Supporting Information

Experimental Section

Protein expression and purification

Wild type human Hsp90β and its mutants were expressed in *Escherichia coli* BL21 (DE3) Codon Plus strain. Cultures were grown in LB medium at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.6. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM, followed by incubation for 18-20 hours at 18 °C. Cells were harvested by centrifugation at 6000 rpm for 8 minutes and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole, 0.2 mM EDTA, and 1 mM PMSF. Cell lysis was performed using sonication. The lysate was clarified by centrifugation at 15000 rpm for 30 minutes at 4 °C, and the supernatant was applied to a Ni-NTA affinity column. The column was washed with wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl) containing increasing concentrations of imidazole (20, 50, and 80 mM; 2-3 column volumes each). The target protein was eluted with elution buffer comprising 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 500 mM imidazole. Subsequently, the protein was subjected to buffer exchange into a low-salt buffer (20 mM Tris-HCl, pH 7.6) using ultrafiltration. Further purification was achieved using an ion-exchange chromatography column (Q Sepharose, GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 7.6). The protein was eluted with a linear gradient of 0–600 mM NaCl. The purified protein was exchanged into storage buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.6), concentrated to approximately 100-200 μM, and stored at −20 °C.

Synthesis of labels and stock preparation

1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiol sulfonate (MTSL) and 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (M-Prox) were purchased from Santa Cruz Biotechnology Inc. Fluorine labels[1], BrPSPy-DO3A-Gd[2] and BrPS-PyMTA[3] were synthesized according to previously reported protocols. $MgCl_2{\cdot}6H_2O$, ATP-2Na, and ADP-2Na were obtained from Sangon Biotech Co., Ltd. AMP-PNP was purchased from Aladdin Scientific Corp. MTSL, M-Prox, T3, T4 and T5 were dissolved in DMF to create a 100 mM stock solution. BrPSPy-DO3A-Gd, BrPS-PyMTA, and $Gd(NO_3)_3$ were each dissolved in ddH_2O to prepare a 100 mM stock solution. BrPS-PyMTA-Gd was prepared by mixing equal amounts of BrPS-PyMTA and $Gd(NO_3)_3$. The pH of the stock solutions of BrPSPy-DO3A-Gd, BrPS-PyMTA, and BrPS-PyMTA-Gd was adjusted to 6-7 using 1 M NaOH. $MgCl_2$, ATP, ADP, and AMP-PNP were dissolved in ddH_2O to prepare a 100 mM stock solution. The pH of the ATP and ADP stock solutions was also adjusted to 6-7 using 1 M NaOH. All stock solutions were diluted to the appropriate concentrations with their respective solvents prior to use.

Protein labelling

For the wild-type Hsp90 reacting with labels, the protein stock was diluted to 20 μM using either HEPES buffer (50 mM HEPES, 150 mM NaCl, pH 7.0) or Tris buffer (50 mM Tris, 150 mM NaCl, pH 8.0). Subsequently, 0.2 mM of each label was added, and the mixture was incubated at 298 K for 8 hours. For the Hsp90 C366A or E11C/C366A mutants reacting with Gd labels, the protein stock was diluted to 20 μM in Tris buffer (50 mM Tris, 150 mM NaCl, pH 8.0). Then, 0.2 mM final concentration of either BrPSPy-DO3A-Gd or BrPS-PyMTA-Gd was added, and the incubation was carried out at 298 K for 4 to 6 hours. For the Hsp90 6A/E11C mutant reacting with nitroxide labels, the protein stock was diluted to 20 μM in HEPES buffer (50 mM HEPES, 150 mM NaCl, pH 7.0), followed by the addition of 0.1 mM of MTSL or M-Prox, and the mixture was incubated at 298 K for 2 hours. For the wild-type Hsp90 reacting with BrPSPy-DO3A-Gd in Mg^{2+}/nucleotide-bound states, the protein stock was diluted to 20 μM in Tris buffer (100 mM Tris, 150 mM NaCl, pH 8.0). Depending on the specific conditions, 5 mM $MgCl_2$, 5 mM $MgCl_2$ with 5 mM ATP, 5 mM $MgCl_2$ with 5 mM ADP, or 5 mM $MgCl_2$ with 5 mM AMP-PNP was added, and the mixture was incubated at 298 K for 10 minutes. Following this, 0.2 mM final concentration of the BrPSPy-DO3A-Gd(III) label was added, and the final mixture was incubated at 298 K for 150 minutes.

