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A Novel Group of Genes Regulates Susceptibility to Antineoplastic Drugs in Highly Tumorigenic Breast Cancer Cells[S]

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ABSTRACT

Doxorubicin is an anthracycline antibiotic used for cancer chemotherapy. The utility of doxorubicin is limited by its inability to kill all of the cells within a tumor and by resistant cells emerging from the treated population. We have screened for genes that regulate doxorubicin susceptibility in highly tumorigenic breast cancer cells by cDNA microarray and RNA interference (RNAi) analysis, and we have identified genes associated with both proliferation and cell cycle arrest after doxorubicin treatment. We confirmed that MDA-MB-231 cells treated with doxorubicin induce the expression of carbonic anhydrase II (CAII), inhibitor of differentiation/DNA binding 2 (Id2), activating transcription factor 3 (Atf3), and the phosphatidylinositol 3-kinase 55-kDa regulatory subunit p55PIK. These genes were induced at different times and with varying specificities to different chemotherapeutic drugs. In addition to being induced at the transcriptional level, the CAII and clusterin proteins were elevated after doxorubicin treatment. CAII, Id2, p55PIK, and clusterin were not altered by doxorubicin in MCF-7 cells, a weakly tumorigenic cell line used in previous studies of doxorubicin-regulated gene expression. By inhibiting gene expression using RNAi, we found that CAII and clusterin increase cell survival after doxorubicin treatment, whereas Id2 increases susceptibility to doxorubicin. Our results support a model in which highly tumorigenic breast cancer cells induce a transcriptional response to doxorubicin that is distinct from less malignant cells. The induced genes regulate drug susceptibility positively and negatively and may be novel targets for therapeutic intervention.

Solid tumors are typically treated with a regimen that includes DNA replication inhibitors (Chabner et al., 2001). Because tumors replicate at a high rate, the resulting DNA damage reduces the tumor mass and suppresses spread of the tumor. However, DNA replication inhibitors generally fail to kill all of the cells within a tumor, and the surviving cells frequently develop drug resistance. One of the primary goals in cancer research is to develop new ways of inhibiting cancer cell growth, in part by improving the effectiveness of existing cancer treatment regimens.

Doxorubicin (Adriamycin) is an anthracycline antibiotic that is a component of many treatment regimens for solid tumors. Doxorubicin blocks the activity of topoisomerase II, a DNA unwinding protein, causing arrest of the cell cycle or apoptosis (Chabner et al., 2001). Doxorubicin resistance can emerge through altered availability of the drug or through its inactivation, through changes in topoisomerase II, or through changes in pathways mediating DNA repair and apoptosis (Longley and Johnston, 2005). In principle, identification of genes that are induced by doxorubicin could lead to new targets for improving the effectiveness of doxorubicin-based therapies.

Doxorubicin is a mainstay in the treatment of breast cancer, and several groups have used microarrays to screen for

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ABBREVIATIONS: RNAi, RNA inhibition; RT, reverse transcription; PCR, polymerase chain reaction; MTT, 3-[4,5 dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide; Id2, inhibitor of differentiation/DNA binding 2; Atf3, activating transcription factor 3; CAII, carbonic anhydrase II; p55PIK, phosphatidylinositol 3-kinase 55-kDa regulatory subunit; Rb, retinoblastoma tumor suppressor protein; PI3K, phosphatidylinositol 3-kinase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

doxorubicin-regulated genes in breast cancer cell lines. Kudoh et al. (2000) identified 14 genes that are up-regulated and three genes that are down-regulated by doxorubicin in MCF-7 breast cancer cells. MCF-7 cells are a p53/estrogen receptor/progesterone receptor-positive cell line that is nontumorigenic in the absence of estradiol (Soule and McGrath, 1980). In general, genes that were up-regulated by doxorubicin, including cyclin D2 and Cdk6, direct cell cycle progression, whereas genes that were down-regulated, such as Bcl-2, inhibit apoptosis. The study by Kudoh et al. (2000) was performed with a microarray filter containing 5180 genes, and the authors anticipated further analyses when sequencing of the human genome was complete.

More recently, Troester et al. (2004) analyzed global gene expression patterns in two different p53-positive breast cancer cell lines (one line was the same MCF-7 cell line) treated with doxorubicin or 5-fluorouracil. These expression patterns were compared with those of mammary epithelial cells that were immortalized with telomerase (Troester et al., 2004). For MCF-7 cells, the results of Troester et al. (2004) differed from those of Kudoh et al. (2000) in that genes associated with proliferation, such as *CDC2*, cyclin A2, Ki67, and ribonucleotide reductase, were repressed, whereas cell cycle inhibitors such as $p21^{WAF1}$ were induced. Similar results were found more recently by Elmore et al. (2005). All three studies analyzed expression patterns in MCF-7 cells treated with similar doxorubicin doses (1 μM and 1.7 μM, respectively) and similar time points, and two studies detected the induction of epoxide hydrolase by doxorubicin (Kudoh et al., 2000; Troester et al., 2004).

Other groups have used microarray-based screens to search for doxorubicin-regulated genes in hepatoma cells (Moriyama et al., 2003), lung cancer cells (Niiya et al., 2003), lymphoblasts (Hussain et al., 2004), and in breast cancer cells treated with hepatocyte growth factor/scatter factor (Yuan et al., 2001). Because these studies spanned a range of cell types and conditions, doxorubicin-regulated genes varied widely. Additional studies have used microarrays to identify expression patterns associated with doxorubicin-resistant cells. These studies identified midkine (Kang et al., 2004) and eukaryotic translation initiation factor 1A (Kang et al., 2004), among others (Kudoh et al., 2000; Ichikawa et al., 2004), as important in acquired doxorubicin resistance. Finally, other groups have compared doxorubicin-treated cells that display a senescent morphology with cells that have continued to proliferate. Chang et al. (2002) compared arrested and proliferating cells 10 days after a 24-h dose of doxorubicin and identified numerous genes affecting proliferation and arrest. The regulation of many of these genes was p53-dependent (Chang et al., 2002).

Unlike previous studies in MCF-7 cells, we have determined a doxorubicin-specific expression pattern in the highly tumorigenic MDA-MB-231 cell line (Zhang et al., 1991), which is p53/estrogen receptor/progesterone receptor-negative. In MDA-MB-231 cells, doxorubicin induced genes that regulate numerous pathways, including cellular proliferation and survival, deacidification, and membrane signaling. Using our microarray data set as candidate genes, we then inhibited three of the genes using RNAi and tested their roles in antineoplastic drug susceptibility. Our results support a model in which tumorigenic breast cancer cells undergo a doxorubicin-induced change in gene expression that is distinct from less malignant cells, and we have identified multiple genes that regulate antineoplastic drug susceptibility.

Materials and Methods

Cell Culture and Drug Treatments. The HeLa, MCF-7, and MDA-MB-231 cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to their instructions. Cells were maintained in 5% CO_2 at 37°C. Doxorubicin, camptothecin, and etoposide were purchased from Sigma-Aldrich (St. Louis, MO). Mechlorethamine was the kind gift from the laboratory of Dr. Robert Orlowski (University of North Carolina at Chapel Hill, Chapel Hill, NC).

For drug treatments, cells were split to a density of 500,000 cells per 100-mm dish and allowed to attach overnight. Cells were then treated with various drug concentrations and incubated for 24 h. Cells were harvested by scraping from the dish with a rubber policeman, centrifuging, and washing once with phosphate-buffered saline (PBS). Doses of various drugs were chosen because they cause toxicity after a prolonged incubation (data not shown). However, after 24 h, none of these agents caused pronounced cellular rounding, and the fluorescence-activated cell sorting profiles indicated that the cells were viable and largely nonapoptotic (Fig. 1).

Microarray Conditions. Three untreated or three doxorubicin-treated plates of cells were harvested separately, and RNA was purified from each treatment plate using the RNAeasy kit from QIAGEN (Valencia, CA). Each purified RNA was then separately reverse transcribed, labeled, and hybridized to a Hu 133A chip (Affymetrix, Santa Clara, CA) by the University of Kentucky Microarray Core Facility (Lexington, KY). For each probe set, a two-independent sample t test was performed to test for the equality of the mean expression levels between the untreated and doxorubicin-treated cells. The assumption of equal variance between the two treatment groups was made. Of the 22,125 probe sets, 1502 probe sets had significant differences between the mean expression levels of the untreated and doxorubicin-treated cells with a significance of 0.01. Of the 1502 probe sets, 903 had a false discovery rate less than 0.01.

Reverse Transcription-Polymerase Chain Reaction. RNA was purified as described above and reverse transcribed (RT-PCR) using SuperScript II reverse transcriptase and random hexamers (both from Invitrogen, Carlsbad, CA). PCR reactions were performed with *Taq* polymerase (GenScript Corp., Scotch Plains, NJ) in an Eppendorf Mastercycler (Eppendorf North America, Westbury, NY) using 30 to 38 cycles of a program consisting of 94°C for 1 min., 55°C for 1 min., and 72°C for 1 min. PCR reactions contained primers to doxorubicin-induced genes and to actin, which was an internal control for the amount of cDNA template. DNA was then visualized by electrophoresis in 2.5% agarose 1000 (Invitrogen). The primer sequences for the various analyses are available on the web (http://www2.mc.uky.edu/Pharmacology/rjc_research.asp). Images of the agarose gels were captured as jpg files, and the intensities of the bands were quantitated using ImageQuant software (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The ratios of doxorubicin signature genes to actin were calculated, and statistical tests were performed using Microsoft Excel (Microsoft, Redmond, WA).

