SUPPLEMENTAL INFORMATION

Intermolecular interactions between Hsp90 and Hsp70

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Supplementary Results

DnaK-D211, is another residue in the DnaJ binding region of DnaK that was shown to be important for the DnaK interaction with $Hsp90_{Ec}$ [1]. A substitution mutant, DnaK-D211R, was shown to be partially defective in interaction with $Hsp90_{Ec}$ in vivo and in vitro [1]. To test if this residue was located close enough to $Hsp90_{Ec}$-K354C to form a covalent bond, we constructed DnaK-D211C and monitored crosslinking with $Hsp90_{Ec}$-K354C using $CuCl_2$ (Supplementary Fig. S2a). Not only was DnaK-D211C observed to crosslink well to $Hsp90_{Ec}$-K354C, but the D211C substitution improved the interaction with both $Hsp90_{Ec}$ wild-type and K354C compared to DnaK wild-type in a bacterial two hybrid assay (Supplementary Fig. S2c) and with purified protein in a pull-down assay (Supplementary Fig. S2d). Additionally, DnaK-D211C could collaborate with both $Hsp90_{Ec}$ wild-type and $Hsp90_{Ec}$-K354C in the reactivation of heat-denatured GFP better than DnaK wild-type with $Hsp90_{Ec}$ (Supplementary Fig. S2e). The observations that DnaK-D211R and D211C have different properties in vivo and in vitro suggest that diverse amino acid substitutions affect the DnaK–$Hsp90_{Ec}$ interaction differently (Supplementary Fig. S2c and d) [1]. We also tested the ability of DnaK-E209C and $Hsp90_{Ec}$-K354C to function in GFP reactivation and observed little collaboration between the two proteins (Supplementary Fig. S2f). This result was consistent with a weaker interaction between the two mutant chaperones, as observed in the bacterial two-hybrid and pull-down assays (Fig. 2c and 2d).

