Cell Reports

Supplemental Information

Mps1 Mediated Phosphorylation of Hsp90 Confers Renal Cell Carcinoma Sensitivity and Selectivity to Hsp90 Inhibitors

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Supplemental Data

Figure S1

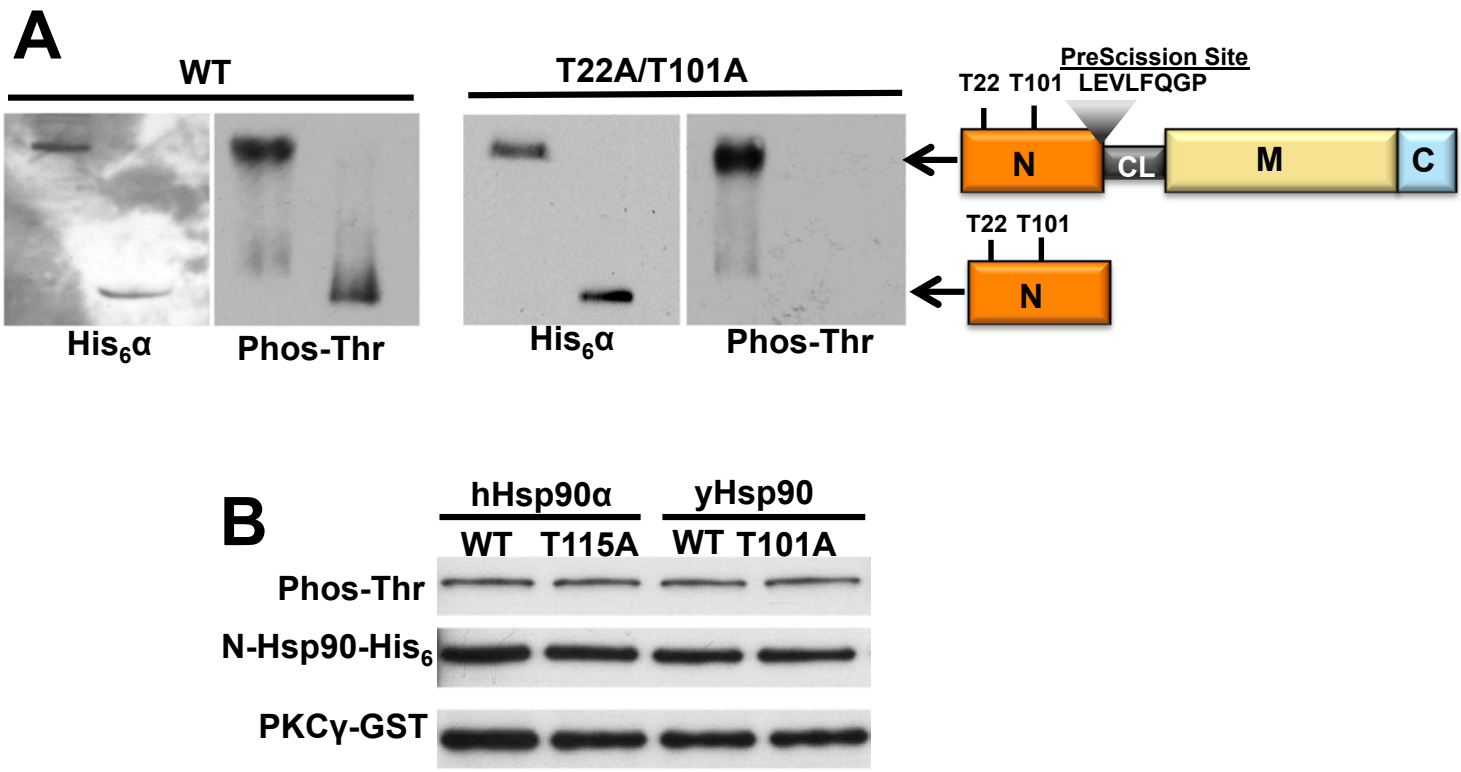
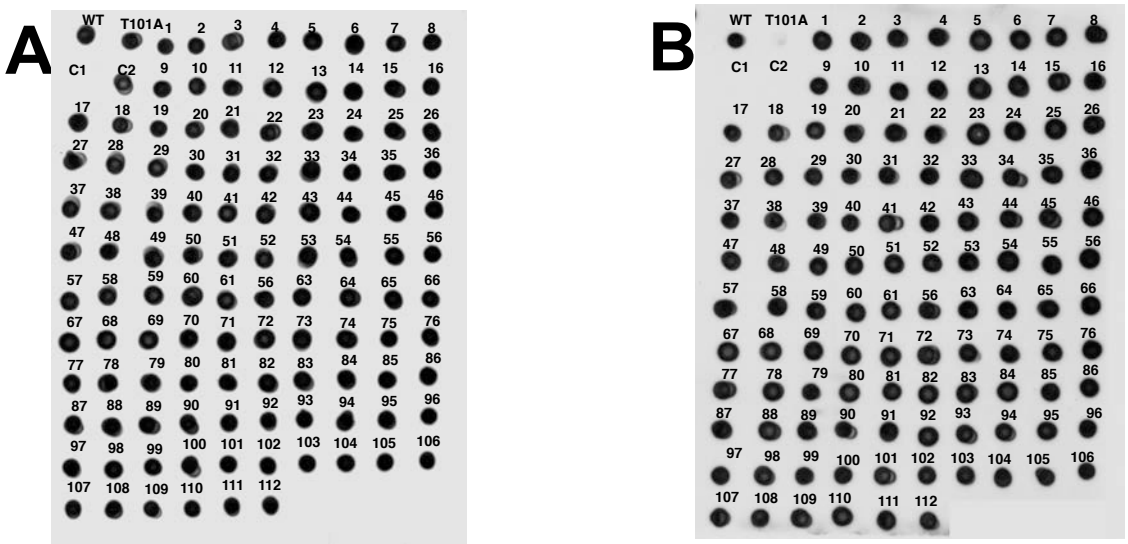


