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Hpr6 (heme-1 domain protein) regulates the susceptibility of cancer cells to chemotherapeutic drugs

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Non-standard abbreviations: Dap1p, damage resistance protein; *hbd*, heme-binding deficient; Hpr6, heme-1 domain protein; IZA, inner zone antigen; RNAi, RNA inhibition

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Abstract

Cancer cells have varying levels of susceptibility to chemotherapeutic agents, and the proteins that direct drug susceptibility are promising targets for intervention in cancer. Hpr6 (heme-1 domain protein)/PGRMC1 (progesterone receptor membrane component 1) is overexpressed in tumors, and Hpr6 is the human homologue of a budding yeast damage resistance gene called Dap1p. Cells lacking Dap1p are damage-sensitive, and we have found that inhibition of Hpr6 expression by RNAi increases sensitivity of breast cancer cells to chemotherapeutic drugs. Hpr6 is composed largely of a cytochrome b_5-related heme-1 domain, and we have found that purified Hpr6 binds to heme, like its yeast and rodent homologues. We generated an aspartate-120-to-glycine (D120G) mutant of Hpr6 at a highly conserved site in the heme-1 domain, and demonstrated that Hpr6-D120G cannot bind to heme. The Hpr6-D120G mutant was named Hpr6^{hbd} for heme binding defective. We prepared an adenovirus encoding Hpr6^{hbd}, and found that Ad-Hpr6^{hbd} increases susceptibility of breast cancer cells to doxorubicin, etoposide, and camptothecin. Our findings support a model in which Hpr6, like its yeast homologue, binds to heme and regulates susceptibility to damaging agents.

Introduction

Treatments for solid tumors usually include a combination of DNA damaging agents. Doxorubicin or adriamycin is an anthracycline antibiotic that is used in combination with other drugs for treating breast cancer (Chabner et al., 2001). Doxorubicin acts through multiple mechanisms that include intercalating within DNA, binding to topoisomerase II, and generating reactive oxygen species (Chabner et al., 2001; Longley and Johnston, 2005). Other chemotherapeutic agents in use for solid tumors include camptothecin analogs, which inhibit topoisomerase I, and nitrogen mustards, which alkylate DNA (Chabner et al., 2001). Because many alkylating agents require activation in the liver, mechlorethamine is frequently used with cultured cells, because it does not require conversion to an active form. The proteins that regulate susceptibility to chemotherapy are potential therapeutic targets for cancer, and altering their expression or activity could improve clinical chemotherapy responsiveness.

Hpr6 is a member of the heme-1 domain family of proteins, which includes the human Hpr6 and Dg6 proteins (Gerdes et al., 1998), the rodent 25-Dx (Selmin et al., 1996) and IZA proteins (Raza et al., 2001), and the budding yeast Dap1p, for *da*mage resistance *p*rotein (Hand et al., 2003). The Hpr6/25-Dx/IZA/Dap1p proteins contain a central heme-1 domain that shares homology with cytochrome b_5 (Mifsud and Bateman, 2002). Like cytochrome b_5, IZA and Dap1p bind to heme (Min et al., 2004; Mallory et al., 2005a). 25-Dx is a variant of IZA (Min et al., 2004) suggesting that it binds to heme, and we show in the present study that Hpr6 binds to heme. Heme is composed of protoporphyrin IX and ferrous iron and is a cofactor in numerous reactions in energy production and metabolism.

Hpr6 is the homologue of Dap1p, and mutants lacking Dap1p are sensitive to a DNA alkylating agent and inhibitors of sterol biosynthesis (Hand et al., 2003). These defects can be suppressed by high levels of heme or the cytochrome P450 protein Cyp51p/Erg11p (Mallory et al., 2005a), a heme binding protein, suggesting a role for Dap1p in heme transport or metabolism. In rodents, IZA binds to

heme (Min et al., 2004) and localizes to the adrenal inner zones (Raza et al., 2001), where it likely up-regulates the steroid biosynthetic activities of cytochrome P450 proteins (Min et al., 2004). Because Dap1p directs resistance to xenobiotic damaging agents, we have tested the extent to which Hpr6 performs a similar function.

Hpr6 is overexpressed in clinical tumor samples, including approximately 50% of breast tumors, and is readily detectable in cancer cell lines (Crudden et al., 2005), suggesting a role for Hpr6 in tumor cell survival. Multiple microarray studies have also identified Hpr6 as an up-regulated gene in tumor samples (Difilippantonio et al., 2003; Kim et al., 2004; Irby et al., 2005). The rodent Hpr6 homologue was originally identified as 25-Dx because it is up-regulated in liver tumors induced by dioxin (Selmin et al., 1996), but the function of Hpr6 in tumors has not been analyzed previously. Human Hpr6 was originally named as a human membrane progesterone receptor (Gerdes et al., 1998) based on its homology to putative progesterone binding proteins (Falkenstein et al., 1996) and is listed in databases as PGRMC1 for *pro*gesterone *r*eceptor *m*embrane *c*omponent 1 or HMPR for *h*uman *m*embrane *p*rogesterone *r*eceptor. Hpr6 is often identified as PGRMC1 in microarray studies, but progesterone binding for this protein has not been demonstrated.

To test the role of Hpr6 in resistance to chemotherapeutic agents, we have inhibited Hpr6 expression and activity in breast cancer cells, and we have measured the susceptibility of cells with inhibited Hpr6 function to chemotherapeutic drugs. In cells where Hpr6 expression was inhibited by RNAi, we detected a significant increase in cell death after chemotherapy. We then found that purified Hpr6 bound to heme, while a mutation in the heme-1 domain inactivates heme binding. Over-expression of the heme binding defective mutant in breast cancer cells increased doxorubicin susceptibility, indicating that heme binding is critical for Hpr6 activity. These findings indicate that Hpr6 mediates resistance to chemotherapeutic agents in breast cancer and is a promising target for therapeutic intervention.

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Materials and Methods

Cell lines, culturing, and infection. MDA-MB-231 human breast cancer cells were a kind gift from Dr. Carolyn Sartor (University of North Carolina at Chapel Hill) and were maintained in Dulbecco's Modified Eagle Medium with 10% Serum Supreme (Fisher, Chicago, IL) and penicillin and streptomycin. Cells were grown at 37^{o}C in a humidified chamber containing 5% CO_2 in air. Cells were visualized using a VistaVision inverted microscope attached to a Sony DCS-F717 digital camera (VWR, Batavia, IL). For infection, cells were incubated with varying doses of Ad-LacZ or Ad-Hpr6^{hbd} for 24 hours prior to drug treatment. Infections were performed in normal culture medium (see above).

