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Interferon Regulatory Factor 5, a Novel Mediator of Cell Cycle Arrest and Cell Death[1]

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ABSTRACT

We have previously shown a critical role for IFN regulatory factor 5 (IRF-5) in the innate immune response to virus infection. For the first time, we now show that although IRF-5 is a direct target of p53, its cell cycle regulatory and proapoptotic effects are p53 independent. IRF-5 inhibits both *in vitro* and *in vivo* B-cell lymphoma tumor growth in the absence of wild-type p53. The molecular mechanism(s) of IRF-5-mediated growth inhibition is associated with a G_2-M cell cycle arrest and modulation of growth regulatory and proapoptotic genes, including *p21*, *Bak*, *DAP kinase 2*, and *Bax*. Taken together, these data indicate that although IRF-5 is a downstream target of p53, its growth inhibitory and proapoptotic effects are independent of p53.

INTRODUCTION

Transcription factors of the IRF[3] family play essential roles in the regulation of genes induced by viral infection, immunostimulation, as well as cell growth regulation. Nine members of this family have been identified to date. The transcription factor IRF-5 was originally identified as a regulator of type I IFN gene expression (1), however, more recent studies have revealed that it plays a role in many aspects of host defense, including induction of cytokines and chemokines involved in the recruitment of T lymphocytes (2). In the context of virus infection, IRF-5 is activated by phosphorylation, leading to the transactivation of cytokine/chemokine genes. Nonetheless, low levels of nuclear IRF-5 and transactivation of *IFN* genes can also be detected in uninfected cells (1, 2). *IRF-5* itself is regulated by type I IFN, indicating an important regulatory pathway for the controlled induction of multiple immunomodulatory genes. The constitutive expression of *IRF-5* is limited to lymphoid organs, dendritic cells, and peripheral blood lymphocytes yet is absent in numerous leukemia and lymphoma cell lines (1), which may indicate a propensity for *IRF-5* gene deletion or possibly silencing by methylation in these malignancies. *IRF-5* has been mapped to chromosome 7q32 that contains a cluster of imprinted genes and known chromosomal aberrations and deletions in lymphoid malignancies, indicating that IRF-5 may also manifest antiproliferative properties (3–5).

Studies on the IRF family members have revealed that many are involved in the regulation of cell growth, differentiation, and oncogenesis (6–11). The precise mechanisms underlying IRF-mediated tumor suppression and oncogenesis remain to be elucidated. IRF-1, IRF-3, and IRF-8 (IFN consensus sequence binding protein) have been shown to function as tumor suppressor proteins and/or mediators of virus-induced apoptosis by activating a set of target genes that could induce tumor suppression such as those responsible for the inhibition of cell growth, induction of apoptosis, and possibly regulation of DNA repair (6–10, 12, 13).

In an effort to gain additional insight into the possible role of *IRF-5* in oncogenesis, we have analyzed the effect of IRF-5 on cell cycle, tumor growth, and apoptosis. We have found that IRF-5 inhibits the growth of tumor cells both *in vitro* and *in vivo*, thus indicating that IRF-5 has tumor suppressor properties. Focusing on the molecular mechanism of this inhibition, we show that IRF-5 induces G_2-M cell cycle arrest, induction of *Bak*, *caspase 8*, *Bax*, and *p21* genes, along with multiple cell cycle regulatory and proapoptotic genes. These studies additionally reveal that although wt p53 stimulates expression of IRF-5, the proapoptotic and cell cycle regulatory effects of IRF-5 are completely independent of p53. In addition, we show that expression of IRF-5 is suppressed in a number of primary hematological malignancies, suggesting a possible role for IRF-5 in lymphomagenesis. Taken together, these data indicate that although IRF-5 is a downstream component of the p53-signaling pathway, its function is independent of p53.

MATERIALS AND METHODS

Cells and Plasmids. Mononuclear cells from healthy donors or patients with CLL, ALL, or AML were obtained from the Johns Hopkins Cancer Center Tumor Procurement Bank. The human BJAB cell lines were maintained in RPMI 1640 (Mediatech, Herndon, VA), and HCT116 cell lines and Saos were maintained in DMEM (Mediatech) supplemented with 10% FBS and gentamicin. BJAB/IRF-5- and IRF-7-overexpressing cells were generated as described previously (2). HCT116 $p53^{-/-}$ cells were generated by genetic recombination to inactivate the p53 gene (14). Flag-tagged IRF-5 and IRF-7, as well as the p21 luciferase reporter and p53 expressing plasmid, were described previously (1, 15–17).

Antibodies. The M2 anti-Flag antibodies were obtained from Sigma (St. Louis, MO); polyclonal anti-IRF-5 antibodies were obtained from Abcam (Cambridge, United Kingdom); monoclonal anti-p21 (OP64), anti-p53 (OP33), anti-p53 (OP43), anti-bak1 (AM03), anti-caspase 8 (AM46), polyclonal anti-bax (PC66), and anti-cyclin B (PC133) antibodies were from Oncogene (Cambridge, MA); polyclonal anti-DAP kinase 2 (251710) antibodies were from Calbiochem (San Diego, CA); monoclonal anti-CD95 antibodies were from BD PharMingen (San Diego, CA).

FACS Analysis and Immunofluorescence. BJAB or HCT116 $p53^{-/-}$ cells (5×10^5) were stained with Hoechst 33258 (Sigma) dye (18) and analyzed by flow cytometry for determination of cell cycle and by fluorescent microscopy for cell morphology. Apoptosis was quantified by single PI (BD PharMingen, Lexington, KY) and/or Hoechst 33258 staining and Annexin-FITC-PI (BD PharMingen) double staining according to standard procedures given by the manufacturers on a Becton Dickinson LSR flow cytometer (San Jose, CA). BJAB cells were incubated with anti-CD95 antibodies for 8–10 h and then stained with Annexin-FITC and PI and analyzed by FACS.

