

Endoplasmic Reticulum Polymers Impair Luminal Protein Mobility and Sensitize to Cellular Stress in Alpha₁-Antitrypsin Deficiency

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Point mutants of alpha₁-antitrypsin (α 1AT) form ordered polymers that are retained as inclusions within the endoplasmic reticulum (ER) of hepatocytes in association with neonatal hepatitis, cirrhosis, and hepatocellular carcinoma. These inclusions cause cell damage and predispose to ER stress in the absence of the classical unfolded protein response (UPR). The pathophysiology underlying this ER stress was explored by generating cell models that conditionally express wild-type (WT) α 1AT, two mutants that cause polymer-mediated inclusions and liver disease (E342K [the Z allele] and H334D) and a truncated mutant (Null Hong Kong; NHK) that induces classical ER stress and is removed by ER-associated degradation. Expression of the polymeric mutants resulted in gross changes in the ER luminal environment that recapitulated the changes observed in liver sections from individuals with PI*ZZ α 1AT deficiency. In contrast, expression of NHK α 1AT caused electron lucent dilatation and expansion of the ER throughout the cell. Photobleaching microscopy in live cells demonstrated a decrease in the mobility of soluble luminal proteins in cells that express E342K and H334D α 1AT, when compared to those that express WT and NHK α 1AT (0.34 ± 0.05 , 0.22 ± 0.03 , 2.83 ± 0.30 , and $2.84 \pm 0.55 \mu\text{m}^2/\text{s}$, respectively). There was no effect on protein mobility within ER membranes, indicating that cisternal connectivity was not disrupted. Polymer expression alone was insufficient to induce the UPR, but the resulting protein overload rendered cells hypersensitive to ER stress induced by either tunicamycin or glucose depletion. **Conclusion:** Changes in protein diffusion provide an explanation for the cellular consequences of ER protein overload in mutants that cause inclusion body formation and α 1AT deficiency. (HEPATOLOGY 2013;57:2049-2060)

Alpha₁-antitrypsin (α 1AT) deficiency results from point mutations that lead to the formation of large, ordered polymers that accumulate within the endoplasmic reticulum (ER) of hepatocytes.¹ The resulting inclusions cause a toxic gain of function that is associated with neonatal hepatitis, cirrhosis, and hepato-

cellular carcinoma.² This is best exemplified by the Z (E342K), King's (H334D), Siiyama (S53F), and Mmalton (Δ 52F) alleles.³⁻⁶ Plasma deficiency in α 1AT deficiency can also result from truncating mutations, such as the Null Hong Kong (NHK) allele that target the protein for ER-associated degradation (ERAD).⁷ This

Abbreviations: α 1AT, α 1-antitrypsin; ATF6, activating transcription factor 6; CHO, Chinese hamster ovary; D_{eff} , effective diffusion coefficient; Dox, doxycycline; ELISA, enzyme-linked immunosorbent assay; EOR, endoplasmic overload response; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; ICC, immunocytochemistry; IRE1, inositol-requiring 1; mRNA, messenger RNA; NHK, Null Hong Kong allele; PAGE, polyacrylamide gel electrophoresis; PERK, protein Kinase-like ER kinase; RFP, red fluorescent protein; RT-PCR, reverse-transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; Tet-On, tetracycline-on; Tm, tunicamycin; UPR, unfolded protein response; XBP-1, X-box binding protein 1; WT, wild type.

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reduces secretion of α 1AT, but does not cause the gain-of-function liver disease. In both cases, the lack of circulating α 1AT predisposes the homozygote to early-onset emphysema.⁸

A wide range of signaling pathways are activated to restore the ER homeostasis in response to an overload of misfolded proteins. An important stress response is the unfolded protein response (UPR) that combines transient attenuation of protein translation with enhancement of the ER's protein-folding capacity, thus preventing uncontrolled aggregation of proteins within its lumen.⁹ This conserved stress response is tightly regulated by the activation of three ER stress sensors operating in parallel: inositol-requiring 1 (IRE1); double-stranded RNA-activated protein kinase-like ER kinase (PERK); and activating transcription factor 6 (ATF6). Under nonstressed conditions, each sensor is held in an inactive state by binding of the ER chaperone, BiP, to its luminal domain. When misfolded protein levels increase during ER stress, the UPR is activated, at least in part, through the titration of BiP away from these sensor molecules.

We aimed to determine the effect of polymers and truncated α 1AT on the ER environment. We show that the accumulation of ordered polymers of α 1AT leads to gross expansion of ER cisternae and impairment of ER luminal mobility. This hinders the organelle's capacity to deal with otherwise minor perturbations of protein folding and thus leads to enhanced sensitivity to ER stress. Our findings provide one of several potential mechanisms for the toxicity associated with ER overload in α 1AT deficiency-related liver disease.

Materials and Methods

Plasmid Constructs. Complementary DNA for human wild-type (WT) α 1AT and two polymeric mutants (E342K and H334D) were subcloned into the pTRE2hyg vector (Clontech, Saint-Germain-en-Laye, France) for the generation of stable Chinese hamster ovary (CHO)-K1 Tet-On (tetracycline-on) α 1AT cell lines. The NHK-truncated α 1AT variant (L318fsX17) was generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) using WT α 1AT as the template and then subcloned into the pTRE2hyg plasmid.

Generation and Characterization of Stable Cell Lines Expressing α 1AT. The CHO-K1 cell line was purchased from Clontech, and stable cell lines were generated by following the manufacturer's instructions. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) Tet/fetal bovine serum, 1% (w/v) nonessential amino acids, 1% (w/v) penicillin/streptomycin, 200 μ g/mL of Geneticin, and 500 μ g/mL of Hygromycin B (both selective antibiotics from Invitrogen, Carlsbad, CA) at 37°C and 5% (v/v) CO₂. α 1AT was typically induced with 1 μ g/mL of doxycycline (Dox) for 48 hours. Whole cell lysates, sodium dodecyl sulfate (SDS) and nondenaturing polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting, enzyme-linked immunosorbent assay (ELISA), and metabolic labeling were performed as detailed previously^{4,10} (see Supporting Materials).

Immunofluorescence, Electron Microscopy, and Immunocytochemistry. Immuno- and electron microscopy of CHO-K1 cells and immunocytochemistry (ICC) of paraffin tissue slides from human subjects were undertaken as described in the Supporting Materials.

Photobleaching Analysis in Live Cells. CHO-K1 Tet-On cells were transiently transfected with an ER-RFP (red fluorescent protein) plasmid¹¹ or a CytERM (cytoplasmic end of an ER signal-anchor membrane protein) msfGFP (monomeric superfolder green fluorescent protein) membrane reporter.¹² Live cells were imaged on a 37°C environmental controlled stage of a confocal microscope system (DuoScan; Carl Zeiss Inc., Thornwood, NY) with a \times 63/1.4NA oil objective and a 40-mW 561-nm diode laser with a 565-nm band-pass filter. For fluorescence recovery after photobleaching (FRAP/FLIP) experiments, a small region of interest was photobleached at full laser power in the 561 line and fluorescence recovery or loss was monitored over 0.2-second time intervals. Effective diffusion coefficient (D_{eff}) measurements were calculated as described previously.¹³ Fluorescence loss curves were obtained by transforming fluorescence intensities into a percentage scale in which the first bleach time point represents 100% of fluorescence intensity. Statistical significance was assessed using the Student *t* test.