Expression plasmids and viral preparation. The 3' end of the *HPR6* open reading frame containing the D120G mutation was amplified from the plasmid IMAGE 3254089 (Research Genetics, Huntsville, AL) using the primers HPR-D120G-F (5'-TACGGGCCCGAGGGGCCGTATGGGGTCTTTGCTGGAAGAGGTGCATCCAGGGGCCTTGC) and HPR-690R-APA (5'-GAGCACTTGCTATAAGTTTCTCGAGGGGCCCACA) and cloned into *Apa*I site of the plasmid pRC38 (Hand and Craven, 2003). The resulting plasmid, pRC42, contained the full-length *HPR6* open reading frame with the D120G mutation. The *HPR6*-D120G open reading frame was cloned into the *Bgl*II and *Xho*I sites of pShuttle-CMV (provided by the University of North Carolina at Chapel Hill Adenoviral Core Facility), resulting in the plasmid pRC45. The Ad-Hpr6^{hbd} adenovirus was isolated by the University of North Carolina at Chapel Hill adenoviral core facility, and the adenovirus was amplified by the Medical College of Wisconsin adenoviral core facility.

RNAi. MDA-MB-231 cells were plated at a density of 500,000 cells per 100 mm tissue culture dish. RNA oligonucleotide duplexes were diluted to 220 nM in 1 ml of Opti-MEM medium (Invitrogen,

Carlsbad, CA). A 1:6 suspension of Oligofectamine (Invitrogen, Carlsbad, CA) in Opti-MEM was added to the RNAi duplex solution according to the manufacturer's instructions, and the mixture was incubated 15-20 minutes. During the incubation, plated MDA-MB-231 cells were washed once and overlaid with 4.4 ml of Opti-MEM. The RNAi suspension was added to the cells to a final concentration of 40 nM RNAi, a dose that was determined to be optimal for MDA-MB-231 cells. After 4 hours at 37^{o}C, 2.8 ml of culture medium containing 30% Serum Supreme (Fisher, Chicago, IL) was added to the cells, and the cells were incubated overnight. The cells were then trypsinized and 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.

Fusion protein plasmids, preparation, and analysis. Hpr6-GST fusion proteins contained amino acids 43-195 of the Hpr6 open reading frame. Hpr6 was amplified using the primers HPR+126F-*Bam* and HPR+566R-*Xho* with the plasmid pRC40 as a template. PCR products were cloned into the pCR2.1 plasmid (InVitrogen, Carlsbad, CA) forming the plasmid pRC44. The Hpr6 fragment was then cloned into the *Bam* HI and *Xho* I sites of pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ), forming the plasmid pRC46. To introduce the D120G mutant, the plasmid pRC46 was digested with *Apa* I and *Xho* I and ligated to the 260 bp *Apa* I-*Xho* I fragment of the plasmid pRC45, forming the plasmid pGC4.

Fusion protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Fisher, Chicago, IL), and cells were lysed in the B-PER reagent (Pierce, Rockford, IL) and bound to glutathione-agarose beads (Pierce, Rockford, IL). At this stage, the GST-Hpr6-bound columns were visibly brown due to bound heme, while the GST-Hpr6-D120G-bound columns were white due to the inability of this mutant to bind heme. After estimating protein quantity by SDS-PAGE electrophoresis, 100 μg of Hpr6 or Hpr6-D120G proteins were liberated by digestion with 1U of thrombin for 2 hours, and a scan of absorbance from 350-550 nm was determined using a

Beckman Coulter DU640 spectrophotometer (Fullerton, CA). Proteins were reduced with the addition of 1 mg of sodium hydrosulfite.

Drug treatments and viability assays. Doxorubicin (Sigma, St. Louis, MO) was added to culture media and incubated for 5 days. Cells were incubated with mechlorethamine (kindly provided by Dr. Robert Orlowski, University of North Carolina at Chapel Hill) or camptothecin (Sigma, St. Louis, MO) for 72 hours. For measurements of cell growth, transfected cells were counted and plated in 96 well dishes, then treated with doxorubicin, camptothecin, or mechlorethamine for 72-96 hours. Cells were then grown in media containing 0.5 mg/ml MTT (3-[4,5 dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) for 1-2 hours. MTT-containing media were then removed and replaced with 100 µl of dimethyl sulfoxide. The plates were incubated for 20 minutes on a rotating platform and the A_{595}/A_{650} was determined using a Dynatech MR600 Microplate Reader. The percent of viable cells 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. Trypan blue assays were performed by incubating cells in 0.4% trypan blue (Sigma, St. Louis, MO) for 5 minutes and counting the percentage of blue cells, which are inviable cells that are unable to exclude the dye. Trypan blue measurements were also performed in triplicate.

Western blots. Western blots of cultured cells were performed as described previously (Yang et al., 2003). The antibodies used were as follows: anti-HA (HA11, BAbCo, Berkeley, CA), tubulin (Fisher, Chicago, IL), PARP (Santa Cruz Biotechnologies, Santa Cruz, CA), Hpr6 (Meyer et al., 1998), and Cyp21 (Corgen LLC, Guilford, CT). The antibody to Cyp1A1 was a kind gift from Dr. Harry Gelboin at the Laboratory of Metabolism at the National Cancer Institute. The intensities of the Hpr6 and

tubulin bands were quantitated by densitometry using Image Quant software (Molecular Dynamics, Sunnyvale, CA), and the ratio of Hpr6 to tubulin was calculated from these values.

RT-PCR. RNA was purified using the RNAeasy kit from Qiagen and reverse transcribed using SuperScipt II Reverse Transcriptase and random hexamers (Invitrogen, Carlsbad, CA). PCR reactions were performed using Taq polymerase (Genscript, Piscataway, NJ) in an Eppendorf Master Cycler (Eppendorf North America, Westbury, NY) using 25-35 cycles of a program consisting of 94^{o}C for 1 min., 55^{o}C for 1 min., and 72^{o}C for 1 min. PCR reactions contained primers to *HPR6* and to actin, which was an internal control for equal loading of the cDNA template. DNA was then visualized by electrophoresis in 2.5% agarose 1000 (Invitrogen, Carlsbad, CA). The primer sequences were HPR+1080F (TCTGGACTGCACTGTTGTCCTTG) and HPR+1370R (GCAAACACCTGTTCCTATTCTG).

