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Sun Yang

Chapman University, syang@chapman.edu

Bobbye J. Misner

University of California, Irvine

Rita Chiu

University of California, Irvine

Frank L. Meyskens Jr.

University of California, Irvine

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Common and Distinct Mechanisms of Different Redox-Active Carcinogens Involved in the Transformation of Mouse JB6P+ Cells

Sun Yang^{1,2}, Bobbye Misner¹, Rita Chiu¹, and Frank L. Meyskens Jr^{1,2,3,*†}

¹Chao Family Comprehensive Cancer Center, University of California, Irvine, Orange, California

²Department of Medicine, University of California, Irvine, Orange, California

³Department of Biological Chemistry, University of California, Irvine, Orange, California

Abstract

We transformed JB6P+ cells with prolonged intermittent low-dose UVB radiation or prolonged exposure to low-dose H₂O₂ or CdCl₂. Stable transformation was confirmed by an anchorage-independence assay. The JB6P+ transformants formed more colonies (~six folds) in soft agar as compared to their JB6P+ parent cells and were associated with increased intracellular reactive oxygen species (ROS) levels. Activating protein-1 (AP-1) is a family of transcription factors that are rapidly activated by elevated intracellular ROS levels, and their composition is important in the process of cellular transformation and/or tumor progression. To investigate if carcinogenesis induced by distinct carcinogens was via similar molecular mechanisms in these transformants, gel mobility shift and immunoblot analyses were utilized to determine the distinct AP-1 compositions. Compared to parent JB6P+ cells, the gain of JunB and Fra-1 in AP-1 DNA binding complexes was markedly increased in all transformed cells, which might contribute to a more proliferative phenotype, while loss of Fra-2 occurred in JB6P+/H₂O₂ and JB6P+/Cd cells. Differential AP-1 components in the transformants suggested that their transformations might be mediated by distinct transcription signalings with distinct AP-1 dimer compositions. However, all three transformants exhibited increased activation of pathways involved in cell proliferation (ERK/Fra-1/AP-1 and JNK/c-jun/AP-1) and anti-apoptosis (Bcl-x1). The development of the JB6P+ transformants (JB6P+/UVB; JB6P+/H₂O₂; JB6P+/Cd) provides a unique tool to study the mechanisms that contribute to different redox-active carcinogens in a single model.

Keywords

JB6P cells; UVB; metal; H₂O₂; AP-1

*Correspondence to: Department of Medicine, School of Medicine, University of California, Irvine, 101 The City Drive South, Bldg. 56, Room 215, Orange, CA 92868.

†Professor of Medicine and Biological Chemistry.

Introduction

The JB6 mouse epidermal cell lines were first established in the early 1980s by Nancy Colburn's group and consist of tumor promotion sensitive JB6P+ and tumor promotion resistant JB6P- cells. These cell lines have been widely utilized as a model of cellular malignant transformation to study mechanisms that underlie tumor promotion [1–3]. In the presence of the tumor promoters, JB6P+ cells undergo a response similar to second stage tumor promotion that is associated with anchorage-independent growth and tumorigenic transformation; these changes do not occur in JB6P- cells.

A growing body of evidence has shown that reactive oxygen species (ROS) at low concentration act as second messengers in intracellular signaling cascades, which induces and maintains the oncogenic phenotype of cancer cells; on the other hand, ROS at high concentrations produce cellular toxicity. This is a common phenomenon in cancer cells, resulting in redox imbalance and chronic oxidative stresses [4]. Many studies have demonstrated that the JB6P+ cells are sensitive to cellular transformation by various types of ROS [5–7].

Ultraviolet light (UV) radiation initiates carcinogenesis by direct DNA damage and generates ROS that act as second messengers responsible for the alteration of gene expressions [8,9]. As H₂O₂ crosses biomembranes freely [10], the exposure of cells to H₂O₂ is a classical model of direct oxidative stress that not only results in lipid, protein, and DNA damage, but also acts as a second messenger in promoting tumor survival [11–12].

Cadmium is a well-studied carcinogenic metal, which is related to air and water pollution and cigarette smoking [13]. Although cadmium itself is not a transition-state metal (it cannot generate ROS directly), it is well documented that its carcinogenicity is mediated by the indirect production of various radicals such as the superoxide radical, hydroxyl radical, and nitric oxide [13,14], which contribute to the activation of JNK/AP-1 signal transduction pathways responsible for the transcription of genes involved in cell growth, as seen, for example, in hepatic cells [15,16].