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Detection of the UPR Markers. Immunoblottings for three main ER luminal chaperone proteins (GRP94, BiP/GRP78, and PDI), the ATF6-reporter luciferase assay, and the reverse-transcriptase polymerase chain reaction (RT-PCR) X-box binding protein 1 (XBP-1) splicing assay were performed as described previously.¹⁰

Results

Conditional Expression of α 1AT. Stable CHO-K1 cell lines were developed using the Tet-On inducible system that enables tight regulation of α 1AT expression. Four clonal cell lines were generated to express four single variants of α 1AT: the WT human α 1AT (M); the polymeric Z [E342K] mutant; the polymeric King's [H334D] mutant (both form-ordered polymers in the ER of hepatocytes in association with liver disease^{3,4}); and an NHK-truncated variant that does not fold properly, preventing the formation of ordered polymers, and instead activates the UPR.⁷ Expression of each variant was readily detected on treating cells with Dox (Figs. 1 and Supporting Fig. 1A-E). Cells expressing M α 1AT showed lower intracellular signal, compared to medium, consistent with its efficient secretion. Nondenaturing PAGE and ELISA assays identified intracellular polymers for only Z and H334D α 1AT. No ELISA signal was detected for the NHK variant, suggesting that the 9C5 epitope that is present in mono- and polymeric forms of α 1AT was removed by deletion of the C-terminal 61 amino acids. Protein-trafficking rate was assayed by metabolic labelling, followed by immunoprecipitation (Fig. 1C). WT α 1AT was almost completely secreted, compared with Z and H334D α 1AT, which were largely retained as polymers. In contrast, NHK α 1AT failed to reach the culture medium, despite being cleared from cell lysates at 4 hours, consistent with its degradation by ERAD.⁷

Accumulation of α 1AT Polymers Causes Disruption of the ER. Immunofluorescence confocal microscopy revealed that both Z and H334D α 1AT colocalized with the ER marker, BiP, although, in many cells, ER structure was distorted from its reticular morphology into a punctate pattern (Fig. 2A). No gross morphological changes were observed in cells expressing either M or NHK α 1AT. Of note, only M α 1AT colocalized with the Golgi marker, p115, indicating efficient exit from the ER (Fig. 2A). Electron microscopy was used to visualize cells expressing WT and mutant α 1AT. The ultrastructure in cells expressing M α 1AT was normal (Fig. 2B,a-b), when they

were compared with mock-transfected cells (Fig. 2B,i-k). The inclusions in cells expressing either Z or H334D α 1AT were associated with the replacement of healthy tubular and lamellar ER by electron-dense vesiculated structures (Fig. 2B,c-f). In contrast, expression of the NHK variant induced a markedly different phenotype with electron lucent dilatation and expansion of the ER throughout the cell (Fig. 2B,g-h). To elucidate whether the alterations observed could be the result of a massive overexpression of α 1AT in our model, we also assessed the distribution and signal of total α 1AT in paraffin-embedded liver sections from a PI*ZZ α 1AT homozygote and control human liver by ICC (Fig. 2C). The PI*ZZ homozygote human liver demonstrated levels of polymer accumulation at least as dramatic as that noted in cultured cells, suggesting that the CHO-K1 Tet-On cells represent a valid model of the accumulation of α 1AT polymers within the ER. Taken together, these data show that the accumulation of ordered polymers within the ER leads to gross changes in the organelle's morphology and, importantly, that these changes differ markedly from those induced by the NHK mutant that causes classical ER stress.

Polymeric Mutants of α 1AT Impair ER Protein Mobility in Live Cells. The effect of polymer formation on the ER luminal environment was determined by monitoring changes in the mobility of the ER-RFP fluorescence marker in live cells. ER-localized RFP is a protein (27-kDa and 2.3-nm hydrodynamic radius) with no known interacting partners that can rapidly sample the entire ER lumen, reporting on both crowdedness and interconnectivity.¹¹ ER-RFP distribution revealed two distinct ER architectures in cells expressing α 1AT variants. Each cell line displayed a typical ER reticular network in the absence of Dox, as did cells expressing M and NHK α 1AT when treated with Dox (Supporting Fig. 2). In contrast, approximately 30%-40% of cells expressing Z and H334D α 1AT developed a characteristic vesiculated architecture when treated with Dox (Supporting Fig. 2), as detected by staining for either α 1AT or BiP (Fig. 2A). Within the same clonal cell lines, there remained a proportion of cells expressing Z and H334D α 1AT that retained the normal reticular pattern of ER, suggesting that formation of inclusion bodies is a stochastic phenomenon (Supporting Fig. 3).

We then assessed ER lumen continuity by FLIP,¹⁴ in which a small region of cell ER-RFP fluorescence was repeatedly photobleached. If the protein is mobile within a continuous compartment, total fluorescence within this compartment will be depleted. Over time,

