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# Correlation of glucocorticoid-mediated E4BP4 upregulation with altered expression of pro- and anti-apoptotic genes in CEM human lymphoblastic leukemia cells

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ABSTRACT

In *Caenorhabditis elegans*, motorneuron apoptosis is regulated via a *ces-2–ces-1–egl-1* pathway. We tested whether human CEM lymphoblastic leukemia cells undergo apoptosis via an analogous pathway. We have previously shown that E4BP4, a *ces-2* ortholog, mediates glucocorticoid (GC)-dependent upregulation of *BIM*, an *egl-1* ortholog, in GC-sensitive CEM C7-14 cells and in CEM C1-15mE#3 cells, which are sensitized to GCs by ectopic expression of E4BP4. In the present study, we demonstrate that the human *ces-1* orthologs, *SLUG* and *SNAIL*, are not significantly repressed in correlation with *E4BP4* expression. Expression of *E4BP4* homologs, the PAR family genes, especially *HLF*, encoding a known anti-apoptotic factor, was inverse to that of *E4BP4* and *BIM*. Expression of pro- and anti-apoptotic genes in CEM cells was analyzed via an apoptosis PCR Array. We identified *BIRC3* and *BIM* as genes whose expression paralleled that of *E4BP4*, while *FASLG*, *TRAF4*, *BCL2A1*, *BCL2L1*, *BCL2L2* and *CD40LG* as genes whose expression was opposite to that of *E4BP4*.



## 1. 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]. The pharmacological actions of GC therapy include, among other effects, the suppression of the immune response and shrinkage of T-lymphocyte population. Loss of T-lymphocytes via apoptosis is a major cause of the immunosuppressive actions of GCs [1], however, the mechanisms by which GCs evoke an apoptotic response is still not clear. Relapse of leukemia after GC therapy is often associated with development of resistant cells that no longer respond to GC-evoked apoptosis [2]. The primary action of GCs is mediated through their interactions with the glucocorticoid receptor (GR), a transcription factor from the nuclear receptor family, which modulates up or downregulation of genes containing GC responsive elements (GRE) or through interactions with other transcription factors, coactivators and corepressors [2]. We have demonstrated that GR transcriptional activity correlates with susceptibility of cells to GC-evoked apoptosis. Several laboratories have analyzed changes in gene expression profiles induced by GCs in an effort to identify candidate genes modulating GC-evoked apoptosis of leukemic lymphoid cells [3,4].

We have established a crucial relationship between GC-dependent upregulation of *E4BP4* and sensitivity to GC-evoked apoptosis in the human leukemic CEM cell culture model [5]. E4BP4 (or NFIL3) is a bZIP transcription factor with a role in anti-inflammatory response, circadian oscillation, apoptosis regulation, and immune cell development [6–8]. *E4BP4* is an evolutionarily conserved homolog of the pro-apoptotic *Caenorhabditis elegans* death specification gene *ces-2*, which is known to downregulate the downstream survival gene *ces-1* to allow the upregulation of the pro-apoptotic gene *egl-1*, resulting in apoptosis in neurosecretory motor neuron cells. An analogous pathway in humans has been speculated (Fig. 1) [9]. Human orthologs of *ces-1*, *SLUG* and *SNAIL*, have pro-survival functions. Expression of *SLUG* is abundant in hematopoietic stem cells (HSCs) and in undifferentiated progenitor cells but not in pro T and pro-B cells, or beyond [10]. Both Slug and Snail have been shown to promote tumor invasiveness, cell survival, protect from apoptosis, and induce chemoresistance [10,11]. The role of Slug and Snail in GC-evoked lymphoid cell apoptosis, or their regulation by E4BP4, has not yet been explored. The human ortholog of egl-1, Bim (a BH-3 only protein from the Bcl-2 family), an important regulator of the intrinsic pathway of

