SUPPLEMENTARY RESULTS

Small Molecule Proteostasis Regulators for Protein Conformational Diseases

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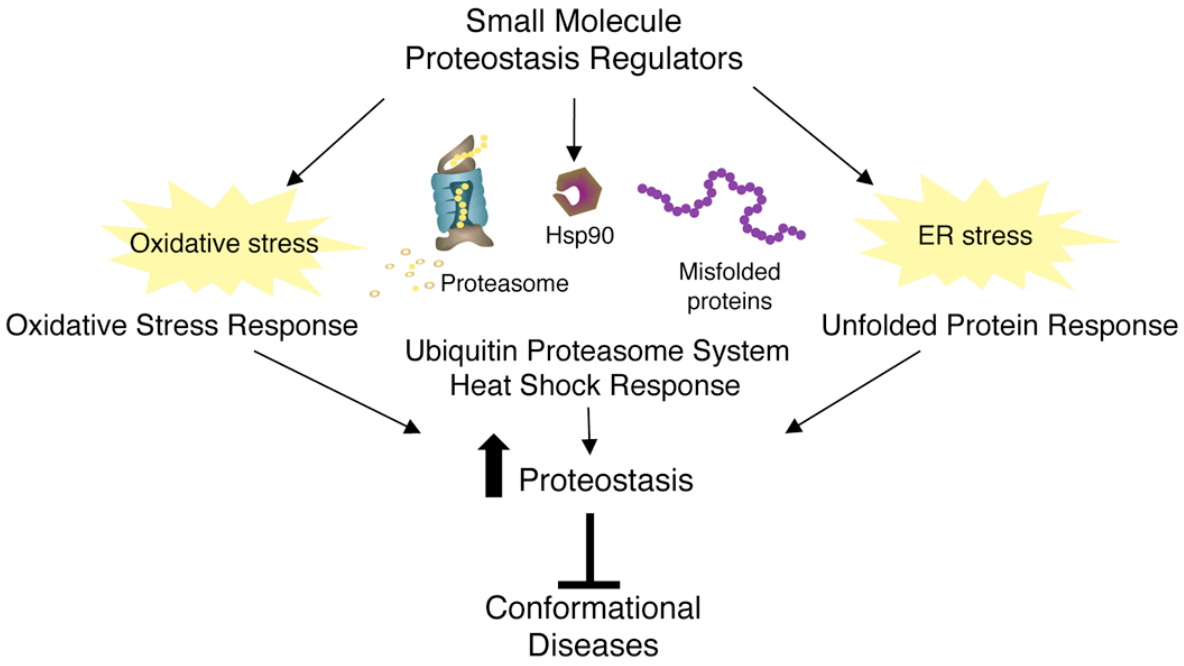
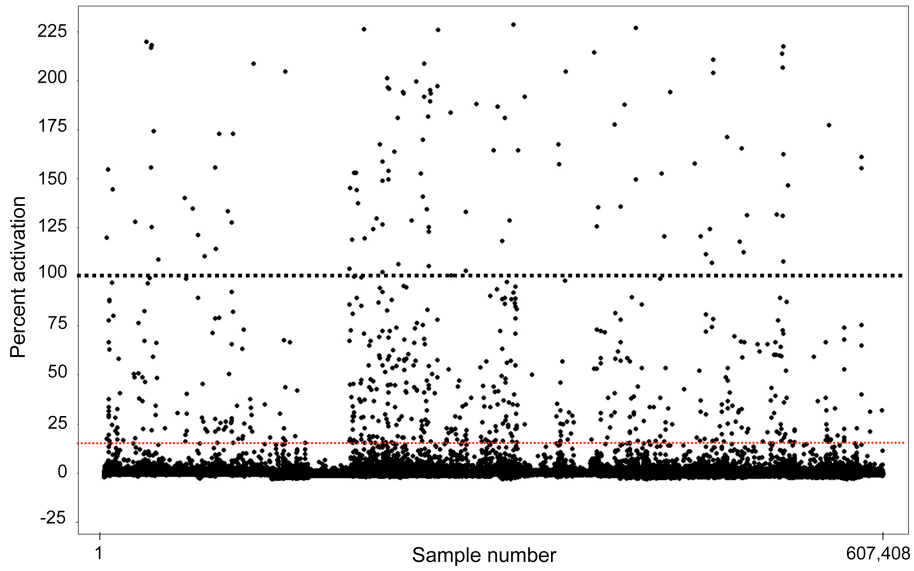
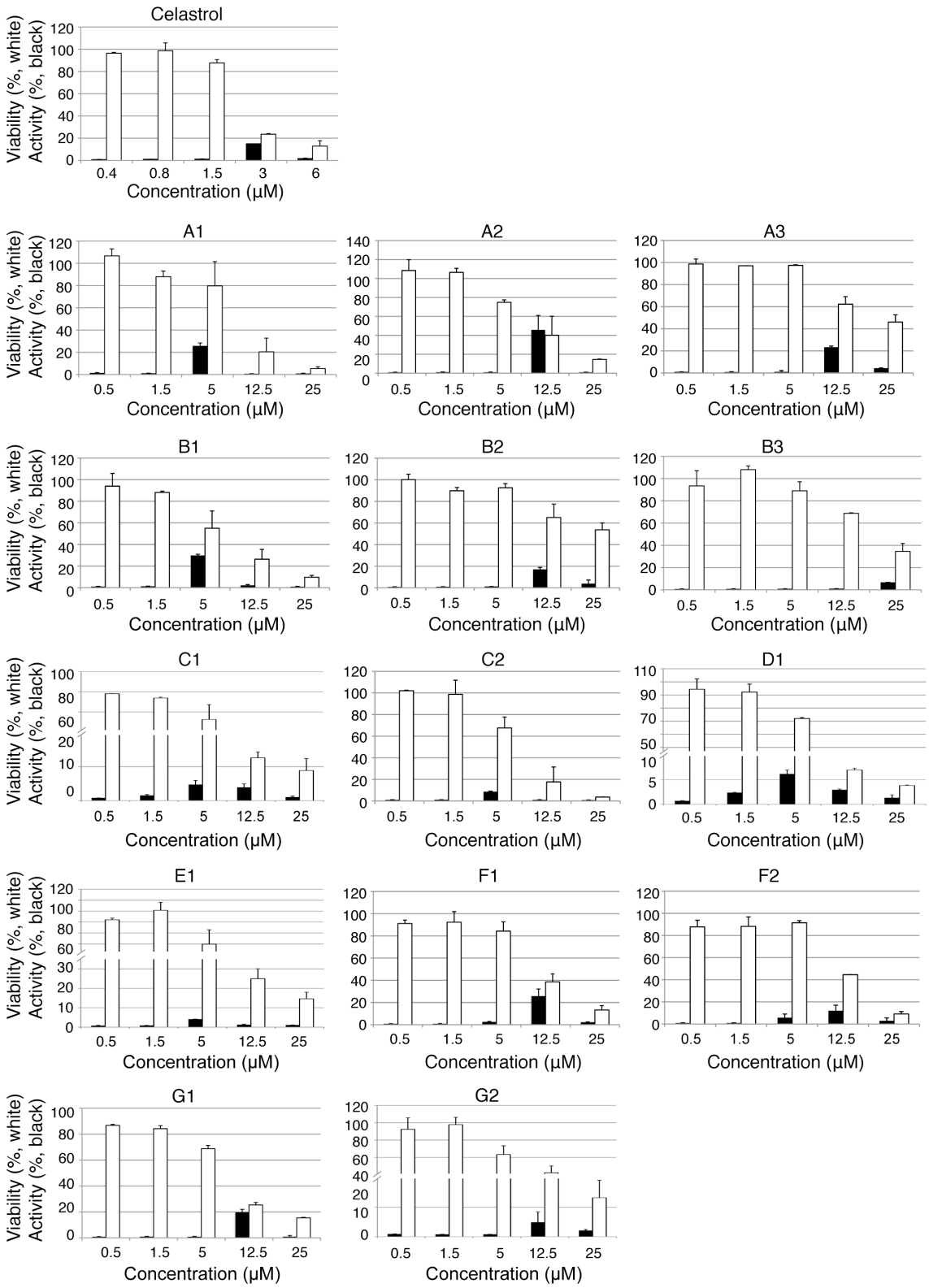