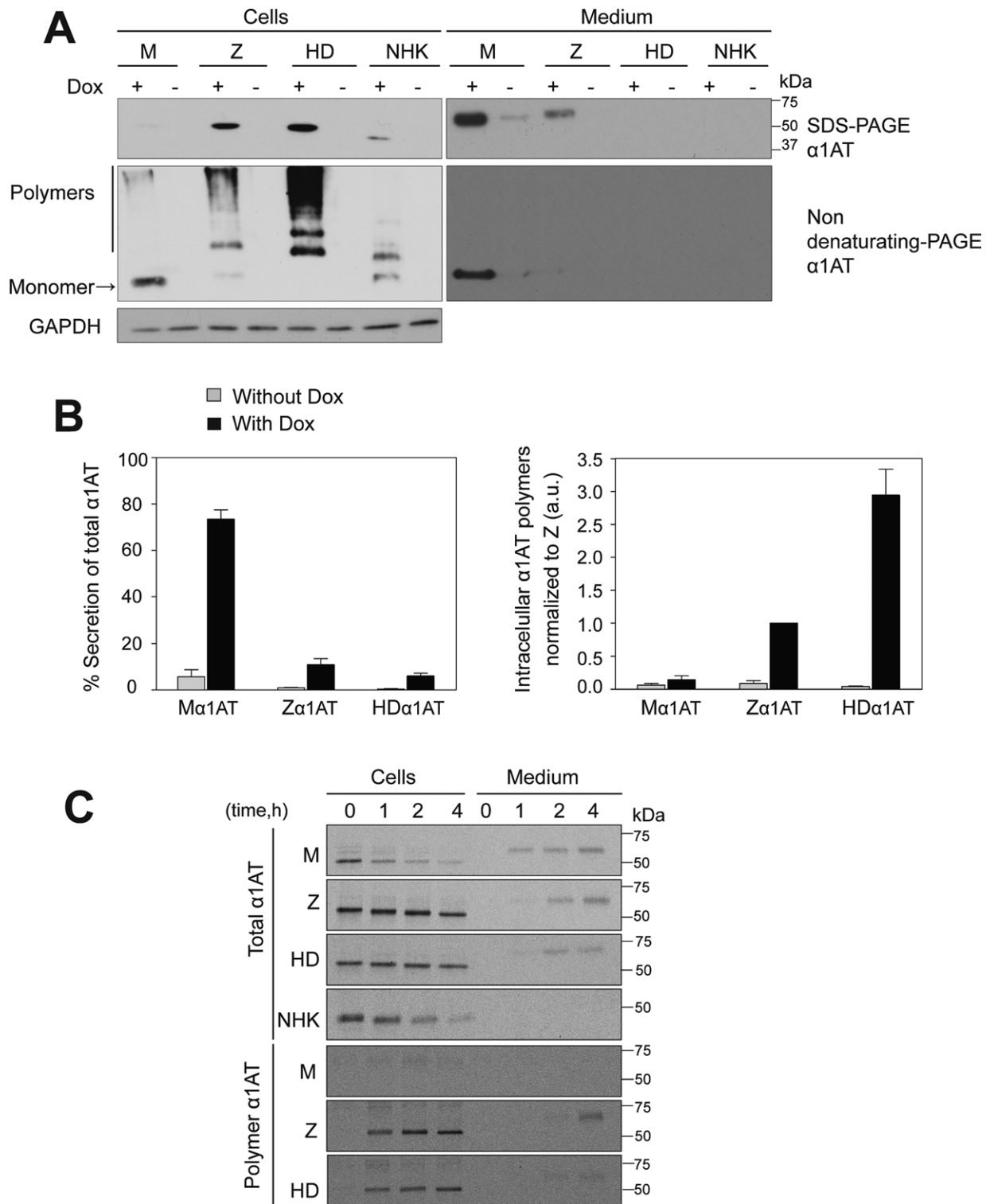


Fig. 1. Characterization of CHO-K1 Tet-On cell lines expressing different variants of α 1AT: WT (M); Z (E342K); HD (H334D); and the truncated NHK variant. (A) Cell lysates and medium were collected after induction of α 1AT with 1 μ g/mL of doxycycline (Dox) for 48 hours and analyzed by 10% (w/v) SDS and nondenaturing PAGE, followed by immunoblotting for α 1AT. (B) Percentage of secreted total α 1AT and quantification of intracellular polymers for M, Z, and HD α 1AT were quantified by sandwich ELISA using the 9C5 (left panel) or 2C1 (right panel) monoclonal antibodies, which recognize all forms of α 1AT or only polymers, respectively. Graphs show mean \pm standard error of the mean (n = 4). (C) CHO-K1 Tet-On cells expressing M, Z, HD, and NHK α 1AT were pulse-labeled with 35 S-Met/Cys for 15 minutes and chased for the times indicated (0-4 hours). α 1AT from cell lysates and medium was immunoprecipitated with a polyclonal antibody (total) or the 2C1 monoclonal antibody (polymers), then samples were resolved by 10% (w/v) SDS-PAGE and detected by autoradiography. Gels are representative of three independent experiments.

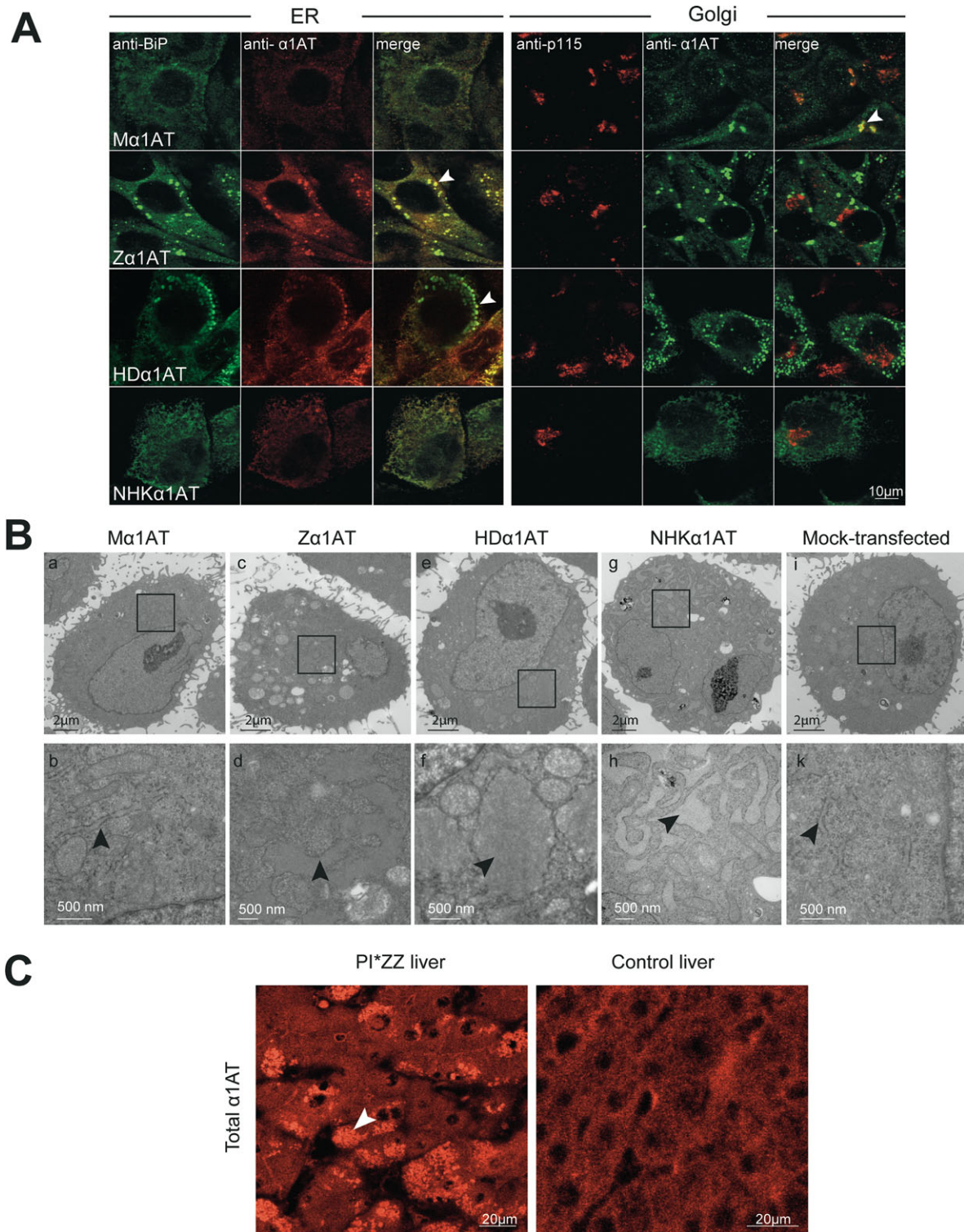


Fig. 2. Intracellular accumulation of α 1AT polymers in inclusion bodies disrupts ER structure. (A) Representative fluorescence images of Tet-On CHO-K1 cells were induced to express M, Z, HD, and NHK α 1AT with Dox for 48 hours, fixed, and coimmunostained with an α 1AT polyclonal antibody and either an ER protein marker (BiP) or a Golgi protein marker (p115). White arrows in merged panels show a punctate pattern of colocalized polymeric forms of α 1AT (Z and HD) with the ER marker (left panel) or WT α 1AT colocalized with the Golgi marker (right panel). (B) Electron microscopy analysis for ER structure in Tet-On CHO-K1 cells expressing α 1AT variants. Upper panels represent whole cells, with higher power images shown in lower panels. Black arrows indicate ER cisterns. Inclusion bodies were only identified in micrographs of polymeric mutants (Z and HD α 1AT). (C) Immunostaining of paraffin-embedded liver sections from a PI*ZZ α 1AT individual and a healthy control liver with a polyclonal antibody to total α 1AT (white arrow).