Doxorubicin staining and flow cytometry analysis. For doxorubicin visualization, MDA-MB-231 cells were plated in 6-well culture slides and infected with 1000 pfu/cell of Ad-LacZ or Ad-Hpr6^{hbd} for 24 hours. The cells were then incubated with 1 μM doxorubicin for 24 hours, washed three times with phosphate-buffered saline, fixed with formaldehyde, and visualized using a Zeiss fluorescence microscope. For FACS analysis, MDA-MB-231 cells were plated at a density of 500,000 cells/100mm^2 dish, then were infected with 1000 pfu/cell of Ad-LacZ or Ad-Hpr6^{hbd} for 24 hours. The cells were then treated with 1 μM doxorubicin for 6 hours, and the drug was removed and replaced with normal medium. The cells were then harvested, washed once with phosphate-buffered saline, and analyzed by fluorescent activated cell sorting at the University of Kentucky Flow Cytometry Facility.

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Results

Hpr6 expression is induced by doxorubicin. Hpr6 is expressed in a number of breast cancer cell lines (Crudden et al., 2005) and we measured the extent to which Hpr6 expression is altered by chemotherapeutic drugs. Hpr6 expression was induced by 0.5 and 1 μM of the topoisomease II inhibitor doxorubicin (Fig. 1, lanes 1-3) and 0.25 μM of the topoisomerase I inhibitor camptothecin (Fig. 1, lane 4) in MDA-MB-231 breast cancer cells. These findings are consistent with our observations with a cDNA microarray that Hpr6 is transcriptionally induced by doxorubicin in MDA-MB-231 cells (Mallory et al., 2005b).

Inhibiting Hpr6 expression increases chemotherapy susceptibility. In some cases, genes that are induced by a damaging agent regulate susceptibility to that agent. To determine the role of Hpr6 in doxorubicin susceptibility, we inhibited Hpr6 expression using RNAi. MDA-MB-231 cells were transiently transfected with oligonucleotide duplexes consisting of a random sequence (Con) or a sequence targeting Hpr6 (Hpr6i), and Hpr6i inhibited Hpr6 transcript levels by 62% relative to actin (Fig. 2A). The viability of Con- and Hpr6i-transfected cells was analyzed by MTT assay four days following transfection and was not significantly different in the two cell populations (P=0.17).

Hpr6 inhibition caused a significant increase in susceptibility to doxorubicin (Fig. 2B, closed triangles) and camptothecin (Fig. 2B, open triangles) compared to control cells (Fig. 2B, open and closed squares). Measurements were performed in triplicate, and error bars indicate standard deviation (Fig. 2B). The difference between Hpr6i- and control-transfected cells was highly significant at doses of 0.5 and 0.125 μM doxorubicin (P=0.006 and 5×10^{-5}, respectively, by *t*-test) and 0.5 μM camptothecin (P= 0.002). We conclude that Hpr6 regulates susceptibility to two different chemotherapeutic drugs. Hpr6 inhibition also caused a moderate increase in susceptibility to

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mechlorethamine (83% ± 0.3 for the control cells vs. 65% ± 7.5 for Hpr6i-transfected cells), but the result was marginally significant (P=0.05).

Hpr6 binds to heme, and the D120G mutation in the heme-1 domain blocks heme-binding activity. Homologues of Hpr6 bind to heme (Min et al., 2004; Mallory et al., 2005a). We purified a GST fusion protein containing amino acids 43-195 of Hpr6 (Fig. 3A), and found that purified Hpr6 had an absorbance peak at 400 nm that shifted to 420 nm after reduction with sodium hydrosulfite (Fig. 3B), as is characteristic of heme binding proteins. Hpr6 and its homologues share the sequence FYGP-x-GPY-x-x-FAG-x-DASR-x-LA within their heme-1 domain (Mifsud and Bateman, 2002), and an Asp-to-Gly mutation in this sequence inactivates the yeast Dap1p protein (Mallory et al., 2005a). We made the analogous D120G mutation in Hpr6 (Fig. 3D) and found that the 400 nm absorbance peak was absent in the Hpr6-D120G protein (Fig. 3C). As a result, we did not detect a peak at 420 nm in the Hpr6-D120G mutant after hydrosulfite treatment. We then measured the A_{400} in triplicate preparations of Hpr6 and Hpr6-D120G proteins, and the difference in absorbance between the two proteins was statistically significant (P=0.03). By adding heme to purified Hpr6, we estimated the K_D for heme as 0.4 mM, while the K_D for Hpr6-D120G was 1.9 mM, a 5-fold decrease. We conclude that Hpr6 is a heme-binding protein, and that Hpr6-D120G is an inactive form of the protein.

The Ad-Hpr6^{hbd} adenovirus increases chemotherapeutic drug susceptibility. To test the role of heme binding in Hpr6-mediated drug resistance, we prepared an adenovirus (called Ad-Hpr6^{hbd}, where "hbd" stands for heme binding-defective) encoding the Hpr6-D120G protein (diagrammed in Fig. 3C). We infected MDA-MB-231 human breast cancer cells with the Ad-Hpr6^{hbd} adenovirus, resulting in efficient, dose-dependent expression of Hpr6^{hbd} (Fig. 4A, top panel, lanes 1-3) that was absent in cells infected with a control Ad-LacZ adenovirus (Fig. 4A, top panel, lane 4). Hpr6^{hbd} was overexpressed

relative to the endogenous Hpr6 protein when detected with antibodies to the HA epitope tag (Fig. 4B, upper panel) and total Hpr6 (Fig. 4B, middle panel).

Ad-Hpr6^{hbd}-infected cells suffered a significant loss of viability after doxorubicin treatment compared to cells infected with a control virus (Fig. 5A-C). Loss of viability in Ad-Hpr6^{hbd}-expressing cells was significant at doses of 0.5 (P=0.0003), 1 (P=0.0002), and 1.5 μM doxorubicin (P=0.008). In the absence of doxorubicin, the Ad-Hpr6^{hbd} adenovirus had no detectable effect on tumor cell survival or proliferation (Fig. 5A-C, 0 dose of doxorubicin). Because the MTT assay measures mitochondrial activity, it is formally possible that Hpr6^{hbd}-expressing cells are viable but have limited mitochondrial activity. To test this possibility, we measured viability of control and Hpr6^{hbd}-expressing cells, before and after doxorubicin treatment, by trypan blue assays (Fig. 5B). As expected, Ad-Hpr6^{hbd} – expressing cells exhibited decreased viability following doxorubicin treatment (Fig. 5B, 1 μM dose of doxorubicin, P=0.0006). Doxorubicin susceptibility was dependent on the dose of the Ad-Hpr6^{hbd} adenovirus (Fig. 5C; P=0.0002 at 1000 pfu/cell and P=0.0001 at 2000 pfu/cell). We conclude that the heme binding activity of Hpr6 regulates doxorubicin susceptibility in MDA-MB-231 breast cancer cells.

