Supporting Information

Franco et al. 10.1073/pnas.1215177110

SI Materials and Methods

Heat shock protein 90 was purified from rat liver. Two hundred grams of rat liver were dissected and homogenized using an Elvehjem-Potter in homogenization buffer (50 mM Tris, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 3.3 mM $CaCl_2$, 0.5 mM PMSF, pH 7.4). The homogenate was centrifuged for 15 min at 1,500 × *g* at 4 °C. The supernatant was spun for 1 h at 10,000 × *g* at 4 °C. Floating fat was removed carefully, and the clear cytosol was dialyzed against dialysis buffer (50 mM Tris, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 25 mM KCl, 2 mM DTT, 0.5 mM PMSF, pH 7.4). After dialysis, the cytosol was centrifuged at 95,000 × *g* at 4 °C for 14 h. Floating fat was removed carefully. The supernatant was collected and filtered with a 0.45-μm filter. The cytosolic fraction was loaded on an ion exchange column (Q Sepharose Fast Flow Amersham Biosciences 17–0510-01, XK16/40) linked to a BioLogic LP Chromatography system (Bio-Rad). The ion exchange column was equilibrated with buffer A (25 mM Tris, 1.25 mM $MgCl_2$, 1.25 mM KH_2PO_4, 12.5 mM KCl, pH 7.4). After the column was washed with 75% buffer A and 25% buffer B (25 mM Tris, 1.25 mM $MgCl_2$, 1.25 mM KH_2PO_4, 12.5 mM KCl, 1 M NaC, pH 7.4), the absorbed proteins were eluted with a linear gradient of 25–100% buffer B. Fractions were collected and analyzed by SDS/PAGE. Fractions containing the highest heat-shock protein 90 (Hsp90) concentration were pooled and dialyzed against 10 mM dialysis buffer (pH 6.8). The pooled Hsp90 fractions were loaded on a hydroxyapatite column (Bio-Rad) equilibrated with 10 mM KH_2PO_4 (pH 6.8). After washing with 10 mM KH_2PO_4 (pH 6.8), proteins were eluted with a linear gradient of 10–400 mM KH_2PO_4 (pH 6.8) buffer. Fractions were collected and analyzed by SDS/PAGE. Fractions containing the highest Hsp90 concentration were pooled. The pooled fractions were dialyzed against 50 mM Tris, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 25 mM KCl, 2 mM DTT, 0.5 mM PMSF, pH 7.4. The protein concentrations were adjusted to 5 mg/mL using Centricon Plus-20 (Millipore). Hsp90 was purified further by gel filtration using a Superdex 200 HiLoad16/60 column (Amersham Biosciences). The column was equilibrated with 50 mM Tris, 2.5 mM $MgCl_2$, 150 mM KCl, KH_2PO_4, pH 7.4. Fractions were collected and analyzed with SDS/PAGE.

