Supporting Information

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SI Methods

Proteins. Plasmids for the overproduction of wild-type and mutant *Escherichia coli* ($Hsp90_{Ec}$) were transformed into *E. coli* strain BL21(DE3). Cultures were grown at 30 °C to an $OD_{600} \sim 0.8$ and induced for 3.5 h with 1 mM IPTG. Soluble extracts were applied to a HiPrep 16/10 Q Sepharose Fast Flow ion exchange column (GE Healthcare) and proteins were eluted with a 150–600 mM linear gradient of NaCl in 20 mM Tris • HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 10% glycerol (vol/vol). Fractions containing $Hsp90_{Ec}$ were purified further using a HiLoad 16/60 Superdex gel filtration chromatography column (GE Healthcare) in 50 mM Tris • HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 10% glycerol (vol/vol).

Hsp82 expression and purification were performed as described previously (1) with the following modifications. Hsp82 was purified on a HisTrap High-Performance 1-mL column (GE Healthcare) according to the manufacturer's instructions. Fractions containing Hsp82 were purified further using a HiLoad 16/60 Superdex gel filtration chromatography column (GE Healthcare) in 50 mM Tris • HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 10% glycerol (vol/vol).

Heat-Denatured Luciferase Reactivation Assay. Luciferase (80 nM; 120 μL) was heat-denatured at 45 °C for 7 min in 25 mM Hepes, pH 7.5, 50 mM KCl, 15 mM $MgCl_2$, 0.05 mg/mL BSA, 1 mM ATP, 2 mM DTT, and an ATP regenerating system (20 mM creatine phosphate, 0.06 mg/mL creatine kinase). About 99% of the luciferase activity was inactivated by this treatment. After 1 min on ice, DnaK wild type or mutant (0.75 μM), DnaJ or CbpA (0.15 μM), GrpE (0.05 μM) and $Hsp90_{Ec}$ wild type or mutant (0.5 μM) were added as indicated and reaction mixtures (130 μL) were incubated at 24 °C. To determine luciferase activity, 5-μL aliquots were added at the indicated times to 120 μL of 25 mM Hepes buffer, pH 7.5, containing 200 μM D(-)-Luciferin (Roche), 0.5 mM ATP, and 10 mM $MgCl_2$. Light emission was measured using a Victor^3 1420 Multilabel Counter (PerkinElmer). Where indicated, geldanamycin (GA) (15 μM) was added to inhibit $Hsp90_{Ec}$ activity and CbpM (1.6 μM) was added to inhibit the CbpA protein. Background corrections were made by subtracting the light emission measured at $t = 0$. Reactivation was determined compared to an unheated luciferase control.

Chemically Denatured Luciferase Reactivation Assay. Luciferase (14 μM) was denatured in 6 M guanidine hydrochloride for 5 min at 24 °C then diluted 80-fold into mixtures (80 μL) containing 25 mM Hepes, pH 7.5, 50 mM KCl, 2 mM DTT, 0.1 mg/mL BSA, 0.005% Triton X-100 (vol/vol), 4 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 0.03 mg/ml creatine kinase), and 10 mM $MgCl_2$. After 5 min at 24 °C, DnaK (1.3 μM), CbpA (0.2 μM), GrpE (0.1 μM), and $Hsp90_{Ec}$ (1.5 μM) were added as indicated and reactions (100 μL) were incubated at 24 °C. To determine luciferase activity, aliquots (5 μL) were added to 0.1 mL of Luciferase Assay Substrate (Promega, E2510) at the times indicated and 550 nm light emission was measured in a Varian Cary Eclipse fluorescence spectrophotometer using Bio/Chemiluminescence mode with a slit width of 20 nm and gate time of 200 ms. Reactivation was determined compared to a nondenatured luciferase control.

Glucose-6-Phosphate Dehydrogenase (G6PDH) Reactivation. G6PDH (19 μM) was denatured at 47 °C for 5 min in 25 mM Hepes, pH 7.5, 13% glycerol (vol/vol), 30 mM DTT, 5 M urea, then diluted 50-fold with 47 °C folding buffer (FB) buffer [25 mM Hepes, pH 7.5, 50 mM KCl, 10 mM $Mg(OAc)_2$, 0.1 mM EDTA, 5 mM DTT], incubated an additional 2 min at 47 °C, and then chilled on ice for 2 min. Denatured G6PDH (80 μL) was incubated at 24 °C in reactivation assays (100 μL) containing FB buffer, 2 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 0.03 mg/mL creatine kinase), DnaK (1.3 μM), CbpA (0.2 μM), GrpE (0.1 μM), and $Hsp90_{Ec}$ wild type or mutant (1.5 μM) as indicated. G6PDH activity was measured in FB buffer containing BSA (50 μg/mL), 2 mM glucose-6-phosphate and 1 mM NAD by monitoring NADH fluorescence over time at 24 °C using a Varian Cary Eclipse fluorescence spectrophotometer with a well plate reader. Excitation and emission wavelengths were 340 and 468 nm, respectively. Slopes from the initial linear increase in NADH fluorescence were used to calculate the percent reactivation compared to a native G6PDH control. GA (15 μM) was used to inhibit $Hsp90_{Ec}$ where indicated.

