

# Targeting Cdc37 Inhibits Multiple Signaling Pathways and Induces Growth Arrest in Prostate Cancer Cells

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## Abstract

**Members of the 90-kDa heat shock protein (HSP90) family are known to bind and stabilize intermediates in a wide variety of cell signaling pathways and contribute to their dysregulation in cancer. An important intracellular cofactor for HSP90 is Cdc37, a protein with a broad role in fostering the activities of protein kinases. By targeting Cdc37 using RNA interference, we have shown that the loss of Cdc37 function induces irreversible growth arrest in androgen receptor-positive and -negative prostate carcinoma cells. In contrast to HSP90-directed agents, Cdc37 targeting seems to affect cancer cells through a distinct mechanism and does not significantly deplete the intracellular levels of most known HSP90 client proteins. Instead, Cdc37 depletion inhibits cellular kinase activity and flux through growth-promoting signal transduction cascades. We show that the loss of Cdc37 leads to reduced activity of the Erk, Akt, mTOR, and androgen-induced pathways. We have also discovered synergistic interactions between Cdc37 inactivation and the HSP90-inhibitory anti-cancer drug 17-(allylamino)-17-demethoxygeldanamycin (17AAG). These interactions involve enhanced degradation of proteins essential for growth and inhibition of 17AAG-induced expression of the antiapoptotic HSP70. Thus, Cdc37 is essential for maintaining prostate tumor cell growth and may represent a novel target in the search for multitargeted therapies based on the HSP90 chaperone system. [Cancer Res 2007;67(24):11942–50]**

## Introduction

The heat shock proteins (HSP) are the products of a diverse family of genes whose main function is to fold polypeptides into functional configurations (1). These proteins, characterized by such molecular chaperone activity, are highly conserved throughout evolution (2). Although many HSPs are induced primarily during cell stress, a number are ubiquitously expressed in all cell types. Among these is HSP90, which represents 1% to 2% of total cellular protein and is found in complexes with numerous protein cofactors or co-chaperones (3).

One important HSP90 co-chaperone whose functions are just now being described is Cdc37. First identified in yeast as a cell cycle protein, recent studies indicate a role for Cdc37 in targeting

protein kinases to the HSP90 complex (4). Thus, in normal tissues, Cdc37 interacts with newly translated protein kinases, promoting their maturation and recruitment to HSP90 complexes. However, when Cdc37 is overexpressed, novel or cryptic properties emerge, and the protein can perform a number of additional functions both in concert with and independently of HSP90 (5). Analysis of clinical samples has shown abundant expression of Cdc37 in prostate carcinoma compared with relatively low levels in normal prostatic epithelium (6). Indeed, Cdc37 overexpression is sufficient to induce prostatic dysplasia in mice (6). This attribute is unique among the HSP90 co-chaperones and may be related to the fact that Cdc37 is the only such molecule to play an essential role in cell cycle regulation (7). At the molecular level, Cdc37 binds to a conserved motif located within the catalytic domains of a large number of protein kinases including molecules whose functions are commonly dysregulated in cancer, such as the Raf-1, Akt, Aurora B, epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor kinases (8–12). Although the repertoire of Cdc37 clients is largely restricted to protein kinases, one exception exists, which is of considerable relevance to prostate cancer: its activating function in androgen receptor signaling (13). Of further consequence for the biology of malignant cells, many Cdc37 binding partners are key signaling pathway intermediates that become activated in cancer by changes such as mutation, overexpression, and overactivation; although these changes render such proteins prone to aggregation, the elevated levels of Cdc37 in cancer cells may serve to chaperone the unstable states of these clients and maintain them in an active form that permits a role in mediating the autonomous growth characteristic of many cancers (14).

The current strategy of targeting the HSP90 chaperone system for cancer treatment is attractive in permitting simultaneous inhibition of multiple oncoproteins without the accumulating toxicity of using multiple therapies. Numerous HSP90 inhibitors have been developed, including the ansamycins, that specifically inhibit the intrinsic ATPase activity of HSP90 (15). Effective ansamycin derivatives have been isolated, including 17-(allylamino)-17-demethoxygeldanamycin (17AAG) that has recently completed several phase I clinical trials (16–23) and is now undergoing evaluation in phase II. Initial results are encouraging, although problems were noted with formulation and hepatotoxicity. Although the strengths of HSP90 inhibitors in cancer therapy include their versatility in inhibiting a wide range of oncogenic pathways, these qualities may also contribute to some complications of treatment because HSP90 is essential for a range of indispensable functions in normal tissues. Targeting Cdc37 represents a potential alternative to direct HSP90 inhibition that may offer greater specificity (due to its elevated expression in cancer) and an improved side effect profile. Additionally, Cdc37 may be an attractive target in tumor types such as androgen-independent prostate cancer that currently lack highly effective therapies.

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

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In this study, we have examined the role of Cdc37 in the growth of human prostate cancer cells. We show that depletion of Cdc37 using RNA interference leads to irreversible growth arrest in a range of AR<sup>-</sup> and AR<sup>+</sup> prostate cancer cells and sensitizes them to treatment with HSP90 inhibitors. Growth inhibition after Cdc37 depletion seems to involve the inhibition of growth-dependent protein kinase activity. In AR<sup>+</sup> cells, however, Cdc37 depletion also leads to the loss of AR transcriptional activity, and Cdc37 targeting may exert a proportion of its effects through ablation of androgen-dependent gene expression. Our studies further indicate that Cdc37 plays a qualitatively different role in prostate cancer growth compared with that of HSP90, fostering kinase activity rather than protecting client proteins from proteolysis. When we combined the targeting of Cdc37 and HSP90, we observed strong additive cytotoxicity, evidence for their contrasting mechanisms in cell regulation and suggestive of potential joint uses in cancer treatment.

## Materials and Methods

**Cell culture.** LNCaP, Du145, and PC3 cells were obtained from the American Type Culture Collection. LNCaP-HP was developed in-house by repeated passage of LNCaP cells until they developed the ability to grow in low androgen conditions. Du145 and PC3 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin cocktail. LNCaP and LNCaP-HP were grown similarly but in RPMI 1640 (Invitrogen). Cells were grown in a humidified incubator maintained at 37°C with 5% CO<sub>2</sub>. Each of these malignant prostate carcinoma cell lines showed increased levels of Cdc37 expression compared with normal prostatic epithelium (Supplemental Fig. S1).

