

Clearance of protein aggregates during cell division

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eLife Assessment

How misfolded proteins are segregated and cleared is a significant question in cell biology, since clearance of these aggregates can protect against pathologies that may otherwise arise. The authors discover a cell cycle stage-dependent clearing mechanism that involves the ER chaperone BiP, the proteasome, and CDK inactivation, but is curiously independent of the anaphase promoting complex (APC). These are **valuable** and interesting new observations, but the evidence supporting these claims is partially **incomplete**. New experiments and/or toning down the conclusions and highlighting what has not been learned may be appropriate and can then spur more work in the field.

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Abstract

Protein aggregates are spatially organized and regulated in cells to prevent deleterious effects of proteostatic stress. Misfolding of proteins in the endoplasmic reticulum (ER) result in aggregate formation, but how the aggregates are processed especially during cell division is not well understood. Here, we induced proteostatic stress and protein aggregation using a proteostasis reporter, which is prone to misfolding and aggregation in the ER. Unexpectedly, we detected solid-like protein aggregates deposited mainly in the nucleus and surrounded by the ER membrane. The membrane-bound aggregates were then cleared as cells progressed through mitosis and cytokinesis. Aggregate clearance depended on Hsp70 family chaperones in the ER, particularly BiP, and proteasomal activity. The clearance culminated at mitotic exit and required cyclin-dependent kinase 1 (Cdk1) inactivation and ER reorganization but was independent of the anaphase-promoting complex (APC/C). Thus, dividing cells have the capacity to clear protein aggregates to maintain proteostasis in the newly divided cells, which could have implications for human disease development and aging.

Introduction

Nascent polypeptides fold into three-dimensional structures to perform their biological functions (Hipp et al., 2019 [↗](#)). Misfolded proteins tend to have their hydrophobic residues exposed and result in aggregation of proteins (Hartl et al., 2011 [↗](#)). Intramolecular beta-sheets in some proteins are also structural elements prone for protein aggregation (Tyedmers et al., 2010 [↗](#)). Protein misfolding and aggregation not only disrupt the native function of the protein but also could interfere other proteins' functions by co-aggregation (Olzscha et al., 2011 [↗](#); Woerner et al., 2016 [↗](#)). This challenges proteome homeostasis (proteostasis) and causes proteostatic stress in cells, which is a main driver of cellular aging and neurodegenerative disorders (Balch et al., 2008 [↗](#); Labbadia and Morimoto, 2015 [↗](#)).

Cells evolve several protein quality control (PQC) systems to maintain their proteostasis in which components of PQC systems facilitate refolding, degradation, and spatial deposition of misfolded protein aggregates (Jayaraj et al., 2020 [↗](#)). Molecular chaperones are key players in the PQC system. They are involved to fold nascent polypeptides, target misfolded proteins for degradation by ubiquitin-proteasome system or autophagy, and promote solubilization of protein aggregates (Tyedmers et al., 2010 [↗](#)). Eukaryotic cells confine misfolded proteins and smaller aggregates into distinct cellular deposition sites, which reduce the reactivity of these harmful species to the proteome (Kaganovich et al., 2008 [↗](#)). These deposition sites include aggresomes or aggresome-like induced structures locating near centrosomes, INQ (intranuclear quality control compartment) residing in the nucleus, cytosolic CytoQ, and the perivacuolar IPOD (insoluble protein deposit) (Kaganovich et al., 2008 [↗](#)). Confinement of protein aggregates in a deposition site facilitates retention of aggregates in short-lived cells (mother cells) during asymmetric cell division (Aguilaniu et al., 2003 [↗](#); Fuentealba et al., 2008 [↗](#); Rujano et al., 2006 [↗](#); Singhvi and Garriga, 2009 [↗](#); Zhou et al., 2014 [↗](#)).

The endoplasmic reticulum (ER) processes one-third of cellular proteome and possesses stronger ability to maintain aggregation-prone proteins in a non-toxic state than the cytosol owing to its specific molecular chaperone environment (Vincenz-Donnelly et al., 2018 [↗](#)). Chaperones in the ER such as HSPA5/BiP regulates the unfolded protein response (UPR), which blocks instant protein synthesis and activates transcription of genes involved in PQC (Preissler and Ron, 2019 [↗](#); Wiseman et al., 2022 [↗](#)). In addition, the proteostatic stress in the ER triggers ER-associated degradation (ERAD) and ER-phagy to remove misfolded proteins (Mochida and Nakatogawa, 2022 [↗](#); Olzmann et al., 2013 [↗](#)). Despite the high capacity of proteostatic stress response in the ER (Rousseau et al., 2004 [↗](#); Vincenz-Donnelly et al., 2018 [↗](#)), excessive misfolded proteins still lead to formation of protein aggregates in the ER (Melo et al., 2022 [↗](#); Miyata et al., 2020 [↗](#)). In response to protein aggregation in the ER, BiP functions as a disaggregase to promote solubilization of these aggregates. In cells undergoing asymmetric cell division such as in the budding yeast, the ER diffusion barrier and the ER stress surveillance (ERSU) pathway ensure ER protein aggregates are retained preferentially in the short-lived mother cells during cell division (Clay et al., 2014 [↗](#); Pina and Niwa, 2015 [↗](#)). How ER protein aggregates are regulated in dividing cells, especially those that do not undergo asymmetric cell division, is less well studied. We addressed the fate of ER protein aggregates in human cells undergoing cell division symmetrically and identified a clearance mechanism of protein aggregates when cells are exiting mitosis.

Results

Targeting a proteostasis reporter to the ER results in protein aggregate formation in the nucleus

To investigate how cells maintain their proteostasis during cell division, we employed a proteostasis reporter consists of a firefly luciferase mutant prone for protein mis-folding and protein aggregation fused to a green fluorescent protein (FlucDM-eGFP) (Gupta et al., 2011 [\[1\]](#)). Next, we targeted the reporter to the ER by fusing a ER-targeting sequence at its N-terminus (ER-FlucDM-eGFP). Interestingly, we found that ER-FlucDM-eGFP assembled into visible protein aggregates in the nucleus when stably expressed in mammary epithelial MCF10A cells in the absence of any perturbation of the proteome stability (**Figure 1A** [\[2\]](#)). The protein aggregates were below detection limit of our microscopy in first 2 days after lentivirus transduction but progressively accumulated in cells and stabilized in the population several days after lentivirus transduction. This contrasted with a previous study in which the ER-FlucDM-eGFP was transiently expressed in cells and hence did not allow long term tracking of the protein (Sharma et al., 2018 [\[3\]](#)). ER-FlucDM-mCherry localized to the ER network indicated by the ER membrane protein Sec61 β (**Figure 1B** [\[4\]](#)). Since we detected aggregate formation in the nucleus in cells expressing ER-FlucDM-eGFP, we tested if targeting FlucDM-eGFP to the nucleus (NLS-FlucDM-eGFP) would result in the formation of visible protein aggregates. Our data showed that NLS-FlucDM-eGFP did not form protein aggregates in the nucleus and was not co-localized with the ER-FlucDM-mCherry, and accumulated lower protein abundance than ER-FlucDM-eGFP (**Figure S1A-D** [\[5\]](#)). Luciferase activity measurement showed that ER-FlucDM-eGFP has significantly reduced enzymatic activity and was not functional possibly due to mis-folding and protein aggregation (**Figure 1C** [\[6\]](#)). ER-FlucDM-eGFP was also detected in the insoluble fraction after heat stress (**Figure S1E** [\[7\]](#)). Moreover, we observed the colocalization of ER-FlucDM-eGFP aggregates and thioflavin T (ThT), a universal dye to detect amyloid fibril, further supporting these aggregates were misfolded protein aggregates (**Figure S1F** [\[8\]](#)). Thus, expression of ER-FlucDM-eGFP results in proteostatic stress and allows us to study how protein aggregates are regulated in human cells.

Formation of protein aggregates in the nucleus by ER-FlucDM-eGFP was not limited to MCF10A cells. The protein aggregates were also detected in the nucleus when ER-FlucDM-eGFP was expressed in other cell lines such as A549, MDA-MB-231 and U2OS cells (**Figure 1A** [\[9\]](#) and **S1G**). Similar observations were found when a different N-terminal signal peptide sequence was used (**Figure 1D** [\[10\]](#) and **S1H**). In addition, when ER-FlucDM was replaced by ER-HaloDM, which is a modified haloalkane dehalogenase prone to mis-folding and aggregation (Melo et al., 2022 [\[11\]](#)), we observed similar protein aggregates in the nucleus (**Figures 1D** [\[12\]](#) and **S1H**). However, only very low number of cells formed aggregates in the nucleus when intact GFP was targeted to the ER (**Figure S1I** [\[13\]](#)). Thus, ER-FlucDM-eGFP formed protein aggregates in the nucleus independent of cell line, fluorescent protein types, specific ER targeting sequence and FlucDM.

Despite localizing in the nucleus, we found that the protein aggregates were surrounded by the ER membrane as stained by the ER-tracker (**Figure 1E** [\[14\]](#)). To further confirm this, we sectioned the cells and used electron microscopy to observe the protein aggregates. We found that the aggregates were surrounded by a single layer membrane closed to the inner membrane of the nucleus (**Figure 1F** [\[15\]](#)). Thus, the aggregates are intra-nuclear membranous structure surrounded by the ER membrane.

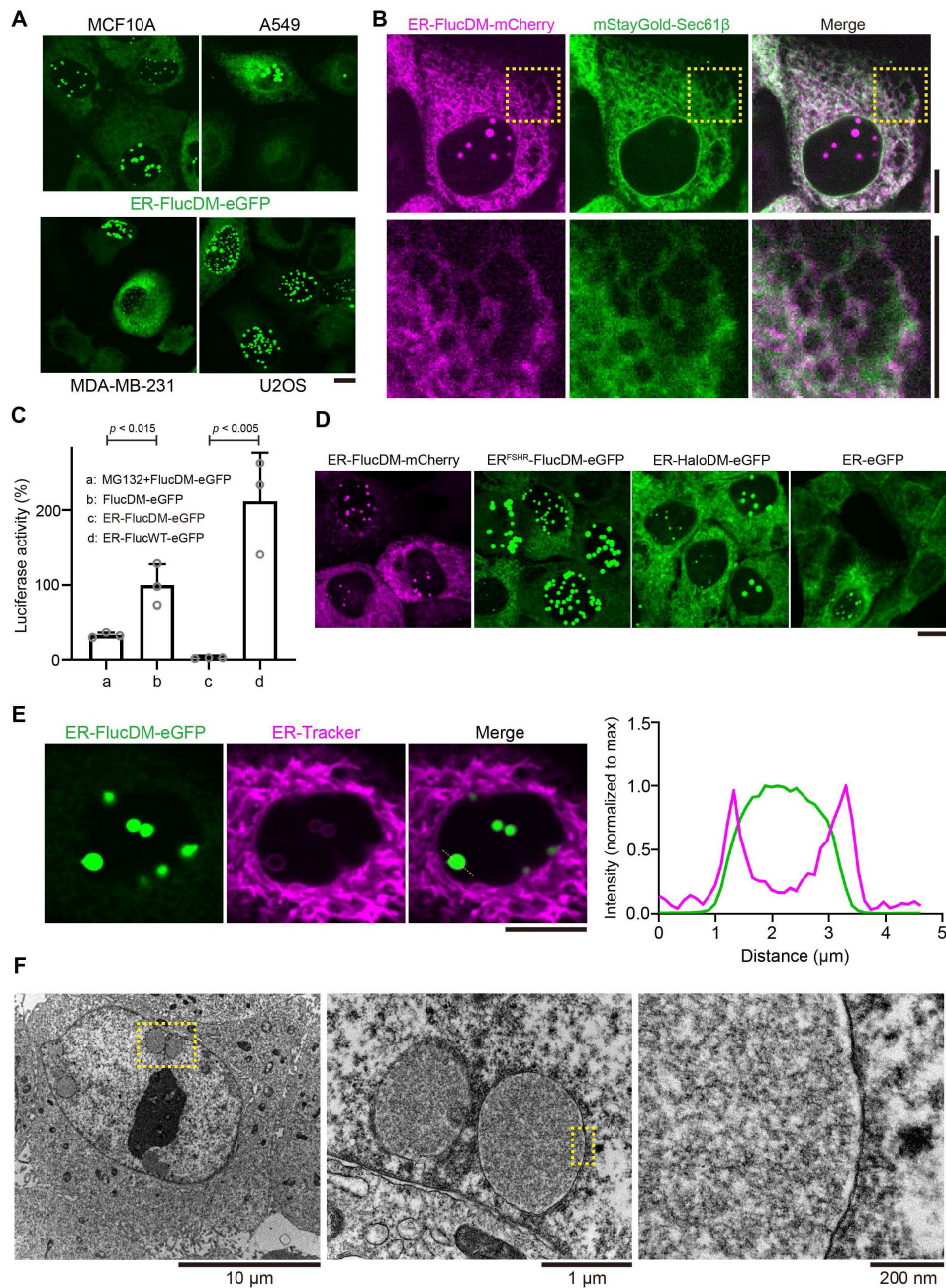


Figure 1.

ER-FlucDM-eGFP forms protein aggregates in the nucleus.

(A) Representative images of ER-FlucDM-eGFP expressed in different cell lines. Maximum projected images were shown.

(B) Microscopy images of cells expressing ER-FlucDM-mCherry and mStayGold-Sec61β. Single Z-slice images were shown.

(C) Quantification of the luciferase activity in MCF10A cells expressing different constructs. Unpaired t-test. Data were collected from three independent experiments. Abundance of GFP fusion proteins was determined using protein blotting and was used in normalization.

(D) Representative images to show MCF10A cells expressing ER-FlucDM-mCherry, ER^{F^{SHR}}-FlucDM-eGFP (signal peptide sequence from the N-terminus of FSHR), ER-HaloDM-eGFP or ER-eGFP. Maximum projected images were shown.

(E) Images of ER-FlucDM-eGFP aggregates surrounded by the ER membrane in a MCF10A cell. Single Z-slice images were shown.

(F) TEM images of sectioned MCF10A interphase cells expressing ER-FlucDM-eGFP.

Data represent mean + SD. Scale bar, 10 μm (A to E).

Differential and slow turnover of protein aggregates in cells at interphase and mitosis

Pathological proteins aggregates such as Z- α 1-antitrypsin accumulated in the ER exhibited minimal turnover (Dickens et al., 2016). To test whether ER-FlucDM-eGFP aggregates has a reduced dynamics as well, we performed fluorescence recovery after photobleaching (FRAP) in cells expressing both ER-FlucDM-eGFP and ER-FlucDM-mCherry. The ER-FlucDM-eGFP signal served as a tracker for the location of aggregates and the ER-FlucDM-mCherry was used to probe the aggregate dynamics. We photobleached ER-FlucDM-mCherry with 561 nm laser while ensuring ER-

FlucDM-eGFP signal was not affected. In interphase cells, ER-FlucDM-mCherry aggregates intensity barely recovered after 4.5 min post-bleaching, indicating that ER-FlucDM-mCherry aggregates possessed minimal turnover similar to that of in other protein aggregates such as Z- α 1-antitrypsin (Figure 2A). Similarly, ER-HaloDM-eGFP aggregates in the nucleus also displayed low recovery after photobleaching (Figure S2A). When ER-FlucDM-mCherry aggregates were photobleached in mitotic cells, the recovery of intensity was also much lesser than the ER-FlucDM-mCherry signal present in the ER network (Figures 2B and 2C). However, ER-FlucDM-mCherry aggregates in mitotic cells showed a higher recovery than that of in interphase cells (Figure 2D; aggregates of interphase cells recovered from 0.131 ± 0.093 to 0.157 ± 0.095 ; aggregates of mitotic cells recovered from 0.196 ± 0.137 to 0.356 ± 0.151).

Consistently, the single-cell analysis revealed that in mitotic cells, there was an increase of the recovery intensity of ER-FlucDM-mCherry in late time-point as compared to that of in early time-points after photobleaching. The increased recovery intensity was not observed in ER-FlucDM-mCherry aggregates of interphase cells (Figure S2B). Taken together, ER-FlucDM-mCherry aggregates in the nucleus have low turnover and the aggregate displays increased turnover in mitotic cells.

