

Modulation of drug resistance by artificial transcription factors

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Abstract

The efficiency of chemotherapeutic treatments in cancer patients is often impaired by the acquisition of drug resistance. Cancer cells develop drug resistance through dysregulation of one or more genes or cellular pathways. To isolate efficient regulators of drug resistance in tumor cells, we have adopted a genome-wide scanning approach based on the screening of large libraries of artificial transcription factors (ATFs) made of three and six randomly assembled zinc finger domains. Zinc finger libraries were linked to a VP64 activation domain and delivered into a paclitaxel-sensitive tumor cell line. Following drug treatment, several ATFs were isolated that promoted drug resistance. One of these ATFs, 3ZF-1-VP, promoted paclitaxel resistance in cell lines having mutated or inactivated p53, such as MDA-MB-435 and Kaposi's sarcoma cell lines. 3ZF-1-VP also induced strong resistance to etoposide, vincristine, and cisplatin. Linkage of a repression domain to the selected ATF resulted in enhanced sensitivity to multiple drugs, particularly vincristine, cisplatin, and 5-fluorouracil. Small interfering RNA-mediated inhibition of p53 revealed

that 3ZF-1-VP activated both p53-dependent and p53-independent mechanisms to promote survival, whereas other ATF required intact p53. Real-time expression analysis and DNA microarrays showed that several ATFs up-regulated targets of p53, such as the cyclin-dependent kinase inhibitor *p21^{WAF1/CIP1}*, and genes participating in the *p14^{ARF}-MDM2-p53* tumor suppressor pathway, such as *hDMP1*. Thus, ATF can be used to map genes and pathways involved in drug resistance phenotypes and have potential as novel therapeutic agents to inhibit drug resistance. [Mol Cancer Ther 2008;7(3):688–97]

Introduction

Acquired resistance to anticancer drugs is a formidable problem that hampers current chemotherapeutic treatments. Tumor cells develop drug resistance by dysregulating expression of one or more genes that affect a variety of mechanisms (1). Commonly dysregulated genes include ABC transporters (e.g., the *MDR1* gene product), genes involved in drug metabolism (e.g., cytochrome *P450*), genes controlling cell cycle progression (e.g., cyclin inhibitors), and antiapoptotic genes (e.g., the *bcl* family members; refs. 2, 3). The *MDR-1* gene confers resistance to several drugs, including paclitaxel, doxorubicin, and vincristine. Inhibitors of *MDR-1* have been developed, including small molecules, ribozymes (4, 5), antisense oligonucleotides (6, 7), small interfering RNA (siRNA; refs. 8, 9), and *de novo* designed artificial transcription factors (ATF; ref. 10). However, the multiplicity of molecular responses that trigger drug resistance in cancer cells, or multifactorial multidrug resistance (1), poses a fundamental limitation for single, directed, gene targeting approaches.

In contrast to inhibitors that target single genes, natural transcription factors typically recognize DNA sequences located in several promoters, thereby orchestrating expression of many genes and subsequent cellular responses. Transcription factors, such as p53, a tumor suppressor lost or mutated in 50% of human tumors (11–13), play a central role in regulating multiple targets in response to many stress responses, including DNA-damaging signals (11–19). Like natural transcription factors, ATFs may recognize DNA sequences located in several promoters and thus can regulate expression of more than one gene. In addition, ATFs can be designed to mediate both up- and down-regulation of targeted genes. When the DNA-binding domain (DBD) of an ATF is linked to an activation domain, the resulting transcriptional activator is able to up-regulate target gene expression, whereas linkage of a repression domain down-regulates target gene expression (20). We reasoned that multiplicity of targeting, together with transcriptional modulation of gene expression mediated by ATFs, could be used to efficiently regulate resistance to

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This work describes the isolation of ATF able to modulate resistance to multiple drugs in tumor cells; these proteins hold promise in cancer therapeutics as modulators of acquired drug resistance.

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anticancer drugs in cancer cell lines. To test the capability of ATFs to down-regulate resistance to anticancer drugs, we have adopted a genome-wide screening strategy. We have described previously the construction of ATF libraries by recombination of sequence-specific zinc finger DBDs (21–23). In this article, we delivered three-finger (3ZF) and six-finger (6ZF) zinc finger activator libraries into HeLa cells to identify and regulate gene pathways involved in drug resistance.

Materials and Methods

Zinc Finger Selection

The pMX-3ZF-library-VP64-IRES-GFP and the pMX-6ZF-library-VP64-IRES-GFP were transfected into 293 cells that express the gag-pol genes as described (21, 23). Viral supernatant was used to infect 3×10^5 and 10^8 HeLa cells, plated in 20×150 cm tissue culture dishes, for the 3ZF and 6ZF libraries, respectively. After 72 h, the cells were treated with 200 nmol/L paclitaxel for 3 days. As a control, mock-transduced cells were treated with the same concentration of paclitaxel. After drug treatment, the cells were washed once with PBS and once with medium and allowed to proliferate for a period of 1 week (to avoid the selection of nonproliferating, terminally differentiated cells). After the drug treatment, no cells survived from mock-transduced plates, but we obtained up to five resistant colonies per plate from the library-transduced cells. Genomic DNA was extracted from surviving cells and the DNA encoding the zinc fingers was extracted by PCR and recloned into the pMX-IRES-GFP vector as described (21, 23). Single clones were sequenced and processed as described below for functional analysis.

Survival Assays

Individual 3ZF or 6ZF proteins expressed in the retroviral vector pMX-ZF-VP64-IRES-GFP were individually transduced into HeLa cells. An XTT (Roche) survival assay to determine the fraction of surviving cells was done following the manufacturer's instructions. The experiment was repeated at least three times with independent transductions to ensure reproducibility. Statistical significance between groups (ATF-transduced clones and control cells) was determined using a Student's *t* test ($P < 0.05$ was considered significant).

DNA-Binding Analysis

The zinc fingers were cloned in pMalC2 vector (NEB), which enables expression of the zinc finger DBD as a COOH-terminal fusion with the bacterial carrier protein maltose-binding protein. The zinc finger DBD-maltose-binding protein were expressed in *Escherichia coli* XL-1 and purified using an amylose resin as described by the manufacturer's instructions (NEB). Biotinylated oligonucleotides comprising the ATF-binding sites were added to streptavidin-coated 96-well ELISA plates as described (24). Purified protein in binding buffer [ZFA buffer containing 5 mmol/L DTT/1% bovine serum albumin/sonicated salmon sperm 140 ng/ μ L (Sigma)] was added at different concentrations in the linear range of this assay. Binding was

normalized to 1 with the cognate substrate signal for each protein. DNA mobility shifts and K_d determinations were done as described (24).

