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Transcriptional activation of the proapoptotic bik gene by E2F proteins in cancer cells

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Abstract BH3-only proteins are required for execution of apoptotic cell death. We have found that one of these proteins, Bik, is strongly induced in cancer cells treated with chemotherapeutic agents. Furthermore, we showed that chemotherapy-induced expression of bik is independent of p53. Consistent with its pro-apoptotic activity, blockade of bik expression reduces the adriamycin-mediated apoptotic cell death. We also found that the bik gene is transcriptionally activated by E2F proteins. Consistently, adriamycin induces the E2F-bik pathway. In addition, E2Fs transactivate bik by a p53-independent mechanism. Thus, our data indicate that transcriptional regulation of bik contributes to the efficient apoptotic response to chemotherapeutic agents.

Keywords: Bik; E2F; Apoptosis; Chemotherapy

1. Introduction

BH3-only proteins serve as upstream sentinels that selectively respond to apoptotic stimuli inducing activation of other members, mainly Bax and Bak, required for execution of cell death [1]. All BH3-only proteins can bind to pro-survival Bcl-2-like proteins. Blockade of expression or activation of different BH3-only proteins has been associated with cancer cell survival. To this end, it has been shown that mice deficient in Bad progress, with aging, to diffuse large B cell lymphoma [2]. Additionally, Hrk is methylated in colorectal and gastric cancer cell lines, which correlates with loss of expression of this proapoptotic gene. Blockade of other BH3-only genes such as Noxa or Bim promotes resistance to chemotherapeutic agents in different tumour cell models [3,4].

A number of transcription factors (p53, FoxO3a, and E2F1 among others) have been shown to promote the expression of different BH3-only genes, including bnip3L, hrk, bim, puma and noxa [5,6]. Most of these transcriptional pathways are activated in response to chemotherapeutic agents.

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Abbreviations: EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; GFP, green fluorescent protein

E2F transcription factors regulate the expression of many genes involved in differentiation, development, proliferation, and apoptosis [7]. Although the pathway to cell cycle progression seems straightforward with a number of growth-promoting E2F target genes having been described, the pathways to apoptosis are less well defined and more complex [8].

We report here a transcriptional mechanism, mediated by E2F proteins, that promotes the expression of bik. Chemotherapeutic agents enhance the activation of the E2F-bik pathway, which contributes to the apoptotic response of cancer cells to chemotherapy.

2. Materials and methods

2.1. Cell culture

SHSY-5Y, NTERA-2 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, CA). HCT116 cells and their p53 (−/−) derivatives [9], a gift from B. Vogelstein, were grown in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal calf serum. SUM159 was incubated as described [6]. Adriamycin and cisplatin were used at the indicated concentrations (Sigma, St. Louis, MO).

2.2. Analysis of apoptosis

Apoptosis was routinely determined by both an enzyme-immunoassay method that quantifies the histone-associated DNA fragments present in the cytosol (Roche, Mannheim, Germany) as described previously [10], and a flow cytometry analysis using a phycoerythrin-labeled annexin V antibody (Becton Dickinson, Madrid, Spain). Values obtained with the immunoassay method were represented as the percentage of apoptosis in comparison with a positive internal control included in each experiment.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5–10 μg of total protein) were incubated with a ^{32}p-labeled double stranded DNA probe from the promoter region of the bik gene containing the E2F site (5′-CGAGCGCGGAATCC-3′). Samples were run on a 5% non-denaturing polyacrylamide gel in 200 mM Tris–borate, 2 mM EDTA. Supershifts were performed using rabbit anti-HA or anti-Flag antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

2.4. Chromatin immunoprecipitation assay (ChIP)

Purification of sonicated nuclear lysates and immunoprecipitation were performed as described elsewhere [11]. Precipitates were heated at 65 °C for at least 4 h to reverse the formaldehyde cross-linking. Proteins were digested in the presence of 20 μg/μl Proteinase K, and then DNA fragments were purified with a QIAquick Spin Kit (Qiagen, Valencia, CA). For PCR, 2 μl from a 40 μl DNA extraction and 21–25 cycles of amplification were used.