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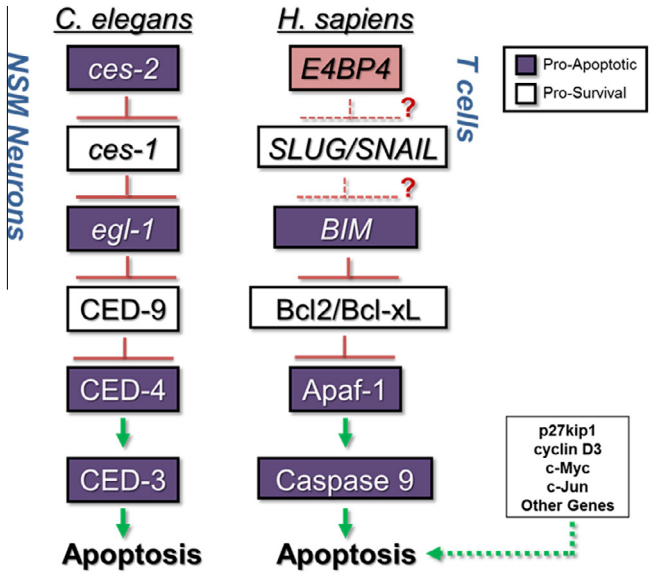
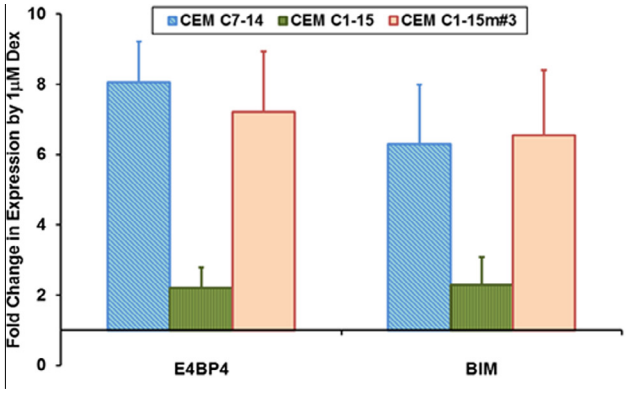


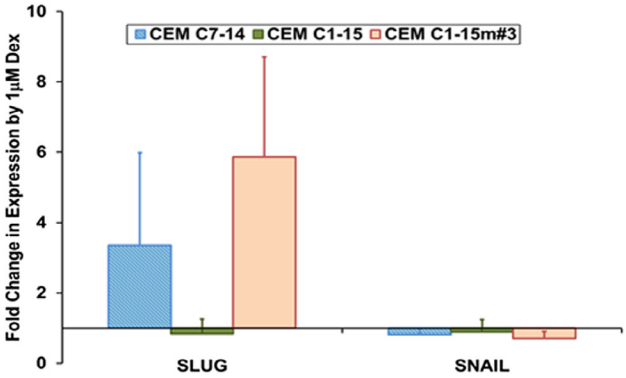

**Fig. 1.** Conserved pathway for apoptosis: analogy between the known pathway for apoptosis in neurosecretory motor neurons of *C. elegans* (left) and the hypothetical pathway in human leukemic T cells (right). In *C. elegans*, *ces-2* upregulates the proapoptotic *egl-1* via repression of the survival gene *ces-1*, to eventually activate CED-3 and trigger apoptosis. In human leukemic T cells, upregulation of *BIM* and subsequent inhibition of anti-apoptotic members of the Bcl-2 family leads to activation of caspases. Expression of the *ces-2* ortholog *E4BP4* has been shown to facilitate GC-mediated *BIM* upregulation, but the role of *SLUG*/*SNAIL* is not clear. Several other proteins including p27kip1, cyclin D3, c-Myc and c-Jun have been shown to facilitate apoptosis.

apoptosis, has been previously shown to play a vital role in apoptosis in response to various stimuli [12,13], including GCs [3,14]. *BIM* is upregulated in GC sensitive human leukemic CEM C7-14 cells in conjunction with GC-evoked apoptosis. We have demonstrated that ectopic E4BP4 expression restores sensitivity to GC-evoked apoptosis in a refractory CEM sister cell line, CEM C1-15, via restoration of *BIM* induction [15].

E4BP4 belongs to the PAR (Proline and Acid Rich) subfamily of bZIP transcription factors, based on its DNA-binding specificity, even though it lacks a PAR region (Fig. 3A) [16]. Other key members of this subfamily include hepatic leukemia factor (HLF), thyrotrophic embryonic factor (TEF) and albumin D-site binding protein (DBP). E4BP4 and PAR proteins regulate transcription by binding to an E4BP4 response element (EBPRE), which has a consensus sequence of (G/A)T(G/T)A(C/T) GTAA (C/T) [21]. Generally, E4BP4 and other PAR proteins have temporally antiparallel expression patterns and antagonize each other's action, as has been extensively studied in the circadian regulation of clock genes [17]. In contrast to the apoptosis inducing action of E4BP4, TEF is reported to have antiapoptotic activity [18], while HLF is highly expressed in leukemic stem cells (LSCs) and has been implicated in chemoresistance [19]. While the PAR family transcription factors have been characterized as transcriptional activators, E4BP4 is a repressor [16]. In several cases E4BP4 has been shown to act as a transactivator, and a transactivation domain (TAD) has been reported just upstream of the transrepressor domain (TRD). E4BP4 has also been recognized as a survival factor in a cell-type specific context. In pro-B lymphocytes, E4BP4 activates downstream genes and stimulates IL-3 (a cytokine) promoting cell survival [20]. Similarly, TEF and DBP have been shown to promote oxidative stress induced