ATPase. Reaction mixtures (50 μL) contained 25 mM Hepes, pH 7.5, 50 mM KCl, 2 mM DTT, 20 mM $MgCl_2$, 80 μM ATP, 0.1 μCi (1 Ci = 37 GBq) of [γ-^{33}P]ATP (3,000 Ci/mmol; Perkin Elmer), and 1 μM $Hsp90_{Ec}$ wild type or mutants. Where indicated, GA (15 μM) or CbpM (1.6 μM) was added. Reaction mixtures were incubated at 37 °C for 60 min and analyzed as described (2). The activity of $Hsp90_{Ec}$ was set to 100%, corresponding to 0.30± 0.02 nmol of ATP hydrolyzed in 60 min. The time point selected, 60 min, was within the linear range of the assay.

Two-Hybrid Analysis. Bacterial two-hybrid experiments were performed as previously described (3). PCR products containing *htpG* or *dnaK* genes were fused in frame with the 3′ end of the genes coding for the two catalytic domains, T18 and T25, of *Bordetella pertussis* adenylate cyclase using plasmids pEB355 and pEB354, respectively (4). The resulting plasmids were cotransformed into *E. coli* strain BTH101 (3), plated on LB plates containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) and incubated at 30 °C. Transformants were used to inoculate cultures of LB selective media containing 0.5 mM IPTG. After incubation at 30 °C overnight, β-galactosidase activity was measured as previously described (3).

In Vitro Protein Binding Assay. To measure the association of $Hsp90_{Ec}$ with DnaK, 0.13 μM [^{3}H]DnaK was incubated at 24 °C for 5 min in reaction mixtures (100 μL) containing 20 mM Tris • HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10% glycerol (vol/vol), 0.05% Triton X-100 (vol/vol), 5 mM DTT, and 1 μM $Hsp90_{Ec}$, Hsp82, or BSA as indicated. Reactions were filtered through Microcon YM-100 filters (Millipore) by centrifugation at 2,700 × *g* for 12 min at 24 °C. Retained proteins were recovered with 10% (vol/vol) SDS and radioactivity was measured. Background corrections were made by subtracting the percentage of [^{3}H]DnaK retained in the presence of buffer alone (19.3%).

1. Panaretou B, et al. (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J* 17:4829–4836.
2. Shacter E (1984) Organic extraction of Pi with isobutanol/toluene. *Anal Biochem* 138:416–420.
3. Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA* 95:5752–5756.
4. Battesti A, Bouveret E (2006) Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. *Mol Microbiol* 62:1048–1063.

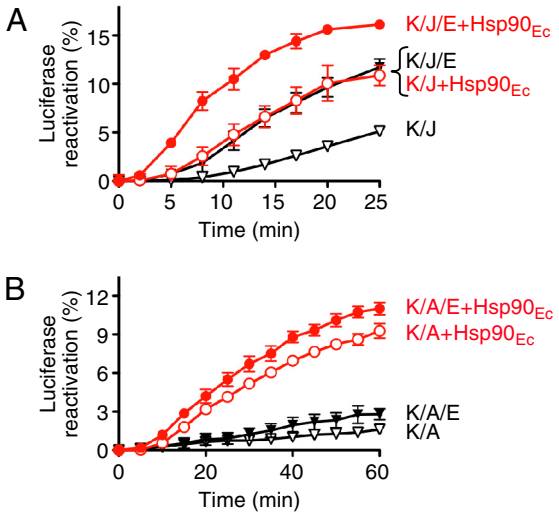

Fig. S1. GrpE stimulates reactivation of heat-denatured luciferase by the DnaK system and $Hsp90_{Ec}$. (*A*) Reactivation of heat-denatured luciferase was measured over time as described in *Methods* in the presence of DnaK and DnaJ without (K/J) or with GrpE (K/J/E); or, $Hsp90_{Ec}$ and K/J or K/J/E. (*B*) Reactivation of heat-denatured luciferase was measured as in *A* except that CbpA was substituted for DnaJ in the DnaK system, referred to here as K/A or K/A/E. Data from three replicates are presented as mean ± SEM.

