Supporting Information

Het, a marine sponge terpenoid, targets TDP-43, a key factor in several neurodegenerative disorders.

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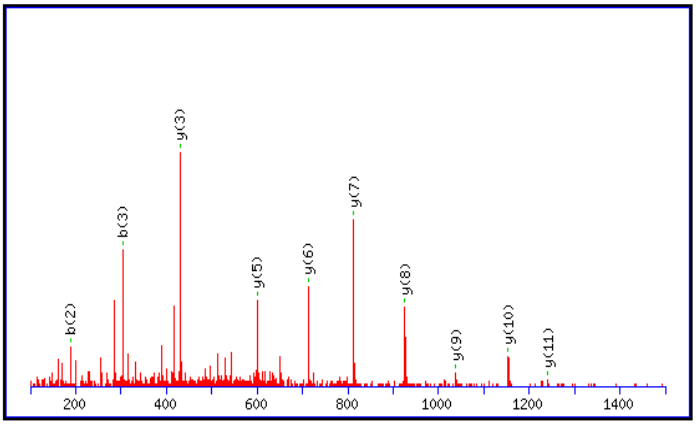
Mascot reports and MSMS spectra for Het-binding partners identification:

Best identification for TDP-43:

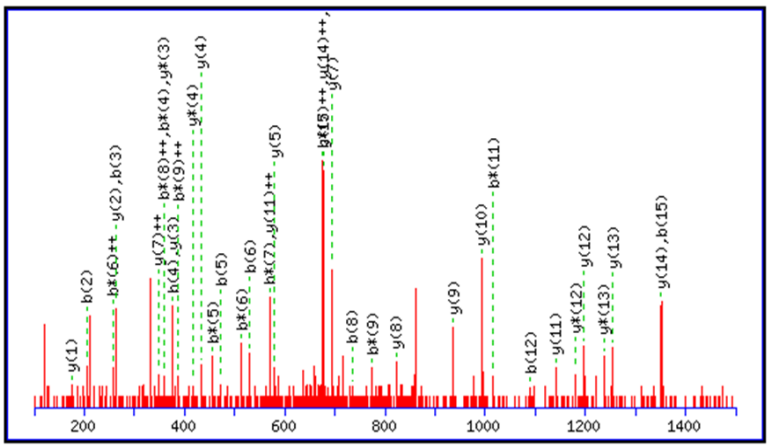
TADBP_HUMAN Mass: 45053 Score: 189 Matches: 6(6) Sequences: 4(4) emPAI: 0.43
TAR DNA-binding protein 43 OS=Homo sapiens GN=TARDBP PE=1 SV=1
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
57	671.4008	1340.7870	1340.7704	12.4	0	68	7.1e-06	1	U	K.TSDLIVLGLPWK.T
58	671.4008	1340.7870	1340.7704	12.4	0	(41)	0.0035	1	U	K.TSDLIVLGLPWK.T
141	863.8845	1725.7544	1725.7608	-3.69	0	77	2.2e-06	1	U	R.FGGNPGGFGNQGGFGNSR.G
142	863.8845	1725.7544	1725.7608	-3.69	0	(55)	0.00036	1	U	R.FGGNPGGFGNQGGFGNSR.G
210	864.7584	2591.2534	2591.2829	-11.40	1	45	0.0042	1	U	K.TTEQDLKEYFSTFGEVLMVQVK.K
213	891.4419	2671.3039	2671.3204	-6.17	0	40	0.012	1	U	R.AFAFVTFADDQIAQSLCGEDLIIK.G

