# Hsp27 Modulates p53 Signaling and Suppresses Cellular Senescence

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## Abstract

**The small heat shock protein Hsp27 is expressed at high levels in many tumors and provides protection against anticancer drugs. Here, we show that expression of recombinant Hsp27 at elevated levels leads to protection of MCF10A human mammary epithelial cells from doxorubicin. The protection was associated with suppression of the doxorubicin-induced senescence, where Hsp27 inhibited p53-mediated induction of p21, the major regulator of the senescence program. Similarly, Hsp27 inhibited accumulation of p21 and suppressed senescence in response to the p53 activator nutlin-3, indicating that Hsp27 has a general effect on the p53 pathway. In line with these findings, down-regulation of Hsp27 in HCT116 human colon carcinoma cells that express this heat shock protein at high levels caused senescence in a population of cells and sensitized the rest of the cells to doxorubicin-induced senescence (at low doses) or apoptosis (at high doses of doxorubicin). Induction of senescence by Hsp27 down-regulation associated with activation of the p53 pathway and induction of p21. Interestingly, depletion of Hsp27 caused neither significant proteotoxic nor genotoxic stress, and therefore this heat shock protein seems to have a specific effect on the p53 signaling. Indeed, Hsp27 down-regulation was associated with destabilization of HDM2 and stabilization of p53. These data suggest that Hsp27 may play a general role in regulation of cellular senescence by modulating the p53 pathway.** [Cancer Res 2007;67(24):11779–88]

## Introduction

One of the important factors associated with tumor drug resistance is the small heat shock protein Hsp27. In normal cells, Hsp27 is ubiquitously expressed at low levels, but treatments with a wide variety of physiologic or environmental stresses (e.g., heat shock or oxidative stress) lead to its massive accumulation. In contrast, many tumors constitutively express Hsp27 at high levels without any stress. This protein is permanently up-regulated in a significant fraction of colon, breast, ovarian, gastric, liver, and prostate cancers (for a review, see ref. 1). Moreover, elevated levels of Hsp27 correlate with poor prognosis in breast, ovarian, head and neck cancer, esophageal squamous cell carcinoma, and leukemia (1). These data suggest that Hsp27 may play a role in development of malignancy and acquiring of the drug resistance. In fact, recent publications directly implicate Hsp27 and its homologue α-B-crystallin in tumor progression and metastases (2–4). Importantly, it was shown that overexpression of Hsp27 associates with resistance to proteasome inhibitor bortezomib/PS-341 (5) and γ radiation (6, 7). Furthermore, reports from several groups have highlighted Hsp27 as a factor conferring resistance to doxorubicin, a chemotherapeutic drug that creates double-strand DNA breaks by inhibiting topoisomerase II. For instance, Hsp27 was found up-regulated in strains of human breast carcinoma MCF-7 cells that were resistant to the drug (8, 9), and overexpression of Hsp27 in human breast adenocarcinoma MDA-MB-231 cells resulted in increased resistance to doxorubicin (9, 10).

In part, the protection of cancer cells from drugs by Hsp27 could be related to its antiapoptotic effect, which has been extensively studied (for review, see refs. 11, 12). Hsp27 was shown to block several steps in the apoptotic pathway, including inhibition of formation of apoptosome by sequestering cytochrome *c* (13, 14). Furthermore, Hsp27 can prevent caspase-8–dependent activation of Bid, a proapoptotic member from the Bcl-2 family (15). Moreover, Hsp27 was shown to inhibit radiation-induced apoptosis via suppression of protein kinase Cδ–mediated reactive oxygen species production (7).

Although, originally, apoptosis was thought to be the major pathway in killing tumor cells by anticancer drugs, it recently became clear that activation of other forms of cell demise may dictate the outcome of cancer therapy. Recent data have shown that low clinically relevant doses of radiation or genotoxic drugs do not cause apoptosis in epithelial tumor cells but instead lead to a mitotic catastrophe or a permanent growth arrest associated with cellular senescence (16), whereas apoptosis is induced mainly in lymphomas. In fact, tumor biopsies from patients undergoing chemotherapy show extensive acidic β-galactosidase staining, which is the hallmark of senescence, and little apoptotic terminal deoxyribonucleotide transferase–mediated nick-end labeling staining (17, 18). Similarly, several reports have shown that treatment of epithelial cancer cell lines in culture with low doses of doxorubicin leads to cellular senescence rather than apoptosis (19). We therefore hypothesized that elevated Hsp27 levels in cancer cells could play a role in suppression of doxorubicin-induced senescence. Here, we show that in fact Hsp27 strongly modulates senescence program activated by doxorubicin by controlling the p53 pathway.

## Materials and Methods

**Cell cultures and reagents.** HCT116 wild-type, HCT116$^{p53-/-}$, and HCT $^{p21-/-}$ were kindly provided by Dr. B. Vogelstein (The Johns Hopkins Medical Institutions, Baltimore, MD). MCF10A, MCF-7, and DU-145 cells were from American Type Culture Collection. HCT116 cells were cultivated in McCoy 5A medium supplemented with 10% fetal bovine serum and streptomycin sulfate (100 μg/mL) and penicillin (100 units/mL). MCF10A cells were grown in DMEM/F12 medium supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone,

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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10 μg/mL insulin, and 100 ng/mL cholera toxin. MCF-7 and DU-145 cells were cultivated in DMEM supplemented with 10% fetal bovine serum and streptomycin sulfate (100 μg/mL) and penicillin (100 units/mL). Doxorubicin was from Biomol; nutlin-3 was from Cayman Biochemicals; and all other reagents were from Sigma.

**Retroviral short hairpin RNA vectors.** For knockout of Hsp27 expression, we used RNAi-Ready pSIREN-RetroQ vector from BD Biosciences. The vector is provided prelinearized for ligation with double-stranded oligonucleotide encoding a short hairpin RNA (shRNA); it also contains a puromycin resistance gene for selection of stable transfectants. Two sequences of human Hsp27 gene *HSPB1* (accession number NM 001540) were selected as targets for RNA interference:

shHsp27(1) (start 435): ACGGUCAAGACCAAGGAUG
shHsp27(2) (start 701): ATCCGATGAGACTGCCGCCAA
shLuciferase (start 773): GTGGATTTCGAGTCGTCTTAAT

For most of the experiments, we used shHsp27(2) if not indicated otherwise.

For production of retroviruses, we cotransfected 293T cells using GenePorter (GTS) or Lipofectamine 2000 (Invitrogen) with plasmids expressing retroviral proteins Gag-Pol, G (VSVG pseudotype), and our constructs or enhanced green fluorescent protein (kindly provided by Jeng-Shin Lee, Harvard Medical School, Boston, MA). At 48 h after transfection, supernatants containing the retrovirus were collected and frozen at −70°C. Cells were infected with twice-diluted supernatant and 10 μg/mL polybrene overnight, washed, and selection with puromycin (0.5 μg/mL) was started 48 h after infection. Retroviral vector expressing enhanced green fluorescent protein was used as a control for infection efficiency: Usually 90% to 100% of cells were fluorescent 2 to 3 days after infection, and only small fraction of cells (<20%) were killed during selection which lasted 2 days.

Human Hsp27 was cloned in PCX-bcr vector and infected MCF10A were selected with blastocidin (10 μg/mL).

The p53-expressing retroviral plasmid in pLXSP vector was a gift from Dr. I. Roninson (Ordway Research Institute, Albany, NY).

**Senescence quantification.** β-Galactosidase assay was done using X-gal (pH 6.0) as described previously (20).

Senescence was also quantified according to morphologic appearance of cells (enlarged, flattened morphology, and vacuolization).

**Viability assays.** For colony survival assays, cells were grown to 50% to 70% confluency, treated with doxorubicin, counted, and plated on 60-mm plates. After 10 days, cells were washed with PBS, fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 30 min, stained with 0.5% crystal violet in 70% ethanol, and macroscopic colonies were counted. Survival was measured as the fraction of cells that were able to form macroscopic colonies upon treatment relative to number of colonies counted in nontreated cells.