Ad-Hpr6^{hbd} also increased susceptibility to the topoisomerase I and II inhibitors camptothecin and etoposide. MDA-MB-231 cells were infected with Ad-LacZ or Ad-Hpr6^{hbd} and treated with 125-1000 nM camptothecin. Ad-Hpr6^{hbd} significantly increased sensitivity to camptothecin at a dose of 125 nM (Fig. 5D, P=0.005). In contrast, Ad-Hpr6^{hbd} had no effect on the susceptibility of MDA-MB-231 cells to the alkylating agent mechlorethamine (data not shown). We conclude that the effects of Ad-Hpr6^{hbd} are relatively specific and do not include all classes of damaging agents and drugs.

Hpr6^{hbd} does not induce cell death via a classical apoptotic pathway. Doxorubicin frequently induces cell death via the apoptotic pathway. We detected rounded cells after infection with Ad-

Hpr6^{hbd} and treatment with doxorubicin, although the majority of the cells had a wasted, necrotic morphology (Fig. 6A, right panel). These cells were less evident in cells infected with a control adenovirus (Fig. 6A, left panel). In spite of the fact the cells were rounded, we were unable to detect classical markers of apoptosis, including cleavage of PARP (Fig. 6B), the focal adhesion kinase (FAK), endonucleolytic cleavage of chromosomal DNA, or nuclear condensation (data not shown).

Hpr6 does not regulate doxorubicin accumulation. We tested the possibility that Hpr6 regulates drug susceptibility by altering intracellular accumulations of chemotherapeutic drugs. To test this, cells were infected with Ad-LacZ or Ad-Hpr6^{hbd}, treated with doxorubicin, and examined by fluorescence microscopy. We were unable to detect any difference in doxorubicin accumulation in Ad-LacZ vs. Ad-Hpr6^{hbd}-infected cells (Fig. 7A). We then measured doxorubicin concentrations after 24 hours of treatment by fluorescence-activated cell sorting (FACS) and were unable to detect any differences in doxorubicin levels (Fig. 7B). We conclude that Hpr6 does not have a significant effect on the intracellular accumulation of doxorubicin. Hpr6^{hbd} expression also had no effect on the stability of the cytochrome P450 proteins Cyp1A1 and Cyp21 (Fig. 6B, second and third panels).

Discussion

Breast cancer is usually treated with a combination of chemotherapeutic agents that includes doxorubicin or related anthracyclines. We have shown that Hpr6 regulates the susceptibility of breast cancer cells to doxorubicin and other chemotherapeutic drugs. We have also shown that Hpr6 is a heme binding protein and that expression of an Hpr6 mutant that cannot bind heme increases chemotherapy susceptibility in breast cancer cells. These findings suggest that Hpr6 increases cancer cell survival during chemotherapy.

One potential model is that Hpr6 alters doxorubicin susceptibility by regulating the activity of cytochrome P450 proteins. In rats, the Hpr6 homologue IZA localizes to the adrenal inner zones, the

zona fasciculata and zone reticularis (Laird et al., 1988), and a monoclonal antibody to IZA inhibited two reactions catalyzed by cytochrome P450 in the adrenal (Laird et al., 1988). Furthermore, IZA elevated Cyp21/steroid 21-hydroxylase activity in transfected cells (Min et al., 2004). In yeast, the Hpr6 homologue Dap1p regulates sterol synthesis and damage resistance (Hand et al., 2003) by stabilizing the cytochrome P450 protein Cyp51p/Erg11p (Mallory et al., 2005a). However, Hpr6 did not alter the expression of the cytochrome P450 proteins Cyp1A1 or Cyp21 in MDA-MB-231 cells (Fig. 6). Thus, Hpr6-mediated chemotherapy susceptibility does not correlate with a role for Hpr6 in regulating cytochrome P450 proteins. One possible explanation is that the Hpr6-related protein Dg6 (Gerdes et al., 1998) supplements the loss of Hpr6 in maintaining cytochrome P450 protein levels, and that Hpr6 performs a damage function that is distinct from Dg6.

The structural basis through which Hpr6^{hbd} disrupts Hpr6 function is unknown. One likely mechanism for Hpr6^{hbd} is competition with endogenous Hpr6 for binding to substrates and associated proteins. However, there is evidence that Hpr6 homologues function as a covalently bound dimer (Min et al., 2004). Thus, Hpr6^{hbd} could bind directly to wild-type Hpr6, forming an inactive complex. Inactive heterodimer formation is a common mechanism among dominant-negative mutants (Rishi et al., 2004; Zhu et al., 2004), including drug resistance proteins (Kage et al., 2002) and cytochrome proteins (Curry et al., 2004). However, covalently bound Hpr6-Hpr6^{hbd} dimers would have been detected on western blots of infected cells (such as those in Fig. 4), and these bands were not observed.

Because the Hpr6 homologue Dap1p regulates sterol synthesis (Hand et al., 2003; Mallory et al., 2005a), it is possible that Hpr6 also regulates the synthesis of sterols. Indeed, Hpr6 binds to the sterol regulatory protein Insig-1 (Yang et al., 2002; Suchanek et al., 2005), which in turn regulates the SREBP (sterol regulatory element binding protein) transcription factor via binding to SCAP (SREBP cleavage activating protein; Yang et al., 2002; Anderson, 2003). If Hpr6 inhibition decreased sterol synthesis, this could alter the turnover of trans-membrane receptors, a process that is dependent on sterol-rich lipid rafts (Le Roy and Wrana, 2005). Several receptors have been implicated in

doxorubicin susceptibility in breast cancer, including the hepatocyte growth factor receptor (Fan et al., 1998; Gao et al., 2001), although scatter factor failed to protect MDA-MB-231 cells from doxorubicin-mediated cell death (Fan et al., 1998). However, the relationship between Hpr6, sterol synthesis, and damage signaling is only speculative at the present.

In conclusion, our results are consistent with a model in which Hpr6 functions in protecting tumor cells from chemotherapeutic agents. In normal tissues, Hpr6 is highly expressed in liver and kidney (Gerdes et al., 1998; Hand and Craven, 2003). When overexpressed in breast tumors, Hpr6 could be corrupted to protect tumor cells from xenobiotic compounds, providing resistance to chemotherapeutic drugs. Our results indicate that inhibiting Hpr6 increases the ability of chemotherapeutic drugs to kill cancer cells, suggesting that Hpr6 is a target for intervention in cancer therapy.