In this study, we transformed the JB6P+ cells with low-dose intermittent UVB radiation or continuous exposure to low-doses of H₂O₂ or cadmium chloride (JB6P+/UVB, JB6P+/H₂O₂, JB6P+/Cd), events which are all mediated by ROS production. The comparisons of subsequent alterations of transcription factors (i.e., activating protein-1, AP-1) or proliferation signalings that are sensitive to oxidative stresses are the major focus of this current study. The development of these new transformants has provided a unique opportunity to study the mechanisms that underlie carcinogenesis in a single model with different redox-active stimuli.

Materials and Methods

JB6P+ Cell Transformation

The JB6P- and the JB6P+ cells were obtained from ATCC (Manassas, Virginia). The JB6P+ cells were seeded at low density (1.5×10^4 cells/mL) in 60 mm dishes. UVB (10 mJ/cm²)

treatments were performed twice a week, 24 h after the cells were re-seeded. JB6P+ cells were incubated with growth media containing H₂O₂ (10 μM) or CdCl₂ (1 μg/mL) for 12 wks and the media were changed at least twice a week. The control JB6P+ cells were treated with the same volume of sterile H₂O at the same time. The cells were allowed to grow to a 60% confluence before splitting. All of the cells were cultured in EMEM medium supplemented with 4% bovine fetal serum, L-glutamine (2 mM), and 25 μg/mL gentamicin then incubated with 5% CO₂ at 37°C. After 12 wks, cell transformations were confirmed by the anchorage-independent growth assay.

Anchorage-Independent Cell Transformation Assay

Cell transformation in the presence or absence of TPA was investigated by soft agar colony formation analysis. Cells (8×10^3 /mL) were exposed to TPA (10 or 20 ng/mL) in 1 mL of 0.33% basal medium Eagle agar containing 10% FBS. The cultures were incubated in a 5% CO₂ atmosphere at 37°C for 14–18 d [2] and the colonies (containing >50 cells) were quantified using a light microscope at 40× magnification.

Protein Extraction and Western Blot Immunoassay

Nuclear extracts from JB6P cells were prepared as described previously with minor changes [17]. Whole cell lysis extracts were prepared according to the directions of the manufacturer (Cell Signaling Technology, Danvers, MA). Using a Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA), protein concentrations of all the samples were precisely measured to confirm equivalent loading. Immunoblot analysis was performed as previously described [17]. Specifically, equal amounts of nuclear protein (20–25 μg) or whole cell protein (50–100 μg) were loaded respectively per sample.

Electrophoretic Mobility Shift Assay (EMSA) of AP-1 and NF-κB DNA-Binding Activity

AP-1 binding activities were determined by Gel Shift Assay Systems (Promega, Madison, WI) with optimizations. Briefly, nuclear extract was incubated in a final volume of 10 μL containing 2 μL gel shift binding buffer (5×), 1 μL ³²P-labeled AP-1 consensus oligos (approx., 6×10^5 cpm). Two microliters of antibodies to AP-1 members (Santa Cruz Biotechnology®, Inc., Santa Cruz, CA) was added to reaction mixtures for supershift. For oligonucleotide competition experiments, each reaction was preincubated with a 50-fold excess unlabeled AP-1 for 20 min before the addition of hot probes. The mixtures were separated on 4–5% nondenaturing polyacrylamide gels. Using autoradiography the shift bands were quantitated by densitometry.

Results and Discussion

JB6 cell lines are a unique model to study the molecular events and mechanisms involved in tumor promotion. The exposure of JB6P+ cells to TPA, EGF, or TNF-α leads to irreversible transformation and tumorigenicity with acquired anchorage independent growth. Many studies have demonstrated that early events induced by ROS stresses, such as activation of AP-1 or NF-κB pathways, play an important role in the transformation of JB6 P+ cells [18]. It is well documented that UVB radiation, H₂O₂, or CdCl₂ either directly or indirectly lead to elevated ROS stresses [9]. In addition, our data consistently showed that in JB6P+ cells a

