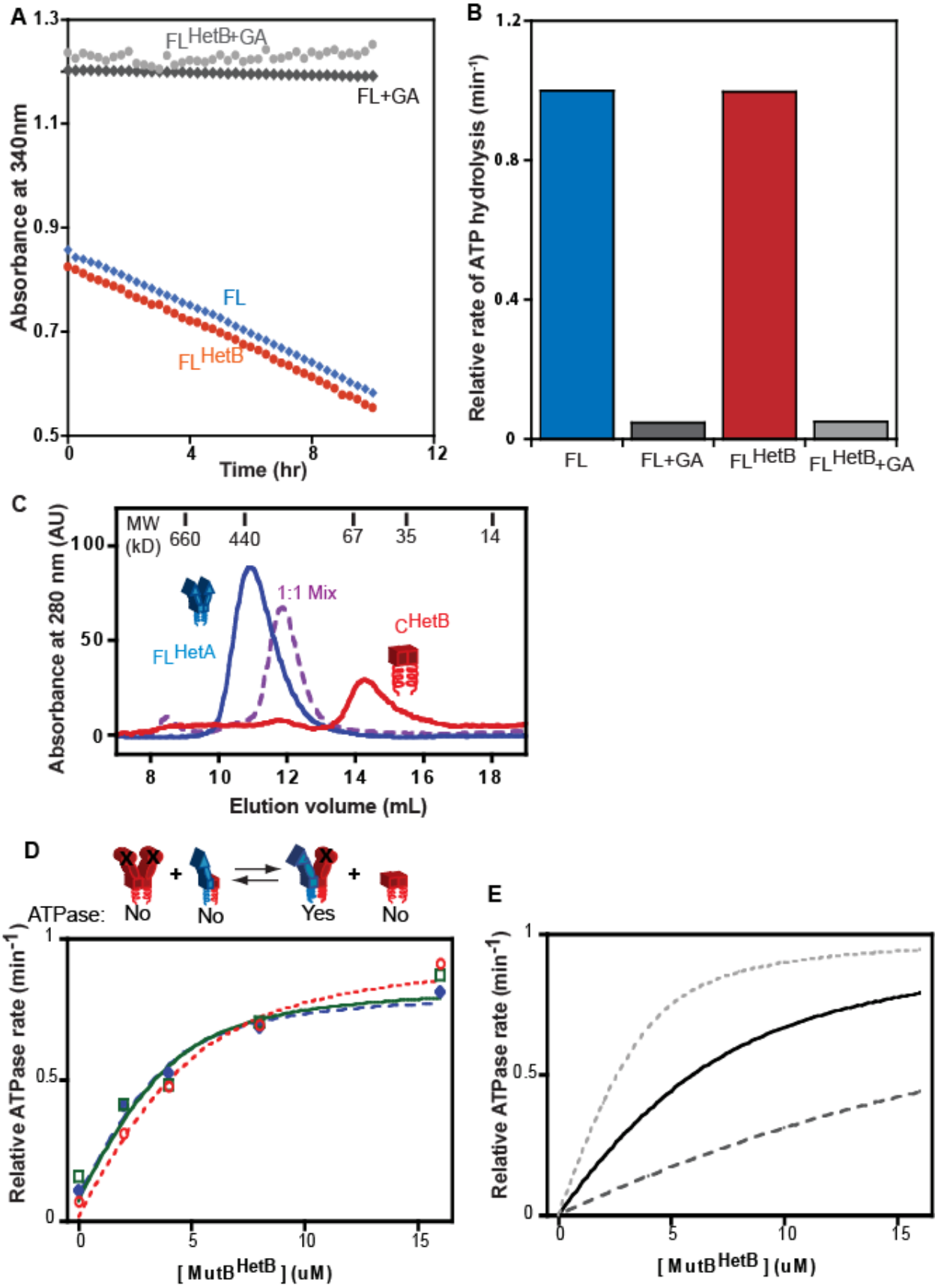
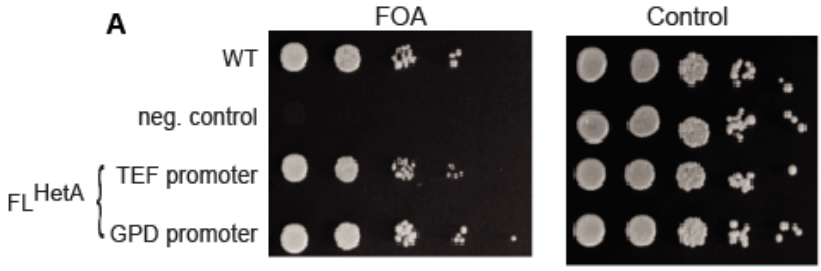
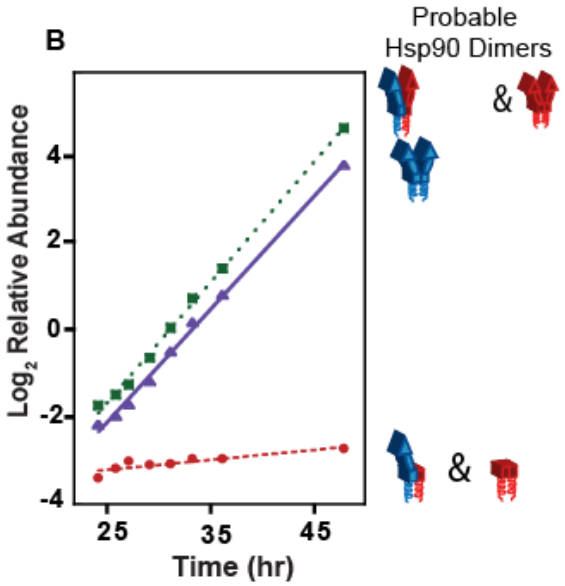