Supplementary Materials and Methods

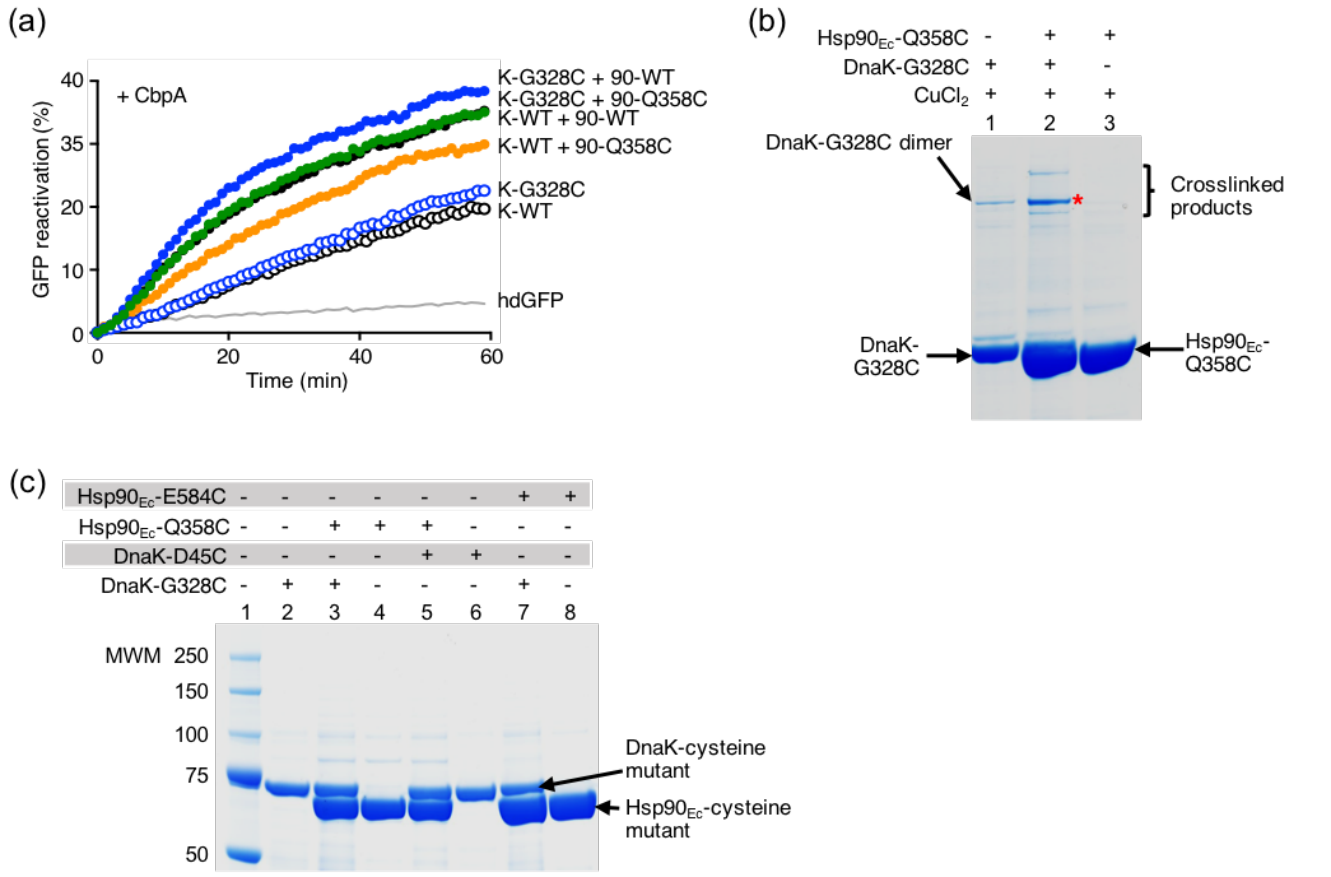
GFP disaggregation- GFP reactivation assays (100 μL) contained 25 mM Hepes, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin (BSA), 4 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 0.3 μg/ml creatine kinase), 10 μL heat-aggregated GFP (4.5 μM GFP in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10% glycerol, 0.1 mM EDTA and 5 mM DTT, heated for 10 min at 80˚C and then frozen on dry ice prior to thawing for use), 1.3 μM wild-type or mutant DnaK, 1 μM CbpA, and where indicated, 1 μM wild-type or mutant $Hsp90_{Ec}$. Reactivation was monitored over time at 23 °C using a Tecan Infinite M200Pro plate reader. Excitation and emission wavelengths were 395 nm and 510 nm, respectively.

Heat-denatured luciferase reactivation - Luciferase reactivation was performed as previously described [2, 3]. 20 nM heat-denatured luciferase, prepared as described [4], was incubated at 24 ˚C in reaction mixtures (70 μL) containing 25 mM Hepes, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin (BSA), 1 mM ATP, an ATP regenerating system (14 mM creatine phosphate, 3 μg creatine kinase), 1.5 μM Ssa1 wild-type or mutant, 0.25 μM Sis1, 0.09 μM Sti1 and 0.5 μM Hsp82, as indicated. Aliquots were removed at the indicated times and light output was measured using a Tecan Infinite M200Pro in luminescence mode with an integration time of 1000 ms following the injection of luciferin (50 μg/ml). Reactivation was determined compared to a non-denatured luciferase control. Luciferase and luciferin were from Promega.

