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Detecting post-translational modifications of Hsp90

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Summary

The molecular chaperone Heat Shock Protein 90 (Hsp90) is essential in eukaryotes. Hsp90 chaperones proteins that are important determinants of multistep carcinogenesis. The chaperone function of Hsp90 is linked to its ability to bind and hydrolyze ATP. Co-chaperones as well as post-translational modifications (phosphorylation, SUMOylation and ubiquitination) are important for its stability and regulation of the ATPase activity. Both mammalian and yeast cells can be used to express and purify Hsp90 and also detect its post-translational modifications by immunoblotting.

Keywords

Heat Shock Protein 90 (Hsp90); Molecular Chaperones; Post-translational modification; Phosphorylation; SUMOylation; Ubiquitination

1. Introduction

Heat Shock Protein 90 (Hsp90) is an essential molecular chaperone in eukaryotes^{1,2}. Its cellular functions have been most clearly identified in mammalian cells, *Drosophila*, and baker's yeast *Saccharomyces cerevisiae*³. Hsp90 creates and maintains the functional conformation of a subset of proteins^{4–6}. These targets (or “clients”) are key components of signal transduction pathways and numerous transcription factors. Hsp90 and a discrete set of co-chaperone proteins “hold” these clients in a state from which they can respond to activating signals⁷.

Hsp90 chaperone activity depends on ATP binding and hydrolysis^{8–10}. This is coupled to a conformational cycle involving the opening and closing of a dimeric “molecular clamp” via

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transient association of Hsp90's N-terminal domain^{11,12}. The N-domain also binds the antitumor antibiotics geldanamycin and radicicol, both Hsp90 inhibitors^{13–17}.

Hsp90 ATPase activity is also regulated by co-chaperones. For example, HopSti1^{18–20}, p50Cdc37^{21–23}, and p23Sba1^{24,25} have an inhibitory effect on the ATPase cycle of Hsp90, while Aha1^{26–30}, and Cpr6^{31,32} have an activating effect.

Hsp90 is post-translationally modified (PTMs) by phosphorylation, acetylation, S-nitrosylation, ubiquitination, and SUMOylation^{33–36}. These PTMs, in concert with co-chaperones, fine-tune Hsp90 chaperone function, which ultimately leads to chaperoning of kinase and non-kinase client proteins of Hsp90^{37,38}. The most extensively studied Hsp90 PTM is phosphorylation^{39–49}. Early work has shown that cells treated with the serine/threonine phosphatase inhibitor, okadaic acid, demonstrate Hsp90 hyperphosphorylation and decreased association of the chaperone with pp60v-Src, suggesting a link between Hsp90 phosphorylation and chaperoning of its target proteins^{50,51}. Subsequent study has shown that c-Src directly phosphorylates Tyr300 of Hsp90 under basal conditions, reducing its ability to chaperone client proteins⁴⁸.

Recent work has shown that Hsp90 is also subject to SUMOylation, which is an addition of a small ubiquitin-like modifier to a lysine residue. This modification affects cellular localization or function of a protein rather than signal for its degradation like ubiquitination. SUMOylation of Lys191 in human Hsp90α (Lys178 in yeast) promotes its binding to the co-chaperone Aha1 and also increases cells' sensitivity to Hsp90 inhibitors³³.

Lack of PTM-specific antibodies has made it difficult to study PTMs of Hsp90. There is currently only one phospho-Hsp90α antibody (Cell Signaling) available for detecting the phosphorylation of Hsp90α-Thr5/7⁵². Also *HSP90* gene knockouts are lethal in mammalian systems, therefore any PTM Hsp90 mutant must be investigated in a background of highly expressed native mammalian Hsp90 proteins.

Simple baker's yeast, *Saccharomyces cerevisiae*, is a well-established and valuable tool for studying various aspects of conserved protein chaperone machinery. The yeast system has provided us with a powerful tool to study Hsp90 phosphorylation, since it readily allows plasmid exchange whereby any introduced Hsp90 gene – provided it is partially functional – can provide 100% of the Hsp90 of the cell (Figure 1). Such genetic modifications are simply not achievable in cultured mammalian cells. This plasmid exchange (Figure 1) was used to isolate temperature sensitive (*ts*) Hsp90 mutants.