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Footnote

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Figure legends

Fig. 1. Hpr6 is induced by doxorubicin. MDA-MB-231 cells were untreated (lane 1), treated with 0.5 or 1 μM doxorubicin (lanes 2 and 3), or 0.25 μM camptothecin (lane 4) for 3 days, and Hpr6 expression was analyzed by western blot. The fold increase in Hpr6 expression (below the upper panel) is derived from the Hpr6: tubulin ratio from each sample. Tubulin is included as a control for equal protein loading (lower panel). The abbreviations are as follows: Hpr6, heme-1 domain protein; dox, doxorubicin; and cmp, camptothecin.

Fig. 2. RNAi targeting Hpr6 inhibits Hpr6 expression and increases chemotherapy susceptibility. (A) The expression of Hpr6 was measured by RT-PCR in cells treated with a control RNAi duplex (Con, lane 2), or an Hpr6-specific duplex (Hpr6i, lane 3). Primers for actin were included in the same reaction as an internal standard for cDNA loading. Lane 1 is a control in which template was omitted. (B) Viability assays for control- (squares) and Hpr6i-transfected cells (triangles) treated with increasing doses of doxorubicin (closed shapes) or camptothecin (open shapes). Cells were transfected, plated in 96 well culture dishes, treated with drugs, and viability was measured by MTT assay. Each point was analyzed in triplicate. *P* values are indicated by one (<0.05), two (<0.01), or three (<0.001) asterisks, respectively.

Fig. 3. Hpr6 binds to heme, and Hpr6-D120G lacks heme binding. (A) Purification of GST-Hpr6 (lane 1) and GST-Hpr6-D120G (lane 2) fusion proteins. Proteins were purified and analyzed by SDS-PAGE electrophoresis. (B) Absorbance scans of Hpr6 before and after reduction with sodium hydrosulfite. Hpr6 exhibited a peak at 400 nm (solid line) that shifted to 420 nm after reduction (dashed line). (C) Absorbance scans of Hpr6 (top line) and Hpr6-D120G (bottom line) from 360-580 nm. The scan shows a peak at 400 nm for Hpr6 that is characteristic of heme-binding proteins. The 400 nm peak was absent in the Hpr6-D120G scan. (D) Diagram of the domain structure of Hpr6 (top), the region of Hpr6 purified as a GST-fusion protein (second from top), the site of the D120G mutation within the

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GST-Hpr6 fusion protein (second from bottom), and the site of the D120G mutation within the Hpr6^{hbd} construct (bottom).

Fig. 4. The Ad-Hpr6^{hbd} adenovirus directs efficient expression of Hpr6^{hbd}. (A) Western blot analysis of MDA-MB-231 cells infected with 500, 1000, or 2000 pfu/cell of Ad-Hpr6^{hbd} (lanes 1-3) or 2000 pfu/cell of Ad-LacZ (lane 4). Hpr6 was detected with an antibody to the HA epitope tag, and tubulin was included as a control for protein loading (bottom panel). (B) The Hpr6^{hbd} protein is overexpressed compared to endogenous Hpr6 following Ad-Hpr6^{hbd} infection. MDA-MB-231 cells were infected with Ad-LacZ (lane 1) or Ad-Hpr6^{hbd} (lane 2) and analyzed by western blots probed for HA (top panel), Hpr6 (middle panel), or tubulin (bottom panel). The positions of the exogenous Hpr6^{hbd} and endogenous Hpr6 protein are indicated in the center panel (top and bottom, respectively).

Fig. 5. Ad-Hpr6^{hbd} acts synergistically with doxorubicin and camptothecin to induce cell death. (A) graph showing viability of MDA-MB-231 cells infected either Ad-LacZ (squares) or Ad-Hpr6^{hbd} (triangles), combined with increasing concentrations of doxorubicin (X axis). Viability was measured by MTT assay. (B) Trypan blue assay showing the viability of MDA-MB-231 cells infected either Ad-LacZ (squares) or Ad-Hpr6^{hbd} (triangles), combined with 0-4 μM doxorubicin. All measurements were performed in triplicate. (C) Graph showing viability of MDA-MB-231 cells treated with 1 μM doxorubicin combined with increasing doses of either Ad-LacZ (squares) or Ad-Hpr6^{hbd} (triangles). Viability was measured by MTT assay. (D) Graph showing viability of MDA-MB-231 cells infected either Ad-LacZ (squares) or Ad-Hpr6^{hbd} (triangles), combined with increasing concentrations of camptothecin (X axis). For all of the assays, error bars represent the standard deviations of triplicate measurements of a single experiment, and the results shown are representative of at least three independent experiments. *P* values are indicated by one (<0.05), two (<0.01), or three (<0.001) asterisks, respectively.

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Fig. 6. Hpr6^{hbd} induces cell death but does not induce apoptosis. (A) Morphology of MDA-MB-231 cells infected with Ad-LacZ (left panel) or Ad-Hpr6^{hbd} (right panel) and treated with 1 μM doxorubicin for 72 hours. Ad-Hpr6^{hbd}-infected, doxorubicin-treated cells generally had a rounded or necrotic morphology. (B) Hpr6^{hbd} does not alter the levels of apoptosis or two cytochrome P450 proteins in doxorubicin-treated cells. MDA-MB-231 cells were infected with Ad-LacZ (lanes 1 and 3) or Ad-Hpr6^{hbd} (lanes 2 and 4) and left untreated (lanes 1 and 2) or were treated with 1 μM doxorubicin (lanes 3 and 4). The cells were then lysed and analyzed for the migration of poly-ADP ribose polymerase (PARP), upper panel, Cyp1A1, second panel, or Cyp21, third panel. Ad-Hpr6^{hbd} did not increase levels of p85^{PARP}, a marker for apoptosis, either before or after treatment with doxorubicin, and Hpr6^{hbd} did not alter the levels of Cyp1A1 or Cyp21.

Fig. 7. Hpr6^{hbd} does not alter the accumulation of doxorubicin. (A) MDA-MB-231 cells were infected with either Ad-LacZ (left panels) or Ad-Hpr6^{hbd} (right panels) and treated with 1 μM doxorubicin for 24 hours. Cells were then fixed and visualized using bright-field microscopy (top panels) or fluorescence (bottom panels). In both groups of infected cells, doxorubicin accumulated in the nucleus to similar extents. (B) MDA-MB-231 cells were infected with either Ad-LacZ (left panels) or Ad-Hpr6^{hbd} (right panels) and left untreated (top panels) or were treated with 1 μM doxorubicin for 24 hours (bottom panels). Cells were then analyzed by fluorescence activated cell sorting.

