Protein synthesis is supported by cellular machineries that ensure polypeptides fold to their native conformation, whilst eliminating misfolded, aggregation prone species. Protein aggregation underlies pathologies including neurodegeneration. Aggregates’ formation is antagonised by molecular chaperones, with cytoplasmic machinery resolving insoluble protein aggregates. However, it is unknown whether an analogous disaggregation system exists in the Endoplasmic Reticulum (ER) where ~30% of the proteome is synthesised. Here we show that the ER of a variety of mammalian cell types, including neurons, is endowed with the capability to resolve protein aggregates under stress. Utilising a purpose-developed protein aggregation probing system with a sub-organellar resolution, we observe steady-state aggregate accumulation in the ER. Pharmacological induction of ER stress does not augment aggregates, but rather stimulate their clearance within hours. We show that this disaggregation activity is catalysed by the stress-responsive ER molecular chaperone – BiP. This work reveals a hitherto unknown, non-redundant strand of the proteostasis-restorative ER stress response.
Newly synthesised polypeptides must attain the functional three-dimensional conformation, thermodynamically encoded by the amino acid sequence1. Protein folding describes the process where polypeptides seek the minimum point in energy-funnel but can be trapped in local minima separated by low barriers (misfolding traps).3 Further, the chemical diversity and crowdedness of cellular compartments create conditions for reactions competing with native folding, inducing local unfolding and stabilising non-functional, misfolding-trapped conformations. Misfolded and misfolded protein species tend to form insoluble aggregates.3 To avoid the accumulation of cytotoxic aggregates, the biosynthetic organelles (i.e. cytoplasm, the Endoplasmic Reticulum, ER, and mitochondria) evolved a multi-layer proteostasis network (PN) that chaperones nascent proteins and rescues or recycles misfolded intermediates.4 The PN is endowed with a capability to feedback to the gene-transcription and translation systems to interactively adjust the protein production rate to its folding-assistance and quality control capability. The ER branch of the PN, the Unfolded Protein Response (UPR), alleviates un/misfolded protein load during stress by transient attenuation of protein synthesis and posttranslational chaperone activation, followed by transcriptional upregulation of protein maturation and quality control factors.5,6

Shortfalls of the PN machinery may lead to pathology: protein aggregation accompanies neurodegenerative diseases such as Alzheimer’s, Parkinson’s, Amyotrophic Lateral Sclerosis and Frontotemporal Dementia.7 Neuronally expressed metastable proteins (e.g. Aβ, Tau, or α-synuclein)—with a tendency to oligomerise and consequently aggregate—underlie these conditions. The late onset of the aggregation-related sub-population neurodegeneration is consistent with age- and environmental factor-dependent decline in the efficiency of the PN machinery. Understanding how the cell handles aggregation-prone proteins on an organellar level is crucial to rationalising the disease-related proteostasis impairment.

The recent discovery of the cytosolic Hsp70 chaperone and its assisting system’s capacity to resolve cytoplasmic aggregates8 (including amyloids9,10) adds another functional layer to the PN for rectifying imperfections of the quality control system or prion-like transition from native folding to aggregation. However, it is unknown whether such a capability exists in the ER—a site responsible for the manufacturing of about one-third of the cell’s protein repertoire (including disease determinants, e.g. amyloid precursor protein, APP). Observations suggest that the chaperone-rich environment of the ER is more immune to protein aggregation than the cytoplasm.11 How the organelle maintains high fidelity protein folding, avoiding aggregation throughout the lifetime of the cell, particularly the non-dividing neurons, remains an open question. Explaining this in terms of protein folding quality control alone would rest on the assumption that the system can achieve zero aggregation and absolute quality control through astronomically large numbers of protein folding cycles.

In this work, we use a purpose-developed protein aggregation probing system with sub-organelle resolution to show accumulation of protein aggregates in the ER. Strikingly, ER stress, induced by N-glycosylation or Ca2+ pump inhibitors, triggers an aggregates’ clearance activity. We find that protein disaggregation is catalysed by the stress-responsive ER molecular chaperone—BiP. Modulating its amount in the ER reveals an inverse correlation between BiP’s presence and the aggregation load. Further, we reconstruct the disaggregation reaction in vitro by a minimal system of ATP-fuelled BiP with a J-domain cofactor.

Results and discussion

Monitoring ER protein aggregation in live cells. We sought an optical single-cell protein aggregate probing approach with subcellular space resolution to surmount limitations associated with biochemical, post-lysis cell population extracts. The lyse-probing techniques can detect aggregation events in a population involving drastic changes in a high proportion of an aggregating protein. However, the heterogeneous population averaging effect in ensemble analyses, combined with the lack of information on the probe’s distribution in space and the reliance on protein status preservation in lysis limit the ability to resolve folded/misfolded/aggregated species of metastable substrate. The folding status of metastable reporter-protein, which can assume either state, can reflect the activity of the cellular protein folding handling machinery. Thus, to address how the ER handles protein folding/aggregation, we established an optical approach to monitoring this process in live cells based on a cell-inert metastable surrogate protein. The protein core of the probe is an E. coli halolalkane dehydrogenase variant destabilised by mutagenesis and covalently modifiable by a solvatochromic fluorescent moiety (Fig. 1a)12 HT-aggr herein. As HT-aggr has no natural function and interacts in the ER, its fate (in terms of folding, aggregation, and degradation) is exclusively reflective of the protein-handling activity in the host organelle.