This chapter describes the isolation and identification of yeast Hsp90 phosphorylation using immunoblotting procedures. Using the yeast system it is possible to show that Hsp90 is constitutively phosphorylated on serine and threonine residues. However, Hsp90 threonine phosphorylation is lost upon either heat shock stress or treatment with the Hsp90 inhibitor GA (Figure 2).

This chapter also describes the isolation and analysis of the human (h)Hsp90-N-domain from mammalian cells. This is achieved by introducing a PreScission protease cleavage site between the N-domain and adjacent charged linker region of hHsp90α, allowing isolation of

the N-domain. Separation of the N-domain containing either wild-type or non-SUMOylated hHsp90 α -K191R mutant from the full-length Hsp90 protein allows for better detection of SUMOylated Hsp90 by immunoblotting (Figure 3).

2. Materials

1. YPD (2% (wt/vol) Bacto peptone, 1% (wt/vol) yeast extract, 2% (wt/vol) glucose, 20 mg/liter adenine).
2. Yeast protein extraction buffer (yEB): 50mM Tris-HCl, pH6.8, 100mM NaCl, 50mM MgCl₂. One tablet of complete EDTA-free protease inhibitor cocktail (Roche) and one tablet of PhosphoSTOP (Roche) are added to 50mL mEB.
3. 425–600 μ m glass beads (acid washed) (Sigma).
4. Dulbecco's Modified Eagle's Medium – high glucose (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS).
5. Mammalian protein extraction buffer (mEB): 0.1% NP-40, 20mM Tris-HCl, pH7.4, 100mM NaCl, 1mM MgCl₂, 20mM Na₂MoO₄. One tablet of complete EDTA-free protease inhibitor cocktail (Roche) and one tablet of PhosphoSTOP (Roche) are added to 50mL mEB. (For detection of SUMO, mEB should also contain 20mM *N*-ethylmaleimide (NEM), see Note 8).
6. *TransIT*-2020 transfection reagent (Mirus).
7. Bio-Rad Protein Assay Dye solution (Bio-Rad).
8. Ni-NTA agarose (Qiagen).
9. Imidazole (Sigma).
10. Anti-FLAG M2 Affinity Gel agarose (Sigma).
11. PreScission protease (GE Healthcare).
12. PreScission protease cleavage buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT (pH 7.0)
13. SDS-PAGE sample buffer (2X): 125 mM Tris-HCl pH6.8, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue, stable at –20°C. Aliquot and avoid freeze-thaw cycles.
14. Protran BA85, 0.45 μ m Nitrocellulose membrane (Whatman).
15. Ponceau S solution (Sigma).
16. Tris-buffered saline (TBS): 150 mM NaCl, 25 mM Tris-Base. Adjust pH to 7.4 using HCl. Sterile filter and incubate at 4°C.
17. Albumin, bovine serum (minimum purity 98%).
18. Dried skimmed milk.
19. Phospho-serine antibody (Sigma).

20. Phospho-threonine antibody (Sigma).
21. Phospho-tyrosine antibody (4G10; Millipore).
22. Acetylated lysine antibody (Cell Signaling).
23. Ubiquitin antibody (Santa Cruz).
24. SUMO-1 or SUMO-2/3 antibody (Cell Signaling).
25. 6X-His antibody (Invitrogen).
26. FLAG epitope antibody (ThermoScientific).
27. Anti-secondary mouse and/or rabbit antibody; ECL™ anti-mouse or anti-rabbit IgG, Horseradish Peroxidase-linked whole antibody (GE Healthcare).
28. ELC plus Western Blotting Detection System (GE Healthcare).
29. X-ray film, X-ray cassette, and X-ray film developing machine.

