Inventory of Supplemental Information

Figure S1- GA causes G1 cell cycle arrest in yeast, providing an explanation for reduced tyrosine phosphorylation of yHsp90 in GA treated yeast cells in Figure 1A.

Figure S2- Tyrosine phosphorylation of yHsp90 in G1 released yeast cells. These data strengthen the conclusion that yHsp90 tyrosine phosphorylation occurs in a cell cycle dependent manner (Figure 2A).
yHsp90-Y24F is not ubiquitinated in the presence of MG132, confirming that Y24 phosphorylation is a prerequisite for yHsp90 ubiquitination and then degradation (Figure 2C, D).

Figure S3- The quality of bacterially expressed and purified yHsp90 and hHsp90α proteins was examined by Coomassie staining prior to their use in *in vitro* kinase assays (Figure 3C, D).

Figure S4- *In vivo* lethality of additional phosphomimic (aspartic acid) mutations complements the data in Figure 4A.

Figure S5- v-Src maturation is cell cycle-associated in yeast, providing additional explanation of the data in Figure 5C, D, where v-Src maturation is absent in yeast cells expressing yHsp90-Y24F or in Δ*swe1* delete yeast.
Quantification of hHsp90-Y38F interaction with client proteins in Figure 5G, H, K & L.

Figure S6- Tyrosine phosphorylation of yHsp90 in “open” and “closed” conformationally restricted mutants, and yHsp90 bound to GA-beads is not tyrosine phosphorylated. These data provide additional support the conclusion made from Figure 7.

SUPPLEMENTAL TABLES AND FIGURE LEGENDS

Table S1. Primer sequences. Mutated sequences are highlighted in bold.

Primer	Sequence
HSP82-Y24F-F	ATCATCAACACCGTC**TTT**TCTAACAAGGAAATT
HSP82-Y24F-R	AATTTCCTTGTTAGA**AAA**GACGGTGTTGATGAT
HSP82-Y47F-F	TTGGATAAAATTAGA**TTC**AAATCTTTGTCTGAT
HSP82-Y47F-R	ATCAGACAAAGATTT**GAA**TCTAATTTTATCCAA
HSP82-Y508F-F	GACCCAATTGATGAA**TTC**GCCTTCACTCAATTG
HSP82-Y508F-R	CAATTGAGTGAAGGC**GAA**TTCATCAATTGGGTC
HSP82-Y606F-F	TCTTCCATGTCCTCC**TTC**ATGTCTTCCAAGAAG
HSP82-Y606F-R	CTTCTTGGAAGACAT**GAA**GGAGGACATGGAAGA
Y24EF	GATCATCAACACCGTC**GAA**TCTAACAAGGAAATT
Y24ER	AATTTCCTTGTTAGA**TTC**GACGGTGTTGATGATC
HSP82-Y24D-F	ATCATCAACACCGTC**GAT**TCTAACAAGGAAATT
HSP82-Y24D-R	AATTTCCTTGTTAGA**ATC**GACGGTGTTGATGAT
SWE1SFHF	ATTGCGTAGTGCTGGGGAAAAGTAAACACACACAGGCGCACACGAGAACAG**TTCGTACGCTGCAGGTCGAC**
SWE1SFHR	CGTGTGGGAAAAAAGTATGTAAATAAAACAAGGTTTTTTGTTCCATTTA**CCACTAGTGGATCTGATATC**
SWE1-200-R	CGGTTGTTATCTGCTACATCT
SWE1-200-F	GTTGAACATTGGCGTGCCCCT
KanR	TGTACGGGCGACAGTCACATC
KanF	ATTTTAATCAAATGTTAGCG
SWE1-HA6-F	TTATCCAGGAAGACGACTTTGGACCTAAGCCAAAATTTTTTATA**TCCGGTTCTGCTGCTAGT**
SWE1-HA6-R	TGGGAAAAAAGTATGTAAATAAAACAAGGTTTTTTGTTCCATTTA**CCTCGAGGCCAGAAGAC**

Figure S1: GA causes G1 cell cycle arrest in yeast, related to Figure 1. Flow cytometry revealed accumulation of G1 cells in asynchronously growing yeast treated with 100 μM GA for 1hr and 2hrs. 34.2% of untreated cells, 59.0% of GA-treated cells (1hr), and 62.7% of GA-treated cells (2hrs) were arrested in G1.