LC-MS spectroscopy

The mass spectrum of the protein samples was recorded on an Agilent 1290 series HPLC system coupled with a 6545 series ESI-Q-TOF mass spectrometer (Agilent). The sample was injected into a C8 column (Agilent ZORBAX 300SB-C8, 2.1 × 150 mm), and the separation was performed at 70°C. The mobile phases consisted of water with 0.1% formic acid (buffer A) and acetonitrile with 0.1% formic acid (buffer B). The gradient elution conditions were as follows: 95% buffer A from 0 to 2 minutes; 95–40% buffer A from 2 to 10 minutes; 40–20% buffer A from 10 to 11 minutes; 20–10% buffer A from 11 to 12 minutes; 10% buffer A from 12 to 14 minutes; 10–95% buffer A from 14 to 14.5 minutes; and 95% buffer A from 14.5 to 16.5 minutes. The flow rate was set to 0.5 mL/min. Data were analysed using Agilent MassHunter Bioconfirm software.

ATPase assay

ATPase activity was assessed using a Na^+-K^+ ATPase Activity Assay Kit (Sangon Biotech Co., Ltd.) according to the manufacturer's instructions. In this assay, ATPase catalyzes the hydrolysis of ATP, resulting in the release of ADP and a free phosphate ion. Subsequently, the released phosphate ion reacts with a phosphate determination reagent, generating a chromophore that can be quantified by measuring the optical density at 660 nm (OD_{660}). The assay was performed using samples containing 50 μM Hsp90 or its mutants in a reaction buffer composed of 20 mM Tris (pH 7.6), 150 mM NaCl, and 5 mM $MgCl_2$. All ATPase measurements were carried out at 37 °C. Each experiment was performed using three independent measurements, and the data are reported as mean ± standard deviation.