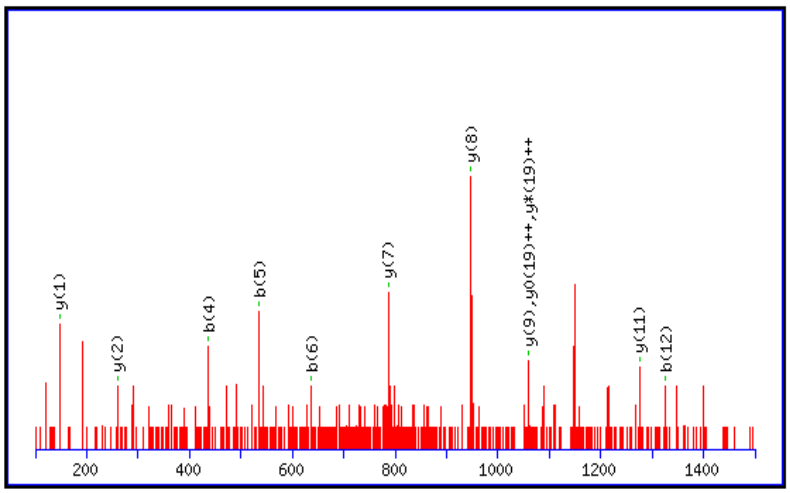
Figure S1: Mascot report of all matched peptides of TDP-43



MS/MS Fragmentation of **TSDLIVLGLPWK**
Found in **TADBP_HUMAN** in **SwissProt**, TAR DNA-binding protein 43 OS=Homo sapiens GN=TARDBP PE=1 SV=1



MS/MS Fragmentation of **FGGNPGGFGNQGGFGNSR**
Found in **TADBP_HUMAN** in **SwissProt**, TAR DNA-binding protein 43 OS=Homo sapiens GN=TARDBP PE=1 SV=1



MS/MS Fragmentation of **AFAFVTFADDQIAQSLCGEDLIIK**
Found in **TADBP_HUMAN** in **SwissProt**, TAR DNA-binding protein 43 OS=Homo sapiens GN=TARDBP PE=1 SV=1

Figure S2: Tandem mass spectra of the TDP-43 identified peptides

Proteasome Subunits alpha:

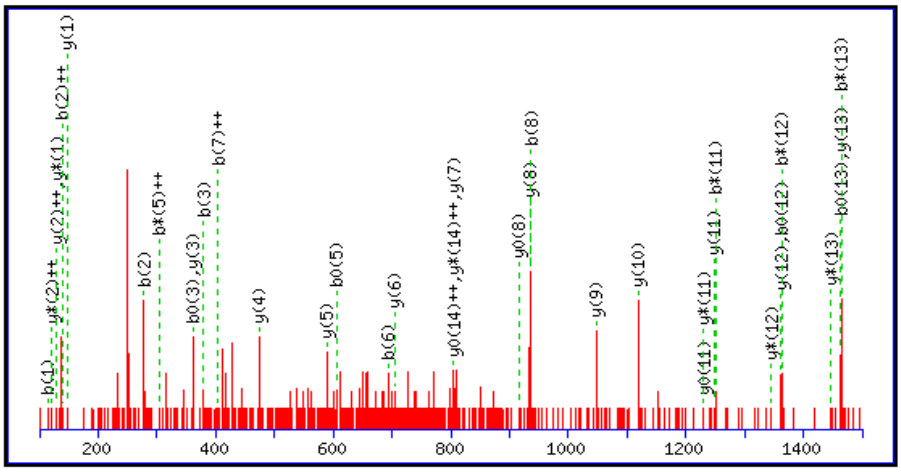
PSA7_HUMAN Mass: 28041 Score: 43 Matches: 1(1) Sequences: 1(1) emPAI: 0.15
Proteasome subunit alpha type-7 OS=Homo sapiens GN=PSMA7 PE=1 SV=1
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
71	870.9009	1739.7872	1739.8101	-13.15	0	43	0.0062	1	U	K.NYTDEAIETDDLTIK.L