ER-RFP fluorescence in the whole cell was steadily and uniformly lost in cells displaying a tubular ER network, whereas fluorescence depletion was substantially slower in cells containing Z α 1AT polymer (Fig. 3A; Supporting Videos 1 and 2). This did not reflect an effect of transfected ER-RFP expression levels, because normalized plots showed that the remaining whole cell fluorescence intensity after 200 seconds was 23.5% and 61.1% for cells retaining a tubular ER network or a polymer-filled ER, respectively (Fig. 3A). Similar results were obtained for cells expressing H334D α 1AT (Fig. 3B). Thus, the presence of vesiculated structures is associated with marked loss of intra-ER luminal communication (i.e., slower ER-RFP mobility between inclusions) that is likely to adversely increase the heterogeneity of the ER luminal environment.

FRAP analysis was used to quantify the change in mobility of the ER-RFP reporter (Fig. 4A). This provides information on local rates of protein diffusion (D_{eff} , $\mu\text{m}^2/\text{s}$) by bleaching a small defined region of a single cell. D_{eff} is affected by local viscosity, size of the reporter protein, and its protein-protein interactions¹⁵ and is inversely proportional to crowdedness of the environment. Moreover, small changes in D_{eff} are likely to be biologically significant.¹¹ No significant differences in mobility of ER-RFP were detected in the different cell lines in the absence of α 1AT expression (Fig. 4B). ER-RFP D_{eff} values in cells expressing NHK and M α 1AT were identical (2.84 ± 0.30 versus $2.83 \pm 0.55 \mu\text{m}^2/\text{s}$). However, cells containing polymers of either Z or H334D α 1AT exhibited significantly lower ER-RFP D_{eff} values, compared with M α 1AT (0.34 ± 0.05 and $0.22 \pm 0.03 \mu\text{m}^2/\text{s}$, respectively; Fig. 4A,B; Supporting Videos 2 and 3).

The striking effect of polymers on ER-RFP diffusion within the lumen of the ER prompted an assessment of their effect on diffusion of an ER transmembrane reporter. We used the CytERM msfGFP plasmid, which encodes a signal domain required to anchor the fluorescent protein into the ER membrane with the sfGFP exposed to the cytoplasm.¹² There is no luminal domain and, consequently, the luminal content has no effect on membrane protein diffusion. There were no significant differences in CytERM msfGFP D_{eff} values in cells containing polymers of H334D α 1AT, compared to cells in which expression was not induced (0.35 ± 0.04 versus $0.40 \pm 0.02 \mu\text{m}^2/\text{s}$) (Fig. 4C).

Taken together, these data demonstrate that inclusions of α 1AT polymers have a remarkable effect on the ER environment, reducing luminal protein diffu-

sion. However, protein mobility within ER membranes is unaffected, indicating that cisternae connectivity is not disrupted.

Increased Susceptibility to ER Stress in Polymer-Expressing Cells. Previous work has shown that the ER accumulation of ordered polymers of mutant α 1AT and another member of the same protein family, neuroserpin, fail to activate the UPR.¹⁶⁻¹⁹ However, a recent report has suggested that Z α 1AT polymer accumulation triggers UPR activation in peripheral blood monocytes.²⁰ We hypothesized that the alteration of the ER luminal environment might destabilize ER homeostasis sufficiently to sensitize the cell to a second insult and so to trigger activation of the UPR. Activation of multiple reporters of UPR signaling was monitored after treatment with tunicamycin (Tm), an inhibitor of N-linked glycosylation. Immunoblotting analysis for three ER luminal chaperones (BiP/GRP78, GRP94, and PDI) confirmed that ordered polymers of α 1AT alone failed to activate the UPR (Fig. 5A, lanes 1-3). In contrast and as expected, synthesis of truncated NHK α 1AT led to robust activation of the UPR (Fig. 5A, lane 4). Remarkably, both polymerogenic α 1AT variants showed a hypersensitive response in UPR activation after treatment with low concentrations of Tm (5-25 ng/mL), when compared to the WT protein (Fig. 5A).

A more-quantitative reporter assay was used to assess the activation of the ATF6 branch of the UPR. Only cells expressing NHK α 1AT showed measureable activation of ATF6 under nonstressed conditions (Fig. 5B). However, cells expressing polymeric variants were more susceptible to activation of ATF6 on treatment with moderate concentrations of Tm (60-300 ng/mL). A similar response was observed when cells were treated with low concentrations of glucose (10.0-0.2 mM), which represents a more-physiological ER stress (Fig. 5C).

IRE1 activation was assessed by the splicing of *XBP1* messenger RNA (mRNA). None of the cell lines showed spliced *XBP1* mRNA under basal conditions. However, Tm (300 ng/mL) efficiently triggered IRE1 in all cell lines, as reported by the appearance of a smaller spliced *XBP1* mRNA band (Fig. 5D). The failure to detect splicing of *XBP1* mRNA in cells induced to express the NHK variant in the absence of Tm reflects the transitory nature of IRE1 activation.²¹ After the induction of ER chaperones, which occurs rapidly on expressing this truncated mutant, ER stress is alleviated and IRE1 returns to its inactive state. Indeed, the elevated level of ER chaperones in NHK-expressing cells served to protect them from