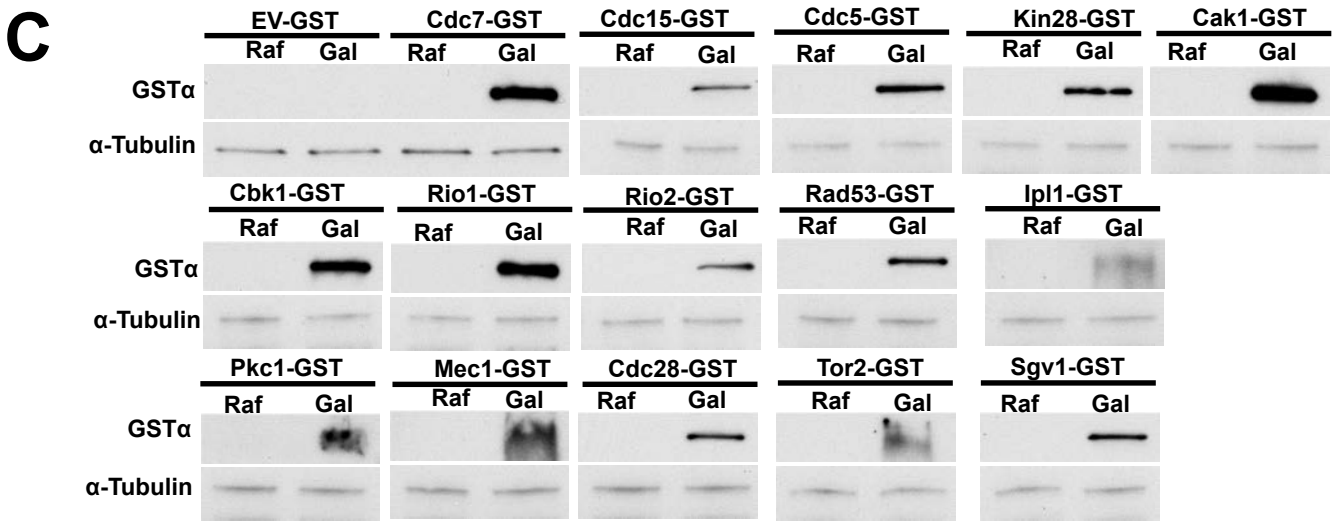
Figure S1, Related to Figure 1. T22 and T101 are the only phosphorylated threonine sites in the yeast Hsp90 N-domain and PKCγ does not phosphorylate T101-yHsp90 and T115-hHsp90.

A) Wild-type yeast strains expressing yHsp90-His6 (WT) or T22A/T101A double mutants containing PreScission site inserted between the N-domain and charged-linker were isolated by Ni-NTA agarose and N-domains were isolated after PreScission protease digest. N-domains were detected by immunoblotting with anti-hexahistidine and threonine phosphorylation was examined by pan-anti-phospho-threonine antibodies.

B) Bacterially expressed and purified yHsp90-His_6, T101A mutant hHsp90a-His6 and T115A mutant were bound to Ni-NTA agarose and in vitro kinase assay was carried by addition of Insect-expressed and purified PKCγ All Hsp90 constructs had PreScission site inserted between the N-domain and charged-linker. Threonine phosphorylation of Hsp90 N-domain was assessed after PreScission digest and immunoblotting using pan-anti-phospho-threonine antibody.

Figure S2


A
WT T101A 1 2 3 4 5 6 7 8
C1 C2 9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36
37 38 39 40 41 42 43 44 45 46
47 48 49 50 51 52 53 54 55 56
57 58 59 60 61 56 63 64 65 66
67 68 69 70 71 72 73 74 75 76
77 78 79 80 81 82 83 84 85 86
87 88 89 90 91 92 93 94 95 96
97 98 99 100 101 102 103 104 105 106
107 108 109 110 111 112
B
WT T101A 1 2 3 4 5 6 7 8
C1 C2 9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36
37 38 39 40 41 42 43 44 45 46
47 48 49 50 51 52 53 54 55 56
57 58 59 60 61 56 63 64 65 66
67 68 69 70 71 72 73 74 75 76
77 78 79 80 81 82 83 84 85 86
87 88 89 90 91 92 93 94 95 96
97 98 99 100 101 102 103 104 105 106
107 108 109 110 111 112


C
EV-GST
Cdc7-GST
Cdc15-GST
Cdc5-GST
Kin28-GST
Cak1-GST
Raf Gal
GSTα
α-Tubulin
Cbk1-GST
Rio1-GST
Rio2-GST
Rad53-GST
Ipl1-GST
Pkc1-GST
Mec1-GST
Cdc28-GST
Tor2-GST
Sgv1-GST

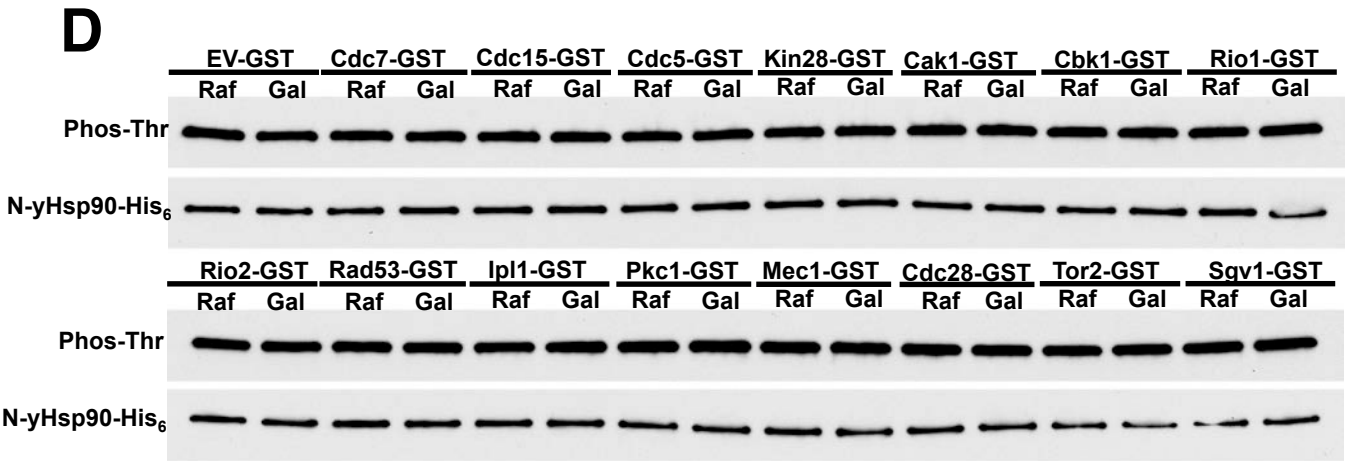

D
EV-GST
Cdc7-GST
Cdc15-GST
Cdc5-GST
Kin28-GST
Cak1-GST
Cbk1-GST
Rio1-GST
Raf Gal
Phos-Thr
N-yHsp90-His6
Rio2-GST
Rad53-GST
Ipl1-GST
Pkc1-GST
Mec1-GST
Cdc28-GST
Tor2-GST
Sgv1-GST

Figure S2, Related to Figure 1. Identification of yMps1 protein kinase targeting T101-yHsp90.