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2.5. Expression analyses

Total RNA was prepared using TRIZOL reagent (Invitrogen). To assess mRNA expression, semiquantitative RT-PCR and quantitative real-time PCR were performed as previously described [6,10]. The generated cDNA was amplified by using primers for human bnip3, bim, bik, bnip3L, β-actin, [6] and puma (5′-ACGACCTCAACGCACAGTACG and 5′-TGGGTAAGGGCAGGAGTCC).

Protein expression was determined by Western blotting as described [6]. Blots were incubated with rabbit anti-Bik, and anti-HA antibodies (Santa Cruz Biotechnology), or mouse anti-β-Tubulin (Sigma) antibodies.

2.6. Gene silencing

HCT116 cells were transfected with a pool of four small interfering RNA (siRNA) duplexes (100 nM) specific for human bik (Dharmacon, Chicago, IL) by using lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. After 24 h of transfection, 2 μM adriamycin was added to the culture and cells were incubated for another 24-h period. A pool of four irrelevant siRNA duplexes was also used as a negative control.

2.7. Transfections and gene reporter assays

A genomic PCR fragment of 596 bp from the promoter region of bik (bik-pt), was cloned into *Kpn*I and *Hin*dIII sites of the pGL2-basic luciferase reporter vector (Promega Corp., Madison, WI). HEK293T cells were co-transfected with 1 μg of pGL2-bik-pt, 1 μg of E2F1, E2F2 or E2F3 cDNAs cloned into the Flag/HA-tag containing pcDNA3 expression vector [12], and 50 ng of pRSV-β-gal by lipofection using Superfect (Qiagen). Twenty-four hour post-transfection, cell extracts were prepared and analyzed for the relative luciferase activity by a dual-light reporter gene assay system (Applied Biosystems, Foster City, CA).

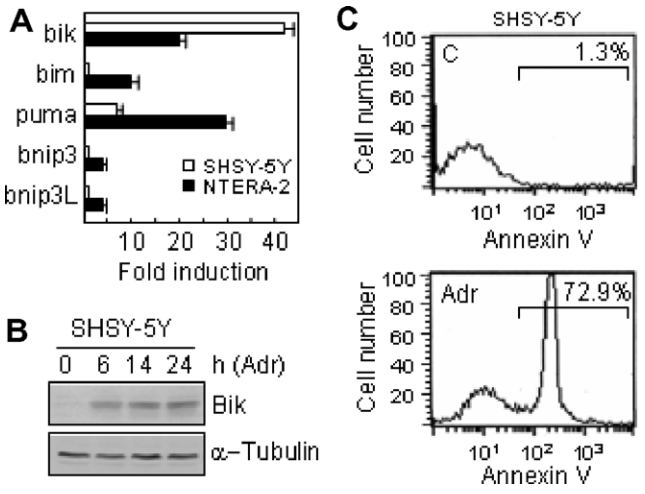

Fig. 1. Bik is upregulated in cancer cells in response to adriamycin. (A) Cells were treated with 1 μM adriamycin for 24 h and then, total RNA was extracted and analyzed for the expression of BH3-only genes by real-time quantitative PCR. Data are shown as fold induction compared with untreated cells. All data points represent the means ± S.D. of three independent experiments. (B) Cells were treated with adriamycin (Adr) and at different time points the protein levels of Bik were determined by Western blotting. The levels of α-Tubulin were also analyzed to assure equal loading. (C) The percentage of apoptotic cells following treatment with adriamycin was determined by flow cytometry with FITC-labeled annexin V. Numbers above the selected regions indicate the percentage of apoptotic cells.

3. Results

3.1. Bik is induced by chemotherapeutic agents in cancer cells through a p53-independent pathway

We searched for pro-apoptotic BH3-only genes differentially expressed in SHSY-5Y and NTERA-2 cancer cells following treatment with chemotherapeutic agents. Adriamycin provoked about a 40-fold and 20-fold induction of bik mRNA in SHSY-5Y and NTERA-2, respectively. The levels of puma were also found to increase 7-fold in SHSY-5Y and 30-fold in NTERA-2; whereas those of bim, bnip3 and bnip3L increased (3–9-fold) only in NTERA-2 cells (Fig. 1A). Furthermore, we observed a time-dependent induction of Bik protein after treatment with adriamycin (Fig. 1B). Consistent with the cell death promoting effect of adriamycin, most SHSY-5Y cells underwent apoptosis within 24 h of treatment (Fig. 1C).