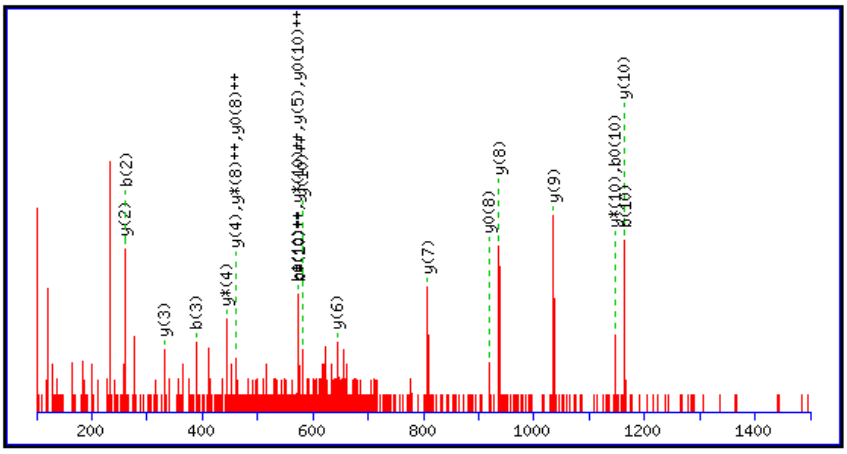
PSA5_HUMAN Mass: 26565 Score: 34 Matches: 1(1) Sequences: 1(1) emPAI: 0.16
Proteasome subunit alpha type-5 OS=Homo sapiens GN=PSMA5 PE=1 SV=3
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
52	712.3948	1422.7750	1422.7758	-0.55	0	34	0.033	1	U	R.LFQVEYAIEAIK.L

Figure S3: Mascot report of all matched peptides of proteasome subunits alpha



MS/MS Fragmentation of NYTDEAIETDDLTIK
Found in PSA7_HUMAN in SwissProt, Proteasome subunit alpha type-7 OS=Homo sapiens GN=PSMA7 PE=1 SV=1



MS/MS Fragmentation of **LFQVEYAIEAIK**
Found in **PSA5_HUMAN** in **SwissProt**, Proteasome subunit alpha type-5 OS=Homo sapiens GN=PSMA5 PE=1 SV=3

Figure S4: Tandem mass spectra of the proteasome alpha-subunits identified peptides

Het and MG132 inhibition of HeLa cells proteasome

An experiment to clarify if Het causes proteasome inhibition in HeLa cells has been performed. In details, proteasome activity was measured upon Het administration to HeLa cells by detecting the proteasome-mediated proteolysis of a fluorogenic peptide substrate, specific for the chimotrypsin catalytic subunit. Since the chimotrypsin-like activity of the HeLa proteasome was not impaired by the treatment with Het for 2 h at 10 µM (Fig. S5), the previously measured biological effect of Het on TDP-43 cellular localization can be evidently attributed to the direct interaction between the counterparts. MG 132, a known proteasome inhibitor, has been used as positive control.

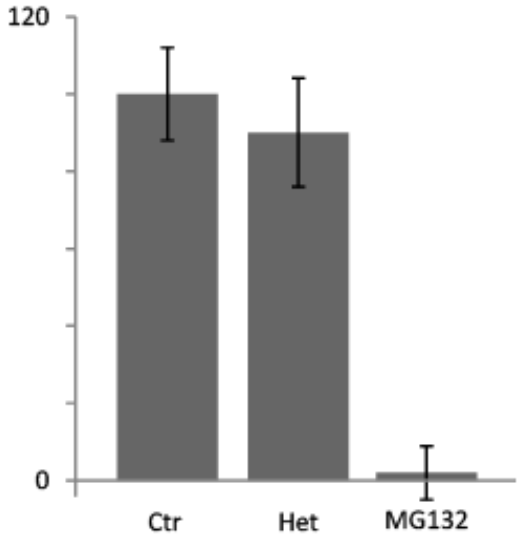


Fig S5: Analysis of proteasomal chimotrypsin-like activity. Inhibition in HeLa cells by Het and MG132

MG132 effects on TDP-43 localization

The effect of MG132, a known proteasome inhibitor, on TDP-43 cellular localization has also been tested. The pre-treatment of the cells with MG-132 before heat shock induced the formation of SGs and TDP-43 positive SGs, even if in a lower extent respect to Het.