Colony Formation Assay and Animal Studies. BJAB cell lines were plated in duplicate with serial dilutions of 10^5 to 10^3 cells/ml in 0.35% agar-media. Colony formation was evaluated after 14–28 days by staining the cells with crystal violet. Female athymic NCr-nude mice (NCI, Frederick, MD), 6–8 weeks old, were injected s.c. with 5×10^6 viable BJAB vector control cells, BJAB/IRF-5 cells, or BJAB/IRF-7 cells. Tumor cell growth was monitored over a 6-week period when tumor size of BJAB-injected control

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[3] The abbreviations used are: IRF, IFN regulatory factor; wt, wild type; CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; AML, acute monocytic leukemia; FACS, fluorescence-activated cell sorting; PI, propidium iodide; RT-PCR, reverse transcription-PCR; RPA, RNase protection assay.

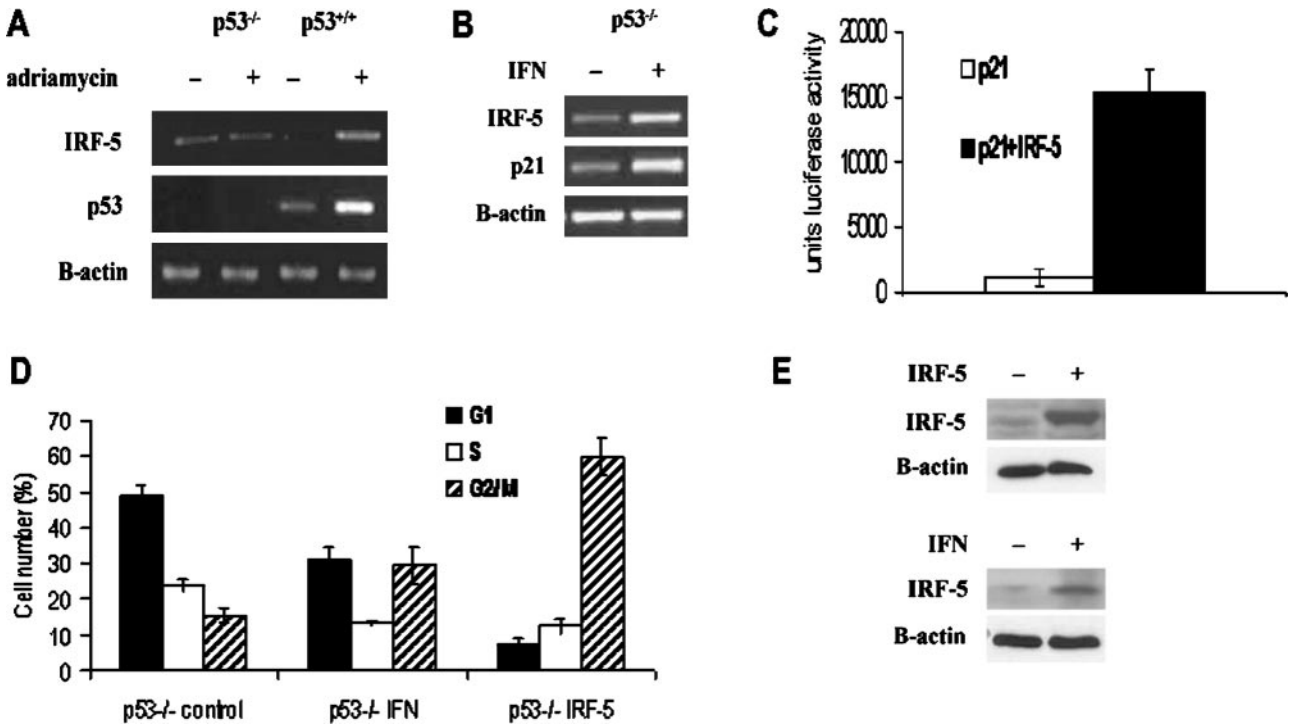

Fig. 1. Stimulation of IRF-5 expression by endogenous p53. *A*, RT-PCR analysis of IRF-5 mRNA in HCT116 $p53^{-/-}$ and HCT116 $p53^{+/+}$ cells before and after treatment with Adriamycin for 24 h. Levels of human β-actin transcripts are shown as a control for RNA levels. *B*, IFN induced expression of the endogenous IRF-5 gene in HCT116 $p53^{-/-}$ cells. β-Actin transcripts are shown as a control for the amount of RNA used. Data presented are representative from a minimum of three separate experiments. *C*, IRF-5 stimulates transcriptional activity of the p21 promoter. Saos cells were cotransfected with equal amounts (1 μg) of the p21 luciferase reporter plasmid and IRF-5-expressing plasmid. The thymidine kinase *Renilla*-luciferase reporter gene was cotransfected for normalization. Cell lysates were prepared at 36 h after transfection, and luciferase activity was measured using a Dual Luciferase Assay System (Promega). Data are expressed as fold induction for a representative experiment from a minimum of three separate experiments, each performed in triplicate. *D*, IFN treatment and IRF-5 overexpression alters cell cycle profile in $p53^{-/-}$ cells. Cells were stained with Hoechst 33258, and cell cycle progression was analyzed by FACS. *E*, levels of IRF-5 protein expressed in $p53^{-/-}$ cells transfected with Flag-tagged IRF-5 (*top panel set*) or treated with IFN (*bottom panel set*); the respective cell lysates were separated on an SDS gel and immunoblotted with polyclonal anti-IRF-5 antibodies.

mice reached ~15 mm. Tumor size and validity were assessed after sacrificing mice, excising tumor nodules for measurement, and RNA isolation.

DNA Microarray Analysis. Total RNA was isolated from BJAB vector control cells and BJAB/IRF-5-expressing cells and transcribed to cDNA. P^{32}-labeled DNA probes were generated and hybridized to the human apoptosis, p53 signaling, and/or cell cycle pathway GEArray Q series microarrays (SuperArray, Inc., Bethesda, MD) that each contained a total of 96 key genes involved with apoptosis or cell cycle regulation. The hybridization data were analyzed using the Image analysis software (Scanalyze by Michael Eisen) and the GEArrayAnalyzer v.1.2.31 (SuperArray, Inc.).