Protein aggregates are cleared during cell division

Since aggregates displayed some levels of turnover during mitosis (Figures 2B, 2C and 2D), we next investigated the aggregate behaviour throughout cell division. To this end, we labelled cells expressing ER-FlucDM-eGFP with SiR-Tubulin to distinguish cells at various stages based on the microtubule organization. We categorized cells into interphase and prophase, prometaphase, metaphase, anaphase, telophase and early G1 cells based on their microtubule structures (Figure 3A). Next, we analyzed the number and area of aggregates in cells of each category. Interestingly, we observed that the number and area of aggregates in telophase and early G1 cells were significantly lower than in interphase cells, suggesting that aggregates are cleared gradually when cells progress through mitosis and cytokinesis (Figure 3B).

To test whether this was the case, we employed time-lapse imaging to profile aggregates in cells undergoing division. To increase the number of dividing cells during imaging, we synchronized cells at G2/M and released them into mitosis prior to imaging. We observed that upon entry into mitosis, the ER-FlucDM-eGFP aggregates were released from the nucleus during nuclear envelope breakdown and gradually decreased in numbers as cells progress through mitosis and cytokinesis (Figure 3C and Video 1). In majority of early divided G1 cells, there was essentially no detectable aggregates (Figure 3D). Interestingly, the aggregates released from the nucleus during mitosis were still surrounded by the ER membrane as indicated by the ER-tracker (Figures S3A and S3B). The decrease of aggregates appears to happen specifically in dividing cells as the number and area of aggregates remained largely unchanged in interphase cells (Figures 3C and 3D). Furthermore, aggregates formed in cells expressing ER-HaloDM-eGFP decreased in

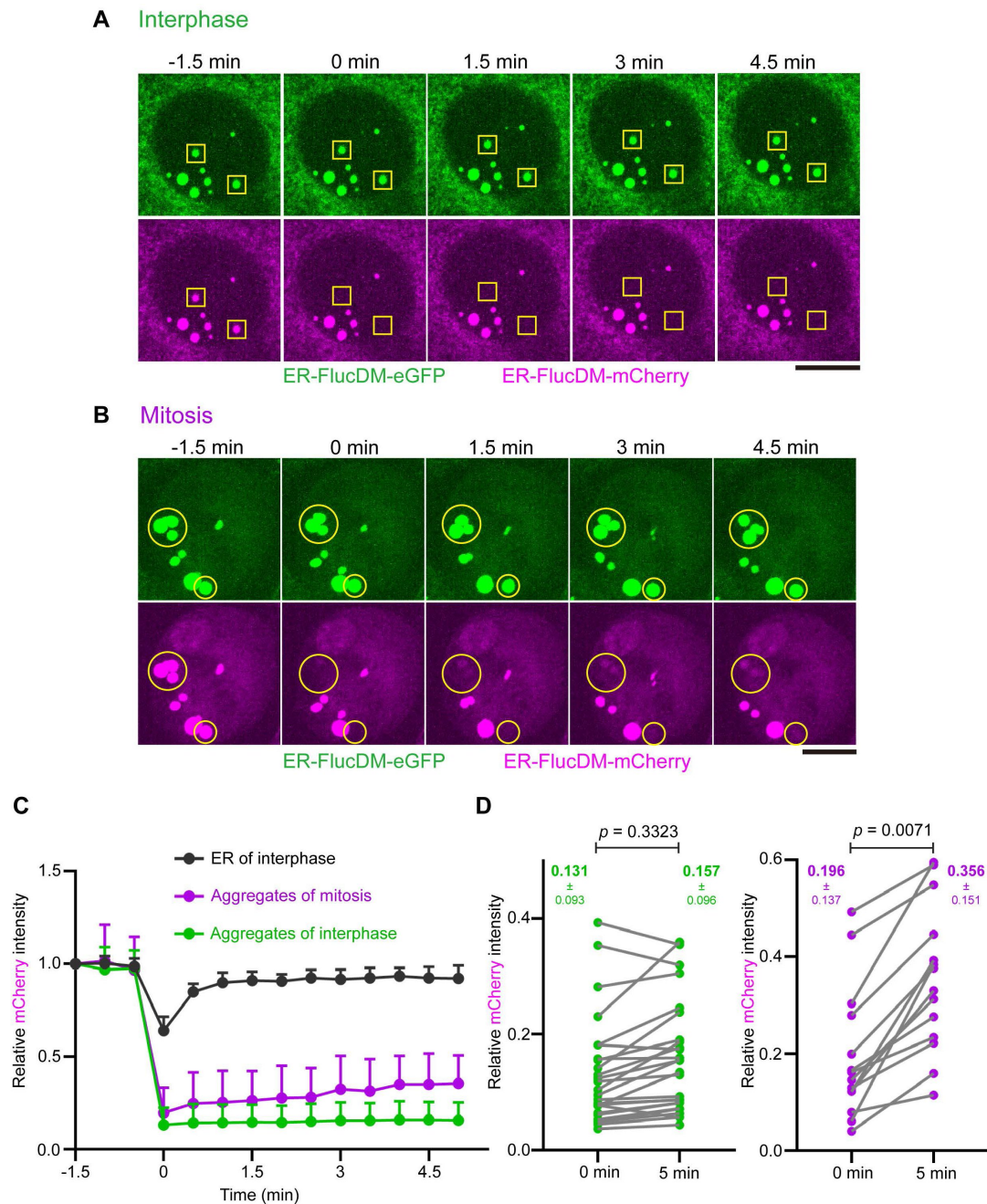


Figure 2.

Differential aggregate dynamics in cells at interphase and mitosis.

(A) Representative images of interphase cells co-expressing ER-FlucDM-eGFP and ER-FlucDM-mCherry before and after photobleaching. Yellow rectangles indicate bleached aggregates. Maximum projected images were shown.

(B) Representative images of mitotic cells co-expressing ER-FlucDM-eGFP and ER-FlucDM-mCherry before and after photobleaching. Yellow circles indicate bleached aggregates. Maximum projected images were shown.

(C) Recovery curves of ER-FlucDM-mCherry intensity after photobleaching. 14 ROIs from the ER of interphase cells, 14 aggregates from mitotic cells, 26 aggregates from interphase cells. Data represent mean + SD. A small region of ER-FlucDM-mCherry in the ER network was chosen as a control for photobleaching.

(D) Quantification of the ER-FlucDM-mCherry intensity of bleached aggregates right after bleaching and after 5 min of recovery. 0 min and 5 min refer to the time points in (C). Paired t-test (two-tailed). Data were collected from four independent experiments. Scale bar, 10 μ m.

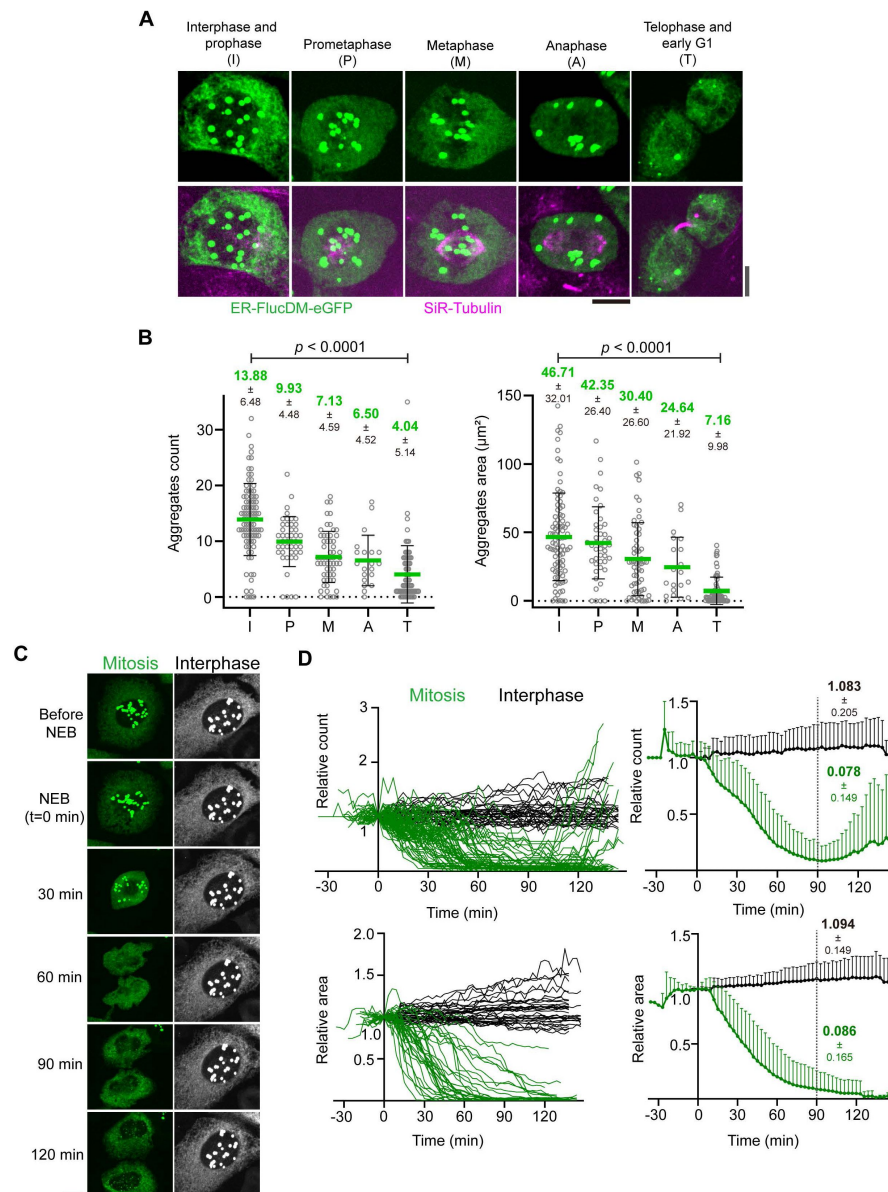


Figure 3.

Aggregates are cleared during cell division.

(A) Representative images of dividing ER-FlucDM-eGFP cells at different cell cycle phases. Grey scale bar: telophase and early G1 cells; black scale bar: interphase and prophase, prometaphase, metaphase, anaphase. Maximum projected images were shown. Scale bar, 10 μm .

(B) Quantification of ER-FlucDM-eGFP aggregates from dividing cells. Count and area are individual cell data. Each grey ring represents aggregates from one cell. Interphase and prophase, 91 cells; prometaphase, 45 cells; metaphase, 60 cells; anaphase, 20 cells; telophase and early G1, 73 cells. From three independent experiments. Unpaired two side Mann-Whitney U-test. Data represent mean $\pm$ SD

(C) Representative time-lapse images of cells expressing ER-FlucDM-eGFP released from the G2/M boundary. NEB (nuclear envelop breakdown) is set to be $t = 0$ min. Maximum projected images were shown. Scale bar, 10 μm

(D) Quantification of aggregate number and area of dividing cells expressing ER-FlucDM-eGFP. Left panel: data of individual cells. Right panel: mean values + SD of single-cell data (left panel). Time point of NEB in each cell was set to 0 min. Aggregate count and total area of each cell at each time point is normalized to its own value at $t = 0$ min. Aggregate count data are from 61 interphase and 73 dividing cells of four independent experiments. Aggregate area of each cell is calculated from 32 interphase and 34 dividing cells of two independent experiments.

numbers when cells progress through mitosis and cytokinesis (**Figure S3C** and Video 2), suggesting that the clearance of protein aggregates during cell division was not specific to ER-FlucDM-eGFP.

Effects of ER stress inducers on aggregate clearance in mitotic cells

Expression of ER-FlucDM-eGFP results in proteostatic stress in cells. Consistently, we observed that gene expression for chaperones and co-chaperones in the ER were upregulated in these cells (**Figure 4A**), indicating cells involved ER proteostasis control during adaptation. Proteomic analysis of cells expressing ER-FlucDM-GFP showed up-regulation of proteins involved in ER stress response (**Figure S4A**). We speculated that acute perturbation to ER homeostasis could exacerbate the proteostatic stress in dividing cells expressing ER-FlucDM-eGFP and affect aggregate clearance during mitosis. To test if this was the case, synchronized cells expressing ER-FlucDM-eGFP were released into mitosis in the presence of either DMSO (control) or 1 μ M thapsigargin (Thaps), which blocks the ER calcium ion pump and causes ER stress. In cells at prometaphase and metaphase (35 min after release), DMSO and Thaps treatments showed comparable number and area of aggregates in cells (**Figures 4B** and **4C**). However, in cells at the late stage of cell division (65 min after release), there were significantly higher number of aggregates retained in the cytosol of Thaps-treated cells than DMSO (**Figures 4B** and **4C**). Consistently, time-lapse microscopy revealed that ER-FlucDM-eGFP aggregates retained in the cytosol after cytokinesis in Thaps-treated cells, whereas the aggregates were largely cleared in control cells completing cytokinesis (**Figures 4D**, **4E**, **S4B** and Video 4). Thus, acute treatment of ER stress inducer Thaps in dividing cells prevents clearance of ER-FlucDM-eGFP aggregates.

A previous study showed that cells with protein aggregates treated for hours with Thaps or Tunicamycin (Tuni), which inhibits protein glycosylation in the ER, promotes aggregate clearance in cells (Melo et al., 2022). To test if prolonged treatment of cells expressing ER-FlucDM-eGFP with Tuni could promote aggregate clearance, we pretreated cells blocked at G2/M with varying concentrations of Tuni for 3 hours and released cells into mitosis. Interestingly, prolonged pretreatment of Tuni prior to mitosis promoted aggregate clearance in dividing cells (**Figure S4C**). Thus, acute treatment of cells expressing ER-FlucDM-eGFP with ER stress inducers prevents aggregate clearance in dividing cells while prolonged treatment with ER stress inducers promotes aggregate clearance.

Hsp70 family protein BiP is required for aggregate clearance during cell division

Since we observed that the expression of a chaperone Hsp70 (HSPA5/BiP) was upregulated in cells expressing ER-FlucDM-eGFP (**Figure 4A**) and Hsp70 is involved in elimination of protein aggregates (Melo et al., 2022; Nillegoda et al., 2015), we next tested whether the clearance of ER-FlucDM-eGFP aggregates in dividing cells was mediated by Hsp70 family proteins. To this end, cells entering mitosis were treated with either DMSO (control) or VER-155008 (VER), an inhibitor of HSP70's ATPase activity (Samanta et al., 2021). We found that VER-treated dividing cells fixed after 65 minutes released from the G2/M boundary retained high number of ER-FlucDM-eGFP aggregates in the cytosol compared to control treatment (**Figures 5A** and **5B**). Time-lapse microscopy showed that when cells were treated with VER, ER-FlucDM-eGFP aggregates released from the nucleus upon entry into mitosis were not cleared as to the same extent as control cells (**Figure 5C** and Video 5).

