Cysteine Reactivity Distinguishes Redox Sensing by the Heat Inducible and Constitutive Forms of Heat Shock Protein 70 (Hsp70)

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Supplemental Materials and Methods

ATP Agarose Binding

Hsp72, MB treated Hsp72, Hsp72 C267D, and Hsp72 C306D were tested for their ability to bind an ATP agarose column. Samples of 6 μM were prepared in Buffer A (25 mM HEPES, 10 mM KCl, 5 mM $MgCl_2$ pH 7.5) and loaded onto a 1mL ATP agarose column. The column was washed with 5 mL of Buffer A, 5 mL of Buffer B (25 mM HEPES, 1 M KCl, 5 mM $MgCl_2$ pH 7.5), 5mL of Buffer A, and then eluted with 3 mL of Buffer A with 3 mM ATP. Fractions were then analyzed using a 10-20% Tris-Tricine pre-cast gel (Invitrogen), transferred to a nitrocellulose membrane and probed using a rabbit anti-Hsp72 antibody (Assay Designs, Ann Arbor, MI) followed by a horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent (Super Signal, Pierce) and HyBlot CL film (Denville Scientific Inc.).

Circular Dichroism

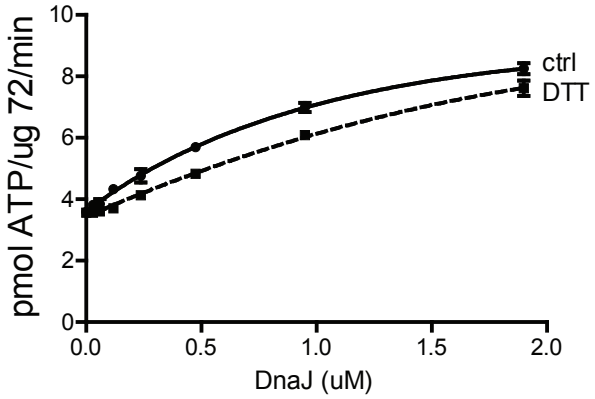
Hsp72, MB treated Hsp72 and Hsp72 mutants were prepared in 50 mM NaF pH 7.5 buffer and spectra collected at 0.1 mg/ml in a 0.1-cm cuvette at room temperature. CD spectra were recorded on a Jasco J-715 spectropolarimeter (Jasco, Easton, MD) at 1-nm intervals from 190 to 260 nm at a scanning speed of 50 nm/min and a 5.0 nm bandwidth. Each spectrum reported is the average of 15 scans after the subtraction of the base-line spectrum (buffer without the addition of Hsp72) and normalization (millidegree cm^2 $dmol^{-1}$).

Partial Proteolysis

The partial proteolysis studies of Hsp72, MB treated Hsp72 and mutants were performed as previously described (Rousaki *et al.* 2011 *J. Mol. Biol.* 411:614).