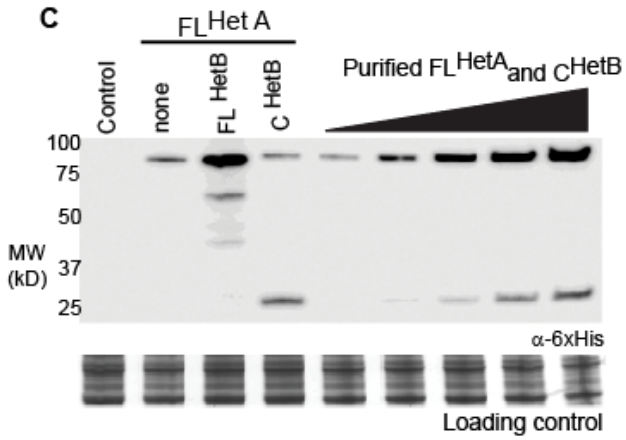
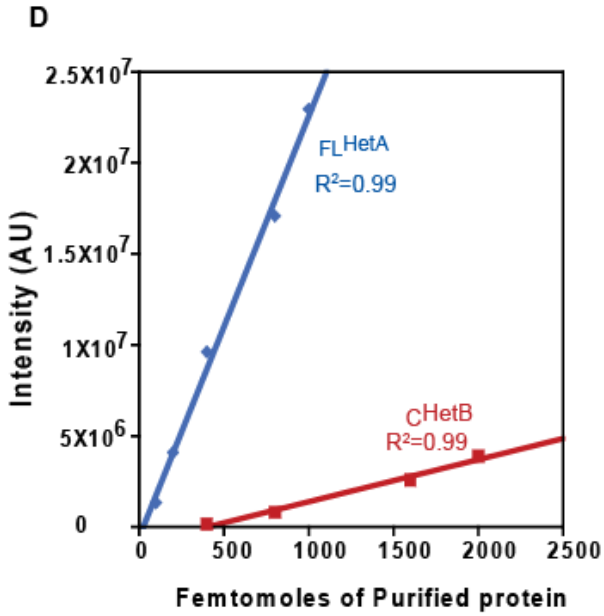

