Fig. S1: Deuteron incorporation into Sti1 in the absence and presence of Hsp90. Unprocessed mass spectra of three peptic peptides of Sti1 TPR1 (55-65), TPR2a (253-272), and TPR2b (422-440) domain before hydrogen exchange and after 30 s in D$_2$O in the absence and presence of Hsp90 as indicated.
Fig. S2: Deuteron incorporation into Sti1 in the absence and presence of Hsp90 after incubation for 30 s in D₂O. (a) Relative hydrogen exchange is plotted for all peptic peptides of Sti1 in the absence (blue bars) and in the presence (red bars) of Hsp90 from the N to the C terminus as indicated to the right. Error bars represent the standard error of the mean of three independent experiments. (b) Cartoon representations of homology models of TPR1 (top panel), TPR2a (middle panel) and TPR2b (bottom panel) on the structures of human Hop (PDB entry codes 1ELW and 1ELR) colored according to the relative exchange after 30 s as indicated. Top panel insert: TPR1 rotated by 90° as indicated.
Fig. S3: Deuteron incorporation into Sti1 or Sti1Δ(TPR2B-DP2) in the absence and presence of Hsp90, Hsp90ΔMEEVD or Hsp90-DDΔMEEVD. (a) Effects of the C-terminal MEEVD-motif and the dimerization domain onto Sti1. Difference plot of deuteron incorporation into Sti1 in the presence of Hsp90 with the MEEVD-motif deleted (Hsp90ΔMEEVD) or the C-terminal dimerization domain of Hsp90 (DD) with the MEEVD-motif deleted (Hsp90-DDΔMEEVD) minus deuteron incorporation into Sti1 in the absence of Hsp90 after 30 s in D₂O. Error bars indicated the standard error of the mean of three independent experiments. (b) Cartoon representation of a homology model of TPR2a and TPR2b onto the crystal structure of human Hop TPR2a in complex with the MEEVD peptide (PDB entry code 1ELR (Arnold et al, 2006; Kiefer et al, 2009; Peitsch, 1995; Scheufler et al, 2000)) colored according to the change in deuteron incorporation in the presence of Hsp90ΔMEEVD as indicated. (c) Sti1 Δ(TPR2B-DP2) still binds to Hsp90. Difference plot of deuteron incorporation into Sti1Δ(TPR2B-DP2) in the presence of Hsp90 minus deuteron incorporation into Sti1Δ(TPR2B-DP2) in the absence of Hsp90 after 30 s in D₂O.
**Fig. S4: Deuteron incorporation into Hsp90 in the absence and presence of Sti1.** Unprocessed mass spectra of three peptic peptides of Hsp90 NBD (residues 90-105, 114-124) and MD (residues 480-492) before hydrogen exchange and after 30, 100, and 1000 s in D$_2$O in the absence and presence of Sti1 as indicated. Brackets indicate signals from other peptides of Hsp90 and Sti1.
Fig. S5: Hydrogen exchange kinetics of segments of Hsp90 in the absence and presence of Sti1. (a) Hydrogen exchange in percent of total numbers of exchangeable amide protons within the peptic fragment indicated in the absence (blue) and presence (red) of Sti1. Error bars indicated the standard error of the mean of 3 independent experiments (note: most error bars are smaller than the symbol).
Fig. S5: Hydrogen exchange kinetics of segments of Hsp90 in the absence and presence of Sti1. (a) continued.
Fig. S5: Hydrogen exchange kinetics of segments of Hsp90 in the absence and presence of Sti1. (b) Difference plot of deuteron incorporation into Hsp90 in the presence of Sti1 minus deuteron incorporation into Hsp90 in the absence of Sti1 after 30 s (cyan), 100 s (marine blue), and 1000 s (dark blue) in D₂O. Error bars indicated the standard error of the mean of 3 independent experiments. (c) Cartoon representation of Hsp90 in the closed conformation (PDB entry code 2CG9) colored according to the number of amide protons protected from hydrogen exchange in the presence of Sti1 relative to the exchange in the absence of Sti1 after 30 s and 1000 s in D₂O as indicated.
Fig. S6: Sti1 reduces conformational dynamics in Hsp90. (a) Comparison of the effect of Sti1 on the conformational dynamics of yeast Hsp90 with the effect of ATP on the conformational dynamics of *E. coli* HtpG (data from Graf et al. 2009). Cartoon representations of yeast Hsp90 (PDB entry code 2CG9) and of a homology model of *E. coli* HtpG onto the structure of yeast Hsp90 colored according to ligand induced changes in hydrogen exchange as indicated; dark blue, protection of more than two amide protons; marine, protection of 1-2 amide protons; cyan, protection of 0.5 to 1 amide protons; yellow, no significant change in deuteron incorporation; gray, peptides covering this region were not detected. Red numbers indicate examples for segments protected in HtpG·ATP but not in Hsp90·Sti1 (numbers in parenthesis, corresponding segments in yeast Hsp90); green numbers, indicate examples for segments protected in Hsp90 in the presence of Sti1 but not in HtpG·ATP (numbers in parenthesis, corresponding segments in HtpG). First and last residue of each structure are indicated where visible.
