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UBR1 promotes protein kinase quality control and sensitizes cells to Hsp90 inhibition

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Abstract

UBR1 and UBR2 are N-recognin ubiquitin ligases that function in the N-end rule degradation pathway. In yeast, the UBR1 homologue also functions by N-end rule independent means to promote degradation of misfolded proteins generated by treatment of cells with geldanamycin, a small molecule inhibitor of Hsp90. Based on these studies we examined the role of mammalian UBR1 and UBR2 in the degradation of protein kinase clients upon Hsp90 inhibition. Our findings show that protein kinase clients Akt and Cdk4 are still degraded in mouse *Ubr1*^{−/−} cells treated with geldanamycin, but that their levels recover much more rapidly than is found in wild type cells. These findings correlate with increased induction of Hsp90 expression in the *Ubr1*^{−/−} cells compared with wild type cells. We also observed a reduction of UBR1 protein levels in geldanamycin-treated mouse embryonic fibroblasts and human breast cancer cells, suggesting that UBR1 is an Hsp90 client. Further studies revealed a functional overlap between UBR1 and the quality control ubiquitin ligase, CHIP. Our findings show that UBR1 function is conserved in controlling the levels of Hsp90-dependent protein kinases upon geldanamycin treatment, and suggest that it plays a role in determining the sensitivity of cancer cells to the chemotherapeutic effects of Hsp90 inhibitors.

Introduction

Quality control processes contribute to the etiology of cancer and other diseases of ageing. These processes regulate proteome health by facilitating polypeptide folding and ensuring that misfolded proteins are targeted for degradation via the ubiquitin/proteasome (UPS) or autophagic systems [1]. Both folding and degradation arms of the quality control process are regulated by molecular chaperones that interact with unfolded or misfolded proteins to determine their fate. The central role played by molecular chaperones to quality control processes is underscored by their importance to several late onset disease states including cancer. The Hsp90 molecular chaperone, for example, is currently of great interest because it is the target of several small molecule chemotherapeutics [2]. These small molecules act by competitive inhibition of Hsp90's ATPase and promote targeting of chaperone clients to the ubiquitin/proteasome system for degradation rather than folding. Since Hsp90 facilitates folding of many client types involved in cellular signaling, including protein kinases and transcription factors, its inhibition has a profound effect on cell growth and the cell cycle, and results in cell death [3].

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A key insight into how chaperones and the UPS collaborate was uncovered with the identification of the C-terminal Hsp Interacting Protein (CHIP), a chaperone binding E3 ubiquitin ligase [4, 5]. CHIP potentiates ubiquitylation and degradation of Hsp90 clients by both inhibitor-dependent and independent means [6–9]. CHIP functions via direct interaction of Hsp70/Hsp90 and also misfolded proteins [39]. In addition to promoting degradation of misfolded proteins, CHIP functions as a general integrator of the stress response. It is a positive regulator of the heat shock response and binds to heat shock transcription factor (Hsf; [10]). CHIP is also important to heat shock recovery, by catalyzing ubiquitylation of induced Hsp70 chaperones [11]. Inhibition of Hsp90 in CHIP^{−/−} cells results in reduced degradation of a client protein kinase, but the effect is not abolished, indicating the existence of functionally related ubiquitin ligases [12]. Recently, Cul5, a RING domain ubiquitin ligase that interacts with both Hsp70 and Hsp90, was also shown to promote degradation of ErbB2 and Hif1 α in cells treated with geldanamycin (GA;[13]).

Ubr1 is an N-recogin ubiquitin ligase that promotes protein degradation via distinct mechanisms [14]. It is a RING domain ubiquitin ligase that functions in the N-end rule of proteins with N-termini that have been processed. Such proteins having basic (type I) or bulky hydrophobic (type II) N-terminal residues are substrates for Ubr1. Other non-N-end rule substrates have been characterized, however, and recent studies showed that Ubr1 appears to have a general role in degradation of misfolded cytosolic proteins [12, 15, 16]. In these studies, all performed in the yeast, *Saccharomyces cerevisiae*, Ubr1 was shown to promote ubiquitylation and degradation of unstable proteins, including Hsp90 clients in the presence of the inhibitor, geldanamycin. The mechanism appears to involve direct interaction of the unfolded proteins with Ubr1 itself [12].