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Figure 1

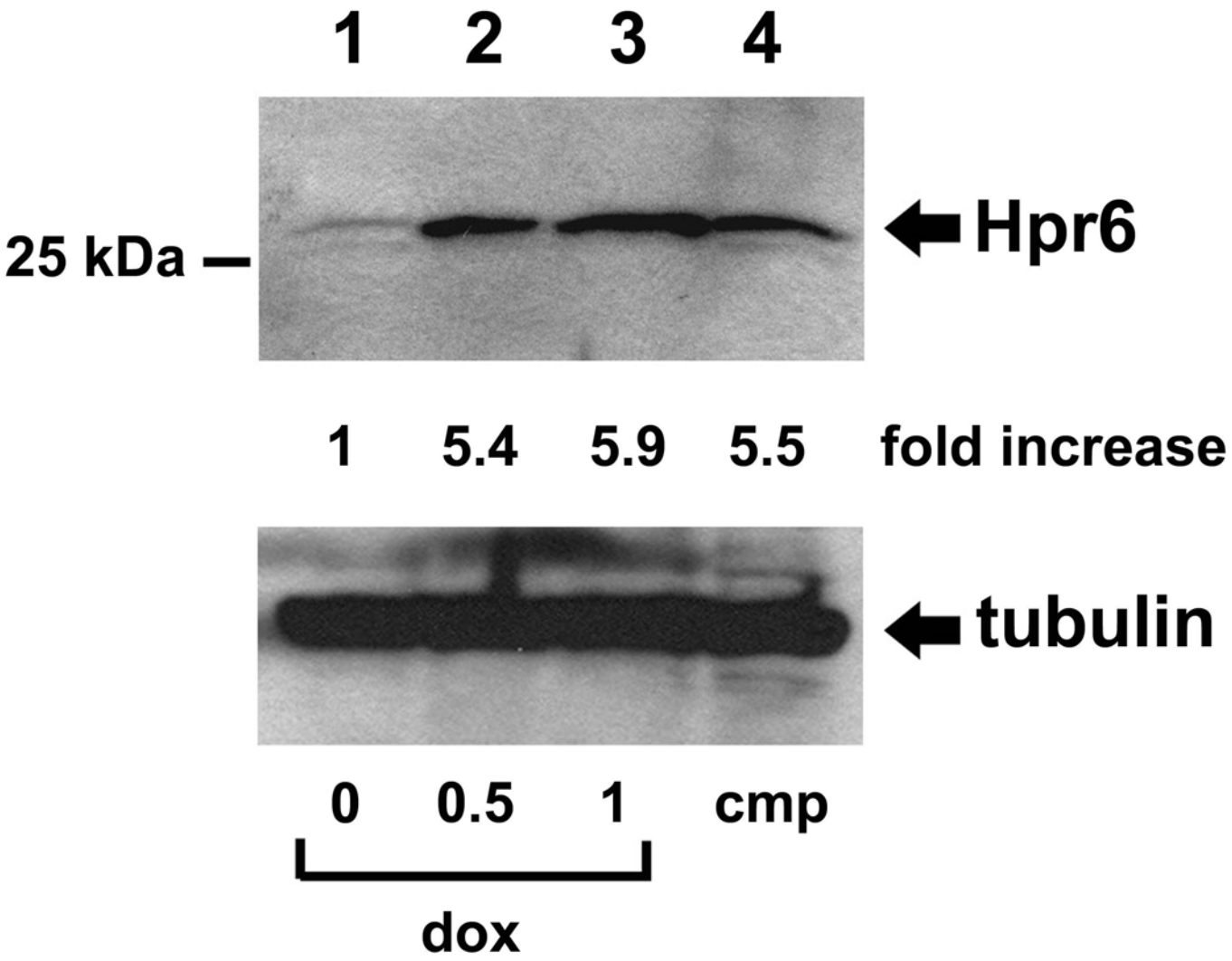

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Figure 2