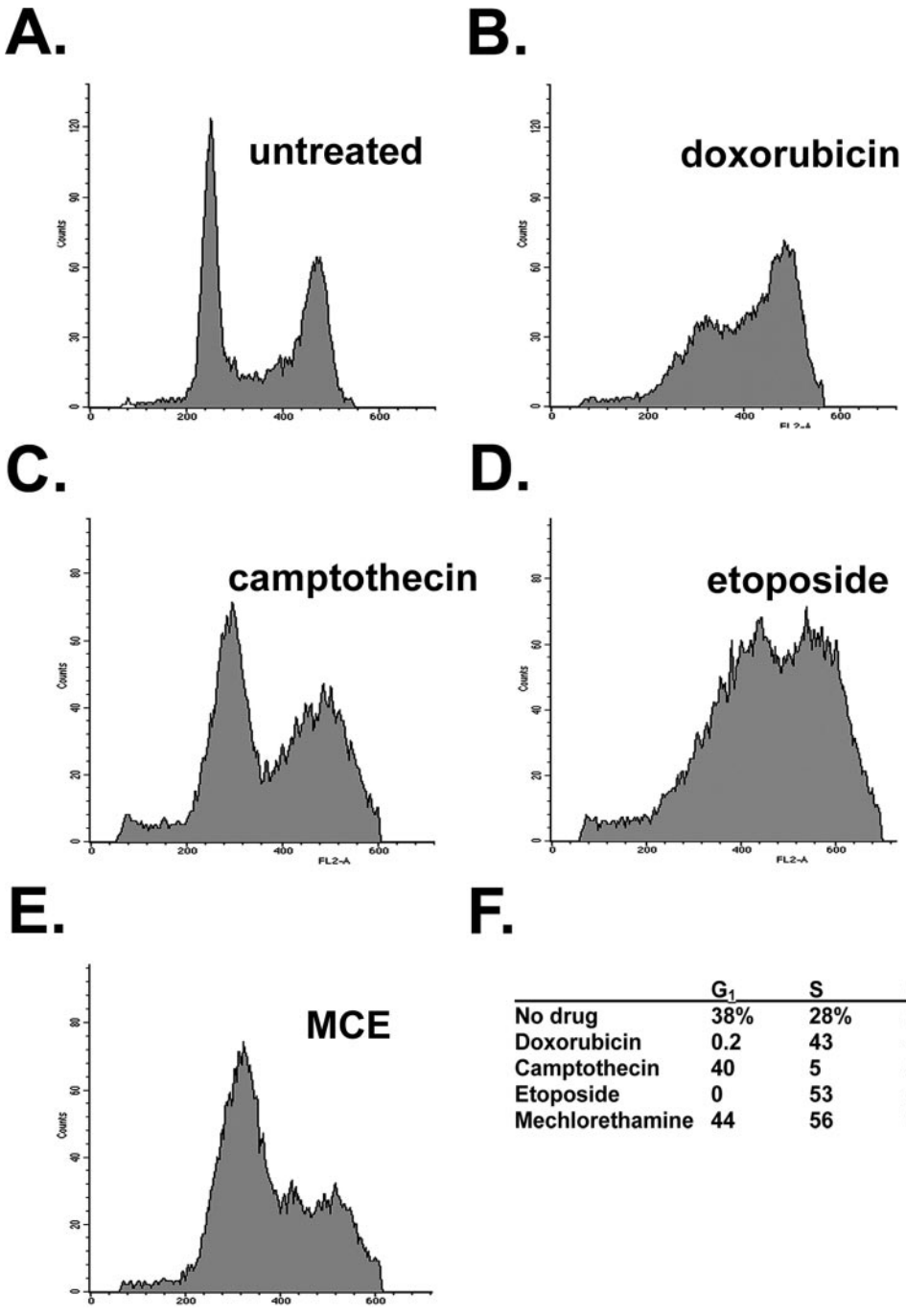
Cell Cycle Analysis. MDA-MB-231 cells were treated with various drugs, harvested, washed with PBS, and fixed with 70% ethanol overnight. After fixing, the cells were washed again with PBS and resuspended at a density of 10^6 cells/ml in 20 μg/ml propidium iodide and 20 μg/ml DNase-free RNase. Samples were analyzed at the University of Kentucky Flow Cytometry Facility. The percentages of cells in the G_1, S, and G_2 phases of the cell cycle were calculated using ModFit Software (Verity Software House, Topsham, ME).

Western Blotting. Cell lysates were prepared by incubating cells in Nonidet P-40 buffer (1% Nonidet P-40, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, pH 7.4, and 10 μg/ml of the protease inhibitors aprotinin and leupeptin) followed by centrifugation at

maximum speed in a microcentrifuge. Lysates were then separated on 8 to 16% SDS-polyacrylamide gel electrophoresis gels. Proteins were then transferred to Immobilon P membranes (Millipore Corporation, Billerica, MA) and probed with various antibodies before visualization with the West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL). The antibodies to clusterin (H-330) and carbonic anhydrase II (H-70) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the antibody to tubulin was from Fisher Scientific Co. (Pittsburgh, PA).

RNAi Transfections. MDA-MB-231 cells were plated at a density of 500,000 cells per 100-mm dish and left to attach overnight. After attachment, RNA oligonucleotide duplexes were diluted to 220 nM in 1 ml of Opti-MEM (Invitrogen) and left for 5 min. A 1:6 suspension of Oligofectamine (Invitrogen) in Opti-MEM was then added to the RNAi duplex solution, and the mixture was incubated at room temperature for 20 min. During the incubation, plated MDA-MB-231 cells were washed once with Opti-MEM and overlaid with 4.4 ml of Opti-MEM I. The RNAi duplex suspension was gently added to the cells, for a final concentration of 40 nM RNAi duplex. After a 4-h incubation at 37°C, 2.8 ml of culture medium containing 30% Serum Supreme (Fisher Scientific Co.) was added to the cells, and the cells were left overnight. The cells were then trypsinized from the dish and were plated at a density of 5000 cells per well in a 96-well dish or 500,000 cells per plate in a 100-mm dish.

Viability Assays. For measurements of cell growth, transfected cells were counted manually, plated in triplicate in 96-well dishes, and then treated with varying doses of doxorubicin, camptothecin, etoposide, or mechlorethamine for 96 h. Media were then removed and replaced with growth media containing 0.5 mg/ml 3-[4,5 dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT; Sigma-



F.

	G_1	S	G_2
No drug	38%	28%	34%
Doxorubicin	0.2	43	57
Camptothecin	40	5	55
Etoposide	0	53	47
Mechlorethamine	44	56	0

Fig. 1. Cell cycle arrest after treatment with four different chemotherapeutic drugs. MDA-MB-231 cells were either left untreated (A) or were treated with 1 μM doxorubicin (B), 1 μM camptothecin (C), 10 μM etoposide (D), or 40 μM mechlorethamine (E) for 24 h. The cells were then fixed and stained for DNA content with propidium iodide. The percentages of cells in G_1, S, and G_2/M were then calculated using ModFit Software and are presented in F. Doxorubicin and etoposide are both inhibitors of topoisomerase II and arrested the cell cycle in S and G_2/M (B, D, and F). Camptothecin is a topoisomerase I inhibitor that caused G_1 and G_2/M arrest, and mechlorethamine is an alkylating agent that arrested the cells in G_1 and S phases.

Aldrich) and incubated for 1 to 2 h. After incubation, MTT-containing media were removed, and 100 ml of dimethyl sulfoxide was added to each well. The cells were then incubated for 20 min on a rotating platform and the A_{595}/A_{650} was determined using a Dynatech MR600 microplate reader (Dynex Technologies, Chantilly, VA). The percentage of viability was calculated as absorbance of cells treated with drug divided by the average absorbance of untreated cells. In each case, the results shown indicate representative results of at least three independent experiments. LD_{50} values were calculated from triplicate MTT assay measurements.

Results

The Doxorubicin Transcriptome in MDA-MB-231 Breast Cancer Cells Includes 903 Genes. To examine doxorubicin-mediated gene expression in a highly tumorigenic cell line, we treated MDA-MB-231 human breast cancer cells with 1 μM doxorubicin for 24 h. At this time and dose, the cells were not apoptotic, but they were arrested in the S and G_2/M phases of the cell cycle (Fig. 1, B and F). The resulting microarray analysis identified 4266 genes of 22,283 that were significantly altered by doxorubicin ($P < 0.01$). A subset of 903 genes was selected as the only genes with a false discovery rate of <1% (see *Materials and Methods*).