Ubr1 is one of seven mammalian genes that contain a signature UBR box that is involved in binding N-end rule substrates [17]. Of these 7, Ubr1 and Ubr2 have a general role in the N-end rule, although they display specificity based on the phenotypes exhibited by their deletion in mice. Deletion of Ubr1 phenocopies human Johanson-Bllizard syndrome, which is characterized by pancreatic insufficiency and developmental abnormalities [18]. Deletion of Ubr2 results in impaired male meiosis while the double knockout strains are embryonic lethal [19]. In the following studies, we show that UBR1 has a specific role in quality control of protein kinases upon Hsp90 inhibition in mammalian cells.

Materials and Methods

Chemicals

Geldanamycin (GA) was purchased from Invivogen (San Diego, CA) and dissolved in 100% DMSO. PU-H71 was also dissolved in DMSO.

Cell Culture and Transfection

Mouse embryonic fibroblast cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Mediatech, Inc, Herdon, VA), 100 units/ml penicillin, 100 μ g/ml streptomycin (MP Biomedicals, LLC, France). BT474 cells stably transfected with control and UBR1 shRNA plasmid were maintained in a 1:1 mixture of DMEM: F12 supplemented with 2mM glutamine, 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin and 1 μ g/ml puromycin (Sigma-Aldrich). All cells were kept at 37° C in 5% CO₂ incubator.

For RNA interference experiments the 21-nucleotide siRNA duplexes were synthesized and purified by IDT. The target sequences of mouse CHIP siRNA are as follows:

CHIP1-1, 5'-AUCUUCAUGACCCUCGUGGTT-3';

CHIP1-2, 5'-UUUAUCGUGCUUGGCCUCATT -3'.

The sequences of control siRNAs are as follows:

5'-CUUCCUCUCUUUCUCUCCCUUGUGA -3'.

To achieve transient suppression of CHIP expression, the duplex siRNAs (400nM) were transfected into WT and Ubr1^{-/-} MEF cells with the Nucleofection system (Amaxa Biosystem, Cologne, Germany) using MEFII transfection kit (Lonza) and A-023 program. To knock down UBR1 in WT MEF cells, the ON-Target plus SMART pool of mouse UBR1 (Thermo Scientific Dharmacon; Cat# L-047034-01) was used. To stably suppress the expression of human UBR1, BT474 cells were transfected with control shRNA plasmid (sc-108060) and UBR1 shRNA plasmid (sc-106918-sh; Santa Cruz Biotechnology, Inc) with the Nucleofection system using solution-V (Lonza) and P-020 program. After 48 hours of transfection, cells were selected with media containing puromycin (1µg/ml). For over expression of rat UBR1, the Ubr1^{-/-} MEF cells were transfected with 5 µg of plasmid DNA as described above. After 22 hours of transfection, cells were treated with different concentrations of GA for another 24 hours and cell viability was measured.

Cell viability assay

Cell viability after GA treatment was measured using the cellTiter-Glo^R Luminescent cell viability assay kit (# G7571; Promega) according to the manufacturer's instructions. Briefly, exponentially growing cells were seeded into 96-well microtiter plates (#3917; Corning) and incubated in medium containing either vehicle control (DMSO) or GA/PU-H71 for 24 hours at 37°C. Plates containing 4 replicate wells per assay condition were seeded at a density of 1000 cells for each cell line in 100 µl medium. After exposure of cells to the Hsp90 inhibitors, 100 µL CellTiter-Glo reagent was added to each well. Plates were incubated for 10 minutes at room temperature. The luminescence signal in each well was measured in microplate luminometer reader using the GloRunner program. The percentage of cell viability was calculated by comparing luminescence readings obtained from treated versus control cells.