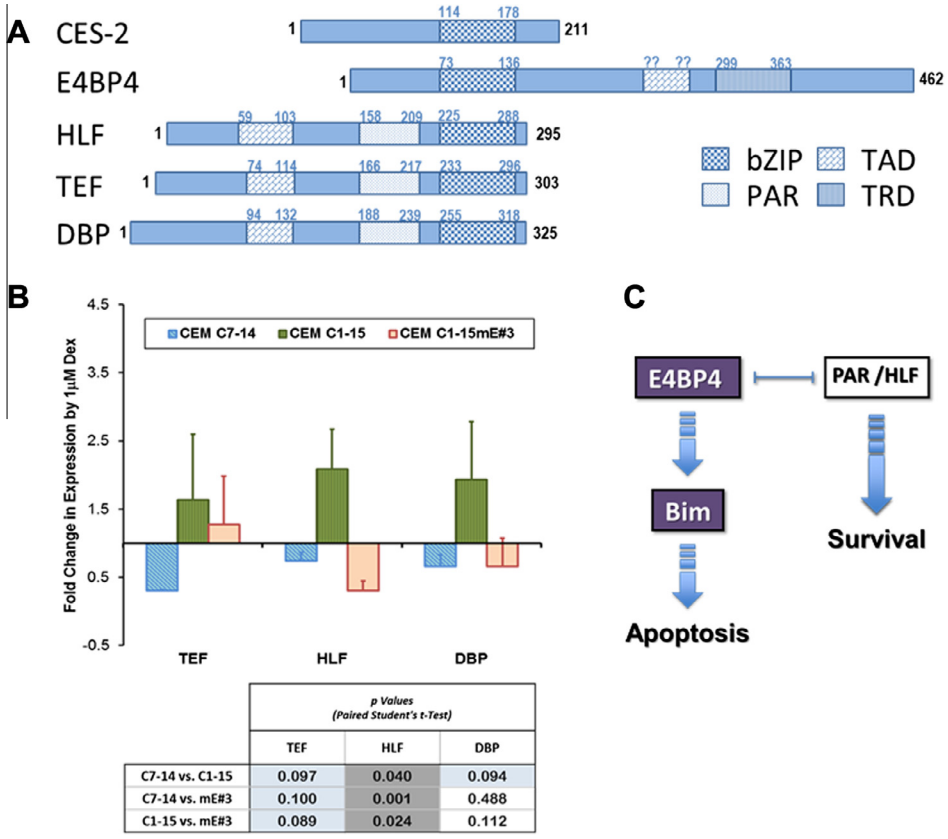



| | p Values (Paired Student's t-Test) | |
|---|---|---|
| | E4BP4 | Bim |
| C7-14 vs. C1-15 | 0.000 | 0.016 |
| C7-14 vs. mE#3 | 0.118 | 0.345 |
| C1-15 vs. mE#3 | 0.002 | 0.012 |




| | p Values (Paired Student's t-Test) | |
|---|---|---|
| | SLUG | SNAIL |
| C7-14 vs. C1-15 | 0.122 | 0.267 |
| C7-14 vs. mE#3 | 0.197 | 0.221 |
| C1-15 vs. mE#3 | 0.036 | 0.166 |

**Fig. 2.** Dex-mediated upregulation of *BIM* and *E4BP4* in sensitive CEM cells does not correlate with downregulation of *SLUG*/*SNAIL*: CEM C7-14, CEM C1-15 and CEM C1-15mE#3 cells were treated with ethanol or 1 μM Dex and real-time qPCR was performed using primers (Table 1) specific for *E4BP4*, *BIM* (Panel A), *SLUG* and *SNAIL* (Panel B). Fold change in expression of each transcript by 1 μM Dex was calculated by the Pfaffl method using *ACTINB* as a reference. Data represent averages ± SD from three independent experiments. Statistical significance was calculated using a paired Student's *t*-test, shown next to the charts. Shaded boxes represent significant differences with *p*-values $\leqslant 0.05$.




| p Values (Paired Student's t-Test) | TEF | HLF | DBP |
|---|---|---|---|
| C7-14 vs. C1-15 | 0.097 | 0.040 | 0.094 |
| C7-14 vs. mE#3 | 0.100 | 0.001 | 0.488 |
| C1-15 vs. mE#3 | 0.089 | 0.024 | 0.112 |

**Fig. 3.** Inverse relationship between E4BP4 and other PAR-family homologs: Panel A: representation of protein structural homology between *C. elegans* CES-2, and human PAR family proteins including E4BP4, HLF, TEF and DBP.TAD = transactivation domain; TRD = transrepression domain; PAR = proline & acid rich domain. Panel B: real-time qPCR was performed on ethanol or 1 μM Dex treated CEM cells and using primers (Table 1) specific for *TEF, HLF* and *DBP*. Fold change in expression was calculated by the Pfaffl method using *ACTINB* as a reference. Data represent averages ± SD from three independent experiments. Statistical significance was calculated using a paired Student's *t*-test, shown below the chart. Dark shaded boxes represent significant differences with *p*-values ⩽0.05, light shaded boxes represent *p* values ⩽0.10. Panel C: proposed relationship between E4BP4 and other PAR genes and their effect on cell survival and apoptosis.

apoptosis via upregulation of BIK, a BH3-only proapoptotic member of the Bcl2 family [21].