The aggregation propensity of the HT-aggr probe is predicted by a relatively low unfolding free energy value ΔG0 ≤ 5.6 kcal/mol in HT-WT to 2.0 kcal/mol in HT-aggr K73T, Supplementary Fig. 1). This metastability—propensity to aggregate, was reflected in fluorescence correlation spectroscopy (FCS) measurements: upon heating in vitro, HT-aggr formed particles with a hydrodynamic radius (Rh) of 48 nm, while HT-WT only partially aggregated under the same conditions displaying a biphasic FCS trace, which represents putative monomers and larger aggregates (the predicted Rh of a globular 26-kDa HT of 2.9 nm agrees with the FCS-measured monomer size of 2–3 nm, Fig. 1b, c, consistent with13). Further, we observed an increase in the solvatochromic fluorophore’s fluorescence lifetime attached to aggregating probe species (Fig. 1d, e). The native HT-aggr and HT-WT showed a unimodal distribution of lifetimes peaking at ~3.3 ns that broadened and shifted upon heating to longer lifetime values with a jagged plateau between 4.9 and 7.6 ns for HT-aggr (Fig. 1d) and two distinct peaks for HT-WT (Fig. 1e) corresponding to monomers and large aggregates, apparent in the FCS traces (Fig. 1c). This equivalence between the probe’s fluorescence lifetime and aggregation state (Fig. 1a) provides means for monitoring the state of the aggregation probe in live cells by Fluorescence Lifetime Imaging Microscopy (FLIM). This methodology offers an accurate and absolute measure of the probe’s state, free from the confounding effects of intensity calibrations and photo-bleaching14–16, thus removing the limits associated with the intensity-based readouts. Based on the probe’s in vitro performance, we sought to establish FLIM-based measurements of its folding/aggregation status in live cells. Consistent with the in vitro measurements, heat-stress triggered aggregation of HT-aggr, transiently expressed in the cytoplasm or ER, as registered by an increase in probes’ fluorescence lifetime (Fig. 1f, g). This behaviour was not observed for HT-WTER (Supplementary Fig. 2). In line with previous observations11,17, the ER appeared more resilient than the cytoplasm in maintaining HT-aggr soluble in heat-shocked cells, as reflected by an attenuated shift towards longer fluorescence lifetime in the ER, compared to cytoplasm-resident probe. Notably, the increase in fluorescence lifetime of the ER-targeted probe upon heat shock is considerably more heterogeneous in magnitude across the cell population than that of the cytoplasmic probe (Fig. 1h). This points to greater resilience of the ER to protein aggregation in some cells (compare HT-aggrER to HT-aggrK73T in Fig. 1h, note the presence of more/less heat-sensitive HT-aggrER cells reflected in their yellow/green FLIM-appearance, respectively). As
ER-localised proteins undergo N-glycosylation if their sequence contains an exposed Asp-X(except Pro)-Ser/Thr tripeptide, a modification that could alter HT-aggr properties, we confirmed that HT sequence is devoid of this signal.

To assess the long-term kinetics of the HT-aggrER aggregation, we delivered its encoding vector in a retroviral vehicle driving genomic integration and thus stable expression. Tracking the aggregation status post-infection, we observed that prolonged expression of the probe in the ER led to spontaneous HT-aggr aggregation with an accumulation of large aggregate particles (apparent as red puncta—lifetime >5 ns, similar to the aggregates' values in vitro, Fig. 2a). Notably, the population of pixels with regular (non-punctate) ER-pattern also showed a longer lifetime (pixels in the yellow spectrum), presumably reflecting smaller
sub-diffraction limit aggregate intermediates/oligomers mixed with native probe (mixture of green and red, Fig. 2a, b). The fraction of cells with FLIM-detectable aggregates (red puncta) increased with time up to 9 days (Fig. 2c). From that point on, the size distribution of the aggregates drifted with time towards larger particles (Fig. 2d), indicating a further accumulation of aggregated material.