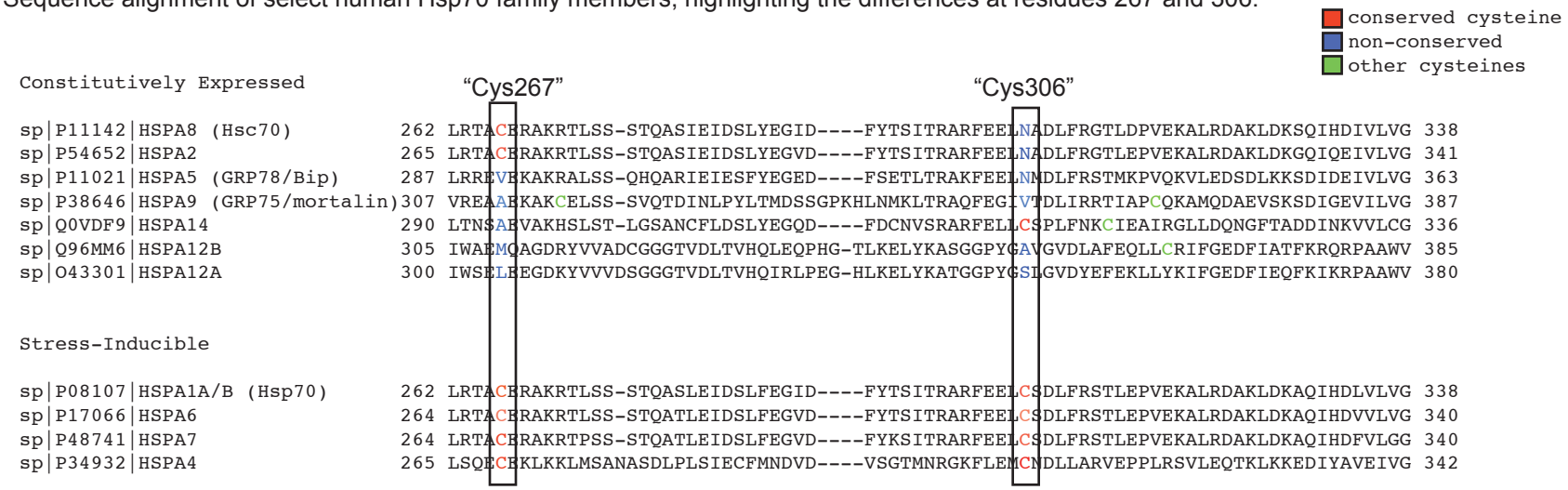
Luciferase refolding assay

The refolding of chemically denatured firefly luciferase was monitored as described (Chang *et al.* 2011 *Chem. Biol*. 18:210).

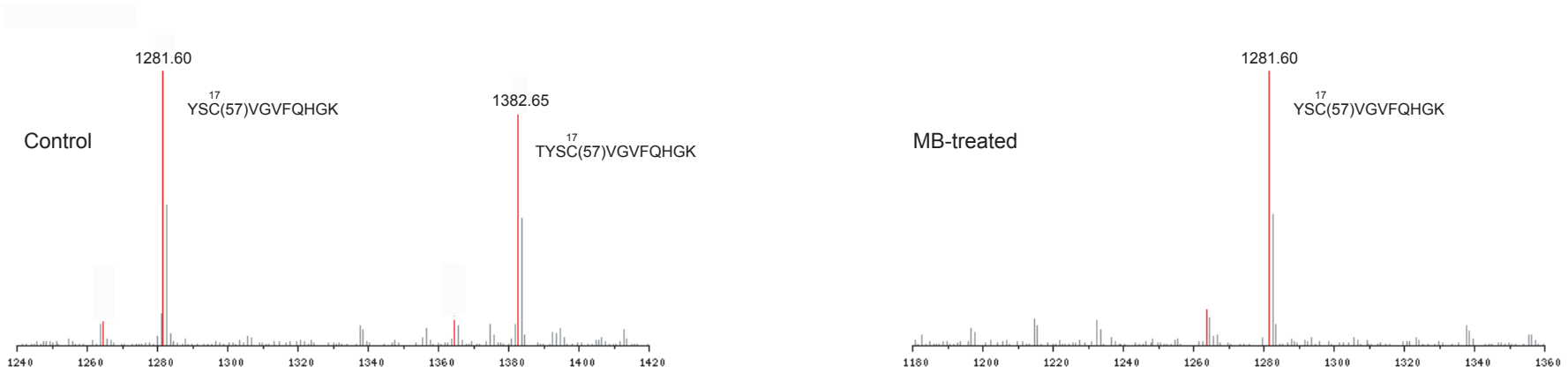


Supplemental Figure 1. (A) MB-mediated oxidation of Hsp72 is irreversible. Hsp72 was treated with MB, dialyzed to remove the compound and then treated with DTT for 30 minutes. ATPase activity was measured as in Figure 1A. Results are the average of two experiments performed in triplicate. Error bars represent standard error of the mean. Correlates with Fig. 2 of the main text.

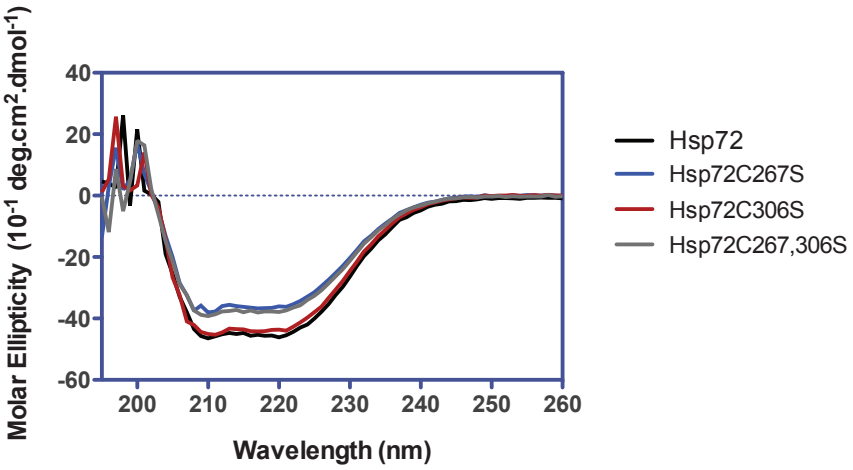
a Sequence alignment of select human Hsp70 family members, highlighting the differences at residues 267 and 306.



