1    **Allosteric activation of Hsp70 reduces mutant huntingtin levels, the**

2    **clustering of N-terminal fragments, and their nuclear accumulation**

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**Abstract**

Aims: Huntington's disease (HD) is caused by a mutant huntingtin protein that misfolds, yields toxic N-terminal fragments, aggregates, and disrupts proteostasis. The Hsp70 chaperone is a potential therapeutic target as it prevents proteotoxicity by favouring protein folding, disaggregation, or degradation. We tested the hypothesis that allosteric Hsp70 activation with a pharmacological mimetic of the Hsp70 co-chaperone Hip, YM-1, could modulate huntingtin proteostasis.

Main methods: We used HD cell models expressing either N-terminal or full-length huntingtin. Using single-cell analysis we studied huntingtin aggregation in different cellular compartments by fluorescence microscopy. Protein interaction was evaluated by immunoprecipitation, while protein levels were quantified by immunofluorescence and western-blot.

Key findings: N-terminal huntingtin interacted with Hsp70 and increased its levels. Treatment with YM-1 reduced N-terminal huntingtin clustering and nuclear aggregation. Full-length mutant huntingtin also interacted with Hsp70, and treatment with YM-1 reduced huntingtin levels when combined with Hsp70 induction by heat shock. Mechanistically, YM-1 increases the Hsp70 affinity for substrates, promoting their proteasomal degradation. Consistently, YM-1 reduced the levels of ubiquitinated proteins. Interestingly, YM-1 accumulated in mitochondria, interfered with its Hsp70 isoform involved in protein import, and increased NRF1 levels, a regulator of proteasome genes. We thus suggest that YM-1 may trigger the coordination of mitochondrial and cytosolic proteostasis, enhancing protein degradation.

Significance: Our findings show that the strategy of allosteric Hsp70 activation holds potential for HD. While drug efficacy may be limited to tissues with elevated Hsp70, combined therapies with Hsp70 elevating strategies could harness the full potential of allosteric Hsp70 activators for HD.

**Keywords**

Neurodegeneration; Huntington's disease; proteostasis; Hsp70; mitochondria; ubiquitin-proteasome system.


**Abbreviations**

CHIP, C-terminal Hsp70-interacting protein; HD, Huntington's disease; Hip, Hsp70 interacting protein; HSF1, heat shock factor 1; Htt, huntingtin; mut-Htt, mutant

56   huntingtin; NRF1, nuclear factor erythroid 2-related factor 1 (not to be confused with

57   nuclear respiratory factor 1); polyQ, polyglutamine.

## 1. Introduction

Huntington's disease (HD) is a neurodegenerative disorder without effective treatment caused by a polyglutamine (polyQ) expansion mutation in the N-terminal region of the huntingtin (Htt) protein [1, 2]. Mutant huntingtin (mut-Htt) is highly prone to fragmentation, which leads to the accumulation of toxic small N-terminal fragments that can misfold and aggregate [2, 3]. Chronic expression of mut-Htt overwhelms the chaperone machinery and protein degradation pathways, inducing the collapse of the proteostasis network [4].

Molecular chaperones are key players of the proteostasis network that minimize misfolding and aggregation events [5]. The Hsp70 family, which comprises isoforms with different cellular localizations, promote the folding of newly synthesized proteins, solubilize aggregated proteins and prevent aggregation by refolding misfolded proteins or by cooperating with protein degradation pathways to clear aberrant proteins [6, 7]. ATP binding and hydrolysis in the Hsp70 nucleotide-binding domain regulate the interaction of Hsp70 with client proteins: while ATP binding reduces the affinity of the substrate-binding domain to the substrates, ATP hydrolysis into ADP increases the affinity of Hsp70 to the substrates. This nucleotide cycle of Hsp70 is further regulated by co-chaperones, including the Hsp70-interacting protein (Hip) [6, 8, 9]. Hip stabilizes the ADP-bound state, delaying the substrate release. This delay favours the ubiquitination of the Hsp70-bound substrates by the C-terminal Hsp70-interacting protein (CHIP), and targeting for proteasomal degradation [7, 9].

Promoting Hsp70 activity through its overexpression or the overexpression of its co-chaperone Hip reduced aggregation of mut-Htt in HD models:  Hsp70 overexpression reduced mut-Htt aggregation in cells [10, 11], and in early disease stages of the R6/2 mice [12]; Hip overexpression reduced the size and number of mut-Htt aggregates in cultured cortical neurons expressing N-terminal Htt [13, 14]. The opposite strategy of inhibiting Hsp70 activity, using the ATP-competitive inhibitor VER-155008 [15], accelerated the degeneration of cells expressing mut-Htt [16]. Collectively, these data indicate that strategies that promote the activity of Hsp70, such as the overexpression of Hsp70 or of its co-chaperone Hip, are potential therapeutic strategies in HD.

YM-1 is an allosteric activator of Hsp70 that mimics the co-chaperone Hip, favouring the accumulation of the ADP-bound form of Hsp70 [17].  This promotes a longer interaction between Hsp70 and its substrates [17], enhancing CHIP-dependent

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ubiquitination of the substrates and their proteasomal degradation [7]. Treatment with YM-1 decreased the levels of the mutant-androgen receptor oligomers and aggregates in a cellular model of Kennedy's disease [17], and decreased the levels of the misfolded protein tau in models of tauopathy [18, 19]. It is currently unknown if YM-1 reduces protein aggregation in other misfolding diseases, such as HD.

In the present study, we test the hypothesis that Hsp70 allosteric activation with YM-1 may reduce mut-Htt levels and aggregation. As in HD mut-Htt occurs in full-length and in aggregate-prone N-terminal species, and as Hsp70 levels vary with the progression of the disease [12], we test the efficacy of YM-1 in N-terminal and in full-length cellular HD models, with or without heat shock treatment to induce the expression of Hsp70.

## 2. Materials and Methods

### 2.1. Cellular models of HD

*N-Terminal Htt model*. Previous studies investigated N-terminal mut-Htt aggregation in transfected U2OS cells [20, 21]. The efficient transfection and the optimal morphological features of U2OS cells - large and flat, allowing imaging markers such as protein aggregates in different compartments - explain their widespread use in neuroscience studies [22, 23]. Here, U2OS cells (ATCC) were grown as we previously described [24], using Dulbecco's Modified Eagle's Medium (DMEM; A1443001, Gibco), supplemented with 10 mM galactose, 5 mM HEPES and 1 mM sodium pyruvate (Sigma-Aldrich), plus 2 mM glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin (Gibco). In order to express the N-terminal Htt fragment, cells were transfected with plasmids encoding either 'wild-type' EGFP-Htt[ex1]Q23 or 'mutant' EGFP-Htt[ex1]Q74 (40261 and 40262, Addgene), using the Lipofectamine LTX Reagent (Invitrogen) as we previously described [24]. U2OS cells were seeded at 2 x $10^4$ cells/cm$^2$ in 13 mm diameter coverslips (Thermo Scientific) and in 6-well plates (TPP) for immunofluorescence and protein extraction, respectively. For live imaging, cells were seeded at 4.5 x $10^4$ cells/cm$^2$ in 8-well glass-bottom μ-slides (Ibidi).

   *Full-length Htt model*. Previous studies investigated the proteostasis of full-length Htt in PC12 cells containing the full Htt transgene, overcoming the low transfection rates of a large gene such as Htt [25, 26]. PC12 cells have also been used to study the effects of Hsp70 modulation on the proteostasis of mutant androgen receptor - another polyQ containing protein [17]. Here, we used PC12 cells with inducible expression of full-length Htt containing 23 (wild-type, CH00285) or 145 (mutant, CH00289) polyQ repeats (Coriell Cell Repository). Cells were cultured in DMEM (2430054, Gibco), supplemented with: 15% horse serum, 2.5% fetal bovine serum, 0.2 mg/ml geneticin, and 1% penicillin/streptomycin (Gibco), and with 0.2 mg/ml zeocin (Invivogen). To induce Htt expression, cells were incubated with 5 μM ponasterone A (Sigma-Aldrich) for 24 or 96 h. Cells were seeded at 4.2 x $10^4$ cells/cm$^2$ in 6-well plates (TPP) for protein extraction, and at 1.0 x $10^4$ cells/cm$^2$ in 13 mm diameter coverslips (Thermo Scientific) for immunofluorescence.