HSPA5/BiP is a major Hsp70 family member in regulating ER proteostasis. We next tested whether inhibition of BiP function could affect clearance of aggregates in dividing cells. Similar to VER treatment, inhibition of BiP's ATPase activity by YUM-70 (Samanta et al., 2021) led to a dose-dependent accumulation of ER-FlucDM-eGFP aggregates in cells (**Figures 5D** and **5E**). Moreover, by using immunofluorescence staining, we found a strong colocalization of BiP and ER-

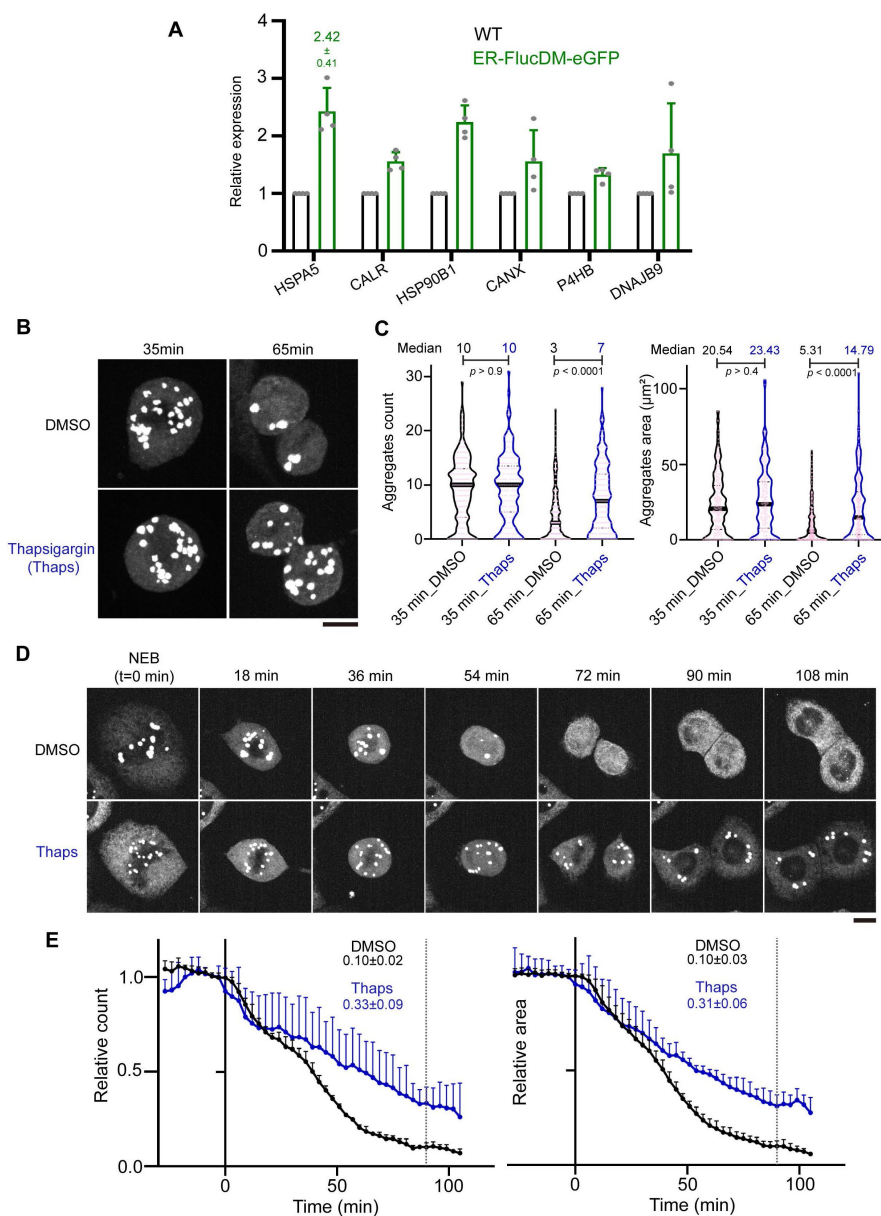


Figure 4.

Effects of ER stressors on aggregate clearance.

(A) Relative gene expression levels of chaperones and co-chaperones in cells expressing ER-FlucDM-eGFP. Four independent experiments.

(B) Images of cells treated with DMSO or 1 μM Thaps and fixed at 35 min or 65 min after released from the G2/M boundary. Maximum projected images were shown.

(C) Quantification of cells treated with DMSO or 1 μM Thaps and fixed at 35 min or 65 min after released from the G2/M boundary. 35 min_DMSO, 207 cells; 35 min_Thaps, 173 cells; 65 min_DMSO, 244 cells; 65 min_Thaps, 209 cells. Unpaired two side Mann-Whitney U-test. The solid black line in violin plot indicates median of data in the column. Gray dotted lines are quartiles. Each light pink circle indicates measurements of individual cells.

(D) Representative time-lapse images of cells expressing ER-FlucDM-eGFP treated with DMSO or 1 μM Thaps after released from the G2/M boundary. Maximum projected images were shown.

(E) Quantification of cells expressing ER-FlucDM-eGFP treated with DMSO or 1 μM Thaps. Time point of NEB in each cell was set to 0 min. Aggregates count and total area of each cell is normalized to its own value at t = 0 min. Black dotted lines are 90 min since NEB. DMSO, 89 cells; Thaps, 65 cells.

Scale bar, 10 μm . Three (C and E) or four (A) independent experiments.

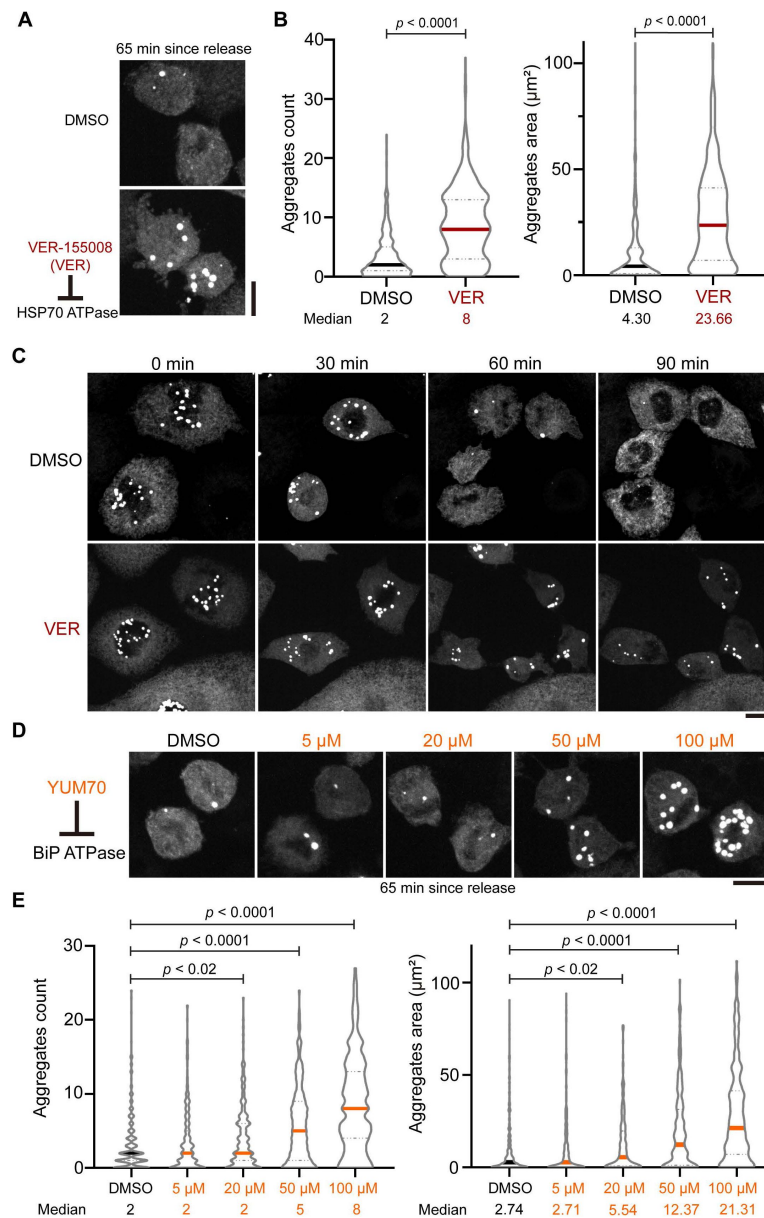


Figure 5.

Inhibition of HSP70 and BiP prevents aggregate clearance.

(A) Representative images of cells expressing ER-FlucDM-eGFP treated with DMSO or 50 μM VER and fixed at 65 min after released from the G2/M boundary. Maximum projected images were shown.

(B) Quantification of cells expressing ER-FlucDM-eGFP treated with DMSO or 50 μM VER and fixed at 65 min after released from the G2/M boundary. DMSO, 357 cells; VER, 465 cells.

(C) Time-lapse images of dividing cells expressing ER-FlucDM-eGFP treated with DMSO or 50 μM VER. Maximum projected images were shown.

(D) Representative images of cells expressing ER-FlucDM-eGFP treated with DMSO or different concentrations of YUM70. Maximum projected images were shown.

(E) Quantification of cells expressing ER-FlucDM-eGFP treated with DMSO or YUM70 and fixed at 65 min after released from the G2/M boundary. DMSO, 310 cells; 5 μM YUM70, 258 cells; 20 μM YUM70, 215 cells; 50 μM YUM70, 209 cells; 100 μM YUM70, 287 cells.

Scale bar, 10 μm . Unpaired two side Mann-Whitney U-test. Each solid line in violin plot indicates median of data in the column. Gray dotted lines are quartiles. A, B, D and E are from three independent experiments. C is from two independent experiments

FlucDM-eGFP, consistent with a role of BiP in regulating ER-FlucDM-eGFP aggregates (Figures S5A to S5C). Collectively, our data demonstrated that the clearance of ER-FlucDM-eGFP aggregates was regulated by the HSP70 family proteins, particularly BiP.

Aggregate clearance is a mitotic exit event but is independent of the anaphase promoting complex (APC/C)

Protein aggregates are subjected to proteasomal degradation in cells (Tyedmers et al., 2010). To test if aggregate clearance during cell division involves proteasomes, we inhibited the proteasome activity with MG132 in dividing cells expressing ER-FlucDM-eGFP. After treating cells released from the G2/M boundary with low or high concentrations of MG132 for 55 minutes, majority of mitotic cells were arrested at metaphase and accumulated high number of aggregates as compared to control cells treated with DMSO (Figures S6A and S6B). Next, we inhibited the proteasome with MG132 in cells at late anaphase or telophase and examined the aggregates 35 minutes after MG132 treatment (Figures S6C and S6F). In cells completing cytokinesis, there were significantly high number of aggregates in the early divided cells treated with MG132 versus the control (Figures S6D, 6E, S6G and S6H).

Since protein aggregates are cleared when cells progress through mitosis and cytokinesis, which are the cell cycle stage with low Cyclin B/CDK1 activity, we speculated that inactivation of CDK1 activity in MG132-treated and metaphase-arrested cells could lead to clearance of ER-FlucDM-eGFP aggregates in the cytosol. To test if this was the case, we released cells into mitosis in the presence of MG132 for 55 minutes and treated metaphase-arrested cells with RO-3306 to inhibit Cdk1 activity (Figure 6A). Interestingly, ER-FlucDM-eGFP aggregates were cleared in cells treated with RO-3306 while the aggregates were retained in cells treated with DMSO (Figures 6B and S6I). Consistently, live cell imaging demonstrated similar accelerated clearance in Cdk1 inhibited cells (Figures 6C and 6D). Thus, clearance of the aggregates happens when cells exit from mitosis.

We established that clearance of aggregates during cell division requires proteasomes and CDK inactivation, it is possible that the anaphase promoting complex (APC/C), which is a E3 ubiquitin ligase regulating mitotic exit (Watson et al., 2019), is involved in clearing the aggregates. To test if this was the case, we inhibited APC/C activity using a cocktail of APC/C inhibitors (APC/Ci) consists of Apcin and ProTAME in dividing cells expressing ER-FlucDM-eGFP (Sackton et al., 2014). Interestingly, inhibition of APC/C did not affect aggregate clearance as APC/Ci-treated cells that have arrested at metaphase have comparable number of aggregates as in control DMSO-treated cells (Figures 6E and 6F). When cells at late anaphase or telophase were treated with APC/Ci, the newly divided cells have similar number of aggregates as in control cells (Figures 6G, 6H and 6I). Lastly, we verified the efficacy of APC/C inhibition using cells expressing the APC/C reporter derived from Geminin (Figure S6J). Consistently, APC/Ci treatment has prevented the removal of APC/C reporter in newly divided cells indicating that the APC/C was inhibited by the dosage of APC/Ci used in our experiments (Figure S6K). Taken together, our data showed that clearance of ER-FlucDM-eGFP aggregates happens when cells exit from mitosis, is proteasome dependent but does not involve APC/C.

To examine if ER-FlucDM-eGFP decreased in abundance while cells progressing through mitosis, we inhibited protein translation using cycloheximide (CHX) in cells released from G2/M boundary. Short half-life proteins like p53 showed a rapid decrease of abundance upon CHX treatment, indicating CHX prevented new protein synthesis in our treatment (Figure S7A and S7B). ER-FlucDM-eGFP did not show significant change of its abundance after CHX treatment, suggesting that ER-FlucDM-eGFP aggregates were not cleared by the protein degradation mechanism directly (Figure S7A and S7C). Consistently, when the total GFP intensity of ER-FlucDM-eGFP was quantitated in dividing cells 20-30 min (early) or 85-95 min (late) released from G2/M boundary,

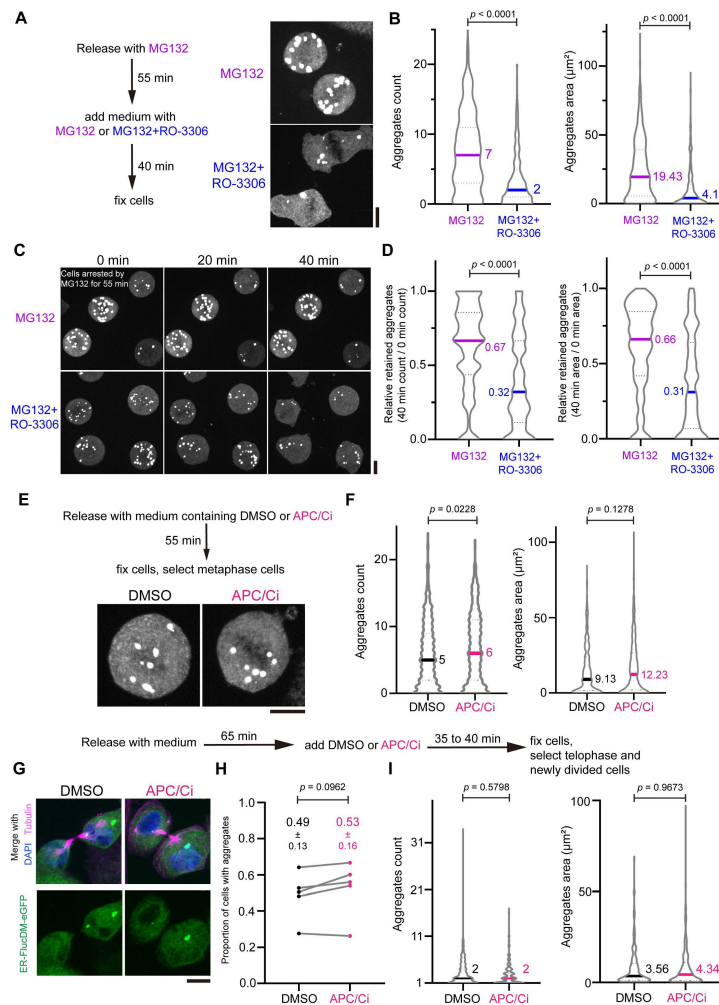


Figure 6.

Effects of MG132, APCi and RO-3306 (Cdk1i) on aggregate clearance.

(A) Representative images of cells expressing ER-FlucDM-eGFP arrested at mitosis by MG132 and further treated with Cdk1i to induce mitotic exit. Cells were first treated with 25 μ M MG132 for 55 min and mitotic arrested cells were further treated with 25 μ M MG132 plus 10 μ M RO-3306 or 25 μ M MG132 only. Cells were fixed 40 min after the second treatment. Maximum projected images were shown.

(B) Quantification of (A). MG132 only, 442 cells; MG132 + RO-3306, 374 cells.

(C) Time-lapse images of cells expressing ER-FlucDM-eGFP arrested at mitosis by MG132 and further treated with RO-3306 to induce mitotic exit. Cells were treated with 25 μ M MG132 for 55 min, and further treated with 25 μ M MG132 plus 10 μ M RO-3306 or 25 μ M MG132 before imaging. Maximum projected images were shown.

(D) Quantification of (C). Remaining aggregates at 40 min were calculated for their aggregates count or area and compared to that of 0 min. MG132 only, 125 cells; MG132 + RO-3306, 134 cells.

(E) Images of cells expressing ER-FlucDM-eGFP treated with DMSO or APCi (20 μ M proTAME and 300 μ M Apcin) and fixed at 55 min after released from the G2/M boundary. Maximum projected images were shown.