To measure apoptosis, cells were collected and lysed in buffer containing 4 mol/L urea, 10% (v/v) glycerol, 2% SDS, 5% (v/v) 2-mercaptoethanol, and 0.01% bromophenol blue. Samples were heated for 3 min at 100°C and passed through a 27 G1/2 syringe to decrease viscosity. After PAGE and immunoblotting with anti-poly(ADP)ribose polymerase (PARP) antibody (PharMingen), blots were developed with enhanced chemiluminescence reagent (Amersham), films were scanned, and cleavage of PARP was quantified using Quantity One software (Bio-Rad).

**Immunoblotting.** Cells were washed twice with PBS, aspirated, and lysed in lysis buffer [40 mmol/L HEPES (pH 7.5); 50 mmol/L KCl; 1% Triton X-100; 1 mmol/L $Na_3VO_4$; 50 mmol/L β-glycerophosphate; 50 mmol/L NaF; 5 mmol/L EDTA; 5 mmol/L EGTA; 1 mmol/L phenylmethylsulfonyl fluoride; 1 mmol/L benzamidine; and 5 μg/mL each of leupeptin, pepstatin A, and aprotinin]. Total protein concentration was measured by Bio-Rad Protein Assay, after which they were diluted with lysis buffer to achieve equal protein concentration in all samples. Total cell extracts were separated by SDS-PAGE, transferred to nitrocellulose membrane, and analyzed using the following antibodies: anti-Hsp27 (Stressgen), anti–β-actin (Sigma, clone AC-15), anti-PARP, anti-p21 (PharMingen), anti-p53 DO-1 and survivin (Santa Cruz Biotechnology), anti–phospho-H2AX $Ser^{139}$ (Upstate Cell Signaling Solutions), anti-ubiquitin (Zymed), anti-cdc2 (Cell Signaling), and anti-Mdm2 (Ab-4, Calbiochem).

**Protein oxidation.** Detection of oxidatively damaged proteins was done with Oxiblot-Oxidative Protein detection kit from Chemicon International, according to manufacturer's recommendation.

**Transfection and reporter plasmid assay.** Transient transfections were done with Lipofectamine 2000 according to manufacturer's recommendations. Cells were transfected with reporter plasmids encoding luciferase under HSP70B promoters (a gift from Dr. Calderwood, Beth Israel Deaconess Medical Center, Boston, MA), p21 promoter or Hdm2 promoters (gifts from Dr. Z. Xiao, Boston University, Boston, MA), or cytomegalovirus (CMV) promoter in triplicate. In each experiment, a separate set of plates was transfected with plasmid expressing luciferase under CMV promoter to normalize for efficiency of transfection in retroQ and shHsp27 cells. Forty-eight hours after the start of transfection, cells were washed twice in ice-cold PBS, lysed with cell lysis buffer (Promega), and stored at −80°C. After thawing, protein concentration was measured (Bio-Rad Protein Assay) and samples were diluted with cell lysis reagent to achieve equal protein content. Luciferase activity was measured using the Luciferase assay system kit (Promega).

**Luciferase refolding assay.** For refolding assay, cells were transfected with CMV-luciferase plasmid as described above. Forty-eight hours after transfection, cells were subjected to heat shock at 43°C for 15 min and then allowed to recover for the indicated times. Samples were collected and processed as indicated above.

**Protein stability assay.** For assessing of p53 and Hdm2 stability, cells were treated with protein synthesis inhibitor emetine (10 μmol/L), and samples were taken at regular intervals for 1.5 to 2 h and subjected to immunoblotting as described above.

**Fluorescence-activated cell sorting analysis.** Cells were grown to 60% to 70% confluence, fixed in 63% iced-cold ethanol/PBS overnight at −20°C, stained with 50 μg/mL propidium iodine in the presence of 100 μg/mL RNase A, and analyzed using Becton Dickinson FACScan Cytometer.

**Statistical analysis.** Data shown are means ± SE of three to six independent experiments. Statistical significance was assessed by Student's *t* test.

## Results

**Hsp27 overexpression protects MCF10A human mammary epithelium cells from doxorubicin-induced cytotoxicity via suppression of cellular senescence.** Previously, it was reported that overexpression of Hsp27 can protect cells from doxorubicin treatments (9, 10, 21, 22). To investigate the mechanism of this phenomenon, we overexpressed Hsp27 in MCF10A normal human breast epithelial cells using retroviral expression vector. MCF10A cells have relatively low background levels of Hsp27, and infection with the retrovirus led to ~3-fold increase on Hsp27 expression (Fig. 1*A*). Control (vector only) and Hsp27-MCF10A cells were treated with low (clinically relevant) doses of doxorubicin for 48 h, and their clonogenic ability was assessed after 2 weeks. As seen in Fig. 1*B*, and in line with previous reports (9, 10, 21, 22), Hsp27 overexpression led to a significant increase in survival after doxorubicin treatment. Importantly, under these conditions, the main mechanism of the loss of clonogenic ability was activation of the senescence program rather than apoptosis or mitotic catastrophe. In fact, we did not observe signs of mitotic catastrophe (i.e., micronuclei formation, not shown) or apoptosis (caspase activation) after treatment with low doses of doxorubicin (10–200 nmol/L), whereas high doses of the drug (1–2 μmol/L) clearly induced apoptosis, as judged by PARP cleavage (Supplementary Fig. S1*A*). Instead, cells treated with doxorubicin at low doses ceased to divide but remained strongly attached to the substrate. Most of them acquired enlarged flat morphology typical for senescence and

