Supporting Information

S1 Material and Methods

Cloning and synthesis of bacterial HtpGs. For collecting PsHtpG and XcHtpG genes genomic DNA from *Pseudomonas syringae* and *Xanthomonas campestris* were obtained from DSMZ (Braunschweig) and the DNA was amplified by PCR using primer pairs (5´-ATG AGT GTG GAA ACT CAA AAG GAA AC-3´; 5´-TCA AAC CGA CAG TTC TAC CAG CAA CT-3´ for PsHtpG and 5´- ATG ACC GTT GAT ACC GAC AAG CAG AC-3´; 5´-TTA AGC CGA CAA CTC CAA CAA CAA CT-3´ for XcHtpG) and a DNA polymerase from Jenabioscience (Jena, Germany). The PCR products were purified (Peqlab) and the PCR products were cloned into a pETSUMO-Topo vector (Invitrogen). This gave the pETSUMO-PsHtpG and pETSUMO-XcHtpG fusion constructs within the frame of the N-terminal hexahistidine and the SUMO protease cleavage site, respectively. Selected clones were sequenced to confirm the sequence and the insertion. The *Escherichia coli* strain BL21 (DE3) from Invitrogen was used to host the plasmid pETSUMO containing the PsHtpG or XcHtpG as described before by Schax et al., 2014.[22] All protein purification steps were carried out at 4 °C and 0.005 % (w/v) protease inhibitor (P-8465 from Sigma-Aldrich) were freshly added to lysis buffers (20 mM Tris-HCl, pH 8.0, 500 mM KCl, 2 mM β-mercaptoethanol, 2 mM imidazole, 10 % (v/v) glycerol). Additionally, all buffers were supplemented with 2 mM β-mercaptoethanol. Cell pellets were lysed by two French Press cycles at 12,000 p.s.i. and adjusted to a volume of 100 ml lysis buffer. Soluble proteins were separated from cellular debris by centrifugation at 25,000 x g for 45 min at 4 °C. The recombinant protein was purified from the supernatant by the metal chelating resin Talon from Clontech after incubation for 2h on ice. The N-terminal tag of the recombinant protein was removed by SUMO protease from Promega. The dialysis was performed against a 500-fold volume of buffer containing 20 mM Tris pH 8.0, 20 mM KCl, 2 mM β-mercaptoethanol and 10 % (v/v) glycerol at 4 °C for 24 h. The protein was concentrated with an YM30 centrifugal filter device, Amicon (EMD Millipore Corporation) and purified by SEC16/60 chromatography in 20 mM Tris-HCl pH 8.0, 500 mM KCl and 2 mM β-mercaptoethanol. Finally, the protein was concentrated with an YM30 centrifugal filter device, Amicon (EMD Millipore Corporation) to 1 ml and diluted twice to 10 ml of storage buffer 20 mM Tris pH 7.5, 50 mM KCl, 6 mM β-mercaptoethanol, 10 % (v/v) glycerol, and samples were frozen at a concentration of 3 mg ml^{-1} in liquid nitrogen and stored at –80 °C.

Direct Competitive Microarray Assay Proteins were spotted in the storage buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM β-mercaptoethanol, 10 % (v/v) glycerol) in 4 x 5 matrices on the UniSart® 3D nitro slide (Sartorius Stedim Biotech S.A.) using a contactless GeSim Nano-Plotter™ (GeSim) with a nanotip pipette as described before by Schax et al., (2014)[22]. The slide was air dried for 30 min at room temperature. Before printed protein arrays were subjected to the ATP-binding and direct competitive assay, unspecific sites were blocked with 1 % (w/v) BSA in the same buffer as before for 45 min at room temperature. For binding, the slides were incubated directly with 100 nM dye labelled ATP as indicated and unlabeled ATP or inhibitors with diluted concentrations of 100 µM to 10 pM in binding buffer (20 mM HEPES-KOH, pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, 0.01% (v/v) Tween 20, 2 % (v/v) DMSO, 0.1 mg ml^{-1} BSA) for 16 h at 4°C. The mix of binding buffer, 100 nM dye-labelled ATP and 500 nM radicicol served as positive control while the negative control contained the binding buffer and 100 nM Cy5-ATP in the absence of potential inhibitors. 16 well hybridizations chambers of Nexterion (Schott, Nexterion) were used to separate the subarrays. After incubation, the slides were washed three times for 5 min with binding buffer. Binding one of Cy5-labelled ATP[23] from Jena Bioscience GmbH was determined by GenePix 4000B Laser Scanner (Molecular Devices, Inc.) with 635 nm excitation wavelength, laser power 10 %, PMT gain 380 and calculated with ImaGene 5 of BioDiscovery, Inc. Evaluation of displacement was done with the EC_{50} value. The dose-response curves were calculated with Origin 7G (OriginLab Corporation) and fitted with the non-linear function logistic, A1= 0, A2= 1. Quality validation of the microarray performed by calculating the mean (sd) of 10 spots as described before using a binding buffer with 100 nM dye-labelled ATP.[11]