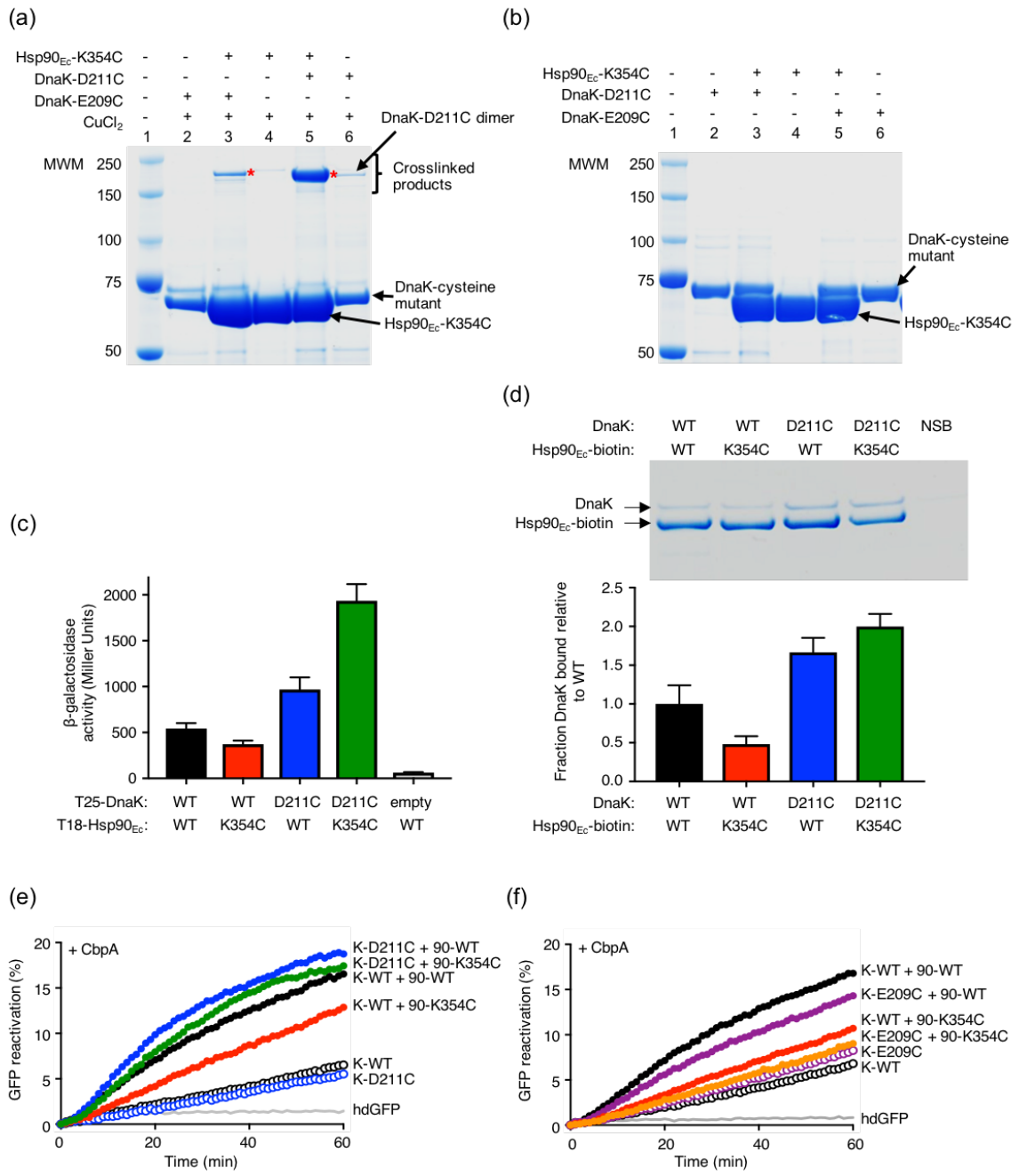
Partial proteolysis - DnaK wild-type or mutant, Ssa1 wild-type or mutant and Hsp82 wild-type or mutant (15 μg), were incubated at 37 °C in reaction mixtures (50 μl) containing 25 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$ and 2 mM DTT in the presence of trypsin (15 ng). At 0, 5, 10, 30 and 60 min 9.5 μl of the reaction mixture was removed, and reactions were stopped with the addition of LDS buffer and the samples analyzed by Coomassie staining following SDS-PAGE.