one-time exposure of UVB (40 mJ/cm²) radiation led to a significant increase of ROS production in JB6P+ cells compared to that of the control [19] and that the elevation of ROS was measurable for at least 24 h. A similar increase in DCF density was also observed with CdCl₂ treatment. The peak occurred shortly after 5 min of exposure, followed by a quick reduction to normal levels by 30 min. An anchorage-independent cell growth assay was performed to determine if cellular transformation had occurred by chronic repeated exposure of UVB or persistent H₂O₂ or Cd exposure. The JB6P- cells did not form colonies in soft agar either in the presence or absence of the tumor promoter TPA. However, a small amount of JB6P+ colonies were evident, and were remarkably increased by TPA stimulation. For all of the transformants, in the absence of TPA, colonies formed at a frequency of 5–10-folds that of unstimulated JB6P+ parent cells and 1–2-folds that of TPA-stimulated ones [19].

Based on these observations, we propose that although the malignant transformations were induced by distinct types of carcinogens (UVB, Cd, or H₂O₂), their effects are all mediated by elevated ROS production. As many cell signalings are very sensitive to oxidative stresses, we further delineated whether these transformations were preceded through the regulation of similar or different downstream targets. Specifically, AP-1 is a family of transcription factors that are rapidly activated by elevated intracellular ROS levels [9,20]. The AP-1 comprises a large family of basic-region leucine zipper (bZIP) transcription factors (including *c-jun*, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2) and forms distinct heterodimers or homodimers, which differentially regulate downstream target genes. The dynamic changes in Jun and Fos compositions depend on the initiating stresses, which play a key role in defining whether cells undergo apoptosis, survival, or senescence through subsequent downstream shift of the signaling pathways (for recent reviews see References [21,22]). Therefore, it is important to evaluate the AP-1 components in different JB6 cells. In our current study, we therefore, utilized gel mobility supershift and Western blot assays to identify the distinct compositions of AP-1 complexes among JB6P- cells, JB6P+ cells, and the new transformants.

Nuclear extracts, prepared from different JB6 cells, were incubated with ³²P-AP-1 in the presence of antibodies to detect specific AP-1 member proteins. As shown in Figure 1A, additions of anti-JunD and anti-Fra-2 antibodies resulted in a strong supershift of AP-1 bands, indicating the presence of JunD and Fra-2 in the AP-1 DNA binding complexes in JB6P- cells. Similar supershifts were also observed in other JB6 cells (as shown in Figure 1B–E) and the results were summarized in Table 1. Our EMSA analysis revealed a remarkable gain of JunB and Fra-1 in all the transformants compared to their parent JB6P+ cells. Fra-2 was observed in JB6P+ and JB6P+/UVB cells but absent in JB6P+/H₂O₂ and JB6P+/Cd extracts. In contrast to promoter resistant JB6P- cells, *c-jun* antibody produced a retarded AP-1 band in JB6P+ cells as well as in all the transformants extracts. However, compared to *c-jun*, antibodies to JunB, JunD, and Fra-1 produced more pronounced supershift bands, indicating that they might be the major constituents in AP-1 complexes. JunD was detected in all JB6P cells, while c-Fos and FosB were absent in all of these cells.

Induction of Fra-1 expression was shown in all of our transformants and JB6P+ cells compared to JB6P- by immunoblotting assay (Figure 2A), while supershifts of AP-1 DNA binding by Fra-1 antibody only occurred in transformed cells (Figure 1), indicating the

involvement of Fra-1 in AP-1 complexes. Different from c-Fos, the prototype of the Fos family, Fra-1 alone does not exhibit any transformation activity [21,23]; However, Fra-1 does contribute to tumor progression through distinct mechanisms and many of its downstream target genes are involved in cell survival, proliferation, and invasiveness [24]. Elevated Fra-1 expression has been reported in many cancer cells and has been suggested to serve as an important target for cancer prevention or intervention [25].

EMSA revealed that JunB binding occurred in all of our transformed JB6P+ cells. Although there are studies showing that JunB exhibits anti-oncogenic activities and acts as an antagonist of *c-jun* both in vitro and in vivo [21,22,26,27], elevated JunB expression levels have been detected in many human tumors such as lymphomas and breast cancer cells, which correspond to cell cycle promoter cyclin D1 [28–30]. This dramatic increase of JunB in AP-1 complexes suggests a possible common role of JunB in transformation.