Microscale thermophoresis analysis XcHtpG was labelled according to Cy5 mono-reactive dye protein assay (GE Healthcare, Germany) and a stock solution of 200 µL was adjusted to a final concentration of 100 nM. To estimate signal intensity a pre-run was performed at a protein concentration of 50 nM in a MST glass capillary and checked a proper LED power on Monolith NT.115 to yield low florescence between 200 and 1500 counts. Taking the stock solution a 12 step dilution series of Gambogic acid, in MST buffer containing 20 mM Hepes, 50 mM KCL, 5 mM $MgCl_2$ x $6H_20$, 0.1 mg/ml BSA, 0.05 % Tween20, 2 % DMSO (v/v), 20 mM Na_2MoO_4 x $2H_20$, 1 mM ATP pH7.3, starting from 874.5 µM down to 53 nM was prepared. The ratio between each of two adjacent dilution steps was 1:1 at a final volume of 10 µL. Ten µl of labelled XcHtpG solution was filled into the same 12 tubes and mixed very well. The mixed sample was incubated in the dark at room temperature for 20 minutes. After an adequate incubation time all of the samples were transferred into Monolith

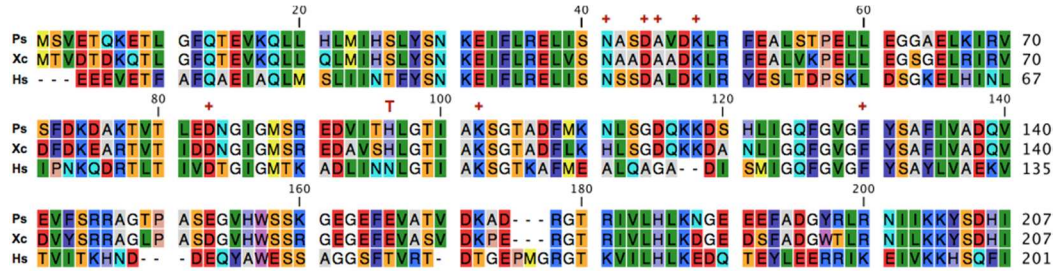
NT capillaries. The capillaries were inserted into the slots on the sample tray, and the measurements were started. The measurements were performed at a LED power of 30% using a MST power of 20%, 40% and 60% and a final protein concentration of 50 nM at different Gambogic acid concentrations. Afterwards the capillaries scan and MST measurement started with three repetitions. Data analysis and calculation of Kd was done by NT analysis software.

Inhibitors Reblastatin derivatives were synthesized as described before (Schax et al., 2014; Hermane et al., 2015; Mohammadi et al., 2018)[22,23,25]. Gambogic acid was purchased from Combi Blocks (San Diego, USA).

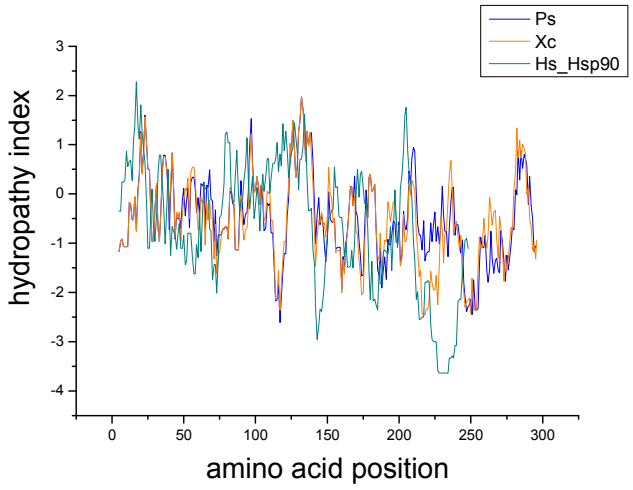
S2 Figures

Figure S1 (a) Sequence alignment of HtpG related proteins. b) Hydropathy plot of heat shock proteins. c) Interactome of Hsp90 and HtpG proteins computed by the STRING database.