The doxorubicin-regulated genes are composed of multiple classes of genes, some of which are listed in Table 1. When sorted by annotation of biological function, there was a significant up- and down-regulation of metabolic genes and genes regulating cell cycle progression (see Supplemental data). Genes regulating DNA synthesis and chromatin assembly were significantly induced (Fisher's exact test, $P = 0.03$, respectively), whereas repressed genes included regulators of chemotaxis ($P = 0.0005$), ion transport ($P = 0.03$), and the inflammatory response ($P = 0.04$; see Supplemental data). Because of the global patterns of transcriptional changes, we have analyzed the expression of two transcription factors previously associated with proliferation, inhibition of differentiation/DNA binding 2 (Id2) and Atf3, and two metabolic genes regulating acid-base metabolism (carbonic anhydrase II; CAII) and phospholipid synthesis (phosphatidylinositol 3-kinase 55-kDa regulatory subunit p55PIK). We note that the microarray predicted that the most strongly up-regulated gene was *Kreisler Maf-related leucine zipper homolog*; Table 1), but we were unable to replicate this result in independent PCR analyses (data not shown).

Doxorubicin-Regulated Genes Are Induced with Varying Kinetics and Drug Specificities. To examine the kinetics of gene induction, we analyzed RNA levels by RT-PCR using actin as an internal standard for cDNA loading. CAII and Id2 were induced quickly by doxorubicin, with a detectable increase 2 to 6 h after treatment (Fig. 2, A and B). In contrast, p55PIK and Atf3 were induced more slowly, reaching peak expression only after 24 h (Fig. 2, C and D, respectively). These results suggest that doxorubicin-regulated genes are induced at different rates.

Doxorubicin causes arrest of the cell cycle in the S and G_2/M phases (Fig. 1, B and F). We determined whether induction of doxorubicin signature genes is cell cycle phase-specific. Like doxorubicin, the topoisomerase II inhibitor etoposide (Chabner et al., 2001) caused arrest in S and G_2/M (Fig. 1, B, D, and F, respectively), whereas the topoisomerase I inhibitor camptothecin (Chabner et al., 2001) arrested cells in G_1 and G_2/M (Fig. 1, C and F). The alkylating agent mechlorethamine, the active form of cyclophosphamide, which requires activation in the liver (Chabner et al., 2001), arrested cells primarily in G_1 and S (Fig. 1, E and F).

Expression was analyzed after low or high doses of drugs (Fig. 3, A–D), and then the same assays were performed in triplicate for statistical analysis (Fig. 3, E–H). CAII was induced efficiently by multiple drugs (Fig. 3A). The induction of CAII by doxorubicin, camptothecin, etoposide, and mechlorethamine was significant compared with that of untreated cells ($P \leq 0.01$ for each drug), but not when CAII induction by any two drugs (i.e., doxorubicin and camptothecin) was compared ($P > 0.05$ for any pair). Id2 induction by doxorubicin was highly significant (Fig. 3B, lanes 1–3; $P = 2 \times 10^{-6}$ for untreated versus treated cells; Fig. 3F), and Id2 was induced to a lesser degree by camptothecin (Fig. 3B, lanes 4 and 5; 5-fold), etoposide (Fig. 3B, lanes 6 and 7; 8-fold), and mechlorethamine (Fig. 3B, lanes 8 and 9; 10-fold). In each case, the levels of induction by doxorubicin were significantly higher than for camptothecin, etoposide, and mechlorethamine ($P < 0.0001$ for each drug; Fig. 3F).

We observed similar results for p55PIK and Atf3, where doxorubicin specifically induced p55PIK (Fig. 3, C and G) and Atf3 (Fig. 3, D and H). In these cases, relatively low levels of

TABLE 1

Genes regulated by doxorubicin in MDA-MB-231 cells

-Fold change indicates relative expression in doxorubicin-treated versus untreated cells.

Gene Name	-Fold Change	*P* Value
Transcription factors		
Fos-b	80	0.001
Id2	64	0.0002
Atf3	20	2×10^{-9}
c-Fos	6	0.0001
c-Myb	6	0.0001
EHF, Ets homologous factor	−13	0.0002
Cell signaling		
p55PIK	12	0.0006
Rac3, Rho family GTP binding protein	−13	0.0004
EGFR, epidermal growth factor receptor	−5	0.0004
DNA structure		
Histone H3	46	1×10^{-7}
Histone H2A	14	6×10^{-6}
DNA repair		
GADD-45β	13	0.0002
GADD-45γ	8	0.0001
NBS1, nibrin	−8	3×10^{-5}
OGG1	−5	0.0001
RAD50	−4	0.0003
Cell death		
Clusterin; CLI, cytolysis inhibitor	64	0.0007
TRAIL-R3	8	0.0006
Death-preventing kinase	7	2×10^{-6}
TRAIL-R4	5	0.0004
Cell cycle regulation		
Cyclin-dependent kinase inhibitor 1, p21	10	0.0001
Cyclin-dependent kinase inhibitor 2, p18	9	0.0009
Cell division cycle 25C	4	0.0008
Cyclin A1	4	9×10^{-5}
Xenobiotic metabolism and drug transport		
ABCF2, ATP binding cassette (GCN20)	62	6×10^{-5}
MDRTAP, ATP binding cassette	8	6×10^{-6}
Epoxide hydrolase	8	0.008
Cyp1B1, cytochrome P450 protein 1B1	6	0.0004
Cyp1A1, cytochrome P450 protein 1A1	5	0.0001
NADPH-dependent cytochrome P450 reductase	5	3×10^{-6}
Iza1/Hpr6.6, cytochrome P450 activator	3	0.001
Deacidification/solute transport		
Carbonic anhydrase II	55	6×10^{-7}
AQP3, aquaporin 3	18	9×10^{-5}

induction were detected in cells treated with camptothecin, etoposide, and mechlorethamine. The results indicate that doxorubicin induces some genes with a remarkable degree of specificity, whereas other genes are induced by DNA-damaging agents that do not share the cell cycle arrest profile of doxorubicin.

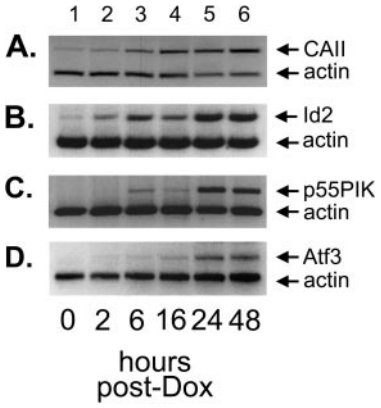


Fig. 2. Analysis of doxorubicin-induced genes by RT-PCR. MDA-MB-231 breast cancer cells were treated with 1 μM doxorubicin and harvested at 0, 2, 6, 16, 24, and 48 h after treatment (lanes 1–6). At each time point, RNA was purified and reverse transcribed. cDNA was then amplified with primers to actin (bottom bands) as a loading control and with primers to CAII (A), Id2 (B), p55PIK (C), or Atf3 (D). The earliest time of induction varied among the four genes, with Id2 and CAII being stably induced within 6 h, whereas p55PIK and Atf3 were induced strongly after 24 h.

The CAII and Clusterin Proteins Are Induced by Doxorubicin. As a consequence of its transcriptional regulation by doxorubicin, the CAII protein was induced by 1 μM doxorubicin (Fig. 4A) and by camptothecin (Fig. 4C, lane 3). CAII levels after treatment with etoposide or mechlorethamine were lower than levels after doxorubicin and camptothecin treatment (Fig. 4C, lanes 4 and 5), which is consistent with the RNA analysis for CAII (Fig. 3). Clusterin regulates chemotherapy resistance in osteosarcoma (Trougakos et al., 2004), and we found that clusterin was highly induced by 0.1 μM doxorubicin and was nearly saturated by a 0.5 μM dose (Fig. 4B). In addition, clusterin was highly induced by all four chemotherapeutic agents that we tested (Fig. 4D). We did not analyze Id2, Atf3, or p55PIK by Western blot because commercially available antibodies were not sufficiently specific.

Most of the Doxorubicin-Regulated Genes in MDA-MB-231 Cells Are Not Altered by Doxorubicin in MCF-7 Cells. Previous microarray analyses of doxorubicin-treated breast cancer cells used MCF-7 cells and did not detect the majority of the genes identified in our screen. To examine differences in gene regulation in MDA-MB-231 and MCF-7 cells directly, we treated both cell lines with doxorubicin and analyzed differences in gene expression. In some cases, we also analyzed changes in HeLa cells, which have been widely used in studying drug resistance. MCF-7 cells expressed high levels of CAII (Fig. 5A, lane 1), and CAII levels did not change with doxorubicin treatment (Fig. 5A, lane 2). In con-