ATPase activity- Steady state ATP hydrolysis was measured at 37 ˚C in 25 mM Hepes, pH 7.5, 20 mM KCl, 5 mM DTT, 5 mM $MgCl_2$ and 2 mM ATP using a pyruvate kinase/lactate dehydrogenase enzyme-coupled assay as described [5] and using 1 μM Hsp82 wild-type or mutant in the absence or presence of 1 μM Sti1 or 1 μM Ssa1 wild-type or mutant in the absence or presence of 1 or 2 μM Ydj1, as indicated.

Supplementary Figures and Figure Legends

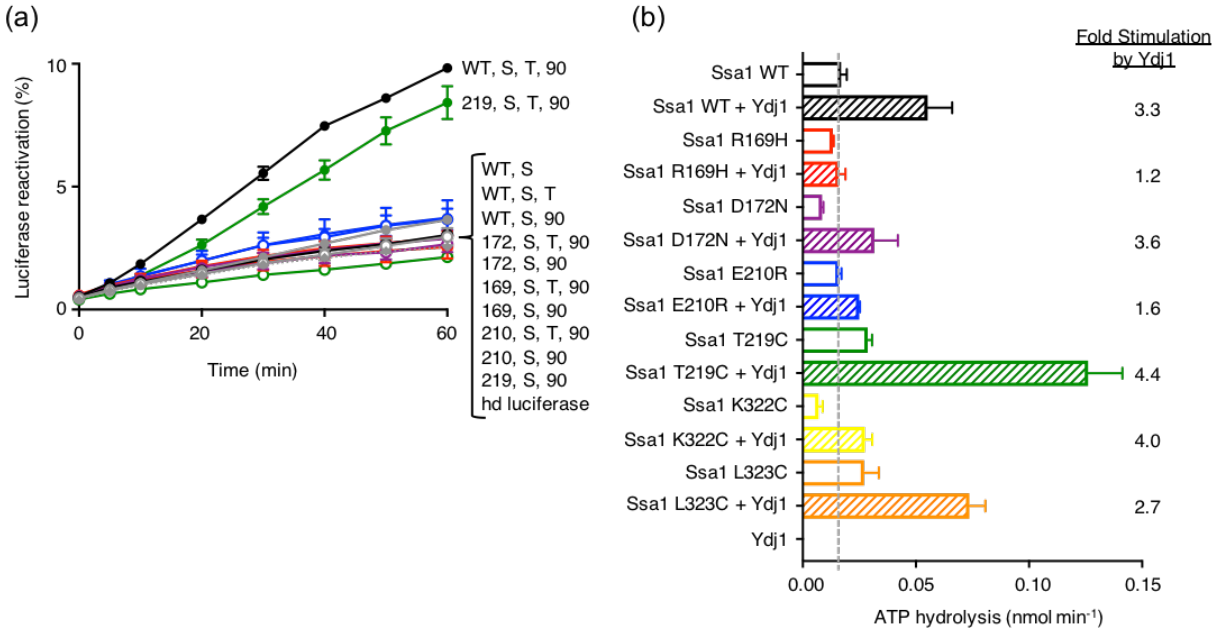


Supplementary Fig. S1. DnaK mutant properties and crosslinking controls. (a) Heat-denatured GFP was reactivated by DnaK wild-type or mutant, G328C, in the presence of CbpA and ATP with or without $Hsp90_{Ec}$ wild-type or Q358C, as indicated and described in Supplementary Materials and Methods. GFP reactivation was measured by monitoring the increase in fluorescence intensity over time. Results from a representative experiment of three or more replicates are shown. (b) $Hsp90_{Ec}$-Q358C (4 μM) and DnaK-G328C (4 μM) were treated with $CuCl_2$ (~2 Å linker) alone and in a mixture together and covalent bond formation was monitored by SDS-PAGE followed by Coomassie blue staining. The major crosslinked product (*) was consistent with crosslinked $Hsp90_{Ec}$-Q358C and DnaK-G328C (lane 2). A small amount of a slowly migrating species was likely a covalently linked DnaK-G328C dimer, since it was observed in reactions without $Hsp90_{Ec}$ (lane 1). Additional higher molecular weight and lower molecular weight species (lane 2) were not characterized. In (b), (*) indicates crosslinked band of interest, and a representative gel is shown of three or more replicates. (c) DnaK mutants including, DnaK-G328C and D45C (4 μM), and $Hsp90_{Ec}$ mutants including $Hsp90_{Ec}$-Q358C and E584C (4 μM) were incubated in the absence of crosslinker alone and in mixtures together as indicated and proteins were monitored by SDS-PAGE followed by Coomassie blue staining.