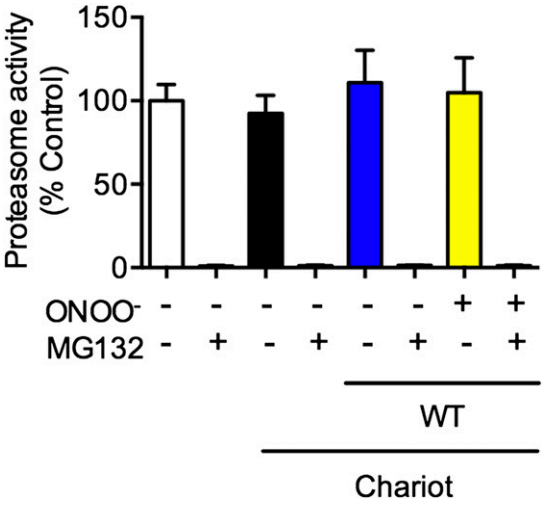
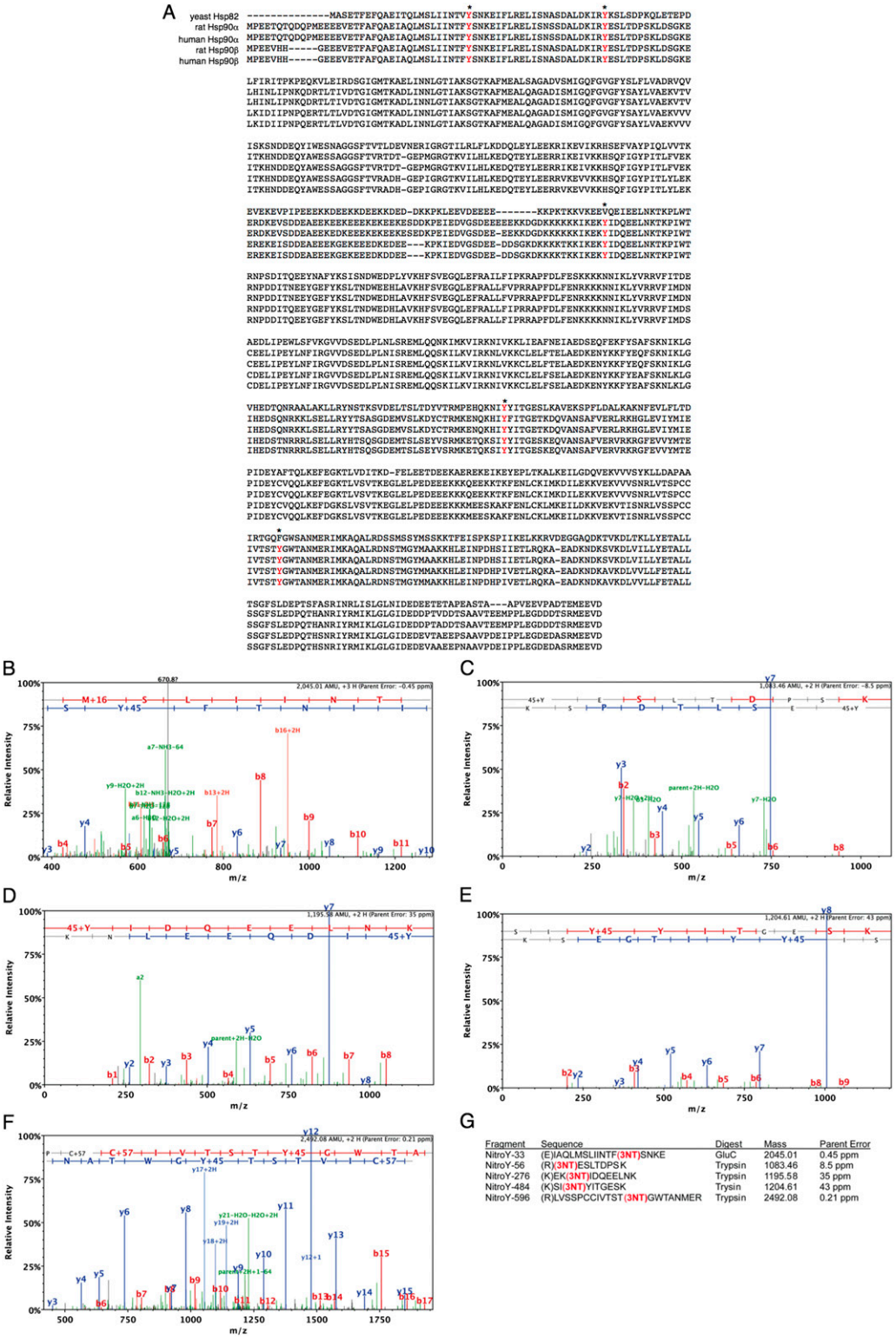


Fig. S1. Peroxynitrite-treated Hsp90 does not affect the proteasome activity in PC12 cells. The chymotrypsin-like activity of the proteasome was determined by a luminescent cell-based assay after the delivery of Hsp90 (WT) or peroxynitrite-treated Hsp90 (WT+$ONOO^-$). The proteasome inhibitor MG132 (1 μM) was used to verify the specificity of the assay.