A
Absorbance at 340nm
Time (hr)
FL HetB+GA
FL+GA
FL
FL HetB
B
Relative rate of ATP hydrolysis (min-1)
FL
FL+GA
FL HetB
FL HetB+GA
C
Absorbance at 280 nm (AU)
MW (kD)
660
440
67
35
14
1:1 Mix
FL HetA
C HetB
Elution volume (mL)
D
ATPase:
No
No
Yes
No
Relative ATPase rate (min-1)
[MutB HetB] (uM)
E
Relative ATPase rate (min-1)
[MutB HetB] (uM)

Figure S1. Appending asymmetric coiled coils to Hsp90 preferentially stabilizes heterodimers, but does not alter its biochemical properties, related to Figure 2. (A) ATPase activity of 3uM of FL (blue) and FL^{HetB} (red) in absence of Geldanamycin and in presence of Geldanamycin (black and grey) for the respective Hsp90 proteins. (B) Bar graph indicating the rate of ATP hydrolysis for the FL and FL^{HetB} with and without Geldanamycin. (C) Size exclusion chromatography indicates that the asymmetric coiled-coil causes the preferential accumulation of Hsp90 heterodimers. (D) Investigation of ATPase mutant impacts on heterodimerization. Titration of FL^{HetB} ATPase mutants (where blue, green and red indicate the titration curves for $E33A^{HetB}$, $D79N^{HetB}$ and $R380A^{HetB}$ respectively) into a mixture of WT^{HetA} and C^{HetB} indicates that all of the FL^{HetB} ATPase mutants readily cross dimerize with WT^{HetA}. Heterodimers are slightly favored for all variants (Keq of 3.4 for E33A, 3.1 for D79N, and 1.9 for R380A). (E) Theoretical model of titrations with FL^{HetB} mutants that altered heterodimer preference (either plus four-fold in light grey dash, no preference in solid black, or minus four-fold in dark grey dash) indicate that the assay conditions stringently test dimer preference.


A
FOA
Control
WT
neg. control
FLHetA
TEF promoter
GPD promoter


B
Probable
Hsp90 Dimers
&
&
Log2 Relative Abundance
4
2
0
-2
-4
25
35
45
Time (hr)


C
FLHet A
Control
none
FL HetB
C HetB
Purified FLHetA and CHetB
100
75
50
MW
(kD)
37
25
α-6xHis
Loading control


D
2.5X10^7
2X10^7
1.5X10^7
1X10^7
5X10^6
0
Intensity (AU)
FLHetA
R²=0.99
CHetB
R²=0.99
0
500
1000
1500
2000
2500
Femtomoles of Purified protein