Figure S2: yHsp90 tyrosine phosphorylation occurs in a cell cycle dependent manner and yHsp90-Y24F is not ubiquitinated in the presence of MG132, related to Figure 2. Temporal parameters of Hsp90 tyrosine phosphorylation was monitored after arresting yeast cells in G1 with α-factor and then releasing them by removal of α-factor.

Yeast cells expressing either wt or Y24F His6-tagged yHsp90, or empty plasmid (C), were treated with the proteasome inhibitor MG132 (50 μM for 1 h), and yHsp90 tyrosine phosphorylation and ubiquitination were determined. Ub-yHsp90-His_6 was detected using anti-ubiquitin antibody.

Figure S3: Bacterially expressed and purified yHsp90-His_6 or hHsp90-His_6 and their mutants, related to Figure 3. Ni-NTA purification of bacterially expressed yHsp90-His_6, yHsp90-His_6-Y24F, hHsp90-His_6, and hHsp90-His_6-Y24F proteins. The quality of purified proteins was examined by Coomassie staining of SDS-PAGE gels.

Figure S4: *In vivo* effect of phosphomimic (aspartic acid) mutations, related to Figure 4. The phosphomimic mutants yHsp90-Y24D and hHsp90α–Y38D abolish viability on de-selection of the y*HSP90* gene.

Figure S5: v-Src maturation is cell cycle-associated in yeast and quantification of hHsp90-Y38F interaction with client proteins, related to Figure 5.

Yeast cells expressing v-Src under *GAL1* promoter were grown on raffinose (raf) and arrested in G1-phase (with α-factor), in S-phase (with hydroxyurea, HU), and in M-phase (with nocodazole, NOC). After addition of galactose (gal) for 30 min., total phosphotyrosine and v-Src expression were analyzed.

Average densitometry scans of the results shown in Figure 5G, H, I & J. Gels were scanned and where necessary normalized against total intensity. The data are expressed as mean density relative to wt. Standard deviation derived from three independent experiments.

Figure S6: Tyrosine phosphorylation of yHsp90 in "open" and "closed" conformationally restricted mutants, and yHsp90 bound to GA-beads is not tyrosine phosphorylated (related to Figure 7). yHsp90 tyrosine phsophorylation was detected in the D79N "open" conformation mutant, but was absent in the E33A "closed" conformation mutant. Tyrosine phosphorylation was detected by immunoblotting.

GA-bead pulldown and detection of yHsp90 tyrosine phosphorylation status in yeast lysates from wild type and *swe1*Δ cells, or of yHsp90-Y24F mutants. yHsp90His_6 pulldown was used as a control (C).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids

Mutant forms of yeast expressed pHsp82 (yHsp90) and pHsp90α plasmids as well as the bacterially expressed Hsp82-pRSETA and hHsp90α pRSETA plasmids (Millson et al., 2007; Panaretou et al., 1998) were derived by QuickChange®II site directed mutagenesis kit (Stratagene) of these plasmids with primers listed on Table S1. Mutations were checked by DNA sequencing.
GAL1-SWE1-GST and *GAL1-CDC14*-GST were generously provided by Dr. M. Snyder, (Ptacek et al., 2005).
SWE1 was deleted in these PP30 using KanMX4 cassette as described previously (Guldener et al., 1996). Primers for the gene deletions are listed on Table S1,

