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ORIGINAL ARTICLE

C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances

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Heat shock proteins (Hsp)90 and Hsp70 facilitate protein folding but can also direct proteins for ubiquitin-mediated degradation. The mechanisms regulating these opposite activities involve Hsp binding to co-chaperones including CHIP and HOP at their C-termini. We demonstrated that the extreme C-termini of Hsp70 and Hsp90 contain phosphorylation sites targeted by kinases including CK1, CK2 and GSK3-β *in vitro*. The phosphorylation of Hsp90 and Hsp70 prevents binding to CHIP and thus enhances binding to HOP. Highly proliferative cells contain phosphorylated chaperones in complex with HOP and phospho-mimetic and non-phosphorylable Hsp mutant proteins show that phosphorylation is directly associated with increased proliferation rate. We also demonstrate that primary human cancers contain high levels of phosphorylated chaperones and show increased levels of HOP protein and mRNA. These data identify C-terminal phosphorylation of Hsp70 and Hsp90 as a switch for regulating co-chaperone binding and indicate that cancer cells possess an elevated protein folding environment by the concerted action of co-chaperone expression and chaperone modifications. In addition to identifying the pathway responsible for regulating chaperone-mediated protein folding/degradation balances in normal cells, the data provide novel mechanisms to account for the aberrant chaperone activities observed in human cancer cells and have implications for the application of anti-chaperone therapies in cancer treatment.

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INTRODUCTION

Chaperones are required for the folding and conformational regulation of proteins. Two of the major cytosolic chaperones, heat shock protein (Hsp)70 and Hsp90, are particularly responsible for the production of signal transduction proteins and cell cycle regulators.[1–3] However, their roles extend beyond the correct folding of client proteins to regulate degradation of unfolded clients.[3,4] These degradation and folding properties of chaperones are essential to maintain native proteins and prevent aggregation of denatured proteins.[5] To achieve folding or degradation of client proteins, Hsp90 and Hsp70 cooperate with co-chaperones and Hsp90 is known to interact with at least 20 co-chaperones that are variously involved in coordinating the interaction of Hsp90 with Hsp70, in regulating the ATPase activity of Hsp90, and in the recruitment of specific client proteins into the chaperone system.[6]

Although co-chaperones may bind to the N-terminal or middle regions of HSPs, the largest group of co-chaperones bind to the highly conserved sequence EEVD-COOH in the C-terminal domains of Hsp90 and Hsp70.[7–9] These co-chaperones contain a so-called tetratricopeptide repeat (TPR) domain that interacts with this C-terminal sequence. Within the group of TPR-containing co-chaperones are proteins that can assist with protein folding activity, such as HOP, or that have ubiquitin ligase activity, such as CHIP. Thus, the balance between the binding of Hsp's to these co-chaperones is a potential mechanism that may contribute to the regulation of whether proteins are folded and stabilized or are targeted for degradation.[10,11] Such a process must be highly regulated to ensure correct cell functionality, but how the dynamic interactions between chaperones and TPR-containing co-chaperones are regulated are unclear.

Here, we provide evidence that chaperone binding to co-chaperones is a regulated process and searched for posttranslational modifications in the C-terminus of Hsp70 and Hsp90 that regulate binding to the CHIP or HOP TPR domains. Our results show that phosphorylation of the C-terminal region of both chaperones is critical for the decision between folding or degradation of client proteins by altering the binding of CHIP and HOP. We also show increased phosphorylation in cancer cells together with increased levels of the co-chaperone HOP. The data define a physiological pathway for the regulation of protein folding of importance for normal cellular growth and for cancer.

RESULTS

Prediction of phosphorylation of C-terminus of Hsp70 and Hsp90

As it is known that the C-terminal regions of Hsp70 and Hsp90 are critical for binding to the TPR domain of co-chaperones, we scanned this region and identified a highly homologous region at the extreme C-terminus of both Hsp70 and Hsp90 that contains serine and threonine residues that are potential phosphorylation sites for CK2[12] (Figures 1a and b). The evolutionarily younger Hsp90α protein contains a second potential phosphorylation site that is not present in vertebrate Hsp90β or in Hsp90 of non-vertebrate species. We also used NetPhosK 1.0 and NetPhos 2.0

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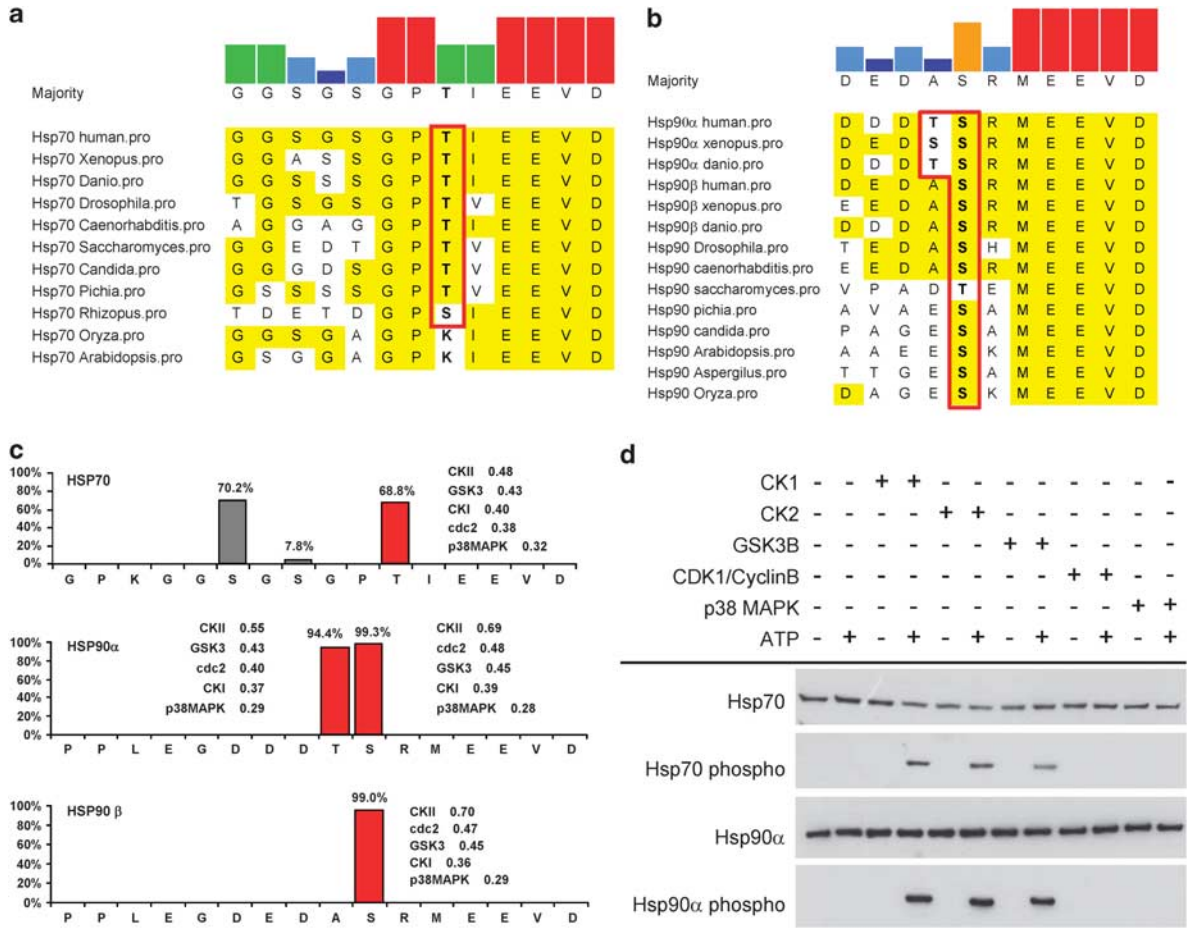

Figure 1. Phosphorylation of Hsp70 and Hsp90 C-terminus. (**a**) Alignment of eukaryotic Hsp90 sequences. Potentially phosphorylated serines or threonines at seventh and eighth position from C-terminus of Hsp90 are lined with red. (**b**) Alignment of eukaryotic Hsp90 sequences. Eukaryotic Hsp70, except green plants, contains serine or threonine at sixth position from its C-terminus. (**c**) Prediction of phosphorylation of C-terminus of Hsp70 and Hsp90. The graph shows the probability of phosphorylation of C-terminal residues of Hsp70 and Hsp90 based on neural network prediction. The index 0–1 of listed kinases reflects the probability that the kinase phosphorylates the serine or threonine. (**d**) *In vitro* phosphorylation of recombinant Hsp70 and Hsp90α protein. The phosphorylation of Hsp70 and Hsp90α was detected using phospho-specific monoclonal antibodies GGS2.1 and GDD8.2, respectively.