### 2.2. Chaperone modulation

*Pharmacological modulation* - We used YM-1, an allosteric activator of Hsp70 that increases its substrate binding affinity [17]. Previous studies in cell models of tau pathology identified 3 µM YM-1 as an effective concentration [18]. We measured the concentration-dependent effects of YM-1 on cellular resazurin metabolism, and confirmed 3 µM as a bioactive concentration without significant toxicity. Cells were thus treated with either solvent (0.1% DMSO) or 3 µM YM-1 for 24 h: treatment of the N-terminal Htt model started upon completion of the transfection protocol; treatment of the full-length Htt model started 72 h (3 days) after induction of Htt expression. The study of resazurin (Sigma-Aldrich) metabolism was performed as we previously described [24].

*Heat shock modulation* - Heat shock was previously used to induce Hsp70 [27]. We induced heat shock at 16 h post-treatment (YM-1 or solvent control) by incubating cells for 1 h at 42 °C [28]. Cells were then returned to the standard 37 °C for a 7 h recovery period before protein extraction or immunofluorescence at 24 h post-treatment. To control for the effects of heat shock, we used cells continuously maintained at 37 ºC.

## 2.3. Immunoprecipitation and western blot

*Protein extraction and quantification* - was performed as we previously described [29]. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed in buffer (pH = 7.4) containing 50 mM Tris (NZYTech), 150 mM NaCl (Merck), 1 mM EDTA, 1% IGEPAL CA-630, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (Sigma-Aldrich), and protease inhibitor cocktail (Thermo Scientific). After homogenization with Precellys® Evolution (5800 rpm, 2 x 15 s and pauses of 30 s) and 3 freeze-thaw cycles, the lysates were centrifuged at 600 x *g*, for 10 min, at 4°C. Supernatant protein concentrations were quantified via the Bradford method (Bio-Rad).

*Immunoprecipitation*. SureBeads$^{TM}$ Protein G Magnetic Beads (Bio-Rad) were incubated with either anti-Hsp70 (PA5-28003, Invitrogen; 1:500 for 10 min) or anti-GFP (MA5-15256, Invitrogen; 1:200 for 30 min) – to selectively immunoprecipitate N-terminal Htt from the transfected cells. After washing (PBS with 0.1% Tween 20; Sigma-Aldrich), SureBeads were incubated with test samples containing 500 µg of protein for 1 h [29]. After 3 washes, bound proteins were eluted in NuPAGE LDS sample buffer (Invitrogen), for 10 min at 70°C, and then processed for western blot.

*Western blot*. Samples were denatured at 70 °C for 10 min (in NuPAGE LDS buffer supplemented with NuPAGE reducing agent; Invitrogen), loaded (20-25 µg protein) into polyacrylamide gels (3-8 % Tris-acetate or 4-12 % Bis-Tris NuPAGE

169 precast gels, Invitrogen), electrophoresed (150 V, 50 min for 3-8 % gels; 200 V, 30 min

170 for 4-12 % gels), and electro-blotted to PVDF membranes (25 V for 6-8 min; iBlot-2 dry

171 blotting system; Invitrogen). Membranes were blocked with 5 % bovine serum albumin

172 (BSA, NZYTech) in PBS with 0.05 % Tween 20, then incubated with primary and

173 respective secondary antibodies. Primary antibodies: anti-Htt-NT (1:500; 4-19 N-

174 terminal; CH00146, Coriell Institute), anti-polyQ (1:3000; anti-polyQ clone 5TF1-1C2;

175 MAB1574, Merck Millipore), anti-ubiquitin (1:1000; sc-8017, Santa Cruz

176 Biotechnology), anti-Hsp70 (1:1000; sc-66049, Santa Cruz Biotechnology / 1:3000; PA5-

177 28003, Invitrogen), and anti-Grp75 (1:1000; mitochondrial Hsp70; sc-133137, Santa

178 Cruz Biotechnology). Secondary antibodies (Invitrogen): horseradish peroxidase-

179 conjugated anti-mouse (1:4000, G-21040) and anti-rabbit (1:4000, G-21234). Detection

180 was performed using Novex ECL Chemiluminescent kit (Invitrogen) and a Chemidoc

181 MP imager (Bio-Rad). Membranes were stained with coomassie for protein loading

182 control.

183

## 2.4. Fluorescent probes and Immunolabeling

185 To label polarized mitochondria and indirectly evaluate the mitochondrial membrane

186 potential with single-cell resolution microscopy, cells were incubated (30 min at 37 ºC)

187 with 10 nM TMRM$^+$ (Invitrogen) - before live imaging; or with 300 nM MitoTracker

188 Deep Red (Invitrogen) - before fixation. To label nuclei/DNA, cells were incubated with

189 5 µg/ml Hoechst 34580 (Sigma-Aldrich) for 5 min and washed in PBS. For

190 immunolabeling, cells in coverslips were fixed with 4 % paraformaldehyde for 15 min at

191 37 °C, washed in PBS, and permeabilized and blocked with 0.1 % Triton X-100 (Sigma-

192 Aldrich) and 3 % BSA in PBS for 30 min. Cells were then incubated with primary

193 antibody for 1 h at room temperature or overnight at 4 ºC: anti-GFP (1:2000; MA5-15256,

194 Invitrogen); anti-Htt-NT (1:200; CH00146, Coriell Institute), anti-Hsp70 (1:200; PA5-

195 28003, Invitrogen); anti-Grp75 (1:200; 14887-1-AP, Proteintech); and anti-Hsp90

196 (1:200; sc-13119, Santa Cruz Biotechnology). Cells were then washed with 0.1 % Triton

197 X-100 in PBS and incubated for 1 h with the respective AlexaFluor-488 (anti-mouse, A-

198 11029) and -568 (anti-rabbit, A-11036) conjugated secondary antibodies (1:200;

199 Invitrogen). Coverslips were then assembled in a fluorescent mounting medium (Dako).

200

## 2.5. Fluorescence microscopy

202  Image acquisition was performed using non-saturating identical equipment settings to

203  allow intensity comparisons between treatments. Images were acquired in an inverted

204  microscope (Eclipse TE300, Nikon) with a motorized stage (ProScan, Prior), a

205  monochromator (Polychrome II, Photonics), and a CCD camera (ORCA-ER,

206  Hamamatsu), all controlled by the Micro-Manager 2.0 software [24]. Additionally, high-

207  resolution images were acquired using an inverted confocal laser scanning microscope

208  with Airyscan processing (Zeiss, LSM 880). AlexaFluor-488 and EGFP were excited at

209  488 nm, AlexaFluor-568 and TMRM$^+$ at 557 nm, MitoTracker Deep Red at 640 nm, and

210  Hoechst 34580 at 380 nm. YM-1 fluorescence was detected with excitation at 488 and

211  525 nm. Emissions were collected using band pass filters (Chroma) for DAPI (Hoechst),

212  FITC (EGFP, AlexaFluor-488, YM-1), TRITC (TMRM$^+$, AlexaFluor-568, YM-1), and

213  CY5 (MitoTracker Deep Red).

214

215  **2.6. Image processing and data analysis**

216  Image background subtraction and densitometric analyses of immunoblots were

217  performed with Image*J* (http://rsbweb.nih. gov/ij/; National Institutes of Health). The

218  analyses of U2OS cell images were automated in CellProfiler 3.1.8

219  (https://cellprofiler.org; Broad Institute) to identify: transfected cells and huntingtin

220  aggregates (GFP staining); cell compartments (nucleus – Hoechst staining; cytoplasm –

221  Mitotracker Deep Red staining); and extract quantitative measurements of intensity and

222  shape. Aggregates were detected inside cells as GFP-containing particles with a minimum

223  diameter of 2.75 μm. Classification of Htt-GFP expressing cells into 'diffuse' or

224  'aggregated' was performed with machine-learning in CellProfiler Analyst 2.2.1. Data

225  calculations were performed with pivot tables (Excel, Microsoft). Graphical

226  representations and statistical analysis were performed with Prism 8.0 (GraphPad

227  Software) or R (https://www.r-project.org). The Shapiro-Wilk normality test was used to

228  assess data distributions. Comparisons between two groups were performed using *t*-test.