A) EUROSCARF collection of haploid non-essential kinase deletes in BY4741 were transformed with yHsp90-His_6-Presc, expressed, isolated, and cleaved with PreScission protease. Pull-down proteins were spotted on to nitrocellulose membrane. N-domain yHsp90 and
B) Threonine phosphorylation was detected by immunoblotting with anti-hexahistidine and pan-anti-phospho threonine antibodies respectively. Empty plasmid (C1) and T101A mutant was used as negative controls. N-domain wild type (WT) yHsp90 and N-domain T22AT101A (C2) were also used as controls.
C) Yeast essential kinases were over-expressed and detected by immunoblotting with anti-GST antibody.
D) Threonine phosphorylation of the N-domain Hsp90 was examined by immunoblotting with pan-anti-phopho-threonine antibody.

Figure S3

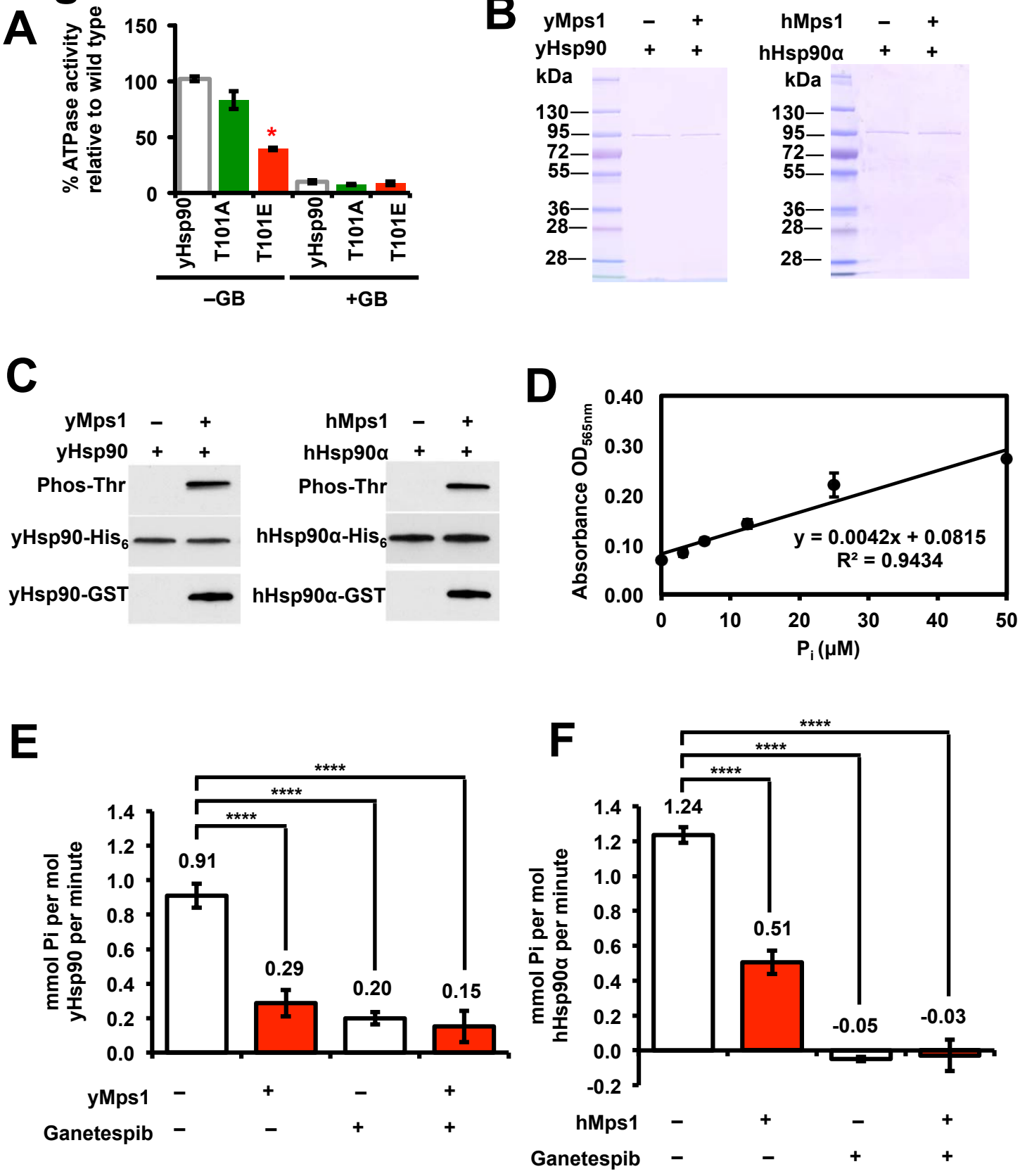

A
% ATPase activity relative to wild type
150
100
50
0
*
yHsp90
T101A
T101E
yHsp90
T101A
T101E
–GB
+GB
B
yMps1 – +
yHsp90 + +
kDa
130
95
72
55
36
28
28
hMps1 – +
hHsp90α + +
kDa
130
95
72
55
36
28
28
C
yMps1 – +
yHsp90 + +
Phos-Thr
yHsp90-His6
yHsp90-GST
hMps1 – +
hHsp90α + +
Phos-Thr
hHsp90α-His6
hHsp90α-GST
D
Absorbance OD565nm
0.40
0.30
0.20
0.10
0.00
y = 0.0042x + 0.0815
R² = 0.9434
0
10
20
30
40
50
Pi (μM)
E
mmol Pi per mol yHsp90 per minute
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
0.91
0.29
0.20
0.15
yMps1 – + – +
Ganetespib – – + +
F
mmol Pi per mol hHsp90α per minute
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
****
****
****
1.24
0.51
-0.05
-0.03
hMps1 – + – +
Ganetespib – – + +

Figure S3, Related to Figure 2. Impact of yMps1 mediate phosphorylation of T101-yHsp90 and T115-hHsp90α on the ATPase activity.

A) Relative ATPase activity of wild-type yHsp90-His6, T101A, and T101E mutants expressed and isolated from yeast. ATPase activity was inhibited by addition 10μM ganetespib (GB). Error bars represent standard deviation of three independent experiments. *$p < 0.05$.
B) 50ng of the recombinant yHsp90-His_6 and hHsp90-His_6 proteins before and after *in vitro* phosphorylation by Mps1 were resolved on the SDS-PAGE gel and stained with Coomassie stain.
C) Phosphorylation of T101-yHsp90 and T115-hHsp90α were confirmed by immunblotting.
D) Inorganic phosphate (Pi) standard curve. The x-axis shows μM of Pi per assay and the y-axis shows absorbance at 565nm. Mean ± S.D. from values obtained in three independent experiments.
E) and
F) ATPase activity of Hsp90 from B) and C) with or without 10μM ganetespib. Mean ± S.D. presented from three independent experiments. Mean ± S.D. from values obtained in three independent experiments with **$p < 0.0001$.