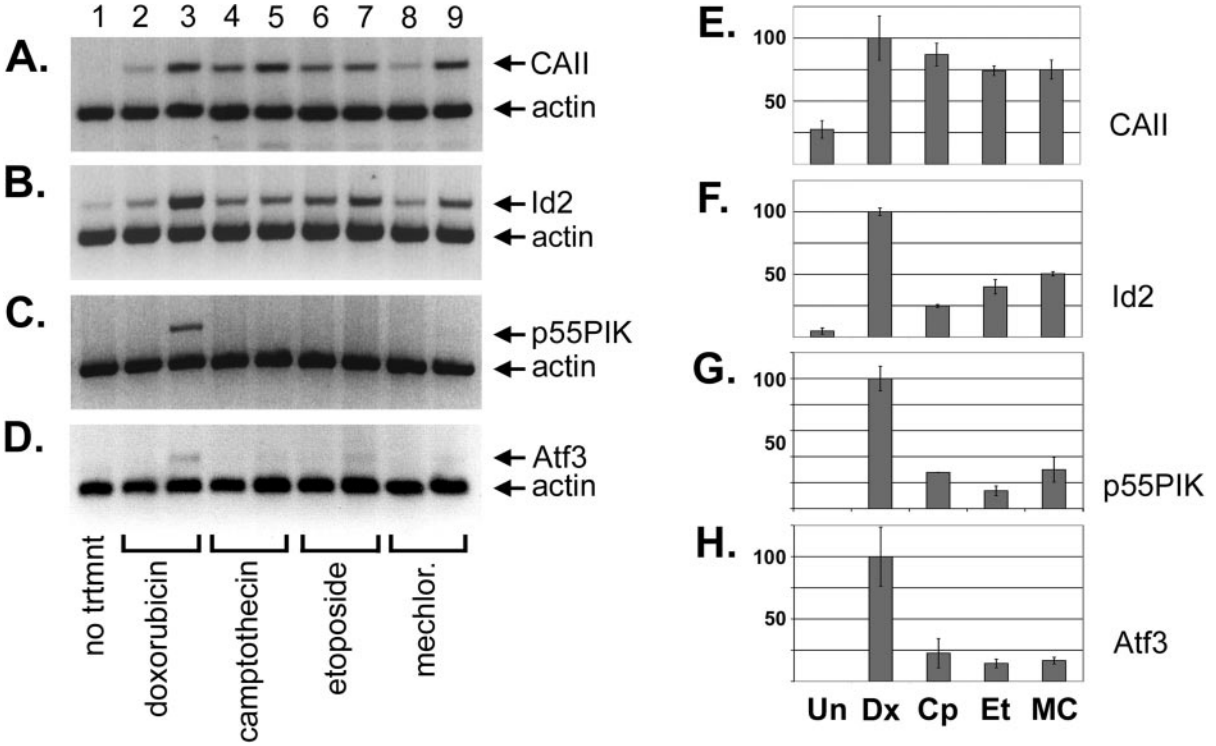


Fig. 3. Doxorubicin-induced genes differ in drug specificity. MDA-MB-231 cells were either left untreated (lanes 1) or were treated with 0.2 μM doxorubicin (lanes 2), 1 μM doxorubicin (lanes 3), 0.2 μM camptothecin (lanes 4), 1 μM camptothecin (lanes 5), 2 μM etoposide (lanes 6), 10 μM etoposide (lanes 7), 10 μM mechlorethamine (lanes 8), or 40 μM mechlorethamine (lanes 9). RNA was then purified and reverse transcribed. cDNA was then amplified with primers to actin (bottom bands) and with primers to CAII (A), Id2 (B), p55PIK (C), or Atf3 (D). In E to H, RT-PCR reactions for doxorubicin-induced genes were performed in triplicate and quantitated, and the ratios of gene signal to actin were calculated. The genes analyzed were CAII (E), Id2 (F), p55PIK (G), and Atf3 (H). Cells were untreated (first columns; Un) or treated with 1 μM doxorubicin (second columns; Dx), 1 μM camptothecin (third columns; Cp), 10 μM etoposide (fourth columns; Et), or 40 μM mechlorethamine (fifth columns; MC). Each of the four genes was induced most highly by doxorubicin, and the ratio of each gene to actin in doxorubicin-treated cells is expressed as 100%. Error bars represent the standard deviation in three independent assays. The results showed that CAII is induced by multiple drugs, whereas Id2, Atf3, and p55PIK were induced primarily by doxorubicin.

trast, MDA-MB-231 and HeLa cells expressed low levels of CAII (Fig. 5A, lanes 3 and 5) and induced CAII expression after doxorubicin treatment (Fig. 5A, lanes 4 and 6). CAII expression was so much higher in MCF-7 cells that the Western blot had to be dissected and developed separately. In contrast, clusterin was not expressed in MCF-7 cells before or after doxorubicin treatment (Fig. 5B, lanes 1 and 2), but it was efficiently induced by doxorubicin in both MDA-MB-231 and HeLa cells (Fig. 5B, lanes 3–6).

Like CAII, Id2 was expressed highly in MCF-7 cells (Fig. 5C, lane 3), and its expression did not increase after doxorubicin treatment (Fig. 5C, lane 4). Similar to clusterin, p55PIK was expressed at low levels in MCF-7 cells (Fig. 5D, lane 3), and p55PIK expression decreased in MCF-7 cells after doxorubicin treatment (Fig. 5D, lane 4). The only gene with a similar regulation in MDA-MB-231 and MCF-7 cells was Atf3, which was minimally expressed in both cell lines (Fig. 5E, lanes 1 and 3) and was induced by doxorubicin (Fig. 5E, lanes 2 and 4).

CAII, Id2, Atf3, and Clusterin Regulate Drug Susceptibility. To determine the roles of doxorubicin-induced genes, we attenuated the expression of several genes using RNAi oligonucleotide duplexes (Elbashir et al., 2001). CAII, Id2, and clusterin expression was inhibited by transiently transfecting MDA-MB-231 cells with RNAi duplexes targeting the genes (CAIIi, Id2i, or CLSi) or with a nonspecific

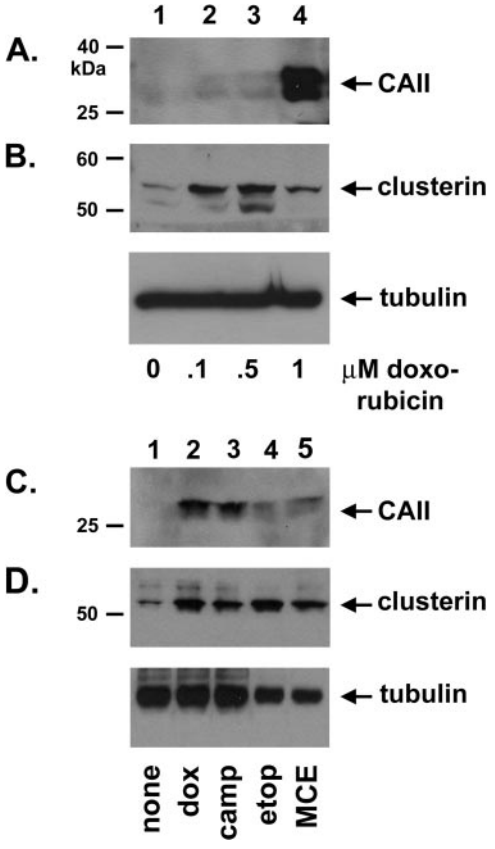


Fig. 4. Western blot analysis of proteins encoded by doxorubicin-induced genes. For A and B, MDA-MB-231 cells were treated with 0, 0.1, 0.5, or 1 μM doxorubicin (lanes 1–4, respectively) for 24 h, and then they were lysed and analyzed by Western blot for CAII (A), clusterin (B), or tubulin (bottom) as a control for loading. The numbers to the left of each panel refer to the migration of stained molecular mass standards in kilodaltons. Doxorubicin increased the expression of both proteins. For C and D, MDA-MB-231 breast cancer cells were left untreated (lanes 1) or treated with 1 μM doxorubicin (lanes 2), 1 μM camptothecin (lanes 3), 10 μM etoposide (lanes 4), or 40 μM mechlorethamine (lanes 5). The blot was probed for CAII (C), clusterin (D), or tubulin (bottom).