Real-time Reverse Transcription-PCR and DNA Microarray Expression Analysis

Cells were collected and the RNA was extracted using the RNeasy extraction kit (Qiagen) and reverse transcribed using the high-capacity cDNA Archive kit (Applied Biosystems). Real-time PCR conditions and DNA microarray experiments are described in Supplementary Material.⁵

Luciferase Assays

The *WAF1/CIP1* promoter reporter plasmid was kindly provided by Carol Prives (Columbia University). For the construction of a human DMP1 promoter reporter, we amplified a 2.3-kb genomic fragment 5' of exon 1 using a PCR approach and oligonucleotide primers (forward 5'-GAGCTCTTCATTCTCCATTAGCACAGCAATCTCCATCAGC-3' and reverse 5'-CTCGAGTCCGGGCACTTTGGAAGAACCAGGATGGAAGCTC-3'); the primer used incorporated *SacI* and *XhoI* restriction sites into the fragment. The resulting promoter fragment was cloned into the *SacI* and *XhoI* sites of the pGL2-Basic vector (Promega). For transfection experiments, 293T cells were seeded into a 24-well plate at a density of 3×10^3 cells per well. After 24 h, cells were transfected using a modified calcium phosphate method (25). For the reporter assay, 100 ng reporter plasmid was cotransfected with 50 or 200 ng zinc finger expression plasmid or pcDNA3.1 vector. pRL-TK *Renilla* luciferase vector (4 ng) was cotransfected in each experiment as an internal control for transfection efficiency. After 48 h, cell lysates were prepared and luciferase activity was measured according to the manufacturer's Dual-Luciferase reporter assay protocol (Promega). Each transfection was done in triplicate and data are presented as the mean \pm SE.

Proliferation Analysis

Individual clones of ATF-transduced cells were plated in triplicate in a 96-well format tissue culture plates (2,000 cells per well). Cells were allowed to proliferate for 4 days. Growth was assessed by XTT survival analysis (Roche), done every 24 h, according to the manufacturer's instructions.

Cell Cycle Analysis

Individual clones of ATF-transduced cells were harvested ($\sim 10^6$ cells per clone) and treated with 0.5 mL PBS containing RNase A (0.02 mg/mL; Qiagen) for 30 min in the dark and then chilled on ice for 10 min. A 25 μ L aliquot of 1 mg/mL propidium iodide (Sigma) was added. Cells were incubated in the propidium iodide solution for 1 h at room temperature in the dark and then analyzed by flow cytometry using a FACSCalibur (BD). The percentages of cells in the G_0 - G_1 , S, and G_2 -M phases of the cell cycle were determined using CellQuest (BD) and ModFit software (BD). The experiment was done three times with three independent transductions to ensure reproducibility.

⁵ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Results

Selection of ATF That Promote Drug Resistance

Three- and six-finger libraries linked to the VP64 activation domain were delivered into HeLa cells using the pMX-IRES-GFP retroviral vector (26). We chose HeLa cells because this cell line is sensitive to the drug paclitaxel and expresses wild-type p53. The conditions described below were the same as described for peptide library selections using retroviral libraries in HeLa cells (27). As described elsewhere (21), a 3ZF library with a complexity of $\sim 10^4$ different clones can recognize most 9-bp sequences of type 5'-(G/A/TNN)₃-3'. A 6ZF library of complexity 7.4×10^7 allows recognition of most 18 bp of sequences of the type 5'-(G/A/TNN)₆-3'. We transduced 3×10^5 HeLa cells with a 3ZF library and 10^8 HeLa cells with a 6ZF library. After 72 h, the transduced cells were treated with 200 nmol/L paclitaxel. After an additional 72 h, virtually all untransduced cells became apoptotic and detached from the plate. However, several 3ZF- and 6ZF-transduced colonies were able to survive to paclitaxel treatment. Retroviral clones that survived in this assay were replated and propagated for a period of 1 week in absence of drug as described (28). The genes encoding the 3ZF and 6ZF DBDs were extracted by PCR using genomic DNA from the transduced cells and recloned into the pMX-IRES-GFP vector as described (21, 23).

Individual ATF clones were reintroduced into HeLa cells and individually challenged with paclitaxel. From a total of 15 3ZF clones and 30 6ZF clones tested individually, we isolated several ATFs able to promote paclitaxel resistance in a survival assay (Table 1; Fig. 1A). One of the 6ZF sequences was identical from two independent clones, indicating selective pressure for particular ATF members. As shown in Fig. 1A and Supplementary Fig. S1,⁵ selected ATF induced different degrees of paclitaxel resistance. The strongest effect was achieved with a 3ZF protein, 3ZF-1-VP. Each of the ATF that promoted paclitaxel resistance also

protected HeLa cells from the drug etoposide, a topoisomerase II inhibitor (Fig. 1B; Supplementary Fig. S2)⁵ at concentrations higher than 5 μ mol/L. The ATF did not have a significant effect on toxicity of the drug doxorubicin (data not shown). In contrast to results from a recent report (29), we were not able to select ATFs that imparted significant resistance to paclitaxel or other anticancer drugs by using transient expression vectors, even after five rounds of selection.

To investigate whether the induction of drug resistance phenotype by ATFs depended on the nature of the effector domain, we expressed the DBDs as fusions with a strong transcriptional repression domain (Kruppel domain). All selected proteins, except ATF 3ZF-6-KD, had no effect on the drug resistance phenotype relative to the cell line (Supplementary Fig. S3).⁵ ATF 3ZF-6 promoted drug resistance independently of the effector domain; this zinc finger protein might act either through simple DNA binding and competition with endogenous proteins or through post-transcriptional regulation. The other ATF selected in this screen required the activation domain, and therefore transcriptional gene activation, for phenotype induction.