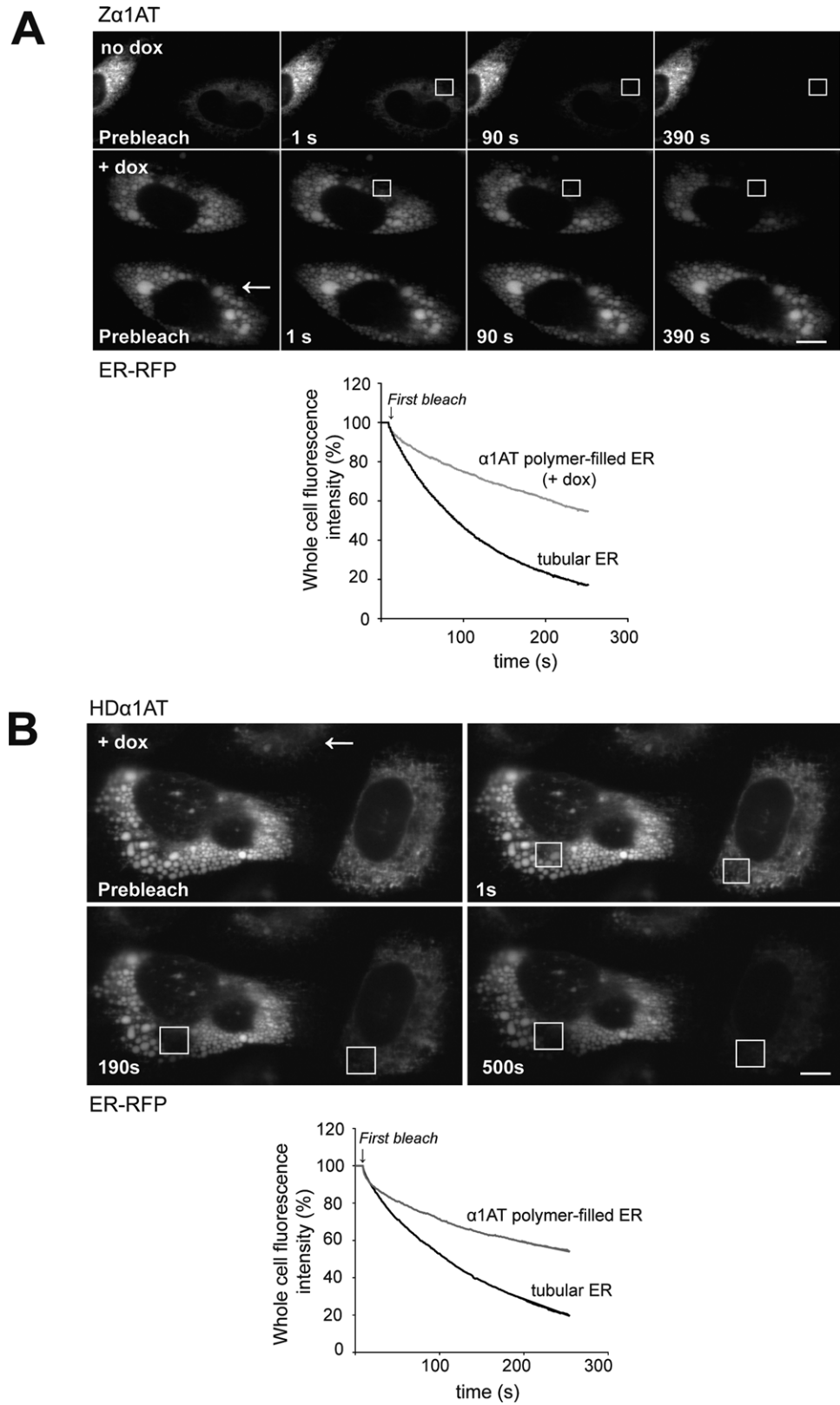


Fig. 3. Luminal α 1AT inclusion bodies impair protein mobility within the ER lumen. (A) FLIP analysis of Z α 1AT cells expressing ER-RFP within both a tubular ER network cell (without Dox) and an α 1AT polymer-filled ER (with Dox for 48 hours). FLIP normalized plots setting the prebleach intensity as 100%. (B) FLIP of H334D α 1AT cells expressing ER-RFP within both a tubular ER network cell and an α 1AT polymer-filled ER from the same sample after treatment with dox for 48 hours. Normalized plots from FLIP analysis as described in (A). Fluorescence loss was specific, because adjacent cells remained fluorescent (white arrow). White boxes represent the region subjected to photobleaching.

subsequent ER stress, as evidenced by the blunted response of *XBP1* splicing noted in these cells (Fig. 5D). In contrast, on treatment with Tm, M-, Z-, and H334D-expressing cells showed rapid splicing of *XBP1*

mRNA. These data indicate that the presence of polymers does not affect the intrinsic activity of IRE1. However, they do impair the cell's ability to restore ER homeostasis, because higher levels of spliced *XBP1*