Western blotting and antibodies

Cells were grown to 70–80% confluence and exposed to GA, PU-H71 or DMSO vehicle for indicated times. Lysates were prepared using lysis buffer containing 0.1% NP-40, 20 mM HEPES (pH 7.5), 0.12 M NaCl, 1 mM EDTA, 2.5 mM glycerophosphate, 1mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na₃VO₄ and protease inhibitors (Complete mini, Roche Diagnostics, Indianapolis, IN). Protein concentration was determined using Bradford method. Samples of 20 g were analyzed in SDS-polyacrylamide gels, transfer to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) and blocked for 30 minutes at room temperature with 5% nonfat dry milk in TTBS buffer (20 mM Tris-HCl pH-7.5, 0.25 M NaCl, 0.05% Tween-20). Incubation with primary antibodies (usually diluted 1:1000 in antibody dilution buffer; 1x phosphate-buffered saline, 3% bovine serum albumin, 0.05% Tween-20 and 0.1% Thimerosol) was done at room temperature for 2 hour or overnight at 4°C. After three washes with TTBS the membranes were incubated with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit or Licor goat anti-rabbit IRDye 800CW and goat anti-mouse IRDye 680 diluted 1: 15,000 in antibody dilution buffer) for 2 hours at room temperature. After three more washes the blots were treated with the enhanced chemiluminescence reagents (Pierce) and exposed to x-ray film (Kodak) for detection or detected using the Licor Odyssey Infrared imaging system. Antibodies used were: Akt, p-Akt, ErbB2, Cdk4 (Cell signaling, Beverly, MA), Hsp70 (SPA-822), anti-Raf1 (KAP-MA020C) (Stressgene, Victoria, Canada), UBR1 (Abcam Inc, Boston, MA), Actin (Sigma-Aldrich).

Results

Based on previous studies in the yeast system we hypothesized that mammalian Ubr1 homologs would have a conserved function in the quality control of protein kinases that misfold upon inhibition of the Hsp90 molecular chaperone [12]. While yeast has just one N-end rule ubiquitin ligase, Ubr1, mammalian cells have several, of which UBR1 and UBR2 are most similar to their yeast ortholog, and have a general pattern of expression. We used embryonic fibroblasts (MEFs) from Ubr1^{-/-}, Ubr2^{-/-} and double deletion (DKO) mice to test the hypothesis that such cells would have an altered response to Hsp90 inhibition compared with MEF cells from wild type mice [17]. Initial studies focused on two well-established Hsp90 protein kinase clients, Akt and Cdk4 [20, 21]. In the presence of 1 μ M geldanamycin (GA), levels of both protein kinases diminished rapidly in wild type MEF cells, beginning within 6 hours of treatment (Fig. 1A). A similar drop in Akt and Cdk4 levels was also observed in Ubr1^{-/-} cells at 6–18 hours. Akt and Cdk4 Degradation were inhibited in the presence of the proteasome inhibitor, MG132, suggesting that even in the Ubr1^{-/-} cells the normal pathways for protein disposal are operating (not shown). However, the effect of GA was clearly diminished at subsequent times in the Ubr1^{-/-} cells and the levels of both protein kinases returned beginning at 12–18 hours after treatment (Fig. 1A, B and C). Akt and Cdk4 levels decreased in response to drug treatment in both Ubr2^{-/-} and the DKO cells in a similar manner to the wild type cells (Fig. 1D).

The results shown above suggested that protein kinase quality control upon Hsp90 inhibition was dependent to some extent on UBR1, but not UBR2. This was confirmed in dose-response experiments. For these studies, we analyzed all four MEF cell-lines with different amounts of GA (0–1 μ M) over a 24-hour period. We observed reduced degradation of Akt and Cdk4 (not shown) in the Ubr1^{-/-} and to a lesser extent in the DKO cells, but not in the Ubr2^{-/-} cells (Fig. 1E). These combined data show that UBR1, but not UBR2, affects the dose response of GA with respect to protein kinase degradation, although there is a small level of stabilization of phospho-Akt in the Ubr2^{-/-} mutant alone compared with the wild type and DKO cells (Fig. 1E).

