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Supplemental Information

Dynamic Interaction of USP14 with the Chaperone HSC70 Mediates Crosstalk between the Proteasome, ER Signaling, and Autophagy

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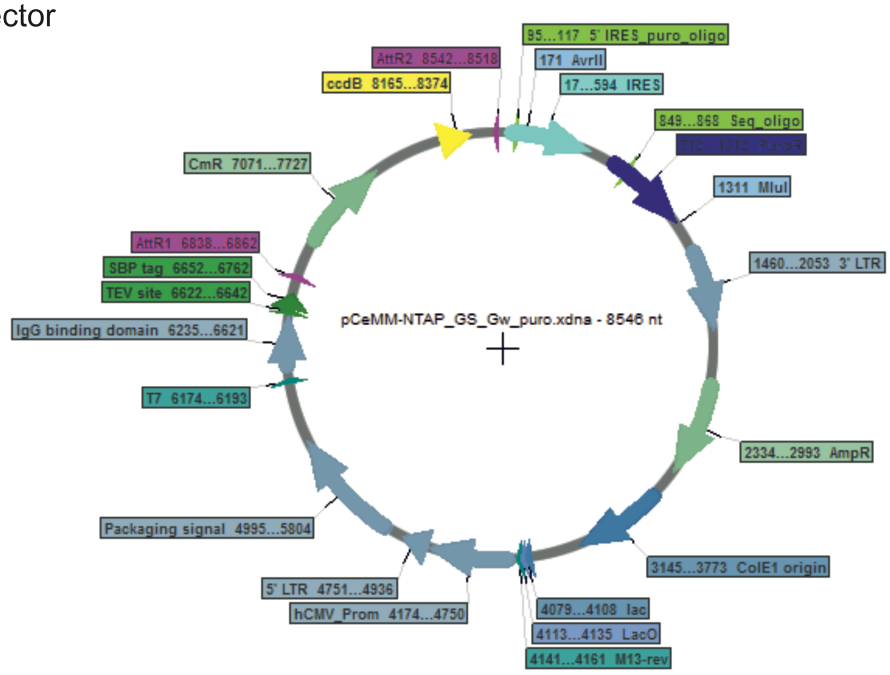
Supplemental Items