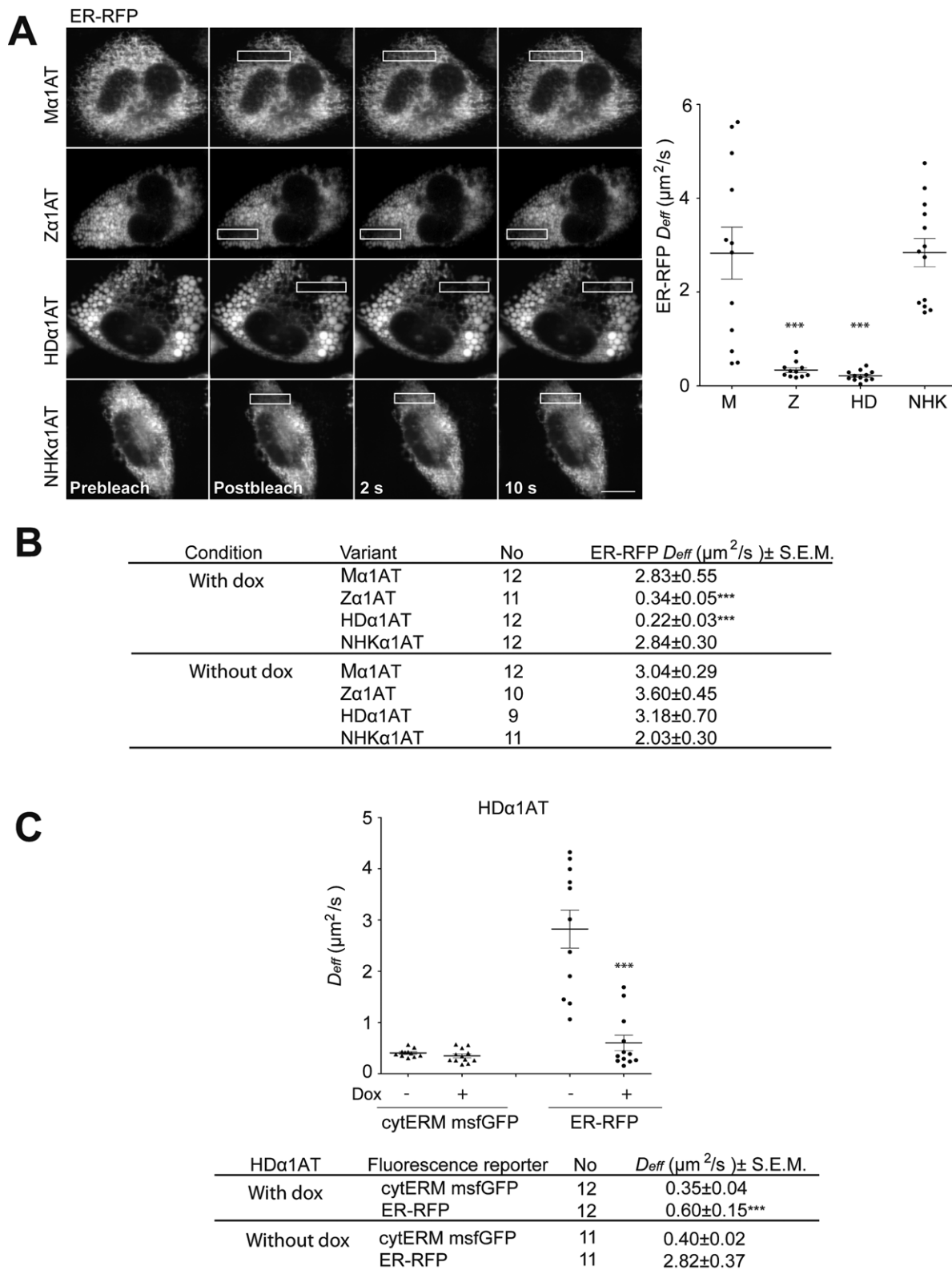


Fig. 4. Polymers of α 1AT modify the ER luminal environment decreasing ER protein mobility. (A) Left: FRAP series of Tet-On CHO-K1 cells induced to express M, Z, HD, and NHK α 1AT with Dox for 48 hours and transiently cotransfected with ER-RFP. Cells expressing either Z or HD α 1AT show a vesiculated pattern in which fluorescence recovery was significantly reduced. All images were captured immediately before (prebleach), immediately after (postbleach), and at times after photobleaching in the white box. Scale bar: 10 μm . Right: D_{eff} values from FRAP analysis in individual cells. (B) Mean values \pm standard error of the mean (SEM) for each condition and variant in (A). (C) Upper panel: D_{eff} values from FRAP analysis in individual Tet-On HD CHO-K1 cells treated with or without Dox for 48 hours and transiently cotransfected with both ER-RFP and CytERM msfGFP membrane reporter. Lower panel: mean \pm SEM for each condition and construct. *** $P < 0.0001$ in a two-tailed Student t test.

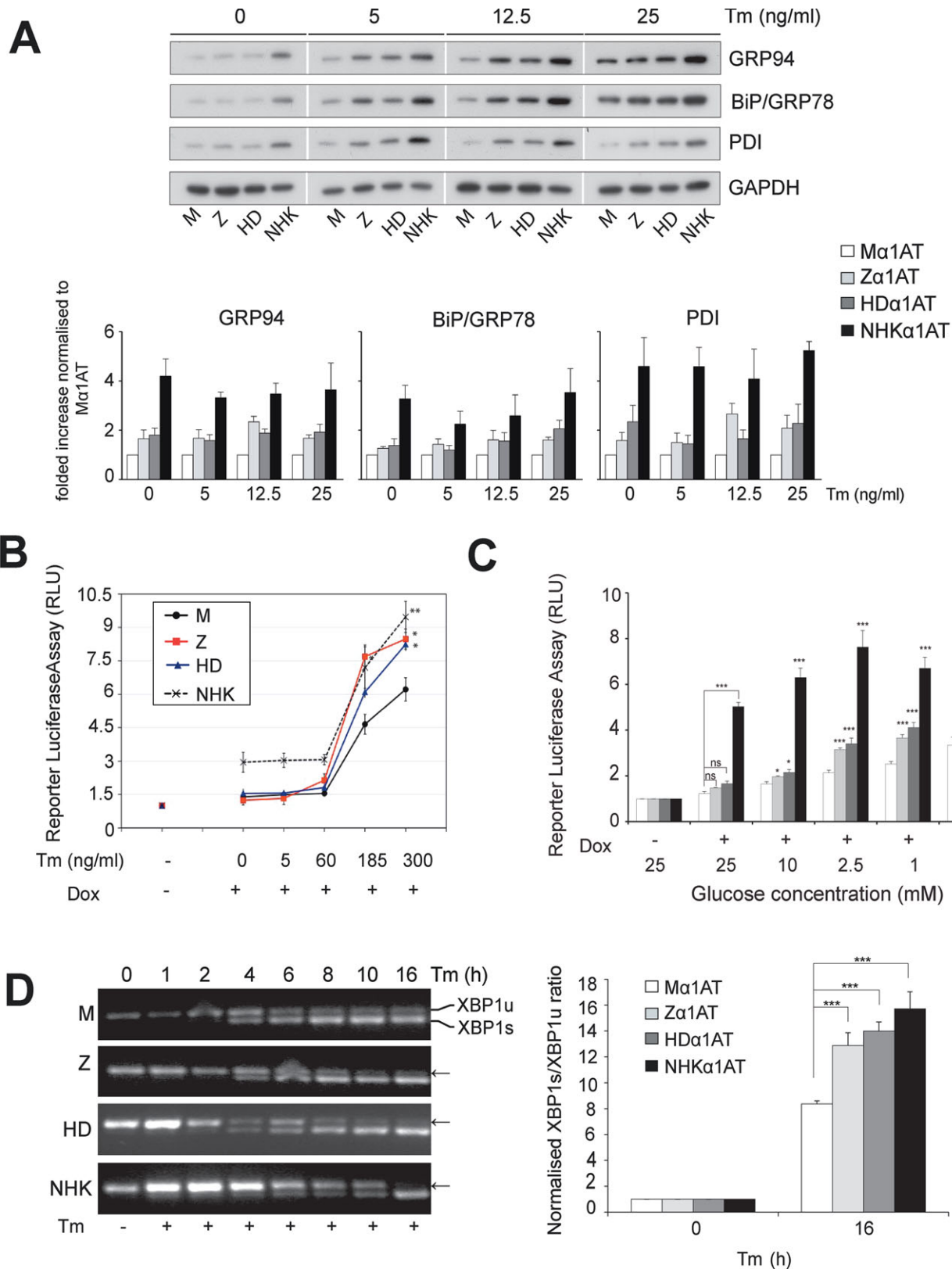


Fig. 5. Differential activation of three UPR sensors in polymeric forms of α 1AT reveals an increased sensitivity to UPR after a second *hit*. (A) Immunoblotting of whole cells lysates from CHO-K1 Tet-On cells induced with Dox for 48 hours and treated with the indicated doses of Tm for 16 hours. Grouping of separated lanes within the same gel was performed and indicated by dividing white lines. Histogram representation of four independent replicates of experiment (A) showing fold increase of GRP94, BiP/GRP78, or PDI normalized to loading control and then to WT variant for each concentration of tunicamycin (mean \pm standard error of the mean; SEM). (B) and (C) ATF6 activation during induction of ER stress. α 1AT expression was induced with Dox for 48 hours. Twenty-four hours before lysis, cells were cotransfected for 6 hours with a p5 \times ATF6-Luc encoding firefly luciferase under the control of a UPR response element and with pRL-TK *Renilla* luciferase as a normalization control, then treated with the indicated doses of Tm (B) or glucose (C) for 16 hours. Graphs show firefly luciferase normalized to *Renilla* luciferase as mean \pm SEM (n = 3). (D) *XBP1* processing during ER stress induction. Cells were induced with Dox for 48 hours and subjected to a time course of Tm treatment (300 ng/mL). RT-PCR was used to amplify *XBP1* mRNA, and PCR products were resolved by 2% (w/v) agarose gel electrophoresis. Ratio of spliced/unspliced *XBP1* mRNA for each α 1AT variant after 16-hour Tm is represented graphically as mean \pm SEM (n = 3). **P* < 0.05; ***P* < 0.01; ****P* < 0.001, according to analysis of variance test, followed by Bonferroni's post-hoc test.