Server, which uses artificial neural network methods,[13] to provide additional evidence for potential phosphorylation of these sites in the C-terminus of Hsp70 and Hsp90. Figure 1c shows that the probability of phosphorylation of C-terminal residues of Hsp70 and Hsp90 is between 60 and 99% and it is predicted that Hsp90α will be phosphorylated at both sites. NetPhos 1.0 also predicted that CK1 and GSK3-β are capable of phosphorylating the C-termini of Hsp70 and Hsp90.

CK1, CK2 and GSK3-β phosphorylate Hsp70 and Hsp90 *in vitro*

To investigate whether CK2 can phosphorylate the C-terminus of Hsp70 and/or Hsp90, we performed *in vitro* phosphorylation assays using recombinant Hsp70 and Hsp90α and CK2. We used mass spectrometry coupled with sequential proteolytic digestion (Supplementary Data S2) to detect C-terminal phosphorylation. The MS/MS analysis of recombinant Hsp90α phosphorylated by CK2 *in vitro* confirmed that both serines and threonines in close proximity to the EEVD sequence are phosphorylated. Using mass spectrometry we could not detect the C-terminal peptide of Hsp70. Therefore, we developed monoclonal antibodies against phosphorylated peptides specific for either Hsp70 or Hsp90. These monoclonal antibodies were rigorously tested for specificity to recognize C-terminal phosphorylation of Hsp70 or of Hsp90α and were shown to be highly selective for the phosphorylated forms (see Supplementary Data S1). The purified monoclonal antibodies were also used to demonstrate *in vitro* phosphorylation of C-terminal Hsp70 and Hsp90α by CK1, CK2, GSK3β, but not by p38 mitogen-activated protein kinase (MAPK) or cyclin-dependent kinase (CDK)1/cyclinB (Figure 1d).

Phosphorylation of Hsp70 and Hsp90 alters their binding to co-chaperones

To test the influence of C-terminal phosphorylation of Hsp70 and Hsp90 on their ability to bind HOP and CHIP, biotin-labeled Hsp70 and Hsp90 peptides and phosphopeptides were adsorbed to streptavidin-coated plates and recombinant glutathione *S*-transferase (GST)-tagged HOP and CHIP proteins were added. Figure 2a shows that the non-phosphorylated C-terminal peptides of both Hsp70 and Hsp90 interact more strongly with CHIP than the phosphorylated forms of these peptides. In contrast, HOP binds more strongly to the phosphorylated peptide of Hsp90α although the interaction between Hsp70 and HOP was not significantly influenced by phosphorylation (Figure 2b). As an independent method to assess the strength of binding of CHIP and HOP to the C-terminal phosphorylated forms of Hsp70 and Hsp90, we used fluorescence polarization assay to confirm the above results

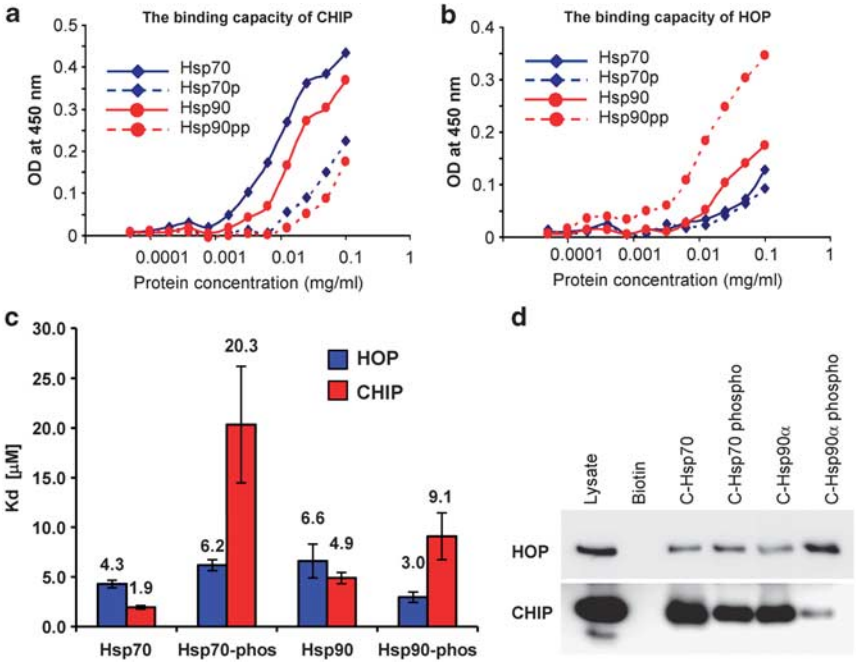

Figure 2. The impact of C-terminal phosphorylation of Hsp70 and Hsp90 on binding of CHIP and HOP proteins. Enzyme-linked immunosorbent assay analysis of complex formation between C-terminal peptides of Hsp70 and Hsp90 with CHIP and HOP proteins. Streptavidin-coated plates were incubated with biotinylated peptides corresponding to phosphorylated and non-phosphorylated C-terminal region of Hsp70 and Hsp90. Purified recombinant protein GST-CHIP (**a**) and GST-HOP (**b**) were tested for interaction with these peptides. (**c**) Equilibrium dissociation constants between proteins HOP and CHIP and phosphorylated or non-phosphorylated fluorescent peptides were measured by fluorescence polarization binding assay. (**d**) Peptide pull-down assay of CHIP and HOP protein from cell lysate. Biotinylated phospho- and non-phosphorylated C-terminal peptides of Hsp70 and Hsp90 were incubated with A375 cell lysates. The figure shows the level of HOP and CHIP in cell lysate and their interaction with C-terminal peptides of Hsp70 and Hsp90 origin. HOP and CHIP proteins were detected by monoclonal antibodies HOP1.1 and CHIP3.1.

and to quantify the dissociation constant between Hsp's C-terminal peptides and HOP and CHIP proteins (Supplementary Data S3). The data from fluorescence polarization are consistent with data obtained by enzyme-linked immunosorbent assay. The calculated dissociation constants indicate that phosphorylated Hsp70 has an ~20-fold lower affinity for CHIP than non-phospho-Hsp70, whereas the affinity of HOP for Hsp70 is not increased by phosphorylation of Hsp70. For Hsp90, the phosphorylated C-terminal peptide shows both a higher affinity for HOP and a lower affinity for CHIP (Figure 2c). To test whether these observations are also seen *in vivo*, A375 cell lysates were incubated with magnetic beads coated with peptides corresponding to the C-terminus of Hsp70 and Hsp90 and the amounts of bound CHIP and HOP measured by western blotting. As with the *in vitro* data, CHIP preferentially binds to the non-phosphorylated C-terminal peptides of both Hsp70 and Hsp90, whereas HOP is preferentially bound to the phosphorylated C-terminal peptide of Hsp90 and phosphorylated and non-phosphorylated Hsp70 peptides bind equivalent levels of HOP (Figure 2d). These results indicate that the abilities of CHIP and HOP to bind Hsp70 and Hsp90 are influenced by C-terminal phosphorylation of the chaperones.