Figure S1

A

p-ES-NTAP-Puro vector



B

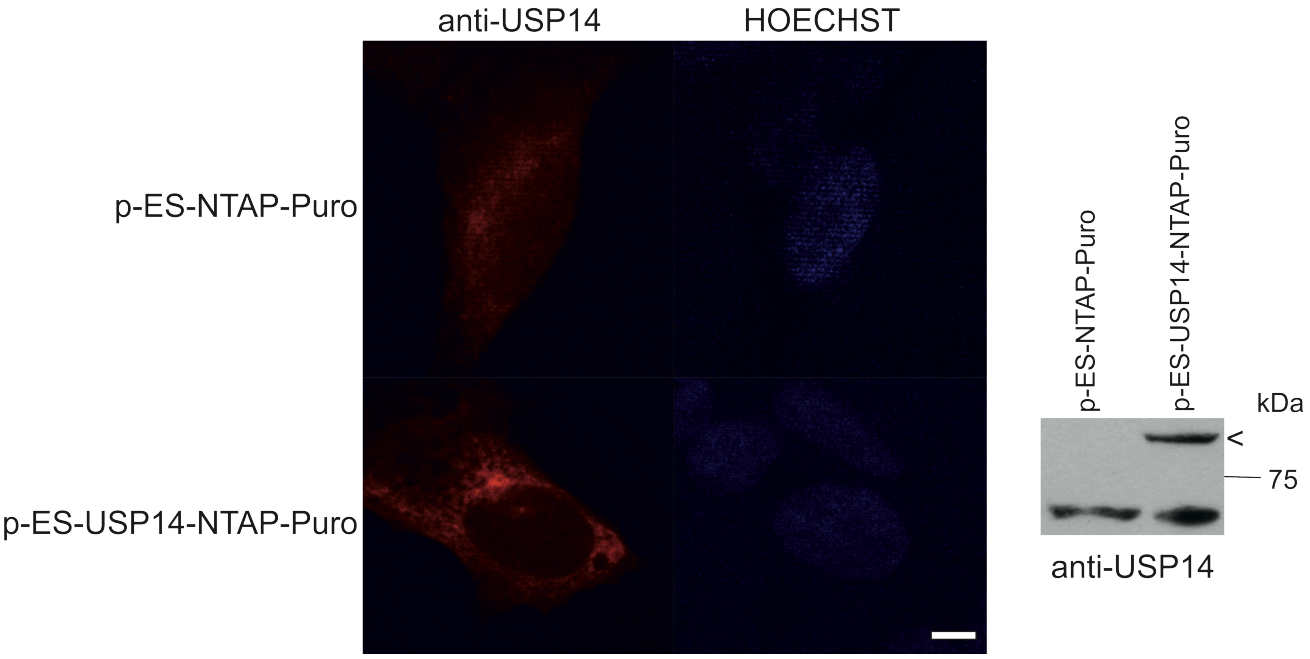
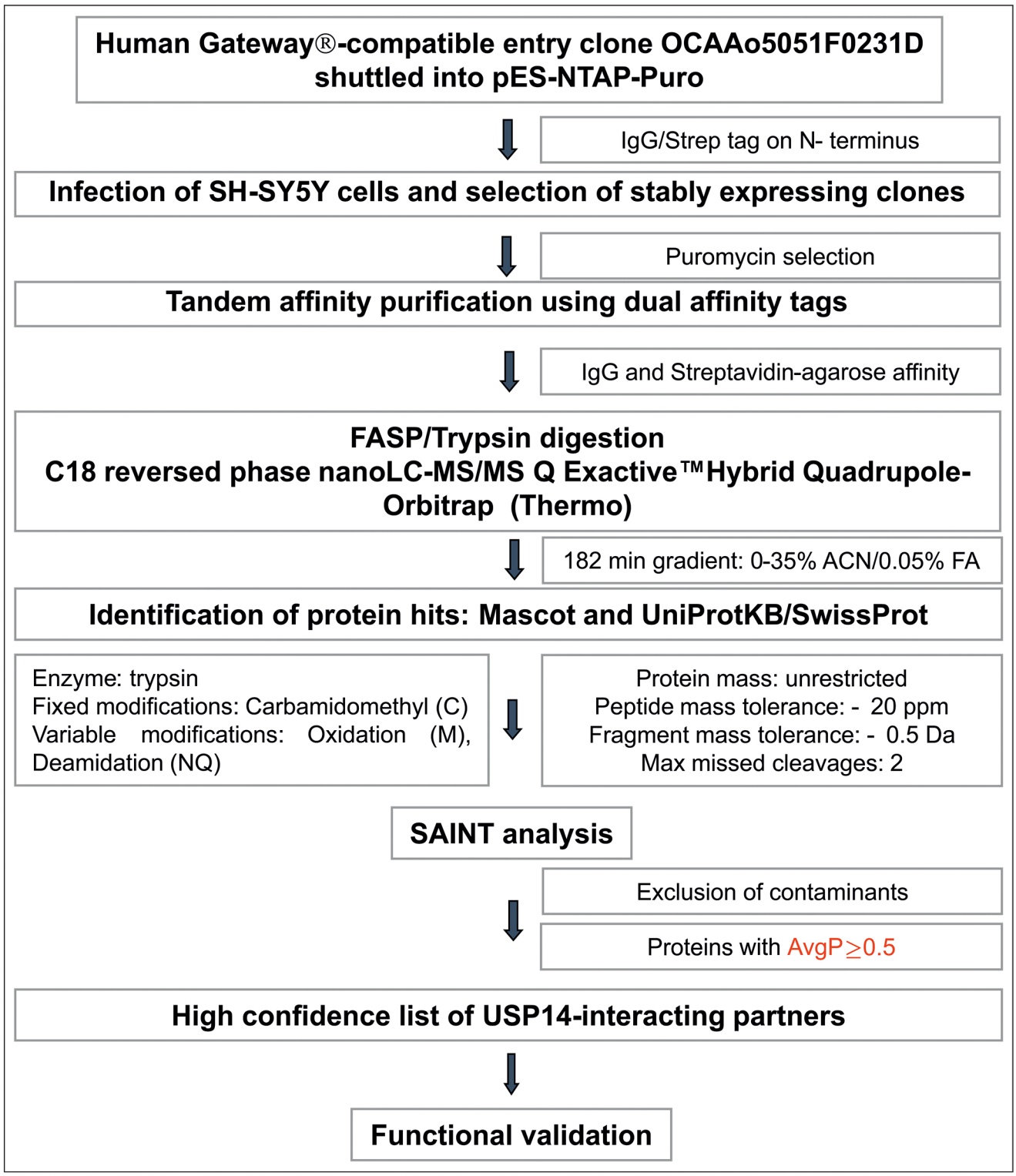