Hsp90 is a negative regulator of the heat shock response, and its inhibition with GA results in de-repression of heat shock transcription factor [22]. This de-repression results in induction of Hsp70 and to a lesser extent, Hsp90. To determine whether cells lacking UBR1 have a similar or altered heat shock response upon Hsp90 inhibition, we analyzed levels of both Hsp70 and Hsp90 in Ubr1^{-/-}, Ubr2^{-/-} and DKO cells. As shown in Fig. 2, Hsp70 induction is very similar in the absence of Ubr1 and Ubr2 compared with the wild type. By contrast, there is a sharp increase in Hsp90 induction in the Ubr1^{-/-} cells compared with the wild type and Ubr2^{-/-} cells. These findings demonstrate a positive correlation between induced Hsp90 expression and decreased sensitivity of the Ubr1^{-/-} cells to Hsp90 inhibitors. To further investigate this correlation, we determined whether the resurgence of protein kinase levels that occurs in Ubr1^{-/-} cells upon GA treatment represented new synthesis. This was accomplished by treating Ubr1^{-/-} cells with and without cycloheximide to inhibit further translation. The experimental approach was to incubate the Ubr1^{-/-} cells with GA for 12 hours before addition of cycloheximide (Fig. 3A). Aliquots of cells were taken at the 12 hour time point and then subsequently upon 3, 6 and 8 hours after cycloheximide addition. Western blot analysis of Akt and Cdk4 revealed that there was a further decrease in kinase levels between 15–20 hours of GA treatment in the cycloheximide treated cells. This effect was much more dramatic for Cdk4 than for Akt. By contrast, steady state kinase levels in the absence of cycloheximide were stabilized over the same time period. These findings suggest that the effect of GA becomes diminished in the Ubr1^{-/-} cells such that newly synthesized protein kinases are not as rapidly degraded. This could be

due to increased Hsp90 expression as described above, or because the activity of GA as an inhibitor is reduced.

In previous studies it was shown that cells can become resistant to GA or its derivatives. For example, a clinically useful derivative of GA, 17-AAG becomes less effective upon decreased expression of NAD(P)H/quinone oxidoreductase I (NQO1) [23, 24]. To determine whether the resistance of UBR1^{-/-} cells to GA treatment was compound-specific we analyzed a chemically distinct Hsp90 inhibitor PU-H71, developed by Chiosis and colleagues [25]. In dose response experiments, there was decreased efficacy of PU-H71 in 0.25–0.5 μ M range after 24 hours in the Ubr1^{-/-} cells that was evident in the levels of Akt protein kinase and its phosphorylated form (Fig. 4A). The effect was less noticeable for Cdk4, and this was confirmed in a time course analysis, shown in Fig. 4B. In this case, Cdk4 levels were largely unaffected up to 30 hours post treatment with 500 nM PU-H71. Akt acted much like it did in the presence of GA, since its levels dropped within 6 hours of treatment only to recover within 18 hours. Levels of phosphorylated Akt were also higher in the Ubr1^{-/-} cells treated with PU-H71 compared with the wild type MEF cells. Similar findings were also recorded for Raf-1 (Fig. 4B).

The findings shown above suggest that UBR1 plays a role in sensitizing cells to Hsp90 inhibitors. To test this more directly we performed a growth analysis of cells with and without Ubr1/Ubr2 in the absence and presence of GA and PU-H71 (Fig. 5 A and B). The viability of wild type MEF cells decreased in a concentration dependent manner over a 24-hour period in the presence of either Hsp90 inhibitor, as expected, with the effect of GA being greater than for PU-H71. However, cells deleted for either Ubr1 or Ubr2 exhibited reduced sensitivity to both compounds. In the case of GA, the greatest resistance was observed for Ubr1^{-/-} cells. We also observed resistance of the Ubr2^{-/-} cells to Hsp90 inhibitors even though we did not observe any effects of deleting this N-recognin on Akt or Cdk4 levels (Fig. 1). Overexpression of a plasmid encoding rat Ubr1 [26] in Ubr1^{-/-} cells partially suppressed the resistance phenotype by approximately 30%, which is very similar to the transfection efficiency of these cells (Fig. 5C).