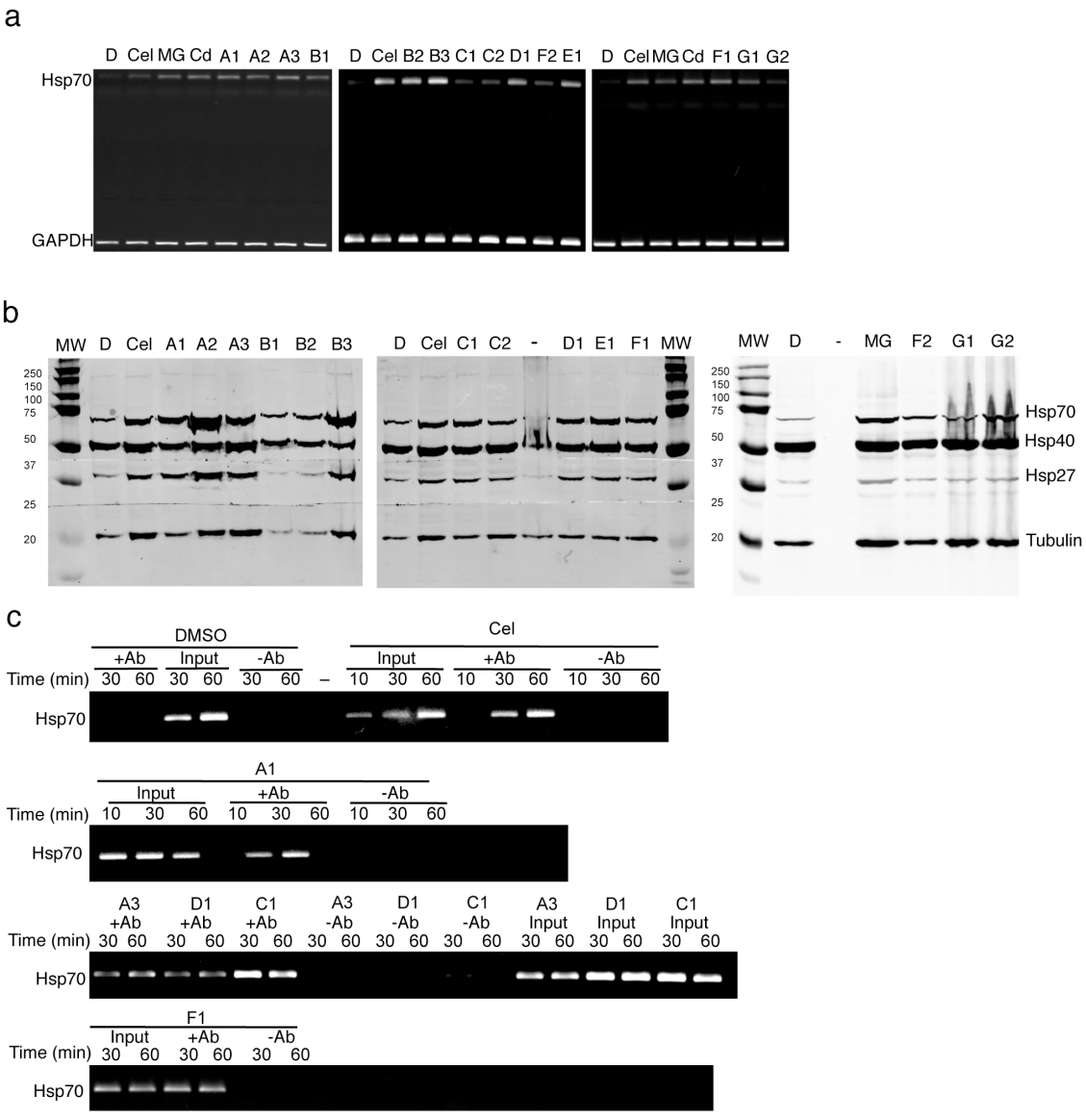
Supplementary Scheme 1



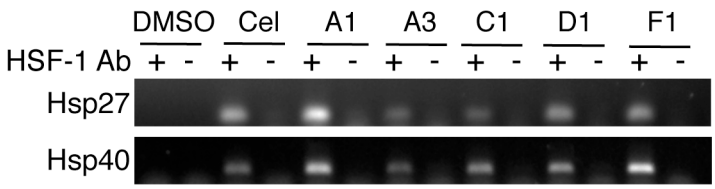
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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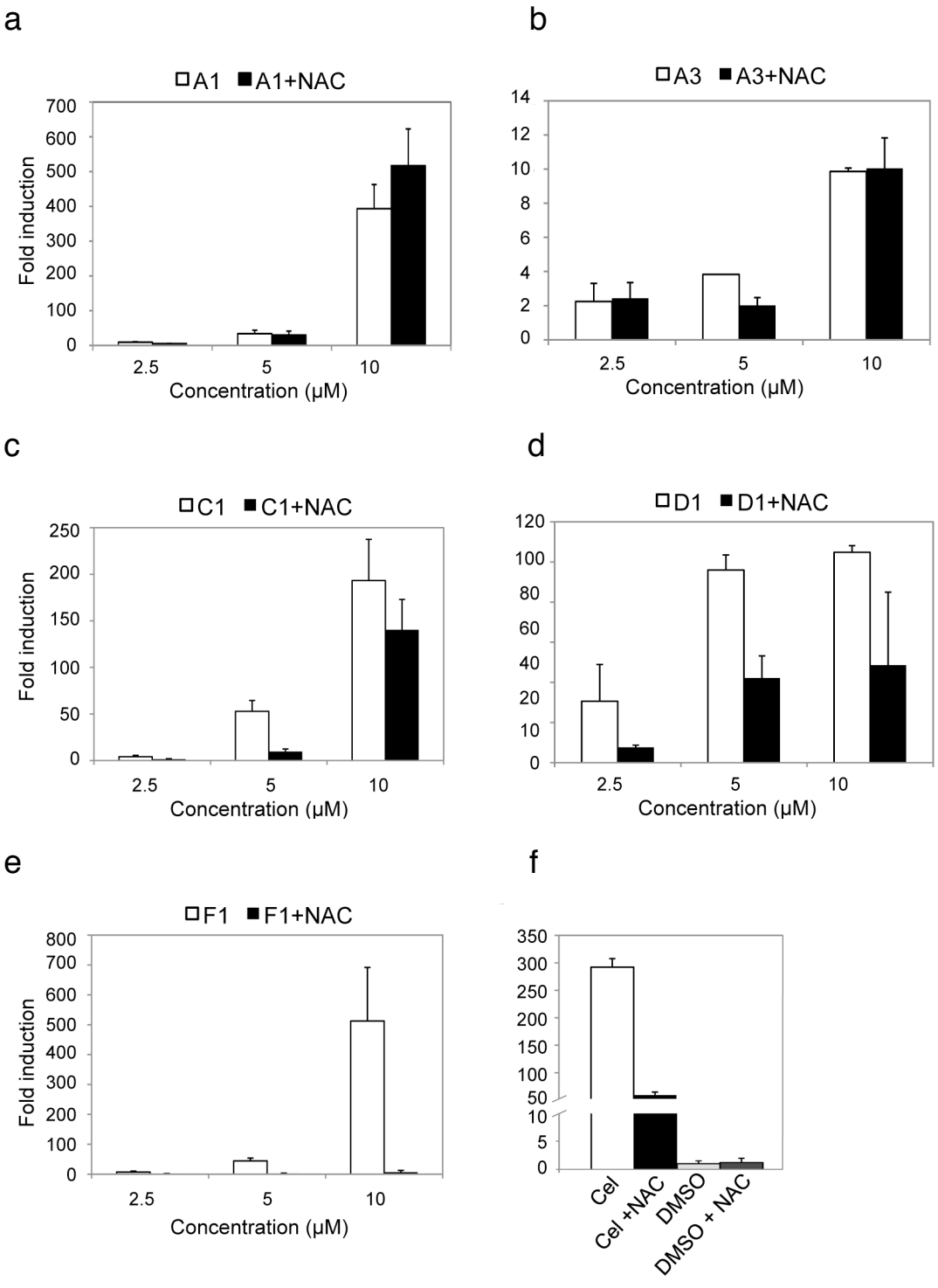
a
A1
A1+NAC
Fold induction
700
600
500
400
300
200
100
0
2.5
5
10
Concentration (μM)
b
A3
A3+NAC
14
12
10
8
6
4
2
0
2.5
5
10
Concentration (μM)
c
C1
C1+NAC
Fold induction
250
200
150
100
50
0
2.5
5
10
Concentration (μM)
d
D1
D1+NAC
120
100
80
60
40
20
10
0
2.5
5
10
Concentration (μM)
e
F1
F1+NAC
Fold induction
800
700
600
500
400
300
200
100
0
2.5
5
10
Concentration (μM)
f
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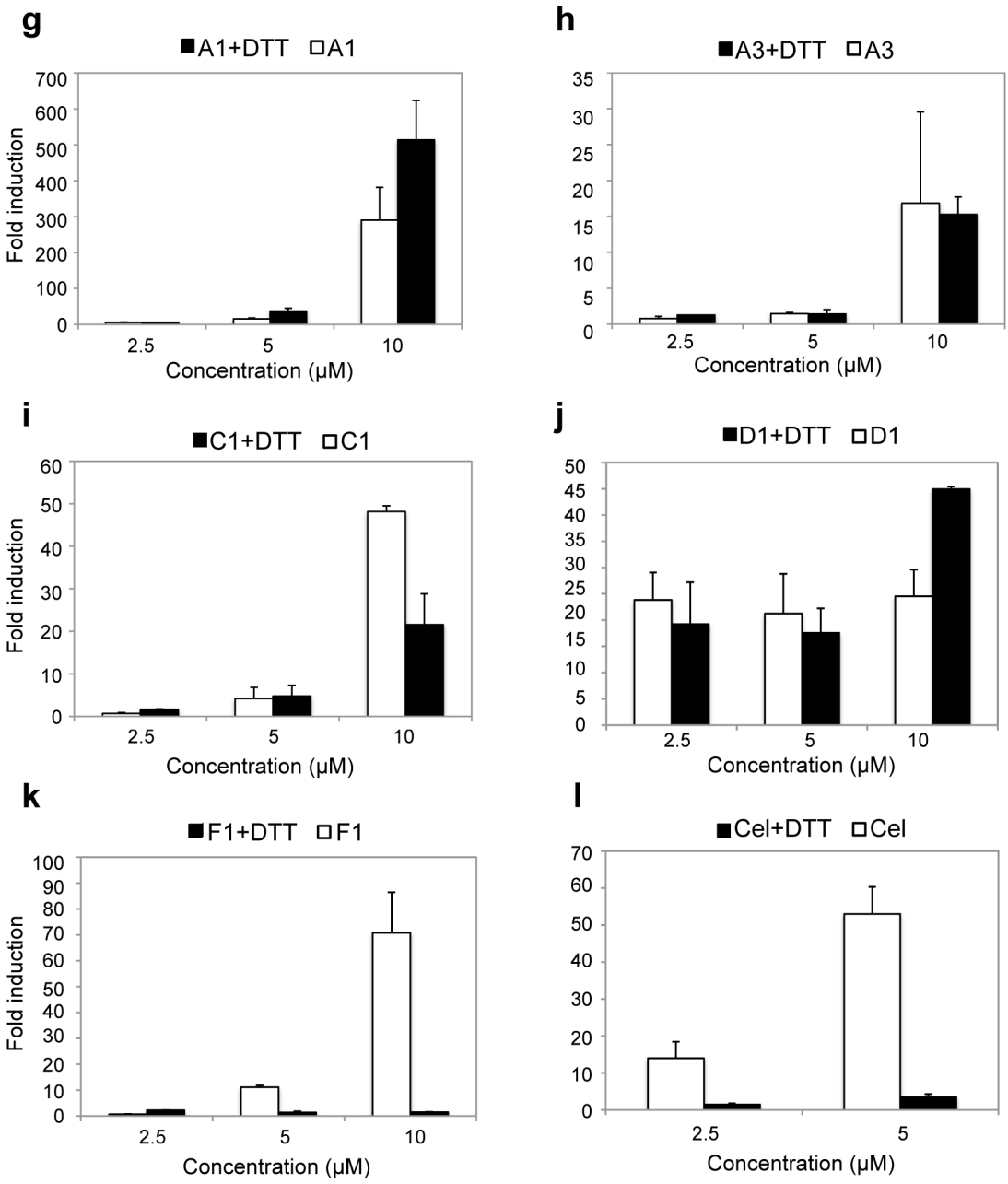
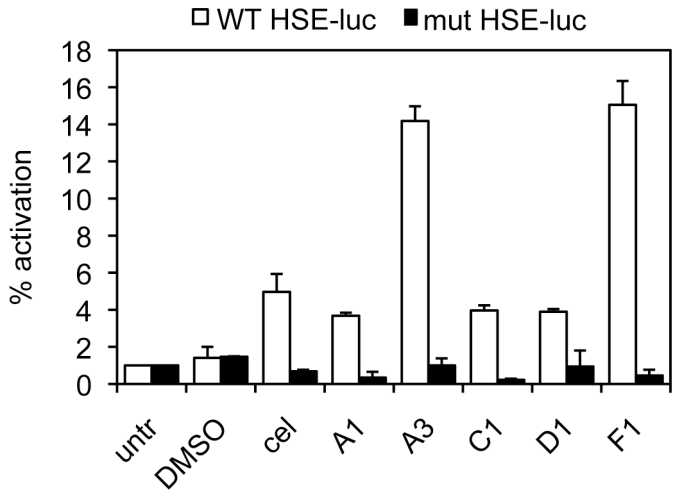
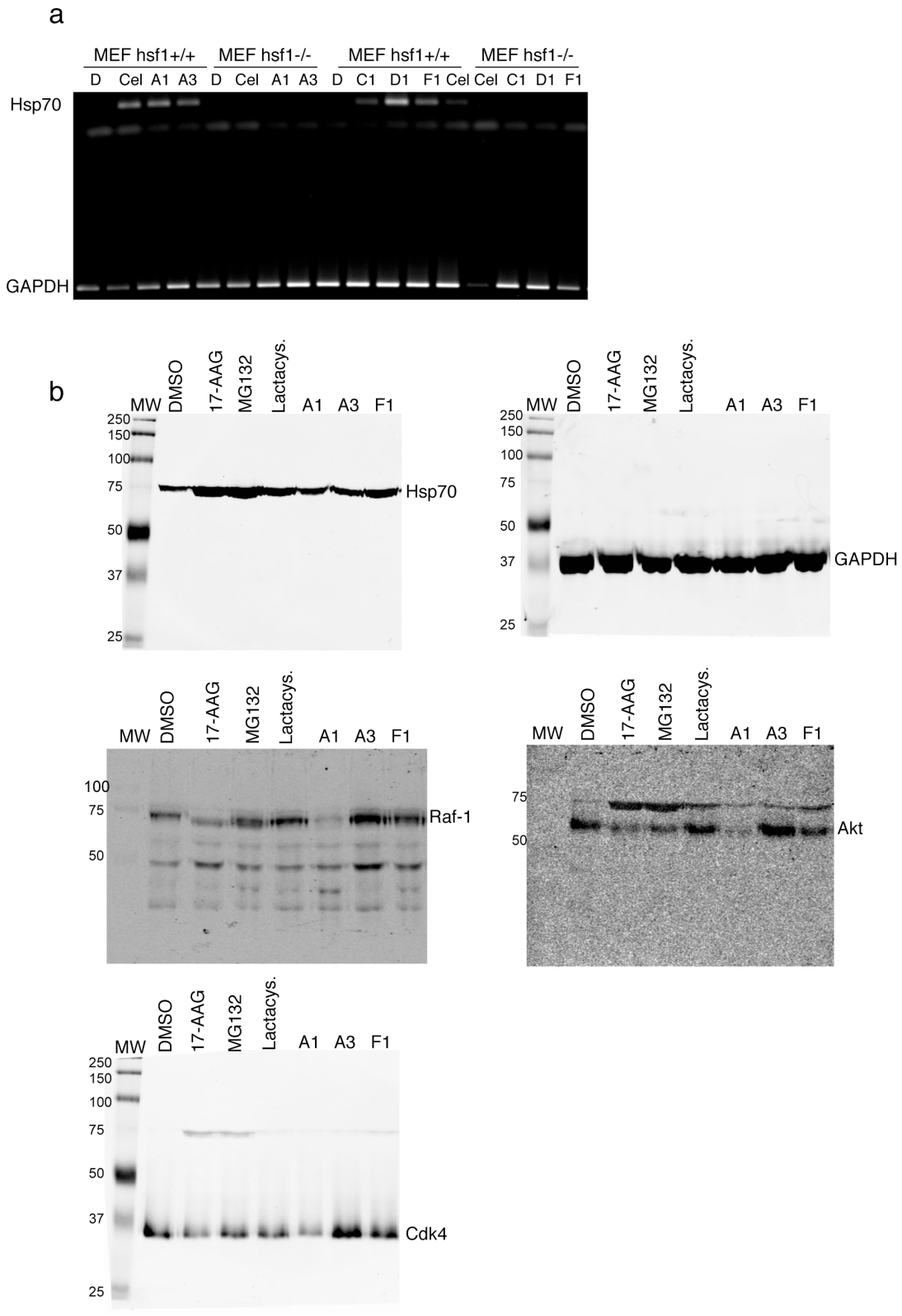
300
250
200
150
100
50
10
5
0
Cel
Cel +NAC
DMSO
DMSO + NAC