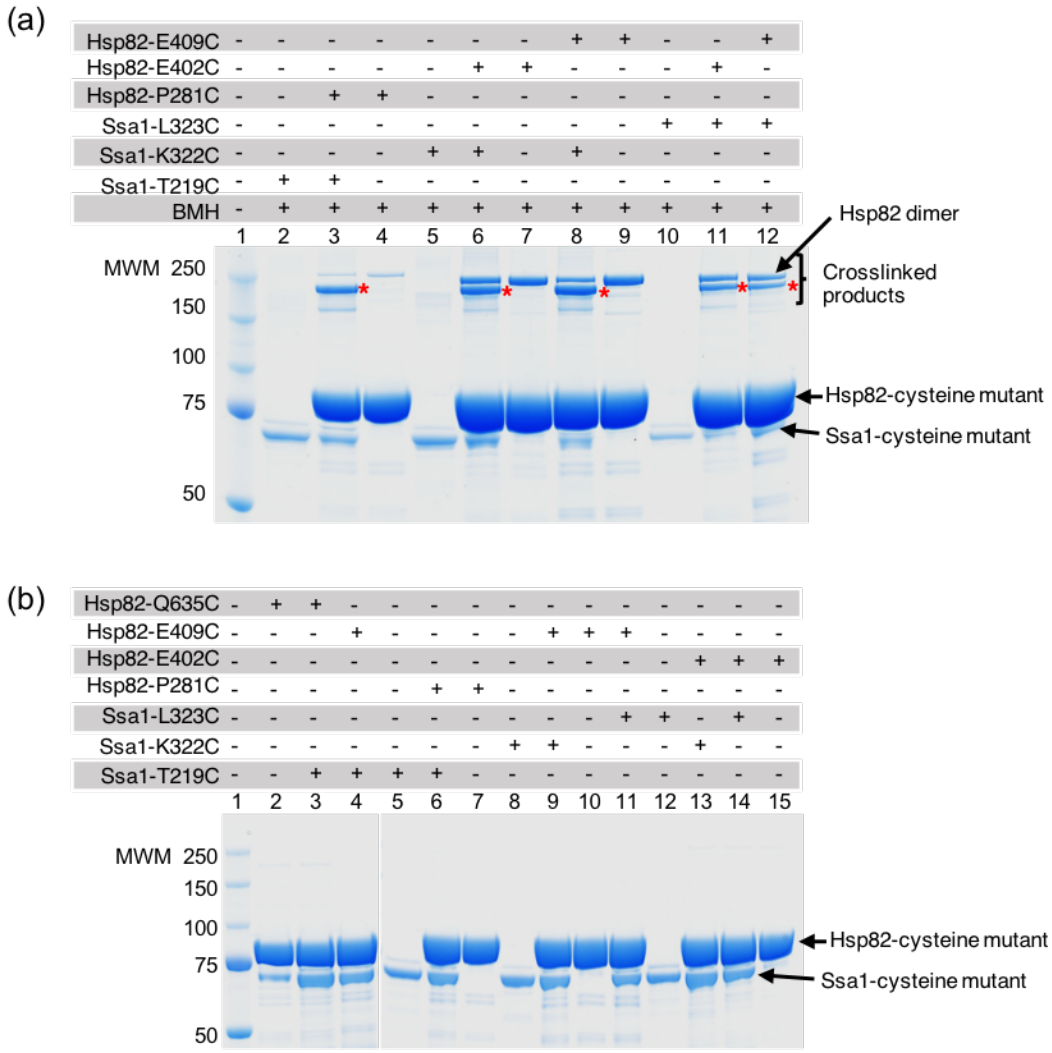


Supplementary Fig. S2. DnaK mutants and $Hsp90_{Ec}$ mutants with substitutions in residues important for interaction can interact in vitro and in vivo. (a) $Hsp90_{Ec}$-K354C and either DnaK-E209C (also shown in Fig. 2a) or D211C (4 μM each) are treated with $CuCl_2$ (~2 Å linker) alone and in mixtures together as indicated and covalent bond formation is monitored by SDS-PAGE followed by Coomassie blue staining. The major crosslinked products (*) are consistent with crosslinked $Hsp90_{Ec}$-K354C and DnaK-D209C (lane 3) and $Hsp90_{Ec}$-K354C and DnaK-D211C (lane 5). A small amount of a slowly migrating species (lane 4) was likely a covalently linked $Hsp90_{Ec}$-K354C dimer as it was observed in reactions without DnaK; a potential covalently linked DnaK-D211C dimer was also seen (lane 6), since it was observed in

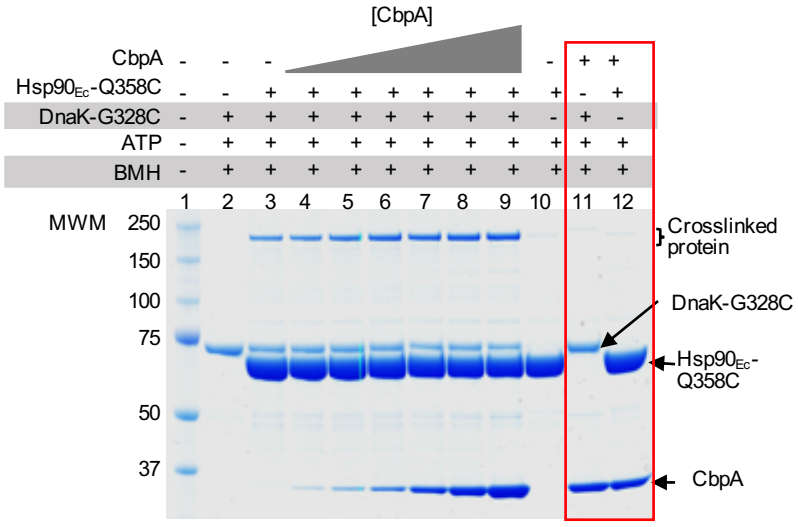
reactions without $Hsp90_{Ec}$. A representative gel is shown of three or more replicates. (*) indicates crosslinked band of interest in lanes 3 and 5. Crosslinked dimers of $Hsp90_{Ec}$-K354C (lane 4) and DnaK-D211C (lane 6) can be observed; DnaK has been shown to form dimers at high concentration [6-9]. (b) DnaK mutants, including DnaK-E209C and D211C (4 μM), and $Hsp90_{Ec}$-K354C (4 μM) were incubated in the absence of crosslinker alone and in mixtures together as