To establish whether the aggregated species accumulate in the ER lumen or post-retro-translocated into the cytoplasm, we implemented the Lightening super-resolution approach coupled to 3D FLIM enabled through a considerable improvement in the speed of the time-correlated single-photon counting of this modality. Both the large, puncta-like aggregates (lifetime range in red) and the intermediates (lifetime range in yellow) showed a complete intra-ER localisation in the high-resolution fast 3D FLIM (Fig. 2e, Supplementary Movie 1). Consistently, inhibition of retro-translocation did not abolish the aggregates’ formation (Supplementary Fig. 3a, b). Further, co-localisation analyses of the aggregates’ signal with an ER marker in untreated cells and upon inhibition of retro-translocation showed a similar ER population in both conditions (Supplementary Fig. 3a, b). A high degree of co-localisation between an ER marker and the whole HT-aggrER population was also measurable in normal conditions and upon retro-translocation inhibition (Supplementary Fig. 3c). Thus, HT-aggrER does not accumulate in the cytoplasm post-retro-translocation (unsurprisingly, as for most ER proteins, degradation is coupled to retro-translocation of ER-associated degradation, ERAD).

Further, the extent of intracellular aggregation of the HT-aggrER variants (cell area fraction occupied by aggregates) correlated linearly with their unfolding equilibrium constant, a measure of their unfolding fraction (Fig. 2f-h). The differences in thermodynamic stability of HT-aggr variants explain the degree of aggregation-propensity of the HT-aggr variants Fig. 2h, inset). The properties of HT-aggr K73T suggest it as a useful variant for monitoring the capacity of the cellular protein folding quality control: This milder aggregation-prone probe showed expression levels at a steady-state similar to HT-WT (Supplementary Movie 2 for HT-aggr K73T and Supplementary Movie 3 for HT-WT), and a relatively low, but detectable ER aggregates’ load. Thus, FLIM of HT-aggr provides a space/time-resolved measure of aggregation in live cells, revealing that the chaperone-rich ER is not immune to protein aggregation—unfolded/misfolded intermediates of a metastable substrate may evade the folding quality control system accumulating over time in sizeable intra-ER aggregate particles.

The unfolded/misfolded intermediates of the probes and the aggregates that they give rise to were well tolerated with no cost to cell proliferation rates (Fig. 2i) and did not trigger a measurable increase in UPR activity at any point of the aggregate accumulation period (Supplementary Fig. 4).

**ER stress-induced aggregates’ clearance.** Surprisingly, imposing ER stress pharmacologically did not increase the aggregated fraction of the probe (Fig. 3). In these experiments, we pulse-labelled the probe with its fluorescent reporter-ligand and chased the fate of its labelled sub-population over time in the presence or absence of various treatments (i.e. stress inducers, degradation blockers). This mode allows dissecting the aggregation, disaggregation and degradation events which the model protein is undergoing. Namely, short exposure to the fluorescent HT-ligand labels a sub-population of pre-existing probe species, the fate of which is then traced, unmixed from the total population that includes species produced during the chase period (the dark probe). Strikingly, ER stress induction decreased the intra-ER aggregation: a pulse-labelled population of HT-aggrER showed a substantial shortening of fluorescence lifetime (Fig. 3a, b). The aggregated fraction substantially reduced in a time-dependent manner following the induction of unfolded protein stress, through the inhibition of nascent proteins’ N-glycosylation by tunicamycin (Tm, Fig. 3c, d, see ER stress markers’ induction and dose/time tests in Supplementary Fig. 5a, b, respectively). The diminished aggregation load manifested in a lower percentage of cells that show large puncta-like aggregate particles (lifetime > 5 ns, stand out as red colour coded pixels, Fig. 3a, c), and shrinking of the area occupied by these large aggregates (Fig. 3d). Moreover, in the FLIM images of tunicamycin treated cells, the entire distribution of pixels’ fluorescence lifetime gradually shifted towards lower values in nearly all the cells (Fig. 3b). This indicates that the longer lifetime oligomeric intermediates of the probe (too small to be detected as puncta) are also cleared. A similar effect was observed for the most aggregation-prone HT-aggrER variant M21K-F86L (Supplementary Fig. 6). Further, the probe faced a similar fate in human cortical neurons derived from induced pluripotent stem cells (iPSC): in these cells, too, HT-aggrER formed puncta-like aggregates with a longer lifetime, whilst ER stress induction by tunicamycin lowered the aggregation load (Supplementary Fig. 7). Next, we examined the same aggregation parameters in cells prior and after induction of ER stress by other means—depleting ER calcium through its SERCA-ATPase pumps inhibition by thapsigargin. The aggregates clearance in these ER stress conditions was also evident through a shift of probe’s fluorescence lifetime pixel distributions towards shorter values, decreased fractions of cells with aggregates and a contraction of the cell area occupied by aggregates (Fig. 3e-h).