Supplementary Figure 5

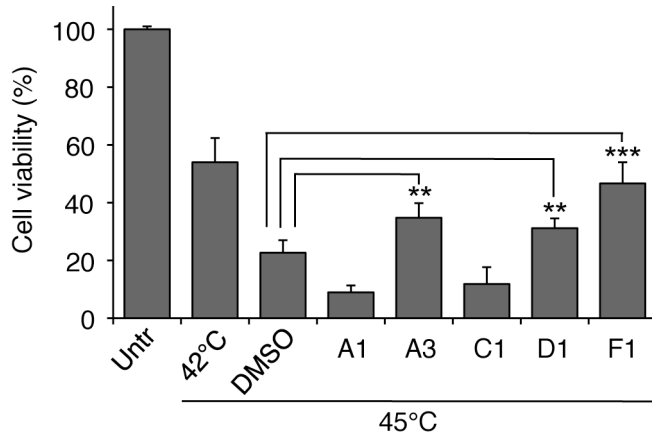


Supplementary Figure 6

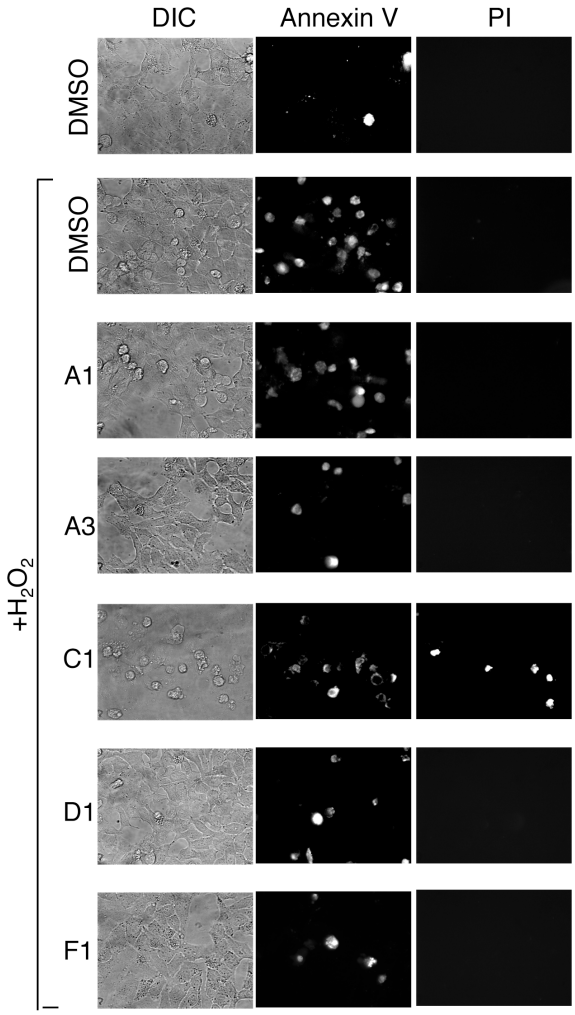


Supplementary Figure 7

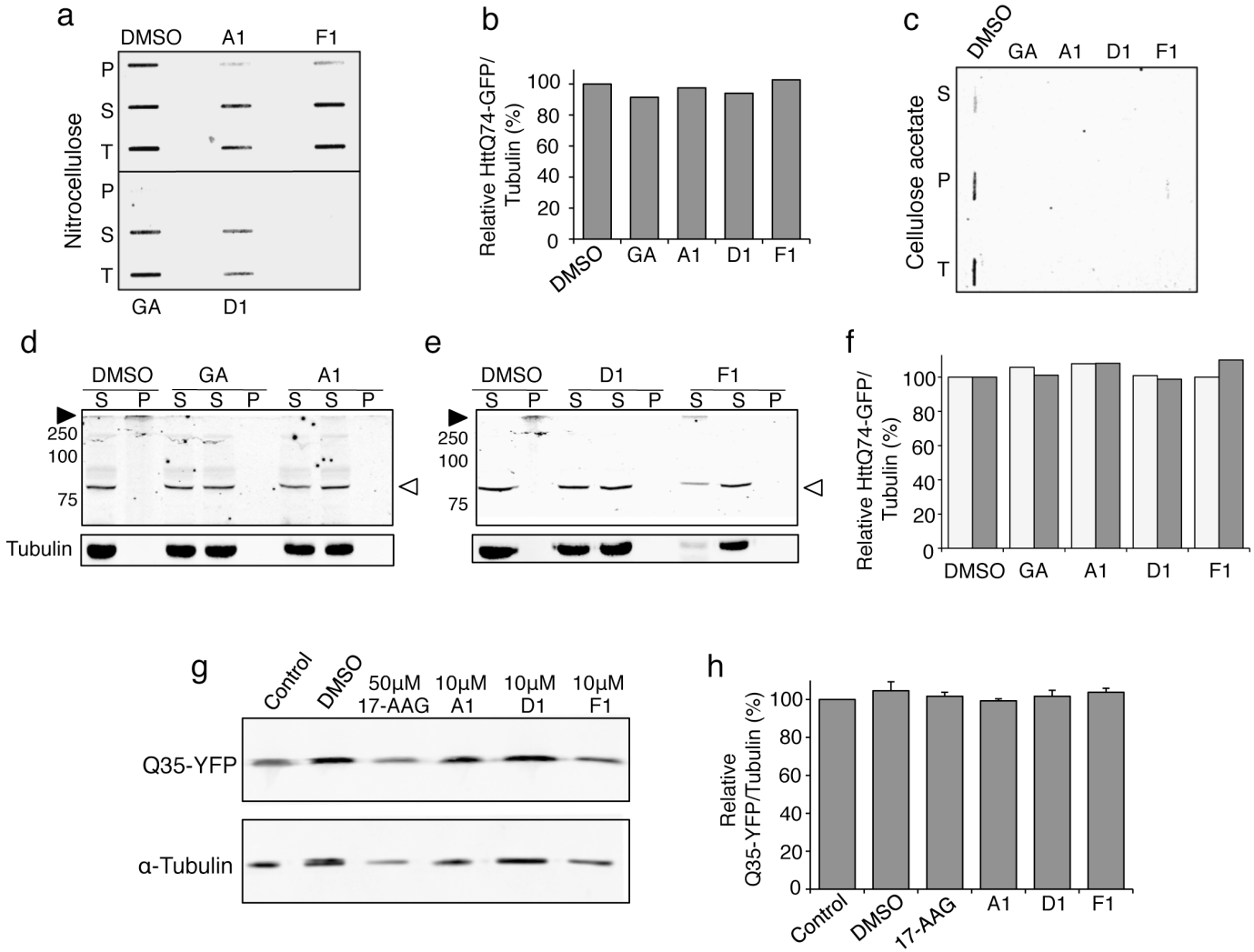
a



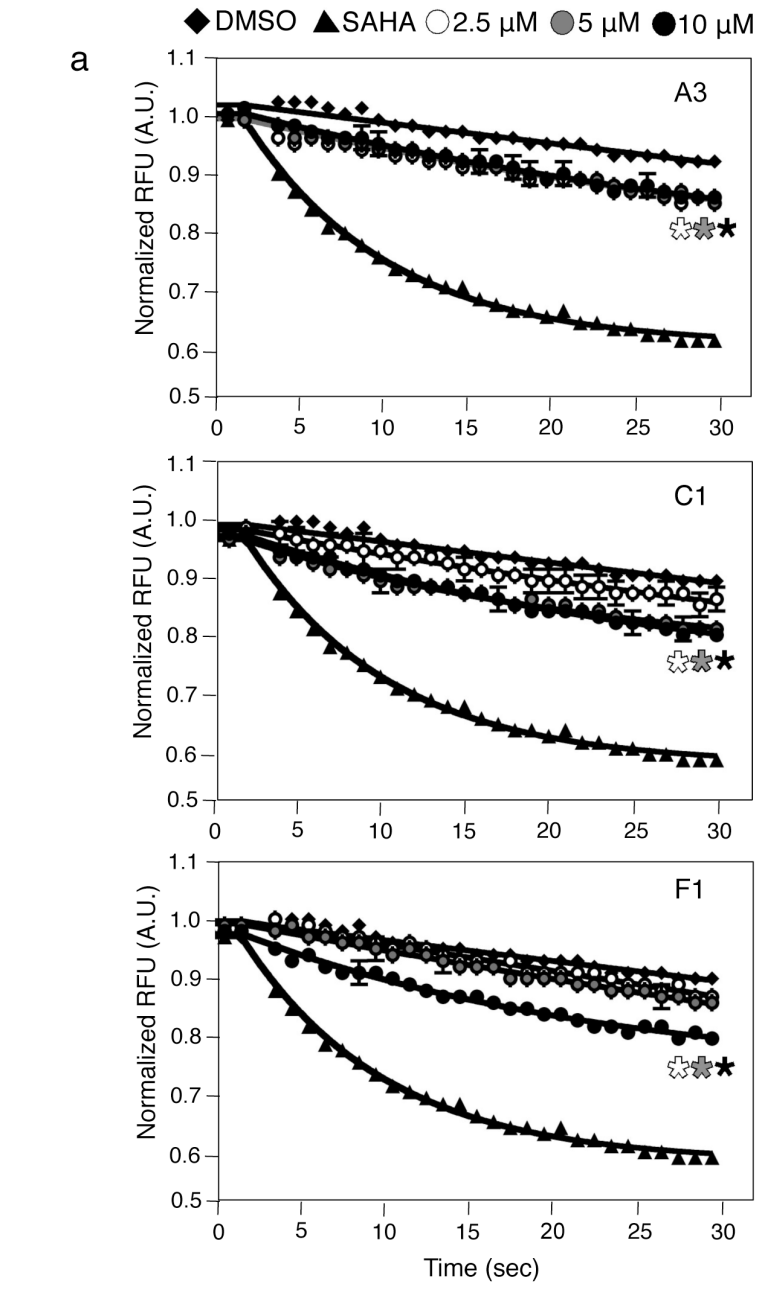
b



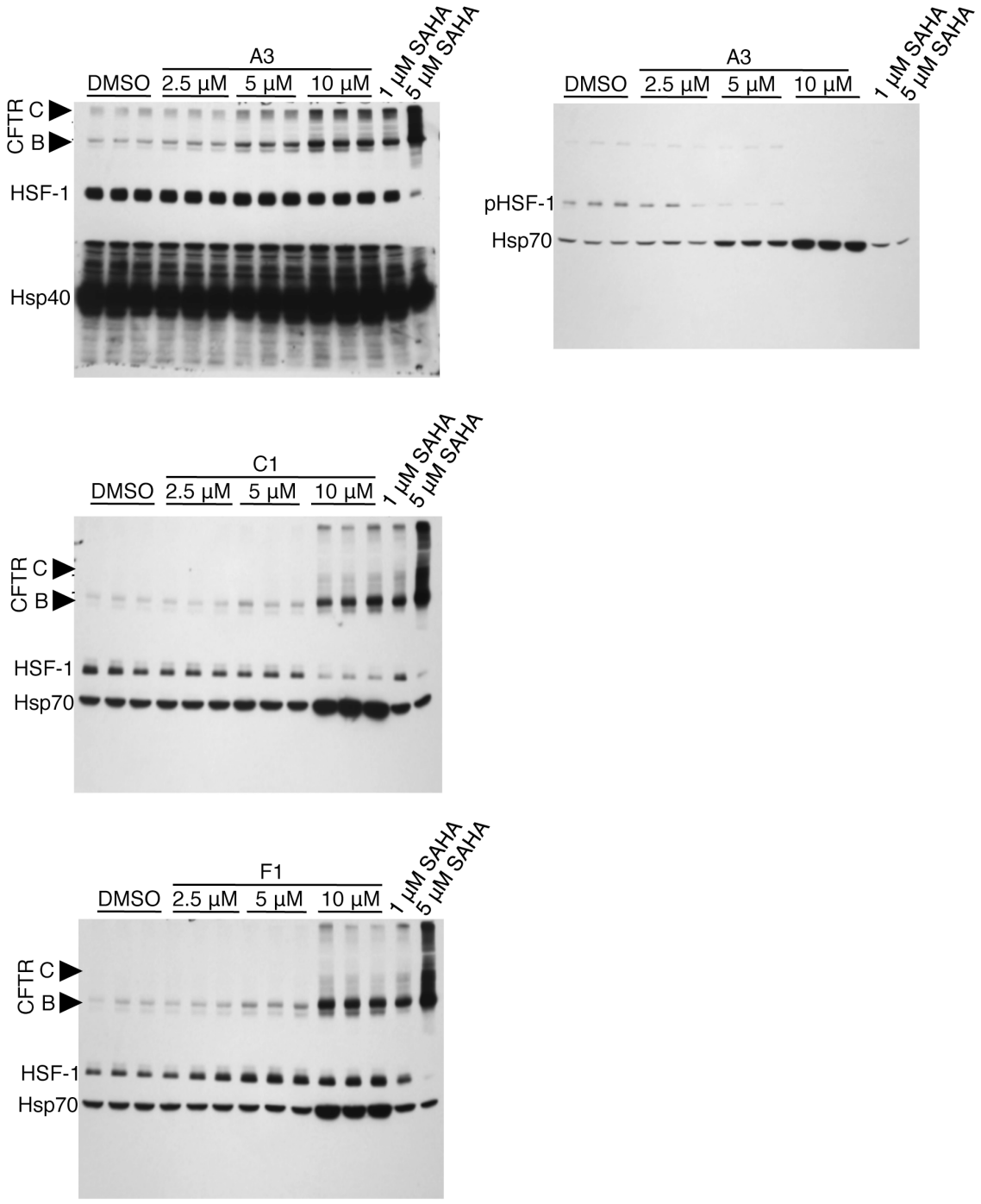
Supplementary Figure 8



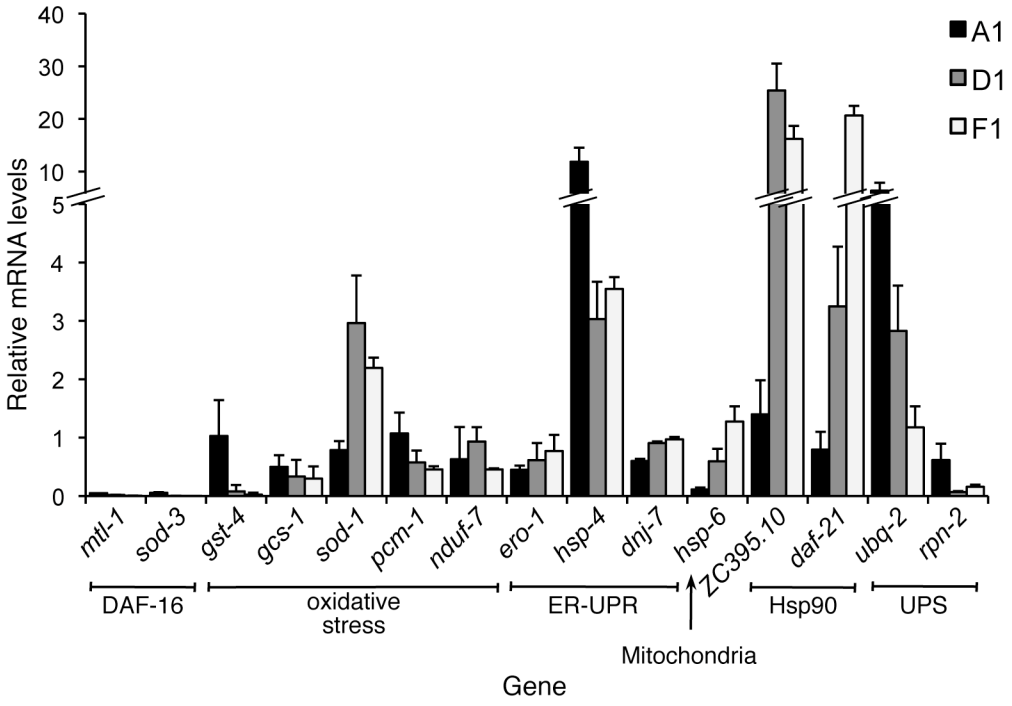
Supplementary Figure 9



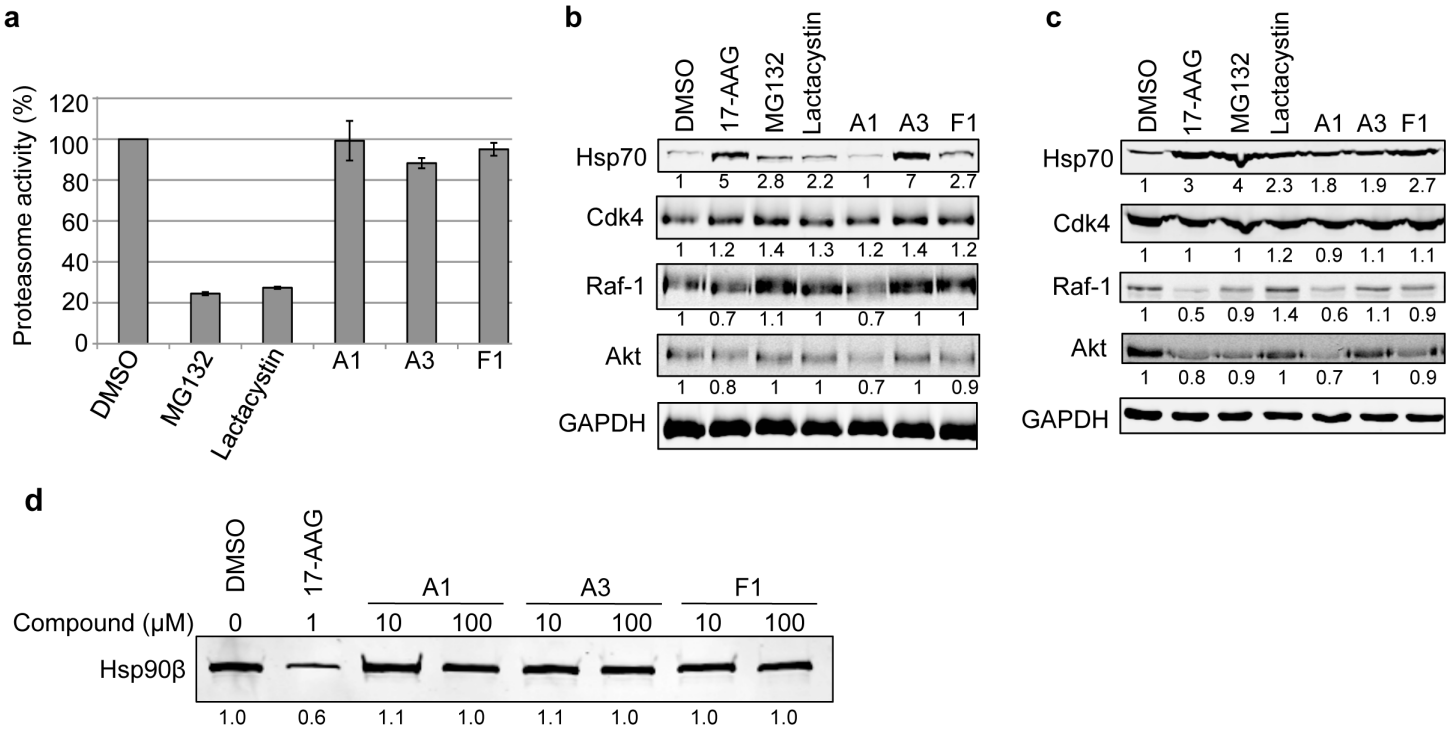
Supplementary Figure 10



Supplementary Figure 11



Supplementary Figure 12



Supplementary Figure 13

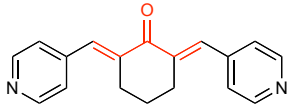
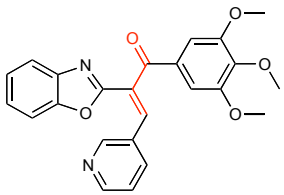
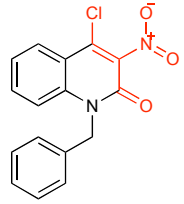
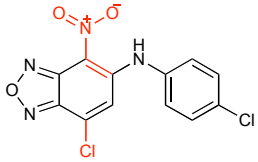
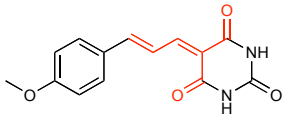
Supplementary Table 1. Assay protocol in 384 and 1,536-well plate formats.