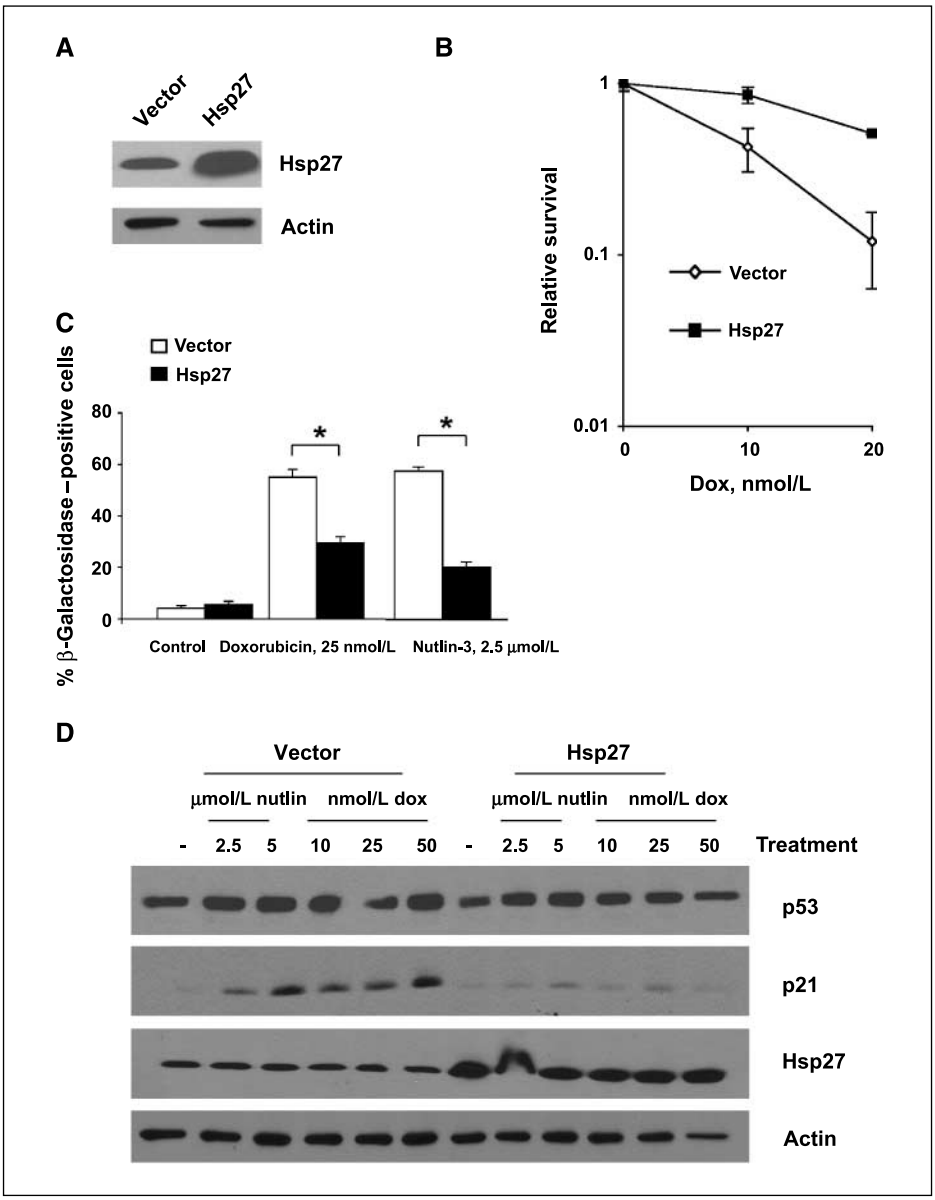


**Figure 1.** Hsp27 overexpression protects MCF10A cells from doxorubicin-induced cytotoxicity via suppression of cellular senescence. *A,* overexpression of Hsp27 in MCF10A cells. MCF10A cells were infected with retroviruses expressing Hsp27 or empty vector, and 48 h later blasticidin S (10 μg/mL) was added to select infected cells. After a 2-d selection, samples were collected and levels of Hsp27 were assayed by immunoblotting. *B,* sensitivity of control and Hsp27-overexpressing cells to doxorubicin (*Dox*) treatment. Control (vector-infected) and Hsp27 MCF10A cells were treated for 48 h with doxorubicin. Then, cells were plated to assess the colony-forming ability, as described in Materials and Methods. *C,* senescence following doxorubicin treatment in control and Hsp27-overexpressing cells. Control and Hsp27-overexpressing cells were treated for 48 h with 25 nmol/L doxorubicin or 2.5 μmol/L nutlin-3, then cells were washed, fixed, and stained for acidic β-galactosidase activity, as described in Materials and Methods. *Columns,* means; *bars,* SE. *, $P < 0.05$, Student's *t* test. *D,* accumulation of p53 and p21 in Hsp27-overexpressing cells upon treatment with doxorubicin or nutlin-3. Control and Hsp27-overexpressing MCF10A cells were treated with indicated doses of doxorubicin or nutlin-3 for 48 h, and levels of p53, p21, Hsp27, and actin (loading control) were assessed by immunoblotting.

developed acidic β-galactosidase activity (Supplementary Fig. S1*B* and S1*C*). Furthermore, they showed elevated expression of genes characteristic to senescent cells (see below). The increase in survival after doxorubicin treatment in Hsp27-overexpressing cells apparently associated with suppression of the doxorubicin-induced senescence, because significantly fewer Hsp27-MCF10A cells showed expression of acidic β-galactosidase and senescence morphology after treatment with doxorubicin, compared with control MCF10A cells (Fig. 1*C*). As reported previously, doxorubicin stimulates senescence program mostly via activation of the p53 pathway and induction of p21 (19). Therefore, we further investigated whether Hsp27 can modulate the p53 response after doxorubicin treatment.

**Hsp27 is a suppressor of p53-mediated induction of p21.** To establish whether Hsp27 can modulate the p53 response, MCF10A control and Hsp27-overexpresing cells were treated with various doses of doxorubicin for 48 h and accumulation of p53 and p21 was measured by immunoblotting. Doxorubicin led to a pronounced accumulation of p21 in control cells but this increase was markedly suppressed in Hsp27-MCF10A cells, whereas there were no significant differences in p53 accumulation (Fig. 1*D*). To better understand the mechanism of this phenomenon, we compared activation of p53 in control versus Hsp27-MCF10A cells by nutlin-3, a small molecule that antagonizes Hdm2, the major p53 ubiquitin ligase (23). It is important to note that activation of p53 by nutlin-3, in contrast to doxorubicin-induced activation, is not associated with DNA damage or activation of DNA-damage signaling pathways (24). As seen in Fig. 1*D*, nutlin-3 treatment for 48 h led to accumulation of p21 in control cells, but this accumulation was suppressed in Hsp27-overexpressing cells. These data show that Hsp27-mediated suppression of p21 induction can be seen in the absence of genotoxic stress and seems to be independent of DNA damage signaling. Because nutlin-3 can activate cellular senescence (25), we investigated whether overexpression of Hsp27 can suppress the nutlin-3–induced senescence. Control and Hsp27-MCF10A cells were treated with 5 μmol/L nutlin-3, and after

48 h cells were stained for acidic β-galactosidase expression. As seen in Fig. 1*C*, elevated levels of Hsp27 had a profound inhibitory effect on the nutlin-3–induced senescence, because whereas 80% of control cells became β-galactosidase positive upon the nutlin-3 treatment, Hsp27-MCF10A cells largely remained β-galactosidase negative. In line with this observation, the majority of the Hsp27-MCF10A cells, in contrast to control cells, did not develop the enlarged and flat morphology upon nutlin-3 treatment (not shown).

Finally, we studied whether Hsp27 can protect MCF10A cells from direct toxic effects of p53 expression. As expected, infection of control cells with p53 retrovirus leads to severe loss of clonogenic ability, whereas Hsp27 increased colony formation (Supplementary Fig. S1*C* and S1*D*). Importantly, loss of the clonogenic ability upon p53 expression was associated with p21 accumulation and senescence (not shown) rather than with apoptosis (Supplementary Fig. S1*E*).

Therefore, up-regulation of Hsp27 seems to have a general effect on suppression of the p53/p21 signaling and development of cell senescence, which can account for the Hsp27-mediated protection from doxorubicin.

**Hsp27 depletion reduces cell viability and sensitizes HCT116 human colon carcinoma cells to doxorubicin.** As mentioned in Introduction, many cancer cells constitutively express Hsp27 at elevated levels, which associates with drug resistance. Because overexpression of recombinant Hsp27 suppressed the senescence program and protected cells from doxorubicin, we investigated the effects of Hsp27 depletion on response of human cancer cells to this drug. As a model, we chose a human colon carcinoma HCT116 cell line, because these cells have highly elevated levels of Hsp27 (Supplementary Fig. S2*A*) and because a senescence response to doxorubicin treatment is especially well studied in this line (19, 26, 27). Specific down-regulation of Hsp27 was achieved by a