Figure S2

A


Human Gateway®-compatible entry clone OCAAo5051F0231D shuttled into pES-NTAP-Puro
IgG/Strep tag on N- terminus
Infection of SH-SY5Y cells and selection of stably expressing clones
Puromycin selection
Tandem affinity purification using dual affinity tags
IgG and Streptavidin-agarose affinity
FASP/Trypsin digestion
C18 reversed phase nanoLC-MS/MS Q Exactive™Hybrid Quadrupole-Orbitrap (Thermo)
182 min gradient: 0-35% ACN/0.05% FA
Identification of protein hits: Mascot and UniProtKB/SwissProt
Enzyme: trypsin
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M), Deamidation (NQ)
Protein mass: unrestricted
Peptide mass tolerance: - 20 ppm
Fragment mass tolerance: - 0.5 Da
Max missed cleavages: 2
SAINT analysis
Exclusion of contaminants
Proteins with AvgP≥0.5
High confidence list of USP14-interacting partners
Functional validation

Supplemental Figure Legends

Supplemental Figure 1 (Figure S1). Construction of p-ES-NTAP-Puro vector. Related to Figure 2.

(A) The plasmid map of pCeMM-NTAP(GS)-Gw-Puro (p-ES-NTAP-Puro) is represented. Details of vector construction are described in the Materials and methods.

(B) Expression of p-ES-NTAP-Puro-USP14 in SH-SY5Y cells was verified by immunostaining against USP14 (red). Nuclei were stained with Hoechst dye (blue). Scale bar- 100 μm. Right panel- expression of the p-ES-NTAP-Puro-USP14 was verified by immunoblotting with USP14 antibody. The arrowhead marks the overexpressed NTAP-USP14 protein migrating at ~80 kDa, showing similar expression level as an endogenous USP14 (at ~55 kDa) in both conditions. No band at ~80 kDa is seen in SH-SY5Y cells expressing p-ES-NTAP-Puro vector only.

Supplemental Figure 2 (Figure S2). The workflow depicting the affinity purification and bioinformatic analysis of USP14 interacting partners. Related to Figure 2.

Retroviral infection compatible vector, pES-NTAP-Puro (USP14-NTAP-Puro) was utilized to infect SH-SY5Y cells and generate stably expressing clones upon puromycin selection. Cytoplasmic extracts were subjected to tandem affinity purification using dual affinity tags followed by FASP and tryptic digestion. Peptides were analyzed by tandem mass spectrometry and the results processed using Mascot and SAINT software. Contaminants were excluded based on the AvgP threshold resulting in a high confidence list of USP14-interacting partners (Supplemental Table 1). The identified interaction was further validated in Co-IP and functional experiments.

Supplemental Table 1: SAINT analysis of USP14 interacting partners in pES-NTAP-Puro-USP14 stably expressing SHSYSY-5Y cells recovered by TAP-MS. Related to Figure 2.