**Virus production and transduction.** Cdc37 small hairpin RNA (shRNA) and control plasmids were purchased from Open Biosystems. All pLKO.1 plasmids were developed by the RNAi consortium. Packaging and envelope plasmids were obtained from Addgene. To produce virus, pLKO.1 plasmids, the packaging plasmid psPAX2, and the envelope plasmid pCMV-VSVg were simultaneously transfected into 293FT cells (Invitrogen). Eighteen hours after transfection, cells were re-fed DMEM + 30% fetal bovine serum. Virus containing media was subsequently removed in two aliquots at 24 and 48 h and frozen at -80°C.

Virus transduction was completed by adding the appropriate amount of virus-containing media and 8 µg/mL hexadimethrine bromide to growth media containing nonattached cells. Upon completion of transduction and attachment 24 h later, virus-containing media was removed and replaced with fresh media containing 2.5 µg/mL puromycin. Transduced cells were selected for at least 2 days before use. Transduction efficiencies typically exceeded 95% in most cell types. The following TRC designated plasmids were tested: TRCN0000116632 (5'-CCGGGCCAATTCAAGTCTCTGCTTCTCGAGAAAGCAGAGACTTGAATGGGCTTTTGG-3'), TRCN0000116633 (5'-CCGGCCGCGAGTTCTTCACTAAGATTCTCGAGAATCTTAGTGAA-GAATGCCGTTTGG-3'), TRCN0000116634 (5'-CCGGCCGCGCAGTTCTTCACTAAGATCTCGAGATCTTAGTGAAGAAGTCCGCGTTTGG-3'), TRCN0000116635 (5'-CCGGCCAGACAATCGTCATGCAATTCTCGAGAATTGCATGACGATTGTCTGGTTTGG-3'), and TRCN0000116636 (5'-CCGGGACAGCAATTACCTGGTCATCTCGAGATGACCAGGTAATTGGCTGTCTTTTGG-3'). It was determined that TRCN0000116633 did consistently better than the other clones in repeated experiments in all cells tested and is heretofore referred to simply as "Cdc37 shRNA".

**Antibodies and reagents.** I7AAG was obtained from Assay Designs. Puromycin and RNase A were obtained from Sigma Aldrich. Normal prostate lysate was obtained from ProSci. The following antibodies were purchased from Cell Signaling Technologies: Akt, phospho-Akt (Ser<sup>473</sup>), Aurora B, phospho-Gsk3β (Ser<sup>9</sup>), Gsk3β, phospho-p90RSK (Ser<sup>380</sup>), p90RSK, phospho-p42/44 mitogen-activated protein kinase (MAPK; Thr<sup>202</sup>/Tyr<sup>204</sup>), p42/44 MAPK, phospho-S6 ribosomal protein (Ser<sup>235/236</sup>), and S6 ribosomal protein. Antibodies to Raf-1 and β-actin were purchased from Sigma Aldrich. Anti-Cdc37 was purchased from Abcam. Horseradish

peroxidase (HRP)-conjugated secondary antibodies were purchased from Invitrogen. Pan-HSP70 antibody was created in-house as previously described (24).

**Cell counting and colony formation assays.** For cell counting assays, 200,000 cells were plated in 10-cm dishes in triplicate and grown as described for the indicated time periods. Cells were trypsinized and counted thrice per data point on a hemocytometer excluding dead cells with trypan blue (total of nine counts per time point). For colony assays, an appropriate amount of cells were plated in six-well plates (each data point in triplicate) and grown as described for 9 to 14 days until visible colonies were seen. Cells were then fixed in cold methanol, stained with 1% crystal violet, and washed in dH<sub>2</sub>O. Colonies containing >50 cells were then scored by a blinded observer.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and data analysis.** Four thousand to 6,000 Cdc37-knockdown and control cells were plated in triplicate into 96-well plates containing serial dilutions of the drug. Cells were incubated for 4 days, after which Cell-Titer 96 Aqueous One solution (Promega) was added per manufacturer's instructions. Absorbance was read on a plate reader, background was subtracted, and survival was set relative to wells containing no drug. Sigmoid curves were fitted to collected data using SigmaPlot X (Systat). Statistical analysis was also conducted using SigmaPlot.

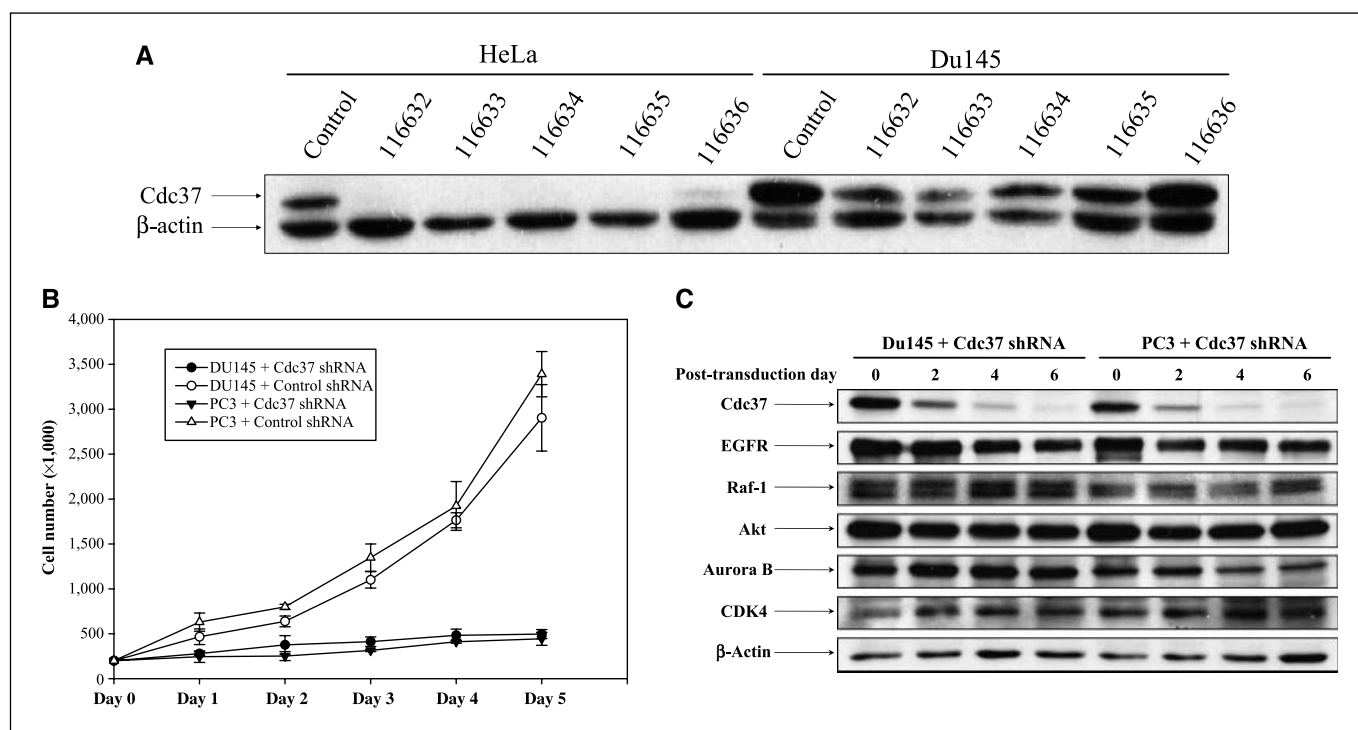
**Cell transfection and reporter assays.** All cells were transfected using Fugene-HD reagent (Roche) per manufacturer's instructions. The HSP70B-luc reporter has been previously described (25). The ARE-luc reporter has also been previously described (26). HSP70B-luc/ARE-luc and pCMV-β-Gal were co-transfected into cells 24 h before treatment. Upon assay completion, cells were harvested and lysed in 1× reporter lysis buffer (Promega) and then assayed with Promega's luciferase or β-Gal assay kits per manufacturer's instructions.

