

Supporting Information

Experimental Section

Protein expression and purification

Wild type human Hsp90 β 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 (OD₆₀₀) 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 μ M, 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 labels¹, BrPSPy-DO3A-Gd² and BrPS-PyMTA³ were synthesized according to previously reported protocols. MgCl₂·6H₂O, 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(NO₃)₃ were each dissolved in ddH₂O to prepare a 100 mM stock solution. BrPS-PyMTA-Gd was prepared by mixing equal amounts of BrPS-PyMTA and Gd(NO₃)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. MgCl₂, ATP, ADP, and AMP-PNP were dissolved in ddH₂O 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 μ M 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 μ M 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 μ M 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 Mg²⁺/nucleotide-bound states, the protein stock was diluted to 20 μ M in Tris buffer (100 mM Tris, 150 mM NaCl, pH 8.0). Depending on the specific conditions, 5 mM MgCl₂, 5 mM MgCl₂ with 5 mM ATP, 5 mM MgCl₂ with 5 mM ADP, or 5 mM MgCl₂ 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 (OD₆₆₀). 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 MgCl₂. 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.

Hsp90 α		MPETQTQDQPM.....EEEVETFAFQAEIAQLMSL	32
Hsp90 β		MPPEVHHG.....EEEVETFAFQAEIAQLMSL	27
TRAP1		MARELRALLWGRRLRPLRALAPAAVPGGKPLLCPRRTTAQAGPRRNPAWSQAQGLFTQTGEDKEEPLHHSIISSSTESVQSGTSKHFEQAEITKLLDI	100
GRP94		MRALWVLG...LCVVLLTFGSVRADVDVDTGVETEDLGKSGREGSRDTDE...VVQREAEATQDGLGNASQIRELRSEKFAFQAEVNRMMKL	88
Hsp90 α		IINTFYSMKEHIRELISNSDALOKIRYESLTDPSKLSGKELHINLIENKQDRTLTIYDTGSGMTKADINILGTTAKSGTKAFMEALQAG.....AD	127
Hsp90 β		IINTFYSMKEHIRELISNSDALOKIRYESLTDPSKLSGKELKIDIIENPQERTLTLYDTGSGMTKADINILGTTAKSGTKAFMEALQAG.....AD	122
TRAP1		VARSISYSEKFIRELISNSDALOKIRHKLVSYDGOALP...EYEHQLQNAKSGTTITQDTGSGMOQEBVSNLGTIAPSGSKAEMLDALQO...AERAS	194
GRP94		IINSIYNKEHIRELISNSDALOKIRLISLTDENALSGNEETTVKIKCDKKNLLHVDVTGSGMREEBVKNLGTTAKSGTSEFLNKMTEAQEDGGOST	188
Hsp90 α		ISMIGQFGVGFYSAYLVAEKVVIITKHNDDEQYAMESSAGGSTVTVDTCGPMGRGKVIILHLEDQTEYLEERRRIKEIIVKHSQFEGYPTITL FVEKERD	227
Hsp90 β		ISMIGQFGVGFYSAYLVAEKVVIITKHNDDEQYAMESSAGGSTVTVDTCGPMGRGKVIILHLEDQTEYLEERRRIKEIIVKHSQFEGYPTITL FVEKERD	222
TRAP1		SKIIGQFGVGFYSAPMAADRVGVYSRGAAPGSLGYQMLSDGSGVFEIAEASGVRTGKIIHLASDCKEFSSEARVDDVVKYSNEVSEPLVNGRR...	291
GRP94		SELIGQFGVGFYSAYLVAEKVVIITKHNNDTQHIMESDSNEFSVIADPRGNTILGRGTITITLVLEASDYLEDLTIKNLVKKISQFINFPIHYVMSSKTET	288