RT-PCR Analysis and RPA. BJAB or HCT116 cells were treated with 0.2 μg/ml Adriamycin for 16 h or 500 units/ml IFN-α for 16 h. Total RNA was reverse transcribed to cDNA using oligo(dT) primers. From this mixture of cDNAs, IRF-3, IRF-5, IRF-7, and β-actin were amplified by semiquantitative PCR under conditions that give a linear response, as described previously (1, 2). p21 was amplified with the following primers: sense, 5′-CCTCTTCGGC-CCGGTGGAC-3′; antisense, 5′-CCGTTTTCGACCCTGAGAG-3′. Total RNA was also analyzed by the RPA as previously described (2) using the hAPO-1c, hAPO-2b, or hStress-1 multiprobe template sets (BD PharMingen).

RESULTS

wt p53 Induces IRF-5. While our laboratory was in the process of characterizing the multiple functions of IRF-5 in immune regulation and growth inhibition, Mori *et al.* (19) published that IRF-5 is a direct target of p53. However, the potential function of IRF-5 as a regulator of cell growth in the absence of p53 was not addressed, nor was a mechanism proposed to explain their findings of growth inhibition. As a result, we have confirmed and extended their findings to further elucidate the multiple functions of *IRF-5* in cell growth regulation by using HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells in which the *p53* gene was inactivated by genetic recombination (14). Fig. 1*A* reveals that although low levels of endogenous IRF-5 could be detected in both the untreated and Adriamycin-treated $p53^{-/-}$ cells, Adriamycin stimulated expression of IRF-5 (~4-fold) and p53 in $p53^{+/+}$ cells. However, Adriamycin did not induce IRF-5 in $p53^{-/-}$ cells. In addition, treatment of cells with Irinotecan, another inducer of p53, stimulated expression of both *IRF-5* and *p21* genes. Under the same conditions, the levels of IRF-3 transcripts were unaffected by Irinotecan in these cells.[4] These results indicate that expression of IRF-5 is enhanced by Adriamycin- or Irinotecan-induced p53.

Because type I IFN has been shown to stimulate IRF-5 expression, we next examined whether IFN treatment would mediate cell arrest by the induction of IRF-5. For these studies, we have used the HCT116 $p53^{-/-}$ cells, where the modulation of cell cycle is a direct effect of IRF-5. As shown in Fig. 1*B*, treatment of these cells with IFN-α significantly increased the levels of *IRF-5* and *p21* transcripts, as well as the percentage of HCT116 $p53^{-/-}$ cells arrested in G_2-M (Fig. 1*D*). Cotransfection of an IRF-5 expression plasmid with a p21 luciferase reporter in Saos cells ($p53^{-/-}$) resulted in a 7-fold increase in the transactivation of the p21 promoter (Fig. 1*C*). Transfection of an IRF-5-expressing plasmid to HCT116 $p53^{-/-}$ cells resulted in a substantially more effective G_2-M cell arrest than by IFN treatment alone (Fig. 1*D*). The levels of IRF-5 protein expressed in transfected cells were ~2.5-fold higher than the levels induced by IFN (Fig. 1*E*). These data indicate that the G_2-M cell arrest is related to the levels of IRF-5 expressed in the cells. Furthermore, although p53 induced IRF-5 expression, overexpression of IRF-5 alone in the absence of wt

[4] B. J. Barnes and R. Ravi, unpublished data.

p53 was sufficient to trigger cell cycle arrest. These data also suggest that IRF-5 may be one of the factors that plays a role in IFN-mediated cell growth inhibition (20–23).

IRF-5-induced G_2-M Arrest and Apoptosis Is Independent of p53. We next used the human BJAB B-cell lymphoma line to examine closely the function of IRF-5 in cell growth regulation. As shown in Fig. 2*A*, although BJAB cells expressed nearly undetectable levels of constitutive IRF-5 transcripts, expression of IRF-3 and IRF-7 could be readily detected. Treatment of BJAB cells with IFNα (Fig. 2*A*, *Lane 3*) revealed that expression of IRF-5 could be stimulated (Fig. 2*A*). As shown in Fig. 2, *B* and *C*, although BJAB cells express high levels of p53, the fact that Adriamycin did not stimulate expression of