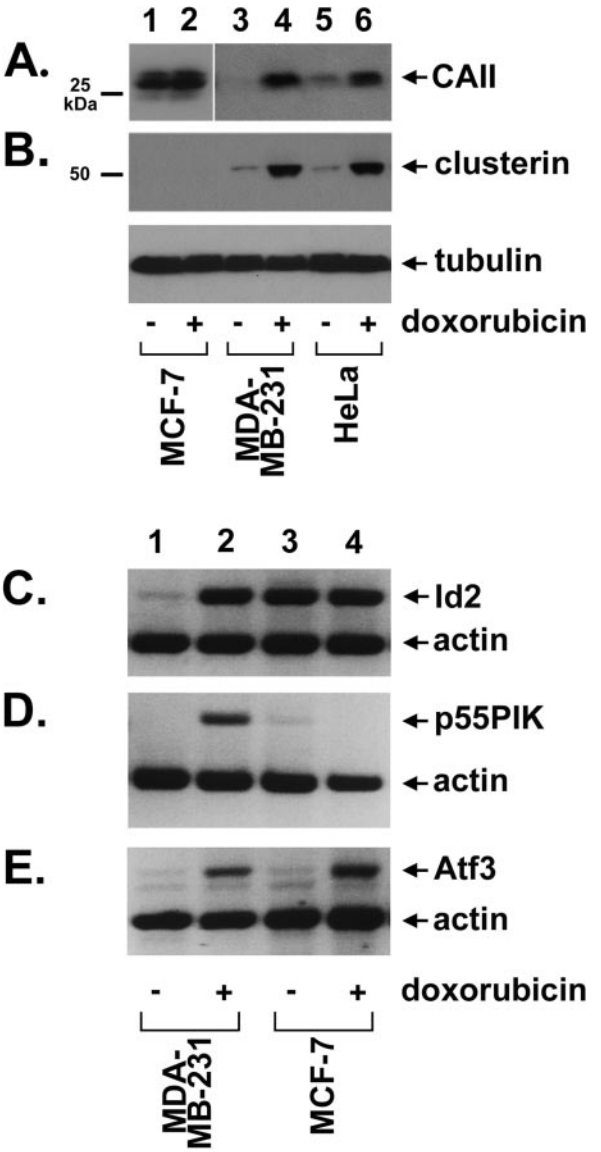


Fig. 5. Four doxorubicin-induced genes are not altered in MCF-7 cells. A and B, proteins were analyzed by Western blot in MCF-7 (lanes 1 and 2), MDA-MB-231 (lanes 3 and 4), and HeLa cells (lanes 5 and 6) that were untreated (odd lanes) or treated with 1 μM doxorubicin for 24 h (even lanes). The blot was probed for CAII (A), clusterin (B), or tubulin (bottom). CAII expression was significantly higher in MCF-7 cells without doxorubicin treatment (A, lane 1), and CAII expression did not change after doxorubicin (A, lane 2). In contrast, CAII was weakly expressed in MDA-MB-231 and HeLa cells (A, lanes 3 and 5), but both cells induced CAII expression after doxorubicin treatment (A, lanes 4 and 6). Clusterin was similarly induced by doxorubicin in MDA-MB-231 and HeLa cells (B, lanes 3–6), but it was neither expressed constitutively nor induced by doxorubicin in MCF-7 cells (B, lanes 1 and 2). For C to E, RNA levels in MDA-MB-231 (lanes 1 and 2) and MCF-7 (lanes 3 and 4) cells were analyzed by RT-PCR using actin as an internal standard, before and after treatment with 1 μM doxorubicin. (C) Id2 expression increased with doxorubicin treatment in MDA-MB-231 cells (lanes 1 and 2), but it was elevated and did not change with treatment in MCF-7 cells (lanes 3 and 4). (D) p55PIK expression increased with doxorubicin exposure in MDA-MB-231 cells (lanes 1 and 2), but it was weak in MCF-7 cells (lane 3) and decreased with doxorubicin (lane 4). (E) Atf3 was minimally expressed in MDA-MB-231 and MCF-7 cells under normal growth conditions (lanes 1 and 3) and increased in both cell lines upon doxorubicin treatment (lanes 2 and 4).

control sequence (Fig. 6D, con). RNAi molecules targeting CAII, Id2, and clusterin inhibited the basal expression of Id2 (Fig. 6B, compare lanes 1 and 3) or attenuated the induction of each gene after doxorubicin treatment (Fig. 6, A–C, compare lanes 2 and 4). Furthermore, CAII protein levels were markedly decreased before (Fig. 6D, lanes 1 and 2) and after doxorubicin treatment (Fig. 6D, lanes 3 and 4) in CAIIi-transfected cells.

Cells with inhibited clusterin expression exhibited a loss of viability compared with control-transfected cells after doxorubicin treatment (Fig. 7A). The change in viability in CLSi- versus control-transfected cells at 0.08 to 2 μM doxorubicin was significant ($P \leq 0.007$ for each dose). There was no change in viability in CLSi-transfected cells without drug treatment, consistent with the low levels of basal clusterin expression in this cell line (Figs. 4B, lane 1; 5B, lane 3; and 6C, lane 1). The loss of viability in CLSi-transfected cells resulted in a significant decrease in LD_{50} compared with control cells after treatment with doxorubicin, camptothecin, etoposide, and mechlorethamine (Fig. 7C; $P \leq 0.01$ for each drug). We conclude that clusterin suppresses susceptibility to multiple chemotherapeutic drugs in MDA-MB-231 cells.

Inhibition of CAII expression increased susceptibility of MDA-MB-231 cells to doxorubicin, camptothecin, etoposide (Fig. 7B, solid line), and mechlorethamine. The differences between control- and CAIIi-transfected cells were highly significant at doses of 1.25 μM ($P = 0.04$), 5 μM ($P = 0.0001$), and 20 μM etoposide ($P = 0.002$). Similar to clusterin, the LD_{50} values for cells with inhibited CAII expression were significantly lower than those of control cells after treatment with doxorubicin, camptothecin, etoposide, and mechlorethamine (Fig. 7C, $P \leq 0.005$ for each drug). It is likely that this is a conservative estimate of the effect of CAII on drug susceptibility, because there was a low level of CAII expression in the CAIIi-transfected cells (Fig. 6D, lane 4). We conclude that CAII expression is associated with decreased susceptibility to multiple chemotherapeutic drugs.

Id2 inhibition decreased cell proliferation (Fig. 8A, black columns), even in the absence of doxorubicin. Growth inhibi-

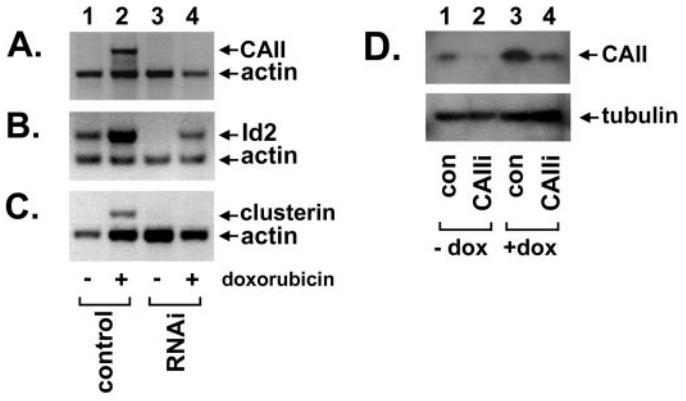


Fig. 6. Doxorubicin-induced genes are inhibited by RNAi. Gene expression was analyzed by RT-PCR, which showed diminished expression of CAII (A), Id2 (B), and clusterin (C). In each case, the inhibited gene is the top band, and the expression of actin (bottom band) served as a control for loading. In lanes 1 and 2, expression was analyzed in cells transfected with a control oligonucleotide duplex in the absence (lane 1) or presence (lane 2) of 1 μM doxorubicin for 24 h. In lanes 3 and 4, expression was analyzed in cells transfected with gene-specific RNAi duplexes in the absence (lane 3) or presence (lane 4) of 1 μM doxorubicin. In D, the inhibition of CAII was confirmed by Western blot for CAII (top), with tubulin as a control for loading (bottom). Expression was analyzed in cells transfected with a control oligonucleotide duplex (con, lanes 1 and 3) in the absence (lane 1) or presence (lane 3) of 1 μM doxorubicin for 24 h. In lanes 2 and 4, expression was analyzed in cells transfected with gene-specific RNAi duplexes in the absence (lane 2) or presence (lane 4) of 1 μM doxorubicin. The results show that the various RNAi duplexes inhibit doxorubicin-induced gene expression.