In vitro Characterization of 3ZF and 6ZF DBD

Next, we determined whether the selected 3ZF and 6ZF DBDs were able to bind their predicted DNA substrates *in vitro*. These substrates were defined according to the nature of the DNA-binding α -helical sequences indicated in Table 1. DBDs were expressed as COOH-terminal fusions with bacterial maltose-binding protein. Electrophoretic mobility shift assays and DNA-binding specificity ELISA were used to determine binding affinities and specificities. As shown in Fig. 2, all zinc finger-maltose-binding protein fusions bound predicted target sites with high sequence selectivity. These results show that ATFs isolated from functional phenotypic screens retain their predicted substrate specificity. The affinities of the zinc finger proteins

Table 1. Zinc finger sequences selected for Taxol resistance in HeLa cells

	TFSZF			Zinc finger helices*			Predicted target sites [†]		K_d (nmol/L)
	F6	F5	F4	F3	F2	F1	Half-site 1	Half-site 2	
6ZF-1-VP	DPGNLVR	QAGHLAS	QSSNLAS	TSGNLVR	DCRDLAR	RSDVLVR	5-GACTGATAA	-GATGCCGTG-3	ND
6ZF-5-VP	QAGHLAS	QAGHLAS	RSDHLTT	DPGALVR	RSDVLVR	SDHLTNH	5-TGAGCGGCT	-TGAGGGGTC-3	ND
6ZF-8-VP	RSDNLVR	QAGHLAS	QSSNLAS	QSSNLVR	TSGNLVR	DPGHLVR	5-GAGTGATGA	-GAAGATGGC-3	ND
6ZF-20-VP	QAGHLAS	QSGDLRR	TSGSLVR	QAGHLAS	DCRDLAR	QRANLRA	5-TGAGCAGTT	-TGAGCCAAA-3	ND
6ZF-24-VP	QSGDLRR	TSGNLVR	QSSNLAS	DPGHLVR	TSGHLVR	QSSNLAS	5-GCAGATTAA	-GGCGGTAA-3	1.23 ± 0.087
6ZF-30-VP	QSSNLVR	QRAHLER	TSGELVR	RSDDLVR	TSGNLVR	RSDKLVR	5-GAAGGAGCT	-GCGGATGGG-3	1.22 ± 0.12
3ZF-1-VP				QSSHLVR	TSGSLVR	RSDTSSN		5-GGAGTTAAG-3	3.34 ± 0.32
3ZF-5-VP				REDNLHT	TSGSLVR	RSDNLVR		5-TAGGTTGAG-3	ND
3ZF-6-VP				QSSSLVR	QSSNLVR	QSSHLVR		5-GTAGAAGGA-3	ND
3ZF-16-VP				TSGSLVR	RSDTLN	RSDNLVR		5-GTTAAGGAG-3	ND

NOTE: The predicted 18-bp DNA-binding site (6ZF library selections; top) and 9-bp binding site (3ZF library selections; bottom) are indicated. Predicted target DNA sequences are presented in the 5 to 8 orientation. K_d , dissociation constant, calculated by gel retardation (22); ND, not determined.

*Zinc finger helices are positioned in the antiparallel orientation (COOH-F6 to F1-NH2) relatively to the DNA target sequence. Amino acid position -1 to +6 of each DNA.

[†]Recognition helix is shown. Top, 6ZF proteins; bottom, 3ZF proteins.

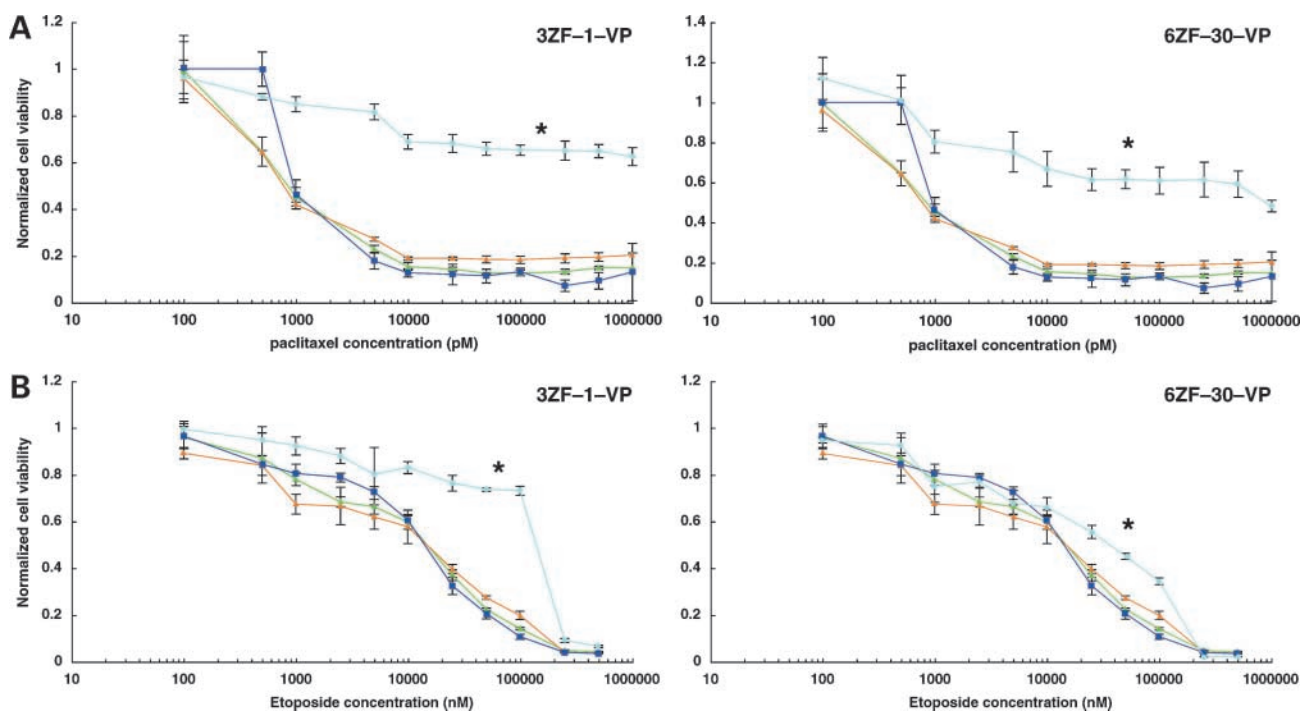


Figure 1. Survival assays of HeLa cells transduced with individual ATF isolated from a paclitaxel resistance screen. For simplicity, data from only two ATF (3ZF-1-VP and 6ZF-30-VP) are shown; data from each ATF tested are plotted in Supplementary Figs. S1 and S2.⁵ Cells were transduced with an ATF (3ZF-8-VP; *green*) that did not impart drug resistance, with a construct lacking an activation domain (*dark blue*), with the 6ZF library (*orange*), and with 3ZF and 6ZF proteins selected from the drug screen (*light blue*). Dark blue, mock-transduced cells. Single clones of transduced cells were seeded in a 96-well tissue culture plate and treated with the indicated concentrations of paclitaxel (**A**) and etoposide (**B**). Survival was determined by an XTT assay (Roche). Average of three wells \pm SE. Data were normalized to results from untreated cells in each clone set. The experiment was repeated three times in independent transductions. Cells were efficiently transduced (70-99%) as assessed by flow cytometry using green fluorescent protein as a control. *, $P < 0.05$, ATF-transduced groups (*light blue*) versus control cells (cells transduced with library; *orange*).

were in the 1 to 5 nmol/L range (Table 1). As expected, 6ZF proteins had higher affinities than 3ZF proteins due to the presence of additional zinc finger-DNA interactions.