Yeast Growth Media

Yeast was grown on YPD (2% (wt/vol) Bacto peptone, 1% yeast extract, 2% glucose, 20 mg/liter adenine), YPGal (2% (wt/vol) Bacto peptone, 1% yeast extract, 2% galactose, 20 mg/liter adenine) or YPRaf (2% (wt/vol) Bacto peptone, 1% yeast extract, 2% raffinose, 20 mg/liter adenine). Selective growth was on dropout 2% glucose (DO) medium with appropriate amino acids (Adams et al., 1997). The medium pH was adjusted to 6.8 with NaOH before autoclaving. 5-Fluoroorotic Acid (5-FOA) plates were prepared as previously described (Adams et al., 1997). The cell cycle arrest was achieved by addition of 2.5 µg/ml alpha1-mating factor (Sigma), 200 mM Hydroxyurea (Sigma), 20Mg/ml Nocodazole (Sigma), 100µM Latrunculin B (BIOMOL). Proteasome inhibition was achieved by treating the cells with 50 µM Z-Leu-Leu-Leu-al-MG132. Tyrosine phosphatase inhibition was done by treating the yeast cells with 100µM Potassium Bisperoxo(1,10-phenanthroline)oxovanadate (bpVphen), (Calbiochem). YPD cultures were diluted to an optical density at 600 nm of 0.5, and 5~µl aliquots of a 10-fold dilution series were spotted onto YPD–2.0% agar plates supplemented with the indicated level of GA. Growth was monitored over 3 to 5 days at 25°C.

Cell Culture

The human prostate cancer cell line PC3 (ATCC) was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 Uml^{-1} penicillin (Invitrogen) and 100 μgml^{-1} streptomycin (Invitrogen). The human

cervical cancer cell line HeLa (ATCC) was cultured in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 Uml^{-1} penicillin (Invitrogen) and 100 μgml^{-1} streptomycin (Invitrogen). All cell lines were propagated at 37°C in an atmosphere containing 5% CO_2.

Yeast Nuclear Extract

Nuclear and cytoplasmic protein extracts were prepared from yeast cells. For each condition, PP30-yHsp90His_6 cells were grown in 3 liters YPD to mid-log and then harvested and resuspended in 500ml YPD. Cells were treated with 200mM HU or 50µM MG132. The quality of nuclear and cytoplasmic protein fractions was assessed by monitoring Nop1 (anti-Nop1p monoclonal antibody, Santa Cruz Biotechnology) and α-tubulin (anti-tubulin monoclonal antibody, Calbiochem), respectively.

Immunoprecipitation and Immunoblotting

For immunoprecipitation, mammalian cell lysates were incubated with anti-FLAG antibody conjugated beads (Sigma) for 2 hr at 4ºC, or incubated with either anti-v-Src-AB1 (Calbiochem) antibody 1 hr and protein G agarose for 2 hr at 4ºC.
Yeast cell lysates were incubated with Ni-NTA agarose (Qiagen) for 2 hr at 4ºC, or anti-Hsp90 antibody (H90-10, Abcam) 1 hr and protein G agarose for 2 hr at 4ºC. Immunopellets were washed 4 times with fresh lysis buffer (20mM HEPES (pH7.0), 100 mM NaCl, 1 mM $MgCl_2$, 0.1% NP40 protease inhibitor cocktail (Roche), and PhosSTOP (Roche) or proteins bound to Ni-NTA agarose washed with 50 mM imidazole in lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) and eluted with either 300 mM imidazole in lysis buffer or with 5X Laemmli buffer. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Co-immunoprecipitated proteins were detected by immunoblotting with indicated antibodies recognizing Tetra-His (Qiagen), phosphotyrosine-clone 4G10 (Millipore), Nop1 (Santa Cruz), Chicken-Tubulin (Affinity Bioreagents), MPK1 (yC-20) (Santa Cruz), GST (GE-Healthcare), HA (Covance), Hsp86N-domain (Affinity BioReagents), $Cdc28^{Cdc2}$-PSTAIRE (Sigma), phospho-Cdc2 (Tyr15, Cell Signaling), Wee1-B11 (Santa Cruz), ErbB2 (Santa Cruz), GR (Santa Cruz), AR (Santa Cruz), $p60^{Hop}$ (Cell Signaling), $p50^{cdc37}$ (Neomarkers), p23 (Affinity Bioreagents), Aha1 (Rockland), cleaved Caspase-3 (Cell Signaling) and cleaved PARP (Cell signaling). Yeast co-chaperones were detected with Sti^{Hop} (kind gift from Dr Daniel C. Masison), $Cdc37^{p50}$ (Neomarkers), Sba1 and Aha1 (Institute of Cancer Research).