**Real-time reverse transcription-PCR.** Total RNA from cultured cell lines was isolated from cells using the RNeasy purification kit (Qiagen). Normal prostate RNA was purchased from Clontech. Reverse transcription was done on 1 µg of RNA using iScript (Bio-Rad). Primers used for Cdc37 were forward: 5'-GGGAGCAGAAAGACAAGACC-3', reverse: 5'-GTGGACGT-TGTCTGACAGGT-3' and, for β-actin, were forward: 5'-AAAAGCCACCC-CACCTTCTCT-3', reverse: 5'-CTCAAGTTGGGGGACAAAAA-3'. Real-time PCR was done in triplicate using an ABI 7300 (Applied Biosystems) and iQ SYBR green master mix (Bio-Rad). Data was directly analyzed with ABI provided software using the ddCT method. Relative Cdc37 levels were normalized to β-actin levels.

**Western blot analysis.** Protein from harvested cells was lysed in 1× radioimmunoprecipitation assay buffer (Boston Bioproducts) supplemented with 1× protease cocktail (Roche). Protein sample concentration was determined using the detergent-compatible protein assay (Bio-Rad). Twenty to 60 µg of protein was prepared in 1× SDS-PAGE sample buffer and run on a 4% to 12% polyacrylamide gel (Boston Bioproducts). Gels were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) using a Bio-Rad wet transfer apparatus. Membranes were blocked in 5% nonfat milk or bovine serum albumin for 1 h and then probed with various antibodies according to the manufacturer's instructions. HRP-conjugated secondary antibodies were visualized using enhanced chemiluminescence (ECL) reagents (Boston Bioproducts) and X-ray film. Quantitation was done using ImageJ software (27) and graphed using Microsoft Excel 2007.

## Results

**Cdc37 is overexpressed in prostate cancer cell lines and is necessary for proliferation.** We aimed to examine the role of Cdc37 in growth and survival of prostate carcinoma cells using RNA interference to deplete intracellular concentrations. Figure 1A shows the effectiveness of a range of shRNAs in reducing Cdc37 in cells expressing either low (HeLa) or high (Du145) Cdc37 concentrations. A number of these constructs proved effective, and we selected one (116633, see Materials and Methods) that was packaged into lentiviral vectors and used to infect a number of



**Figure 1.** Cdc37 is required for the growth of prostate carcinoma cell lines, but its loss does not affect the stability of clients. *A*, effects of lentivirus bearing various Cdc37-targeted shRNA constructs or control shRNA on Cdc37 expression in HeLa and Du145 cells. Cells were selected with puromycin for 3 d before harvesting and assay of Cdc37 levels by immunoblot. Each Cdc37 shRNA-bearing virus reduced Cdc37 levels compared with control shRNA viruses with clone number 116633 showing the greatest depletion of Cdc37 in repeated experiments. *B*, effects of Cdc37 depletion by RNA interference on prostate cancer cell growth. Du145 (circles) and PC3 cells (triangles) were transduced with Cdc37 shRNA virus 116633 (closed shapes) or control virus (open shapes) and selected with puromycin for 7 d. Two hundred thousand cells were then plated in 10-cm dishes in triplicate (day 0), and a fraction were harvested and counted by trypan dye exclusion at each indicated time point. Points, means; bars, SD. *C*, Western blot analysis of protein levels in cells transduced with Cdc37 shRNA virus (day 0) and selected with puromycin (beginning day 1) for the indicated times. At each time point, relative levels of Cdc37, EGFR, Raf-1, Akt, Aurora B, CDK4, and  $\beta$ -actin were assayed by immunoblot. All experiments in this study were done at least twice with reproducible findings.

prostate carcinoma cell lines. Infection with shRNA-containing lentivirus inhibited the growth of PC3 and Du145 cells, whereas a control virus containing a scrambled sequence was ineffective (Fig. 1B). Cdc37 levels in the shRNA-treated cells are subsequently indicated in Fig. 1C (top) and show a progressive decline to essentially undetectable levels by day 6. Total growth inhibition was also observed in the androgen-dependent LNCaP cell line after Cdc37 depletion. Infection of LNCaP cells with shRNA targeting Cdc37 virus led to a complete loss of ability to form colonies in our colony growth assays (data not shown).

**Cdc37 knockdown inhibits multiple signaling pathways.** We next investigated molecular changes ensuing from Cdc37 depletion that could potentially mediate the growth inhibition shown in Fig. 1B. One of the mechanisms by which HSP90 inhibitors reduce cancer cell growth is by triggering degradation of HSP90 client proteins, many of which are arrayed along key growth-promoting and antiapoptotic pathways (28). We therefore examined levels of some HSP90/Cdc37 client proteins after Cdc37 depletion. However, in contrast to the known effects of HSP90 inhibition, Cdc37 depletion did not markedly reduce the levels of the majority of these proteins as judged by the immunoblot assay (Fig. 1C). The levels of the protein kinases EGFR, Raf-1, Akt, Aurora B, and cell division kinase 4 (CDK4), which are almost completely depleted by growth-inhibitory concentrations of HSP90 drugs, were not markedly affected over the 6-day course of the experiment. (Exceptions were a small reduction in Aurora B levels seen in PC3 cells and a marginal reduction in EGFR levels in both lines).

