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Supplementary material for: N-terminal and C-terminal modulation of hsp90 have produce different phenotypes

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MATERIALS AND METHODS

Cell lines and cell culture. HCT116 human colorectal carcinoma (CCL-247) cell line was obtained from ATCC (Manassas, Virginia, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with supplements as proscribed by the manufacturer, and incubated in a humidified chamber at 37 ^{0}C with 5% CO_2.

Reagents and antibodies. Stock solutions of 17-N-allylamino-17-demethoxygeldanamycin (17-AAG; Sigma Aldrich) and SM122 (SRM laboratory) were prepared by dissolving the solid compound in dimethyl sulfoxide (DMSO, Sigma Aldrich). Primary antibodies to HSF-1 (1:2,000), and Hsp27 (1:2,000) were purchased from Abcam; Hsp90 (1:2,000) and Hsp70 (1:2,000) were purchased from Enzo Life Sciences; and Actin (1:4,000) was obtained from Santa Cruz Biotechnology. Secondary antibodies to goat anti-mouse HRP (1:2,000), goat anti-rabbit HRP (1:2,000), and rabbit anti-goat HRP (1:2,000) were obtained from Abcam.

Immunoblotting. HCT116 cells were seeded in 6-well plates (5×10^5 cells per well) and incubated for 24 h before treatments. Cells were treated with indicated drugs for 24 h and then lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate and 0.5% NP40) supplemented with cocktail protease inhibitors (Roche) for another 24 h. The total protein concentrations of lysates were determined by the bicinchoninic acid (BCA) method with the BCA kit (Pierce) following the manufacturer's instructions. 100 mg of total protein were separated by 4 ~ 20% Tris-Glycine gel (Life Technologies) and transferred to a PVDF membrane (Thermo Fisher Scientific). Membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline containing 0.1% Tween-20) for 2 h and incubated with respective primary antibodies in 2.5% non-fat milk (in TBST) at 4 ^{0}C overnight. After wash with cold TBST membranes were incubated with respective HRP-conjugated secondary antibodies at 4 ^{0}C for 2 h, following by three-time wash with cold TBST and one wash with cold TBS (Tris-buffered saline). Immunoblotting was performed using chemiluminescent substrates (Thermo scientific) and the images were captured by ImageQuant LAS 4010 digital imaging system (GE Healthcare). Data was quantified by ImageJ software.

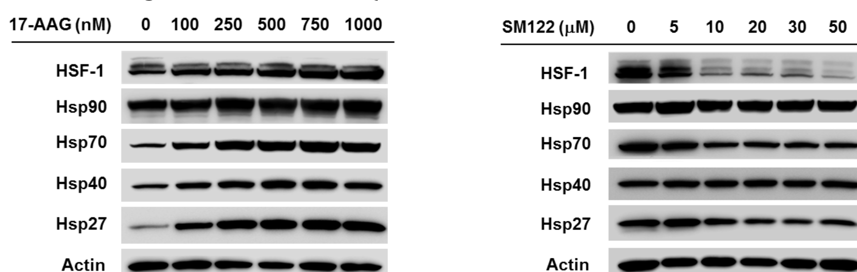
Luciferase refolding assay. Firefly luciferase (12.5 mg/mL; Novus Biologicals) was diluted to a concentration of 2 mg/mL in stability buffer (25 mM Tricine, pH 7.8, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 10% (v/v) glycerol, and 10 mg/mL bovine serum albumin), and was heat denatured at 41 ^{0}C for 30 min. The denatured protein was further diluted (1:20, v/v) in stability buffer to form 0.1 mg/mL stock solution and placed on ice before refolding. 0.5 mL of drugs with indicated concentrations or DMSO (Sigma-Aldrich) as a control was incubated with 48.5 mL of 50% diluted rabbit reticulocyte lysate (RRL; Promega) in Mili-Q water at 30 ^{0}C for 5 h. Refolding was initiated by adding 1.0 mL of the denatured luciferase stock into the RRL refolding system, treated either with drugs or DMSO in advance. Reactions were performed at 30 ^{0}C. After 120 min, 5 mL of each reaction mixture was removed and added to 45 mL of Bright-GloTM luciferase assay buffer (Promega) mixed with Bright-GloTM luciferase assay substrate (Promega), which was preloaded in a white, flat-bottomed, 96-well plate (Greiner Bio-One). After incubating for 5 min at room temperature in dark, the luminescence was measured using a luminometer (Berthold Orion Microplate Luminometer). Luciferase activity in refolding reactions at each time point was calculated by the formula:

$$\text{Luciferase activity (\%)} = (LI_{sample} / LI_{DMSO} \text{ at 120 min}) \times 100$$

where LI indicates the luminescence intensity in each reaction. The luciferase activity in the refolding reaction with DMSO (control) at 120 min was considered as 100% refolding.