Detection of yHsp90-His_6 Tyrosine Phosphorylation

0.5-1.0 mg of yHsp90-His_6 or its mutants were precipitated with Ni-NTA agarose (Qiagen) as described above. Pulldown protein separated by SDS-PAGE and transferred to nitrocellulose membranes and then incubate in 2% non-fat milk dissolved in TBS-0.01%Teen20 (Sigma), (TBS-T), for 20 minutes. Membranes were washed 3X with TBS-T and then incubated with 2% BSA (Sigma) dissolved

in TBS-T containing phosphotyrosine-clone 4G10 (Millipore) overnight at 4ºC. Membranes were then washed 3X with TBS-T and then incubated with 2% non-fat milk dissolved in TBS-T, containing ECL-anti-mouse IgG (GE-Healthcare) for 1 hr at room temperature. Membranes were washed 3X with TBS-T and then detected by ECL-plus Western blotting detection system (GE-Healthcare).

HSE-LacZ and GR Activation Assays *In Vivo*

Yeast strains of the appropriate genotype were transformed with a) the centromeric *HIS3* vector, pHCA/rGR (Garabedian and Yamamoto, 1992), constitutively expressing glucocoticoid receptor (GR) under control of the Alcohol dehydrogenase promoter (*ADH1*), and b) the GR reporter vector pΔS26X, a *URA3* vector which expresses β-galactosidase (encoded by *lacz*) as a reporter gene under control of a promoter bearing 3×GR response elements (Schena et al., 1989). Transformants were selected by SD medium supplemented with appropriate amino acids without leucine, uracil and histidine. Yeast cells were grown overnight to exponential phase with a cell density of 2-3×10^6 cells per ml in 50ml of the same medium at 30°C. Then, dexamethasone (DEX) was added to a final concentration of 30μM, followed by incubation at 30°C for 2.5h to activate the receptors. Cells were collected by centrifugation (2000×g; 5 minutes), washed once with ddH_2O, and frozen at -80°C. The proteins were extracted as previously described, except for exclusion of EDTA in the extraction buffer. β-Galactosidase activities of GR or HSE were measured as previously described (Nathan and Lindquist, 1995). Cell lysate (10μl) was mixed with equal volume of 2×buffer Z (0.12M Na2HPO4.7H2O, 0.08M NaH2PO4.H2O, 0.02M KCl, 0.002M MgSO4) pH 7.0. The mixture was added to 700μl of 2mg/ml ONPG solution in 1×buffer Z prewarmed at 30°C and incubated at 30°C for 5-30 minutes. The reaction was stopped by adding 500μl of 1M Sodium Carbonate. The optical density at 420nm (OD_{420}) of each reaction mixture was determined. The protein concentration of the lysate was determined by the BioRad assay (BioRad). The β-galactosidase activity was calculated using the following formula: Enzyme Activity = 1000×OD_{420}/minute/[10μl×protein concentration (μg/μl)].