Structural comparison of the HOP and CHIP TPR domains

To address the question of how the interactions between CHIP and the C-termini of Hsp70 and Hsp90 are disrupted by C-terminal phosphorylation, we compared the crystal structures of the HOP TPR (PDB: 1ELW and 1ELR) and CHIP TPR (PDB: 2C2L) domains.[14,15] Despite limited amino-acid sequence similarity, the alignment of CHIP TPR with HOP TPR1 and TPR2A shows high structural similarity, especially in the N-terminal parts of the domain that are responsible for binding the most C-terminal part of EEVD-COOH peptides.[16] Figure 3a demonstrates the conserved basic residues that are responsible for anchorage of the C-terminal carboxyl groups of the EEVD domain. The first five helices of the TPR domains contain conservative basic residues responsible for interaction with C-termini of the EEVD domains. However, the structure of the TPR domain of CHIP differs from HOP in the C-terminal part. The most prominent difference is loop 6, which connects helix 6 and 7 of the TPR domain of CHIP and also interacts with serine and threonine of the Hsp90 C-terminus. This loop is longer than the corresponding loop of HOP TPR1 or TPR2A and protrudes into the groove of the TPR domain in CHIP (Figure 3b). Most importantly, the asparagine 131 of loop 6 in the CHIP TPR is able to form hydrogen bonds with the C-terminal serine and threonine of Hsp90α. Phosphorylation of the C-terminal serine and threonine of Hsp90 or Hsp70 will prevent the formation of hydrogen bonds with loop 6 of the CHIP TPR domain and disrupt protein binding.

With regard to the regulation of binding to HOP, phosphorylated groups in Hsp90 can contact one or more of the basic groups in the groove of the elongated HOP TPR. Among the basic groups protruding into the space in close proximity to the phosphorylated serine and threonine of Hsp90 are lysines 237, 238 and 239 of the human HOP TPR2A domain. Thus, phosphorylation of Hsp90 is predicted to enhance binding to these lysines in the HOP TPR2A domain (Figure 3b). Despite an overall similarity of structure in the TPR domains of HOP and CHIP, these three lysines in loop 1 of HOP TPR2A are not present in the corresponding parts of CHIP TPR and HOP TPR1, so that phosphorylation of Hsp90 or Hsp70 is predicted not to increase the interaction with CHIP or with the TPR1 domain of HOP, to which Hsp70 binds. Thus, phosphorylation is predicted to enhance Hsp90 binding to HOP but not to directly influence binding of Hsp70, in keeping with the findings above.

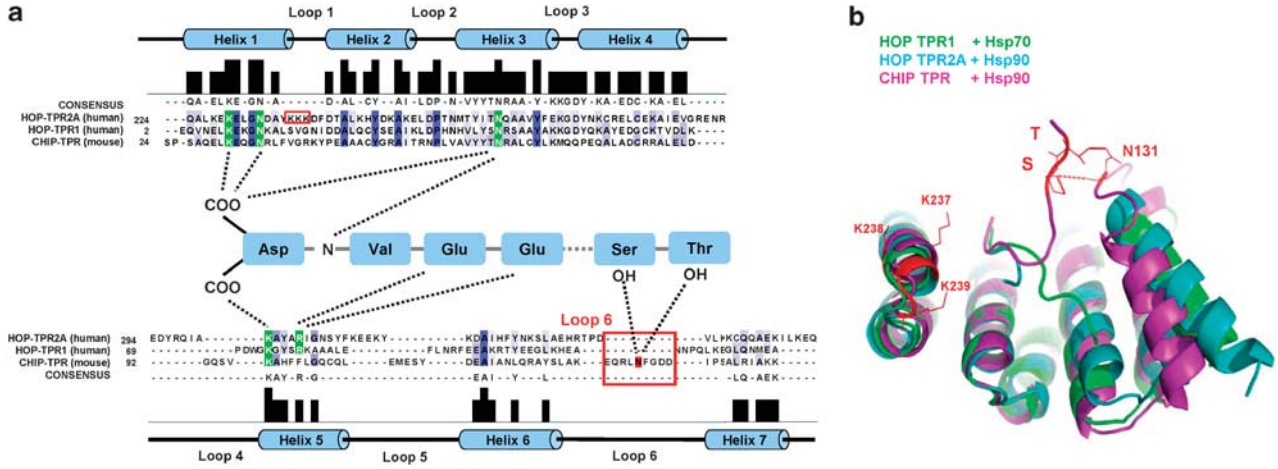

Figure 3. Structural alignment of the CHIP TPR, HOP TPR1 and HOP TPR2A domains. (**a**) The alignment of TPR domains shows the conservative residues responsible for binding of EEVD of Hsp70 or Hsp90. The loop 6 of protein CHIP TPR domain is highlighted to show the hydrogen bonds between asparagine and C-terminal serine and threonine of Hsp90α. (**b**) Alignment of PDB structures 2c2L (complex between CHIP TPR and Hsp90α peptide DTSRMEEVD), 1ELR (complex between HOP TPR2A and Hsp90 peptide AceMEEVD) and 1ELW (complex between HOP TPR1 and Hsp70 peptide GPTIEEVD). The lysines K238-239 of HOP TPR2A are highlighted.

Hsp C-terminal phosphorylations are higher in proliferating cells

These data demonstrate that phosphorylation of the C-termini of Hsp70 and Hsp90 alters their binding to co-chaperones with opposite functions in protein folding and stability. Proliferating cells are known to require high levels of protein synthesis to allow increased cell growth, reflected by an increase in ribosomal mass in proliferating cells compared with non-proliferating cells.[17] To assess the physiological relevance of our observations we therefore initially tested the association of Hsp C-terminal phosphorylation with proliferative state in A375 melanoma cells cultured in medium containing 10% fetal bovine serum or without serum. We performed dephosphorylation of each sample to demonstrate the specificity of the phospho-Hsp antibodies. Figure 4a shows that the level of C-terminal phosphorylation of both Hsp70 and Hsp90 is markedly increased in proliferating cells compared with serum-starved cells. The specificity of phospho-specific antibodies was also confirmed by the absence of signal following alkaline phosphatase treatment. These data indicate that C-terminal phosphorylation of Hsp70 and Hsp90 is altered according to cellular environment. Co-immunoprecipitation of Hsp70 or Hsp90 demonstrated that Hsp90 preferentially binds HOP in proliferating cells, in contrast to serum-starved cells in which both Hsp's preferentially complex with CHIP (Figures 4b and c). We also used proximity ligation assay as another independent method to confirm that Hsp90 and Hsp70 in proliferating cells preferentially bind HOP in contrast to CHIP (Figure 4d and Supplementary Data S4). These data indicate that Hsp binding to these two co-chaperones is a regulated process in cells.