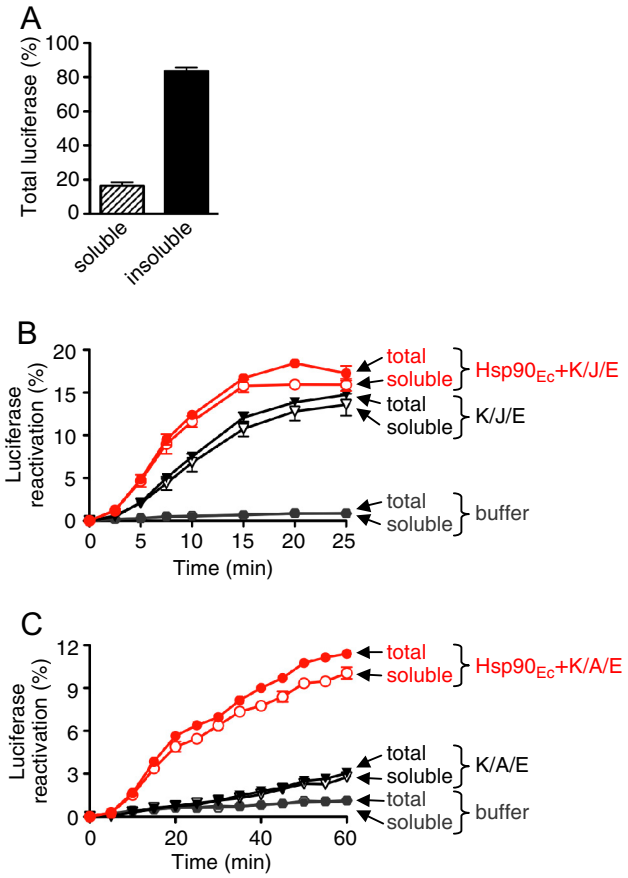

Fig. S2. $Hsp90_{Ec}$ and the DnaK system reactivate the soluble fraction of heat-denatured luciferase. (*A*) Quantification of the soluble and insoluble fractions of heat-denatured luciferase. Luciferase was heat-denatured as described in *Methods*. After 1 min on ice, mixtures were centrifuged at 16,000 × *g* for 5 min at 4 °C. The amount of luciferase present in the supernatant (soluble fraction) was quantified by densitometry from Western blots using luciferase antibody (Promega G7451) and presented as the percentage of the total luciferase measured before denaturation. (*B*) Reactivation of total (closed symbols) or the soluble fraction (open symbols) of heat-denatured luciferase (prepared as described in *A*) was measured over time as described in *Methods* in the presence of $Hsp90_{Ec}$ and DnaK, DnaJ, and GrpE (K/J/E); K/J/E; or buffer. (*C*) Reactivation of heat-denatured luciferase was measured as in *B* except that CbpA was substituted for DnaJ in the DnaK system, referred to as K/A/E. Data from three or more replicates are presented as mean ± SEM.

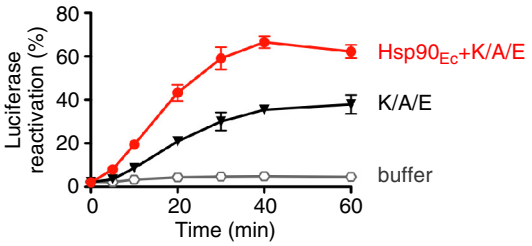

Fig. S3. $Hsp90_{Ec}$ reactivates chemically denatured luciferase in collaboration with the DnaK chaperone system. Reactivation of chemically denatured luciferase was measured over time as described in *SI Methods* in the presence of DnaK, CbpA, and GrpE (K/A/E); $Hsp90_{Ec}$ and K/A/E; or buffer. Data from three replicates are presented as mean ± SEM.

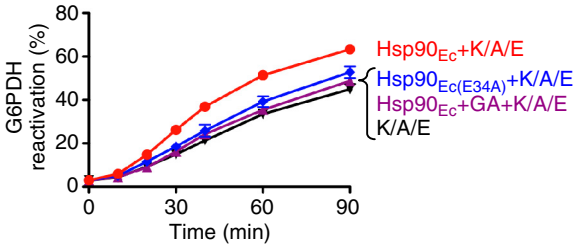

Fig. S4. $Hsp90_{Ec}$ ATPase activity is required for G6PDH remodeling in collaboration with the DnaK system. Reactivation of denatured G6PDH was measured over time as described in *Methods* in the presence of DnaK, CbpA, and GrpE (K/A/E); $Hsp90_{Ec}$ and K/A/E without or with GA; or $Hsp90_{Ec(E34A)}$ and K/A/E. Data from at least three replicates are presented as mean ± SEM.