Flow Cytometric Analysis (FACS Analysis)

Apoptosis was monitored by FACS analysis. Briefly, cells were cultured in 100-mm plates and collected by centrifugation. Pellets were fixed with 70% ethanol for 4 h at -20°C and centrifuged again. Pellets were resuspended in phosphate–citrate buffer (0.2 M Na_2HPO_4 and 4 mM citric acid) and incubated for 20 min at room temperature. After centrifugation, the pellets were resuspended in DNA staining solution (50 mg/ml PI and 10 μg/ml RNaseA) and incubated for 20 min at RT. Samples were scanned with a FACSCalibur (Becton Dickinson) cell sorter under conditions to measure only specific PI-mediated fluorescence. Apoptotic cells appeared as a hypodiploid peak due to nuclear fragmentation and loss of DNA.

Isothermal Titration Calorimetry (ITC) and K_d Determinations

Heat of interaction was measured on a MSC system (Microcal), with a cell volume of 1.458 ml, under the same buffer conditions (20 mM Tris pH 8.0 containing 1 mM EDTA, 5 mM NaCl and 7 mM $MgCl_2$) at 30^{o}C. For AMPPNP interactions, 20 aliquots of 14.8 μl of 1 mM AMPPNP were injected into 50 μM wild type or mutant Y24E or Y24F yeast Hsp90. For GA binding the buffer system contained 2% DMSO. Heat of dilution was determined in a separate experiment by diluting protein into buffer, and the corrected data fitted using a nonlinear least square curve-fitting algorithm (Microcal Origin) with three floating variables: stoichiometry, binding constant and change in enthalpy of interaction.

SUPPLEMENTAL REFERENCES

Adams, A., Gottschling, D.E., Kaiser, C.A., and Stearns, T. (1997). Methods in Yeast Genetics. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Garabedian, M.J., and Yamamoto, K.R. (1992). Genetic dissection of the signaling domain of a mammalian steroid receptor in yeast. Mol Biol Cell *3*, 1245-1257.

Guldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J.H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res *24*, 2519-2524.

Millson, S.H., Truman, A.W., Racz, A., Hu, B., Panaretou, B., Nuttall, J., Mollapour, M., Soti, C., and Piper, P.W. (2007). Expressed as the sole Hsp90 of yeast, the alpha and beta isoforms of human Hsp90 differ with regard to their capacities for activation of certain client proteins, whereas only Hsp90beta generates sensitivity to the Hsp90 inhibitor radicicol. FEBS J *274*, 4453-4463.

Nathan, D.F., and Lindquist, S. (1995). Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. Mol Cell Biol *15*, 3917-3925.

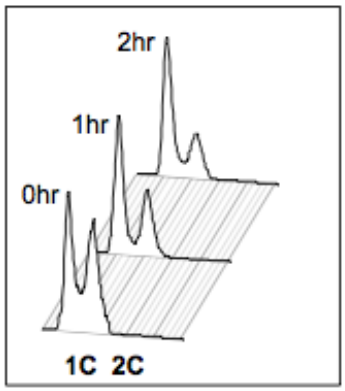
Panaretou, B., Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W., and Pearl, L.H. (1998). ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. EMBO J *17*, 4829-4836.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R.*, et al.* (2005). Global analysis of protein phosphorylation in yeast. Nature *438*, 679-684.

Schena, M., Freedman, L.P., and Yamamoto, K.R. (1989). Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Genes Dev *3*, 1590-1601.