Puma is a well known p53 target gene; however, the expression of bik has been reported to be both p53-dependent and p53-independent. We treated p53-deficient and wild type HCT116 cells with adriamycin or cisplatin, and showed that the mRNA levels of bik increased in response to chemotherapy regardless of the presence of p53 (Fig. 2A). However, puma was only upregulated in wild type cells. Furthermore, p53-deficient cells showed much lower levels of apoptosis than those detected in wild type cells (Fig. 2B). To study the relevance of bik in chemotherapy-induced apoptosis we used an RNA interference approach that blocks bik expression. Specific siRNA decreased 2–3-fold the mRNA expression of bik as determined by semiquantitative (Fig. 2C) and real-time RT-PCR (data not shown). This reduction was also confirmed at the protein level by western blot analysis (Fig. 2D), and resulted in a 20–30% loss of the apoptotic response to adriamycin as assessed by an ELISA method that quantifies the histone-associated DNA fragments (Fig. 2E) and by flow cytometry with annexin V (Fig. 2F).

3.2. E2F proteins transcriptionally regulate bik gene expression

We found a putative binding sequence for E2F in the bik gene promoter, 104 bases upstream from the transcription start site. Then, a 596 bp fragment from the promoter region, encompassing 40 nucleotides of exon 1, was cloned into a luciferase reporter vector (Bik-Luc). HEK293T is a highly efficient cell model for transient transfection experiments. Thus, we determined the levels of luciferase activity in these cells transfected with Bik-Luc in the presence of E2Fs. As shown in Fig. 3A, all E2F proteins increased luciferase activity more than 20-fold. To directly prove the binding of E2F proteins to the bik promoter, HEK293T cells were transfected with E2F-containing vectors and then nuclear extracts were subjected to EMSA. As expected, in all cases we detected a protein–DNA complex (Fig. 3B). If an antibody reacted with E2F bound to the probe, it was expected that the shifted band either would have slower mobility (supershift) or would disappear as a result of dissociation of E2F from the probe. Fig. 3B shows that the E2F–DNA complex was removed in the presence of antibodies against the N-terminal HA/Flag epitope used as a tag. Additionally the interaction of endogenous E2F protein with the bik promoter was demonstrated by a ChIP assay. We observed a significant enrichment of the promoter (amplified fragment spans the region from −236 to −86) when using the anti-E2F1 antibody (Fig. 3C). Moreover, overexpression of E2F1 promoted an 18-fold induction of bik mRNA levels. On the contrary, bim and puma did not show significant variations. Similar results were obtained with SUM159 (Fig. 4A). In addition, treatment with adriamycin enhanced the formation of E2F1-bik promoter binding com-

