

1 **A biosensor to gauge protein homeostasis resilience differences in the nucleus compared to
2 cytosol of mammalian cells**

3 Candice B. Raeburn¹, Angelique Ormsby¹, Nagaraj S. Moily¹, Dezerae Cox¹, Simon Ebbinghaus², Alex
4 Dickson³, Gawain McColl⁴ and Danny M. Hatters^{1,*}

5 ¹Department of Biochemistry and Pharmacology; and Bio21 Molecular Science and Biotechnology
6 Institute, The University of Melbourne, VIC 3010. Australia

7 ²Physical and Theoretical Chemistry, TU Braunschweig, 38106 Germany and Braunschweig
8 Integrated Centre of Systems Biology, 38106 Germany

9 ³Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI,
10 48824, USA

11 ⁴Melbourne Dementia Research Centre, Florey Institute of Neuroscience and Mental Health and
12 University of Melbourne, Parkville, VIC 3052, Australia.

13 *Correspondence: dhatters@unimelb.edu (D.M. Hatters).

14

15 **Abstract**

16 An extensive network of chaperones and other proteins maintain protein homeostasis and guard
17 against inappropriate protein aggregation that is a hallmark of neurodegenerative diseases. Using a
18 fluorescence resonance energy-based biosensor that simultaneously reports on intact cellular
19 chaperone holdase activity and detrimental aggregation propensity, we investigated the buffering
20 capacity of the systems managing protein homeostasis in the nucleus of the human cell line HEK293
21 compared to the cytosol. We found that the nucleus showed lower net holdase activity and reduced
22 capacity to suppress protein aggregation, suggesting that the nuclear quality control resources are
23 less effective compared to those in the cytosol. Aggregation of mutant huntingtin exon 1 protein
24 (Httex1) in the cytosol appeared to deplete cytosolic chaperone supply by depleting holdase activity.
25 Unexpectedly, the same stress increased holdase activity in the nucleus suggesting that proteostasis
26 stress can trigger a rebalance of chaperone supply in different subcellular compartments.
27 Collectively the findings suggest the cytosol has more capacity to manage imbalances in proteome
28 foldedness than the nucleus, but chaperone supply can be redirected into the nucleus under
29 conditions of proteostasis stress caused by cytosolic protein aggregation.

30

31 **Introduction**

32 Protein homeostasis involves a network of quality control systems that ensures the proteome is
33 properly translated, folded, delivered to the correct cellular location and turned over at appropriate
34 times. When protein homeostasis becomes unbalanced, proteins become prone to misfolding
35 leading to their mislocalisation and accumulation as aggregates (1,2). The imbalance of protein
36 homeostasis is hypothesized to underlie the inappropriate protein misfolding and aggregation that
37 arise in the brain of patients with common neurodegenerative diseases including Alzheimer's,
38 Parkinson's, Huntington's and motor neuron diseases (3,4). Tools that can measure proteostasis
39 imbalance therefore offer capacity to explore the mechanisms involved.

40 Previously we developed a method that enabled a measure of the effectiveness of the quality
41 control system in maintaining protein homeostasis (5). The method involved the use of a biosensor
42 that comprised of a model protein that engages with protein quality control machinery such as
43 chaperones. The biosensor reported on amount of the model protein bound to quality control
44 proteins (which we call hereon call holdase activity) and on the ability of quality control proteins to
45 repress inappropriate protein aggregation of the model protein. The model protein was a
46 catalytically inactive variant of the prokaryotic RNase barnase. Unfolded barnase is both permissive
47 to aggregation and able to bind to Hsp70 and Hsp40 family chaperones (6). Barnase folding
48 resembles a 2-state mechanism and the proportion of unfolded barnase relative to folded barnase
49 can be predictably tuned by mutation (7). Hence a panel of barnase variants enable different
50 biosensors tuned to different ratios of folded versus unfolded proteins. When the biosensor is
51 expressed in cells the proportion of folded proteins can be measured by fluorescence resonance
52 energy transfer (FRET) through N- and C-terminal fusions to fluorescent protein donors and
53 acceptors (**Fig 1A**). We previously showed that the abundances of unfolded barnase is increased in
54 cells relative to that predicted by analysis of purified proteins due to quality control machinery
55 forming complexes with the unfolded-like conformations of the biosensor and partitioning it from
56 the equilibrium of folding (6). Therefore, alterations in quality control levels influence the total
57 abundance of unfolded-like barnase, which we can detect by FRET, and therefore determine
58 changes in the overall quality control supply available as a measure of proteostasis capacity.

59 The amount of biosensor aggregation can also provide a measure of overall chaperone activity. Our
60 prior work devised a strategy that quantitatively measured aggregation as a complementary
61 approach to foldedness (6). Here, we applied our biosensor system to examine how proteostasis
62 balance is affected specifically within the nucleus and cytosol. We examine these local proteostasis
63 changes that result from different triggers of stress either globally to the cell or locally within the
64 cytoplasm or nucleus.

65

66 **Results**

67 **Generation of nuclear targeted biosensors and validation of folding stabilities**

68 The biosensor system comprises a suite of constructs whereby the barnase moiety has been
69 mutated to display different standard free energies of folding (ΔG^0_F), which define the
70 thermodynamic equilibrium of folding K_f (**Fig 1B**). These constructs contained a nuclear export
71 sequence (NES), which leads to them being restricted to a cytosolic localization (6). To direct the
72 constructs to the nucleus we removed the NES and fused a nuclear localization sequence (NLS) from
73 the SV40 protein to the N-terminus (**Fig 1A**; **Table 1** for sequences used). The biosensor containing
74 wild-type* (WT*) barnase (which is marked with * to denote it contains the catalytic inactivation
75 mutation H102A that is used in all our constructs) was efficiently targeted into the nucleus (**Fig 1C**).
76 All mutants of barnase showed a similar result (not shown).

77 Because the NLS and NES could themselves affect the folding equilibrium, we measured their effect
78 on folding by a urea denaturation curve which showed no noticeable difference (**Fig 1D**). Hence, we
79 concluded that the biosensors with NLS and NES are amenable to directly measure and compare
80 protein homeostasis balances between the cytosol and nucleus.

81 The strategy to monitor both the abundances of unfolded barnase and aggregation behaviour
82 involved a flow cytometry protocol we previously developed (6). In essence, cells expressing the
83 biosensor bifurcate into two distinct FRET populations when cells are gated on acceptor
84 fluorescence versus donor fluorescence. The donor fluorescence levels are proportional to
85 expression level and FRET and both are approximately linearly dependent on the acceptor
86 fluorescence. One of the populations comprises a “lower slope” FRET population that contains cells
87 with only soluble barnase biosensor (i.e. a mixture of medium and low FRET states; **Fig 1A and E**),
88 whereas the other contains an “upper slope” FRET population, which contains cells with aggregated
89 biosensor (i.e. dominated by high FRET states) (6). The gradient of the lower slope population is
90 proportional to the actual FRET value and hence informative to the abundance of unfolded barnase
91 versus folded barnase (i.e. the average signal of low and medium FRET states from **Fig 1A**). We had
92 previously shown that quality control machinery such as Hsp70 and Hsp40 proteins HSPA8 and
93 DNAJB1, respectively, can bind to unfolded biosensor and hold it in an unfolded-like state that has a
94 low FRET signal (6) as summarized conceptually in **Fig 1A**. This binding creates a pool of chaperone-
95 bound biosensor that is partitioned from the equilibrium of folding. Greater partitioning leads to
96 lower FRET signals, which can thus be used as a measure of the capacity of the quality control
97 system to engage with the biosensor.