Hsp90 α		KEVSDDEAEKEKEDKEEKEKESKEDKPEIEDVSGSDEBEKKDKGKKKKKIKEKYIDQEEINKTKEITRNDDITNEBYGSEYKSLTNDWEDHLAVK	327
Hsp90 β		KEI8DDEAEKEKG...EKEEEDKDDEKPKIEDVSGSDEEDDSGKDKKKKTKKIKEKYIDQEEINKTKEITRNDDITNEBYGSEYKSLTNDWEDHLAVK	319
TRAP1	EKEESDDE.....AAVEEBEEEE...KKPKTKKVEKTVWDWELMNDIKFELQRPSEKEVEDYKAEYKSFESKESDDPMAYI	329
GRP94		VEEPMEEBEEAAKE.....EKEESDDE.....AAVEEBEEEE...KKPKTKKVEKTVWDWELMNDIKFELQRPSEKEVEDYKAEYKSFESKESDDPMAYI	373
Hsp90 α		FEVSEGQLEFRALLFVPRAPFDLFENR...KKNNIKLYPRPFIIDNCEELIPEINFRGVVDSDELPLNIRSEMLOQSKILKVIENKLVKKCLELFT	425
Hsp90 β		FEVSEGQLEFRALLFVPRAPFDLFENK...KKNNIKLYPRPFIIDNCEELIPEINFRGVVDSDELPLNIRSEMLOQSKILKVIENKLVKKCLELFS	417
TRAP1		RYKTADPLNIRSIFYVPMKPSMFVSR...ELGSSVALYSKVIQTKATDIIKEMRFRGVVDSDELPLNIRSEMLOQSKILKVIENKLVKKCLELFT	427
GRP94		FEVTRAGEVTEKSIILFVPTSAFNGLEFDEYGSKKSDYIKLYRRRGIIDDPHDMMEKVLNFKVGVDSDELPLNIRSEMLOQSKILKVIENKLVKKCLELFT	473
Hsp90 α		ELAE.DKENYKKFVEQFSKNIKLGIHED...SQNRKKLSIELLRYTTSASGD.EMVSLADYCTRMKENQKHIYITGETKDQVANSFAVEELRHGHGEVIYV	521
Hsp90 β		ELAE.DKENYKKFVEAFSKNLKLGITHED...STWRRLSELRVHTTSASGD.EMTSEYVSRLMETQKSIXYITGESKEQVANSFAVEELRHGHGEVIYV	513
TRAP1		DQSKDAEKYARFEDYGLFMREGIVTATEQEVKEDIAKDLRYESLPSGQLTSEYASRMFAGTNNIYLCAPNRHDAHSPYIEMMRKDTDEVLCF	527
GRP94		KIAD.DKYN.DTFMKFEGTNIKLGIVIED...HSNRTRLAKLLRFQSSHHPT.DITSLDQYVERMKKEKQDKLFEMAGSSRKEAESPFVDELLKGYEVIYL	568
Hsp90 α		TEPIDEYCVQQLKEEFGKPLVSTKTEGLELPEDEEKKKQE.....EKKTKFENLCKIMKD.IDEKKVEKVVVSNRLVTPSCIVTSTYGTWTANMERIMK	615
Hsp90 β		TEPIDEYCVQQLKEEDGKSLVSTKTEGLELPEDEEKKKME.....ESKAKFENLCKLKE.IDDKKVEKVTINRLVSPCCIVTSTYGTWTANMERIMK	607
TRAP1		FEQDELTLHIREPDKKKLISVETDVIVDHYHEKFEEDRSPAECLSKETSEELVAMWNN.VDSSRVTVNVKVTIRLDTHEAMVTVLEWGAARHFLRMOQ	626
GRP94		TEPVDEYCIQALPEEDDGKRFQNVAKEGVKFDESEKTKESRE.....AVEKEFEPLNMMKDKADKDKIEKAVVSQRLTESPCALVASQTSQSGNMERIMK	663
Hsp90 α		AQALR...DNSTMGYNAAKKHLEINPDHSIIETLRQKAEADKNKSVKVDVILLYEPALLSSGFSLEDPQTHAIRVIRMIKLGIDIDEDDDPADDTSAAV	712
Hsp90 β		AQALR...DNSTMGYMAKKHLEINPDHSIIETLRQKAEADKNKAVKDVILLFEPALLSSGFSLEDPQTHAIRVIRMIKLGIDIDEDDEVAEEFNAAV	704
TRAP1		LAKTQ.....FEAQQLLOPTLEINPRHALIKKLNOIRASEPG.LAQLEVDQIYENMTAASLVDDPRAMVGRINELLIVKALERH	704
GRP94		AQAYQTGKDISINYASQKKTEINPRHFLIRDMLRIKDEDDDKTVLLIDAVLVFEPATLRSKILLPITKAYGDIHERMLRLSLNIDPDAKVVEEPEEPEP	763
Hsp90 α		TEEMPPLEGDDDDTSRMEEVD	732
Hsp90 β		FDEIPPLEGDEEDASRMEEVD	724
TRAP1		EETAEDTTTETQDDEEEMDVGTDBEEETAKESTARKDE	802
GRP94			

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

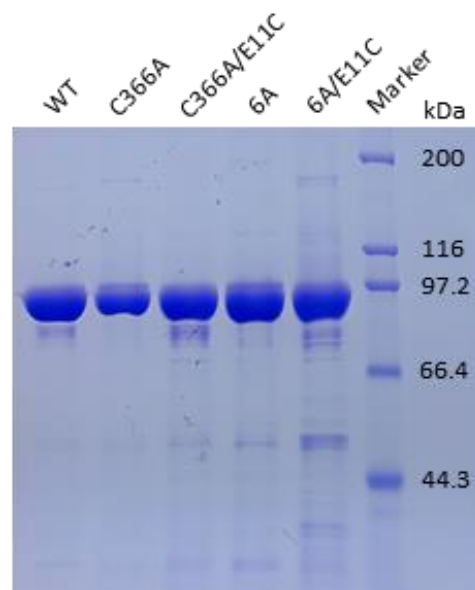


Fig S2 SDS-PAGE analysis of human Hsp90 β and its mutants.