(F) Quantifications of cells expressing ER-FlucDM-eGFP treated with DMSO or APCi (20 μ M proTAME and 300 μ M Apcin). DMSO, 450 cells; APCi, 549 cells.

(G) Images of cells at telophase or cells of newly divided cells upon treatment of DMSO or APCi for 35 to 40 min. Maximum projected images were shown.

(H) Cells at telophase or newly divided cells that have aggregates upon treatment of DMSO or APCi were counted.

(I) Aggregates number and area were counted in cells at telophase or in newly divided cells after treated with DMSO or APCi. Only cells with aggregates were included in the quantification. DMSO, 297 cells; APCi, 319 cells.

Scale bar, 10 μ m. Data are from at least three independent experiments and analysed by unpaired two side Mann-Whitney U-test (B, D, F and I) or paired t-test (H).

there was no significant change of GFP fluorescence intensity between early and late dividing cells (**Figure S7D** [↗](#)), further supported that the protein degradation mechanism was not responsible for the ER-FlucDM-eGFP aggregate clearance.

Aggregate clearance depends on the ER reorganization during cell division

Since we showed that ER-FlucDM-eGFP aggregates are not primarily cleared by the protein degradation mechanism, we considered the possibility that aggregate clearance requires reorganization of the ER that happens in cells exiting mitosis. To this end, we used CRISPR interference (CRISPRi) technique to knock down ER membrane fusion proteins Atlastin 2 (ATL2) and Atlastin 3 (ATL3) in cells expressing ER-FlucDM-eGFP and quantitated the aggregate numbers in cells exiting mitosis (65 min after release). Interestingly, dividing cells depleted of ATL2 and ATL3 accumulated more ER-FlucDM-eGFP aggregates than in control cells (**Figure 7A** [↗](#) and **B** [↗](#)).

The mitotic kinase Aurora A has been recently shown to remodel the ER network during mitosis (Zhang et al., 2024 [↗](#)). We next tested if blocking Aurora A activity could affect aggregate clearance. When mitotic cells expressing ER-FlucDM-eGFP were treated with Aurora A inhibitor, MLN-8237, aggregate clearance was prevented compared to cells treated with DMSO (**Figure 7C** [↗](#) and **D** [↗](#)). Since the ER reorganization involves microtubule dynamics, we also perturbed microtubule networks by treating cells expressing ER-FlucDM-eGFP by nocodazole (**Figure 7E** [↗](#) and **F** [↗](#)). Our data showed that mitotic cells treated with nocodazole accumulated higher number of aggregates. Taken together, we found that clearance of ER-FlucDM-eGFP aggregates involves reorganization of the ER during mitotic exit (Lee et al., 1989 [↗](#)).

Discussion

We showed that stably expression of ER-FlucDM-eGFP leads to proteostatic stress and formation of protein aggregates in human cells without the use of additional proteostatic stressors. ER-FlucDM-eGFP aggregates may derive from the invagination of the inner nuclear membrane. Morris et al reported a similar aggregate upon BiP overexpression (Morris et al., 1997 [↗](#)). This fluorescent based live-cell imaging reporter enables us to study how protein aggregates are regulated in dividing cells, which are sensitive to genetic or environmental perturbations. Conventional methods that use proteasomal inhibitors to cause proteome imbalance are not ideal to investigate proteostasis in dividing cells given an essential role of proteasomes in driving mitotic exit (Ghislain et al., 1993 [↗](#); Glotzer et al., 1991 [↗](#)). Additionally, methods such as heat shock also perturb mitotic progression, especially in human cells (Kakihana et al., 2019 [↗](#)). By using ER-FlucDM-eGFP that does not perturb cell cycle progression significantly, we reveal an unexpected process of clearing protein aggregates in cells progressing through mitosis and cytokinesis.

Previous studies showed that the proteome of mitotic cells have higher structural stability and less aggregation prone compared to that of in interphase cells (Becher et al., 2018 [↗](#); Wirth et al., 2013 [↗](#)). Post-translational modifications such as phosphorylation of mitotic proteins are suggested to be responsible for the protein stability during cell division (Becher et al., 2018 [↗](#)). The aggregate clearance we identified in this study could represent an active mechanism that increases proteome stability in dividing cells. We found that aggregate clearance requires Cdk1 inactivation, which is a hallmark of mitotic exit, however, does not involve APC/C that is important in driving mitotic exit through protein degradation. It is possible that APC/C targets mostly cytosolic proteins whereas ER-FlucDM-eGFP aggregates are confined in a membrane during mitosis. Furthermore, the clearance of protein aggregates coincides with ER reorganization that happens throughout mitosis and cytokinesis, which may contribute to the aggregate clearance (Bergman et al., 2015 [↗](#); Schlaitz et al., 2013 [↗](#)). A previous study showed that the size of protein aggregates is limited by the ER tubules (Parashar et al., 2021 [↗](#)). In fact, when the ER

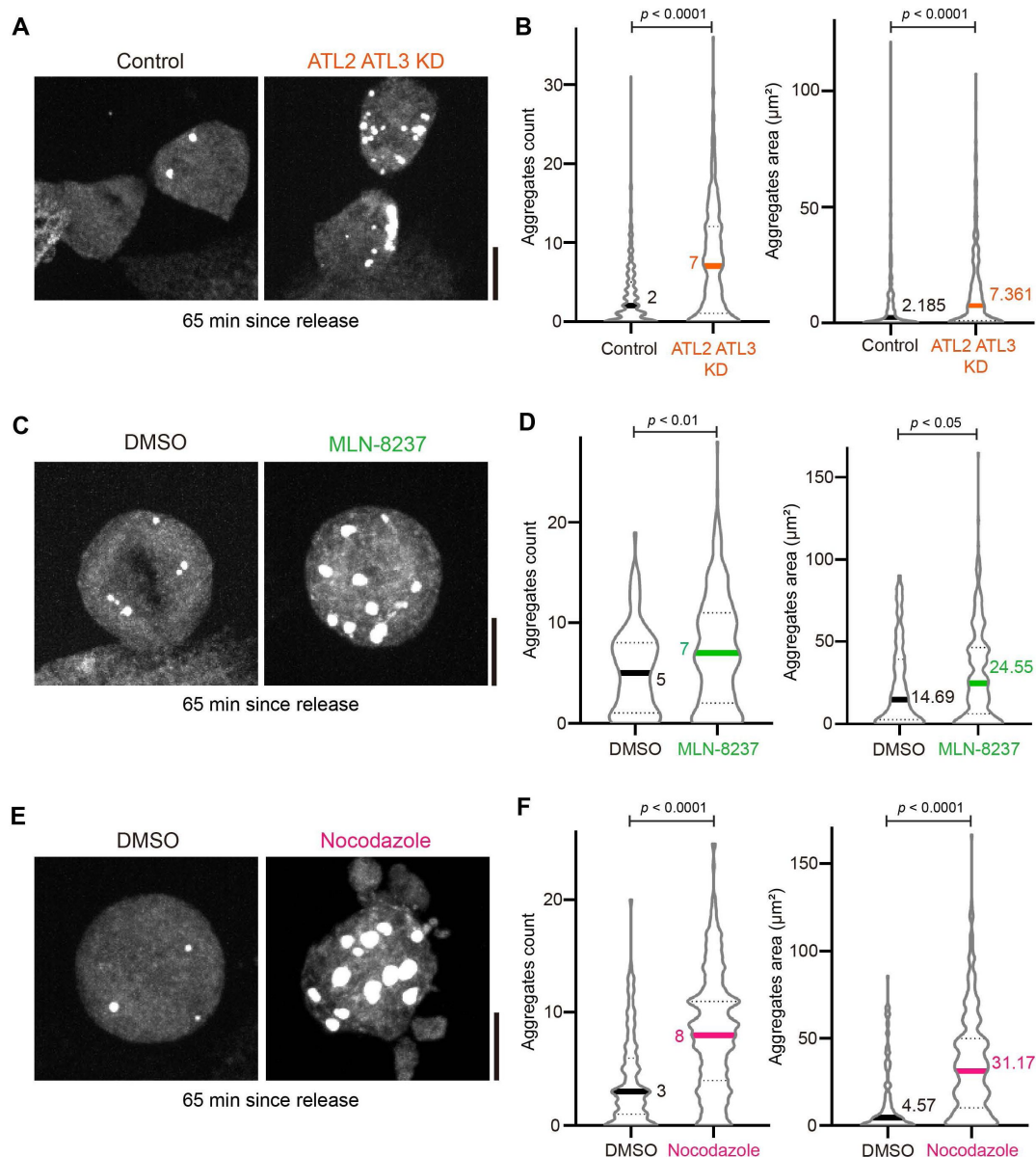


Figure 7.

Perturbation of proteins that regulate ER reorganization in aggregate clearance

(A) Images of cells expressing ER-FlucDM-eGFP after depletion of Atlastin 2 and Atlastin 3 and fixed at 65 min after released from the G2/M boundary. Maximum projected images were shown.

(B) Quantification of cells expressing ER-FlucDM-eGFP after depletion of Atlastin 2 and Atlastin 3 and fixed at 65 min after released from the G2/M boundary. Control, 396 cells; ATL2/ATL3 KD, 362 cells.

(C) Images of cells expressing ER-FlucDM-eGFP treated with DMSO or 100 nM MLN-8237 and fixed at 65 min after released from the G2/M boundary. Maximum projected images were shown.

(D) Quantification of cells expressing ER-FlucDM-eGFP treated with DMSO or 100 nM MLN-8237 and fixed at 65 min after released from the G2/M boundary. Only round mitotic cells were counted. DMSO, 102 cells; MLN-8237, 288 cells.

(E) Images of cells expressing ER-FlucDM-eGFP treated with DMSO or 331nM nocodazole and fixed at 65 min after released from the G2/M boundary. Maximum projected images were shown.

(F) Quantification of cells expressing ER-FlucDM-eGFP treated with DMSO or 331nM nocodazole and fixed at 65 min after released from the G2/M boundary. Only round mitotic cells were counted. DMSO, 122 cells; nocodazole, 320 cells.

Scale bar, 10 μm . Data are from three independent experiments and analysed by unpaired two side Mann-Whitney U-test.

reorganization is perturbed by depleting ER membrane fusion proteins ATL2 and 3, clearance of ER-FlucDM-eGFP aggregates is affected. Consistently, ER-FlucDM-eGFP proteins do not turn over significantly in cells progressing through mitosis and cytokinesis. Thus, it is less likely that ER-FlucDM-eGFP is ubiquitinated by APC/C and targeted for protein degradation.

Misfolded or damaged proteins form protein aggregates and are spatially organized into specific subcellular sites to sequester them from other cellular processes (Hill et al., 2017 [↗](#)). These aggregate deposition sites are localized in the cytosol or in the nucleus to confine misfolded proteins and aggregates for elimination from cells (Kaganovich et al., 2008 [↗](#)). Similarly, we identified confinement of ER-FlucDM-eGFP aggregates in the nucleus and are surrounded by a single layer membrane. These intranuclear membranous structures contain ER membranes and BiP, which is a key Hsp70 family chaperone in the ER and translocates into the nucleus under stress (Liu et al., 2023 [↗](#)). How protein aggregates are targeted and assembled into the intranuclear membranous structure awaits future investigation. Interestingly, upon entry into mitosis, concomitant with the nuclear membrane breakdown, the membranous structure is released from the nucleus and the aggregates are cleared when cells progress through mitosis till early G1 phase of the daughter cells. The confinement and clearance of protein aggregates during cell division presumably protect dividing cells from harmful effects of protein misfolding and aggregation and to ensure reliable inheritance of genetic materials. It has been reported that aggregates are asymmetrically retained in one of the daughter cells that are usually short-lived (Rujano et al., 2006 [↗](#)). The aggregate clearance mechanism we report here may provide an additional cellular defense strategy to overcome proteostatic stress and to maintain proteome integrity during cell division. Proteomic analysis of cells expressing ER-FlucDM-eGFP identified up-regulation of multiple proteins involved in ER stress response, indicating that cells experience significant proteostatic stress upon expression of ER-FlucDM-eGFP. Moreover, several proteins regulating cell cycle displayed higher abundance in the proteome, which together with proteostatic stress indicate a perturbed cellular health.

Hsp70 chaperones regulate proteostasis by preventing protein aggregation, promoting disaggregation and subjecting solubilized aggregates to degradation or refolding (Rosenzweig et al., 2019 [↗](#)). Our proteomic analysis revealed significant expression of BiP (HSPA5) in cells expressing ER-FlucDM-eGFP but not other Hsp70 family members. A recent study showed that BiP solubilizes aggregates and drives aggregate clearance in the ER (Melo et al., 2022 [↗](#)). Our study of mitotic aggregate clearance in dividing cells further extend the role of BiP in disaggregation. Consistently, we found that Cdk1 inactivation in the presence of the proteasomal inhibitor could still lead to aggregate clearance, suggesting a partial role of the proteasome in clearing aggregates and the disaggregation breaks down the aggregates into smaller and possibly soluble species. The role of proteasome in regulating ER-FlucDM-eGFP aggregate clearance appears indirect as ER-FlucDM-eGFP proteins are stable throughout cell division. It is possible that players involved in ER-FlucDM-eGFP aggregate clearance are regulated by proteasomal degradation, which are affected when proteasomes are inhibited. Interestingly, Melo et al. showed that aggregate clearance is promoted by treating ER-cells with ER stressors as the treatment triggers UPR, which in turn leads to higher activity of BiP to cope with the ER stress. We observed differential responses of aggregate clearance to ER stressors depending on the duration of the treatments. It is possible that acute treatment of the ER stressor on dividing cells does not provide sufficient time for cells to accumulate abundant amount of BiP given that mitotic cells are not very transcriptionally active (Palozola et al., 2017 [↗](#)). Also, degradation of BiP could be accelerated upon the treatment of ER stressors, which in turn undermines the abundance of BiP in disaggregation (Shim et al., 2018 [↗](#)). Thus, short term treatment of the ER stressor in cells exacerbates the ER stress and affects aggregate clearance during cell division, whereas longer treatment of the ER stressor promotes aggregate clearance. Mechanistic understanding of aggregate clearance during cell division could offer new insights to understand disaggregation in proteostasis control.

Materials and methods

Cell lines and culture

MCF10A cells were cultured in DMEM F-12 (Sigma) supplemented with 1% Glutamax (Gibco), 5% horse serum (Biological Industries), 20 ng/ml EGF (Gibco), 0.5 mg/ml Hydrocortisone (MedChemExpress), 100 ng/ml Cholera toxin (Sigma), 10 µg/ml Insulin (Biological Industries), and 1% Pen/Strep (Biological Industries). U2OS, A549, and HEK293Ta cells were maintained in DMEM (Sigma) with Glutamax (Gibco), 6% fetal bovine serum (Biological Industries), and 1% Pen/Strep (Biological Industries). MDA-MB-231 cells were cultured in DMEM F-12 (Sigma) with 1% Glutamax (Gibco) and 1% Pen/Strep (Biological Industries). All cell cultures were maintained at 37°C with 5% CO₂ in a humidified incubator. Cells expressing ER-FlucDM-eGFP, ER-eGFP, ER-HaloDM-eGFP, ER-FlucWT-eGFP, and ER^{F_{SHR}}-FlucDM-eGFP, ER-FlucDM-mCherry, NLS-FlucDM-eGFP were generated through lentivirus transduction and selected with 5 µg/mL puromycin (Sangon Biotech). MCF10A cells expressing mStayGold-Sec61β were prepared by lentivirus transduction, selected with 50 µg/mL hygromycin B and only used in three days after transduction (Ando et al., 2024 [DOI](#)). MCF10A cells expressing CRISPRi plasmids to knock down ATL2 and ATL3 were selected by 6 µg/mL Blasticidin (Solarbio, 703X023) and only used in five days after transduction. MCF10A cells co-expressing ER-FlucDM-eGFP and ER-FlucDM-mCherry for FRAP or co-expressing ER-FlucDM-mCherry and mGreenLantern-hGeminin (degron) or co-expressing ER-FlucDM-mCherry and NLS-FlucDM-eGFP were generated by lentivirus transduction and selected by 5 µg/mL puromycin and followed by fluorescence-activated cell sorting (FACS) to enrich double-positive cells.