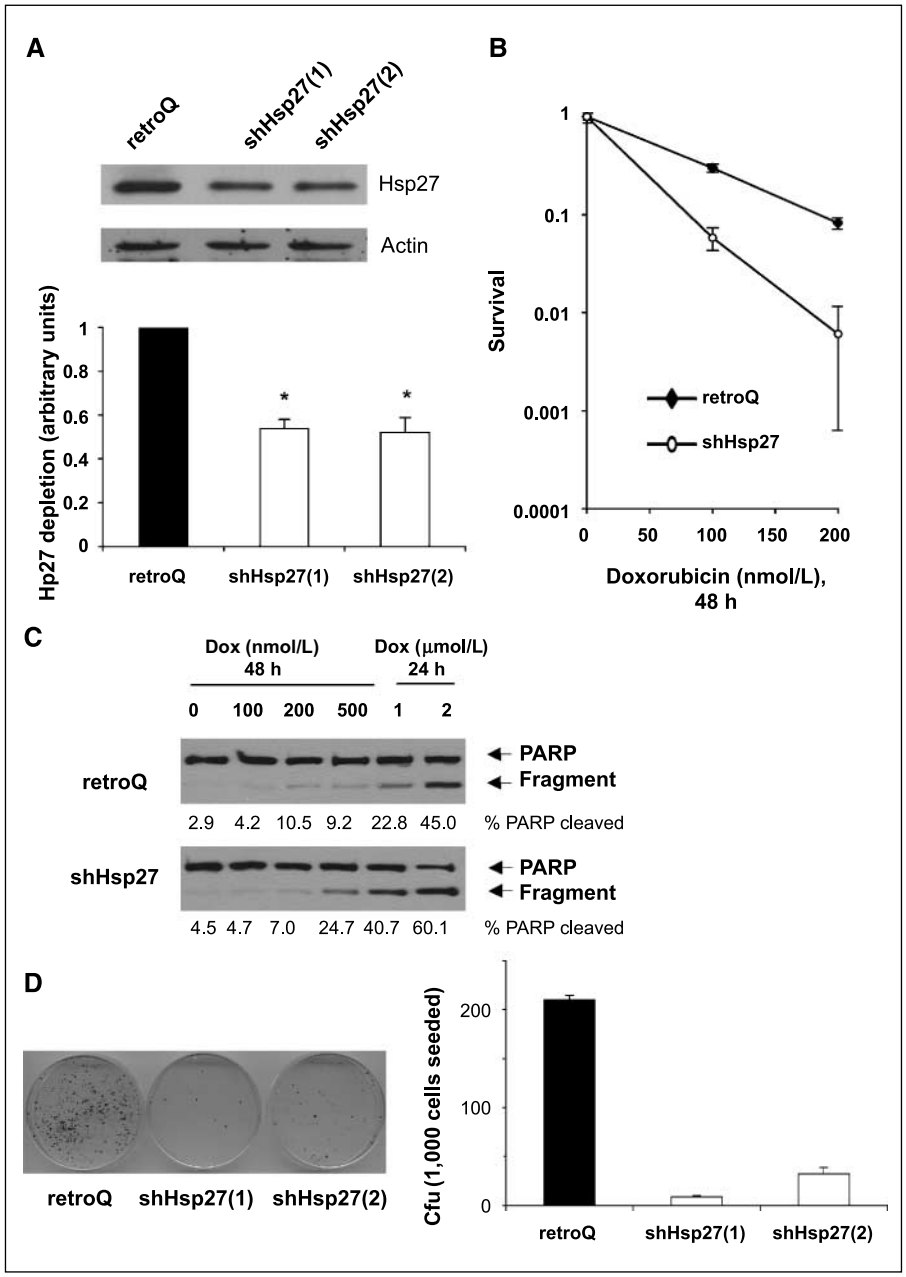


**Figure 2.** Hsp27 depletion sensitizes HCT116 human colon carcinoma cells to doxorubicin. *A,* depletion of Hsp27 in HCT116 colon carcinoma cells. HCT116 cells were infected with retroviruses expressing shRNA to two unrelated sequences in the coding region of Hsp27 [shHsp27(1) or shHsp27(2)], or empty vector (*retroQ*), and 48 h after infection, puromycin (0.5 μg/mL) was added to select infected cells. Samples were collected 4 d postselection, and levels of Hsp27 were assessed by immunoblotting (*top*) and quantified (*bottom*). *, $P < 0.01$. *B,* sensitivity of control (retroQ) and shHsp27 cells to doxorubicin treatment. RetroQ and shHsp27 cells were treated for 48 h with 100 to 200 nmol/L of doxorubicin, and cells were plated to assess their colony-forming ability, as described in Materials and Methods. *C,* effect of Hsp27 down-regulation on apoptosis. Control and shHsp27-infected cells were treated with indicated doses of doxorubicin for 24 or 48 h, and apoptotic PARP cleavage was determined by immunoblotting and was quantified. *D,* effect of Hsp27 down-regulation on clonogenic ability of cells. HCT116 retroQ and shHsp27 cells were seeded 6 d after infection at a density of 1,000 cells per 60 mm plate. Ten days later, colonies were fixed, stained (*left*), and counted (*right*). *Cfu,* colony-forming units.

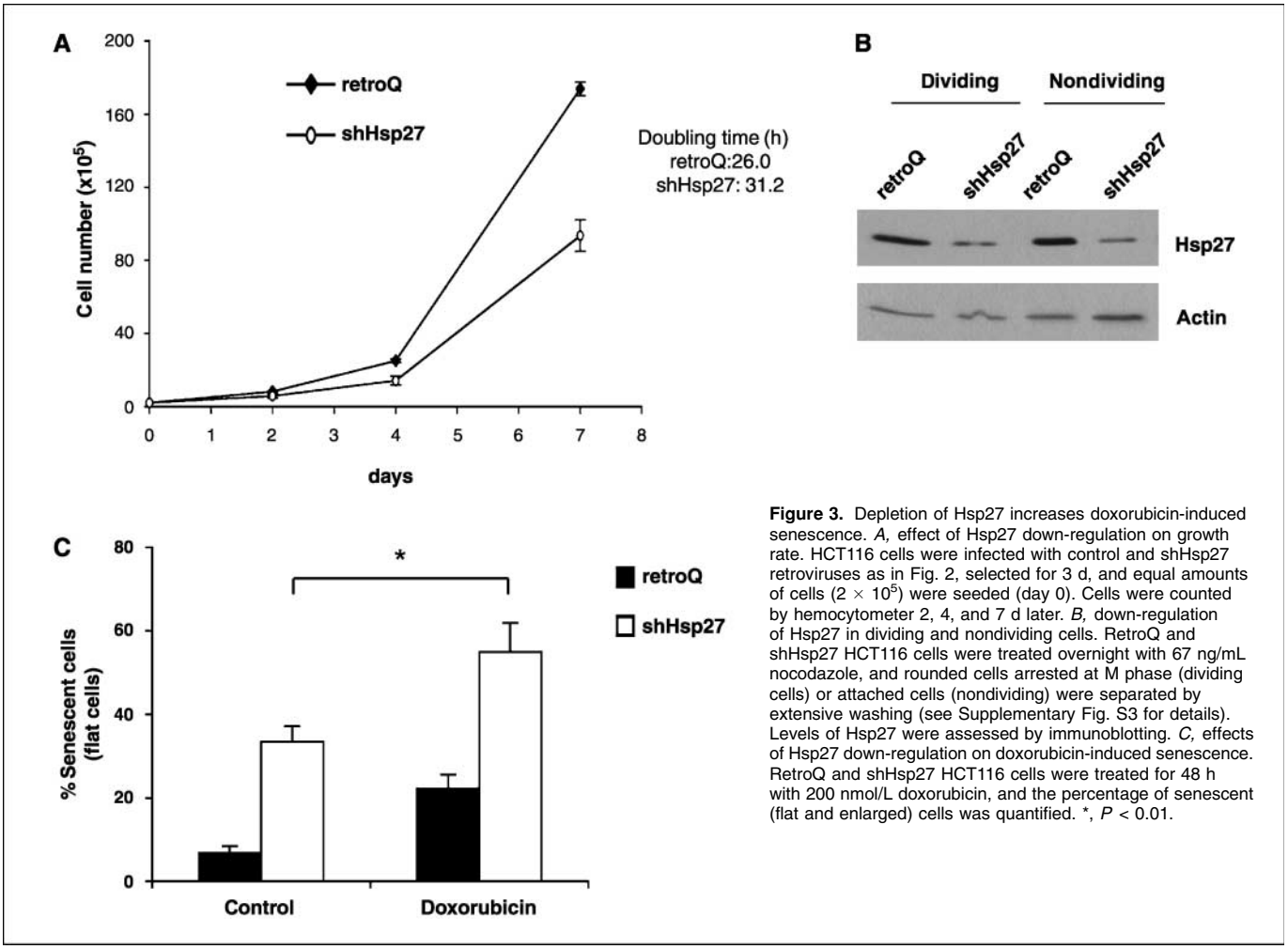


**Figure 3.** Depletion of Hsp27 increases doxorubicin-induced senescence. *A,* effect of Hsp27 down-regulation on growth rate. HCT116 cells were infected with control and shHsp27 retroviruses as in Fig. 2, selected for 3 d, and equal amounts of cells ($2 \times 10^5$) were seeded (day 0). Cells were counted by hemocytometer 2, 4, and 7 d later. *B,* down-regulation of Hsp27 in dividing and nondividing cells. RetroQ and shHsp27 HCT116 cells were treated overnight with 67 ng/mL nocodazole, and rounded cells arrested at M phase (dividing cells) or attached cells (nondividing) were separated by extensive washing (see Supplementary Fig. S3 for details). Levels of Hsp27 were assessed by immunoblotting. *C,* effects of Hsp27 down-regulation on doxorubicin-induced senescence. RetroQ and shHsp27 HCT116 cells were treated for 48 h with 200 nmol/L doxorubicin, and the percentage of senescent (flat and enlarged) cells was quantified. *, $P < 0.01$.