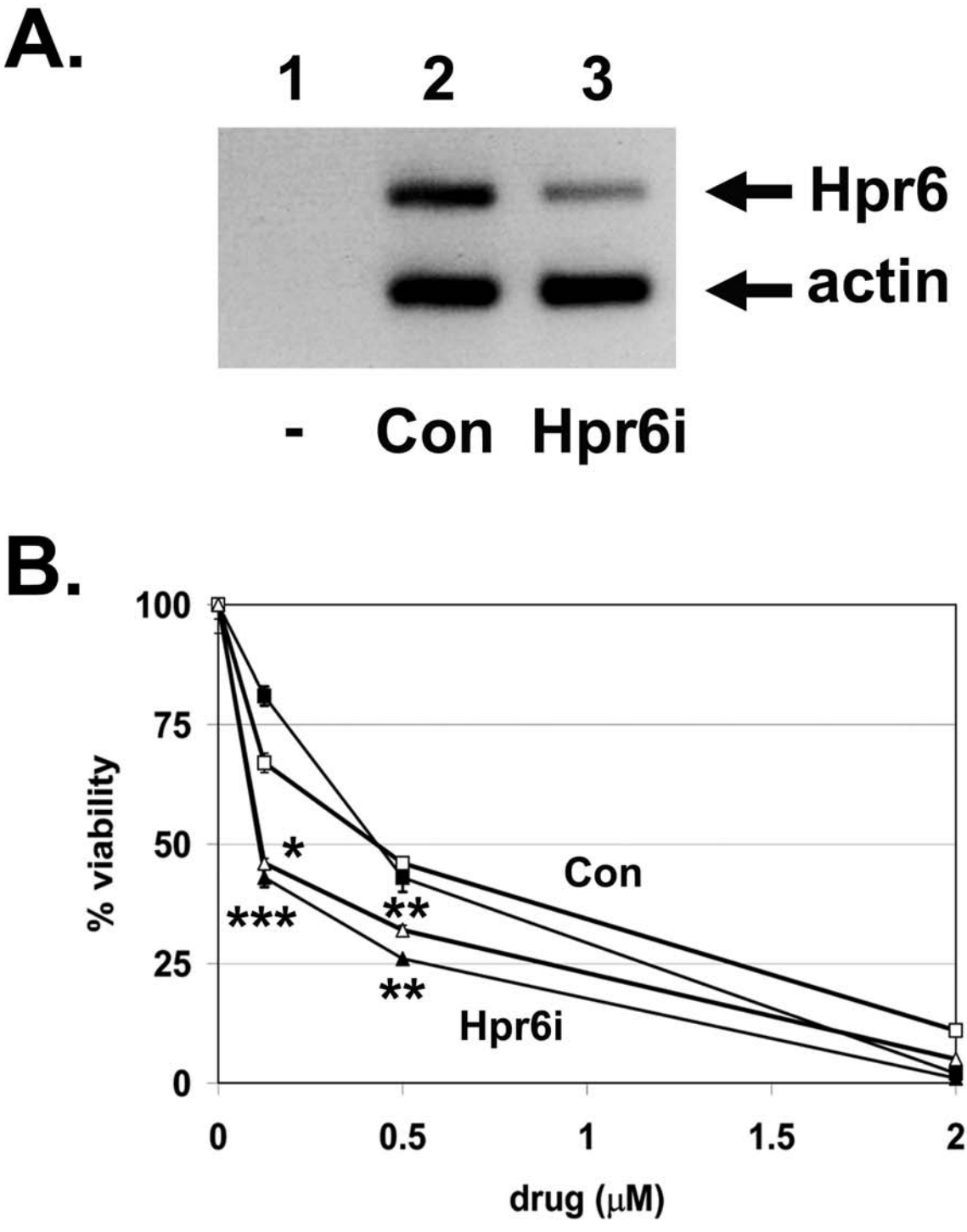

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Figure 3

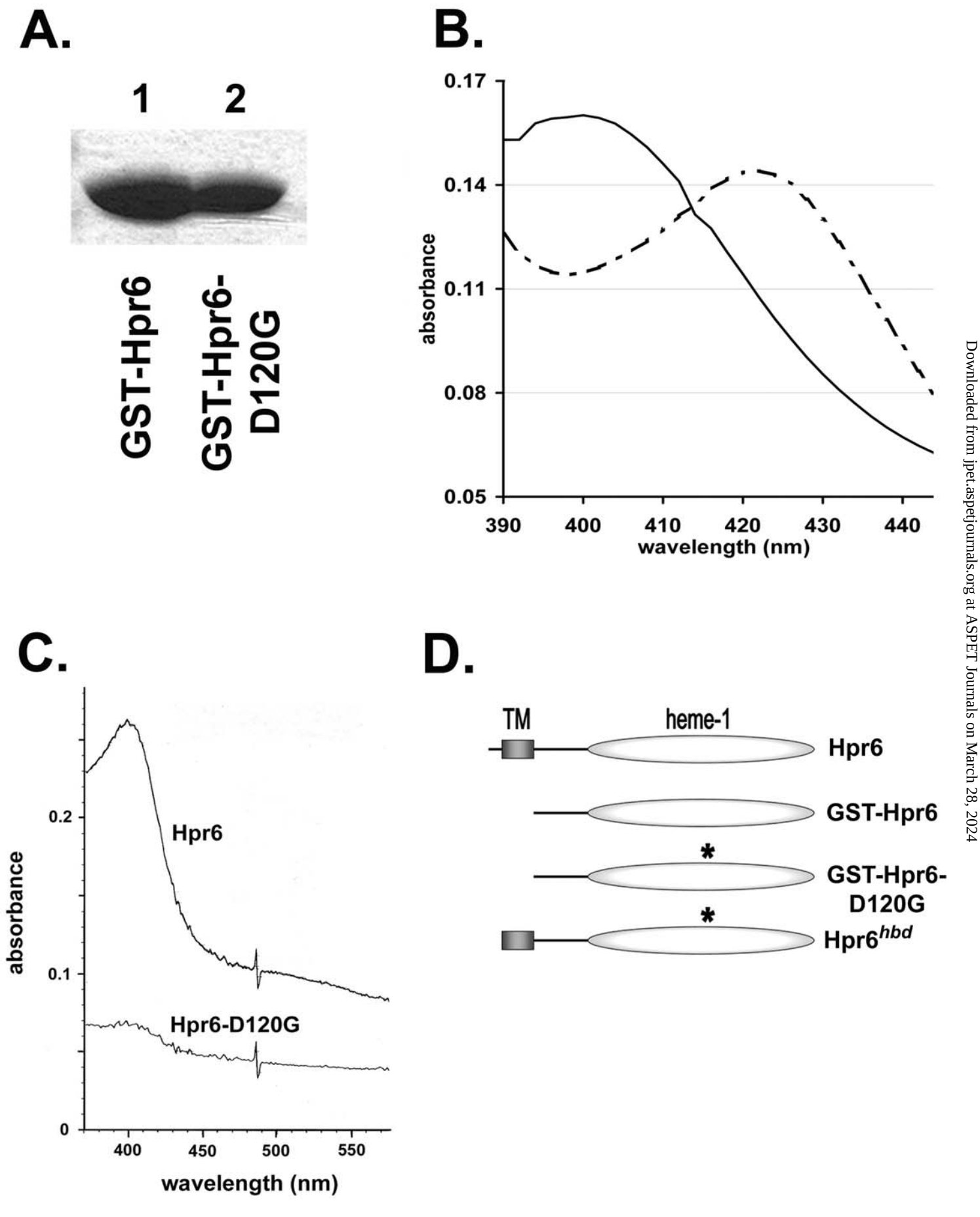

Figure 4

Figure 5

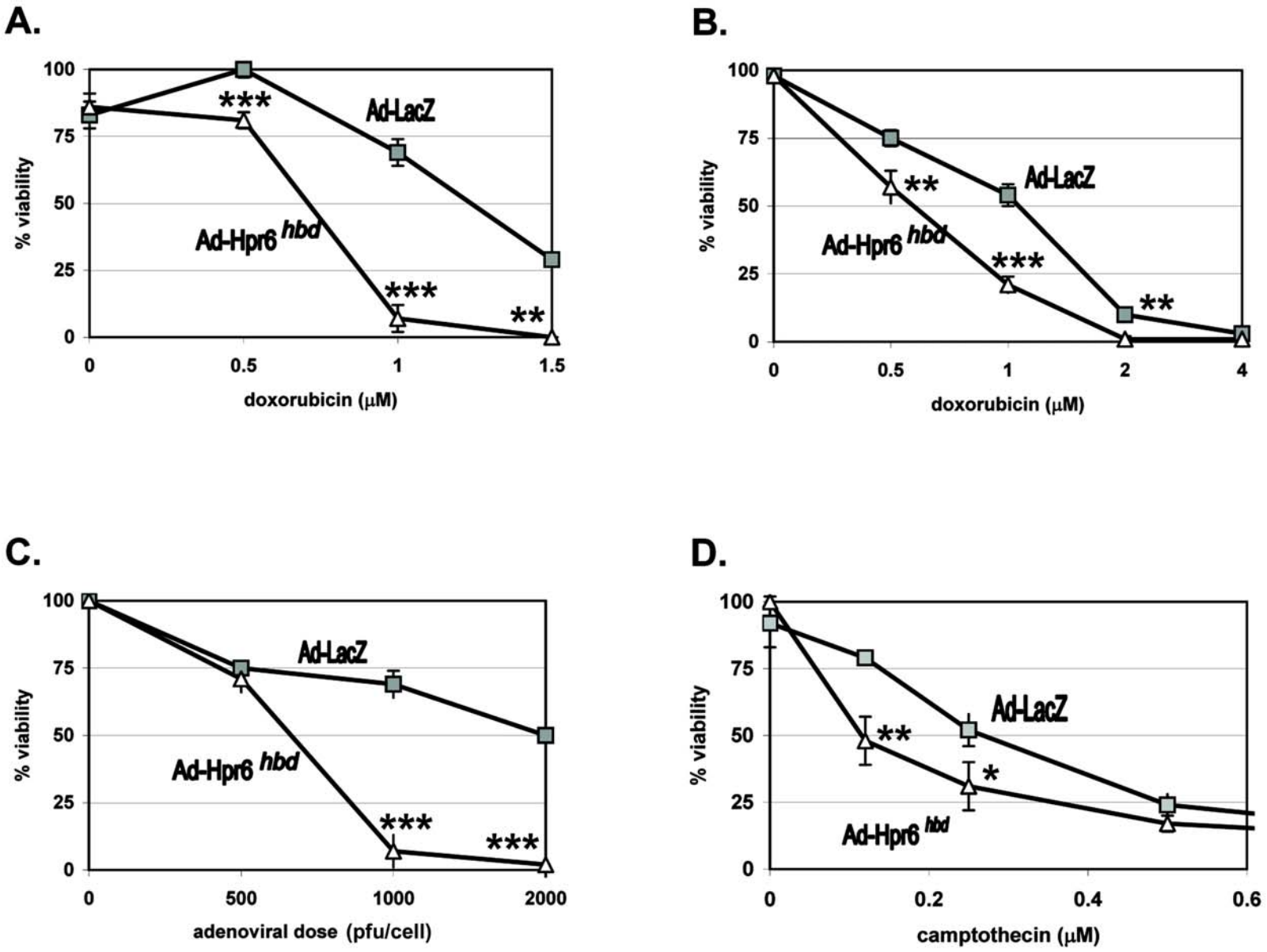

