Z-α₁-antitrypsin polymers impose molecular filtration in the endoplasmic reticulum after undergoing phase transition to a solid state

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Misfolding of secretory proteins in the endoplasmic reticulum (ER) features in many human diseases. In α₁-antitrypsin deficiency, the pathogenic Z variant aberrantly assembles into polymers in the hepatocyte ER, leading to cirrhosis. We show that α₁-antitrypsin polymers undergo a liquid-solid phase transition, forming a protein matrix that retards mobility of ER proteins by size-dependent molecular filtration. The Z-α₁-antitrypsin phase transition is promoted during ER stress by an ATF6-mediated unfolded protein response. Furthermore, the ER chaperone calreticulin promotes Z-α₁-antitrypsin solidification and increases protein matrix stiffness. Single-particle tracking reveals that solidification initiates in cells with normal ER morphology, previously assumed to represent a healthy pool. We show that Z-α₁-antitrypsin–induced hypersensitivity to ER stress can be explained by immobilization of ER chaperones within the polymer matrix. This previously unidentified mechanism of ER dysfunction provides a template for understanding a diverse group of related proteinopathies and identifies ER chaperones as potential therapeutic targets.

INTRODUCTION

A wide variety of conformational disorders arises when protein folding is compromised by gene mutation, aberrant posttranslational modification, or imbalances in the stoichiometry of protein complexes (1, 2). α₁-antitrypsin deficiency is an archetypal serine protease inhibitor (serpin) conformational disorder that results from mutations in the SERPINA1 gene and is associated with both loss-of-function toxicity and toxic gain of function (3). α₁-antitrypsin is produced predominantly by hepatocytes and acts to control inflammation and matrix degradation in distant tissues, most notably in the lungs, by inhibiting proteases released by neutrophils (4). The most common E342K pathogenic variant of α₁-antitrypsin is the Z allele (Z-α₁-antitrypsin). Wild-type M-α₁-antitrypsin is not thought to form higher-order assemblies, but the Z-α₁-antitrypsin mutant allele generates a protein that can form ordered linear polymers (5). Polymerization occurs in the lumen of the endoplasmic reticulum (ER) where proteins destined for the cell surface or secretion fold to acquire their native structure. Accumulation of Z-α₁-antitrypsin polymers is accompanied by fragmentation of the ER into large vesicular structures referred to as ER inclusions. ER inclusions are associated with toxic gain of function, which causes liver cirrhosis that accounts for 10% of deaths in individuals homozygous for the Z allele (PiZZ) (6–8). In addition, the Z allele is overrepresented in patients requiring liver transplantation for both nonalcoholic steatohepatitis and alcoholic liver cirrhosis (9), indicating its involvement in common diseases.

We hypothesized that accumulation of polymerized α₁-antitrypsin could alter ER function by changing the biophysical properties of the protein milieu within the lumen. To investigate this, we characterized the mobility of α₁-antitrypsin and other proteins in the ER of live cells and found that Z-α₁-antitrypsin can form a solid matrix of protein polymers that fills ER inclusions and impedes the mobility of other ER proteins, acting as a size-dependent molecular sieve. Furthermore, we provide evidence that calreticulin, an abundant lectin chaperone that interacts with α₁-antitrypsin during folding, promotes the formation of solid-phase Z-α₁-antitrypsin, increasing Z-α₁-antitrypsin polymer length and, in turn, the stiffness of the protein matrix, which immobilizes the chaperone. Solidification of Z-α₁-antitrypsin was also promoted by ER stress–induced activation of the unfolded protein response (UPR), a collection of pathways that defend cells against partially folded and misfolded ER proteins (10). Z-α₁-antitrypsin solidification during ER stress was ameliorated by inhibition of the activating transcription factor 6 (ATF6) branch of the UPR, which up-regulates calreticulin. Notably, the UPR is not activated by the accumulation of Z-α₁-antitrypsin polymers alone (11–14), but cells bearing the Z-α₁-antitrypsin allele are hypersensitive to orthogonal stresses that induce the UPR. The mechanism for this hypersensitivity has remained mysterious but has been suggested as a cause for hepatotoxicity in α₁-antitrypsin deficiency (12, 13). We propose that the bidirectional relationship between UPR-induced

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chaperones and the solid state of Z-α₁-antitrypsin explains the heightened sensitivity of Z-α₁-antitrypsin–expressing cells to ER stress. The filtration of ER proteins imposed by solid-phase Z-α₁-antitrypsin provides rare insight into the consequences of protein phase transitions within living cells, offering additional mechanistic understanding of the pathology of proteinopathies, and suggests new therapeutic targets.

RESULTS
Z-α₁-antitrypsin accumulation alters biophysical properties within ER inclusions
Cells expressing Z-α₁-antitrypsin display a range of ER morphologies, from a normal reticular ER to complete ER fragmentation (15). We previously established a CHO-K1 cell model expressing α₁-antitrypsin fused to fluorescent proteins to facilitate imaging studies (16). We further validated the CHO-K1 model by comparison with our published hepatocyte-like cells (HLCs) derived from induced pluripotent stem cells (iPSCs) (15). CHO-K1 cells made to express yellow fluorescent protein (YFP)–tagged Z-α₁-antitrypsin (YFP-Z) displayed a spectrum of ER morphologies, faithfully reproducing those observed in iP*ZZ iPSC-derived HLCs with similar frequency (Fig. 1, A to C) (15).

A major function of the ER is to support protein folding, which is dependent on molecular motion. The rate of movement of amino acid side chains during protein folding is strongly influenced by environmental microviscosity (17). At another level, translational motion of proteins facilitates binding and release of molecular chaperones to nascent proteins to promote correct protein folding (18, 19), which is governed both by microviscosity and crowding/confined by other macromolecules (20). To investigate the effect of α₁-antitrypsin accumulation on the biophysical properties of the contents of the ER, we assessed ER microviscosity in live cells expressing M- or Z-α₁-antitrypsin using rotor-based organelle viscosity imaging (ROVI). A small cell-permeable fluorescent molecular rotor was covalently bound to a genetically encoded HaloTag that was targeted to the ER using a signal sequence (Fig. 1D) (21–23). Fluorescence lifetime of the rotor reports on local microviscosity (23). ER lumen–targeted ROVI was applied to CHO-K1 cells expressing either wild-type M-α₁-antitrypsin or polymersogenic Z-α₁-antitrypsin (Fig. 1, E to I). As previously reported, expression of Z-α₁-antitrypsin caused variable fragmentation of the ER network (Fig. 1G, bottom) (13, 16). Fluorescence lifetime imaging microscopy (FLIM) of the rotor revealed a significant increase in ER luminal microviscosity in Z-α₁-antitrypsin–expressing cells compared with cells expressing M- or no α₁-antitrypsin (Fig. 1, G to I). This observation demonstrates that Z-α₁-antitrypsin accumulation affects molecular motion in the ER lumen.