To investigate the role of E4BP4 in GC-evoked apoptosis of human leukemic cells, we have previously created a cell line, CEM C1-15mE#3, that ectopically expresses mouse E4BP4 in the GC-resistant CEM C1-15 cells [15]. These cells are sensitized to GC-evoked apoptosis and *BIM* upregulation, analogous to the GC-sensitive sister cell line, CEM-C7-14. In the present study, we have further investigated GC-mediated regulation of *SLUG, SNAIL, HLF, TEF* and *DBP* in all three cell lines, in an effort to understand the role of these genes in GC-evoked apoptosis, and to evaluate the contribution of E4BP4 in their expression. Additionally, we have evaluated the GC-dependent regulation of transcripts of genes implicated in apoptosis using the RT$^2$ Profiler™ PCR Array from SA Biosciences.

## 2. Materials & methods

### 2.1. Reagents

Dexamethasone (Dex) was purchased from EMD Biosciences (Madison, WI). RNA was extracted using TRIzol reagent (Cat #15596-026) from Invitrogen (La Jolla, CA). Reagents for reverse transcription (RT), including M-MLV reverse transcriptase, oligo(dT)$_{15}$ primer, RNasin®Ribonuclease inhibitor, dNTP mix were purchased from Promega Life Sciences (Madison, WI). SYBR®Jump-Start™*Taq* Ready Mix (Cat #4438) for real-time qPCR was from Sigma–Aldrich (St. Louis, MO). RNeasy® Mini Kit (Cat #74104) and DNase Column (Cat #79254) were from Qiagen (Valencia, CA,). RT$^2$ First Strand Kit (Cat #C-03), RT$^2$ RNA Quality Control (QC) PCR Array (Cat #PAHS-999) and RT$^2$ Profiler™ modified human apoptosis PCR Arrays (CAPH09369) were from SA Biosciences. Other reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma–Aldrich.

### 2.2. Cell culture

The CCRF-CEM derived human T-ALL cell lines CEM C7-14 and CEM C1-15 are sensitive and resistant to GCs, respectively, and are generous gifts from Dr. E.B. Thompson, University of Texas Medical Branch, Galveston. The CEM C1-15m#3 subclone, stably transfected with mouse E4BP4, has been characterized previously and is sensitive to GC-evoked apoptosis [15]. Cells were maintained in log phase at 37 °C in a 5% $CO_2$ incubator in RPMI 1640 containing L-Glutamine (Catalog #50-020-PB) from Cellgro (Manassas, VA) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Cat #S11150) from Atlanta Biologicals (Lawrenceville, GA).

### 2.3. Reverse transcription and RT-qPCR (real-time-quantitative PCR) analysis

Cells were treated for 24 h at a density of $5 \times 10^5$ cells/ml with either absolute ethanol (control/vehicle) or 1 μM Dex and total RNA was extracted in 1 ml of TRIzol reagent, according to instructions from the manufacturer. Reverse transcription was carried out

**Table 1**
PCR primers.

| Transcript | Forward primer | Reverse primer | Product size (bp) |
|---|---|---|---|
| E4BP4 | 5′ATGGGGAATTCTTTCTCTGG3′ | 5′CTTTGATCCGGAGCTTGTGT3′ | 250 |
| Bim | 5′CAGATATGCGCCCAGAGATA3′ | 5′ACCAGGCGGACAATGTAAC3′ | 163 |
| Slug | 5′CCCTGAAGATGCATATTCGGAC3′ | 5′CTTCTCCCCCGTGTGAGTTCTA3′ | 116 |
| Snail | 5′GCTGCAGGACTCTAATCCAGAGTT3′ | 5′GACAGAGTCCCAGATGAGCATTG3′ | 130 |
| HLF | 5′GCGTGCTCAGGTCCCTGCTG3′ | 5′AGGCCCCAGGAATGCCGACT3′ | 143 |
| TEF | 5′GCTCTCCAGTGTGCCAGGCG3′ | 5′CGGGCATCCCGTGACCGTTT3′ | 189 |
| DBP | 5′TCCAGGTGCCGGAGGAGCAG3′ | 5′GTTCTCCTTGAGCCGCCGGG3′ | 104 |
| β-Actin | 5′AGTCCTCTCCCAAGTCCACA3′ | 5′CACGAAGGCTCATCATTCAA3′ | 130 |

by incubating 7 μg of RNA for 3 h at 42 °C with 0.5 mg of oligo(dT)$_{15}$, 1 μl (~200 U) of M-MLV reverse transcriptase, 0.5 mM dNTP mix, and 100 U of RNase inhibitor. For RT-qPCR, SYBR® Green JumpStart™ Taq ReadyMix was mixed with 250 nM each of forward and reverse primers (Table 1) and 1 μl of reverse transcription product in a final volume of 25 μl, and run on an ABI Prism 7300 Real Time PCR System.