3. Methods

3.1. Extraction of Total Yeast Protein

1. Grow PP30 cells ⁸ expressing His₆ linked at the N domain of Hsp82 (yHsp90) on 150ml YPD overnight at 28°C.
2. Harvest and wash cells 2–3 times in ice-cold deionized water (dH₂O).
3. Transfer the cell pellet into a screw cap 2ml tube.
4. Pellet the cells and remove the supernatant (*see* Note 1).
5. Add equal volume of cell pellets, ice-cold glass beads.
6. Add half the volume of pellet/glass beads, yEB.
7. Bead beat the cells using the mini-beadbeater (BioSpec Products, Inc.) for 30 sec.
8. Incubate the cells on ice for 30 sec.
9. Repeat (7 and 8), 10–12 times.
10. Centrifuge the tubes at (10,000*g*) for 10 min at 4°C (*see* Note 2).
11. Transfer the supernatants into fresh 1.5ml micro-centrifuge tubes,
12. Centrifuge the tubes at (10,000*g*) for 10 min at 4°C (*see* Note 2).
13. Transfer the supernatants (soluble protein) into fresh 1.5ml micro-centrifuge tubes.
14. Determine protein concentrations using Bio-Rad Protein Assay solution (Bio-Rad).
15. Transfer 40 µl of Ni-NTA Agarose slurry into a 1.5ml micro-centrifuge tube. (*see* Note 3).

16. Add 1.0 ml of yEB to the Ni-NTA Agarose and spin at 10,000g for 1min at 4°C.
17. Remove supernatant and add 1.0 ml of yEB to the Ni-NTA Agarose (*see Note 4*).
18. Repeat step (16–17) four times.
19. Re-suspend the Ni-NTA Agarose in 30 μ M imidazole in yEB and incubate at 4°C for 30 min (*see Note 5*).
20. Repeat step 16–17 twice and remove supernatant.
21. Add 1mg of total protein to the Ni-NTA Agarose in a total volume of 600 μ L.
22. Incubate the total proteins/Ni-NTA Agarose at 4°C for 2 hr (*see Note 6*).
23. Centrifuge the tubes at (1,000g) for 1 min at 4°C.
24. Gently remove the supernatant (*see Note 7*).
25. Add 1 ml of yEB to the Ni-NTA Agarose.
26. Repeat (23–25) five times.
27. Wash the Ni-NTA agarose with 30 μ M imidazole in yEB.
28. Wash the Ni-NTA agarose with yEB once.
29. Centrifuge the micro-centrifuge tube at 15,000g for 1 min at 4°C.
30. Remove as much supernatant as possible.
31. Add 40 μ L of the protein sample buffer.
32. Boil the samples for 3–5 min.
33. Proceed to section 3.3 for Western blotting and PTM detection.

3.2 Extraction of total protein from HEK293 cells and immunoprecipitation (IP) of hHsp90:

1. Transfect HEK293 cells (~40% confluent in a 10cm dish; growing in DMEM +10% FBS) with 2 μ g hHSP90-FLAG using *TransIT-2020* reagent (Mirus) and incubate overnight at 37°C, 5% CO₂.
2. Place plates on ice and aspirate media. Wash 2x with cold PBS. Remove all remaining PBS from plate.
3. Add 200 μ L cold mEB to plate. Scrape cells and transfer to 1.5mL micro-centrifuge tube on ice (*see Note 8*).
4. Sonicate the lysate for 15 seconds. Incubate on ice for 15 seconds. Repeat ten times.
5. Centrifuge the tubes at (10,000xg) for 8min at 4°C (*see Note 2*).
6. Transfer supernatants (soluble protein) into fresh 1.5mL micro-centrifuge tubes.
7. Determine protein concentrations using Bio-Rad Protein Assay solution (Bio-Rad).

8. Transfer 50 μ L anti-FLAG M2 Affinity Gel agarose (Sigma) into a 1.5mL microcentrifuge tube.
9. Add 500 μ L mEB to the anti-FLAG agarose and spin at 10,000xg for 1 min. Remove supernatant (*see* Note 4).
10. Repeat step 9 four times.
11. Add 1mg of total protein to the anti-FLAG agarose in a total volume of 500 μ L.
12. Incubate the total protein/anti-FLAG agarose at 4°C for 2hr on a rotator (*see* Note 6).
13. Centrifuge the tubes at 1,000xg for 1 min.
14. Gently remove the supernatant (*see* Note 7).
15. Add 500 μ L mEB to the anti-FLAG agarose.
16. Repeat (steps 13–15) five times.
17. Add 500 μ L mEB to the anti-FLAG agarose.
18. Centrifuge at 15,000xg for 1min.
19. Remove as much supernatant as possible. (*see* Note 9 for optional PreScission protease cleavage, Figure 3).
20. Add 40 μ L of the protein sample buffer.
21. Boil the samples for 3–5min.
22. Proceed to section 3.3 for Western blotting and PTM detection.