Figure S4

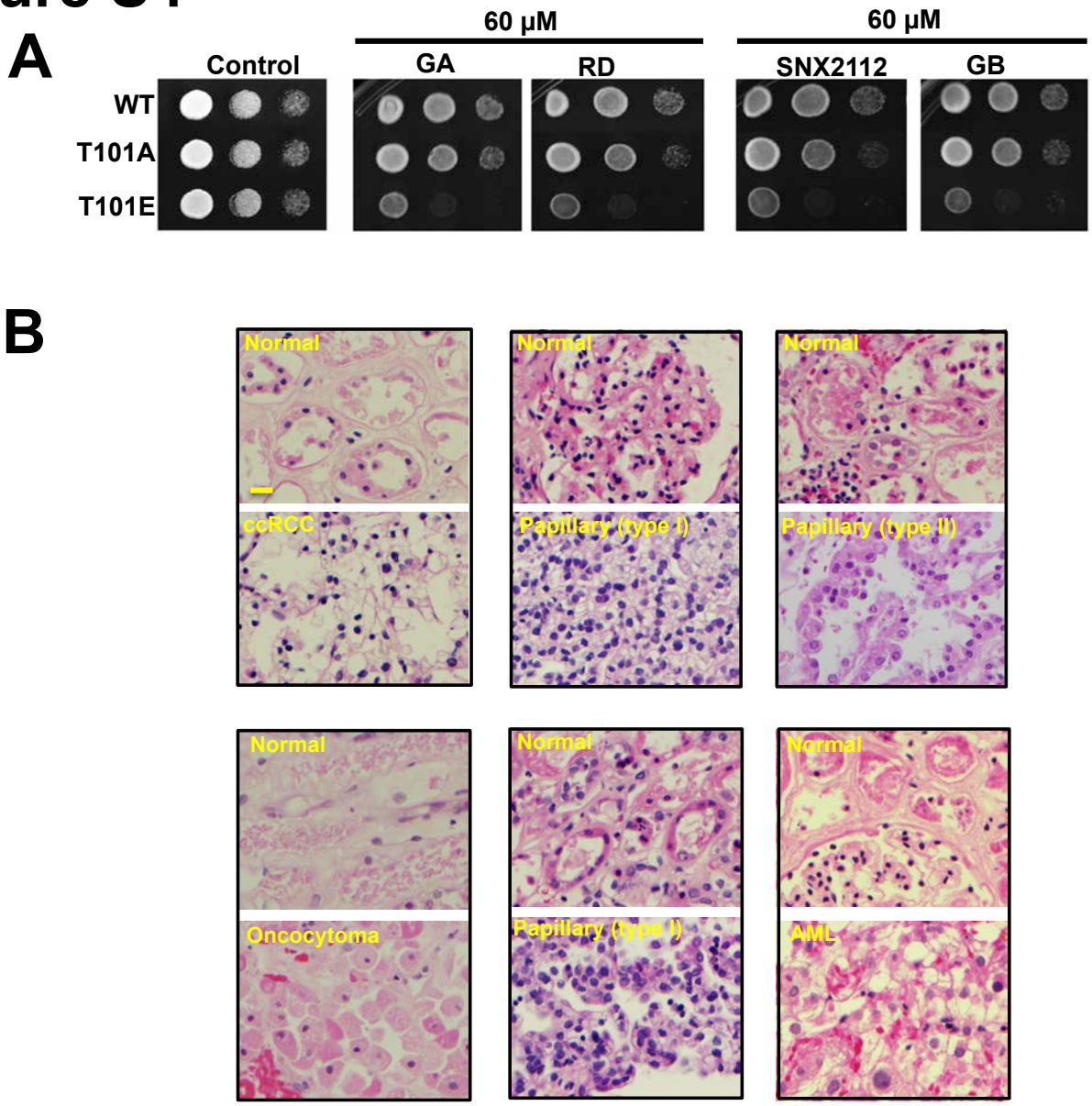


Figure S4, Related to Figure 7. Mps1 phosphorylation of Hsp90 sensitizes yeast cells to Hsp90 inhibitors and H&E staining of the renal cell carcinoma tumors.

A) Yeast expressing yHsp90-His_6 (WT), and indicated phospho-T101 mutants were spotted at a 1:10 dilution series of 10^7cells/ml on YPDA agar containing 60μM Hsp90 inhibitors geldanamycin (GA), and radicicol (RD), or ganetespib (GB) and SNX2112. Plates were incubated at 28˚C for 4 days.

B) Hematoxylin and eosin (H&E) staining of human renal cell carcinoma (RCC) tumors. Clear cell renal cell carcinoma (ccRCC), Papillary type I, type II, Oncocytoma, and angiomyolipoma (AML) were used for staining. Scale bar represents 20μm.

Table S1. Non-essential kinase deleted yeast strains used for screening of yHsp90-T101 phosphorylation. Related to Figure 1 and S2.