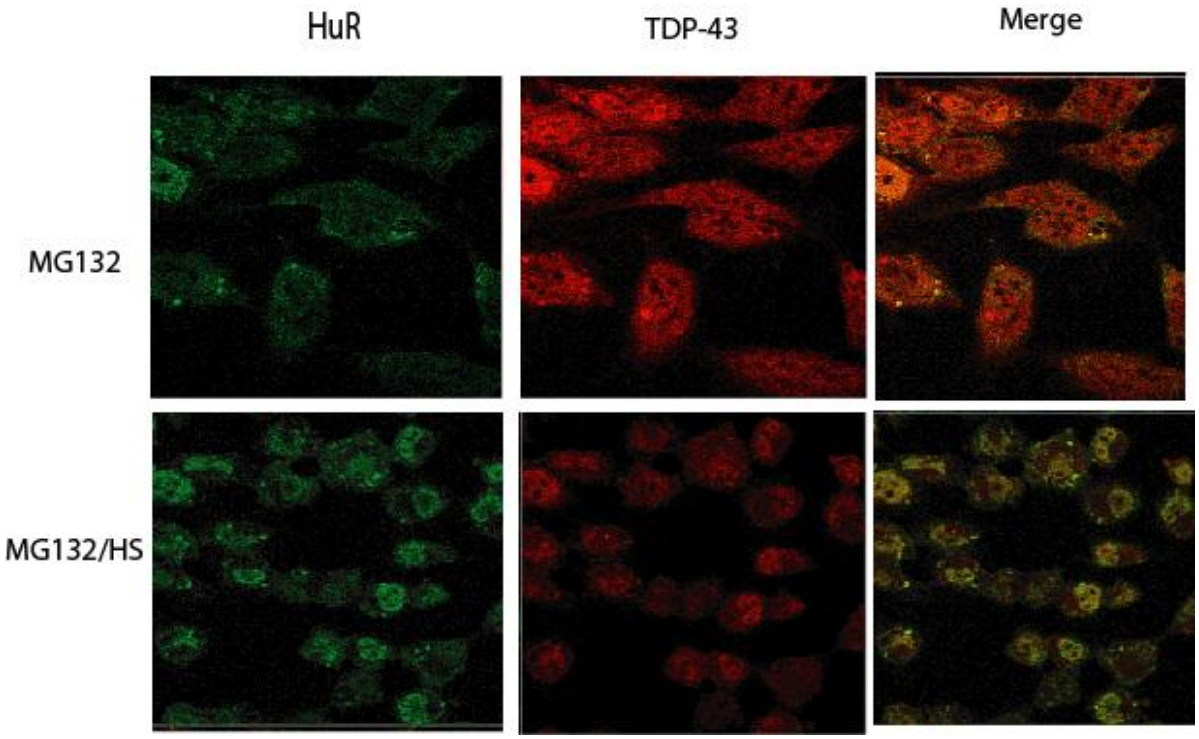


Fig. S6. HeLa cells cultured on cover slips were pre-treated with 50 μM MG132 and then 43 °C heat shocked (HS), subsequently immuno-labeled for TDP-43 and the SG marker HuR. TDP-43 localization to SGs is indicated by the merged images showing the overlap between red and green signals.

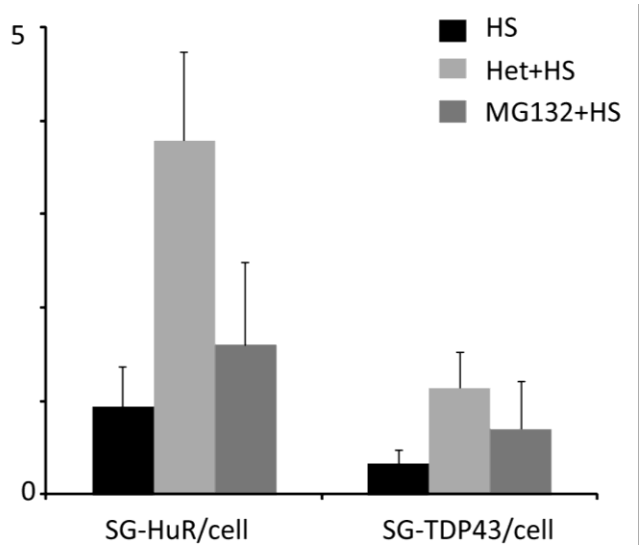


Fig. S7: TDP-43 and HuR-positive SGs were counted in HS, Het+HS and MG132 treated cells. A minimum of 200 cells were counted across multiple fields of view (and multiple coverslips) for each treatment. The total number of HuR or TDP-43 positive SGs was divided by the total number of cells to provide a mean measure of SGs per cell. Data were expressed as fold induction of HuR positive mean SGs in HS cells set to 1