To quantitate the relative expression levels, the cycle threshold (Ct) values for each sample were used to calculate fold inductions using the Pfaffl method formula: $(E)\Delta^{\text{CTtarget(control-sample)}}/(E)\Delta^{\text{CTreference(control-sample)}}$, where E represents primer efficiency which was calculated for each sample reaction using LinRegPCR software. The reference gene was β-actin. Statistical significance was calculated using a paired Student's *t*-test, with a $p \leqslant 0.05$ denoting high significance and $p \leqslant 0.1$ denoting moderate significance.

### 2.4. RT$^2$ Profiler™ PCR Array

CEM-C7-14, CEM-C1-15, and CEM-C1-15m#3 cells were treated at a density of $5.0 \times 10^5$ cells/ml with ethanol or 1 μM Dex for a 24-h period. Approximately 20 ml of cell suspension (~$1.0 \times 10^7$ cells) were harvested using the Qiagen RNeasy® Mini Kit following the manufacture's protocol. Residual DNA was removed using a DNase column. First strand cDNA synthesis was completed with the RT$^2$ First Strand Kit, using 1.5 μg of total RNA. RNA quality was assessed on the ABI Prism 7300 using the RT$^2$ RNA QC PCR Array according to the manufacturer's protocol. Each RT sample that passed the quality control check was analyzed using a modified human apoptosis PCR Array. Data were analyzed using $\Delta\Delta C_t$ method through the SABioscience PCR Array Data Analysis Web Portal. Data presented are averages of two independent experiments.

## 3. Results and discussion

We have previously demonstrated that E4BP4-dependent upregulation of Bim contributes to GC-evoked apoptosis of human leukemic CEM cells, using a cell line derived from the GC-resistant CEM C1-15 cells that ectopically expresses mouse E4BP4 (CEM C1-15mE#3) [15]. Here, we have extended our studies to evaluate the role of E4BP4 in GC-dependent regulation of genes whose protein products are known to crosstalk with E4BP4, and genes implicated in apoptosis.

### 3.1. Parallel regulation of E4BP4 and BIM by Dex

As reported previously, significant Dex-mediated upregulation of *E4BP4* (8.1 and 7.2-fold) and *BIM* (6.3 and 6.5) transcripts was seen in CEM C7-14 and CEM C1-15mE#3 cells respectively, while CEM-C1-15 cells showed a distinctly blunted response for both *E4BP4* (2.2-fold) and *BIM* (2.3-fold) (Fig. 2A). One thousand base pairs of the *BIM* promoter was scanned for the presence of EBPRE consensus sequences using transcription factor binding site prediction programs, AliBaba 2.1 and TRANSFAC, but none were detected (data not shown), suggesting that E4BP4 must regulate *BIM* expression either through other factors such as Slug/Snail or in conjunction with other transcription factors.

### 3.2. E4BP4 does not upregulate BIM via inhibition of SLUG and SNAIL expression

To test whether E4BP4-dependent apoptosis follows an *E4BP4* > *SLUG/SNAIL* > *BIM* pathway parallel to the evolutionarily conserved *ces-2* > *ces-1* > *egl-1* pathway in CEM cells (Fig. 1), we tested whether expression of the survival genes *SLUG* and *SNAIL* had an inverse relationship with *E4BP4* and *BIM*. In contrast to this hypothesis, *SLUG* transcripts were upregulated by 1 μM Dex in CEM C7-14 and CEM C1-15mE#3 cells by 3.4 and 5.9-fold respectively, and were marginally downregulated (0.84-fold) in CEM C1-15 cells. Data between individual cell lines were not statistically significant, as determined by a paired Student's *t*-test, except for CEM C1-15 and CEM C1-15mE#3, where *p* was 0.036 (Fig. 2B). *SNAIL* expression was minimally repressed by 1 μM Dex in all three cell lines (0.7 to 0.9-fold) (Fig. 2B), and again the difference between pairs of cell lines was not significant, but the extent of repression (compared to ethanol treated controls) for CEM C7-14 and CEM C1-15mE#3 cells was marginally significant with a *p* value of 0.09 and 0.06 respectively. These data suggest that Dex-evoked *SNAIL* downregulation may facilitate *BIM* upregulation.