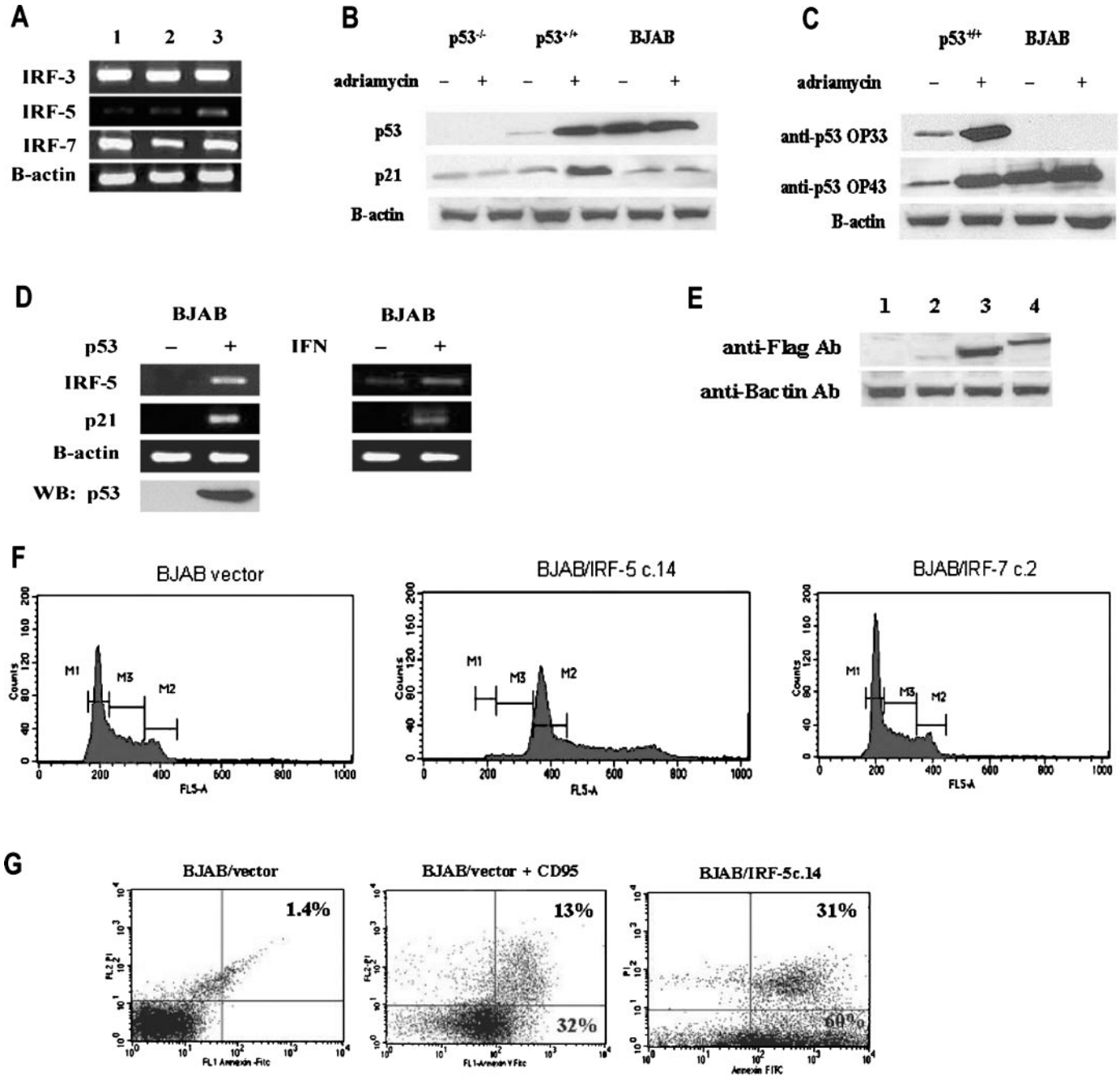

Fig. 2. IRF-5 induces G_2-M arrest and apoptosis in BJAB cells. *A*, levels of endogenous IRF expression in BJAB cells. IRF-3, IRF-5, IRF-7, and β-actin transcripts were amplified by RT-PCR. *Lane 1*, untreated BJAB cells; *Lane 2*, BJAB cells infected with Newcastle Disease Virus (NDV); and *Lane 3*, BJAB cells treated with type I IFN. *B*, BJAB cells express high levels of p53 protein but do not induce p21. Levels of p53, p21, and β-actin in lysates of HCT116 and BJAB cells were determined by immunoblot analysis. *C*, BJAB cells are deficient in the expression of functional p53. Wild-type and mutant p53 were immunoprecipitated with OP43 antibodies from cell lysates of HCT116 and BJAB cells. The wt p53 present in the immunoprecipitates was then detected by immunoblot analysis with OP33 antibodies. *D*, p53- and IFN-induced expression of the endogenous IRF-5 gene in BJAB cells. β-Actin transcripts are shown as a control for the amount of RNA used. Levels of p53 in transfected cells were determined by immunoblot analysis with OP33 antibodies. *E*, differential expression of Flag-tagged IRF-5 or IRF-7 proteins in stable-transfected BJAB clonal lines. *Lane 1*, BJAB vector control; *Lane 2*, BJAB/IRF-5 c.3 (low expressor); *Lane 3*, BJAB/IRF-5 c.14 (high expressor); and *Lane 4*, BJAB/IRF-7. *F*, effect of ectopic expression of IRF-5 or IRF-7 on cell cycle progression in BJAB cells. Histogram plots shown are gated (G_1-gated) on the log-phase growth population (G_1, S, and G_2-M) and thus the sub-G_1 cell population is not represented. M1 indicates G_1; M3, S phase; and M2, G_2-M. Results were confirmed by three to four independent experiments. *G*, FACS analysis of PI and Annexin-FITC-labeled BJAB cells expressing IRF-5. BJAB control cells were also treated with anti-CD95 antibodies to induce apoptosis. The percentage of cells stained positive for Annexin-FITC is shown in *bottom right quadrant*, and percentage of cells double stained with Annexin-FITC and PI is shown in *top right quadrant*.

p53 or p21 indicates that BJAB cells express mutant nonfunctional p53 (16). As a control, we show that Adriamycin induces p53 expression in $p53^{+/+}$ cells and not in $p53^{-/-}$ cells. Furthermore, by immunoblot analysis using monoclonal antibodies recognizing both a mutant and wt p53 (OP43) or wt p53 alone (OP33), wt p53 was detected only in lysates from $p53^{+/+}$ cells and not in BJAB cells (Fig. 2*C*).

Finally, we have shown that reconstitution of wt p53 expression in BJAB cells resulted in the induction of both *IRF-5* and *p21* genes, thus demonstrating that the p53-mediated signaling pathway in these cells is active. Treatment of BJAB cells with type I IFN also resulted in an enhancement of both IRF-5 and p21 transcript levels in the absence of functional p53 (Fig. 2*D*). Furthermore, Irinotecan (p53 inducer) enhanced the IFN-mediated expression of IRF-5 (from 2- to 5-fold induction) in $p53^{+/+}$ cells but not in $p53^{-/-}$ cells (3). These results indicate that p53 and IFN-mediated induction of IRF-5 is synergistic.

On the basis of these findings, we constructed BJAB B-lymphoma clonal lines overexpressing Flag-tagged IRF-5. For comparison, we also generated BJAB cells overexpressing Flag-tagged IRF-7 (Fig. 2*E*). During passage of these cells in culture, we noticed a continuous reduction (~10–30%) in the growth rate of IRF-5-expressing cells as compared with BJAB control cells over a 48–72-h time period, as determined by the number of viable cells. Whereas the growth rate of IRF-7-expressing BJAB cells and vector control cells did not change (data not shown). To examine the effect of ectopic IRF-5 or IRF-7 expression on cell cycle progression, cells were stained with Hoechst 33258, and the G_1-gated population of viable cells was examined by FACS. Although the majority (~45–50%) of BJAB cells transfected with empty vector or IRF-7-expressing plasmid were in the G_1 phase of the cell cycle (fraction of cells represented as M1), BJAB/IRF-5-expressing cells revealed an explicit G_2-M arrest (fraction of cells represented as M2; Fig. 2*F*). When BJAB vector control cells and BJAB/IRF-5-expressing cells were stained with Hoechst 33258 and examined by fluorescent microscopy, there was a distinct morphological difference between the BJAB control cells and BJAB/IRF-5 cells. Interestingly, IRF-5-expressing cells, but not control cells, showed the presence of apoptotic cell bodies (~30%), as determined in the sub-G_0-G_1 fraction (PI positive) of ungated IRF-5-expressing cells by FACS analysis (data not shown).