Z-α₁-antitrypsin reduces protein mobility within ER inclusions
To test directly whether the changes in ER microviscosity are accompanied by altered protein mobility, we next used fluorescence correlation spectroscopy (FCS) to measure protein diffusion in the ER lumen. To circumvent confounding effects of complex ER network geometry (24), we limited our analyses to large ER inclusions (greater than 1 μm in diameter). FCS uses fluctuations in fluorescence intensity within femtoliter volumes to report on diffusion with millisecond resolution (25). To discern the effect of Z-α₁-antitrypsin on small-protein mobility in ER inclusions, FCS was used to measure diffusion of the small inert protein HaloTag, localized to the ER lumen with a C-terminal KDEL motif (HaloTag–KDEL) in ER inclusions of YFP-Z and in rare inclusions of YFP-tagged M-α₁-antitrypsin (YFP-M), producing autocorrelation curves with good fit (Fig. 2A to C). HaloTag–KDEL displayed a lower effective diffusion coefficient (D_eff) in inclusions of Z-α₁-antitrypsin–expressing cells compared to those expressing M-α₁-antitrypsin (2.6 ± 0.3 μm²/s versus 4.7 ± 0.5 μm²/s; Fig. 2D). For diffusion-dominated molecular motion, the mean-squared displacement <R²(t)> scales as a power law with time. The exponent α (denoting an anomalous diffusion parameter) can be used to broadly classify motion as “diffusive” (α = 1), “superdiffusive” (α > 1), or “subdiffusive” (α < 1) (derived in Materials and Methods) (26). Protein mobility in cellular environments can often display motion consistent with anomalous diffusion as a result of crowding or confinement (27, 28). The reduction in D_eff of HaloTag–KDEL was accompanied by a significant reduction in α from 0.68 ± 0.01 in YFP-M–expressing cells to 0.58 ± 0.01 in YFP-Z–expressing cells (Fig. 2E), indicating increased molecular confinement in the presence of Z-α₁-antitrypsin. In the context of the geometry of large ER inclusions (in both Z- and M-α₁-antitrypsin–expressing cells), such confinement cannot be attributed to direct collision of proteins with the encapsulating ER membrane. Hence, these data suggested that Z-α₁-antitrypsin itself might directly restrict and confine the mobility of small proteins in the ER lumen.

As many chaperones and folding factors that support ER protein folding assemble into higher-order complexes (29–30), we next assessed the effect of Z-α₁-antitrypsin expression on mobility of larger protein assemblies. To this end, we generated an ER-localizing *Aquificae aeolicus* lumazine synthase scaffold (ER-AqLs), a spherical genetically encoded multimeric nanoparticle (GEM) with diameter of 20 nm (Fig. 2F) (31). ER-AqLs punctate particles could readily be observed to colocalize with an ER marker protein in cells imaged by highly inclined and laminated optical sheet (HILO) microscopy (Fig. 2, G to J). ER-AqLs was then expressed in CHO-K1 cells together with HaloTagged α₁-antitrypsin (16) and imaged at a frame rate of 50 Hz. Kymographs display ER-AqLs intensity fluctuations over time through a linear region of interest (ROI) bisecting the inclusion (Fig. 2, K to N). In inclusions of cells expressing HaloTag–M-α₁-antitrypsin, mobile ER-AqLs GEMs could be visualized within ER inclusions [Fig. 2, K and L, and movie S1 (top)]. In some cells expressing HaloTag–Z-α₁-antitrypsin, ER-AqLs displayed relatively homogeneous distribution of fluorescence (fig. S1), consistent with a rapid GEM diffusion or high GEM density preventing particle resolution. However, a subpopulation of HaloTag–Z-α₁-antitrypsin–expressing cells displayed ER-AqLs puncta that remained static throughout the course of the experiment [Fig. 2, M and N, and movie S1 (bottom)]. This suggests that large protein complexes can become immobilized by physical confinement in Z-α₁-antitrypsin inclusions. Together, these FCS and HILO data indicate that Z-α₁-antitrypsin reduces the mobility of both small proteins and large protein assemblies within ER inclusions. The different degrees to which these inert protein species of differing sizes are immobilized suggested a model whereby Z-α₁-antitrypsin imposes size-dependent molecular filtration on the contents of ER inclusions.

Z-α₁-antitrypsin adopts a low mobility state in the ER
We previously reported that YFP-Z recovers slowly from photobleaching within ER inclusions (16). This, combined with the effects of Z-α₁-antitrypsin on ER microviscosity and protein mobility, led us to explore the mobility of Z-α₁-antitrypsin in large ER inclusions.
The application of fluorescence recovery after photobleaching (FRAP) to assess α₁-antitrypsin mobility within individual ER inclusions is hampered by variable connectivity between inclusions and the rest of the ER network (Fig. 3A, “Bleached ROI intensity” graphs) (16). To overcome this problem, we implemented a modified FRAP protocol, referred to here as intensity differential FRAP (ID-FRAP), to report on intracinclusion protein mobility with minimal confounding influence from ER connectivity. ID-FRAP used the intensity differential method to measure the mobility of a protein of interest within ER inclusions. This approach allows for more accurate quantification of mobility without the interference of ER connectivity. The results show that ID-FRAP provides a more reliable method for assessing protein mobility within ER inclusions compared to traditional FRAP.
Fig. 2. Reduced mobility of inert proteins in ER inclusions suggests molecular filtration imposed by Z-α1-antitrypsin. CHO-K1 cells were transfected with YFP-M or YFP-Z and a HaloTagged ER protein 48 hours before FCS in large ER inclusions. (A) Schematic showing FCS detection volume (DV) within an ER inclusion being traversed by a fluorescent particle (FP). (B and C) Example FCS autocorrelation curves (top) and residuals (bottom) for HaloTag-KDEL in cells expressing (B) YFP-M or (C) YFP-Z. (D) Measured effective diffusion coefficients ($D_{eff}$) for HaloTag-KDEL and (E) the corresponding anomalous parameter of diffusion ($\alpha$). A minimum of 25 cells were analyzed, acquired over three independent experiments. (F) Domain organization of ER-AqLs-Sapphire; preprolactin signal sequence (PrSS), the *Aquifex aeolicus* lumazine synthase scaffold (AqLs), and Sapphire fluorescent protein (aA denotes number of amino acids). (G to J) HILO micrographs of ER in a CHO-K1 cell coexpressing (G) Sec61TA-HaloTag (JF646 labeled) and (H) AqLs-Sapphire, merged in (I). (J) Maximum intensity projection of AqLs intensity from 1000 frames acquired over 20 s. (K to N) ER inclusion containing (K) HaloTagged–M-α1-antitrypsin (Halo-M) or (M) HaloTagged–Z-α1-antitrypsin (Halo-Z) (JF646 labeled) and AqLs-Sapphire. Dashed lines denote ROIs used to generate fluorescence kymographs [(L) and (N), respectively], displaying a 20-s imaging period. Images are representative of three independent repeats.
Fig. 3. Z-α₁-antitrypsin adopts a spectrum of mobilities in ER inclusions. (A) Schematic depicting photobleaching of fluorescent protein (green) within an ER inclusion that can exchange fluorescent protein with the ER network. Solid circle represents a bleached ROI, while the dashed-line circle represents a control ROI. Represented are two situations, showing a fluorescent protein with relatively high (top) or low (bottom) mobility. Cartooned graphs show the bleached ROI intensity (left) versus control ROI intensity minus bleached ROI intensity (right) as used in intensity differential (ΔI) FRAP (ID-FRAP). (B to D) Representative examples of CHO-K1 cell large ER inclusions containing (B) mobile, (C) semi-mobile, and (D) immobile YFP-Z before and at 0 or 80 s after bleach. ΔI (Intensity\text{control} − Intensity\text{bleached}) is shown as a percentage of the initial fluorescence intensity. (E) Distribution of YFP-M or YFP-Z mobilities (coexpressed with mCherry-KDEL) in CHO-K1 cell large ER inclusions determined by ID-FRAP, shown as a percentage of cells analyzed. (F) A representative example of large ER inclusions containing mobile M-α₁-antitrypsin. (G) A representative example of large ER inclusions containing immobile YFP-Z coexpressed with mCherry-KDEL in a MEF. (H) A representative example of a large ER inclusion containing immobile mEmerald-tagged neuroserpin(G392E) in a CHO-K1 cell, coexpressed with mCherry-KDEL.
The spread of YFP-Z mobilities in large inclusions of CHO cells remained greater than 10% after \( \Delta \) (Fig. 3C), while those in which \( \Delta \) failed to exceed 10% were described as “semi-mobile” (Fig. 3C), while those in which \( \Delta \) remained greater than 10% after 80 s were described as “immobile” contents (Fig. 3B); those in which \( \Delta \) recovered to below 10% within 80 s were described as “semi-mobile” (Fig. 3C), while those in which \( \Delta \) remained greater than 10% after 80 s were described as “immobile” contents (Fig. 3D).