It is quite striking that after 6 days of shRNA expression, when Cdc37 is essentially undetectable, these HSP90 client proteins are still present (Fig. 1C). Similar experiments with control virus showed no change in Cdc37 levels or client levels (data not shown).

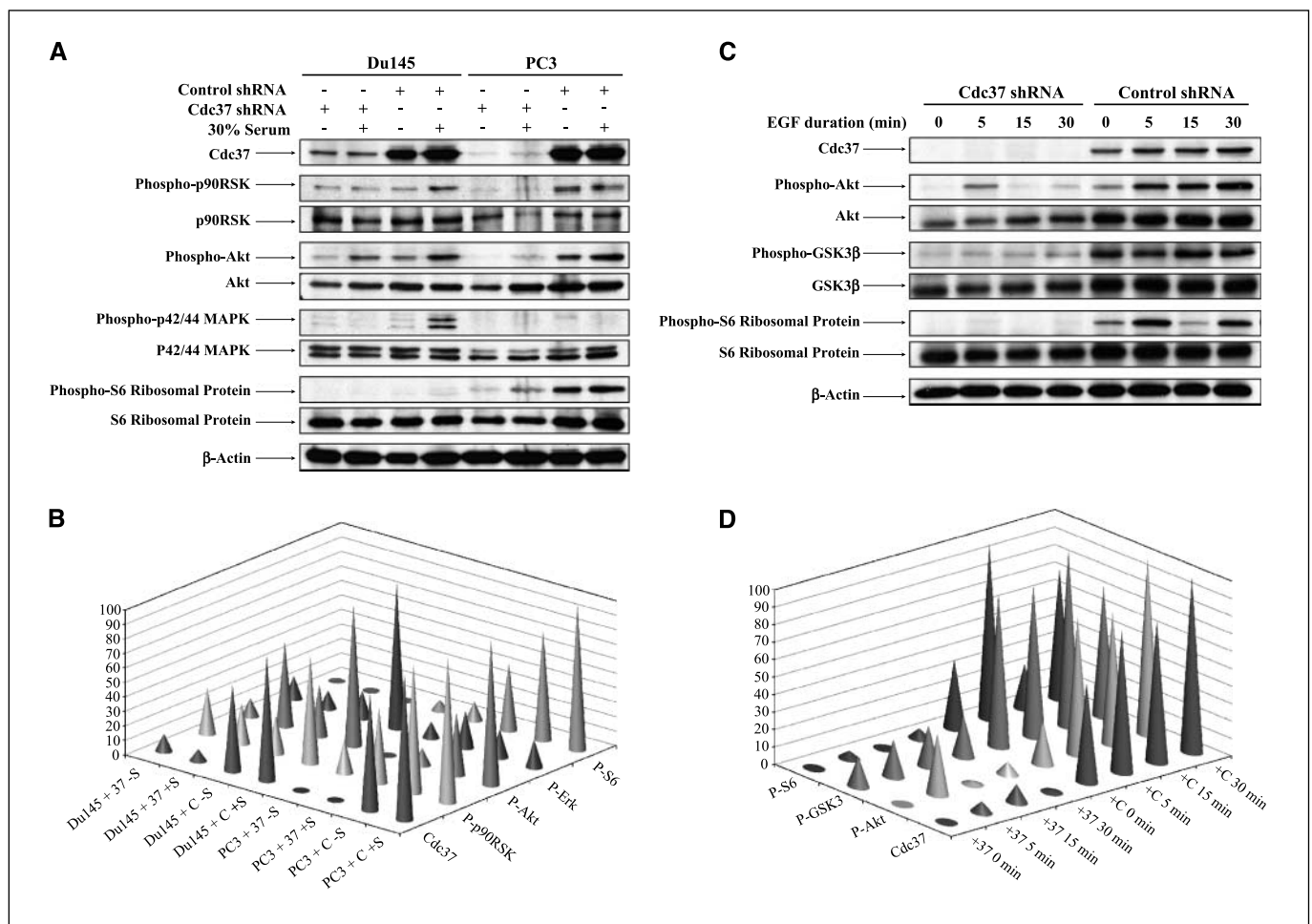
Because Cdc37 depletion only marginally affects the concentrations of its client proteins, we next asked whether cell growth inhibition might result from inhibition of the enzymatic activities of these protein kinases rather than their destabilization and destruction. We therefore examined whether Cdc37 targeting inhibits flux through mitogen-induced protein kinase cascades that regulate prostate cancer growth. To examine multiple intermediates in network signaling, we used antibodies that detect phosphorylation at regulatory sites on such kinases rather than enzymatic activity itself. Such phosphorylation is, in most cases, directly correlated with activity. We examined protein kinases and their substrates that are intermediates in two main pathways of mitogenic signaling, including the Erk/MAPK pathway (Erk itself, p90-RSK, S6) and the Akt pathway (Akt itself and S6). Cdc37 knockdown and control cells (Du145 and PC3) were serum starved overnight and then briefly incubated in medium containing either 30% serum (mitogenic stimulation) or serum-free controls for 10 min before being harvested, lysed, and assayed by Western blot for multiple phospho-proteins (Fig. 2A and B). In Du145 cells, with a characteristically active Erk/MAPK pathway, Cdc37 depletion inhibited serum-induced phosphorylation of p42/44 MAPK as well as the downstream effector kinase p90RSK while not markedly affecting the levels of P42/44MAPK or RSK. In PTEN null PC3 cells,

in which enhanced Akt activity plays a major role in growth, Cdc37 depletion eliminated basal phosphorylation of Akt as well as serum-induced phosphorylation. These effects were also apparently fed down through the mTOR pathway as indicated by reduced basal and serum-stimulated phosphorylation of the S6 ribosomal protein. There also seems to be constitutive phosphorylation of p90RSK in PC3 cells, and these levels were also reduced by Cdc37 inhibition. Thus, although Cdc37 targeting does not markedly alter the levels of HSP90 clients, it does inhibit flux through key kinase cascades presumably through the loss of the chaperoning power of Cdc37 and prevention of complete kinase maturation. Of interesting note is the fact that the addition of growth factors seems to moderately increase the detectable levels of Cdc37 in the control cells, although the exact mechanism behind this is not entirely clear and is currently under investigation.