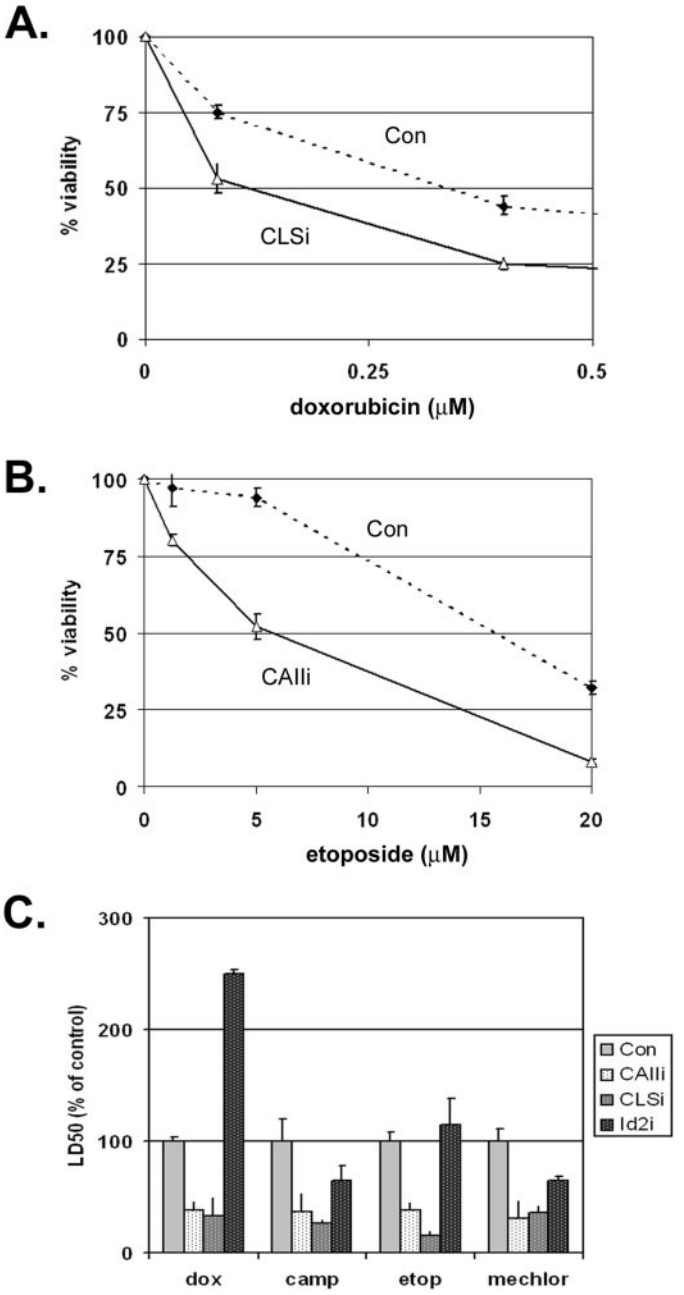


Fig. 7. Clusterin, CAII, and Id2 regulate viability after treatment with chemotherapeutic drugs. A, inhibition of clusterin increases susceptibility to chemotherapeutic drugs, including doxorubicin. Equal numbers of MDA-MB-231 cells were transfected with control (Con, dashed line) or clusterin-specific (CLSi, solid line) oligonucleotide duplexes and then treated with increasing doses of doxorubicin for 72 h. Viability was measured by MTT assay (see *Materials and Methods*). B, inhibition of CAII increases susceptibility to multiple drugs, including etoposide. Cells were transfected as for A, and viability was measured by MTT assay. In A and B, all points were analyzed in triplicate and were repeated in three separate experiments. C, LD_{50} values for MDA-MB-231 cells treated with a control RNAi duplex (left column), CAIIi (second column), CLSi (third column), or Id2i (right column). Id2i decreased susceptibility to doxorubicin ($P = 0.0005$), whereas CLSi and CAIIi increased susceptibility to all four drugs ($P \leq 0.01$) for each.

tion was highly significant at 25 nM ($P = 2 \times 10^{-7}$) or 100 nM ($P = 8 \times 10^{-5}$) doses and was reflected in a 46% decrease in the viable cell count at 96 h after transfection. Id2 inhibition did not induce a significant increase in cell death, because we detected only a modest increase in the number of dead cells (5 ± 2 for control-transfected cells compared with 15 ± 5 for Id2i-transfected cells) by trypan blue exclusion assay. In contrast, the Id2i-transfected population contained a 12% increase in the number of cells arrested in S, G_2, and M phase (data not shown) when measured by fluorescence-activated cell sorter analysis. Id2i-transfected cells exhibited decreased sensitivity to doxorubicin (Fig. 8B). At the 0.3 μM dose of doxorubicin, Id2i-transfected cells were significantly ($P = 0.002$) less sensitive than cells transfected with the control RNAi duplex (Fig. 8B). As a result, Id2i-transfected cells had LD_{50} values greater than control cells for doxorubicin (Fig. 7C) but not for camptothecin, etoposide, or mechlorethamine. We conclude that Id2 expression is associated with doxorubicin susceptibility in MDA-MB-231 cells.

Discussion

We have found that highly tumorigenic MDA-MB-231 breast cancer cells treated with chemotherapeutic drugs initiate a transcriptional response that is distinct from that of MCF-7 cells. Even in the presence of extensive damage and delayed cell cycle progression, cells induce the expression of genes associated with proliferation and survival. Genes induced by doxorubicin positively or negatively regulate drug susceptibility and also regulate cell proliferation. We discuss these genes below.

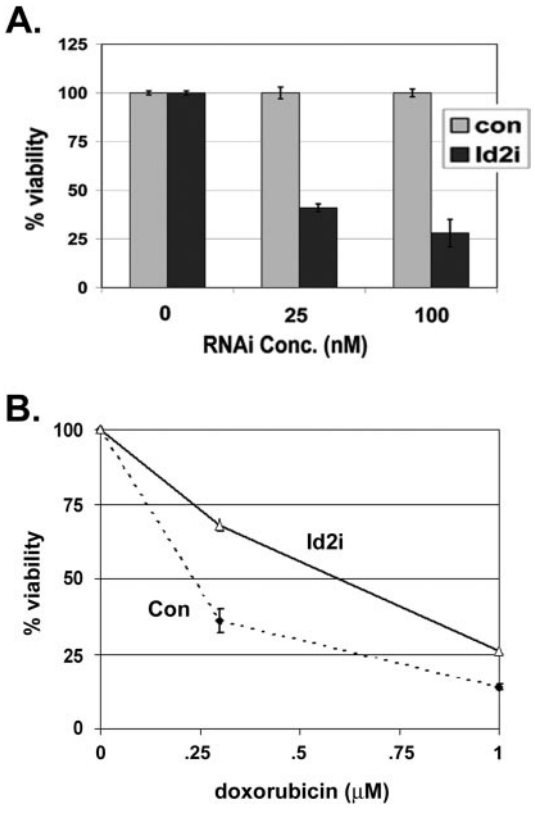


Fig. 8. Id2 positively regulates proliferation and doxorubicin susceptibility. A, MDA-MB-231 cells were transfected with a control RNAi (light gray columns) or Id2i (black columns), and viability was determined by MTT assay. Id2i inhibited growth at doses of 25 or 100 nM. B, equal numbers of MDA-MB-231 cells transfected with a control RNAi duplex or Id2i were treated with increasing doses of doxorubicin and incubated for 72 h. Viability was then measured by MTT assay. The results show that cells with inhibited Id2 expression (solid line) are significantly less susceptible to doxorubicin ($P = 0.003$ at 0.3 μM dose) than cells transfected with the control RNAi (dashed line). The results are representative of five separate experiments.

Carbonic anhydrase II regulates the cellular acid/base balance by catalyzing the reaction $H^+ + HCO_3^- \rightleftarrows H_2O + CO_2$. This reaction allows for efficient secretion of acid (Potter and Harris, 2003). Tumors have an acidic extracellular pH, which may contribute to drug resistance by reducing the uptake and cytotoxicity of weak bases such as doxorubicin (Raghunand et al., 1999). Our results are consistent with a model in which MDA-MB-231 cells induce CAII to improve survival, perhaps by limiting doxorubicin uptake. However, we note that CAII contributed to survival after treatment with camptothecin, etoposide, and mechlorethamine, suggesting a more universal mechanism for CAII. For example, CAII may influence water or ion exchange with the extracellular environment. Numerous genes regulating ion and water balance were altered after doxorubicin treatment, including Aqp3/aquaporin and multiple solute carrier proteins (Table 1), suggesting that changes in membrane permeability and ion balance contribute to chemotherapeutic responsiveness. Finally, CAII was expressed more strongly in MCF-7 cells than in MDA-MB-231 cells and was not induced by doxorubicin in the former cell line (Fig. 5A), suggesting that distinct pathways regulate CAII transcription in the two cell lines.

Several genes with reported antiapoptotic functions were induced by doxorubicin in MDA-MB-231 cells. Clusterin/apolipoprotein J/complement lysis inhibitor is a secreted protein that is induced by chemotherapy (Biroccio et al., 2003). Clusterin is overexpressed in tumors (Redondo et al., 2000; Chen et al., 2003) and has antiapoptotic functions in some cell types (Trougakos and Gonos, 2002; July et al., 2004). Although we did not detect a net effect on proliferation after clusterin inhibition (data not shown), clusterin inhibition had a pronounced effect on drug susceptibility in MDA-MB-231 cells (Fig. 7). Furthermore, clusterin was neither expressed constitutively nor induced in MCF-7 cells, suggesting that strategies targeting clusterin may be effective in highly tumorigenic cells. For all RNAi studies, we emphasize that changes in viability are conservative estimates, because transient transfections inhibit gene expression in less than 100% of the transfected cells.

Id2 is a basic helix-loop-helix protein that lacks a DNA binding domain and is capable of forming inactive heterodimers with other basic helix-loop-helix proteins (Sikder et al., 2003). Id2 is associated with proliferation, is up-regulated in cancers, and extends keratinocyte life span (Sikder et al., 2003). Id2 induction by doxorubicin was reported in previous studies of murine fibroblasts, where it interfered with activation of the MyoD muscle-specific transcription factor (Kurabayashi et al., 1994). In developing breast tissue, Id2 has been associated with proliferation (Mori et al., 2000) and differentiation (Parrinello et al., 2001; Miyoshi et al., 2002), but in human breast tumor samples, Id2 is frequently down-regulated (Itahana et al., 2003), and Id2 expression is associated with positive prognosis in breast cancer (Stighall et al., 2005). To clarify the role of Id2 in breast cell proliferation, Itahana et al. (2003) expressed Id2 in MDA-MB-231 cells under a constitutive promoter, and Id2 suppressed cell

cycle progression and invasiveness. Our results using RNAi for Id2 are inconsistent with these overexpression studies, because Id2 inhibition suppressed proliferation. In some cases, constitutive overexpression of a gene phenotypically resembles the loss of the same gene, suggesting a sensitive regulation of the gene product. Indeed, Id2 expression is transcriptionally regulated during the cell cycle (Barone et al., 1994), and Id2 is phosphorylated by multiple kinases (Nagata et al., 1995; Hara et al., 1997), suggesting that constitutive expression of Id2 during G_1 phase could delay cell cycle progression.