C-terminal phosphorylation increases proliferation rate

To further investigate the physiological relevance of Hsp C-terminal phosphorylation in regulating cellular activities and the relationship with cell proliferation, we determined whether phosphorylation directly influences cell proliferation. We designed mutations of Hsp70, Hsp90α and Hsp90β to mimic the phosphorylated and non-phosphorylated status of their C-termini. Hsp70, Hsp90 and their phospho-mimetic variants were produced as N-terminal streptavidin-binding protein-tagged proteins in cells. Figure 5a shows that both co-chaperones CHIP and HOP specifically bind to Hsp70 and Hsp90 in contrast to GFP, which served as a negative control. The ratios between HOP and CHIP indicate their affinity for wild-type, non-phospho- or phospho-mimetic forms of Hsp70, Hsp90α and Hsp90β. Phosphorylated Hsp90 has increased affinity to HOP compared with non-phosphorylated forms of Hsp90 proteins, whereas CHIP has higher affinity for non-phosphorylated than for the negatively charged phosphorylated C-terminus of both Hsp70 and Hsp90. To investigate the effects of C-terminal Hsp90 phosphorylation on proliferation, we transfected cells with non-tagged Hsp70, Hsp90α and Hsp90β and their phospho-mimetic mutants. Expression of the non-phosphorylable Hsp70 and Hsp90 mutants significantly prolonged the doubling time compared with cells transfected with wild-type protein ($P<0.001$ for each), whereas expression of phospho-mimetic mutants of Hsp90 reduced the doubling time ($P<0.01$ for Hsp90α and Hsp90β phospho mimetics; Figure 5b). Doubling times and statistical testing were computed from exponential growth curves described in Supplementary Data S5.

The regulation of C-terminal phosphorylation of Hsp70 and Hsp90 *in vivo* involves numerous kinases and phosphatase activity

We had identified that CK1, CK2 and GSK3β are able to directly phosphorylate the C-terminus of Hsp70 and Hsp90 *in vitro*. To investigate whether other kinases are responsible for C-terminal phosphorylation *in vivo*, we screened a panel of kinase inhibitors applied to proliferating cells and measured C-terminal phosphorylation of Hsp90 and Hsp70 by western blotting with phospho-specific monoclonal antibodies (Figure 6a). Inhibition of phosphatidylinositol 3-kinase, mitogen-activated protein kinase)/extracellular signal-regulated kinase or AKT but not tyrosine kinase or CDKs reduced C-terminal Hsp90 phosphorylation, while Hsp70 phosphorylation was reduced in the presence of phosphatidylinositol 3-kinase inhibitors only. Treatment with a specific inhibitor of p38MAPK, SB202190, was a very effective inhibitor of Hsp90α C-terminal phosphorylation and of Hsp70 to a lesser extent (Figure 6b). These data identify a complexity of the regulation of Hsp70 and Hsp90 C-terminal phosphorylation that is modified by a variety of kinases, and a prominent role for p38MAPK in selectively regulating the C-terminal phosphorylation of Hsp90, despite the inability of p38MAPK to directly phosphorylate these residues (Figure 1d).

To investigate whether the *in vivo* phosphorylation status of Hsp70 and Hsp90 is also subject to regulation by protein phosphatases we treated cells with serine/threonine phosphatase inhibitors Calyculin A (inhibits PP1 and PP2A) and Okadaic acid

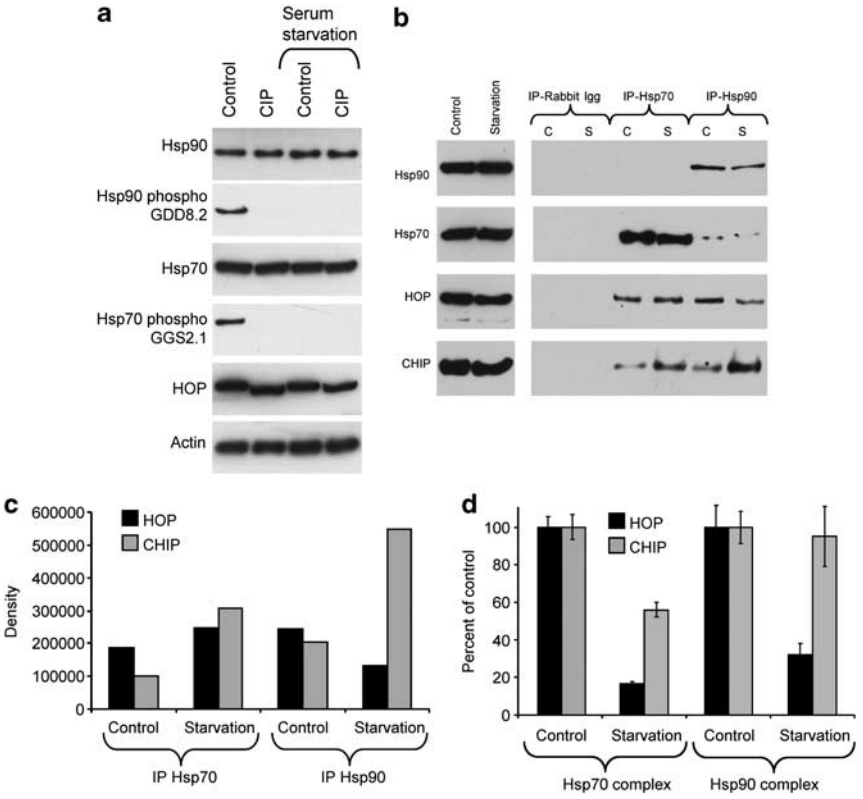

Figure 4. Phosphorylation of Hsp70 and Hsp90 are regulated by proliferation. (**a**) The lysates of proliferating cells or cells grown without serum were treated by alkaline phosphatase. The C-terminal phosphorylations of Hsp70 and Hsp90α were detected by phospho-specific monoclonal antibodies GGS2.1 and GDD8.2. (**b**) Differential binding of chaperones to co-chaperones in proliferating and serum-starved cells. Co-immunoprecipitation of complexes between Hsp's and CHIP and HOP using rabbit polyclonal antibodies recognizing Hsp70 or Hsp90. (**c**) The intensity of CHIP and HOP was quantified by densitometry. (**d**) The individual complexes of Hsp70-CHIP, HSP70-HOP, Hsp90-HOP and Hsp90-CHIP were analyzed using proximity ligation assay *in situ* and rolling circle amplification. The graph shows the mean number of signals per cell.

(selective for PP2A). Figure 6c shows that treatment with Calyculin A in contrast to Okadaic acid increased the steady state levels of C-terminal phosphorylation of both Hsp70 and Hsp90, indicating a selective role for PP1 in regulating Hsp C-terminal phosphorylation *in vivo*.

Inhibiting phosphorylation synergizes with Hsp90 inhibition to reduce client protein stability

Our data indicate that C-terminal phosphorylation prevents the interaction of Hsp's with the ubiquitin ligase CHIP and should therefore prolong the half-life of Hsp90 clients by inhibiting their degradation. In particular, C-terminal phosphorylation should restrict degradation after inhibition of Hsp90 by 17AAG. To investigate this possibility, we initially analyzed the stability of Hsp90 clients in T47D breast cancer cells that express estrogen receptor, CDK4 and mutant p53, all well-studied Hsp90 client proteins. Figure 7a shows that inhibiting Hsp90 with 17-AAG reduces the levels of these three client proteins, confirming the involvement of Hsp90 in regulating their production and stability. Inhibition of p38 MAPK by SB202190 reduces Hsp90 phosphorylation and reduces the levels of client proteins. Most notably, combined treatment causes a further reduction in protein levels, indicating synergistic effects of inhibiting Hsp90 phosphorylation and inhibiting Hsp90 activity.

However, these experiments only show an association between phosphorylation and client protein levels and do not show a direct role for C-terminal phosphorylation. Therefore, we performed experiments with point mutants of Hsp70 and Hsp90α to directly investigate the effect of C-terminal phosphorylation on client protein stability. For these experiments, we used the p53 mutant R175H as a model client protein that relies on Hsp90 for stability.[18] The p53-null cell line H1299 was co-transfected with p53 mutant R175H, Hsp70, Hsp90α and their phospho-mimetic or non-phosphorylable mutants. Figure 7b shows that the level of p53 mutant is decreased when coexpressed with non-phosphorylable alanine mutants of Hsp70 and Hsp90α but is stabilized in the presence of phospho-mimetic mutants.