Fragment	Sequence	Digest	Mass	Parent Error
NitroY-33	(E)IAQLMSLIINTF(3NT)SNKE	GluC	2045.01	0.45 ppm
NitroY-56	(R)(3NT)ESLTDPSK	Trypsin	1083.46	8.5 ppm
NitroY-276	(K)EK(3NT)IDQEELNK	Trypsin	1195.58	35 ppm
NitroY-484	(K)SI(3NT)YITGESK	Trysin	1204.61	43 ppm
NitroY-596	(R)LVSSPCCIVTST(3NT)GWTANMER	Trypsin	2492.08	0.21 ppm

Fig. S2. Amino acid sequence alignment of yeast 82-kDa heat-shock protein (Hsp82) and rat and human Hsp90α and -β and confirmation of the site-specific incorporation of nitrotyrosine residues into human Hsp90β. (*A*) The amino acid sequences corresponding to yeast Hsp82 and rat and human Hsp90α and -β were aligned using the Multiple Sequence Alignment (MSA) tool from the Expert Protein Analysis System (ExPASy) Proteomics Server. The asterisks denote the position of the five tyrosine residues prone to nitration (in red). Of note are the regions with a high degree of homology surrounding the tyrosine residues of interest and the high degree of homology between the human and rat Hsp90 sequences. (*B–F*) To confirm the site-specific incorporation of nitrotyrosine into recombinant human Hsp90β, in-gel digests were performed on pure proteins with trypsin or gluC, and the resulting fragments were analyzed by tandem mass spectrometry (MS/MS) fragmentation in two independent facilities. Shown are spectra of the sequence-specific MS/MS fragmentation of each peptide containing nitrotyrosine for each of the genetically nitrated Hsp90 proteins. *A* through *E* show spectra of relevant peptides from digests of nitrotyrosine at sites 33, 56, 276, 484, and 596, respectively. (*G*) Table showing the digest method and expected fragments detected for *A–E* with the altered site labeled as 3NT (in red). These spectra confirm 3-nitrotryosine incorporation at the expected positions.