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Figure 6

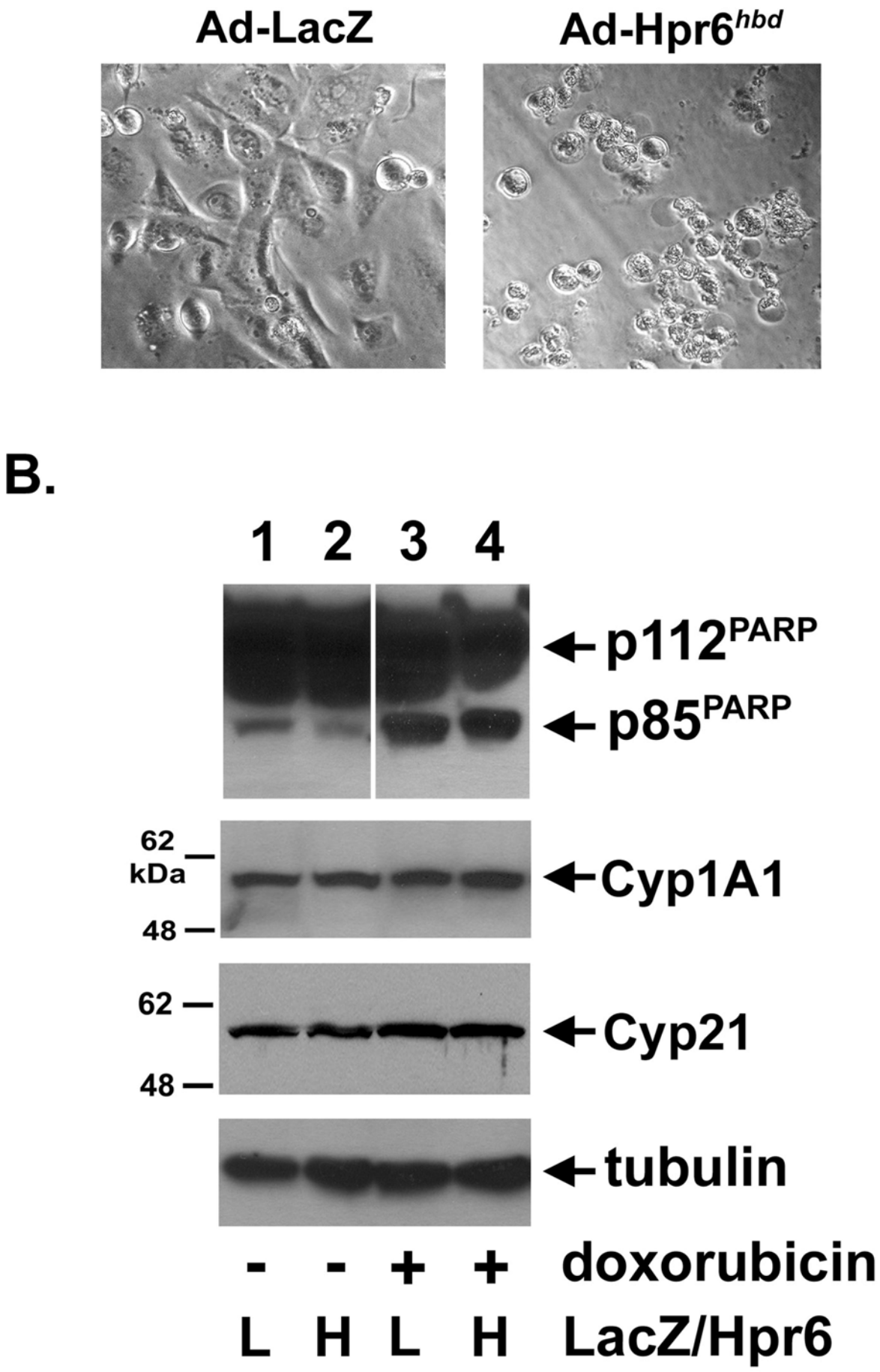

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Figure 7