98 First, we examined whether the cytosolic and nuclear environments differentially affected the FRET
99 readouts. This was achieved by expressing a FRET construct in which barnase was replaced with a
100 short linker sequence that was not expected to be affected by changes in conformation or other
101 ligand binding events. As such this linker should render the biosensor insensitive to folding-related
102 effects and hence measures off-target influences on FRET signal as previously described (6). The
103 linker control revealed a small (1.7%) but significant ($p < 0.0001$, Student's t-test; 2-tailed) decrease
104 in FRET in the nucleus compared to the cytosol between the NLS and NES tagged variants (**Fig 2A**).
105 To correct for this influence, all the subsequent analyses involving the barnase biosensors were
106 corrected for differences using the NLS and NES linker construct controls.

107 Next, we examined the effectiveness of protein quality control systems to interact with the
108 biosensor in the nucleus versus the cytosol. For this we examined four previously characterized
109 mutants of barnase in addition to wild-type* that have variable ΔG^0_F values and therefore different
110 proportions of folded to unfolded barnase at equilibrium (**Fig 1B**). After correction for off-target
111 FRET changes with the linker control, all nuclear-targeted biosensor variants had overall significantly
112 higher FRET values for the lower slope populations than the cytosol-targeted biosensors, except for
113 the most destabilised variant (I25A I96G), which is predicted to be substantially unfolded and
114 therefore possibly outside the dynamic range that can be detected (**Fig 2B**). The higher FRET values
115 in the nucleus are therefore indicative of less unfolded-like barnase conformations being held in
116 complex with chaperones that would otherwise be partitioned from the equilibrium of folding. The
117 results therefore suggested that the pool of chaperones that can bind to the biosensor is lower in
118 the nucleus than the cytosol.

119 To examine the aggregation propensity of the barnase biosensors, we applied our previously devised
120 method of determining the concentration of barnase at which 50% of the cells contain aggregates
121 ($A_{50\%}$) (6). $A_{50\%}$ values are derived from plots of the proportions of cells partitioning in the upper
122 slope for a given expression level of barnase in cells (**Fig 2C-D**). Consistent with prior findings (6),
123 WT* barnase did not aggregate and the less stable mutants (i.e. those with higher ΔG^0_F values) were
124 more sensitive to aggregation as determined by lower $A_{50\%}$ values (**Fig 2D**). For all the barnase
125 variants that aggregated, the $A_{50\%}$ values were lower in the nucleus compared to the cytosol (**Fig 2C-D**). These results indicated that barnase is inherently more aggregation prone in the nucleus than
127 the cytosol, and therefore strengthens the conclusion that there are less quality control proteins in
128 the nucleus that are able to bind to and stabilize barnase and prevent aggregation.

129 **Hsp70 and Hsp40 chaperone systems more robustly mitigate unfolded proteins from aggregating**
130 **in the cytosol than the nucleus**

131 Hsp70 isoforms HSPA1B and HSPA8 were previously found in immunoprecipitation experiments as
132 major chaperone interactors to the destabilized barnase mutants (6). We found that related Hsp70
133 family member HSPA1A could modulate both the amount of unfolded-like barnase and the amount
134 of barnase aggregation, suggesting it could bind to and stabilize an unfolded-like conformation of
135 barnase (6). While these Hsp70 isoforms are highly abundant in the cytosol (8), it was unclear as to
136 how modulating their supply might propagate changes in the nucleus or cytosol.

137 To examine this question, we co-transfected HSPA1A and a specific Hsp40 cofactor DNAJB1 (9) with
138 the barnase biosensors and analysed the cells after 48 hours culture. The transfected HSPA1A and
139 DNAJB1 showed a mostly cytosolic enrichment (**Fig 3A**). The co-transfected chaperones significantly
140 reduced the biosensor lower slope gradients in the cytosol (**Fig 3B**), consistent with a greater
141 abundance of chaperone bound to unfolded barnase. The treatments also increased the $A_{50\%}$ values
142 indicating that the chaperones effectively suppressed inappropriate aggregation (**Fig 3C**). However,
143 these effects appeared more muted in the nucleus indicating that chaperone overexpression
144 preferentially deepens the pool of chaperone supply in the cytosol, which likely is explained by the
145 transfected chaperone being mostly restricted to the cytosol.

146 To further probe the role of Hsp70 activity we inhibited Hsp70 on cells (without overexpressed
147 chaperones) using the small molecule inhibitor VER-155008, which competitively binds to the ATP-
148 binding pocket of Hsp70 family proteins and impairs substrate binding (10). This treatment increased
149 the FRET values of the lower slope populations in the nucleus but not the cytosol (**Fig 3D**). This result
150 suggested that while Hsp40 and 70 proteins are more effective at binding barnase in the cytosol,
151 there was higher redundancy and flexibility in the cytosol to absorb a reduced Hsp70 activity than in
152 the nucleus. Hence the network appeared more vulnerable to collapse in the nucleus upon stresses
153 to proteostasis systems. However, the increased sensitivity to proteostasis imbalance in the nucleus
154 was not seen in terms of aggregation. Indeed, aggregation of the biosensor was far more
155 disproportionately enhanced in the cytosol than the nucleus (**Fig 3E**). These findings suggested that
156 the correlation of holdase activity and aggregation can be decoupled when specific elements of the
157 proteostasis network are impaired and that this effect may arise through redundant holdase activity
158 in the cytosol from non-Hsp70 chaperones that are overall less effective at preventing aggregation
159 than Hsp70.

160 **Aggregation of mutant Htt exon 1 in the cytosol propagates proteostasis imbalances in the cytosol
161 and nucleus**

162 Next we investigated the quality control supply in the nucleus and cytosol in the context of disease-
163 related protein misfolding and aggregation. For this we co-expressed the biosensors with mutant

164 Huntington exon 1 fragment containing 97 glutamines in the polyglutamine repeat sequence
165 (Httex1_{97Q}) fused to GFP, which forms cytosolic perinuclear inclusions in HEK293T cells (11). Mutant
166 Httex1 fragments with polyglutamine sequences longer than 36 glutamines accumulate into
167 inclusion bodies in neurons of Huntington Disease patients and have been implicated to direct a
168 maladaptation in protein quality control (12). Because the fluorescence of GFP interferes with our
169 capacity to monitor FRET, we used a variant of GFP that is non-fluorescent as characterized
170 previously (13) and also added a 6 amino acid tetracysteine motif for post hoc detection by ReAsH
171 biarsenical dye binding (14). Live cells were examined for inclusions, which were detectable as
172 spherical pearl-like structures under transmission imaging of confocal microscopy, imaged for FRET
173 and then post hoc analyzed by ReAsH staining to validate the inclusion structure. Because we
174 needed to fix the cells after imaging, which reduces ReAsH staining, we were not able to ascertain
175 cells containing only diffuse cytosolic Httex1_{97Q}. Using this approach, we observed the barnase
176 biosensor as enriched at the periphery of the inclusions suggesting a degree of co-aggregation or co-
177 recruitment to the inclusions (**Fig 4A**). This was both true for the WT* barnase biosensor, which does
178 not aggregate by its own volition, and for the nucleus-targeted biosensors suggesting that the
179 biosensors were kinetically trapped on the surface of the Httex1 inclusion. To assess whether the
180 biosensor was self-aggregated at the molecular scale we determined their FRET using a ratiometric
181 analysis of the fluorescence (**Fig 4A**). Indeed, the biosensor enriched at the inclusion periphery
182 appeared to have higher FRET than when more distal from the inclusions in either the nucleus or
183 cytosol. We further assessed the aggregation state using fluorescence recovery after photobleaching
184 (FRAP) (**Fig 4B**). A small section of the biosensor was targeted for bleaching on the periphery of the
185 Httex1 inclusion. Both nucleus and cytosol targeted biosensors showed little to no recovery after
186 bleaching, indicating the protein was in an immobile state on the seconds timescale (**Fig 4C**).
187 Because the aggregation of the biosensor at the periphery of the inclusion is a confounding factor in
188 whole cell fluorescence analysis by our flow cytometry methods, we instead measured FRET by
189 microscopy targeting small subregions of the cells away from the inclusions. This analysis revealed
190 that the presence of Httex1_{97Q} inclusions had no bearing on the FRET of the WT* barnase biosensor
191 outside that associated with the inclusion periphery (**Fig 4D**). By contrast, the I88G barnase
192 biosensor showed significantly changed FRET values regions outside the Httex1_{97Q} inclusions
193 compared to cells without inclusions at all. In the case of the I88G barnase biosensor targeted to the
194 cytosol, the FRET was increased, which suggested a reduced overall holdase activity of chaperones in
195 the cytosol arising from Httex1_{97Q} aggregation. However, in the case of the I88G barnase biosensor
196 targeted to the nucleus the FRET was decreased. This result is consistent with an elevated holdase
197 activity in the nucleus when Httex1_{97Q} aggregates form. This result therefore suggests when protein