Lentivirus packaging

Lentiviral particles were generated using the 3rd generation lentiviral packaging system. Packaging plasmids pRSV-Rev, pMDLg/pRRE, pMD2.G (kindly provided by Didier Trono) and the transfer plasmid were chemically transfected using GeneTwin transfection reagent (Biomed, TG101) into the HEK293Ta packaging cell line (Genecopoeia, LT008). The cell culture medium was harvested after 2 days and filtered through a 0.45 µm filter. The filtrate was concentrated using a 5X precipitation solution (250 g/L PEG 8,000 and 43.83 g/L NaCl in ddH₂O) for overnight, and precipitated by centrifuging at 4000 × g for 25 min at 4°C. Lentiviral particles were resuspended in phosphate buffered saline (PBS) and added to the culture medium, which were supplemented with 1 µg/mL polybrene (HanBio).

Lentivirus expression constructs

Lentiviral transfer plasmids used in this study were listed below: pTGL0563 containing FlucDM-eGFP in which FlucDM was mutated from FlucWT (a gift from Dr. Mikael Bjorklund); pTGL0645 containing ER-FlucDM-eGFP-KDEL; pTGL0662 containing NLS-FlucDM-eGFP; pTGL0673 containing ER-FlucDM-mCherry-KDEL; pTGL0698 containing ER-FlucWT-eGFP-KDEL; pTGL0703 containing ER-HaloDM-eGFP-KDEL in which HaloDM was mutated from HaloWT; pTGL0707 containing ER-HaloWT-eGFP-KDEL; pTGL0733 containing ER-eGFP-KDEL; pTGL0743 containing ER^{F_{SHR}}-FlucDM-eGFP; pTGL0828 containing mGreenLantern-hGeminin (degron); pTGL0891 was the empty plasmid used in constructing pTGL0645 and used as a control for proteomics; pTGL0914 containing mStayGold-Sec61β; pTGL0386 was the empty plasmid in constructing CRISPRi plasmids; pCRISPRi0249 to knockdown ATL2 containing gRNA 5' GAGGGCAGCAACCGCACCAG 3'; pCRISPRi0252 to knockdown ATL3 containing gRNA 5' GAGCAGGGGTGCAGAGGAGA 3'.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, RC112-01). Subsequently, the RNA was reversely transcribed into complementary DNA (cDNA) using the HiScript II Q RT SuperMix for qPCR (Vazyme, R223-01). The levels of the cDNAs were quantified using real-time PCR with the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-02). The PCR reaction mix was prepared on a hard-shell PCR plate (Bio-Rad, HSP9655), sealed with Microseal 'B' seals (Bio-Rad, MSB1001), and conducted in the CFX96 Touch Real-Time PCR Detection System (Bio-RAD, C1000). Primers used in the qPCR: GAPDH, 5' CAGGAGGCATTGCTGATGAT 3' and 5' GAAGGCTGGGGCTCATTT 3'; HSPA5, 5' CACAGTGGTGCCTACCAAGA 3' and 5' TGTCTTTTGTTCAGGGGTCTTT 3'; Calreticulin, 5' ATAAAGGTTTGCAGACAAGC 3' and 5' CCACAGTCGATGTTCTGCTC 3'; HSP90B1, 5' TCCAGCAGAAAAGAGGCTGA 3' and 5' CAAATTCGGAAGGGCCTGA 3'; CANX, 5' GCACCTATTCTGGAGGCGAG 3' and 5' ACAGCAACCACTTCCCTTCC 3'; P4HB, 5' TTCAGGAATGGAGACACGGC 3' and 5' TCCACGTCCTTGAAGAAGCC 3'; DNAJB9, 5' GTGGAGGAGCAGCAGTAGTC 3' and 5' CGCTCTGATGCCGATTTTGG 3'; ATL2, 5' CTGTTCCATTGCTGCTTGC 3' and 5' CTTCAGCTGTTGCCTGAAGC 3'; ATL3, 5' TCACCCCAAGTCCATGCTTC 3' and 5' CTCCCCACAAACCTCTTCC 3'.. The C_T value for GAPDH was used for normalization to obtain the relative expression level.

Protein immunoblotting

Adherent cells were lysed with 1x LDS sample buffer (Beyotime) and boiled at 95°C for 10 min. The 1x LDS sample buffer was prepared by diluting 4 X LDS sample buffer (Beyotime) in 100 mM Tris-HCl (pH 7.4) with 2.5% v/v β -mercaptoethanol. Protein samples were separated by pre-cast SDS-PAGE gels (GenScript) and blotted to a PVDF membrane (EMD Millipore). Blots were blocked with Quick Block buffer (Beyotime) for at least 20 min at room temperature and were incubated at 4°C overnight with the primary antibody. After rinsing blots with TBS containing 0.05% Tween-20 for four times (5 min each time), blots were incubated at room temperature with the secondary antibody for 1h, followed by washing with TBS containing 0.05% Tween-20. Odyssey CLX was employed to image the blots and LI-COR Image Studio software was used to quantify the band intensity. To incubate different primary antibodies in the same blot, the blot was vigorously rinsed with the stripping buffer (CWBIO, 01427/12724) and washed 3 times with TBS containing 0.05% Tween-20 and processed as described before.

To examine the solubility of ER-FlucDM-eGFP in **Figure S1E**, the lysis buffer containing 100 mM Tris-HCl (pH 7.4), 1% protease inhibitor cocktail (Lablead, C0101) and different concentrations of NP-40 (Sigma) was used. To prepare supernatant and pellet fractions of ER-FlucDM-eGFP, cells were lysed with the lysis buffer containing 0.2%, 0.5% or 1.0% v/v NP-40. The lysate was then centrifuged at 14,000 $\times$ g for 10 min at 4°C and the supernatant was collected. The cell pellet was further lysed with 1x LDS buffer on ice for 40 min and vortexed vigorously for 10 seconds every 10 min.

Primary antibodies used were: 1:3000 anti-GFP (Huabio, ET1601-13); 1:5000 anti- β -actin (Genscript, A00702); 1:1000 anti-Cyclin B1 (Proteintech, 55004-1-AP); 1:1000 anti-p53 (Huabio, ET1601-13). Secondary antibodies were goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody conjugated to Alexa Fluor™ Plus 800 (Invitrogen, A32735) and goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody conjugated to Alexa Fluor™ Plus 680 (Invitrogen, A32729).

Proteomics

MCF10A cells transduced with pTGL0891 (empty vector) or pTGL0645 (ER-FlucDM-eGFP) for six days were trypsinized, centrifuged and washed twice with ice-cold PBS. The pellets were quickly frozen by liquid nitrogen and then sent to Tsingke (Wuhan, China) for further processing for

proteomics. In brief, cell pellets were lysed with buffer containing 8 M urea, 1 mM PMSF and 2 mM EDTA. Samples were subjected to ultrasound sonication on ice for five minutes and followed by centrifugation at 15,000 xg for 10 minutes. The supernatants were then collected, and their concentrations were measured using BCA method (Beyotime). Solutions containing 100 µg was topped up to 200 µl with 8M urea and DTT (final concentration 5 mM) was added before incubation at 37°C for 45 min. Protein samples were alkylated using iodoacetamide (final concentration 11 mM) in a dark room at room temperature for 15 min. The solutions were then subjected to trypsin digestion and further analyzed using LC-MS/MS analysis. The MS/MS data were processed using DIA-NN (v1.8.1). The protein quantification of DIA-NN software is done by MaxLFQ algorithm.

Luciferase activity test

Each well of 96-well plates (Biosharp, BS-MP-96W for luminescence, BS-MP-96B for fluorescence) was seeded with 10,000 cells in 100 µL of medium. After incubation for 18 hours, 5 µM MG132 was added to the MG132+FlucDM-eGFP group, while other untreated wells remained unchanged.

Following an additional 6 hours, cells were washed once with PBS.

For luminescence detection, cell lysis was achieved by adding 50 µL of Steady-Glo Luciferase Assay System buffer (Promega, E2510) to the wells, followed by a 15-minute incubation at room temperature. Luminescence in each well was then recorded three times over 5 minutes using the Spark® Multimode Microplate Reader (Tecan, 1000 ms exposure). Luminescence intensity in each well was calculated by normalizing these three measurements and subtracting the background signal from empty wells.

To determine abundance of luciferase in each treatments, 1×10^5 MCF10A cells were seeded in 12-well for 24 h. The cells were lysed with 1x LDS sample buffer (Beyotime) and protein immunoblotting using anti-β-actin and anti-GFP, respectively was performed. β-actin was first used to normalized the GFP intensity, and the value was used to normalize the luminescence to calculate the luciferase activity.

Mitotic cell preparation

To increase the quantity of dividing cells, cells were first seeded on imaging chambers (ibidi, 80826) and were synchronized to the G2/M boundary by treating cells with 7.5 µM RO-3306 (Selleckchem, S7747) for 16 to 20 hours. Cells were released to enter into mitosis synchronously by washing once with pre-warmed fresh medium.

Drug treatments

The following drugs were used in this study: Thapsigargin (Abcam, ab120286), Tunicamycin (GlpBio, GC16738), MG132 (CSN Pharm, CSN11436), YUM70 (Aladdin, Y413413), VER-155008 (CSN Pharm, CSN13116), Apcin (Sigma, SML1503), proTAME (Cayman Chemical, 25835), Cycloheximide (AbMole, M4879), Nocodazole (Beyotime, S1765), MLN-8237 (TargetMol, T2241).

Immunofluorescence staining

For HSPA5/BiP staining, MCF10A cells expressing ER-FlucDM-eGFP were seeded in 8-well ibidi plates, fixed with 100% methanol for 10 minutes and permeabilized with 0.5% Triton X-100 for 15 minutes. The fixed cells were blocked with 5% FBS in PBS for 30 minutes. Subsequently, cells were incubated with the mouse anti-BiP antibody (HuaBio, HA601076) at 1:200 dilution in 1% FBS overnight at 4°C. The secondary antibody used was Donkey Anti-Mouse IgG H&L (Alexa Fluor® 568, ab175472, Abcam) at 1:2000 dilution for one hour. For Tubulin staining, cells were fixed using 4% PFA for 20 minutes and permeabilized with 0.5% Triton X-100 for 15 minutes. After blocking the fixed cells with 5% FBS in PBS for 30 minutes, cells were stained with the mouse anti-α-tubulin

monoclonal antibody (Proteintech, 66031-1-IG) at 1:200 dilution. The secondary antibody used was Donkey Anti-Mouse IgG H&L (Alexa Fluor® 568, ab175472, Abcam) at 1:2000 dilution for one hour. To stain DNA, DAPI Staining Solution (Sangon Biotech, E607303, 5 mg/L) was diluted in PBS to 50 ng/mL and applied to cells for 10 minutes. After the DAPI solution was removed, fixed cells were washed with PBS twice before imaging.

Transmission Electron Microscopy (TEM)

To prepare samples for TEM, MCF10A cells expressing ER-FlucDM-eGFP were synchronized to the G2/M boundary by treating cells with RO-3306 for 16 to 20 h and were released into mitosis by removing RO-3306. After 45 min released from the G2/M boundary, cells were trypsinized for 10 min before centrifugation. The cell pellet was fixed by 2.5% paraformaldehyde (in PBS) for 30 min at room temperature before overnight at 4°C. Fixed pellet was further fixed by 1% osmium tetroxide and then 2% uranium acetate. Ethanol and acetone were used to dehydrate samples before embedding them into epoxy resin. Sectioned samples were observed by Tecnai G2 spirit 120kV transmission electron microscopy (Thermo FEI).

Live-cell staining

The live-cell staining dyes used are as follow: 100 nM SiR-Tubulin (Cytoskeleton, CY-SC002), 1 μM ER-tracker Red (Beyotime, C1041S) or 10 μM Thioflavin T (ThT) in the medium to label cells for about 30 min in the incubator before imaging. For ThT staining, about ten percent aggregates could be stained by ThT in ER-FlucDM-mCherry cells.

Spinning-disk confocal microscopy imaging

The hardware configuration of the spinning-disk confocal microscopy system was as described previously (Wang et al., 2023 [4](#)). Imaging utilized a z-step size of 0.5 μm, with a x-y plane resolution of 183.3 nm/pixel for the 60× lens and 110 nm/pixel for the 100× lens. Fluorophores were excited using laser lines at wavelengths of 405, 488, 561, or 640 nm.

For FRAP analysis, images were acquired with 488 nm and 561 nm laser lines through the 100× lens. The procedure involved acquiring three pre-bleach images every 30 seconds, followed by bleaching the mCherry signal using the 561 nm laser. Subsequently, one image was acquired immediately post-bleaching, followed by ten images acquired every 30 seconds. In total, fourteen time-point images were acquired, and the total duration of each FRAP experiment was 6.5 minutes.

Image analysis and processing

Fiji was used to process images. Z-stack images for quantification were first projected using max-intensity projection (Fiji/image/stacks/Z project). To quantify aggregates, projected images were classified by Trainable Weka Segmentation (Fiji/Plugins/Segmentation/Trainable Weka Segmentation). The segmented images were processed by using the ImageJ Macro commands: run(“8-bit”); setOption(“BlackBackground”, true); run(“Convert to Mask”); run(“Watershed”) before using Analyze Particles (Fiji/Analyze/Analyze Particles). For time-lapse images except images from FRAP, the segmented images were processed by using the ImageJ Macro commands: run(“8-bit”); setThreshold(0, 120); run(“Make Binary”, “method=Default background=Light black”); run(“Invert LUT”); run(“Watershed”, “stack”) before using Analyze Particles (Fiji/Analyze/Analyze Particles).

To quantify total GFP intensities in live cells, Z-stack images (24 μm) were acquired and projected using sum slices projection. Cell outlines were segmented using polygon selection in Fiji. To quantitate the background intensity, the mean intensity of a small dark region of the field-of-view was measured and multiplied by the area of the segmented cell. The background was subtracted from the GFP intensity of segmented cells.

For FRAP analysis of ER-eGFP and ER-HaloDM-eGFP, region-of-interests (ROI) in projected images were manually selected and the average fluorescence intensity of ROIs was measured in Fiji. For FRAP analysis of aggregates in cells co-expressing ER-FlucDM-eGFP and ER-FlucDM-mCherry (both interphase and mitosis), the aggregates were first segmented by Weka Segmentation. The unsegmented images from the mCherry channel were processed by Analyze Particles (Fiji/Analyze/Analyze Particles) based on the corresponding segmented images from the GFP channel. For fluorescence intensity quantification, background in all FRAP experiments were subtracted by one hundreds.

Quantification and statistical analysis

Datasets were analysed by Student's t test or Mann-Whitney test. Prism 8 (GraphPad) was used for the statistical analysis and data plotting. Statistical details are indicated in the figure legends.