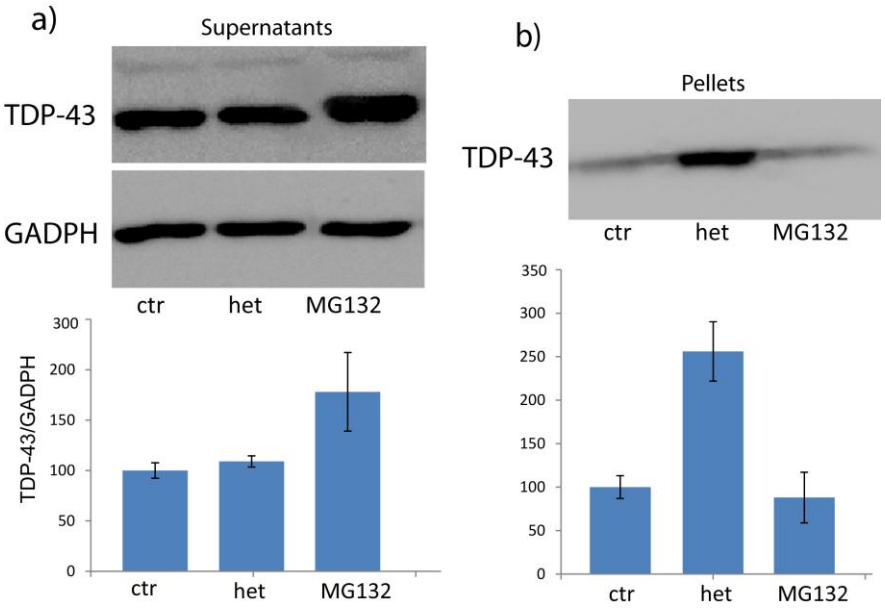


Fig. S8: a) Western blotting analysis of TDP43 found in pellet (a) and supernatant (soluble proteins, b) fractions of HeLa cell lysates treated or not with HeT 10 µM and MG132 50 µM. Histograms were the results of image quantification analysis of three independent experiments.

Materials and Methods

Generation of the Het functional matrix

Epoxy-activated Sepharose™ 6B matrix was swollen with water (200 µL/mg) for 60 min. 2 mg of HET (4,1 µmol) were diluted in 450 µL of 78% MeCN/ 22% $NaHCO_3$ at pH 8.0 and added to 200 µL of matrix at room temperature for 2.5 hours with continuous shaking. A control matrix was obtained in the same experimental conditions without the marine metabolite. The amount of immobilized HET was estimated by integrating the peaks of the free HET after HPLC injections of supernatants at t=0 and 2.5 h in a 1100 Series Chromatographer (Agilent). HPLC runs were carried out on a C18 column (Jupiter proteo C18 5µ 250 x 2.00 mm, Phenomenex, Torrance, CA) at a flow rate of 200 mL*min-1. The gradient (solution A: H_2O and TFA (0.1%); solution B: MeCN and TFA (0.1%)) was 10–95% B over 25 min. Both resins were washed 3 times with 1 mL of phosphate saline buffer (PBS; sodium phosphate 50 mM, NaCl 150 mM, pH 7.5) and then incubated with a mixture of water/isopropanol (1:2) for 4 h at room temperature to inactivate the epoxy free groups. Then, the matrices were washed extensively with PBS to remove traces of isopropanol and stored at 4°C in PBS 30% of ethanol.