As the pulse-chase mode of imaging excludes the contribution of changes in aggregates formation from newly synthesised species, the surprising effect of imposed ER stress on the abundance of the probe’s aggregated fraction is explainable by the activation of mechanisms that enhanced protein degradation by autophagosomal/lysosomal machinery or by a disaggregation activity in the ER (hitherto unknown). Presumably, proteasome-mediated elimination of the ER-aggregated probe should also require its disaggregation prior to retro-translocation to the cytoplasm, where proteasomes reside. To distinguish between the two scenarios, we first investigated the turnover of HT-aggrER under non-stress and stress conditions. The ER quality control machinery attends to the misfolding-prone substrates—it lowers the variants’ expression by increasing their turnover (Table 1, Supplementary Fig. 8,
However, the induction of ER stress slowed down the degradation rates of the WT-HT and HT-aggrER probes’ variants (Table 1, Supplementary Fig. 8, Supplementary Movie 4 and Supplementary Movie 5). Besides, aggregate clearance evolved on a faster time scale than the probes’ degradation in stress (Fig. 3, Table 1). Furthermore, the aggregate reduction effect of the ER stress was insensitive to autophagosomal/lysosomal blockers (Supplementary Fig. 9). Therefore, the protein aggregation-antagonising effect of ER stress cannot be explained by enhanced autophagosomal/proteasomal degradation but consistent with the organelle’s active disaggregation activity.
Fig. 2 Intra-ER accumulation of non-toxic aggregates of the metastable HT-aggr. a Time series of FLIM micrographs of CHO-K1 cells post stable retroviral infection with ER-targeted HT-aggr probe (K73T), labelled with P1 fluorophore. ER-targeting of HT as in ref. 36. b Plot of mean fluorescence lifetime values of HT-aggrER/P1 for the time points in (a). c Plot of the percentage of cells with FLIM-detectable aggregates (lifetime > 5 ns, apparent as red puncta, the threshold set to visualise aggregates in nearly monochromatic) from image-series as in (a). d Frequency distribution of aggregates’ sizes extracted from FLIM time-series as in (a) using the Aggregates Detector, Icy algorithm. (shown in (b, c) are values of mean ± SEM, n = 7 independent fields of view with at least 20 cells each). e High-resolution fast 3D FLIM image projections of CHO-K1 cells with HT-aggrER/P1. f FLIM images of live CHO-K1 cells stably expressing HT-WTER or HT-aggrER variants with various degrees of metastability, as indicated by Gibbs free energy values of unfolding (ΔG0Unf, inset). g Plot of relative cell area occupied by FLIM-detected aggregates (lifetime > 5 ns, red puncta) quantified for the HT-aggrER variants from images as in (f). (mean ± SEM, n = 3 independent fields of view, 60, 42, 55 and 45 cells analysed for WT, K73T, L172Q and M2IK FB6L variants, respectively). h Plot of relative cell area with aggregates (a measure of aggregation from (g) for each variant) as a function of its metastability, represented by the equilibrium constant of unfolding, expressed as Kdiss = exp(−ΔG0Unf/RT), where ΔG0Unf is the free energy of unfolding (inset). i Growth curves for CHO-K1 cells expressing the HT-aggrER variants (WT, cyan; K73T, yellow; L172Q, orange; M2IKFB6L, red) along with parental cells (magenta). Solid lines are the fits for the exponential growth according to the equation % conft0 2 (t/td), where td is the doubling time. Individual values and standard deviations of td calculated from triplicates are shown in the inset. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, unpaired T test (two-tailed).

Table 1 Turnover of HTER variants, labelled with tetrathymethyl-rhodamine Halotag ligand (TMR, 250 nM, 6 h), following the label removal, the samples were chase-imaged in the absence or presence of an ER stressor, tunicamycin (Tm, 0.5 μg/mL).

| HTER variant | Untreated t1/2 (hours) | Tm-induced stress (hours) |
|--------------|------------------------|---------------------------|
| WT           | 41 ± 9                 | 174 ± 21                  |
| K73T         | 32 ± 3                 | 58 ± 7                    |
| L172Q        | 7 ± 2                  | 27 ± 5                    |
| M2IKFB6L     | 28 ± 11                | 15 ± 3                    |

The time of the decrease of TMR fluorescence to 50% of the initial intensity (half-life, t1/2) was calculated from triplicates by fitting the curves to an exponential decay function (Supplementary Fig. 8, see Methods for details).