229  Single-factor analyses were performed using One-Way ANOVA with Dunnett's (vs.

230  control) or Sidak's (between groups) post-Hoc tests. Multifactorial analyses (two- or

231  three-way ANOVA) were used to test interactions and main effects of the different

232  factors. Correlation analyses were performed with the linear model function in R. For all

233  analyses, a *P* value under 0.05 was taken as statistically significant. Unless otherwise

234  stated, data are mean ± standard error of the mean (SEM), or median and interquartile

235  range (boxplots), of at least 3 independent experiments (*n* specified in figure legends).

236 Analysis of imaging data involved a minimum of 50 cells per condition in each
237 independent experiment.
238

## 3. Results

### 3.1. N-terminal huntingtin species interact with Hsp70 and increase its levels

Hsp70 chaperones are key players in cellular proteostasis. They interact with misfolded proteins, promoting their correct folding or degradation, and preventing aggregation [7, 9]. To evaluate Hsp70 chaperone levels in the N-terminal Htt cellular model, we quantified protein expression by immunofluorescence. Specifically, we quantified: the cytosolic and nuclear Hsp70 (hereafter referred collectively as 'Hsp70' - a combination of constitutive Hsc70 and stress-inducible Hsp72 with over 80% identity; [30]); the mitochondrial Hsp70 isoform (hereafter referred to as 'Grp75'); and the associated levels of either wild-type or mutant N-terminal Htt (Fig. 1). Hsp70 presented a nuclear and cytosolic distribution (Fig. 1A*i*), whereas Grp75 presented a selective mitochondrial distribution: Grp75 presented a cellular distribution similar to the mitochondrial probe MitoTracker (Fig. 1A*ii*). Using single-cell analysis of cells expressing either wild-type or mutant N-terminal Htt species, we found that the levels of N-terminal Htt positively correlated with Hsp70 levels (Fig. 1B). Overall, Hsp70 levels were significantly elevated in cells expressing N-terminal Htt species *vs.* control (Fig. 1C). This induction effect was selective for Hsp70, as shown by the unaltered levels of Grp75 (Fig. 1B), and prompts the hypothesis that the N-terminal Htt species interact with Hsp70. Consistently, Hsp70 co-immunoprecipitated with either wild-type or mutant N-terminal Htt (Fig. 1D). Thus, in this HD cellular model, the N-terminal Htt species interact with Hsp70 and this is associated with a compensatory increase in the levels of this chaperone.

### 3.2. Mutant N-terminal huntingtin aggregates recruit Hsp70

To characterize the interaction between N-terminal mut-Htt and Hsp70, we investigated the spatial distribution of mut-Htt aggregates and its association with Hsp70. Cells expressing mutant N-terminal Htt formed aggregates in the nucleus and cytosol, which were surrounded by a more intense pattern of Hsp70 distribution than areas without aggregates (Fig. 2A, B). In contrast, the other major cellular chaperone − Hsp90 − was not particularly enriched in the vicinity of aggregates (Fig. 2A), indicating that mut-Htt aggregates selectively recruit Hsp70. Additionally, the levels of Hsp70 (Fig. 2C*i*) and the heterogeneity of its distribution (Fig. 2C*ii*) were significantly higher in cells presenting mut-Htt aggregates *vs.* those expressing only diffuse mut-Htt. Taken together, these results show that Hsp70 interacts with mutant N-terminal Htt, being recruited into

273 aggregates, and suggesting that the allosteric activation of Hsp70 could potentially affect
274 aggregate formation or their cellular distribution.

275

**3.3. YM-1 decreases mutant N-terminal huntingtin levels in aggregates and their nuclear accumulation**

278 Before examining the effects of the allosteric Hsp70 activator YM-1 on Htt proteostasis,
279 we confirmed YM-1 bioactivity and cellular internalization (taking advantage of YM-1
280 autofluorescence [31]). YM-1 was bioactive, evoking a concentration-dependent
281 decrease in resazurin metabolism (Fig. 3A). The previously reported effective
282 concentration of 3 μM YM-1 for Hsp70 modulation [18] induced a minor decrease in
283 metabolism (~20%) (Fig. 3A*ii*) without detectable toxicity (cells maintain normal
284 morphology, mitochondrial polarization, and nuclear integrity; Fig. 3B, C). 3 μM YM-1
285 was internalized by live cells, where YM-1 autofluorescence overlapped with that of the
286 mitochondrial membrane potential sensitive probe TMRM$^+$ (Fig. 3B), consistent with the
287 cation YM-1 accumulating in the negatively charged mitochondria. We thus used 3 μM
288 YM-1 [18] in all our subsequent experiments, fixing cells after the treatments, and
289 permeabilizing with Triton X-100 to allow antibody penetration and eliminate YM-1
290 autofluorescence, while maintaining nuclear staining with Hoechst (Fig. 3C) and
291 allowing the detection of huntingtin-GFP or MitoTracker Deep Red (Fig. 4D).

292 We next investigated whether treatment with YM-1 influenced the levels of
293 Hsp70, using heat shock as a positive control for Hsp70 induction [27]. Heat shock
294 treatment significantly elevated Hsp70, whereas YM-1 alone maintained Hsp70 levels
295 (Fig. 4A). Given that YM-1 accumulates in mitochondria, we tested if it affected the
296 mitochondrial Grp75 levels, and the MitoTracker Deep Red intensity, which is indicative
297 of mitochondrial levels and polarization status [32]. Treatment with YM-1 significantly
298 elevated Grp75 (Fig. 4B), without detectable changes in MitoTracker Deep Red intensity
299 (Fig. 4C, D).

300 To test the efficacy of YM-1 in promoting protein degradation, we quantified the
301 levels of ubiquitinated proteins in cells expressing mutant N-terminal Htt. Treatment with
302 YM-1 significantly decreased the levels of ubiquitinated proteins (Fig. 5A), suggesting
303 increased proteasome-mediated degradation. We also investigated whether YM-1
304 treatment increases the expression of nuclear factor erythroid 2-related factor 1 (NRF1;
305 not to be confused with nuclear respiratory factor 1), a master transcription factor of
306 proteasome genes [33]. YM-1 treatment increased the levels of NRF1 in cells expressing

mutant N-terminal Htt (Supplementary Fig. 1), suggesting that YM-1 triggers a transcriptional response involving NRF1, promoting protein clearance by the ubiquitin-proteasome system.

As results suggest that YM-1 promotes protein degradation, we tested the effects of YM-1 upon mut-Htt aggregation. Cells expressing mutant N-terminal Htt continued to form aggregates when treated with YM-1. However, YM-1 significantly decreased the levels of Htt in the aggregates (reduction of the Htt staining intensity in the aggregates, Fig. 5B, C), and reduced the proportion of aggregates found in the nucleus (Fig. 5D). This reduction of nuclear aggregates seems critically dependent on the allosteric activation of Hsp70 with YM-1. Indeed, although heat shock alone suffices to induce Hsp70 (Fig. 4A), heat shock only reduces the proportion of nuclear aggregates when combined with YM-1 (Fig. 5D - Heat Shock). Moreover, as there was a corresponding increase in the proportion of cytosolic aggregates (Fig. 5D), these data suggest that allosteric Hsp70 activation with YM-1 retains mut-Htt in the cytosol, reducing its accumulation and aggregation in the nucleus.

