect T- cells from GC-evoked apoptosis [33], RCAN1-1-mediated inhibition of calcineurin activity promotes apoptosis. Overexpression of RCAN1 has also been shown to activate caspase-3 activity to induce apoptosis [20]. In some models, such as cardiomyocytes, calcineurin facilitates Ca²⁺ dependent apoptosis, hence activation of RCAN1-4 has been shown to protect cells from apoptosis [23, 24].

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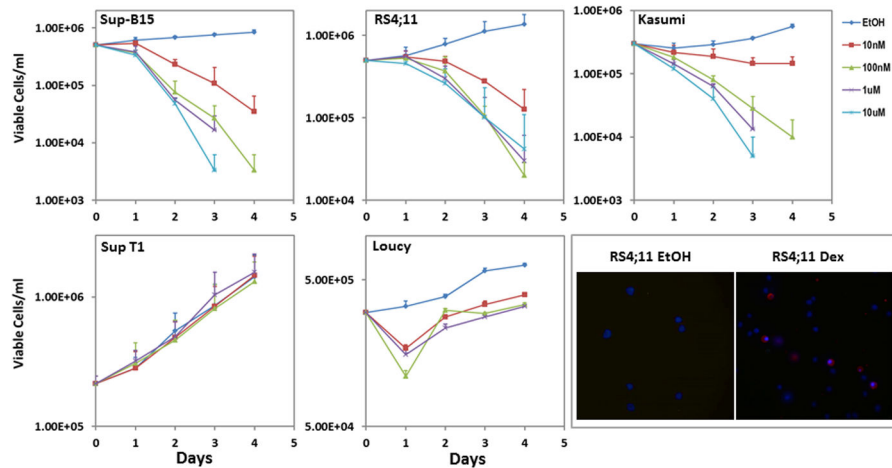


Figure 1. Sensitivity of Leukemia Cell lines to Dex

Sup B-15, RS4;11, Kasumi-1, Sup-T1 and Loucy cells were plated at a density of 1 to 5×10^5 cells/ml and treated for 4 days with either ethanol or 10nM, 100nM, 1µM or 10µM Dex. Cells were incubated at 37°C in a humidified 5% CO₂ incubator and trypan blue excluding viable cells were counted at 24 hour intervals. Each point represents a mean \pm SD of three independent experiments conducted in duplicates. Lower right panel shows representative epifluorescence images of ethanol and Dex treated RS4;11 cells stained for 30 min with CF 594 Annexin V and Hoechst 33342, to detect apoptosis. Cells with phosphatidylserine membrane eversion show a red rim.