indicated and proteins were monitored by SDS-PAGE followed by Coomassie blue staining. (c) The in vivo interaction between $Hsp90_{Ec}$ wild-type or K354C and DnaK wild-type or D211C using a bacterial two-hybrid system was measured by monitoring beta-galactosidase activity in liquid assays. β-galactosidase activity is shown as mean ± SEM (n=3). (d) The in vitro interaction between biotinylated $Hsp90_{Ec}$ wild-type or K354C (2 μM) and DnaK wild-type or D211C (4 μM) using a pull-down assay in the presence of L2 is monitored by SDS-PAGE followed by Coomassie blue staining. Quantification of DnaK wild-type or D211C associated with biotinylated $Hsp90_{Ec}$ wild-type or K354C as described in Materials and Methods is shown as a bar graph and the data were normalized to $Hsp90_{Ec}$-biotin and the ratio of DnaK mutant to wild-type was plotted as the mean ± SEM (n=3). NSB stands for non-specific binding. (e, f) Heat-denatured GFP is reactivated by DnaK wild-type or mutant, D211C (e) or E209C (f), in the presence of CbpA and ATP with or without $Hsp90_{Ec}$ wild-type or K354C, as indicated and described in Supplementary Materials and Methods. GFP reactivation is measured by monitoring the increase in fluorescence intensity over time as described in Supplementary Materials and Methods. In (e) and (f) a representative experiment is shown of three or more replicates.