Bait	Prey	PreyGene	Spec	SpecSum	AvgSpec	NumReplicates	ctrlCounts	AvgP	MaxP	TopoAvgP	TopoMaxP	SaintScore	logOddsScore	FoldChange	BFDR	maxSpec	PreyGeneID	PreyGeneName	Project_Frequency	Shared_Frequency	BioGrid
USP14	HSP7C_HUMAN	HSPA8	2|4	6	3	2	0|0	0,99	1	1	1	1	6,02	30	0	4	3312	HSPA8	76,92	100	novel
USP14	IGHG2_HUMAN	IGHG2	3|3	6	3	2	0|0	1	1	1	1	1	5,41	30	0	3	3501	IGHG2	92,31	100	TAP contaminant
USP14	PRS4_HUMAN	PSMC1	10|16	26	13	2	0|0	1	1	1	1	1	38,9	130	0	16	5700	PSMC1		100	reported
USP14	PRS7_HUMAN	PSMC2	7|11	18	9	2	0|0	1	1	1	1	1	31,4	90	0	11	5701	PSMC2	23,08	100	reported
USP14	PRS6A_HUMAN	PSMC3	4|11	15	7,5	2	0|0	1	1	1	1	1	25,77	75	0	11	5702	PSMC3	15,38	100	reported
USP14	PRS8_HUMAN	PSMC5	5|6	11	5,5	2	0|0	1	1	1	1	1	29,21	55	0	6	5705	PSMC5	15,38	100	reported
USP14	PSMD1_HUMAN	PSMD1	4|14	18	9	2	0|0	1	1	1	1	1	24,72	90	0	14	5707	PSMD1	15,38	100	reported
USP14	PSMD2_HUMAN	PSMD2	9|16	25	12,5	2	0|0	1	1	1	1	1	35,95	125	0	16	5708	PSMD2	15,38	100	reported
USP14	PSMD3_HUMAN	PSMD3	8|12	20	10	2	0|0	1	1	1	1	1	35,09	100	0	12	5709	PSMD3		100	reported
USP14	PSMD4_HUMAN	PSMD4	3|5	8	4	2	0|0	1	1	1	1	1	23,33	40	0	5	5710	PSMD4		100	reported
USP14	HORN_HUMAN	HRNR	8|4	12	6	2	0|3	0,84	0,96	0,69	0,9	0,84	-0,07	4	0	8	388697	HRNR	84,62	100	statistical contaminant
USP14	ADRM1_HUMAN	ADRM1	1|5	6	3	2	0|0	0,5	1	0,5	1	0,5	15,24	30	0,12	5	11047	ADRM1		100	reported
USP14	PSA1_HUMAN	PSMA1	0|2	2	1	2	0|0	0,49	0,99	0,5	1	0,5	3,59	10	0,28	2	5682	PSMA1		100	novel
USP14	PSA2_HUMAN	PSMA2	1|4	5	2,5	2	0|0	0,5	1	0,5	1	0,5	13,48	25	0,17	4	5683	PSMA2	7,69	100	reported
USP14	PSA3_HUMAN	PSMA3	1|4	5	2,5	2	0|0	0,5	1	0,5	1	0,5	16,29	25	0,17	4	5684	PSMA3		100	reported
USP14	PSA5_HUMAN	PSMA5	0|3	3	1,5	2	0|0	0,5	1	0,5	1	0,5	5,7	15	0,2	3	5686	PSMA5	15,38	100	reported
USP14	PSA6_HUMAN	PSMA6	0|3	3	1,5	2	0|0	0,5	1	0,5	1	0,5	8,51	15	0,2	3	5687	PSMA6		100	reported
USP14	PSA7_HUMAN	PSMA7	0|6	6	3	2	0|0	0,5	1	0,5	1	0,5	6,4	30	0,09	6	5688	PSMA7		100	novel
USP14	PSB1_HUMAN	PSMB1	0|2	2	1	2	0|0	0,49	0,99	0,5	1	0,5	5	10	0,28	2	5689	PSMB1		100	reported
USP14	PSB5_HUMAN	PSMB5	0|8	8	4	2	0|0	0,5	1	0,5	1	0,5	9,92	40	0,02	8	5693	PSMB5		100	reported
USP14	PSB6_HUMAN	PSMB6	0|3	3	1,5	2	0|0	0,5	1	0,5	1	0,5	7,11	15	0,2	3	5694	PSMB6		100	novel
USP14	PSB7_HUMAN	PSMB7	0|3	3	1,5	2	0|0	0,5	1	0,5	1	0,5	7,81	15	0,2	3	5695	PSMB7		100	novel
USP14	PRS6B_HUMAN	PSMC4	0|5	5	2,5	2	0|0	0,5	1	0,5	1	0,5	9,92	25	0,12	5	5704	PSMC4	23,08	100	reported
USP14	PRS10_HUMAN	PSMC6	1|8	9	4,5	2	0|0	0,5	1	0,5	1	0,5	16,64	45	0,02	8	5706	PSMC6		100	reported
USP14	PSD11_HUMAN	PSMD11	2|1	3	1,5	2	0|0	0,49	0,99	0,5	1	0,5	18,05	15	0,28	2	5717	PSMD11		100	reported
USP14	PSD12_HUMAN	PSMD12	0|3	3	1,5	2	0|0	0,5	1	0,5	1	0,5	5	15	0,2	3	5718	PSMD12	15,38	100	reported
USP14	PSMD5_HUMAN	PSMD5	2|1	3	1,5	2	0|0	0,49	0,99	0,5	1	0,5	13,13	15	0,28	2	5711	PSMD5		100	novel
USP14	PSMD8_HUMAN	PSMD8	0|2	2	1	2	0|0	0,49	0,99	0,5	1	0,5	2,89	10	0,28	2	5714	PSMD8		100	novel
USP14	RS27A_HUMAN	RPS27A	0|3	3	1,5	2	0|0	0,5	1	0,5	1	0,5	-0,97	15	0,2	3	6233	RPS27A	92,31	100	statistical contaminant

Supplement Table Legend

Supplemental Table 1. List of interacting partners in USP14 overexpressing neuroblastoma cells. Related to Figure 2.