ATFs Impart Resistance to Several Anticancer Drugs

Next, we investigated whether the ATF enhanced survival of HeLa cells treated with anticancer drugs with unrelated mechanisms of action. We focused on ATF 3ZF-1-VP because this protein had the strongest activity and protected cells from higher concentrations of paclitaxel than the other ATFs evaluated (Fig. 1; Supplementary Fig. S1).⁵ We challenged HeLa cells retrovirally transduced with 3ZF-1-VP with several commonly used anticancer drugs. As shown in Fig. 3, 3ZF-1-VP enhanced drug resistance to cisplatin, a DNA-damaging drug, and vincristine, a microtubule-destabilizing drug. ATF 3ZF-1-VP did not alter susceptibility to 5-fluorouracil. The results of these experiments showed that the ATF 3ZF-1-VP protected HeLa cells not only from paclitaxel (the drug used in the selection) but also from anticancer drugs with unrelated mechanisms of action.

Dependence on the Effector Domain in ATF-Mediated Drug Resistance

Our results suggested that ATF 3ZF-1-VP was able to promote multidrug resistance in a manner dependent on the linked transactivation domain. We investigated whe-

ther this same DBD linked to a transcriptional repressor, the Kruppel domain, could down-regulate the drug resistance phenotype. As shown in Fig. 3, HeLa cells transduced with ATF 3ZF-1-KD exhibited enhanced sensitivity to several drugs, particularly vincristine, cisplatin, and 5-fluorouracil, compared with cells transduced with a control retrovirus. ATF 3ZF-1-KD did not significantly enhance sensitivity to carboplatin. This may indicate that specific mechanisms of drug resistance are associated with certain drugs.

ATF 3ZF-1-VP Activates Both p53-Dependent and p53-Independent Pathways

We next attempted to determine the molecular mechanisms by which 3ZF 1-VP induces drug resistance. We first studied whether intact p53 was necessary for 3ZF-1-VP-mediated drug resistance. The p53 protein is mutated in 50% of human tumors and has been shown to play a central role in inducing growth arrest and apoptosis in response to DNA-damaging signals (11-13). Overexpression of known targets of p53, such as $p21^{WAF1/CIP1}$, is correlated with antiproliferative responses, G_0 - G_1 cell cycle arrest (12, 13, 30, 31) and responsiveness to chemotherapy (14, 17, 32). The p53 pathway is inactivated in HeLa cells as the papillomavirus E6 protein binds to the p53 protein and targets it for ubiquitin-mediated degradation (33).

It has been reported, however, that p53 can be reactivated in HeLa cells by expression of the papillomavirus E2 protein or by treatment with leptomycin B or actinomycin D (34).

To determine whether drug resistance mediated by this 3ZF-1-VP required p53, we first studied the effect of ATF on drug resistance in cell lines other than the HeLa. As shown in Fig. 4A, ATF 3ZF-1-VP was able to enhance paclitaxel resistance in the MDA-MB-435 cell line and in a Kaposi's sarcoma line. A mutated form of p53 is expressed in MDA-MB-435 cells (35) and the p53 pathway is inactivated by herpesvirus proteins in the Kaposi's line (36). The fact that ATF 3ZF-1-VP had some activity in cells with mutated or inactivated p53 suggests that the mechanism of ATF 3ZF-1-VP does not completely depend on p53.

This was further verified by inhibition of p53 expression using a siRNA. HeLa cells were transduced with a lentiviral vector expressing a siRNA complementary to a region

of *p53* mRNA (37). HeLa cells stably expressing this construct showed 90% to 95% reduction in *p53* mRNA expression, compared with the parental HeLa cell line, as assessed by real-time reverse transcription-PCR quantification (Fig. 4C). HeLa cells transduced with a lentiviral vector encoding a p53 siRNA or with a control lentiviral vector that expressed a non-p53 siRNA were first treated with hygromycin to select for cells that had stably integrated vectors. These cells were next transduced with vector that allowed expression of ATF 3ZF-1-VP and were then challenged with paclitaxel. The percentage of cells surviving the drug was assessed by XTT assay. ATF expression in HeLa cells was verified by flow cytometry using GFP as a control.

As shown in Fig. 4B (top), the p53 siRNA had little effect on survival of cells as concentrations of paclitaxel were increased. This was expected because the p53 tumor suppressor is inactivated at the protein level in HeLa cells. However, when p53 siRNA was coexpressed with ATF 3ZF-1-VP, there was a significant reduction (20-40%) in drug resistance relative to cells that expressed only 3ZF-1-VP (Fig. 4B, bottom). This indicates that ATF 3ZF-1-VP activates both p53-dependent and p53-independent pathways to induce the drug-resistant phenotype. Thus, ATF 3ZF-1-VP could activate some targets involved in control of cell growth that are independent of the p53 pathway or targets downstream of p53 itself. The p53 dependence was also observed in other ATF-transduced cells, such as ATF 3ZF-1-VP, where the p53-siRNA significantly affected the ability of transduced cells to survive paclitaxel treatment. Consistent with this p53 dependence, we found that ATF-transduced cells exhibited lower proliferation rates relative to control cells (Supplementary Fig. S4).⁵

Real-time Expression Analysis of Genes in Cells That Express ATFs

The experiments described above suggested that the selected ATF could activate p53-dependent pathways to promote survival. We then investigated the expression of genes participating in p53-dependent pathways in ATF-transduced cells by real-time expression analysis (Fig. 5A). We analyzed the expression of two targets of p53: the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and the zinc finger protein *wild-type p53-induced gene 1 (wig-1)*. $p21^{WAF1/CIP1}$ induces cell cycle arrest and antiproliferative responses and is up-regulated in cell lines resistant to paclitaxel (14–16, 32) and in breast tumors exposed to chemotherapy (17). *wig-1* is a zinc finger, RNA-binding protein that mediates antiproliferative responses dependent on p53 (38–40). We also evaluated p53 mRNA levels and mRNA levels of another important tumor suppressor $p14^{ARF}$. $p14^{ARF}$ stabilizes and activates p53 by binding and neutralizing the functions of a negative regulator of p53, the protein MDM2, resulting in growth arrest (11). The transcription factor *hDMP1* is a direct activator of $p14^{ARF}$ (41, 42).