To further confirm these findings, Annexin-FITC and PI-labeled BJAB cells and BJAB/IRF-5-expressing cells were analyzed by FACS. In BJAB cells, only 1.4% of the population stained positive for FITC and PI (Fig. 2*G*). Treatment of these cells with anti-CD95 antibody for 6 h induced apoptosis, and ~32% of the cells were positive for FITC and 13% for FITC and PI, indicating induction off early-to-late stages of apoptosis. Analysis of BJAB/IRF-5-expressing cells have shown that ~31% of the population was stained positive for both FITC and PI and 60% for FITC alone. Thus, similar to the treatment of BJAB cells with anti-CD95 antibodies, overexpression of IRF-5-induced apoptosis in BJAB cells.

Overexpression of IRF-5 but not IRF-7 Inhibits Growth of BJAB Tumor Cells in Soft Agar and *in Vivo*. BJAB cells originated from a B-cell lymphoma that can form colonies in soft agar and tumors in nude mice. We therefore examined the effect of IRF-5 and IRF-7 on the ability of BJAB cells to form colonies in soft agar. As shown in Fig. 3*A*, BJAB/IRF-5 c.14 was unable to grow in soft agar although both BJAB/IRF-7 c.2 and BJAB control cells formed colonies efficiently. The inhibition of colony formation was related to the level of IRF-5 expression because c.14 (a high expressor) was unable to grow, whereas c.3 (a low expressor; Fig. 2*E*, *Lane 2*) was able to grow in soft agar, although with lower efficiency than BJAB control cells (Fig. 3*A*). The number of colonies was reduced by ~70–80% as compared with BJAB control colony formation.

To determine whether overexpression of IRF-5 in BJAB cells inhibits the ability of these cells to grow as a tumor *in vivo*, we injected nude mice s.c. with BJAB cells (group 1), BJAB/IRF-7 c.2 (group 2), or BJAB/IRF-5 c.14 (group 3). Tumor formation was monitored over 6 weeks when the tumor size in the control group reached ~15 mm. Although multiple tumors were detected in group 1, only single tumors were detected in group 2. However, both groups 1 and 2 had formed tumors as early as 3 weeks after injection, and tumors ranged in size from 15 to 20 mm after a 6-week incubation period. Group 3 did not show any tumor growth by external visual inspection (Fig. 3*B*). However, upon internal inspection, three of the six mice in group 3 had very small tumors (<5 mm in size). To determine whether tumors obtained from groups 2 and 3 still expressed the human IRF-7 or IRF-5, respectively, RNA isolated from these tumors was analyzed by RT-PCR. The analysis revealed that two of three tumors excised from mice in group 3 lost expression of

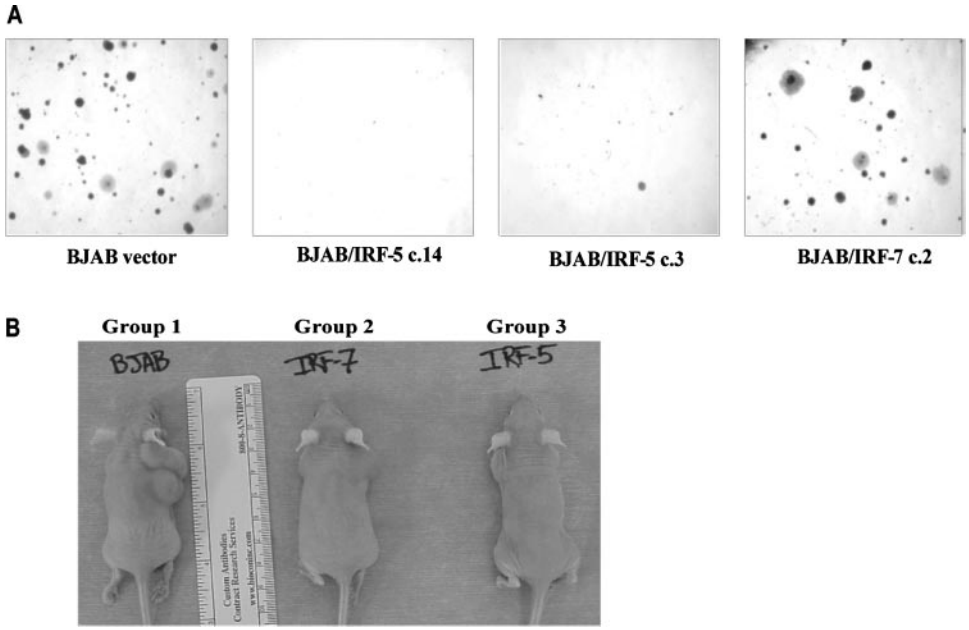

Fig. 3. Overexpression of IRF-5 inhibits *in vitro* and *in vivo* BJAB tumor cell growth. *A*, colony formation in soft agar of BJAB control cells, BJAB/IRF-5 c.14, BJAB/IRF-5 c.3, and BJAB/IRF-7 c.2. Each experiment was repeated at least three times using duplicate samples. *B*, IRF-5 inhibits tumor cell growth in athymic nude mice. Dorsal view of mice 47 days after injection. Group 1 was injected with viable BJAB/vector control cells, group 2 with BJAB/IRF-7 c.2 cells, and group 3 with BJAB/IRF-5 c.14 cells. A representative mouse from each group of six mice is shown.

IRF-5, whereas all tumors from group 2 mice expressed high levels of *IRF-7* (data not shown). These results indicate that IRF-5 was a substantially more effective inhibitor of tumor growth than IRF-7 because IRF-7-expressing tumors were still present in each mouse. Furthermore, IRF-7 expression did not result in the modulation of cell cycle or growth of BJAB cells in soft agar, whereas IRF-5-expressing cells were arrested at G_2-M and unable to grow as tumors.