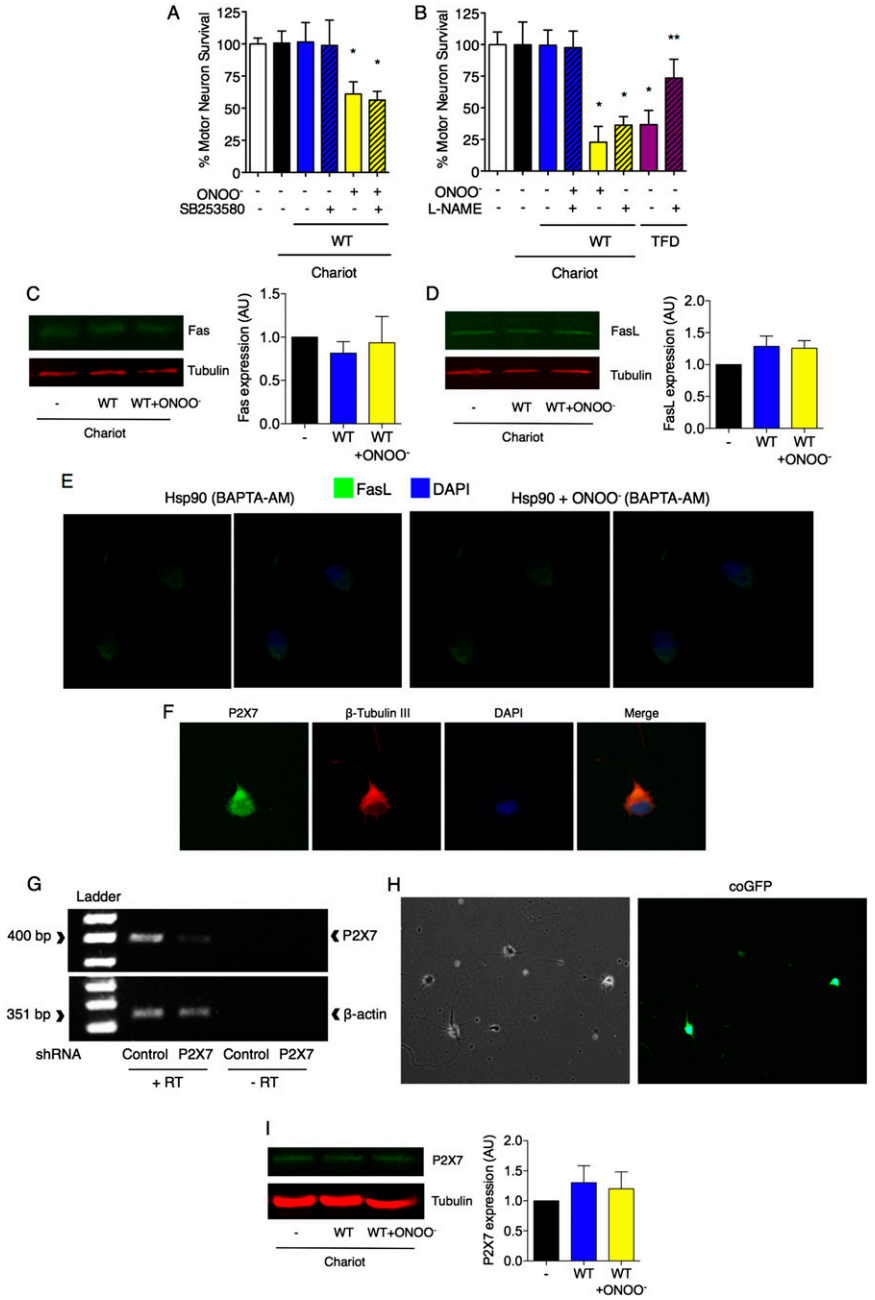


Fig. S3. Peroxynitrite-treated Hsp90 does not activate the DAXX component of the Fas pathway or alter the expression of FasL and P2X7 in motor neurons but does induce the mobilization of FasL to the plasma membrane. (*A* and *B*) The p38 inhibitor SB253580 (1 μM) (*A*), and the NOS inhibitor L-NG-nitroarginine methyl ester (L-NAME) (100 μM) (*B*) did not protect motor neurons from nitrated Hsp90-induced cell death. The motor neurons were incubated with SB253580 and L-NAME for 24 h after intracellular delivery of peroxynitrite-treated Hsp90. $*P < 0.05$ versus WT, $**P < 0.05$ versus WT + $ONOO^-$ by ANOVA followed by Bonferroni multiple comparison test. (*C* and *D*) WT Hsp90 and peroxynitrite-treated Hsp90 (WT+$ONOO^-$) do not alter the expression of Fas receptor (*C*) or FasL (*D*) in motor neurons, as determined by Western blot with infrared detection. The graphs on the right of the panels show the ratio between the Fas receptor or FasL and tubulin signals ($n = 3$) with respect to the control incubated with Chariot alone (–). (*E*) Calcium chelation decreased the mobilization of FasL to the plasma membrane upon the intracellular delivery of peroxynitrite-treated Hsp90. The motor neurons were incubated for 16 h in the presence of 5 μM BAPTA-AM after the intracellular delivery of unmodified (Hsp90) or peroxynitrite-treated Hsp90 (Hsp90 + $ONOO^-$). The cells then were stained for FasL (green). (*F*) P2X7 (in green) is expressed in motor neurons. The cytoskeleton was stained for β-tubulin III (red); DAPI corresponds to the nuclear staining (blue). "Merge" shows the superposition of the signals. (*G*) RT-PCR showing the P2X7 receptor knockdown in motor neurons upon transduction with lentiviral particles expressing P2X7 shRNA. Expression of a scrambled shRNA sequence was used as a control (control shRNA). The motor neurons were incubated further for 84 h after the addition of the lentiviral particles. +RT and −RT indicate that the samples were incubated in the presence or absence of retrotranscriptase, respectively. (*H*) The transduction efficiency was 90–95%, as shown 84 h after transduction of motor neurons with lentiviral particles expressing coGFP. (*I*) WT Hsp90 and peroxynitrite-treated Hsp90 (WT+$ONOO^-$) do not alter the expression of P2X7 receptor in motor neurons, as determined by Western blot with infrared detection. The graph on the right shows the ratio between the P2X7 and tubulin signals ($n = 3$) with respect to the control (–).

A

Peptides	Code	Position
SLIINTFYSNKEIFLREL	1A	Tyr33
SLIINTFnYSNKEIFLREL	1B	NitroTyr33
SDALDKIRYESLTDPSK	2A	Tyr56
SDALDKIRnYESLTDPSK	2B	NitroTyr56
TKKIKEKYIDQEELNKTK	3A	Tyr276
TKKIKEKnYIDQEELNKTK	3B	NitroTyr276
KETQKSIYYITGESKEQ	4A	Tyr484
KETQKSInYYITGESKEQ	4B	NitroTyr484
IVTSTYGWTANMERI	5A	Tyr596
IVTSTnYGWTANMERI	5B	NitroTyr596