Fra-2 has been described as a less potent trans-activator than c-Fos with a weak transforming efficacy [31]. Recent studies using a knock-out mice model have showed that Fra-2 is required for postnatal survival [32], and in breast cancer cells overexpression of Fra-2 was associated with a more aggressive tumor phenotype [33]. Dimerization with *c-jun*, Fra-2 rescued cells from experimentally induced growth arrest [34]. On the other hand, studies have also suggested a role of Fra-2 in cellular differentiation in ovarian granulosa, osteoblasts, muscle, and melanoma [35]. The loss of Fra-2 in AP-1 complexes in H₂O₂- and CdCl₂-transformed JB6P+ cells might contribute to the development to a proliferative phenotype.

Interestingly, our immunoblot results were not correlated to the EMSA findings. The inductions of c-Fos that occurred in all of the transformants as measured by immunoblotting were consistent with the finding of studies in other tumor cells [30,36]. Notably, the JB6P+/UVB transformant exhibited a similar pattern of elevated phospho-*c-jun*, JunB, and c-Fos as reported with one single dose of UVB radiation, which was because of the MAPKs pathways (ERK, JNK, and p38) activated by UVB-generated ROS [37–39]. However, we failed to observe any c-Fos-specific shifted bands by EMSA. A similar inconsistency was seen with Fra-1 in JB6P+ cells. In contrast to immunoblotting, the EMSA gel shift assay mimics AP-1 DNA bindings in vitro and identifies DNA-bound proteins using specific antibodies in a non-denaturing condition.

We further explored the distinct gene expressions associated with proliferation and apoptosis among these cells. The role of MAPK signaling in proliferation is well characterized [40]. As shown in Table 2 and Figure 2B, our study revealed increases of phosphorylated ERK and JNK in all the transformants (JB6P+/UVB, 1.5- and 1.9-fold; JB6P+/H₂O₂, 2.2- and 1.5-fold; JB6P+/Cd, 1.9- and 1.5-fold respectively) as compared to JB6P+ cells (1.2- and 0.9-fold respectively, levels in JB6P– standardized as 1.0). These findings coincided and supported other observations, which have suggested the critical involvement of MAPK in tumor promotion and carcinogenesis [18,41,42]. Notably, no significant alterations of p38 activities were observed (data not shown); these results are in contrast to the reports indicating the involvement of p38 after UV exposure [43,44]. The explanation might be related to the differential UV exposure among studies.

Fra-1 is regulated at both transcriptional and posttranscriptional levels. Characterized as an immediate early response gene, Fra-1 is activated by the MAPK and PI3K/AKT pathways. Additionally, ERK plays a critical role in the stabilization of Fra-1 protein upon phosphorylation to prevent degradation by the proteasome [45,46]. Overexpression of Fra-1 was found in many cancer cells displaying high ERK activities [47] and TPA-induced Fra-1 activities were ERK-dependent [48]. Therefore, the level of activated ERK is very important for regulating Fra-1/AP-1 activities. The remarkable activations of ERK in transformants identified by our study might be closely related to the enhanced Fra-1 activities.

Phosphorylated JNK is the active form of JNK, which subsequently phosphorylates *c-jun* and leads to an increase of its DNA binding activities. Increased ratios of phosphorylated JNK/JNK were seen in all the transformants (Table 2). Consistently, phosphorylated *c-jun* was also found elevated in the transformants (Table 2, Figure 2B), which contributed to a significant increase of AP-1 transcriptional activities evident in transformants compared to the parent cells [19]. *c-jun* has been shown to perform an important function in the proliferation of cancer cells [21]. The critical role of the activation of AP-1 transcription factors in carcinogenesis was recently detailed [18] and for this reason, the JB6 model is unique in defining molecular events in the MAPK/ AP-1 and NF- κ B pathway that lead to tumor promotion. No remarkable differences of Cdk2, a well-known protein found throughout the G phase to the S phase of the cell cycle, were observed among these cell lines.