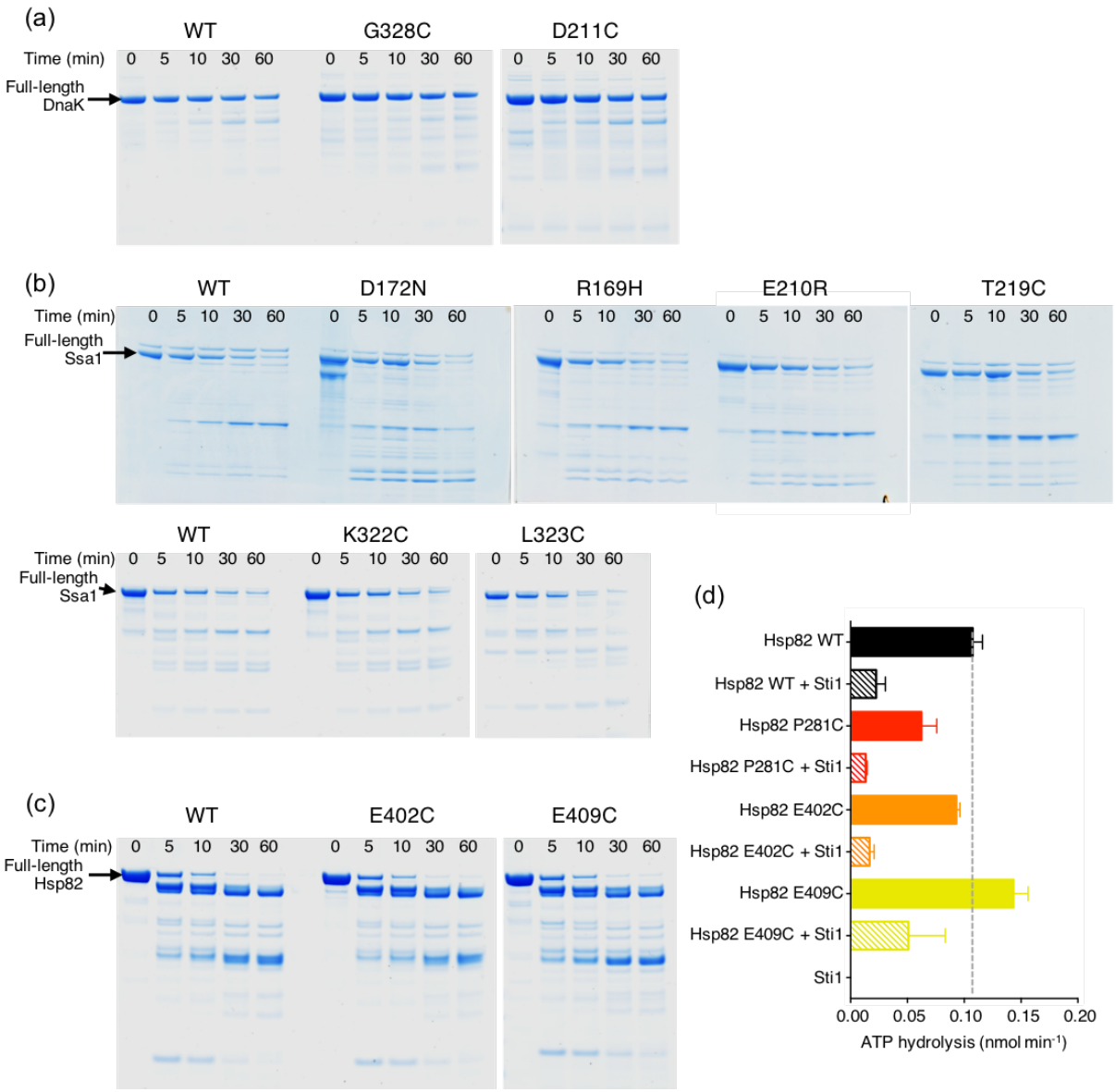
Supplementary Fig. S3. Functional properties of Ssa1 mutants. (a) Heat-inactivated luciferase reactivation by Ssa1 wild-type or mutant, Sis1 (S), Sti1 (T) and Hsp82 (90) as indicated and described in Supplementary Materials and Methods. For some points, the symbols obscure the error bars. (b) ATP hydrolysis by Ssa1 wild-type or mutant in the absence or presence of Ydj1 was measured as described in Materials and Methods. The fold stimulation in ATPase activity by Ydj1 is shown. The condition used for testing the stimulation of ATPase activity by Ydj1 is not limiting for Ydj1 and may mask Ssa1 mutant defects. This is in contrast to the limiting Ydj1 concentration used for the reactivation of luciferase (Fig. 3e) where sever defects were observed for 3 of the 4 Ssa1 mutants tested. The dashed line indicates the basal rate of ATP hydrolysis by Ssa1 wild-type and is meant to aid the eye. In (a) and (b), data from three or more replicates are presented as mean ± SEM.