Impact of modulating Bip levels on the aggregation load in the ER. To this point, monitoring the fate of the HT-aggrER reveals an inducible capability of the ER to resolve protein-aggregates formed from a fraction that eluded the organelle’s quality control. This raises the question as to the identity of the ER disaggregation catalysis(s). In the cytoplasm, the chaperone network of Hsp10022 (absent in metazoans) and Hsp70, fuelled by ATP, can catalyse the solubilisation of protein aggregates8–10. The notions that the latter is the only currently known mammalian machinery with evidence of disaggregation activity through a plausible energetics/mechanics8–10, and that parallel functioning between cytoplasmic and ER chaperone-analogs are common, lead to the ER analogs as candidate-disaggerease machinery. Therefore, we hypothesised that the essential Hsp70-analogous ER chaperone Bip can be responsible for the organelle’s disaggregation activity. Bip’s candidacy for this role is further supported by its posttranslational/transcriptional...
response to ER stress, a condition in which we observe the disaggregation activity (Fig. 3). Namely, the UPR upscapes BiP abundance and modulates its activity by amending the chaperone’s posttranslational modification and oligomerisation status prior to its upregulation and ER expansion. Specific elimination of this key endogenous molecular chaperone by treating cells with Subtilase cytotoxin (SubAB) considerably augmented the probe’s aggregated fraction (SubAB is a toxin from Shiga toxigenic strains of E. coli with a proteolytic A subunit that specifically targets BiP and B subunit responsible for internalisation and retrograde trafficking of the toxin to the ER), Fig. 4a–c). Elimination of calreticulin, a central chaperone of the parallel BiP ER protein folding-assisting system, by KO did not affect the HT-aggrER aggregation load (Supplementary Fig. 10), arguing against the possibility of a generic aggregation enhancement of compromised protein folding. The aggregation-enhancing effect of BiP neutralisation with SubAB was exacerbated by stressing the ER with tunicamycin (Fig. 4a–c).

To test whether elevation of BiP expression would reduce the aggregation load, we enhanced transcription from its genomic locus using CRISPRa-mediated activation (CRISPRa). This approach allowed the expression enhancement of targets with high endogenous expression levels, such as BiP (unlike ectopic, exogenous cDNA in-plasmid delivery), and achieved approximately a three-fold increase in BiP expression at the RNA level (Supplementary Fig. 11a–c). The CRISPRa upregulation led to a significant decrease in the probe’s aggregation load (Fig. 4d, e). These observations support the BiP’s role in disaggregation.

In vitro reconstruction of BiP-mediated disaggregation reaction. To further ascertain BiP’s capacity to facilitate disaggregation, we sought to reconstruct this reaction in vitro. Since J-Domain proteins (DnaJ/ERdj) modulate HSP70/BiP activity and are crucial for recruiting the chaperone to its substrates, we fused the HT-aggr probe to a minimal generic J-Domain (HT-aggr-JD), a solution to enhance BiP ATP hydrolysis mimicking natural BiP/co-chaperone/substrate dynamics (see substrates Supplementary Table 1). Cells were grown overnight at 37 °C and then incubated at 32 °C for 24 h in fresh media containing 1% BSA. Media containing the retroviral particles was collected, at 37 °C and then incubated at 32 °C for 24 h in fresh media containing 1% BSA. Media containing the retroviral particles was collected, filtered through a 0.45 µm filter and stored at 4 °C. Fresh media containing 1% BSA was added to the cells kept at 32 °C to collect the second batch of retroviral particles 24 h later. Recipient CHO-K1 cells 25% confluent were incubated at 37 °C with media containing retroviral particles diluted 1:2 in F12 media supplemented with 2 µg/mL of polybrene for around 2 h. After this period, cells were incubated in fresh media for 24 h before adding puromycin at 6 µg/mL. After 48 h, media was replaced, and puromycin concentration increased to 8 µg/mL for selection of stable expressers. Afterwards (24–48 h), cells were labelled with HT fluorescent ligand TMR (Promega) and sorted for stable HT expressors. WT and calreticulin KO cells with stable probe expression were generated by the same viral delivery method. For CRISPRa the 20-nucleotide sequence in the guide RNA (gRNA) targeting the transcription start site of BiP (Hspa5) was designed using the tool (http://crispr.targeting.stanford.edu) and sub-cloned into the gRNA expression vector (pCRISPRa_gRNA vector). CHO-K1 cells stably expressing HT-aggrER were co-transfected with pCRISPRa_gRNA vector and the dCas9-VP64 and Synergistic Activation Mediators (SAMs) expression vector, and 24 h later cells expressing both gRNA and Cas9 (indicated by tag BFP and mCherry fluorescence, respectively) were sorted (FACS Melody, BD Biosciences, San Jose, CA) and subjected to qRT-PCR (description below). After confirming upregulation of BiP transcription, the same gRNA sequence was sub-cloned into the vector that expresses both gRNA and dCas9-VP64/SAM (pCRISPRa_all-in-one). The effect of BiP enhancement onto HT-aggrER aggregation was quantified as described below on image analysis. Engineered cell lines obtained in this study are available upon request.