Id2 inhibition improved cell survival after doxorubicin treatment, suggesting that MDA-MB-231 cells induce Id2 as part of a pathway that leads to cell death. The mechanism through which Id2 regulated cell survival may include displacing other proteins from the Rb complex, which has been linked to chemotherapy susceptibility. However, doxorubicin-treated cells accumulate in S and G_2/M (Fig. 1), a stage where Rb is hyperphosphorylated and has minimal protein interactions through its pocket domain. An alternate model is that Id2 up-regulates cell cycle progression through binding to helix-loop-helix proteins. As a result, loss of Id2 expression decreases proliferation and improves survival, perhaps by allowing cells with damaged DNA more time to complete DNA repair.

MCF-7 cells undergo a senescent-like change when treated with doxorubicin, whereas MDA-MB-231 cells do not (Elmore et al., 2002). A doxorubicin-resistant MCF-7 subclone overcame senescence by limiting the repression of positive cell cycle regulators (Elmore et al., 2005), including cdc2 and cyclin E2, which are normally suppressed by doxorubicin (Elmore et al., 2005). In contrast, Id2 (a gene associated with proliferation) caused decreased survival in MDA-MB-231 cells, the opposite result. We conclude that MCF-7 cells suppress chemotherapy susceptibility through a distinct mechanism from MDA-MB-231 cells.

Phosphatidylinositol 3-kinase (PI3K) has antiapoptotic functions in response to numerous stimuli (Fresno Vara et al., 2004), and we detected marked up-regulation of the PI3K regulatory subunit p55PIK/PI3K-p55γ (Pons et al., 1995) by doxorubicin. Although the role of p55PIK in growth regulation is poorly understood, p55PIK binds to the Rb tumor suppressor protein (Xia et al., 2003), and overexpression of the amino-terminal Rb binding sequence of p55PIK causes growth arrest (Hu et al., 2005). p55PIK is also implicated in insulin and cytokine signaling (Mothe et al., 1997; Takahashi-Tezuka et al., 1997; Dey et al., 1998). In addition, we note that p55PIK was not induced by doxorubicin in MCF-7 cells, suggesting genetic factors that are altered in MDA-MB-231 cells regulate p55PIK expression.

Several other genes that are associated with proliferation were induced by doxorubicin. Atf3 is a member of the activating transcription factor/cAMP-responsive element binding protein family of bZip transcription factors (Hai and Hartman, 2001). Atf3 binds to the DNA sequence TGACGTCA and is associated with neoplastic transformation and the response to serum, stress, and damage (Yu et al., 1996; Amundson et al., 1999; Shtil et al., 1999; Hai and Hartman, 2001). Atf3 was up-regulated by doxorubicin in MDA-MB-231 and MCF-7 cells, suggesting that strategies targeting Atf3 would be unlikely to be specific for aggressive cancers.

Other prosurvival genes that were induced by doxorubicin include tumor necrosis factor-related apoptosis inducing ligand receptors (TRAIL-R3 and TRAIL-R4, which are "decoy receptors" for TRAIL (Kim and Seol, 2003). TRAIL-R3 and TRAIL-R4 bind to TRAIL, but they lack cytoplasmic death domains and do not induce apoptosis when engaged by TRAIL (Sheridan et al., 1997). Instead, TRAIL-R3 and TRAIL-R4 have antiapoptotic functions (Meng et al., 2000; Bernard et al., 2001). TRAIL-R3 has been reported previously as a doxorubicin-responsive gene in breast cancer (Ruiz de Almodovar et al., 2004), and our findings also implicate TRAIL-R4 in resistance to doxorubicin-mediated cell death. In addition, numerous genes that modulate cell death were down-regulated by doxorubicin, including multiple interleukins. Among these genes, interleukin-1α has been investigated previously for its ability to enhance the anticancer activity of doxorubicin (Nakamura et al., 1991; Monti et al., 1993).

Doxorubicin also induced the expression of proteins that metabolize xenobiotic compounds, including the cytochrome P450 proteins Cyp1A1 and Cyp1B1, the cytochrome P450 reductase, and the cytochrome b_5-related P450 activator Iza1/Hpr6.6 (Table 1). Cyp1A1 and Cyp1B1 are induced in response to numerous aromatic compounds with similar structures to doxorubicin (Nebert and Russell, 2002). Hpr6.6 homologues activate and stabilize cytochrome P450 proteins (Min et al., 2004; Mallory et al., 2005), and Hpr6.6 regulates cell death after oxidative damage (Hand and Craven, 2003), which is generated by doxorubicin. As a group, these proteins probably modify doxorubicin or cellular metabolites induced by doxorubicin. Cytochrome P450 proteins probably act in concert with the drug transporter proteins GCN20/ABCF2 and MDRTAP, which were induced by doxorubicin (Table 1), in minimizing the effective concentrations of doxorubicin within the cell.

In summary, we have identified a group of genes that are induced by chemotherapeutic agents and that regulate drug susceptibility. The functions of individual genes could not be predicted based on their transcriptional pattern, because genes that were induced by doxorubicin had both positive and negative effects on doxorubicin susceptibility. In spite of this limitation, we detected two genes, CAII and clusterin, that were associated with cell survival after chemotherapeutic drug treatment. Because these genes were not transcriptionally altered in less tumorigenic cells, there may be a "therapeutic window" in which their activity can be inhibited in tumor cells without disrupting their function in nontumorigenic cells. A third gene, Id2, is associated with increased doxorubicin susceptibility, suggesting that Id2 might serve as positive prognostic indicator when it is induced by doxorubicin in clinical tumor samples.

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References

Amundson SA, Bittner M, Chen Y, Trent J, Meltzer P, and Fornace AJ Jr (1999) Fluorescent cDNA microarray hybridization reveals complexity and heterogeneity of cellular genotoxic stress responses. *Oncogene* **18:**3666–3672.