Primary cancers show altered C-terminal phosphorylated chaperones and increased levels of HOP

The previous experiments had shown that C-terminal phosphorylation of Hsp70 and Hsp90 induces preferential binding of HOP and directly influences growth of tumor cells. To detect differences in phosphorylation of Hsp's in primary tumors, we used surgically removed human breast tissues from which we took a sample of healthy and cancerous tissue. Western blotting shows that both cancer and normal cells express comparable levels of Hsp70 and Hsp90α. However, the C-terminal phosphorylation of Hsp's is significantly increased in cancer cells compared with normal breast tissue (Figure 8a). Previous work had suggested that co-chaperone balances may be disrupted in cancer cells,[19] prompting us to investigate whether the levels of CHIP or HOP are different between normal and tumor tissues. Unlike previous data,[19] we did not find decreased levels of CHIP protein in cancer compared with normal tissues but levels of HOP were considerably increased in cancer cells. We also interrogated the expression profiling of cancer tissues contained with the Oncomine database[20] for altered expression of CHIP and HOP

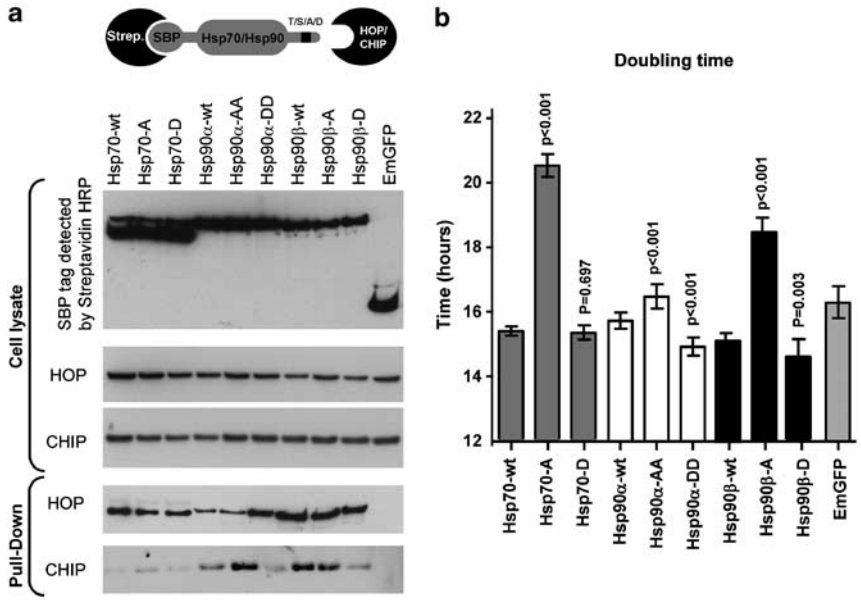

Figure 5. Point mutations of C-terminal serine and threonine of Hsp70, Hsp90α and Hsp90β influence co-chaperone binding and cell proliferation. (**a**) Pull-down assay of complexes between Hsp90 and Hsp70 phospho-mimetic mutants and co-chaperones HOP and CHIP. Streptavidin-binding protein (SBP)-tagged GFP, Hsp70 wild-type, non-phosphorylable Hsp70 mutant T636A (Hsp70A), phospho-mimetic Hsp70 mutant T636D (Hsp70D), Hsp90α wild-type, non-phosphorylable Hsp90α mutant T725A, S726A (Hsp90α AA), phospho-mimetic Hsp90α mutant T725D, S726D (Hsp90α DD), Hsp90β wild-type, non-phosphorylable Hsp90β mutant S718A (Hsp90βA) and phospho-mimetic Hsp90β mutant S718D (Hsp90βD) were transfected into HEK-293 cells to perform pull-down assays on streptavidin sepharose. The interaction of these proteins with HOP and CHIP was assessed by monoclonal antibodies against CHIP and HOP using western blotting. (**b**) The effect of point mutations of Hsp70 and Hsp90 was assessed in transfected HEK293 cells using the xCELLigence System monitoring cell proliferation in real time. The graph shows the doubling time extrapolated from growth curve. The *P*-value shows the statistical significance of difference between point mutants and appropriate wild-type.

mRNAs. These data confirmed that HOP (*STIP1*) mRNA is overexpressed in many human cancers compared with corresponding normal tissues (melanoma, bladder, gastric, lung, breast and pancreas), whereas the levels of CHIP (*STUB1*) mRNA are not commonly altered (Supplementary Material S6). We also showed that the levels of HOP mRNA are higher in proliferating cells compared with serum-starved cells ($P<0.05$) but there was no difference in CHIP mRNA levels (Figure 8b).

DISCUSSION

Chaperones act to promote the folding and activation of polypeptide substrates, but are also involved in targeting proteins for degradation.[3,4] Given their crucial importance for cellular function, chaperone activities must be tightly regulated according to cellular requirements for proliferation and survival. Previous data have shown that Hsp90 is phosphorylated in the N-terminal and linker regions by protein kinases including CK2, Akt, Wee1 and DNA-dependent protein kinase.[21–23] CK2 also phosphorylates the co-chaperone Cdc37 to enhance the interaction with Hsp90, leading to increased activity of numerous kinases that are specifically chaperoned by Cdc37 and have pro-proliferative actions.[21]

However, numerous co-chaperones with different activities bind to the C-termini of Hsp70 and Hsp90 through the TPR domains of the co-chaperones. Two such C-terminal binding co-chaperones, CHIP and HOP, represent a paradigm for the overall protein folding or protein degradation activities of Hsp70/Hsp90.[24] HOP is important in coordinating protein folding and binds Hsp70 and Hsp90 together only when Hsp90 is in an open, client-free state.[25,26] In contrast, the co-chaperone CHIP targets client proteins for degradation. Thus, we hypothesized that interactions between the Hsp's and TPR-domain co-chaperones represent an important regulator of chaperone activities.

Initially, we predicted that evolutionarily conserved phosphorylation sites in the C-termini of Hsp70 and Hsp90 might influence differential binding activities and showed that the interactions of Hsp70 and Hsp90 with CHIP and HOP are altered by different growth conditions (Figure 9). We have shown that a variety of kinases including CK1, CK1 and GSK3-β are able to directly phosphorylate the C-termini of Hsp70 and Hsp90 *in vitro* and that p38MAPK also regulates the levels of C-terminal phosphorylated Hsps *in vivo* but does not directly phosphorylate these residues *in vitro*. Together with the observations that phosphatases are involved in regulating phosphorylation levels *in vivo*, these findings indicate a complex regulation of C-terminal Hsp70 and Hsp90 phosphorylation that involves both direct and indirect regulatory factors, as would be expected for a fundamental physiological process involved in determining the overall protein production/degradation balance in cells. In view of this complexity, it will be a challenging but important task to identify the roles of these and potentially other kinases and phosphatases in Hsp C-terminal phosphorylation in different physiological and pathological states *in vivo*. However, to confirm the physiological relevance of Hsp C-terminal phosphorylation, we demonstrated that proliferating cells contain higher levels of C-terminally phosphorylated Hsp70 and Hsp90. Moreover, the introduction of phospho-mimetic or non-phosphorylatable Hsp70 and Hsp90 proteins demonstrated that C-terminal phosphorylation directly affects proliferation. As these forms are preferentially bound to HOP rather than CHIP, the data indicate that dividing cells exhibit a pro-folding environment, in keeping with the requirement for increased protein production in proliferating cells.[17] Finally, we demonstrated that Hsp70 and Hsp90 exist predominantly as C-terminal phosphorylated forms in primary tumor tissues compared with non-neoplastic tissue, suggesting that tumors exhibit a dominant folding environment. The importance of the