Methods

cell culture, HT transfection and CRISPRa. Monkey African green kidney cell line COS7, ATCC CRL-1651, Hamster Chinese ovary cell line (CHO-K1, ATCC CCL-61) and mouse embryonic fibroblasts (MEFs) lacking calreticulin and their wild type counterparts (from Marek Michalak, University of Alberta, Alberta, Canada) were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 1% pen/strep (100 IU/mL and 100 µg/mL, respectively) and 1% L-glutamine (10 mM). For human embryonic kidney 293 cells (HEK 293T, ATCC CRL-3216), 2-Mercaptoethanol at 50 µM final concentration and non-essential amino acids (1:100) were used as additional supplements. Induced pluripotent stem cells (iPSC, from Michael E. Ward, National Institute of Health) were cultured as in25. Transient transfection of COS7 cells was performed using the Neon Transfection System (Invitrogen), applying 5 µg of plasmid per 1 x 106 cells. Stable CHO-K1 cell lines expressing HT WT and variants were obtained by retroviral infection. Retroviral particles were produced in HEK 293T cells using Transit IT transfection reagent (Mirus Bio) and plasmids SP_H6_Halo_pBabeEr (5 µg), pLV5-VG (1.5 µg), pK3 (5 µg) and pCMV_TAT_HIV (0.6 µg) (see plasmids Supplementary Table 1). Cells were grown overnight at 37 °C and then incubated at 32 °C for 24 h in fresh media containing 1% BSA. Media containing the retroviral particles was collected, filtered, then diluted 1:2 in F12 media supplemented with 2 µg/mL of polybrene for around 12 h. After this period, cells were incubated in fresh media for 24 h before adding puromycin at 6 µg/mL. After 48 h, media was replaced, and puromycin concentration increased to 8 µg/mL for selection of stable expressers. Afterwards (24–48 h), cells were labelled with HT fluorescent ligand TMR (Promega) and sorted for stable HT expressors. WT and calreticulin KO cells with stable probe expression were generated by the same viral delivery method. For CRISPRa the 20-nucleotide sequence in the guide RNA (gRNA) targeting the transcription start site of BiP (Hspa5) was designed using the tool (http://crispr.targeting.stanford.edu) and sub-cloned into the gRNA expression vector (pCRISPRa_gRNA vector). CHO-K1 cells stably expressing HT-aggrER were co-transfected with pCRISPRa_gRNA vector and the dCas9-VP64 and Synergistic Activation Mediators (SAMs) expression vector, and 24 h later cells expressing both gRNA and Cas9 (indicated by tag BFP and mCherry fluorescence, respectively) were sorted (FACS Melody, BD Biosciences, San Jose, CA) and subjected to qRT-PCR (description below). After confirming upregulation of BiP transcription, the same gRNA sequence was sub-cloned into the vector that expresses both gRNA and dCas9-VP64/SAM (pCRISPRa_all-in-one). The effect of BiP enhancement onto HT-aggrER aggregation was quantified as described below on image analysis. Engineered cell lines obtained in this study are available upon request.

Quantitative RT-PCR. Total RNA extraction and reverse transcription were performed using RNaseasy plus micro kit (Qiagen) and GoScript™ Reverse Transcription Mix (Promega), respectively. Real-time PCR was carried out using PowerUp™
SYBR™ Green Master Mix and QuantStudio™ 5 (Applied Biosystems). Primer sequences (Fw and Rev, respectively) were: BiP (Hspa5), 5′-GTGCA-GAAACTTCGTCGTGA-3′ and 5′-TTCTGGACTGGCTTCATGGT-3′; Gapdh, 5′-GACATGTCGCCTGGAGAAAC-3′ and 5′-CACTGTTGAAGTCGCAGGAG-3′. Relative gene expression of BiP between the mock and the CRISPRa transfected cells was calculated using the delta-delta Ct method.

Confocal microscopy, FLIM, fast 3D FLIM and image analysis. Cells were imaged in coverslips bottom dishes in complete medium with a laser confocal microscopy system (LSM 710, Carl Zeiss) equipped with an Argon laser and a Plan-Apo-chromat 60x oil immersion lens (NA 1.4) coupled to a microscope incubator, maintaining standard cell culture conditions. FLIM images of Halotag labelled with the P1 fluorophore12 (see excitation/emission spectra of P1 in PBS).
acquired in a Fluoromax spectrofluorimeter from Horiba Scientific. Supplementary Fig. 13), were acquired with a time-correlated single-photon counting PicoQuant module coupled to the LSM 710 above using a pulsed, 20 MHz repetition rate, 440 nm diode laser for excitation, with constant intensity level, an MBS 485 nm dichroic and an emission filter of LP520. Instrument Response Function (IRF) was recorded by light scattered on a clean coverslip. Fluorescence lifetimes and colour coded FLIM images were analysed by the FastFLIM function of Sympho Time 64 PicoQuant analysis software (version 2.4) using the average TCSPC channel of the decay to the average TCSPC channel of the IRF channel of the decay as measure for the average lifetime. The distance of the fluorescence decay was fitted to an exponential function of the form $I(t) = I_0 e^{-t/\tau}$, where $I(t)$ is the fluorescence intensity at time $t$, $I_0$ is the initial intensity, and $\tau$ is the decay time. The fluorescence lifetime $\tau$ was used to calculate the hydrodynamic diameter ($D_h$) of P2-labelled HT-aggr-JD (pre-aggregated) for the same time course and trace's colours as in (e). Fluorescence was measured in discrete data points, 0.3 mL each. Dynamic light scattering measurements showing the hydrodynamic diameter ($D_h$) of P2-labelled HT-aggr-JD (pre-aggregated) for the same time course were plotted in the inset to unveil the growth of the aggregates in the absence of BiP and explain why aggregates are retained in the pre-filter of the column leading to a decreased amount eluted over time. The measurements in (a-f) were performed in the presence of ATP (3–9 mM), which elutes at 20.2 mL, fractions omitted from the chromatogram.