shRNA targeted against *HSPB1* gene, the major form of Hsp27, expressed from a retroviral vector. Retroviruses encoding two shRNA constructs that target two unrelated sequences in the coding region of Hsp27, along with a puromycin resistance gene, were generated: shHsp27(1) and shHsp27(2). As control, we used retroviruses that either do not encode shRNA (retroQ) or encodes an unrelated sequence that target luciferase (shLuc). As seen in Fig. 2*A*, infection of HCT116 cells with shHsp27(1) or shHsp27(2) resulted in >50% reduction of Hsp27 levels, whereas infection with retroQ or shLuc (not shown) did not affect Hsp27. Hsp27 depletion developed over several days postinfection, and levels of Hsp27 reached minimum on day 6 (not shown).

To assess the sensitivity of HCT116 cells to doxorubicin upon partial Hsp27 depletion, control and shHsp27 cells were treated with different doses of the drug for 48 h, and their survival was measured in a colony-forming assay. Interestingly, down-regulation of Hsp27 resulted in >3- to 10-fold reduction of the colony-forming ability even in naïve cells (see Fig. 2*D*), indicating that this heat shock protein is critical for the overall survival of HCT116 cells that naturally have elevated levels of Hsp27. The doxorubicin dose-dependent decrease in the number of emerging colonies was much stronger in Hsp27-depleted cells compared with control cells (Fig. 2*B*), indicating that elevated endogenous levels of Hsp27 provide protection of HCT116 cells from this anticancer drug. Importantly, on the graph at Fig. 2*B*, the colony count was normalized on original survival data before doxorubicin treatment (these data without normalization are presented in Supplementary Fig. S2*C*, to highlight the overall effect of Hsp27 depletion). In other words, the number of colonies corresponding to basal survival level at zero doxorubicin concentration in control cells is actually four times higher than the basal survival level in shHsp27-2 cells (Fig. 2*D*), and therefore the overall survival of the Hsp27-depleted cells was reduced after the doxorubicin treatment was even stronger. As with MCF10A cells, low doses of doxorubicin (100–500 nmol/L) did not cause significant apoptosis (assessed by PARP cleavage) in HCT116 cells (Fig. 2*C*), and therefore loss of the clonogenicity was associated with a distinct mode of death (e.g., senescence; see below). Interestingly, down-regulation of Hsp27 also significantly aggravated apoptosis, which was seen at higher doses of doxorubicin (1–2 μmol/L).

As mentioned above, loss of the clonogenic ability of HCT116 cells after low doses of doxorubicin treatment is mainly associated with senescence (27). Therefore, sensitization of these cells by depletion of Hsp27 could be associated with stronger stimulation of the senescence program. The population of the Hsp27-depleted cells that lost their clonogenic ability even before doxorubicin treatment could also be senescent. In fact, we observed that following Hsp27 down-regulation, a significant fraction of HCT116 cells (~40%) showed major hallmarks of cellular senescence, including flat morphology, enlargement, vacuolization, and β-galactosidase