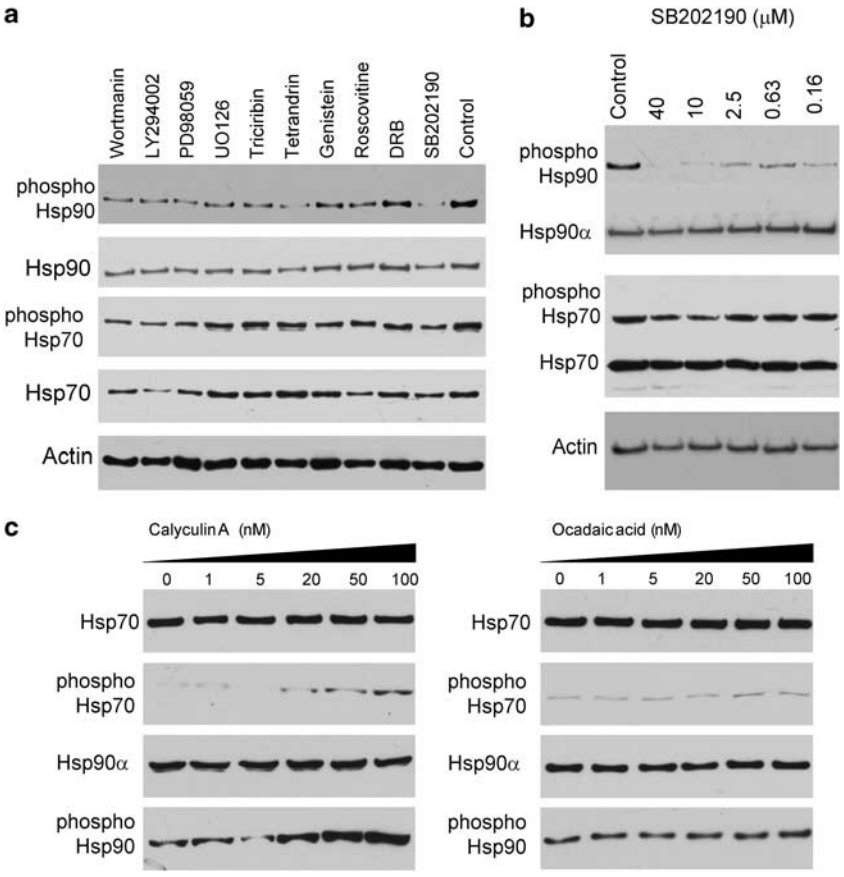

Figure 6. Inhibition of phosphatases and p38MAPK influence the phosphorylation of Hsp70 and Hsp90. (**a**) Detection of C-terminal phosphorylation in A375 cells treated with different protein kinase inhibitors for 8 h. (**b**) Detection of phosphorylation of Hsp70 and Hsp90α in A375 cells treated with different concentrations of SB202190. (**c**) Detection of C-terminal phosphorylation of Hsp70 and Hsp90α in A375 cells treated with different concentration of phosphatase inhibitors Calyculin A and Okadaic acid for 1 h.

altered interactions between Hsp70, Hsp90, CHIP and HOP in cancers is supported by evidence for additional mechanisms that disrupt this pathway. Thus, HOP mRNA and protein are higher in primary human cancers compared with matched normal tissues, reinforcing the pro-folding environment. In addition, CHIP has been suggested to function as a tumor suppressor and can inhibit metastatic spread.[19] Our data indicated that the levels of CHIP protein were lower in proliferating cells than in non-proliferating cells but mRNA levels were similar, suggesting posttranslational regulation. However, we could not confirm from our own data or from publicly available gene expression profiling databases that CHIP protein or mRNA levels are commonly lower in human cancers. Taken together, the data indicate that the upregulation of Hsp90 chaperone activity that is observed in cancer cells is related both to increased levels of HOP and the increased proportions of C-terminally phosphorylated Hsp70 and Hsp90, together with a reduced amount of Hsp90-bound CHIP. In some tumors, there may also be a reduction in CHIP levels to allow for a more folding environment.[19]

In conclusion, it is known that Hsp90α is selectively activated in human cancer and that cancer cells become addicted to increased chaperoning activity, such that cancers can be effectively and selectively treated with agents that inhibit Hsp90 or Hsp70.[27–29] We have identified that a key mechanism for the dynamic regulation of chaperone activity is C-terminal phosphorylation, which regulates binding to co-chaperones that either fold or degrade client proteins. These co-chaperones are themselves regulated, such that replicating tumor cells possess a dominant pro-folding environment and non-proliferating cells exhibit a dominant protein degradation phenotype (summarized in Table 1). In addition to influencing co-chaperone binding, it is possible that C-terminal phosphorylation of Hsps might influence other properties such as selective binding of clients and/or ATPase activity, either directly or indirectly. Finally, the data provide a framework both for understanding normal protein homeostasis and the disruptions that occur to chaperone activity in cancer, and allow for the identification of novel chemotherapies that can restore the normal balance of protein folding/degradation in cancer.

MATERIALS AND METHODS

Cell culture and treatment

Human cancer cell lines A375, H1299, T47D and HEK-293 were cultured in Dulbecco's modified Eagle's medium, 10% fetal bovine serum and 300 mg/l L-glutamine and penicillin/streptomycin. Cells were grown to 80% confluence before experimental treatments. In specified experiments serum was removed for 72 h to block the cell cycle at G_0 phase.

Antibodies

The antibodies used in the study are described in Supplementary Data S1.

Plasmid constructs

CHIP and HOP cDNAs were cloned into pDEST-15 that is designed to express the proteins containing an N-terminal fusion with GST in *Escherichia coli*. Hsp70 and Hsp90α cDNAs were cloned into pDEST17, which expresses the proteins with N-terminal 6 × His tag in *E. coli*. All

vectors designed for expression in *E. coli* contained TEV protease cleavage site ENLYFQG between N-terminal tag and first methionine.

The vectors designed for pull-down assay contained CMV promoter and streptavidin-binding protein tag MDEKTTGWRGGHVVEGLAGELEQLRARLE HHPQGQREPS[30] fused to N-terminus of EmGFP, Hsp's and their point mutants.

The phospho-mimetic and non-phosphorylable point mutants of Hsp70, Hsp90α and Hsp90β were constructed by changing their C-terminal serines or threonines to alanine (Hsp70A, Hsp90αAA and Hsp90βA) or to aspartic acid (Hsp70D, Hsp90αDD and Hsp90βD) using the QuikChange Site-Directed Mutagenesis Kit (Agilent). We used primer pair 5′-ctgggtcaggcccc gacattgaggaggtag-3′ and 5′-ctacctcctcaatgtcggggcctgacccag-3′ to make phospho-mimetic mutant of Hsp70 (T636D), primer pair 5′-ctgggtcaggcccc gccattgaggaggtag-3′ and 5′-ctacctcctcaatggcggggcctgacccag-3′ to make non-phosphorylable mutant of Hsp70 (T636A), primer pair 5′-gaaggagatga cgacgatgatcgcatggaagaagta-3′ and 5′-tacttcttccatgcgatcatcgtcgtcatctcct tc-3′ to make phospho-mimetic mutant of Hsp90α (T725D, S726D), primer pair 5′-gaaggagatgacgacgcagcacgcatggaagaagta-3′ and 5′-tacttcttc-catgcgtgctgcgtcgtcatctccttc-3′ to make non-phosphorylable mutant of Hsp90α (T725A, S726A), primer pair 5′-gcgatgaggatgcggaccgcatggaagaag-3′ and 5′-cttcttccatgcggtccgcatcctcatcgc-3′ to make phospho-mimetic mutant of Hsp90β (S718D) and primer pair 5′-gcgatgaggatgcggctcgcatg-gaagaag-3′ and 5′-cttcttccatgcgagccgcatcctcatcgc-3′ to make non-phos-phorylable mutant of Hsp90β (S718A).