To further investigate the role of Cdc37 in intracellular signal transduction, we examined the phosphorylation state of inter-

mediates along the Akt pathway after mitogenic stimulation with epidermal growth factor (EGF). PC3 cells were serum starved overnight and then incubated with 25 ng/mL recombinant EGF (Fig. 2C and D). Control cells exposed to EGF showed rapid flux through the Akt pathway, whereas cells depleted of Cdc37 exhibited a blunted response to EGF, with only a brief spike of Akt phosphorylation, which was quickly dissipated. These effects of Cdc37 inactivation seemed to be amplified downstream in the kinase cascade as evidenced by drastically reduced levels of phospho-GSK3, a direct target of Akt, and phospho-S6, a downstream target of the mTOR pathway. It should also be noted that in both these experiments, serum deprivation seemed to induce minor but apparent decreases in several kinases in Cdc37-depleted cells not observed in cells growing in full medium.

Our experiments indicate that Cdc37 inhibition has a profound effect on signal transduction propagated through protein phosphorylation cascades. We have concentrated in these experiments



**Figure 2.** Targeting Cdc37 inhibits the kinase cascades that underlie mitogenic signaling. **A**, Western blot analysis of proteins in Cdc37 knockdown or control cells after serum starvation overnight and then re-feeding with media containing either 30% serum or controls with 0% serum as indicated for 10 min. Cells were harvested, and relative levels of signal transduction intermediates and their phosphorylated counterparts were assayed by immunoblot with specific antibodies. Lysates were probed sequentially with antibodies to Cdc37, p90RSK, phospho-S380-p90RSK, Akt, phospho-S473-Akt, p42/44MAPK, phospho-T202/Y204-p42/44, S6, and phospho-S235/236-S6 protein.  $\beta$ -Actin levels were measured in extracts for a loading control. **B**, graph representing densitometric quantitation of select bands in **A**. +37, addition of Cdc37 shRNA; +C, addition of control shRNA. +/- S, addition or absence of serum. *Value axis*, arbitrary densitometric units indexed to the highest value band for each species. **C**, Western blot analysis of proteins in Cdc37 knockdown or control PC3 cells serum starved overnight and then fed medium containing 25 ng/mL recombinant human EGF for the times indicated. Cells were then harvested and blotted for Cdc37 and signal transduction proteins involved in the PI3K/Akt/mTOR signaling pathway, including Akt, phospho-S473-Akt, GSK3 $\beta$ , phospho-S9-GSK3 $\beta$ , S6, and phospho-S235/236-S6 protein.  $\beta$ -Actin levels were assayed as control for loading. **D**, graph representing densitometric quantitation of select bands in **C**. +37, addition of Cdc37 shRNA; +C, addition of control shRNA. 0 to 30 min, time from addition of EGF. *Value axis*, arbitrary densitometric units indexed to the highest value band for each species.

on mitogenic signaling, although it is anticipated that protein kinases involved in other aspects of cell regulation such as cell cycle progression and other processes will also be affected by Cdc37 loss.

**Cdc37 also inhibits androgen signaling in androgen-responsive prostate cancer cells.** Androgen receptor-positive LNCaP cells are dependent on many of the signaling pathways that we show above are blunted by Cdc37 depletion in other cell lines (Fig. 2); such effects are therefore likely to contribute to decreased growth after Cdc37 depletion in LNCaP cells. However, the dependence of LNCaP cells on androgens for growth may also make them vulnerable. AR is the only known non-kinase client described for Cdc37, and Cdc37 participates in AR signaling as shown in previous experiments using a forced-expression system (13). Therefore, we next investigated the effects of Cdc37 targeting on AR function in LNCaP cells. Indeed, depletion of Cdc37 induced a cell morphology characteristic of androgen-dependent prostate carcinoma undergoing androgen depletion (29)—the acquisition of a neuroendocrine-like phenotype (Fig. 3A, *left*) characterized by the formation of filamentous pseudopodia and dendrite-like intercellular connections. Although cells infected with Cdc37 shRNA-expressing virus exhibited this morphology, transduction of nontargeting control virus had no marked effect on LNCaP cell morphology (Fig. 3A, *right*). Thus, Cdc37 targeting mimics some of the morphologic effects of androgen depletion.

We next examined the molecular effects of Cdc37 depletion on AR function in LNCaP cells. We first investigated androgen-dependent transcription using a reporter construct containing an androgen receptor element (ARE) promoter coupled to the luciferase gene. This experimental promoter contains three copies of the full-length promoter from the *psa* gene arranged in tandem. Although the androgen, dihydrotestosterone (DHT), strongly activated this *psa* promoter-reporter construct when transfected into LNCaP cells, depletion of Cdc37 significantly reduced these transcriptional responses to androgenic stimulation (Fig. 3B). We also examined the effects of Cdc37 depletion on expression of the native *psa* gene by measuring the concentration of intracellular PSA protein by immunoblot assay. Exposure to DHT caused a dose-dependent increase in PSA levels in control cells. However, Cdc37 knockdown markedly reduced PSA levels both in unstimulated cells and after exposure to DHT (Fig. 3C). Thus, AR and androgen-dependent transcription are targets in growth inhibition by Cdc37 depletion.

**Cdc37 knockdown sensitizes cells to the HSP90 inhibitor 17AAG.** As our previous experiments suggested that Cdc37 and HSP90 have distinct mechanisms for kinome maintenance and for fostering cell growth, we next examined the potential of combining Cdc37 targeting with HSP90 drugs. Combination of Cdc37 targeting with the HSP90 inhibitor 17AAG was highly effective in inhibiting the growth of PC3 and DU145 cells (Fig. 4A). Serial dilution 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay indicates that Cdc37 knockdown sensitizes both cell lines to killing by 17AAG by nearly a full log (Fig. 4A). Additive inhibition of growth by HSP90 targeting drugs and Cdc37 depletion was then confirmed by colony formation assay (Fig. 4B). Cdc37 depletion markedly sensitized both DU-145 and PC3 cells to 17AAG and led to a profound loss of clonogenicity.

Because 17AAG acts primarily by promoting the degradation of HSP90 client proteins, we next examined whether Cdc37 targeting might modulate these effects of HSP90 inhibition. We therefore treated Cdc37 knockdown and control cells with increasing doses of 17AAG and investigated the effects on protein kinase levels by

Western blot (Fig. 4C). Akt, a well-characterized HSP90/Cdc37 client, undergoes dose-dependent degradation in response to increasing doses of 17AAG. However, these effects were greatly potentiated in cells depleted of Cdc37, and quantitative loss of the protein occurred (Fig. 4C). Thus, although Cdc37 depletion does not in itself cause marked depletion of client proteins, it does potentiate client protein loss when HSP90 is inhibited by 17AAG.