Automated microscopy measurements: growth curves and HT turnover rates. CHO-K1 stably expressing HTs were plated in a 6 well plate at low density (5 × 10^4 cells) and cell confluence imaged with the 10× objective every 4 h using the phase-contrast channel of the Sartorius Incucyte live-cell imaging system (Göttingen, Germany). Exponential growth was fitted to the equation $% conft = % conft_{0} e^{(kt)}$, where $t_d$ is the time for a cell division (doubling time). To measure the turnover of HT, cells were plated at the same low density, grown overnight and then labelled with 0.25 mM TMR fluorophore (Promega) in media for 6 h. After that, media was removed, cells washed with PBS, and fresh media added to start imaging the red fluorescence channel every 4 h with the 10× objective. Phase-contrast and fluorescence images were analysed using the Sartorius Incucyte analysis software, version 2021 C. TMR fluorescence decay was fitted to an exponential decay using the equation $(TMR\text{ Int.}_n/\text{conft}_n) = (TMR\text{ Int.}_0/\text{conft}_0) \exp(-kt_d t_n)$ where $TMR\text{ Int.}_n$ is the TMR integrated fluorescence intensity corresponding to the amount of HT labelled, $k_d$ is cell confluence, and $k_d$ is the HT degradation rate constant. Half-lifetimes were calculated by $t_{1/2} = \ln\frac{1}{2}/k_d$. TMR fluorescence needs to be divided by cell confluence to account for cell content sharing between daughter cells as doubling time is significantly faster than HT turnover for most variants and conditions.

Fig. 5 ATP-driven HT-aggr disaggregation by BiP in vitro. a Protein absorption traces (280 nm) of BiP WT (50 µM) alone (grey), with native HT-aggr (dark blue) or native HT-aggr, fused to J-domain (HT-aggr-JD, 10 µM) (light blue), resolved by gel filtration chromatography (10–600 kDa fractionation range). b Traces as in (a) of native HT-aggr (green), native HT-aggr-JD (light blue) and heat-aggregated HT-aggr-JD (53 °C, 20 min, 10 µM) (magenta). c, Traces as in (a, b) of aggregated as in (b) HT-aggr-JD, incubated with BiP WT/ATP (magenta) or V461F/ATP (orange), a substrate-binding deficient BiP mutant. Note the increase in the main peak of the aggregated HT-aggr-JD (pale red) upon the addition of BiP WT/ATP only, indicative of its interaction with BiP and thus disaggregation. d, e Fluorescence traces of chromatograms as in (d) this time exclusively detecting HT-aggr-JD (pre-aggregated) through the fluorescence of its P2-halo ligand label (HT-aggr-JD, pale red; 4h, magenta; 24 h, orange; 48 h, light blue). e Fluorescence traces of chromatograms as in (d) this time exclusively detecting HT-aggr-JD (pre-aggregated) in the absence of BiP for the same time course and trace's colours as in (e). Fluorescence was measured for discrete data points, 0.3 mL each. Dynamic light scattering measurements showing the hydrodynamic diameter ($D_h$) of P2-labelled HT-aggr-JD (pre-aggregated) for the same time course were plotted in the inset to unveil the growth of the aggregates in the absence of BiP and explain why aggregates are retained in the pre-filter of the column leading to a decreased amount eluted over time. The measurements in (a-f) were performed in the presence of ATP (3–9 mM), which elutes at 20.2 mL, fractions omitted from the chromatogram.

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Flow cytometry. The effect of the Unfolded Protein Response was studied by a viral infection of CHO-K1 CHO-CP-GFP and Xbp1-MTurquoise reporter cells line11 (from Dr D. Ron, Cambridge Institute for Medical Research) expressing HT-WT and HT-aggr. To identify the expressing cells, TRM labelling was performed. To evaluate whether the HT-aggr induces the UPR response, cells were analysed after infection for 48 h, 72 h, and then for 7 and 9 days. Where indicated, cells were treated with the ER stress-inducing agent tunicamycin (0.5 µM) for 24 h to induce the UPR. Cells were analysed using a four-channel CytoFLEX flow cytometer (Beckman Coulter), GFP (excitation laser 488 nm, filter 525/40), M-Turquoise (excitation laser 405 nm, filter 450/45) and TMR signals (excitation laser 561, filter 585/42) were detected (CyExpert software, version 2.3.0.84). The data were processed using the FlowJo software, version 10.6.0 (BD).