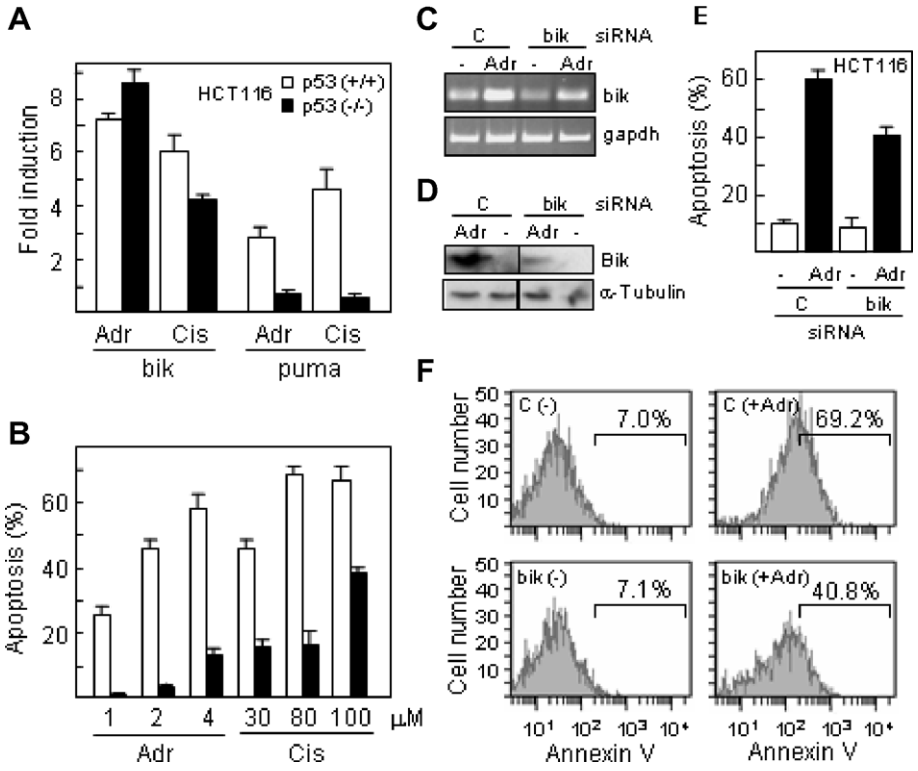

Fig. 2. Bik is induced in a p53-independent manner and contributes to the apoptotic response to chemotherapy. (A) Wild type and p53-deficient HCT116 cells were treated with 2 μM adriamycin (Adr) or 30 μM cisplatin (Cis). After 24 h of treatment, total RNA was extracted and analyzed for the expression of bik and puma by real-time quantitative PCR. (B) Apoptotic cell death was also determined in HCT116 cells treated with increasing concentrations of chemotherapeutic agents by an ELISA method that quantifies the histone-associated DNA fragments present in the cytosol. Wild type HCT116 cells transfected with irrelevant (control) or bik-specific siRNA duplexes were treated with 2 μM adriamycin, and after 24 h bik expression was determined by semiquantitative RT-PCR (C), and Western blot (D) analyses, and apoptosis was analyzed by the immunoassay method (E) and by flow cytometry with FITC-labeled annexin V (F). gapdh mRNA was included as an amplification control. All histograms represent the means ± S.D. of three independent experiments.

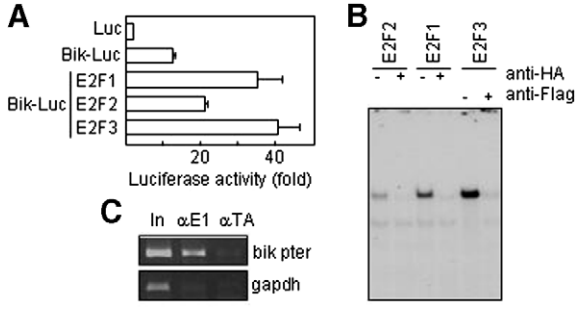

Fig. 3. E2F proteins transactivate the bik promoter. (A) HEK293T cells were transfected with Bik-Luc either in the presence or in the absence of E2F1, E2F2 or E2F3. Following 24 h of transfection, cell extracts were prepared and analyzed for the relative luciferase activity. Results were normalized for transfection efficiency with values obtained with pRSV-β-gal. Histograms represent the means ± S.D. of three independent experiments. (B) Formation of E2Fs–DNA binding complexes was determined by EMSA using a radiolabeled probe from the bik promoter. Anti-HA and anti-Flag antibodies were used to show the specific binding of transfected proteins. (C) A ChIP assay was performed using SHSY-5Y cells. Cross-linked chromatin was incubated with an antibody against E2F1 (αE1), or with an irrelevant antibody against the T antigen (αTA). Immunoprecipitates from each sample were analyzed by PCR using primers specific for the bik promoter and for the gapdh coding region. As a positive control, a sample representing 0.1% of the total input chromatin (In) was included.

plexes (Fig. 4B). Consistently, adriamycin promoted a 2-fold induction of luciferase activity in cells transfected with Bik-Luc compared with untreated cells (Fig. 4C).