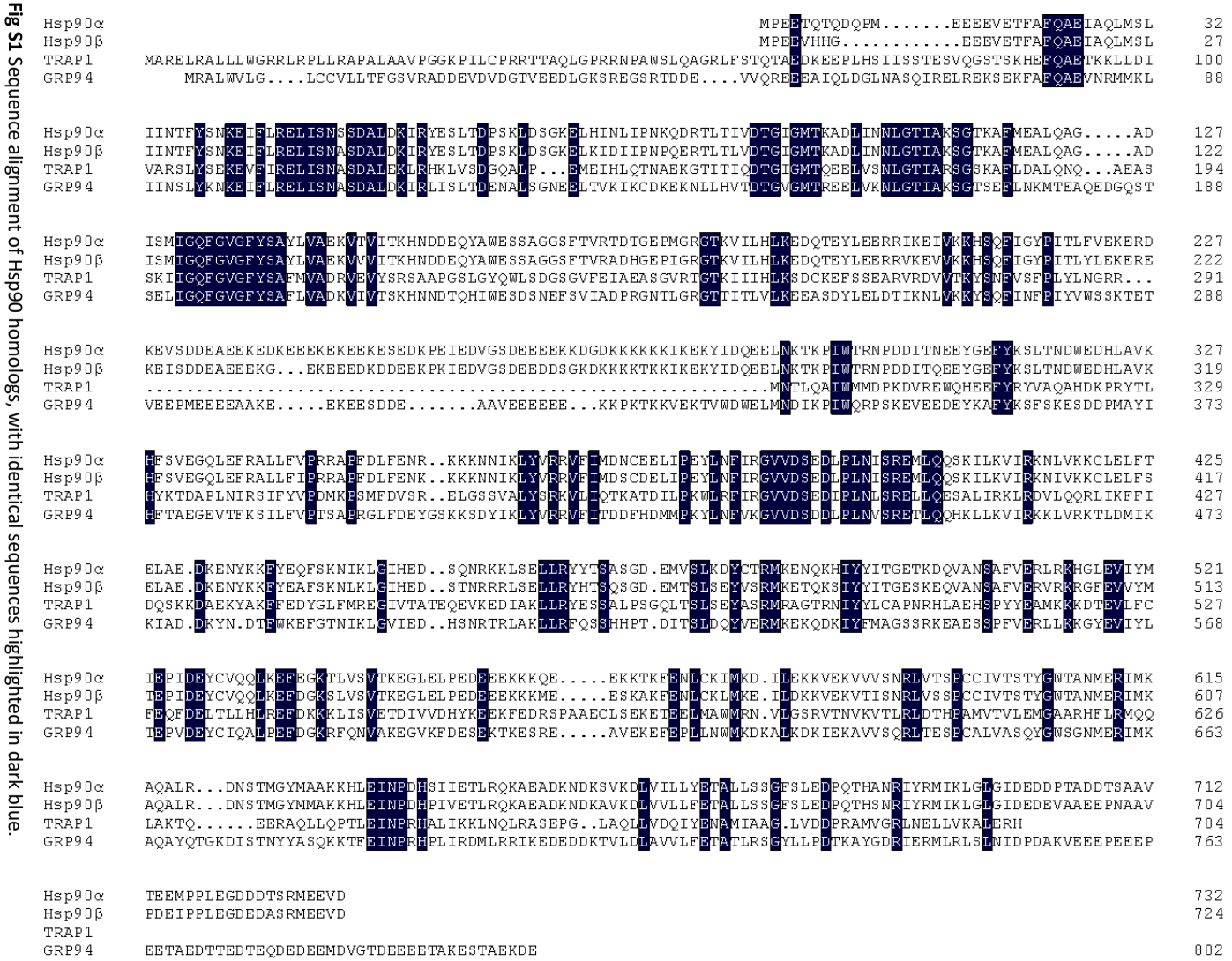

Fig S1 Sequence alignment of Hsp90 homologs, with identical sequences highlighted in dark blue.

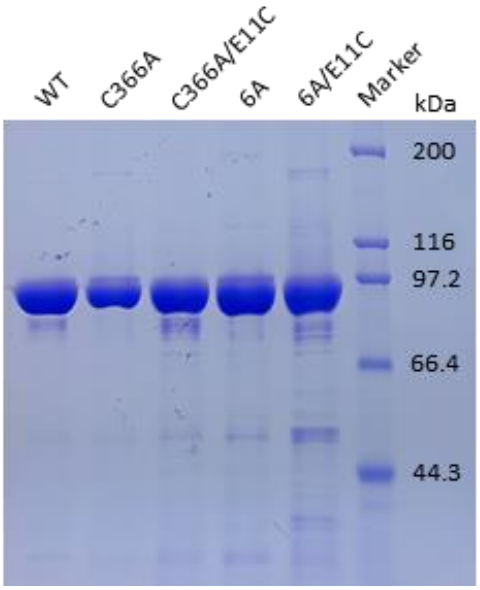

Fig S2 SDS-PAGE analysis of human Hsp90β and its mutants.