No	ORF	Gene	No	ORF	Gene	No	ORF	Gene	No	ORF	Gene
1	YBR059C	AKL1	29	YLR113W	HOG1	57	YOL100W	PKH2	85	YDR523C	SPS1
2	YGL021W	ALK1	30	YOR267C	HRK1	58	YDR466W	PKH3	86	YNR031C	SSK2
3	YBL009W	ALK2	31	YKL101W	HSL1	59	YIL042C	PKP1	87	YCR073C	SSK22
4	YNL020C	ARK1	32	YPL204W	HRR25	60	YGL059W	PKP2	88	YPL042C	SSN3
5	YGL180W	ATG1	33	YJL106W	IME2	61	YIL095W	PRK1	89	YLR362W	STE11
6	YJL095W	BCK1	34	YHR079C	IRE1	62	YKL116C	PRR1	90	YHL007C	STE20
7	YGR188C	BUB1	35	YPR106W	ISR1	63	YDL214C	PRR2	91	YDL159W	STE7
8	YGR262C	BUD32	36	YCL024W	KCC4	64	YAL017W	PSK1	92	YJL187C	SWE1
9	YBR274W	CHK1	37	YKL161C	KDX1	65	YOL045W	PSK2	93	YBL088C	TEL1
10	YIL035C	CKA1	38	YDR122W	KIN1	66	YKL198C	PTK1	94	YJR066W	TOR1
11	YOR061W	CKA2	39	YLR096W	KIN2	67	YJR059W	PTK2	95	YGL179C	TOS3
12	YNL298W	CLA4	40	YAR018C	KIN3	68	YGL158W	RCK1	96	YJL164C	TPK1
13	YFR014C	CMK1	41	YOR233W	KIN4	69	YLR248W	RCK2	97	YPL203W	TPK2
14	YOL016C	CMK2	42	YCR091W	KIN82	70	YMR139W	RIM11	98	YKL166C	TPK3
15	YKL139W	CTK1	43	YKL168C	KKQ8	71	YFL033C	RIM15	99	YDR247W	VHS1
16	YJL006C	CTK2	44	YLL019C	KNS1	72	YDL025C	RTK1	100	YBR097W	VPS15
17	YML112W	CTK3	45	YHR082C	KSP1	73	YER129W	SAK1	101	YLR240W	VPS34
18	YGR092W	DBF2	46	YGR040W	KSS1	74	YCR008W	SAT4	102	YJL141C	YAK1
19	YPR111W	DBF20	47	YNL307C	MCK1	75	YHR205W	SCH9	103	YBR028C	YPK3
20	YDL101C	DUN1	48	YOR351C	MEK1	76	YDR422C	SIP1	104	YHR135C	YCK1
21	YKL048C	ELM1	49	YOR231W	MKK1	77	YGL208W	SIP2	105	YNL154C	YCK2
22	YNR047W	FPK1	50	YPL140C	MKK2	78	YOL113W	SKM1	106	YER123W	YCK3
23	YPL141C	FRK1	51	YDL079C	MRK1	79	YPL026C	SKS1	107	YOL128C	YGK3
24	YBL016W	FUS3	52	YKL171W	NNK1	80	YMR216C	SKY1	108	YMR291W	YMR291W
25	YER027C	GAL83	53	YNL183C	NPR1	81	YHR030C	SLT2	109	YKL126W	YPK1
26	YDR283C	GCN2	54	YJL128C	PBS2	82	YPR054W	SMK1	110	YMR104C	YPK2
27	YDR507C	GIN4	55	YPL031C	PHO85	83	YDR477W	SNF1	111	YPL150W	YPL150W
28	YJL165C	HAL5	56	YDR490C	PKH1	84	YGL115W	SNF4	112	YPL236C	ENV7

Table S2. Interactors of yHsp90-His_6 and their associated interaction change upon T101 Phosphorylation (Log2 Ratio yHsp90-His_6-T101E/ yHsp90-His_6-T101A). Related to Figure 5.

Gene-Name	Log2 Ratio E/A yHsp90 Norm
PAL1	3.70
JLP1	3.26
PMU1	2.95
RPS9B	2.52
YRA1	2.51
MET3	2.14
SHM1	1.99
STM1	1.93
ASC1	1.80
YGR283C	1.69
RPL30	1.60
TSR1	1.45
RPA135	1.43
GUA1	1.34
KRS1	1.29
MDJ1	1.27
CPS1	1.21
MSN4	1.20
NPL3	1.17
NCL1	1.16
CDC55	1.13
SDH2	1.06
ATP3	0.97
AMD1	0.78
VMA1	0.70
CUE5	0.68
AIM32	0.67
UBA4	0.67
YMR265C	0.64
YMR087W	0.61
GND1	0.54
TUF1	0.52
NMD3	0.50
REB1	0.46
CLU1	0.38
CPR6	0.31
AST2	0.30
YCK2	0.30
BDH1	0.27
NOB1	0.26
TES1	0.25
PRO1	0.22
ABF1	0.20
DEF1	0.19
CLB3	0.17
RPS10B	0.15
RIB4	0.14
ARC1	0.14
MET17	0.14
DET1	0.11
KCS1	0.10
ALY2	0.07
HSP82	0.00
ESC1	0.00
DIG1	-0.04
SSD1	-0.06
TPS1	-0.08
RPS27A	-0.10
CDC19	-0.14
RPC40	-0.14
RSP5	-0.16
HYP2	-0.17
BUL1	-0.19
RPS8A	-0.21
PMA1	-0.22
RPS31	-0.25
THG1	-0.26
MRPS18	-0.29
RCL1	-0.30
YGR250C	-0.35
TDH3	-0.38
ILV2	-0.39
YEF3	-0.39
PAA1	-0.40
RPS22A	-0.42
RPL9A	-0.47
SSA1	-0.50
RPS3	-0.52
SGN1	-0.54
PDC1	-0.56
MSB1	-0.60
CDC48	-0.62
GTS1	-0.62
PPZ1	-0.63
RPS7B	-0.65
HBT1	-0.66
ASK10	-0.66
SSC1	-0.69
YPT1	-0.70
CAF20	-0.70
SSA2	-0.71
RPS5	-0.74
RPL31A	-0.75
RPL6B	-0.77
PGK1	-0.77
YMR102C	-0.78
URA2	-0.82
BOI1	-0.82
BMH1	-0.83
KSP1	-0.83
HRR25	-0.85
RPL40A	-0.87
FPK1	-0.87
PSP2	-0.87
PET9	-0.88
RPS0A	-0.88
SRO9	-0.88
RNH1	-0.95
IST2	-0.95
PWP1	-0.98
GPD2	-0.98
PIG2	-0.99
KAR2	-1.03
YMR045C	-1.04
DOT6	-1.06
RPS1A	-1.07
RPS1B	-1.08
MOT3	-1.08
AKL1	-1.10

RPL2A	-1.10
LGE1	-1.10
RPL29	-1.12
RGI1	-1.13
RPS23A	-1.14
UTP11	-1.15
GYP1	-1.17
LSM12	-1.18
RPL42A	-1.18
ATP1	-1.20
PBP1	-1.21
YHR097C	-1.21
RPL3	-1.21
PBP4	-1.22
ATP2	-1.25
SSB2	-1.25
ENO2	-1.26
EFT1	-1.27
RPS13	-1.29
RRB1	-1.30
RPS4A	-1.30
BRE5	-1.30
CDC14	-1.30
RPL37B	-1.35
RPL23A	-1.39
RPS29A	-1.41
RIM4	-1.42

YGR237C	-1.42
RPS18A	-1.42
SAR1	-1.44
PRP43	-1.44
RPL10	-1.49
RPL43A	-1.51
UBP3	-1.52
MRPL32	-1.55
MRM1	-1.57
TIF1	-1.59
ACC1	-1.60
RPL21A	-1.66
POR1	-1.71
HRK1	-1.71
RPL25	-1.78
SCS2	-1.78
RPL28	-1.80
RPS29B	-1.80
YGR266W	-1.80
BEM2	-1.80
RTK1	-1.84
CYR1	-1.84
YCL019W	-1.86
IPP1	-1.91
RPL19A	-2.00
ROM2	-2.00
RPL5	-2.03