Supplementary figures

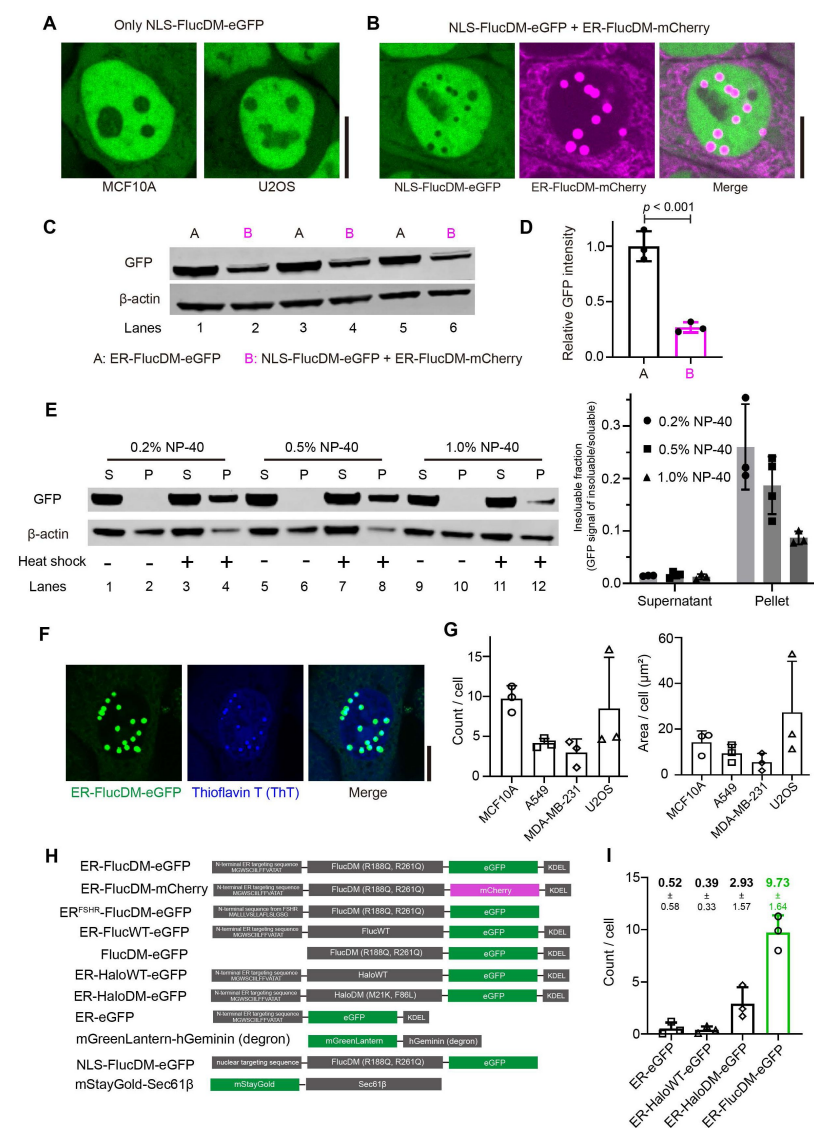


Figure S1.

ER-FlucDM-eGFP forms protein aggregates, related to Figure 1

(A) Microscopy images of cells expressing NLS-FlucDM-eGFP. Two cell lines were used to express NLS-FlucDM-eGFP. Single Z-slice images were shown.

(B) Microscopy images of cells expressing NLS-FlucDM-eGFP and ER-FlucDM-mCherry in MCF10A cells. Single Z-slice images were shown.

(C) Protein blots for ER-FlucDM-eGFP and NLS-FlucDM-eGFP using anti-GFP or anti-β-actin antibodies.

(D) Relative GFP protein intensity in S1(C) was quantitated and plotted. The intensity of β-actin was used in normalization.

(E) Protein blotting to examine solubility of ER-FlucDM-eGFP under different concentrations of detergents, NP-40. Cells expressing ER-FlucDM-eGFP were subjected to 45 °C for 1 hour before harvesting cell lysates in heat shock experiments. Insoluble fractions were quantitated and plotted.

(F) Colocalization of ER-FlucDM-eGFP and Thioflavin T (ThT), a marker for protein aggregates. Single Z-slice images were shown.

(G) Quantification of ER-FlucDM-eGFP aggregate number (left) and total aggregate area (right) of individual cells in different cell lines. MCF10A, 516 cells; A549, 395 cells; MDA-MB-231, 369 cells; U2OS, 528 cells.

(H) Schematic representation of different constructs used in this study.

(I) Quantification of aggregate number in cells expressing ER-eGFP, ER-HaloWT-eGFP, ER-HaloDM-eGFP. ER-eGFP, 612 cells; ER-HaloWT-eGFP, 405 cells; 546 ER-HaloDM-eGFP, 546 cells; ER-FlucDM-eGFP, 516 cells.

Data are from three independent experiments, analysed by unpaired t-test and represented by mean + SD. Scale bar, 10 μm.

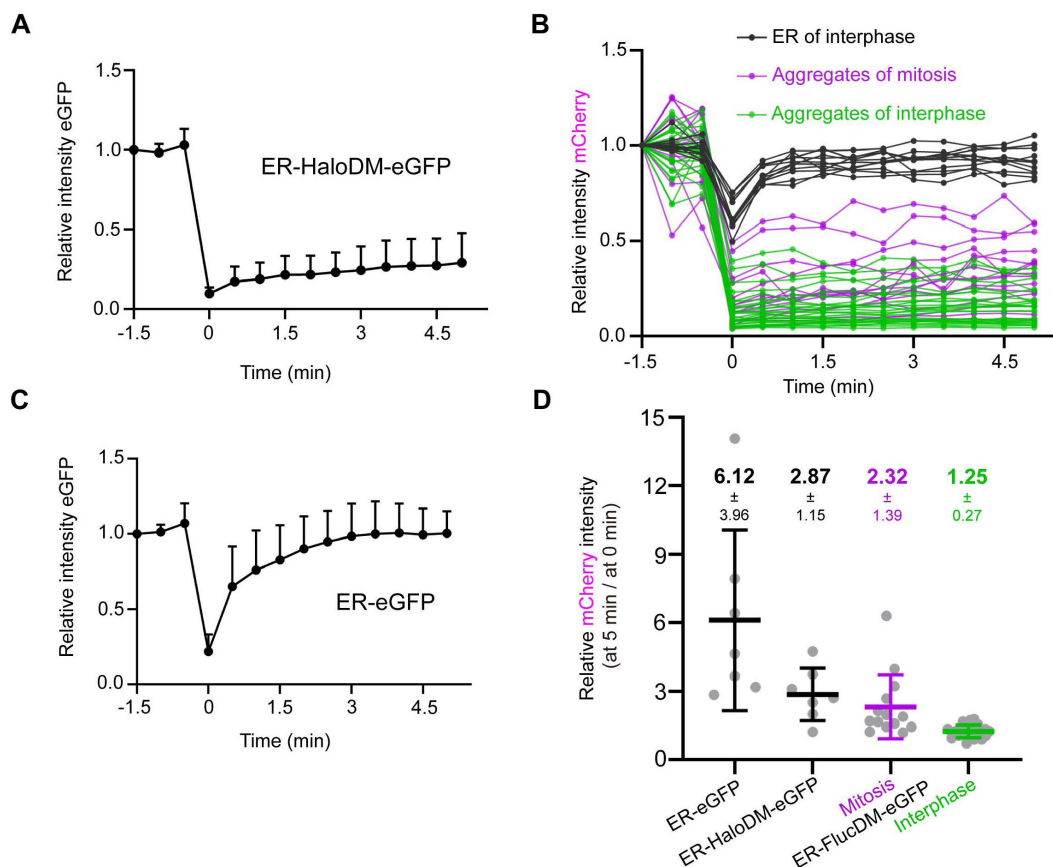


Figure S2.

FRAP analysis of protein aggregates, related to Figure 2.

(A) Recovery curve of ER-HaloDM-eGFP before and after photobleaching. Seven interphase cells from three independent experiments. Data represent mean + SD.

(B) Recovery curve of individual cell data in ER-FlucDM-eGFP before and after photobleaching. 14 ROIs from the ER of interphase cell, 14 aggregates from cells at mitosis, 26 aggregates from cells at interphase. Data were collected from four independent experiments.

(C) Recovery curve of ER-eGFP before and after photobleaching. Seven interphase cells from three independent experiments. Data represent mean + SD.

(D) Quantification of protein fluorescence intensity of different constructs right after photobleaching and 5 min after recovery. 0 min is the protein intensity right after photobleaching and 5 min is the protein intensity 5 min after recovery from photobleaching. At least three independent experiments. Data represent mean ± SD.

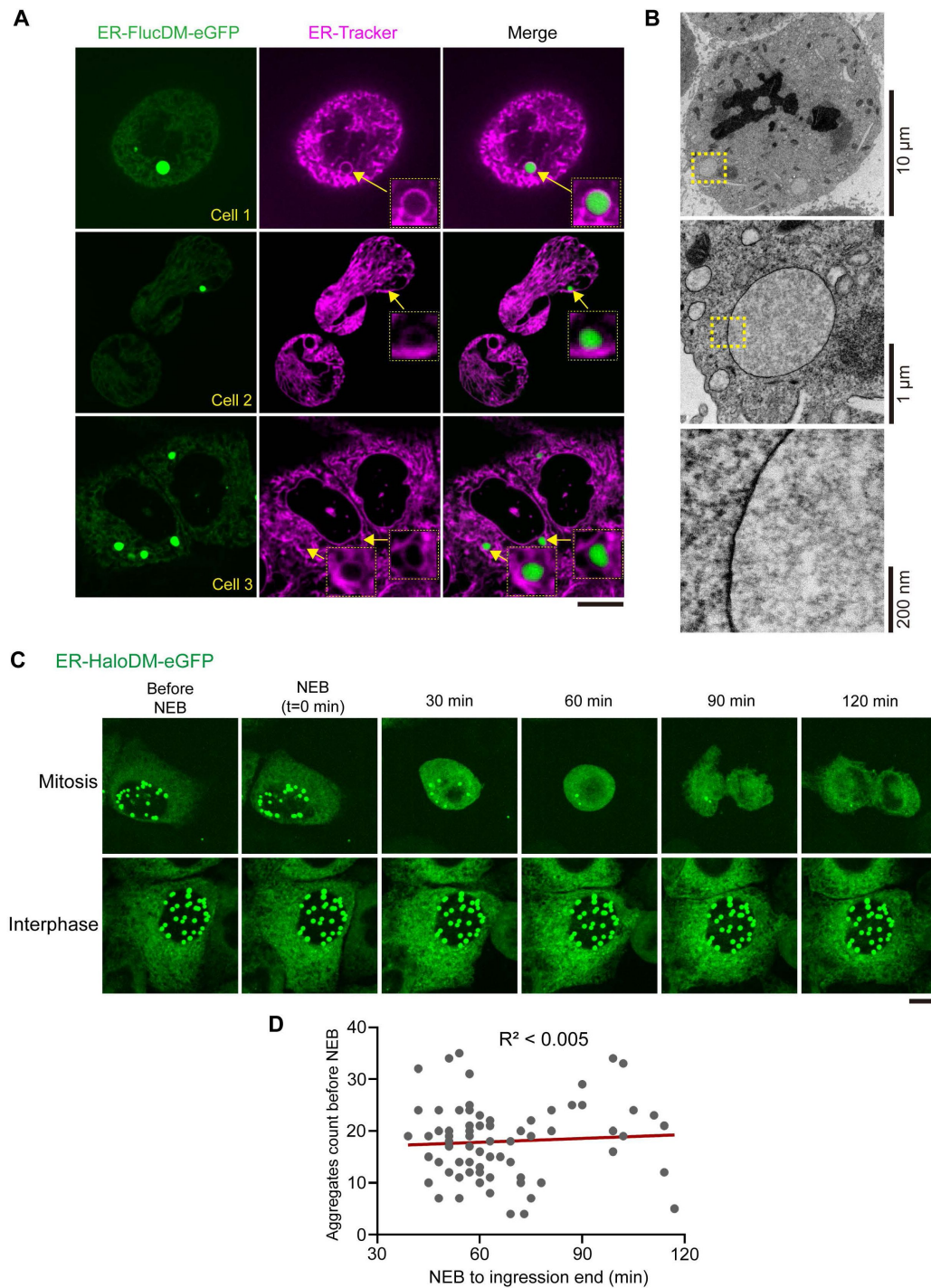


Figure S3.

Aggregates in dividing cells, related to Figure 3 [↗](#).

(A(A) Images to show ER-FlucDM-eGFP aggregates of dividing cells were surrounded by the membrane stained by the ER-tracker. Scale bar, 10 μ m. Single Z-slice images were shown.

(B) TEM images of sectioned MCF10A mitotic cells expressing ER-FlucDM-eGFP.

(C) Time-lapse images of cells expressing ER-HaloDM-eGFP released from the G2/M boundary. NEB (nuclear envelop breakdown) is set to be t = 0 min. Maximum projected images were shown. Scale bar, 10 μ m.

(D) Linear regression analysis to show that cell division process is not linearly dependent on aggregates quantity. 74 dividing cells are from four independent experiments.

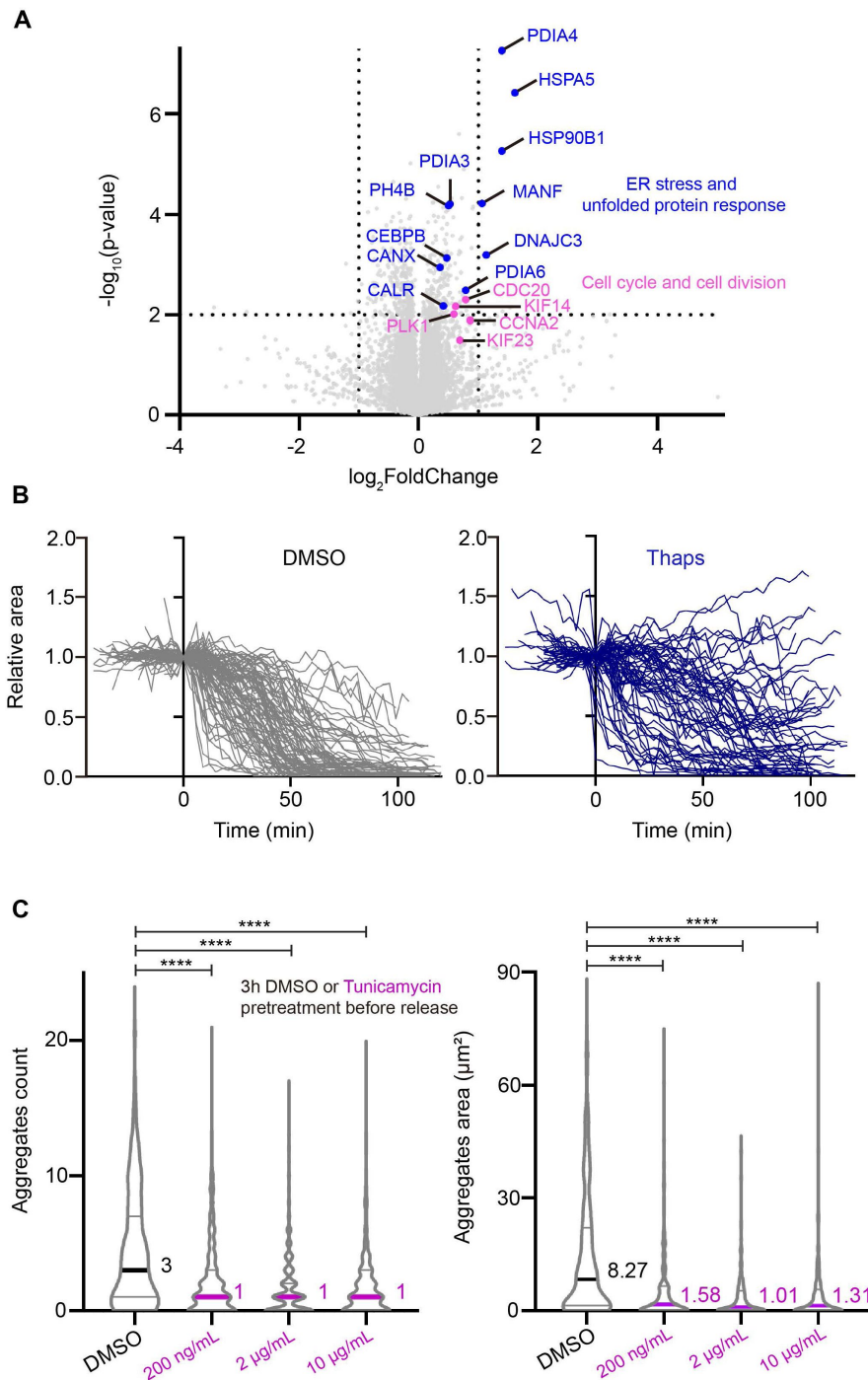


Figure S4.

Effect of Thapsigargin or Tunicamycin on aggregates clearance, related to Figure 4 [↗](#).

(A) Proteomic analysis of cells expressing ER-FlucDM-eGFP. Three independent experiments. Proteins involved in ER stress and unfolded protein response (blue) or cell cycle and cell division (pink) were highlighted.