IRF-5 Targets Genes Regulating Cell Cycle and Apoptosis. To elucidate the molecular mechanism by which IRF-5 induces cell arrest and cell death, we examined the potential impact of IRF-5 expression on the transcription of cellular genes associated with cell cycle regulation and apoptosis. To this effect, we used RNA from BJAB, BJAB/IRF-5, HCT116 $p53^{-/-}$, and HCT116 $p53^{-/-}$/IRF-5-expressing cells and examined gene expression using the human apoptosis, p53 signaling, and cell cycle-specific cDNA microarrays. Table 1 shows a group of selected genes that were regulated by at least 2.5-fold in the presence of IRF-5 and the absence of wt p53. Although a number of the IRF-5-regulated genes such as *p21*, *Bax*, *caspase 6*, *B99*, and *Bcl-2* represent a subset of known *p53* target genes, a large group of unique genes (18 of 24 regulated genes) such as *Bak1*, *caspase 8* and *Dap-kinase 2* were up-regulated by *IRF-5* alone (Table 1). On the other hand, a number of *p53* target genes, including *PUMA*, *14-3-3*, and *Bcl-x* were not targeted by *IRF-5* (data not shown). These results suggest that although there is some overlap in gene regulation by *IRF-5* and *p53*, the pathways emanating from these two factors are not redundant.

To confirm genes predicted by microarray analysis, we next examined the expression levels of several of these genes by the RPA and/or immunoblot analysis (Fig. 4). Fig. 4*A* reveals that each of the analyzed gene transcripts predicted by microarray to be modulated by *IRF-5* were indeed up-regulated or down-regulated by the RPA. Finally, by immunoblot analysis, we show that the IRF-5-mediated modulation of gene transcription also results in an alteration of the relative levels of respective proteins (Fig. 4*B*). Taken together, these results indicate that IRF-5-induced gene regulation is direct and independent of wt p53 and places IRF-5 downstream of p53 (Fig. 4*C*).

IRF-5, similar to other IRF family members, is a DNA binding protein that binds to the IRF-E consensus sequence in the promoters of target genes as homodimers or heterodimers with IRF-3 (2, 24). We have therefore examined whether the promoters of the identified *IRF-5* target genes contain IRF-E binding sites (GAAANN, AANNGAAA). As shown in Table 1, a number of the *IRF-5* target genes contained IRF-E sites indicating that IRF-5 may be able to bind to the promoter and participate in the transcriptional regulation of these genes. By transient cotransfection assay, IRF-5 indeed stimulated the transcriptional activity of the p21 promoter in Saos ($p53^{-/-}$) cells, indicating that the IRF-E in the p21 promoter is recognized by IRF-5 and activation of the p21 promoter does not require p53 (Fig. 1*C*).

IRF-5 Gene Expression Is Lacking in a Large Number of Primary Hematological Malignancies. By Northern blot analysis, we have previously shown that the constitutive expression of IRF-5 occurs primarily in lymphoid tissue, peripheral blood lymphocytes, and dendritic cells yet was not detected in a number of immortalized B- and T-cell leukemias (1). Here, we have additionally extended and confirmed these findings by comparing IRF-5 expression levels in mononuclear cells from healthy donors or from patients with CLL, ALL, or AML. For comparison, we have examined expression of the IRF-7 gene because it is also expressed primarily in lymphoid tissue (25). Furthermore, expression of the IRF-7 gene was found to be silenced by hypermethylation in some tumor cell lines (26).

Expression of IRF-5 and IRF-7 was detected in mononuclear cells from all healthy donors (Fig. 5, *Lanes 1–6*). We also detected constitutive expression of IRF-5 and IRF-7 in purified human activated monocytes and B- or T cells by intracellular flow, indicating that IRF-5 expression is not down-regulated in activated cells.[5, 6] In contrast, IRF-5 mRNA was not detected in the majority of leukemia cells from patients with hematological malignancies (Fig. 5). The most striking defect in IRF-5 expression was seen in patients with ALL where none of the 10 examined primary tumors expressed IRF-5 (Fig. 5, *Lanes 7–12*). However, IRF-7 was expressed in all of the tumors examined although the relative levels of expression have shown large variability. These data indicate that the silencing of IRF-5 expression in hematological malignancies may be associated with leukemogenesis.

Table 1 *IRF-5-modulated genes involved in cell cycle arrest and apoptosis*

IRF-5-responsive genes were identified by microarray analysis using the SuperArray GEArray Q series gene arrays. RNA isolated from BJAB cells or HCT116 $p53^{-/-}$ cells expressing IRF-5 was used for these studies. Levels of induction or repression were determined by statistical analyses using background subtractions and data normalization with control genes on each microarray. The table lists genes of interest that were modulated by IRF-5. Text in bold represents genes confirmed by RPA and/or immunoblot analysis. The asterisk denotes p53 target genes (36, 52). [a] (+) ≥2.5–4.99-fold induction; (++) ≥5.0–9.99-fold induction; (+++) ≥10.0; (−) ≤0.0. The data are representative of two to three independent microarray gene array experiments. The number(s) of IRF-E consensus sequence(s) in the promoter is indicated, except in those cases where sequences have not been deposited in GenBank (NA, not available).

Gene name	Genbank no.	Induction (+) Repression (−)	No. of IRF-E consensus sequence (promoter/coding region)
Bak1	**AA923639**	**++**	**NA**
p21Waf1 (p21Cip1)*	**L47233**	**+**	**4**
Bax*	**L22474**	**+**	**1**
Caspase 6*	**U20537**	**+**	**NA**
Caspase 8	**NM_001228**	**++**	**0**
CIDE-B	NM_014430	+++	1
TNFRSF12 (DR3)	U74611	+	NA
TNFRSF8 (CD30)	NM_001243	++	1
TNFRSF10B (DR5)*	AF016266	+	3
DAP-kinase 2	**NM_014326**	**++**	**NA**
TNFRSF10A (TRAIL-R)	U90875	+	NA
Chk2 (RAD53)	NM_007194	+	NA
Cyclin G2	L49506	+	NA
Chk1	AF016582	+	NA
BRCA1	U68041	+	1
ATM	NM_000051	++	1
D5S346	M73547	++	NA
B99*	NM_016426	++	NA
p57Kip2	U22398	++	1
CyclinB1	**M25753**	**−**	**2**
Cdc2 (cdk1)	NM_001786	−	0
Bcl-2*	**M14745**	**−**	**NA**
API3/XIAP	NM_001167	−	NA
Survivin (API4)	U75285	−	0

[a] B. J. Barnes, unpublished data.