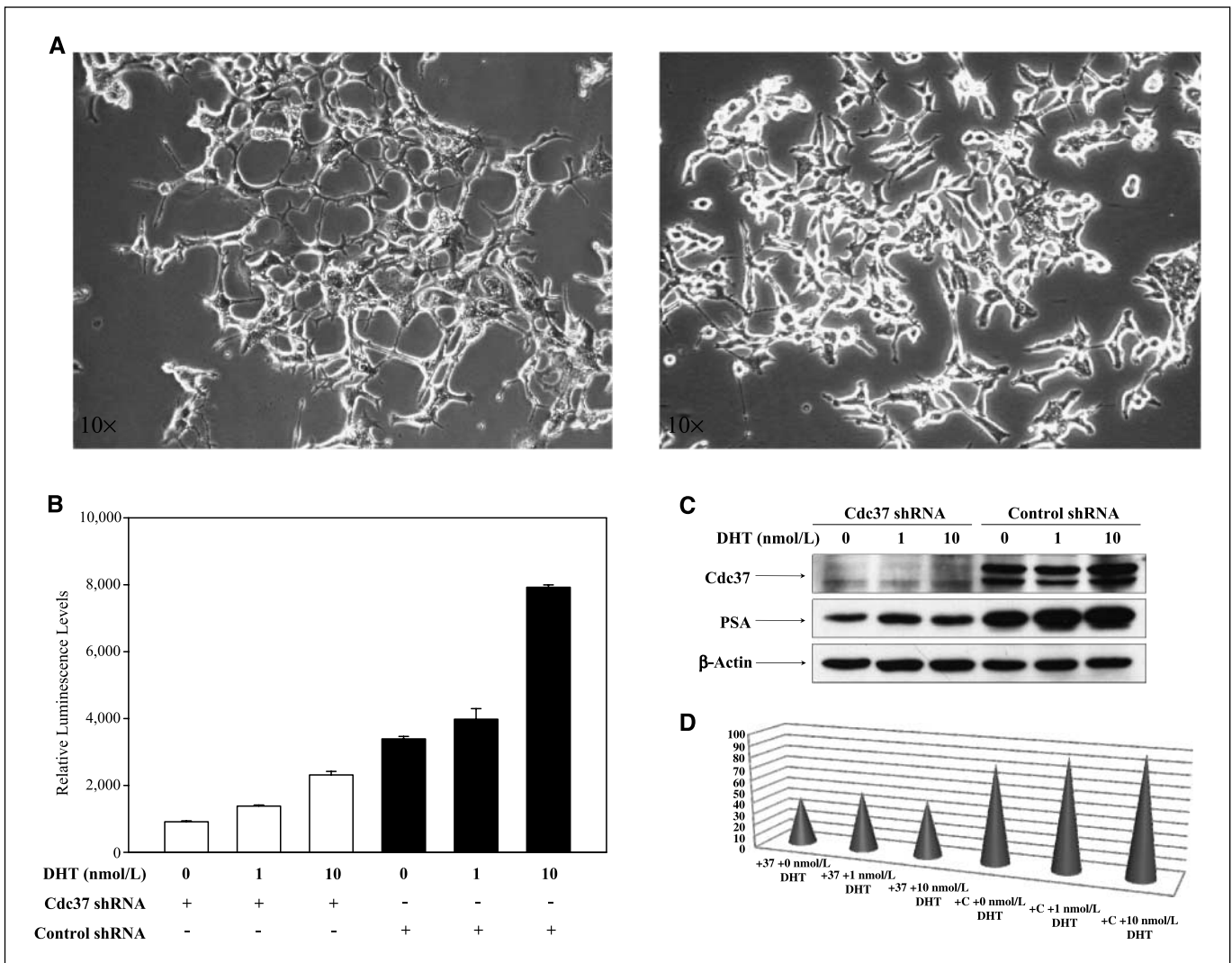
We also examined another potential pathway through which Cdc37 depletion might lead to potentiation of cell killing by 17AAG. We investigated one of the known pathways of resistance to HSP90 inhibitors, the induction of HSP70 by chemical inhibitors of HSP90 (30). HSP70 mediates resistance to treatment-induced death pathways through its inhibitory effects on intracellular caspase cascades (14). We therefore measured HSP70 levels in prostate cancer cells treated with HSP90 inhibitors with or without Cdc37 depletion (Fig. 4C). We used the antibody Ab46-4 that detects a number of HSP70 family members, including the constitutive protein HSC70 that migrates with an apparent  $M_r$  of 73 kDa and the stress-inducible 72-kDa isoform HSP72, which is also present at basal levels in prostate cancer cells (31). In cells depleted of Cdc37, both baseline and 17AAG-stimulated levels of the HSP72 isoform are greatly reduced, although expression of the more slowly migrating HSC70 is not affected (Fig. 4C). The induction of HSP72, which occurs even with low doses of HSP90 inhibitors, is prevented at each dose of 17AAG in the Cdc37-depleted cells. The constitutive HSC70 is unaffected under all conditions (Fig. 4C).

To determine additional mechanisms by which Cdc37 ablation decreases HSP72 induction by 17AAG, we next examined whether Cdc37 depletion affects HSP70 transcription. HSP70 induction through HSP90 inhibition involves the transcription factor HSF1 (heat shock factor 1), a sequence-specific factor that regulates expression of *HSP* genes through response elements in their promoters (HSE; ref. 31). For these experiments, we employed a promoter-luciferase reporter construct based on the *HSP70B* gene, an *HSP70* family gene that is activated by HSF1 with high specificity. Transcription of *HSP70B* was strongly induced by 17AAG, as expected from previous studies (Fig. 4D). However, following Cdc37 depletion, we observe significantly reduced basal *HSP70B* activity as well as inhibition of the induced transcriptional activity in cells treated with increasing doses of 17AAG (Fig. 4D). This inhibitory effect of Cdc37 depletion on the induction of HSF1 activity was most clearly observed in Du145 cells, but could also be seen in PC3 cells at high doses of 17AAG. Thus, Cdc37 seems to be required for the trans-activation of HSF1 by 17AAG. Our studies therefore suggest that sensitization of cells to 17AAG by Cdc37 depletion involves at least two mechanisms, including increased loss of proteins required for growth and survival and inhibition of induction of antiapoptotic protein HSP72.

## Discussion

These studies indicate that Cdc37 is essential for growth and survival of both AR<sup>+</sup> and AR<sup>-</sup> human prostate carcinoma cells and suggest the possibility of targeting Cdc37 for treatment of prostate cancer. Because the majority of prostate tumors and cell lines have elevated levels of Cdc37 (Supplementary Fig. S1), which seem essential for their growth, these studies suggest a strategy for selective inhibition of prostate cancer growth.