To quantitatively analyze RNA expression, we first transduced HeLa cells with ATF-encoding retroviruses and control viruses containing no zinc fingers. RNA was

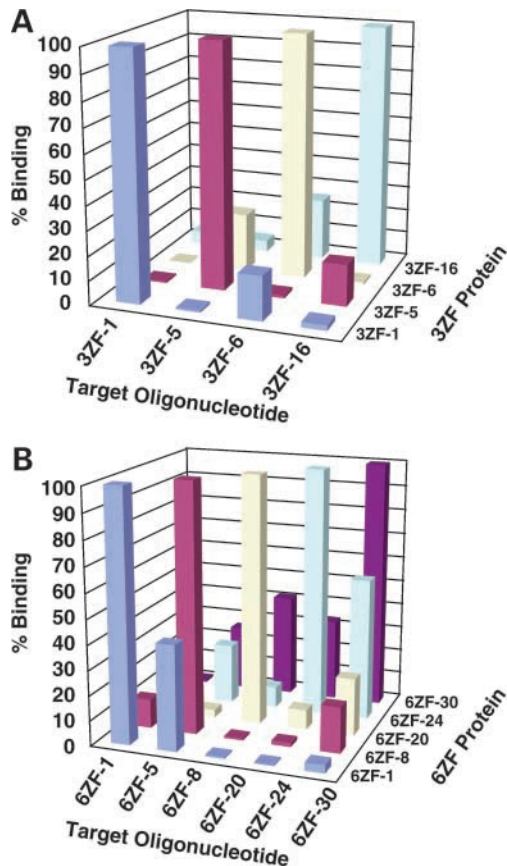


Figure 2. DNA-binding ELISA assay of purified 3ZF (A) and 6ZF (B) proteins. Proteins were incubated with their predicted double-stranded biotinylated substrate DNA oligonucleotides (predicted recognition sites are shown in Table 1) in streptavidin-coated ELISA plates. Complexes were incubated with anti-maltose-binding protein antibody and a secondary antibody conjugated with alkaline phosphatase. Data were done in duplicate with six serial dilutions of protein (60 nmol/L concentration). Background levels were determined using nonspecific double-stranded DNA oligonucleotide-coated wells and bovine serum albumin-coated wells.

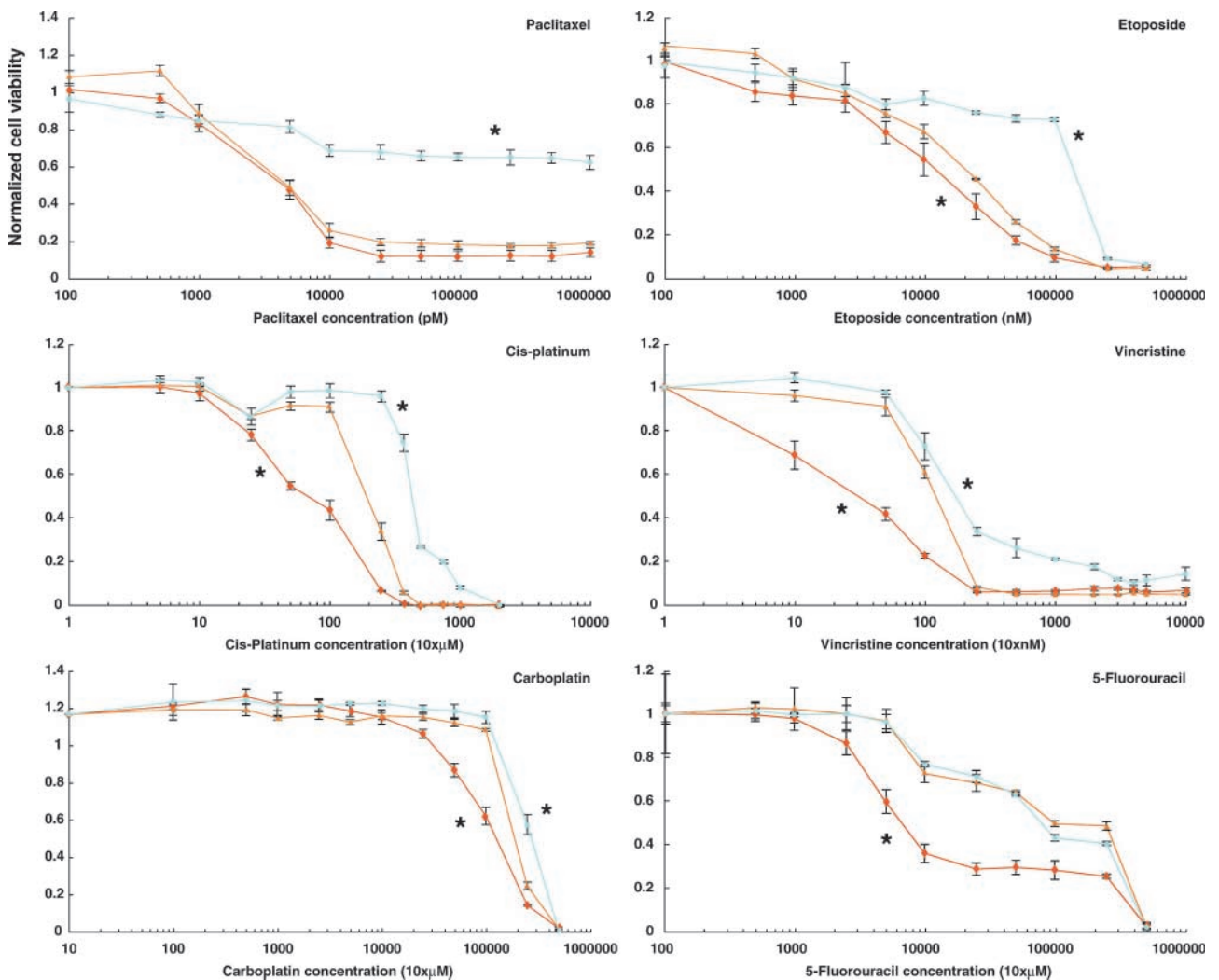


Figure 3. Survival assays of HeLa cells transduced with ATF 3ZF-1. The 3ZF-1 DBDs were linked to a VP64 activation domain (3ZF-1-VP; blue) or to the Kruppel domain, a strong transcriptional repressor (3ZF-1-KD; red). The control was a retrovirus lacking the VP64 activation domain (orange). Transduced cells were seeded in 96-well tissue culture plates and challenged with different concentrations of the indicated anticancer drugs. The fraction of cells surviving the drugs was determined by XTT ELISA as described in Materials and Methods. *, $P < 0.05$, ATF-transduced groups (blue) versus control cells (cells transduced with libraries; orange).

extracted from transduced cells and processed for real-time expression analysis. Data were normalized to the sample that did not express a zinc finger. As shown in Fig. 5B, some of the ATFs that induced drug resistance up-regulated genes in the $p14^{ARF}$ -MDM2- $p53$ pathway. We did not see significant variations in MDM2 mRNA levels in ATF-transduced cells (data not shown).