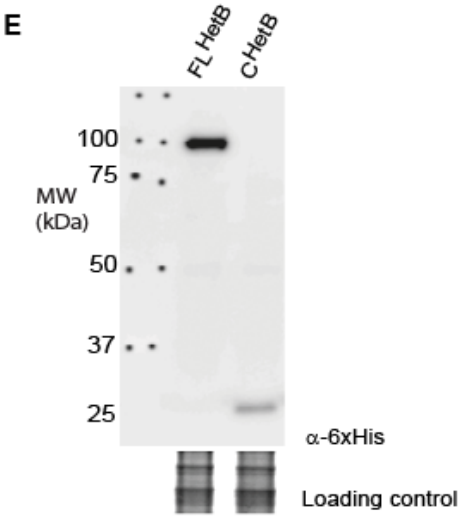

E
FL HetB
CHetB
100
75
MW
(kDa)
50
37
25
α-6xHis
Loading control

Figure S2. Growth of yeast and relative expression analysis of FL^{HetA} and C^{HetB} constructs, related to Figure 3. (A) Comparison of the growth of yeast expressing different levels of FL^{HetA} Hsp90. The reduced expression level from chromosomally integrated Hsp90 with a TEF promoter results in slightly slower growth compared to the analogous strain with a stronger GPD promoter. Under the GPD promoter, the FL^{HetA} Hsp90 strain grows indistinguishable from a strain expressing WT Hsp90 at endogenous levels. The negative control is a strain that cannot generate active Hsp90 dimers in the presence of FOA. Equivalent serial dilutions were made on a plate lacking FOA as a control. (B) Yeast strains expressing heterodimer with FL versions of Hsp90 (FL^{HetA}/FL^{HetB}) shown in green, heterodimers of FL and C-domain (FL^{HetA}/C^{HetB}) shown in red and homodimers of FL^{HetA} shown in purple. (C) Western blot (anti-6xHis) of whole cell lysates to assess the expression level of the different Hsp90-coil constructs. Purified proteins of FL^{HetA} and C^{HetB} were used as standards to quantify the expression levels in cell lysates. (D) Standard plot drawn from the quantification of bands in Panel C of increasing concentration of FL^{HetB} (blue) and C^{HetB} (red) standards. (E) Anti-6xHis antibody does not detect any non-specific bands at the Molecular weights of the FL^{HetB} and C^{HetB} proteins. Western blot with anti-His antibody showing molecular weight markers in lane 1, FL^{HetB} in lane 2 and C^{HetB} in lane 3. Loading control is a 10% coomassie stained SDS-gel with FL^{HetB} and C^{HetB} diluted in 12ug of the yeast cell lysates lacking any His tagged protein.