Affinity purification and identification of Het partners

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/mlpenicillin, 100 mg/ml streptomycin, at 37°C in a 5% CO_2 atmosphere (all reagents were from Sigma–Aldrich). Cells were collected by centrifugation (600*g*, 5 min), washed twice with PBS and resuspended in 1X ice cooled PBS containing Igepal (0.1%), supplemented with a protease inhibitor cocktail. The obtained suspensions were sonicated for 2 min

with Vibracell (Sonics) setting an amplitude of 30% and cellular debris were removed by centrifugation at 10000*g* for 10 min at 4 °C. Protein concentration was determined using Bradford assay and adjusted to 1 mg/ml. Het-bound bead suspension (50 µL) and the same amount of the control unbound matrix were separately incubated with 1 mg of HeLa total protein extract under continuous shaking (16 h, 4°C). The beads were collected by centrifugation (865*g*, 1 min, 4°C) and washed six times with PBS (pH 7.4). The bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 0.001% bromophenol blue, **10%** glycerol, 2% 2-mercaptoethanol). The eluted proteins were separated on 10% SDS-PAGE, and stained with Coomassie G-250 (Bio-Rad, Hercules, CA).

Het partners identification

Three bands from SDS-PAGE gel lane relative to the Het-based and control experiments were cut and digested. The experiment has been repeated twice using an opportune control matrix bearing the linker without any metabolite. Each piece was washed with ultrapure water and CH_3CN and subjected to in situ protein digestion as described by Shevchenko.[27] Briefly, each slice was reduced with 10 mM 1,4-dithiothreitol (DTT) and alkylated with 54 mM iodoacetamide, then washed and rehydrated in trypsin solution (12 ng/mL) on ice for 1 h. After the addition of ammonium bicarbonate (30 µL, 50 mM, pH 7.5), proteins digestion was allowed to proceed overnight at 37°C. The supernatant was collected and peptides were extracted from the slice using 100% MeCN and both supernatants were combined. The peptide sample was dried and dissolved in formic acid (FA, 10%) before MS analysis. The peptide mixture (5 µL) was injected into a nano-ACQUITY UPLC system (Waters). Peptides were separated on a 1.7 mm BEH C18 column (Waters) at a flow rate of 400 nL/min. Peptide elution was achieved with a linear gradient (solution A: 95 % H_2O ,5 % MeCN, 0.1% FA; solution B: 95 % MeCN, 5 % H_2O, 0.1 % FA); 15–50% B over 55 min). MS and MS/MS data were acquired with a Q-TOF Premier mass spectrometer (Waters). The five most intense doubly and triply charged peptide ions were chosen by MassLynx software (Waters) and fragmented. The resulting MS data were processed by ProteinLynx (Waters) software to generate peak lists for protein identifications. Database searches were carried out on the Mascot server (http://www.matrixscience.com/). The SwissProt database (release 2012_03, 21 March 2012, 535248 sequence entries, 189901164 amino acids abstracted from 208076 references) was employed (settings: two missed cleavages; carbamidomethyl (C) as fixed modification and oxidation (M) and phosphorylation (ST) as variable modifications; peptide tolerance 80 ppm; MS/MS tolerance 0.8 Da).

***In vitro* validation of TDP43–Het interaction**

Het-modified beads suspension (50 µL) and the same amount of the unmodified matrix were treated as described above and the eluates were analyzed by Western blotting. Briefly, each sample was resolved on a 10% SDS-PAGE gel, and transferred onto a nitrocellulose membrane. The membrane was incubated for 1 h in a blocking solution containing 25 mM Tris pH 8, 125 mM NaCl, 0.1% Tween-20, 5% nonfat dried milk, then incubated overnight at 4°C with primary monoclonal antibody raised against TDP43 (Sigma-ALdrich, code T1705), at 1:8000 dilution. Membranes were then incubated for 1 h with rabbit peroxidase-conjugated secondary antibody (1:5000; Thermo Scientific). TDP43 was detected using an enhanced chemiluminescent substrate (ECL) and LAS 4000 (GE Healthcare, Waukesha, WI, USA) digital imaging system.