Tandem affinity analyses of USP14 interacting proteins were done as described in Transparent Methods section. Peptides obtained were analyzed using Mascot and SAINT software. Contaminants were excluded based on the AvgP threshold resulting in a high confidence list of USP14-interacting partners. Several proteasome interactors of USP14 have been reported, whilst the binding to HSPA8/HSC70 was previously unrecognized.

Transparent Methods

Cell culture and transfections

SH-SY5Y human and Neuro2A mouse neuroblastoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) supplemented with 10% fetal bovine serum (Gibco), 7.5% NaHCO3, 100 mM NA-glutamine (Gibco) and 100 mM penicillin-streptomycin (Gibco) at 37°C in 5% CO_2. Striatal cell lines (kind gift from Prof. Tönis Timmusk, Tallin University of Technology, Estonia) derived from mice expressing different huntingtin (Htt) genotypes and immortalized with a temperature sensitive large T antigen were used as previously described (Kannike et al., 2014). Control cells expressed 7-polyglutamine (7Q) repeats while the mutant cells expressed 109-polyglutamine (109Q) repeats. Cells were cultured in DMEM supplemented with 10 % fetal bovine serum (Gibco), 7.5% NaHCO3, 100 mM NA-glutamine (Gibco) and 100 mM penicillin-streptomycin (Gibco) at 33°C in 5% CO_2.

Primary neuron cultures were obtained from brain cortices of embryonic E17-old rat pups, and cultured as described (Do et al., 2013). Briefly, the cortices were enzymatically dissociated by trypsin and further processed using DNase I (Roche). The solution was then centrifuged and the pellet resuspended in fresh Neurobasal solution (Gibco). The appropriate number of cells were plated onto cell culture dishes coated with poly-L-ornithine (Sigma) and cultured in Neurobasal (Gibco) medium supplemented with 50x B27 (Gibco), 25 mM glutamine (Gibco) and 100 mM penicillin-streptomycin (Gibco) at 37°C in 5% CO_2. Half of the medium was replaced every two days and cells were incubated for 8 days before experiments.

Different compounds were added to the cells, including 20 μM MG132 (Calbiochem), 0.5 μM bortezomib (Calbiochem) or 5 μM Lactacystin (Sigma) for 5-6 h to inhibit the proteasome. To inhibit autophagy, 200 nM bafilomycin A1 (Sigma) or 50 μM chloroquine (Sigma) were added for 5 h and to block the activity of HSC70, 25 or 50 μM VER-155008 (Tocris Biosciences) was added for 24 h. In some experiments, 2.5 μg/ml tunicamycin (Sigma) was added for 6 h to induce the unfolded protein response (UPR). Linear Polyethylenimine 25.000 (PEI) (Polysciences, Inc.) was used for transient transfection of neurons based on the manufacturer's instructions. PEI was prepared at a stock concentration of 1 mg/ml. DNA and PEI were combined at a ratio of 1:3 for complexing. Following 24 - 48 h of transfection, different treatments were applied as above.

Cell viability assay

Cell viability was assayed essentially as described previously (Hyrskyluoto et al., 2013). In brief, striatal cells expressing 7Q or 109Q Htt protein were treated with 25 μM or 50 μM VER-155008 for 24 h and cell death was measured by utilizing tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT, Sigma) substrate added to cells for 2 h. The insoluble formazan substrate was then reconstituted in a solvent of 0.1 M HCl-isopropanol and incubated for 30 min. with gentle agitation. Absorbance was measured at 560 nm and the value reflected the relative number of surviving cells following each treatment.

Expression vectors cDNAs and transfection

Wild type human pRK-FLAG-USP14 (Flag-WT-USP14) and mutant pRK-FLAG-USP14 C114A plasmids were a kind gift from Dr Yihong Ye (Wang et al., 2006). XBP1 (Myc-DDK-tagged)-Human X-box binding protein 1 (XBP1), transcript variant 1 (hereafter referred to as XBP1u) was obtained from Origene. pcDNA5/FRT/TO-GFP-HSPA8 (GFP-WT-HSC70), Flag-HA-GFP and Flag-HA-USP14 were obtained from Addgene. eGFP-WT-USP14 (GFP-WT-USP14) was generated by amplifying USP14 cDNA from pRK-FLAG-WT-USP14 and subcloning it into eGFPC1 vector. pDestEGFP-GABARAP (EGFP-GABARAP) plasmid was a kind gift from Dr. Nikolai Engedal (University of Oslo, Norway). The point mutation of WT-USP14 to W58A-USP14 was made using the QuickChange lightning site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions.