Calreticulin drives the immobilization of Z-\( \alpha_1 \)-antitrypsin

Calreticulin is a highly abundant soluble ER lectin chaperone that interacts with client proteins via N-linked glycans and is up-regulated by ATF6 (34). It is known to engage Z-\( \alpha_1 \)-antitrypsin (36), which is glycosylated at three sites (37, 38). Knockdown of calreticulin has been shown to decrease intracellular levels of Z-\( \alpha_1 \)-antitrypsin by promoting its secretion (39). Hence, we postulated that calreticulin is a likely contributor to Z-\( \alpha_1 \)-antitrypsin immobilization. Using two-color ID-FRAP, we assessed the mobility of Z-\( \alpha_1 \)-antitrypsin (YFP-Z) coexpressed with HaloTagged calreticulin (HaloTag-CRT) in ER inclusions. Expression of wild-type HaloTag-CRT increased the proportion of cells with immobile YFP-Z from 17 to 55% (Fig. 4C). By contrast, expression of the HaloTag-CRT-G392E mutant, which binds neither substrate glycans nor the calreticulin-cofactor ERP57 (40, 41), failed to drive YFP-Z immobilization (Fig. 4C). Similarly, neither wild-type HaloTag-CRT nor HaloTag-CRT-G392E affected the mobility of glycosylation-incompetent YFP-Z-N46A,N83A,N247A (Fig. 4C). These results implicate calreticulin as an ATF6-transcriptional target that promotes Z-\( \alpha_1 \)-antitrypsin immobilization.

In addition to assessing YFP-Z mobility, ID-FRAP recovery times [time taken for fluorescence intensity differential (\( \Delta I \)) of a protein between control and bleach ROIs within the same inclusion to fall below 10%] were measured for coexpressed fluorescent proteins. Once again, YFP-Z had no effect on the small fluorescent protein mCherry-KDEL, which homogenized within 1 s after bleach (fig. S3A). By contrast, homogenization of HaloTag-CRT was notably slower in inclusions containing immobile YFP-Z (Fig. 4D). Although expression of HaloTag-CRT-G392E did not promote the immobilization state of YFP-Z (Fig. 4C), the mobility of this inactive calreticulin mutant was reduced in cells with immobile YFP-Z to a similar extent as had been seen for wild-type HaloTag-CRT (Fig. 4D versus Fig. 4E). These effects on ER protein mobility did not require Z-\( \alpha_1 \)-antitrypsin glycosylation, as similar values were observed in cells expressing nonglycosylated YFP-Z-N46A,N83A,N247A (Fig. 4, F and G, and fig. S3B). Together, these data suggested that the reduction in the mobility of calreticulin in the presence of immobile YFP-Z was not likely to represent a chaperone-client interaction but was more likely the result of chaperone confinement by immobile YFP-Z.

To ensure that the fluorescent protein tag on Z-\( \alpha_1 \)-antitrypsin did not contribute to reduced calreticulin mobility, a single-color ID-FRAP experiment was performed in a CHO-K1 stable cell line expressing untagged Z-\( \alpha_1 \)-antitrypsin under a dox-inducible promoter (13). Recovery time distributions for coexpressed HaloTag-CRT and HaloTag-CRT-G392E were similar in the presence of untagged or YFP-tagged Z-\( \alpha_1 \)-antitrypsin, exonerating the fluorescent protein tag from driving reduced calreticulin mobility (fig. S3C). Next, to assess whether the effect of calreticulin overexpression translated to other polymeric serpin family proteins, the mobility of mEmerald-tagged neuroserpin was assessed by two-color ID-FRAP. When coexpressed with mCherry-KDEL, mEmerald-tagged neuroserpin-G392E showed a spread of mobilities (11% immobile, 54% semi-mobile, 35% mobile).

Z-\( \alpha_1 \)-antitrypsin immobilization is promoted by the UPR

The reported hypersensitivity of Z-\( \alpha_1 \)-antitrypsin–expressing cells to ER stress (12, 13) led us to question whether an imbalance in ER proteostasis might contribute to formation of the immobile Z-\( \alpha_1 \)-antitrypsin. Accordingly, CHO-K1 cells expressing YFP-Z and mCherry-KDEL for 48 hours were exposed to ER stress for a further 8 hours, either by treatment with tunicamycin, which inhibits N-linked glycosylation, or thapsigargin, which depletes ER calcium. Neither tunicamycin nor thapsigargin had a detectable effect on mobility of the inert ER marker mCherry-KDEL as reported by ID-FRAP recovery time (fig. S2), but both agents markedly increased the proportion of cells with immobile YFP-Z from 19.2 to 54.8% with tunicamycin and 39.6% with thapsigargin (Fig. 4A).

ER stress is accompanied by UPR activation that increases ER protein folding capacity, in part, through up-regulation of molecular chaperones (10). There are three canonical branches of the UPR, and the ATF6 branch drives a large proportion of UPR-induced chaperone up-regulation (33, 34). In an attempt to uncouple the driver of ER stress (protein misfolding) from its downstream effects (via UPR activation), cells expressing YFP-Z were treated with tunicamycin in the presence of Cepain A7, which inhibits the ATF6 branch of the UPR (35). ATF6 inhibition abolished the YFP-Z immobilizing effects of tunicamycin treatment (Fig. 4B), suggesting that induction of chaperones by the endogenous stress response may drive Z-\( \alpha_1 \)-antitrypsin into the immobile state. Moreover, inhibition of basal ATF6 activity in the absence of ER stress decreased the basal proportion of cells displaying immobile YFP-Z and increased the proportion with the mobile phenotype (Fig. 4B). These data implicate ATF6-induced factors in the formation of a low mobility state of Z-\( \alpha_1 \)-antitrypsin.
and 35% mobile), similar to that seen for YFP-Z (Fig. 4H versus Fig. 4C), while coexpressed mCherry-KDEL was recovered within 1 s after bleach (Fig. 4I). Upon overexpression of calreticulin, neuroserpin G392E adopted the immobile phenotype in all cells analyzed (Fig. 4H) and was accompanied by slow recovery of HaloTag-calreticulin fluorescence (Fig. 4I), indicating a generalizable effect of calreticulin on immobilization of polymerogenic serpins in the ER.

Together, these data show that calreticulin mobility is retarded through confinement imposed by an immobile matrix of Z-α1-antitrypsin. As calreticulin chaperone activity is implicated in the immobilization of Z-α1-antitrypsin, it is likely that the Z-α1-antitrypsin immobilization observed during ER stress (Fig. 4, A and B) is, at least in part, the result of calreticulin up-regulation by ATF6 (Fig. S4). The more pronounced effect on mobility of calreticulin and large protein assemblies (Fig. 2, F to N) compared with a small ER marker protein (Fig. 2, A to E, and figs. S2 and S3, A and B) indicates a size-dependent filtration effect imposed by the immobile Z-α1-antitrypsin matrix.
Z-α₁-antitrypsin immobilization is associated with larger polymeric species

The notable effect of calreticulin expression on Z-α₁-antitrypsin mobility led us to investigate mechanistic drivers of this process. Polymerization of Z-α₁-antitrypsin is thought to occur via a C-terminal domain swap (42, 43), proceeding via a late folding intermediate of the native folding pathway that is stabilized by the E342K Z-mutation (44). Accordingly, this folding intermediate is predicted to have an unfolded C-terminal domain (Fig. 5A, shown in blue), consisting of s1C, s4B, and s5B that are yet to insert into the pocket residing behind β sheet A (45). A structural model of this intermediate showed that the unfolded C-terminal domain would likely have the flexibility to come within close proximity to all three N-linked glycans of antitrypsin and hence could plausibly interact with calreticulin bound at these positions (Fig. 5A). Furthermore, the unfolded C terminus is populated with hydrophobic residues (Fig. 5B) that represent a plausible target for chaperone binding (46). We therefore hypothesized that increased calreticulin levels, leading to a higher frequency of chaperone-binding events, might stabilize a polymerogenic intermediate and thus promote polymerization. Z-α₁-antitrypsin