To further address the role of UBR1 in protein kinase quality control upon Hsp90 inhibition we used a human breast cancer cell line. BT474 cells were used because they overexpress ErbB2 and are very sensitive to Hsp90 inhibitors. As shown in Fig. 6, knockdown of UBR1 with shRNA did not affect ErbB2 levels in the very short term compared with control treated cells, but kinase levels resurged 12–18 hours later, in similarity with the Ubr1^{-/-} MEF cells (Fig. 1A–C). We also noted that BT474 cells with reduced UBR1 had greater levels of Akt, including the active form, and Cdk4 compared with cells having normal levels of UBR1. Furthermore, UBR1 protein levels appeared to be reduced upon GA treatment, suggesting that it may be a client of Hsp90. The reduced levels of UBR1 were more evident in the cells having reduced UBR1 to begin with, and these levels resurged at later times of GA treatment as did ErbB2 (Fig. 6A; UBR1shRNA lanes). To further address the possibility that UBR1 levels are related to Hsp90 activity, we examined UBR1 protein levels in wild type MEF cells (Fig. 6B). GA treatment of wild type MEF cells resulted in decreased UBR1 protein levels as assessed by Western blot analysis, and this decrease was more profound when UBR1 expression was diminished due to siRNA treatment. As expected, siRNA treatment of wild type MEF cells for UBR1 also resulted in increased levels of Akt upon GA treatment compared with cells treated with control siRNA (Fig. 6B and 6C).

It was established previously that the E3 ligase CHIP has a role in the degradation of at least some Hsp90 client kinases, including Akt and ErbB2 [7, 27]. This suggests that UBR1 may act in concert with CHIP based on the studies described above. To address this hypothesis we knocked down CHIP levels in Ubr1^{-/-} cells and measured the effect of GA on protein

kinase degradation. As shown in Fig. 7A, CHIP levels were efficiently knocked down with siRNA, but there was little consequence to the degradation of either Akt or Cdk4 in the presence of GA in wild type MEF cells. By contrast, there was a marked effect of reducing CHIP levels in the Ubr1^{-/-} MEF cells. In this case, there was much greater resistance to GA and a corresponding accumulation of both Akt and Cdk4 compared with the Ubr1^{-/-} cells with normal CHIP levels (Fig. 7B and 7C). These findings suggest that UBR1 and CHIP share a functional relationship in protein kinase quality control.

Discussion

The role of Hsp90 inhibitors as effective chemotherapeutics depends on their ability to promote rapid degradation of oncogenic protein kinases and transcription factors via the ubiquitin proteasome system. Previous studies demonstrated that the ubiquitin ligase, CHIP, played a role in this process via direct interaction with Hsp70 and Hsp90 molecular chaperones and misfolded protein substrates [28]. In CHIP knockout cells, however, the oncogenic protein kinase ErbB2 was still degraded upon Hsp90 inhibition but at a reduced rate [7], suggesting a role for other E3s in this process. Cul5 was recently shown to fulfill this role [13]. Based on our previous studies in a yeast model system [12], UBR1 also appeared to be a good candidate for such an E3, and the results of our studies shown above suggest that this is the case. For example, there are increased levels of protein kinases in cells deleted for Ubr1 after treatment with two different Hsp90 inhibitors, and this correlates with increased viability of the treated cells. Although we failed to observe similar effects of UBR2 on protein kinase stability, we noted that the Ubr2^{-/-} cells were moderately resistant to GA and PU-H71 with respect to viability. This may represent substrate specificity between these two ubiquitin ligases.