Taken together, these findings indicate that treatment with the allosteric Hsp70 activator YM-1 increases NRF1 levels, promotes protein degradation and modifies the proteostasis of mutant N-terminal Htt, leading to aggregates with lower mut-Htt levels (less compact), and reducing their formation in the nucleus. The YM-1 internalization in mitochondria and the interference with Grp75-dependent protein import may explain increased NRF-1 levels [33, 34], enhancing proteasomal capacity and thus protein degradation. We next investigated whether and how treatment with YM-1 influenced the proteostasis of full-length Htt, in a cellular model without aggregation.

**3.4. YM-1 lowers mutant full-length huntingtin levels when Hsp70 is induced by heat shock**

To investigate whether YM-1 also modulates full-length Htt proteostasis, we tested YM-1 in a full-length HD model: cells with ponasterone A-inducible wild-type or mutant full-length Htt expression. After induction with ponasterone A, wild-type and mutant Htt expression increased over time, without formation of mut-Htt aggregates (Fig. 6A, B). In this full-length HD cell model, cells expressing mut-Htt presented higher levels of Hsp70, while cells expressing wild-type Htt presented similar levels of Hsp70 to non-induced cells (Fig. 6C). Accordingly, only mut-Htt co-immunoprecipitated with Hsp70 (Fig. 6D), indicating that the mutant full-length Htt interacts with Hsp70, increasing its levels.

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Similar to the N-terminal HD model, heat shock treatment increased the levels of Hsp70 in the full-length HD model (Fig. 7A, B). In cells expressing wild-type full-length Htt, YM-1 did not change the levels of ubiquitinated proteins or wild-type Htt (Fig. 7A, C, D). In cells expressing mutant full-length Htt, YM-1 reduced the levels of ubiquitinated proteins when cells were also subjected to heat shock treatment (Fig. 7A, C), which was the condition with the highest level of Hsp70 expression (Fig. 7B). Together with the reduction in ubiquitinated proteins, YM-1 treatment also decreased the levels of mutant full-length Htt (Fig. 7A, E). The key findings and proposed mechanisms are summarized in Fig. 8.

## 4. Discussion

The pathophysiology of HD is predominantly associated with a toxic gain-of-function of mut-Htt, which has a widespread distribution in the organism. Therapeutic strategies that decrease mut-Htt may benefit HD patients [2, 35]. Such strategies involve reducing mut-Htt production or increasing its clearance. Genetic approaches to reduce mut-Htt production have been tested in clinical trials, with a set of challenges related to invasive drug delivery to the central nervous system [1, 36]. Small molecule drugs that interact with key players of the proteostasis network and promote mut-Htt clearance constitute alternative or complementary therapeutic strategies for HD [37].

Htt is a client protein of Hsp70 [38, 39], which supports the study of Hsp70 modulation in HD [9]. Here we show that the N-terminal and full-length mut-Htt species expressed by the cellular HD models interact as clients with Hsp70 and increase its levels. Mechanistically, competition with increased client proteins releases the heat shock factor 1 (HSF1) from the latency control of Hsp70. Free HSF1 promotes the expression of Hsp70, in a compensatory feedback that protects cells from proteotoxic stress [40-43]. However, increasing Hsp70 levels may be insufficient to attenuate Htt proteotoxicity, particularly in advanced HD stages with a high misfolded protein load [44, 45], or the compensatory feedback may fail in cases of low HSF1 levels or activity [40, 46, 47]. This led us to investigate strategies to potentiate the activity of Hsp70, namely its allosteric activation.

Our data show that treatment with the Hsp70 allosteric activator YM-1 decreased the levels of full-length mut-Htt. YM-1 also reduced the clustering of N-terminal mut-Htt and the accumulation of aggregates in the nucleus. Mechanistically, YM-1 mimics the co-chaperone Hip [17, 48] increasing the affinity of Hsp70 for client proteins, which delays their release and enhances their ubiquitination by CHIP. Htt presents N-terminal lysine residues - mainly K6 and K9 - that can undergo the ubiquitination that targets Htt for proteasomal degradation [49, 50], decreasing its levels.

Regarding Htt aggregation, we propose that YM-1 reduces the levels of N-terminal mut-Htt in aggregates by preventing aggregation or promoting disaggregation. YM-1 can prevent aggregation by activating Hsp70 and thus prolonging its interaction with mut-Htt. Activated Hsp70 can promote disaggregation in cooperation with other chaperones that assemble a disaggregase complex [7]. Treatment with YM-1 also reduced the proportion of nuclear aggregates, which are linked with transcriptional dysregulation

[51]. Mechanistically, YM-1 retains mut-Htt in the cytosol through the binding to Hsp70. This retention by Hsp70 also predicts a reduction in the mut-Htt that is available to interact with nucleoporins and disrupt nuclear integrity [52, 53], thereby reducing mut-Htt access to and aggregation in the nucleus.

Mitochondria may have a role in the effects of YM-1. Although YM-1 is prevalent in cytosolic fractions [31], its cation nature predicts the internalization of YM-1 in the electronegative environment of mitochondria. Indeed, we show that YM-1 accumulates in mitochondria and increases the levels of the local Hsp70 isoform (Grp75) - a likely feedback response to Grp75 inhibition. Grp75 integrates the translocase of the inner mitochondrial membrane, contributing to the import and folding of nuclear-encoded mitochondrial proteins [54]. The binding of YM-1 to Grp75 may stabilize the ADP-bound form of this chaperone, delaying the release and import of substrates. Perturbations to mitochondrial protein import can trigger a transcriptional response that enhances proteasomal capacity and restores proteostasis, as shown in yeast and mammalian cells [34, 55]. In yeast, this response is mediated by the transcription factor Rpn4, in a protective process called UPR$^{am}$ (*unfolded protein response activated by mistargeting of proteins*) [34, 56]. In mammals, a similar process may be mediated by NRF1 (nuclear factor erythroid 2-related factor 1; not to be confused with nuclear respiratory factor 1), which executes comparable functions to Rpn4 as a master transcription factor of proteasome genes [33, 34, 57]. We thus propose that mitochondria-localized YM-1 may trigger a pathway that enhances proteasomal capacity, explaining the associated decrease in ubiquitinated proteins, and contributing to mut-Htt clearance. YM-1 could thus be a valuable pharmacological tool to investigate the currently emerging topic of inter-coordination of cytosolic and mitochondrial proteostasis.

Notwithstanding a potential mitochondrial-dependent contribution, the primary effects of YM-1 are dependent on Hsp70 levels. The dependency on Hsp70 levels was also reported for the co-chaperone Hip, which only facilitated protein refolding when co-overexpressed with Hsp70 [14]. Thus, Hsp70 levels are a predictive factor for the efficacy of Hip mimetic allosteric activators such as YM-1. As the levels of chaperones differ among organs and tissues affected by HD, YM-1 may be more potent in tissues with high levels of Hsp70 such as muscle, or when combined with Hsp70 elevating treatments such as Hsp90 inhibition [58, 59].

**5. Conclusion**

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Here we show that allosteric Hsp70 activation with YM-1 reduces full-length mut-Htt levels, and the clustering and nuclear aggregation of its N-terminal fragments. These findings extend previous knowledge of YM-1 effects on misfolding diseases, being tested here for the first time in HD models. Mechanistically, we propose that YM-1 increases the retention of mut-Htt by Hsp70, favouring its ubiquitination and proteasomal degradation. Additionally, we show that YM-1 targets mitochondria, where its interaction with the mitochondrial Hsp70 may interfere with the protein import to mitochondria, triggering a transcriptional response via NRF1 and activating protein clearance machinery. We propose that YM-1 could be an interesting pharmacological tool to investigate the coordination between mitochondrial and cytosolic proteostasis. As our results suggest that Hsp70 levels are a predictive factor of YM-1 efficacy, combined therapies with Hsp70 elevating strategies will likely be necessary to harness the full potential of allosteric Hsp70 activators.

**Declaration of Competing Interest**

The authors declare that they have no conflicts of interest.