Fig. 5. Calreticulin promotes larger Z-α₁-antitrypsin polymer-containing species. (A) A structural model of a predicted late folding intermediate of Z-α₁-antitrypsin, showing three Glcα1–3Manα1–2Manα1–2Man glycans at residues N46, N83, and N247. The C-terminal region is shown in blue. (B) An enlargement of the C terminus shows hydrophobic amino acid side chains in red (and in inset peptide sequence). (C) Whole-cell lysates of CHO-K1 cells transiently transfected to express untagged M- or Z-α₁-antitrypsin (M- or Z-A1AT) with mCherry-KDEL and Z-A1AT with either HaloTag-calreticulin (Halo-CRT) or HaloTag-calreticulin(92A,92A) (Halo-CRT) were separated by native-PAGE, and Western blots were probed with the α₁-antitrypsin polymer-specific mAb2C1. The same samples were separated by SDS-PAGE and blotted for total α₁-antitrypsin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Quantitation was performed on (D) total α₁-antitrypsin on SDS-PAGE and (E) total lane intensity of native-PAGE mAb 2C1 signal, from three independent experiments, with means and SEs shown. (F) mAb 2C1 signal intensity quantification on the blot shown in (C) was profiled from bottom to top of the gel. The X axis reflects increasing polymer size. (G) CHO-K1 cell lysates prepared as in (C) were centrifuged at 16,100g before both supernatant and pellet fractions were separated by native-PAGE. Western blots were probed with polymer-specific mAb2C1 antiserum. Representative gel of three experiments.
polymers can be visualized by native polyacrylamide gel electrophoresis (PAGE). Western blot using the conformation-specific antibody mAb2C1 (47). Polymer analysis by native PAGE was carried out on lysates of CHO-K1 cells expressing untagged Z-α1-antitrypsin with various other proteins (Fig. 5C). Cells coexpressing mCherry-KDEL with M-α1-antitrypsin produced negligible mAb2C1 reactivity compared to those expressing mCherry-KDEL with Z-α1-antitrypsin, where a ladder of mAb2C1-immunoreactive polymers was seen (Fig. 5C).

Coexpression of calreticulin led to a significant increase in both total cellular Z-α1-antitrypsin (Fig. 5, C and D) and of Z-α1-antitrypsin polymer accumulation, which was greatly diminished in cells expressing the nonfunctional mutant calreticulin (7) (Fig. 5, C and E). Furthermore, calreticulin-induced mAb2C1-reactive polymers dominated the upper region of the gel, indicating that they represent larger protein species (Fig. 5F). Centrifugation of lysates was then used to separate soluble and insoluble material, and only cells overexpressing calreticulin showed significant polymer accumulation in the pellet (Fig. 5G). Notably, lower-molecular weight polymers, which were similarly abundant in whole-cell lysates of cells expressing wild-type or mutant calreticulin (Fig. 5, C and F), were pelleted by centrifugation only from cells overexpressing wild-type calreticulin (Fig. 5G). This suggests that shorter-length polymers are also retained within the immobile Z-α1-antitrypsin matrix even upon cell lysis.

These data indicate that increased calreticulin levels, which promote Z-α1-antitrypsin immobilization, give rise to larger polymer-containing species that retain ER proteins. Next, we chose to investigate the physical properties of immobile Z-α1-antitrypsin.

**Z-α1-antitrypsin undergoes transition to a solid state in the ER lumen**

Formation of immobile Z-α1-antitrypsin could plausibly be attributed to several phenomena. These include agglomeration of Z-α1-antitrypsin polymers with unfolded/misfolded proteins, changes to polymer organization or structure, or an increase in macromolecular crowding in the ER resulting from impaired protein export or degradation. To investigate these possibilities, we used hypotonic shock to induce osmotic swelling of the ER and thereby reduce macromolecular crowding (48). The mobility of mEmerald-tagged Z-α1-antitrypsin was assessed by FRAP (Fig. 5A) before hypotonic shock. Improved photostability of mEmerald over YFP aided its continuous imaging during hypotonic-driven ER dilatation. Osmotic swelling of semi-mobile Z-α1-antitrypsin inclusions led to homogeneous dispersion of both mEmerald-Z and an ER-marker protein HaloTag-KDEL throughout the expanded ER volume (Fig. 5A). This was also seen in rare cells with inclusions of mEmerald-M (Fig. S5B). However, in cells with immobile mEmerald-Z inclusions, while ER swelling was again accompanied by homogeneous dispersion of HaloTag-KDEL, mEmerald-Z remained in discrete structures (Fig. 5B and movie S2). During swelling, immobile mEmerald-Z puncta appeared to remain tethered to the inclusion membrane, suggesting possible interaction with constituents of the ER membrane (Fig. 5C), but the puncta did not disperse or dissolve. These observations indicated that the immobility of Z-α1-antitrypsin in inclusions is not caused by tight packing of accumulated Z-α1-antitrypsin, confined by the ER membrane, but rather that Z-α1-antitrypsin undergoes a physical state change from a monodispersed to a solid condensed phase.

Material states that result in low protein mobility within cells include liquid-liquid phase separations (LLPSs) and transitions to solid states such as hydrogels, soft glasses, or crystalline aggregates (49). LLPS was deemed unlikely to account for Z-α1-antitrypsin immobilization, as immobile mEmerald-Z was unaffected by treatment with the small aliphatic alcohols 1,6-hexanediol (Fig. 6D) or propylene glycol (Fig. 6A), which disrupt many LLPS protein condensates in cells (50). By contrast, green fluorescent protein (GFP)-tagged fused in sarcoma (FUS-GFP) LLPS condensates formed in the nucleus upon mild hypertonic stress readily dissolved under these conditions (Fig. S6, B and C) (51, 52). Next, cells expressing mEmerald-tagged α1-antitrypsin were treated with the detergent-like compound saponin, which partially permeabilizes cellular membranes. Saponin treatment led to a rapid ER depletion of both mEmerald-M and the small ER luminal marker HaloTag-KDEL, as expected for soluble proteins (Fig. 6E). By contrast, mEmerald-Z remained within structures that morphologically resembled their parent ER inclusions despite ER membrane solubilization (as indicated by loss of HaloTag-KDEL fluorescence) (Fig. 6F). In cells expressing mEmerald-Z and HaloTag-calreticulin, both proteins endured upon saponin treatment (Fig. 6G), consistent with retention of calreticulin within the Z-α1-antitrypsin matrix (Fig. 4D). These observations are consistent with formation of a continuous solid phase of Z-α1-antitrypsin that is sufficiently porous to allow the transit of small proteins but traps HaloTag-calreticulin.

Confocal images of saponin-treated mEmerald-Z inclusions suggested underlying structure within the retained solid matrix [Fig. 6, F and G (bottom)]. To assess how matrix organization might influence protein complex distribution within inclusions, mEmerald-Z was coexpressed in CHO cells with ER-localized AqLs fused to HaloTag, labeled with the bright, photostable, and fluorogenic ligand JF646. Lattice SIM superresolution microscopy was performed on saponin-treated cells, and images of inclusions were reconstructed using Zeiss’ SIM² algorithm (Fig. 6, H to J). Strong anticorrelation was observed between mEmerald-Z and AqLs-HaloTag fluorescence intensity, implying that large protein complexes (with dimensions of approximately 20 nm) may become trapped between high-density regions of the Z-α1-antitrypsin solid matrix (Fig. 6K).