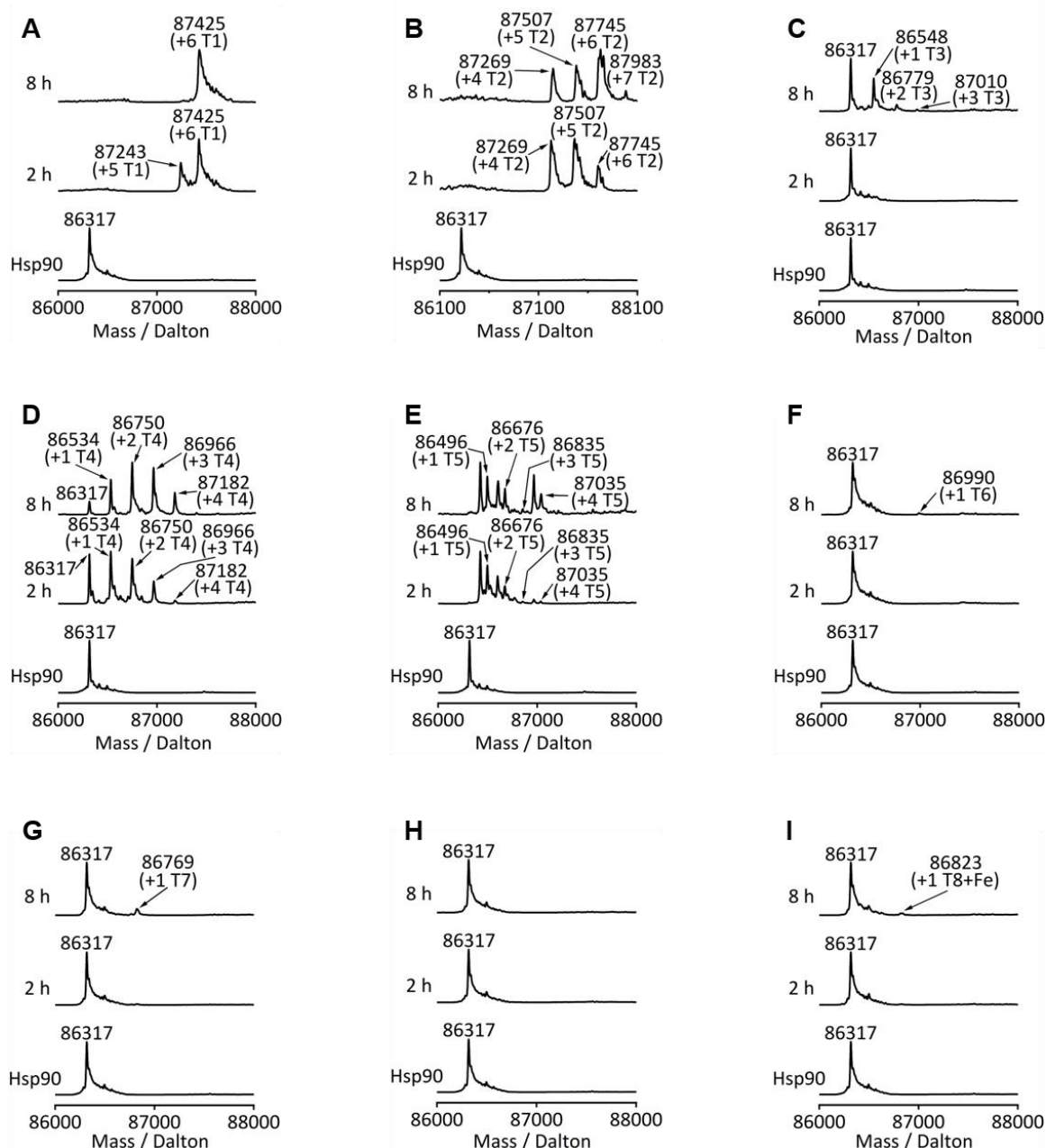


Fig. S3 ESI-Q-TOF mass spectrometry analysis of wild-type Hsp90 modification by functional tags. Deconvoluted mass spectra of wild-type Hsp90 β incubated with labels T1–T8. Spectra illustrate the native protein (bottom trace) and the corresponding reaction products following incubation with (A) T1, (B) T2, (C) T3, (D) T4, (E) T5, (F) T6, (G) T7 and (H–I) T8. Reaction conditions: (A–E) 20 μ M 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.