3.3 Western blotting and detection of Hsp90 PTMs:

1. Centrifuge the samples at 1,000g and load the supernatant on to a 7.5% SDS-PAGE Tris-HCl gel (*see* Note 10).
2. Transfer the proteins from SDS-PAGE gel on to ProtranBA85, 0.45 μ m nitrocellulose membrane (Whatman) (*see* Note 11).
3. Examine the quality and efficiency of the transfer by staining the membrane with Ponceau S solution (Sigma) for 2 min (*see* Note 12).
4. Wash the membrane with dH₂O.
5. Incubate the membrane in 5% milk in TBS-T for 15–20 min at room temperature.
6. Wash the membrane with 1XTBS-T for 5 min at room temperature.
7. Repeat (38) three times
8. Incubate the membrane with primary antibody (*see* Table 1).
9. Wash the membrane three times with 1XTBS-T for 5 min at room temperature.

10. Incubate the membrane with 1:2000 dilution of secondary anti-mouse or anti-rabbit antibody in 5% milk-TBS-T for 1 h at room temperature.
11. Wash the membrane three times with 1XTBS-T for 5 min at room temperature.
12. Remove 1XTBS-T and then apply ECL plus (GE Healthcare) to nitrocellulose membrane for 2–3min.
13. Drain nitrocellulose membrane of excess developing solution (do not let dry).
14. Wrap the blot in saran wrap.
15. Place the blot in the X-ray film cassette (*see* Note 13).
16. Expose the blots to X-ray films by placing X-ray film directly against the western blot at different length of time.

Notes

1. The cell pellet must be kept on ice.
2. At this stage, Bio-Rad Protein Assay solution (Bio-Rad) should be prepared.
3. Ni-NTA Agarose is precharged with Ni²⁺ ions and appears blue in color. It is provided as a 50% slurry in 30% ethanol.
4. Do not disturb the Ni-NTA or anti-FLAG Agarose pellet.
5. Imidazole at low concentrations is commonly used in the binding and wash buffer to minimize binding of unwanted host cell proteins.
6. Use Eppendorf Thermomixer R to gently mix total proteins/Ni-NTA Agarose solution.
7. Avoid disturbing the Ni-NTA or anti-FLAG Agarose.
8. For detection of SUMO, mEB should always contain 20mM *N*-ethylmaleimide (NEM).
9. PreScission protease cleavage: incubate hHsp90 α -FLAG bound to anti-FLAG agarose with 2 units of PreScission Protease in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT (pH 7.0) at 10°C for 16 h.
10. Criterion precast gels from Bio-Rad are suitable for this purpose.
11. The high MW setting on the Bio-Rad Trans-Blot Turbo transfer system is suitable for this purpose.
12. Prepare 5% dry milk (LabScientific Inc.) in 1x TBS-T (0.1% Tween-20, Sigma) buffer before examining the membrane.
13. This procedure must be performed in the dark.