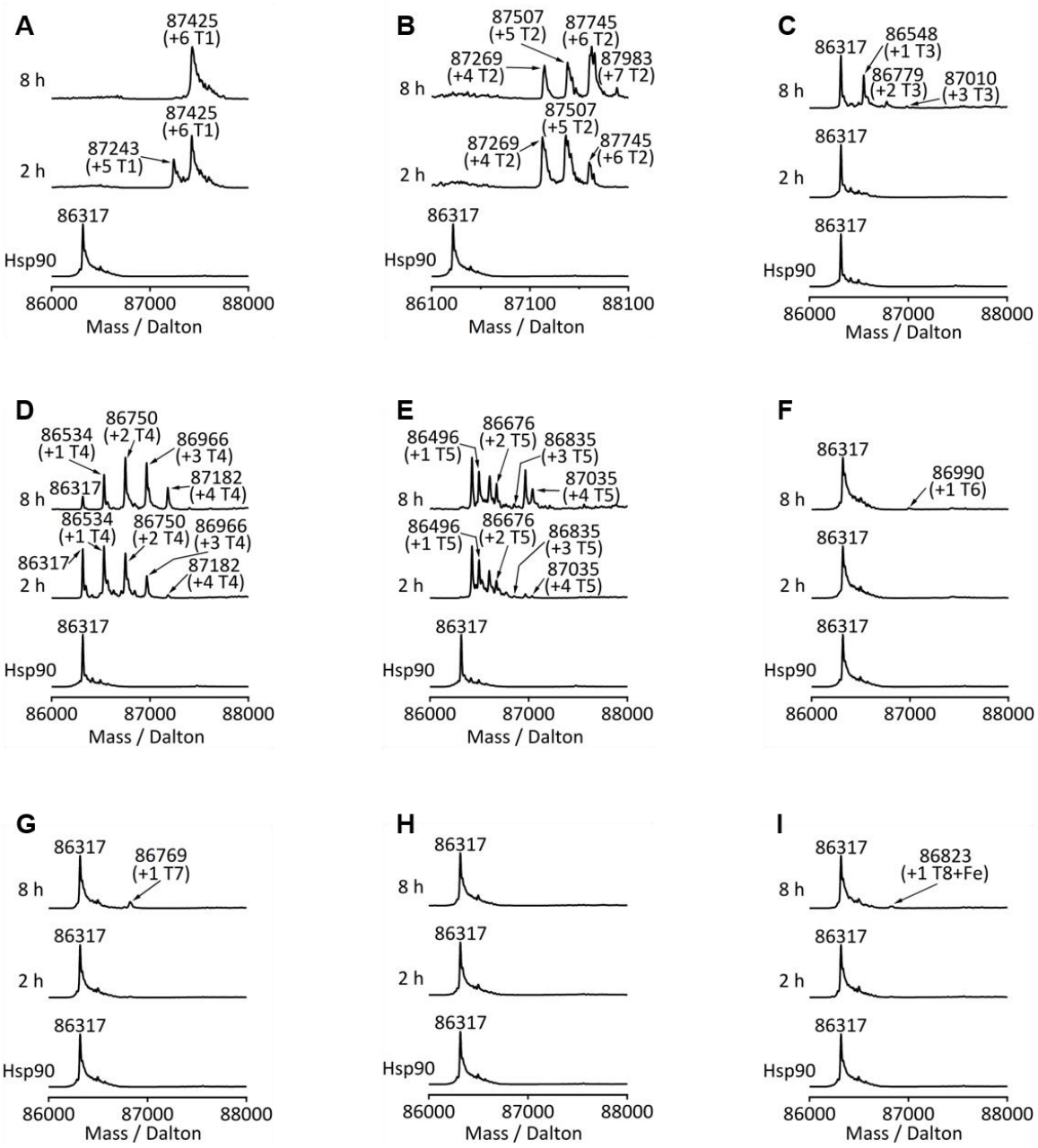

Fig. S3 ESI-Q-TOF mass spectrometry analysis of wild-type Hsp90 modification by functional tags. Deconvoluted mass spectra of wild-type Hsp90β incubated with labels T1–T8. Spectra illustrate the native protein (bottom trace) and the corresponding reaction products following incubation with (A) T1, (B) T2, (C) T3, (D) T4, (E) T5, (F) T6, (G) T7 and (H-I) T8. Reaction conditions: (A-E) 20 µM Hsp90 with 0.2 mM tag in 50 mM HEPES, 150 mM NaCl pH 8.0, (F-H) 50 mM Tris-HCl, 150 mM NaCl pH 7.0, and (I) 50 mM Tris-HCl, 150 mM NaCl pH 8.0 at 298 K. Peak labels indicate the number of conjugated tags.