	Order	Step	Condition	Comments
384-well plate format (Southern Res. Inst.)	1	Cell dispensing	20 µL/well	1,875 cells/well
	2	Primary incubation time	16-18 h	37°C, 5%CO_2
	3	Compound addition	50 nL/well in 5 µL medium	Test concentration: 2 µg/mL; final DMSO concentration: 0.2%
	4	Secondary incubation time	24 h	37°C, 5%CO_2
	5	Luciferase detection reagent addition	25 µL/well	
	6	Tertiary incubation	5 min	Room temperature
1,536-well plate format (Scripps)	1	Cell dispensing	5 µL/well	3,750 cells/well
	2	Primary incubation time	4 h	37°C, 5%CO_2
	3	Compound addition	50 nL/well	Test concentration: 10 µM; final DMSO concentration: 1%
	4	Secondary incubation time	16 h	37°C, 5%CO_2
	5	Luciferase detection reagent addition	5 µL/well	
	6	Tertiary incubation	15 min	Room temperature

Supplementary Table 2. Summary of the high-throughput screen results.

Screen details	Scripps Drug Discovery Library	MLPCN Library	NINDS Library
Number of compounds	607,408	196,179	100,000
Compound concentration	10 μM	10 μM	2 μg/ml
Positive control	MG132 (30 μM)	MG132 (30 μM)	$CdCl_2$ (50 μM)
Plate format	1,536 wells	1,536 wells	384 wells
Z' value	0.63	0.62	0.68
Primary hit cut-off (%)	15.86	15.86	5
Primary hit rate (%)	0.11	0.04	0.04
Number of primary hits	677	82	37
Number of confirmed hits	218	12	33

Supplementary Table 3. The PRs contain potentially reactive groups (indicated in red).

Compound structure					
Compound ID	A1	A3	C1	D1	F1

SUPPLEMENTARY METHODS

Compounds. All the compounds were obtained from the commercial vendors indicated below and were used without further purification. **A1**: (2E,6E)-2,6-Bis(4-pyridinylmethylene)-Cyclohexanone, CAS 871361-88-5, ChemDiv: C151-0032; **A2**: (3E,5E)-3,5-Bis[(2,4-dimethoxyphenyl)methylene]-1-methyl-4-Piperidinone, CAS 1207456-48-1, Enamine T0514-8318; **A3**: 2-(2-Benzoxazolyl)-3-(3-pyridinyl)-1-(3,4,5-trimethoxyphenyl)-2-Propen-1-one, CAS 496011-51-9, ChemBridge 7355596; **B1**: 6-Bromo-2-(4-ethoxy-3-methoxyphenyl)-8-methoxy-3-nitro-2H-1-Benzopyran, CAS 448189-54-6, ChemBridge, 8880788; **B2**: 1,4-Dimethoxy-2-methyl-5-(2-nitroethenyl)-Benzene, CAS 25505-64-0, ChemBridge 6561455; **B3**: 2-Ethoxy-6-iodo-4-(2-nitroethenyl)-Phenol, CAS 662160-43-2, ChemBridge 7251856; **C1**: 4-Chloro-3-nitro-1-(phenylmethyl)-2(1H)-Quinolinone, CAS 385387-83-7, ChemBridge 6935964; **C2**: 4,5-Dichloro-2-(4,6-dimethyl-2-pyrimidinyl)-3(2H)-Pyridazinone, CAS 78403-59-5, T0507-9111; **D1**: 7-Chloro-N-(4-chlorophenyl)-4-nitro-2,1,3-Benzoxadiazol-5-amine, CAS 313966-69-7, ChemBridge 5241454; **E1**: N-(4,5-Dichloro-2-benzothiazolyl)-5-nitro-2-Furan-carboxamide, CAS 797776-58-0, Asinex BAS 08207501; **F1**: 5-[3-(4-Methoxyphenyl)-2-propen-1-ylidene]-2,4,6(1H,3H,5H)-Pyrimidinetrione, CAS 100872-83-1, ChemBridge 5772104; **F2**: 5-[[5-(3-Bromophenyl)-2-furanyl]methylene]-2,4,6(1H,3H,5H)-Pyrimidinetrione, CAS 331464-02-9, Asinex BAS 00336306; **G1**: 2-Cyano-5-(2-nitrophenyl)-N-phenyl-2,4-Pentadienamide, CAS 733044-10-5, Enamine T0516-4089; **G2**: 2-Cyano-N-cyclohexyl-5-(2-nitrophenyl)-2,4-Pentadienamide, CAS 737819-53-3, Enamine T0516-8259.

Cell Cultures. Mammalian cell lines used in this study were HeLa cells, a stable HeLa cell line containing a heat shock-inducible reporter construct that consist of the

Hsp70.1 promoter sequence fused to a luciferase reporter gene (HeLa-luc)[1], WT and *hsf-1*-/- MEF cells[2], inducible PC12 cells expressing HttQ74-GFP[3] and human bronchial epithelial cells stably expressing the ΔF508-CFTR as well as the H148Q-YFP mutant[4] (CFBE41o- -YFP). HeLa and HeLa-luc cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) with phenol red buffered with HEPES and supplemented with 10% v/v fetal bovine serum (FBS), 1% L-glutamine, and 100 U/ml penicillin/streptomycin. HeLa-luc cells were also supplemented with 100 μg/ml of G418. WT and *hsf-1*-/- MEFs were maintained on DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 100 U/ml penicillin/streptomycin, and 55 μM 2-mercaptoethanol. HttQ74-GFP PC12 cells were previously described[3] and were maintained on DMEM supplemented with 5% v/v Tet-approved FBS, 10% v/v horse serum, 100 μg/ml G418, 75 μg/ml hygromycin B, and 100 U/ml penicillin/streptomycin. Cells co-expressing ΔF508-CFTR and CFBE41o- -YFP were grown in α-MEM containing 100 U/ml penicillin/streptomycin, 10% v/v FBS, 2 mM L-glutamine, 2 μg/ml puromycin and 0.75 mg/ml G418. Cells were maintained at 37°C with 5% CO_2 atmosphere until they were ready for passage or harvest.

Cell-based High-throughput Assays. The automated primary screens were performed according to the following steps. For the 100,000 compound library screened at the Southern Research Institute, the assay was optimized for a 384-well plate format and resulted in a Z'-value >0.6 using $CdCl_2$ as positive control (50 μM). HeLa-luc cells were dispensed into white tissue culture treated 384-well plates at a density of 1,875 cells/well in 20 μL assay medium using a WellMate with a small bore cassette head (Matrix Technologies Corp., Thermo Fisher Scientific, Hudson, NH). The assay plates were incubated for 16-18 hours at 37°C and 5% CO_2 before compound addition. Compound addition was performed in a two step process with the positive control added

to control wells and an equal volume of medium added to remaining wells with a Biomek FX liquid handling system (Beckman Coulter Inc., Brea, CA). The compounds were then delivered without dilution in 50 nL with 5 µL medium at a concentration of 2 µg/mL using an Echo 550 liquid handler (Labcyte Inc., Sunnyvale, CA). After 24 h incubation with compounds, the assay plates were equilibrated to room temperature before endpoint addition. 25 µl of Bright-Glo Luciferase Assay System (Promega, Madison, WI) was added to the plates using a Multidrop dispenser (Titertek Instruments Inc., Huntsville, AL) and luminescence signal was read using an Envision multi-label plate reader (Perkin Elmer, Waltham, MA). The assay was run in three 107-plate batches run in serial. Every step of the assay process, along with every reagent and instrument used throughout the assay process was tracked using a Uniflow Laboratory Information Management System to ensure consistency between screening runs and to facilitate any trouble shooting that may arise along the way. Following each screening batch, data was imported into IDBS ActivityBase data management system. Because of the low hit rate and the relatively low response elicited by the library compounds when compared to the response to $CdCl_2$, the hit criteria was defined as >5% signal induction of the positive control. Using this criterion, 37 compounds were identified as hits (0.04% hit rate). Dose-response experiments were then performed in triplicate using a 1:2 serial dilution of the hit compounds in DMSO starting from 20 µg/ml. Because of the narrow active concentration range of compounds tested in dose response (data not shown), the list of compounds to be tested in dose response was expanded to 169 based on structural relationships to the hits identified in the primary screen. The majority of the compounds screened in dose response demonstrated very narrow concentration ranges for which they were active. Therefore the criterion for confirmed activity was defined as a compound demonstrating maximum % activation >10% of $CdCl_2$. Using this criterion, 56 compounds (33%) demonstrated confirmed activity in the dose response screen. Of the

56 confirmed active compounds, 33 were hits selected from the primary screen, resulting in a hit confirmation rate of 89%.

For the 607,408 Scripps Drug Discovery unique compound library and for the 196,179 compound MLPCN library screened at the SRIMSC, the assay was optimized for a 1,536-well plate format and resulted in a Z' coefficient >0.6 using MG132 (30 μM) as positive control. A detailed description of the protocols used is available on the PubChem website (http://pubchem.ncbi.nlm.nih.gov/; PubChem AIDs 1203 and 1252).

Cytotoxicity Assays. For the hit compounds identified at the Southern Research Institute, the following procedure was used. HeLa cells were plated at a density of 10,000 cells per well in black 96-well plates in 100 μl of DMEM supplemented with 10% FBS and 1% Pen/Strep/Neo. Plates were incubated for 16 hours at 37°C, 5% CO_2 and 95% relative humidity before compound addition. 1 μL of hit compounds in DMSO or DMSO alone were added to the sample or control wells, respectively. Plates were then placed back in the incubator for 24 hours. After incubation, cells were washed 2x with 200 μl of PBS and 200 μl of a solution of 1 μg/ml of calcein AM (Invitrogen, Carlsbad, CA) was added to each well. Cells were then incubated for 45 min at 37°C, 5% CO_2 before fluorescence measurement using an Analyst GT multimode reader (Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was expressed relative to wells containing cells treated with DMSO only (100%).

A detailed description of the protocol used at the SRIMSC is available at the PubChem website (http://pubchem.ncbi.nlm.nih.gov/; PubChem AIDs 1259).