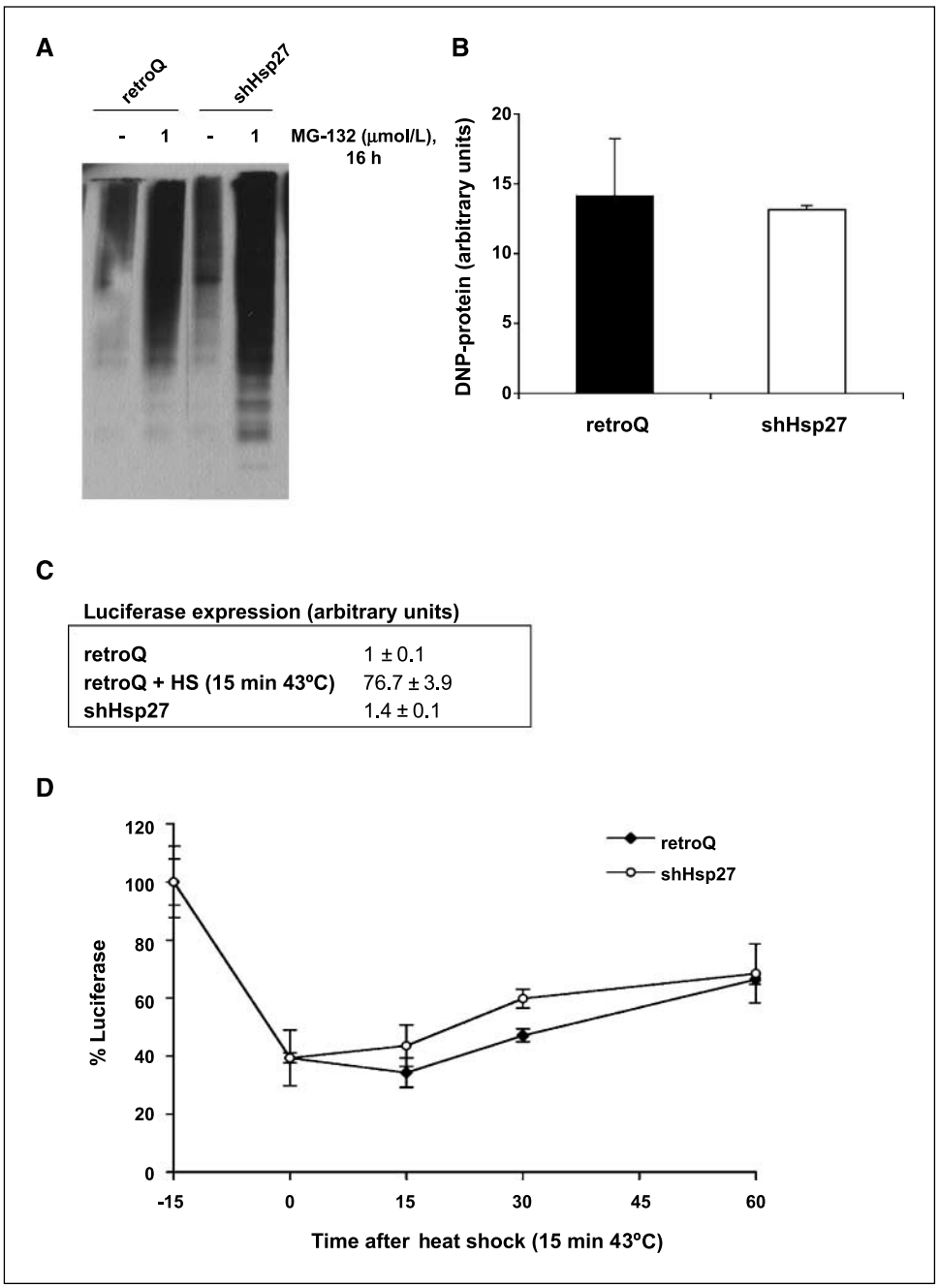


**Figure 4.** Hsp27 depletion does not lead to proteotoxic stress. *A,* protein ubiquitination in control and siHsp27 HCT116 cells. Protein ubiquitination was measured in cell lysates from retroQ or siHsp27-infected cells at day 8 postinfection by immunoblotting with antiubiquitin antibodies. As positive control, retroQ and siHsp27 cells were treated with 1 μmol/L proteasome inhibitor MG-132 for 16 h. *B,* protein oxidation in retroQ and siHsp27 HCT116 cells. Protein oxidation was assessed in cell lysates from control or shHsp27-infected cells by Oxiblot. *C,* Hsp27 down-regulation does not lead to activation of the heat shock response. Activation of the heat shock response was measured by a reporter plasmid assay: retroQ and shHsp27 cells at day 8 postinfection were transfected with a plasmid containing luciferase under the control of *Hsp70B* promoter, and 48 h later cells were collected and the luciferase activity was measured by a luminometer. As a positive control, retroQ cells were heat shocked for 15 min at 43°C and allowed to recover overnight at 37°C. Data are means ± SE; $P > 0.05$, Student's *t* test between retroQ and siHsp27 cells. *D,* luciferase refolding in Hsp27-depleted cells after mild heat shock. Control and shHsp27-infected cells at day 8 postinfection were transfected with a plasmid encoding luciferase under the control of the *CMV* promoter. Twenty-four hours later, cells were heat shocked at 43°C for 15 min and samples were taken immediately after heat shock and at the indicated times of recovery.

staining (Supplementary Figs. S2 and S3*C*, and data not shown). Similar activation of senescence by Hsp27 down-regulation was also observed in MCF-7 human breast carcinoma cells (Supplementary Fig. S2), but not in MCF10A untransformed breast cells (not shown). It seems, therefore, that high endogenous levels of Hsp27 are important for suppression of a default senescence program in tumor cells, allowing them to proliferate.

It is important to emphasize that only ~40% of HCT116 cells became truly senescent and stopped dividing when Hsp27 was reduced (see Fig. 3*C*), whereas the remaining population was still able to proliferate, albeit slower (Fig. 3*A*). We investigated whether these two populations of cells are different in the degree of Hsp27 depletion. To address this question, we incubated shHsp27 cells with nocodazole for 16 h, and as a result of this treatment, the population of dividing cells reached the M phase of the cell cycle and was arrested there (Supplementary Fig. S3*A*). Rounded and poorly attached M-phase cells (Supplementary Fig. S3*B*) were separated from the rest of the population by the shake off, and the levels of Hsp27 in the M-phase cells and the remaining attached cells were assayed by immunoblotting. Figure 3*B* shows that these two populations of cells had similar levels of Hsp27, indicating that probably there are some other endogenous factors that affect ability of HCT116 cells to undergo senescence after Hsp27 depletion, thus resulting in the population heterogeneity.

Although treatment of control cells with 200 nmol/L doxorubicin for 48 h led to senescence of ~20% of cells, treatment of Hsp27-depleted cells with 200 nmol/L of doxorubicin caused senescence of almost 60% of cells, as assayed by the appearance of enlarged

and flat morphology (Fig. 3*C*). Apparently, the population of dividing cells upon Hsp27 down-regulation became sensitive to doxorubicin in terms of activation of the senescence program. Therefore, Hsp27 depletion seems to cause senescence in a significant fraction of cells, and the remaining population, while being able to proliferate, could be easily committed to senescence by treatment with low doses of doxorubicin, and most likely represents a presenescent state.

**Depletion of Hsp27 does not result in proteotoxic or genotoxic stresses.** Because Hsp27 is a molecular chaperone, we hypothesized that depletion of Hsp27 may lead to reduction of the overall protein refolding capacity in cells and accumulation of damaged proteins, which, in turn, being stressful to cells, may result in cell senescence and increased sensitivity to doxorubicin. To test this possibility, we assessed different variables associated with general protein damage (Fig. 4). Overall protein ubiquitination was similar in control and shHsp27 cells (Fig. 4*A*), indicating that depletion of Hsp27 does not result in a massive increase in damaged proteins that are targeted for degradation by the proteasome. Similarly, no significant differences in control and shHsp27 cells were seen with Oxiblot (Fig. 4*B*), indicating that depletion of Hsp27 does not result in bulk accumulation of the oxidatively damaged proteins. To further evaluate whether Hsp27 depletion can cause a general proteotoxic stress, we assessed activation of the heat shock response, an important hallmark of protein damage. Accordingly, we transfected control and shHsp27 cells with a reporter plasmid containing luciferase under the control of the highly inducible Hsp70B promoter. Both control and Hsp27-depleted cells showed similar luciferase expression, indicating that depletion of Hsp27 does not lead to activation of the heat shock response (Fig. 4*C*). In this experiment, mild heat treatment (as a control) caused almost 80-fold increase in the luciferase expression (Fig. 4*C*).

We also directly assessed the refolding capacity of cells upon Hsp27 reduction by measuring the ability of control and shHsp27 cells to refold heat-denatured luciferase *in vivo*. Control and Hsp27-depleted cells were transfected with a vector expressing luciferase under a CMV promoter. Cells were exposed to mild heat shock (43°C, 15 min) that causes significant luciferase denaturation, and samples were collected at different times after the insult (Fig. 4*D*). The rate of luciferase refolding was assessed as the degree of recovery of the luciferase activity after heat shock. In this experiment, luciferase activity dropped to <40% immediately after heat shock, and then gradually recovered to >60% within 1 h (Fig. 4*D*). Interestingly, we did not detect a reduced rate of luciferase refolding in Hsp27-depleted cells compared with control cells (Fig. 4*D*), indicating that Hsp27 is dispensable for efficient luciferase refolding in HCT116 cells. Most likely, high levels of other chaperones, like αB-crystalline or Hsp72, can compensate for the deficit in Hsp27. Overall, these data indicate that down-regulation of Hsp27 does not result in significant suppression of the ability of the cell to handle abnormal proteins or in a general protein damage, and therefore such damage apparently cannot account for activation of p53 pathway under these conditions.

Because depletion of Hsp27 promoted senescence induced by the DNA-damaging agent doxorubicin, we evaluated whether DNA integrity or DNA damage signaling pathways are affected by depletion of Hsp27. Accordingly, we measured phosphorylation of histone H2AX, a major marker of double-strand breaks. As seen in Supplementary Fig. S4*A*, no increase in phosphorylation of histone H2AX was seen in cells with reduced levels of Hsp27, whereas doxorubicin clearly activated H2AX phosphorylation. In