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**Figure Legends**


**Figure 1. N-terminal huntingtin species interact with Hsp70 and increase its levels.**
Data are from U2OS cells at 24 h post-transfection with N-terminal Htt constructs: wild-type (wt-Htt; EGFP-Htt$^{ex1}$Q23) or mutant (mut-Htt; EGFP-Htt$^{ex1}$Q74). **(A)** Representative immunofluorescence showing nuclei (*blue;* Hoechst); N-terminal Htt in diffuse or aggregated form (*green*; AlexaFluor-488); mitochondria (*magenta*; MitoTracker Deep Red); and Hsp70 (*i - top*) or Grp75 (*ii - bottom*; mitochondrial Hsp70) in red (AlexaFluor-568). **(B)** Correlation analysis between the immunofluorescence intensity levels of N-terminal Htt (*i* - wild-type; *ii* - mutant) and Hsp70 (*red line*) or Grp75 (*black line*); a.f.u. - arbitrary fluorescence units; *$P$ < 0.05, $n$ = 200 cells per condition, from 4 independent experiments. **(C)** Immunofluorescence quantification: Hsp70 levels in cells expressing wt- or mut-Htt (*grey bars*) in percentage of control (*white bar*, without N-terminal Htt); *$P$ < 0.05, $n$ = 4 independent experiments, each with ≥ 50 cells per condition. **(D)** Western blots of complete protein samples (Input; *left*) and immunoprecipitated samples (IP; *right*; Pull-down with anti-GFP). Note that: anti-Htt-NT detects N-terminal wt-Htt, whereas anti-polyQ detects N-terminal mut-Htt; Hsp70 co-immunoprecipitates with both N-terminal Htt species.


**Figure 2. Mutant N-terminal huntingtin aggregates recruit Hsp70.**
Data are from U2OS cells at 24 h post-transfection with the mutant N-terminal Htt construct (EGFP-Htt$^{ex1}$Q74). **(A)** High-resolution immunofluorescence imaging of chaperones (Hsp70 or Hsp90; *red*) and mutant Htt aggregates (*green*) - shown with either full dynamic range (non-saturating) or partial dynamic range (to visualize fainter staining); white-dashed lines depict the nuclear borders; note how Hsp70 (but not Hsp90) levels increase specifically in the proximity of nuclear and cytosolic aggregates. **(B)** Representative images of a cell with aggregates (green) and Hsp70 immunofluorescence (red) along the Z axis - depth (**i**); the dashed line labeled X was used to derive fluorescence intensity profiles (**ii**); note how Hsp70 levels increase in proximity to aggregates. **(C)** Quantification of Hsp70 levels in cells expressing only diffuse N-terminal Htt (Diff; grey bars) *vs*. cells containing aggregates (Aggr; black bars); $n$ = 4 independent experiments, each with ≥ 50 cells; *$P$ < 0.05. Note that cells with aggregates show higher Hsp70 levels (**i**) with higher heterogeneity due to recruitment (**ii**; higher SD/mean).

**Figure 3. YM-1 shows concentration-dependent bioactivity and mitochondrial localization.**

**(A)** Chemical structure of the Hsp70 allosteric activator YM-1 (**i**); concentration-dependent effects of YM-1 on resazurin metabolism of U2OS cells (**ii**); data are mean ± SD in % control (solvent-treated cells). **(B)** Live imaging of U2OS cells transfected with the EGFP-Htt$^{ex1}$Q23 (green) and treated with YM-1 (3 μM, 24 h), and then incubated with the mitochondrial membrane potential indicator TMRM$^+$ (10 nM; red) for 30 min; Note that in cells incubated with TMRM$^+$ without YM-1 (solvent control) there is red TMRM$^+$ fluorescence (mitochondria), but no green fluorescence in non-transfected cells. When YM-1 is present, there is a green YM-1 autofluorescence in all cells, which overlaps with the red signal of TMRM$^+$ (mitochondria). **(C)** Representative images of U2OS cells treated with solvent or 3 μM YM-1 (without TMRM$^+$), imaged with 488 and 525 nm excitation to detect YM-1 autofluorescence in the green and red emission filters; note that permeabilization (0.1% Triton X-100, 30 min) completely removes the YM-1 green/red signals, while maintaining the nuclear staining with Hoechst (blue).

**Figure 4. Heat shock increases Hsp70, whereas YM-1 increases Grp75 levels.**

Data are from immunofluorescence assays with U2OS cells expressing wild-type (wt-Htt) or mutant (mut-Htt) N-terminal Htt, treated with solvent (white bars) or YM-1 (3 μM; blue bars), with or without heat shock (42 ºC, 1 h), as described in the Chaperone modulation section of Materials & Methods. **(A)** Hsp70 levels. **(B)** Grp75 - mitochondrial Hsp70 - levels. **(C)** MitoTracker Deep Red intensity levels - indicative of mitochondrial levels and polarization status; $n = 4$ independent experiments, each with $\geq$ 50 cells per condition. Factorial ANOVA: $^*P < 0.05$, heat shock effect; $^\#P < 0.05$, YM-1 effect; $^\S P < 0.05$, mut-Htt effect. **(D)** Representative images of Grp75 immunolabeling and MitoTracker staining in cells expressing N-terminal wt-Htt treated with solvent or 3 μM YM-1.

**Figure 5. YM-1 decreases the levels of ubiquitinated proteins, mutant N-terminal huntingtin levels in aggregates and their nuclear accumulation.**

Data are from U2OS cells expressing mutant N-terminal Htt, treated with solvent or YM-1 (3 μM; blue color), with or without heat shock (42 ºC, 1 h), as described in the Chaperone modulation section of Materials & Methods. **(A)** Ubiquitinated-proteins: **(i)** ubiquitinated proteins levels - in percentage of cells treated with solvent - quantified by

western blot; **(ii)** representative blot; *n* = 5 independent experiments; *P* < 0.05. **(B)** Representative image of cells showing mutant Htt (green) in diffuse form or aggregated; white-dashed lines delimit nuclei; arrows signal representative aggregate locations: nuclear, cytosolic, or in the nuclear-cytosolic interface. **(C)** Htt levels in aggregates quantified by the intensity of Htt staining (anti-GFP) by immunofluorescence - in percentage of the Htt levels from cytosolic aggregates of control cells (treated with solvent, without heat shock); *P* < 0.05 *vs*. respective aggregates in control cells; the left-pointing arrows denote that the boxplots in the heat shock condition (right) are statistically compared with the boxplots in the control condition (left; solvent without heat shock). **(D)** Proportion of aggregates per location: cytosolic, nuclear, or nuclear-cytosolic interface; *P* < 0.05, effect of YM-1 *vs*. solvent. **(C, D)** Data are from 4 independent experiments, each with ≥ 50 cells per condition.

**Figure 6. Mutant full-length huntingtin interacts with Hsp70 and increases its levels.** Data are from inducible PC12 cells expressing full-length huntingtin (wild-type: wt-Htt; mutant: mut-Htt). **(A-*i*)** Protocol: cells were treated with either solvent (control) or inducer (ponasterone A) for 4 days before protein extraction (a 1 day induction condition was included to assess the time-dependence of Htt expression). **(A-*ii*)** Representative immunofluorescence images of cells with anti-Htt-NT (green) and nuclei (blue) at 4 days post-induction. **(A-*iii*)** Representative western blots with anti-Htt-NT (stronger recognition of wt-Htt), anti-polyQ (stronger recognition of expanded polyQ in mut-Htt), and anti-Hsp70; Coomassie: loading control. **(B)** Levels of **(i)** wt- and **(ii)** mut-Htt, respectively quantified with anti-Htt-NT and anti-polyQ by Western Blot, at 1 and 4 days post-induction. *n* = 4 independent experiments. **(C)** Levels of Hsp70 quantified by western blot in control (non-induced; white bar) *vs*. induced cells (grey bars) at 4 days post-induction; *n* = 4 independent experiments; *P* < 0.05 *vs*. control. **(D)** Western blots of complete protein samples (Input; *left*) and immunoprecipitated samples (IP; *right*; Pull-down with anti-Hsp70); note that Hsp70 co-immunoprecipitated with full-length mut-Htt (detected with anti-polyQ), but not with wt-Htt (detected only in the Input with anti-Htt-NT).