3.3. p53 does not modify the E2F-dependent induction of bik

E2F proteins may induce pro-apoptotic genes by activation of a p53-dependent transcriptional pathway. Thus, we assessed whether E2F promoted the expression of bik through p53. First, we co-transfected SHSY-5Y cells with Bik-Luc and either E2F1 or p53. E2F1 promoted a significant increase (about 11-fold) in the levels of luciferase activity (Fig. 5A). On the contrary, p53 did not modify the activity of the reporter gene, but increased about 25-fold the luciferase activity when a positive control promoter was used (Fig. 5A). To further confirm this, p53-deficient and wild type HCT116 cells were co-transfected with Bik-Luc and E2Fs. As shown in Fig. 5B, luciferase activity was similarly induced in both cell lines.

It has been described that ectopic expression of several of the E2Fs results in apoptotic cell death [13]. We transiently cotransfected SHSY-5Y cells with E2F1 and a green fluorescent protein (GFP)-containing vector. After transfection, no apoptosis was observed in the GFP-positive cell population (Fig. 5C). To confirm this result, HCT116 was transfected with E2F1 or E2F2 (Fig. 5D). By 30 h of transfection no evidence of apoptosis was observed as determined by an

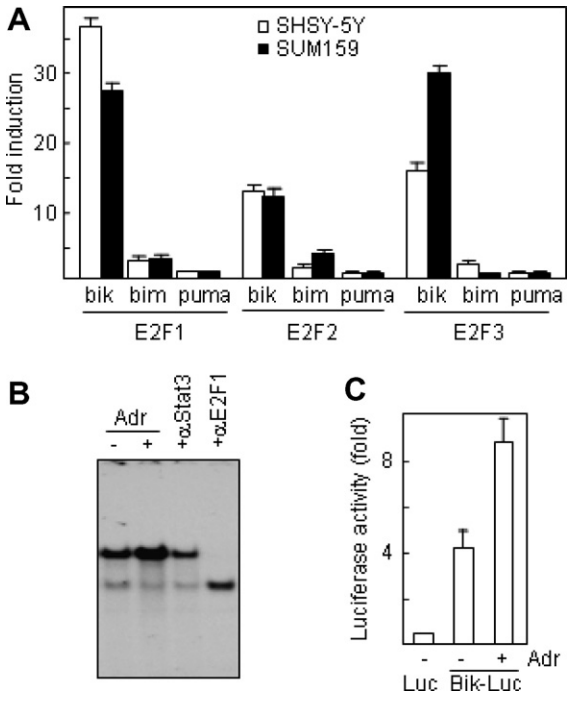

Fig. 4. E2F proteins induce endogenous bik and activates the bik promoter in response to adriamycin. (A) Cell lines were transfected with E2F1, E2F2 or E2F3. Following 48 h of culture, transfected cells were analyzed for expression of BH3-only genes by real-time quantitative PCR. Data are shown as fold induction compared with cells transfected with the empty vector. (B) SUM159 cells were treated with 1 μM adriamycin for 3 h, and the formation of E2F1–DNA binding complexes was determined by EMSA as in Fig. 3B. Nuclear extracts were preincubated with the indicated antibodies. (C) SUM159 cells were transfected with Bik-Luc and treated with 10 μM adriamycin for 16 h. Cell extracts were analyzed for the relative luciferase activity as in Fig. 3A.