The strongest activation of the $p53$ and $p14^{ARF}$ pathways was mediated by ATF 3ZF-6-VP, 3ZF-16-VP, and 6ZF-30-VP. Because $hDMP1\alpha$, the full-length isoform expressed in humans (42), initializes an upstream genetic cascade by transactivating $p14^{ARF}$ (41, 42), we looked for a direct interaction of the ATF with the proximal promoter of $hDMP1\alpha$. As shown in Fig. 5C, ATF 6ZF-30-VP and, to a lesser extent, 3ZF-1-VP were able to activate the proximal $hDMP1\alpha$ promoter in a reporter transactivation assay. This activation

was specific because these ATFs were not able to transactivate the $p21^{WAF1/CIP1}$ promoter. A search for 6ZF-30-VP-binding sites in the $hDMP1\alpha$ promoter revealed an 18-bp sequence located 10 bp upstream of the transcription start site that matched 13 of the 18 bp in the predicted 6ZF-30-VP recognition site. Very close to that site (at position -20) was another potential 6ZF-30-VP-binding site that matched 11 of 18 bp in the predicted 6ZF-30-VP site (Fig. 5C). A deletion of the promoter region comprising these two potential binding sites totally abolished the transactivation of the promoter by 6ZF-30-VP in a luciferase assay (data not shown). These results further suggest that the $hDMP1\alpha$ promoter is a direct target of 6ZF-30-VP.

ATF 3ZF-1-VP weakly transactivated the $hDMP1\alpha$ reporter. A search for 3ZF-1-VP-binding sites revealed a potential 3ZF-1-VP site located at position -1,042. This

transactivation was apparently too weak to efficiently activate the endogenous gene because no up-regulation was detected by real-time analysis. *wig-1* was slightly, but significantly, up-regulated by ATF 3ZF-1-VP. This up-regulation was confirmed by DNA microarray experiments (Table 2; Supplementary Material) that showed a 3-fold increase in expression of this target in ATF 3ZF-1-VP-transduced HeLa cells compared with control cells. As shown in Table 2 (Supplementary Material), ATF 3ZF-1-VP also up-regulated p53-independent targets. This could explain the activity of this ATF in different cell lines with different drugs. Some of these targets are directly associated with inhibition of cell growth, such as the tumor suppressor retinoic acid responder-1. The up-regulation of

β -tubulin may explain the enhanced resistance to the microtubule-destabilizing drugs, paclitaxel and vincristine. These drugs act by binding to β -tubulin and inducing a blockade in the cytokinesis step of mitosis, resulting in apoptosis (43).

Discussion

Resistance to anticancer drugs represents a major problem for the long-term effectiveness of chemotherapy. In cancer cells, dysregulation of several different genetic cascades, including both p53- and p53-independent responses (1–3), can lead to drug resistance. Thus, novel therapeutic interventions that inhibit the mechanism of drug resistance