(B) Changes of aggregate number and total area as a function of time after cells expressing ER-FlucDM-eGFP were treated with DMSO or 1 μM Thaps.

(C) Quantification of aggregate number and total area of cells expressing ER-FlucDM-eGFP treated with DMSO or Tunicamycin (Tuni) during mitosis. DMSO, 542 cells; 200 ng/mL Tuni, 385 cells; 2 $\mu\text{g/mL}$ Tuni, 471 cells; 10 $\mu\text{g/mL}$ Tuni, 439 cells. Four independent experiments. Unpaired two side Mann-Whitney U-test. Each solid thick line in violin plot indicates median of data in the column. Gray lines are quartiles.

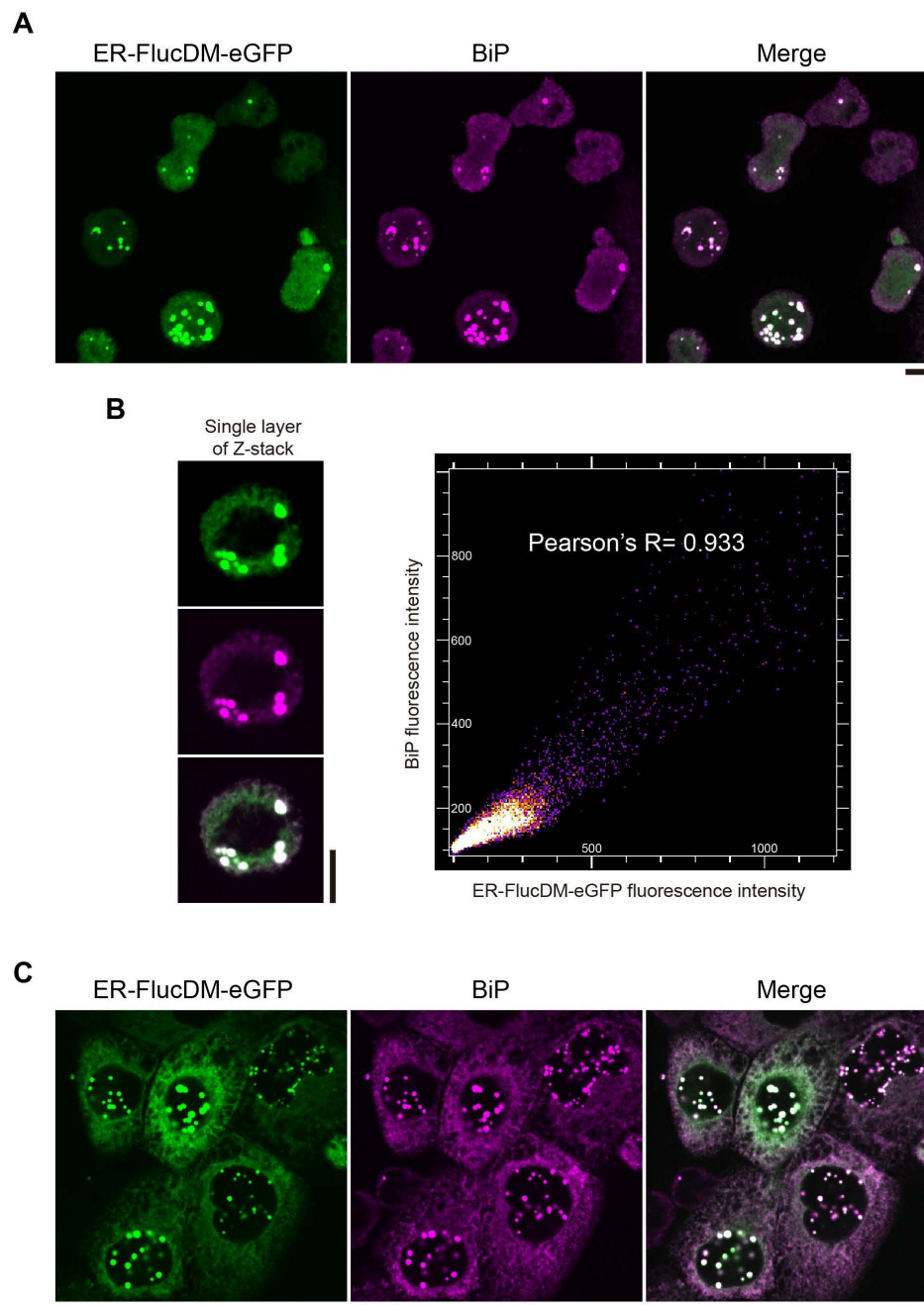


Figure S5.

Colocalization of BiP and ER-FlucDM-eGFP, related to Figure 5

(A) Immunofluorescence staining of BiP and ER-FlucDM-eGFP in dividing cells. Maximum projected images were shown.

(B) Colocalization analysis of BiP and ER-FlucDM-eGFP. R represents Pearson correlation coefficient (r).

(C) Immunofluorescence staining of BiP and ER-FlucDM-eGFP in interphase cells. Single Z-slice images were shown.

Scale bar, 10 μ m. Three independent experiments.

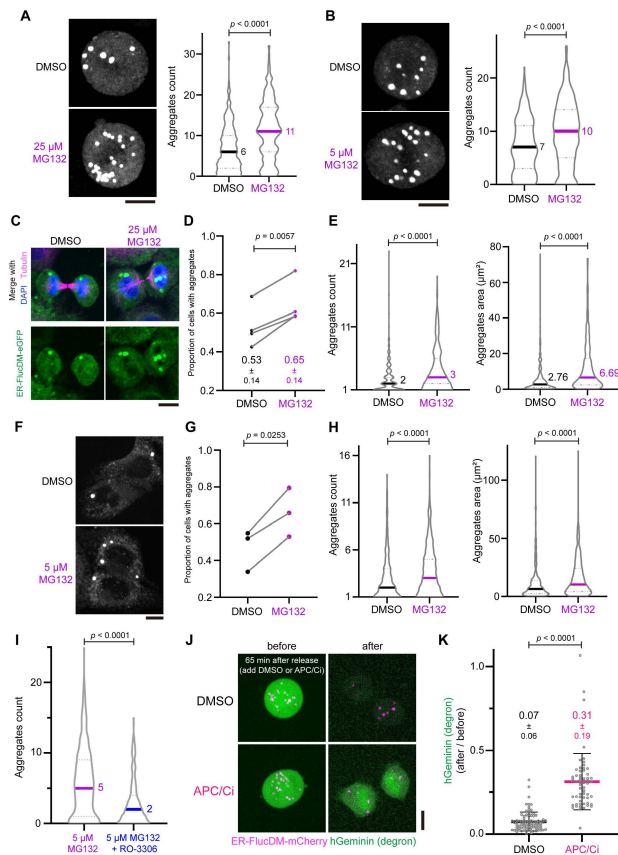


Figure S6.

MG132, APCi and RO-3306 treatments on aggregate clearance, related to Figure 6

(A) Quantification of the aggregate number in cells expressing ER-FlucDM-eGFP treated with DMSO or 25 μ M MG132 and fixed at 55 min after released from the G2/M boundary. DMSO, 293 cells; MG132, 350 cells. Maximum projected images were shown.

(B) Quantification of the aggregate number in cells expressing ER-FlucDM-eGFP treated with DMSO or 5 μ M MG132 and fixed at 55 min since release. DMSO, 295 cells; MG132, 436 cells. Maximum projected images were shown.

(C) Images of cells at telophase or newly divided cells after treated with DMSO or 25 μ M MG132 for 35 min. Maximum projected images were shown.

(D) Cells at telophase or newly divided cells that have aggregates upon treatment of 25 μ M MG132 were counted. DMSO, 455 cells; MG132, 393 cells.

(E) Aggregates number and area were counted in cells at telophase or in newly divided cells after treated with DMSO or 25 μ M MG132. Only cells with aggregates were included in the quantification. DMSO, 294 cells; MG132, 339 cells.

(F) Images of cells at telophase and newly divided cells after treated with DMSO or 5 μ M MG132 for 35 min. Maximum projected images were shown.

(G) Cells at telophase or newly divided cells that have aggregates upon treatment of DMSO or 5 μ M MG132. DMSO, 676 cells; APCi, 646 cells.

(H) Aggregates number and area were counted in cells at telophase or in newly divided cells after treated with DMSO or 5 μ M MG132. Only cells with aggregates were included in the quantification. DMSO, 227 cells; MG132, 297 cells.

(I) Quantification of the aggregate number in cells expressing ER-FlucDM-eGFP treated with 5 μ M MG132 or 5 μ M MG132 + RO-3306.

(J) Images of cells co-expressing ER-FlucDM-mCherry and mGreenLantern-hGeminin (degron).

Left panel: cells were treated with DMSO or APC/Ci after 65 min released from the G2/M. Right panel: after 65 min released from the G2/M, cells were treated with DMSO or APC/Ci for 40 minutes using live-cell imaging. Maximum projected images were shown.

(K) Quantification of mGreenLantern-hGeminin (degron) intensity of cells treated with DMSO or APC/Ci for 40 min.

Scale bar, 10 μ m. Data are from at least three independent experiments and analysed by unpaired two side Mann-Whitney U-test (A, B, E, H and I), unpaired t-test (K) and paired t-test (D and G).

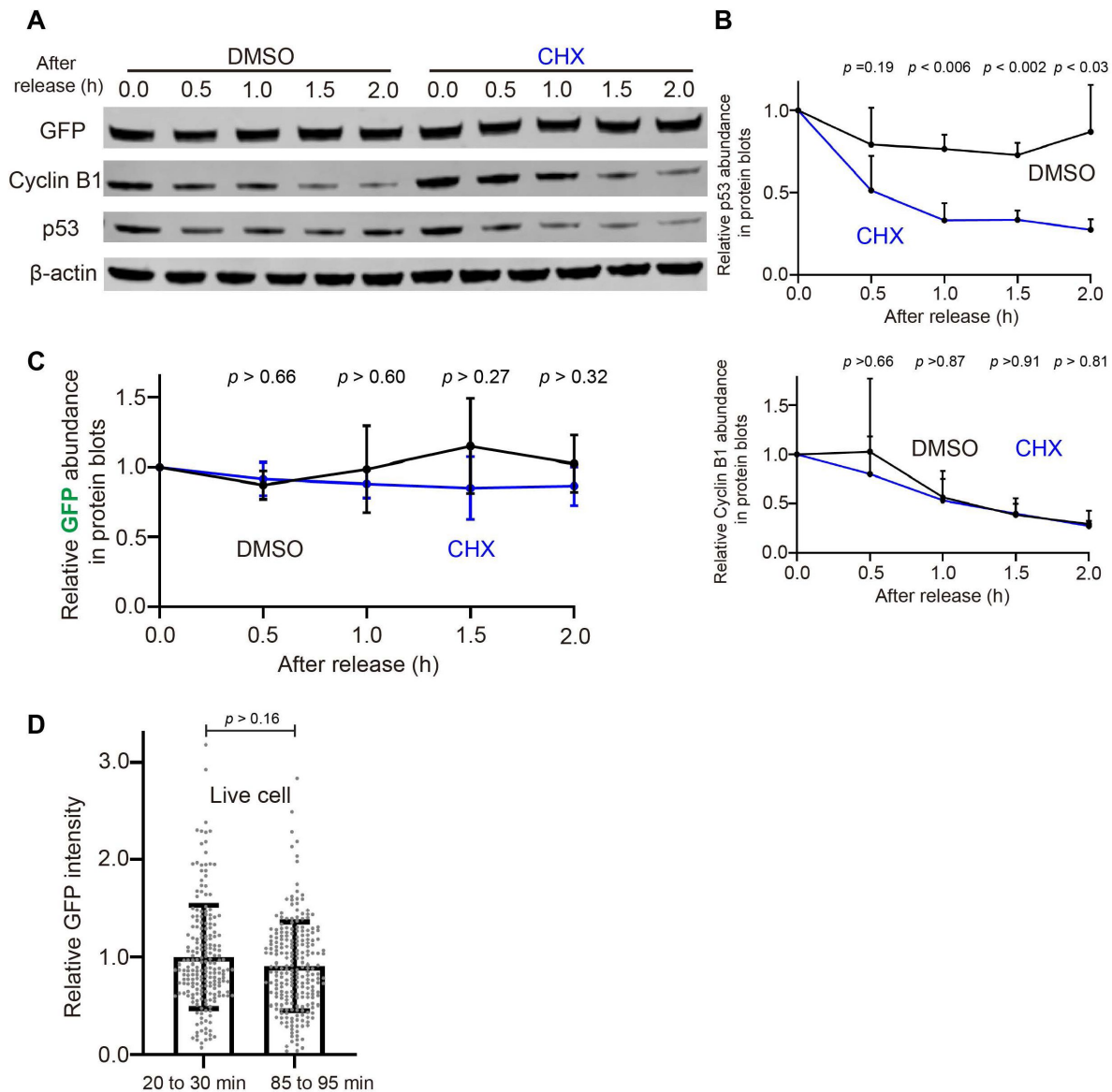


Figure S7.

Cycloheximide chase experiment and microscopy quantification of cells expressing ER-FlucDM-eGFP, related to Figure 6 [↗](#).

(A(A) Protein blots of ER-FlucDM-eGFP, Cyclin B1, p53 and β-actin under DMSO or 200 μM CHX treatments. Cells were synchronized to the G2/M boundary and released into medium containing DMSO or CHX and lysates were harvested.

(B) Protein levels of p53 and Cyclin B1 in different time points after DMSO or CHX treatments were measured and plotted. Levels of β-actin were used in normalization.

(C) Protein levels of ER-FlucDM-eGFP in different time points after DMSO or CHX treatments were measured and plotted. Levels of β-actin were used in normalization.

(D) Relative intensity of ER-FlucDM-eGFP in early and late dividing cells.

Scale bar, 10 μm. Data are from three independent experiments and analysed by unpaired t-test.

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Additional information

Author contributions

S.D conceived the study, designed and performed experiments, and analyzed data. Y.W, B.C, S.X, K.Y.C provided some reagents used in the study. D.H participated in the discussion during project development. T.G.C. conceived the study, designed experiments and supervised the study. D.S and T.G.C prepared the manuscript with inputs from D.H.

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Reviewer #1 (Public review):

Du et al. address the cell cycle-dependent clearance of misfolded protein aggregates mediated by the endoplasmic reticulum (ER) associated Hsp70 chaperone family and ER reorganisation. The observations are interesting and impactful to the field.

Strength:

The manuscript addresses the connection between the clearance of misfolded protein aggregates and the cell cycle using a proteostasis reporter targeted to ER in multiple cell lines. Through imaging and some biochemical assays, they establish the role of BiP, an Hsp70 family chaperone, and Cdk1 inactivation in aggregate clearance upon mitotic exit. Furthermore, the authors present an initial analysis of the role of ER reorganisation in this clearance. These are important correlations and could have implications for ageing-associated pathologies. Overall, the results are convincing and impactful to the field.

Weakness:

The manuscript still lacks a mechanistic understanding of aggregate clearance. Even though the authors have provided the role of different cellular components, such as BiP, Cdk1 and ATL2/3 through specific inhibitors, at least an outline establishing the sequence of events leading to clearance is missing. Moreover, the authors show that the levels of ER-FlucDM-eGFP do not change significantly throughout the cell cycle, indicating that protein degradation is not in play. Therefore, addressing/elaborating on the mechanism of disassembly can add value to the work. Also, the physiological relevance of aggregate clearance upon mitotic exit has not been tested, nor have the cellular targets of this mode of clearance been identified or discussed.

<https://doi.org/10.7554/eLife.96675.2.sa3>

Reviewer #2 (Public review):

This paper describes an interesting observation that ER-targeted misfolded proteins are trapped within vesicles inside nucleus to facilitate quality control during cell division. This work supports the concept that transient sequestration of misfolded proteins is a fundamental mechanism of protein quality control. The authors satisfactorily addressed several points asked in the review of first submission. The manuscript is improved but still unable to fully address the mechanisms.

Strengths:

The observations in this manuscript are very interesting and open up many questions on proteostasis biology.