b Representative cysteine-containing fragment from human Hsc70 LC-MS/MS spectra after treatment with MB, showing that this isoform is not sensitive to oxidation.

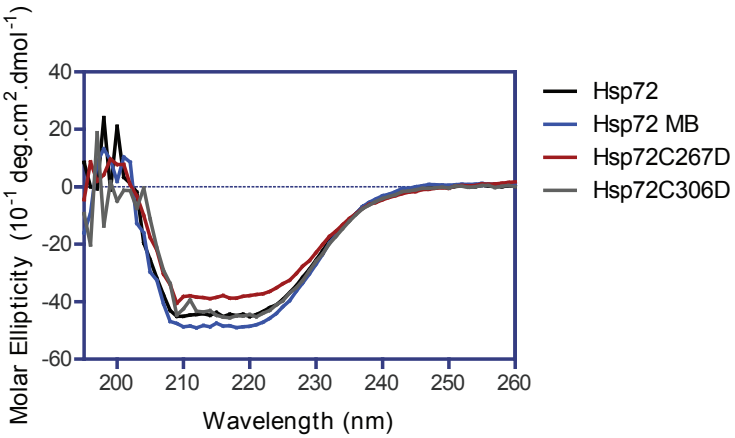


Supplemental Figure 2. Stress inducible, but not constitutive forms of the Hsp70 family contain a unique, reactive cysteine at position 306. (a) Sequence alignment of select human Hsp70 family members, showing conservation of cysteine 306 in stress inducible, but not constitutive forms. The 267 and 306 numbers are derived from human Hsp72 (HSP1A1). For more information see Fujikawa et al. (2010) Cell Stress Chaperones 15:193-204. (b) Representative trypsin fragment of human Hsc70 treated with MB (200 μM), as in Fig. 2, showing that Cys17 is not oxidized. In addition, no other fragments in the Hsc70 sequence were oxidized. As discussed in the text, the fragment containing 267 was not observed in the spectra. Correlates with Fig 1 of the main text.

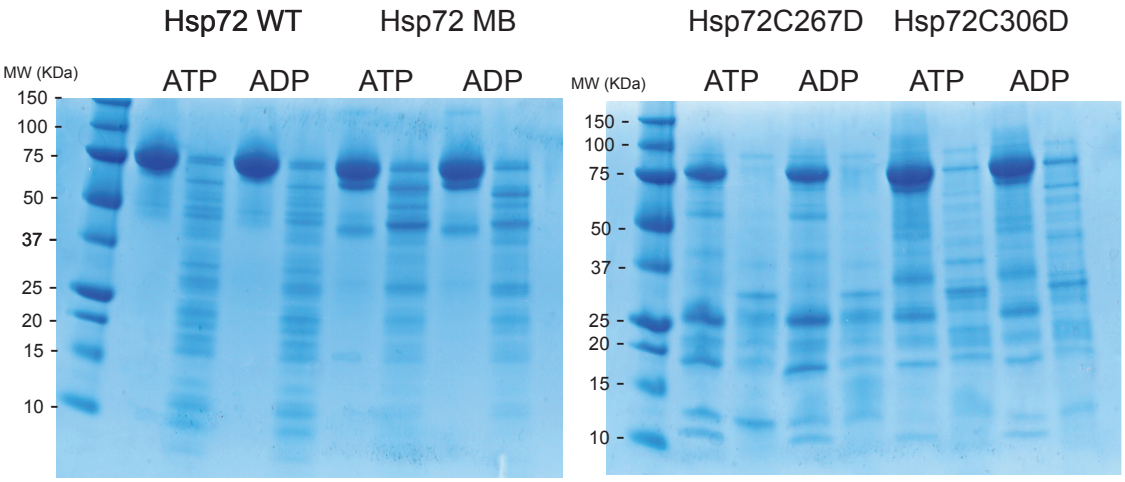


Supplemental Figure 3. Serine point mutants Hsp72 C267S and C306S and the double mutant C267/306S) are properly folded, as measured by circular dichroism. Correlates with Fig 3 of the main text.