### 3.3. Inverse regulation of PAR-family transcripts in comparison to E4BP4

HLF, TEF and DBP share the same bZIP domain and DNA binding specificity as E4BP4, but lack a TRD (Fig. 3A), and are known to have an opposite outcome on transcription of target genes compared to E4BP4. They have been shown to promote cell survival and chemoresistance, and their expression profile is opposite to that of E4BP4. To determine whether HLF, TEF and DBP have a role in sensitivity or resistance to GCs in the CEM cell model, we determined the effect of 1 μM Dex on transcript levels of all three genes in GC-sensitive CEM C7-14 and CEM C1-15mE#3 cells and in GC-resistant CEM C1-15 cells (Fig. 3B). All three transcripts were upregulated by Dex in the GC-resistant CEM C1-15 cells, and downregulated in GC-sensitive CEM C7-14 cells. In CEM C1-15mE#3 cells, both *HLF* and *DBP* were downregulated, while *TEF* was marginally upregulated. All data were significant for *HLF* with $p \leqslant 0.05$, and for *TEF* between CEM C7-14 and CEM C1-15 cells, with $p \leqslant 0.10$. While *E4BP4* is upregulated by Dex in all three cell lines, the extent of upregulation is 4-fold higher for the sensitive lines than for the resistant line (Fig. 2A). Our results suggest a mutually antagonistic relationship between PAR proteins and

**Table 2**
Genes regulated 2-fold or higher by 1 μM dexamethasone

| Table 2: Genes Regulated Two-Fold or Higher by 1μM Dexamethasone | | | | | | | |
|---|---|---|---|---|---|---|---|
| Symbol | Unigene | RefSeq | Description | Gene Name | CEM C7-14 | CEM C1-15 | C1-15 mE#3 |
| **A: Genes Upregulated in CEM C7-14 cells** | | | | | | | |
| BIRC3 | Hs.127799 | NM_001165 | Baculoviral IAP repeat-containing 3 | AIP1/API2 | 9.80 | 3.28 | 13.83 |
| BCL2L11 (BIM | Hs.469658 | NM_006538 | BCL2-like 11 (apoptosis facilitator) | BAM/BIM | 8.81 | 2.90 | 5.45 |
| TNFSF10 | Hs.478275 | NM_003810 | Tumor necrosis factor (ligand) superfamily, member 10 | APO2L/Apo-2L | 2.07 | -1.06 | -1.56 |
| **B: Genes Upregulated in CEM C1-15 cells** | | | | | | | |
| CD27 | Hs.355307 | NM_001242 | CD27 molecule | S152/T14 | -1.07 | 3.15 | 2.08 |
| CFLAR | Hs.390736 | NM_003879 | CASP8 and FADD-like apoptosis regulator | CASH/CASP8AP | 1.16 | 2.41 | 1.47 |
| BCL2L2 | Hs.410026 | NM_004050 | BCL2-like 2 | BCL-W/BCLW | -1.46 | 2.23 | -2.01 |
| BAD | Hs.370254 | NM_004322 | BCL2-associated agonist of cell death | BBC2/BCL2L8 | 1.12 | 2.17 | 1.40 |
| CD40LG | Hs.592244 | NM_000074 | CD40 ligand | CD154/CD40L | -1.33 | 2.15 | -2.14 |
| AKT1 | Hs.525622 | NM_005163 | V-akt murine thymoma viral oncogene homolog 1 | AKT/PKB | -1.41 | 2.03 | -1.01 |
| **C: Genes Downregulated in CEM C7-14 cells** | | | | | | | |
| FASLG | Hs.2007 | NM_000639 | Fas ligand (TNF superfamily, member 6) | APT1LG1/CD178 | -4.37 | -3.29 | -11.04 |
| BCL2A1 | Hs.227817 | NM_004049 | BCL2-related protein A1 | ACC-1/ACC-2 | -2.32 | 1.42 | -1.85 |
| TRAF4 | Hs.8375 | NM_004295 | TNF receptor-associated factor 4 | CART1/MLN62 | -2.02 | 1.25 | -1.82 |
| **D: Genes Downregulated in CEM C1-15mE#3 cells** | | | | | | | |
| SNAI1(SNAIL) | Hs.48029 | NM_005985 | Snail Homolog 1 (Drosophila) | SNAIL | -1.15 | 1.09 | -5.05 |
| CIDEA | Hs.249129 | NM_001279 | Cell death-inducing DFFA-like effector a | CIDE-A | -1.19 | 1.44 | -4.13 |
| TNFRSF25 | Hs.462529 | NM_003790 | Tumor necrosis factor receptor superfamily, member 25 | APO-3/DDR3 | -1.27 | 1.96 | -2.80 |
| PYCARD | Hs.499094 | NM_013258 | PYD and CARD domain containing | ASC/CARD5 | 1.43 | 1.54 | -2.65 |
| NOL3 | Hs.513667 | NM_003946 | Nucleolar protein 3 (apoptosis repressor with CARD dor | ARC/CARD2 | -1.14 | 1.14 | -2.02 |
| BCL2L1 | Hs.516966 | NM_138578 | BCL2-like 1 | BCL-XL/S | -1.42 | 1.93 | -2.01 |

Genes upregulated >2-fold are in red; genes downregulated >2-fold are in blue.