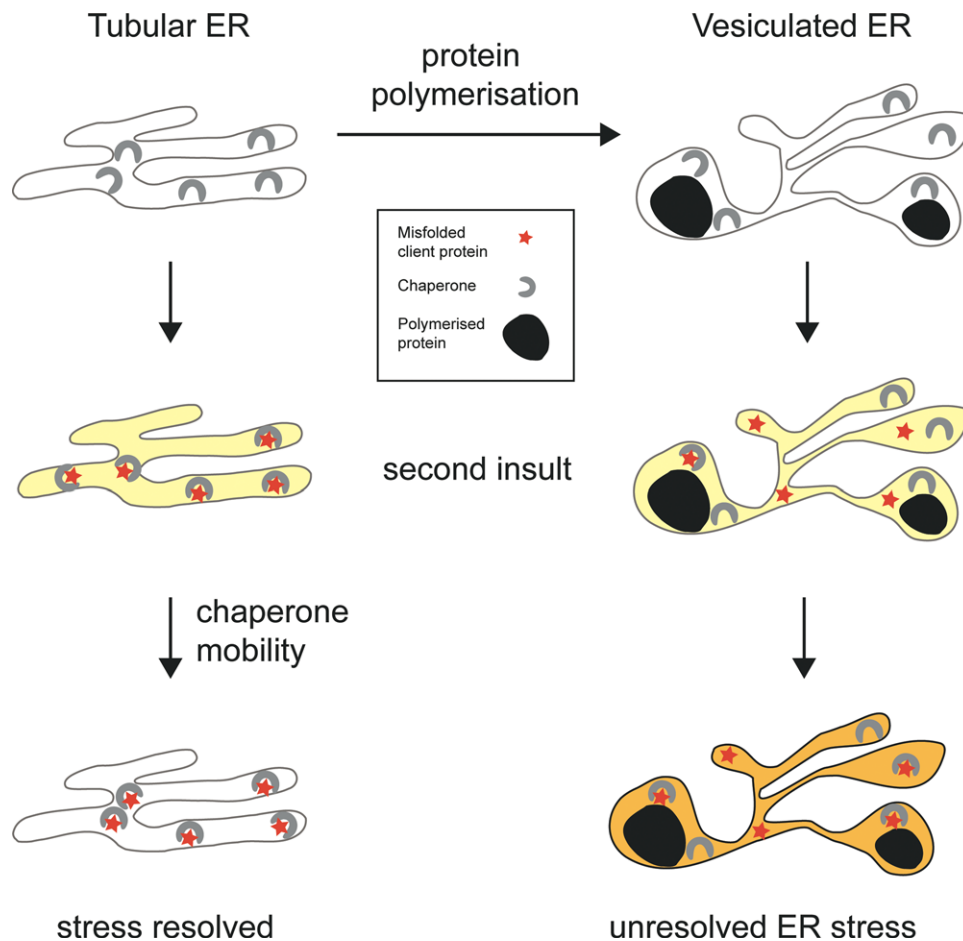


Fig. 6. Model for enhanced sensitivity to ER stress observed in cells experiencing ER overload. An insult resulting in misfolded client protein is effectively buffered by chaperone diffusion in healthy cells. This prevents ER stress ("resolved"). In contrast, in cells that accumulate polymerized protein, the global ER environment is affected, which impairs chaperone access to misfolded proteins, thereby increasing the cell's propensity to experience ER stress ("unresolved"). Yellow represents a resolved ER stress, whereas orange corresponds to an unresolved ER response to ER overload.

mRNA persisted after 16-hour treatment with Tm in cells expressing both Z and H334D α 1AT, compared to the WT protein.

It is possible that differences in sensitivity to Tm might be responsible for the enhanced UPR activation in cells that express polymers of α 1AT. However, this was not the case because treatment with Tm resulted in equal levels of a slower migrating glycosylated form of α 1AT and a faster migrating nonglycosylated form in each α 1AT-expressing cell line (Supporting Fig. 4).

Discussion

It is well recognized that point mutations of α 1AT form ordered polymers that are retained as inclusions within the ER, resulting in ER dysfunction and liver disease.⁸ These inclusions form in the face of effective disposal of misfolded monomer and polymers by ERAD^{18,22} and autophagy.²³ One of the cellular consequences of the accumulation of these ordered polymers is ER overload response (EOR), which results in the release of ER calcium and the activation of nuclear factor kappa B, a central mediator of inflamma-

tion.^{19,24} This pathway is distinct from the canonical UPR, which is triggered by the accumulation of misfolded protein and results in the inhibition of protein synthesis, induction of ER chaperones, and ER stress.²⁵ EOR and UPR pathways often occur together, but mutants of α 1AT provide ideal tools to probe these pathways because the polymer-forming mutants activate only the EOR,¹⁶⁻¹⁹ whereas truncated mutants activate only the UPR.⁷

We have used two polymeric mutants of α 1AT (E342K and H334D) to determine the mechanism by which ER overload can affect organization of the ER environment and the cell's susceptibility to ER stress. Our data reveal that polymers of α 1AT accumulate in spherical, apparently vesiculated ER, concomitantly with loss of the normal branching tubule network. Similar ER vesiculation has been reported to be associated with other cellular stresses, including mechanical injury and elevated cytosolic calcium concentration, although the physiological relevance of this remains unclear.^{26,27} Live-cell imaging revealed that accumulation of polymers in vesiculated ER leads to a marked, specific impairment of luminal protein mobility

resulting from an increased ER heterogeneity, which might reflect a change in either luminal viscosity and/or in tortuosity for ER-RFP diffusion within and between spherical inclusions. Interestingly, the truncated NHK mutant that causes classical ER stress (UPR) and is efficiently degraded by the proteasome showed a different ultrastructural change characterized by gross expansion of ER cisternae. This had no effect on luminal protein mobility. Current technology limits the area of photobleaching to slightly larger than the size of an inclusion. However, because effects of photobleaching were examined by FLIP throughout the cell, we are confident in these findings. It will be valuable, when the technology has improved sufficiently, to revisit the diffusion between individual inclusions.

In agreement with previous studies,¹⁶⁻¹⁹ we report that cells expressing polymers of either Z or H334D α 1AT do not show constitutive activation of the UPR. However, these cells do exhibit a hypersensitivity to both pharmacological and physiological ER stressors. To date, most studies that have attempted to detect ER stress in cells expressing mutant α 1AT have made use of methods such as the up-regulation of ER chaperones. Although providing important insights, such approaches have been unable to address how ER overload with polymers of α 1AT might affect molecular organization of the ER organelle. Our observation that enhanced sensitivity to ER stress after expression of polymerogenic α 1AT mutants correlates with the presence of marked changes in the biophysical features of the ER suggests a mechanism for this phenomenon (Fig. 6). Chaperones can readily diffuse to sites of protein misfolding in cells possessing a reticular, highly interconnected ER. In contrast, in cells experiencing ER overload, a comparable level of misfolded client protein cannot diffuse freely, decreasing their accessibility to quality-control proteins required for folding and transport. Therefore, we propose a model in which decreased mobility or availability of ER chaperones resulting from changes in diffusive features and/or obstruction caused by protein overload sensitizes the cell to subsequent activation of the UPR by a second hit that further increases the unfolded secretory protein burden. Our findings are directly relevant to the liver disease associated with polymerogenic mutants of α 1AT. However, although polymer accumulation involves a gain of function associated with liver dysfunction, epidemiological studies have shown that only 10% of patients with α 1AT deficiency develop clinically apparent liver disease.²⁸ It appears that additional, incompletely understood genetic and environmental factors contribute to this heterogeneity in

hepatic phenotype.^{29,30} Our results suggest that polymer accumulation increases the vulnerability of the cell to a “second hit” that ultimately initiates liver disease in individuals with α 1AT deficiency. Moreover, the mechanism proposed is likely to be relevant to other serpinopathies, such as the neuronal toxicity associated with polymers of neuroserpin in the dementia, familial encephalopathy with neuroserpin inclusion bodies.³¹