SNF4	-2.08
BRE1	-2.10
ADE8	-2.19
SNF1	-2.21
RPL35A	-2.22
RPL14A	-2.24
DUF1	-2.25
PUF3	-2.26
PHO88	-2.33
SRV2	-2.39
RPL4A	-2.40
RPP2A	-2.40
RPL8A	-2.42
MHP1	-2.45
SIP2	-2.47
RPS2	-2.49
RPS24A	-2.51
RPL13B	-2.53
NMA1	-2.55
RPL4B	-2.59
RPL36B	-2.60
GFA1	-2.64
RPP2B	-2.72
RPP1B	-2.74
RPL33A	-2.92
RPP1A	-4.01

Table S3. Primer sequences. Mutated sequences are highlighted in red. Restriction sites are green. Epitope sequences are highlighted in blue. Short flanking region (SFH) for yMps1 are underlined.

Primer	Sequence
yMps1-Hind-myc-F	GTCAGCATAAGCTTATGGAACAAAAGTTAATCTCCGAAGAAGAT TTAGAGTTTTCAACAAACTCATTCCAT
yMps1-Xho1-R	TTCAGCTCTCGAGCTAAATTTTGTAATCTGCAAATTTCC
yMps1-myc-SFHF	GATGTGGTAGACACTGTTTTAAGGAAATTTGCAGATTACAAAAT TTCCGGTTCTGCTGCTAGT
yMps1-myc-SFHR	AATGTATTTATGTTCATAACTGGCACATGCTTTTCTTCCTTATGC GGCTCTTCCTCGAGGCCAGAAGAC
yMps1-200F	ACTACCAAGGGTTGCAAAGAG
yMps1-200R	ATATAATATTTCACTGAGCGCCG
KanR	TGTACGGGCGACAGTCACATC
KanF	ATTTTAATCAAATGTTAGCG
hMps1-F-BamHI-FLAG	GTACCTTGGATCCATG**GATTACAAAGACGATGACGATAAG**GAA TCCGAGGATTTAAGTGGC
hMps1-RXho1	GGATCGTCTCGAGTCATTTTTTTCCCCTTTTTTTTTCAAAAGTCT TGGAGGAT
HSP82T101AF	CATTGCCAAGTCTGGTGCCAAAGCCTTCATGGAAG
HSP82T101AR	CTTCCATGAAGGCTTTGGCACCAGACTTGGCAATG
HSP82T101EF	CATTGCCAAGTCTGGTGAAAAAGCCTTCATGGAAG
HSP82T101ER	CTTCCATGAAGGCTTTTTCACCAGACTTGGCAATG
hHsp90αT115A-F	ATCGCCAAGTCTGGGGCCAAAGCGTTCATGGAA
hHsp90αT115A-R	TTCCATGAACGCTTTGGCCCCAGACTTGGCGAT
hHsp90αT115E-F	ATCGCCAAGTCTGGGGAGAAAGCGTTCATGGAA
hHsp90αT115E-R	TTCCATGAACGCTTTCTCCCCAGACTTGGCGAT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

Yeast and bacterial expression plasmids containing pHsp82 (yHsp90) and pHsp90α (hHsp90) with or without a PreScission protease cleavage site, (Panaretou et al. 1998; Millson et al. 2007) were derived using a QuickChange®II site directed mutagenesis kit (Stratagene) with primers listed in Table S3. Mutations were checked by DNA sequencing. GAL-hMps1-Myc-pYES2, pCDNA3-hMps1-FLAG plasmids were constructed with primers in Table S3.

Yeast Growth Media

Yeast were grown on YPDA (2% (wt/vol) Bacto peptone, 1% yeast extract, 2% glucose, 20mg/liter adenine), YPGal (2% (wt/vol) Bacto peptone, 1% yeast extract, 2% galactose, 20mg/liter adenine), YPRaf (2% (wt/vol) Bacto peptone, 1% yeast extract, 2% raffinose, 20mg/liter adenine). Selective growth was on dropout 2% glucose (DO) medium with appropriate amino acids (Adams et al. 1997). 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). Cell cycle arrest was achieved by addition of 2.5μg/ml α1-mating factor (Sigma) or 20μg/ml nocodazole (Sigma).

Mammalian Cell Culture

The human breast cancer cell line SkBr3 (ATCC) was cultured in McCoy's 5A media (GIBCO) supplemented with 10% fetal bovine serum (FBS, Invitrogen). The human embryonal kidney cell line HEK293 were grown in Dulbecco's modified Eagle's minimal

essential medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum. All cell lines were propagated at 37°C in an atmosphere containing 5% CO_2.

Immunoprecipitation, PreScission Protease Cleavage and Immunoblotting

Protein extraction from both yeast and mammalian cells was carried out using methods previously described (Mollapour and Neckers 2011). For immunoprecipitation, mammalian cell lysates were incubated with Anti-FLAG M2 agarose (Sigma) for 2hr at 4ºC. Yeast cell lysates were incubated with Ni-NTA agarose (Qiagen) for 2hr at 4ºC. Immunopellets were washed 4 times with fresh lysis buffer (20mM HEPES (pH 7.0), 100mM NaCl, 1mM $MgCl_2$, 0.1% NP40, protease inhibitor cocktail (Roche) and PhosSTOP (Roche)). Proteins bound to Ni-NTA agarose were washed with 50mM imidazole in lysis buffer (20mM Tris-HCl (pH 7.5), 100mM NaCl, protease inhibitor cocktail and PhosSTOP) and eluted with either 300mM 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 Hexa-His and pan-phospho-threonine (Qiagen), α-tubulin (Affinity Bioreagents), Hsp86 N-domain (Affinity BioReagents), FLAG (Sigma-Aldrich), GR, Cdk4, ErbB2, Mpk1/Slt2, Cdc14, Mps1 (Santa Cruz), c-Myc, GST, $p60^{Hop}$, $p50^{cdc37}$ (Cell Signalling Technology), p23 (Affinity Bioreagents), and hAha1 (StressMarq). Yeast co-chaperones were detected with antibody to $Sti1^{Hop}$ (a kind gift from Dr. Daniel C. Masison, NCI, USA), $Cdc37^{p50}$ and yAha1 (NCI, USA), Sba1 (Institute of Cancer Research, UK). PreScission Protease cleavage was achieved by incubating $yHsp90His_6$ bound to Ni-NTA agarose with 2 units

of PreScission Protease in 50mM Tris-HCl, 150mM NaCl, 1 M EDTA, 1mM DTT (pH 7.0) at 5°C for 16hr.