Weaknesses:

Despite inclusions of several protein-level experiments, the manuscript remained a microscopy-driven work and missed the opportunity to work out the mechanisms behind the observations.

<https://doi.org/10.7554/eLife.96675.2.sa2>

Reviewer #3 (Public review):

This paper describes a new mechanism for the clearance of protein aggregates associated to endoplasmic reticulum re-organization that occurs during mitosis.

Experimental data showing clearance of protein aggregates during mitosis is solid, statistically significant, and very interesting. The authors made several new experiments included in the revised version to address the concerns raised by reviewers. A new proteomic analysis, co-localization of the aggregates with the ER membrane Sec61beta protein, expression of the aggregate-prone protein in the nucleus does not result in accumulation of aggregates, detection of protein aggregates in the insoluble fraction after cell disruption and mostly importantly knockdown of ATL proteins involved in the organization of ER shape and structure impaired the clearance mechanism. This last observation addresses one of the weakest points of the original version which was the lack of experimental correlation between ER structure capability to re-shape and the clearance mechanism.

In conclusion, this new mechanism of protein aggregate clearance from the ER was not completely understood in this work but the manuscript presented, particularly in the revised version, an ensemble of solid observations and mechanistic information to scaffold future studies that clarify more details of this mechanism. As stated by the authors: "How protein aggregates are targeted and assembled into the intranuclear membranous structure waits for future investigation". This new mechanism of aggregate clearance from the ER is not expected to be fully understood in a single work but this paper may constitute one step to better comprehend the cell capability to resolve protein aggregates in different cell compartments.

<https://doi.org/10.7554/eLife.96675.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Reviews:

Reviewer #1 (Public Review):

Strengths:

The manuscript utilizes a previously reported misfolding-prone reporter to assess its behaviour in ER in different cell line models. They make two interesting observations:

- (1) Upon prolonged incubation, the reporter accumulates in nuclear aggregates.*
- (2) The aggregates are cleared during mitosis. They further provide some insight into the role of chaperones and ER stressors in aggregate clearance. These observations provide a starting point for addressing the role of mitosis in aggregate clearance. Needless to say, going ahead understanding the impact of aggregate clearance on cell division will be equally important.*

Weaknesses:

The study almost entirely relies on an imaging approach to address the issue of aggregate clearance. A complementary biochemical approach would be more insightful. The intriguing observations pertaining to aggregates in the nucleus and their clearance during mitosis lack mechanistic understanding. The issue pertaining to the functional relevance of aggregation clearance or its lack thereof has not been addressed. Experiments addressing these issues would be a terrific addition to this manuscript.

We have performed protein blotting and proteomics to characterize ER-FlucDM-eGFP expressing cells. We have also provided evidence to support the role of ER reorganization in regulating aggregate clearance. Our proteomic analysis provided a global view of the cellular state of cells expressing ER-FlucDM-eGFP, which potentially revealed functional relevance of ER-FlucDM-eGFP. Details are explained in the following comments.

Reviewer #2 (Public Review):

Summary:

The authors provide an interesting observation that ER-targeted excess misfolded proteins localize to the nucleus within membrane-entrapped vesicles for further quality control during cell division. This is useful information indicating transient nuclear compartmentalization as a quality control strategy for misfolded ER proteins in mitotic cells, although endogenous substrates of this pathway are yet to be identified.

Strengths:

This microscopy-based study reports unique membrane-based compartments of ER-targeted misfolded proteins within the nucleus. Quarantining aggregating proteins in membrane-less compartments is a widely accepted protein quality control mechanism. This work highlights the importance of membrane-bound quarantining strategies for aggregating proteins. These observations open up multiple questions on proteostasis biology. How do these membrane-bound bodies enter the nucleus? How are the single-layer membranes formed? How exactly are these membrane-bound aggregates degraded? Are similar membrane-bound nuclear deposits present in post-mitotic cells that are relevant in age-related proteostasis diseases? Etc. Thus, the observations reported here are potentially interesting.

Weaknesses:

This study, like many other studies, used a set of model misfolding-prone proteins to uncover the interesting nuclear-compartment-based quality control of ER proteins. The endogenous ER-proteins that reach a similar stage of overdose of misfolding during ER stress remain unknown.

We have included a previous study that showed accumulation of BiP aggregates in the nucleus upon overexpression of BiP (Morris et al., 1997; DOI: 10.1074/jbc.272.7.4327) in the discussion (Line 299).

The mechanism of disaggregation of membrane-trapped misfolded proteins is unclear. Do these come out of the membrane traps? The authors report a few vesicles in living cells. This may suggest that membrane-untrapped proteins are disaggregated while trapped proteins remain aggregates within membranes.

We initially made mStayGold-Sec61 β to image the ER structures and ER-FlucDM-eGFP aggregates. However, we could not obtain convincing time-lapse images to show the release of ER-FlucDM-eGFP aggregates from the ER membrane as there are abundant ER structures present close to the aggregates during mitosis, preventing the differentiation of the membrane encapsulating aggregates from the ER structures.

The authors figure out the involvement of proteasome and Hsp70 during the disaggregation process. However, the detailed mechanisms including the ubiquitin ligases are not identified. Also, is the protein ubiquitinated at this stage?

We performed cycloheximide chase experiments in cells released from the G2/M and found that ER-FlucDM-eGFP protein level did not fluctuate significantly when cells progressed through mitosis and cytokinesis. Thus, we did not consider protein ubiquitination and degradation of ER-FlucDM-eGFP as a major mechanism for its clearance. We have included this observation in the results (Figure S7A; Line 266) and in the discussion (Line 324) of the revised manuscript.

This paper suffers from a lack of cellular biochemistry. Western blots confirming the solubility and insolubility of the misfolded proteins are required. This will also help to calculate the specific activity of luciferase more accurately than estimating the fluorescence intensities of soluble and aggregated/compartmentalized proteins.

We performed solubility test in cells expressing ER-FlucDM-eGFP and detected insoluble ERFlucDM-eGFP after heat stress (Figure S1E; Line 102). We have also performed protein blotting to detect ER-FlucDM-eGFP to normalize the luciferase activity (Line 609). We have updated the method section for luciferase measurement (Line 494).

Microscopy suggested the dissolution of the membrane-based compartments and probably disaggregation of the protein. This data should be substantiated using Western blots. Degradation can only be confirmed by Western blots. The authors should try time course experiments to correlate with microscopy data. Cycloheximide chase experiments will be useful.

We performed cycloheximide chase experiments in cells released from the G2/M and found that ER-FlucDM-eGFP protein level did not fluctuate significantly when cells progressed through mitosis and cytokinesis (Figure S7A to S7C). Also, live-cell imaging of cells released from the G2/M indicated no significant change of total fluorescence intensity of ER-FlucDM-eGFP (Figure S7D). Thus, we do not think that protein degradation of ER-FlucDM-eGFP is the major mechanism for its clearance.

The cell models express the ER-targeted misfolded proteins constitutively that may already reprogram the proteostasis. The authors may try one experiment with inducible overexpression.

We have re-transduced fresh MCF10A cells with lentiviral particles to induce expression of ER-FlucDM-eGFP. The aggregates started to form after 24 h post-transduction. We made similar observations as described in the manuscript (e.g. aggregate clearance) two days after re-transduction.

It is clear that a saturating dose of ER-targeted misfolded proteins activates the pathway.

The authors performed a few RT-PCR experiments to indicate the proteostasis-sensitivity.

Proteome-based experiments will be better to substantiate proteostasis saturation.

We have performed proteomic analysis in cells expressing ER-FlucDM-eGFP and observed up-regulation of multiple proteins involved in the ER stress response, indicating that cells expressing ER-FlucDM-eGFP experience proteostatic stress (Figure S4A; Line 179).

The authors should immunostain the nuclear compartments for other ER-membrane resident proteins that span either the bilayer or a single layer. The data may be discussed.

We have co-expressed ER-FlucDM-mCherry and mStayGold-Sec61 β and detected mStayGold-Sec61 β around ER-FlucDM-mCherry aggregates (Figure 1B).

All microscopy figures should include control cells with similarly aggregating proteins or without aggregates as appropriate. For example, is the nuclear-targeted FlucDM-EGFP similarly entrapped? A control experiment will be interesting. Expression of control proteins should be estimated by western blots.

We targeted FlucDM-eGFP to the nucleus by expressing NLS-FlucDM-eGFP (Figure S1A). We found that the nuclear FlucDM-eGFP did not co-localize with the ER-FlucDM-mCherry aggregates (Figure S1B; Line 96). We have also determined the expression levels of NLSFlucDM-eGFP and ER-FlucDM-mCherry (Figure S1C and S1D).

There are few more points that may be out of the scope of the manuscript. For example, how do these compartments enter the nucleus? Whether similar entry mechanisms/events are ever reported? What do the authors speculate? Also, the bilayer membrane becomes a single layer. This is potentially interesting and should be discussed with probable mechanisms. Also, do these nuclear compartments interfere with transcription and thereby deregulate cell division? What about post-mitotic cells? Similar deposits may be potentially toxic in the absence of cell division. All these may be discussed.

Thank you for interesting suggestions for our study. We speculated that ER-FlucDM-eGFP aggregates may derive from the invagination of the inner nuclear membrane given that the aggregates are in close proximity to the inner nuclear membrane in interpase cells (Line 299). We have included a previous study that reported a similar aggregate upon BiP overexpression (Morris et al., 1997; DOI: 10.1074/jbc.272.7.4327; Line 300). Our proteomic analysis showed that cells expressing ER-FlucDM-eGFP have several up-regulated proteins related to cell cycle regulation (Figure S4A; Line 346).

Reviewer #3 (Public Review):

Summary:

This paper describes a new mechanism of clearance of protein aggregates occurring during mitosis.

The authors have observed that animal cells can clear misfolded aggregated proteins at the end of mitosis. The images and data gathered are solid, convincing, and statistically significant. However, there is a lack of insight into the underlying mechanism. They show the involvement of the ER, ATPase-dependent, BiP chaperone, and the requirement of Cdk1 inactivation (a hallmark of mitotic exit) in the process. They also show that the mechanism seems to be independent of the APC/C complex (anaphase-promoting complex). Several points need to be clarified regarding the mechanism that clears the aggregates during mitosis:

- What happens in the cell substructure during mitosis to explain the recruitment of BiP towards the aggregates, which seem to be relocated to the cytoplasm surrounded by the ER membrane.*

We have included images to show that BiP co-localizes with ER-FlucDM-eGFP aggregates in interphase cells (Figure S5C). We think that BiP participates in the formation of ER-FlucDM-eGFP during interphase instead of getting recruited to the aggregates during mitosis.

- How the changes in the cell substructure during mitosis explain the relocation of protein aggregates during mitosis.*

We provided evidence to show that clearance of ER-FlucDM-eGFP aggregates involves the ER remodeling process. We depleted ER membrane fusion proteins ATL2 and ATL3 to perturb the distribution of ER sheets or tubules and found that cells were defective in clearing the aggregates (Figure 7A and B; Line 278).

- Why BiP seems to be the main player of this mechanism and not the cyto Hsp70 first described to be involved in protein disaggregation.*

In our proteomic analysis, we found that BiP (HSPA5) but not other Hsp70 family members were up-regulated in cells expressing ER-FlucDM-eGFP (Line 352; Figure S4A). This explains why BiP is the main player of the ER-FlucDM-eGFP aggregate clearance.

Strengths:

Experimental data showing clearance of protein aggregates during mitosis is solid, statistically significant, and very interesting.

Weaknesses:

Weak mechanistic insight to explain the process of protein disaggregation, particularly the interconnection between what happens in the cell substructure during mitosis to trigger and drive clearance of protein aggregates.

In our revised manuscript, we now provided evidence to show that ER-FlucDM-eGFP aggregate clearance involved remodeling of the ER structures during mitotic exit. This is added as a new Figure 7 in the revised manuscript and is described in the result section (Line 278) and in the discussion section (Line 323). We believe that this addition has provided mechanistic insights into ER-FlucDM-eGFP aggregate clearance.

Recommendations for the authors:

Reviewing Editor comments:

I have read these reviews in detail and would like to recommend that the authors perform the experiments according to the reviewers' suggestions, as well as provide the appropriate controls raised by the reviewers.

I think there are not that many requests and they all seem very reasonable and easily doable. I would recommend that the authors carry out the suggested experiments to develop a stronger story where the evidence transitions from being incomplete presently to a "more complete" standard.

We have addressed questions raised by three reviewers and updated our manuscript (labeled in red in the main text).

Reviewer #1 (Recommendations For The Authors):

The manuscript makes exciting observations about the accumulation of reporter protein aggregates in the nucleus and its clearance during mitosis. It also provides some insight into the role of chaperons in aggregate clearance. These observations provide a good platform to perform in-depth analysis of the underlying mechanism and its functional relevance which perhaps the authors will plan over the long term. However, the below suggestions will help improve the current version of the manuscript:

(1) Although it is assumed that the aggregates are cleared by the protein degradation mechanism, clear evidence supporting this assumption in the author's experiments is lacking and needs to be provided. Is it possible that mitosis induces disassembly of these aggregates instead of degradation?

We performed two experiments to verify whether ER-FlucDM-eGFP aggregates are cleared by the protein degradation mechanism. In the first experiment, we treated cells expressing ER-FlucDM-eGFP released from the G2/M boundary with cycloheximide (CHX) and found that ER-FlucDM-eGFP did not decrease in protein abundance in cells progressing through mitosis (Figure S7A to S7C). In the second experiment, we measured the intensity of ERFlucDM-eGFP in early dividing cells and late dividing cells after release from the G2/M boundary and found that there was no significant difference between early and late dividing cells (Figure S7D). Thus, we concluded that protein degradation of ER-FlucDM-eGFP is not the primary mechanism of its clearance during cell division (Line 324). Furthermore, we included new data to show that the ER-FlucDM-eGFP aggregate clearance depends on ER reorganization during cell division, so mitotic exit induces disassembly of the aggregates instead of protein degradation.

(2) It is intriguing that the aggregates are nuclear. Is the nuclear localization mediated by localization to ER? A time course analysis would reveal this and would provide credence to the idea that the reporter was originally expressed in the ER. It is currently unclear if the reporter ever gets expressed in ER.

We showed that in interphase cells, ER-FlucDM-eGFP co-localizes with mStayGold-Sec61 β , which labels the ER structures (Figure 1B). So, ER-FlucDM-eGFP is expressed and present in the ER network and invaginates into the inner nuclear membrane as aggregates. We attempted to image ER-FlucDM-eGFP for its formation; however it was technically challenging as the aggregates appeared very small and not too visible after clearance under our microscopy system.

(3) It would be expected that the persistence of these aggregates would impact cell division and cellular health. An experiment addressing this hypothesis would be very useful in establishing the functional relevance of this observation in the context of the current study.

We have performed proteomic analysis on cell expressing ER-FlucDM-eGFP and found that multiple proteins involved in the ER stress response were up-regulated (Figure S4A). Additionally, proteins related to cell cycle regulation were up-regulated upon expression of ER-FlucDM-eGFP (Figure S4A). The increase of these proteins may indicate a perturbed cellular health (Line 344).

(4) A recent report (PMID: 34467852) identified the role of ER tubules in controlling the size of certain misfolded condensates. Would specific ER substructures affect the nuclear localization and/or clearance of the FlucDM aggregates? This is tied to point#2 and would provide insights into the connection between ER and the nuclear aggregates.

Thank you for your suggestions. We perturbed the ER remodeling process by knocking down ATL2 and ATL3, which are ER membrane fusion proteins, and found that clearance of ER-FlucDM-eGFP aggregates was affected (Figure 7A and B). Hence, perturbation of the distribution of ER tubules and ER sheets affects ER-FlucDM-eGFP aggregate clearance. We have also added the recent paper about ER tubule size in regulating the sizes of misfolded condensates in the discussion (Line 321)

Reviewer #2 (Recommendations For The Authors):

I expect that the images indicate z-sections. Should be indicated in legends as applicable.

We have indicated whether the images are Z-stack or single Z-slices in the figure legends.

Small point: the control region (outside inclusion) that was bleached in 2c may be clearly indicated.

We have added the explanation in the figure legend of Figure 2C.

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