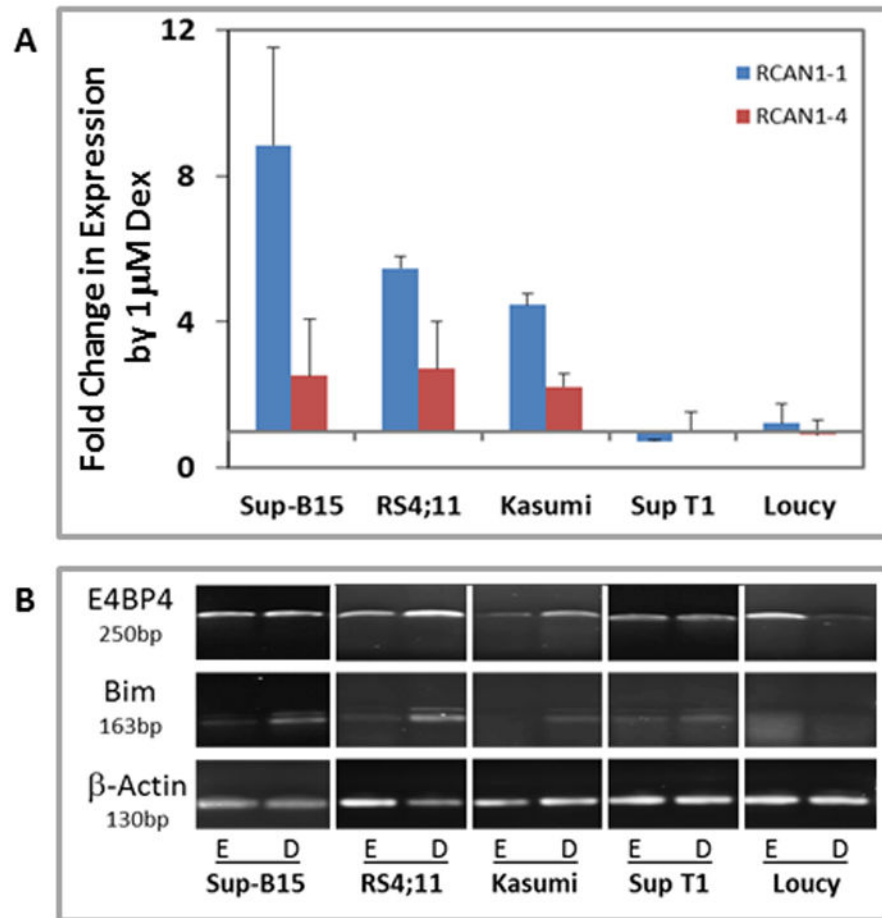


Figure 2. Dex-mediated upregulation of *RCAN1-1*, *E4BP4* and *BIM* in leukemic cells correlates with their ability to undergo apoptosis

Sup-B15, RS4;11, Kasumi-1, Sup T1 and Loucy cells were treated with ethanol or 1 μM Dex and real-time qPCR (Panel A) or endpoint PCR (Panel B) was performed using primers (Table 1) specific for *RCAN1-1* and *RCAN1-4* (Panel A), and *E4BP4* and *BIM* (Panel B). For Panel A, fold change in expression of each transcript by 1 μM Dex was calculated by the Pfaffl method using *β-Actin* as a reference. Data represent averages ± SD from three independent experiments. Panel B shows representative qualitative endpoint PCR data from one of two independent experiments using *β-Actin* as a reference.

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-1580 gagggcatgcccatgacaggcctcaggaagtctgaggacatgtaccaaggtgatcaaggcaca
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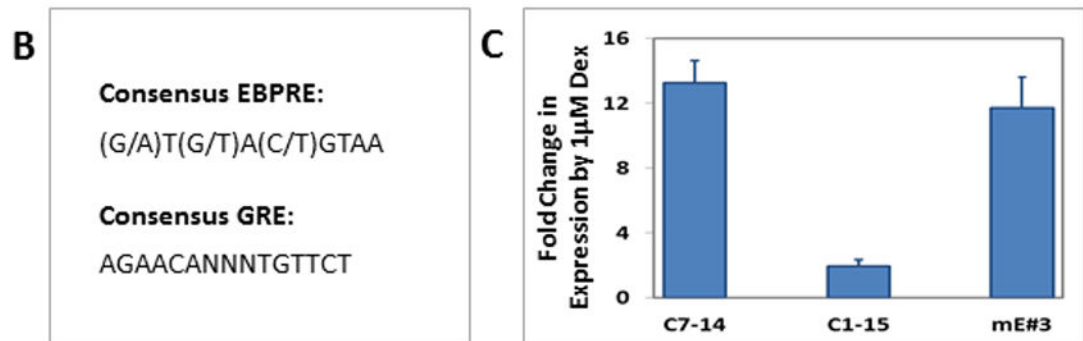


Figure 3. E4BP4 may be a regulator of *RCAN1-1* expression

Panel A: Nucleotide sequence of the human *RCAN1-1* gene promoter and first exon.

Sequence is derived from GenBank Accession #NG_007071. Transcription start site (+1) is highlighted in yellow, and is according to Sun *et al.*[20]. The first exon is in uppercase green, with the two start codons representing the RCAN1-1L and RCAN1-1S isoforms are in black. The putative GRE (Red and Pink) and EBPRES sequences (grey and blue) are highlighted. The red and grey sequences are those reported by Sun *et al.*[20]. **Panel B:** Consensus GRE and EBPRES sequences. **Panel C:** CEM C7-14, CEM C1-15 and CEM C1-15mE#3 cells were treated with ethanol or 1µM Dex and real-time qPCR was performed

using primers (Table 1) specific for *RCANI-1*. Fold change in expression was calculated by the Pfaffl method using β -Actin as a reference. Data represent averages \pm SD from three independent experiments.