Gel Mobility Shift Analysis (EMSA). Electrophoretic mobility shift analysis was performed using a ^{32}P-labeled probe containing the proximal heat shock element from the human Hsp70.1 gene promoter. The whole cell extract-labeled probe mixtures were

incubated room temperature for 20 minutes and analyzed by native electrophoresis on 4% polyacrylamide gels. The intensities of the shifted bands were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For competition experiments, a 200-fold molar excess of the same unlabeled double stranded oligonucleotide was used. Supershifts were performed by incubating 1 µl of polyclonal antibodies specific for HSF-1[5] with the whole cell extracts for 20 minutes at room temperature prior to the HSF-1-heat shock element binding reaction.

Chromatin Immunoprecipitation Assays (ChIP). Nuclear cell extracts obtained from about 3×10^7 HeLa cells were used for each tested condition, cross-linked for 10 minutes at room temperature with a 37% formaldehyde solution and sonicated for 30 sec at 4°C. Samples were immunoprecipitated with 10 µl of a rabbit polyclonal HSF-1 #47[1] at 4°C overnight. Primers used for the human Hsp70.1, Hsp40, Hsp27 and dihydrofolate reductase promoters were the following:
(forward) 5′-GGCGAAACCCCTGGAATATTCCCGA-3′ and (reverse) 5′-AGCCTTGGGACAACGGGAG-3’; (forward) 5′-GTGGTACCCTCCTCCGACCTGTG-3’ and (reverse) 5’-TACTCGAGACCCCCTCCTGCG-3’; (forward) 5′-CTATCTCACACGCGTGTGGTTCC-3′ and (reverse) 5′-TTAAGGAGGACAGAGCCAGACAG-3’**.** Primers used for the human dihydrofolate reductase promoter were: 5′-GGCCTCGCCTGCACAAATAGGG-3′ (forward) and 5′-GGGCAGAAATCAGCAACGGGC-3′ (reverse).

Multiplex Gene Expression Analysis. WT and *hsf-1-/-* MEFs were seeded at a density of 12,000 cells/well in 96-well plates with an overnight incubation at 37°C, 5% CO_2. Cells were treated with serially diluted compounds in a 7-point dose dependent manner. Cell lysis with 50% [v/v] Panomics Lysis Mixture (Lysis Mixture + 10 µl/ml

Proteinase K) was performed 6 hours post-compound treatment. Lysed cells were heated at 50°C to ensure appropriate lysing and the plates were then frozen at -80°C. Cell lysates, thawed at room temperature on the day of the assay, were pooled with mouse 8-gene multiplex probe sets and with 8 different sets of magnetic capture beads (Luminex Technology, Austin, TX) in a 100 µl/well volume. Biomek FX was used at every liquid transfer step to reduce variability by human error. The eight plates containing lysate-probe-bead mixtures were incubated at 54°C ± 1°C on a shaking platform for an overnight incubation in the dark (18-20 hours). The following day the hybridization plates were compressed by transferring the hybridized lysates into a single magnetic capture plate. The plate was kept on a magnet to hold the beads and then washed with Panomics Wash Buffer 2.0 on a BioTek ELx405 select plate washer to remove any unbound sample. This step was followed by serial hybridizations and washings of the bDNA pre-amplifier (1 hour, 50°C), bDNA amplifier (1 hour, 50°C), label probe (1 hour, 50°C), and streptavidin-phycoerythrin (SAPE, 30 minutes, room temperature)[6]. The plate was then washed with SAPE wash buffer to remove unbound SAPE and each well was analyzed with the Luminex FlexMap3D (Luminex, Austin, TX). SAPE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads[7]. Fold changes in gene expression were obtained for each gene per well by normalizing the raw data first to the DMSO control and then to a housekeeping gene (TBP).

HeLa cell transfection with wild type and mutant HSE-luc. HeLa cells were plated in a white 96-well plate and transfected with a wild type and mutant HSE-luc plasmid using FuGENE HD transfection reagent (Roche, Indianapolis, IN). Cells were treated with PRs A1, A3, C1, D1, and F1 (10 µM) and then luciferase activity was measured 24 h later.

Cytoprotection and Apoptosis Analysis. HeLa cells were plated in black 96-well plates at a density of 10,000 cells/well. The cells were treated with DMSO or the PRs A1, A3, C1, D1 and F1 (10 μM) for 1 h and then recovered for 8 h before a 45°C heat shock treatment for 35 min. As a control, the cells were pretreated with a 42°C heat shock for 1 h. Cell death was assessed 24 h later by using the Calcein AM dye. Assessment of apoptotic and necrotic events was performed using the Annexin V-FLUOS/PI Staining Kit (Roche, Indianapolis, IN) accordingly to the manufacturer's protocol. In brief, cells were washed twice with ice-cold PBS, resuspended in binding buffer, and incubated for 15 min in the dark at room temperature with Annexin V-fluorescein and PI labeling solution. Fluorescence images were acquired using an Axiovert S100 TV fluorescence microscope (Carl Zeiss, Germany) equipped with a 40x objective and a CoolSNAP HQ camera system (Photometrics, Tucson, AZ). All image processing and analysis was performed using functions in the MetaMorph software (Molecular Devices, Inc., Sunnyvale, CA). For quantification of Annexin V-stained cells, approximately 200 cells were counted for each treatment.

Filter Trap Assay and Immunoblotting. The filter trap assay was performed accordingly to Wanker et al.[8]. In brief, cells were lysed on ice for 30 min in filter trap lysis buffer [50 mM Tris-HCl, pH 8.8; 100 mM NaCl; 5 mM $MgCl_2$; 0.5% (v/v) Nonidet P-40; and 1 mM EDTA] in presence of protease inhibitors. Insoluble material was pelleted by centrifugation and resuspended in 100 μl of 20 mM Tris-HCl, 15 mM $MgCl_2$, 0.5 mg/ml DNase I for 2 h at 37°C. Protein concentration of total, soluble and insoluble fractions was determined by the protein *dot*Metric assay (G-Biosciences, St. Louis, MO). 20 μg of soluble and insoluble material was diluted into 2% SDS, boiled and added to a 48-well blot apparatus (Bio-Rad Laboratories, Hercules, CA) containing either a nitrocellulose or

cellulose acetate membrane. For western blotting, protein samples were electrophoresed by SDS-PAGE and transferred to PVDF membranes using a semi-dry apparatus. Membranes were then blocked in 5% milk for 1 h and then probed with the following primary antibodies: a goat monoclonal anti-GFP IR dye 800 (Rockland Immunochemicals, Gilbertsville, PA) at 1:4,000 and a mouse monoclonal anti-tubulin at 1:5,000 (Sigma, St. Louis, MO). The secondary antibody was an Alexa Fluor 680 goat anti mouse IgG diluted 1:5,000 (Invitrogen, Carlsbad, CA). Western analysis was performed with the Odyssey system (Li-COR, Lincoln, NE).

ΔF508-CFTR YFP Quenching Assay. Human bronchial epithelial cells (CFBE41o-) stably expressing ΔF508-CFTR as well as H148Q/I152L-YFP (CFBE41o- -YFP) were added to a 96-well black walled plate and grown to confluency in growth media (α-MEM containing 100 U/ml penicillin, 100 U/ml streptomycin, 10% v/v FBS, 2mM L-glutamine, 2 μg/ml puromycin and 0.75 μg/ml G418). Cells were treated with the indicated concentration of compounds in complete growth media and incubated at 37^{o}C, 5% CO_2 for 24 h. Cells were subsequently washed three times with 200 μl of PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 0.7 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and equilibrated in 40 μl of PBS pH 7.4 and maintained at 37^{o}C throughout. Cells were stimulated with a final concentration of 10 μM forskolin (fsk) and 50 μM genistein (gen) for 15 min prior to addition of PBS + NaI (replacement of NaCl with 137 mM NaI). Fluorescence was monitored every second for a total of 30 seconds (3 seconds prior to addition of NaI and 27 seconds after addition of NaI). Data were normalized to the initial fluorescence to account for variations in the overall starting fluorescence. To ensure that the observed H148Q/I152L-YFP fluorescence quenching was the result of ΔF508-CFTR activation and not the action of additional halide channels, the CFTR specific inhibitor (CFInh-172) was used.

ΔF508-CFTR Transport Assay. CFBE41o- cells stably expressing ΔF508-CFTR were plated in 12-well dishes and grown to confluency in growth media (α-MEM containing 100 U/ml penicillin, 100 U/ml streptomycin, 10% v/v FBS, 2 mM L-glutamine and 2 μg/ml puromycin). Cells were treated with the indicated concentration of compound in complete growth media and maintained at 37^{o}C, 5% CO_2 for 24 hrs. Cells were lysed on ice in 50 μl of TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) + 1% (v/v) Triton X-100 containing 2 mg/ml of complete protease inhibitor cocktail (Roche, Switzerland) for 30 min. The lysates were harvested and cleared at 20,000xg for 20 min (4^{o}C) and the supernatant was collected for analysis. Equal amount of total protein (15 μg) was separated by SDS-PAGE (8% gel) and transferred to nitrocellulose. The blot was probed overnight at 4^{o}C for CFTR (3G11 rat monoclonal antibody at 1:500 dilution) and indicated chaperone proteins.

***C. elegans* Strains**. Worms were maintained according to standard methods, at 20°C on nematode growth media (NGM) with OP50 *E. coli* (Brenner 1974). The following strains were obtained from the *C. elegans* Genetic Center (CGC): wild-type (wt) Bristol strain N2, HSF-1 mutant *hsf-1(sy441)* (PS3551), temperature sensitive strains *unc-52(e669su250)* and *unc-45(e286)* (HE250 and CB286, respectively). The polyglutamine strain expressing 35 CAG-repeats fused with YFP (Q35::YFP) was described elsewhere (AM140 in CGC)[9].

***C. elegans* Assays for Aggregation and Motility Defects**. The treatment with chemical compounds was performed in a 96-well plate format, in liquid culture[10]. Each well contained a final volume of 60 μL, comprising 20 to 25 L2 (larval 2 stage) age-synchronized animals, compound at the appropriate concentration, and OP50 bacteria to

a final OD_{595nm} of 0.8 in the microtiter plate. Animals and bacteria were resuspended in S-medium supplemented with streptomycin, penicillin, and nystatin (Sigma, St. Louis, MO). To obtain the age synchronized population of L2 larvae, gravid adults were bleached with a NaOCl solution [250 mM NaOH and 1:4 (v/v) dilution of commercial bleach] and the eggs were allowed to hatch in M9 buffer overnight at 20°C. The first larval stage (L1) animals were transferred to OP50 plates to allow them to develop into L2 stage (20°C). The animals were then washed with M9 buffer, resuspended in S-medium to the appropriate concentration, and transferred into the 96-well plates. The compounds were dissolved and diluted in 100% DMSO, and the animals were incubated at a maximum concentration of 1.5% DMSO to avoid solvent-specific developmental defects and toxicity. The range of concentrations tested was 0, 1, 5, 10 and 15 μM (final concentration in the well). OP50-only and DMSO-only controls were used. In addition, the compound 17-(allylamino)-17-demethoxygeldanamycin (17-AAG, Biomol, Plymouth Meeting, PA) (at the concentrations 0.5, 1, 5 and 50 μM) was utilized as positive control for induction of the HSR. The plates were incubated at 20°C for 4 days. The animals were scored for changes in aggregation (number of fluorescent foci) using the stereomicroscope Leica MZ16FA equipped for epifluorescence (Leica Microsystems, Switzerland). Suppression of aggregation was scored positive when ≥50% of the worms had a reduction in fluorescent foci number, without loss of body-wall fluorescence, compared to the DMSO control (experiments done at least in triplicate). Other phenotypes such as thrashing in liquid, body size and progeny number were also taken into account, as indicative of animal health. Simultaneous treatment with RNA interference (RNAi) and compounds was also performed in liquid culture (commercial RNAi library GeneServiceTM, USA). RNAi for *hsf-1* or the empty-vector (EV) control L4440 bacterial cultures were grown over-night in LB-ampicillin 50 μg/ml at 37°C, and induced with 0.7 mM isopropyl β-D-thiogalatoside (IPTG) for 2.5 hours at 37°C. Bacteria

was then pelleted and resuspended in supplemented S-medium to a final OD_{595nm} of 0.8 in the microtiter plate. Assay plates were setup as before, with L2 age synchronized animals and 4 days incubation at 20°C. Fluorescent microscopy images were taken using an Axiovert 200 microscope with a Hamamatsu digital camera C4742-98 (Carl Zeiss, Germany).