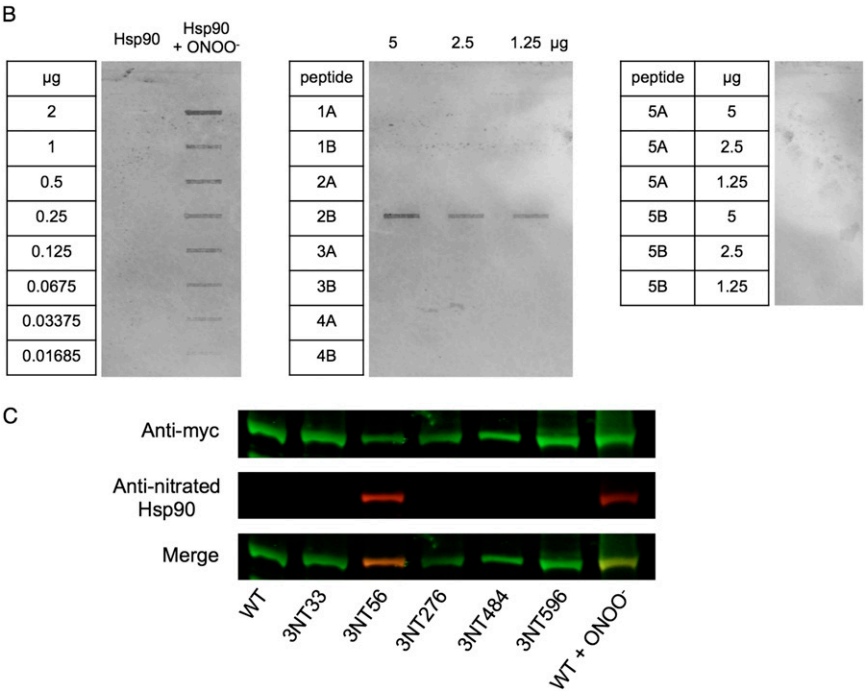


Fig. S4. Characterization of the intramolecular specificity of the antibody raised against nitrated Hsp90. The antibody specifically recognizes Hsp90 when nitrated at position 56. (*A*) Sequences of peptides synthesized with the human Hsp90β isomer for each nitration site in Hsp90 with tyrosine (Y, in red) or nitrotyrosine (nY, in red). The sequence of the peptides was corroborated by mass spectrometry. (*B*) The peptides were transferred to a membrane using a Slot blot device (Bio-Rad) as identified by the legend on the left, and were incubated with a monoclonal antibody (1:2,000 dilution) raised to recognize nitrated Hsp90. The primary antibody then was visualized using the Odyssey system (Licor) after incubation with a corresponding infrared fluorescent secondary antibody. The antibody recognized only peroxynitrite-treated Hsp90 and the peptide 2B corresponding to tyrosine 56 in Hsp90β. (*C*) The specificity of the antibody was corroborated by Western blot. The myc-tagged recombinant Hsp90β treated or not treated with peroxynitrite (WT + $ONOO^-$ and WT, respectively) and the five modified proteins containing a single nitrotyrosine in the positions prone to nitration (3NT) were loaded into a SDS/PAGE. A polyclonal antibody for the myc tag was used to detect total Hsp90β, and the monoclonal antibody was used to detect nitrated Hsp90. "Merge" shows the superposition of both signals. These results show that the antibody against nitrated Hsp90 and used for immunohistochemistry in Fig. 4 recognized only Hsp90 with nitrotyrosine in position 56.

Table S1. Putative nitrated proteins in PC12 cells after treatment with peroxynitrite

Protein	Score	Matched peptides
β-Tubulin	103	8
β-Actin	123	7
Elongation factor 1α-S	122	8
Hsp90	131	9
Hsp70	99	7
Lamin A	138	10
DnaK-mitochondrial molecular chaperone	79	5
Mortalin mot-2	79	6
Myosin Ic	96	8
Malate synthase	66	5
Histone H1.1	96	8
MHC class I antigen	54	7
Fructose-biphosphate aldolase	66	7
Growth inhibitor gene	68	6
RNA polymerase subunit P3	59	6
Quinolinate synthase	55	7
Amidohydrolase	54	6

The nitrated proteins were identified by MALDI-TOF mass spectrometry analysis. At least one of the matched peptides was sequenced for each protein.