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References

- Lomas DA, Carrell RW. Serpinopathies and the conformational dementias. *Nat Rev Genet* 2002;3:759-768.
- Wu Y, Whitman I, Molmenti E, Moore K, Hippenmeyer P, Perlmutter DH. A lag in intracellular degradation of mutant alpha 1-antitrypsin correlates with the liver disease phenotype in homozygous PiZZ alpha 1-antitrypsin deficiency. *Proc Natl Acad Sci U S A* 1994;91:9014-9018.
- Lomas DA, Evans DL, Finch JT, Carrell RW. The mechanism of Z alpha 1-antitrypsin accumulation in the liver. *Nature* 1992;357:605-607.
- Miranda E, Pérez J, Ekeowa UI, Hadzic N, Kalsheker N, Gooptu B, et al. A novel monoclonal antibody to characterize pathogenic polymers in liver disease associated with alpha1-antitrypsin deficiency. *HEPATOLOGY* 2010;52:1078-1088.
- Lomas DA, Finch JT, Seyama K, Nukiwa T, Carrell RW. Alpha 1-antitrypsin Siiyama (Ser53->Phe). Further evidence for intracellular loop-sheet polymerization. *J Biol Chem* 1993;268:15333-15335.
- Lomas DA, Elliott PR, Sidhar SK, Foreman RC, Finch JT, Cox DW, et al. alpha 1-Antitrypsin Mmalton (Phe52-deleted) forms loop-sheet polymers *in vivo*. Evidence for the C sheet mechanism of polymerization. *J Biol Chem* 1995;270:16864-16870.
- Sifers RN, Brashears-Macatee S, Kidd VJ, Muensch H, Woo SL. A frameshift mutation results in a truncated alpha 1-antitrypsin that is retained within the rough endoplasmic reticulum. *J Biol Chem* 1988;263:7330-7335.
- Gooptu B, Lomas DA. Polymers and inflammation: disease mechanisms of the serpinopathies. *J Exp Med* 2008;205:1529-1534.
- Marciniak SJ, Ron D. Endoplasmic reticulum stress signaling in disease. *Physiol Rev* 2006;86:1133-1149.
- Irving JA, Ekeowa UI, Belorgey D, Haq I, Gooptu B, Miranda E, et al. The serpinopathies studying serpin polymerization *in vivo*. *Methods Enzymol* 2011;501:421-466.
- Snapp EL, Sharma A, Lippincott-Schwartz J, Hegde RS. Monitoring chaperone engagement of substrates in the endoplasmic reticulum of live cells. *Proc Natl Acad Sci U S A* 2006;103:6536-6541.
- Costantini LM, Fossati M, Francolini M, Snapp EL. Assessing the tendency of fluorescent proteins to oligomerize under physiologic conditions. *Traffic* 2012;13:643-649.
- Snapp EL. Fluorescent proteins: a cell biologist's user guide. *Trends Cell Biol* 2009;19:649-655.
- Snapp EL, Altan N, Lippincott-Schwartz J. Measuring protein mobility by photobleaching GFP chimeras in living cells. *Curr Protoc Cell Biol* 2003;Chapter 21:Unit 21.1.
- Lippincott-Schwartz J, Snapp E, Kenworthy A. Studying protein dynamics in living cells. *Nat Rev Mol Cell Biol* 2001;2:444-456.

16. Graham KS, Le A, Sifers RN. Accumulation of the insoluble PiZ variant of human alpha 1-antitrypsin within the hepatic endoplasmic reticulum does not elevate the steady-state level of grp78/BiP. *J Biol Chem* 1990;265:20463-20468.
17. Hidvegi T, Schmidt BZ, Hale P, Perlmutter DH. Accumulation of mutant alpha1-antitrypsin Z in the endoplasmic reticulum activates caspases-4 and -12, NFkappaB, and BAP31 but not the unfolded protein response. *J Biol Chem* 2005;280:39002-39015.
18. Kroeger H, Miranda E, MacLeod I, Pérez J, Crowther DC, Marciniak SJ, Lomas DA. Endoplasmic reticulum-associated degradation (ERAD) and autophagy cooperate to degrade polymerogenic mutant serpins. *J Biol Chem* 2009;284:22793-22802.
19. Davies MJ, Miranda E, Roussel BD, Kaufman RJ, Marciniak SJ, Lomas DA. Neuroserpin polymers activate NF-kappaB by a calcium signaling pathway that is independent of the unfolded protein response. *J Biol Chem* 2009;284:18202-18209.
20. Carroll TP, Greene CM, O'Connor CA, Nolan AM, O'Neill SJ, McElvaney NG. Evidence for unfolded protein response activation in monocytes from individuals with alpha-1 antitrypsin deficiency. *J Immunol* 2010;184:4538-4546.
21. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, et al. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* 2004;18:3066-3077.
22. Teckman JH, Burrows J, Hidvegi T, Schmidt B, Hale PD, Perlmutter DH. The proteasome participates in degradation of mutant alpha 1-antitrypsin Z in the endoplasmic reticulum of hepatoma-derived hepatocytes. *J Biol Chem* 2001;276:44865-44872.
23. Hidvegi T, Ewing M, Hale P, Dippold C, Beckett C, Kemp C, et al. An autophagy-enhancing drug promotes degradation of mutant alpha1-antitrypsin Z and reduces hepatic fibrosis. *Science* 2010;329:229-232.
24. Pahl HL, Baeuerle PA. The ER-overload response: activation of NF-kappa B. *Trends Biochem Sci* 1997;22:63-67.
25. Kaufman RJ. Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 2002;110:1389-1398.
26. Raeymaekers L, Lariviere E. Vesicularization of the endoplasmic reticulum is a fast response to plasma membrane injury. *Biochem Biophys Res Commun* 2011;414:246-251.
27. Subramanian K, Meyer T. Calcium-induced restructuring of nuclear envelope and endoplasmic reticulum calcium stores. *Cell* 1997;89:963-971.
28. Sveger T. The natural history of liver disease in alpha1-antitrypsin deficient children. *Acta Paediatr Scand* 1995;77:847-851.
29. Pan S, Huang L, McPherson J, Muzny D, Rouhani F, Brantly M, et al. Single nucleotide polymorphism-mediated translational suppression of endoplasmic reticulum mannosidase I modifies the onset of end-stage liver disease in alpha1-antitrypsin deficiency. *HEPATOLOGY* 2009;50:275-281.
30. Perlmutter DH. Liver injury in alpha1-antitrypsin deficiency: an aggregated protein induces mitochondrial injury. *J Clin Invest* 2002;110:579-1583.
31. Davis RL, Shrimpton AE, Holohan PD, Bradshaw C, Feiglin D, Collins GH, et al. Familial dementia caused by polymerization of mutant neuroserpin. *Nature* 1999;401:376-379.