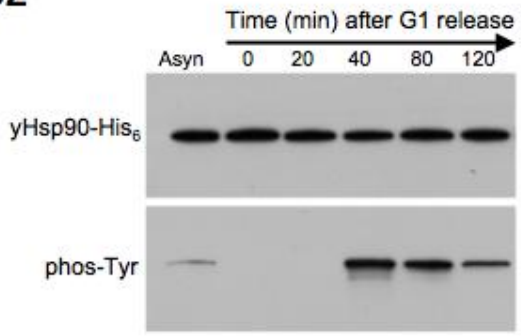
SUPPLEMENTARY FIGURES

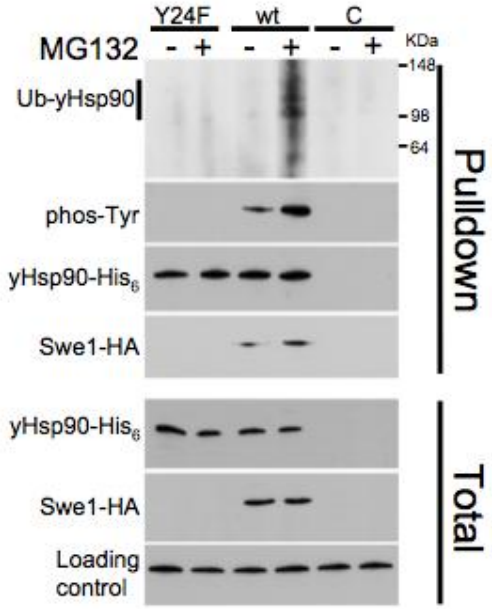
S1



SUPPLEMENTARY FIGURES

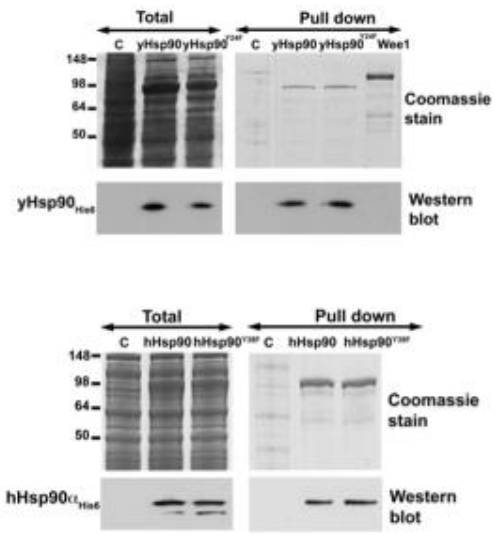
S2





SUPPLEMENTARY FIGURES

S3



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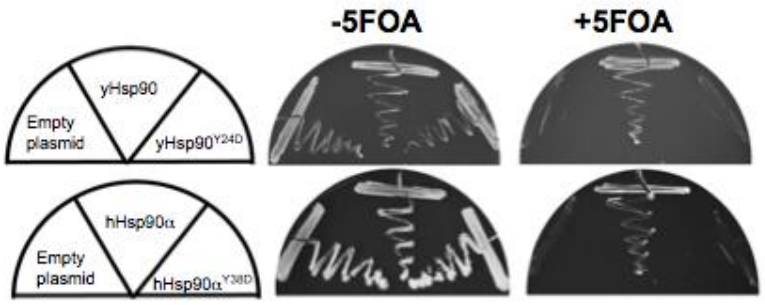

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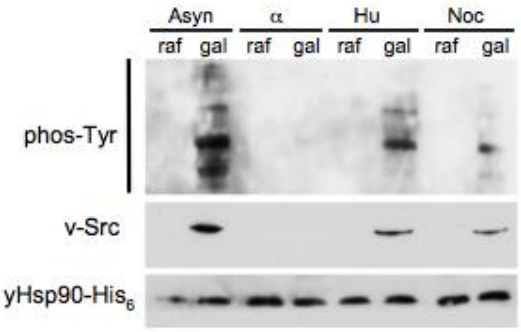
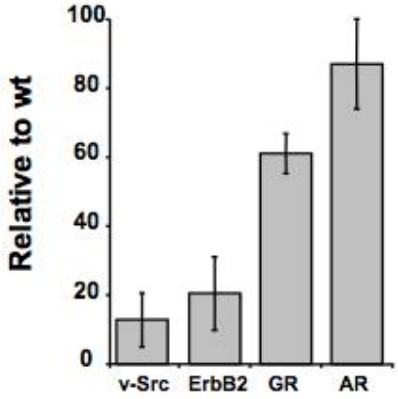
S5





SUPPLEMENTARY FIGURES