DISCUSSION

The data presented here demonstrate that expression of IRF-5 in uninfected cells is associated with the inhibition of cell cycle and induction of apoptosis. The observation that IRF-5 expression is silenced in primary hematological malignancies indicates that IRF-5 may function as a tumor suppressor gene. Although the IRF-7 gene was expressed in all primary tumors examined, expression of IRF-5 was lacking in these malignancies. The most striking absence of IRF-5 expression was in patients with ALL where none of the 10 examined tumors expressed IRF-5. Whether the IRF-5 gene is deleted in these tumors or silenced by hypermethylation is currently under investigation.

[5] A. Izaguirre, B. J. Barnes, S. Amrute, W. S. Yeow, N. Megjugorac, J. Dai, D. Feng, E. Chung, P. M. Pitha, and P. A. Fitzgerald-Bocarsly. Comparative analysis of IRF and IFN-α expression in human plasmacytoid and monocyte derived dendritic cells. J. Leuk. Biol., in press, 2003.

[6] S. Amrute and B. J. Barnes, unpublished data.

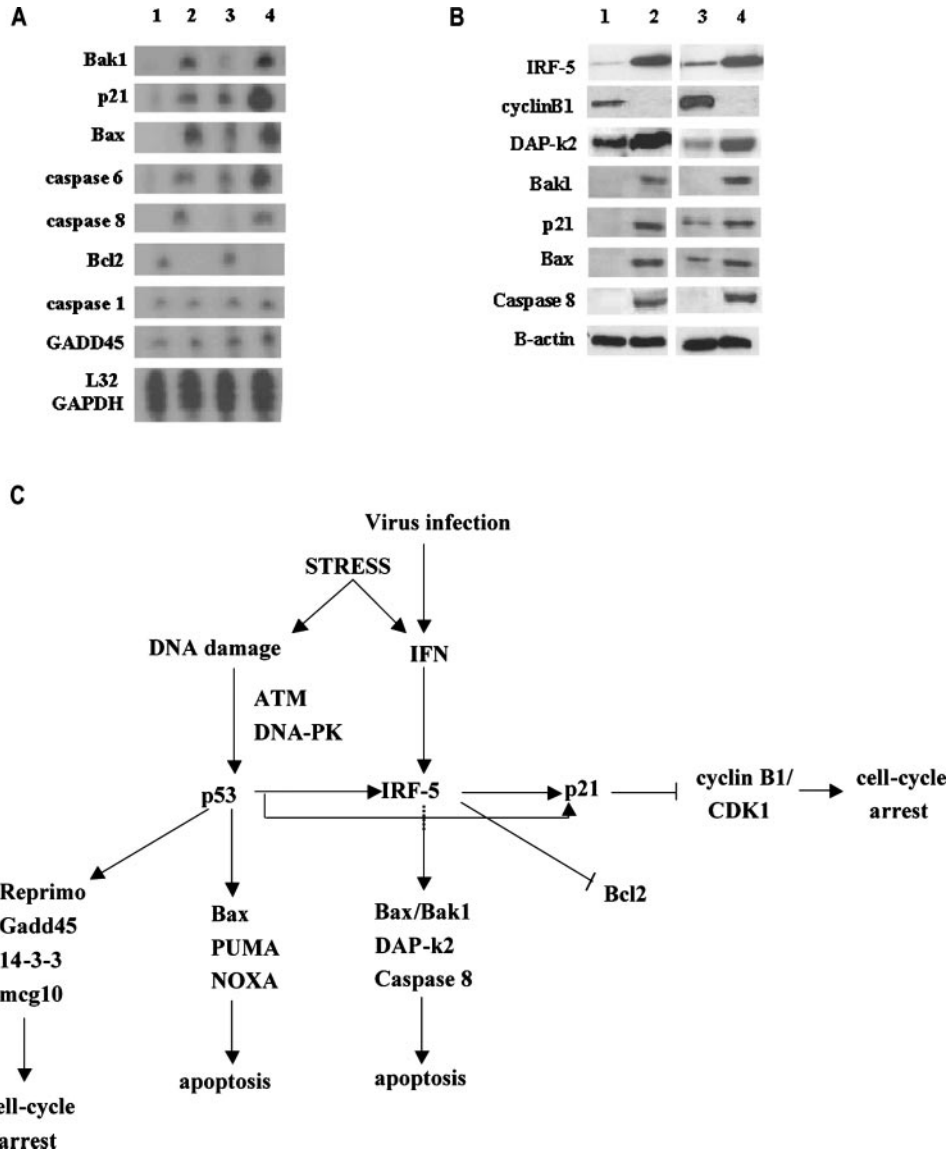

Fig. 4. IRF-5 targets genes regulating cell cycle arrest and apoptosis. *A*, analysis of p21, Bcl2 family members, and caspase transcript levels in BJAB and $p53^{-/-}$ cells by the RPA. *Lane 1*, BJAB cells; *Lane 2*, BJAB/IRF-5 c.14-expressing cells; *Lane 3*, HCT116 $p53^{-/-}$ cells; *Lane 4*, $p53^{-/-}$/IRF-5-expressing cells. Levels of L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown as internal controls. *B*, immunoblot analysis of proteins regulated by IRF-5. Cell lysate from BJAB cells (*Lane 1*), BJAB/IRF-5 c.14-expressing cells (*Lane 2*), HCT116 $p53^{-/-}$ cells (*Lane 3*), and $p53^{-/-}$/IRF-5-expressing cells (*Lane 4*) were analyzed by immunoblot using antibodies described in experimental procedures. Levels of transfected Flag-tagged IRF-5 are shown. *C*, schematic representation of the role of IRF-5 in cell cycle regulation and apoptosis. The extrinsic pathways for IRF-5 gene induction are triggered by wt p53 or IFN. Activation of the stress-signaling network stimulates induction of p53 and consequently an increase in IRF-5 transcript levels. The expression of several target genes is then activated by IRF-5. These genes are involved in processes that either slow progression through the cell cycle (cell arrest) or increase cell death (apoptosis).