pCeMM-NTAP(GS)-Gw-Puro cloning strategy

The details of pCeMM-CTAP(GS)-Gw-Puro (pES-CTAP) vector preparation were previously described (Scifo et al., 2013). In the first step, a fragment containing the IRES sequence and the puromycin ORF was amplified from pES-CTAP via PCR using the following primers: 5'-gttattttccaccatattgccg (5'-IRES_puro) and 5'-tcctcc<u>accggtacgcgt</u>caggcaccgggcttgcg (IRES_puro-3'; AgeI and MluI sites are underlined). The PCR fragment was then digested with AvrII (singular site in IRES) and AgeI (introduced by the PCR primer). This fragment was then ligated into the AvrII-BspEI site of the vector pCeMM-NTAP(GS)Gw (Burckstummer et al., 2006), thus replacing IRES-GFP by IRES-Puro casette from pES-CTAP vector. To achieve it, IRES-GFP was first removed from pCeMM-NTAP-GS-Gw by digesting the vector with AvrII (singular site in IRES) and Kpn2I (isoschizomer of BspEI, partial digest) thus isolating the BspEI (nt 1348) – AvrII vector band (size 7.4 kb). Potential clones were sequence verified using the following primers: 5'-gttattttccaccatattgccg (5'-IRES_puro) and 5'-caccgagctgcaagaactc (ML_Seq_1). The sequence and map of the pCeMM-NTAP(GS)-Gw-Puro vector are provided in the supplemental material.

Full length USP14 (OCAAo5051F0231D) sequence verified entry clone in pENTR221 vector, purchased from Source Biosource (<u>OCAAo5051F0231D</u>) was shuttled into pES-NTAP-Puro, for TAP-MS experiments, as described (Scifo et al., 2013). Recombination of DNA fragments was

performed using the LR clonase reaction (Life Technologies Europe BV, Espoo, Finland) and analyzed with BsrGI restriction enzyme.

Construct for shRNA against USP14

SHC002 Non-Mammalian (scramble shRNA) Negative control and constructs against human USP14 were purchased from TRC1 library in a backbone of pLKO.1 vector (Sigma). Five shRNA constructs were tested for efficient USP14 knockdown (KD) by immunoblotting for USP14 and the best constructs were then chosen for generation of SH-SY5Y USP14 KD cells. These constructs were TRCN0000007425 (USP14 shRNA1) and TRCN0000007428 (USP14 shRNA2).

Generation of SH-SY5Y human neuroblastoma cells with knockdown of USP14

Lentivirus particles expressing shRNA constructs were generated at the Biomedicum Functional Genomics Unit, University of Helsinki, Finland. SH-SY5Y cells were seeded into 6-well plates and transduced with lentiviral particles containing shRNA the following day. Following 48 h of virus infection, the antibiotic selection was performed by changing medium with fresh DMEM containing 1 µg/ml puromycin (Gibco, Life technologies). The shRNA constructs exhibiting the best USP14 KD efficiency were further chosen for expansion, referred to as USP14 shRNA1 and USP14 shRNA2 cell clones. SH-SY5Y cells transduced with scramble shRNA expressing viruses were used as a control. Selection pressure with puromycin was maintained during propagation and the medium was changed every 3 days with fresh DMEM containing puromycin.

TAP purification

Monolayer cells were harvested from three 150 mm plates of USP14-NTAP-Puro or NTAP-Puro infected SH-SY5Y cells, grown to 80% confluency (1×10^8 cells). Preparation of cytoplasmic extract from the cells was performed as previously described (Scifo et al., 2013), while the TAP purification procedure followed by FASP preparation prior to MS analysis as in (Scifo et al., 2015a). The Lys-C and trypsin peptide digests were processed on Zip-Tip C18 reversed phase (Merck Millipore) according to manufacturer protocol. Peptide digests were resuspended in 1% TFA and sonicated in a water bath for 1 min. The peptide mixture was applied onto a C-18 reverse phase pre-column (nanoACQUITY Symmetry® C18, Waters) using 0.1% TFA as mobile phase and then separated on a nano-UPLC C-18 column (nanoACQUITY BEH C18, Waters) with an 0.05% formic acid in a gradient of acetonitrile (0–35% ACN in 182 min), at a flow rate of 250 nl/min, and analyzed on Q Exactive™Hybrid Quadrupole-Orbitrap (Thermo Scientific), as described (Scifo et al., 2015a). MS/MS peak lists or spectral data were searched with the Mascot Daemon interface (version 2.4.0;