**Figure 7. YM-1 decreases mutant full-length huntingtin and ubiquitinated proteins when Hsp70 is induced by heat shock.**

Data are from induced PC12 cells expressing full-length huntingtin (wild-type: wt-Htt; mutant: mut-Htt). Htt expression was induced with ponasterone A for 4 days; at 3 days post-induction, cells were treated with solvent or YM-1 (3 µM) for 24 h, with or without heat shock (42 ºC, 1 h), as described in the Chaperone modulation section of Materials & Methods. **(A)** Representative western blots with anti-Hsp70, anti-Htt-NT (stronger recognition of wt-Htt), anti-polyQ (stronger recognition of expanded polyQ in mut-Htt), and anti-ubiquitin; Coomassie: loading control. **(B)** Western blot quantification of Hsp70 levels; $n = 3$ independent experiments; Factorial ANOVA: $^*P < 0.05$, heat shock effect; $^\S P < 0.05$, mut-Htt effect. **(C)** Western blot quantification of ubiquitin-conjugated proteins (Ub-proteins); $n = 3$ independent experiments; Factorial ANOVA: $^\#P < 0.05$. **(D, E)** Western blot quantification of Htt levels; $n = 4$ independent experiments; Factorial ANOVA: $^\#P < 0.05$.

**Figure 8: Putative model of mutant huntingtin effects on cellular proteostasis and the mechanisms of action of YM-1. (A)** Mutant full-length Htt undergoes proteolysis and generates N-terminal fragments containing the expanded polyQ tract. N-terminal Htt fragments can oligomerize and form Htt aggregates [2, 3]. **(B)** N-terminal Htt fragments disrupt the nuclear membrane by interacting with nucleoporins [52, 53], facilitating the entry of N-terminal Htt to the nucleus where it aggregates. **(C)** The allosteric activator YM-1 inhibits the ATPase domain of Hsp70, stabilizing Hsp70 in its ADP-bound conformation, which has higher affinity for substrates. Thus, YM-1 delays the release of Hsp70 substrates, favouring their ubiquitination by CHIP and subsequent proteasomal degradation [17, 48]. **(D)** YM-1 accumulates in mitochondria where it interacts with the mitochondrial Hsp70 - Grp75 - a subunit of the translocase of the inner mitochondrial membrane (TIM) [54]. Stabilization of the Grp75 ADP-bound conformation by YM-1 slows down the substrate release, disturbing mitochondrial protein import, which can increase the levels of NRF1 (nuclear factor erythroid 2-related factor 1; not to be confused with nuclear respiratory factor 1) and induce proteasomal degradation [33, 34].

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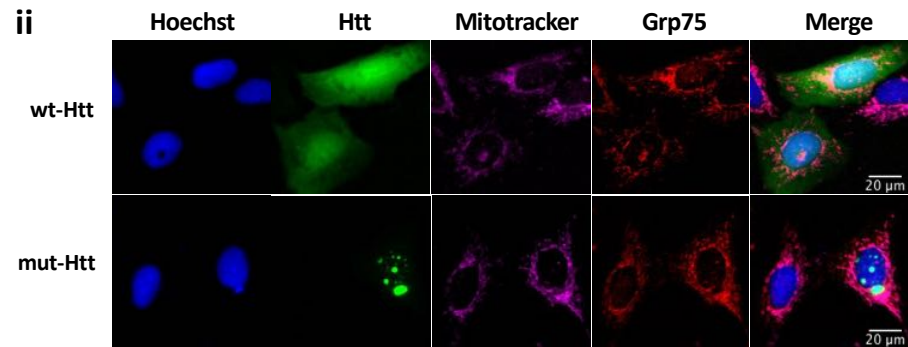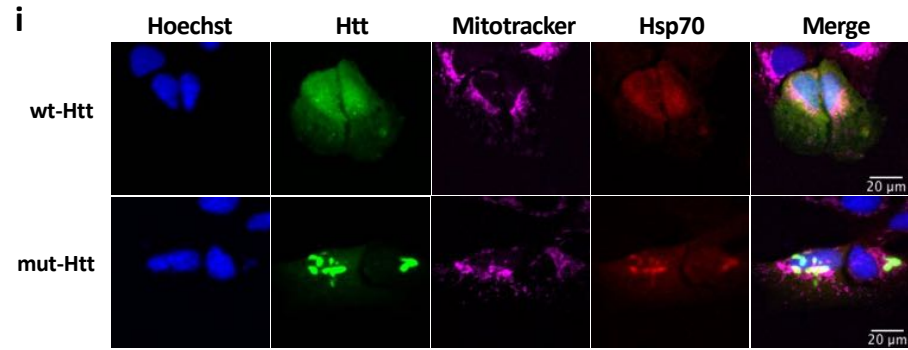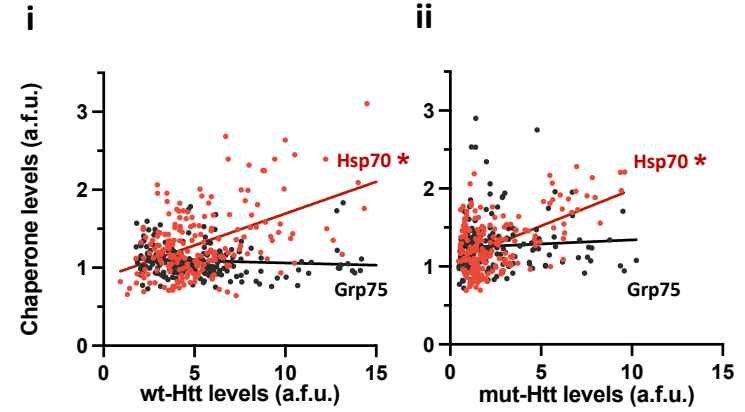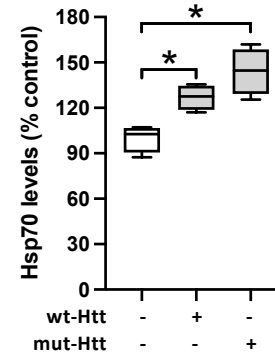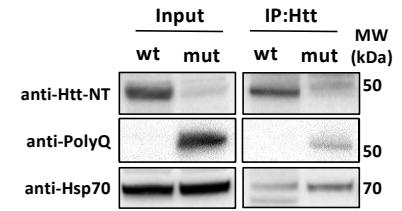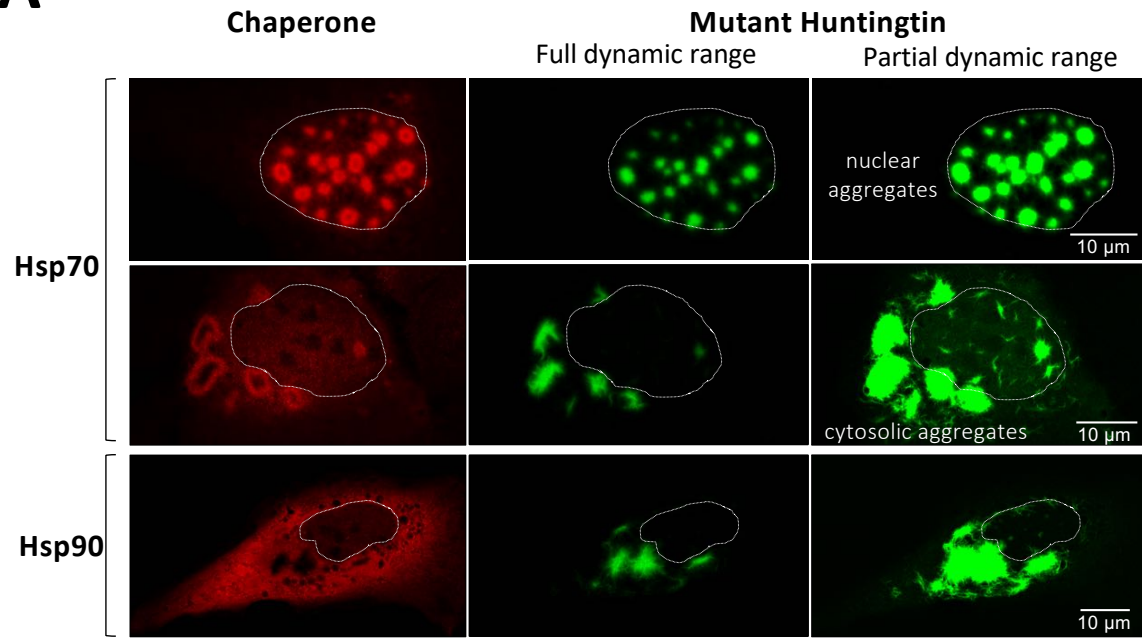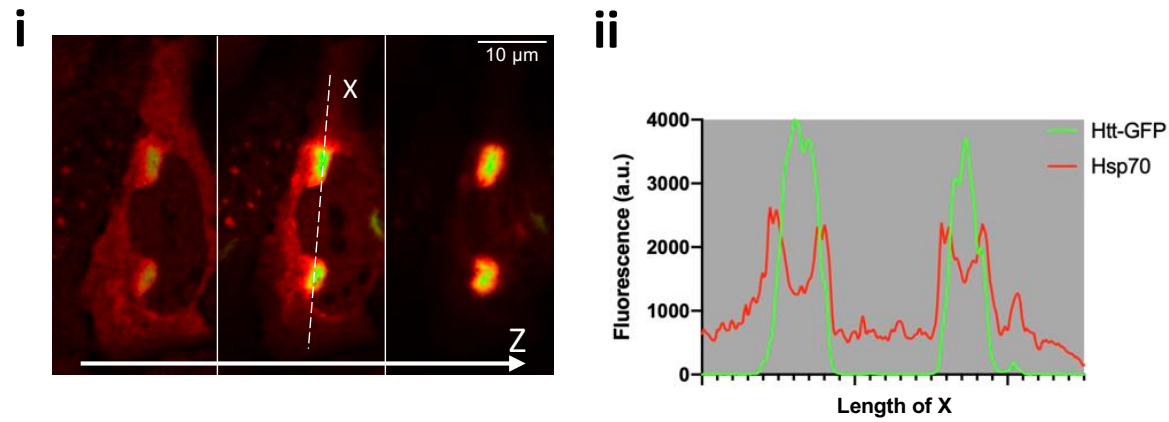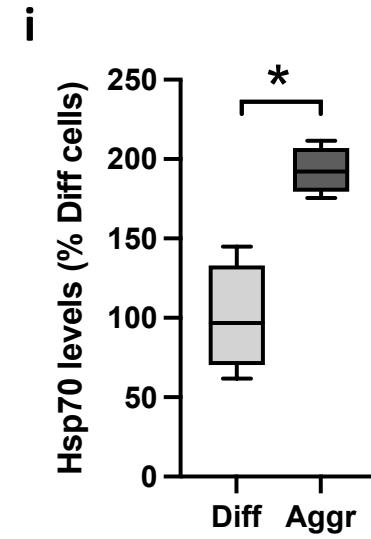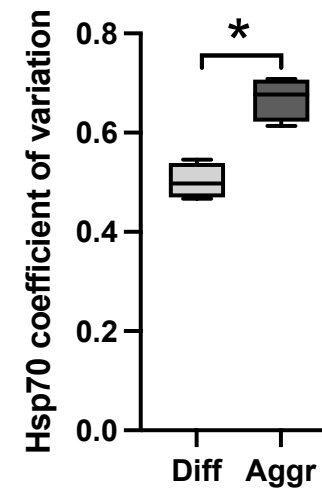
# Figure 1