Our findings, however, are not so straightforward that we can propose a simple and direct effect of UBR1 on protein kinase ubiquitylation. For example, GA promotes rapid degradation of both Akt and Cdk4 very soon after administration in both wild type and Ubr1^{-/-} MEFs. What distinguishes Ubr1^{-/-} cells is that the effect wears off after ~18 hours resulting in a resurgence of Akt and Cdk4 levels. This resurgence does not occur in cycloheximide treated cells confirming that the increase in kinase levels represents new synthesis rather than resolubilization from an aggregated state (Fig. 3). The acquired resistance phenotype of Ubr1^{-/-} cells does not reflect metabolism of the drug, since it occurs with two distinct Hsp90 inhibitors (Fig. 4). Each drug also appears to enter cells efficiently, since the initial response is robust (Fig. 1). The resistance of Ubr1^{-/-} MEF cells to Hsp90 inhibitors was also observed in BT474 breast cancer cells with knocked down UBR1, suggesting the effect is related to the E3 levels. These combined findings suggest that UBR1 acts directly or indirectly to help in the reprogramming of the proteostasis network to promote efficient clearance of client protein kinases when Hsp90 is inhibited. In the absence of UBR1, the effect of Hsp90 inhibitors is blunted. One possible mechanism by which this reprogramming might take place relates to our finding that Hsp90 levels were more highly induced upon GA treatment in the Ubr1^{-/-} cells compared to wild type MEFs (Fig. 2). This induction could help to explain why GA becomes relatively ineffective. Furthermore, we noted that UBR1 levels are themselves sensitive to GA treatment in both human BT474 breast cancer cells and MEF cells (Fig. 6). These findings suggest that UBR1 is a client of Hsp90, thereby indicating the existence of a novel feedback loop, where UBR1 negatively controls Hsp90 expression, while Hsp90 controls UBR1 stability.

The model proposed above suggests that UBR1 acts to integrate the cellular response to Hsp90 inhibitors to generate a sustained effect. Our studies with CHIP further suggest that this effect is related to other components of the quality control apparatus. In wild type MEF cells there is little effect of CHIP knockdown on the sensitivity of the cells to GA treatment

(Fig. 7). One possibility is that CHIP is redundant with UBR1, and this is supported by the finding that knockdown of CHIP in the *Ubr1*^{-/-} cells led to reduced protein kinase degradation in the presence of GA. We therefore propose that while CHIP and UBR1 have some functional overlap with respect to their E3 activities, UBR1 also affects the function of the Hsp90 chaperone machinery. This hypothesis could also reflect the existence of distinct pools of Hsp90 that differ in their responsiveness to the inhibitors. Indeed, Kamal et al [29] showed that Hsp90 in cancer cells have a 100-fold higher affinity for both ATP and inhibitors, and was more highly organized into complexes with co-chaperones. Furthermore, co-chaperones themselves can affect the sensitivity of cells to Hsp90 inhibitors. Aha1, for example, stimulates Hsp90's ATPase and its knockdown results in increased cellular sensitivity to Hsp90 inhibitors [30]. Overexpression of Aha1 was also recently shown to suppress the effect of mutating Thr22 in Hsp90, which resides in the ATPase domain and is phosphorylated by Casein kinase II [31]. The complex interplay between co-chaperone activity and chaperone post-translational modification can therefore result in changes to the cellular sensitivity of Hsp90 inhibitors. The mechanisms by which UBR1 affects this process in association with other E3s such as CHIP remain to be understood.

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Figure-1. Ubr1^{-/-} cells are less sensitive to treatment with geldanamycin

A. WT and Ubr1^{-/-} MEF cells were treated with 1μM of GA for different times. 20 μg of total protein from each cell line was fractionated by SDS-PAGE and probed with antisera for total Akt (t-Akt), Cdk4 and Actin. **B** and **C.** Quantification of t-Akt (**B**) and Cdk4(**C**) levels in extracts from cells treated with GA. n = 5 +/- standard error. Statistical analysis of the difference between WT and UBR1^{-/-} at a given concentration was calculated by paired t test (* p<.05 and ** p<.005) **D.** Ubr2^{-/-} and Ubr1^{-/-} Ubr2^{-/-} MEF cells were treated with 1μM of GA for the indicated time points. Extracts were probed with antisera to Akt (t-Akt), Cdk4 and Actin. **E.** All four MEF cell lines were treated with different concentrations of GA

for 24 hours. 20 µg of total protein from each cell line was fractionated by SDS-PAGE and probed for t-Akt, phospho-Akt (pAkt; S473) and Actin.