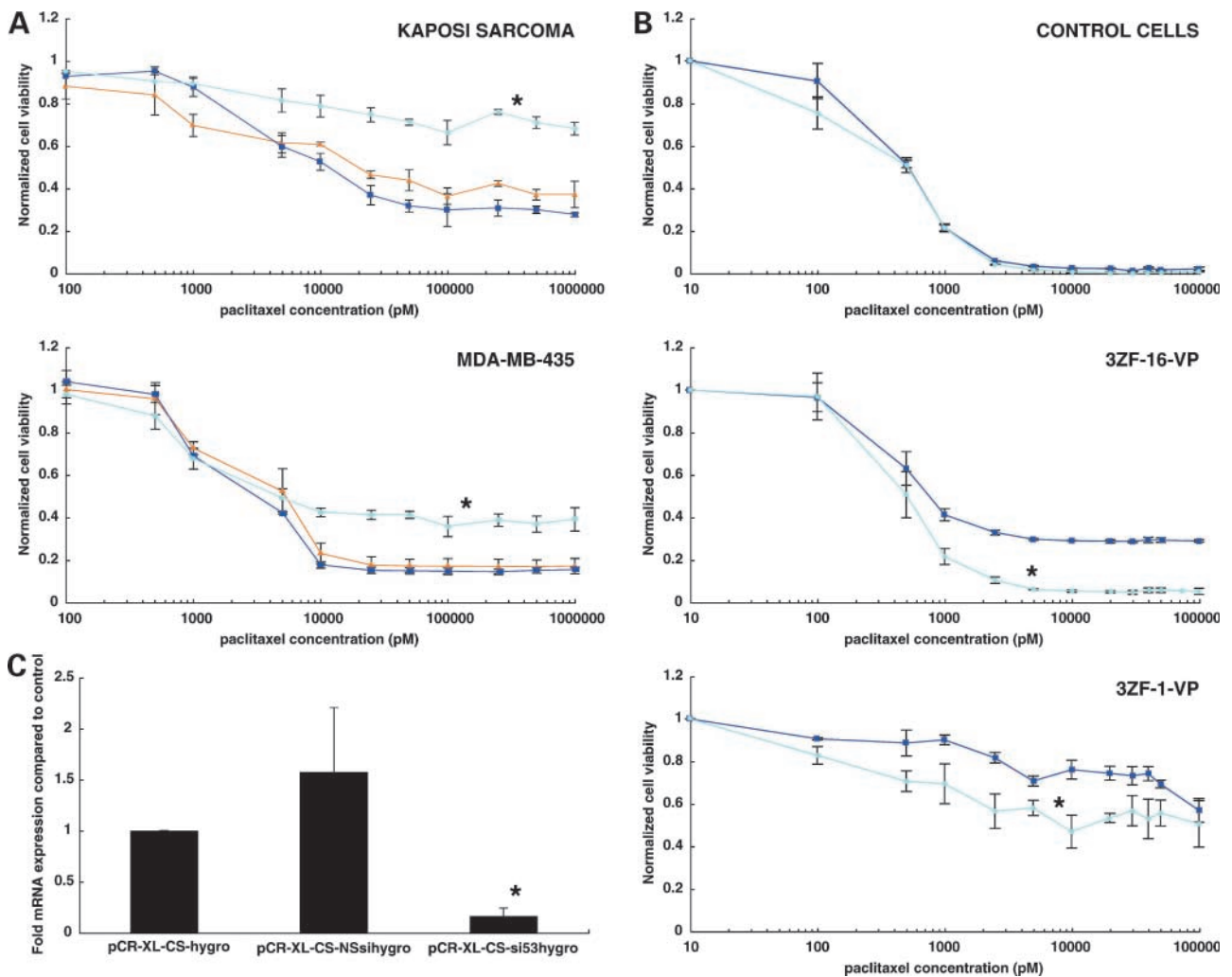


Figure 4. Dependence of p53 in ATF-mediated drug resistance. **A**, survival assays of cancer cells having mutated or inactivated p53 pathways transduced with ATF 3ZF-1-VP (light blue), with control retroviruses encoding 6ZF-VP library (orange), or with a control retrovirus in absence of activation domain (dark blue). Cells were then challenged with the indicated concentrations of paclitaxel and the fraction of cells surviving the drug was determined by XTT assay. **B**, survival assays of HeLa cells transduced with a lentiviral vector expressing a siRNA specific for p53 (light blue) or with a control lentiviral vector that expressed a nonspecific siRNA (dark blue). These cells were transduced with the indicated ATF (ATF 3ZF-1-VP and ATF 3ZF-16-VP) or with a control retrovirus without DBD. **C**, real-time quantification of p53 mRNA levels in HeLa cells transduced with a lentiviral vector expressing a siRNA specific for p53 or cells transduced with a control lentiviral vector expressing a nonspecific siRNA (control siRNA). *, $P < 0.05$, ATF-transduced groups (light blue) versus control cells (cells transduced with library; orange).

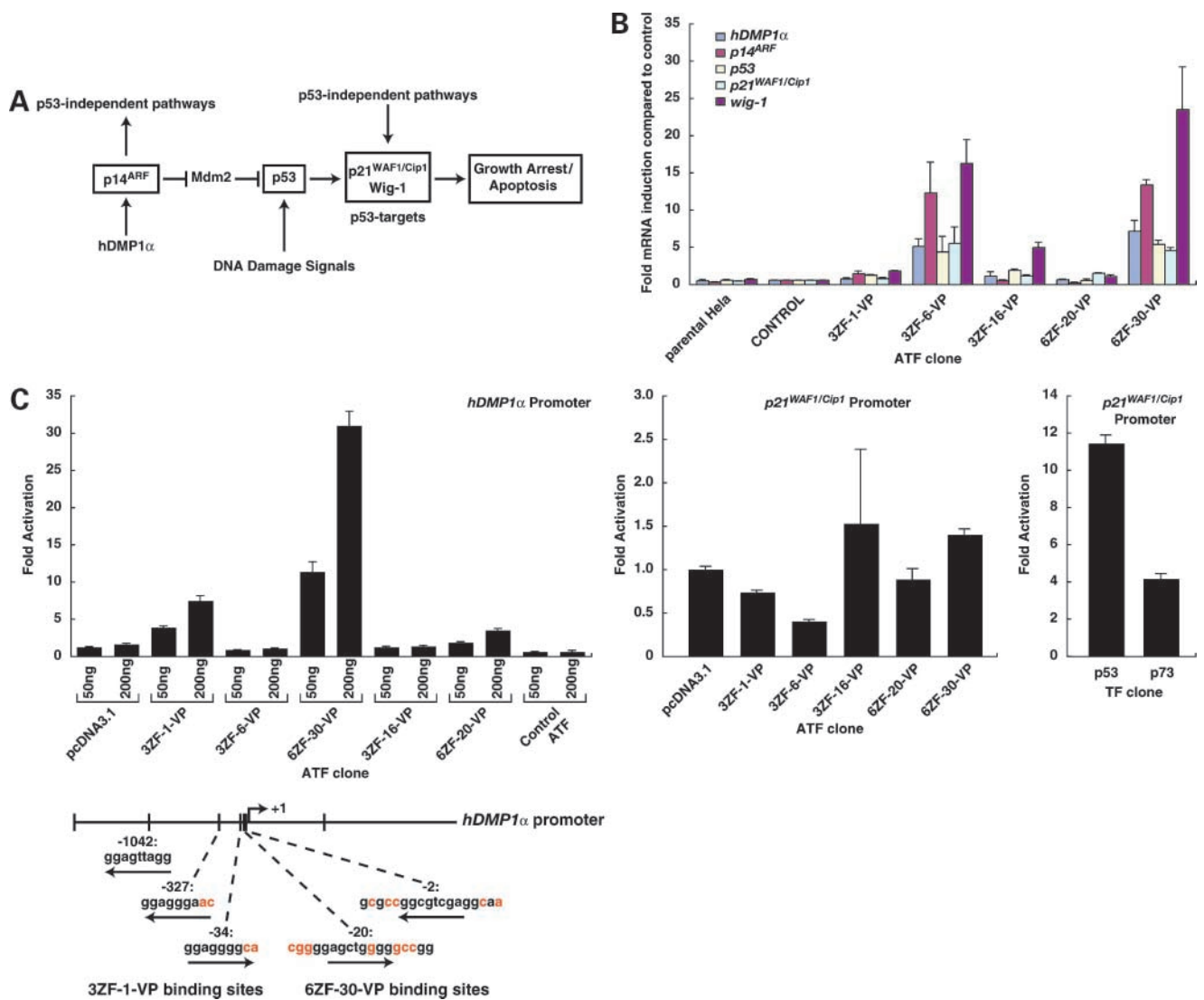


Figure 5. ATF activate genes participating in the $p14^{ARF}$ - $MDM2$ - $p53$ tumor suppressor pathway. **A**, schematic view of genes participating in the $p14^{ARF}$ - $MDM2$ - $p53$ tumor suppressor pathway. **B**, real-time expression analysis of genes participating in p53-dependent responses in HeLa cells. Cells were transfected with the indicated ATF promoting drug resistance or with a control retroviral vector expressing no zinc finger DBD. **C**, luciferase transactivation assays of the proximal $p21^{WAF1/CIP1}$ and $hDMP1\alpha$ gene promoters with the indicated ATF. Control ATF was a 6ZF activator protein (144-29) that activated a VE-cadherin gene (21). As a positive control for $p21^{WAF1/CIP1}$ assay, cells were cotransfected with 200 ng of a $p53$ -coding plasmid as indicated in Materials and Methods. Putative 3ZF-1-VP-responsive and 6ZF-30-VP-responsive elements in the proximal $hDMP1\alpha$ promoter are indicated. Red nucleotides represent mismatches from the hypothetical ATF-binding sites (assuming the zinc finger specificities shown Table 1).

are desired. ATF that target many pathways simultaneously would enable a multifunctional and effective regulation of the drug resistance phenotype. To obtain ATF regulators of drug resistance, we have used a genome-wide genetic approach. We delivered libraries of 3ZF and 6ZF ATF domains into a paclitaxel-sensitive cell line and then challenged the transduced population with paclitaxel. Surviving cells provided information on mechanisms of chemoresistance and the selected ATF may ultimately serve as therapeutic agents.