198 aggregation occurs in the cytosol that cells can move the pool of quality control machinery from the
199 cytosol into the nucleus as part of a global coordinated stress response.

200

201 **Discussion**

202 Our studies show that the balance of resources required to manage proteostasis is different in the
203 cytosol relative to the nucleus. We find evidence for there being a lower supply of chaperone
204 capacity in the nucleus that is able to bind to the unfolded barnase and prevent its aggregation.

205 When we supplemented the cells with additional Hsp70 and Hsp40 protein by their overexpression
206 (HSPA1A and DNAJB1), we increased holdase activity in the cytosol and lowered the aggregation
207 potential consistent with these chaperones exerting a critical activity to bind unfolded proteins and
208 prevent their aggregation. When we pharmacologically inhibited the Hsp70 chaperone system we
209 observed a disproportionate impact on aggregation in the cytosol, concordant with the cytosol being
210 more richly dependent on Hsp40 and Hsp70 chaperone-based networks to prevent protein
211 aggregation.

212 Overall, these results are consistent with the greater requirement of the Hsp70 chaperone system to
213 engage with unfolded or aggregation-prone proteins in the cytosol. This finding is consistent with
214 the high abundance of these chaperones in the cytosol (8), which is not surprising given that most
215 proteins are synthesized in the cytosol or endoplasmic reticulum.

216 Our findings with mutant Httex1_{97Q} indicate that the aggregation in the cytosol can manifest
217 dysfunction in quality control capacity in both the cytosol and nucleus. Consistent with prior findings
218 that protein aggregation can sequester quality control resources away from “housekeeping”
219 activities and lead to metastably-folded proteins aggregating (15), we found that the pool of
220 resources binding to unfolded barnase biosensor decreased in the cytosol. Prior studies have found
221 that Hsp70 and Hsp40 proteins are recruited into inclusions formed by Httex1_{97Q} and similar proteins
222 with expanded polyglutamine sequences (16-19). One function for this recruitment may be
223 disaggregation, in light of recent findings showing the Hsp70 based chaperone machinery can
224 dissociate amyloid fibrils (20). More unexpected however was the finding that there was an increase
225 in unfolded barnase in the nucleus, which suggests that chaperones are redirected into the nucleus
226 under stress. Hsp70 is known to translocate from the cytosol into the nucleus upon heat shock (21-
227 23), suggesting there is a dynamic capacity for quality control machinery activity in the nucleus
228 under times of stress. This translocation is regulated by the Hikeshi nuclear import carrier, which is
229 crucial for cells to recover from heat shock stress (24). DNAJB1 can also deliver misfolded protein
230 into the nucleus for degradation (25).

231 The other notable result from our study was the recruitment of WT* barnase biosensor to the
232 Httex1_{97Q} inclusion. We have never observed the wild-type* biosensor to aggregate when expressed
233 on its own suggesting that the inclusion provides a mechanism to recruit this protein to the surface.
234 One interesting possibility is that a small fraction of the biosensor remains in complex with
235 chaperones; and that these complexes are recruited to the surface of the inclusion by Hsp70 -based
236 triage mechanisms that more generally handle misfolded proteins in the cell. The different extent of
237 WT* biosensor foldedness in the nucleus compared to the cytoplasm supports the conclusion that
238 some of the wild-type barnase is partitioned from the equilibrium of folding in an unfolded-like
239 state. Discrete bodies containing misfolded protein including the JUNQ, aggresome and Q-bodies
240 have been proposed as cellular depots for processing protein aggregates, and are enriched with
241 different Hsp70 and Hsp40 proteins (26,27). In addition, Hsp70 has been proposed to engage with
242 the surface of protein aggregates to act as a disaggregase (20). Hence, the capture of wild-type*
243 biosensor may be indicative of a wider network of chaperone client interactions, protein aggregate
244 bodies in the cell and a broader interconnected quality control network. And thus chaperones may
245 have a broader function as a kind of lubricant constantly interfacing with unfolded proteins and
246 aggregating proteins.

247 **Materials and Methods**

248 **Expression constructs.** The cytosolic FRET barnase biosensor library expressed in the pTriEx4 vector
249 were prepared as previously described (5). In brief, the barnase moiety was flanked by circularly
250 permuted mTFP1 cp175 and Venus cp173 fluorescent proteins. Nuclear localised FRET barnase was
251 generated by the addition of a N-terminal SV40 NLS sequence to the original cytosolic barnase using
252 a synthesized gene cassette containing the relevant localization sequences (GeneArt (Thermofisher),
253 Waltham, Massachusetts) and standard restriction endonuclease-based ligation methods. For
254 generation of individual mutants of targeting biosensor, the WT* barnase biosensor kernel was
255 replaced by the barnase mutant of choice. This was achieved by double-digestion of both the
256 desired barnase mutant and nuclear targeting construct plasmids at BamHI and KpnI restriction
257 sites. The tetracysteine tagged Httex1 construct containing a tetracysteine tag at the C-terminus of
258 the Httex1 (TC1 (28)), and a non-fluorescent mutant of Emerald fluorescent protein (Em), Y66L (13),
259 was generated in-house to yield a plasmid named Httex1_{97Q}TC1-Em Y66L in the pT-Rex vector
260 (Invitrogen). The pT-Rex Em Y66L construct alone was also generated in-house as described
261 previously (13). V5-tagged chaperone proteins were overexpressed from pcDNA5/FRT/TO V5
262 DNAJB1 and pcDNA5/FRT/TO V5 HSPA1A provided as gifts from Harm Kampinga (29).via Addgene,
263 Watertown, Massachusetts.

264 **Cell culture.** HEK293T cells were maintained in DMEM supplemented with 10% (w/v) fetal calf
265 serum and 1 mM glutamine in a 37°C humidified incubator with 5% v/v atmospheric CO₂. Cells were
266 seeded in poly-L-lysine coated plates. For microscopy experiments cells were plated at 3 × 10⁵
267 cells/ml in an 8 well μ-slide (Ibidi, Martinsreid, Germany). For flow cytometry experiments cells were
268 seeded at 1.1 × 10⁵ cells/ml in a 48 well plate. Cells were transiently transfected with Lipofectamine
269 3000 reagent as per manufacturer's instructions (Life Technologies, ThermoFisher). For Barnase and
270 Httex1 co-transfections, the transfection was done in a way to decouple the expression of the two
271 plasmids.

272 HSP70 was inhibited with 20 μM VER-155008 (cat #SML0271, Sigma-Aldrich, St. Louis, Missouri) in
273 culture media for 18 h.