Assays for Hsp90 Client Activity

Yeast strains were transformed with either *HSE-lacZ* (Mollapour et al. 2010), or co-transformed with the centromeric *HIS3* vector pHCA/rGR constitutively expressing glucocorticoid receptor (GR) under control of the alcohol dehydrogenase promoter (*ADH1*) (Garabedian and Yamamoto 1992), and 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. Dexamethasone (DEX) was added to a final concentration of 30μM, followed by incubation at 30°C for 2.5hr to activate the receptors. Cells were collected by centrifugation (2000×g; 5min), washed once with dH_2O, and frozen at -80°C. 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 an equal volume of 2× buffer Z (0.12M $Na_2HPO_4•7H_2O$, 0.08M $NaH_2PO_4•H_2O$, 0.02M KCl, 0.002M $MgSO_4$, pH 7.0). The mixture was added to 700μl of 2mg/ml ONPG solution in 1× buffer Z pre-warmed at 30°C and incubated at 30°C for 5-30min. The reaction was stopped by adding 500μl of 1M sodium carbonate. The optical density (OD_{420}) of each

reaction mixture was determined. The protein concentration of the lysate was determined using a BioRad protein assay (BioRad). β-galactosidase activity was calculated using the following formula: enzyme activity = $1000 \times OD_{420}$/minute/[10 μl×protein concentration (μg/μl)]. Ste11ΔN induction was analyzed as described previously (Louvion et al. 1998; Flom et al. 2008). Ste11ΔN plasmid was a gift of Dr Jill Johnson.

Isothermal Titration Calorimetry and K_d Measurments.

Heat of interaction was measured on a MSC system (Microcal), with a cell volume of 1.458ml, under the same buffer conditions (20mM Tris pH 8.0 containing 1mM EDTA, 5mM NaCl and 7mM $MgCl_2$) at 30°C. For AMPPNP interactions, 20 aliquots of 14.8μl of 1mM AMPPNP were injected into 50μM wild type or mutant T101A or T101E yeast Hsp90. For geldanamycin binding the buffer system contained 2% DMSO. In total 10 aliquots of 27μl of 150μM geldanamycin were injected into 10μM yeast Hsp90 wild type or mutant T101A or T101E. 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.

Flow Cytometric Analysis (FACS Analysis)

FACS analysis was performed as described previously (Mollapour et al. 2010). Yeast cells ($1x10^7$) were collected by centrifugation (16,000×g; 30s) and washed with 1ml ice-cold water and fixed with 1ml of 70% ethanol. Fixed cells (0.3ml) were pelleted by

centrifugation (16,000×g; 30s) and washed with 1ml of 50mM sodium citrate. The cells were pelleted by centrifugation (16,000×g; 30s) and resuspended with 0.5ml of 50mM sodium citrate containing 0.1mg/ml RNase. The cell suspension was incubated for 2 hours at 37°C, followed by addition of 0.5ml of 50mM sodium citrate containing 4μg/ml propidium iodide. The stained cells were analyzed via a FACScan flow cytometer (BD LSRFORTESSA).

Hsp90 ATPase Activity *in vitro*

ATPase activities were measured using a method previously described (Panaretou et al. 1998). This is an enzyme-coupled assay using typically 2μM pure yeast Hsp90 and T101A and T101E mutants. All activities are averages of three separate measurements (Prodromou et al. 2000).

Hsp90 ATPase Activity *in vivo*

ATPase activity of yHsp90-His6, T101A and T101E mutants isolated from yeast was measured as previously described (Kamal et al. 2003) with the following exceptions. Following protein extraction and pull-down experiments as described in the Materials and Methods, protein-bound Ni-NTA (Qiagen) were washed five times in 0.5M NaCl and 1% NP40 buffer. Proteins were competed off the beads with 300μM imidizole, at 4°C for 1hr with agitation. Protein was then concentrated with Amicon® Ultra-2 ml, 10K centrifugal filters (Millipore). Using the Micro BCA™ Protein Assay Kit (Thermo Scientific), protein was quantified and 1μg was run on an SDS-PAGE gel as described previously to standardize the amount of protein used in the assay. Assay was

performed as described in the P_iPer™ Phosphate Assay Kit instructions for use (Life Technologies). Standard curve with linear fit line was created from 0-100μM final concentration reactions. 2.5μg of yHsp90 and phospho-T101 mutants were incubated at 37°C for 1hr with 100mM ATP as substrate, with or without 10μM ganetespib (Synta Pharmaceuticals). ATP turnover was calculated as mmol P_i per mol yHsp90 per minute, and relative ATPase activity was calculated from those values, with the value of Hsp90α alone representing 100% activity.

Purification of the yHsp90 Interactome From Yeast

Three biological replicates of 100ml of yeasts expressing either yHsp90-T101A-His_6 or yHsp90-T101E-His_6 were grown on YPDA to an OD_{600} of 1. Cells were harvested and HIS_6-tagged yHsp90, along with the associated interactome was isolated as follows: Protein was extracted via bead beating in 500μl Binding/Wash Buffer (50mM Na-phosphate pH 8.0, 300mM NaCl, 0.01% Tween-20). 200μg of protein extract was incubated with 50μl His-Tag Dynabeads (Invitrogen) at 4°C for 15min. Dynabeads were collected by magnet then washed 5 times with 500μl Binding/Wash buffer. After final wash, buffer was aspirated and beads were incubated with 100μl Elution buffer (300mM imidazole, 50mM Na-phosphate pH 8.0, 300mM NaCl, 0.01% Tween-20) for 20min, then beads were collected via magnet. The supernatant containing purified yHsp90-His_6 and associated interactome was transferred to a fresh tube, 25μl of 5x SDS-PAGE sample buffer was added and the sample was denatured by boiling for 5min at 95°C. 10μl of sample was analyzed by SDS-PAGE.