Matrix Science, Uni. of Helsinki) against the Swiss-Prot 2018_05 database (20350 sequences), with taxonomy set to Homo sapiens. Carbamidomethyl-Cys, met oxidation and N/Q residues deamidation were used as fixed and variable modifications, respectively. Mass tolerance of the precursor ions was set to 20 ppm, and of MS/MS fragment ions to 0.5 Da. The peptide charges were set to 1-3, and 2 tryptic mis-cleavages were allowed. SAINT analysis was performed as previously described (Scifo et al., 2013), except that two biological replicates were used for the bait (USP14) and negative control (empty pES-NTAP-Puro vector). Protein hits that passed the threshold of an AvgP ≥0.5 were considered as true USP14 interactors, after exclusion of an empty pES-NTAP-Puro vector contaminants (Scifo et al., 2013; Scifo et al., 2015a, b), statistical contaminants (www.crapome.org) and checking for those matching the list of previously identified USP14 interacting partners (see compendium in BioGRID 3.5 database; Biogrid/homo-sapiens/usp14).

Immunoprecipitation

Cells were lysed in a modified RIPA lysis buffer containing 50 mM Tris-HCl pH 7.7, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate for 30 min. on ice. Following the lysis, the tubes were rotated for 15-30 min. at 4°C and centrifuged at 10,000g for 10 min. at 4°C. The supernatant was collected, and protein concentration was measured using the BCA protein assay kit (Pierce, Thermo Fisher Scientific). Equal amounts of protein lysates were taken and precleared using 20 µl of prewashed Protein A/G agarose beads (Sigma) for one hour at 4°C. Pre-cleared lysate was incubated with the Anti-Flag M2 (Sigma) or Anti-USP14 (Sigma) overnight in a rotor at 4°C. The following day, BSA blocked agarose beads were added to the overnight antibody conjugated lysate mixture and was left to rotate at 4°C for 2-3 h. The antibody complexed beads were centrifuged at 9000xg for 1 min. at 4°C and washed with the lysis buffer 3-4 times on a rotator at 4°C, 5 min. each. Immunoprecipitated proteins were eluted by adding 35 µl of 2x denaturing Laemmli buffer and heated at 95°C for 5 min. The heat-denatured samples were centrifuged for 3 min. at 10.000xg and the supernatant was collected. The 35 µl supernatant was loaded to PAGE and the immunoprecipitated complexes were analyzed using Western blot for detecting the immunoprecipitated protein and its interacting partners. 40 µg of lysate was used as the input control.

Immunoblotting

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 1% SDS, 50 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors (Roche) and phosphatase inhibitor (Phosphostop, Roche). Protein concentration measurements were performed with the BCA protein assay kit (Pierce, Thermo Fisher Scientific),

and equal amounts of protein were loaded to PAGE and blotted onto nitrocellulose membrane filters (Amersham Biosciences, Helsinki, Finland). The membranes were incubated for 1h in 5% skimmed milk or 5% bovine serum albumin, in TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and then with the primary antibodies overnight at 4°C with gentle agitation. Primary antibodies used were as follows: anti-USP14 (1:500, Abgent, AP2142a); anti-USP14 (1:1000, Sigma, J6111-6D6); anti-HSC70 (1:2000, SantaCruz Biotechnology, sc7298); anti-XBP1 (detects both XBP1s running at 56 kDa and XBP1u, at 29 kDa as indicated in respective IBs and figure legends), 1:2000, Cell signaling technologies, D2C1F); anti-Actin (1:10000,Sigma, A2066); Anti-GFP (1:2000, Sigma, 11814460001); anti-Flag (1:2000, Sigma); Anti-pIRE1α (phosphor S724; 1:2500, Abcam, ab48187), Anti-IRE1α (1:2500, Abcam, ab37073); Anti-PSMD2 (1:1000, Thermo Fisher Scientific); anti-GABARAP (E14JE) (1:2000, CST, 13733). Following washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2500, Jackson Immunoresearch Laboratories) at room temperature for 1 h with gentle agitation. Protein signals were detected using enhanced chemiluminescence substrate (Pierce, Thermo Fisher Scientific). Immunoblots were quantified with ImageJ (NIH) quantification software.