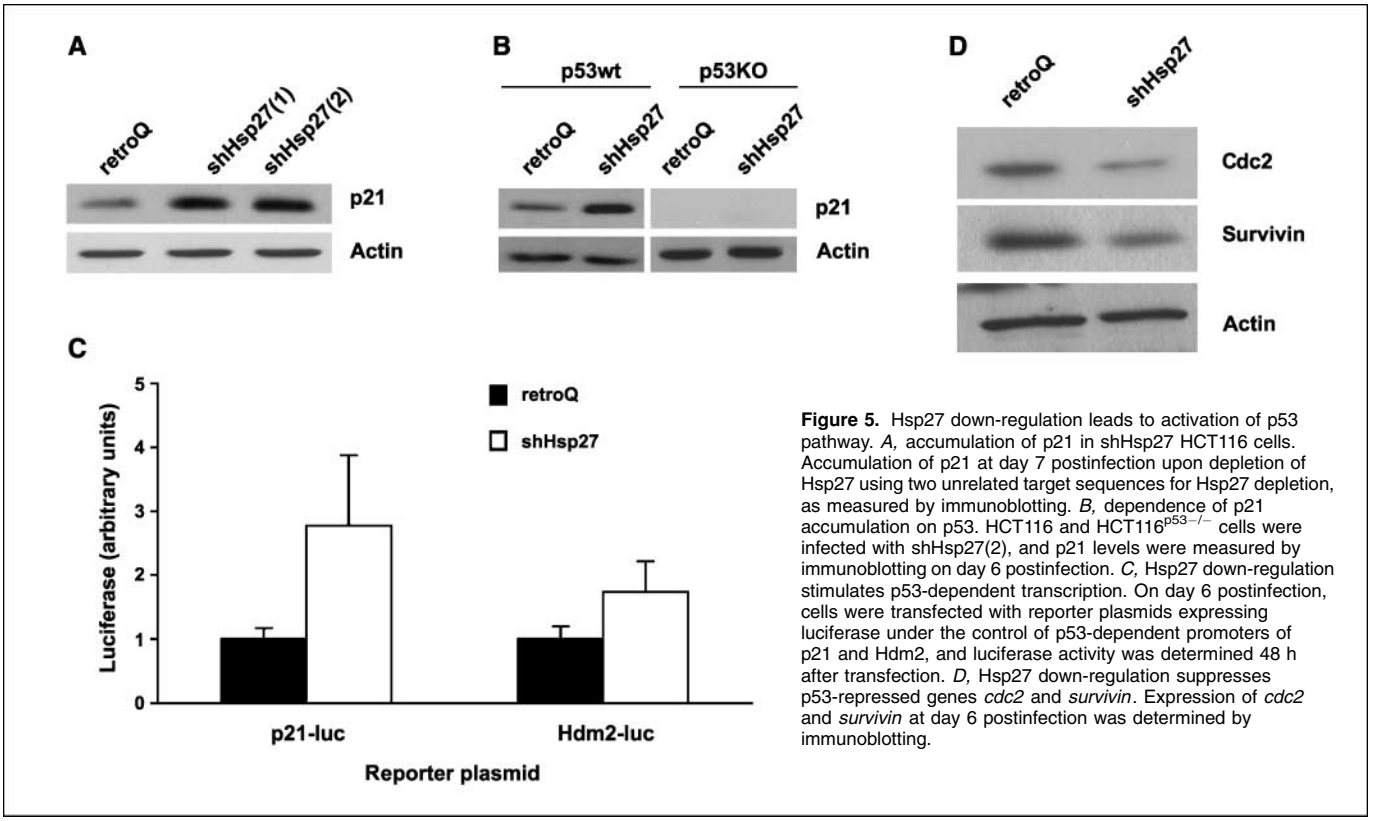


**Figure 5.** Hsp27 down-regulation leads to activation of p53 pathway. *A,* accumulation of p21 in shHsp27 HCT116 cells. Accumulation of p21 at day 7 postinfection upon depletion of Hsp27 using two unrelated target sequences for Hsp27 depletion, as measured by immunoblotting. *B,* dependence of p21 accumulation on p53. HCT116 and HCT116$^{p53-/-}$ cells were infected with shHsp27(2), and p21 levels were measured by immunoblotting on day 6 postinfection. *C,* Hsp27 down-regulation stimulates p53-dependent transcription. On day 6 postinfection, cells were transfected with reporter plasmids expressing luciferase under the control of p53-dependent promoters of p21 and Hdm2, and luciferase activity was determined 48 h after transfection. *D,* Hsp27 down-regulation suppresses p53-repressed genes *cdc2* and *survivin*. Expression of *cdc2* and *survivin* at day 6 postinfection was determined by immunoblotting.

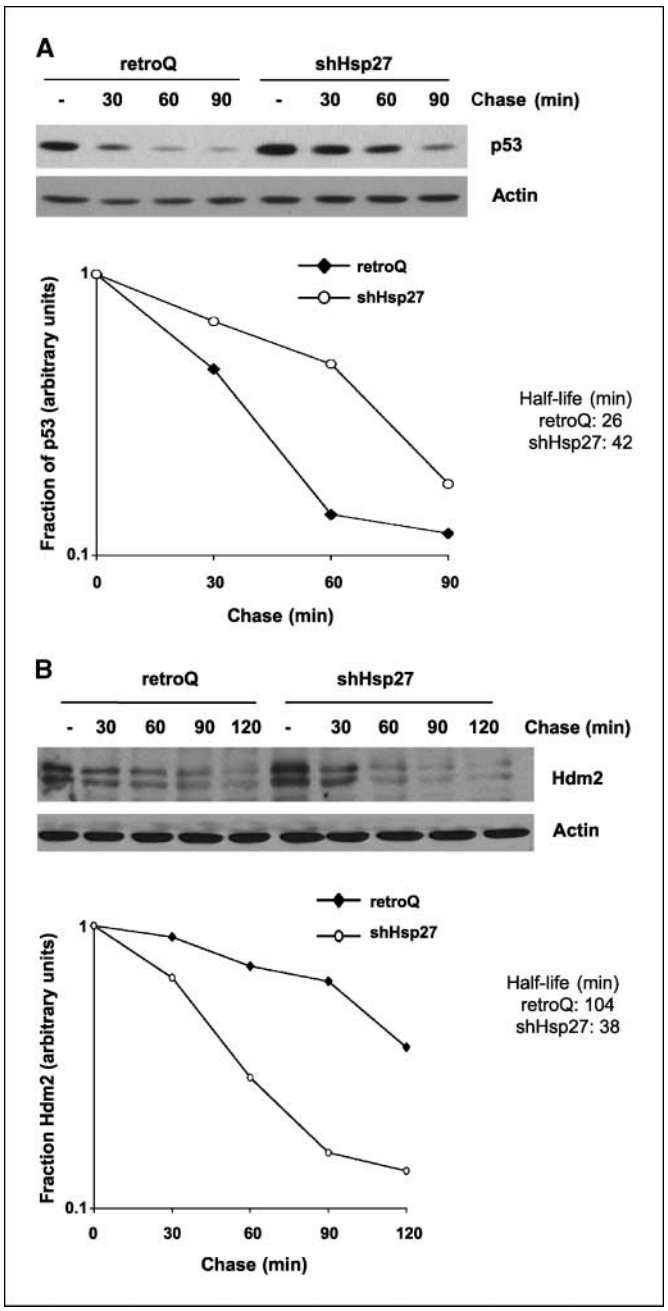


**Figure 6.** Regulation of p53 and Hdm2 stability by Hsp27 depletion. *A,* stabilization of p53 in Hsp27-depleted cells. RetroQ and shHsp27 HCT116 cells at day 6 postinfection were incubated with 10 μmol/L emetine for the indicated times. Samples were collected and the extent of remaining p53 was assessed by immunoblotting (*top*). *Bottom,* quantification of results presented at the top panel. The fraction of p53 remaining after emetine treatment was expressed relative to initial p53 levels in retroQ and shHsp27 cells, and half-life was calculated. *B,* destabilization of Hdm2 in Hsp27-depleted cells. HDM2 stability was assessed as in *A*.

parallel, we directly measured the extent of DNA damage by the alkaline comet assay. This assay measures double-strand breaks as well as single-strand breaks and alkali labile sites. Supplementary Figure S4*B* shows that there is no increase in the comet tails after Hsp27 depletion, further proving that Hsp27 down-regulation does not result in DNA damage. The absence of apparent proteotoxic and genotoxic stresses in shHsp27 cells indicates that the activation of the senescence program in these cells may be due to a specific role of Hsp27 in p53 signaling, rather than due to increased cellular stress.