E4BP4 in modulating cell survival and apoptosis, respectively (Fig. 3C).

### 3.4. Regulation of genes implicated in apoptosis

In an effort to identify additional genes playing a role in GC-evoked apoptosis, and their dependence on E4BP4, gene expression profiles for CEM-C7-14, CEM-C1-15, and CEM-C1-15mE#3 cells were analyzed using the Human RT$^2$ Profiler™ Apoptosis PCR Array. Total RNA was extracted from cells treated for 24 h with ethanol or 1 μM Dex, and first-strand synthesis was performed and PCR Arrays were run as described in the Section 2. Two independent experiments, with six groups each (control and treated groups for each of three cell types) were performed. The software, RT$^2$ Profiler™ PCR Data Analysis v3.5, available through the SA Biosciences website, was used for data analysis. Briefly, control and Dex-treated threshold cycles for each gene were normalized to the average of three housekeeping genes, and the expression levels were calculated using the ΔΔCt method. Only those genes that passed the software's quality check for all groups were analyzed. Transcripts with a Ct value greater than 35 in any sample were excluded from the analysis. Additionally, any gene that showed a variation of greater than 3 standard deviations within the two replicates for any group was eliminated from the analysis.

For a gene to be considered as regulated by GCs in an E4BP4-dependent manner, it must be reproducibly up- or downregulated by GCs in the GC-sensitive CEM C7-14 and CEM C1-15mE#3 cells but not in the GC-resistant CEM C1-15 cells, suggesting that ectopic expression of E4BP4 restores GC-dependent transcriptional regulation in CEM C1-15mE#3 cells. Following these criteria, two genes, *BCL2L11 (BIM)* and *BIRC3* showed a significantly greater upregulation by 1 μM Dex in the sensitive cells compared to the resistant cells (Table 2A), although both these genes were upregulated >2-fold in CEM C1-15 cells. CEM C1-15 cells express functional GR, which has been shown to be transcriptionally active with a blunted response to GC stimulus, hence modest regulation of GC-dependent genes is to be expected [4]. Bim is a well-known mediator of GC-evoked apoptosis of lymphoid leukemic cells [14], and we have shown that its expression correlates with *E4BP4* expression [15] (Fig. 2A). BIRC3 or IAP-1 (Inhibitor of Apoptosis-1) is known to bind to TRAFs (Tumor necrosis factor Receptor Associated Factors) and inhibit apoptosis by interfering with activation of caspases. *BIRC3* has been previously shown to be upregulated in correlation with GC-evoked apoptosis, and has been proposed as a

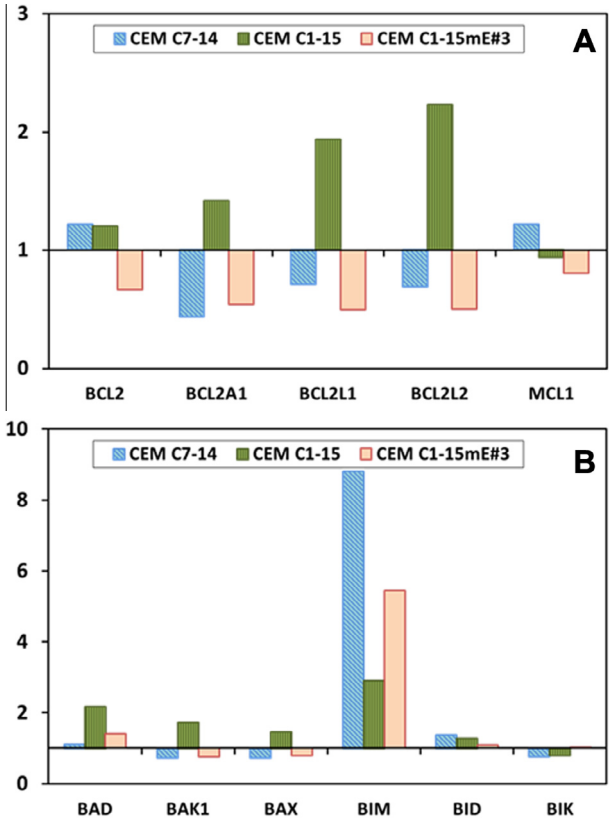



**Fig. 4.** Correlation of sensitivity to GCs and expression of Bcl-2 family genes: Data for anti-apoptotic (Panel A) and pro-apoptotic (Panel B) members of the Bcl-2 family were extracted from the RT$^2$ Profiler™ Apoptosis PCR Array.

rescue mechanism activated in cells trying to escape apoptosis [4]. TNFSF10, a gene encoding a member of the TNF family, that has been shown to induce apoptosis in tumor cells by activating caspases, was upregulated only in CEM C7-14 cells, suggesting that its upregulation by Dex is not mediated by E4BP4.