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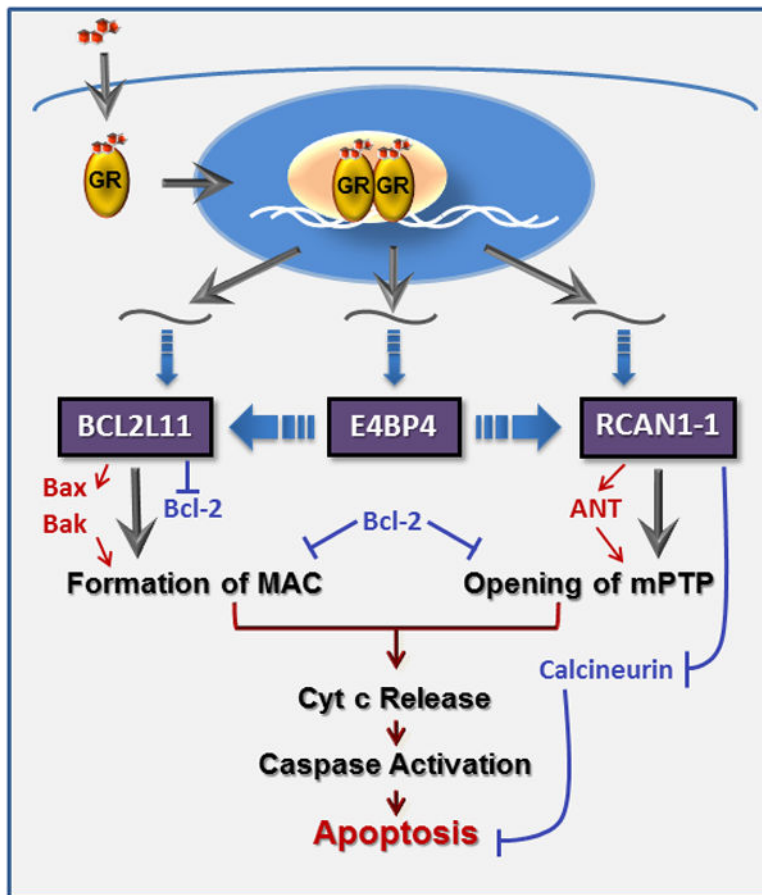


Figure 4. Genetic control of GC-evoked apoptosis

GCs bind to the GR, which dimerizes and translocates to the nucleus (blue oval), where it alters expression of target genes in conjunction with a coregulatory complex (beige oval). Three genes we focus on here are *Bcl2L11* (*BIM*), *E4BP4* and *RCAN1-1*, all of which are direct targets of GR. *E4BP4* may modulate *BIM* and *RCAN1-1* transcription. *Bim* plays a crucial role in formation of MAC, via activation of *Bax/Bak* and inhibition of *Bcl-2*. *RCAN1-1* binds to and activates *ANT* to facilitate opening of the mPTP. *Bcl-2* inhibits both MAC and mPTP formation. Opening of both channels leads to release of cytochrome c, driving the classic apoptosis cascade.

Table 1

Primers used for PCR

Transcript	Forward Primer	Reverse Primer	Product Size
RCAN1-1	5'-ACCATCGCCTGTCACCTGGA-3'	5'-GGTGATGTCCTTGTCATACGTCCT-3'	96bp
RCAN1-4	5'-CTCCCTGATTGCCTGTGTGG-3'	5'-TTCCTCTTCTTCCTCCTTCT-3'	484bp
E4BP4	5'ATGGGGAATTCTTCTCTGG3'	5'CTTGATCCGGAGCTTGTGT3'	250bp
BIM	5'CAGATATGCGCCAGAGATA3'	5'ACCAGGCGGACAATGTAAC3'	163bp
β -actin	5'AGTCCTCTCCCAAGTCCACA3'	5'CACGAAGGCTCATCATCAA3'	130bp

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