As calreticulin overexpression increased the unit size of Z-α1-antitrypsin species observed by native PAGE (Fig. 5, C to G), we hypothesized that this could plausibly affect the mechanical properties of the Z-α1-antitrypsin solid matrix. To explore this, puncta of solid Z-α1-antitrypsin were liberated from cells by detergent, purified by differential centrifugation, before assessing their mechanical properties using atomic force microscopy (AFM). Force curves of isolated YFP-Z puncta, deposited on a glass substrate, were obtained (Fig. S7). YFP-Z puncta extracted from cells coexpressing HaloTag-calreticulin showed increased stiffness compared to those extracted from cells coexpressing HaloTag-KDEL (Fig. 6L). These data indicate that calreticulin not only promotes formation of the solid phase of Z-α1-antitrypsin but also modulates the mechanical properties of the solid matrix to a more rigid conformation.

**Z-α1-antitrypsin mobility is reduced in ER tubules in a stress-induced manner**

While ER inclusions are a hallmark of liver biopsies from PiZZ individuals, many hepatocytes remain morphologically normal with no inclusions (6), generally assumed to represent a healthy pool of cells. However, the influence of Z-α1-antitrypsin expression on the reticular ER network has not been formally addressed. To investigate this, we optimized a single-particle tracking methodology for...
**Fig. 6. Immobile Z-α₁-antitrypsin forms a solid matrix.** CHO-K1 cells expressing mEmerald-tagged Z-α₁-antitrypsin (mEmerald-Z) and HaloTag-KDEL (Halo-KDEL) labeled with TMR HaloTag ligand were analyzed by ID-FRAP to assign α₁-antitrypsin mobility phenotype. (A) Semi-mobile mEmerald-Z or (B) immobile mEmerald-Z inclusions were imaged before (top) and after (bottom) 5 min of treatment with hypotonic buffer, leading to ER swelling. Images are representative of three independent experiments. Example inclusions marked “i” and “ii” are expanded in (C), as three-dimensional renderings from Z-stacked confocal image series, 7 min after hypotonic shock. (D) CHO-K1 cells were transiently transfected with expression plasmids encoding mEmerald-Z and HaloTag-KDEL and were analyzed by ID-FRAP. Images show ER inclusions of immobile mEmerald-Z before (left) and immediately after photobleach (middle). Cells were subsequently treated with 4% (w/v) 1,6-hexanediol (1,6-Hex) for 20 min before ID-FRAP assessment of Z-α₁-antitrypsin mobility in the same inclusion (right). Images are representative of all 14 cells analyzed over three experiments. (E to G) CHO cells expressing (E) mEmerald-M and Halo-KDEL, (F) mEmerald-Z and Halo-KDEL, and (G) mEmerald-Z and Halo-CRT were imaged before (top) and after (bottom) saponin treatment. (H to J) Lattice SIM images of mEmerald-Z puncta detergent-extracted from cells coexpressing AqLs-HaloTag labeled with JF646 ligand. Images were reconstructed using the Zeiss SIM² algorithm. The white line overlaid on the merged channel image (J) represents a linear ROI used to produce the histogram of fluorescence intensity gray values [kilogray (kGy)] along the length of the ROI (distance) shown in (K). (L) Apparent Young’s moduli of YFP-Z puncta extracted from cells expressing YFP-Z with either Halo-KDEL or Halo-CRT, assessed by atomic force microscopy (AFM) on a glass substrate. P value was assigned by Student’s t test.
assessing protein mobility in tubular ER networks (48). Experiments were performed in COS7 cells, which are more resistant to ER fragmentation than CHO-K1 cells despite accumulation of mAb221-immunoreactive Z-α1-antitrypsin polymers (47). Cells were made to express either mEmerald-M or mEmerald-Z, and the mobility of the small inert protein HaloTag-KDEL was assessed using the photoactivatable far-red fluorescent HaloTag ligand PA-JF646 (53). Images were acquired at a frame rate of 167 Hz with sufficiently low HaloTag-KDEL particle density to operate in a single-molecule regime. Fidelity of spot detection and track assignment were assessed by simultaneously imaging the ensemble fluorescence of ER-localized mEmerald (fused to α1-antitrypsin or KDEL) to scaffold ER structure, revealing that HaloTag-KDEL tracks consistently mapped to the ER and ER tubules remained within the focal plane of imaging (Fig. 7, A and B, and movie S3). Mean track velocities of HaloTag-KDEL molecules were assessed in sections of predominantly tubular ER in cells expressing mEmerald-M or mEmerald-Z. The distributions of track velocities appeared bimodal, with peaks at approximately 20 and 45 μm/s (Fig. 7, C and D). Expression of mEmerald-Z produced a pronounced increase in the proportion of low-velocity tracks compared to cells expressing mEmerald-M (Fig. 7, A and B, right), (C and D), equating to a reduction in mean cell $D_{\text{eff}}$ of HaloTag-KDEL from 2.0 μm$^2$/s in cells expressing mEmerald-M to 1.4 μm$^2$/s in cells expressing mEmerald-Z (Fig. 7E). These data indicate that Z-α1-antitrypsin restricts movement of ER proteins in tubular ER and in ER inclusions.

We next assessed the effect of chaperone overexpression on the mobility of Z-α1-antitrypsin in itself in the tubular network, by analyzing motion of HaloTag–Z–α1-antitrypsin particles. Recapitulating the effects seen in ER inclusions, the mean track velocity of HaloTag–Z–α1-antitrypsin particles was reduced below during overexpression of calreticulin (Fig. 8A) and after 8 hours of ER stress induction (Fig. 8B and movies S4 and S5). By contrast, ER stress induction alone did not affect the mobility of the inert protein HaloTag-KDEL (Fig. 8C). These relationships were confirmed by assessment of protein effective diffusion coefficients extracted from the mean instantaneous velocity of tracked particles in each cell analyzed (Fig. 8, D and E). Similar architecture of tubular ER in stressed and unstressed cells suggested that the observed effects on protein mobility are unlikely to be dominated by altered network geometries but rather by changes to ER luminal biophysics (Fig. 8, F to H; note the shortened, slower tracks of Halo–Z–A1AT in stressed ER). These data parallel our findings using ID–FRAP and FCS in ER inclusions. Furthermore, the notion that Z-α1-antitrypsin also reduces mobility of ER proteins in the tubular ER network suggests that hepatocytes of Pi²ZZ individuals with morphologically normal ER may represent a population relevant to liver pathology. Moreover, calreticulin overexpression, as well as stress induction that drives up-regulation of chaperones including calreticulin, reduces movement of Z-α1-antitrypsin itself. Together, these data indicate that Z-α1-antitrypsin immobilization is likely to initiate in the morphologically normal tubular ER, which represents a significant proportion of the ER in the hepatocytes of α1-antitrypsin-deficient individuals.