Barone MV, Pepperkok R, Peverali FA, and Philipson L (1994) Id proteins control growth induction in mammalian cells. *Proc Natl Acad Sci USA* **91:**4985–4988.
Bernard D, Quatannens B, Vandenbunder B, and Abbadie C (2001) Rel/NF-κB transcription factors protect against tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by up-regulating the TRAIL decoy receptor DcR1. *J Biol Chem* **276:**27322–27328.
Biroccio A, Leonetti C, and Zupi G (2003) The future of antisense therapy: combination with anticancer treatments. *Oncogene* **22:**6579–6588.
Chabner BA, Ryan DP, Paz-Ares L, Garcia-Carbonero R, and Calabresi P (2001) Antineoplastic agents, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (Hardman JG and Limbard LE eds), McGraw-Hill Companies, New York.
Chang BD, Swift ME, Shen M, Fang J, Broude EV, and Roninson IB (2002) Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. *Proc Natl Acad Sci USA* **99:**389–394.
Chen X, Halberg RB, Ehrhardt WM, Torrealba J, and Dove WF (2003) Clusterin as a biomarker in murine and human intestinal neoplasia. *Proc Natl Acad Sci USA* **100:**9530–9535.
Dey BR, Furlanetto RW, and Nissley SP (1998) Cloning of human p55 gamma, a regulatory subunit of phosphatidylinositol 3-kinase, by a yeast two-hybrid library screen with the insulin-like growth factor-I receptor. *Gene (Amst)* **209:**175–183.
Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature (Lond)* **411:**494–498.
Elmore LW, Di X, Dumur C, Holt SE, and Gewirtz DA (2005) Evasion of a single-step, chemotherapy-induced senescence in breast cancer cells: implications for treatment response. *Clin Cancer Res* **11:**2637–2643.
Elmore LW, Rehder CW, Di X, McChesney PA, Jackson-Cook CK, Gewirtz DA, and Holt SE (2002) Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction. *J Biol Chem* **277:**35509–35515.
Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, and Gonzalez-Baron M (2004) PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* **30:**193–204.
Hai T and Hartman MG (2001) The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* **273:**1–11.
Hand RA and Craven RJ (2003) Hpr6.6 protein mediates cell death from oxidative damage in MCF-7 human breast cancer cells. *J Cell Biochem* **90:**534–547.
Hara E, Hall M, and Peters G (1997) Cdk2-dependent phosphorylation of Id2 modulates activity of E2A-related transcription factors. *EMBO (Eur Mol Biol Organ) J* **16:**332–342.
Hu J, Liu S, Wang J, Luo X, Gao X, Xia X, Feng Y, Tao D, Wang G, Li X, et al. (2005) Overexpression of the N-terminal end of the p55gamma regulatory subunit of phosphatidylinositol 3-kinase blocks cell cycle progression in gastric carcinoma cells. *Int J Oncol* **26:**1321–1327.
Hussain SP, Amstad P, He P, Robles A, Lupold S, Kaneko I, Ichimiya M, Sengupta S, Mechanic L, Okamura S, et al. (2004) p53-induced up-regulation of MnSOD and GPx but not catalase increases oxidative stress and apoptosis. *Cancer Res* **64:**2350–2356.
Ichikawa Y, Hirokawa M, Aiba N, Fujishima N, Komatsuda A, Saitoh H, Kume M, Miura I, and Sawada K (2004) Monitoring the expression profiles of doxorubicin-resistant K562 human leukemia cells by serial analysis of gene expression. *Int J Hematol* **79:**276–282.
Itahana Y, Singh J, Sumida T, Coppe JP, Parrinello S, Bennington JL, and Desprez PY (2003) Role of Id-2 in the maintenance of a differentiated and noninvasive phenotype in breast cancer cells. *Cancer Res* **63:**7098–7105.
July LV, Beraldi E, So A, Fazli L, Evans K, English JC, and Gleave ME (2004) Nucleotide-based therapies targeting clusterin chemosensitize human lung adenocarcinoma cells both in vitro and in vivo. *Mol Cancer Ther* **3:**223–232.
Kang HC, Kim IJ, Park JH, Shin Y, Ku JL, Jung MS, Yoo BC, Kim HK, and Park JG (2004) Identification of genes with differential expression in acquired drug-resistant gastric cancer cells using high-density oligonucleotide microarrays. *Clin Cancer Res* **10:**272–284.
Kim Y and Seol DW (2003) TRAIL, a mighty apoptosis inducer. *Mol Cell* **15:**283–293.
Kudoh K, Ramanna M, Ravatn R, Elkahloun AG, Bittner ML, Meltzer PS, Trent JM, Dalton WS, and Chin KV (2000) Monitoring the expression profiles of doxorubicin-induced and doxorubicin-resistant cancer cells by cDNA microarray. *Cancer Res* **60:**4161–4166.
Kurabayashi M, Jeyaseelan R, and Kedes L (1994) Doxorubicin represses the function of the myogenic helix-loop-helix transcription factor MyoD. Involvement of Id gene induction. *J Biol Chem* **269:**6031–6039.
Longley DB and Johnston PG (2005) Molecular mechanisms of drug resistance. *J Pathol* **205:**275–292.
Mallory JC, Crudden G, Johnson BL, Mo C, Pierson CA, Bard M, and Craven RJ (2005) Dap1p, a heme-binding protein that regulates the cytochrome P450 protein Erg11p/Cyp51p in Saccharomyces cerevisiae. *Mol Cell Biol* **25:**1669–1679.
Meng RD, McDonald ER, 3rd, Sheikh MS, Fornace AJ Jr, and El-Deiry WS (2000) The TRAIL decoy receptor TRUNDD (DcR2, TRAIL-R4) is induced by adenovirus-p53 overexpression and can delay TRAIL-, p53- and KILLER/DR5-dependent colon cancer apoptosis. *Mol Ther* **1:**130–144.
Min L, Takemori H, Nonaka Y, Katoh Y, Doi J, Horike N, Osamu H, Raza FS, Vinson GP, and Okamoto M (2004) Characterization of the adrenal-specific antigen IZA (inner zone antigen) and its role in the steroidogenesis. *Mol Cell Endocrinol* **215:**143–148.
Miyoshi K, Meyer B, Gruss P, Cui Y, Renou JP, Morgan FV, Smith GH, Reichenstein M, Shani M, Hennighausen L, et al. (2002) Mammary epithelial cells are not able to undergo pregnancy-dependent differentiation in the absence of the helix-loop-helix inhibitor Id2. *Mol Endocrinol* **16:**2892–2901.
Monti E, Mimnaugh EG, and Sinha BK (1993) Synergistic antiproliferative effects of interleukin-1 alpha and doxorubicin against the human ovarian carcinoma cell line (NIH:OVCAR-3). *Biochem Pharmacol* **45:**2099–2107.
Mori S, Nishikawa SI, and Yokota Y (2000) Lactation defect in mice lacking the helix-loop-helix inhibitor Id2. *EMBO (Eur Mol Biol Organ) J* **19:**5772–5781.
Moriyama M, Hoshida Y, Otsuka M, Nishimura S, Kato N, Goto T, Taniguchi H, Shiratori Y, Seki N, and Omata M (2003) Relevance network between chemosensitivity and transcriptome in human hepatoma cells. *Mol Cancer Ther* **2:**199–205.
Mothe I, Delahaye L, Filloux C, Pons S, White MF, and Van Obberghen E (1997) Interaction of wild type and dominant-negative p55PIK regulatory subunit of phosphatidylinositol 3-kinase with insulin-like growth factor-1 signaling proteins. *Mol Endocrinol* **11:**1911–1923.
Nagata Y, Shoji W, Obinata M, and Todokoro K (1995) Phosphorylation of helix-loop-helix proteins ID1, ID2 and ID3. *Biochem Biophys Res Commun* **207:**916–926.
Nakamura S, Kashimoto S, Kajikawa F, and Nakata K (1991) Combination effect of recombinant human interleukin 1 alpha with antitumor drugs on syngeneic tumors in mice. *Cancer Res* **51:**215–221.
Nebert DW and Russell DW (2002) Clinical importance of the cytochromes P450. *Lancet* **360:**1155–1162.
Niiya M, Niiya K, Kiguchi T, Shibakura M, Asaumi N, Shinagawa K, Ishimaru F, Kiura K, Ikeda K, Ueoka H, et al. (2003) Induction of TNF-alpha, uPA, IL-8 and MCP-1 by doxorubicin in human lung carcinoma cells. *Cancer Chemother Pharmacol* **52:**391–398.
Parrinello S, Lin CQ, Murata K, Itahana Y, Singh J, Krtolica A, Campisi J, and Desprez PY (2001) Id-1, ITF-2, and Id-2 comprise a network of helix-loop-helix proteins that regulate mammary epithelial cell proliferation, differentiation and apoptosis. *J Biol Chem* **276:**39213–39219.
Pons S, Asano T, Glasheen E, Miralpeix M, Zhang Y, Fisher TL, Myers MG Jr, Sun XJ, and White MF (1995) The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol* **15:**4453–4465.
Potter CP and Harris AL (2003) Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer. *Br J Cancer* **89:**2–7.
Raghunand N, He X, van Sluis R, Mahoney B, Baggett B, Taylor CW, Paine-Murrieta G, Roe D, Bhujwalla ZM, and Gillies RJ (1999) Enhancement of chemotherapy by manipulation of tumour pH. *Br J Cancer* **80:**1005–1011.
Redondo M, Villar E, Torres-Munoz J, Tellez T, Morell M, and Petito CK (2000) Overexpression of clusterin in human breast carcinoma. *Am J Pathol* **157:**393–399.
Ruiz de Almodovar C, Ruiz-Ruiz C, Rodriguez A, Ortiz-Ferron G, Redondo JM, and Lopez-Rivas A (2004) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) decoy receptor TRAIL-R3 is up-regulated by p53 in breast tumor cells through a mechanism involving an intronic p53-binding site. *J Biol Chem* **279:**4093–4101.
Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, et al. (1997) Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science (Wash DC)* **277:**818–821.
Shtil AA, Mandlekar S, Yu R, Walter RJ, Hagen K, Tan TH, Roninson IB, and Kong AN (1999) Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. *Oncogene* **18:**377–384.
Sikder HA, Devlin MK, Dunlap S, Ryu B, and Alani RM (2003) Id proteins in cell growth and tumorigenesis. *Cancer Cell* **3:**525–530.
Soule HD and McGrath CM (1980) Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett* **10:**177–189.
Stighall M, Manetopoulos C, Axelson H, and Landberg G (2005) High ID2 protein expression correlates with a favourable prognosis in patients with primary breast cancer and reduces cellular invasiveness of breast cancer cells. *Int J Cancer* **115:**403–411.
Takahashi-Tezuka M, Hibi M, Fujitani Y, Fukada T, Yamaguchi T, and Hirano T (1997) Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. *Oncogene* **14:**2273–2282.
Troester MA, Hoadley KA, Sorlie T, Herbert BS, Borresen-Dale AL, Lonning PE, Shay JW, Kaufmann WK, and Perou CM (2004) Cell-type-specific responses to chemotherapeutics in breast cancer. *Cancer Res* **64:**4218–4226.
Trougakos IP and Gonos ES (2002) Clusterin/apolipoprotein J in human aging and cancer. *Int J Biochem Cell Biol* **34:**1430–1448.
Trougakos IP, So A, Jansen B, Gleave ME, and Gonos ES (2004) Silencing expression of the clusterin/apolipoprotein j gene in human cancer cells using small interfering RNA induces spontaneous apoptosis, reduced growth ability and cell sensitization to genotoxic and oxidative stress. *Cancer Res* **64:**1834–1842.
Xia X, Cheng A, Akinmade D, and Hamburger AW (2003) The N-terminal 24 amino acids of the p55 gamma regulatory subunit of phosphoinositide 3-kinase binds Rb and induces cell cycle arrest. *Mol Cell Biol* **23:**1717–1725.
Yu R, Shtil AA, Tan TH, Roninson IB, and Kong AN (1996) Adriamycin activates c-jun N-terminal kinase in human leukemia cells: a relevance to apoptosis. *Cancer Lett* **107:**73–81.
Yuan R, Fan S, Achary M, Stewart DM, Goldberg ID, and Rosen EM (2001) Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor in the setting of DNA damage. *Cancer Res* **61:**8022–8031.
Zhang RD, Fidler IJ, and Price JE (1991) Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. *Invasion Metastasis* **11:**204–215.

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