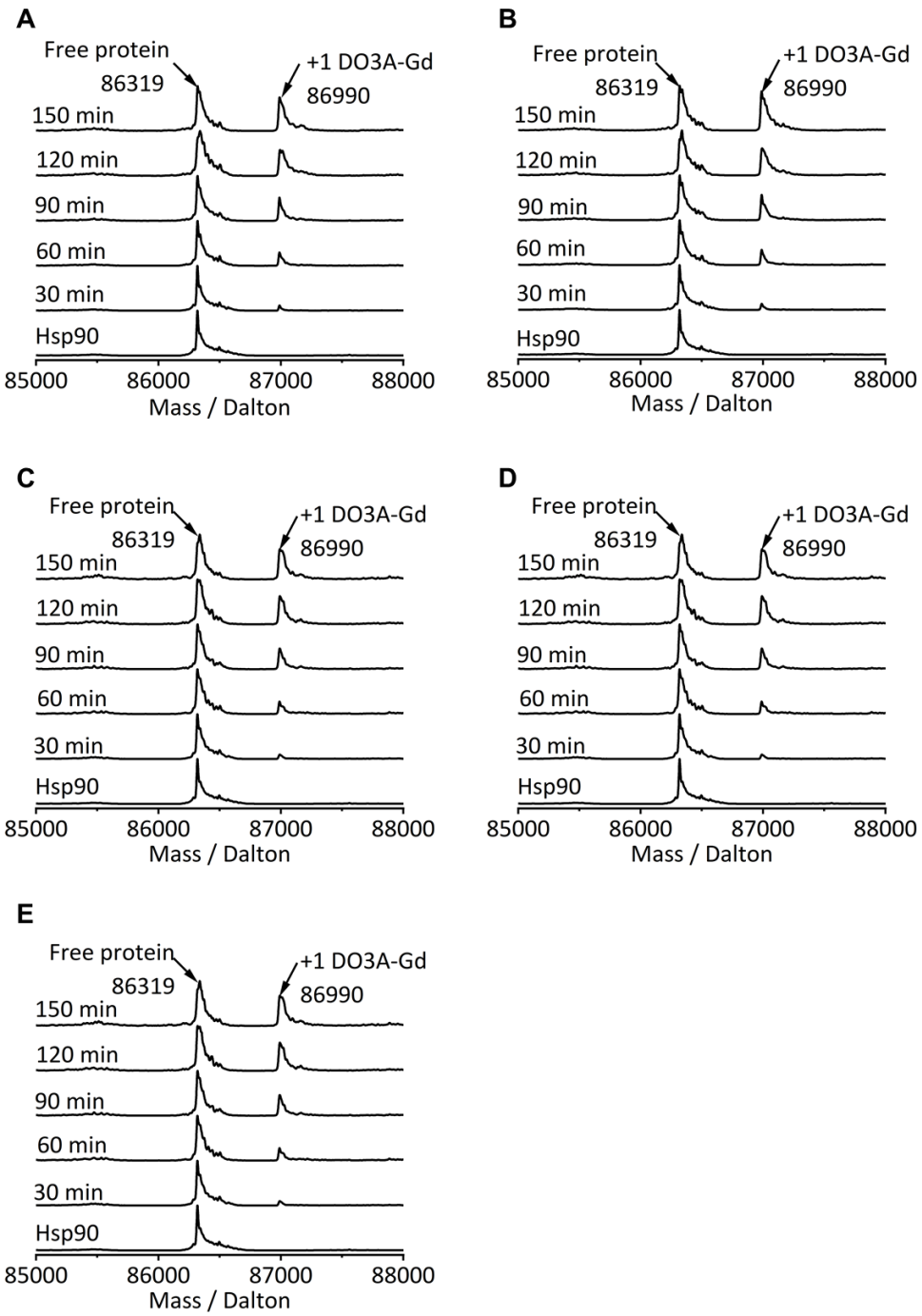

Fig. S4 Labelling of Hsp90 with BrPSPy-DO3A-Gd under nucleotide-bound states. Deconvoluted ESI-Q-TOF mass spectra of wild-type Hsp90β incubated with T6. Spectra illustrate the native protein (bottom trace) and the corresponding reaction products following incubation with (A) Apo-Hsp90, (B) + $MgCl_2$, (C) + $MgCl_2$ + ATP, (D) + $MgCl_2$ + ADP, (E) + $MgCl_2$ + AMP-PNP. Reaction conditions: 20 μM Hsp90 with 0.2 mM T6, 5 mM $MgCl_2$ and 5 mM nucleotide in 100 mM Tris-HCl, 150 mM NaCl pH 8.0 at 298 K.

Reference

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4 E. Etienne, N. Le Breton, M. Martinho, E. Mileo, V. Belle, *Magn. Reson.Chem.*, 2017, **55**, 714–719.