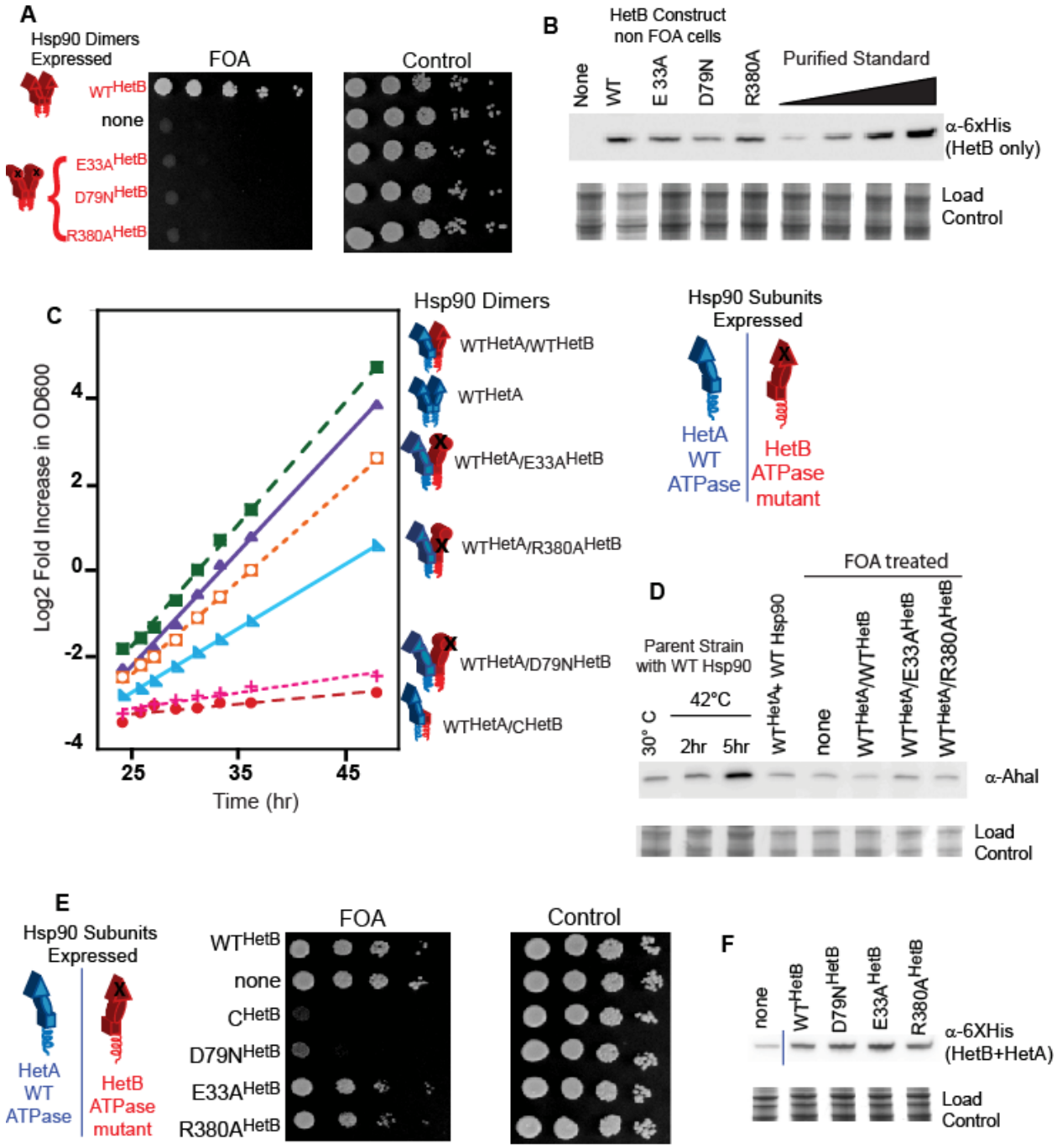


Figure S3. Growth of yeast in liquid media and controls with ATPase mutants as the sole Hsp90, related to Figure 4. (A) Growth on plates of yeast with ATPase mutants as the sole version of Hsp90. (B) Analyses of the expression level of HetB constructs in cells co-expressing

untagged WT Hsp90 (control cells in panel B) determined by a Western blot. (C) Growth in liquid culture of yeast strains expressing WT^{HetA} together with Mutant ^{HetB} constructs. (D) Western analysis of Aha1 levels in different yeast strains. (E) Growth of DBY519 yeast with HetB subunits expressed from a plasmid encoded TEF promoter (reduced expression level relative to analogous experiments shown in Figure 4). The similar relative growth properties of all HetB variants in both of these experiments indicates that the system is robust to changes in the expression level of the HetB constructs and that the observed growth properties are primarily dependent on the biochemical properties of the HetA/HetB dimers. (F) Western analysis of the steady state expression of Hsp90 variants in the yeast strains from panel E. Intervening lanes were removed for presentation purpose. Of note, both the chromosomally integrated HetA subunit and the mutant HetB variants of Hsp90 are detected with the α-6XHis antibody.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid and strain construction of Hsp90 variants

The DBY519 strain (trp1 ura3 hsp82 hsc82 ho::pTef1-Hsp90^{HetA}-His3 pKAT6) used in this study is a W303 derivative that was generated from Ecu cells (Nathan et al., 1997) by integration of the Hsp90^{HetA} construct at the HO locus. The pKAT6 plasmid is *URA3* marked and expresses the wild type Hsp82 gene. The designed Hsp90 heterodimers were generated by inserting either the viiA (GGTSSVKELEDKNEELLSRIAHERNEVARLKKLVGERTG) or viiB (GGTSSVKELEDKNEELLSELAHLKNEVARLKKLVGERTG) coiled coil sequence (Havranek and Harbury, 2003) and a His_6 tag (GGHHHHHHGGH) after amino acid 678 in the Hsp82 gene. Hsp90^{HetB} constructs were generated in p414GPD and included the terminator sequence from Cyc1. Western blotting indicated that the integrated Hsp90^{HetA} gene that lacked a terminator expressed to about 1/15 the level of the plasmid encoded Hsp90^{HetB} constructs (Supplementary Figure S2C,D). Single amino acid mutant constructs of Hsp90^{HetB} were generated by site directed mutagenesis and confirmed by sequencing of the entire open reading frame.

Yeast Growth rate

Hsp90^{HetB} constructs on plasmids were transformed into DBY519 cells using standard approaches and media recipes (Gietz et al., 1995). Transformants were selected on synthetic dextrose plates lacking tryptophan and uracil (SD-W-U). Single colonies were then grown in SD-W-U liquid media to an OD600 of about 0.8. These cells were then diluted into SD-W media and grown at 30 °C for 16 hours to an OD600 of about 0.2. Cells were finally diluted into SD-W+FOA to select for loss of the pKAT6 plasmid either in plates or liquid culture. For the liquid culture growth experiments, cells were maintained in log phase (OD600 between 0.1 to 0.8) by

periodic dilution. Growth of the cultures was estimated based on OD600 measurements taking into account dilutions. The log2 of OD600 versus time was fit to a linear equation to determine growth rates.