We reasoned that a 3ZF library with 9-bp binding sites would be able to regulate a larger number of genomic

targets than the complex 6ZF library that interacts with 18-bp genomic sites. Any given 3ZF has the potential to bind $\sim 10,000$ sites in the genome. Indeed, we found that the most effective ATF regulator isolated from our screen was a 3ZF protein, ATF 3ZF-1-VP. ATF 3ZF-1-VP was able to induce paclitaxel resistance in both p53-deficient and p53-mutant backgrounds. The additional result that siRNA-mediated knockdown of p53 in ATF 3ZF-1-VP-transduced cells diminished the drug resistance activity of this protein by 20% to 40% indicated that ATF 3ZF-1-VP used both p53-dependent and p53-independent pathways to promote survival. The activity in absence of $p53$ mRNA was consistent

with the activity of this protein in p53-deficient backgrounds.

We used real-time expression analysis to determine whether targets or regulators of the p53 tumor suppressor were up-regulated in ATF-expressing cells. Notably, *p21^{WAF1/CIP1}* and *wig-1* were up-regulated in several ATF-transduced cells compared with controls. *p21^{WAF1/CIP1}* is a cyclin-dependent kinase inhibitor and its overexpression has been associated with resistance to paclitaxel (14, 32) and other anticancer drugs (15, 16). In particular, ErbB-2 overexpression in human breast carcinomas has been correlated with up-regulation of *p21^{WAF1/CIP1}* and poor response to chemotherapy (16). *wig-1* encodes a putative RNA-binding zinc finger protein activated by p53 that induces growth arrest in colony formation assays (38, 39). Other proteins up-regulated by the ATF included the tumor suppressors *p14^{ARF}* and *hDMP1 α* , a transcription factor that directly activates *p14^{ARF}* transcription (41, 42). It has been shown that *hDMP1 α* induces cell cycle arrest in primary mouse fibroblast in an ARF-dependent manner. *p14^{ARF}* acts to stabilize p53 at protein level by interference with MDM2 function (44). *p14^{ARF}* expression induces both p53-dependent and p53-independent antiproliferative genes. Both *wig-1* and *p21^{WAF1/CIP1}* are up-regulated by ectopic expression of *p14^{ARF}* (43). We observed specific up-regulation of *hDMP1 α* , *p14^{ARF}*, *p21^{WAF1/CIP1}*, and *wig-1* in cells transduced with ATF 3ZF-6-VP and 6ZF-30-VP.

Because *hDMP1 α* is an upstream regulator of *p14^{ARF}*, we investigated whether these ATFs directly transactivated the *hDMP1 α* gene promoter. We showed strong transactivation of the *hDMP1 α* promoter by ATF 6ZF-30-VP. This transactivation was specific because this ATF was not able to activate the proximal *p21^{WAF1/CIP1}* promoter. An analysis of the DNA sequence of the *hDMP1* promoter revealed a putative 18-bp site at position -10 that matched 13 of 18 bp in the predicted binding site for ATF 6ZF-30-VP and a closely related sequence located 20 bp upstream of the first site that matched 11 of 18 bp in the predicted binding site. It is likely that activation requires occupancy of both sites to compensate for reduced binding at these imperfect sites. Consistent with activation of p53 genes, several of the selected ATF decreased the proliferation rate of HeLa cells and induced G₀-G₁ cell cycle arrest.

Treatment with microtubule-stabilizing drugs, such as paclitaxel or vincristine, the topoisomerase II drug, etoposide, or DNA-damaging drugs results in a G₂-M arrest and induction of apoptosis (14, 44). Paclitaxel acts by binding β -tubulin and preventing microtubule depolymerization and results in incomplete mitosis and induction of apoptosis (43). Cyclin-dependent kinases play an essential role in controlling cell cycle transitions (45). One possible mechanism used to escape the mitotic arrest and induction of apoptosis mediated by anticancer drugs is to dysregulate the cell cycle checkpoints to prevent or delay the entry to mitosis. In breast cancer, ErbB-2 up-regulation is associated with enhanced *p21^{WAF1/CIP1}* expression (16), inhibition of the cyclin p34^{cdc2} (16), and paclitaxel resistance (45). Overexpression of *ErbB2* oncoprotein also results in arrest

in the G₀-G₁ phases of the cell cycle (16). In light of this, it may be that the selected ATF cause dysregulation of the cell cycle leading to G₀-G₁ arrest.

DNA microarray analysis of ATF 3ZF-1-VP, our most effective regulator, showed regulation of p53-dependent genes, such as *wig-1*, and p53-independent genes with different mechanisms of action, such as β -tubulin and tumor suppressor genes. Our array analysis also revealed specific regulation of important signaling molecules involved in tumor progression, such as ephrin-A4 (46). The multigene nature of the ATF 3ZF-1-VP-mediated transcriptional response probably reflects the effectiveness of this ATF in regulating resistance to many drugs, including paclitaxel, etoposide, vincristine, cisplatin, and carboplatin.

Importantly, we found that linkage of the DBD of ATF 3ZF-1-VP with a transcriptional repression domain (Kruppel domain) resulted in down-regulation of the drug resistance phenotype, particularly for the microtubule-destabilizing drug vincristine and the DNA-damaging drugs cisplatin and 5-fluorouracil. Increased sensitivity to 5-fluorouracil, Tomudex, and Taxol has been described by Dolnick et al. by treating colon cancer cells with 3-oxododecanoyl homoserine lactone [3-oxo-C12-(L)-HSL; ref. 47]. The effects of this compound were associated with changes in microtubule and cytoskeleton proteins (α -tubulin and β -actin expression). More experiments are required to determine which genes are targeted by the repressor ATF.

Our observations raise the possibility that ATF might be used not only to block activation of multiple pathways associated with drug resistance but also to sensitize cells to chemotherapy treatments. In conclusion, our data show that ATF can be used to regulate drug resistance by activation of p53-dependent and p53-independent pathways. Furthermore, our analysis proves that ATF libraries, in combination with siRNA technology, can be used to precisely map promoters, genes, and pathways involved in drug resistance.

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