Trypsin Digests of yHsp90 Complexes From SDS-PAGE Gels

Purified yHsp90-T101A-His_6 or yHsp90-T101E-His_6 complexes were loaded onto a 12% MOPS buffered SDS-PAGE gel (Invitrogen) and run for 10min at 200v resulting in a ~2cm "gel plug". The gel was stained with 25ml Imperial Stain (Pierce) at room temperature, and destained overnight in dH_2O at 4°C. The gel plugs for each sample to be analyzed were excised by sterile razor blade, divided into 2 sections ~1cm each, and chopped into ~1mm^3 pieces. Each section was washed in dH_2O and destained using 100mM NH_4HCO_3 pH 7.5 in 50% acetonitrile. A reduction step was performed by addition of 100µl 50mM NH_4HCO_3 pH 7.5 and 10µl of 200mM Tris (2-carboxyethyl) phosphine HCl at 37°C for 30min. The proteins were alkylated by addition of 100µl of 50mM iodoacetamide prepared fresh in 50mM NH_4HCO_3 pH 7.5 buffer, and allowed to react in the dark at 20°C for 30min. Gel sections were washed in water, then acetonitrile, and vacuum dried. Trypsin digestion was carried out overnight at 37°C with 1:50-1:100 enzyme–protein ratio of sequencing grade-modified trypsin (Promega) in 50mM NH_4HCO_3 pH 7.5, and 20mM $CaCl_2$. Peptides were extracted with 5% formic acid and vacuum dried.

Isotopic labeling of trypsin-digested yHsp90 complexes

Peptide digests were reconstituted with 70µl of Tris–HCl Buffer Solution (10mM of Tris–HCl, 150mM NaCl, 20mM $CaCl_2$, pH 7.6), vortexed for at least 20min to reconstitute the peptide mixture, then split into two vials of 30µl each (^{16}O vial and ^{18}O vial), and 10ul was retained unlabeled and stored in -80˚C. In a separate vial Mag-Trypsin beads (Clontech) were prepared as follows. 30ul mag-trypsin beads per rxn (^{16}O or ^{18}O) were

pooled and washed 3 times with 800µl of Tris–HCl Buffer Solution, then brought back up in 30µl/sample of Tris-HCl buffer, and aliquoted to a new 1.5ml vial in the quantity of 30µl, vortexing lightly after each aliquot to keep the mag-trypsin beads in suspension. Using a magnetic rack, the Tris-HCl buffer was removed from the beads, and the 30µl of sample digest from above was added to the beads and vacuum dried. 30µl of either ^{16}O H_2O or 97% ^{18}O H_2O (Cambridge Isotopes Laboratories) was added to the respective ^{16}O or ^{18}O prepared Mag-Trypsin bead vial and vortexed for 20min to reconstitute the peptide mixture, and allowed to exchange overnight at 37°C. After ^{18}O exchange, the solution was transferred to a new vial and any free trypsin in solution was inactivated with 1mM PMSF for 30min at 4°C. For each sample section, the (yHsp90-T101E/yHsp9-T101A) digests were combined 1:1 as follows: Forward (FWD) Sample Set: (F)-^{16}O:(E)-^{18}O and Reversed (REV) Sample Set: (E)-^{16}O:(F)-^{18}O, dried and stored at −80°C until analysis. Three biological replicate experiments were performed.

HPLC for mass spectrometry

All samples were re-suspended in Burdick & Jackson HPLC-grade water containing 0.2% formic acid (Fluka), 0.1% TFA (Pierce), and 0.002% Zwittergent 3–16 (Calbiochem), a sulfobetaine detergent that contributes the following distinct peaks at the end of chromatograms: MH^+ at 392, and in-source dimer [2 M + H^+] at 783, and some minor impurities of Zwittergent 3–12 seen as MH^+ at 336. The peptide samples were loaded to a 0.25µl C_8 OptiPak trapping cartridge custom-packed with Michrom Magic (Optimize Technologies) C8, washed, then switched in-line with a 20cm by 75µm C_{18} packed spray tip nano column packed with Michrom Magic C18AQ, for a 2-step

gradient. Mobile phase A was water/acetonitrile/formic acid (98/2/0.2) and mobile phase B was acetonitrile/isopropanol/water/formic acid (80/10/10/0.2). Using a flow rate of 350nl/min, a 90min, 2-step LC gradient was run from 5% B to 50% B in 60min, followed by 50%–95% B over the next 10min, hold 10min at 95% B, back to starting conditions and re-equilibrated.

LC–MS/MS analysis

The samples were analyzed via electrospray tandem mass spectrometry (LC–MS/MS) on a Thermo Q-Exactive Orbitrap mass spectrometer, using a 70,000 RP survey scan in profile mode, m/z 360–2000 Da, with lockmasses, followed by 10 MS/MS HCD fragmentation scans at 17,500 resolution on doubly and triply charged precursors. Single charged ions were excluded, and ions selected for MS/MS were placed on an exclusion list for 60s.

LC–MS/MS data analysis, statistical analysis

Interactomes of yHsp90-T101A-His_6 and yHsp90-T101E-His_6 were analyzed as described previously (Truman et al. 2012). All LC-MS/MS *.raw Data files were analyzed with MaxQuant version 1.2.2, searching against the SPROT Human database using the following criteria: ^{18}O heavy label was selected for quantitation with a min of 1 high confidence peptide to assign quantitation H/L ratio. Trypsin was selected as the protease with max miss cleavage set to 2. Carbamiodomethyl (C) was selected as a fixed modification. Variable modifications were set to Oxidization (M), Formylation (n-term), and Phosphorylation (STY). Orbitrap mass spectrometer was selected using an

MS error of 20ppm and a MS/MS error of 0.5Da. 1% FDR cutoff was selected for peptide, protein, and site identifications.

Ratios were reported based on the MS level light and heavy peak areas determined by MaxQuant and reported in the proteinGroups.txt file as heavy/light or (T101E/T101A mutant). Proteins were removed from this results file if they were flagged by MaxQuant as “Contaminants”, “Reverse” or “Only identified by site”. Complete three biological replicates were performed, with each biological replicate split into two technical replicates (^{18}O forward (FWD) labeling, and ^{18}O reverse (REV) labeling). The abundance data from each biological replicate were normalized to the ratio of the bait protein in that run (e.g. normalized to the yHsp90-His_6 ratio). Light and Heavy peak intensities were analyzed in each run to determine protein hits that fell into the category of either yHsp90-T101E-only hits or yHsp90-T101A-only hits and retained if they confirmed to this state across all 6 runs. In the case of yHsp90-T101E-only or yHsp90-T101E-only protein hits, spectra counts can be used as a proxy for abundance as these would not of been assigned a quantitation ratio. This produced a list of yHsp90 interactors and their respective quantitated changes between yHsp90-T101E and yHsp90-T101A.

Further statistical analysis was performed using the R statistical package (http://www.r-project.org/). Proteins with three out of the six observations were retained. Missing values were imputed using row mean imputation. An ANOVA test was then performed to identify proteins that indicate significant variability (p-value < 0.05) between biological replicates within each group. These were removed from consideration. A list of proteins identified and corresponding ratios can be found in Table S1. The mass spectrometry

proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD001969.

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