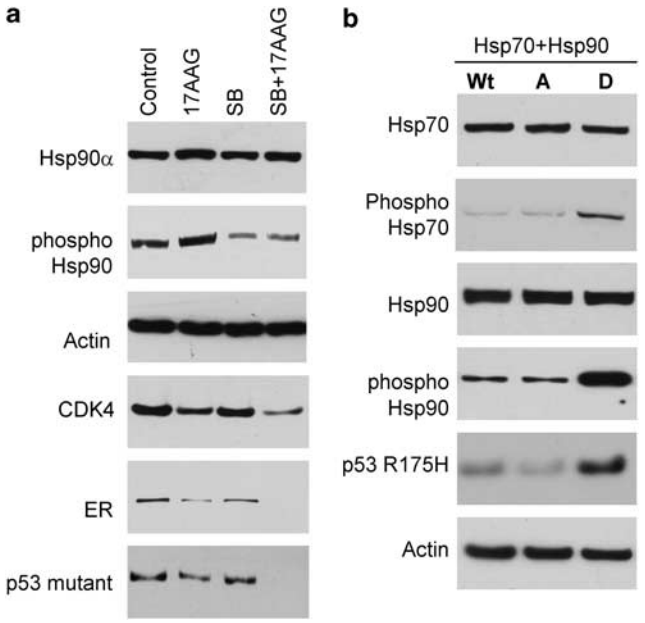

Figure 7. The effect of C-terminal phosphorylation on stability of Hsp90 clients. (**a**) The effect of inhibition of Hsp90 by 17AAG and inhibition of phosphorylation by SB202190 on the degradation of CDK4, mutant p53 and estrogen receptor in T47D cells. (**b**) The effect of point mutations mimicking phosphorylated or non-phosphorylated status of Hsp70 or Hsp90 C-terminus in p53-null H1299 cells. Mutant p53 was co-transfected with Hsp70 and Hsp90a and their point mutants. The levels of 53 were detected by DO-1 antibody. The phospho-mimetic mutants of Hsp70 and Hsp90 were detected by phospho-specific antibodies GGS2.1 and GDD8.2, respectively.

Enzyme-linked immunosorbent assay

Biotinylated peptides biotin-GGSGSPTIEEVD, biotin-GGSGSP(pT)IEEVD, biotin-GDDTSRMEEVD and biotin-GDDD(pT)(pS)RMEEVD correspond to the non-phosphorylated and phosphorylated C-termini of human Hsp70

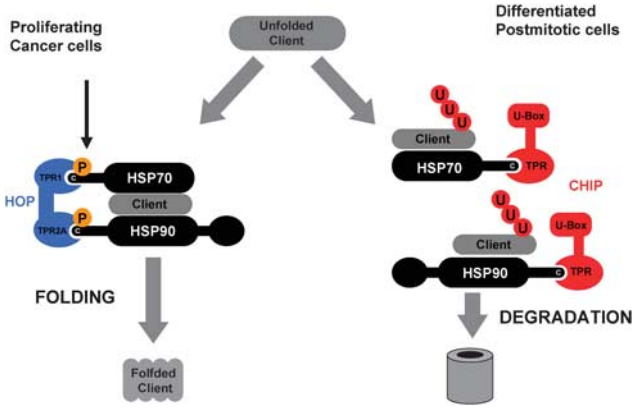

Figure 9. The balance between folding and degradation. The C-terminal phosphorylation of Hsp70 and hsp90 in proliferating cancer cells enhance the assembly with co-chaperone HOP. The non-phosphorylated chaperones preferentially bind CHIP mediating degradation of client proteins.

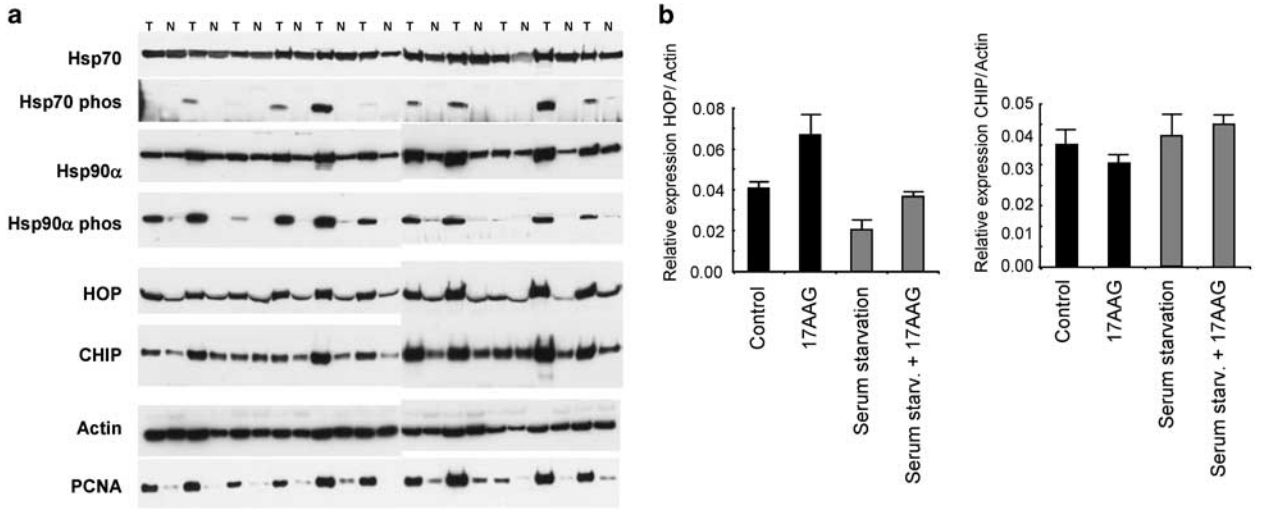

Figure 8. The chaperone status in cancer versus normal cells. (**a**) The expression levels of Hsp90α, Hsp70, their C-terminal phosphorylations and the expression levels of co-chaperone HOP and CHIP in primary human breast tumors (T) and paired normal tissues (N). Actin was detected as a loading control, proliferating cell nuclear antigen (PCNA) was detected as a marker of proliferation.

Table 1. Differences in Hsp70 and Hsp90 chaperones in normal and cancer cells

Proliferating cancer cells	*Differentiated post-mitotic cells*
Higher expression levels of Hsp70, Hsp90 and HOP	Lower expression of Hsp70, Hsp90 and HOP, higher expression of CHIP
C-terminal phosphorylation of Hsp70 and Hsp90	Dephosphorylation of Hsp70 and Hsp90 C-terminus
Hsp70 and Hsp90 preferentially bind HOP	Hsp70 and Hsp90 preferentially bind CHIP
Formation of multichaperone complexes	The connection of Hsp70 and Hsp90 is reduced
Stabilization and folding of oncogenic client proteins	Degradation of unfolded proteins
High folding capacity is essential to buffer genetic instability	Hsp-mediated degradation maintains protein homeostasis
Malignant transformation, proliferation and metastasis	Prevention of protein aggregation

(NP_005336) and non-phosphorylated and phosphorylated C-termini of human Hsp90α (NP_005339), respectively. Streptavidin-coated 96-well plates were incubated for 1 h with biotinylated peptides (50 μl per well at a final concentration 2 μM) dissolved in buffer-containing phosphate-buffered saline, 0.1% NP-40 and 3% immunoglobulin-free Albumin (Sigma A3803). The plates were washed three times using 200 μl washing buffer (phosphate-buffered saline + 0.1% Tween 20). The peptide-coated plates were incubated for 2 h with purified GST-tagged HOP and CHIP proteins. After three washes in washing buffer the protein binding was detected by horseradish peroxidase-conjugated anti-GST antibody diluted 1 μg/ml in buffer-containing phosphate-buffered saline, 0.1% Tween 20 and 3% Albumin. The signal was detected using the TMB Substrate Kit (Thermo Pierce) following the manufacturer instructions.