References

1. Prodromou C Mechanisms of Hsp90 regulation. *Biochem J* 2016;473:2439–52. [PubMed: 27515256]
2. Rohl A, Rohrborg J, Buchner J. The chaperone Hsp90: changing partners for demanding clients. *Trends Biochem Sci* 2013;38:253–62. [PubMed: 23507089]
3. Taipale M, Jarosz DF, Lindquist S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat Rev Mol Cell Biol* 2010;11:515–28. [PubMed: 20531426]
4. Karagoz GE, Rudiger SG. Hsp90 interaction with clients. *Trends Biochem Sci* 2015;40:117–25. [PubMed: 25579468]
5. Oroz J, Kim JH, Chang BJ, Zweckstetter M. Mechanistic basis for the recognition of a misfolded protein by the molecular chaperone Hsp90. *Nat Struct Mol Biol* 2017.
6. Zierer BK, Rubbelke M, Tippel F, et al. Importance of cycle timing for the function of the molecular chaperone Hsp90. *Nat Struct Mol Biol* 2016;23:1020–8. [PubMed: 27723736]
7. Verba KA, Wang RY, Arakawa A, et al. Atomic structure of Hsp90-Cdc37-Cdk4 reveals that Hsp90 traps and stabilizes an unfolded kinase. *Science* 2016;352:1542–7. [PubMed: 27339980]
8. Panaretou B, Prodromou C, Roe SM, et al. ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J* 1998;17:4829–36. [PubMed: 9707442]
9. Obermann WM, Sondermann H, Russo AA, Pavletich NP, Hartl FU. In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *J Cell Biol* 1998;143:901–10. [PubMed: 9817749]
10. Grenert JP, Johnson BD, Toft DO. The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes. *J Biol Chem* 1999;274:17525–33. [PubMed: 10364185]
11. Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 1997;90:65–75. [PubMed: 9230303]
12. Grenert JP, Sullivan WP, Fadden P, et al. The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J Biol Chem* 1997;272:23843–50. [PubMed: 9295332]
13. Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* 1997;89:239–50. [PubMed: 9108479]
14. Roe SM, Prodromou C, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem* 1999;42:260–6. [PubMed: 9925731]
15. Workman P, Burrows F, Neckers L, Rosen N. Drugging the cancer chaperone HSP90: combinatorial therapeutic exploitation of oncogene addiction and tumor stress. *Ann N Y Acad Sci* 2007;1113:202–16. [PubMed: 17513464]
16. Neckers L, Trepel JB. Stressing the development of small molecules targeting HSP90. *Clin Cancer Res* 2014;20:275–7. [PubMed: 24166908]
17. Neckers L, Workman P. Hsp90 molecular chaperone inhibitors: are we there yet? *Clin Cancer Res* 2012;18:64–76. [PubMed: 22215907]
18. Schmid AB, Lagleder S, Grawert MA, et al. The architecture of functional modules in the Hsp90 co-chaperone Sti1/Hop. *EMBO J* 2012;31:1506–17. [PubMed: 22227520]
19. Richter K, Muschler P, Hainzl O, Reinstein J, Buchner J. Sti1 is a non-competitive inhibitor of the Hsp90 ATPase. Binding prevents the N-terminal dimerization reaction during the atpase cycle. *J Biol Chem* 2003;278:10328–33. [PubMed: 12525481]
20. Song Y, Masison DC. Independent regulation of Hsp70 and Hsp90 chaperones by Hsp70/Hsp90-organizing protein Sti1 (Hop1). *J Biol Chem* 2005;280:34178–85. [PubMed: 16100115]
21. Lee P, Shabbir A, Cardozo C, Caplan AJ. Sti1 and Cdc37 can stabilize Hsp90 in chaperone complexes with a protein kinase. *Mol Biol Cell* 2004;15:1785–92. [PubMed: 14742721]
22. MacLean M, Picard D. Cdc37 goes beyond Hsp90 and kinases. *Cell Stress Chaperones* 2003;8:114–9. [PubMed: 14627196]