Loss of Cdc37 seems to inhibit prostate tumor cell growth through mechanisms that contrast with those that accompany growth inhibition by HSP90 targeting (Figs. 1 and 2). Our finding



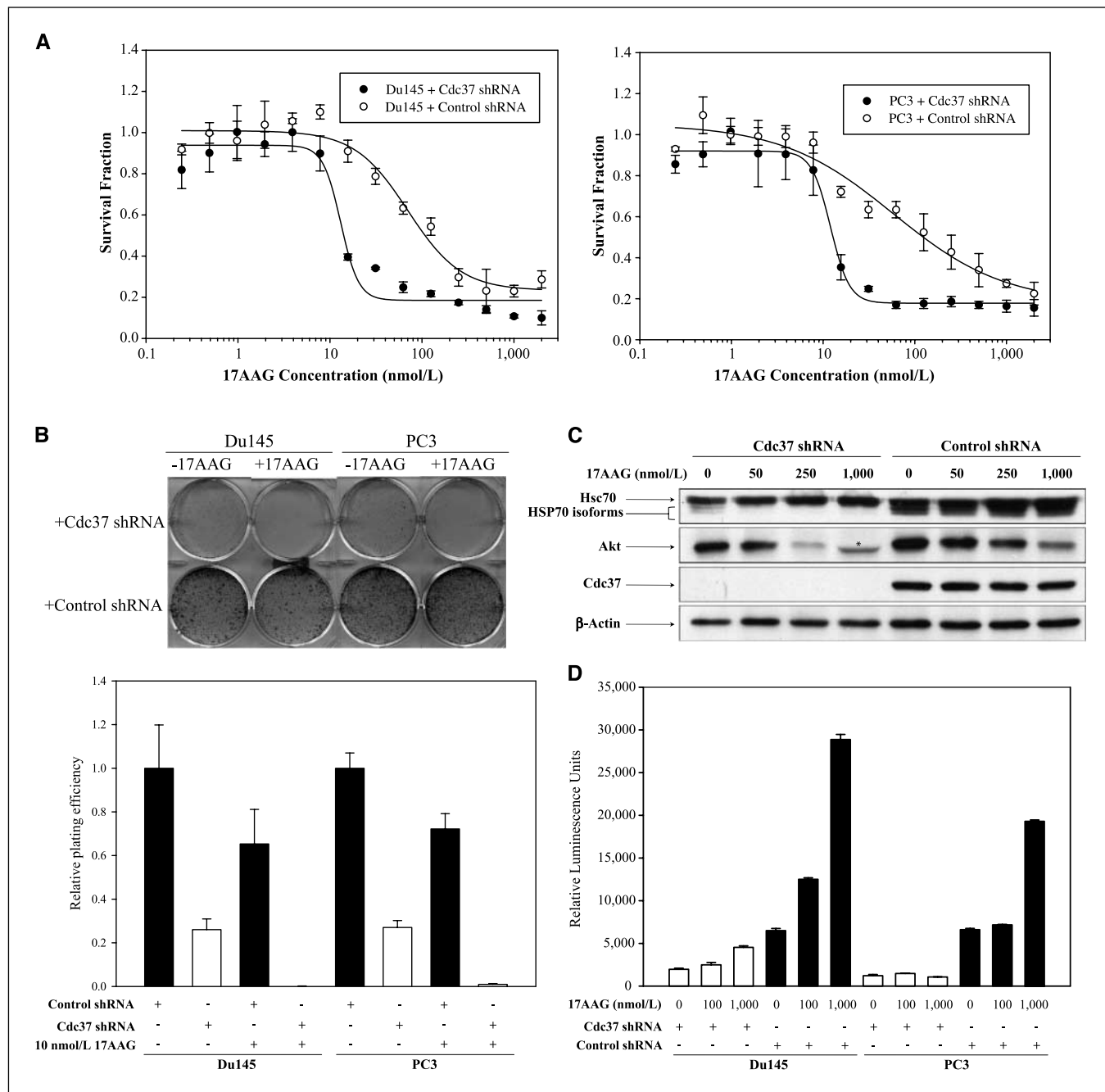
**Figure 3.** Cdc37 knockdown inhibits androgen signaling. *A, left*, phase-contrast microscopic images of LNCaP cells infected for 6 d with Cdc37 shRNA-expressing lentivirus. Infected cells were grown under normal conditions in the presence of serum. Cells infected with control virus are shown for comparison (*right*). *B*, depletion of Cdc37 inhibits trans-activation of the PSA promoter by the chemical androgen DHT. LNCaP cells transduced with Cdc37 or control virus were transfected with an ARE-luciferase reporter plasmid and then grown for 24 h in 10% charcoal-dextran–stripped serum-containing media. Cells were then stimulated with increasing doses of DHT for 24 h and assayed for luciferase levels. Relative luminescence levels are normalized to a co-transfected  $\beta$ -galactosidase expression construct to correct for cell number and transfection efficiency. *Columns*, means; *bars*, SD. *C*, Cdc37 depletion reduces basal and DHT-stimulated levels of PSA protein. Cells grown for 24 h in charcoal-dextran–stripped serum were stimulated with the indicated amounts of DHT for 24 h before being harvested and subjected to sequential Western blot analysis with anti-Cdc37, anti-PSA and anti- $\beta$ -actin antibodies. *D*, graph representing densitometric quantitation of select bands in *C*. +37, addition of Cdc37 shRNA; +C, addition of control shRNA. *Value axis*, arbitrary densitometric units indexed to the highest value band.

that Cdc37 depletion does not induce global degradation of its clients was unanticipated because the widely reported effects of HSP90 inhibitors on tumor growth invoke such a mechanism. Our studies are also somewhat in discord with those employing model systems to study Cdc37 in other species such as yeast and *Caenorhabditis elegans*, which indicate that Cdc37 depletion can reduce the levels of some clients (32, 33) and others in *Drosophila* SL2 cells, which show that depletion of Cdc37 leads to decreased levels of Aurora B (10). However, previous experiments in human cancer cells accord with our findings and show that inhibition of the HSP90/Cdc37/client protein complex did not lead to Aurora B degradation in some tumor types (10). Species and tissue differences in the precise intracellular roles of Cdc37 may thus exist. It has been shown in normal mammalian cells that Cdc37 is responsible for recruiting newly formed kinases to the HSP90

machinery (4). However, in a background characterized by Cdc37 overexpression and overloading of HSP90 seen in many malignant cells, this paradigm may be perturbed, and cryptic functions for Cdc37 may be exposed. The elevated expression of Cdc37 observed in cancer cells leads to partial chaperoning of client proteins independently of HSP90 and the rescue of cells containing lethal kinase mutations (34). In addition, only a small proportion of the overexpressed Cdc37 molecules are found in complexes with HSP90. Thus, in a setting where the HSP90 system is overworked by the increased concentrations of unstable proteins characteristic of cancer, overexpressed Cdc37 may function by buffering essential kinases in an active state with reduced input from HSP90. Indeed, Cdc37 seems to be especially important for kinase maturation (35). In this study, Mandal et al. showed that functional Cdc37 promotes optimal kinase activity for a number of clients without affecting the

overall levels of these proteins. Hinz et al. also recently reported that Cdc37 is important for the maturation and enzymatic competence of IKK complexes, but is not necessary for their assembly or stability (36).