Western blotting

To determine the expression level of Hsp90 variants, yeast strains were grown in SD-W-U to an OD600 of about 0.5 and collected by centrifugation. These cell pellets were lysed by vortexing with glass beads in lysis buffer (50 mM Tris, pH 7.5, 5 mM EDTA and 10 mM PMSF) followed by addition of SDS to 2%. Lysed cells were then centrifuged at 14000g for 1 minute and protein concentrations in the lysates estimated using the BCA assay (Pierce Inc.). Lysate containing 12 μg of protein was separated by SDS-PAGE and the expression level quantified by blotting using α-HisG antibody (Invitrogen Inc.). A standard curve was generated using purified Hsp90 constructs added to yeast lysates from cells that did not express any epitope tagged Hsp90. Analyses of Aha1 levels in different yeast strains were investigated as described above following FOA treatment to remove WT Hsp90 and using a rabbit polyclonal Aha1p antibody (StressMarq SPC-204D).

Production and purification of Hsp90 protein

Hsp90 constructs were expressed individually in BLR(DE3) cells from T7 promoters. Bacterial cells were grown at 37 °C to an OD600 of 1.0, followed by induction with 1 mM IPTG at 30 °C for 5 hours. Cells were harvested by centrifugation and re-suspended in Wash Buffer (20 mM potassium phosphate, pH 8.0, 300 mM potassium chloride, 10 mM imidazole). Cell lysis was performed by treatment with lysozyme and sonication. These cell lysates were incubated with Ni^{2+}NTA agarose (Qiagen) for 1 hour at 4 °C. After rinsing the nickel resin extensively with Wash Buffer, Hsp90 protein was collected in Elution Buffer (200 mM imidazole, pH 7.5). EDTA

and DTT were added to 10 mM, and constructs were further purified on a Phenyl Sepharose column using a linear gradient of ammonium sulfate from 60 mg/mL to 0 mg/mL in 20 mM potassium phosphate, pH 8.0, 1 mM EDTA, and 5 mM DTT. The Hsp90 constructs were further purified by anion exchange chromatography using a Q sepharose HP column (GE) eluted with a linear gradient from 0 to 500 mM potassium chloride in 20 mM potassium phosphate, pH 8.0, 1 mM EDTA. Finally the proteins were dialyzed in 20 mM Potassium phosphate, pH 7.0, 2 mM DTT, and 1 mM EDTA. Proteins were concentrated in the same buffer to approximately 2 mg/ml using Amicon Ultra concentrators (Millipore). Protein concentrations were determined spectroscopically using extinction coefficients based on amino acid composition calculated with the program Sednterp (Amgen).

Analytical Size Exclusion Chromatography

100 μL samples of 28 μM protein were analyzed using SEC buffer (20 mM potassium phosphate, pH 7.0, 2 mM DTT, 1 mM EDTA and 100 mM potassium chloride) and a Superdex200 column (GE). Absorbance at 280 nm was used to monitor the elution profile.

Enzymatically coupled ATPase assay

Hydrolysis of ATP was enzymatically linked to NADH oxidation and monitored spectroscopically as previously described (Norby, 1988). ATPase assays were performed at 37 °C. Hsp90 constructs were mixed and equilibrated at 37 °C for 2 hours prior to ATPase analyses. Using a Bio50 Spectrophotometer equipped with a Peltier temperature control unit (Cary) and a 1 cm pathlength cuvette, absorbance at 340 nm was measured at 15 second intervals for 10 minutes. ATPase reactions contained 20 mM Tris, pH 7.5, 5 mM magnesium chloride, 100 mM potassium chloride, 1 mM ATP (Sigma), 0.17 mM NADH (Sigma), 0.67 mM phosphoenolpyruvate (Sigma), 0.01 mg/mL Pyruvate Kinase (Sigma), and 0.02 mg/mL Lactose

Dehydrogenase (EMD Biosciences). ATPase rates were determined by fitting the change in absorbance versus time to a linear model, converting absorbance units to concentration of NADH oxidized and normalizing to the concentration of Hsp90.