274 **Microscopy.** Cells were imaged on a TCS SP5 confocal microscope (Leica Biosystems, Nussloch,
275 Germany). For immunofluorescence, cells were fixed in 4% w/v paraformaldehyde for 15 mins at
276 room temperature, washed with phosphate buffered saline (PBS), and permeabilized in 0.5% v/v
277 Triton X-100 in PBS (Sigma-Aldrich) for 30 mins. After incubation in blocking solution (5% w/v bovine
278 serum albumin in 0.3% v/v Triton X-100 in PBS), cells were incubated with anti-V5 (1:250 dilution in
279 1% w/v bovine serum albumin in 0.3% v/v Triton X-100 in PBS) (cat #ab27671, Abcam, Cambridge,
280 United Kingdom) overnight at 4°C. Cells were then washed in 1% w/v ovine serum albumin in 0.3%
281 v/v Triton X-100 in PBS before being stained with anti-mouse cy5 (1:500 dilution in PBS) for 30 min
282 at room temperature. Prior to confocal imaging, cells were stained with Hoechst 33342
283 (ThermoFisher).

284 **Image analysis.** Confocal images were analysed using custom analysis scripts for FIJI (30) and
285 Python (v 3.6.7), available alongside example datasets at doi.org/10.5281/zenodo.4686851.

286 In the case of immunofluorescence measurements, whole cells and nuclei were initially identified
287 using the machine learning package CellPose (31). Segmentation was performed on the Cy5-labelled
288 anti-V5 antibody and Hoechst channels (633 nm excitation, 650-750 nm emission and 405 nm
289 excitation, 410-450 nm emission respectively) to identify the whole cell and nuclei regions of
290 interest (ROI) respectively. Per-pixel information for each ROI was then collected and the nuclei ROIs
291 removed from the whole cell to yield the cytosolic population. Finally, the fluorescence intensity for
292 the nucleus and cytosol was determined from the mean of all pixels in each compartment.

293 To quantify fluorescence recovery after photobleaching (FRAP), ROI for individual bleach spots were
294 defined via automatic Otsu thresholding of the first bleaching frame. Identical ROI's were then
295 manually placed for the adjacent (non-bleached) and background regions. Where necessary, ROI
296 positions were manually adjusted across timepoints to account for cellular drift. The mean intensity

297 was calculated for each ROI, and both bleached and non-bleached ROIs were then corrected against
298 the corresponding background ROI for each time point, generating B_{corr} and NB_{corr} respectively. The
299 ratio of B_{corr} / NB_{corr} at each time point was finally normalised to the pre-bleach ratio of B_{corr} / NB_{corr}
300 to yield the relative recovery.

301 In the case of FRET measurements, whole cells, nuclei and Httex1 inclusions were initially identified
302 using CellPose (31) as described above. In this case, segmentation was performed on the acceptor
303 channel (488 nm excitation, 510–560 nm emission), computationally inverted acceptor channel and
304 ReAsH channel (561 nm excitation, 610–680 nm emission) for whole cells, nuclei and inclusions
305 respectively. After manual inspection to ensure the accuracy of each round of segmentation, per-
306 pixel intensity values for each ROI were collected. In the case of cytosolic barnase variants, both the
307 nuclei and aggregate features were excluded from the whole cell to yield the diffuse barnase
308 population. In the case of nuclear barnase variants, any aggregate ROI within the nuclei ROI were
309 similarly excluded to yield the diffuse barnase population. Finally, the relative FRET for each ROI was
310 calculated as the mean per-pixel intensity in the FRET channel (458 nm excitation, 510–560 nm
311 emission).

312 **Flow cytometry.** After 24 h (drug treatments) or 48 h (co-transfections) post-transfection, cells were
313 washed and harvested by gentle pipetting in PBS. Cells were analysed via flow cytometry as
314 described previously (6). In short, 150 μ l of cell suspension was analysed at flowrate of 3 μ l/sec on a
315 BD LSRFortessa cell analyser (BD Biosciences, North Ryde, NSW, Australia). Acceptor (Venus)
316 fluorescence was collected with the 488 nm laser and FITC (530/30) filter. Acceptor sensitized
317 emission (FRET) and donor (mTFP1) fluorescence were collected with the 405 nm laser with PE
318 (575/25) and V500 (525/50) filters, respectively. All flow cytometry data were processed with FlowJo
319 (version 10, Tree Star Inc, Ashland, Oregon) to exclude cell debris, cell aggregates and untransfected
320 cells. The Venus channel was compensated to remove bleed through from mTFP1 and FRET channels
321 using donor only. Data were analysed in MATLAB (version 9, MathWorks, Natick, Massachusetts).
322 The gating strategy and associated data analysis protocols are detailed previously (32).

323 **Urea denaturation curves.** Urea denaturation curves were measured on cell lysates expressing the
324 biosensors in 96 well format. In essence, 80 μ l of samples were prepared containing 0 M to 8 M urea
325 in PBS. Lysates were prepared from cells 24 h after transfection (wild-type* with NES and NLS tags)
326 by pipetting in 20 mM Tris pH 8.0, 2 mM MgCl₂, 1% v/v Triton X-100, 1 \times EDTA-free protease inhibitor
327 (Roche, Basel, Switzerland), 150 mM NaCl, 20 U ml⁻¹ benzonase, 1 mM PMSF. Aggregates and cell
328 debris were pelleted by centrifugation at 16,000 g for 10 min at 4 °C. 5 μ l supernatant was added to
329 each urea concentration. As the measurements were ratiometric and both fluorophores were on the
330 same molecule, samples were not matched for protein concentration. Fluorescence readings

331 (430 nm excitation, 492 nm emission and 532 nm emission) were measured at 23 °C using a
332 Clariostar microplate reader (BMG Labtech, Mornington, Victoria, Australia) every 15 min for 4 h.
333 Relative FRET efficiencies (calculated as Acceptor fluorescence/[Donor fluorescence + Acceptor
334 fluorescence]) were averaged across readings and fit to a two-state unfolding model as described
335 previously (6).