TDP43–Het binding affinity by surface plasmon resonance

TDP43 was immobilized onto a CM5 sensor chip by using standard amine coupling procedures. 1X PBS was used as running buffer. The carboxymethyl dextran surface was activated with a 5 min injection of a 1:1 ratio of EDC and NHS (100 mM at 5 µL/min). TDP43 was diluted to 30 ng/mL in potassium acetate (10 mM, pH 4.5) and injected onto the activated chip surface (flow rate 5 µL/min) until reaching ~15 000 RU. Remaining active groups were blocked with a 7 min injection of ethanolamine·HCl (1.0 M, pH 8.5) at 5 µL/min. For these biosensor experiments, HET (0.01–25 µM) was diluted in PBS containing 2 % DMSO. Each concentration was tested at least three times. Since Het dissociated to baseline within a reasonable time, no regeneration was required. The interaction experiments were carried out at a flow rate of 10 µL/min over a 3 min injection time. The dissociation time was set at 600 s. Rate constants for association (ka), dissociation (kd) and the dissociation constant (K_D) were obtained by globally fitting data from all the injection of different concentrations of each compound by using BIAevaluation software (GE Healthcare) with a simple 1:1 Langmuir binding model. The same experiment was carried out immobilizing the following proteins that didn't bind Het: Heat Shock Protein 90 and bovine serum Albumin.

In vitro effect of Het on TDP43 aggregation

TDP-43 stock solution (25 µM in 33% glycerol, 80 mM Tris-HCl, 0.1% SDS 1 mM DTT) was diluted in 1:500 in PBS containing 2 mM DTT and 5 % glycerol at a final concentration of 50 nM and incubated with a 50 molar fold of Het. Since the small molecule was withdrawn from 50X stock solutions in DMSO, a control experiment was performed adding an equal amount of DMSO to protein solution. Samples were then centrifuged for 30 min at 16000 g; pellets and supernatants were carefully separated and a solution of 8 M urea and 2 M thiourea was added to each sample to reach a final concentration of 2.6 M Urea and 0.67 M thiourea. Following 10 min of incubation on ice, 10 µL of each sample were diluted with 5 µL of loading buffer and ran on a 10% SDS-PAGE. Western blotting analysis was then performed as reported above.

Alpha-screen assays to monitor TDP43 binding to bt-TAR32-DNA

TDP-43 with a glutathione S-transferase (GST) tag on the C-terminus was purchased from Abnova. Single-stranded DNA oligonucleotide was synthesized by LifeTechnologies and biotinylated (bt) at the 5′ end. The TAR-32 sequence is 5′-CTG CTT TTT GCC TGT ACT GGG TCT CTG TGG TT-3′ and corresponds to the first 32 nucleotides of the sequence identified by Ou et al. to bind to TDP-43. AlphaScreen GST detection kit was purchased from PerkinElmer Life Sciences. Assay mixtures contained TDP-43 at final concentration of 0.2 nM, bt-TAR-32 5 nM, 10 µg/mL of AlphaScreen streptavidin donor beads and anti-GST acceptor beads were diluted in a total volume of 40 µL assay buffer (25 mM Tris [pH 7.4], 0.1% chaps) in a 384-well plates. Assays were incubated in the dark at room temperature for 3 h to ensure the binding reaction was at equilibrium, and then the AlphaScreen signal was measured on EnSpire Alpha plate reader (PerkinElmer, Waltham, MA, USA). More in details, TDP-43 (0.2 nM) was preincubated with 20 µg/mL anti-GST acceptor beads in assay buffer for 30 min at room temperature and then added to assays containing 10 nM bt-TAR-32 also preincubated with 20 µg/mL streptavidin donor beads for 30 min at room temperature. After incubation in the dark at room temperature for 3 h to ensure the binding reaction was at equilibrium, the AlphaScreen signal was measured on a EnSpire Alpha plate reader. Different

concentrations of bt-TAR-32 were tested to optimize the measurements. Then, Het (0.05 nM-50 µM) was preincubated with TDP-43 (0.2 nM) prebound to AlphaScreen anti-GST acceptor beads for 30 min at room temperature. DMSO concentrations were less than 1% in each sample. Assays were initiated by the addition of bt-TAR-32 at 10 nM prebound to AlphaScreen strepavidin donor beads. After incubation in the dark at room temperature for 3 h, the AlphaScreen signal was measured. IC_{50} values were determined from nonlinear regression fits of the data in GraphPad Prism.