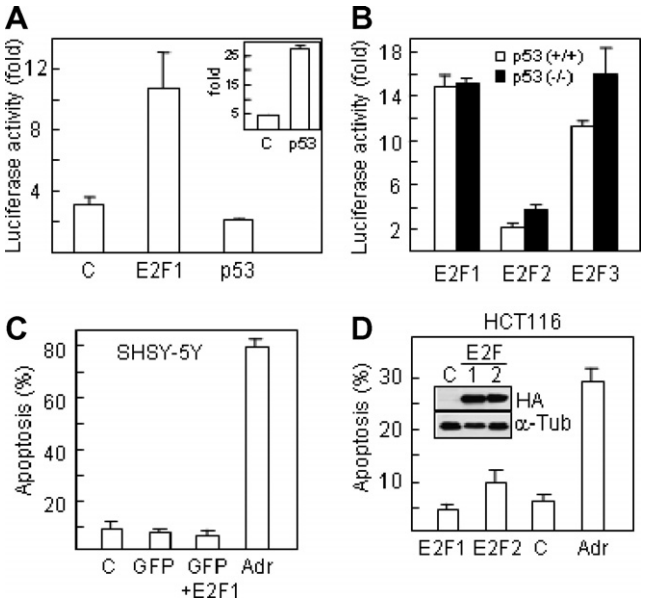

Fig. 5. E2F proteins induce bik by a p53-independent pathway and do not promote apoptosis. (A) SHSY-5Y cells were transfected with Bik-Luc and E2F1 or p53, and after 24 h of incubation, cell extracts were analyzed for the relative luciferase activity. Cells were also transfected with a luciferase reporter vector containing the mdm2 promoter in the presence of p53. (B) p53 null and wild type HCT116 cells were transfected with Bik-Luc and E2Fs. By 24 h of transfection, the luciferase activity was analyzed as in Fig. 3A. (C) Cells were transfected with a GFP-containing vector in the presence or in the absence of E2F1, and the percentage of GFP-positive apoptotic cells were determined by flow cytometry with phycoerythrin-labeled annexin V. (D) Wild type HCT116 cells were transfected with E2F1 or E2F2 and apoptosis was analyzed as in (C). Expression of exogenous proteins in cell extracts were also determined by Western blotting with anti-HA antibodies. C, untreated cells; All data points represent the means ± S.D. of three independent experiments.

immunoenzymatic assay and flow cytometry analysis (Fig. 5D and data not shown). Thus, at least in these cell systems, overexpression of E2F proteins is not sufficient to promote apoptosis.

4. Discussion

We have shown that adriamycin induces bik in a p53-independent manner. By contrast, it has been previously described that p53 independently induced bik [14]. However, in line with our results, bik is induced in p53-deficient lymphoma cells following treatment with camptothecin [15]. Consistent with this apparent discrepancy is that there is marked variation in the intrinsic ability of cancer cells to activate the p53 apoptotic pathway in response to chemotherapy [16], which suggests that other transcriptional mechanisms may contribute to the expression of bik.

We found that E2Fs transactivate the bik gene. In line with this, E2Fs have been shown to induce the expression of a number of BH3-only genes including puma and bim [5]. Moreover, we described that a partial blockade of bik expression reduced the levels of chemotherapy-induced apoptosis. However, the presence of active p53 appeared to be necessary for an efficient apoptotic response to chemotherapeutic agents, which is in agreement with the well known participation of this tumour suppressor in apoptosis [17]. Overexpression of bik has been previously shown to promote apoptosis [18]. By contrast, we found that although E2Fs efficiently upregulated bik, this transcriptional mechanism had no effect on cell survival. Consistent with our result is the argument that Bik is not a direct initiator of apoptosis, but sensitizes cells to death stimuli by reducing the level of free pro-survival Bcl-2-family members [19]. An alternative explanation for the lack of killing activity is that bik cannot neutralize all the pro-survival proteins expressed substantially in the studied cells. In support of this, recent data have shown that Noxa BH3, which selectively binds Mcl-1, potently enhances killing by Bik, which preferentially binds Bcl-xL, reflecting complementary BH3 function [20]. Thus, a likely model is that E2F proteins are activating bik, among other apoptotic genes, through a p53-independent mechanism, and this transcriptional pathway may act in conjunction with p53 and its apoptotic target genes for a complete response to chemotherapeutic agents. Consistently, our knock-down experiments indicate that bik contributes to chemosensitization. Based on these data, bik seems to participate in a regulatory network of factors that control apoptosis in response to chemotherapy. In line with this, it is generally accepted that multiple pro-survival proteins must be inactivated to unleash Bax and Bak, which drive apoptosis [21]. Some BH3-only members bind and block all the pro-survival proteins, whereas others engage distinct subsets and exhibit complementary killing. Hence, multiple BH3-only proteins are most likely activated to mount an

efficient apoptotic response, and this orchestrated response to chemotherapy appears to be cell type dependent.

Together, this study shows a role of E2F proteins in the transcriptional regulation of bik, and supports that this pathway can be activated by chemotherapeutic agents, thus contributing to the apoptotic response to chemotherapy. Further studies will need to deepen the understanding of the physiological relevance of this transcriptional pathway in normal and cancer cells.

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