336

337 **References**

- 338 1. Schneider, K., and Bertolotti, A. (2015) Surviving protein quality control catastrophes--from
339 cells to organisms. *J. Cell Sci.* **128**, 3861-3869
- 340 2. Knowles, T. P., Vendruscolo, M., and Dobson, C. M. (2014) The amyloid state and its
341 association with protein misfolding diseases. *Nat Rev Mol Cell Biol* **15**, 384-396
- 342 3. Bertram, L., and Tanzi, R. E. (2005) The genetic epidemiology of neurodegenerative disease.
343 *J. Clin. Invest.* **115**, 1449-1457
- 344 4. Hipp, M. S., Park, S. H., and Hartl, F. U. (2014) Proteostasis impairment in protein-misfolding
345 and -aggregation diseases. *Trends Cell Biol.* **24**, 506-514
- 346 5. Wood, R. J., Ormsby, A. R., Radwan, M., Cox, D., Sharma, A., Vöpel, T., Ebbinghaus, S.,
347 Oliveberg, M., Reid, G. E., Dickson, A., and Hatters, D. M. (2018) A biosensor-based
348 framework to measure latent proteostasis capacity. *Nature Communications* **9**, 287
- 349 6. Wood, R. J., Ormsby, A. R., Radwan, M., Cox, D., Sharma, A., Vöpel, T., Ebbinghaus, S.,
350 Oliveberg, M., Reid, G. E., Dickson, A., and Hatters, D. M. (2018) A biosensor-based
351 framework to measure latent proteostasis capacity. *Nat Commun* **9**, 287
- 352 7. Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A., and Fersht, A. R. (1992) The folding of
353 an enzyme. II. Substructure of barnase and the contribution of different interactions to
354 protein stability. *J. Mol. Biol.* **224**, 783-804
- 355 8. Finka, A., and Goloubinoff, P. (2013) Proteomic data from human cell cultures refine
356 mechanisms of chaperone-mediated protein homeostasis. *Cell Stress Chaperones* **18**, 591-
357 605
- 358 9. Kampinga, H. H., and Craig, E. A. (2010) The HSP70 chaperone machinery: J proteins as
359 drivers of functional specificity. *Nature Reviews Molecular Cell Biology* **11**, 579-592
- 360 10. Schlecht, R., Scholz, S. R., Dahmen, H., Wegener, A., Sirrenberg, C., Musil, D., Bomke, J.,
361 Eggenweiler, H. M., Mayer, M. P., and Bukau, B. (2013) Functional analysis of Hsp70
362 inhibitors. *PLoS One* **8**, e78443
- 363 11. Ramdzan, Y. M., Trubetskoy, M. M., Ormsby, A. R., Newcombe, E. A., Sui, X., Tobin, M. J.,
364 Bongiovanni, M. N., Gras, S. L., Dewson, G., Miller, J. M. L., Finkbeiner, S., Moily, N. S., Niclis,
365 J., Parish, C. L., Purcell, A. W., Baker, M. J., Wilce, J. A., Waris, S., Stojanovski, D., Bocking, T.,
366 Ang, C. S., Ascher, D. B., Reid, G. E., and Hatters, D. M. (2017) Huntington Inclusions Trigger
367 Cellular Quiescence, Deactivate Apoptosis, and Lead to Delayed Necrosis. *Cell Rep* **19**, 919-
368 927
- 369 12. Cox, D., Raeburn, C., Sui, X., and Hatters, D. M. (2020) Protein aggregation in cell biology: An
370 aggregomics perspective of health and disease. *Semin. Cell Dev. Biol.* **99**, 40-54
- 371 13. Olshina, M. A., Angley, L. M., Ramdzan, Y. M., Tang, J., Bailey, M. F., Hill, A. F., and Hatters, D.
372 M. (2010) Tracking mutant huntingtin aggregation kinetics in cells reveals three major
373 populations that include an invariant oligomer pool. *J. Biol. Chem.* **285**, 21807-21816
- 374 14. Ramdzan, Y. M., Polling, S., Chia, C. P., Ng, I. H., Ormsby, A. R., Croft, N. P., Purcell, A. W.,
375 Bogoyevitch, M. A., Ng, D. C., Gleeson, P. A., and Hatters, D. M. (2012) Tracking protein
376 aggregation and mislocalization in cells with flow cytometry. *Nat Methods* **9**, 467-470
- 377 15. Gidalevitz, T., Ben-Zvi, A., Ho, K. H., Brignull, H. R., and Morimoto, R. I. (2006) Progressive
378 disruption of cellular protein folding in models of polyglutamine diseases. *Science* **311**, 1471-
379 1474
- 380 16. Mitsui, K., Nakayama, H., Akagi, T., Nekooki, M., Ohtawa, K., Takio, K., Hashikawa, T., and
381 Nukina, N. (2002) Purification of polyglutamine aggregates and identification of elongation
382 factor-1alpha and heat shock protein 84 as aggregate-interacting proteins. *J. Neurosci.* **22**,
383 9267-9277
- 384 17. Hay, D. G., Sathasivam, K., Tobaben, S., Stahl, B., Marber, M., Mestril, R., Mahal, A., Smith, D.
385 L., Woodman, B., and Bates, G. P. (2004) Progressive decrease in chaperone protein levels in
386 a mouse model of Huntington's disease and induction of stress proteins as a therapeutic
387 approach. *Hum. Mol. Genet.* **13**, 1389-1405

388 18. Seidel, K., Meister, M., Dugbartey, G. J., Zijlstra, M. P., Vinet, J., Brunt, E. R., van Leeuwen, F.
389 W., Rub, U., Kampinga, H. H., and den Dunnen, W. F. (2012) Cellular protein quality control
390 and the evolution of aggregates in spinocerebellar ataxia type 3 (SCA3). *Neuropathol. Appl.*
391 *Neurobiol.* **38**, 548-558

392 19. Radwan, M., Lilley, J. D., Ang, C. S., Reid, G. E., and Hatters, D. M. (2020) Immiscible inclusion
393 bodies formed by polyglutamine and poly(glycine-alanine) are enriched with distinct
394 proteomes but converge in proteins that are risk factors for disease and involved in protein
395 degradation. *PLoS One* **15**, e0233247

396 20. Nillegoda, N. B., Kirstein, J., Szlachcic, A., Berynskyy, M., Stank, A., Stengel, F., Arnsburg, K.,
397 Gao, X., Scior, A., Aebersold, R., Guilbride, D. L., Wade, R. C., Morimoto, R. I., Mayer, M. P.,
398 and Bukau, B. (2015) Crucial HSP70 co-chaperone complex unlocks metazoan protein
399 disaggregation. *Nature* **524**, 247-251

400 21. Pelham, H. R. (1984) Hsp70 accelerates the recovery of nucleolar morphology after heat
401 shock. *EMBO J.* **3**, 3095-3100

402 22. Welch, W. J., and Feramisco, J. R. (1984) Nuclear and nucleolar localization of the 72,000-
403 dalton heat shock protein in heat-shocked mammalian cells. *Journal of Biological Chemistry*
404 **259**, 4501-4513

405 23. Velazquez, J. M., and Lindquist, S. (1984) hsp70: nuclear concentration during environmental
406 stress and cytoplasmic storage during recovery. *Cell* **36**, 655-662

407 24. Kose, S., Furuta, M., and Imamoto, N. (2012) Hikeshi, a nuclear import carrier for Hsp70s,
408 protects cells from heat shock-induced nuclear damage. *Cell* **149**, 578-589

409 25. Park, S. H., Kukushkin, Y., Gupta, R., Chen, T., Konagai, A., Hipp, M. S., Hayer-Hartl, M., and
410 Hartl, F. U. (2013) PolyQ proteins interfere with nuclear degradation of cytosolic proteins by
411 sequestering the Sis1p chaperone. *Cell* **154**, 134-145

412 26. Kaganovich, D., Kopito, R., and Frydman, J. (2008) Misfolded proteins partition between two
413 distinct quality control compartments. *Nature* **454**, 1088-1095

414 27. Sontag, E. M., Vonk, W. I. M., and Frydman, J. (2014) Sorting out the trash: the spatial nature
415 of eukaryotic protein quality control. *Curr. Opin. Cell Biol.* **26**, 139-146

416 28. Ramdzan, Y. M., Nisbet, R. M., Miller, J., Finkbeiner, S., Hill, A. F., and Hatters, D. M. (2010)
417 Conformation sensors that distinguish monomeric proteins from oligomers in live cells.
Chem. Biol. **17**, 371-379

418 29. Hageman, J., and Kampinga, H. H. (2009) Computational analysis of the human
419 HSPH/HSPA/DNAJ family and cloning of a human HSPH/HSPA/DNAJ expression library. *Cell*
420 *Stress Chaperones* **14**, 1-21

421 30. Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., and
422 Eliceiri, K. W. (2017) ImageJ2: ImageJ for the next generation of scientific image data. *BMC*
423 *Bioinformatics* **18**, 529

424 31. Stringer, C., Wang, T., Michaelos, M., and Pachitariu, M. (2021) Cellpose: a generalist
425 algorithm for cellular segmentation. *Nat Methods* **18**, 100-106

426 32. Hatters Danny, Wood Rebecca, Raeburn Candice, Ormsby Angelique, Mikael Oliveberg, and
427 Alex, D. (2018) Measuring proteostasis capacity using transiently transfected bait proteins by
428 flow cytometry. *28 March 2018, Protocol Exchange (Version 1) available at Protocol*
429 *Exchange*