**Hsp27 depletion leads to activation of p53 pathway and accumulation of p21.** Activation of the senescence program usually involves up-regulation of cyclin-dependent kinase inhibitors p21, p16, or p27, and therefore we monitored levels of these proteins in cells after down-regulation of Hsp27. We could not observe any significant changes in p16 (which is silenced in HCT116 cells) or p27 levels (not shown), but we did see strong accumulation of p21 in Hsp27-depleted cells (Fig. 5*A*). Infection of cells with either shHsp27(1) or shHsp27(2) retroviruses produced similar up-regulation of p21, whereas infection with control retroviruses did not affect p21 levels, indicating that Hsp27 depletion specifically induces p21 (Fig. 5*A*). Accumulation of p21 under these conditions was dependent on p53, because p21 levels remained low in HCT116$^{p53-/-}$ knockout cells upon depletion of Hsp27 (Fig. 5*B*), indicating that Hsp27 can control the p53 pathway. Furthermore, no accumulation of p21 was observed in DU-145 prostate carcinoma (Supplementary Fig. S5) or MDA-MB231 breast carcinoma (not shown) with mutant p53, but Hsp27 down-regulation profoundly induced p21 in MCF7 breast carcinoma with wtp53 (Supplementary Fig. S5).

To directly assess whether Hsp27 depletion activates p53-dependent transcription, cells were transfected with luciferase reporter under the control of p53-regulated promoters, p21 and Hdm2. To control the efficiency of transfection, Hsp27-depleted and control cells were transfected with luciferase under the control of p53-independent CMV promoter (CMV-Luc). Hsp27 down-regulation increased transcriptional activation of p21 ~3-fold, and of HDM2 ~2-fold (Fig. 5*C*). In addition to being an inducer of transcription, p53 can also repress certain genes (26, 28, 29). Accordingly, we observed a suppression of known downstream targets of p53, survivin, and cdc2 in shHsp27 cells (Fig. 5*D*).

p53 is highly unstable under normal conditions, whereas, upon stress, p53 undergoes modifications (e.g., phosphorylation, acetylation, ubiquitination, or SUMOylation) and gets stabilized, leading to its accumulation and induction of the target genes (e.g., *p21*). We hypothesized that Hsp27 down-regulation can cause stabilization of p53. In fact, direct measurement of the degradation rates showed that the half-life of p53 in shHsp27 cells increased from 26 to 42 min (Fig. 6*A*).

It well known that the major role in stabilization of p53 both under normal and stressful conditions is played by Hdm2, the stability (and activity) of which is, in turn, regulated mostly by self-ubiquitination followed by degradation (30). Accordingly, we assessed effects of down-regulation of Hsp27 on levels and stability of Hdm2. Levels of Hdm2 were even slightly higher in these cells, probably reflecting transcriptional activation of the *HDM2* gene by active p53 (see Fig. 5*C* above). At the same time, stability (half-life) of HDM2 was reduced from 104 to 38 min (Fig. 6*B*). The destabilization of Hdm2 reflects its inability to ubiquitinate p53 associated with faster self-ubiquitination and subsequent degradation. These data indicate that Hsp27 seems to affect Hdm2, which regulates stability of p53, in turn controlling expression of p21, and subsequent activation of the senescence program and sensitization to doxorubicin.

Of note, p53 plays an important role in senescence caused by depletion of Hsp27. In fact, in HCT$^{p53-/-}$ cells, senescence was reduced by ~50% compared with parental cells (see Supplementary Fig. S5*C*). Similar suppression was seen in HCT$^{p21-/-}$ cells.

Furthermore, in DU145 cells that lack p53, depletion of Hsp27 did not cause significant senescence (not shown).

## Discussion

Here, we addressed the role of Hsp27 in resistance of tumors to the chemotherapeutic drug doxorubicin and provided insights in the molecular mechanisms underlying this phenomenon. At low concentrations, doxorubicin reduces cell viability by activation of signaling pathways leading to cellular senescence, and, only at higher doses, it causes apoptotic cell death (Figs. 1 and 2). It is important that clinically relevant doses of doxorubicin do not kill cells within a tumor, especially in solid tumors, but rather permanently arrest growth of cancer cells by activation of senescence pathways (17, 18). Therefore, we designed an experimental protocol where treatment with doxorubicin caused cellular senescence rather than apoptosis. In HCT116 colon carcinoma cells, depletion of Hsp27 dramatically exacerbated doxorubicin-induced senescence in the dividing population of cells (Fig. 3*C*). In line with these findings, MCF10A cells that overexpress recombinant Hsp27 become protected from doxorubicin, which was accompanied by suppression of the drug-induced senescence (Fig. 1). Remarkably, we also found that Hsp27 suppresses senescence induced by nutlin-3, an Hdm2 antagonist that does not cause genotoxic stress, suggesting that Hsp27 interferes with p53 activation or function rather than with DNA damage signaling (Fig. 1). Overall, these data indicate that Hsp27 can protect from genotoxic insults such as doxorubicin by suppression of drug-induced senescence. Because doxorubicin and other major anticancer drugs at clinically relevant doses activate cell senescence rather than apoptosis in epithelial cancers, suppression of senescence by Hsp27 may significantly jeopardize the therapy. The fact that depletion of Hsp27 results in sensitization of cells to anticancer agents is a proof of principle for the development of inhibitors of Hsp27 as enhancers of conventional DNA-damaging drugs.

In addition to sensitization to doxorubicin, depletion of Hsp27 by itself led to growth arrest and cellular senescence in a population of cells (Fig. 3). Here, we show that these alterations result from activation of the p53/p21 pathway. Apparently, elevated levels Hsp27 in tumor cells with wtp53 (e.g., HCT116 and MCF7) can suppress the p53 pathway and thus allows cells to avoid default senescence (Figs. 5 and 6). Down-regulation of Hsp27 has previously been shown to induce apoptosis in LNCaP prostate carcinoma (3), and we also found apoptosis rather than senescence in this cell line.[1] Interestingly, LNCaP cells has wtp53, but, unlike HCT116 and MCF7 cells, other inducers of p53, nutlin-3, and doxorubicin even at low does are unable induce senescence in these cells, and they undergo apoptosis (see also ref. 29).[2] Thus, it seems that consequence of p53/p21 pathway activation (senescence or apoptosis) upon Hsp27 down-regulation may be partially cell specific.

Activation of p53 and p21 upon down-regulation of Hsp27 does not seem to be a consequence of impaired chaperone activity of the cell or genotoxic stress (Fig. 4). To our surprise, depletion of Hsp27 in HCT116 cells did not result in significant proteotoxicity. This result was somewhat unexpected because Hsp27 is regarded as one of the major cellular chaperones. This finding suggests that the increased chaperone load often seen in cancer cells does not serve as a buffering system for endogenous protein damage. Instead, it seems that elevated levels of Hsp27 serve as specific suppressors of p53 activation in cancer cells.

Similar activities in suppression of the p53 pathway and the default senescence program were attributed to certain other chaperones of the Hsp70 family: Hsp70-2 (31), Hsp72 (32), and Grp75 (33). Interestingly, we recently found that activation of p53 pathway upon Hsp72 down-regulation is also associated with destabilization of Hdm2 (32). Because HCT116 cells express high levels of both Hsp27 and Hsp72, and down-regulation of either protein evokes similar effects on Hdm2, p53, p21, and senescence, it seems that both these proteins play a role in the Hdm2 activity, for example, by working as cochaperones of Hdm2 or its regulator(s). Indeed, as we found here, in tumor cells with high levels of Hsp27 and Hsp72 (HCT116), Hdm2 is relatively stable (half-life $\sim$100 min), whereas in normal cells (MCF10A) it is extremely unstable (half-life $\sim$10 min).[3]

It is well known that tumors often overexpress the heat shock transcription factor Hsf1 and several Hsps, including Hsp27 (1, 34). It seems that down-regulation of the p53 signaling pathway by diverse chaperones may be critical for progression of tumors that have normal p53 (35).

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[1] C. O'Callaghan-Sunol et al., unpublished data.
[2] C. O'Callaghan-Sunol et al., unpublished data.
[3] C. O'Callaghan-Sunol et al., unpublished data.

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# Hsp27 Modulates p53 Signaling and Suppresses Cellular Senescence

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