Fluorescence polarization binding assay

The equilibrium bindings between a fixed concentration (30 nM) of a fluorescent ligand and increasing concentrations of either HOP or CHIP in buffer containing 150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.2, 1 mM dithiothreitol and 0.05% Tween-20 were monitored by both total fluorescence intensity and fluorescence polarization on the plate reader Filtermax F5 (Molecular Devices). The ligands corresponding to C-terminal peptide of Hsp70 (GGSGSPTIEEVD), phospho-Hsp70 (GGSGSP(pT)IEEVD), Hsp90α (GDDTSRMEEVD) or phospho-Hsp90α (GDDD(pT)(pS)RMEEVD) were labeled by fluorescein on its N-terminus. The equilibrium dissociation constant (K_d) was then calculated by fitting the sigmoidal dose-dependent FP increases as a function of protein concentrations using Graphpad Prism.[31]

Pull-down assay

Pull-down assay of streptavidin-binding protein-tagged GFP, Hsp70, Hsp90α and Hsp90β was performed in H1299 and HEK-293 cells transfected with plasmids expressing the corresponding proteins or their point mutants. Cells were lysed in buffer containing 0.5% Tween 20, 150 mM NaCl, 50 mM HEPES (pH 7.2) and protease and phosphatase inhibitors. Cell lysate (200 μl) containing 200–800 μg of total protein was incubated with 15 μl of high capacity streptavidin agarose resin (Thermo Scientific) or with 50 μl of streptavidin-conjugated magnetic beads (Invitrogen). After three washes in lysis buffer, the beads were resuspended in 60 μl of NuPAGE LDS Sample Buffer (Invitrogen). A total of 8 μl of prepared samples were used for electrophoresis.

Co-immunoprecipitation

Cell pellets containing 10^7 were lysed in 600 μl lysis buffer containing 0.5% 3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate, 0.1% NP-40, 100 mM KCl, 50 mM Na-HEPES pH 7.3, 1 mM $MgCl_2$, phosphatase and protease inhibitor cocktails and 25 U Benzonase (Sigma-Aldrich). The cell lysate was sonicated and centrifuged at 14 000 *g* for 15 min. The supernatant was separated and the total protein concentration was adjusted to 5 mg/ml in each sample. Protein G Sepharose (GE Healthcare) was pre-incubated with excess rabbit polyclonal antibody and washed in washing buffer containing 0.1% NP-40, 100 mM KCl and 50 mM Na-HEPES pH 7.3. Protein lysate (180 μl) was incubated with 20 μl protein G sepharose for 2 h at 4 °C. The immunoprecipitates were washed four times in washing buffer (1 ml), eluted in 60 μl of NuPAGE LDS Sample Buffer (Invitrogen) and 10 μl used for electrophoresis.

Staining of protein complexes by proximity ligation assay *in situ*

Cells grown on poly-lysine-coated glass slides were fixed in Acetone-Methanol (1:1) at − 20 °C for 10 min. Slides were rehydrated in phosphate-buffered saline and incubated for 1 h in blocking solution (Duolink II-Olink Bioscience). The slides were incubated with the mixture of primary antibodies containing 1 μg/ml of mouse monoclonal antibody recognizing CHIP or HOP and rabbit polyclonal sera (1:10 000) recognizing Hsp70 or Hsp90. Protein complexes were detected and visualized by proximity ligation assay and rolling circle amplification using the Duolink II kit (Olink Bioscience). The fluorescent signals were quantified using the Nikon Eclipse Ti fluorescence microscope and NIS-Elements image analysis software.

Protein purification

His–Hsp70 and His–Hsp90α fusion proteins were produced in BL21-CodonPlus (DE3)-RIPL (Agilent) and purified using the Ni–NTA bead (Qiagen). Cells were lysed in buffer containing 300 mM NaCl, 50 mM Tris pH 8.0, 20 mM Imidazole, 0.3 mM tris(2-carboxyethyl)phosphine, 1 mM phenylmethylsulfonyl fluoride and lysozyme (1 mg/ml). The lysates were sonicated and centrifuged (14 000 *g*) before separation on the Ni-NTA columns. The His-tagged proteins were eluted in Imidazole gradient (20–500 mM). GST-CHIP and GST-HOP proteins were produced in BL21-CodonPlus (DE3)-RIPL (Agilent) and purified using glutathione sepharose (GE Healthcare). The cells were lysed in buffer containing 150 mM NaCl, 50 mM HEPES pH 7.5, 0.3 mM tris(2-carboxyethyl)phosphine and 1 mM phenylmethylsulfonyl fluoride and lysozyme (1 mg/ml). The lysate was incubated with glutathione sepharose after sonication and centrifugation (14 000 *g*). The GST-fusion proteins were eluted in buffer containing 150 mM NaCl, 50 mM HEPES pH 7.5, 0.3 mM tris(2-carboxyethyl)phosphine and 15 mM glutathione.

In vitro phosphorylation

The phosphorylation reaction mediated by CK1, CK2, GSK3B, CDK1/cyclinB (New England Biolabs) and activated p38 (Biaffin GmbH) contained 1 μg of recombinant Hsp90α or Hsp70, reaction buffer supplied by the manufacturer, 500 U of enzyme and 300 μM ATP in total volume 25 μl. The reaction incubated at 30 °C was stopped after 30 min by addition of the NuPAGE LDS Sample Buffer (Invitrogen).

Protein dephosphorylation

Cell pellet containing 5×10^6 cells was lysed in 200 μl lysis buffer containing 0.04% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 150 mM NaCl, 2 mM $MgCl_2$, 50 mM HEPES pH 7.3, protease inhibitor cocktail (Sigma Aldrich) and 1 mM *N*-ethylmaleimide for irreversible inhibition of natural phosphatases. *N*-ethylmaleimide was neutralized after 0.5 h by addition of 2 mM dithiothreitol. Nucleic acids were digested by 250 U of Benzonase Nuclease (Sigma Aldrich) for 0.5 h. The soluble fraction of lysate was separated by centrifugation (14 000 *g*). We used Zeba Spin Desalting Columns, 7K MWCO (Thermo Pierce) to remove low-molecular-weight compounds and exchange the lysis buffer to calf intestinal phosphate reaction buffer (100 mM NaCl, 50 mM Tris–HCl, 10 mM MgCl2, 1 mM dithiothreitol and pH 7.9). The samples containing proteins dissolved in reaction buffer were treated by 50 U of alkaline phosphatase calf intestinal phosphate (New England Biolabs) for 1 h at 30 °C. The reaction was stopped by addition of the NuPAGE LDS Sample Buffer (Invitrogen).

Kinase inhibitor screen

The kinase screening was performed with phosphatidylinositol 3-kinase inhibitors Wortmanin (10 μM) and LY294002 (10 μM), mitogen-activated protein kinase)/extracellular signal-regulated kinase inhibitors PD98059 (100 μM) and UO126 (50 μM), AKT inhibitors Triciribin (10 μM) and Tetrandrin (10 μM), tyrosine kinase inhibitor Genistein (50 μM), CDK inhibitor Roscovitine (50 μM), CDK9 and CK2 inhibitor DRB (50 μM) and p38 MAPK inhibitor SB202190 (50 μM).

Human tumor tissues

Breast tissues after cancer surgery were divided into pieces that consist of tumor or healthy tissue. These tissue samples were lysed by homogenization (TissueLyser, Qiagen) in radioimmuno precipitation assay buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X 100 and protease and phosphatase inhibitor cocktails (Sigma-Aldrich; nos. P8340, P0044 and P5726). The soluble fraction was separated by centrifugation at 14 000 *g* and the total concentration of soluble protein was adjusted to 2 mg/ml in all samples.

CONFLICT OF INTEREST

Borivoj Vojtesek and Petr Muller work as consultants for Moravian Biotechnologies that has developed some antibodies used in this study.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)