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Table 1. Sequence overview of the barnase biosensor constructs*

pTriEx4 barnase WT* + NES

MAHHHHHHGSGEQKLISEEDLGSGSGSGGGHHRVDFKTIYRAKKAVKLPDYHFVDHRIEILNHDKDYNKVTVYE
SAVARNSTDGMDELYKGASGGMVKGEETTMGVIKPDMKIKLKMENGVNGHAFVIEGEGEKGKPYDGTNTIN
LEVKEGAPLFSYDILTTAFAYGNRAFTKYPDDIPNYFKQSFPEGYSWERTMTFEDKGIVVKVSDISMEEDSFYIE
IHLKGENFPNGPVMQKKTTGWDASTERMYVRDGVLKGDVKHKLLEGSGAQVINTFDGVADYLQTYHKLPD
NYITKSEAQALGWVASKGNLADVAPGKSIGGDIIFSNREGKLPGKSGRTWREADINYSGFRNSDRILYSSDWLIYK
TTDAYQTFTKIRAAAMDGGVQLADHYQQNTPIGDGPVLLPDNHLYSYQSALKDPNEKRDHMLVLEFVTAAGIT
LGMDELYKGGSGGMVKGEELFTGVVPILVLDGDVNGHKFSVSGEGEGDATYGKLTLCIITGKLPVPWPTLV
TTLGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNI
LGHKLEYNNNSHNVYITADKQKNGIKANFKIRHNIEGTDILQKKLEELDE

pTriEx4 barnase WT* + NLS

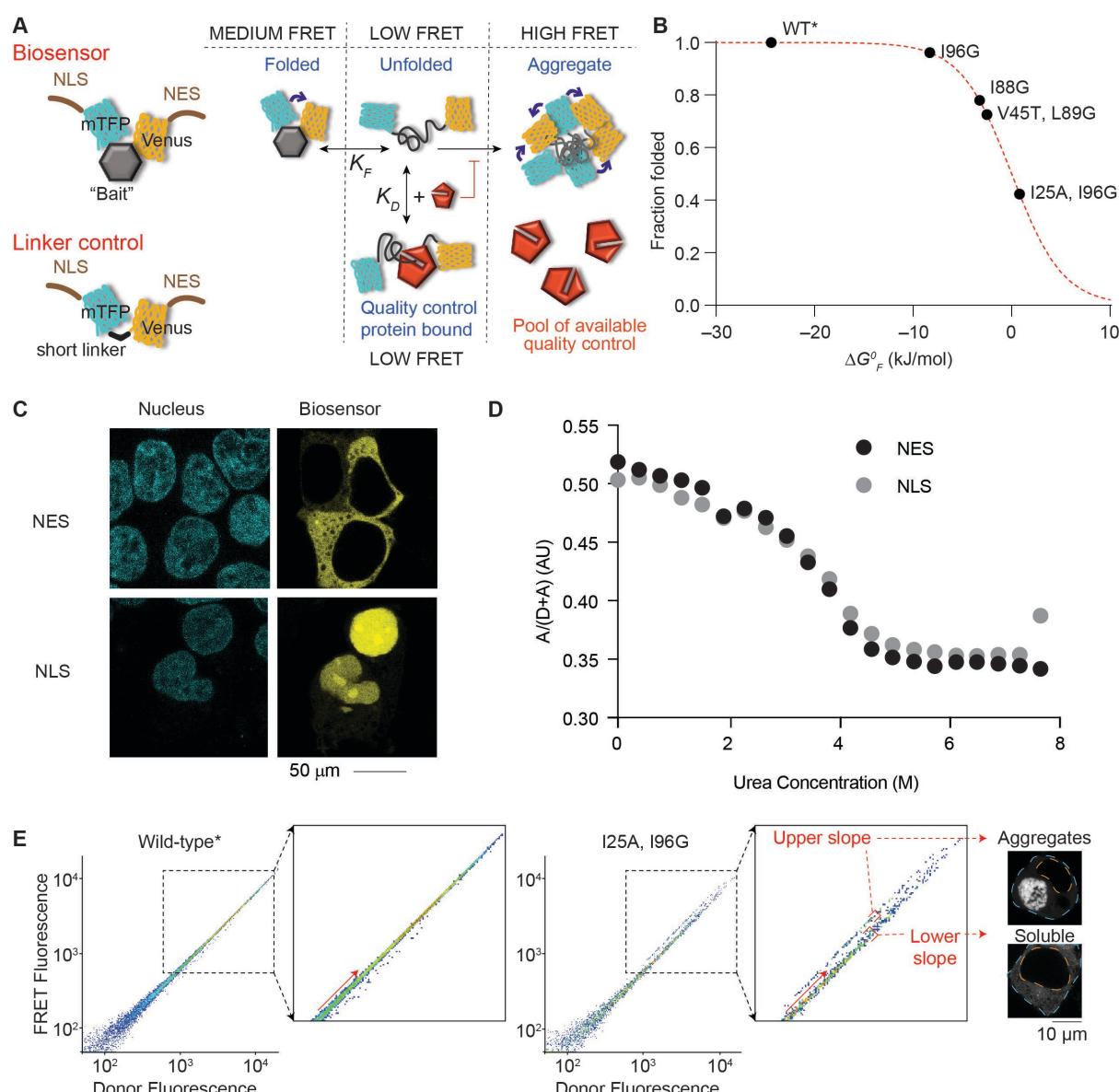
MCGGGPKKKRKVEDPGSGSGGGHHRVDFKTIYRAKKAVKLPDYHFVDHRIEILNHDKDYNKVTVYESAVARN
STDGMDELYKGASGGMVKGEETTMGVIKPDMKIKLKMENGVNGHAFVIEGEGEKGKPYDGTNTINLEVKEG
APLPFSYDILTTAFAYGNRAFTKYPDDIPNYFKQSFPEGYSWERTMTFEDKGIVVKVSDISMEEDSFYIEIHLKGE
NFPPNGPVMQKKTTGWDASTERMYVRDGVLKGDVKHKLLEGSGAQVINTFDGVADYLQTYHKLPDNYITKS
EAQALGWVASKGNLADVAPGKSIGGDIIFSNREGKLPGKSGRTWREADINYSGFRNSDRILYSSDWLIYKTTDAY
QTFTKIRAAAMDGGVQLADHYQQNTPIGDGPVLLPDNHLYSYQSALKDPNEKRDHMLVLEFVTAAGITLGMD
ELYKGGSGGMVKGEELFTGVVPILVLDGDVNGHKFSVSGEGEGDATYGKLTLCIITGKLPVPWPTLVTTLGY
GLMCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNI GHKL
EYNYNNSHNVYITADKQKNGIKANFKIRHNIEGTKDEL

436 *Sequences coded as: **mTFP1 fluorescent protein (cp 175); WT* barnase; Venus fluorescent protein**