Immunocytochemistry

SH-SY5Y cells or Striatal neuronal cells plated on coverslips were fixed using 4% paraformaldehyde at RT for 10 min. Fixed cells were permeabilized with 0.1% Triton-X-100 containing phosphate buffered saline (PBS-T) and blocked with 5% bovine serum albumin (Sigma) diluted in PBS-T for 1 h. The cells were incubated in XBP1u antibody (1:500; SAB2102720, Sigma) diluted in 5% BSA containing PBS-T overnight at 4°C. The coverslips were washed in PBS-T and incubated with fluorophore conjugated Alexa Fluor® 488 or 594 secondary antibody diluted in 5% BSA containing PBS-T (1:600, Invitrogen) for 2 h in dark at room temperature. Subsequently, the coverslips were washed in PBS-T and nuclei stained with Hoechst 33342 (Thermo Fisher Scientific, 62249). The coverslips were mounted onto glass slides with Mowiwol (Calbiochem) containing DABCO gel mounting media. The cells were imaged using Zeiss Axioplan2 imaging universal light microscope.

Live cell imaging for EGFP-GABARAP positive autophagic structures

Striatal neuronal cells transfected with EGFP-GABARAP in combination with empty vector or Flag-W58A-USP14 were imaged using the EVOS FL cell imaging system (Thermo Fisher Scientific) with a 40X objective. Imaging of GABARAP positive autophagosome at a higher resolution was performed using a Nikon Eclipse Ti-E inverted widefield microscope with full environmental chamber, Hamamatsu Orca Flash 4.0 V2 B&W camera for fluorescence and Lumencor Spectra X

light engine (Biomedicum imagine unit, Medicum, University of Helsinki). Briefly, striatal neurons were plated on µ-Slide ibiTreat polymer bottom plates (80826, ibidi, Germany) and transfected with EGFP-GABARAP in combination with empty vector or Flag-W58A-USP14. Following the treatment with VER-155008 (25µM, 24 h) or DMSO, cells were imaged with a 100X Plan Apo VC objective (Numerical aperture, 1.40) and LED-FITC-A filter. Images were acquired using NIS-Elements advanced research with 6D image acquisition module software from Nikon.
Each experiment contained images from several fields of view. Acquired images were analyzed by ImageJ particle analysis.

Analysis for XBP1u ALIS and EGFP-GABARAP positive structures

XBP1u ALIS and EGFP-GABARAP autophagic structures were considered as particles and analyzed using ImageJ ComDet v.0.4.1 plugin. Briefly, the region of interest (ROI) was defined around the cell boundary. The plugin was applied to the defined ROI to obtain the number of particles and their respective integrated intensities. To negate the cell size dependent differences in the number of particles, average integrated intensity/particle was obtained and used as a measure of changes in XBP1u ALIS or EGFP-GABARAP signal intensities between samples.

Deubiquitination activity assay

The assay was performed as described earlier with a few modifications (Borodovsky et al., 2002). Neuro2A cells overexpressing Flag-WT-USP14, Flag-C114A-USP14, and Flag-W58A-USP14 were lysed in 50 mM Tris (pH 7.4), 250 mM sucrose, 5 mM $MgCl_2$, 1mM DTT and 1mM ATP for 1h at 4°C. 50 µg of lysates was then incubated with 3 mM ubiquitin vinyl methyl ester (HA tag; Enzo Life Science) for 3 h at 37°C. The samples were then boiled 95°C for 5 min in denaturing Laemmli buffer and analyzed by immunoblotting with anti-Flag antibodies.

RNA isolation and Quantitative PCR

RNA was extracted from control 7Q and mutant 109Q expressing striatal cells using the RNeasy mini kit (Qiagen). cDNA was synthesized with SuperScript VILO (Invitrogen, Thermo Fisher Scientific Scientific) following the manufacturer's instructions. LightCycler 480 SYBR Green I MASTER (Roche Applied Science) was utilized to perform real time quantitative PCR on a LightCycler 480 (Roche Applied Science) with a 96-well block as described previously (Pham et al., 2016; Pham et al., 2019). PCR conditions were as follows: Initial incubation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s, 63 °C for 20 s and 72 °C for 10 s. Each sample was analyzed in triplicates and from three independent experiments and normalized to glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) using the ΔΔCt (threshold cycle) method. The amplified product was checked by melting curve analysis spanning the temperature range from 65 °C to 95 °C with a ramping rate of 0.03 °C/s. The following primer sequences were used: USP14, forward, 5'GGCGAACAAGGGCAGTATC3', and reverse, 5'TCTGTTGCAGGACTCTCATCA3';
GAPDH, forward, 5'GGGTTCCTATAAATACGGACTGC3', and reverse, 5'CCATTTTGTCTACGGGACGA3'.

Statistical analysis

Statistical comparison was performed using Student's t-test or one-way/two-way ANOVA, depending on the experimental design. p value, $p < 0.05$ was considered as statistically significant. Statistical analysis and graph design were performed using GraphPad PRISM software.

Supplemental References

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