Fig. S7: Effects of Sti1 on the conformational dynamics in Hsp90 middle domain. Deuteron incorporation of Hsp90-MD (16 µM) in the presence of Sti1 (20 µM) minus deuteron incorporation in the absence of Sti1 after 30 s in D₂O. Error bars are standard error of the mean of three independent experiments.
Fig. S8: Sba1 does not interfere with position 57 in Hsp90. Left panel, Space-filling representation of the crystal structure of yeast Hsp90 in complex with Sba1 and AMPPNP (PDB entry code 2CG9). Right panel, cartoon representation of the crystal structure of yeast Hsp90 in complex with Sba1 and AMPPNP (ANP) rotated 90° as compared to left panel as indicated. Hsp90 colored in cyan and green, Sba1 in yellow and orange. Glu57 is highlighted in red.
Fig. S9: Hsp90-E57C-BPA cross-links to TPR2b in Sti1. MALDI-TOF MSMS spectrum with interpretation of individual peaks. Hsp90-E57C with N-terminal Strep-tag was labeled with the cleavable thiol-specific, UV-activatable heterobifunctional cross-linker benzophenone-4-carboxamidocysteine methanethiosulfonate (BPC), incubated with His-tagged-Sti1 and cross-linked by UV irradiation at 360 nm for 10 min. The cross-linking products were purified by successive streptactin and Ni²⁺ IDA affinity chromatography, cleaved with DTT, carbamidomethylated with iodoacetamide, and digested with trypsin in the presence of 2 M urea. The tryptic peptides were analyzed by liquid chromatography and MALDI-TOF-TOF tandem mass spectrometry. Since the cross-linking reaction involves the benzophenone radical, which attacks any C-H bond, the cross-linking product consists of a mixture of peptides labeled by the cross-linker at different amino acids. Consequently, the mass spectrum is a mixture of product ion peaks of at least two peptide species each with the identical amino acid sequence EIDQLYYKASQQR (identical precursor ion mass), but each modified by the cross-linker at a different amino acid. The spectrum in the lower panel shows a zoom of the spectrum in the upper panel. The peaks are labeled according to the nomenclature of Roepstorff & Fohlman (1984 Biomed Mass Spectrom. 11, 601) with “a” indicating the N-terminal product ion of the precursor fragmented in the peptide backbone between the Cα and the carbonyl carbon, “b” and “y” indicating N- and C-terminal product ions of the precursor fragmented in the peptide bond. Large numbers designate the place of fragmentation, counted from the N-terminus (a- and b-ions) and C-terminus (y-ions), respectively. In laser induced dissociation (LID) loss of water (-18) and ammonia (-17) and gain of water (+18) is observed and indicated accordingly (e.g. “a-18”). The asterisk marks fragments that carry the cross-linker. Black labels indicate fragments that do not contain the cross-linker; red labels indicate fragments of the N-terminal half of the peptide that carry the cross-linker; blue labels indicate fragments of the C-terminal half of the peptide that carry the cross-linker; green label designates a fragment that originated from the precursor ion through loss of ammonia. The unassigned peaks most likely originate from the precursor or a fragment through neutral loss at the cross-linker.
Fig. S10: Hsp90-E57C-BPA cross-links to TPR2a in Sti1. MALDI-TOF MSMS spectrum with interpretation of the different peaks. Hsp90-E57C with N-terminal Strep-tag was labeled with the cleavable thiol-specific, UV-activatable heterobifunctional cross-linker benzophenone-4-carboxamidocysteine methanethiosulfonate (BPC), incubated with His-tagged-Sti1 and cross-linked by UV irradiation at 360 nm for 10 min. The cross-linking products were purified by successive streptactin and Ni²⁺ IDA affinity chromatography, cleaved with DTT, carbamidomethylated with iodoacetamide, and digested with trypsin in the presence of 2 M urea. The tryptic peptides were analyzed by liquid chromatography and MALDI-TOF-TOF tandem mass spectrometry.

Since the cross-linking reaction involves the benzophenone radical, which attacks any C-H bond, the cross-linking product consists of a mixture of peptides labeled by the cross-linker at different amino acids. The mass spectrum shown here is a mixture of product ion peaks of four peptide species (with identical precursor ion mass), each with the identical amino acid sequence VISK, carbamylated at the C-terminal lysine, but each modified by the cross-linker at a different amino acid indicated by the asterisk. For clarity reasons the same mass spectrum is shown in all four panels, in each panel labeled only with those peaks that belong to the peptide precursor shown above the panel. Nomenclature of peak labels as in Fig. S9. Black labels indicate fragments that do not carry the cross-linker; red labels designate fragments that carry the cross-linker. The unassigned peaks most likely originate from the precursor or a fragment through neutral loss at the cross-linker.
Fig. S11: Sti1 TPR2a and TPR2b cannot be cross-linked to the NBD of Hsp90\textsubscript{MEEVD}. \textit{α}-FLAG (upper panel) and \textit{α}-HA immunoblot (lower panel) of SDS-PAGE separated cross-linking reactions containing the component as indicated above the panels. Samples of lanes 7 to 12 were treated with TEV protease after UV irradiation.