Six genes were upregulated by GCs in the resistant CEM C1-15 cells (Table 2B). *CD27*, was also upregulated in CEM C1-15mE#3 cells, hence may not have any correlation with E4BP4 expression. CD27 is a member of the TNF receptor superfamily, and is thought to play a role in survival of activated T cells [22]. Three genes, *CFLAR*, *BAD* and *AKT1* are only upregulated in CEM C1-15 cells, and are not significantly regulated in the sensitive cells, and may contribute to resistance to GCs in CEM C1-15 cells. CFLAR is structurally similar to caspase-8, but lacks caspase activity and inhibits apoptosis [23]. BAD is a known proapoptotic member of the Bcl2 family [24], and CEM C1-15 cells resist apoptosis in spite of BAD upregulation. The proapoptotic activity of BAD is inhibited by its phosphorylation via AKT and MAP kinase [24]. It is possible that in CEM C1-15 cells, BAD is inactivated by phosphorylation. In support of this hypothesis, *AKT1*, encoding a serine/threonine-protein kinase survival factor known to phosphorylate BAD (31), is also upregulated only in the resistant cells. Two genes, *BCL2L2* and *CD40LG* are upregulated >2-fold in CEM C1-15 cells but downregulated >2-fold in CEM C1-15mE#3 cells, suggesting that E4BP4 may play a role in their repression. BCL2L2 encodes the anti-apoptotic member of the Bcl2 family, Bcl-w, and has been shown to block GC-induced apoptosis [25]. CD40LG is a negative regulator of apoptosis, is expressed on T-cell surfaces, and engages CD40 on the B cell surface to facilitate immunoglobulin class switching. It has been shown to be negatively regulated by E4BP4 [26].

Of the three genes downregulated >2-fold in CEM-C7-14 cells (Table 2C), *FASLG* was also downregulated in the other two cell lines, but most strongly in CEM C1-15mE#3 cells. It is a member of the TNF superfamily, known to antagonize with GCs in regulating T cell apoptosis [27]. *BCL2A1* and *TRAF4* were downregulated in CEM C7-14 cells >2-fold, in CEM C1-15mE#3 cells by about 1.8-fold and were not significantly altered in CEM C1-15 cells, thus E4BP4 may contribute to their repression. BCL2A1 encodes an anti-apoptotic member of the Bcl2 family, Bfl1 [25], and its downregulation concurs with sensitivity of CEM cells. TRAF4 is a TNF receptor associated factor and a negative regulator of NFκB activation and apoptosis [28], thus its downregulation promotes apoptosis.

Six genes were downregulated significantly (>2-fold) only in CEM C1-15mE#3 cells, suggesting that ectopic E4BP4 expression may facilitate their repression. Notable among these was *SNAI1*encoding Snail, an ortholog of *C. elegans* ces-1 gene which is a survival factor, and may be an intermediate in E4BP4-dependent upregulation of *BIM* (Fig. 1). This is in agreement with our data in Fig. 2B. Another notable gene in this group was *BCL2L1*, an anti-apoptotic member of the Bcl2 family, was upregulated 1.93-fold in CEM C1-15 cells, and its downregulation via E4BP4 may sensitize these cells to GC-evoked apoptosis.

### 3.5. Regulation of Bcl-2 family genes

The RT$^2$ Profiler™ Apoptosis PCR Array included several pro- and anti-apoptotic genes belonging to the Bcl-2 family. Because of the critical role these genes are known to play in the execution of apoptosis, data corresponding to these genes was extracted and charted separately (Fig. 4). Of the anti-apoptotic genes analyzed, all except *MCL1* were upregulated in the GC-resistant CEM C1-15 cells, although only *BCL2L1* and *BCL2L2* were upregulated approximately 2-fold (Fig. 4A). The sensitive cells showed a general downregulation of anti-apoptotic genes, with a 2-fold repression of *BCL2L1* and *BCL2L2* in CEM C1-15mE#3 cells, suggesting that E4BP4 expression played a role in their repression. Among the pro-apoptotic genes analyzed, only *BIM* was markedly upregulated in the two sensitive cell lines compared to the resistant line (Fig. 4B). Since these data are from duplicate arrays, they are not statistically significant, however the trends are informative.

Our studies demonstrate that E4BP4-mediated upregulation of *BIM* may be independent of *SLUG/SNAIL* repression, unlike the orthologous pathway in *C. elegans*. Expression patterns of the PAR family genes *TEF, HLF* and *DBP* are opposite to that of *E4BP4*, with *HLF* showing the most statistical significance. Ectopic expression of E4BP4 in the resistant CEM C1-15 cells restores GC-mediated regulation of key pro-and anti-apoptotic genes, depicting a complex network of gene regulatory signals that ultimately result in apoptosis.

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