Supplementary Fig. S4. Hsp82 and Ssa1 mutants with cysteine substitutions in residues important for the interaction between the chaperones can be crosslinked. (a) Hsp82 mutants including Hsp82-P281C, E402C and E409C (4 μM), and Ssa1 mutants including Ssa1-T219C, K322C and L323C (4 μM), are treated with BMH (13 Å linker) alone and in mixtures together as indicated and covalent bond formation is monitored by SDS-PAGE followed by Coomassie blue staining. (*) indicates crosslinked band of interest in lanes 3, 6, 8, 11 and 12. Crosslinked dimers of Hsp82-P281C (lane 3 and 4), E402C (lane 6, 7 and 11) and E409C (lane 8, 9 and 12) can be observed; the higher quantity of crosslinked dimer for E402C and E409C could be due to a decrease in the distance between the residue on each protomer during the large conformational changes that Hsp82 undergoes. A representative gel is shown of three or more replicates. (b) Hsp82 mutants, including Hsp82-P281C, E402C, E409C and Q635C (4 μM), and Ssa1 mutants including Ssa1-T219C, K322C and L323C (4 μM) were incubated in the absence of crosslinker alone and in mixtures together as indicated and proteins were monitored by SDS-PAGE followed by Coomassie blue staining.



Supplementary Fig. S5. CbpA does not promote homodimerization of $Hsp90_{Ec}$-Q358C or DnaK-G328C. In lanes 11 and 12, $Hsp90_{Ec}$-Q358C (4 μM) and CbpA (4 μM) or DnaK-G328C (4 μM) and CbpA (4 μM) (no endogenous cysteines) as indicated were treated with BMH (13 Å linker) and covalent bond formation was monitored by SDS-PAGE followed by Coomassie blue staining. The red box highlights the lanes of interest. Lanes 1-10 were shown in the main text as Fig. 5b. A representative gel is shown of three replicates.



Supplementary Fig. S6. Properties of DnaK, Ssa1 and Hsp82 mutants. Partial proteolysis of (a) DnaK wild-type and mutants (b) Ssa1 wild-type and mutants and (c) Hsp82 wild-type and mutants. Trypsin digestion was carried out over time as described in Supplementary Materials and Methods and proteolytic products were analyzed by Coomassie blue staining of gels following SDS-PAGE. (d) ATP hydrolysis by Hsp82 wild-type or mutant (1 μM) in the absence or presence of Sti1 (1 μM) was measured as described in Materials and Methods. Data from three or more replicates are presented as mean ± SEM. The dashed line indicates the basal rate of ATP hydrolysis by Hsp82 and is meant to aid the eye.

Supplementary References

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