**DISCUSSION**

Here, we show that Z-α1-antitrypsin undergoes a phase transition to a solid state within the lumen of the ER, leading to molecular filtration of ER proteins in a size-dependent manner. While many examples of phase-state transitions have been shown in living cells, interpretation of their biological consequences is achieved less frequently (51, 52, 54, 55). The filtration of soluble proteins within the luminal space of a cellular organelle represents a previously undescribed mechanism through which a change in protein phase state can influence cellular processes. This observation has potential significance to a wide range of pathologies. Relevant to the ER, FENIB arises from mutations in

![Fig. 7. Single-particle tracking reveals reduced mobility in the tubular ER of Z-α1-antitrypsin–expressing cells.](image-url)

COS7 cells were transfected with mEmerald-tagged α1-antitrypsin and HaloTag-KDEL labeled with PA-JF646 ligand. Shown are fluorescence intensity micrograph of ([A], left) mEmerald-M or ([B], left) mEmerald-Z alongside projections of single-particle tracks (SPTs) acquired over 15,000 frames (corresponding right panels). Tracks are color-coded by mean track velocity. Frequency distribution histograms show mean track velocity of HaloTag-KDEL molecules collated from ([C] 38 cells expressing mEmerald-M and ([D] 50 cells expressing mEmerald-Z, fitted with a bimodal Gaussian distribution model color-coded for the low velocity peak (blue), high velocity peak (red), and combined fit (black). ([E] Mean effective diffusion coefficients ($D_{\text{eff}}$) of HaloTag-KDEL particles in each cell analyzed in ([C] and ([D], with mean and SE shown. $P$ values were assigned by Student’s $t$ test.)
neuroserpin that lead to an autosomal dominant form of dementia, characterized by the accumulation of protein polymers in a fragmented ER network in neurons (56, 57). Here, we show that molecular filtration in the ER is likely a consequence of the disease-associated mutant neuroserpin G392E (Fig. 4, H to J). Furthermore, in diabetes insipidus, specific mutants of vasopressin form fibrillar aggregates that accumulate within the ER, distorting its morphology (58). The extent to which molecular filtration is involved in this and other disease states is a question for future studies.

The folding pathway of α1-antitrypsin is altered by the E342K Z-mutation, which is thought to prolong occupancy of a folding intermediate conformation through which polymerization is favored (43, 59). This likely involves delayed insertion of the C-terminal domain into a pocket at the B-C barrel, arising from local destabilizing effects of E342K (Fig. 4A) (45). Of the mechanisms proposed for Z-α1-antitrypsin polymerization, compelling evidence supports a domain swap wherein the C-terminal domain of one protomer inserts into its native fold site of a second protomer (42, 43, 45), likely promoted by the exceptionally high endogenous expression levels of α1-antitrypsin in hepatocytes (60). The C-terminal domain contains a high proportion of hydrophobic residues (Fig. 5B) that facilitate its engagement in the native (or domain swapped) structure.
(43). Hence, an exposed C-terminal domain conformation could plausibly be stabilized by ER chaperone interactions, which occur at exposed hydrophobic peptide sequences (61–63). It is probable that increased chaperone levels would prolong the duration of an unfolded and exposed C-terminal domain, as chaperone binding is thought to stabilize non-native conformations (64). Structural modeling of α1-antitrypsin reveals intriguing features relating to the positioning of putative glycosylation sites relevant to lectin chaperone interactions (65–67). In addition, the glycosylation site at N46 is positioned at the opening of the hydrophobic cavity that accommodates β sheet B in the final stages of folding (Fig. 5A). Calreticulin binding at this site could plausibly delay β sheet B completion either by direct binding of the hydrophobic C-terminal peptide sequence or by affecting the structure of the B-C barrel that accepts the C terminus in the native fold. Calreticulin binds to hydrophobic peptide sequences providing protection from aggregation (46, 68, 69), reportedly mediated through binding sites both proximal (41) and distal (70) to glycan binding, in addition to recruiting the chaperones ERP57 (71) and ERp27 (72). Furthermore, sequence analysis of the C-terminal 20 residues of α1-antitrypsin reveals a number of predicted binding sites for the ER-localized Hsp70 family chaperone, BiP (73), suggesting that calreticulin interaction might cooperate with other ER chaperones in prolonging residency of the polymericogenic folding intermediate state. Notably, calreticulin has an ER membrane–anchored homolog, calnexin, which also interacts with substrates via N-linked glycans. While membrane localization of calnexin would prevent it from accessing regions of the Z-α1-antitrypsin solid matrix distal to the ER membrane, it is plausible that calnexin may also promote polymerization proximal to the ER membrane.

The UPР maintains ER proteostasis by balancing the load of unfolded client proteins with the chaperone machinery required to fold them (10). The expression of Z-α1-antitrypsin is not accompanied by strong induction of the UPР (11–13), supporting the notion that polymers of Z-α1-antitrypsin are assemblies of well-folded proteins that do not sequester a substantial pool of chaperones. However, the immobile matrix of Z-α1-antitrypsin, promoted by calreticulin, retarded the movement of ER proteins including calreticulin itself. Given that calreticulin can interact with substrates in both a glycan-dependent and glycan-independent manner (74), we cannot exclude the possibility that direct binding of the chaperone to immobile Z-α1-antitrypsin contributes to reduced HaloTag-CRT mobility in the presence of immobile YFP-Z. However, our data describing reduced mobility of inert proteins (HaloTag-KDEL and ER-AqLs) better supports a model whereby calreticulin mobility is reduced via molecular filtration by a solid matrix formed of Z-α1-antitrypsin polymers. We propose that this effect is likely to be further enhanced by the increased stiffness of the Z-α1-antitrypsin solid matrix that is promoted by calreticulin (Fig. 6L). Protein folding is governed by diffusion at both the intramolecular level, as nascent chains fold to their native conformation, and the intermolecular level, as chaperones and client proteins interact through random collisions. Hence, chaperone immobilization could plausibly alter protein folding efficiency in the ER leading to ER stress. Our data suggest that in cells expressing polymericogenic antitrypsin, up-regulation of chaperones during ER stress may further compromise ER protein folding in a positive feedback mechanism by immobilizing ER chaperones within the solid Z-α1-antitrypsin matrix (fig. S8). This model provides a rationale for the heightened sensitivity of Z-α1-antitrypsin–expressing cells to ER stress despite their absence of basal UPР activation (12, 13).

The observations reported here do not exclude a protective role for the encapsulation of Z-α1-antitrypsin within ER inclusions, as occurs for the heavy chain of immunoglobulin M (μ), which, in the absence of its light-chain binding partner, accumulates in dilated ER, reminiscent of Z-α1-antitrypsin inclusions (75). This accumulation of μ, appears to be well tolerated, so immobilization of Z-α1-antitrypsin, promoted by the UPР, might potentially corrupt a protective mechanism of the proteostatic machinery. Our observation that ER stress reduces Z-α1-antitrypsin mobility in ER tubules (Fig. 8, B and D) is compatible with a model of protective ER vesiculation, which would be predicted to reduce confinement of proteins in the ER. The survival of cells that show a high degree of ER vesiculation indicates that ER function is maintained despite these profound structural changes.

Our findings offer an explanation for the hypersensitivity to ER stress experienced by Z-α1-antitrypsin–expressing cells, suggesting that a positive feedback relationship exists between ER stress and the solidification of Z-α1-antitrypsin polymers. Accordingly, physiological ER stress in hepatocytes provokes a UPР that initially promotes up-regulation of chaperones, including calreticulin, that serve to ameliorate the stress. However, in this context, an increased abundance of calreticulin promotes solidification of Z-α1-antitrypsin polymers via a mechanism that requires Z-α1-antitrypsin glycosylation. The result of polymer solidification is the immobilization of ER proteins, including the ER chaperones required to resolve ER stress.

Our discovery that a transcriptional output of the UPР influences the physical state of accumulating Z-α1-antitrypsin poses an opportunity to design novel therapeutic strategies with which to combat α1-antitrypsin deficiency. Understanding the relevance of this disease mechanism to other proteinopathies, both in the ER and other compartments, can provide insight into a broad spectrum of disease states.