Fluorescence correlation spectroscopy and static and dynamic light scattering. The FCS measurements were performed with a Microtime 200 setup from PicoQuant GmbH (Germany). The setup is equipped with a pulsed diode laser excitation source (LDH-P-C-485 PicoQuant, 482 nm, with a repetition rate of 20 MHz) coupled to an Olympus IX-71 inverted microscope. The laser beam was focused ~10 µm deep into the sample solution by a 60x water immersion objective with a numerical aperture of 1.2 (UPLSAPO 60XW, Olympus). Fluorescence was collected by the same objective and passed through a dichroic beam-splitter (485DRLP, Omega) to clean up the back-scattered light from laser excitation. The detected light is further selected by using a 510ALP long-pass filter (Omega). The collected signal is then focused by a detection tube lens to the 50 µm pinhole. The re-collimated beam is divided by means of a 50/50 non-polarising beam-splitter cube and is detected by two single-photon avalanche diode detectors (SPCM-AQR-13, Perkin Elmer). Time-traces of fluorescence intensity from the two detectors were cross-correlate to avoid after-pulsing artefacts. The output signal was computer-processed by a TimeHarp 200 TCSPCI card (PicoQuant). The focal area and the detection volume were calibrated at the beginning of each set of measurements using Atto655-COOH with an assumed diffusion coefficient, D, of 425 µm²/s in water at 25 °C. The autocorrelation curves with the variable ‘Lag time’ being the time shift used in the correlation function were fitted with a pure diffusional model to determine the diffusion coefficient, D. A larger fluorescent molecule such as an aggregate takes longer to cross the confocal observation volume and therefore the decay time of the autocorrelation function occurs at longer ‘Lag time’. The diffusion coefficient was then used to estimate the hydrodynamic radius Rθ by the Stokes-Einstein equation. The dynamic light scattering measurements were carried in a Zetasizer nano-ZS (Malvern), and the autocorrelation curves as a function of delay time were fitted to an exponential function using the Zetasizer software to extract the diffusion coefficient from which the hydrodynamic diameter Dh can be calculated by using the Stokes-Einstein equation. Static light scattering was carried out in a fluoroscan-4 spectrofluorometer (Horiba Scientific), coupled to a water bath used to increase the temperature, using excitation and emission wavelengths of 500 nm.

Protein purification and stability measurements. For in vitro assays, HT, HT-aggr fused to a J-domain (HT-aggr-JD) and BiP were expressed in the BL21 (DE3) strain, purified with Ni-nitrilotriacetic acid (NTA) affinity chromatography (HisTrap HP columns from GE Healthcare) in an AKTA purifier (GE Healthcare) and imidazole was removed by dialysis. Protein purity was checked by SDS-PAGE (Coomassie blue) with a detection limit of 0.1%. For in vitro assays, HT-aggr-JD was then treated with the ER stress-inducing agent tunicamycin (0.5 µM) for 24 h to induce the UPR. Cells were analysed using a four-channel CytoFLEX flow cytometer (Beckman Coulter), GFP (excitation laser 488 nm, filter 525/40), M-Turquoise (excitation laser 405 nm, filter 450/45) and TMR signals (excitation laser 561, filter 585/42) were detected (CyExpert software, version 2.3.0.84). The data were processed using the FlowJo software, version 10.6.0 (BD).

Thermodynamic stability. To determine thermodynamic stability, we used a two-state folding model wherein the Gibbs free energy of unfolding at a given urea concentration can be determined by Eq. 1:

\[ \Delta G^0 = -RT \ln \left( \frac{f_p}{f_u} \right) \]  

where \( f_p \) is the fraction of denatured protein, \( R = 8.314 \text{ J mol}^{-1} \text{K}^{-1}, T = 298 \text{ K} \). Subsequently, \( \Delta G^0 \) was plotted as a function of urea concentration to determine the thermodynamics of unfolding using Eq. 2:

\[ \Delta G_{\text{urea}} = \Delta G^0_{\text{water}} - m[\text{urea}] \]  

where \( \Delta G_{\text{urea}} \) is the Gibbs free energy of unfolding in water.

Gel filtration chromatography and in vitro HT (dis)aggregation assays. All the gel filtration chromatograms were run in a Superdex 200 increase 10/300 column (fractionation range 10–600 kDa, GE Healthcare) coupled to an AKTA purifier, under isocratic elution in HKM buffer (Hepes 20 mM pH 7.4, NaCl 150 mM and MgCl₂ 10 mM). For the assays with aggregated HT-aggr-JD, firstly HT was labelled to 40% with the P2 solvatochromic fluorophore and then protein aggregation was promoted by heating a 50 µM HT-aggr-JD solution to 53 °C for 20 min. Aggregated HT-aggr-JD was then filtered (0.22 µm), mixed with the other components to final concentrations of 10 µM HT-aggr-JD, 9 mM ATP and 50 µM BiP and incubated on a rotating wheel at RT.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available from the corresponding authors upon reasonable request. Plasmids and sequence information generated in this study is available through Addgene. Source data are provided with this paper.

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