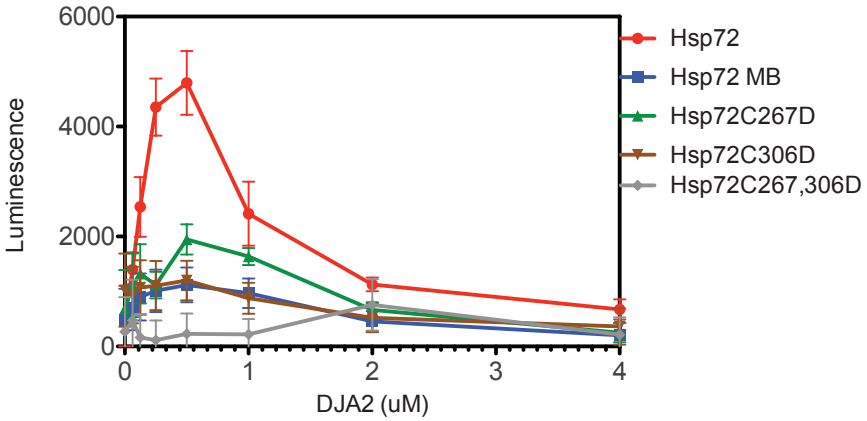
(a) Aspartic acid mutants are properly folded.



(b) Hsp72 C267D and C306D mutants are more susceptible to degradation



(c) MB treatment or aspartic acid mutants damage the ability to refold denatured firefly luciferase



Supplemental Figure 4. C267D and C306D mutants have impaired ATP binding and are more flexible. (a) Circular dichroism results indicate that the mutants have similar global structure to the wildtype. Likewise, MB treatment does not cause major changes in structure, as determined by this method. All proteins were used at 2 μM in 50 mM NaF buffer. (b) Hsp70 variants (6 μM) were treated with nucleotide (5 mM) for 30 minutes and then digested with trypsin for 30 min at rt. Reactions were quenched with loading dye, bands separated on 10-20% Tris-Tricine gels and imaged by Coomasie stain. Results are representative of experiments performed in duplicate. (c) Hsp72 and DJA2 could robustly refold denatured luciferase, but Hsp72 treated with MB (50 μM) or Hsp72 with Asp mutants had greatly reduced activity. Results are the average of at least three independent expeirments performed in triplicate. Error is SEM. Correlates with Fig 6 of the main text.

Hydrogen bond and hydrophobic interactions are lost in Hsp72C267D modeled structure

	Residue	3JXU (Å)	Robetta Model (Å)	ΔÅ
Hydrogen Bond with ADP	**T14**	2.7	4.8	2.1
	T14	2.8	4.3	1.5
	Y15	2.9	4.5	1.6
	G202	2.7	2.3	-0.4
	E268	2.9	4.2	1.3
	K271	2.8	2.7	-0.1
	S275	2.5	2.3	-0.2
	G339	3.1	4.2	1.1
Hydrogen Bond with Phosphate	**T13**	2.5	5.1	2.6
	T13	2.8	4.3	1.5
	T13	3.1	5.5	2.4
	K71	2.7	1.9	-0.8
	E175	2.7	3.2	0.5
	T204	2.5	3.6	1.1
Hydrophobic Interactions	**Y15**	3.8	3.7	-0.1
	G201	3.8	4.0	0.2
	G202	3.8	3.3	-0.5
	G202	3.6	3.3	-0.3
	G202	3.8	3.7	-0.1
	R272	3.9	4.6	0.7
	R272	3.5	5.2	1.7
	R272	3.2	4.7	1.5
	R272	3.7	4.9	1.2
	R272	3.9	5.1	1.2
	G339	3.7	4.2	0.5
	R342	3.8	5.0	1.2
	R342	3.8	5.7	1.9
	R342	3.8	5.3	1.5
	I343	3.6	3.8	0.2
			Total (Å) :	**25.0**