Proteasome assay

100-mm plates of HeLa cells were separately treated with Het 10 µM for 2 h and MG132 50 µM for 3 h whereas cells exposed to vehicle were utilized as control. Then, cells were abundantly washed, scraped off and harvested by centrifugation. The cell pellets were incubated with lysis buffer (PBS 1X, 0.1% Igepal) for 5 min on ice and sonicated for two pulses of 10 s, at 30% output power, using a *Vibracell* sonicator. Lysates were then centrifuged for 10 min at 10000g and 4 °C to remove cellular debris, and the protein concentration was determined according to Bradford using BSA as standard. 10 µg of each sample were diluted in 100 µl assay buffer (25 mM HEPES pH 7.4, 0.5 mM EDTA, 1 mM ATP) and fluorogenic peptides Suc-LLVY-amc (10 µM) was used to measure the chymotrypsin-like activities of the 20S proteasome. The release of 7-amino-4- methylcoumarin (amc) was monitored for 2 hours by emission at 460 nm (excitation 380 nm) using a multi-well plate Perkin Elmer LS55 Fluorescence Spectrometer. The experiments were performed in triplicate.

Het influence on TDP43 cellular localization

For immunofluorescence analysis, $4x10^4$ cells/well were seeded on cover slips in 24-well plastic plates. After 24h, cells were treated with 10 µM Het (2h, 37 °C) or 50 µM MG132 (3h, 37 °C, see SI) followed or not by heat shock treatment (1h, 43°C). At the end of the treatments, cells were fixed in 4% paraformaldehyde (PFA) in PBS and subsequently permeabilized with 0.5% Triton X-100 for 15 min. Cover slips were blocked with 1% bovine serum albumin (BSA) and 10% normal goat serum in PBS for 1 h and incubated with antibodies against TDP-43 (1:2000; Sigma T1705) and HuR (1:200; Santa Cruz sc-5261), diluted in blocking buffer for 2 h at room temperature. Labelling was visualized with the fluorescently conjugated secondary antibodies DyLight 594 anti-rabbit and DyLight 488 anti-mouse (Jackson). All secondary antibodies were diluted 1:2000 in PBS. Images were collected on a Zeiss LSM 510 confocal microscope using LSM software. Images shown are representative of multiple fields and triplicate cover slips per experiment. TDP-43 and HuR-positive SGs were counted in cultures where indicated. A minimum of 200 cells was counted across multiple fields of view (and multiple coverslips) for each treatment. The number of TDP-43 and HuR-positive SGs were counted in each cell. The total number of SGs was divided by the total number of cells to provide a measure of mean SGs per cell. SGs were not observed in untreated cells.

Het influence on TDP43 cellular aggregation

HeLa cells were separately treated with 10 µM Het, 50 µM MG132 (see SI) or vehicle (less than 1% DMSO) as control and incubated for 3 hours at 37°C in a 5% CO_2 atmosphere. Subsequently, cells were harvested and lysed by sonication as described above. Resulting lysates were centrifuged for 10 min at 10000g and 4 °C to separate lysis buffer-soluble and insoluble fractions. Supernatants

were collected and the protein concentration was determined according to Bradford using BSA as standard. The lysis buffer-insoluble fractions were re-suspended in a denaturing buffer (Tris 30mM, Urea 8M, thiourea 2 M and chaps 4%) and solubilization was favored over night under continuous shaking. Urea-soluble samples (5 µL) and lysis buffer-soluble extracts (10 µg) were resolved on a 10% SDS-PAGE gel and subjected to western blotting analysis using anti-TDP-43 antibody as reported above. The experiments were conducted in triplicate and GADPH antibody was used for normalization.