We also examined the expression levels of Bcl-xl and Bax. Bcl-xl was not detected in the JB6P⁻ cells, but showed a substantial increase in the JB6P⁺ transformants as compared to the JB6P⁺ parent cells. No significant changes in Bax protein levels were observed among these cell lines. Bcl-xl is a target gene of the NF- κ B pathway, which has been implicated broadly in tumorigenesis. Remarkable activation of NF- κ B was evident both in basal and UVB-treated JB6P⁺ cells [18,49], as well as in H₂O₂- and Cd-treated cells [9,50]. This might lead to the induction of Bcl-xl in order to enhance survival and contribute to the transformation process. In JB6-derived RT-101 cells, TNF- α -induced apoptosis was because of a decrease in anti-apoptotic Bcl-xl [51].

The differential AP-1 protein compositions exhibited in the JB6P⁺ variants suggest that these cells were transformed by different mechanisms; however, our data also suggested that the JNK/*c-jun*/AP-1 and ERK/Fra-1/AP-1 pathways are being extensively activated in these JB6P⁺ variants even though the transformation was induced by distinct carcinogens. Based on our observations, it is suggested that active MAPKs inhibitors might exhibit potential chemopreventive activities by blocking the transformation induced by a broad range of carcinogens.

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Abbreviation used

AP-1	activator protein-1
AKT	protein kinase B
DCF	2',7'-dichlorofluorescein
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
HE	dihydroethidium
JNK	jun N-terminal kinase
MAPK	mitogen-activated protein (MAP) kinases
NF-κB	nuclear factor kappa B
PI3K	phosphoinositide 3-kinase
ROS	reactive oxygen species
TPA	12-O-tetradecanoyl-phorbol-13-acetate
UVB	ultraviolet light (290–320 nm)

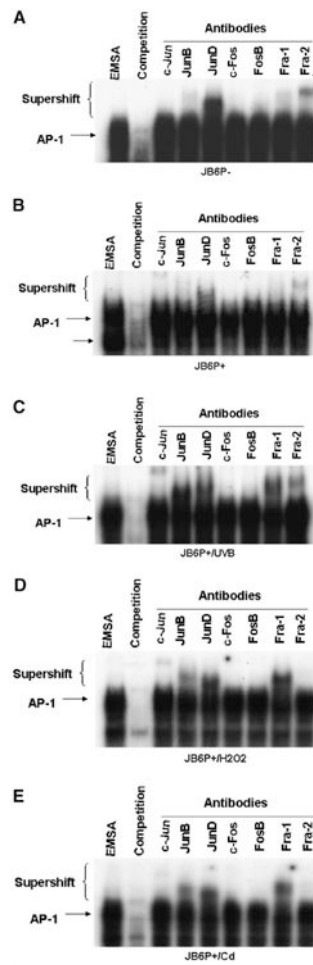


Figure 1.

EMSA and supershift analysis of AP-1 DNA-binding activities and composition in JB6 cells. EMSA with ^{32}P -labeled AP-1 oligonucleotide probe was performed as described in “Materials and Methods” and 50 \times excess unlabeled AP-1 was added for the competition. The nuclear extracts were subjected to EMSA in the absence or presence of antibodies to the indicated AP-1 proteins. JB6 cell lines were grown in their EMEM growth medium as detailed in the “Materials and Methods.” (A–E) JB6P–, JB6P+ cells, JB6P+/UVB, JB6P+/H₂O₂, JB6P+/Cd cells respectively.