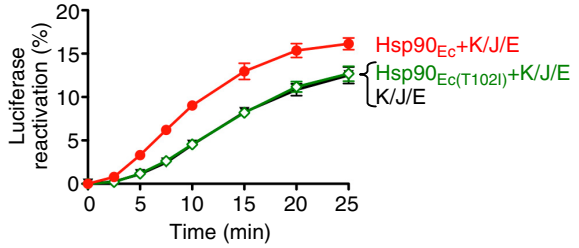

Fig. S5. $Hsp90_{Ec}$ active site lid mutant, $Hsp90_{Ec(T102I)}$ does not stimulate reactivation of heat-denatured luciferase by DnaK, DnaJ, and GrpE. Reactivation of heat-denatured luciferase was measured over time as described in *Methods* in the presence of DnaK, DnaJ, and GrpE (K/J/E); $Hsp90_{Ec}$ and K/J/E; or $Hsp90_{Ec(T102I)}$ and K/J/E. Data from three replicates are presented as mean ± SEM.

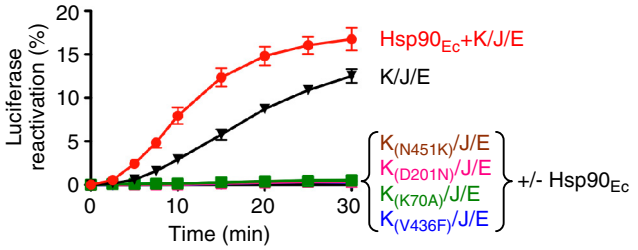

Fig. S6. Several DnaK mutants are unable to function in luciferase reactivation. Reactivation of heat-denatured luciferase was measured over time as described in *Methods* with DnaK, DnaJ, and GrpE (K/J/E), $DnaK_{(K70A)}$/J/E, $DnaK_{(D201N)}$/J/E, $K_{(N451K)}$/J/E, or $DnaK_{(V436F)}$/J/E with or without $Hsp90_{Ec}$. Data from two replicates are presented as mean ± SEM. Some error bars are covered by plot symbols.