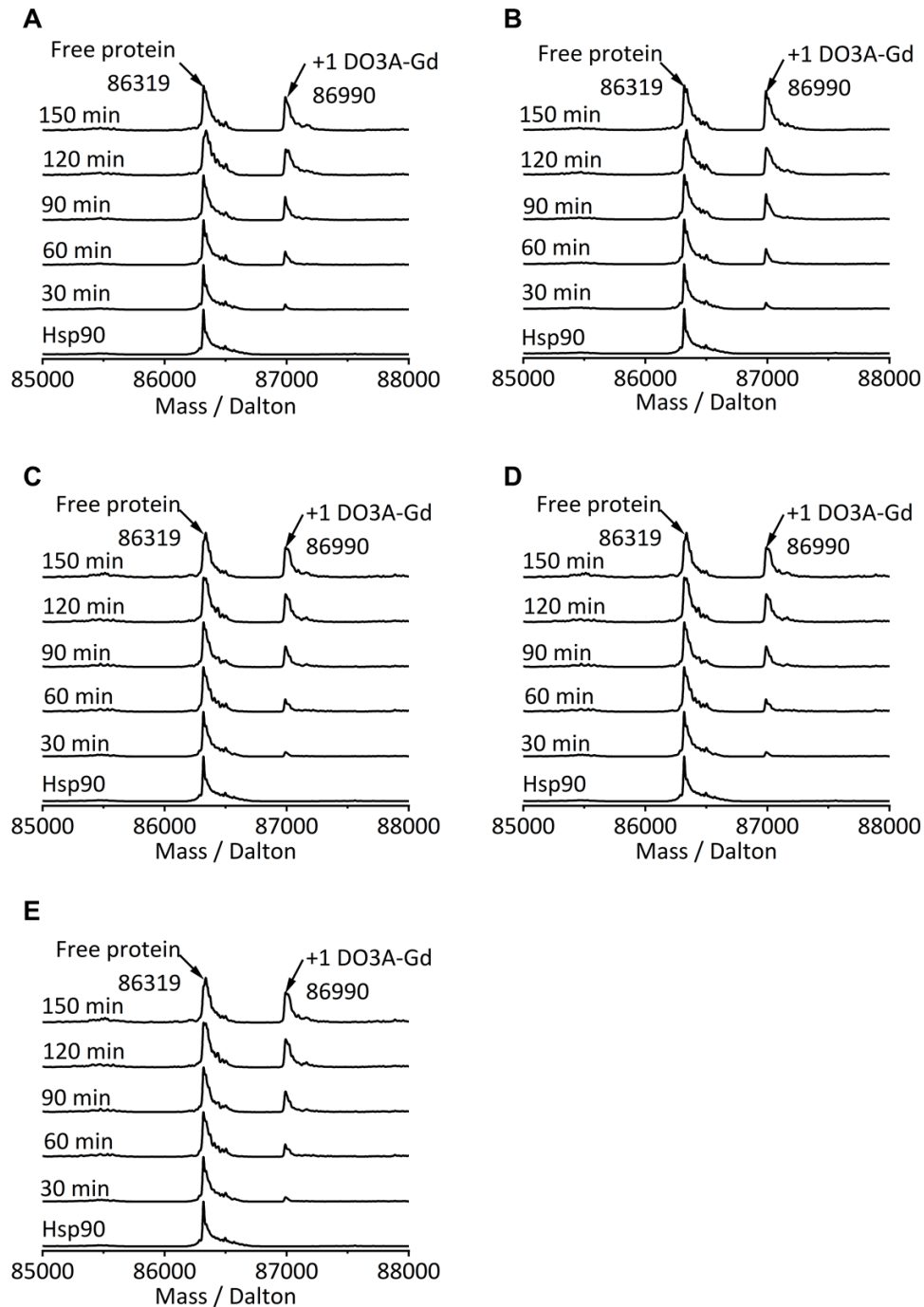


Fig. S4 Labelling of Hsp90 with BrPSPy-DO3A-Gd under nucleotide-bound states. Deconvoluted ESI-Q-TOF mass spectra of wild-type Hsp90 β incubated with T6. Spectra illustrate the native protein (bottom trace) and the corresponding reaction products following incubation with (A) Apo-Hsp90, (B) + MgCl₂, (C) + MgCl₂ + ATP, (D) + MgCl₂ + ADP, (E) + MgCl₂ + AMP-PNP. Reaction conditions: 20 μ M Hsp90 with 0.2 mM T6, 5 mM MgCl₂ and 5 mM nucleotide in 100 mM Tris-HCl, 150 mM NaCl pH 8.0 at 298 K.

Reference

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