MATERIALS AND METHODS

Plasmids, antibodies, and fluorescent labels
Mammalian expression plasmids encoding α1-antitrypsin tagged at its N terminus with either YFP or HaloTag, separated by a flexible linker, were generated from pcDNA3.1 constructs encoding α1-antitrypsin, as described previously in (16). α1-antitrypsin N-terminally tagged with mEmerald was generated by replacing the YFP coding sequence of YFP-M and YFP-Z with that of mEmerald by Gibson assembly. The glycosylation-null variant of α1-antitrypsin was made by Gibson assembly to insert a synthesized gene fragment (GeneArt, Thermo Fisher Scientific, USA) encoding the region between H67 and A274 of unprocessed α1-antitrypsin, containing N70A, N107A, and N271A substitutions (N46A, N83A, and N247A in the signal peptide-cleavage protein and named as such here). ER-AqLs-Sapphire was made by designing a gene fragment (Twist Bioscience, USA) encoding the signal sequence of human prepro-lactin upstream of the AqLs-coding sequence, codon-optimized for mammalian expression. This fragment was used to replace the PfV coding sequence in pCMV-PfV-Sapphire-IRES-DsRed [pLH1337 (31); Liam Holt laboratory, New York University]. ER-AqLs–HaloTag was generated by replacing Sapphire with HaloTag by Gibson assembly. Plasmids encoding mCherry-KDEL (16), HaloTag-KDEL (21), FUS-GFP (76), HaloTag fused to human calreticulin (48), and mEmerald-KDEL (77) were reported previously. HaloTag-calreticulin
was generated by site-directed mutagenesis of HaloTag fused to human calreticulin. Calrecticulin-GFP was expressed from a plasmid encoding rat calreticulin C-terminally fused to GFP (78). A list of primers and plasmids can be found in table S1.

Antibodies in this study were raised against total α1-antitrypsin (A0409, Sigma-Aldrich), glyceraldehyde-3-phosphate dehydrogenase (2118, Cell Signaling Technology), and the α1-antitrypsin polymer–specific mADDC1 (HM2289, Hycult Biotech). HaloTag ligands JF646 and PA-JF646 (79) were a gift from the Luke Lavis laboratory (Janelia, USA). TMR HaloTag ligand was purchased (Promega, USA).

**Mammalian cell culture**

CHO-K1 cells were cultured as described previously (16), in F12 Ham nutrient mixture (Merck, Germany) supplemented with 10% fetal bovine serum (FBS) and GlutaMAX (Thermo Fisher Scientific, USA). Tet-ON CHO-K1 lines with inducible expression of α1-antitrypsin (16) were cultured as for CHO-K1 cells, but supplemented with tetracycline-free FBS (PAN-Biotech, UK). COS7 cells (catalog no. 87021302-1VL, Sigma-Aldrich, UK) were cultured in Dulbecco’s modified Eagle’s medium with glucose (4500 mg/liter; Sigma-Aldrich, UK) supplemented with 10% FBS. Transient transfections in CHO-K1 cells were performed using Lipofectamine LTX (Thermo Fisher Scientific, USA) at a ratio of 4 μl per 1 μg of DNA and in COS7 cells using FuGENE 6 (Promega, USA) at a ratio of 3 μl per 1 μg of DNA.

**Measurement of ER microviscosity by ROVI**

ROVI was performed as described previously (21). Briefly, CHO-K1 cells were seeded in eight-well glass bottom chamber slides (Lab-Tek II Chamber Coverglass) and transiently transfected 48 hours before imaging. Cells were labeled with 0.1 μM BODIPY-O2-HaloLigand (a molecular rotor based on meso-substituted boron dipyrrin) for 30 min in phosphate-buffered saline (PBS) before image acquisition by time-correlated single-photon counting. FLIM was performed using a confocal laser scanning microscope (Leica, SP5 II) with the Ti:sapphire laser in two-photon excitation mode operated at 880 nm. A PMC-100-1 photomultiplier tube (Hamamatsu) and an SPC-830 single-photon counting card (Becker & Hickl) were used for data acquisition. Fluorescence was collected between 500 and 580 nm. The instrument response factor was obtained by measuring second-harmonic generation signal from urea crystals on a glass cover slide.

**Intensity differential fluorescence recovery after photobleaching**

A modified FRAP protocol, here named ID-FRAP, was performed on “large” ER inclusions, being those with a diameter greater than 1 μm. CHO-K1 cells were seeded at 5 × 10⁴ cells per 35-mm dish on 25-mm glass cover slips, and transfection was performed 2 to 6 hours later. Cells were imaged 48 hours after transfection. Fluorophores were excited at 488 nm (mEmerald), 514 nm (YFP), 561 nm [HaloTag-tetramethylrhodamine (TMR) and mCherry], and 633 nm (HaloTag-JF646) and were imaged using a Zeiss LSM 780 confocal microscope with GaAsP detectors using a ×63 1.4 numerical aperture (NA) oil immersion lens. Images were captured with 512 × 512 pixel frame size, line switching between channels, with a two-color frame acquisition time of 0.928 s. Photobleaching was achieved using excitation lasers at 100% power for 25 scan iterations. The circular bleached ROI was chosen to be approximately one-fourth of a diameter of the ER inclusion. An unbleached control ROI of the same dimensions was placed within the same inclusion approximately one ROI diameter away from the bleached ROI. An image series was acquired continuously and was ended between 80 and 120 s after bleach, often dictated by inclusion movement outside of the field of view. For quantitation purposes, inclusion movement during the period of imaging was corrected by adjustment of ROI position relative to the inclusion’s perimeter (fig. S9). After normalization to starting ROI intensities, the intensity differential (ΔI) between the two ROIs in a single inclusion, bleached versus unbleached control, was calculated (ΔI = Intensitycontrol – Intensitybleached) and was used to define the following categories of protein mobility: mobile, if the FRAP bleach did not cause an intensity difference larger than 10% of initial intensity (Fig. 3B); semi-mobile, if the bleach caused the intensity difference between the bleached and control region larger than 10% but, within 80 s after bleach, the intensity difference dropped below the homogenization threshold value of 10% (Fig. 3C); and immobile, if the intensity difference between the bleached and control region was greater than 10% and did not recover below this threshold within 80 s after bleach (Fig. 3D). Hence, ID-FRAP reported on intra-inclusion protein mobility with minimal confounding influence from ER connectivity. ID-FRAP recovery times report the time taken for fluorescence intensity differential (ΔI) of a protein between control and bleach ROIs within the same inclusion to fall below 10%.

**Fluorescence correlation spectroscopy**

All FCS measurements were performed with HaloTag proteins labeled with TMR ligand (Promega, USA). CHO-K1 cells were seeded at 5 × 10⁴ cells per 35-mm dish on 25-mm glass coverslips (high precision no. 1.5H, Marienfeld), and transfection were performed 2 to 6 hours later. Cells were imaged at the center of large ER inclusions 48 hours after transfection using a ×40 1.2 NA water objective on a Zeiss LSM 780 confocal microscope with Zen 2.6 software package (Black edition). Axial and lateral focal radii of the microscope point spread function (PSF) was calibrated using an aqueous solution of rhodamine B excited at 561 nm, calculated by Zen 2.6 software using a reference diffusion coefficient for rhodamine B of 602.6 μm²/s at 37°C (as reported in FCS application notes; PicoQuant, Germany). Before FCS measurement, cells were bleached by 514- and 561-nm lasers to reduce the signal to 10 to 200 kilocounts/s corresponding to a density of 3 to 350 particles within the PSF. FCS was performed using 0.1% laser power to minimize bleaching, producing 1 to 10 kilocounts/s per molecule. The parameters used for fitting of data were as follows: Lag time analyzed, 9.6 μs to 1.68 s; amplitude (fixed = 1); axial focus radius = 1.123 μm; and one component fit with anomalous mode of diffusion (anomalous parameter set free to be determined by the software). Zen 2.6 Black edition software generated the anomalous parameter and diffusion coefficient by fitting the experimental data to the function describing diffusion with set parameters (described above). As the anomalous parameter and diffusion coefficient were set “free,” the software manipulated these parameters and provided the values that gave the best fit of the data (80). While inclusions were selected to be large enough to fit the entire PSF volume within, the pronounced noise in residual traces (Fig. 2, B and C) is likely a product of measurement within a tightly confined regime enforced by the surrounding membrane of the ER inclusion. These operations were made according to the microscope usage manual issued by Zeiss, Germany.
The formula for fitting the autocorrelation curve was as follows