For the motility assay, animals were incubated in liquid culture with the chemical compounds as described above. Six days old animals were transferred from the liquid culture onto a NGM plate seeded with OP50 bacteria (equilibrated at 20°C) and allowed to acclimate for 1 h. At this point the animals' movement was digitally recorded using a Leica M205 FA microscope with a Hamamatsu digital camera C10600-10B (Orca-R2, Leica Microsystems, Switzerland), and the Hamamatsu Simple PCI Imaging software. Movies of 45 sec were captured at 5 frames/s, for each condition, for a minimum of 50 animals, and the experiment was done at least in triplicate (n≥150). Captured frames were merged into **.avi* format and were imported directly into ImageJ[11]. Using the LOCI bio-formats plugin and a custom stack deflicker plugin (http://www.loci.wisc.edu/bio-formats/imagej), light average intensity was normalized for each frame. To enhance the definition of the animals in the movies, the difference between each frame and the constant background was calculated, using the Maximum Z stack projection. The resulting movie was converted to binary format using Otsu Thresholding 2. Binary objects representing the animals were tracked using custom ImageJ plugin, wrMTrck (based on "MTrack2" by Nico Stuurman)[12]. The average speed of each animal was calculated by dividing its body length by the duration of each track (body length per second, or BLPS). Motility measurements are given as a percentage of the wild type control strain motility, N2. Statistically significant changes in motility were obtained with ***p*-value < 0.01 and ****p*-value<0.001 (Student's T-test). The wrMTrck plugin and scripts for automated analysis are open-source and publicly available at

http://www.phage.dk/plugins.

Semi-quantitative RT-PCR (qPCR). The small molecule treatment, with or without RNAi, was performed at least in triplicate for either N2(wt), Q35 or *hsf-1(sy441)* animals. Animals were collected from the liquid cultures (96-well plates) into eppendorf tubes, washed with M9 buffer, pelleted at 3,000 xg (Eppendorf centrifuge 5424, Eppendorf) and re-suspended in Trizol (Invitrogen, Carlsbad, CA). Samples were homogenized by vortexing and incubated on ice for 10 min. 50µl of chloroform were added to each sample, followed by vortexing and centrifugation at 13,500g for 15 min (4°C). A volume of 150µl of 2-propanol was added to each aqueous layer (10 min incubation) and total RNA was spun-down at 18,000g (4°C) for 10 min. The pellets were washed with 75% (v/v) ethanol, air-dried and re-suspended in nuclease free water at 60°C for 10 min. RNA aliquots of 10µg were used for DNase treatment (Applied Biosystems, Carlsbad, CA). 1µg of purified RNA was used for cDNA synthesis (Bio-Rad, Hercules, CA). cDNA samples were diluted to a final volume of 500µl in water and 1.5 µl (~10 ng) were used for real-time PCR amplification. PCR measurements were performed for HSP-70 (C12C8.1, F44E5.4 and C30C11.4), small HSPs (*hsp-16.1* and *hsp-16.49*) mRNA, ER HSP-70 (*hsp-4*), oxidative stress *sod-1*, HSP-90 chaperone and co-chaperone (*daf-21* and ZC395.10) and ubiquitin protein (*ubq-2*) with the following primers:

C12C8.1.fw 5'-ACTCATGTGTCGGTATTTATCA-3';

C12C8.1.rev 5'-ACGGGCTTTCCTTGTTTT-3';

C30C11.4.fw 5'-GCTTCGTACTATTGTGGAATCTC-3';

C30C11.4.rev 5'-GGAGATCTTGCTTGTAGATTCC-3';

F44E5.4.fw 5'-GTCTTCATGCAAAGCTATTGGTATC-3';

F44E5.4.rev 5'-CGTCGTCCAATCAATCCTTTTGCATC-3';

hsp-16.1.fw 5'-ACTTTACCACTATTTCCGTCCAGC-3';

hsp-16.1.rev 5’-GATAATGTATGTCCATCCAAATTA-3’;

hsp-16.49 fw 5’-CCATATTGGAGAAATGCTGATCAC-3’;

hsp-16.49 rev 5’-CTTCTGGTAGAAGAATCATTTTTG-3’;

hsp-4.fw 5’-GCAGATGATCAAGCCCAAAAAG-3’

hsp-4.rev 5’-GGAGACGATTGGTTGAACAACAG-3’;

sod-1.fw 5’-GGACTTACTCCCGGTCTTCATG-3’;

sod-1.rev 5’-GTAAAGCGTGACGAGCGTGTCG-3’;

daf-21.fw 5’-CTTGACAAGATTCGCTACCAG-3’;

daf-21.rev 5’-GCTTGAAGAGCCTCCATGAAGG-3’;

ZC395.10.fw 5’-CTTGTCTATCTGACCATCGAGG-3’;

ZC395.10.rev 5’-GGGTCTTCTTCTGAACTGTGATC-3’;

ubq-2.fw 5’-GAATCCCACCAGATCAGCAAAG-3’;

ubq-2.rev 5’-GTCGCAGTTGTACTTCTGGG-3’;

mtl-1 fw 5’-GGCTTGCAAGTGTGACTGCAAAAACAAGC-3’

mtl-1 rev 5’-TTAATGAGCCGCAGCAGTTCCCTGGTGTTGATGG-3’

sod-3 fw 5’-gcttcaaagcttgttcaaccggttgcg-3’

sod-3 rev 5’-CAGCGCTGGTTGGAGAGCAATTGC-3’

gcs-1 fw 5’-gtgcaagtgtcgacgatcgtac-3’

gcs-1 rev 5’-GCGAATATGTTTTGCCAGTGGCTC-3’

gst-4 fw 5’-cgactccatttggccagc-3’

gst-4 rev 5’-GATCAGCGTCACTTCTAG-3’

pcm-1 fw 5’-cggtttctgcacctcacatg-3’

pcm-1 rev 5’-cacgttccaattgttcactg-3’

nduf-7 fw 5’-GAG GTT CAA TTT GGC CAC TGA C-3’

nduf-7 rev 5’-CAT ATA TGC GGC GAA GTG CAG-3

dnj-7 fw 5’-gagcacggatagtacggac-3’

dnj-7 rev 5'-GAGGCAAGCCATCCAGTC-3'

ero-1 fw 5'-cgacgaaatgcagtgaatatg-3'

ero-1 rev 5'-gtgtagcgttccggattcttcg-3'

hsp-6 fw 5'-caggccgttaccaactctgc-3'

hsp-6 rev 5'-GCAGTTTCCTTCATCTTCATC-3'

rpn-2 fw 5'-GCC TGC AGA TTT GAC CAT CAA G-3'

rpn-2 rev 5'-CTC GTA ATC GTG TCA GCA GC-3'

As an internal control, we amplified mRNA of act-1 (actin) with the primers:

act-1.fw 5'-ATCACCGCTCTTGCCCCATC-3';

act-1.rev 5'-GGCCGGACTCGTCGTATTCTTG.

Assay for TS Phenotypes. The temperature sensitive mutant (TS) animals were age-synchronized to L1 stage, and grown in liquid media with or without compounds, as described before. Plates were either incubated at the permissive temperature 15°C, or incubated at 15°C until animals reached L4 stage and then transferred to the restrictive temperature, 25°C. TS phenotypes were scored 2 days post-transfer to 25°C [13,14]. For abnormal body shape (or stiff paralysis) in the *unc-52(e669su250)*, partially paralyzed animals with moving heads and stick-like bodies were scored. For the egg laying defect in the *unc-45(e286)* animals were scored for accumulation of eggs in the gonad and enlargement of the animal belly. For control conditions, L1 animals were grown on OP50 bacteria and DMSO at either 15°C or 25°C and scored 2 days later for the TS phenotype. Experiment was performed in triplicate.

Proteasome Inhibition Assay. The assay was essentially performed as described by Kisselev et al.[15]. In brief, HeLa cells were incubated with either DMSO (negative control), the positive controls MG132 (10 μM) and lactacystin (6 μM) or the PRs A1, A3

and F1 for 3 and 6 hours and then harvested. Cells were lysed in homogenization buffer (50 mM Tris-HCl, pH7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA, 0.025% digitonin) for 5 min on ice, and total protein concentration of whole cell extract was determined by Bradford assay. 3 µg of whole cell extracts were combined with assay buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 0.05 mg/ml BSA) in a black 96-well plate and the reaction was initiated by the addition of a 2x (200 µM) fluorogenic peptide substrate Suc-LLVY-AMC (EMD Chemicals Inc., Gibbstown, NJ). Fluorescence was measured every 10 min using a Synergy H4 multi-mode microplate reader (BioTek, Winooski, VT).

Hsp90 Client Protein Degradation. Analysis of Hsp90 client protein degradation was carried out using HeLa cells that were treated with the indicated compounds for 6, 16 and 24 h. The same procedure described for the Western Blot analysis was followed. The following primary antibodies were used: a mouse monoclonal Cdk-4 (DCS-31, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a rabbit polyclonal Raf-1 (C-12, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a rabbit polyclonal Akt (Cell Signaling Technology, Inc., Danvers, MA) and a mouse monoclonal GAPDH (AbCam, Cambridge, MA). The primary antibodies were used at the following dilutions: Cdk-4, 1:200; Raf-1, 1:500; Akt, 1:1,000; and GAPDH, 1:5,000.

Luciferase Renaturation Assay in Rabbit Reticulocyte Lysate. The luciferase renaturation assay was performed essentially as described previously[16,17]. Diluted RRL was pre-incubated with either DMSO (0.2%), 17-AAG (2 µM) or PR A1 (10 µM) for 30 minutes at RT before addition of denatured luciferase.

Geldanamycin Competition Assay. Human recombinant Hsp90β (2 μg) in Hsp90 binding buffer (10 mM Tris, 50 mM KCl, 5 mM $MgCl_2$, 20 mM $NaMoO_4$, 0.01% NP40) was preincubated with either DMSO, 17-AAG (1 μM) or PRs A1, A3 and F1 (10 and 100 μM) for 30 min at 4°C before the addition of 1 μM geldanamycin-biotin (Enzo Life Sciences, Farmingdale, NY). Bound Hsp90 was then captured with neutravidin-agarose beads for 30 min at 4°C. The beads were washed with Hsp90 binding buffer, and Hsp90 was eluted from the beads by incubating in Laemmli sample buffer at 95°C for 5 min.