**Figure 2**

**Figure 3**

# Figure 4

# Figure 5

**A**

**i**



**ii**



**B**



**C**



**D**

# Figure 6

## A

### i

| Induction (days) | Extraction time (days) |
|---|---|
| 0 | 4 |
| 1 | 1 |
| 4 | 4 |

■ Induction

Time (days)

0  1  2  3  4

### ii

| | Hoechst | Htt | Merge |
|---|---|---|---|
| wt-Htt | | | |
| mut-Htt | | | |

20 µm

20 µm

### iii

|  | wt-Htt | | | mut-Htt | | | |
|---|---|---|---|---|---|---|---|
| Induction (days) | 0 | 1 | 4 | 0 | 1 | 4 | kDa |
| Extraction time (days) | 4 | 1 | 4 | 4 | 1 | 4 | |
| Anti-Htt-NT | | | | | | | >225 |
| Anti-PolyQ | | | | | | | >225 |
| Coomassie | | | | | | | 85 |
| Anti-Hsp70 | | | | | | | 70 |
| Coomassie | | | | | | | 85 |

## B

### i

wt-Htt

Htt levels (% 1 day)

200
150
100
50
0

0  1  2  3  4  5

Time (days)

### ii

mut-Htt

Htt levels (% 1 day)

200
150
100
50
0

0  1  2  3  4  5

Time (days)

## C

Hsp70 levels (% control)