437 (*cp 173*); NLS; NES

438

439



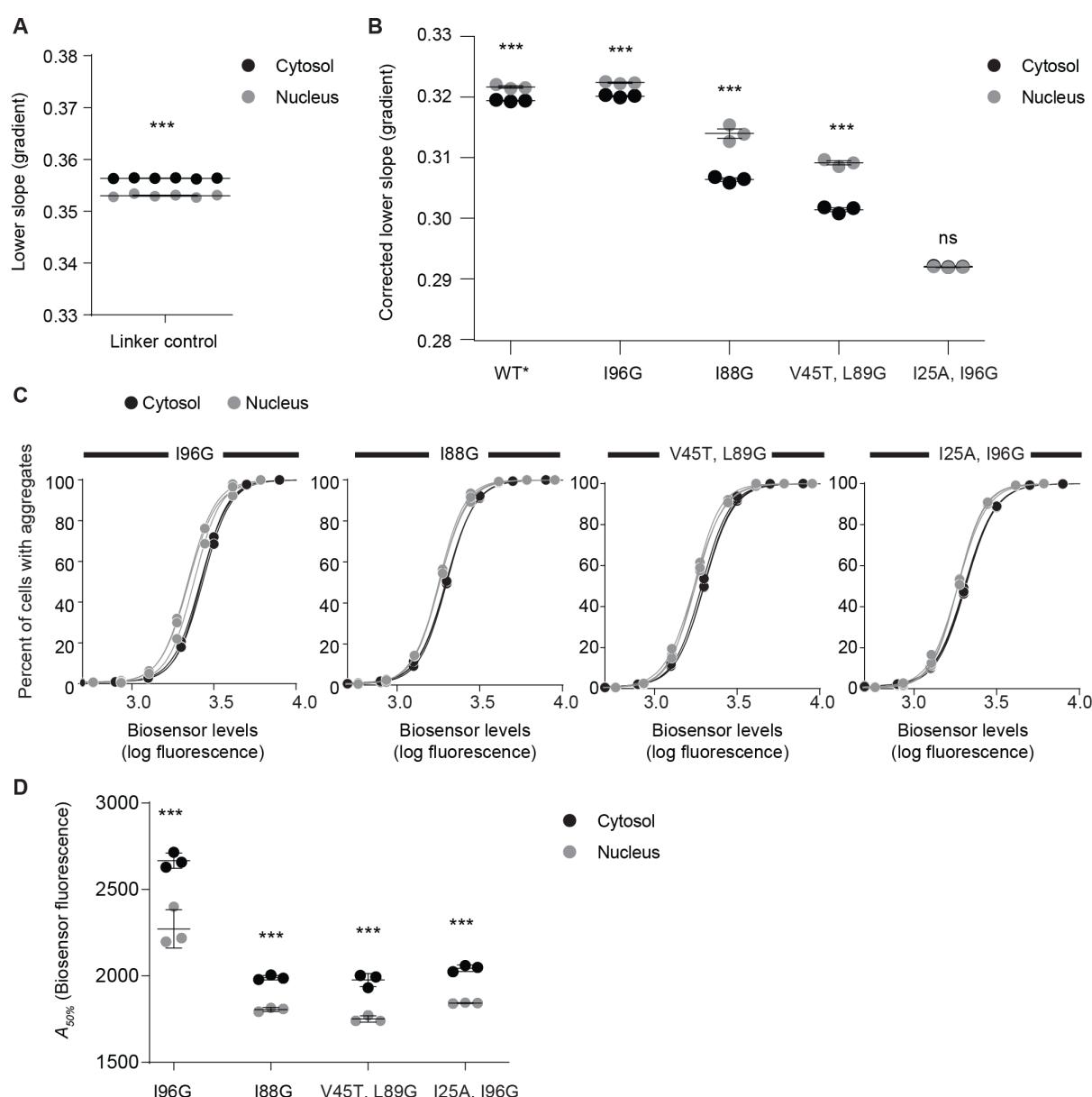
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441 **Figure 1: Targeting the barnase biosensor into the cytosol and nucleus.** **A.** Schematic of how the
 442 biosensor works. The barnase protein is used as the “bait” for chaperones and is flanked with
 443 fluorescence proteins for Fluorescence Resonance Energy Transfer (FRET) measurements. A nuclear
 444 localization sequence (NLS) or nuclear export sequence (NES) is appended to the construct. The
 445 linker control has the barnase module omitted. **B.** Shown is the relationship between mutations in
 446 barnase, the effect on standard free energy of folding (ΔG_F° at 20 °C), and predicted fraction folded
 447 for the various biosensor variants used in the study. Wild-type (WT) barnase is marked with * to
 448 denote it contains the catalytic inactivation mutation H102A. This mutation is present in all
 449 constructs in the study. **C.** Confocal images of HEK293T cells transiently transfected with either
 450 nuclear- or cytosol-targeting biosensor variant of the WT* barnase biosensor. The nucleus was
 451 visualized by Hoechst 33342 stain (cyan) and biosensor by Venus fluorescent protein fluorescence
 452 (yellow). **D.** Urea denaturation curves of WT* barnase biosensor variants as measured in cell lysates

453 by FRET. **E.** Flow cytometry strategy for monitoring foldedness and aggregation. Here the donor and
454 acceptor fluorescence of cells were measured by channels (FRET and Donor fluorescence was gated
455 by the PE (575/25) and V500 (525/50) filters, respectively with the 405 nm laser). The inset
456 highlights the changes that arise for cells bifurcated into “upper” and “lower” slope populations
457 (division shown with red arrow). Representative cells collected from gates corresponding to the
458 upper and lower slope populations imaged by confocal microscopy (grayscale). The orange dashed
459 line denotes the nucleus boundary and the cyan dashed line the cell boundary.