REFERENCES CITED

1. Westerheide, S.D. et al. Celastrols as inducers of the heat shock response and cytoprotection. *J Biol Chem* **279**, 56053-60 (2004).
2. McMillan, D.R., Xiao, X., Shao, L., Graves, K. & Benjamin, I.J. Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J Biol Chem* **273**, 7523-8 (1998).
3. Wyttenbach, A. et al. Polyglutamine expansions cause decreased CRE-mediated transcription and early gene expression changes prior to cell death in an inducible cell model of Huntington's disease. *Hum Mol Genet* **10**, 1829-45 (2001).
4. Galietta, L.J., Haggie, P.M. & Verkman, A.S. Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. *FEBS Lett* **499**, 220-4 (2001).
5. Sarge, K.D., Murphy, S.P. & Morimoto, R.I. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Mol Cell Biol* **13**, 1392-407 (1993).
6. Zhang, A. et al. Small interfering RNA and gene expression analysis using a multiplex branched DNA assay without RNA purification. *J Biomol Screen* **10**, 549-56 (2005).
7. Zheng, Z., Luo, Y. & McMaster, G.K. Sensitive and quantitative measurement of gene expression directly from a small amount of whole blood. *Clin Chem* **52**, 1294-302 (2006).
8. Wanker, E.E. et al. Membrane filter assay for detection of amyloid-like polyglutamine-containing protein aggregates. *Methods Enzymol* **309**, 375-86 (1999).
9. Morley, J.F., Brignull, H.R., Weyers, J.J. & Morimoto, R.I. The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **99**, 10417-22 (2002).
10. Voisine, C. et al. Identification of potential therapeutic drugs for huntington's disease using Caenorhabditis elegans. *PLoS One* **2**, e504 (2007).
11. Collins, T.J. ImageJ for microscopy. *Biotechniques* **43**, 25-30 (2007).
12. Klopfenstein, D.R. & Vale, R.D. The lipid binding pleckstrin homology domain in UNC-104 kinesin is necessary for synaptic vesicle transport in Caenorhabditis elegans. *Mol Biol Cell* **15**, 3729-39 (2004).
13. Gidalevitz, T., Ben-Zvi, A., Ho, K.H., Brignull, H.R. & Morimoto, R.I. Progressive disruption of cellular protein folding in models of polyglutamine diseases. *Science* **311**, 1471-4 (2006).
14. Garcia, S.M., Casanueva, M.O., Silva, M.C., Amaral, M.D. & Morimoto, R.I. Neuronal signaling modulates protein homeostasis in Caenorhabditis elegans post-synaptic muscle cells. *Genes Dev* **21**, 3006-16 (2007).
15. Kisselev, A.F. & Goldberg, A.L. Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates. *Methods Enzymol* **398**, 364-78 (2005).
16. Nimmesgern, E. & Hartl, F.U. ATP-dependent protein refolding activity in reticulocyte lysate. Evidence for the participation of different chaperone components. *FEBS Lett* **331**, 25-30 (1993).
17. Thulasiraman, V. & Matts, R.L. Luciferase renaturation assays of chaperones and chaperone antagonists. *Methods Mol Biol* **102**, 129-41 (1998).

18. Rowlands, M.G. et al. High-throughput screening assay for inhibitors of heat-shock protein 90 ATPase activity. *Anal Biochem* **327**, 176-83 (2004).

SUPPLEMENTAL FIGURE LEGENDS

Supplementary Scheme 1. Model Depicting the Proposed Mechanism by which the Small Molecules PRs Ameliorate Conformational Diseases. The small molecules PRs work by activating some of the major PN machineries therefore inducing components of the quality control machinery and other cytoprotective stress response genes.

Supplementary Figure 1. Graphical representation of the results of the miniaturized HeLa-luc assay screened against a collection of 607,408 compounds of the Scripps Drug Discovery library. Each of the 476 different 1,536-well plates run for this assay contained both positive (black dotted line) and negative internal controls (not shown). The red dotted line represents the activity cutoff, which was calculated at 15.86% activation. Compounds with percent activation higher than the calculated cutoff were designated as primary hits.

Supplementary Figure 2. Dose-response activity and toxicity profiles for the selected small molecule PRs. HeLa-luc cells were treated with increasing concentrations (0.5, 1.5, 5, 12.5 and 25 μM) of the selected PRs (A1, A2, A3, B1, B2, B3, C1, C2, D1, E1, F1, F2, G1 and G2) for 24 h. Celastrol was used as positive control. Compound activity (black columns) and toxicity (white columns) were measured as described in Supplemental Materials and Methods.

Supplementary Figure 3. Original gel pictures for PCR, western blots and ChIP experiments. (**a**) RT-PCR results as in Fig. 2a. (**b**) Western blot analysis of Hsps as in Fig. 2b. (**c**) ChIP analysis of HSF-1 binding to its promoter as in Fig. 2d.

Supplementary Figure 4. Original gel pictures for PCR and western blots experiments. (**a**) RT-PCR results as in Fig. 3a. (**b**) Western blot analysis of Hsp90 client protein degradation as in Fig. 7c

Supplementary Figure 5. The PRs induce HSF-1 binding to the Hsp40 and Hsp27 promoters. Chromatin immunoprecipitation experiments show that both celastrol and selected PRs induce HSF-1 binding to the Hsp40 and Hsp27 promoters *ex vivo*. HeLa cells were treated with DMSO, celastrol (3 μM) and selected PRs (10 μM) for 60 min and then chromatin was cross-linked, harvested, and immunoprecipitated with an antibody specific for HSF-1 (+Ab). The samples were then analyzed by PCR with primers specific for the Hsp40 and Hsp27 promoters. A no antibody control (-Ab) is shown.

Supplementary Figure 6. Effect of the reducing agents NAC and DTT on the PR activity. HeLa-luc cells were treated with either DMSO, 2.5 μM of celastrol (Cel) or 2.5, 5, and 10 μM selected PRs for 24 h in the absence or presence of either 2 mM NAC (**a-f**) or 250 μM DTT (**g-l**). Celastrol and DMSO were used as positive and negative controls, respectively.

Supplementary Figure 7. The PRs do not activate a reporter gene lacking the correct HSE sequence. HeLa cells were transfected with a luciferase reporter gene fused to either a wild type or mutant HSE promoter. Cells were treated with DMSO (negative control), celastrol (3 μM) or the indicated PRs (10 μM) for 8 h before measuring luminescence. Only cells expressing the wild type promoter were able to induce luciferase expression upon compound treatment.

Supplementary Figure 8. The PRs protect cells from stress-induced cell death. HeLa cells were either left untreated or pretreated with heat shock (42°C) or the indicated PRs for 1 h, washed three times and given fresh medium, and then recovered for 8 h prior to a 35 min 45°C heat shock treatment. 24 h later, the percentage of cell death was determined by Calcein AM assay. The differences in group means were compared by the Student's t test; **p value <0.01; ***p value <0.001.

Supplementary Figure 9. The PRs suppress expanded polyQ aggregation without altering the levels of soluble and total protein. (**a**) Treatment of cells with the indicated PRs for 48 h reduced the insoluble form of the protein compared to the DMSO-treated samples. Nitrocellulose slot-blot was used to detect total (T), soluble (S) and pellet (P) forms of HttQ74-GFP. Equivalent amounts of protein were loaded. (**b**) Quantification of total HttQ74-GFP protein from (a) with respect to tubulin loading control was done by densiometric analysis. The DMSO-treated samples were arbitrarily set as 100. (**c**) Insoluble HttQ74-GFP was detected by slot-blot using a cellulose acetate membrane (filter trap assay). (**d-e**) Soluble (S) and insoluble (P) HttQ74-GFP was detected by western blot. Open and closed arrowheads indicate, respectively soluble and insoluble HttQ74-GFP protein (detected in the stacking gel portion of the western blot). (**f**) Quantification of soluble HttQ74-GFP protein from (e) with respect to tubulin loading control was done by densiometric analysis. The DMSO-treated samples were arbitrarily set as 100. (**g**) Western-blot of total polyQ protein from *C. elegans* extracts. (**h**) Quantification of total polyQ protein from (g) with respect to tubulin loading control was done by densiometric analysis. The control samples were arbitrarily set as 100.

Supplementary Figure 10. Dose-response curve for the PRs A3, C1 and F1 and enzymatic digestion of CFTR Golgi-processed glycoform by Endoglycosidase H. (**a**) CFBE41o- YFP cells were treated with 0.1% DMSO (diamonds), the positive control 5μM SAHA (triangles) and the indicated PRs at 2.5 (open circles), 5 (grey circles) and 10μM (black circles) for 24 h. Fluorescence quenching is indicative of restored ΔF508-CFTR trafficking (mean ± s.e.m.; n = 3). The open, grey and black asterisks indicate statistically significant differences from DMSO control at the 30s time point. The SAHA treatment yields a significant difference from DMSO (see Figure 5c). (**b**) Endoglycosidase H resistance assay was used to confirm that band C in the CFTR analysis represents a Golgi modified glycoform. The blot confirms that the ER derived band B glycoform is completely sensitive to EndoH indicated by the appearance of the faster migrating band A, whereas the band C, which is present in all PR treated samples exhibits resistance to endoH.

Supplementary Figure 11. Western blot analysis of CFTR and Hsp70 in CFBE41o- cells. Cells were treated with DMSO or the indicated dose of PRs A3, C1, F1 and the positive control SAHA for 24 h. Blots were cut at the appropriate MW and immunoblotted (IB) with the indicated antibody.

Supplementary Figure 12. Real-time qPCR measurement of stress related gene levels upon treatment with the PRs A1, D1 and F1. Data are relative to the negative control (DMSO). The genes tested are grouped according to stress response or pathway (indicated at the bottom of the figure): DAF-16/FOXO pathway, oxidative stress, ER and mitochondrial UPR, Hsp90 (*daf-21)* chaperone and co-chaperone (*ZC395.10*), and UPS components. Standard deviation in shown.

Supplementary Figure 13. (**a**) HeLa cells were incubated with either DMSO, MG132 (10 μM), lactacystin (6 μM) and the PRs A1, A3 and F1 (10 μM) for 3 h. Proteasome-associated chymotrypsin activity was assessed using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (suc-LLVY-AMC) as described in Materials and Methods. (**b-c**) HeLa cells were treated with DMSO, 17-AAG (2 μM), MG132 (10 μM), lactacystin (6 μM) or the PRs A1, A3 and F1 (10 μM) for 6 (b) and 16 h (c). Protein levels of various Hsp90 client proteins (Cdk-4, Raf-1 and Akt) in equal amounts of whole-cell lysates were assessed by western blot analysis. GAPDH was used as loading control. (**d**) Purified Hsp90β (2 μg) was incubated with either DMSO, 17-AAG (1 μM) or the indicated PRs (10 and 100 μM) for 30 min at 4°C before addition of 1 μM geldanamycin-biotin for 1 h at 4°C. Hsp90 bound to geldanamycin-biotin was captured with neutravidin-agarose beads and analyzed by immunoblotting.