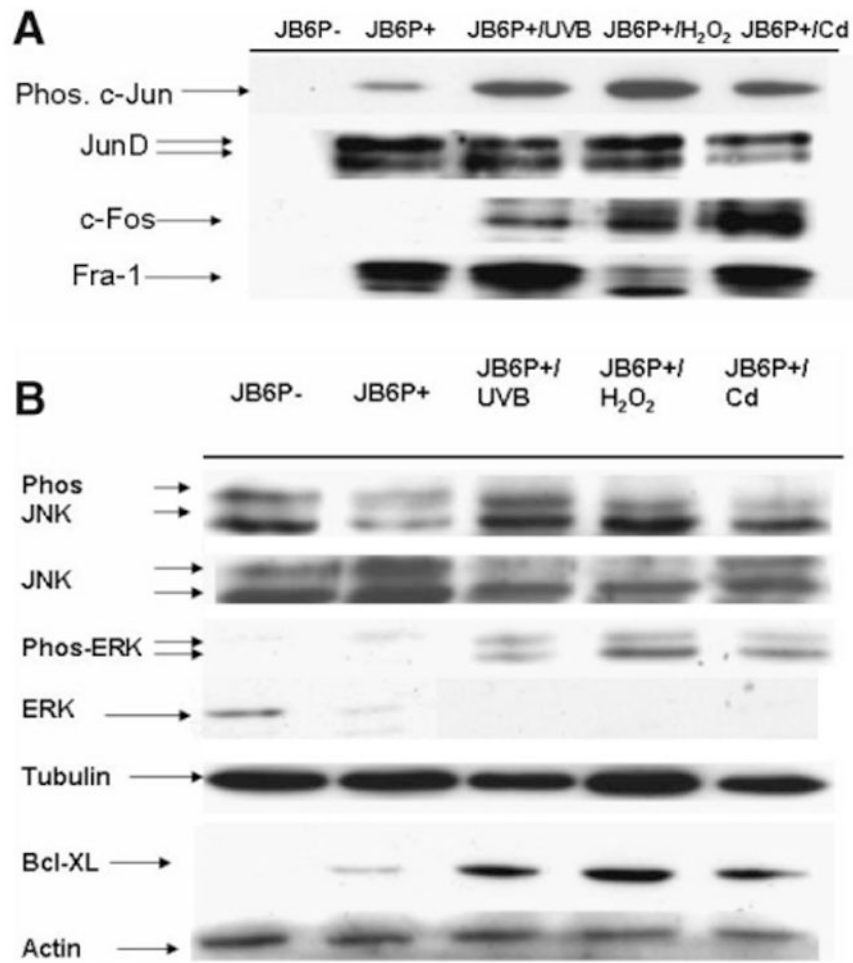


Figure 2. The representative results of AP-1 member protein (A) and related genes (B) expressions detected by Western immunoblotting analysis. Cells were collected under normal culture condition and nuclear protein extracts were extracted as described in “Material and Methods.” Protein expression was determined by Western blotting with antibodies specific for phospho-*c-jun*, JunB, JunD, c-Fos, Fra-1, phospho-MAPK, MAPK, Bcl-XL, Bax, actin, and tubulin respectively. Arrows indicate specific bands.

Table 1
AP-1 Complex Components in JB6P+, JB6P-, JB6P+/UVB, JB6P+/H₂O₂, JB6P+/Cd Cell Lines

	<i>c-jun</i>	JunB	JunD	c-Fos	FosB	Fra-1	Fra-2
JB6P-			▲				▲
JB6P+	▲		▲				▲
JB6P+/UVB	▲	▲	▲			▲	▲
JB6P+/H ₂ O ₂	▲	▲	▲			▲	▲
JB6P+/Cd	▲	▲	▲				▲

▲ represents the presence of certain AP-1 members in DNA-binding complexes.

Table 2
Relative Densitometric Measurements of Basal Levels of Phospho-JNK/JNK, Phospho-ERK/ERK, Phospho-p38/p38 MAPK, Cdk2, Bcl-xl, and Bax Proteins as Determined by Western Immunoblotting

	JB6P-	JB6P+	JB6P+/UVB	JB6P+/H ₂ O ₂	JB6P+/Cd
Phospho-JNK	1.0	0.94	1.9	1.5	1.5
JNK	1.0	1.4	0.8	0.5	0.7
Ratio phos. JNK/JNK	1.0	0.7	2.4	3.0	2.1
Phospho-c-jun	0.0	1.0	2.3	2.9	1.9
Phospho-ERK	1.0	1.2	1.5	2.2	1.9
ERK	1.0	0.6	0.5	0.5	0.3
Ratio phos. ERK/ERK	1.0	2.0	3.0	4.4	6.3
Phospho-p38 MAPK	1.0	1.2	1.3	1.6	1.8
p38 MAPK	1.0	1.4	1.6	1.8	2.0
Ratio phos. p38/p38	1.0	0.9	0.8	0.9	0.9
Cdk2	1.0	0.9	1.4	1.0	1.1
Bcl-xl	0	1.0	2.2	3.4	2.1
Bax	1	1.1	1.0	1.3	1.2

Densitometric measurements of immunoblotted proteins were normalized to the JB6P- cells. As Bcl-xl was not detected in the JB6P- cells, this protein was normalized to the JB6P+ results.