23. Vaughan CK, Mollapour M, Smith JR, et al. Hsp90-dependent activation of protein kinases is regulated by chaperone-targeted dephosphorylation of Cdc37. *Mol Cell* 2008;31:886–95. [PubMed: 18922470]
24. McLaughlin SH, Sobott F, Yao ZP, et al. The co-chaperone p23 arrests the Hsp90 ATPase cycle to trap client proteins. *J Mol Biol* 2006;356:746–58. [PubMed: 16403413]
25. Picard D Intracellular dynamics of the Hsp90 co-chaperone p23 is dictated by Hsp90. *Exp Cell Res* 2006;312:198–204. [PubMed: 16289154]
26. Lotz GP, Lin H, Harst A, Obermann WM. Aha1 binds to the middle domain of Hsp90, contributes to client protein activation, and stimulates the ATPase activity of the molecular chaperone. *J Biol Chem* 2003;278:17228–35. [PubMed: 12604615]
27. Meyer P, Prodromou C, Liao C, et al. Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery. *EMBO J* 2004;23:511–9. [PubMed: 14739935]
28. Panaretou B, Siligardi G, Meyer P, et al. Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. *Mol Cell* 2002;10:1307–18. [PubMed: 12504007]
29. Wolmarans A, Lee B, Spyropoulos L, LaPointe P. The Mechanism of Hsp90 ATPase Stimulation by Aha1. *Scientific reports* 2016;6:33179. [PubMed: 27615124]
30. Retzlaff M, Hagn F, Mitschke L, et al. Asymmetric activation of the hsp90 dimer by its cochaperone aha1. *Mol Cell* 2010;37:344–54. [PubMed: 20159554]
31. Johnson JL, Halas A, Flom G. Nucleotide-dependent interaction of *Saccharomyces cerevisiae* Hsp90 with the cochaperone proteins Sti1, Cpr6, and Sba1. *Mol Cell Biol* 2007;27:768–76. [PubMed: 17101799]
32. Mayr C, Richter K, Lilie H, Buchner J. Cpr6 and Cpr7, two closely related Hsp90-associated immunophilins from *Saccharomyces cerevisiae*, differ in their functional properties. *J Biol Chem* 2000;275:34140–6. [PubMed: 10942767]
33. Mollapour M, Bourboulia D, Beebe K, et al. Asymmetric Hsp90 N Domain SUMOylation Recruits Aha1 and ATP-Competitive Inhibitors. *Mol Cell* 2014;53:317–29. [PubMed: 24462205]
34. Woodford MR, Dunn D, Miller JB, Jamal S, Neckers L, Mollapour M. Impact of Posttranslational Modifications on the Anticancer Activity of Hsp90 Inhibitors. *Adv Cancer Res* 2016;129:31–50. [PubMed: 26916000]
35. Mollapour M, Neckers L. Post-translational modifications of Hsp90 and their contributions to chaperone regulation. *Biochim Biophys Acta* 2012;1823:648–55. [PubMed: 21856339]
36. Preuss KD, Pfreundschuh M, Weigert M, Fadle N, Regitz E, Kubuschok B. Sumoylated HSP90 is a dominantly inherited plasma cell dyscrasias risk factor. *J Clin Invest* 2015;125:316–23. [PubMed: 25485683]
37. Soroka J, Wandinger SK, Mausbacher N, et al. Conformational switching of the molecular chaperone Hsp90 via regulated phosphorylation. *Mol Cell* 2012;45:517–28. [PubMed: 22365831]
38. Xu W, Mollapour M, Prodromou C, et al. Dynamic tyrosine phosphorylation modulates cycling of the HSP90-P50(CDC37)-AHA1 chaperone machine. *Mol Cell* 2012;47:434–43. [PubMed: 22727666]
39. Nguyen MT, Kniess RA, Daturpalli S, et al. Isoform-Specific Phosphorylation in Human Hsp90 β Affects Interaction with Clients and the Cochaperone Cdc37. *J Mol Biol* 2017;429:732–52. [PubMed: 28109840]
40. Woodford MR, Truman AW, Dunn DM, et al. Mps1 Mediated Phosphorylation of Hsp90 Confers Renal Cell Carcinoma Sensitivity and Selectivity to Hsp90 Inhibitors. *Cell reports* 2016;14:872–84. [PubMed: 26804907]
41. Muller P, Ruckova E, Halada P, et al. C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. *Oncogene* 2012.
42. Scroggins BT, Neckers L. Post-translational modification of heat shock protein 90: impact on chaperone function. *Expert Opin Drug Discov* 2007;2:1403–14. [PubMed: 23484535]
43. Garnier C, Lafitte D, Jorgensen TJ, Jensen ON, Briand C, Peyrot V. Phosphorylation and oligomerization states of native pig brain HSP90 studied by mass spectrometry. *Eur J Biochem* 2001;268:2402–7. [PubMed: 11298759]