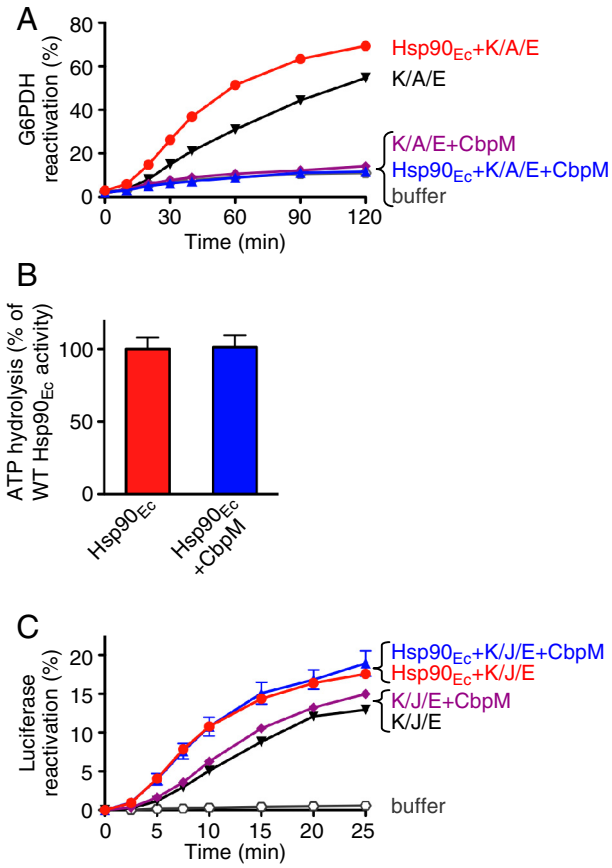

Fig. S7. Effects of CbpM. (*A*) Effect of CbpM on the reactivation of denatured G6PDH. Reactivation of denatured G6PDH was measured over time as described in *Methods* in the presence of DnaK, CbpA, and GrpE (K/A/E) with or without CbpM; $Hsp90_{Ec}$ and K/A/E with or without CbpM; or buffer. (*B*) CbpM does not affect $Hsp90_{Ec}$ ATPase activity. $Hsp90_{Ec}$ ATPase activity was measured as described in *Methods* with or without CbpM. ATPase activities were represented as percentage of $Hsp90_{Ec}$ ATPase activity without CbpM. (*C*) CbpM does not inhibit luciferase reactivation by DnaK, DnaJ, and GrpE in collaboration with $Hsp90_{Ec}$. Reactivation of thermally denatured luciferase was measured over time as described in *Methods* in the presence of DnaK, DnaJ, and GrpE (K/J/E) without or with CbpM; $Hsp90_{Ec}$ and K/J/E without or with CbpM; or buffer. Data from at least three replicates are presented as mean ± SEM.

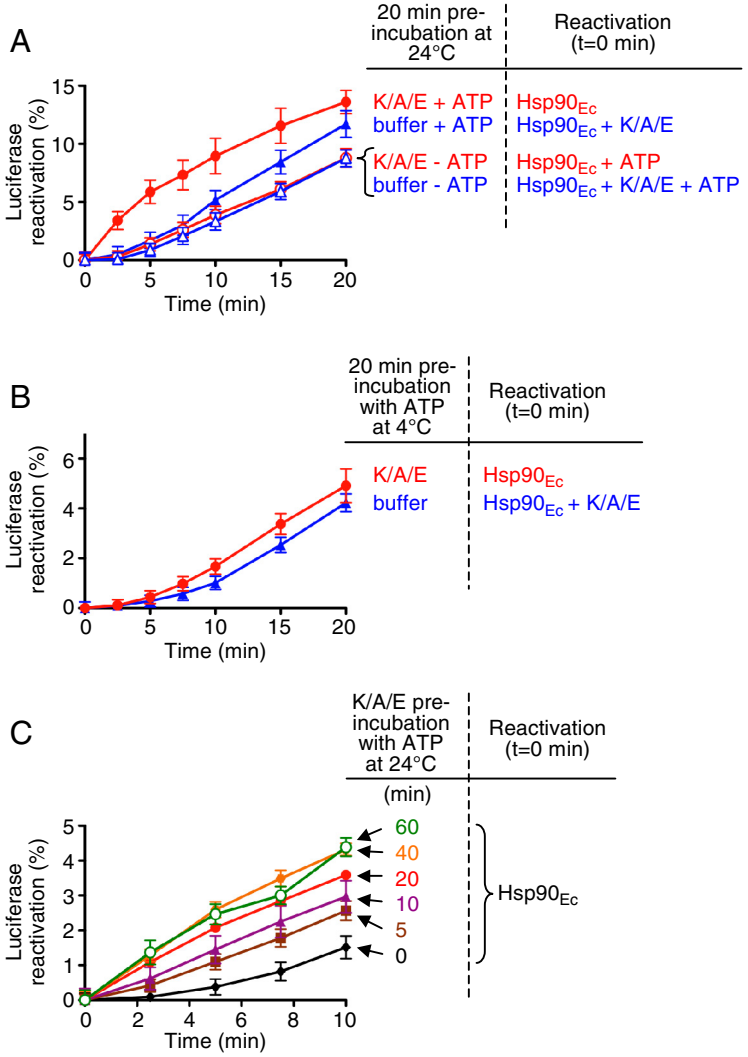

Fig. S8. Chaperone activity of the DnaK system is required prior to $Hsp90_{Ec}$. (*A*) Heat-denatured luciferase was preincubated at 24 °C with or without ATP (2 mM) in the presence of DnaK, CbpA, and GrpE (K/A/E), or buffer for 20 min. After preincubation, $Hsp90_{Ec}$ or $Hsp90_{Ec}$ and K/A/E with or without ATP were added at $t = 0$ and luciferase reactivation was measured over time as described in *Methods*. (*B*) Heat-denatured luciferase was preincubated with DnaK, CbpA, and GrpE (K/A/E), or buffer for 20 min at 4 °C in the presence of ATP. After preincubation, $Hsp90_{Ec}$ or $Hsp90_{Ec}$ and K/A/E were added at $t = 0$ and luciferase reactivation was measured over time as described in *Methods*. (*C*) Heat-denatured luciferase was preincubated with K/A/E and ATP at 24 °C for the times indicated. After preincubation, $Hsp90_{Ec}$ was added at $t = 0$ and luciferase reactivation was measured over time as described in *Methods*. Data from two or more replicates are presented as mean ± SEM.