Equilibrium dimer distribution model 1

In order to estimate heterodimer preference from the C-domain titration and ATPase measurements, we consider two heterodimers swapping partners to generate homodimers (2AB←→AA+BB; where A represents a full-length Hsp90 subunit and B represents a C-domain subunit). For the partner exchange reaction $K_{eq} = [AA][BB]/[AB]^2$. If the free energy of dimerization is equivalent for all three species, then based on random collisions we would expect a 1:2:1 ratio of [AA]:[AB]:[BB] and a K_{eq} of 0.25. To fit the ATPase data (Figure 2A), we assume that full-length homodimers (AA in our equilibrium model) are the dominant contributor to ATPase activity. Starting with the equation for K_{eq}, as well as equations describing the total amount of each subunit (e.g., $A_{total} = 2[AA] + [AB]$), we derived a quadratic solution (shown below) to determine the concentration of ATPase active full-length AA homodimers as a function of K (K_{eq}), A (the total concentration of full-length subunits) and B (the total concentration of C-domain subunits). We used this equation to fit the data in Figure 2 and define heterodimer preference as the ratio of the observed K_{eq} to that for unbiased mixing.

$$[AA] = \frac{A - B - 8KA \pm \sqrt{(A-B)^2 + 16KAB}}{4 - 16K}$$

Equilibrium dimer distribution model 2

In order to investigate potential impacts of ATPase mutants on heterodimer preference, we developed a dimerization competition assay as outlined in Supplementary Figure S1D. These experiments utilized a constant concentration of WT^{HetA} (3 μM), and C^{HetB} (6 μM) and standard

ATPase conditions (with 1 mM ATP). Experiments were performed over a range of Mut^{HetB} concentrations and ATPase activity was utilized to readout the extent of dimer exchange. To estimate the fraction of exchange that had occurred, the raw ATPase rates were normalized to the activity of WT/Mut heterodimers that we had previously measured (Figure 2). We fit the data shown in Supplementary Figure S1C using a dimer exchange model:

$WT^{HetA}/C^{HetB} + Mut^{HetB}/Mut^{HetB} \leftrightarrow WT^{HetA}/Mut^{HetB} + C^{HetB}/C^{HetB}$

$Keq = [WT^{HetA}/Mut^{HetB}][\ C^{HetB}/C^{HetB}] / [WT^{HetA}/C^{HetB}][\ Mut^{HetB}/Mut^{HetB}]$

Substitution with equations describing the total concentration of each species (and making the simplifying assumption that monomer species were negligible), resulted in the following solution to a quadratic equation that was used to estimate heterodimer preference as K for the exchange reaction:

$$Arel = Ao + \Delta A\frac{-(MK + 3K + 6) \pm \sqrt{(MK + 3K + 6)^2 - 12(1 + K)KM}}{2(K + 1)}$$

Where Arel is ATPase activity relative to the activity of isolated WT^{HetA}/Mut^{HetB} heterodimers, Ao is the residual ATPase rate, ΔA is 1-Ao, M is the total concentration of Mut^{HetB} in μM, and K is Keq for the dimer exchange reaction.

Native gel

Mixtures of Hsp90 constructs were equilibrated at 37 °C for 2 hours. Protein samples at 5 μM were prepared in 2.5 mM Hepes, pH 7.5, 10% sucrose, 0.01% bromphenol blue, and loaded onto a polyacrylamide gel (30 mM Hepes, 30 mM imidazole, pH 7.0, 6% polyacrylamide, 1 mM EDTA). Gels were run at 30 mAmp for one hour. Proteins in the gel were visualized using Coomassie Brilliant Blue staining.

SUPPLEMENTAL REFERENCES

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