200
150
100
50
0

*

| wt-Htt | - | + | - |
|---|---|---|---|
| mut-Htt | - | - | + |

## D

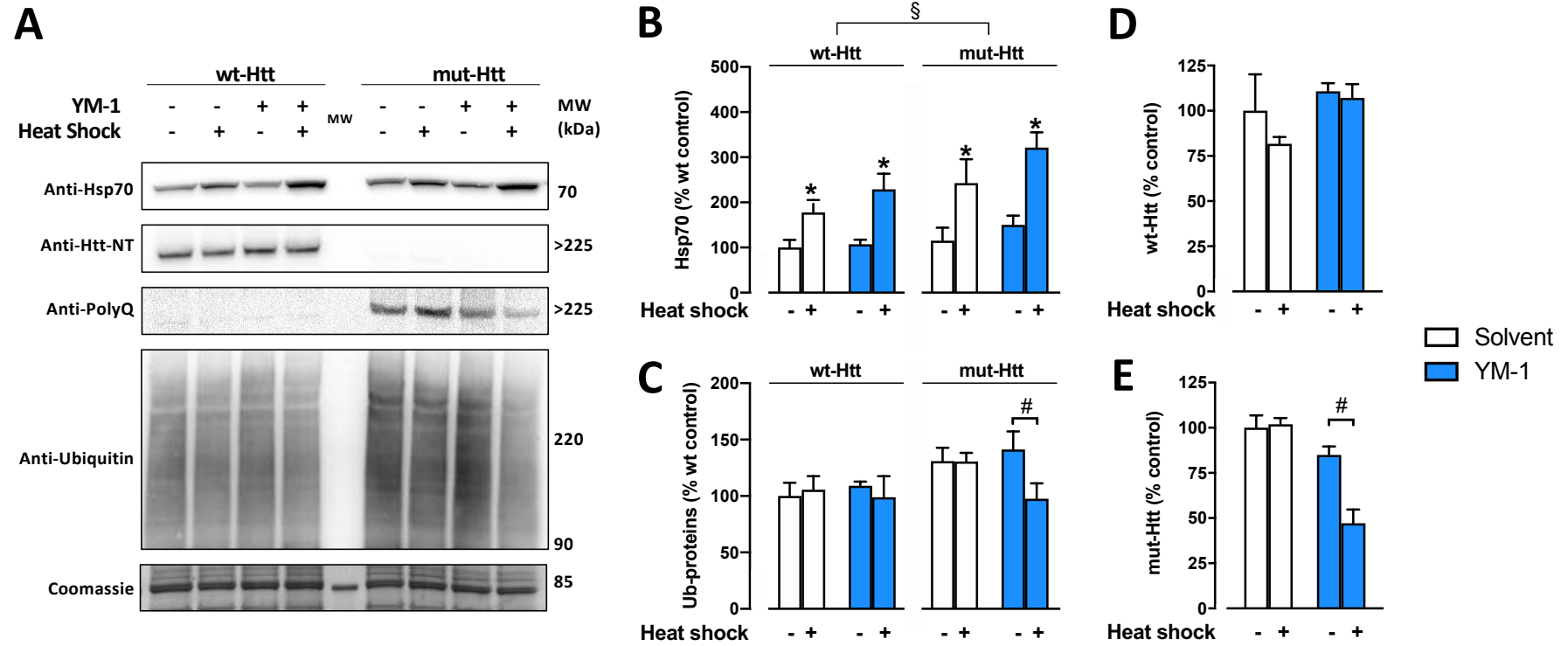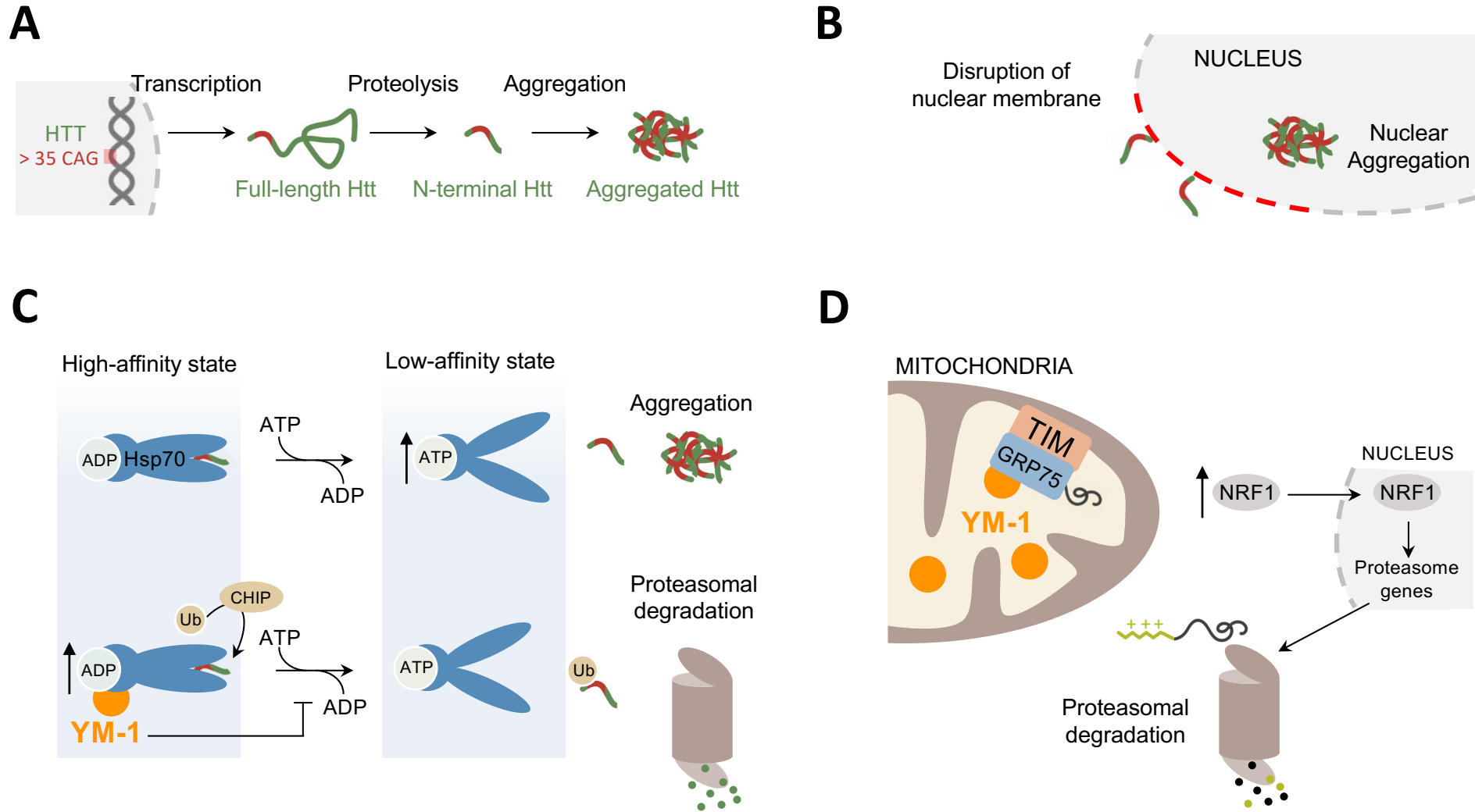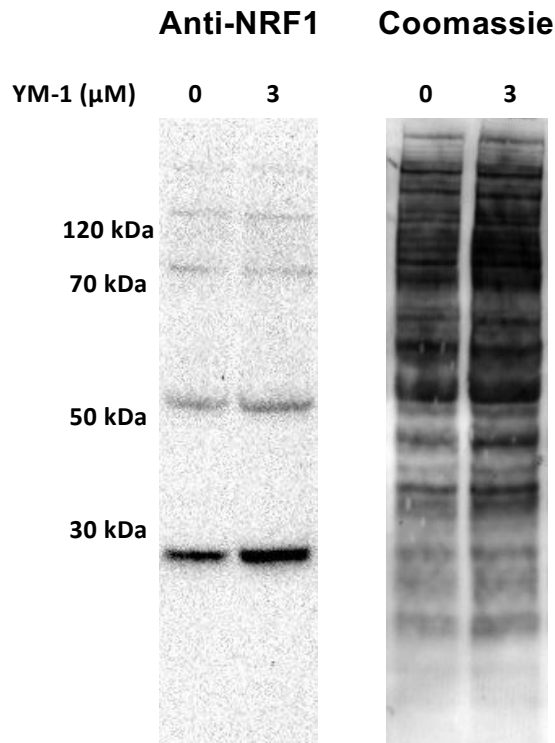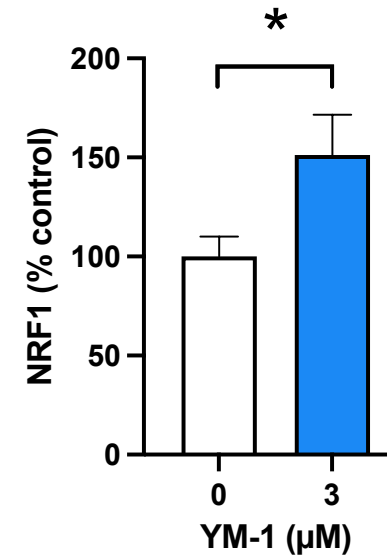| | Input | | IP: Hsp70 | | |
|---|---|---|---|---|---|
| | wt | mut | wt | mut | kDa |
| Anti-Hsp70 | | | | | 70 |
| Anti-Htt-NT | | | | | >225 |
| Anti-PolyQ | | | | | >225 |

## Figure 7

**Figure 8**

**Supplementary Figure 1**. **YM-1 increases the levels of NRF1.** PC12 cells with inducible expression of N-terminal huntingtin fragment containing 74 polyglutamine repeats (Wyttenbach et al. 2001; DOI: 10.1093/hmg/10.17.1829) were simultaneously treated with doxycycline (to induce huntingtin expression) and solvent/YM-1 for 48h. Levels of NRF1 (nuclear factor erythroid 2-related factor 1; not to be confused with nuclear respiratory factor 1) were quantified by western blot using the primary antibody anti-NRF1 (1:500; sc-28379; Santa Cruz Biotechnology) and membrane staining with coomassie was used for protein loading control. **(A)** Representative blot. **(B)** NRF1 levels (sum of detected NRF1 isoforms) in percentage of cells treated with solvent (control); $n = 3$ independent experiments; $*P < 0.05$, ratio paired $t$-test.