\[ G(\tau) = 1 + A \cdot G_d(\tau) \]

where \( G_d(\tau) \) is the translation and \( A \) is the amplitude. Translation is calculated by

\[ G_d(\tau) = \sum_{i=1}^{3} \left(1 + \left(\frac{\tau}{\tau_i}\right)^{0.5}\right) \cdot \left(1 + \left(\frac{\tau}{\tau_0}\right)^{0.5}\right) \cdot e^0 \cdot \left(1 + \left(\frac{\tau}{\tau_0}\right)^{0.5}\right) \]

\[ \Phi_i = \frac{f_i \cdot \eta_i^2}{\sum_{i=1}^{3} (f_i \cdot \eta_i)^2} \]

\[ S = \frac{\alpha_x}{\alpha_y} \]

where \( i \) is the index of component (1,2,3), \( \Phi_i \) is the fractional intensity, \( f_i \) is the fraction of molecules, \( \eta_i \) is the molecular brightness, \( \tau_{0,i} \) is the diffusional correlation time, \( S \) is the structural parameter, \( \alpha_x \) is the axial focus radius (1.123 \( \mu \)m), \( \alpha_y \) is the lateral focus radius (0.206 \( \mu \)m), and \( \alpha_z \) is the anomaly parameter.

For one-component diffusion (\( i = 1 \) and \( f_1 = 1 \)) and the three-dimensional diffusion (\( e_{d1} = 1 \) and \( e_{d2} = 1 \))

\[ \Phi = \frac{f \cdot \eta^2}{(f \cdot \eta)^2} = \frac{1 \cdot \eta^2}{(1 \cdot \eta)^2} = \frac{\eta^2}{\eta^2} = 1 \]

\[ G_d(\tau) = \frac{1}{1 + \left(\frac{\tau}{\tau_0}\right)^{0.5}\left(1 + \left(\frac{\tau}{\tau_0}\right)^{0.5}\right)} \]

Amplitude is calculated by

\[ A = \frac{\gamma}{N} \]

where \( \gamma \) is the geometric factor for PSF (\( \gamma = 1 \) for cylindrical PSF) and \( N \) is the average number of molecules in the observation volume.

**Total internal reflection fluorescence microscopy imaging of AqLs**

Total internal reflection fluorescence (TIRF) microscopy images were acquired using a Zeiss Elyra7 wide-field microscope using a \( \times 63 \) 1.46 NA oil immersion TIRF objective. Dual-channel synchronous capture was achieved using an OptoSplit beam splitter (Cairn Research Ltd., UK) and two pco.edge sCMOS cameras (PCO, Germany). Fluorophores were excited at 488 nm (Sapphire, mEmerald) and 642 nm (HaloTag PA-JF646). Images were captured with an exposure time of 4 ms at a frame rate of 167 Hz. Photoactivation of PA-JF646 bound to HaloTag-KDEL was achieved by continuous exposure to 405-nm laser light to tune an appropriate density of particles within the 128 \( \times \) 128 pixel frame to permit tracking (see movie S5 for particle density). A Laplacian of Gaussian filter was applied to HaloTagged protein images, and single spot detection was performed with subpixel localization. Particle tracks were assigned using the simple Linear Assignment Problem (LAP) tracker of the TrackMate ImageJ plugin (81), and a threshold minimum number of spots within a track was set to 30 to increase spot linking confidence. Mean track velocity was extracted for each assigned particle track and collated with equal weighting across all cells in an experimental group. The mean particle step size was calculated across all tracked particles in individual cells, from which the mean squared displacement was calculated. Mean effective diffusion coefficients were calculated from the mean step size of particles per cell, accounting for the mean localization precision error of imaging.

**Native and SDS-PAGE**

CHO-K1 cells were grown to approximately 80 to 100% confluency in a six-well cell culture plate (Greiner Bio-One, UK). Wells were washed with 5 ml of PBS prechilled on ice. Two hundred microliters of ice-cold lysis buffer [10 mM Heps, 50 mM NaCl, 560 mM sucrose, 0.5% (v/v) Triton, 0.4 mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail (Roche, Switzerland)] was applied to each well, and the homogenate was collected. Cell lysates were sonicated in the water bath sonicator for 30 min at 4°C. One hundred microliters of the sonicated total cell lysate was centrifuged at 16,100 g for 10 min in a bench-top centrifuge at 4°C, and supernatant was separated from pelleted material. The pellet was resuspended in 100 µl of the homogenization buffer by sonication in the water bath sonicator for 30 min at 4°C. Samples were separated by SDS-PAGE under reducing conditions and by native PAGE as described previously (13).

**Osmotic manipulation of cultured cells during imaging**

Cells were prepared and imaged as described for FRAP experiments. Hypotonic swelling was achieved by addition of 6 volumes of Milli-Q water to 1 volume of culture medium to achieve an osmolality of 44 mosmol/kg. Images were acquired 5 min after addition of hypotonic buffer.

**Lattice SIM**

Lattice SIM imaging was performed using a Zeiss Elyra7 microscope using a \( \times 63 \) 1.4 NA oil immersion objective. mEmerald and HaloTag labeled with JF646 were excited at 488 and 642 nm, respectively, and images were captured simultaneously with 40-ms exposure time using an OptoSplit beam splitter and two pco.edge sCMOS cameras. Cells were imaged live, and Z-stacks were acquired with 55-nm sectioning. Images were processed using Zeiss’ SIM² algorithm in three dimensions using the “standard – live” settings with the sectioning set at 92 and intensity scaled with the original image.
A linear background subtraction was performed on both channels of the processed image, and pixel number was increased fourfold. Linear ROI intensity was measured using Fiji (ImageJ).

**AFM of purified YFP-Z puncta**

YFP-Z puncta were extracted from ER inclusions by incubation of CHO cells in lysis buffer [150 mM NaCl, 10 mM tris (pH 7.5), 0.5 mM EDTA, and 0.5% Triton X-100] containing complete protease inhibitors (Roche, CH) 48 hours after transient transfection with expression vectors encoding YFP-Z and either HaloTag-KDEL or HaloTag-calreticulin and subjected to centrifugation at 13,000g for 15 min to isolate cellular debris. Supernatant was discarded, and the pellet was resuspended in fractionation buffer [50 mM NaCl, 560 mM sucrose, 10 mM Heps (pH 7.5), and 0.5% Triton X-100] and spun twice at 800g for 5 min to remove nuclei. YFP-Z puncta were pelleted by centrifugation at 13,000g for 10 min and resuspended in resuspension buffer [150 mM NaCl and 20 mM Heps (pH 7.5), incubated for 16 hours at 4°C, before the sedimented material was discarded and, the supernatant fraction was retained for AFM analysis.

The samples were deposited on glass coverslips and incubated at room temperature for 15 min. The samples were then washed three times with Heps buffer and loaded on the AFM sample holder. AFM measurements were performed on a Bioscope Resolve AFM (Bruker), operated in PeakForce QNM mode, using precalibrated PeakForce QNM-Live Cell probes, with an average spring constant of 0.07 N/m. The force curves were then fitted to a Hertz model, Linker length affects photostability of BODIPY-HaloLigand (figs. S10 to S12) was synthesized as detailed of the puncta.

Linear ROI intensity was measured using Fiji (ImageJ). A linear background subtraction was performed on both channels and the supernatant fraction was retained for AFM analysis.

**REFERENCES AND NOTES**

BODIPY-HaloLigand synthesis

BODIPY-HaloLigand (figs. S10 to S12) was synthesized as detailed in the Supplementary Materials.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abm2094

**View request a protocol for this paper from Bio-protocol.**

**REFERENCES AND NOTES**

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