Figure-2. Induction of Hsp70 and Hsp90 in Ubr1^{-/-}, Ubr2^{-/-} and double knockout Cells
Cells as indicated were treated with 1 μM GA for the times indicated and analyzed by Western blot for the levels of Hsp70 and Hsp90. Phosphatidyl inositol 3 kinase (PI3K) was used as a loading control

Figure-3. Effect of Cycloheximide on Protein Kinase levels in Ubr1^{-/-} Cells

A. Schematic of experimental approach. Cells were treated with GA for 12 hours before subsequent treatment with or without cycloheximide. Aliquots of cells were taken at 15, 18 and 20 hours after initial geldanamycin treatment for Western blot analysis. **B.** Western blot analysis of total Akt (t-Akt), Cdk4 and actin (loading control).

Figure-4. Effects of the Hsp90 inhibitor PU-H71

A. WT and Ubr1^{-/-} cells were treated for 24 hours with indicated concentrations of PU-H71 and cell extracts from each cell line analyzed by western blot for t-Akt, p-Akt (S473), Cdk4 and Actin. **B.** Time course analysis of WT and UBR1^{-/-} MEF cells after treatment with 500 nM of PU-H71. Western blot for t-Akt, p-Akt (S473), Cdk4 and Raf-1. Actin was used as a loading control.

Figure-5. UBR1 and UBR2 promote sensitivity to Hsp90 inhibitors

A. WT, Ubr1^{-/-}, Ubr2^{-/-} and Ubr1^{-/-} Ubr2^{-/-} cells were treated with indicated concentrations of GA for 24 hours and cell viability was measured as described in materials and methods. B. Same as A except the Hsp90 inhibitor used was PU-H71. C. Ubr1^{-/-} cells were mock transfected (grey bars) and transfected with a plasmid encoding rat Ubr1 (rUbr1; black bars). After 22 hours of transfection, cells were treated with different concentrations of GA for 24 hours and cell viability was analyzed as in A. Experiments were performed in quadruplicate and the bars represent the mean from three independent experiments. Error bars indicate standard error. Statistical analysis of the difference between WT and UBR1^{-/-},

UBR2^{-/-}, Ubr1^{-/-} Ubr2^{-/-} (A and B), mock and rUbr1 transfected cells (C) at a given concentration was calculated by paired t test (* p<.05 and ** p<.005).

Figure-6. Effects of UBR1 knockdown in the breast cancer cell line BT474 and wild type MEF cells upon Hsp90 inhibition

A. BT474 cells were stably transfected with control and UBR1 shRNA. Transfected cells were treated with 250 nM GA for 0, 6, 12, 18, 24, and 36 hours. Cell extracts were probed with antisera to p-Akt (Ser 473), Akt, Cdk4, ErbB2, UBR1 and Actin. **B.** Effect of siRNA knockdown of Ubr1 in MEF cells. Panels show Western blot analysis of total Akt (t-Akt), Ubr1 and actin as a loading control after 24 hours of GA treatment at the concentrations indicated. **C.** Quantification of the levels of total Akt in control and Ubr1 siRNA treated cells after geldanamycin treatment. N =3 +/- SE; *p <0.05.

Figure-7. Functional relationship between CHIP and UBR1

A. WT and Ubr1^{-/-} cells were transfected with control and CHIP siRNA. After 22 hours of transfection, cells were treated with different concentrations of GA and DMSO for 24 hours. 20 μg of total proteins was fractionated by SDS-PAGE and probed with CHIP, Akt, pAkt, Cdk4, and Actin. **B.** Quantification of t-Akt normalized against Actin. The bars show the remaining amounts of t-Akt after GA treatment in 3 independent experiments. Error bars indicate the standard error (SE). **C.** Quantification of Cdk4 level. Details as in B. Statistical analysis of the difference between Ubr1^{-/-} plus Control siRNA and Ubr1^{-/-} +CHIP siRNA at a given concentration was calculated by unpaired t test (* p<.05, ** p<.005 and *** p<.0005).