The tumor suppressing activity of IRF-5 was further demonstrated in BJAB B-cell lymphoma, where ectopic expression of IRF-5 induced a G_2-M arrest, inhibited cell growth in soft agar, and tumor formation in nude mice. While addressing the molecular nature of this inhibition, our studies revealed that IRF-5 stimulates expression of the cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ and represses cyclin B1, both of which are responsible in part for the regulation of a G_1-S and/or G_2-M cell cycle checkpoint. In addition, IRF-5 stimulates expression of several proapoptotic genes such as *Bak1*, *Bax*, *caspase 8*, and *DAP kinase-2*. The ability of IRF-5 to induce cell arrest and cell death is also one of the multifaceted activities of p53; yet, our data indicate that IRF-5 and p53 induce both a set of overlapping and distinct genes. As shown in this study, the IRF-5-mediated growth inhibition and proapoptotic effects are p53 independent. These results and previous data (2) suggest that IRF-5 functions as an intermediate in the information cascade initiating from p53 or IFN to effector genes that either induce a program of cell arrest, cell death, or immunomodulation (Fig. 4*C*).

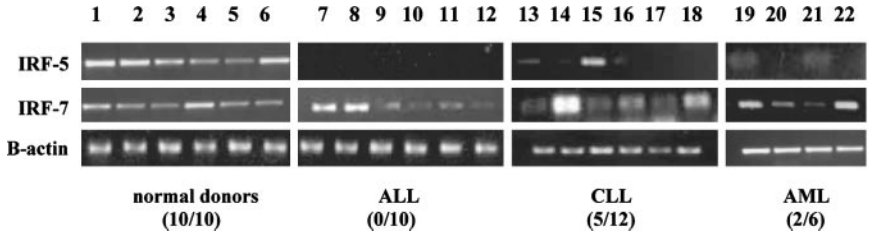

Fig. 5. *IRF-5* gene expression is lacking in primary hematological malignancies. Relative levels of endogenous IRF-5 transcripts present in primary mononuclear cells from healthy donors or from patients with ALL, CLL, or AML. Mononuclear cells from patients with leukemia have a very high percentage of uniformity because of the presence of malignant cells. IRF-5, IRF-7, and β-actin transcripts were amplified by RT-PCR. *Lanes 1–6*, mononuclear cells from healthy donors; *Lanes 7–12*, leukemia cells from patients with ALL; *Lanes 13–18*, patients with CLL; and *Lanes 19–22*, patients with AML. Ratios listed at the bottom indicate number of samples expressing IRF-5/total number of samples tested.

The critical role of p21 and cyclin B1 in growth arrest has been well established (14, 16, 27, 28). Although most of the studies on p21-regulated checkpoints have been focused on the G_1-S transition, several previous observations point to the importance of this gene in G_2-M (14, 27, 29–32). For instance, tumor cells lacking functional p21 were shown to be defective in the control mechanism at the G_2-M checkpoint and thus proceed through multiple rounds of S phase (33). Furthermore, *B99*, *BRCA1*, and *Chk1/Chk2* expression, which was also stimulated by IRF-5, play an important role in G_2-M cell arrest (34–39). Thus, results reported herein implicate *IRF-5* as a trigger for cell cycle arrest within the G_2 phase. Available evidence, thus far, would suggest that this is achieved, at least in part, by IRF-5-induced expression of p21. However, additional studies are necessary to determine the contribution of p21 and other growth regulatory genes induced by IRF-5 to the observed cell cycle arrest.

The ability of *IRF-5* to induce expression of proteins involved in cell growth regulation and immunomodulation represents an important defense mechanism against extracellular stress, including viral infection. We have performed detailed microarray studies to examine more closely the genes regulated by *IRF-5*. Preliminary results indicate that in addition to cell cycle regulatory and proapoptotic genes, IRF-5 induces a large number of immune response genes, IFN-induced proteins, and ubiquitin enzymes (data not shown). Interestingly, a number of these *IRF-5* target genes were induced in both $p53^{-/-}$ and $p53^{+/+}$ cells, indicating the importance of IRF-5 in p53-dependent immune response (data not shown). Viral oncoproteins encoded by many DNA tumor viruses are able to bind p53. The adenovirus-encoded E1A protein induces a rapid p53-dependent apoptotic response, whereas the E1B protein binds p53 and inhibits its function (40, 41). Human cytomegalovirus major IE proteins (42), EBV nuclear antigen (43) and hepatitis B virus X protein (44), all form complexes with the p53 protein and inhibit its transactivation function. Lastly, the human papillomavirus E6 protein induces the degradation of p53 (45). Thus, all of these viruses developed mechanisms by which they can override the host response against infection. In a similar manner, many viruses encode proteins that target IRF function and the antiviral response (46–49). Taken together, these data indicate that both p53 and IRF have important roles in host response to viral infection.

The role of two IRF family members in the induction of apoptosis has been well established. IRF-1-targeted genes include *p21*, *caspase 1*, and *lysyl oxidase* genes (12, 50, 51), and IRF-3 mediates Sendai-virus-induced apoptosis through stimulation of caspases 8 and 3 (7). In this study, we show that *IRF-5* modulates the expression of a number of factors involved in cell cycle regulation and apoptosis, independent of viral infection. Additional studies are necessary to determine the exact position IRF-5 holds in the apoptotic signaling pathway. The observation that some of the growth regulatory and proapoptotic functions of p53 can be replaced by IRF-5 suggests that a closer analysis of pathways leading to and from IRF-5 may provide novel strategies for cell growth management. Clinically, the loss of p53 function is associated with resistance to apoptosis and treatment failure. By demonstrating the proapoptotic and antitumor activity of IRF-5 in the absence of functional p53, our data suggests that induction of IRF-5 expression may lead to novel therapeutic strategies for treatment of cancers deficient in p53 function. Furthermore, the induction of IRF-5 expression may increase the sensitivity of p53-deficient tumors to drug-induced apoptosis. We are currently exploring both of these possibilities.

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