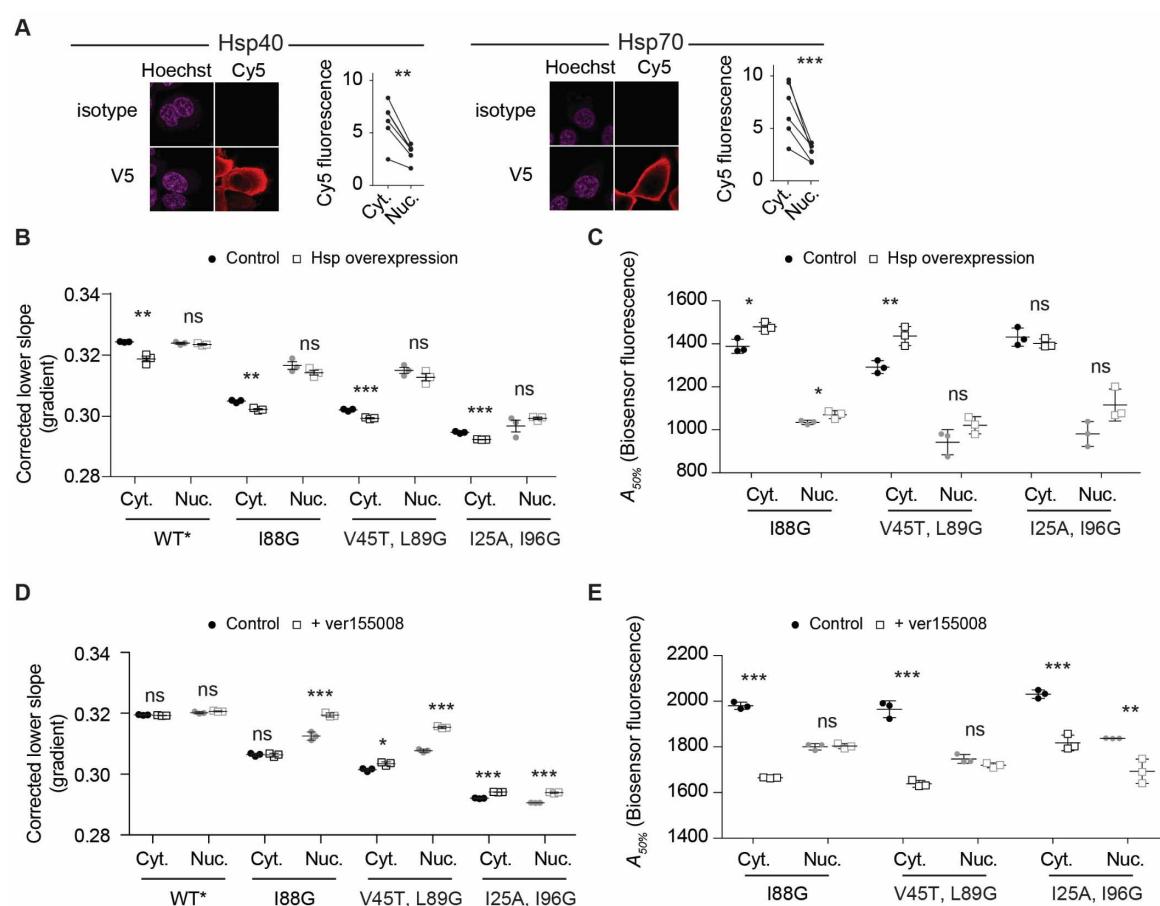
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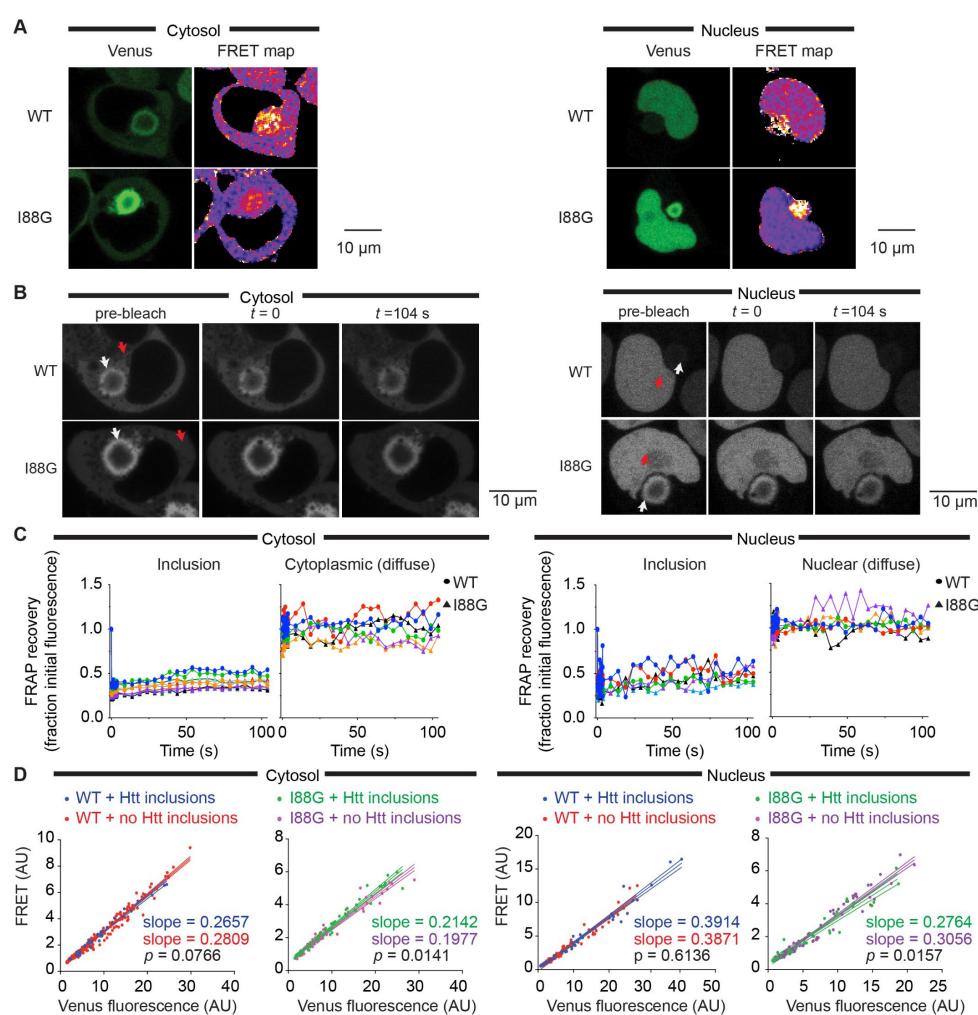
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463 **Figure 2: Reduced proteostasis resilience in the nucleus compared to the cytosol.** All data in this
 464 figure relate to NLS or NES-tagged biosensor constructs transfected in HEK293 cells and analysed by
 465 flow cytometry. Individual biological replicates shown with means \pm S.D. and with differences
 466 (nucleus v cytosol) assessed by Student's t-test (2-tailed); *** $p < 0.001$, ns $\Rightarrow 0.05$. **A.** Effect of
 467 intrinsic FRET differences in nucleus versus cytosol assessed with the linker control. **B.** Analysis of
 468 different barnase mutations. All data were corrected for background effects using the linker control.
 469 **C.** Assessment of aggregation. Shown are cells binned into different biosensor levels (based on
 470 Venus fluorescence) and each bin assessed for percent in upper slope versus lower slope
 471 populations. Curves are fits to Hill equation. Data were fitted independently within each replicate
 472 dataset ($n=3$). **D.** Shown are biosensor concentrations at which 50% of cells have aggregates ($A_{50\%}$),
 473 derived from the Hill equation fits shown in panel C.



474

475 **Figure 3: Cytosolic Hsp70 and Hsp40 activity provide depth in proteostasis resistance against**
476 **protein misfolding and aggregation. A.** Shown are immunofluorescence micrographs of HEK293T
477 cells transiently transfected with either V5-tagged Hsp40 or Hsp70 proteins (DNAJB1 and HSPA1A
478 respectively). The nucleus is stained with Hoechst 33342 and chaperone with Cy5 labelled anti-V5
479 antibody (or isotype control for specificity). Graphs indicate quantitation with paired Student's t-test
480 results shown (2-tailed, paired); *** p < 0.001, ** p < 0.01. Data points represent
481 immunofluorescence intensity in single cells (paired by mean cytosol and mean nucleus). **B.** Lower
482 slope analysis by flow cytometry of HEK293 cells co transfected with the biosensors, DNAJB1 and
483 HSPA1A or control (a non-fluorescent derivative of GFP (Y66L Emerald (13))) for 48 hours. Data points
484 indicate biological replicates, bars indicate means \pm S.D. Student's t-test results are shown (2-tailed;
485 control v overexpression); *** p < 0.001, ** p < 0.01, * p < 0.05, ns > 0.05. **C.** Aggregation analysis
486 ($A_{50\%}$) using the same treatments and conditions as for panel B. **D.** Lower slope analysis of HEK293
487 cells after transfection with the biosensors for 18 hours and a further treatment with 20 μ M Hsp70
488 inhibitor VER-155008 for 18 h (versus vehicle control). Data is presented as per panel B. **E.**
489 Aggregation analysis as presented for the other panels above.



490

491 **Figure 4: Huntington exon 1 aggregation in the cytosol manifests proteostasis imbalances in the**
 492 **nucleus and cytoplasm. A.** Confocal micrographs showing the values proportional to the
 493 fluorescence ratio of acceptor/donor (Venus/mTFP) of the biosensors co-expressed with mutant
 494 Httex1_{97Q} fused to a non-fluorescent mutant of GFP. Selected cells are those with Httex1 inclusions,
 495 identified post-hoc as described in the methods. Nuclear targeted biosensors are on the right and
 496 cytosolic targeted biosensors on the left (same format for each panel). The scale of the FRET map is
 497 colour coded from blue to magenta to yellow corresponding to lowest to highest FRET. **B.**
 498 Fluorescence recovery after photobleaching (FRAP) of biosensor at the periphery of the Httex1_{97Q}
 499 inclusion. Arrows indicate region of bleaching. **C.** Quantitation of the data in panel B, tracking the
 500 recovery of fluorescence in the bleached zone. Each colour depicts the time course of an individual
 501 cell. **D.** Confocal microscopy FRET fluorescence values within cells distal to the inclusion. FRET
 502 fluorescence was measured by exciting at 458nm (mTFP1 excitation) and collecting the emission at
 503 510-560 nm (Venus emission). Each dot represents the average fluorescence derived from a single
 504 cell value. Solid lines show line of best fit from a linear regression with dashed lines showing 95%
 505 confidence intervals. P-value was determined by two-tailed t-test.