a)



b)



c)

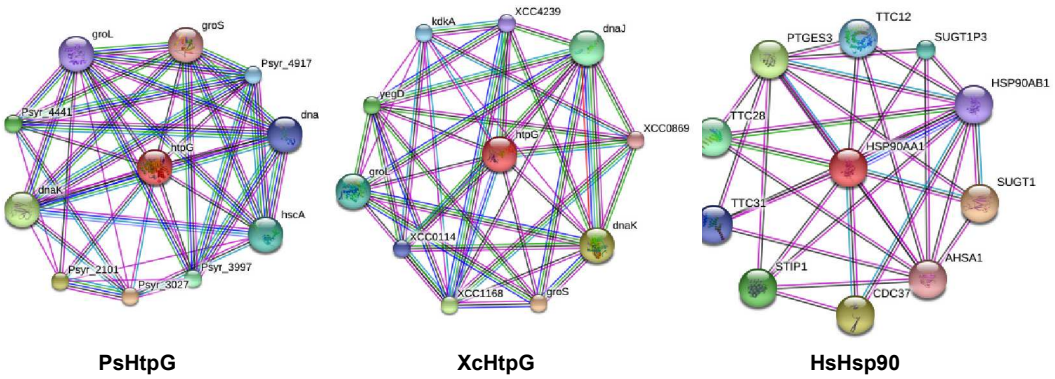


Figure S2 MST-traces are displayed in the mode of Thermophoresis + T-jump. The colour of blue and red represent F_{cold} and F_{hot} respectively, which show the positions of data collection.

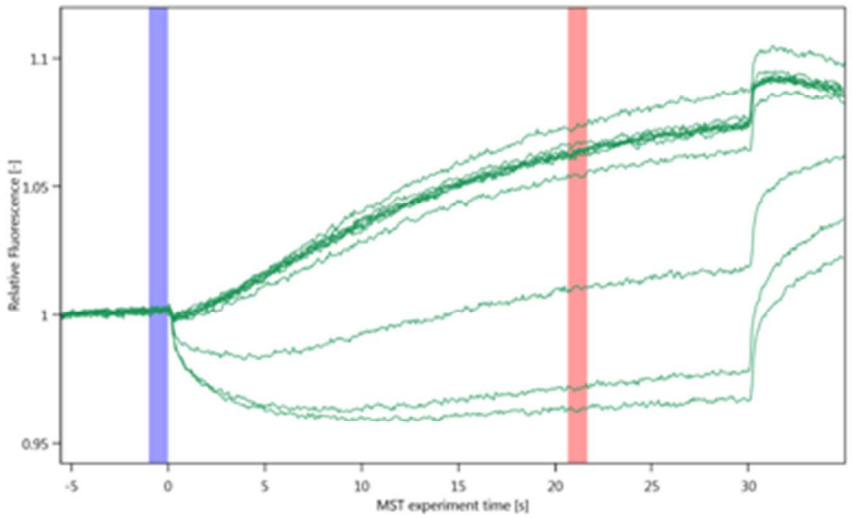


Figure S3. Sequence and structural alignments of putative GBD binding sites. (A) The two putative GBA binding sites of human Hsp90β/ and or α identified before[6,7] were aligned against XcHtpG. The motif XXLIPEYXX described by Yim et al[6] identified in Hsp90β does not fully match with XcHtpG, but the sequence alignment indicate that there is some degree of homology. The positions P371 and Y373 of human Hsp90 described by Davenport[7] exactly match those in the structure of XcHtpG (B). The XcHtpG (green) structure obtained by Swiss Modeler homology modeling was aligned with pdb data from human Hsp90β (cyan).