Our studies also suggest that AR-dependent prostate cancer cells may be sensitive to Cdc37 loss (Fig. 3). Heightened sensitivity may reflect the elevated Cdc37 levels in these cells (Supplementary Fig. S1) and the dependence of AR function on Cdc37 (Fig. 3).



**Figure 4.** Cdc37 depletion strongly potentiates cell killing by the HSP90 inhibitor 17AAG. **A**, MTT cell survival assays for Cdc37 knockdown and control cells treated with serial dilutions of the HSP90 inhibitor 17AAG. Cdc37 depletion reduced the IC<sub>50</sub> in Du145 cells from 108 to 14 nmol/L and in PC3 cells from 125 to 13 nmol/L. Bars, SD. **B**, colony formation assay on cells transfected with Cdc37 shRNA and control shRNA and grown in the presence of 10 nmol/L 17AAG. Single cells were plated at various densities in 17AAG-containing medium for 3 d following virus transduction and then incubated for a further 14 d to permit colony formation. The image is representative of the complete assay, which was run in triplicate. In addition, we show a histogram depicting relative plating efficiency of cells subjected to the various treatments and plated for the colony growth assay. Colonies containing >50 cells were scored blinded in triplicate. Columns, mean relative plating efficiency; bars, SD. The combination of 17AAG treatment and Cdc37 depletion significantly inhibited colony formation in comparison to both Cdc37 depletion alone and 17AAG treatment alone ( $P < 0.001$ ). **C**, protein expression in Du145 cells treated with 17AAG, without or with Cdc37 depletion. Du145 cells were treated with various doses of 17AAG for 48 h. Proteins were extracted, and relative levels of Hsp70, Akt, Cdc37, and β-actin were determined by immunoblot. \*, band migrates aberrantly and may represent a partially degraded protein due to the high amount of dead and floating cells seen in this data point. **D**, effects of 17AAG on the activation of a HSF1-dependent promoter. Du145 and PC3 cells with or without Cdc37 shRNA virus were selected in puromycin for 1 wk and then transfected with a luciferase reporter construct driven by the promoter region of *HSP70B*. Twenty-four hours after transfection, the cells were treated with the indicated doses of 17AAG and incubated for an additional 24 h before being harvested and subjected to a luciferase assay. Relative luminescence levels are normalized to a co-transfected β-galactosidase expression construct to correct for cell number and transfection efficiency. Bars, SD.

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A regulatory role for Cdc37 in optimal androgen signaling was anticipated from previous investigations in yeast model systems (13). However, our study is the first to show the requirement for Cdc37 in AR signaling and prostate cancer cell growth. Optimal AR activation requires the input of a number of protein kinases, and decreased AR activity after Cdc37 depletion may involve targeting such kinases. However, given previous publications suggesting direct interaction of Cdc37 with AR, it is likely that inhibition of AR-dependent transcription involves both Cdc37 binding to AR and the indirect effects of the protein kinase targeting mentioned above (13, 37). Because the bulk of early prostate cancers are androgen responsive, Cdc37 inhibition could be an effective therapy for this subgroup and might be developed into a therapy aimed at preventing the conversion of early-stage tumors into more difficult to treat metastatic androgen-independent types.

The distinct properties of Cdc37 and HSP90 in fostering client protein activity may underlie the ability of Cdc37 targeting to sensitize tumor cells to HSP90 inhibition (Fig. 4). The finding that Cdc37 depletion amplifies the effects of HSP90 inhibition was initially unanticipated because HSP90 and Cdc37 perform cooperative/overlapping functions. However, in cells depleted of Cdc37, the recruitment of protein kinases to HSP90 complexes might be maintained through alternative co-chaperone interactions, including a possible role for Hsc70, a structural homologue of Cdc37 (38). Thus, despite the overtaxing of the HSP90 system in many cancers, cells may retain the residual ability to compensate for the loss of Cdc37 and prevent complete degradation of clients through parallel folding pathways. However, without the high levels of Cdc37 needed for kinase maturation and other molecular chaperone functions in cancer cells, clients evidently become exquisitely sensitive to HSP90 inhibition (Fig. 4C).

Our findings that HSF1 activity and HSP70 synthesis require Cdc37 and are inhibited by Cdc37 depletion offer further rationale

for combined targeting of Cdc37 and Hsp90 (Fig. 4). HSF1 regulates expression of HSP27 and HSP70, which are potent and versatile inhibitors of both programmed cell death and treatment-induced killing (14). HSF1 activity depends on signaling through the phosphoinositide-3-kinase (PI3K)/Akt pathway and the Ras/Erk-MAPK cascades (39). However, key intermediates in both these pathways are degraded in the presence of HSP90 inhibitors, and they are unlikely to be the major effectors in this case. We have previously found that Cdc37 directly interacts with HSF1.<sup>1</sup> Cdc37 may therefore participate in the release of HSF1 from HSP90 complexes, an essential step in HSF1 activation (40). Loss of HSP70 induction may contribute to the increased 17AAG-induced killing in Cdc37-depleted cells (Fig. 4). Because increased levels of HSP70 and other HSPs contribute to the development of HSP90 inhibitor resistance in cancer, combining HSP90 drugs with Cdc37 depletion might be an attractive clinical option.

Overall, therefore, Cdc37 depletion irreversibly inhibits the growth of prostate carcinoma cells, significantly affecting both AR<sup>+</sup> and AR<sup>-</sup> cells. The effects of Cdc37 depletion seem to involve both inhibition of growth-dependent signaling cascades and antagonism of AR function. The effectiveness of Cdc37 depletion in inhibiting growth of a wide range of prostate carcinoma cells suggests a potentially novel treatment strategy for prostate cancer through the pharmacologic development of Cdc37 inhibitors.

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<sup>1</sup> N. Grammatikakis and S.K. Calderwood, unpublished data.

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