44. Zhao YG, Gilmore R, Leone G, Coffey MC, Weber B, Lee PW. Hsp90 phosphorylation is linked to its chaperoning function. Assembly of the reovirus cell attachment protein. *J Biol Chem* 2001;276:32822–7. [PubMed: 11438552]
45. Fontana J, Fulton D, Chen Y, et al. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res* 2002;90:866–73. [PubMed: 11988487]
46. Adinolfi E, Kim M, Young MT, Di Virgilio F, Surprenant A. Tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. *J Biol Chem* 2003;278:37344–51. [PubMed: 12869560]
47. Wang C, Chen J. Phosphorylation and hsp90 binding mediate heat shock stabilization of p53. *J Biol Chem* 2003;278:2066–71. [PubMed: 12427754]
48. Duval M, Le Boeuf F, Huot J, Gratton JP. Src-mediated phosphorylation of Hsp90 in response to vascular endothelial growth factor (VEGF) is required for VEGF receptor-2 signaling to endothelial NO synthase. *Mol Biol Cell* 2007;18:4659–68. [PubMed: 17855507]
49. Miyata Y, Yahara I. The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. *J Biol Chem* 1992;267:7042–7. [PubMed: 1551911]
50. Mimnaugh EG, Worland PJ, Whitesell L, Neckers LM. Possible role for serine/threonine phosphorylation in the regulation of the heteroprotein complex between the hsp90 stress protein and the pp60v-src tyrosine kinase. *J Biol Chem* 1995;270:28654–9. [PubMed: 7499384]
51. Ogiso H, Kagi N, Matsumoto E, et al. Phosphorylation analysis of 90 kDa heat shock protein within the cytosolic arylhydrocarbon receptor complex. *Biochemistry* 2004;43:15510–9. [PubMed: 15581363]
52. Solier S, Kohn KW, Scroggins B, et al. Feature Article: Heat shock protein 90alpha (HSP90alpha), a substrate and chaperone of DNA-PK necessary for the apoptotic response. *Proc Natl Acad Sci U S A* 2012;109:12866–72. [PubMed: 22753480]

Figure 1.

With plasmid shuffling, a mutant *hsp90* gene can be made to provide all the Hsp90 of the yeast cell ($yHsp90=Hsp82$ and $yHsc90=Hsc82$). This involves introducing the mutation into *yHsp90* on *Leu2* plasmid and then introducing it into haploid yeast cells ($yHsp90$, $yHsp90$). Growth of these cells on 5-fluoroorotic acid (5-FOA) will “cure” the yeast cells of the wild type *yHsc90* therefore creating *hsp90* mutant.

Figure 2.

Yeast Hsp90 phosphorylation on serine (phos-S) and threonine (p-T) residues. yHsp90-His₆ was purified from yeast cell that were heat shocked at 39°C for 40 min or treated with 100 μM GA for 1hr. Wild type cells containing the empty plasmid were used as negative control.

Figure 3.

Schematic representation of Hsp90-FLAG showing the amino (N-), charged linker (CL), middle (M-) and carboxy (C-) domains. Mammalian lysate with the Hsp90-FLAG will be attached to anti-FLAG agarose and Hsp90 N-domain can be isolated by PreScission protease digestion.

Table 1.

Post-translational modifications antibodies for immunoblotting. o/n (over-night).

PTM	Antibody	Manufacturer	Dilution	Diluent	Time and Temperature	Species
Phosphorylation	Phospho-serine (PSR-45)	Sigma (cat no. P5747)	1:500–1:1000	1% BSA in TBS-T	o/n 4°C	Mouse
	Phospho-threonine (PTR-8)	Sigma (cat no. P6623)	1:500–1:1000	1% BSA in TBS-T	o/n 4°C	Mouse
	Phospho-tyrosine (4G10)	Millipore (cat no. 05–321)	1:2000	5% milk in TBS-T	o/n 4°C	Mouse
Acetylation	Acetylated lysine	Cell Signaling (#9441)				Rabbit
Ubiquitination	Ubiquitin (P4D1)	Santa Cruz (sc-8017)	1:500–1:2000	5% milk in TBS-T	o/n 4°C	Mouse
SUMOylation	SUMO-1 (2A12)	Cell Signaling (#5718)				Mouse
	SUMO-2/3 (18H8)	Cell Signaling (#4971)				Rabbit