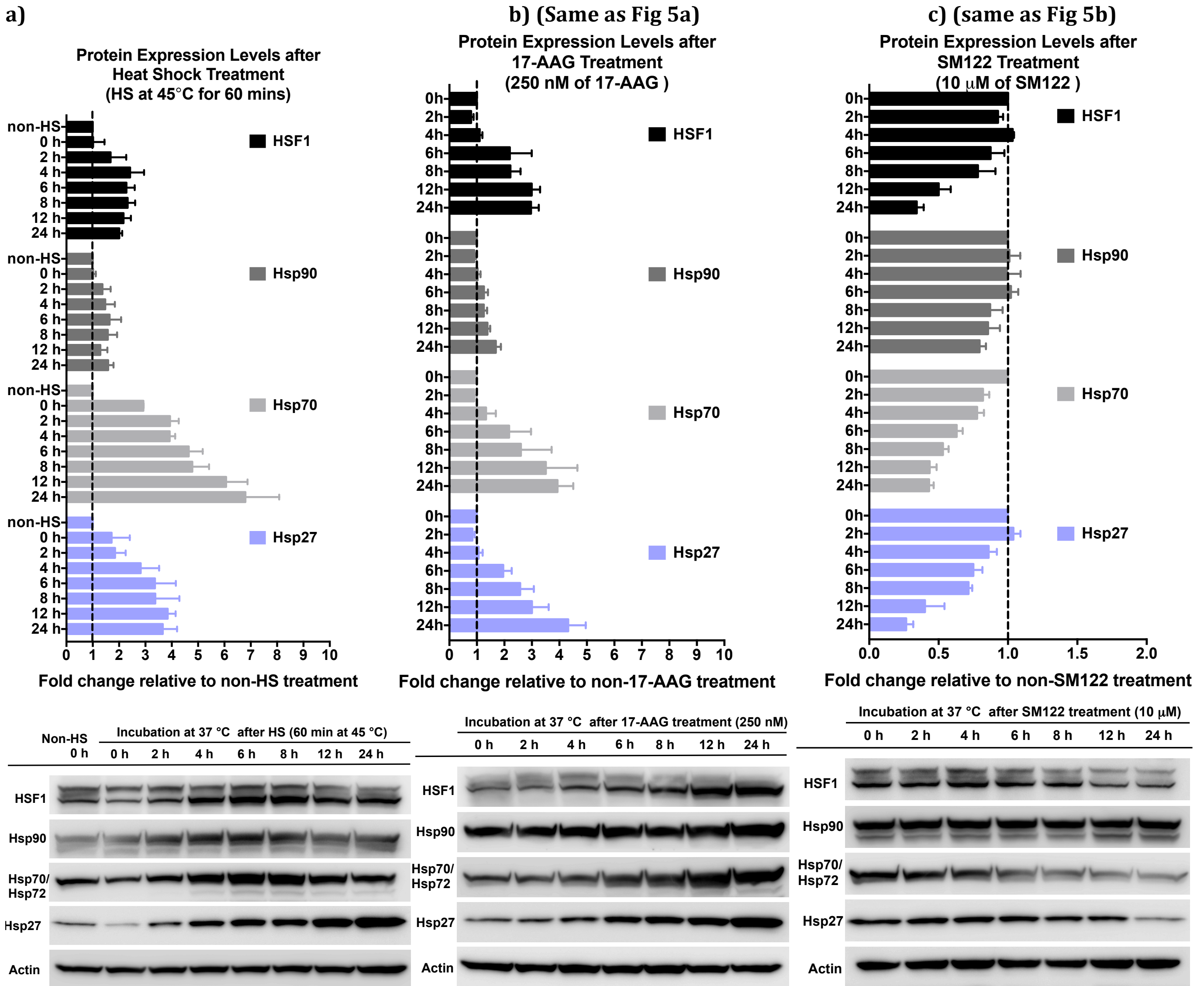
Quantitative real-time PCR assays. HCT116 cells were seeded into 6-well plates with a density of 5×10^5 cells per well 24 h before treatments. Cells were exposed to the indicated drugs for another 24 h at 37 ^{0}C. Total RNA from each treatment (sample) was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. 1 mg of RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR (qRT-PCR) was performed in ViiA 7-Real Time PCR system (Life Technologies) using QuantiFast SYBR Green PCR Kit (Qiagen). Primers of *HSPA8*, *HSPA1A*, and *HSPB1*(housekeeping gene) were purchased from Qiagen. Data were analysed by ViiA 7 Software.

Statistical analysis. To determine the statistical significance of experimental data, the unpaired Student *t* test was conducted using GraphPad Prism 6.0 (GraphPad Software Inc). Data were represented as mean ± s.e.m. from at least three independent experiments. Differences are indicated with *P* values, which less than 0.05 were considered statistically significant relative to indicated comparison and designated with asterisk (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****, $P < 0.001$).



Supplementary Figure S1: Raw data for graphs shown in Figure 4a and 4b respectively. Immunoblot for the proteins of interest in the lysate of HCT116 cells treated over 24 hours with the HS, DMSO (control, shown as 0 μM or 0 nM of indicated inhibitor), 17-AAG, and SM122 at indicated conditions. Immunoblot images of 17-AAG or SM122 treatments after 24 hours, respectively. Actin was used as the protein loading control. Experiments were performed in triplicate, and representative results are shown in western blots.

Values are presented as fold change in specific treatment relative to the controls. Data are represented as average ± s.e.m. from three independent experiments.



Supplementary Figure S2: Raw data for figure 5a and b**.** Immunoblot for the proteins of interest in the lysate of HCT116 cells treated over 24 hours with the HS, DMSO (control, shown as 0 μM or 0 nM of indicated inhibitor), 17-AAG, and SM122 at indicated conditions. (a) The expression levels of indicated proteins in the cells heated at 45°C for 60 minutes were monitored over 24 hours. (b) Cells were treated with 250 nM of 17-AAG and the expression levels of indicated proteins were monitored over 24 hours. (c) Cells treated with 10 μM of SM122 and the expression levels of indicated proteins were monitored over 24 hours. Experiments were performed in triplicate, and representative results are shown in western blots below, with actin as a protein loading control. Values are presented as fold change in specific treatment relative to the controls. Data are represented as average ± s.e.m. from three independent experiments.